ANALYSIS OF NOVEL CANDIDATE ‘BH3-ONLY’ PROTEINS

by

Prerna Suri

A thesis submitted to The Johns Hopkins University in conformity with the requirements

for the degree of Master of Science

Baltimore, Maryland

April 2017

Abstract

Apoptosis is a form of programmed cell death common to metazoan species. BH3-only proteins can be thought of as 'linchpins' in the apoptotic cell death pathway. This is because of their ability to respond to a variety of cell stress signals and to promote apoptosis by interacting with both pro- and anti-apoptotic members of the BCL-2 protein family, making them master regulators of apoptosis. Apart from the short BH3 motif found in BCL-2 homologs, there are eight canonical human BH3-only proteins that share no amino acid sequence similarity with other members of the BCL-2 family or with each other. The development of chemical or peptide mimetics of the BH3 motif to promote apoptotic cell death of infected or transformed cells as drugs against viruses, parasites like *schistosomes* as well as against cancer has become an important goal for both pharmaceutical industries and in academic settings (Shamas-Din, Brahmbhatt, Leber, & Andrews, 2011). However, because of the absence of a 'signature sequence' that rigorously defines the BH3 motif, it has not been possible to systematically screen for BH3-only proteins in the proteome. BH3-only proteins function by either neutralizing anti-apoptotic BCL-2 proteins or directly activating the pro-apoptotic BCL-2 proteins. Th BH3 motif can be considered as bearing Short Linear Motifs (SLiMs) that is 8-12 amino acids long with two 'hot-spot' residues: Asp (D) and Leu (L), which are essential for binding to the hydrophobic cleft of BCL-2 family member proteins (Aouacheria, Combet, Tompa, & Hardwick, 2015). This thesis project focuses on 10 candidate novel BH3-only proteins that were identified by Dr. Abdel Aouacheria and his colleagues using proprietary computational methods to search the entire available proteome. Plasmids expressing these candidate BH3-only proteins, both

wild type (WT) as well as mutants (M) that have point mutations in the two hotspot residues, intended to abolish their binding activity to the hydrophobic groove of members of the BCL-2 family, were provided from Dr. Abdel Aouacheria.

These candidate novel BH3-only proteins stem from diverse non-mammalian organisms including parasites and bacteria. Candidate BH3-only proteins were tested to determine if they have any pro-death function when expressed in mammalian cells. Three of the ten proteins were cytotoxic and localized to mitochondria, similar to many mammalian apoptosis regulators. Importantly, this cytotoxicity was reduced by the hot-spot mutations, despite wild type and mutant proteins being expressed at similar levels. These candidates were also tested to determine if their cytotoxicity could be inhibited by their putative mammalian binding partners, namely the anti-apoptotic BCL-2 family proteins BCL-xL and BCL-W. I found that both BCL-xL and BCL-W could rescue mammalian cells from the cytotoxic activity of the three killer BH3-only proteins, implying a functional interaction. The remaining seven non-toxic candidate BH3-containing proteins did not localize to mitochondria and could not be activated to induce cell death by the presence of the mammalian BH3-only protein tBID, which itself is highly toxic. These seven candidates also did not prevent anti-death proteins BCL-xL and BCL-W from rescuing cells from the tBID death signal. Overall, these results provide evidence for the existence of functional BH3 motifs in at least three of ten candidate proteins of diverse origin. These findings raise the possibility of evolutionary conservation of the BH3 motif as an ancient protein-protein interaction motif. More importantly, the algorithm opens the door to the possibility of accurately predicting short sequence motifs of degenerate nature but that have functional significance not previously possible.

Thesis Advisor: Dr. J Marie Hardwick

Thesis Reader: Dr. Anne Hamacher-Brady

Acknowledgments

Completion of my Master's degree would not have been possible without the hard work, support, encouragement and advice of several people throughout the course of my work both in the Hardwick lab and outside. The time spent working towards achieving this degree has taught me the value of mentorship, guidance, resilience and camaraderie. It has helped me grow both as a person and a scientist.

I would like to start by thanking my thesis and academic advisor Dr. J Marie Hardwick for her innumerable ideas, support, advice and encouragement throughout this project. I am grateful for her patience and mentorship through these two years. Thank you for giving me a chance to be a part of your wonderful lab and making my experience particularly meaningful.

I would like to thank my thesis reader Dr. Anne Hamacher-Brady for her knowledgeable inputs on my thesis.

I would also like to thank Dr. Abdel Aouacheria and members of his lab for providing plasmids of all the candidate proteins and their mutants and for his input and critique through this project.

I would like to thank the members of the Hardwick lab, both past and present for their suggestions, advice, patience and assistance. I would specially like to thank Dr. Heather Lamb for her support and mentorship, Jason Huska and Tim Sen Wang for training me and guiding me through the project and Zachary Stolp for his advice throughout the project. I would not have been able to complete my work without their support. They also created a very enjoyable lab environment in which to work and learn. I would also like to thank past

member, master of science student Arthur A. Reyes for providing constructive feedback of my work during lab meetings.

Above all, I would like to thank my family, especially my Mother without whom the dream of earning a master's degree would have remained a dream. I greatly appreciate her constant encouragement and moral support.

Lastly, I would like to thank my friends Purnima Ravisankara, Gibran Nasir, Rebecca Medina, Joudeh Freij and Winter Okoth for their support and encouragement. I would especially like to thank Yajas Shah for his ideas, constructive criticisms and stimulating conversations through the duration of this project.

Table of Contents

Abstract	ii
Acknowledgements	v
Table of contents	vii
List of Tables	viii
List of Figures	ix
Introduction	1
Materials and Methods	11
Results	15
Discussion	42
References	47
Curriculum vitae	58

List of Tables

Table 1. Genus and species names of Source organism encoding a candidate BH3-only protein **16**

Table 2. Genus and species names of Source organism encoding a candidate BH3-only protein with corresponding predicted size (GFP fusion protein) compared to the observed size on a blot. **20**

List of Figures

Fig. 1. Cytotoxicity of a subset of candidate BH3-only proteins in mammalian cells **17**

Fig. 2. Western blot analyses showing size of candidate BH3-only proteins **20**

Fig. 3. ER localization of five candidate BH3-only proteins. **22**

Fig. 4. Nuclear localization of two candidate BH3-only proteins **25**

Fig. 5. Mitochondrial localization of 3 candidate BH3-only proteins **27**

Fig. 6. Interaction of cytotoxic proteins with BCL-xL and BCL-W **33**

Fig. 7. Rescue of cell death by BCL-xL and BCL-W **34**

Fig. 8. Localization of toxic candidate BH3-only proteins with BCL-xL and BCL-W **35**

Fig. 9. tBID death assay **39**

Fig. 10. tBID vs candidate BH3-only proteins **40**

Introduction

Apoptosis

A large body of evidence indicates that the individual cells of multi-cellular organisms can sense defective cellular functions and environmental effects, such as DNA damage, unfolded proteins, metabolic events resulting from the lack of nutrients or other environmental stressors or phenomena that cause the cell to activate programmed cell death pathways prior to cell exhaustion (Czabotar, Lessene, Strasser, & Adams, 2014). Cell death is an inevitable part of life, and the human body eliminates billions of cells daily, for example to sustain homeostatic functions and selection of functional immune cells. There are over a dozen different cell death pathways reported and partially characterized (Kroemer et al., 2009). Thus far, only three of these are sufficiently well understood at a molecular level to distinguish them as distinct death pathways, apoptosis, necroptosis and pyroptosis. This project focuses on apoptotic cell death.

The term apoptosis was first coined in 1972 by Kerr, Wyllie and Curie in a monumental paper distinguishing deliberate cell death from necrosis, what was presumed at the time to be death by assault (no contribution from the dying cell), based on morphological changes and characteristics of cells (Kerr, Wyllie, & Currie, 1972). Subsequent decades of research have demonstrated that apoptosis is a tightly regulated process that leads to cell death mediated by a set of enzymes known as caspases (cysteine-aspartic proteases), caspase-3 in particular (Elmore, 2007). Apoptosis is a highly complex and energy driven process that can be broadly categorized into the intrinsic (mitochondria-mediated) or extrinsic (death receptor-mediated) pathways (Hotchkiss, Strasser, McDunn, & Swanson, 2009) The

‘extrinsic’ pathway is characterized by the specific binding of ligands, for example Tumor Necrosis Factor-alpha (TNFα), to ‘death receptors’ expressed on the surface of the plasma membrane, which can signal caspase-dependent cell death (Elmore, 2007). The ‘intrinsic pathway’ or mitochondrial apoptotic pathway on the other hand is regulated by interaction between the pro and anti-death members of the BCL-2 family, described below. This pathway can be triggered by stress such as DNA damage or even by cytokine activation, leading to mitochondrial outer membrane permeabilisation (MOMP) (Green & Reed, 1998). The permeablized outer membrane then releases cytochrome *c*, leading to the formation of the apoptosome, which directly activates caspase-9 that in turn cleaves and activates caspase 3 to cause apoptotic cell death (Riedl & Salvesen, 2007). Dysregulation of the apoptotic pathway can lead to deleterious effects such as tumors or even autoimmune diseases (Thompson, 1995).

The BCL-2 family of proteins

The BCL-2 family of proteins consists of several structurally and functionally related proteins. The members of this protein family that regulate apoptosis consist of both pro- and anti-apoptotic proteins. (Cory & Adams, 2002)The founding member of this family, BCL-2, was first identified as a novel proto-oncogene in follicular B-cell lymphomas near the defining 14 (q32);18 (q21) translocation, linking the immunoglobulin heavy chain locus to a novel gene to alter its expression pattern (Tsujimoto, Finger, Yunis, Nowell, & Croce, 1984). This gene was named B cell lymphoma/leukemia-2 or BCL-2. Unexpectedly, BCL-2 was shown to prevent cell death as opposed to increase cell proliferation (Vaux, Cory, & Adams, 1988). The over-expression of BCL-2 in many cancers leads to resistance

to therapeutics and evasion of cell death by upregulating anti-apoptotic BCL-2 proteins is considered a “hallmark” of cancer (Hanahan & Weinberg, 2000).

Contributing importantly to our understanding of human BCL-2 was the simultaneous discovery of the functions of CED-9 and CED-3 in the nematode *C.elegans*. CED-9 is the homolog of BCL-2 and CED-3 is the worm caspase. CED-9 was identified as the gene responsible for preventing developmental cell death due to CED-3 (Hengartner & Horvitz, 1994a). This and other work from the Horvitz group identifying many other components of the apoptosis pathway proved to be evolutionarily conserved across different species and was honored with the Nobel prize in 2002 (Ellis & Horvitz, 1986; Hengartner & Horvitz, 1994b; Yuan & Horvitz, 1990).

In contrast to worms, humans have several BCL-2 homologs with pro- and anti-apoptotic functions. These proteins share sequence and structural similarity, adopting a single alpha-helical globular domain and lack sequence similarity to other protein families. Within the BCL-2 family, there are four regions of greatest similarity known as BCL-2 homology (BH) motifs BH1-4 (Adams & Cory, 1998; Czabotar et al., 2014; Muchmore et al., 1996). Based on this similarity, the BCL-2 family of proteins can be divided into two categories. Anti-death proteins BCL-2, BCL-xL, BCL-W, MCL-1, A1 (BFL-1 in humans) and BCL2L10 share all 4 BH motifs (Hanada, Aimé-Sempé, Sato, & Reed, 1995), although the existence and definition of the BH motif is debated(Aouacheria, de Laval, Combet, & Hardwick, 2013a). Pro-death proteins BAK, BAX and BOK, share three BH motifs. The third category of proteins often grouped with members of the BCL-2 family shares only one of the four BH3 motifs, the BH3, and lacks other sequence similarity with members of the BCL-2 family of proteins (Hanada et al., 1995). This group of proteins is known as

BH3-only proteins, and is often further divided into two groups depending on whether they directly activate BAX or BAK (BID, BIM, and PUMA), or whether they promote cell death by inhibiting the anti-apoptotic proteins (NOXA, BAD, BIK, HRK, BMF). The BH3 motif of BH3-only proteins and of pro-apoptotic BCL-2 homologs can bind into a hydrophobic binding cleft on anti-apoptotic BCL-2 family proteins (Aouacheria et al., 2015; Czabotar et al., 2014). Many of the BCL-2 homologs also possess C-terminal hydrophobic tails that allow them to insert into cellular membranes, particularly the mitochondria and endoplasmic reticulum (ER) (Gross, McDonnell, & Korsmeyer, 1999).

The BH3-only group of proteins

BH3-only proteins regulate cell death by binding to and influencing both the anti- and pro-death members of the BCL-2 family of proteins (Youle & Strasser, 2008). Downstream of an intrinsic death signal, the pro- and anti-apoptotic BCL-2 family members interact which can eventually lead to oligomerization of pro-apoptotic proteins BAX and BAX, leading to MOMP and eventually, caspase mediated cell death(Elmore, 2007; Llambi et al., 2011). BH3-only proteins are usually inactive at organellar membranes unless activated at a transcriptional level and by post translational modifications which influence their binding with other members of the BCL-2 family of proteins (Huang & Strasser, 2000a).

Based on their selective binding, BH3-only proteins are also classified as sensitizers versus activators(Shamas-Din et al., 2011). Depending on their sequence and structure, the 8 canonical BH3-only proteins have differential binding to anti-apoptotic members of the BCL-2 family. Activators are shown to bind to BCL-2, BCL-xL, BCL-W, A1 as well as MCL-1, the proteins BID, BIM and PUMA fall into this category(Chen et al., 2005a). Sensitizers on the other hand selectively bind to MCL-1 or A1 (Letai et al., 2002). This

selective binding is important for BH3 mimetics (Lee et al., 2011). There are currently two different models for activation of BAX/BAK by BH3-only proteins (Letai et al., 2002). The direct activation model involves binding of activator proteins to the anti-apoptotic members of the BCL-2 family, thereby "sensitizing" the cells for death. The sensitizer BH3-only proteins can then bind to BAK/BAK, leading to MOMP, the "point of no return" for apoptosis (Shamas-Din et al., 2011). The indirect activation model involves competitive binding of BH3-only proteins to anti-apoptotic members of the BCL-2 family of proteins such that they either prevent binding of or displace BAK/BAX from the hydrophobic groove on the anti-apoptotic proteins (Kuwana et al., 2005). The free BAK and BAX can now oligomerize, leading to MOMP and eventually, caspase mediated cell death (Chen et al., 2005b).

BH3-only proteins in homeostasis and disease

Apart from regulation of apoptosis, BH3-only proteins and BCL-2 homologs also have functions in normal healthy cells prior to induction of apoptosis (Gross & Katz, 2017; Hardwick, Chen, & Jonas, 2012). In addition to cell death, BH3-only proteins are involved in other functions such as, homeostasis and DNA damage (Lomonosova & Chinnadurai, 2008a). A disturbance in the balance between BH3-only proteins and other members of the BCL-2 family of proteins could lead to tumorigenesis and auto-immune diseases(Thompson, 1995). For example, Bouillet et al showed that progeny of *Bim*$^{+/-}$ was half that of wild type *Bim* mice (Bouillet et al., 1999). They showed that Bim$^{-/-}$ fetuses died before day 10 of the embryonic stage, this was however strain dependent and may be attributed to genetic background as well. Mice lacking *Bim* also have a two-fold increase in the numbers of B-lymphocytes, monocytes, granulocytes and T-lymphocytes,

suggesting a role in the development of the immune system as well (Bouillet et al., 1999). Studies have also shown that ablation of one copy of *Bim* leads to an increase in c-myc expression, which leads to formation of lymphomas (Egle, Harris, Bouillet, & Cory, 2004). The BH3-only protein BAD was shown to play a role in the regulation of cell cycle via interaction with BCL-xL(Chattopadhyay, Chiang, & Yang, 2001). Bad also acts as a regulator of glucose levels in pancreatic islets (Danial et al., 2003). *Bmf* has been shown to play a role in colon, lung and breast cancer(Wick et al., 1996). *Hrk* plays a role in gastric cancer(Obata et al., 2003). *Bik* mRNA levels are highly upregulated in human lung, prostrate and renal cancers (Glab, Mbogo, & Puthalakath, 2017). Puma$^{-/-}$ mice were shown to be resistant to apoptosis induced by external cytotoxic stimuli (Lomonosova & Chinnadurai, 2008a). It was also shown that in response to DNA-damaging agents, *Puma* is upregulated in many cancers (Han et al., 2001). Although *Noxa*$^{-/-}$ mice did not seem to show any detectable phenotypic developmental abnormalities, NOXA is mutated or silenced in diffuse large B-cell lymphomas (Mestre-Escorihuela et al., 2007).

