CHARACTERIZATION OF BCL-2 INTERACTING PARTNERS AT THE ENDOPLASMIC RETICULUM
IDENTIFICATION AND CHARACTERIZATION OF BCL-2 INTERACTING PARTNERS
AT THE ENDOPLASMIC RETICULUM

By

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TITLE: Identification and Characterization of Bcl-2 Interacting Partners at the Endoplasmic Reticulum  

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ABSTRACT

Cancer occurs when cells acquire a number of mutations that trigger uncontrolled cell growth. The normal cellular response to this dysregulation of growth is the activation of programmed cell death. While focus in cancer research has been mainly concentrated in the mechanism of programmed cell death at the mitochondria, endoplasmic reticulum is slowly emerging as an essential platform for this regulatory mechanism.

Bcl-2 is the founding member of the Bcl-2 family of protein, which contributes to the regulation of cell death at the mitochondria and at the endoplasmic reticulum. Previously in our lab, we have shown using MCF-7 cells stably expressing Bcl-2 targeted to the endoplasmic reticulum; they were protected from estrogen deprivation induced cell death. Thus the regulatory mechanism of Bcl-2 at the endoplasmic reticulum represents an interesting avenue to improve current cancer therapeutics.

Two approaches were utilized to identify and characterize Bcl-2 and its interacting partners at the endoplasmic reticulum. Using an affinity tag fused to Bcl-2 that has been engineered to target the endoplasmic reticulum, tandem affinity purification was utilized to identify novel Bcl-2 interacting partners when estrogen receptor positive cells are treated with estrogen deprivation. Using fluorescent protein fused to the proteins of interest, Fluorescent Lifetime Imaging Measurement (FLIM) was used to characterize the interactions of Bcl-2 and its known interacting partner at the endoplasmic reticulum. The findings of this thesis verify the
applications of the two aforementioned methods in the study of Bcl-2 interacting proteins at the endoplasmic reticulum.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ActA</td>
<td>Carboxyl terminal insertion sequence of the ActA protein</td>
</tr>
<tr>
<td>α MEM</td>
<td>Alpha-minimal essential medium</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl2 associated death promoter</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic assay</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl2 homology</td>
</tr>
<tr>
<td>BID</td>
<td>Bcl2 interacting domain</td>
</tr>
<tr>
<td>BIK</td>
<td>Bcl2 interacting killer</td>
</tr>
<tr>
<td>Cb5</td>
<td>Endoplasmic reticulum specific isoform of cytochrome b5</td>
</tr>
<tr>
<td>CBP</td>
<td>Chitin binding peptide</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfate</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>DHPC</td>
<td>1,2-diheptanoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Etyhlenediaminetetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>Est-</td>
<td>Estrogen deprivation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-5-isothiocyanate</td>
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<tr>
<td>FLIM</td>
<td>Fluorescence Lifetime Imaging Microscopy</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IB</td>
<td>Immunoblot</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>NAF1</td>
<td>Nutrient-deprivation autophagy factor-1</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIN</td>
<td>Protease inhibitor cocktail</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotation per minutes</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>S.d</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulphate polyacrylamine gel electrophoresis</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Buffer for washing Western Blot membranes; contains Tris-HCl, NaCl, and Tween-20</td>
</tr>
<tr>
<td>TLB</td>
<td>Tricene SDS-PAGE loading buffer</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
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<tr>
<td>UPR</td>
<td>Unfolded Protein response</td>
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1 Introduction

1.1 Breast Cancer

Each year, it is estimated that there will be 177,800 new cases of cancer across Canada which 13% being breast related (Canadian Cancer Statistics, 2011). Breast cancer is the most common form of cancer amongst women. As the name suggests, BC is the form of cancer that propagates in the tissue of the breast. There are two main types of breast cancer; Ductal carcinoma (the most common form of breast cancer which originates from the tubes that are responsible for the transport of milk from the breast to the nipple) or Lobular carcinoma (where carcinogenesis initiates in the part of the breast that produces milk). It is approximated that 1 in 9 women will be diagnosed with breast cancer in their life time and 3.4% of those diagnosed will die as a result of the disease (Canadian Cancer Statistics, 2011).

Currently, breast cancer maybe treated with chemotherapy, radiation therapy and surgery (Leong et al, 2011). Many types of breast cancer are sensitive to estrogen, which allows tumor proliferation through the effects of the estrogen receptor (ER) (Tyson et al, 2011; Williams, 2010). Hormone therapy is prescribed to women with estrogen receptor positive breast cancer. Anti-estrogen drug such as Tamoxifen or Fulvestrant are designed to antagonize the binding of estrogen to the estrogen receptor, effectively shutting down estrogen receptor downstream signaling pathway, which contributes to the proliferation and carcinogenesis of estrogen receptor positive breast cancer (Ali et al, 2011; Tyson et al, 2011; Williams, 2010). Unfortunately, some patients eventually develop resistance to the hormone
therapy and cancer proliferates. Furthermore, this type of treatment is useless for patients with estrogen receptor negative breast cancer. This has created a demand for further investigation into the downstream signaling pathways of estrogen in order to create an effective therapeutic treatment for breast cancer that bypasses the requirement of the estrogen receptor.

1.2 Programmed Cell death

Most cancers arise from an accumulation of harmful genomic mutations that lead to defects in the regulation of cellular proliferation and homeostasis, resulting in uncontrolled cell growth. Under normal circumstances, the activation of a tightly regulated cellular process known as programmed cell death prevents tumors from developing by killing these defective cells.

The term “Apoptosis” was first introduced in 1972 to describe the phenomenon of programmed cell death (Kerr et al, 1972). Apoptosis is a suicide mechanism that is regulated by a variety of cell signaling pathways to maintain the homeostatic balance within an organism. It plays a critical role in embryogenesis, tissue homeostasis, and the maturation of the immune system (Walsh & Edinger, 2010). The Bcl-2 (B-cell lymphoma 2) family of proteins tightly regulates this delicate process. The deregulation of the Bcl-2 proteins has many implications in human disease. Evasion of apoptosis allows cancerous cells to escape death leading to the formation of both solid and hematopoietic malignancies (reviewed in Look, 1997; Green et al, 2002). Conversely, excessive activation of apoptosis in neurons, as shown in patients with stroke, can cause the permanent lost of neurological function.
(Friedlander, 2003). Thus, understanding the molecular mechanism by which the Bcl-2 family of proteins regulates apoptosis presents an avenue for the development and the discovery of novel cancer therapy that is heavily pursued by researchers.

1.3 The Bcl-2 family of proteins

The Bcl-2 protein family consists of anti- and pro-apoptotic members, all of which contain at least one or more Bcl-2 Homology (BH) regions, BH1-BH4 (Levine et al, 2008). There are three-difference classes of Bcl-2 proteins classified by their function and their BH regions. The first class of the Bcl-2 proteins consists of anti-apoptotic proteins such as Bcl-2, Bcl-xL and Bcl-w, comprising of all four BH domains (Figure 1). The second class of the Bcl-2 proteins contains pro-apoptotic members such as Bax (Bcl-2 associated protein X) and Bak (Bcl-2-antagonist/killer), which contain BH regions 1-3. These proteins structurally lack the BH4 domain compared to the anti-apoptotic members and function to oppose their anti-apoptotic counterparts by permeabilizing membranes thereby promoting cellular demise (Figure 1). The third class of Bcl-2 proteins comprises the pro-apoptotic BH3-only proteins such as Bid, BIM and Puma, which are either sensitizers or activators of the anti-apoptotic and pro-apoptotic members respectively (Bogner et al, 2010).
Together, the three classes of Bcl-2 family of proteins regulate the activation or inhibition of MOMP (mitochondrial outer membrane permeabilization). MOMP is considered the point of no return in apoptosis and once it occurs, the cell is committed to death (Leber et al., 2007). Under apoptotic stimuli, Bax and Bak can be recruited to the MOM (mitochondrial outer membrane) by Bid (or another activator) to form pores. MOMP result in the release of various mitochondrial
intermembrane space (IMS) proteins such as cytochrome c and SMAC/DIABLO, leading to the activation of the downstream caspase cascade (Ciechomska et al, 2008; Djavaheri-Mergny et al, 2010). The activated caspases result in the subsequent cleavage of various intracellular substrates and ultimately result in cell death. Under normal conditions, the anti-apoptotic members normally inhibit MOMP by binding to and preventing the activation of the pro-apoptotic members.

1.4 Models for regulation of apoptosis by Bcl-2 family proteins

Currently the molecular mechanism of MOMP is still controversial and the field is divided between two different models; the “direct activation” and the “derepression” models. The conflict between the two models lies in the nature of Bax/Bak activation. The direct activation model proposes that the BH3-only proteins directly activate Bax and Bak, while the role of the anti-apoptotic proteins is to sequester the Bax/Bak activators (Bogner et al, 2010; Leber et al, 2007). Sensitizer BH3-only proteins can bind to the anti-apoptotic proteins and displace BH3-only activators, which would then be freed to activate Bax/Bak (Bogner et al, 2010; Leber et al, 2007). Conversely, the Derepressor model suggests that Bax and Bak are intrinsically active and anti-apoptotic Bcl-2 proteins are constitutively bound to Bax/Bak to prevent apoptosis (Leber et al, 2007). A third model that is gaining momentum in the field is called “Embedded Together” (Leber et al, 2007). It combines features of both the Direct Activation and the Derepressor models with an added emphasis on the dynamic protein-protein interactions that occur within the
MOM. This model specifies the role of anti-apoptotic members of the Bcl-2 family proteins to bind and sequester both BH3-only activators and activated Bax/Bak.

Figure 2. Three competitive models of apoptosis regulation by Bcl-2 family of proteins. A) Derepressor model proposes that Bax/Bak (shown only as Bax) are constitutively active and the anti-apoptotic proteins (shown here as Bcl-xL) sequester Bax/Bak, and sensitizer BH3-only protein can inhibit anti-apoptotic proteins. B) Direct activation model proposes that BH3-only protein directly bind to and activate Bax/Bak. The role of the anti-apoptotic proteins is to sequester BH3-only activators but not Bax/Bak. BH3-only sensitizers are able to bind to and inhibit the anti-apoptotic proteins, allowing the BH3-only sensitizers to activate Bax. C) Embedded Together model stresses the importance of the membrane where Bcl-2 family members undergo conformational changes upon insertion into the membrane. The activator and sensitizer BH3-only protein can recruit not only Bax/Bak but also anti-apoptotic proteins to the membrane as well. The role of the anti-apoptotic protein is to sequester both BH3-only activators and activated Bax and Bak and anti-apoptotic proteins can be displaced by BH3 sensitizers to promote apoptosis.
The Embedded Together model proposed that the interaction of the anti-apoptotic Bcl-2 family members with BH3-only proteins or activated Bax/Bak induces a conformational change in the anti-apoptotic members, allowing their insertion into the MOM where they exert their pro-survival functions (Leber et al, 2007). This model elaborates on the importance of the Bcl-2 interaction within the membrane, postulating that Bcl-2 family of proteins undergo distinct conformational changes upon binding to the membrane as a requirement for the functional interactions of all these proteins leading to MOMP.

1.5 Bcl-2 proteins at the endoplasmic reticulum

In addition to the mitochondria, Bcl-2 proteins also localize to the endoplasmic reticulum and the nucleus (Zamzami et al, 1998; Zhu et al, 2010). While the role of Bcl-2 family proteins at the MOM has been studied extensively, further investigation into their role(s) at other subcellular compartments is advantageous in understanding carcinogenesis. Recent advances in the development of cancer drugs have revealed the potential to kill cancer cells through another kind of cell death pathway elicited primarily at the endoplasmic reticulum (Chen et al, 2011; Pattingre et al, 2006). Bcl-2 proteins have been shown to be involved in ER stress, regulation of Ca\textsuperscript{2+} level and macroautophagy (Edinger et al, 2003; Glick et al, 2010; Naidoo, 2009a). These stress processes were shown to be regulated by Bcl-2 at the endoplasmic reticulum and its interaction with other proteins at this subcellular compartment represents a new frontier in cancer research.
1.6 Bcl-2 as modulator of Ca^{2+} level at the endoplasmic reticulum

The endoplasmic reticulum is the main site of intracellular calcium storage - and several proteins that interact with Bcl-2 regulate this delicate calcium gradient. BIK (Bcl-2-interacting killer) is a BH3- only pro-apoptotic protein, which under apoptotic stimuli, interacts with Bcl-2 and Bcl-xL at the endoplasmic reticulum to antagonize their anti-apoptotic function (Chang et al, 2010; Chinnadurai et al, 2009; Rashmi et al, 2008). The inhibition of Bcl-2 and Bcl-xL by BIK allows Bax/Bak to proceed with their death inducing function normally prevented by the anti-

Figure 3. Simplified schematic of the model of Ca^{2+} modulation by BIK at the endoplasmic reticulum. BIK interaction with Bcl-2 and Bcl-xL allows the activation of Bax as shown in the boxed panel. Subsequent remodeling of the mitochondrial cristae and the release of cytochrome c after Ca^{2+} release is illustrated as described in the literature (Heath-Engel et al, 2008; Zhou et al, 2011).
apoptotic proteins (Heath-Engel et al, 2008; Zhou et al, 2011). Activation of Bax/Bak leads to the permeabilization of the endoplasmic reticulum in a Bax/Bak dependent manner leading to the release of Ca\(^{2+}\) into the cytosol (Heath-Engel et al, 2008; Mathai et al, 2005). The release of endoplasmic reticulum Ca\(^{2+}\) into the cytosol was shown by Mathai et al to induce the remodeling of inner mitochondrial membrane cristae, leading to the opening of the cristae and the release of cytochrome c through organelle fragmentation (Heath-Engel et al, 2008; Mathai et al, 2005).

