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Inhibition of Caspase Activation by Small Heat Shock Protein, HspB2

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ABSTRACT

Inhibition of Caspase Activation by the Small Heat Shock Protein, HspB2

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HspB2 was discovered as a chaperone of muscular dystrophy protein kinase (DMPK) and originally named, DMPK binding protein (MKBP) and presently called HspB2. Functionally, MKBP binds to DMPK and stabilizes its kinase activity, thereby accelerating the progression of muscular dystrophy. Although other binding partners of HspB2 have been found, the MKBP-DMPK interaction is only case where the function of HspB2 has been carefully analyzed. As one of ten small heat shock proteins, HspB2 shares high homology to both Hsp27 and αB-crystallin. These and other heat shock proteins have been shown to accelerate breast cancer progression through inhibition of apoptosis. In contrast to Hsp27 and αB-crystallin, the role of HspB2 in apoptosis has not been examined. The work detailed in this dissertation establishes a new anti-apoptotic function of HspB2 in a breast cancer model. Overexpression of HspB2 results in a novel inhibition of apoptosis in breast cancer cell lines shown by nuclear morphology assays and FACS analysis when stimulated with TRAIL or TNFα. In correlation with in vitro experiments, expression of HspB2 confers resistance to xenograft tumor regression following treatment with TRAIL. Inhibition of both upstream and downstream caspases was demonstrated by fluorogenic caspase activity assays and western blotting of TRAIL treated cells expressing

HspB2. In contrast, mitochondrial cytochrome c release measured by western blotting and ELISA was not prevented by expression of HspB2. Furthermore, the presence of HspB2 conferred partial protection from apoptosis induced by tBid expression. These experiments aided in defining an anti-apoptotic function for HspB2 in downstream caspase inhibition. These results suggest that HspB2 may associate with caspase-3, subsequently inhibiting apoptosis. In summary, HspB2 expression in breast cancer cells inhibits apoptosis induced by extrinsic pathway stimulants and this anti-apoptotic activity has been mapped to caspases downstream of the mitochondria. These studies describe a novel anti-apoptotic function for HspB2 as an inhibitor of caspases in breast cancer.