There has been intense interest in finding candidate novel BH3-only proteins in mammals as well as pathogenic organisms, such as *schistosomes* and *leishmania* to try and understand the functioning of their BH3 motifs and to develop BH3 mimetics as drugs against diseases causes by the parasites and viruses (Lee et al., 2011; Lee, Young, Lim, Gasser, & Fairlie, 2014; Rosenberg, 2011).

BH3-only proteins as SLiMs

The BH3 motif is a 9-16 amino acid long region that folds into an amphipathic alpha helix. This alpha helix is what binds to the hydrophobic cleft of the anti-apoptotic members of the BCL-2 family of proteins(Huang & Strasser, 2000b). After Sattler et al showed that the

BH3 motif is required for binding of BH3-only proteins to BCL-xL(Sattler et al., 1997), other groups showed that mutations in BH3 motif of the BH3-only proteins resulted not only in a loss of pro-death function but also an inability to bind other BCL-2 family members ((Lomonosova & Chinnadurai, 2008b; Sattler et al., 1997). The core BH3 motif consists of a hexameric sequence roughly defined as L-XXX-G-D where X is any amino acid, however, even the glycine (G) is not well conserved between the 8 canonical BH3-only proteins (Lanave, Santamaria, & Saccone, 2004). Leucine (L) and aspartate (D) are the major conserved residues, referred to as 'hotspot' residues, which are essential for the binding of a BH3 alpha helix to anti-apoptotic members of the BCL-2 family. Given the importance of BH3-only proteins in homeostasis, development, apoptosis, cancer and other diseases, the need to find BH3 mimetics is growing. However, with the absence of a valid signature sequence confirmed by informatics standards, this is a difficult task relying primarily on visual inspection of protein sequences (Aouacheria, de Laval, Combet, & Hardwick, 2013b). Computational methods so far have failed to to reliably identify even the known BH3-only proteins in the proteome due to the limited sequence information available in this motif(Aouacheria et al., 2015; Billard, 2013).

Other than BH3 motifs, there are many other short linear motifs, e.g. the SH3 motif, that are difficult to define based for similar reasons(Diella et al., 2008). Motifs like these can be defined as Short Linear Motifs or SLiM Eukaryotic Linear Motifs (ELMs). SLiMs and ELMs are primarily defined based on primary sequence determinants whereas MoRFs relate to the structural dynamics characteristics of BH3 peptide binding to the hydrophobic pocket present on the surface of Bcl-2 homologous proteins.(Aouacheria et al., 2015). These motifs serve as sites of protein-protein interaction or modification sites that are

critical for function. If we think of the BH3 motif as a SLiM, we can have better insight into its functional roles. Like SLiMs, BH3s are short motifs (9-16 amino acids long) (Lomonosova & Chinnadurai, 2008a) that are mostly found in disordered proteins (Aouacheria et al., 2015), as in the case of canonical BH3-only proteins (Rautureau, Day, & Hinds, 2010). Also like SLiMs, BH3s can be regulated post-transcriptionally (Glab et al., 2017). They lack sufficient sequence similarity and are 9-16 amino acids long(Lomonosova & Chinnadurai, 2008a). Also like SliMs, BH3s can be regulated post-transcriptionally (Glab et al., 2017)

The unresolved problem is that even if the BH3 motif is viewed as a SLiM, computational methods to find them remain elusive. (Aouacheria et al., 2013a; Tompa, Davey, Gibson, & Babu, 2014).

BH3 mimetics

BH3 mimetics are small molecules or peptides that mimic the BH3 motif and function by binding into the hydrophobic cleft of anti-death members of the BCL-2 family to promote cell death (Baell & Huang, 2002). It has been known for many years that BCL-2 family proteins are dysregulated in many diseases including cancer(Thomadaki & Scorilas, 2006; Thompson, 1995) . MCL-1 has been shown to be upregulated in acute myeloid leukemia (AML) and is essential for survival of these cells (Xiang et al., 2010). Pancreatic tumors have increased levels of BCL-xL(Sharma et al., 2005) . BCL-2 is over expressed in neuroblastomas and follicular lymphomas (Lamers et al., 2012). Over expression of BCL-2 family proteins has also been shown to increase resistance to radiation and other forms of chemotherapy used to treat tumors (Cory, Huang, & Adams, 2003; Coultas & Strasser, 2003; Delbridge & Strasser, 2015). Since the first solved crystal structure of BCL-xL

(Muchmore et al., 1996) , many labs and companies have been successful in developing small molecules that bind specifically to anti-apoptotic members of the BCL-2 family (Billard, 2013; Delbridge, Grabow, Strasser, & Vaux, 2016) . These small molecules mimic the alpha helical BH3-motif of BH3-only proteins and can bind to the binding pocket formed by folding of BH1 BH2 and BH3 motif of the anti-death proteins (Billard, 2013). Many *in silico* and *in vitro* screens are being performed to find more mimetics (Billard, 2013; Burrer, Foight, Keating, & Chan, 2016; Lomonosova & Chinnadurai, 2008a) The selective binding of BH3-only proteins to anti-death proteins is a key requirement in developing these mimetics(Chen et al., 2005a). Previously characterized mimetics were path leaders for understanding how BH3 mimetics work(Billard, 2013). (Billard, 2013). Obatoclax was suggested to be a BH3 mimetic, however, it binds to all five anti-death proteins but with low affinity, and may instead be non-specific as it has similar killing activity in both BAX/BAK double knockout cells as well as wild type cells makes its use as a mimetic questionable (Vogler et al., 2009). ABT-737 and its orally bioavailable form ABT-263 have a higher affinity for BCL-xL, BCL-2 and BCL-W but does not bind appreciably to MCL-1 (Billard, 2013; van Delft et al., 2006). Venetoclax (ABT 199), which was recently approved by the FDA, has high affinity only for BCL-2 and has 200 times lower affinity for BCL-xL (Roberts et al., 2016). S63845, is a new small molecule inhibitor that is highly selective for MCL-1, and induces BAX/BAK-dependent killing both *in vitro* and *in* vivo in protection against cancers with upregulated levels of MCL-1(Kotschy et al., 2016). There are a number of other compounds in clinical trials(Billard, 2013), thus BH3 mimetics and BH3 profiling are important for understanding the BH3 motif and will only help the field move forward.

Material and methods

Plasmid constructs

N-terminal GFP-tagged full-length constructs of ten candidate BH3-only proteins and their mutants in pEGFP-c1 vectors were provided by Dr. Abdel Aouacheria, Université de Montpellier, France. Plasmids expressing tBID, BCL-W, BCL-xL and dominant negative caspase-9 were all generated by current or previous members of the lab in SG5 vectors.

Cell Culture

HeLa cervical cancer cells (ATCC® CCL-2) were grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco®) supplemented with 10% FBS, 1U Penicillin/1 µg Streptomycin (Invitrogen®) and 2 mM L-glutamine. Cells were incubated at 37 °C and 5% CO_2 in a humidified chamber.

Immunostaining and microscopy

Cells were seeded in 24 well plates (353043, Falcon®) on coverslips (Fisher®) at a density of ~4 x 10^4 cells per well. The next day, cells were transfected with 175 ng of total plasmid using 1.5 µl of Lipofectamine 2000 (Invitrogen®) and 50 µl of Opti-MEM (Gibco®) per manufacturer's instructions. Cells were processed for immunofluorescence the next day.

At 18-24 h post transfection, cells were washed 3 times with Phosphate Buffered Solution (PBS) and fixed with 4% para-formaldehyde (PFA) for 10 min at room temperature. Fixed cells were permeabilized using 0.2% Triton-X100 in PBS for 5 min. Cells were then washed 3 times with PBS and blocked for 1 h in 2% goat serum (in PBS). After blocking,

cells were incubated with primary antibody [GFP Anitbody IgG2a (Life Technologies A11129, Calnexin Antibody, pAb, A01234-40 Rabbit (GenScript®), Tom20 Antibody (FL-145): sc-11415, mouse (Santa Cruz®)] in a 1:2000 dilution with 2% goat serum in PBS for 1 h at room temperature. Secondary antibody [1:1000 goat anti-mouse Alexa Fluor 488 (Life Technologies®) or goat anti-rabbit Alexa flour 594 (Life Technologies®) was diluted in 2% goat serum in PBS and cells were incubated for 1 h at room temperature, in the dark. Cells were washed 3 times with PBS and incubated for 15 min with a 1:4000 dilution of Hoechst nuclear dye (Invitrogen®) in PBS. Cells were washed 3 times with cold PBS. Coverslips were mounted onto a slide using Prolong (R) Gold (Cell Signaling Technologies®). Coverslips were imaged using the Nikon 90i inverted fluorescence microscope at 40X air objective. Cells had about 70% transfection efficiency.

Cell death assay

Cells in 24-well plates were transfected with a total of 175 ng plasmid (35 ng tBID + 105 ng BH3-only protein plasmid + 35 ng of BCL-xL/BCL-W empty vector) using 1.5µl of Lipofectamine 2000 (Invitrogen®) and 50 µl of Opti-MEM (Gibco®) according to manufacturer's instructions. At 16 h post transfection, cell viability was determined using Trypan blue dye. Media from each well was collected, floating cells were pelleted at 800 rpm for 7 min and the supernatant was aspirated. Adherent cells were incubated with 100 µl of Trypsin-EDTA for 10 minutes or until all cells detached from the dish. 300µl of PBS was added to each well and the trypsinised cells with PBS were then added to the pelleted floating cells. Cells were spun down at 800 rpm for 7 minutes and cells were re-suspended in 100 µl of PBS. 100 µl of Trypan blue, 0.4% (Gibco®) was added to stain dead cells. Cells were counted within 30 s of staining using a hemocytometer and the percent total

cells that were dead was determined (number of Trypan blue-stained cells divided by total number of cells (live + dead)).

Lysate preparation and Western Blot analysis

Cells were seeded overnight in a 12-well plate at a density of ~8.0 X 10^5 cells per well. Cells were transfected with a total of 350 ng plasmid per well using Lipofectamine 2000 (Invitrogen®) as per manufacturer's instructions. Cells were harvested 24 h post transfection. 150 µl of 6x sample buffer (for 100 ml: 70 ml 0.5M Tris, pH 6.8, 10% SDS, 5 ml BME, 30 ml glycerol, 12.5 mg Bromophenol Blue) was diluted to a 1Xworking concentration in RIPA buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate,0.1% SDS, 140 mM NaCl, 1mM PMSF] was added to each well of washed cells, and cells were scraped off. Lysates were collected in a 1.5 ml Eppendorf tube, heated to 95°C for 5 minutes and stored at -20ºC.

Protein lysates were resolved by SDS-PAGE on 12% gels at 110V. Proteins were transferred using the transblot™ system (BioRad®) to a Poly Vinyl Diflouride (PVDF) membrane. Membranes were blocked in TBS-T [20 mM Tris, 150 mM NaCl, pH7.6, 0.1% Tween®20] with 5% milk (Nestle® Carnation™) for 2 h. Membranes were incubated with primary antibody overnight [1:2000 GFP Antibody A6455, rabbit (Invitrogen®) ,1:10000 GAPDH (6C5) sc-32233 (Santa Cruz®) , 1:1000 HA-probe (Y-11) sc-805, rabbit (Santa Cruz®), 1:1000 Actin, clone 4, mouse (Life Technologies®), washed the next morning with TBS-T 3 times x 5 min each, incubated with secondary antibody [1:10000 ECL™ Anti-mouse IgG HRP linked whole antibody or Anti-rabbit HRP linked whole antibody (GE

Healthcare®)and imaged on a BioRad® Universal Hood III using Amersham™ ECL™ prime (GE Healthcare®) developing reagent.

Results

Testing for the pro-death function of candidate novel BH3-only proteins

The eight canonical mammalian BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, PUMA and NOXA) as well as the worm homolog in C. elegans, EGL-1, are all known to have pro-apoptotic activity and most are regulated by transcriptional and post-transcriptional modifications (Bouillet & Strasser, 2002) as well as by anti-death members of the BCL-2 family (e.g. BCL-W, BCL-xL, MCL-1, BCL-2) (D. C. Huang & Strasser, 2000).

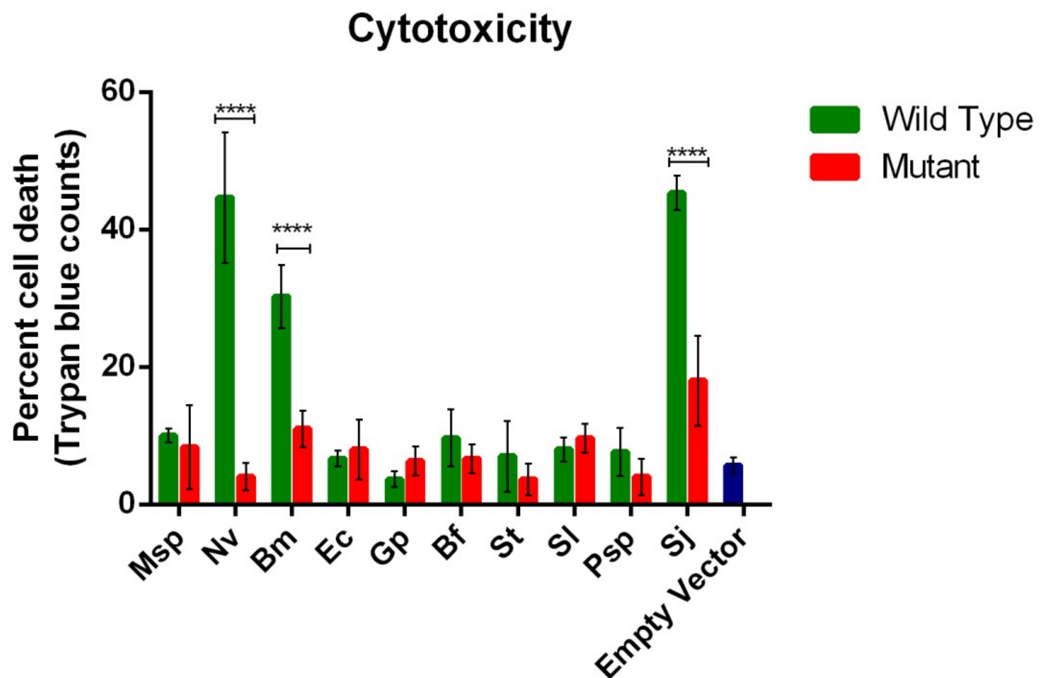
Mammalian expression plasmids for the top ten hits from an *in silico* screen for novel BH3 motifs were provided by Dr. Abdel Aouacheria (University of Montpellier). These plasmids encode N-terminal GFP-fusions of the ten full-length proteins and the corresponding ten mutants (mut) in which the two Leu and Asp residues that define the BH3 motif were changed to Ala (LxxxxD/AxxxxA). These ten candidate BH3-only proteins are encoded by six diverse species of bacteria and four metazoans, including a sea anemone (*Nematostella vectensis*), a filarial worm (*Brugia malayi*), the Florida lancelet (*Branchiostoma floridae*) and the flat-worm commonly known as a blood-fluke (*Schistosoma japonicum*) (Table 1). Among these 10, three are human pathogens that cause schistosomiasis (*Schistosoma japonicum*), tuberculosis (*Mycobacterium sp*) and filariasis (*Brugia malayi).* To explore the possibility that these candidate BH3-only proteins might engage the mammalian apoptosis pathway, all 20 proteins were transiently expressed in HeLa cells to test for the ability to induce cytotoxicity similar to mammalian BH3-only proteins.