In addition to the permeabilization of the endoplasmic reticulum mediated by BIK, Bap31 (B-cell associated protein 31) is another endoplasmic reticulum membrane protein that can cause the release of Ca\(^{2+}\) into the cytosol (Breckenridge et al, 2003; Heath-Engel et al, 2011). Bap31 is a polytopic transmembrane protein that functions as an escort factor in protein trafficking within the endoplasmic reticulum under normal conditions (Ladasky et al, 2006; Wakana et al, 2008). It was shown that when expressed ectopically Bap31 is cleaved by caspase-8 at the c-terminus (Breckenridge et al, 2003; Heath-Engel et al, 2011), resulting in a p20 fragment recently shown to induce a non-apoptotic form of cell death with paratosis-like morphology characterized by the formation of vacuoles in the cytoplasm along with mitochondrial swelling. (Breckenridge et al, 2003; Heath-Engel et al, 2011; Stojanovic et al, 2005). This novel form of cell death was shown to be inhibited by Bcl-2 that was restricted to the endoplasmic reticulum, further extending the function of Bcl-2 in the regulation of cell death at this sub-cellular compartment as an intracellular Ca\(^{2+}\) modulator.
1.7 Bcl-2 in endoplasmic reticulum stress

Endoplasmic reticulum primarily functions as the site of protein folding, post-translational modification, lipid biosynthesis and calcium storage within a cell. Conditions that disrupt the luminal environment of endoplasmic reticulum core functions such as oxidative stress, disruption of Ca$^{2+}$ level or the accumulation of unfolded/misfolded proteins can cause the activation of a self-preservation mechanism called UPR (Unfolded Protein Response) (Bogner et al, 2010; Chinnadurai et al, 2009; Maiuri et al, 2007). Upon stress conditions, UPR can activate three signaling pathways that act to reestablish the normal homeostatic condition through the decrease in protein translation, increased expression of chaperone proteins to help with proper protein folding and increasing the rate of degradation of misfolded proteins (Benbrook et al, 2012). IRE1 (Inositol Requiring kinase 1) is a transmembrane protein at the endoplasmic reticulum and one of the proteins that is activated in response to endoplasmic reticulum stress (Naidoo, 2009a; Naidoo, 2009b). IRE1 functions in a Bax/Bak dependent manner where upon release by endoplasmic reticulum chaperone proteins, IRE1 will oligomerize and its C-terminus will bind to the same position where BH3 only protein binds to Bax/Bak (Naidoo N, 2009a). The binding of Bax/Bak to the C-terminal of IRE1 is essential in the sustained activation of IRE1 in cell survival during UPR, leading to the upregulation of chaperone proteins, endoplasmic reticulum associated protein degradation (ERAD) and autophagy (reviewed in Bateman et al, 2009).
Tumor cells are shown to rely heavily on the UPR to counteract the effects of endoplasmic reticulum stress as a result of the tumor microenvironment or cancer derived from secretory cells with high protein synthesis such as multiple myeloma (Naidoo N, 2009a). Thus, Bax/Bak interaction with IRE1 has the potential to be a useful therapeutic target for treating cancers with constitutive UPR activation.

1.8 Macroautophagy: a self-preservation response

Macroautophagy (referred to as autophagy from here on) is a highly conserved catabolic process in which long-lived proteins and damaged organelles are degraded in an intracellular manner (Levine et al, 2008). Unlike apoptosis, autophagy serves as an emergency response to nutrient starvation and metabolic stress through the recycling of energy and nutrients to prevent cellular demise (Chen et al, 2011; Edinger et al, 2003). Autophagy operates in a caspase-independent manner characterized by the formation of autophagic vacuoles in the cytosol, degradation of the Golgi and the endoplasmic reticulum before the breakdown of the nucleus (Levine et al, 2008).

This multi-step process is highly regulated by numerous autophagy-related genes (ATG), which play a crucial role in autophagosome formation and the regulation of autophagy. These ATGs have also been shown to be associated with important pathological process such as cancer initiation and progression. Logically, autophagy can function as a tumor suppressor through the abolishment of damaged cells, which protects the organism from carcinogenesis by maintaining genomic stability and cellular homeostasis. This cytoprotective effect of autophagy can be
detrimental (Edinger et al, 2003; Levine et al, 2008) as studies have shown that autophagy can promote tumor cell resistance to chemotherapy, contributing to tumor development under stress conditions (Edinger et al, 2003).
Figure 4. Simplified schematic of the mechanism of autophagy. The autophagic pathway is initiated through nutrient deprivation and the inhibition of mTOR. This process can be inhibited by Bcl-2 restricted to the endoplasmic reticulum by preventing the vesicle formation by Beclin1. 1. Vesicle formation – Vps34 is activated at the initiation of vesicle nucleation to generate phosphatidylinositol-3-phosphate complex. Its activation depends on the formation of a multiprotein complex consisting of Beclin1 and its accessory proteins. 2. Retrieval – The process of which targeted organelles are retrieved into the isolation membrane remains elusive. 3. Docking and fusion – Upon the completion of the autophagosome after engulfment of these cytoplasmic materials by the isolation membrane, the autophagosome fuses with lysosomes to create autolysosomes. 4. Autolysosome – the inner membrane as well as the luminal content of the autolysosome are subsequently degraded by the lysosomal enzymes. Autophagy can destroy large proportions of the cytosol and organelles that beyond a certain limit would cause irreversible cellular atrophy with a consequent collapse of vital cellular functions, leading to cellular demise.
The mechanism of autophagy is still not clearly understood in the field. Emerging evidence has suggested that the preautophagosomal vesicles are generated at an endoplasmic reticulum-associated compartment, the omegasome, which is a specialized PI3P (phosphatidylinositol 3-phosphate) enriched endoplasmic reticulum membrane platform that serves as a phagosomal initiation complex (Axe et al, 2008). The recruitment of Vps34 (class III Phosphoinitsotide kinase) to this platform is essential in order for Vps34 to bind to Beclin1 and other accessory proteins to create a functional PI3K complex (Kihara et al, 2001). Beclin1 is essential for the initiation of autophagy at the endoplasmic reticulum as its involvement in a complex with Vps34 is required for the formation of autophagosome. This budding of pre-autophagosome initiated by Beclin1 and its associated protein complex can be inhibited by Bcl-2 binding to Beclin1 at the endoplasmic reticulum, serving as a regulatory control of autophagy in the absence of nutrient starvation (Liang et al, 1999).

Beclin1 was initially classified as a binding partner of Bcl-2 at the endoplasmic reticulum and was later identified to contain a BH3- domain (Pattingre et al, 2005). Studies have shown that a canonical BH3-only protein such as Bad can displace Beclin1 from endoplasmic reticulum restricted Bcl-2, effectively promoting autophagy (Maiuri et al, 2007). This interaction between Bcl-2 and Beclin1 represents a possible convergence point between the apoptotic and autophagy pathways. Interestingly, Beclin1 was shown to be cleaved into 2 - fragments by caspases under growth factor withdrawn conditions (Wirawan et al, 2010). The
amino-terminal fragment of cleaved Beclin1 was shown to translocate to the nucleus while the C-terminal fragment localizes to the mitochondria (Cho et al., 2009; Wirawan et al., 2010; Zhu et al., 2010). The cleavage of Beclin1 dissipated its interaction with Bcl-2 at the endoplasmic reticulum and the cleaved fragment was shown to lack the autophagy inducing capability of the full length Beclin1. Furthermore, Wirawan et al was able to show that the C-terminal fragment of Beclin1 was shown to cause the release of cytochrome C in isolated mitochondria (Wirawan et al., 2010). Of note, the BH3 domain of Beclin1 remains with the amino-terminal fragment, which showed no apparent pro-apoptotic activity (Ciechomska et al., 2009; Zhu et al., 2010).

Recently, another novel protein was discovered to mediate the interaction between Beclin1 and Bcl-2 at the endoplasmic reticulum (Chang et al., 2010). Previously linked to the neurodegenerative disorder Wolfram syndrome 2 (Amr et al., 2007), NAF1 (Nutrient-deprivation autophagy factor 1) was shown to be required for Bcl-2 at the endoplasmic reticulum to antagonize Beclin1 mediated autophagy in lung cancer cells undergoing nutrient deprivation (Chang et al., 2010). Unlike most Bcl-2 interacting-proteins, NAF1 interacts with Bcl-2 in the absence of a BH3 only domain. Instead, the cytosolic two iron-two-sulfur coordinating CDGSH domain was shown to be essential in mediating the interaction (Chang et al., 2010). This interaction can be disrupted in the presence of BIK overexpression; which binds to Bcl-2 via the BH3 binding groove (Chang et al., 2010). Thus, it is likely that Bcl-2 undergoes conformational changes at the endoplasmic reticulum membrane.
upon binding of BIK, which results in the displacement of NAF1. These recent advances in the understanding of Bcl-2 at the endoplasmic reticulum provides important insight into autophagy and apoptosis with Beclin1 and NAF1 being the emphasis.

1.9 Targeting Bcl-2 interaction as a strategy for cancer therapy

The targeting of Bcl-2 family protein interaction as a strategy of cancer therapy is well practiced with the development of BH3 mimetic drugs such as ABT-263 and Obatoclax (Whitecross et al, 2009; Paik et al, 2010). These drugs have the ability to displace Bax and BH3-only activators from Bcl-2 or Bcl-xL to induce apoptosis through MOMP and the release of cytochrome c from the mitochondria leading to caspase activation (Paik et al, 2010). While these two drugs have shown promise in clinical trials, they are far from ideal therapies as the side effects of these treatments include diarrhea, nausea and thrombocytopenia (Whitecross et al, 2009). Tumors that show resistance to these drugs are shown to have elevated expression of anti-apoptotic proteins such as Mcl-1, thus further research is required to generate a more efficient treatment (Whitecross et al, 2009).

With the emergence of the endoplasmic reticulum as a central player in many apoptotic pathways, the essential role of Bcl-2 family members at this location is becoming increasingly apparent as discussed. Ca\(^{2+}\) modulation, endoplasmic reticulum stress and autophagy are all viable pathways for which Bcl-2 proteins play an intricate role in the act of self-preservation. Knowledge of the molecular mechanism of Bcl-2 at the endoplasmic reticulum along with its interacting partners
is still in its infancy compared to the mitochondrial pathway. The interaction between Bcl-2 and non-BH3 protein NAF1 in the regulation of autophagy and the revolutionary role of BH3- only protein Beclin1 to be a player in both autophagy and apoptosis shows promise that further research in Bcl-2 function, mechanism and binding partners at the endoplasmic reticulum can be exploited to develop a new generation of cancer targets.

1.10 Background of the Project

Previous studies in the Andrews lab by Fei Geng have shown evidence that estrogen deprivation treatment can induce endoplasmic reticulum mediated cell death in MCF-7 estrogen receptor positive cells stably expressing a functional mutant of Bcl-2 exclusively targeted to the mitochondria (Bcl2-ActA). Conversely, MCF-7 estrogen receptor positive stable cells expressing Bcl-2 targeted to the endoplasmic reticulum (Bcl2-cb5) are resistant to this type of cell death suggesting that cell death is mediated at the endoplasmic reticulum.

Both Bcl2-ActA and Bcl2-cb5 have previously been shown to be equally as active as the wild type Bcl-2 in preventing the corresponding organelle specific cell death (Zhu et al, 1996). When fetal bovine serum was charcoal stripped with activated carbons, non-polar materials such as certain growth factor, hormones and cytokines were removed. Cells expressing the vector control (CMV) or Bcl2-ActA were killed under treatment with the charcoal stripped media while the Bcl2-cb5 expressing cells proliferated. Supplementing the charcoal stripped medium with estrogen was sufficient in preventing this form of cell death in Bcl2-ActA cells. These
results suggest that Bcl-2 at the endoplasmic reticulum protects cells from estrogen deprivation mediated cell death.