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ABBREVIATIONS

AIF.....apoptosis inducing factor

APAF-1.....apoptotic peptidase activating factor 1

ATP.....adenosine triphosphate

BAD.....bcl-2 antagonist of cell death

BAG.....bcl-2 associated athanogene

BAK.....bcl-2 homologous antagonist/killer

BAX.....bcl-2 associated X protein

BCL-2.....b-cell leukemia/lymphoma 2

BCL-x_L.....b-cell leukemia/lymphoma X_L

BH.....bcl-2 homology domain

CAD.....caspase activated deoxyribonuclease

CARD.....caspase Recruitment Domain

CLK4.....cdc2/cdc28-like 4

CDM.....congenital muscular dystrophy

DcR1/2.....decoy receptor 1/2

DD.....death domain

DED.....death effector domain

DISC.....death inducing signaling complex

DKO..... double knock out

DM.....myotonic dystrophy

DMPK.....myotonic dystrophy protein kinase

DMS.....dimethylsuberimidate

DR.....death receptor

EDTA.....ethylene-diamine-tetra-acetic acid

EGF.....epidermal growth factor

ER.....estrogen receptor

FADD.....fas associated death domain

FLIP.....FLICE-inhibitory protein

FRET.....fluorescence resonance energy transfer

GFP.....green fluorescent protein

GM.....geldamycin

GST.....glutathione S-transferase

HEK.....human embryonic kidney

HER-2.....human epidermal growth factor receptor 2

HOP.....hsp70/hsp90 organizing protein

HSE.....heat shock response element

HSP.....heat shock protein

HTT.....huntingtin protein

IAP.....inhibitor of apoptosis protein

ICAD.....inhibitor of caspase activated deoxyribonuclease

IGFBP-3.....insulin-like growth factor binding protein 3

JNK.....jun n-terminal kinase

LIT.....lymphocyte inhibitor of tnf receptor apoptosis inducing ligand

MAPK.....mitogen-activated protein kinase

MAPKAP.....mitogen-activated protein kinase-activated protein

MKBP.....myotonic dystrophy protein kinase binding protein

MM.....multiple myeloma

MPT.....mitochondrial pore transition

NaSCN.....sodium thiocyanate

NFκB.....nuclear factor kappa b

OPG.....osteoprogesterone

PARP.....poly(ADP-ribose)polymerase

PBS.....phosphate-buffered saline

PKC.....protein kinase c

PLAD.....preligand assembly domain

PMSF.....phenylmethylsulphonyl fluoride

PROMM.....proximal myotonic myopathy

RIP.....receptor interacting protein

RIPA.....radio immunoprecipitation assay

RPTC..... renal proximal tubule cells

SDS-PAGE.....sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMAC/DIABLO.....second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI

TNF.....tumor necrosis factor

TNFR.....tumor necrosis factor receptor

TR3/4.....tumor necrosis-related apoptosis inducing ligand receptor

TRADD.....tumor necrosis-related associated death domain

TRAIL.....tumor necrosis-related apoptosis inducing ligand

TRAILR.....tumor necrosis-related apoptosis inducing ligand receptor

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1. INTRODUCTION

1.1 APOPTOSIS

Apoptosis or programmed cell death has a wide variety of functions both in normal development and in disease. Under normal physiologic conditions, apoptosis ensures proper development by removing interdigital mesenchymal cells to shape limbs as well as maintaining the immune system through removal of activated T-cells (Chen and Zhao, 1998; Danial and Korsmeyer, 2004; Ellis and Horvitz, 1986; Schultz and Harrington, 2003; Vaux and Strasser, 1996). Early apoptotic studies conducted in *Caenorhabditis elegans* revealed an elegant system of checks and balances regulating development (Ellis and Horvitz, 1986). Four proteins comprise this system: the pro-apoptotic proteins, Egl-1, Ced-3, and Ced-4, and anti-apoptotic protein, Ced-9. These proteins are homologous to mammalian apoptotic BH3-only proteins, caspases, Apaf-1, and Bcl-2 proteins respectively. Egl-1 interacts with anti-apoptotic Ced-9 which is bound to Ced-4 (Chinnaiyan et al., 1997b; Conradt and Horvitz, 1998). This interaction releases Ced-4 which activates Ced-3 and leads to apoptosis (Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Yuan et al., 1993). Mammalian cells posses a more complex system with multiple caspases and survival proteins (Strasser et al., 2000). Apoptosis also differentially affects disease progression. In neurological disorders, an increase in the rate of apoptosis leads to a poor prognosis (Friedlander, 2003). For example, Huntington's Disease is a neurological disorder caused by an expanded CAG repeat in the huntingtin protein (Htt) which is a major anti-apoptotic protein in the brain (Pattison et al., 2006). Accumulation of these CAG repeats in Htt

results in the loss of its protective effects and apoptosis ensues to contribute to decreased motor skills, memory, and speech (Pattison et al., 2006). In many types of carcinoma, the lack of apoptotic activity seems to increase the severity of tumorigenesis by maintaining mutated proteins such as p53 and c-Myc, when they should targeted for removal (Hanahan and Weinberg, 2000; Jaattela, 1999).