Trypan blue counts show that three of the ten BH3-only candidates (three of the four metazoan proteins), from *Nematostella vectensis, Brugia malayi and Schistosoma japonicum,* are cytotoxic (Fig. 1). If the BH3 motif is important in promoting cell death, then the BH3 mutations should abolish or decrease the toxicity. Strikingly, the BH3 mutants of all three had significantly diminished ability to cause cell death (Fig. 1), which could not be explained by insufficient protein expression, as the corresponding BH3 mutants are expressed as well or better than wild type in HeLa cells (Fig. 2b, c and j). Similarly, failure of other BH3-only candidates to promote cell death was not due to lack of expression (Fig. 2a, d-i).

Source Organism	**Protein ID**	**Localization-WT**	**Localization-M**
Brachistoma floridae	*Bf*	ER+ Nuclear	ER+ Nuclear
Brugia malayi	*Bm*	Mitochondrial	Cytoplasmic
Escherichia coli	*Ec*	Nuclear	Nuclear
Gamma proteobacterium	*Gp*	Nuclear	Nuclear
Mycobacterium sp.	*Msp*	ER	ER
Nematostella vectensis	*Nv*	Mitochondrial	Non-mitochondrial aggregates
Paenibacillus sp.	*Psp*	ER	ER
Schistosoma japonicum	*Sj*	Mitochondrial	Mitochondrial
Streptomyces lividans	*Sl*	ER	ER
Sphaerobacter thermophiles	*St*	ER	ER

Table 1. Genus and species names of Source organism encoding a candidate BH3-only protein, and predominant subcellular localization of wild type candidate BH3-only proteins and BH3 motif point mutants.

Fig. 1. Cytotoxicity of a subset of candidate BH3-only proteins in mammalian cells Cell death determined by Trypan blue positivity was determined for HeLa cells transfected for 16 h with candidate BH3-only proteins and corresponding mutants (300ng each). Data shown are the means +/- standard deviation for 3 independent experiments counting ~200 cells per sample. Similar results were also seen in COS-7 cells (data not shown).



To confirm expression of all 20 N-terminal GFP-tagged candidate BH3-only proteins, we performed western blot analysis on HeLa cells transiently transfected with the constructs for WT and mut of all proteins (Fig. 2). All GFP-fusion proteins migrate within 10 kDa of their predicted molecular weights plus GFP (27 kDa) (Table 2). Note that the wild type version of the three toxic proteins from *Nematostella vectensis*, *Brugia malayi* and *Schistosoma japonicum* are expressed at much lower levels relative to their BH3 mutant due to loss of dying cells (Fig. 2b, 2c and 2j).

Subcellular localization of candidate BH3-only proteins

BCL-2 family members are often found at membranes of cell organelles, particularly the ER and mitochondrial membranes(Gross et al., 1999). Seven of the 10 BH3-only candidates, including all three of the toxic proteins from *Brugia malayi, Nematostella vectensis* and *Schistosoma japonicum*), four prokaryotic proteins from *Mycobacterium sp*, *Brachistoma floridae* and *Paenibacillus sp*, and *Streptomyces lividans* have a predicted transmembrane helix using several different prediction tools (see Discussion). Despite being non-mammalian proteins, I determined their subcellular localization in mammalian cells using immunofluorescence (IF) microscopy. I observed several different localization patterns (Table 1 and Figs. 3-5). Four candidates localized to the ER (Fig. 3a-e), the three toxic proteins localized to mitochondria (Fig. 5 a-c), two others localized to the nucleus (Fig. 4 a, b) and one exhibited both ER and nuclear localization (Fig. 3c). All transfected cells for each protein had the same localization (there weren't different phenotypic locations for cells transfected with the same plasmid). It was surprising that none of these

proteins exhibited untargeted, diffuse cytoplasmic localization. However, we cannot rule out the possibility that the GFP tag could contribute non-specifically to nuclear localization by becoming partially trapped in the nucleus. The localization of BH3 mutant forms of candidate BH3-only proteins was unchanged except for two of the three toxic BH3-only candidates from *Brugia malayi* and *Nematostella vectensis*. Interestingly, these BH3 mutations blocked localization to mitochondria (Fig. 5 a and b). Mutation of the predicted BH3 in *Brugia malayi* caused a shift in localization from the mitochondria to a more diffuse cytosolic localization (Fig. 5a upper and lower), raising the possibility that there is a mitochondrial target or receptor for the *Brugia malayi* BH3-only candidate. Mutation of the putative BH3 in the *Nematostella v*ectensis candidate resulted in clustered aggregates in the cytoplasm that did not co-localize with either the mitochondrial or the ER markers, suggesting folding defects (Fig. 5b upper and lower panels). Although the BH3 mutations did not alter localization of the candidate from *Schistosoma japonicum,* these proteins (both WT and mut) caused mitochondria to be much more elongated than usual (Fig. 5c, upper left panel). This could potentially be due to altered mitochondrial dynamics. Although the WT proteins from *Nematostella vectensis*, *Brugia malayi* and *Schistosoma japonicum* are cytotoxic, the representative images show live transfected cells, which were hard to find since most of the transfected cells for these constructs had died and were not visible. Similar localizations were also observe in COS-7 cells (data not shown).

Source Organism	Protein ID	Predicted Size (kDA)	Observed Size (kDa)
Brachistoma floridae	*Bf*	41.1	38
Brugia malayi	*Bm*	38.67	38
Escherichia coli	*Ec*	38.83	37
Gamma proteobacterium	*Gp*	66.02	75
Mycobacterium sp.	*Msp*	74.39	65
Nematostella vectensis	*Nv*	44.27	42
Paenibacillus sp.	*Psp*	87.48	84
Schistosoma japonicum	*Sj*	39	37
Streptomyces lividans	*Sl*	71.1	71
Sphaerobacter thermophiles	*St*	77.93	85

Table 2. Genus and species names of Source organism encoding a candidate BH3-only protein with corresponding predicted size (GFP fusion protein) compared to the observed size on a blot.

Fig. 2. Western blot analyses showing size of candidate BH3-only proteins. HeLa cells were transfected with 300ng of candidate BH3-only proteins and harvested 24h post transfection. Bands seen are of GFP fusion proteins that include a 27 kDa GFP tag, and were detected using anti-GFP antibody. Whole cell lysates were prepared from HeLa cells transfected with each of the 10 candidate BH3-only proteins and 10 corresponding LxxxxD/AxxxxA BH3 mutants at 24 h post transfection. Representative blots from 3 independent experiments are shown, and the relative levels between wild type and mutant versions, as well as between different proteins closely approximates their relative levels.

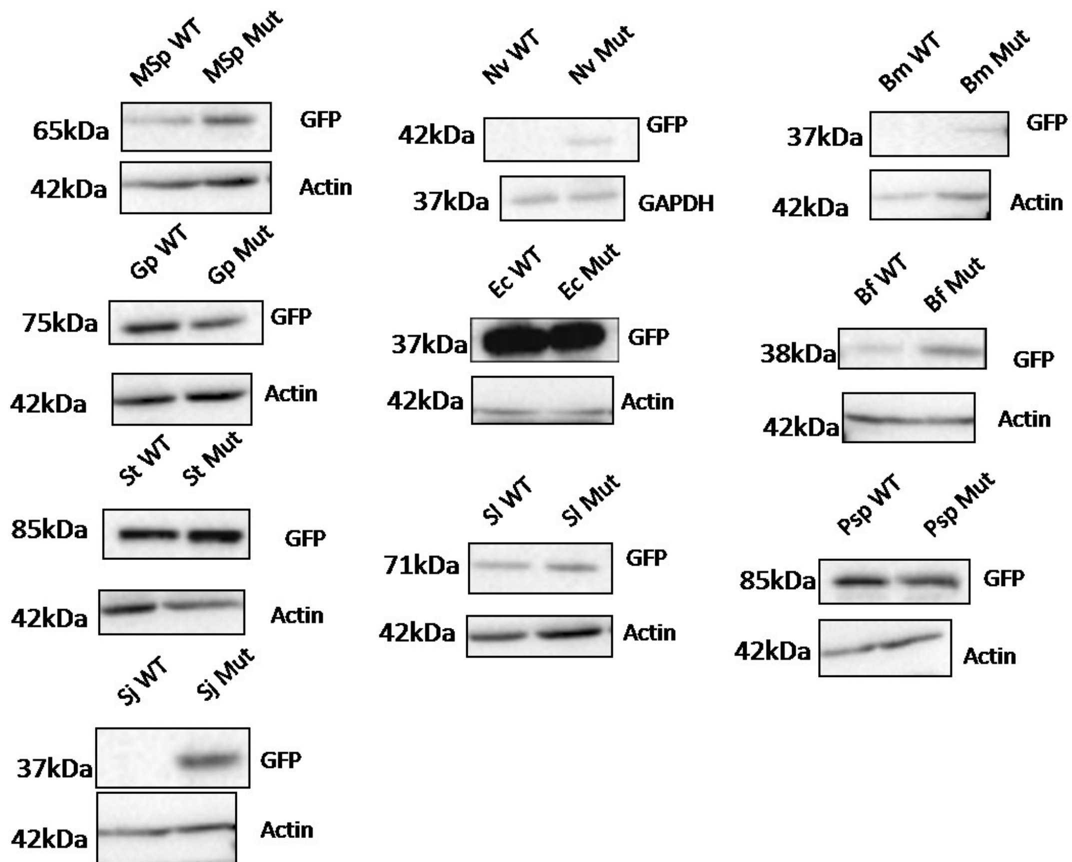
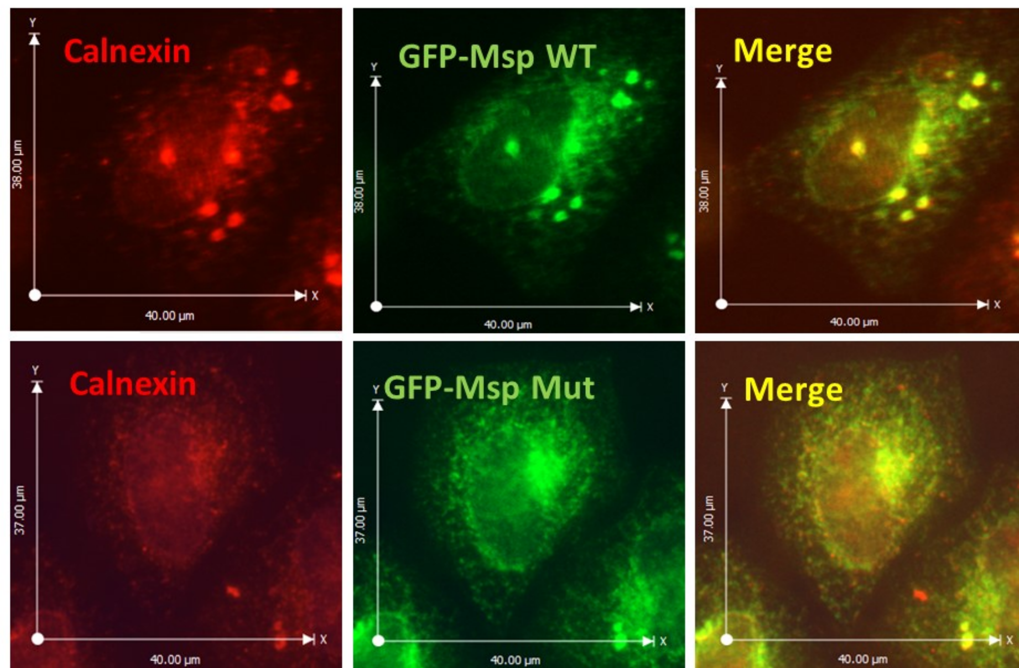


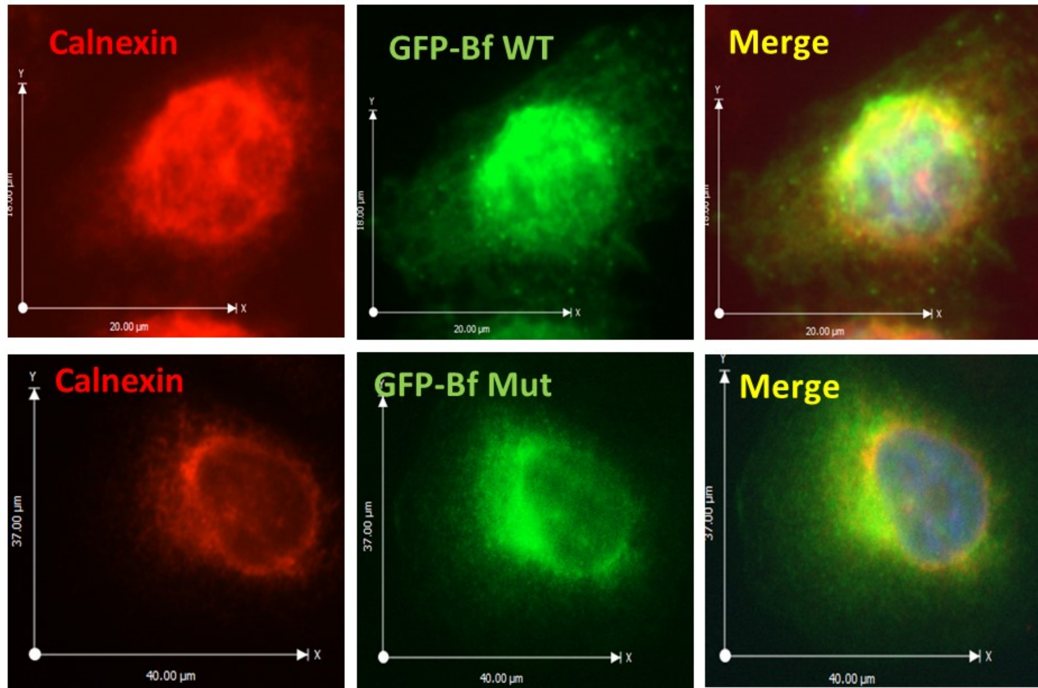
Fig. 3. ER localization of five candidate BH3-only proteins.
Representative immunofluorescence images of HeLa cells processed 24 h post transfection with wild type (WT) and mutant (mut) GFP-BH3-only constructs and stained with anti-GFP antibody. The ER is marked with antibodies to endogenous calnexin, nuclei are stained with Hoechst (blue), and candidate GFP-BH3-only proteins are detected with anti-GFP antibody (green). Markers showing co-localization with candidate BH3-only proteins with different cell organelles are shown (merge). Panels a-e show co-localization of the indicated candidate BH3-only proteins with ER marker calnexin. Scale bars: x-axis: 40nm y-axis: 30nm