![Figure 5. Amino acid sequence of the C-terminal portion of Bcl-2 mutants.](image)

Differential targeting mutant of Bcl-2 were created to target proteins to either the mitochondria (Bcl2-ActA) or the endoplasmic reticulum (Bcl2-cb5)

Estrogen is an important steroid hormone in post-natal, prepubertal and pubertal gland development that binds and activates the estrogen receptor (Tyson et al, 2011). There are two forms of estrogen receptor, ERα and ERβ, with the majority of breast cancer expressing a high level of ERα (Ali et al, 2011). The over expression of ERα in benign breast cancer has been shown to increase the risk of breast cancer which is problematic as ERα expression increases with age (Ali et al, 2011). Estrogen has two major roles in breast cancer cells: cell division and
proliferation. In normal breast cells, the estrogen receptor signaling increases the rate of proliferation via estrogen binding to ERα and increases cyclin D expression by activating its transcription factors (Tyson et al, 2011). The increased expression of cyclin D results in the phosphorylation of the retinoblastoma (Rb) protein, decreasing its inhibitory effects on E2F family of transcription factors which grants breast cancer cells an increased cell cycle rate (Tyson et al, 2011). Furthermore, an estrogen response element (ERE) exists upstream of the Bcl-2 gene (Klinge, 2001), which was shown to yield a 2.5 fold increase in expression of Bcl-2 in MCF-7 breast cancer cells treated with estradiol (E₂) (Klinge, 2001). The increased expression of Bcl-2 in breast cancer cells effectively tips the balance between pro-apoptotic and anti-apoptotic proteins, hindering cells’ ability to trigger apoptosis, preventing programmed cell death.

The observation of Bcl-2 protection against death exclusively at the endoplasmic reticulum downstream of the estrogen receptors suggests a possible novel mechanism or interaction of Bcl-2 at this subcellular compartment. Studies of this interaction could be utilizes to combat breast cancer cells that are resistant to hormone therapy through the lost of functional estrogen receptor; improving the weakness that exist in hormone therapy in their dependence on the estrogen receptor. Thus, we set out to identify and characterize Bcl-2 and its interacting partners at this subcellular compartment in this thesis.

1.11 Identification of novel Bcl-2 interacting partners at the endoplasmic reticulum during estrogen deprivation treatment
Tandem Affinity Purification (TAP) is a method designed to isolate proteins complexes at physiological level under native conditions (Puig et al, 2001). The fusion of the TAP tag to a protein of interest upon introduction into a host cell acts as a bait to capture endogenous interacting partners (Puig et al, 2001). The TAP tag consists of a dual affinity handle made up of a streptavidin binding peptide (SBP) and a calmodulin binding peptide (CBP) (Yifeng, 2010).

Figure 6. A simplified schematic of the tandem affinity purification using the TAP-tag. Bcl2-cb5 fused with an amino-terminal TAP-tag is purified by two consecutive steps under mild conditions to coprecipitate Bcl2-cb5 with its interacting partners

SBP consist of 38 amino acid residues with a dissociation constant of 2.5nM that allows its strong association with the streptavidin resin (Yifeng, 2010). Despite
such strong binding, it can be eluted under mild conditions with 2mM Biotin which has an even higher dissociation constant of $4 \times 10^{-14}$ M (Yifeng, 2010). CBP consists of 26 amino acid residues and binds to the calmodulin resin in a calcium dependent manner (Yifeng, 2010). CBP binds to calmodulin with a dissociation constant of $10^{-9}$ M, a very strong interaction (Yifeng, 2010). When calcium is removed from the environment by chelating agents such as EGTA, calmodulin undergoes conformational changes that result in the release of its ligand. Together, SBP and CBP make the small TAP tag (9kDa) have great specificity that would lower non-specific binding through the course of the purification along with the ability to identify protein interaction.

When fused to a protein of interest, the dual affinity handle allows the target protein along with its binding partners to be isolated in two consecutive purification steps. TAP purification also allows the protein complex to be isolated under mild conditions which preserves the complex integrity and the native conformation of the proteins involved (Puig et al, 2010). The sequential purification yields highly specific isolation with low backgrounds in a “gentle” manner, simplifying the subsequent identification and validation of proteins co-purified with the target using Mass Spectrometry (MS) (Volkel et al, 2010).

1.12 Characterization of known Bcl-2 interacting proteins at the endoplasmic reticulum during Estrogen Deprivation

The term “fluorescence” was first used in 1852 by George Stokes to describe the conversion of invisible ultra violet radiation into visible radiation of longer
wavelength (Lakowicz, 2006, p.5-7). Fluorescence is the emission of light by a fluorophore that had absorbed light or other forms of electromagnetic radiation. When a fluorophore absorbs a photon, it gains energy and enters an excited state (Bastiaens at al, 1999). The fluorophore returns to the ground state by emitting excess energy in the form of heat and the emission of a photon. Since its discovery, there have been numerous advances in the use and the application of fluorescence. Fluorescent proteins have proven to be valuable assets as non-invasive probes in living cells due to their ability to be fused to proteins of interest for investigations of localization, transport, and dynamics.

Fluorescence Resonance Energy Transfer (FRET) occurs between two fluorophores that are in close proximity where the energy from the donor is non-radiatively transferred to the acceptor (Bastiaens et al, 1999; Wallrabe et al, 2005). FRET is an image-based technique that utilizes two fusion proteins that are expressed in cells; one fused to a yellow fluorescence protein (Venus) the other to a red fluorescence protein (mCherry). Energy transfer is measured from the decrease in the fluorescence lifetime of Venus. The detection of FRET provides valuable information about the intracellular spatial relationship of fusion proteins. Efficient energy transfer would only occur if the two fluorescence proteins were within 50 angstrom (Wallrabe et al, 2005). Thus, the fluorescent protein fusions are almost certain to be bound to another. The distance dependency and the rapid reversible response make FRET an attractive way to measure protein: protein interaction in live cells.
FRET can be monitored using confocal or multi-photon microscopy where both the increase of the acceptors’ emission and the decrease of the donor emission are observed. To achieve optimal FRET, the donor and acceptor fluorophore should have large spectral overlap, favorable dipole-dipole orientation, proximity and a large quantum yield for donor, large extinction coefficient for the acceptor (Wallrabe et al, 2005). The spectral properties of fluorescent proteins such as Venus (ex. 515 nm, em. 528 nm) and mCherry (ex. 587 nm, em. 610 nm) are ideal in this respect. However, large spectrum overlaps can lead to spectral bleed through (SBT) where part of donor emission spectrum overlaps with acceptor emission or parts of the acceptor absorption spectrum is excited by donor ’s wavelength, thus SBT must be corrected (Wallrabe et al, 2005).

Fluorescence lifetime is the average time that a fluorophore remains in an excited state before returning to the ground state. Fluorescent Lifetime Imaging (FLIM) measures the rate of decay of the fluorescence emission, which is an intrinsic property of the fluorophore. Hence FLIM measurements are not affected by fluorophore concentration or excitation intensity. FLIM can be measured by two different methods.

Time correlated single photon counting (TCSPC) is a method of measuring lifetime using the time domain. Samples are excited using high repetition rate of laser pulse and the time between excitation and detection of a photon is measured and stored in a histogram. Due to the fact that less than 1 photon is detected per excitation pulse, the histogram generated represent the waveform of the lifetime
decay. (Wallrabe et al, 2005). Lifetime can then be calculated from the slope of the log$I$ vs $t$ graph (Lakowicz, 2006, p.98-99). The frequency domain method excites a fluorophore with intensity-modulated light. Due to the manner of which the fluorophore is excited, the emission is forced to respond at the same modulation frequency. The fluorescence intensity shows a delay or phase-shift with respect to excitation, which can be used to calculate the lifetime of the fluorophore (Lakowicz, 2006, p.98-99). FRET-FLIM allows the detection of the change in lifetime in the donor due to FRET as a result of the close proximity of the acceptor in a concentration and excitation intensity independent manner.

1.13 Investigations presented in this thesis

MCF-7 estrogen receptor positive breast cancer cells stimulated with estrogen deprivation will result in cell death that is preventable by the overexpression of Bcl-2 at the endoplasmic reticulum. This killing effect can be prevented by the over expression of Bcl-2 at the endoplasmic reticulum, but little details are known about Bcl-2 and its interaction at this subcellular compartment.

A biochemical and fluorescence-based assay was developed and used to identify and characterize Bcl-2 interacting partners at the endoplasmic reticulum. Amino-terminal modifications were made to Bcl2-cb5 that allows the protein to be purified with its interacting partner. DHPC was used to solubilize TAP-Bcl2-cb5 from the endoplasmic reticulum membrane and potential binding partners were identified by the tandem affinity purification. BIM was identified to co-purify with Bcl2-cb5 in untreated and estrogen deprived conditions. Fluorescent proteins were
fused to Bcl2-cb5 and its known interacting partners. FLIM was utilized to measure FRET to characterize Bcl-2 interaction in this subcellular compartment. The biochemical assay is a modified technique loosely based off the work of Dr. Felicia Vulcu in the Andrews lab while the fluorescent based assay done through the course of this thesis were built upon the work done by Dr. Alexander Aranovich in the Andrews lab, who pioneered the use of fluorescent lifetime imaging in the studies of Bcl-2 interaction (Aranovich et al, 2012).

Although the enigma surrounding Bcl-2 at the endoplasmic reticulum remains unclear, the assays developed in this thesis laid the foundation of two separate techniques that when utilized together, will shed light on the mystery of Bcl-2 at the endoplasmic reticulum.
2 Materials and Methods

2.1 Materials

Chemicals and tissue culture reagents were obtained from Life technologies or Sigma-Aldrich Chemicals unless otherwise noted. Restriction enzymes were obtained from New England Biolabs. The pNTAP-B cloning vector was obtained from Stratagene. The NAF1-eGFP vector and BCAP31-eGFP vector were obtained from Origene. pmCherry-C1 construct was a gift from Dr. Ray Truant of McMaster University. 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC) was obtained from Avanti Polar Lipids Inc. 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) was obtained from BioRad. Fugene HD transfection reagent was obtained from Promega. GammaBind™ G Sepharose™ resin, Streptavidin sepharose high performance resin and Calmodulin sepharose 4B resin were obtained from GE Healthcare Life Sciences. 25mm Coverslips were obtained from Deckglaser. Complete Mini, EDTA-free Protease inhibitor cocktail tablets were obtained from Roche. Imaging was performed on a multi-photon Leica TCS-SP5 microscope with excitation using a Chameleon Ultra pulsed laser (Coherent Inc). Becker and Hickl GmbH SPCIImage software was acquired from Becker and Hickl GmbH. BioTrace™ Polyvinylidene fluoride transfer membrane (PVDF) 0.45uM was obtained from Pall Life Science. Hoefer SemiPhor semi-dry transfer unit was purchased from Pharmacia Biotech.
2.2 **SDS-PAGE**

Proteins separated by SDS-PAGE using 10% Tricene gels were visualized by Coomassie staining. The gels were electroporated at 30 V for 40 minutes followed by 50 mA per gel for 1 hour and 15 minutes. The gels were placed in Coomassie stain (35% methanol, 0.1% Coomassie blue R-250, 10% acetic acid, 55% dH$_2$O) overnight and then were incubated in destain buffer (35% methanol, 10% acetic acid, 55% dH$_2$O) for at least 3 hours. The gels were visualized using the gel imaging station from Dnr Bio-Imaging System MiniBIS Pro.

2.3 **Antibodies**

Primary polyclonal antibody against Bcl-2 (Stan) was generated against GST-Bcl2-$\Delta$TM in rabbit by Dr. Weija Zhu. Calmodulin binding peptide (CBP) antibody was obtained from EMD Millipore. Antibodies against HRK, BNIP3 and CISD2 were obtained from Abcam Inc. Antibodies against BIK, BAK, Beclin1 (2A4), Bad, BIM, PUMA, BID, BAG1 (3.10G3E2) and ATP2A2/SERCA2 were obtained from Cell signaling. HSP60 antibody was a generous gift from Dr. Radhey Gupta from McMaster University. Monoclonal antibody against Bax (2G2) was generated against a hybrid Bax peptide in mouse. Calnexin antibody was purchased from BD Transduction laboratory. Goat anti-rabbit horseradish-peroxidase (HRP), donkey anti-mouse HRP, donkey anti-sheep HRP, donkey anti-rabbit FITC and goat anti-mouse Alexa568 antibodies were obtained from Jackson Immuno Research Laboratories.
2.4 Immunoblot

Samples were separated by SDS-PAGE electrophoresis using 10 % Tricine gels and then transferred to PVDF membrane for 1.5 hour at 50 mA per membrane using a semi-dry blotter. Membranes were blocked in blocking buffer (10mM K₂PO₄ pH 7.4, 140 mM NaCl, 0.02% NaN₃ and 5g/L powdered milk) at room temperature for at least 45 minutes. The blots were incubated over night at 4°C in primary antibody diluted in Tris-Buffered Saline with 0.2% Tween 20 (TBS-T) with 1% BSA. The blots were washed twice with TBS-T (10mM Tris-HCl pH 7.4, 500mM NaCl and 0.2% Tween-20). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted with TBS-T with 1% BSA for 2 hours at room temperature. The blots were washed three times for five minutes in TBS-T and then developed using the MicroChemi 4.2 immunoblot imager. The dilutions of the primary antibodies used for the immunoblot are illustrated in Table 1, secondary antibody dilutions were always used at 1:10000 unless otherwise specified.
Table 1: List of antibodies and the dilution used in this project.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary Dilution</th>
<th>Second antibody species (1:10000 dilution for all)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stan (Bcl2)</td>
<td>1:30000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Calmodulin Binding peptide</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>BIK</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Bax (2D2)</td>
<td>1:10000</td>
<td>Mouse</td>
</tr>
<tr>
<td>Beclin1</td>
<td>1:5000</td>
<td>Mouse</td>
</tr>
<tr>
<td>NAF1</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>BAP31</td>
<td>1:5000</td>
<td>Mouse</td>
</tr>
<tr>
<td>Bad</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Bak</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>SERCA2</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>BIM</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>PUMA</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>BID</td>
<td>1:5000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>BAG1</td>
<td>1:5000</td>
<td>Mouse</td>
</tr>
<tr>
<td>HSP60</td>
<td>1:5000</td>
<td>Mouse</td>
</tr>
<tr>
<td>Calnexin</td>
<td>1:2000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>1:2000</td>
<td>Sheep</td>
</tr>
</tbody>
</table>