The majority of apoptosis occurs through the activation of two different mechanisms activated by different stimuli. The extrinsic pathway responds to external cellular stimuli and is dependent on ligand binding to a receptor, whereas the intrinsic pathway is ligand independent and is activated by internal signals such as DNA damage or heat stress (Fig 1). The ligands of the extrinsic pathway are all members of the TNF superfamily (Ashkenazi and Dixit, 1999). The most studied receptor-ligand pair is Fas/CD95/Apo-1 and Fas ligand/Apo-1L respectively. Another well studied ligand is TNFα which can bind to its TNF receptor-1 (TNFR-1) and TNFα-related apoptosis-inducing ligand (TRAIL) that binds to its corresponding receptors, DR4 and DR5 (LeBlanc and Ashkenazi, 2003). Ligand binding results in receptor trimerization and subsequent activation. A death domains (DD) located on the internal portion of the receptor recruits adaptor molecules, TRADD (TNFα associated death domain) and FADD (Fas associated death domain), to the cell surface via protein-protein interactions. These adaptor proteins contain death effector domains which attract monomeric molecules of apical procaspases to the cell surface (Tibbetts et al., 2003). The protein complex of death-inducing ligand, death receptor, adaptor molecules, and apical caspases is known as the DISC or death inducing signaling complex (Medema et al., 1997). FasL and TRAIL bind to their respective receptors

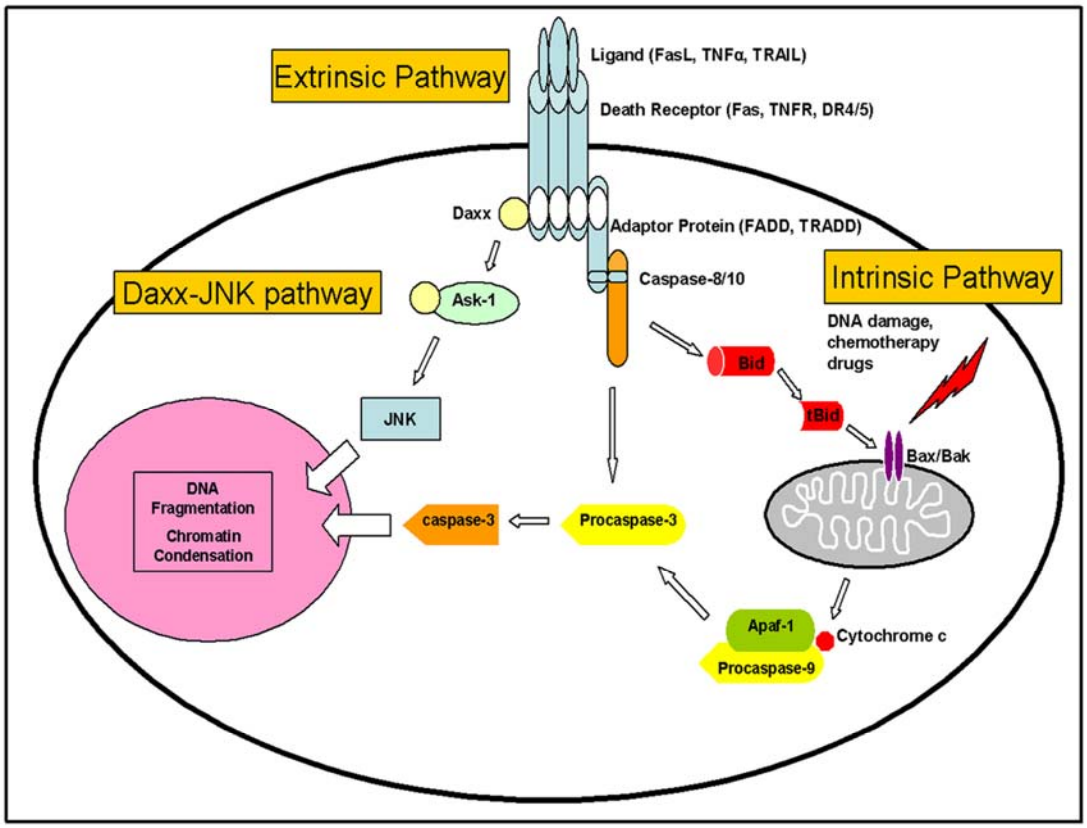


Figure 1: Apoptotic signaling pathways.