Positive colocalization with ER was found for the following constructs: Msp,Bf and St (WT and Mut)

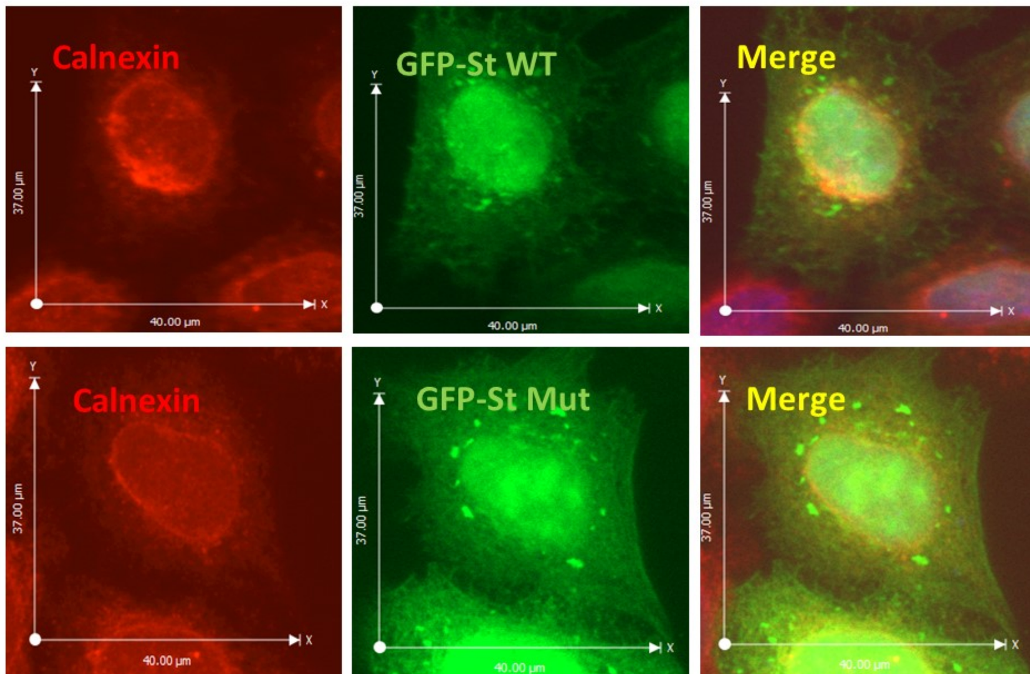
a ***Mycobacterium sp.***



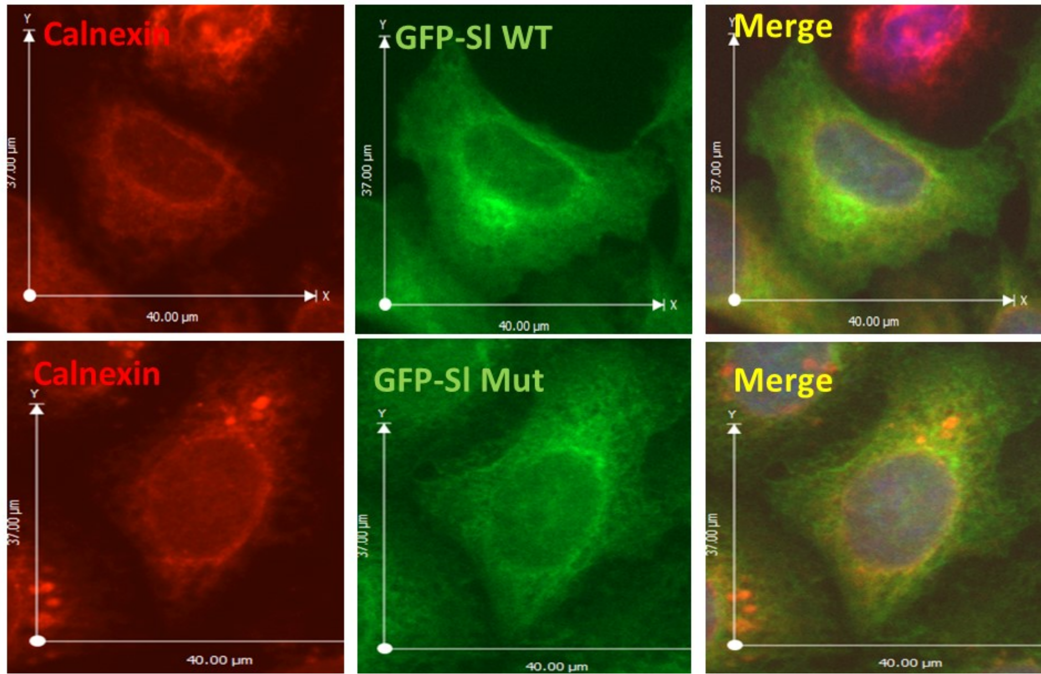
b *Branchistoma floridae*



c *Sphaerobacter thermophilus*



d *Streptomyces lividans*



e *Paenibacillus sp.*

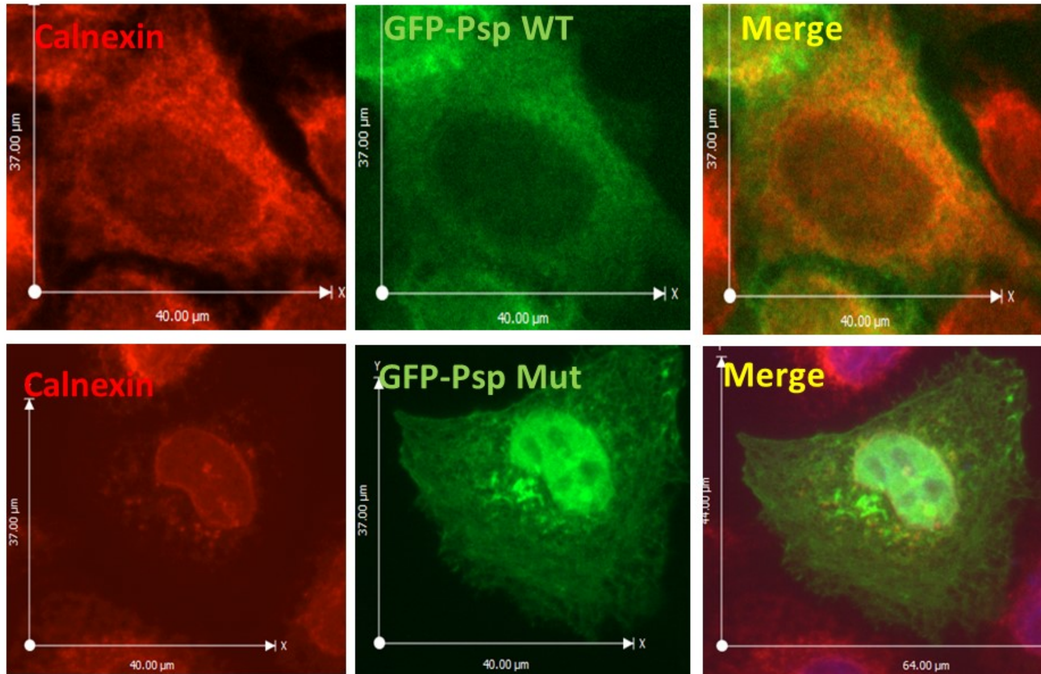
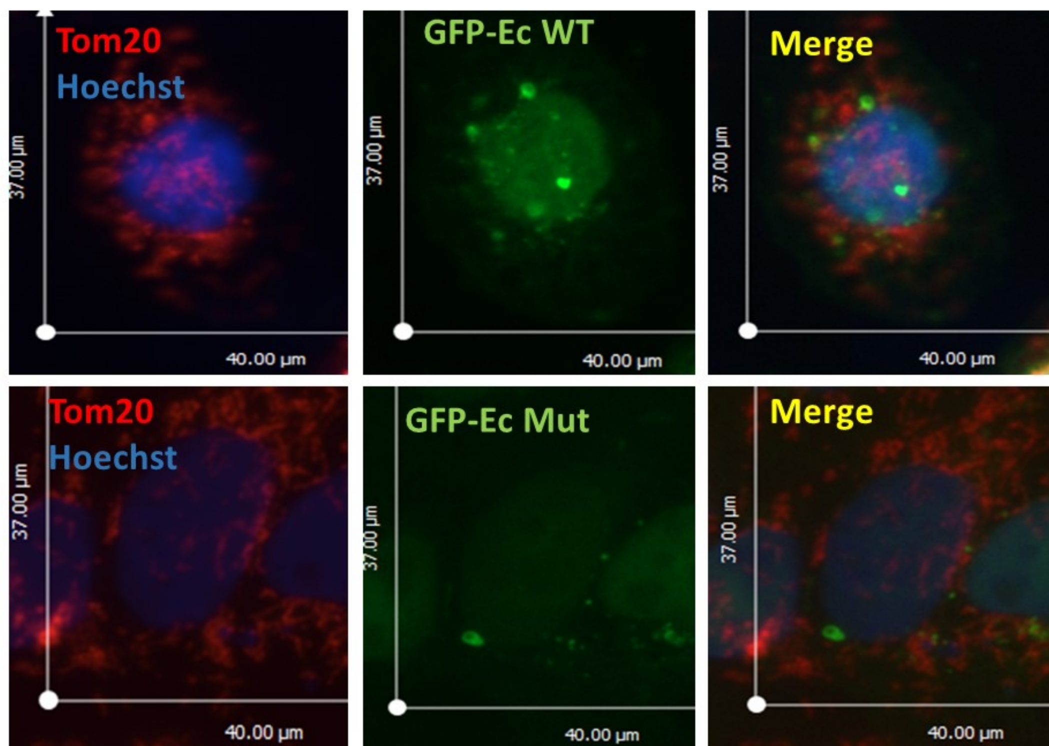


Fig. 4. Nuclear localization of two candidate BH3-only proteins. Representative immunofluorescence images of HeLa cells fixed and stained 24 h post transfection with wild type (WT) and mutant (mut) GFP-BH3-only constructs. The mitochondria were marked with antibodies to Tom20, nuclei is stained with Hoechst (blue), and candidate GFP-BH3-only proteins were detected with anti-GFP antibody (green). Markers showing co-localization with candidate BH3-only proteins with different cell organelles are shown (merge). Panels a,b show co-localization of the indicated candidate BH3-only proteins with nuclear marker. The WT protein is in the upper panel and Mut is in the lower panel. Hoechst. Scale bars: x-axis: 40nm y-axis: 37nm

Positive colocalization with the nucleus was found for the following constructs: Ec and Gp (WT and Mut)

a *Escherichia coli*



b Gamma proteobacterium

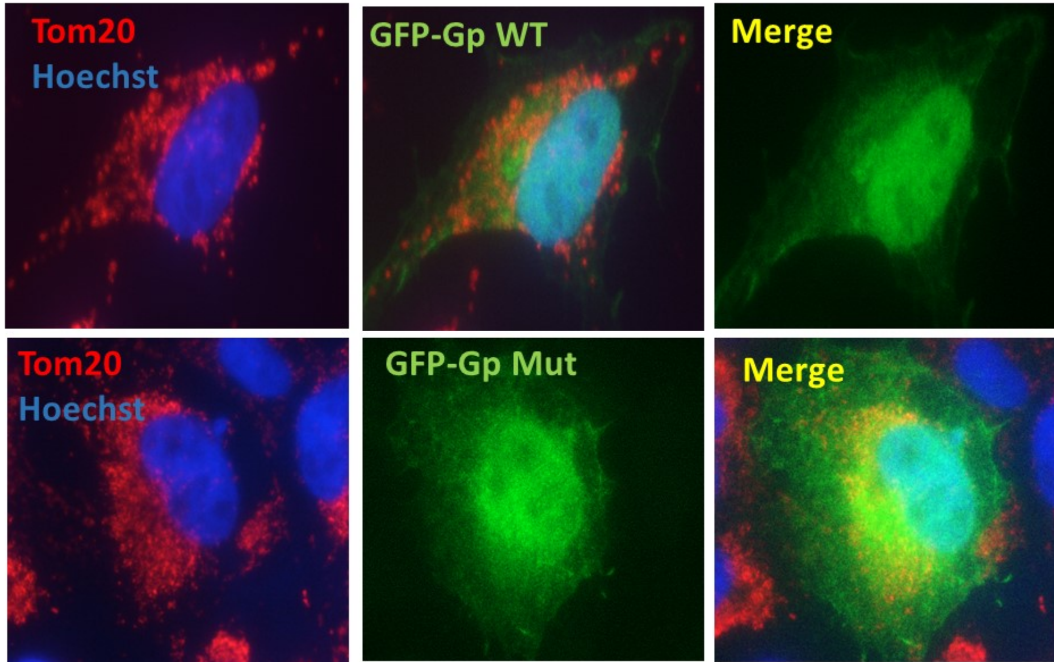
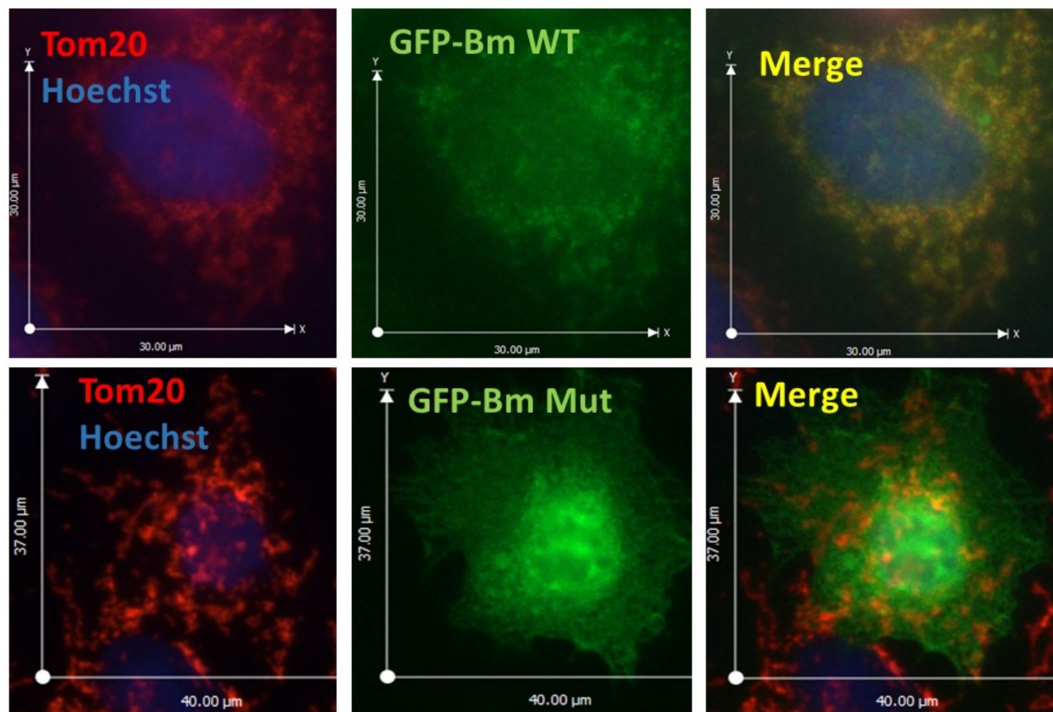