2.5 Plasmids and Cloning

The plasmids used in this project are listed in Table 2. Only plasmids generated for this thesis will be described in detail.
Table 2: Cross-reference between plasmid name and DWA reference numbers of the plasmids used in this project

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>DWA ref. number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNTAP-B</td>
<td>2317</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pNTAP-Bcl2-cb5</td>
<td>2318</td>
<td>Franklin Chan</td>
</tr>
<tr>
<td>Bcl2-cb5. hd3(pSPUTK)</td>
<td>652</td>
<td>Alison Cowie</td>
</tr>
<tr>
<td>pmCherry-C1</td>
<td>2280</td>
<td>Gift from Dr. Truant</td>
</tr>
<tr>
<td>mCherry-ActA</td>
<td>2299</td>
<td>Alexander Aranovich</td>
</tr>
<tr>
<td>mCerulean-BIK</td>
<td>2312</td>
<td>Fei Geng</td>
</tr>
<tr>
<td>mCherry-BIK</td>
<td>2322</td>
<td>Franklin Chan</td>
</tr>
<tr>
<td>mCerulean-Beclin1</td>
<td>2313</td>
<td>Fei Geng</td>
</tr>
<tr>
<td>mCherry-Beclin1</td>
<td>2330</td>
<td>Franklin Chan</td>
</tr>
<tr>
<td>mCherry-Bad</td>
<td>2300</td>
<td>Alexander Aranovich</td>
</tr>
<tr>
<td>NAF1-eGFP</td>
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</tr>
<tr>
<td>NAF1-mCherry</td>
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</tr>
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</tr>
<tr>
<td>BCAP31-mCherry</td>
<td>2351</td>
<td>Franklin Chan</td>
</tr>
<tr>
<td>mCherry-cb5</td>
<td>2354</td>
<td>Franklin Chan</td>
</tr>
</tbody>
</table>

2.5.1 Cloning of TAP-Bcl2-cb5

pSPUTK Bcl2.cb5. hd3 (plasmid#652) was digested with BglII and HindIII while pNTAP-B (plasmid#2317) from Stratagene was digested with BamHI and HindIII. The digestions were visualized using 1% agarose gel. The dropout from the digestion of pSPUTK Bcl2-cb5 hd3 (~760bp) and the linear vector of pNTAP-B (~4.5kb) were physically excised from the agarose gel, which were then use to
perform gel extraction. The products were then ligated using T4 ligase from New England Biolab.

2.5.2 Cloning of mCherry-BIK mammalian expression vector

Plasmids encoding mCherry-Bad (plasmid#2300) and mCerulean-BIK (plasmid#2312) were digested with AgeI and BsrGI restriction enzymes. The digestion products were visualized after electrophoresis using a 1% agarose gel. The dropout from the digestion of the plasmid encoding mCherry-Bad (718bp) and the linearized vector of the plasmid encoding BIK (~4.5bp) were physically excised from the agarose gel and the two DNA fragments were isolated from the gel slices using a kit (Fermentas Gel extraction kit). Afterwards, the DNA fragments were then ligated using T4 ligase from New England Biolabs.

2.5.3 Cloning of mCherry-Beclin1 mammalian expression vector

Plasmid encoding mCherry-Bad(plasmid#2300) and mCerulean-Beclin1 (plasmid#2313) were digested with AgeI and BsrGI restriction enzymes. The digestion products were visualized after electrophoresis using a 1% agarose gel. The dropout from the digestion of the plasmid encoding mCherry-Bad (718bp) and the linearized vector of the plasmid encoding Beclin1 (~5.5bp) were physically excised from the agarose gel and the two DNA fragments were isolated from the gel slices using a kit (Fermentas Gel extraction kit). Afterwards, the DNA fragments were then ligated using T4 ligase from New England Biolabs.

2.5.4 Cloning of NAF1-mCherry mammalian expression vector
Plasmid encoding mCherry with a N-terminal xhoI restriction site and a C-terminal sacII restriction site was generated using mCherry-C1 (plasmid#2280) as the template with the forward primer 5’-AAAAACTCGAGATGGTGAGCAAGGGCGAGG-3’ and reverse primer 5’-TTCCGCGGCTACTTGTACAGCTCGTC-3’. The PCR product and NAF1-eGFP (plasmid#2332) were digested with xhoI and sacII restriction enzymes. The digestion products were visualized after electrophoresis using a 1% agarose gel. The linearized vector (~6 kbp) and mCherry (~700bp) were physically excised from the agarose gel and the two DNA fragments were isolated from the gel slices using a kit (Fermentas Gel extraction kit). Afterwards, the DNA fragments were ligated using T4 ligase from New England Biolab.

2.5.5 Cloning of BAP31-mCherry mammalian expression vector

Plasmid encoding NAF1-mCherry (plasmid#2349) and BAP31-eGFP (plasmid#2350) were digested with BglII and MluI restriction enzyme. The digestion products were visualized after electrophoresis using a 1% agarose gel. The dropout from the digestion of the plasmid encoding BAP31-eGFP (~750bp) and the linearized vector of -mCherry (~6 kbp) were physically excised from the agarose gel and the two DNA fragments were isolated from the gel slices using a kit (Fermentas Gel extraction kit). Afterwards, the DNA fragments were then ligated using T4 ligase from New England Biolab.

2.5.6 Cloning of mCherry-cb5 mammalian expression vector

Plasmid encoding mCherry with a N-terminal NotI restriction site and a C-terminal AflII restriction site was generated using mCherry-C1 (plasmid#2280) as
the template with the forward primer 5’- TATAGCGGCCCGCATGGTGAGCAAGG -3’ and reverse primer 5’- TATACTTAAGAGCTCGAGATCTGAGTCC -3’. The PCR product and the plasmid encoding pNTAP-Bcl2-cb5 (plasmid#2318) were digested with NotI-HF and AflII restriction enzymes. The digestion products were visualized after electrophoresis using a 1% agarose gel. The linearized vector (~4 kbp) and mCherry (~700bp) were physically excised from the agarose gel and the two DNA fragments were isolated from the gel slices using a kit (Fermentas Gel extraction kit). Afterwards, the DNA fragments were ligated using T4 ligase from New England Biolab.

2.6 Immunoprecipitation

100uL reaction containing liposomes (300 uM of lipids) in assay buffer (10mM HEPES (pH 7), 200mM KCl, 5mM MgCl2, 0.2mM EDTA) were prepared and incubated at 37 °C with 100nM of Bcl-xL, 100nM of Bax and 20nM of cBid for 2 hours. Immunoprecipitation of Bcl-xL was performed using the polyclonal Bcl-xL antibody “Fiver” in assay buffer containing either 2% CHAPS, 0.2% Non-ident P 40 or 6mM DHPC. 15uL of GammaBind™ G Sepharose™ resin were added to each reaction for 2 h after which all the reactions were centrifuged at 1000g for 5 minutes to pellet the resin. The supernatant was removed and the pellet was washed three times in assay buffer. 50uL of 2x Tricine loading buffer (TLB) was added to each reaction and 10ul of each samples were used for immunoblot analysis.

2.7 Immunofluorescence
1x10^6 MCF-7 cells were grown with αMEM supplemented with 10 % FBS on cover slips in a 6 well plate for 2 days. Cells were then stained with 500nM of mitotracker deep red for 30 minutes in αMEM supplemented with 10% FBS. Cells were then fixed for 30 minutes with 4% formaldehyde followed by permeabilization with 1% CHAPS in PBS for 15 minutes and incubation with rabbit anti-Bcl2 or mouse anti-calnexin (1:100) in PBS containing 3% BSA for 1 h in a 37°C degree incubator. The samples were washed with PBS with 0.02% tween-20 and secondary antibodies (FITC donkey anti-rabbit (1:30) and Alexa Donkey anti-mouse (1:30)) were added to the samples and incubated for 1 hour in a 37°C degree incubator. Cells were imaged using the SP5 confocal microscope.

2.8 Establishing Stable Cell Line expressing TAP-Bcl2-cb5

MCF-7 estrogen receptor positive breast cancer cells were transfected with TAP-Bcl2-cb5 encoding plasmid (plasmid#2318) using Fugene HD by Roche. The cells were then placed under G418 selection until island colonies were formed. Clones of MCF-7 cells constitutively expressing TAP-Bcl2-cb5 (determined by immunoblotting) were maintained in αMEM medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen).

2.9 Cell Viability assay

1x10^6 MCF-7 cells expressing different Bcl-2 constructs were grown in a 6 well plate for 10 days in charcoal stripped media. Cells were trypsinized with 1x trypsin and collected into a centrifuge tube. Equal volume of αMEM with 10% FBS were added to neutralize the trypsin. The samples were centrifuged to remove
excess supernatant. An equal volume of trypan blue was added to the cells and mixed. The number of viable (unstained) and dead (trypan blue stained) cells within the sample were counted using a hemocytometer.

2.10 Fluorescence Lifetime Measurements

1×10^6 MCF-7 cells were grown with αMEM supplemented with 10 % FBS on coverslips in a 6 well plate for one day. The cells were then transfected with mCherry constructs using Fugene HD. The cells were allowed to express the mCherry construct for 24 hours in a 37°C degree incubator. The coverslips with transfected cells were washed in PBS and placed in a chamber with 500ul of PBS. Steady-state fluorescence images were taken using a Leica TCS SP5 equipped with a 63 × 1.3NA PlanApo glycerol immersion objective. Images of Venus, mCherry and mitotracker deep red were acquired using 514 nm, 561 nm and 633 nm excitation, respectively, and emission was collected between 525-555 nm, 580-670 nm, 650-720 nm respectively. Fluorescence lifetimes were quantified using the Leica TCS SP5 confocal microscope equipped with an integrated SPC-830 Time-Correlated Single Photon Counting (TCSPC) system and Chameleon Ultra pulsed laser. The Ti:Sapphire laser was set to 960 nm which provides the optimal multiphoton-excitation for Venus, and the emission was acquired between 510-555nm. TCSPC-images (256 pixels x 256 pixels x 256 time bins) were recorded for 60 s using Becker and Hickl SPCM acquisition software with the photon count rate set to avoid photon pileup and the laser power set to minimize photobleaching during acquisition. The average
lifetime per pixel of the TCSPC-data was analyzed with the FLIM pixels binned to ensure a peak photon count > 100 and a total photon count > 1,000. The lifetime and photon count for each pixel were then exported for analysis in ImageJ. Lifetime histograms and intensity-weighted fluorescence lifetime images were generated using ImageJ.

2.11 Tandem Affinity Purification

Ten 100mm petri dish of MCF-7 cells stably expressing TAP-Bcl2-cb5 were grown to 80% confluency and pelleted by centrifugation at 3000g for 5 minutes. The pellet was resuspended in PBS and pelleted again to wash off any serum. The pellet was then resuspended in 3mL of nitrogen cavitation buffer (250mM Sucrose, 2mM MgCl₂, 20mM HEPES pH7.5 and 1mM EDTA) and lysed after incubation in nitrogen 150psi for 20 minutes by extrusion followed by dounce homogenization. 40uL of 0.5M EDTA and 7uL of 14.4 β- mercaptoethanol were added to the cell lysate and then the samples were centrifuged at 500g for 5 minutes at 4 degrees to pellet the nuclei, cell debris and unbroken cells. The supernatant was collected (~3mL) and then centrifuged at 120,000g for 1 hour at 4 degrees to pellet the heavy membranes. The heavy membranes were then solubilized with the lysis buffer [150mM NaCl, 25mM Tris-Cl pH 8.0, 6mM DHPC and ½ a protease inhibitor cocktail (complete mini-EDTA-free protease inhibitor) tablet] for at least 45 minutes in ice after which the samples were pelleted at 14,000g at 4 degrees for 15 minute to pellet and remove the insoluble fractions. The supernatant of the DHPC solublization was incubated with 500uL of streptavidin resin that was previously washed twice with
the streptavidin-binding buffer [150mM NaCl, 25mM Tris-Cl pH 8.0, 6mM DHPC, 2mM EDTA and 10mM β- mercaptoethanol]. After 45 minutes incubation at 4 degrees on a rotation shaker, the mixture was centrifuged at 1500g for 5 minutes and the supernatant was removed. The resin was washed twice with 500uL of streptavidin binding buffer before the bound protein was eluted with two 500uL volumes of streptavidin elution buffer [150mM NaCl, 25mM Tris-Cl pH 8.0, 6mM DHPC, 2mM EDTA, 10mM β- mercaptoethanol and 4mM of Biotin]. Magnesium acetate and CaCl₂ were added to the pooled elution to a final concentration of 1mM and 2mM respectively. The samples were incubated with 200uL of calmodulin resin for 45 minutes at 4 degrees. The sample was then centrifuged at 3000 g for 1 minute to pellet the resin and the supernatant was removed. The resin was washed twice with 300uL of calmodulin wash buffer [150mM NaCl, 25mM Tris-Cl pH 8.0, 6mM DHPC, 10mM β-mercaptoethanol, 1mM magnesium acetate, and 1mM imidazole]. The bound protein was then eluted 1mL of calmodulin elution buffer [150mM NaCl, 25mM Tris-Cl pH 8.0, 6mM DHPC, 10mM β- mercaptoethanol, 1mM magnesium acetate, 1mM imidazole and 20mM of EGTA].