The two main pathways of apoptosis are the extrinsic pathway, which uses cell surface receptors, and the internally triggered intrinsic pathway. An additional pathway is also activated via cell surface receptors, but signals through Daxx, Ask-1, and JNK, and is caspase independent.

and recruit adaptor protein FADD in order to form the DISC. Alternatively, TNFα and its receptor have the ability to interact with two adaptor proteins, FADD or TRADD. The canonical pathway of TNFα signaling involves TNFα interacting with its receptor, TNFR-1, which then recruits the adaptor protein TRADD. TRADD then recruits RIP, TRAF2, and FADD which can associate with apical caspase-8 (Chen and Goeddel, 2002). The last step in this pathway is the direct cleavage and activation of downstream effector caspases such as caspase-3 by apical caspases. The active effector caspases then induce degradation of many cellular substrates. Two well characterized caspase substrates are the inhibitor of caspase-activated deoxyribonuclease (ICAD) which releases CAD nuclease and poly (ADP-ribose) polymerase (PARP) (Enari et al., 1998; Kaufmann et al., 1993; Tewari et al., 1995). The targeting of nuclear substrates initiates apoptosis. Internal destruction of cellular contents instigates cell shrinkage and rounding. This is followed by one of hallmarks of apoptosis, condensation of the cell's nucleus. The cellular contents are broken down into small contained apoptotic bodies and are cleared from the body by phagocytosis.

1.2 CASPASES

Caspases (cysteine-aspartic-acid-proteases) are the effectors of the apoptotic cascade. Produced as catalytically inactive zymogens, they become activated at specific aspartic acid residues and cleave their downstream targets, usually additional caspases, resulting in a caspase cascade. Thus far, there are 14 known mammalian caspases named caspases -1 through -14. Although each of these caspases has a unique role, Zheng and colleagues have provided evidence

of functional compensation of caspases -2, -6, and -7 upon deletion of caspases -3 and -9 in hepatocytes from mice (Zheng et al., 2000). Knockout mice of caspases -1, -2, -7, -11, or -12 are still viable, and more importantly, they display minimal developmental apoptotic defects giving further evidence for caspase compensation (Bergeron et al., 1998; Kuida et al., 1995; Kuida et al., 1996; Lakhani et al., 2006; Li et al., 1995; Nakagawa et al., 2000; Wang et al., 1998). Caspase-7 knockout mice show a normal phenotype, suggesting that caspase-3 is sufficient for development (Lakhani et al., 2006). Mice null for caspase-3 live to about three weeks of age, but have neurological morphology defects thus has a greater importance in apoptosis (Kuida et al., 1996). Simultaneous deletion of caspase-3 and caspase-7 removes this compensation, resulting in an embryonic lethal phenotype (Lakhani et al., 2006). Caspase-8 or caspase-9 null mice have severe developmental defects and are embryonic lethal (Hakem et al., 1998; Lakhani et al., 2006; Varfolomeev et al., 1998). Mice without caspase-8 expression are embryonic lethal and display severe defects in heart musculature similar to that of caspase-3 and caspase-7 double knock out mice (Lakhani et al., 2006; Varfolomeev et al., 1998). Deletion of caspase-9 in mice results in embryonic lethality in addition to perturbations in brain development (Hakem et al., 1998).

The caspase family can be divided into two groups based on function. Caspases -1, -4, -5, -11, -12, -14 primarily operate in cytokine development (Strasser et al., 2000). Caspases -2, -3, -6, -7, -8, -9, and -10 have pivotal roles in the two main pathways of apoptosis. These can be further subdivided into initiator and effector caspases (Fig 2). Initiator caspases function as monomers containing long prodomains and either death effector domains (DED), as in caspases-