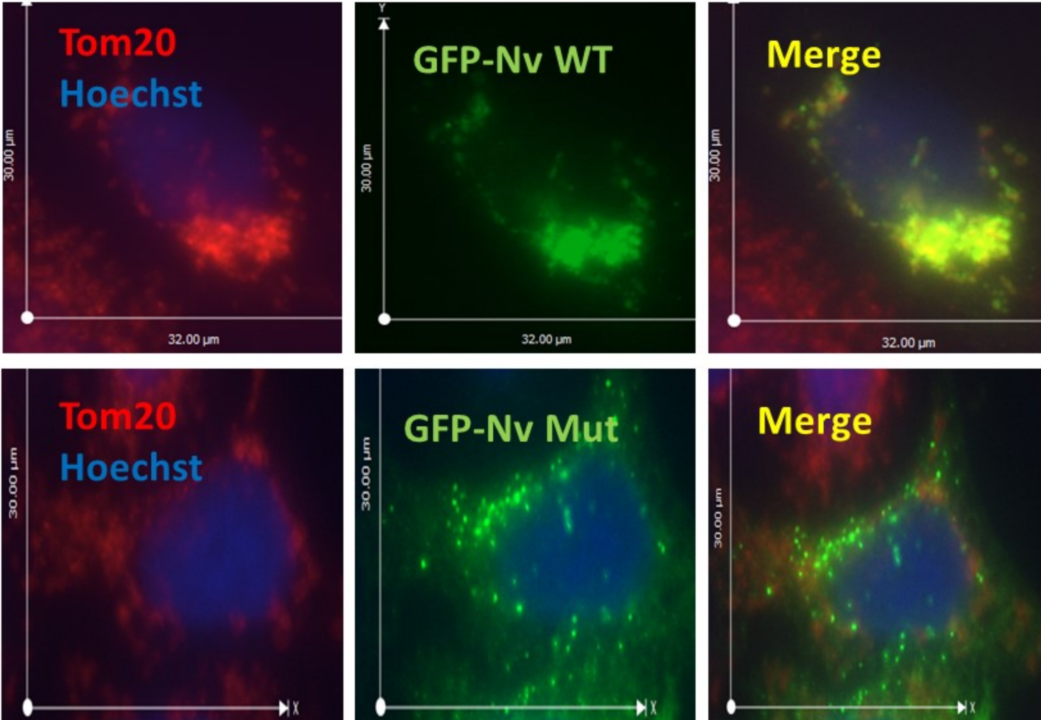
Fig. 5. Mitochondrial localization of 3 candidate BH3-only proteins. Representative immunofluorescence images of HeLa cells fixed and stained 24 h post transfection with wild type (WT) and mutant (mut) GFP-BH3-only constructs. The mitochondria were marked with antibodies to Tom20, nuclei are stained with Hoechst (blue), and candidate GFP-BH3-only proteins were detected with anti-GFP antibody (green). Only those markers showing co-localization with candidate BH3-only proteins with different cell organelles are shown (merge). Panels a-d show co-localization of the indicated candidate BH3-only proteins with nuclear marker. The WT protein is in the upper panel and Mut is in the lower panel. Hoechst. Scale bars: x-axis: 40nm y-axis: 37nm.

Positive colocalization with ER was found for the following constructs: Bm (Wt), Nv (Wt) and Sj (WT and Mut)

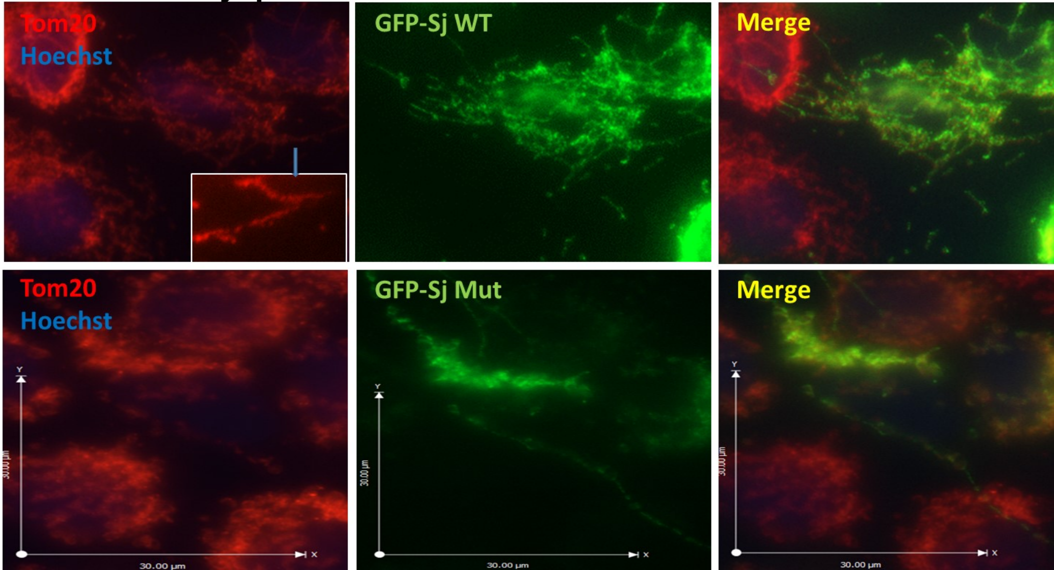
a Brugia malayi



b Nematostella vectensis



c Schistosoma japonicum



Potential Interaction with anti-death members of the BCL-2 family

One way by which BH3-only proteins can trigger apoptosis is by binding to the hydrophobic binding groove on anti-death BCL-2 family members, thereby displacing either mammalian pro-death BCL-2 homologs BAX and BAK or canonical BH3-only proteins(Chen et al., 2005b). Liberated mammalian BH3-only proteins could subsequently induce BAX or BAK oligomerization to cause mitochondrial outer membrane permeabilization (MOMP). MOMP allows the release of pro-death factors such as cytochrome *c* to activate caspase-3-mediated apoptosis (Chen et al., 2005c).

In vitro data indicate that peptides containing the BH3 motif of all 10 candidate BH3-only proteins bind to anti-apoptotic BCL-2 proteins, and can displace the binding of mammalian BH3 peptides (A. Aouacheria, unpublished). Evidence that these BH3 peptides are capable of binding in the hydrophobic groove of anti-apoptotic proteins, rather than other potential binding sites is based on the significantly reduced binding affinity when the BH3 LxxxxD is mutated to AxxxxA (A. Aouacheria, unpublished). To extend these in vitro studies, we tested for the ability of the three toxic BH3-only candidates to inhibit the function of anti-apoptotic BCL-2 and BCL-W. BCL-2 and BCL-W were selected for this assay because they interact with the candidate BH3-only proteins (A. Aouacheria, unpublished). To test for a possible functional interaction between the candidate BH3-only proteins and the anti-death BCL-2 family members, the three cytotoxic BH3-only candidates (from *Nematostella vectensis*, *Brugia malayi* and *Schistosoma japonicum*) were co-expressed

with BCL-xL or BCL-W in HeLa cells. Both BCL-xL and BCL-W could rescue the death phenotype of all three BH3-only candidates, consistent with possible direct interaction. To further address whether the death induced by the three toxic BH3-only candidates was due to caspase-dependent apoptosis, the BH3-only candidates were co-expressed with dominant negative caspase-9 (DNC9), which potently inhibits Bax induced cell death (Srinivasula et al., 1999) (Fig. 7c). Unexpectedly, dominant negative caspase-9 was protective only against one of the three toxic BH3-only candidates from *Brugia malayi* (Fig. 6). Despite being inhibited by anti-apoptotic BCL-2 proteins, dominant negative caspase-9 had no ability to rescue the death induced by expression of the other two BH3-only candidates from *Nematostella vectensis* and *Schistosoma japonicum,* even at different ratios (1:1, 1:2 and 1:3 BH3-only protein: DNC9) (Data not shown). Therefore, it is possible that the death caused by *Nematostella vectensis* and *Schistosoma japonicum* BH3-only candidates may not be apoptotic in nature and may follow some other death pathway. This hypothesis would be supported if these BH3-only candidates were able to kill BAX/BAK double knockout cells, while the *Brugia malayi* BH3 failed to kill BAX/BAK double knockouts, an experiment that is currently being done (in progress).

Further evidence that cell death induced by all three toxic BH3-only candidates is efficiently suppressed by BCL-xL and BCL-W is evident from the Western blot analysis, showing rescue of protein expression of BH3-only candidates due to surviving cells (Fig. 7a,b). The band for the WT cytotoxic proteins from *Nematostella vectensis, Brugia malayi* and *Schistosoma japonicum* can only be detected if co-transfected with either of the anti-death BCL-2 family member proteins, consistent with rescue of the death phenotype (Fig. 7a,b). Furthermore, IF imaging shows co-localization between each of the three candidate

BH3-only proteins and the anti-apoptotic proteins BCL-xL and BCL-W, suggesting the possibility of a functional interaction (Fig. 8a-c). Trypan counts confirmed rescue by anti-death (Fig. 6a, b).

BCL-xL and BCL-W can inhibit cell death induced by mammalian BH3-only proteins, by binding the BH3 motif region into their hydrophobic grooves(Chen et al., 2005a) .A similar mechanism could occur for the non-mammalian BH3-only candidates, preventing the anti-apoptotic proteins from sequestering other endogenous pro-death proteins. Alternatively, the candidate BH3-only proteins could potentially directly activate BAX or BAK, in addition to, or potentially instead of binding to BCL-xL or BCL-W, analogous to direct activator-types of mammalian BH3-only proteins (BID, BIM and PUMA).

The inability of dominant negative caspase-9 to rescue viability of cells expressing two of the three toxic proteins could suggest a different death pathway for these two candidates. In this case, BCL-xL and BCL-W could be protecting cells independently of their canonical anti-apoptotic functions (Gross & Katz, 2017; Hardwick & Soane, 2013). Alternatively, the failure of dominant negative caspase-9 to protect against death induced by candidate BH3-only proteins from *Nematostella vectensis* or *Schistosoma japonicum* could be due to insufficient expression of dominant negative caspase-9 as the flag-tagged dominant negative caspase-9 protein could not be detected on the western blots when co-transfected with any other protein, but could be detected in a control when transfected by itself (data not shown). Experiments are currently being done to tease this apart. It could be possible that the amount of dominant negative caspase-9 being expressed is too low to detect on a western blot, to account for this, experiments are being done using different ratios of BH3-only proteins to dominant negative caspase-9. Another caveat of this experiment is that

dominant negative caspase-9 was unable to protect cells transfected with tBID from dying, which is a known activated apoptotic killer.

Fig. 6. Interaction of cytotoxic proteins with BCL-xL and BCL-W. HeLa cells were transfected with the indicated plasmids and harvested 16h post transfection. Percent cell death was determined by trypan counts. Graph shows percent cell death of both cytotoxic proteins *Nematostella vectensis* and *Schistosoma japonicum* (WT and mut) when cells were transfected with BH3-only protein (88ng) (blue) BH3-only proteins and DNC9 (88ng) (green) BH3-only protein and BCL-xL(88ng) (red) and BH3-only protein with BCL-W(88ng) (yellow). Error bars represent SD.

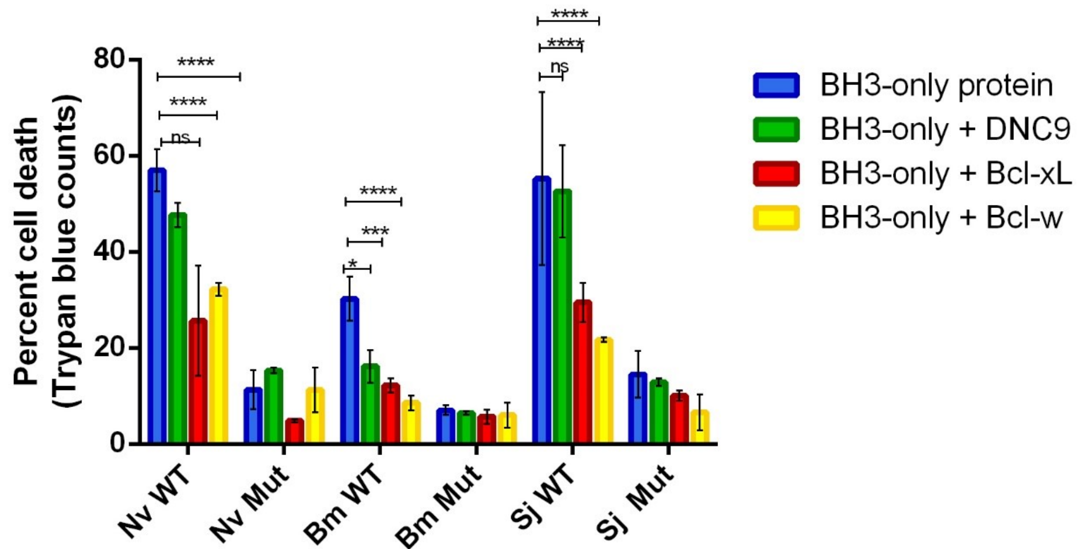


Fig. 7. Rescue of cell death by BCL-xL and BCL-W. HeLa cells were transfected with indicated plasmids and harvested 24h post transfection with 88ng of each plasmid. (a) Rescue of death caused by *Schistosoma japonicum* WT(GFP-tagged) by BCL-xL and BCL-W (HA-tagged) but not dominant negative caspase-9 (DNC9) (Flag tagged) . (b) Rescue of death caused by *Nematostella vectensis* WT(GFP-tagged) by BCL-xL and BCL-W(HA-tagged) but not DNC9(flag-tagged). (c) Rescue of death caused by Flp-BAX(flag-tagged), a known apoptotic killer by DNC9. The names of the antibodies used for detection are listed on the right in each panel.