2.12 Estrogen Deprivation treatment

MCF-7 expressing cells were plated onto a petri dish and incubated overnight in αMEM medium supplemented with 10% FBS to allow the cells to adhere to the plate. The cells were then washed three times with PBS to remove all of the estrogen and then grown for 10 days in αMEM medium supplemented with 10% FBS that had previously been treated with charcoal to strip the media of hormones.
2.13 Titration of DHPC

MCF-7 cells stably expressing TAP-Bcl2-cb5 were grown with αMEM supplemented with 10 % FBS in a 10 cm petri dish until 80% confluency. The cells were then scraped with a cell scraper and pelleted at 5000g for 15 minutes. The pellet was washed twice with PBS and then resuspended in lysis buffer (150mM NaCl, 25 mM Tris-HCl pH 7.2, 5X PIN) with indicated concentration of DHPC. The samples were left on ice for 45 minutes before being pelleted at 15000g for 15 minutes to separate the soluble and insoluble fractions.

2.14 Protein Quantification

The Bicinchoninic Acid assay was performed according to the manufacturer’s protocol to quantify the total protein in the MCF-7 cells lysates. Standard curves of protein concentration were generated using BSA for each individual experiment. Sample concentrations were determined using this curve.

2.15 % FLIM-FRET Efficiency Calculation

The ROIs containing mitochondria and endoplasmic reticulum were selected, and the average lifetime was measured using a customized ImageJ script. Cells were manually discarded from the analysis if the Venus-Bcl2-cb5 signal was too low (and therefore noisy) or the mCherry signal was close to saturated. The ratio of mCherry to Venus was calculated using the intensities of each channel. FLIM-FRET efficiency (E%) was calculated for each ROI as: E% = (1 - τ_i/τ_0)× 100%, where τ_i is the mean lifetime for that ROI, and τ_0 is the average lifetime of all ROIs not expressing detectable mCherry. FLIM FRET efficiency was then distributed into bins of the
same size according to mCherry:Venus ratio (bin size 0.25) and plotted (± se) against mCherry:Venus ratio. The binding curves were fit using GraphPad Prism version 5.0d for Macintosh (GraphPad Software, www.graphpad.com) with the function: 
\[ E\% = E_{max} \times (I_{mCherry} / I_{Venus})h / [K_d + (I_{mCherry} / I_{Venus})h] \]

- \( E_{max} \) is the maximum FLIM FRET efficiency corresponding to saturation of donor binding sites by an acceptor;
- \( I_{mCherry} \) and \( I_{Venus} \) are intensities of mCherry and Venus, respectively;
- \( K_d \) is the relative equilibrium dissociation constant;
- \( h \) is the Hill slope.

3 Results
3.1 Addition of the TAP tag to the amino-terminal of Bcl2-cb5 has negligible effect on its function and subcellular localization.

The protective effect of endoplasmic reticulum localized Bcl-2 against estrogen deprivation is a novel phenomenon that has yet been characterized. Understanding the mechanism and the characterization of Bcl-2 interaction at this subcellular compartment has the potential to be used to enhance breast cancer therapy. The goal of this thesis is to establish an estrogen receptor positive breast cancer cell line expressing Bcl2-cb5, which was either tagged or fused to a fluorescent protein. These addition made to Bcl2-cb5 will be used to investigate the interacting partners of Bcl-2 at the endoplasmic reticulum and characterize their interaction under estrogen deprivation treatment.

Bcl-2 is a tail-anchored protein that localizes to the membrane by inserting its C-terminal tail-anchor helix into the lipid bilayer of mitochondria, endoplasmic reticulum and nucleus. Previous studies have shown that Bcl-2 has different
functions at the mitochondria and at the endoplasmic reticulum (Zhu et al, 1996). Utilizing the endoplasmic reticulum membrane insertion sequence (cb5) of the endoplasmic reticulum specific isoform of cytochrome b5, foreign proteins can be targeted specifically to the endoplasmic reticulum (Pistor et al, 1994). When the endogenous transmembrane sequence of Bcl-2 was replaced with cb5, Bcl2-cb5 was shown to target exclusively to the endoplasmic reticulum (Zhu et al, 1996).

To identify Bcl-2 interacting partners at the endoplasmic reticulum, tandem affinity purification (TAP) was utilized. The Stratagene TAP vector system contains a calmodulin binding peptide (CBP) and a streptavidin binding peptide (SBP) (YiFeng, 2010). Bcl2-cb5 was ligated into an expression vector with a amino-terminal TAP tag to avoid the disruption of the C-terminus of Bcl2-cb5 that is important for the endoplasmic reticulum localization and insertion. MCF-7 estrogen receptor positive breast cancer cells were transfected with the TAP-Bcl2-cb5 expressing vector (plasmid #2318) and clones stably expressing TAP-Bcl2-cb5 (Figure 7) were selected for with G418.
Figure 7. Immunoblot against Bcl-2 in MCF-7 cells stably expressing Tap-Bcl2-cb5. MCF-7 cells were transfected with TAP-Bcl2-cb5 construct and selected for with G418 for 24 days. 19 colonies were picked and lysed. Cell lysates obtained were immunoblotted with Bcl-2 antibody “Stan” (top panel) and calmodulin binding peptide antibody “CBP”. 10μg of proteins were loaded in each lane. The Bcl-2 blots were stripped and then immunoblotted for Actin as a loading control.

To ensure that the addition of the TAP tag to the Bcl2-cb5 did not prevent it protecting MCF-7 cells from estrogen deprivation, cells stably expressing the TAP-Bcl2-cb5 were treated with 10% charcoal stripped fetal bovine serum (CSFBS) with αMEM for ten days. Trypan blue staining was used to analyze the percentage of cells that died as a result of the treatment. As shown in Figure 8, compared to the parental, CMV, Bcl2-ActA (Bcl-2 targeted to the mitochondria) and Bcl2-wt (wild-type Bcl-2) expressing cells, MCF-7 cells stably expressing TAP-Bcl2-cb5 displayed a lower percentage of dead cells under the treatment. However, it does show a 5% increase in the percentage of cell death when compared to cells expressing Bcl2-cb5. Therefore, the addition of the TAP tag to the amino-terminus of Bcl2-cb5 hinders
but does not diminish the protective function of Bcl2-cb5 at the endoplasmic reticulum under estrogen deprivation.

![Bar graph showing cell death percentages for different treatments.]

**Figure 8.** Addition of an amino-terminal tag does not affect the protective ability of Bcl2-cb5 treated with charcoal stripped bovine serum media. $1 \times 10^6$ cells were plated in a 6-well plate and grown in charcoal stripped bovine serum media for 10 days. The cells were then collected and stained with trypan blue. A Hemocytometer was used to count the number of dead and live cells. Error bars are the mean ± standard error of 3 independent experiments.

The subcellular localization of Bcl2-cb5 was also tested to ensure that the addition of the amino-terminal TAP tag did not alter its endoplasmic reticulum localization. Previous studies have shown that MCF-7 cells grown in estrogen free medium would result in the decrease level of endogenous Bcl-2 (Teixeira et al, 1995). To avoid interference of endogenous Bcl-2, MCF-7 cells stably expressing TAP-Bcl2-cb5 were grown on coverslips in 10% CSFBS with αMEM to deplete the level of endogenous Bcl-2 and stained with mitotracker deep red as a mitochondria
marker. After fixation with 4% formaldehyde, the cells were permeablized with 1% CHAPS and then probed against Bcl-2 (Stan) and calnexin antibodies. The cells were then analyzed by immunofluorescence using confocal microscopy (Figure 9).

**Figure 9. Expression and localization of Tap-Bcl2-cb5 in MCF7 cells.** MCF7 stably expressing Tap-bcl2-cb5 were stained with Mitotracker deep red, calnexin and Bcl2 antibody following 4 days growth in aMEM supplemented with CSFBS. Confocal images of the cell stained with Bcl2, Calnexin and mitotracker deep red are shown in the first, second and third column respectively.
The bottom row of figure 9 shows similar staining pattern between TAP-Bcl2-cb5 and the endoplasmic reticulum membrane integral protein calnexin. In contrast, the staining pattern of TAP-Bcl2-cb5 compared to the mitochondrial marker mitotracker deep red does not resemble the mitochondria. Therefore, the addition of the TAP tag does not disruption to the endoplasmic reticulum membrane targeting of Bcl2-cb5.

3.2 Identification of novel Bcl-2 interacting proteins at the endoplasmic reticulum using Tandem Affinity Purification

With the endoplasmic reticulum as the membrane of interest for Bcl-2 interaction, tandem affinity purification was performed using isolated heavy membranes consisting of the mitochondria and endoplasmic reticulum. The presence of mitochondria is desirable because a pull down with a TAP tagged Bcl-2 targeted to the mitochondria would eventually be needed to serve as a control for the estrogen deprivation treatment. MCF-7 cells stably expressing TAP-Bcl2-cb5 were pelleted and lysed using nitrogen cavitation combined with dounce homogenization in detergent-free hypotonic buffer. Cell lysate was centrifuged to separate the nuclear fraction from the lysate. The lysate was then centrifuged again to separate the cytosolic proteins and the heavy membranes.

To solubilize membrane bound proteins, the short-chain phospholipid 1,2-Diheptanoyl-sn-Glycero-3-phosphocholine (DHPC) was added to the buffer used to resuspend the heavy membrane. DHPC is a detergent featuring a lipid backbone that interacts with the lipid bilayer of the membrane and not with membrane proteins.
like traditional detergents (Hauser, 2000). DHPC exerts a wedge-like effect on the neighboring lipids as a result of its bulky polar groups and short hydrocarbon chains, allowing it to destabilize the membrane at a relatively low concentration. Therefore, DHPC would not displace intrinsic membrane lipids from integral membrane proteins, in principle, preserving the native protein structure during the solubilization step (Kessi et al, 1994). Titration was performed to elucidate the effective concentration at which DHPC would solubilize the membrane bound TAP-Bcl2-cb5. Figure 10 shows that 4mM DHPC solubilized the endoplasmic reticulum bound TAP-Bcl2-cb5 evident by the absence of a band in the pellet fraction. Henceforth, 4mM DHPC was added to the buffer to solubilize heavy membranes.
Figure 10. DHPC solubilizes TAP-Bcl2-cb5 at 4mM. MCF-7 cells stably expressing TAP-Bcl2-cb5 were grown to 80% confluency. Increasing concentrations of DHPC were added to the cells for 45 minutes on ice. Samples were centrifuged at 14,000 g for 15 minutes to separate the supernatant (S) and pellet (P) fractions. Samples were analyzed by SDS-PAGE and immunoblotted with (A) Bcl-2 antibody and (B) Calmodulin binding peptide (CBP) antibody. Cells were also lysed with 1% Triton X-100 as a positive control.

Heavy membranes were extracted using a DHPC containing buffer, and subjected to tandem affinity purification. Samples from each step of the purification were collected and resolved by SDS-PAGE in Figure 11A. No band from the CBP elution step was observed in the coomassie stained gel and even the TAP-Bcl2-cb5 was absent (~35kDa). To investigate whether the purification was successful in pulling down TAP-Bcl2-cb5, antibodies against Bcl-2 and CBP was used to immunoblot each step of the TAP purification. The top two panels of figure 11B
shows that TAP-Bcl2-cb5 is in fact being purified indicated by the presence of a band in the immunoblot against Bcl-2 and CBP.