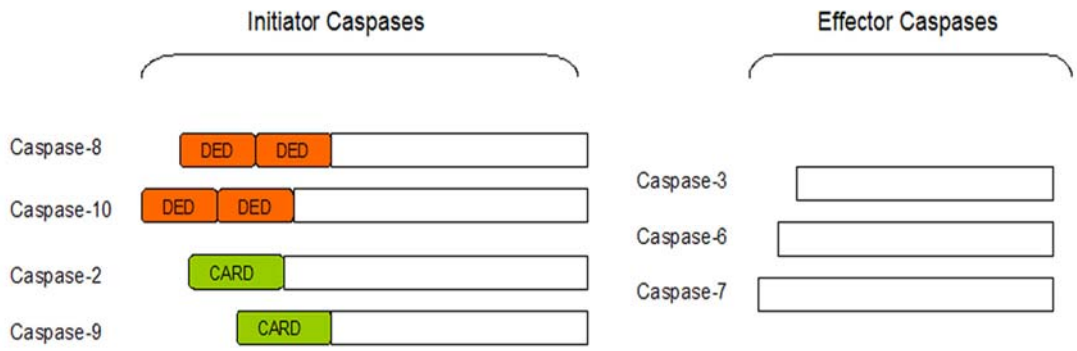


Figure 2: Basic Caspase Structure

Caspases-8 and -10 contain two death effector domains (DED). Caspases-2 and -9 each contain a caspase recruitment domain (CARD). Effector caspases do not contain either DEDs or CARDs and are activated by initiator caspases.

8 and -10, or caspase recruitment domains (CARD), as in caspase-2 and -9 (Hengartner, 2000; Shi, 2002). Effector caspases -3, -6, and -7, act as dimers, contain significantly shorter prodomains, lack DED or CARD domains, and have no intrinsic proteolytic activity (Shi, 2002).

The DISC attracts multiple molecules of the proform of apical caspases-8 and -10. The mechanism of apical caspase activation is controversial. One school of thought is that caspase cleavage is sufficient for caspase activation (Chang et al., 2003). However, other evidence supports a model that caspase cleavage is not necessary for activation. The induced proximity model, originally introduced by Salvensen and Dixit, states that apical caspases are activated by the DISC due to the congregation of the apical caspase homodimers (Salvesen and Dixit, 1999). Although this theory has been disputed over the years, Boatright and colleagues have revisited the induced clustering of initiator caspases (Boatright et al., 2003). They found that dimerization was necessary for activation of caspase-8 through the use of kosmotropes, salts which stabilize protein structure. Furthermore, they demonstrated that in contrast to popular belief, activation can be induced in the absence of cleavage. In this experiment, a processed caspase-8 was unable to bind to an affinity agent designed to capture active caspases. In addition, cleaved caspase-8 mutants that were dimerization defective failed to activate apoptosis thus understating the importance of caspase cleavage (Boatright et al., 2003). It is important to note that these findings are dependent on the formation of the DISC to activate caspase-8. Sohn and colleagues have demonstrated that upon mitochondrial activation of effector caspases -3 and -6, caspase-8 can be cleaved and activated without dimerization by the effector caspases (Sohn et al., 2005). Unlike the apical caspases, the downstream effector caspases exist as homodimeric units and are

activated via proteolytic cleavage (Shi, 2002). However, regardless of method of activation, both initiator and effector caspases require dimerization for activation by classical mechanisms (Liu et al., 2005).

1.3 TRAIL AS AN ACTIVATOR OF THE EXTRINSIC PATHWAY

The cytokine TRAIL (tumor necrosis-related apoptosis inducing ligand), is a member of the TNFα superfamily (Pitti et al., 1996). It is normally expressed by immune natural killer (NK) and T cells, on lymphocyte cell surfaces, and by some cancer cells (Jeremias et al., 1998; Pennica et al., 1984; Pitti et al., 1996; Walczak et al., 1999; Wiley et al., 1995). TRAIL binds five known receptors: two of these are signaling competent, DR4 (TRAIL-R1/TR1) and DR5 (TRAIL-R2/TR5), while the other two receptors display a close homology to DR4 and DR5, but have a decoy function (Ashkenazi and Dixit, 1998; Kischkel et al., 2000; Pan et al., 1997; Walczak et al., 1997). DcR1 (TRAIL-R3/LIT/TRID/TR3) is lacking the transmembrane and death domains while DcR2 (TRAIL-R4/TRUNDD/TR4) retains the transmembrane domain, yet has a truncated death domain (Degli-Esposti et al., 1997a; Degli-Esposti et al., 1997b; Sheridan et al., 1997). Thus, both decoy receptors maintain the ability to bind their ligands; however, without the intact, functional death domain, neither receptor can signal or induce apoptosis. The fifth TRAIL receptor is known as Osteoprotegerin (OPG) and also functions as a decoy receptor (Emery et al., 1998). OPG functions mainly in the bone and increases bone density via inhibition of osteoclastogenesis. Decoy receptors are believed to bind the TRAIL ligand via death domains and prevent formation of the DISC by inhibiting adaptor protein association.