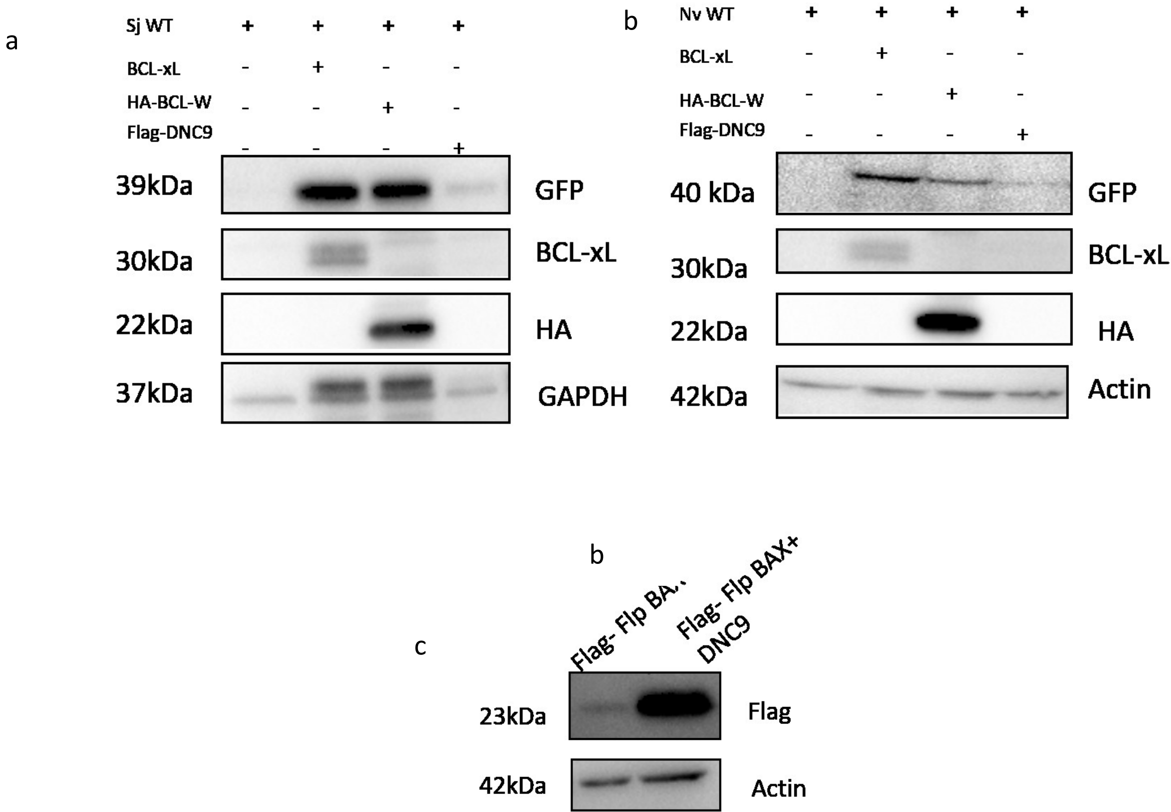
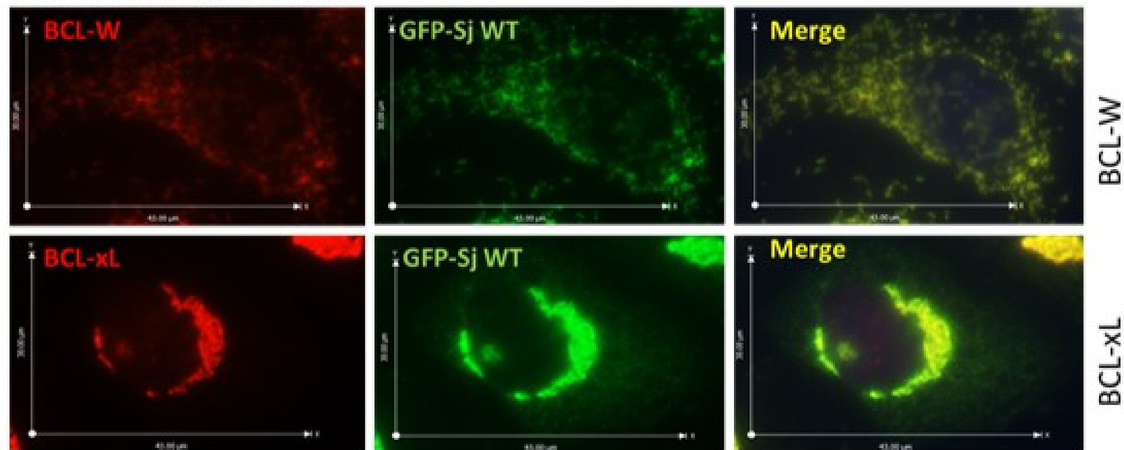
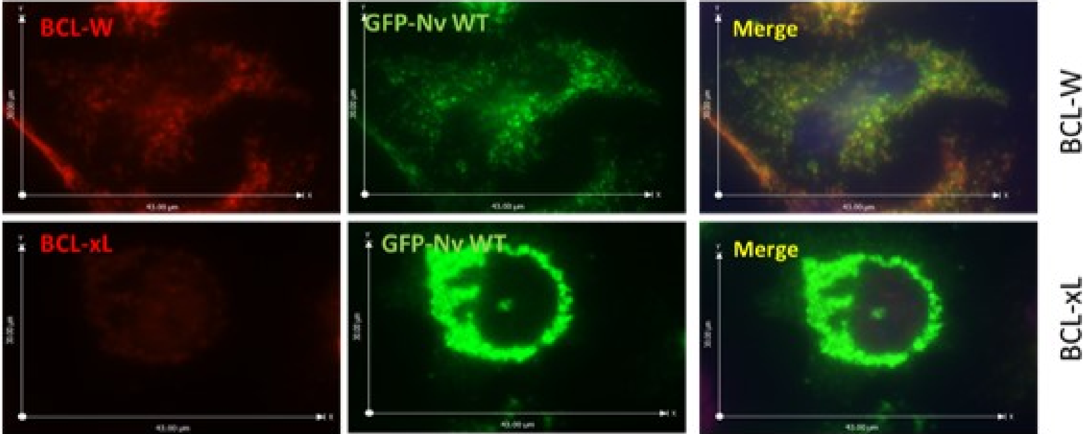


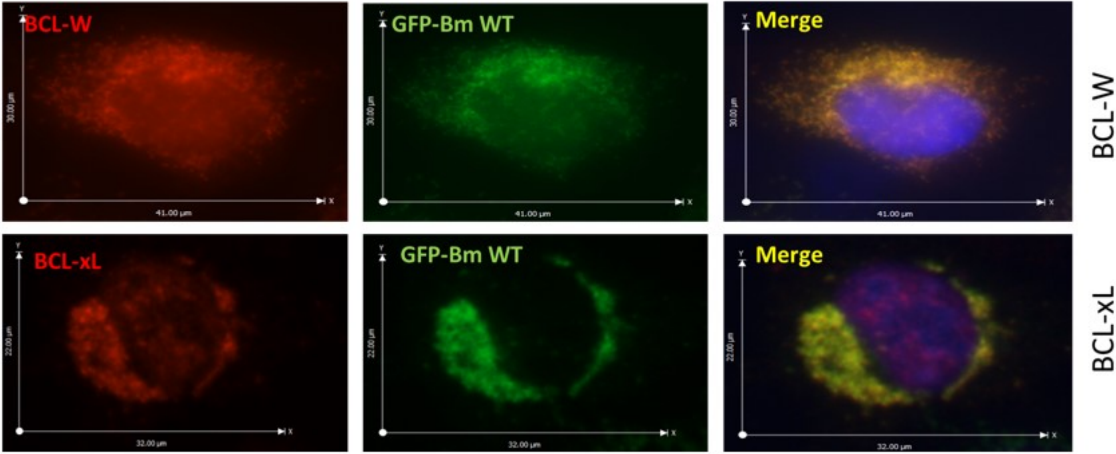
Fig. 8. Localization of toxic candidate BH3-only proteins with BCL-xL and BCL-W. Representative immunofluorescence images of HeLa cells fixed and stained 24 h post transfection with wild type (WT) toxic GFP-BH3-only constructs and BCL-xL (lower panels) or n-terminal HA tagged BCL-W(upper panels). The mitochondria were marked with antibodies to Tom20, nuclei are stained with Hoechst (blue), and candidate GFP-BH3-only proteins were detected with anti-GFP antibody (green). Panel a shows localization of Sj WT (green) with BCL-W (upper right, red) and BCL- xL (lower right, red). Note that co-transfection with anti-death proteins BCL-xL and BCL-W prevent long mitochondrial phenotype. Panel b shows localization of Bm WT (green) with BCL-W (upper right, red) and BCL- xL (lower right, red). Panel c shows localization of Nv WT (green) with BCL-W (upper right, red) and BCL- xL (lower right, red).



b Nematostella vectensis



c Brugia malayi



Potential role of BH3-only candidates to act as "indirect activator" BH3-proteins

We further addressed the possibility that these novel BH3-only candidate proteins could act by displacing mammalian BH3-containing pro-apoptotic proteins from the groove of anti-apoptotic proteins. For this, candidate BH3-only proteins were co-expressed with the toxic caspase-cleavage product of mammalian BH3-only protein tBID (truncated BID) to determine if the candidate BH3-only proteins could suppress the ability of anti-apoptotic BCL-xL or BCL-W to inhibit tBID-induced cell death. The co-expression of wild type or mutant candidate BH3-only proteins from *Mycobacterium sp* (Msp) has no effect on cell viability (Fig. 1), and when co-expressed with human tBID also had no effect on cell death compared to tBID alone (Fig. 10a). However, Msp reduced the ability of BCL-W, but apparently not BCL-xL, to suppress tBID-induced cell death by approximately 20%, raising the possibility that Msp may engage the BCL-W protein (Fig. 10a). However, the BH3 mutant version of Msp also had a similar effect, potentially suggesting non-specific toxicity of Msp revealed by stressing cells with tBID. An alternative potential interpretation is that the Msp proteins cause indirectly activate BAX or BAK. However, this seems unlikely cell death was not observed without the additional stress induced by tBID, as Msp alone is non-toxic (see Fig. 1). Results similar to Msp were also observed with other non-toxic BH3-only candidates *Gamma proteobacterium, Paenibacillus sp.*, *Streptomyces lividans*, *Sphaerobacter thermophiles and possibly E. coli* (Fig. 10d,e,g-i). In contrast, while the non-toxic candidate BH3-only protein Bf had no effect on the anti-apoptotic function of BCL-W, the wild type Bf but not its BH3 mutant completely abrogated the anti-death function of BCL-xL in this experiment (Fig. 10f). This could suggest that the

mutation in its BH3 domain could have abolished its ability to bind to BCL-xL and BCL-W, thereby rendering it unable to displace tBID from the binding groove.

All three cytotoxic BH3-only candidate proteins from *Nematostella vectensis*, *Brugia malayi* and *Schistosoma japonicum* suppressed the anti-apoptotic functions of BCL-xL and BCL-W (Fig. 10b,c,j), consistent with the ability of BCL-xL and BCL-W to suppress cell death induced by these three proteins (see Fig. 6). While the BH3 mutant versions of these three proteins were somewhat less effective. Results from this experiment can be interpreted in many ways, to confirm the possible role of these candidate BH3-only proteins in indirect activation of BAK/BAX, the next step would be to test them in $BAK^{-/-}$/$BAX^{-/-}$ MEFs (see Discussion).

Fig. 9. tBID death assay. tBID is a cytotoxic protein. Cells transfected by tBID itself should have considerable death. Co-transfection with an anti-death BCL-2 family member should rescue this cell death by binding tBID. If a BH3-only protein with some known affinity for the anti-death proteins is added to cells, competitive binding of this protein to the anti-death protein should liberate tBID, causing cells to die.

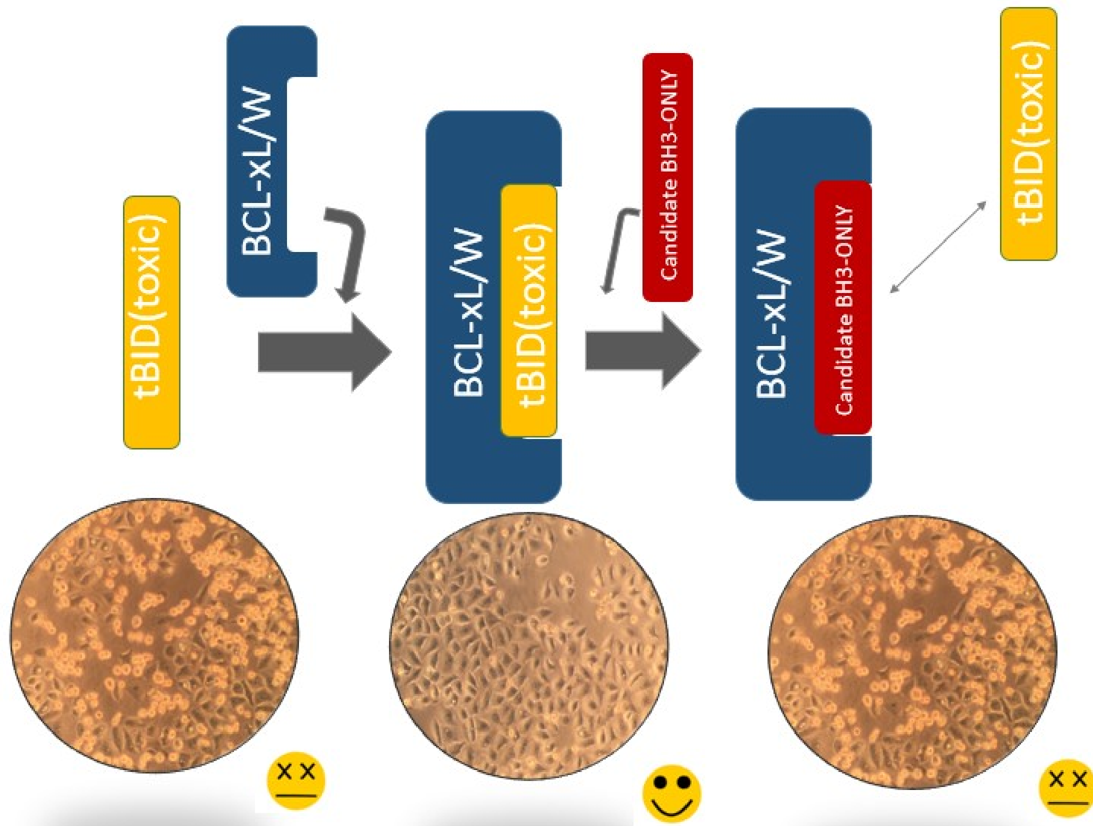
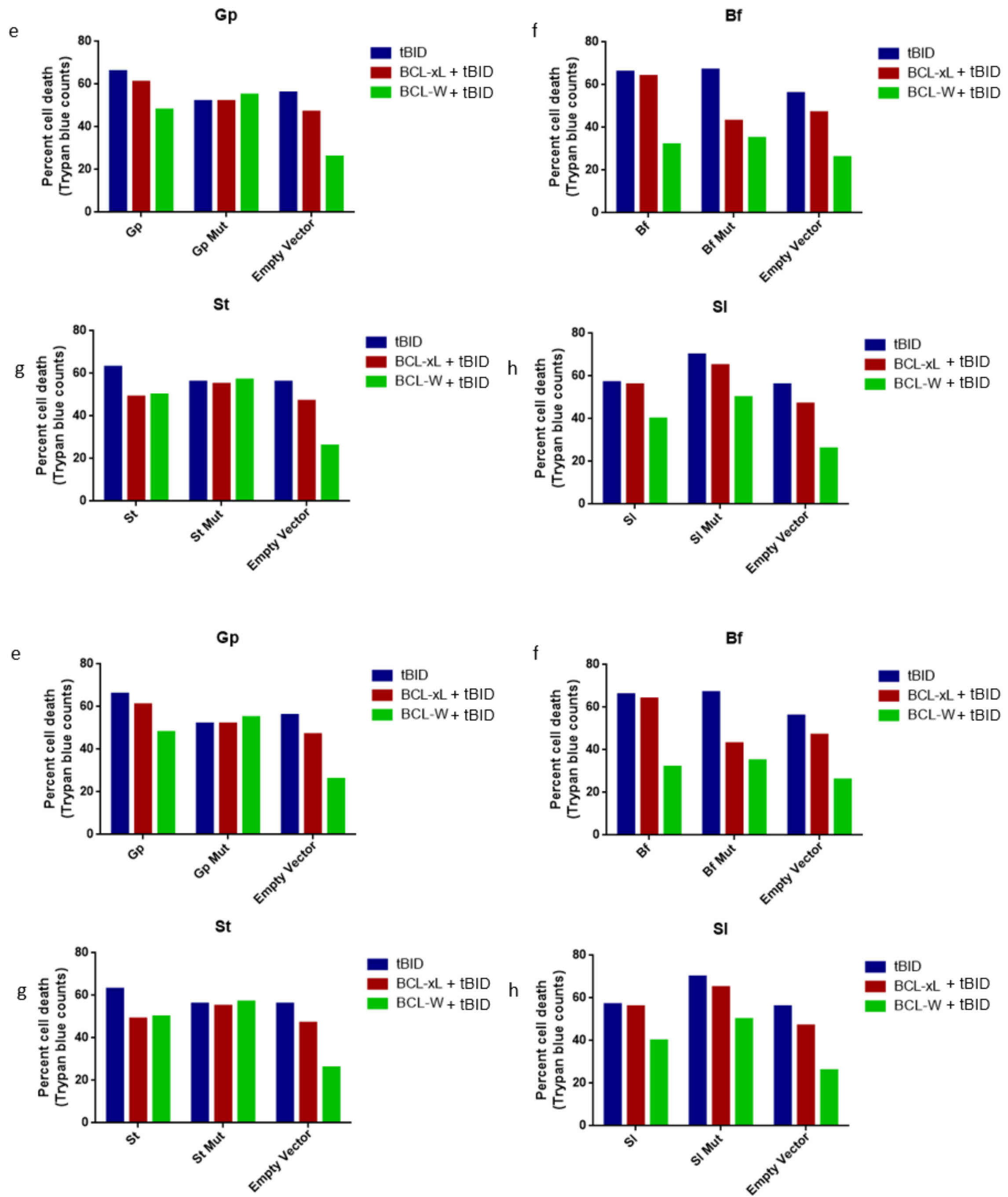
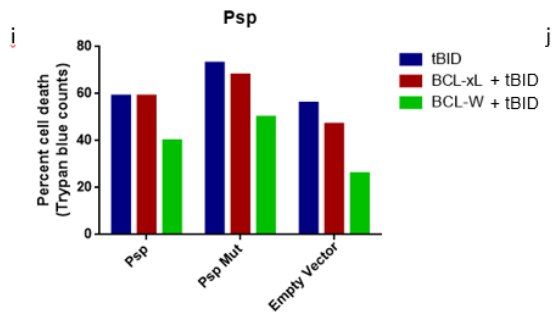