Figure 11. Tandem Affinity purification of TAP-Bcl2-cb5 from MCF-7 cells. MCF-7 breast cancer cell stably expressing TAP-Bcl2-cb5 were lysed with a combination of nitrogen cavitation and dounce homogenization. Heavy membranes containing the mitochondria and the endoplasmic reticulum were isolated and solubilized with 4mM DHPC. The solubilized lysate was then used for the tandem affinity purification. (A) Coomassie of the Tandem Affinity purification of untreated cells resolved on SDS-PAGE gel. Immunoblot analysis of the tandem affinity purification of MCF7 cells in untreated conditions (B) and estrogen deprivation treatment (C) with antibody against Bcl-2 (stan), calmodulin binding peptide (CBP) and various other potential interacting partners of Bcl-2. Negative controls of the purification were immunoblotted against HSP60 (mitochondrial membrane protein), calreticulin (endoplasmic reticulum lumen protein) and Calnexin (endoplasmic reticulum integral membrane protein). MCF-7 cells stably expressing Bcl2-ActA were also subjected to the same treatment as a negative control. Dilution factor was calculated with respected to the total cell lysate. PSM units are at kDa.
<table>
<thead>
<tr>
<th>IB: Stan (Tap-Bcl2-cb5)</th>
<th>IB: CBP (Tap-Bcl2-cb5)</th>
<th>IB: Stan (Bcl2-ActA)</th>
<th>IB: HSP60</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB: Calnexin</td>
<td>IB: Calreticulin</td>
<td>IB: Bad</td>
<td>IB: Bag1</td>
</tr>
<tr>
<td>IB: Bak</td>
<td>IB: Bax</td>
<td>IB: BAP31</td>
<td>IB: Beclin1</td>
</tr>
<tr>
<td>IB: BIK</td>
<td>IB: BIM</td>
<td>IB: NAF1</td>
<td>IB: PUMA</td>
</tr>
<tr>
<td>IB: SERCA2</td>
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Although no band was detected in the coomassie gel, the presence of TAP-Bcl2-cb5 in the CBP elution step proves the purification was successful and that potential partners may be pulled down. Table 3 is a list of potential Bcl-2 interacting partners at the endoplasmic reticulum that previous studies had suggested to interact with Bcl-2. Antibodies against the proteins listed in Table 3 were obtained to immunoblot for their presence at the CBP elution. Bcl-2 and CBP immunoblot showed similar patterns with the protein being present at the CBP elution step. HSP60 (mitochondrial protein), Calnexin (integral endoplasmic reticulum membrane protein) and Calreticulin (endoplasmic reticulum lumen protein) served as negative controls that does not co-purify with TAP-Bcl2-cb5. It should be noted that there is the presence of a band in the DHPC pellet for Bcl-2, calnexin, calreticulin and HSP60 in the untreated conditions compared to the estrogen deprived conditions (figure 11B). This is most likely due to the fact that the heavy membranes were not completely solubilized. Calreticulin was present in the nuclear pellet under estrogen deprivation compared to the untreated condition that indicates the potential lost of endoplasmic reticulum membrane integrity during the nuclear pellet spin.

Presence of Bad, Bag1, Bak, Bax, BAP31, Beclin1, BID, BIK, NAF1, PUMA and SERCA2 were not detected in both the untreated and treated conditions at the final CBP elution step (figure 11B,C) Bad, BAP31, BID and PUMA were no longer present in the DHPC sol. supernatant under estrogen deprivation compared to the untreated controls (figure 11B,C). BIM was the only candidate that was pulled down with TAP-
Bcl2-cb5 under untreated and treated conditions (Figure 11B,C). It is noted that the presence of BIMS (the smallest band) was detected in the purification process under estrogen deprivation when compared to the untreated condition. However, it did not co-purify with TAP-Bcl2-cb5 (figure 11B,C).

<table>
<thead>
<tr>
<th>Bcl-2 interacting protein on the endoplasmic reticulum</th>
<th>Proposed Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bad</td>
<td>Inhibit anti-apoptotic protein such as Bcl-2 or Bcl-xl, promoting apoptosis</td>
<td>Yang et al, 1995</td>
</tr>
<tr>
<td>Bag1</td>
<td>Associates with Bcl-2 and inhibits cell death</td>
<td>Takayama et al, 1995</td>
</tr>
<tr>
<td>Bak, Bax</td>
<td>Promotes Ca²⁺ release at the endoplasmic reticulum and cytochrome c release at the mitochondria, can be inhibited by Bcl-2</td>
<td>Szegedi et al, 2009; Scorrano et al, 2003</td>
</tr>
<tr>
<td>BAP31</td>
<td>Induce mitochondrial cristae remodeling, leading to fission from endoplasmic reticulum Ca²⁺ signal</td>
<td>Breckenridge et al, 2003</td>
</tr>
<tr>
<td>Beclin1</td>
<td>Required for the formation of autophagosome</td>
<td>Sinha et al, 2008</td>
</tr>
<tr>
<td>BID</td>
<td>Cleavage form induces cytochrome c release at the mitochondria</td>
<td>Gross et al, 1999</td>
</tr>
<tr>
<td>BIK</td>
<td>Promotes the release of endoplasmic reticulum Ca²⁺</td>
<td>Mathai et al, 2005</td>
</tr>
<tr>
<td>BIM</td>
<td>Promotes apoptosis by antagonizing Bcl-2 and Bcl-xL</td>
<td>O’Connor et al, 1998</td>
</tr>
<tr>
<td>NAF1</td>
<td>Mediates interaction between Bcl-2 and Beclin1 to inhibit autophagy</td>
<td>Chang et al, 2010</td>
</tr>
<tr>
<td>PUMA</td>
<td>Promotes apoptosis by binding to Bcl-2 or Bcl-xL at the mitochondria</td>
<td>Yu et al, 2001</td>
</tr>
<tr>
<td>SERCA2</td>
<td>Pumps cytosolic Ca²⁺ into the endoplasmic reticulum</td>
<td>Kuo et al, 1998</td>
</tr>
</tbody>
</table>

To examine the changes in the binding profile of Bcl-2 at the endoplasmic reticulum under estrogen deprivation, MCF-7 cells stably expressing TAP-Bcl2-cb5
were treated with 10% CSFBS in αMEM for 10 days before being subject to TAP purification. A similar interaction profile was observed in Figure 11c where BIM is the only candidate that co-eluted with TAP-Bcl2-cb5.

3.3 DHPC activates Bcl-2 family proteins

Presence of detergent has previously been shown to create artificial interactions in the studies of Bcl-2 family proteins (Oltvai et al, 1993). Hsu et al demonstrated that the presence of a non-ionic detergent could cause dimerization of Bcl-2 proteins (Hsu et al, 1997). However, detergents are essential in the isolation of integral membrane proteins from membranes. Thus, to ensure the validity of the results of the TAP purification, the effect of DHPC on Bcl-2 family proteins was assessed.

The presence of a mitochondria-like membrane has been shown to be imperative for Bcl-2 protein function (Billen et al, 2008). It is required for the conformational changes that the Bcl-2 family proteins undergo to effectively perform life and death functions within a cell (Leber et al, 2008). Bcl-xL is an anti-apoptotic protein similar in size and structure to Bcl-2 (Boise et al, 1993). While it is normally cytoplasmic or loosely bound to membranes in live cells (Hsu et al, 1997; Kaufmann et al, 2003; Billen et al, 2008), under apoptotic stimuli, cBid can cause the insertion of Bcl-xL into the membrane (Billen et al, 2008). Due to the fact that native wild type Bcl-2 without modifications has not been successfully purified to date, Bcl-xL is a suitable candidate to test whether the presence DHPC has an undesirable effect to the function of Bcl-2 family proteins.
Bcl-xL, Bax and cBid were incubated in the presence or absence of mitochondria-like liposome as indicated in figure 12. Co-immunoprecipitation was performed using antibody against Bcl-xL to identify if detergent mediated interactions between Bcl-xL and Bax occurred in the presence of DHPC. Figure 12A (lane 2) shows that Bcl-xL co-precipitated with Bax in the absence of the activator protein cBid when DHPC was present. However, the co-immunoprecipitation of Bax and Bcl-xL is diminished in the absence of a membrane as shown in figure 11B (lane 2) based on the intensity of the band between DHPC and the no detergent panel. As stated previously, the presence of a membrane is essential for the function of cBid and its interaction with Bcl-xL and Bax. Faint bands can be observed in figure 12C (lane 1) where cBid co-precipitated with Bcl-xL. The poor intensity of the cBid band is most likely due to the poor quality of the antibody used along with the low amount of cBid within the experiment. Figure 12D (lane 1) shows that the lack of a membrane resulted in the lack of an interaction between Bcl-xL and cBid.

DHPC will cause effects similar to the nonionic detergent NP-40 that was proven to induce a conformational change in Bax required for heterodimerization with Bcl-xL in cells undergoing apoptosis (Hsu et al, 1997; Hsu et al, 1998). However, this artificial activation of Bcl-2 protein appears to be limited to the presence of a lipid membrane.
Figure 12. DHPC causes artificial interaction between Bcl-xL and Bax. 100nM of Bax, Bcl-xL and 20nM of cBid were incubated with (A and C) or without (B and D) liposomes. Samples were immunoprecipitated (IP) in either 2% CHAPS, 0.2% NP-40 or 4mM DHPC, as indicated with specific antibodies and immunoblotted for the indicated protein. Bax immunoprecipitated with Bcl-xL in the presence of DHPC in the presence or absence of mito-like liposome.
3.4 Establishment of MCF-7 cells expressing Venus-Bcl2-cb5 protein

Studies on the interaction of Bcl-2 at the endoplasmic reticulum cannot be accurately assayed by coprecipitation due to the detergent mediated artifacts on Bcl-2 family proteins (figure 12). Hence, FRET FLIM was utilized to characterize interaction between Bcl2-cb5 and its known interacting partners, as this method does not rely on the presence of detergents.

To characterize Bcl2-cb5 and its interactions in live cells, plasmids were constructed to express the protein of interest fused to a fluorescent protein. A MCF-7 breast cancer cell line stably expressing Bcl2-cb5 fused to the C-terminus of Venus was generated (Figure 13). Unlike the wild type version of Bcl-2, Venus-Bcl2-cb5 did not colocalize with the mitochondria marker Mitotracker Deep Red as expected (Figure 14).
Figure 13. Immunoblot against Bcl-2 in MCF7 cells stably expressing Venus-Bcl2 with various c-terminal mutants. MCF7 cells were transfected with Venus-Bcl2 construct with various c-terminal mutants and selected for with G418 for 24 days. Cells were sorted according to Venus expression using Coulter EPICS ALTRA™ Flow cytometer. Cell were lysed with 1% Triton X-100 and the lysis obtained were immunoblotted with Bcl2 antibody “Stan”. 10ug of proteins were loaded in each lane with Actin present as a loading control.

To ascertain that the addition of the amino-terminal fluorescent protein does not disrupt the protective function of Bcl2-cb5 when cells are treated with estrogen deprivation, cells stably expressing Venus-Bcl2-cb5 were treated with 10% charcoal stripped fetal bovine serum (CSFBS) with αMEM for ten days. Trypan blue staining was used to analyze the percentage of cells that died as a result of the treatment. As show in figure 8, compared to the parental, CMV, Bcl2-ActA and Bcl2-wt expressing cell, MCF-7 cells stably expressing Venus-Bcl2-cb5 has a lower percentage of dead cells under the treatment. Therefore, the addition of Venus to the amino-terminus of
Bcl2-cb5 does not interfere with protective function of Bcl2-cb5 at the endoplasmic reticulum under estrogen deprivation treatment.

Figure 14: Subcellular localization of Venus-Bcl2 construct containing either mitochondrion or ER-targeting sequence. MCF7 breast cancer cells stably expressing Venus-Bcl2-ActA (mito) or Venus-Bcl2-cb5 (Endoplasmic reticulum) were plated onto cover slips at 1x10^6 cells per well in a 6 well plate. The cells were then incubated with 500nM of mitotracker deep red for 30 minutes before imaging. Similar staining pattern can be observed between Venus-Bcl2-ActA and mitotracker while no pattern could be observed for Venus-Bcl2-cb5.
3.5 Quantification of binding between Bcl-2 interacting proteins and Bcl2-cb5 by FLIM FRET

To examine the binding of 4 of the best characterized potential interacting partners of Bcl2-cb5 at the endoplasmic reticulum; FLIM FRET was measured for Venus-Bcl2-cb5 with mCherry-Bad, mCherry-BIK, mCherry-Beclin1 and NAF1-mCherry (Figure 15). In our application of FLIM FRET, the fluorescence lifetime of the Venus fluorescence protein fused to the amino-terminus of Bcl2-cb5 changes when the Bcl-2 domain binds to an interacting partner fused to a fluorescence protein such as mCherry that functions as an acceptor for FRET with Venus. This change in lifetime can be used to measure FRET in live cells. The change in the donor lifetime compared to controls with no interaction can be used to measure protein-protein interactions in live cells more robustly than other methods proposed for measuring FRET because FLIM is not affected by spectral bleed-through or change in excitation intensity (Zacharias et al, 2002). The fluorophore concentration plays a critical role in the measurement of FLIM FRET. As the acceptor concentration increases, lifetime changes due to random collision also increases. To distinguish random and authentic interactions, binding curves can be generated to characterize the relationship between FRET FLM efficiency and the acceptor to donor ratio. Random collision will generate a linear relationship in the binding curve along with low FLIM FRET efficiency. For authentic interaction, saturation of the binding curve would be observed at a low acceptor to donor ratio along with high FLIM FRET efficiency.
The FLIM FRET efficiency is the degree of FRET for the donor and acceptor pair and is a measure of the proportion of Venus-Bcl2-cb5 bound to mCherry-Bad/mCherry-BIK/ mCherry-Beclin1/ NAF1-mCherry in that pixel or region of interest in the image (Chen et al, 2003; Periasamy et al, 2005). With a wide range of relative expression levels of the mCherry fusion in different transiently transfected cells, FLIM FRET data together with an estimate of the expression ratio for the two proteins (estimated from steady state intensity images) can be used to plot binding curves for these interacting proteins (Aranovich et al, 2012).