While this function is evident for DcR1 and OPG, a recent study has suggested that DcR2 does not bind to TRAIL, but to DR5 through N-terminal interactions in a TRAIL-independent manner (Clancy et al., 2005). This TRAIL-DR5 complex is formed by interaction of preligand assembly domains (PLADs) with both active and decoy TRAIL receptors. This complex formed independent of ligand binding and prevented the active TRAIL receptor from signaling. Using PLAD deletion mutants and protein interaction studies in Jurkat T cells and 293 fibroblasts a new mechanism was proposed for receptor regulation of TRAIL-induced apoptosis independent of ligand binding (Clancy et al., 2005).

Following functional receptor engagement by TRAIL, formation of the TRAIL DISC begins. Caspase-8 and caspase-10 are recruited to the DISC (Sprick et al., 2002). However, caspase-10 is not a fully functional substitute for caspase-8 in signaling downstream caspases revealing a possible difference in function and signaling (Sprick et al., 2002). This data was contradicted by a different group which found that only caspase-8 was recruited to the TRAIL DISC (Bodmer et al., 2000). Nevertheless, both studies observed caspase-8 recruitment to the TRAIL DISC for subsequent activation (Seol et al., 2001). Similar to FasL, TRAIL signals through the extrinsic pathway by way of FADD to trigger apoptosis. This step has been disputed as the need for FADD to bind both TRAIL receptors is controversial (Harper et al., 2003a; Pan et al., 1997). DR4 does not associate with FADD upon TRAIL stimulation as seen by immunoprecipitation in human embryonic 293 cells; however it is required in MCF-7 breast cancer cells (Pan et al., 1997). Similar experimental studies provided evidence that FADD was

dispensable for TNF-induced DISC formation using DISC immunoprecipitation in Jurkat cells (Harper et al., 2003a).

Downstream of DISC assembly, TRAIL induction of apoptosis also requires mitochondrial proteins. Mitochondrial signaling requires Bax although there is no need for Bak in HCT116 human colon carcinoma lines that are stimulated with TRAIL (LeBlanc et al., 2002). A recent study reveals a novel mechanism for TRAIL-induced mitochondrial protein release. TRAIL activation of caspase-8 or caspase-3 in wild type HCT116 cells induces a degradation of anti-apoptotic Mcl-1 which normally sequesters pro-apoptotic Bim and prevents it from activating Bax (Han et al., 2006). Degradation of Mcl-1 releases Bim to activate Bax and subsequent release of mitochondrial contents (Han et al., 2006). However, TRAIL treatment of HCT116 cells null for Bax increases expression of anti-apoptotic proteins, Mcl-1 and cIAP2 (inhibitor of apoptosis) (Ricci et al., 2007). c-Myc expression increases TRAIL-induced apoptosis in Bax null HCT116 cells through inhibition of Mcl-1 and cIAP2 protein expression (Ricci et al., 2007).