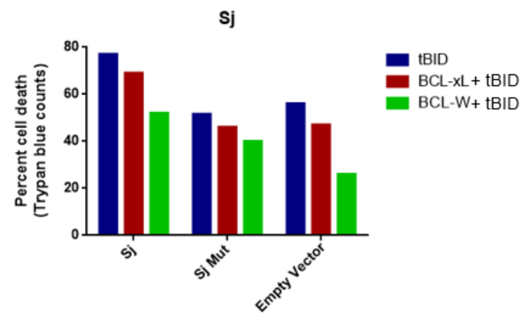
Fig. 10. tBID vs candidate BH3-only proteins. Representative experiment where HeLa cells were transfected with indicated plasmids and harvested 24h post transfection. Y-axis represents cell death in terms of percent of trypan positive cell n=1 with ~ 200 cells counted per sample.



i



j



Discussion

BH3 mimetics can be used against cancer, and have the potential to be used for auto-immune diseases and infectious diseases like schistosomiases, the problem is being able to discover candidate novel BH3-only proteins in the proteome in the absence of a signature sequence with which to search the preoteome, in order to find such targets. Our list of BH3-only proteins that were found using an algorithm implemented by Dr. Abdel Aouacheria (unpublished) are surprisingly all from non-mammalian sources. These sources include bacteria, filarial worms and even parasites like schistosomes (Table 1). The BCL-2 family proteins are not known to exist in these species other than in *Schistosoma japonicum* (Lee et al., 2014) but based on their sequences, all the 10 candidate proteins behaved similarly to 'real BH3-only proteins' when put through different binding assays and tests (Abdel Aouacheria, data unpublished). In our hands, all ten proteins and their mutants were expressed, although the bands for three of the killer WT proteins could not be detected (Fig, 2b, c and j), apparently not due to a lower expression of these proteins but due to their cytotoxic activity. The more minor differences in expression in the other pairs of non-toxic proteins could be due to a difference in transcription level or maybe even due to a difference in number of transfected cells, since the immunofluorescence images do not show a noticeable difference in intensity of GFP signal between different proteins (Figs. 3-5).

All of the candidate BH3-only proteins seemed to go to an intra-cellular membrane, except the BH3 mutants from *Brugia malayi* and *Nematostella vectensis*. However, since the constructs are N-terminally GFP tagged, it is possible that the tag can interfere with localization of the protein, for example, it can get retained in the nucleus. On further analyzing the sequence of BH3-only proteins using different online tools to predict the

presence of transmembrane domains, only seven out of the 10 had predicted transmembrane segments, however this prediction varies depending on which tool was used, since different tools use different algorithms and prediction methods. Only three proteins were consistently predicted to contain transmembrane segments by all three tools: *Mycobacterium sp.* and the toxic proteins from *Schistosoma japonicum* and *Neamtostella vectensis*. Using TMHMM, transmembrane segments were predicted in the proteins from *Mycobacterium sp* and *Branchistoma floridae*, even though their probability score was low. Using another tool, Octopus, transmembrane helices were predicted for the candidates from *Paenibacillus sp*, *Nematostella vectensis* and *Streptomyces lividans*. Using another tool, Phobius, transmembrane helices were predicted in BH3-only candidates from *Mycobacterium sp*. and *Branchistoma floridae*. All three tools predicted a C-terminal transmembrane helix in candidate protein from *Schistosoma japonicum* with high probability of occurrence. It should also be kept in mind that these tools are only prediction tools. There could be another way that these proteins are anchoring themselves onto membranes, possibly via lipid modifications or other mechanisms. It should be noted that the amino acid sequence of both the WT and Mut proteins from *Brugia malayi* have been modified on the Uniprot database after the plasmids for these constructs were made. After speaking with one of the curators from the database website, I learned that 43 amino acids were previously left out on the C-terminal of the protein. Future work for this project includes making new constructs with the complete updated sequence and testing for any difference in function and localization. Candidate BH3-only proteins from *Paenibacillus sp.* also have a predicted N-terminal signal peptide, however since these proteins are N-terminally GFP tagged, this could hinder with the function of the protein's signal peptide.

This construct needs to be cloned into a C-terminally tagged vector to study to function of its signal peptide.

Although all cells transfected with each of the three cytotoxic candidate BH3-only proteins from *Nematostella vectensis, Brugia malayi* and *Schistosoma japonicum* could be rescued by co-transfecting the cells with either BCL-xL or BCL-W (Fig. 6), two out of the three toxic candidate BH3-only proteins from *Nematostella vectensis* and *Schistosoma japonicum* could not be rescued by dominant negative caspase-9. A caveat of this experiment could be that we are not expressing enough dominant negative caspase-9 protein (since it can't be seen on a blot when co-transfected with these proteins either), a future experiment could be do increase the ratio of dominant negative caspase-9 as compared to the BH3-only protein. Another possibility can be that the candidate proteins from these two organisms are such hyper active killers that dominant negative caspase-9 is too slow in comparison to rescue cells from dying. To test this hypothesis, we would have to transfect dominant negative caspase-9 before transfecting either of the two killer proteins. To test whether all three cytotoxic proteins kill in a BAK/BAX dependent manner, future work is being done to express these proteins in $BAK^{-/-}/BAX^{-/-}$ double knockout MEFs. If death induced by the candidate BH3-only proteins from *Schistosom japonicum* and *Nematostella vectensis* is indeed non-apoptotic in nature, future work would include testing for different death pathways that could be initiated by candidate BH3-only proteins from *Nematostella vectensis* and *Schistosoma japonicum*. Some of the ways to test different death pathways could be to use inhibitors of different pathways for example, an iron chelator to test for ferroptosis, necrostatin to check for necrosis as well as spautin to check for autophagic cell death. Future work could also include testing the impact of

mitochondrial integrity, especially for candidate proteins from *Schistosoma japonicum* that causes elongation of mitochondria (Fig. 5 c) by using a Drp1 inhibitor to check for fission defects, test for cytochrome release etc.

The ability of these proteins to interact with tBID, a pro-death protein that it cytotoxic by itself and that binds to the hydrophobic grooves of BCL-xL and BCL-W was also tested (Fig. 10). Even though different proteins showed different results based on their own cytotoxic abilities, their interactions with BCL-xL and BCL-W as well as their functional interaction with tBID, it could be possible that a few of the proteins that caused an increase in cell death even in the presence of an anti-apoptotic protein like BCL-W, which when transfected by itself rescues tBID associated cell death (Fig. 10 a-j) could be doing so by indirectly activating BAX/BAK by binding to these anti-death proteins and neutralizing them. This would lead to a release in tBID which would explain the death even in the presence of anti-death proteins. WT proteins from *Mycobacterium sp.*, *Nematostella vectensis, Brugia Malayi, Sphaerobacter thermophiles* and *Schistosoma japonicum* were able to cause an increased cell death even in the presence of BCL-W (Fig. 10). However, proteins from *Nematostella vectensis, Brugia Malayi* and *Schistosoma japonicum* are toxic themselves and this could explain the increased cytotoxicity even in the presence of BCL-W. Proteins from *E.coli* show similar trends to the empty vector controls (Fig. 10d) suggesting that it may not be able to displace tBID from the binding groove and in-turn may not be able to indirectly activate BAK/BAX. *Gamma proteobacterium* candidate proteins showed a protection with BCL-W but not BCL-xL, similar results were seen for proteins from *Streptomyces lividans* (Fig. 10e,h) whereas the mutant didn't seem to show any protection at all, even in the presence of anti-death proteins (Fig. 10e), a similar result

was seen for proteins from *Sphaerobacter thermophiles* (Fig. 10 g) suggesting that the mutant could have displaced tBID from the binding groove, leading to liberation of tBID and killing of cells. WT proteins from *Branchistoma floridae* and *Paenibacillus sp*. show similar results. While protection is seen in the presence of BCL-W, BCL-xL fails to protect cells from dying (Fig. 10 f,i). Both BCL-xL and BCL-W fail to protect cells in the presence of tBID and WT cytotoxic protein from *Schistosoma japonicum* (Fig. 10j). The mutant however, which by itself is toxic, but not as toxic as its wild type counterpart, seems to show protection with BCL-W (Fig, 10j).

To fully understand and appreciate the functions of these candidate novel BH3-only proteins, we need to understand more about their day jobs and non-death functions(Gross & Katz, 2017).

However, if this algorithm can pull out real BH3-only proteins from the proteome in organisms that before this have no studied BCL-2 proteins, that would open a whole new are in BH3 mimetic development. We could now use BH3 mimetics not only to treat cancer but also bacterial and parasitic infections, which could be the need of the hour given emerging antibiotic resistance in these organisms. Another idea is that the discovery of genuine BH3 motifs in proteins from non-metazoan species poses the question of the identity, structure and function of their endogenous receptors. These natural receptors might also have homologs in metazoan species, which could interact with SLiMs or other motifs. If so, the interaction between BH3 motifs and Bcl-2 family proteins might represent only one instance of a broader interactome.

References

Adams, J. M., & Cory, S. (1998). The bcl-2 protein family: Arbiters of cell survival. *Science (New York, N.Y.), 281*(5381), 1322-1326.

Aouacheria, A., Combet, C., Tompa, P., & Hardwick, J. M. (2015). Redefining the BH3 death domain as a 'short linear motif'. *Trends in Biochemical Sciences, 40*(12), 736-748. doi:10.1016/j.tibs.2015.09.007

Aouacheria, A., de Laval, V. R., Combet, C., & Hardwick, J. M. (2013a). Evolution of bcl-2 homology (BH) motifs – homology versus homoplasy. *Trends in Cell Biology, 23*(3), 103-111. doi:10.1016/j.tcb.2012.10.010

Aouacheria, A., de Laval, V. R., Combet, C., & Hardwick, J. M. (2013b). Evolution of bcl-2 homology (BH) motifs – homology versus homoplasy. *Trends in Cell Biology, 23*(3), 103-111. doi:10.1016/j.tcb.2012.10.010

Baell, J. B., & Huang, D. C. S. (2002). Prospects for targeting the bcl-2 family of proteins to develop novel cytotoxic drugs. *Biochemical Pharmacology, 64*(5-6), 851-863.

Billard, C. (2013). BH3 mimetics: Status of the field and new developments. *Molecular Cancer Therapeutics, 12*(9), 1691. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23974697

Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Köntgen, F., . . . Strasser, A. (1999). Proapoptotic bcl-2 relative bim required for certain apoptotic

responses, leukocyte homeostasis, and to preclude autoimmunity. *Science (New York, N.Y.), 286*(5445), 1735-1738.

Burrer, C. M., Foight, G. W., Keating, A. E., & Chan, G. C. (2016). Selective peptide inhibitors of antiapoptotic cellular and viral bcl-2 proteins lead to cytochrome c release during latent kaposi's sarcoma-associated herpesvirus infection. *Virus Research, 211*, 86-88. doi:10.1016/j.virusres.2015.10.007

Chattopadhyay, A., Chiang, C. W., & Yang, E. (2001). BAD/BCL-[X(L)] heterodimerization leads to bypass of G0/G1 arrest. *Oncogene, 20*(33), 4507-4518. doi:10.1038/sj.onc.1204584

Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., . . . Huang, D. C. S. (2005a). Differential targeting of prosurvival bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Molecular Cell, 17*(3), 393-403. doi:10.1016/j.molcel.2004.12.030

Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., . . . Huang, D. C. S. (2005b). Differential targeting of prosurvival bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Molecular Cell, 17*(3), 393-403. doi:10.1016/j.molcel.2004.12.030

Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., . . . Huang, D. C. S. (2005c). Differential targeting of prosurvival bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Molecular Cell, 17*(3), 393-403. doi:10.1016/j.molcel.2004.12.030

Cory, S., Huang, D., & Adams, J. M. (2003). The bcl-2 family: Roles in cell survival and oncogenesis. *Oncogene, 22*(53), 8590-8607. doi:10.1038/sj.onc.1207102

Cory, S., & Adams, J. M. (2002). The Bcl2 family: Regulators of the cellular life-or-death switch. *Nature Reviews Cancer, 2*(9), 647-656. doi:10.1038/nrc883

Coultas, L., & Strasser, A. (2003). The role of the bcl-2 protein family in cancer. *Seminars in Cancer Biology, 13*(2), 115-123. doi:10.1016/S1044-579X(02)00129-3

Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. *Nature Reviews Molecular Cell Biology, 15*(1), 49-63. doi:10.1038/nrm3722

Danial, N. N., Gramm, C. F., Scorrano, L., Zhang, C., Krauss, S., Ranger, A. M., . . . Korsmeyer, S. J. (2003). BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature, 424*(6951), 952-956. doi:10.1038/nature01825

Delbridge, A. R. D., & Strasser, A. (2015). The BCL-2 protein family, BH3-mimetics and cancer therapy. *Cell Death & Differentiation, 22*(7), 1071-1080. doi:10.1038/cdd.2015.50

Delbridge, A. R. D., Grabow, S., Strasser, A., & Vaux, D. L. (2016). Thirty years of BCL-2: Translating cell death discoveries into novel cancer therapies. *Nature Reviews Cancer, 16*(2), 99-109. doi:10.1038/nrc.2015.17

Diella, F., Haslam, N., Chica, C., Budd, A., Michael, S., Brown, N. P., . . . Gibson, T. J. (2008). Understanding eukaryotic linear motifs and their role in cell signaling and regulation. *Frontiers in Bioscience: A Journal and Virtual Library, 13*, 6580-6603.

Egle, A., Harris, A. W., Bouillet, P., & Cory, S. (2004). Bim is a suppressor of myc-induced mouse B cell leukemia. *Proceedings of the National Academy of Sciences of the United States of America, 101*(16), 6164-6169. doi:10.1073/pnas.0401471101

Ellis, H. M., & Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell, 44*(6), 817-829.