B-cell associated death promoter (Bad) is a 22.1kDa pro-apoptotic BH3-only protein that is cytosolic as it lacks a hydrophobic tail-anchor (Danial, 2009). In response to a pro-apoptotic signal, Bad binds to both Bcl-2 and Bcl-xL to neutralize their anti-apoptotic effects on Bax, thus allowing the permeabilization of the mitochondrial membrane (Danial, 2009; Fernando et al, 2001). Previous work in our lab has shown that the addition of mCherry to the amino-terminus of Bad did not affect its function (Aranovich et al, 2012). The expression of mCherry-Bad in Venus-Bcl2-cb5 expressing cells resulted in a shift in the fluorescence lifetime of Venus-Bcl2-cb5 from 2.8 ns to 2.5 ns (Figure 15). Utilizing images from the same experiment, the average FLIM FRET efficiency was calculated to be 10% for these cells.

Bcl2-interacting killer (BIK) was the first protein identified where cell death activity was linked to the BH3 domain (Boyd et al, 1995); hence it is the first member of the BH3-only pro-apoptotic proteins. At 18kDa, BIK contains a BH3
domain and a TM domain typical of the Bcl-2 family of proteins (Chinnadurai et al, 2009). It is predominately localized to the endoplasmic reticulum where under apoptotic signals BIK interacts with Bcl-2 and Bcl-xL to antagonize their anti-apoptotic abilities, allowing the permeabilization of the endoplasmic reticulum leading to the release of Ca^{2+} into the cytosol (Mathai et al, 2005; Levine et al, 2008). Expression of mCherry-BIK caused a reduction in Venus-Bcl-2-cb5 lifetime from 2.7 ns to 2.5 ns (Figure 15). The average FLIM FRET efficiency in that experiment was 7.4%.

Beclin1 is a 52kDa BH3-only protein essential for autophagy at the endoplasmic reticulum (Chen et al, 2011; Levine et al, 2008). It forms a complex with class III phosphatidylinositol-3-kinase responsible for the formation of the autophagosome that is critical for the initiation of autophagy (Levine et al, 2008). The expression of mCherry-Beclin1 in Venus-Bcl2-cb5 expressing cells resulted in shorter Venus-Bcl2-cb5 fluorescent lifetime from 2.8 ns to 2.4 ns (Figure 15). The average FLIM FRET efficiency was calculated to be 14% for these cells.

NAF-1 is a recently discovered Bcl-2-interacting protein at the endoplasmic reticulum. At 15kDa, it is a small bitopic integral membrane protein whose deregulation had previously been implicated in the neurodegenerative disorder Wolfram syndrome 2. Recently, Chang et al have shown that the presence of NAF-1 is necessary for Bcl-2 at the endoplasmic reticulum to antagonize Beclin1 mediated autophagy in lung cancer cells undergoing nutrient deprivation (Chang et al, 2010). In figure 15, the expression of NAF1-mcherry resulted in a shift in the fluorescent
lifetime of Venus-bcl2-cb5 from 2.7 ns to 2.5 ns. The FLIM FRET efficiency was determined to be 7.4% for these cells.

Binding curves of mCherry-Bad, mCherry-BIK, mCherry-Beclin1 and NAF1-mCherry to Venus-Bcl2-cb5 were generated using the FLIM FRET efficiency (E%) and the ratio of the intensities of the acceptor to the donor (R). These data are plotted as E% vs R in figure 16. Since bound fractions are being measured when generating the binding curve, FLIM FRET efficiency will be dependent on the acceptor concentration as increasing concentration of acceptor will increasing amount of interaction due to random collision. Interaction due to random collision can be characterized by having low FLIM FRET efficiency and a dependent relationship where increasing mCherry/Venus ratio will increase the FLIM FRET efficiency. The cytosolic control mCherry and the mitochondrial control mCherry-ActA displayed binding curves that shows interaction due to random collision. The binding of mCherry-Bad, mCherry-BIK, mCherry-Beclin1 and NAF1-mCherry to Venus-Bcl2-cb5 generated typical binding curves where saturation can be observed in cells expressing levels of the mCherry fusion protein when R > 1, suggesting a binding equilibrium (figure 16C,D, E,F).
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<th>mCherry</th>
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<td>Intensity weighted FLIM</td>
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Figure 15. FLIM-FRET Measurements of the binding of mCherry constructs to venus-Bcl2-cb5 in Live cells. Intensity images of Venus-Bcl2-cb5 (top panel), mCherry (second panel from the top), mitotracker (third panel from the top) and intensity-weighted FLIM image of Venus-Bcl2-cb5 (fourth panel from the top). Lifetime histograms of Venus-Bcl2-cb5 are presented in a continuous pseudo-color scale ranging from 1.9 – 3.0ns in the bottom panel. Occurrence of FRET is indicated by the presence of the red color in the intensity weighted image and the shift in the peak of the lifetime histogram when compared to Venus-Bcl2-cb5 transfected with mCherry. FLIM FRET measurements were made in live MCF-7 breast cancer cells stably expressing Venus-Bcl2- cb5 and transiently expressing mCherry, mCherry-ActA, mCherry-Bad, mCherry-BIK, mCherry-Beclin1 and NAF1-mCherry. The changes in lifetime can be observed by the appearance of a second peak at a lower lifetime in mCherry-Bad, mCherry-BIK, mCherry-Beclin1 and NAF1-mCherry.
**Figure 16. Binding curves of Venus-Bcl2-cb5 and mCherry fusion proteins.** The binding of (A) mCherry, (B) mCherry-ActA, (C) mCherry-Bad, (F) mCherry-BIK, (E) mCherry-Beclin1 and (F) NAF1-mCherry to Venus-Bcl2-cb5. The error bar indicates standard error of 3 independent experiments. mCherry displayed a linear relation with the mCherry/Venus ratio while plateau can be observed for the other mCherry fusion proteins.
4 Discussion and conclusion

4.1 Robustness of the TAP purification

One of the aims of this thesis validate the use of tandem affinity purification and FLIM FRET to identify novel Bcl-2 interacting partners at the endoplasmic reticulum under estrogen deprivation treatment. With sensitivity to estrogen as an important criterion, MCF-7 estrogen receptor positive breast cancer cells were specifically chosen to be the model system. The effects of estrogen on Bcl-2 expression have been well characterized in this adenocarcinoma. The presence of estrogen promotes the expression of Bcl-2 (Teixeria et al, 1995) while estrogen deprivation will result in depletion of endogenous Bcl-2 (Fiebig, 2003). This is an important characteristic in the TAP purification; the depletion of endogenous Bcl-2 will eliminate the competition of endogenous Bcl-2 with bcl-2 interacting partners under the estrogen deprivation treatment. The absence of endogenous Bcl-2 can be observed in figure 9, only endoplasmic reticulum localized TAP-Bcl2-cb5 was observed in the immunofluorescence.

Unfortunately, the elimination of a competitor in binding for Bcl-2 interacting partners did not suffice in yielding a band that could be resolved in the coomassie gel (Figure 11). The absence of a band made identification of unknown Bcl-2 interacting partners difficult, as this is required for mass spectrometry. Experiments were performed to test the functionality of this purification using antibodies against Bcl-2 and CBP. The two blots have similar bands at the same size (35kDa), indicating that TAP-Bcl2-cb5 was precipitated with no size alteration.
Thus, the presence of the Bcl-2 and CBP epitope confirms that it is likely the full-length fusion protein (Figure 11B and C).

Many proteins that were reported to interact with Bcl-2 at the endoplasmic reticulum were immunoblotted with their respective antibody in figures 11B and C. BIM was the only protein that co-purified with TAP-Bcl2-cb5 in the tandem affinity purification. This result is puzzling as known binding partners such as Beclin1 or NAF-1 that previously were shown to bind to Bcl-2 at the endoplasmic reticulum were absent (Chang et al, 2010). This could be a temporal effect of the purification protocol; the samples could simply be in infinite dilution for too long. Bcl2-cb5 complexes that were once present may have been transient or too weak to last the duration of the purification protocol.

BIMEL and BIML were shown in figure 11B and 11C to be the only proteins that co-precipitated with TAP-Bcl2-cb5 from the TAP purification. This result was unexpected since BIM has only been observed interacting with Bcl-2 family members at the mitochondria (O’Connor et al, 1998). Bcl-2-interacting mediator of cell death (BIM) is a pro-apoptotic protein that belongs to the BH3 only group of Bcl-2 family of proteins. BIM was shown to induce apoptosis by sequestering anti-apoptotic Bcl-2 family members (O’Connor et al, 1998). There are three major isoforms of BIM generated by alternative splicing: BIMEL, BIML and BIMS. BIMEL and BIML are normally sequestered to the dynein motor complex through a direct interaction with the dynein light chain (Puthalakath et al, 1999). Under environmental stress, JNK will mediate the phosphorylation of BIM, which releases
BIM from the sequestration of the dynein motor complex. This allows BIM to initiate the mitochondrial apoptotic pathway by sequestering anti-apoptotic Bcl-2 family members to promote apoptosis (Lei and Davis, 2003).

The observation of BIM interacting with Bcl-2 at the endoplasmic reticulum raised the possibility of a role for BIM in regulating Bcl-2 at this subcellular compartment. The nature of the interaction between BIM and Bcl-2 can be assessed by FRET-FLIM using Venus-Bcl2-cb5 and mCherry-BIM in similar fashion to Aranovich et al. Such a method can be used to address questions of whether BIM and Bcl-2 can bind to one another at the endoplasmic reticulum and whether this interaction can be broken by ABT-737 (Aranovich et al, 2012). However, there is a pressing need to validate the authenticity of the interaction between Bcl2-cb5 and BIM observed in the TAP purification. Immunoprecipitation needs to be conducted to investigate the behavior of Bcl-xL with purified BIM in the presence of DHPC. This interaction could be simply dismissed as an artifact of the detergent as figure 12 has shown that DHPC can result in the artificial interaction in bcl-2 family proteins in the presence of a membrane. This finding should be approached with conscious optimism until we can verify its legitimacy.

The most important obstacle that must be overcome in the tandem affinity purification is to optimize the fractionation process, particularly at the separation of the nucleus. Figure 11C shows known Bcl-2 interacting protein at the endoplasmic reticulum are lost in the nuclear pellet along with the nuclei and unbroken cells.

4.2 Effects of Detergent in TAP purification
Detergents are defined as water-soluble surfactants with higher solubility in water than most lipids. The amphiphathic properties of detergents allows them to reduce the surface tension of a liquid when dissolved in it, which is essential for their role in disrupting the membrane. Purification of membrane protein often requires the presence of a detergent to solubilize the membrane and prevent aggregation of membrane protein as it is removed from its lipid environment (Luckey, 2008, p.42-50).

Most detergents rely on the formation of micelles for their action. Micelles are uneven spherical assemblies of detergent where most of the non-polar tails are sequestered from the aqueous environment in a disorganized hydrophobic interior (Luckey, 2008, p.42-50). The self-assembly of detergent into micelles is strongly cooperative and occurs at a defined concentration called the critical micellar concentration (CMC) (Luckey, 2008, p.42-50). When detergents are added to the membrane at a concentration below the CMC, detergent monomers would partition into the bilayer. When the concentration of detergent is at the CMC, dissociation of the bilayer occurs and lipid-protein-detergent micelles are formed. Addition of more detergent beyond the CMC will generate heterogeneous complex of detergent, lipid and proteins. Increasing concentration of detergent beyond the CMC will result in the progressive delipidation of lipid-protein-detergent mixed micelle, where no protein solublization occurs and increases risk in the denaturation of the proteins.

The detergent DHPC was initially chosen for its ability to preserve the activity of solubilized membrane proteins and its minimal interaction with the
integral membrane protein (Kessi et al., 1994). Due to its inability to displace intrinsic membrane lipid from integral membrane protein, DHPC was considered to be the most promising detergent with minimal side effects to the solubilized membrane protein complexes.

DHPC does not appear to be the stimulus that caused the activation of Bcl-2 family protein as shown figure 12B. It is only in the presence of a lipid membrane that Bax co-precipitate with Bcl-xL without the presence of cBid (figure 12A). Further optimization of DHPC must be performed to determine the minimal amount of lipid that Bcl-2 family protein can tolerate before it gets activated. This may prove to be a challenging problem because high detergent concentration will eventually cause the activity of the protein to decline. Should DHPC proves to be unsuitable for the TAP purification, CHAPS should be tested as it had been utilized by Vento et al using TAP-Bcl-xL to identify novel numerous interacting partners in fashion similar to the work in this thesis (Vento et al., 2010).

4.3 Expression profile of Bcl-2 interacting proteins under estrogen deprivation

The effects of estrogen on the expression of Bcl-2 have been well documented by Perillo et al. The presence of estrogen up-regulates the expression of Bcl-2 via the two estrogen-responsive elements that are located upstream of Bcl-2 translation start site (Perillo et al., 2000). The up-regulation of Bcl-2 was shown to prevent apoptosis in MCF-7 cells (Perillo et al., 2000). Concurrently, the expression of several other Bcl-2 family members such as Bad, BIM, Bak, Bcl-x and Beclin1 has
been implicated in experiments where hormones were removed (Hur et al, 2003; Perillo et al, 2000, Wirawan et al, 2010).