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology, 35*(4), 495-516. doi:10.1080/01926230701320337

Glab, J. A., Mbogo, G. W., & Puthalakath, H. (2017). Chapter five - BH3-only proteins in health and disease. In L. Galluzzi (Ed.), *International review of cell and molecular biology* (pp. 163-196) Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/S1937644816300740

Green, D. R., & Reed, J. C. (1998). Mitochondria and apoptosis. *Science; Washington, 281*(5381), 1309-12. Retrieved from http://search.proquest.com/docview/213554268/abstract/E222359CC6004D3FPQ/1

Gross, A., McDonnell, J. M., & Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. *Genes & Development, 13*(15), 1899-1911. doi:10.1101/gad.13.15.1899

Gross, A., & Katz, S. G. (2017). Non-apoptotic functions of BCL-2 family proteins. *Cell Death and Differentiation,* doi:10.1038/cdd.2017.22

Han, J., Flemington, C., Houghton, A. B., Gu, Z., Zambetti, G. P., Lutz, R. J., . . . Chittenden, T. (2001). Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. *Proceedings of the National Academy of Sciences of the United States of America, 98*(20), 11318-11323. doi:10.1073/pnas.201208798

Hanada, M., Aimé-Sempé, C., Sato, T., & Reed, J. C. (1995). Structure-function analysis of bcl-2 protein. identification of conserved domains important for homodimerization with bcl-2 and heterodimerization with bax. *The Journal of Biological Chemistry, 270*(20), 11962-11969.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70. doi:10.1016/S0092-8674(00)81683-9

Hardwick, J. M., Chen, Y., & Jonas, E. A. (2012). Multipolar functions of BCL-2 proteins link energetics to apoptosis. *Trends in Cell Biology, 22*(6), 318-328. doi:10.1016/j.tcb.2012.03.005

Hardwick, J. M., & Soane, L. (2013). Multiple functions of BCL-2 family proteins. *Cold Spring Harbor Perspectives in Biology, 5*(2), a008722. doi:10.1101/cshperspect.a008722

Hengartner, M. O., & Horvitz, H. R. (1994a). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell, 76*(4), 665-676.

Hengartner, M. O., & Horvitz, H. R. (1994b). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell, 76*(4), 665-676.

Huang, D. C. S., & Strasser, A. (2000a). BH3-only Proteins—Essential initiators of apoptotic cell death. *Cell, 103*(6), 839-842. doi:10.1016/S0092-8674(00)00187-2

Huang, D. C. S., & Strasser, A. (2000b). BH3-only Proteins—Essential initiators of apoptotic cell death. *Cell, 103*(6), 839-842. doi:10.1016/S0092-8674(00)00187-2

Kotschy, A., Szlavik, Z., Murray, J., Davidson, J., Maragno, A. L., Le Toumelin-Braizat, G., . . . Geneste, O. (2016). The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature, 538*(7626), 477-482. doi:10.1038/nature19830

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., . . . Melino, G. (2009). Classification of cell death: Recommendations of the nomenclature committee on cell death 2009. *Cell Death and Differentiation, 16*(1), 3-11. doi:10.1038/cdd.2008.150

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., & Newmeyer, D. D. (2005). BH3 domains of BH3-only proteins differentially regulate bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Molecular Cell, 17*(4), 525-535. doi:10.1016/j.molcel.2005.02.003

Lamers, F., Schild, L., den Hartog, Ilona J M, Ebus, M. E., Westerhout, E. M., Ora, I., . . . Molenaar, J. J. (2012). Targeted BCL2 inhibition effectively inhibits neuroblastoma tumour growth. *European Journal of Cancer (Oxford, England: 1990), 48*(16), 3093-3103. doi:10.1016/j.ejca.2012.01.037

Lanave, C., Santamaria, M., & Saccone, C. (2004). Comparative genomics: The evolutionary history of the bcl-2 family. *Gene, 333*, 71-79. doi:10.1016/j.gene.2004.02.017

Lee, E. F., Clarke, O. B., Evangelista, M., Feng, Z., Speed, T. P., Tchoubrieva, E. B., . . . Fairlie, W. D. (2011). Discovery and molecular characterization of a bcl-2–regulated cell death pathway in schistosomes. *Proceedings of the National Academy of Sciences of the United States of America, 108*(17), 6999-7003. doi:10.1073/pnas.1100652108

Lee, E. F., Young, N. D., Lim, N. T. Y., Gasser, R. B., & Fairlie, W. D. (2014). Apoptosis in schistosomes: Toward novel targets for the treatment of schistosomiasis. *Trends in Parasitology, 30*(2), 75-84. doi:10.1016/j.pt.2013.12.005

Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., & Korsmeyer, S. J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell, 2*(3), 183-192. doi:10.1016/S1535-6108(02)00127-7

Llambi, F., Moldoveanu, T., Tait, S. W. G., Bouchier-Hayes, L., Temirov, J., McCormick, L. L., . . . Green, D. R. (2011). A unified model of mammalian BCL-2

protein family interactions at the mitochondria. *Molecular Cell, 44*(4), 517-531. doi:10.1016/j.molcel.2011.10.001

Lomonosova, E., & Chinnadurai, G. (2008a). BH3-only proteins in apoptosis and beyond: An overview. *Oncogene, 27*(Suppl 1), 2. doi:10.1038/onc.2009.39

Lomonosova, E., & Chinnadurai, G. (2008b). BH3-only proteins in apoptosis and beyond: An overview. *Oncogene, 27*(Suppl 1), 2. doi:10.1038/onc.2009.39

Mestre-Escorihuela, C., Rubio-Moscardo, F., Richter, J. A., Siebert, R., Climent, J., Fresquet, V., . . . Martinez-Climent, J. A. (2007). Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. *Blood, 109*(1), 271-280. doi:10.1182/blood-2006-06-026500

Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., . . . Fesik, S. W. (1996). X-ray and NMR structure of human bcl-xL, an inhibitor of programmed cell death. *Nature, 381*(6580), 335-341. doi:10.1038/381335a0

Obata, T., Toyota, M., Satoh, A., Sasaki, Y., Ogi, K., Akino, K., . . . Imai, K. (2003). Identification of HRK as a target of epigenetic inactivation in colorectal and gastric cancer. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 9*(17), 6410-6418.

Rautureau, G. J. P., Day, C. L., & Hinds, M. G. (2010). Intrinsically disordered proteins in bcl-2 regulated apoptosis. *International Journal of Molecular Sciences, 11*(4), 1808-1824. doi:10.3390/ijms11041808

Roberts, A. W., Davids, M. S., Pagel, J. M., Kahl, B. S., Puvvada, S. D., Gerecitano, J. F., . . . Seymour, J. F. (2016). Targeting BCL2 with venetoclax in relapsed chronic lymphocytic leukemia. *New England Journal of Medicine, 374*(4), 311-322. doi:10.1056/NEJMoa1513257

Rosenberg, S. H. (2011). Mammalian apoptosis in a parasitic worm. *Proceedings of the National Academy of Sciences of the United States of America, 108*(17), 6695-6696. doi:10.1073/pnas.1104151108

Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., . . . Fesik, S. W. (1997). Structure of bcl-xL-bak peptide complex: Recognition between regulators of apoptosis. *Science (New York, N.Y.), 275*(5302), 983-986.

Shamas-Din, A., Brahmbhatt, H., Leber, B., & Andrews, D. W. (2011). BH3-only proteins: Orchestrators of apoptosis. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(4), 508-520. doi:10.1016/j.bbamcr.2010.11.024

Sharma, J., Srinivasan, R., Majumdar, S., Mir, S., Radotra, B. D., & Wig, J. D. (2005). Bcl-XL protein levels determine apoptotic index in pancreatic carcinoma. *Pancreas, 30*(4), 337-342.

Srinivasula, S. M., Ahmad, M., Guo, Y., Zhan, Y., Lazebnik, Y., Fernandes-Alnemri, T., & Alnemri, E. S. (1999). Identification of an endogenous dominant-negative short isoform of caspase-9 that can regulate apoptosis. *Cancer Research, 59*(5), 999-1002.

Thomadaki, H., & Scorilas, A. (2006). BCL2 family of apoptosis-related genes: Functions and clinical implications in cancer. *Critical Reviews in Clinical Laboratory Sciences, 43*(1), 1-67. doi:10.1080/10408360500295626

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science; Washington, 267*(5203), 1456. Retrieved from http://search.proquest.com/docview/213548671/abstract/96DE8CDDEE314F00PQ/1

Tompa, P., Davey, N., Gibson, T., & Babu, M. M. (2014). A million peptide motifs for the molecular biologist. *Molecular Cell, 55*(2), 161-169. doi:10.1016/j.molcel.2014.05.032

Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., & Croce, C. M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science (New York, N.Y.), 226*(4678), 1097-1099.

van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P. E., . . . Huang, D. C. S. (2006). The BH3 mimetic ABT-737 targets selective bcl-2 proteins and efficiently induces apoptosis via bak/bax if mcl-1 is neutralized. *Cancer Cell, 10*(5), 389-399. doi:10.1016/j.ccr.2006.08.027

Vaux, D. L., Cory, S., & Adams, J. M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature, 335*(6189), 440-442. doi:10.1038/335440a0

Vogler, M., Weber, K., Dinsdale, D., Schmitz, I., Schulze-Osthoff, K., Dyer, M. J. S., & Cohen, G. M. (2009). Different forms of cell death induced by putative BCL2 inhibitors. *Cell Death and Differentiation, 16*(7), 1030-1039. doi:10.1038/cdd.2009.48

Wick, W., Petersen, I., Schmutzler, R. K., Wolfarth, B., Lenartz, D., Bierhoff, E., . . . von Deimling, A. (1996). Evidence for a novel tumor suppressor gene on chromosome 15 associated with progression to a metastatic stage in breast cancer. *Oncogene, 12*(5), 973-978.

Xiang, Z., Luo, H., Payton, J. E., Cain, J., Ley, T. J., Opferman, J. T., & Tomasson, M. H. (2010). Mcl1 haploinsufficiency protects mice from myc-induced acute myeloid leukemia. *The Journal of Clinical Investigation, 120*(6), 2109-2118. doi:10.1172/JCI39964

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology, 9*(1), 47-59. doi:10.1038/nrm2308

Yuan, J. Y., & Horvitz, H. R. (1990). The caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. *Developmental Biology, 138*(1), 33-41.

PRERNA SURI

New Delhi, India| September 23rd 1993
500 West University Parkway, Apt 6N, Baltimore, MD, 21210
(908)-636-9310| prerna2309@gmail.com| LinkedIn: https://www.linkedin.com/in/prerna-suri-b681a78b

EDUCATION

Master of Science (ScM) in Molecular Microbiology and Immunology **Expected May 2017**
Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Bachelor of Technology (B Tech) in Biotechnology **Jun 2015**
Amity Institute of Biotechnology, Amity University, Noida, UP, India

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD **Oct 2015 - Present**
Department of Molecular Microbiology and Immunology

ScM Graduate Student Researcher

- Thesis research on novel, putative BH-3 only family proteins from non-mammalian organisms, investigating their role in cell death or protection thereof
- Project involves immunostaining, microscopy, cloning and western blots; DNA extraction and isolation techniques, PCR applied among other basic lab techniques including tissue culture, transfection. Working with mammalian cell lines.
- Developing and conducting cell death, proliferation and cell viability assays
- Extensive literature review and trouble shooting

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD **Sep 2015 - Present**
Department of Molecular Microbiology and Immunology

Graduate Research Assistant

- Working with rodent model of malaria, maintenance of an m-cherry tagged, genetically engineered parasite life cycle in rodents and mosquitoes, handling (rearing and maintenance) of mosquitoes including dissection
- Collection of parasites- oocysts and sporozoites from salivary glands and midguts of mosquitoes
- Handling mice- dissection, injections-IP, ID and IV
- Preparation of lysates from various organs
- Blood smears and routine checking of parasite load in mice using a microscope
- Other techniques used include DNA extraction, preparation and PCR
- Maintenance of lab- buffers, chemicals and reagents

Amity University, Noida, UP, India **Jan 2015 – May 2015**
Amity Institute of Virology and Immunology: Thesis research on Survivin - an inhibitor of apoptosis protein

Research Trainee

- Project involved thorough examination of existing literature and studying existing models of protein function and synthesis
- Achieved successful cloning of both human and mouse Survivin into expression vectors, induction of protein synthesis and purification
- Project demanded DNA extraction, PCR, ligation and basic microbiology techniques to be carried out regularly including restriction and digestion of insert into different vectors
- Basic cell culture and maintenance of suspension cell lines also perfumed
- Project involved training on SDS-PAGE at IIT-Delhi following which SDS-PAGE to check for protein expression was routinely performed

Max Hospital, New Delhi, India **May 2014-July 2014**
Department of Pathology- Biochemistry

Research Trainee

- Routinely carried out analysis of urine samples to test for presence of monoclonal free light chains
- Electrophoretically determining presence of these light chains to diagnose the presence of renal dysfunctions in patient samples
- Conducted rapid dot tests for malaria, dengue and HIV
- Other techniques learned include ELISA, reading of flow cytometry outputs and sample collection

Medanta-the Medcity, Gurgaon, Haryana, India May 2013-July 2013

Research trainee, Department of Microbiology

- Worked on a project involving detection of Epstein Barr Virus in liver transplant patients
- Other work done includes basic microbiological assays like streaking, plating and screening for bacterial growth/ contamination in patient urine, blood, CSF and stool samples
- Microscopy and gram staining to confirm bacterial specimen and diagnostics on antibiotic resistance and specificity of positive cultures.
- Basic lab safety training
- Catalogued and managed patient data entry and analysis

PUBLICATIONS

Garg, H., **Suri, P**., Gupta, J. C., Talwar, G. P., & Dubey, S. (2016). Survivin: a unique target for tumor therapy. *Cancer Cell International*, *16* (1), 49

COMMUNITY/SOCIAL SERVICE

Khushi Shelter, India **Jun 2011, 1month**

- Taught verbal and spoken English at an orphanage to children aged 5-8 years

Abhiyaan- Amity Institute of Biotechnology, Social Committee **2012-2015**

- Organized and led school wide donation drives to collect material and help people during times of natural disaster including the Nepal Earthquake, 2014 and Jammu and Kashmir Quake, 2013
- Led a team of over 20 people for Human Values Quarter, 2014 and 2015- Amity University to create awareness about humanitarian values, equality and woman empowerment

OTHER WORK EXPERIENCE

Teaching Assistant- Johns Hopkins Bloomberg School of Public Heath **Oct 2016-Dec 2016**

- Course- Biology of Parasitism
- Work involved teaching laboratory classes, compiling and distributing documents, moderating review of literature discussions and spear heading review sessions
-

Bercos Restaurants Pvt. Ltd, India **May 2011, 1month**

- Worked as a social media and communications intern.
- Conceptualized facebook and other social media pages for the company.

AWARDS, HONORS AND CERITIFICATIONS

- Amity University, India on Admission Scholarship, 2011
- AU-British Standards Institute Six Sigma, Green Belt Certification, 2012
- Shri Baljeet Shashtri Award for "Social and Humanitarian Values", 2015

POSITIONS OF RESPONSIBILITIES

- Johns Hopkins Bloomberg School of Public Health Student Assembly- departmental representative, member of finance and appropriations committee and communications and external affairs committee 2016-17
  Work involved reviewing and approving budgets for Student groups during the school's fiscal year, managing communication and events newsletter for the entire school and serving as a departmental liaison to the student assembly
- Johns Hopkins Bloomberg School of Public Health, Department of Molecular Microbiology and Immunology- Social Coordinator 2016-17
- Indian Graduate Students Association, Johns Hopkins University, Co-Chair of Public Relations, 2016-17
- Amity University, Youth Festival Core Coordinator - Cultural Affairs 2014-15
  Budgeted events at a university level, including sponsorship and awards. Led a group of 15 volunteers for successful coordination and completion of cultural events during the youth festival
- Amity Institute of Biotechnology, Student Body - Head Coordinator, 2013 – 2015