The expression of BIK was absent in the untreated condition (Figure 11B) when compared to treatment under estrogen deprivation (Figure 11C). Although this observation is not conclusive being that the data set is between two separate blots, it is consistent with the findings of Hur et al where the induction of BIK is induced in estrogen starved breast cancer cells (Hur et al, 2003). It would be beneficial to characterize the expression profile of potential Bcl-2 interacting proteins under estrogen deprivation treatment to narrow down the list of potential Bcl-2 interacting protein from the TAP purification.

### 4.4 Characterization of Bcl2 interacting protein at the endoplasmic reticulum using FLIM FRET

With the potential to general artifacts from TAP purification as a result of the detergent, FLIM FRET was used as an alternate method to measure interactions between proteins in MCF-7 cells. The advantage of using FLIM to measure FRET stems from the fact that Bcl-2 interaction will be measured in real time within live cells. It is impossible to have precise control over the expression of the transient transfection of mCherry fusion proteins. However, the measurements made only account for FLIM allows the measurement of FRET to be accurately measured without knowledge of the mCherry:Venus ratio in every pixel of the image. A disadvantage of this technique would be the need to over-express exogenous proteins in the cells, which must be taken into consideration for the interpretation
of the data. Thus, binding curves were required to testify whether the observed change in lifetime is an artifact of over-expression or a legitimate interaction based on the relationship between the mCherry/Venus ratio and the percent FLIM FRET efficiency (Figure 16).

Binding curves are generated by measuring Venus-Bcl2-cb5 with its acceptor partners that are in the endoplasmic reticulum membrane, thus we are effectively measuring FLIM FRET efficiency of a bound fraction in relation to a measured acceptor to donor ratio. When the acceptor does not interact with the donor, a linear relationship can be observed with relatively low FLIM FRET efficiency. This relationship is due random collision that occurs between donor and acceptor within the cell. At a high enough acceptor to donor ratio, the binding curve would plateau between non-interacting partners due to the over saturation of the acceptor within the cell. When an acceptor interacts with a donor, a typical binding curve can be observed where it plateaus at a high FLIM FRET efficiency with a relatively low acceptor to donor ratio.

FLIM FRET was used to characterize interactions between Bcl2-cb5 and four different known Bcl-2 interacting partners. As seen in figure 15, all four proteins interact with Bcl2-cb5 at the endoplasmic reticulum indicated by the decrease in the lifetime of Venus. While BIK, Beclin1 and NAF1 were previously shown to interact with Bcl-2 at this subcellular compartment (Mathai et al, 2005; Levine et al, 2008; Chang et al, 2010), Bad had never been implicated to interact with Bcl-2 at this membrane. The binding curve of mCherry-Bad to Venus-Bcl2-cb5 in MCF-7
generated a binding curve that saturated in cells expressing relatively high level of mCherry-Bad with relatively low FLIM FRET efficiency. It is questionable whether this interaction is real with its low FRET FLIM efficiency; however, this low FRET FLIM efficiency is consistent with the observation made by Aranovich et al when the same experiment was conducted with mCherry-Bad and Venus-Bcl-2 with the wild type transmembrane region (Aranovich et al, 2012). Hence, it is very likely that the observed interaction at the endoplasmic reticulum is legitimate. Colocalizations need to be performed to further authenticate this interaction.

It has been shown that Bad promote cell death by displacing Bax from binding to Bcl-2 or Bcl-xL (Yang et al, 1995; Zha et al, 1996). The apoptotic activity of Bad can be inhibited by a survival factor such as IL-3 that can activate an intracellular signaling pathway to phosphorylate Bad at Ser112 and Ser136 (Zha et al, 1996). Phosphorylation at Ser112 and Ser136 will cause Bad to bind to 14-3-3 proteins, thus preventing Bad from interacting with Bcl-2 or Bcl-xL. As a cytosolic protein, it is not out of the realm of possibility that Bad can be transported to the endoplasmic reticulum to interact with Bcl2-cb5. However, the observation made in figure 15 could be the result of an artifact of FLIM FRET as mCherry-Bad is overexpressed.

With the endoplasmic reticulum membrane as the bilayer of interest in my experiments, an endoplasmic reticulum control must be developed. Since most proteins of interest (BIK, Beclin1 and NAF1) in the FLIM FRET study all have endoplasmic reticulum localization, the over-expression of Venus-Bcl2-cb5 and
mCherry fusion proteins could result in clustering of these exogenous proteins at this subcellular compartment. The number of random collisions between them would also be expected to increase because the compartmental concentration of these proteins is high and the rate of diffusion is low compared to cytosolic diffusion of Bad. It is imperative that a mCherry fusion protein with an endoplasmic reticulum targeting sequence be developed to ensure that the decrease in lifetime observed for the endoplasmic reticulum localized proteins are not a spatial artifact. The cloning of mCherry fused with an endoplasmic reticulum localization signal “cb5” has been started but further work is required to characterize this endoplasmic reticulum control.

B-cell receptor-association protein 31 (BAP31) is a transmembrane protein associated with the endoplasmic reticulum and the intermediate compartment between endoplasmic reticulum and the Golgi (Adachi et al, 1996; Ng et al, 1997). BAP31 has been implicated in protein trafficking and apoptosis (Adachi et al, 1996; Ng et al, 1997). Ng et al showed that BAP31 is cleaved by caspase 8 at the carboxyl-terminal site that contains two identical caspase recognition sites. The amino terminus of the cleaved BAP31 fragments can direct apoptotic signals between the endoplasmic reticulum and the mitochondria, which can be inhibited by Bcl-2 or Bcl-xL (Breckenridge et al, 2003).

To examine binding of BAP31 to Bcl2-cb5 a BAP31-mCherry construct was created and preliminary FLIM FRET was performed to characterize its interaction with Venus-Bcl2-cb5. Although there appears to be a decrease in the lifetime,
indicating the presence of an interaction, the subcellular localization of the BAP31-mCherry does not appear to be endoplasmic reticulum or mitochondria based on visual observation which is puzzling (unpublished data). Further investigation must be performed to characterize the subcellular localization of BAP31-mCherry.

Bodipy Thapsigargin is a green fluorescent molecular probe with the spectral properties similar to fluorescein. Thapsigargin is a naturally occurring compound isolated from the plant Thapsia garganica. It is a tumor promoter that causes an increase in intracellular Ca^{2+} by inhibiting the endoplasmic reticulum Ca^{2+} ATPase (Serca2). This molecular probe could be used to investigate distribution of mCherry fused proteins under treatment condition similar to the way mitotracker red was utilized by Aranovich et al to examine the distribution behavior of Bcl-xL at the mitochondria under various conditions (Aranovich et al, 2012).

Preliminary work on the titration of Bodipy Thapsigargin was conducted to identify a concentration that would not be lethal to the cells for imaging. In figure 17, the nuclear membrane appears to be disrupted at bodiopy thapsigargin concentration of 50nM, 75nM and 100nM. At 10nM, the intensity of the probe is too weak to be detected. 25nM appears to be the optimal concentration of bodipy thapsigargin for this experiment as the nuclear membrane is disturbed while the signals were still visibly detected.
Figure 17. 25mM of Bodipy Thapsigargin is optimal for Imaging. MCF7 cells were plated on cover slips in a 6-well plate at 1x10^6 cells per well and grown over night. The cells were then incubated with various concentrations of Bodipy Thapsigargin Green for 30 minutes before imaging.

4.5 Expansion of FLIM FRET into mitochondria

Pister et al have shown that when the amino terminal sequence of Listeria monocytogenes actin nucleation protein (ActA) was deleted, the protein bound to the outer mitochondria membrane (Pistor et al, 1994). Taking advantage of this knowledge, the carboxyl-terminal transmembrane domain of ActA was used to replace the endogenous transmembrane sequence of Bcl-2 by Zhu et al to target Bcl-2 specifically to the mitochondria to study its function (Zhu et al, 1996).

Venus-Bcl2-ActA was used as a negative control to examine binding of mCherry fused to potential Bcl-2 interacting partners (Bad, BIK, Beclin1 and NAF1). Preliminary work suggested that Venus-Bcl2-ActA could surprisingly interact with mCherry-BIK. Normally endoplasmic reticulum bounded, BIK appears to interact with Bcl-2 at the mitochondria under untreated conditions. This finding is very exciting as Bik has never been shown to interact with Bcl-2 at the mitochondria, hinting at the possibility that either BIK or Bcl-2 can be relocated from the
mitochondria to the endoplasmic reticulum or vice versa. However, this observation should be cautiously evaluated, as the localization of Venus-Bcl2-ActA appears to be questionable in some cells. The venus-Bcl2-ActA construct must be carefully examined and MCF-7 cells with stable expression of Venus-Bcl2-ActA should be reestablished to verify this curious finding.

4.6 Characterizing Beclin1 with FLIM-FRET

Recent work by Wirawan et al in Belgium have shown that in response to the withdrawal of the growth factor interleukin-3 (IL-3), Beclin1 is cleaved by caspase-3 into two separate fragments (Wirawan et al, 2010). The amino-terminal fragment of the cleaved Beclin1 was shown to localize predominately at the nucleus while the C-terminal of the cleaved Beclin1 was shown to localize at the mitochondria (Wirawan et al 2010; Djavaheri-Mergny et al, 2010). The cleavage of Beclin1 resulted in the loss of Beclin1 induced autophagy and promoted apoptosis (Cho et al, 2009; Li et al, 2011; Wirawan et al, 2010; Zhu et al, 2010). Therefore, in my experiments it is possible that Beclin1 interacts with Bcl-2 at the mitochondria was not observed due the mCherry being at the amino-terminal and therefore in the amino-terminal fragment after caspase cleavage. However, if Beclin1 were cleaved by caspase-3, mCherry would be observed in the nucleus in Figure 15 as the amino-terminus should translocate to the nucleus as suggested by Wirawan et al. Since no nuclear membrane localization of mCherry was observed in figure 15 it is unlikely that Beclin 1 was cleaved during when cells were grown in CFBS, although this could also
be the result of mistargeting due to the amino-terminal fusion of a fluorescence protein. Further investigation on the interaction between Bcl-2 and Beclin1 must be conducted with mCherry fused to the C-terminus of Beclin1 to see if the interaction observed using mCherry-Beclin1 would have mitochondrial localization.

High mobility group box 1 (HMGB1) is a chromatin binding protein that manipulates DNA to up-regulate the formation of complexes required for transcription (Cato et al, 2008). It has been shown to have a regulatory role in autophagy and apoptosis, that involves the binding of Beclin1 and the phosphorylation of Bcl-2 in cells following starvation. The latter preventing the association between Beclin1 and Bcl-2 (Livesey et al, 2012; Kang et al, 2010). With the establishment of the FLIM FRET system, interaction between Venus-Bcl2-cb5 and mcherry fused to HMGB1 or Beclin1 can to used to characterize their interaction under starvation treatment in live cells and further shed light on the mechanism by which HMGB1 affects the function of Bcl-2.

4.7 Conclusion and Future work
In this thesis I present two different methods to identify and characterize Bcl-2 interacting partners at the endoplasmic reticulum. The biochemical approach of TAP purification allows the identification of novel Bcl-2 interacting partners at this subcellular compartment while the image based FLIM-FRET approach allows the characterization of Bcl-2 and its known interacting partners in the endoplasmic reticulum under various treatments.
Although the protocols and model systems have been established, there is still room for optimization of the protocols and issues to be addressed. Titration of DHPC should be performed to examine whether the artificial interaction between Bcl-xL and Bax is concentration dependent. A MCF-7 cell line stably expressing TAP-Bcl2-ActA must be established and utilized in the TAP purification as a negative control for the identification of Bcl-2 binding partners that are exclusively at the endoplasmic reticulum. The expression profile of potential Bcl-2 interacting partners under estrogen deprivation should also be assessed to give insight into the outcome of the TAP purification. The incubation time of samples with the SBP and CBP should be further optimized to decrease the amount of time the samples remain in infinite dilution. Lastly, the fractionation process must be optimized to ensure the integrity of the endoplasmic reticulum membrane before solubilization with detergent.

An underlying assumption for the result in the FLIM FRET experiment is that the clustering of protein in that particular membrane would not result in artificial interaction between Bcl-2 and its known binding partners in untreated conditions. mCherry fused with endoplasmic reticulum localizing tail anchor “cb5” must be generated to test the validity of this assumption. Venus-Bcl2-ActA expressing MCF-7 cells must be characterized and established as another control to show the exclusivity of the interaction between Bcl-2 at the endoplasmic reticulum and its interacting partner.
In this thesis, two approaches were taken to investigate potential binding partners of Bcl-2 at the endoplasmic reticulum. Although no novel interacting partner was discovered, the assays and systems developed in this thesis with further troubleshooting, will take us one step closer to uncovering the function and interactions of Bcl-2 at the endoplasmic reticulum.
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