1.3.1 TRAIL as a Therapeutic Agent

While there are many death receptor stimuli of apoptosis, some such as Fas and TNFα have toxic side effects precluding their usage as a therapy for cancer. TRAIL exhibits tremendous potential as a cancer therapy drug given its ability to induce apoptosis almost exclusively in cancer cells and not in normal cells (Marsters et al., 1996; Pitti et al., 1996; Wiley

et al., 1995). TRAIL also displays limited systemic toxicity in normal tissues making it an attractive target for cancer therapy (Ashkenazi et al., 1999; Kelley and Ashkenazi, 2004; LeBlanc and Ashkenazi, 2003; Walczak et al., 1999). Although TRAIL is very effective in many types of cancer cell lines, most breast cancer cell lines are resistant to its effects. The reason behind this resistance remains undetermined. It has been proposed that activation of the protein kinase C (PKC)–mitogen activated protein kinase (MAPK) pathway can inhibit TRAIL-induced apoptosis through a variety of mechanisms ranging from recruitment of apical caspases to post mitochondrial activities (Harper et al., 2003b; Meng et al., 2002; Soderstrom et al., 2002; Zhang et al., 2003). Recent work shows that activation of the PKC-MAPK pathway prevents the mitochondrial translocation Bid, a necessary step in Bid activation, in response to TRAIL stimulation in MCF-7 cells (Ortiz-Ferron et al., 2006). Stimulation with epidermal growth factor (EGF) in HEK 293 and MDA MB 231 cells caused an inhibition of cytochrome c release in response to TRAIL treatment although the exact mechanism was not determined (Gibson et al., 2002). In colon cancer, insulin-like growth factor binding protein 3 (IGFBP-3) increases TRAIL-induced apoptosis via inhibition of NFκB signaling (Williams et al., 2007). Thus determining how to sensitize cancer cells to TRAIL-induced apoptosis is a pertinent issue for the cancer biology community.

Currently, a soluble, recombinant form of TRAIL (PRO1762) is in phase I clinical trials (Genentech, San Francisco, CA; Amgen, Thousand Oaks, CA). TRAIL has been shown to inhibit growth and induce cytotoxicity in many human tumor cell lines (Ashkenazi et al., 1999; Gazitt et al., 1999; Keane et al., 1999). Studies in breast carcinoma lines showed induction of

apoptosis when doxorubicin was used in conjunction with TRAIL treatment (Keane et al., 1999). Using multiple myeloma (MM) cell lines and cells isolated directly from myeloma patients, treatment with TRAIL-induced apoptosis and demonstrated no cytotoxicity in hematopoietic stem cells (Gazitt, 1999). Scheduled treatment of cynomolgus monkeys with recombinant TRAIL showed no lypmphatotoxicity or hepatotoxicity in addition to leukocyte count and serum chemistry over seven days (Ashkenazi et al., 1999). Taken together, these preclinical studies demonstrate the potential of TRAIL as a cancer therapeutic.

Presently, there is promising work being done with human agonistic monoclonal antibodies designed to bind the TRAIL receptors DR4 and DR5. Targeting the DR4 receptor, HGS-ETR1 (mapatumumab; Human Genome Sciences, Rockville, MD), is currently in phase II trials and its effectiveness has been demonstrated in carcinomas from a wide variety of tissues (Pukac et al., 2005). HGS-ETR1 induces apoptosis via both extrinsic and intrinsic pathways in lymphoma cell line ST486 and colon cancer cell line SW480 by binding to DR4 (Pukac et al., 2005). In addition, combinatorial antibody treatment with chemotherapy induced apoptosis in ES2 ovarian and TTn oesophageal cell lines that were not sensitive to HGS-ETR1 alone. Lung, renal, and colon xenograft tumor models were subjected to treatment with HGS-ETR1, which displays a long half life and caused a reduction of growth in these tumor models. Overall, HGS-ETR1 has been shown to be effective both in vitro and in vivo. The agonistic antibodies toward DR5, HGS-ETR2 and HGS-TR2J (Human Genome Sciences, Rockville, MD) have been analyzed in phase I trials and have yielded promising results. HGS-ETR2 induces apoptosis in a variety of primary and cultured lymphoma cell lines (Georgakis et al., 2005). In combination

with doxorubicin or bortezomib, the killing was enhanced in both Jurkat and Hodgkin's lymphoma HD-LM2 cell lines (Georgakis et al., 2005). This apoptotic induction was independent of receptor, Bax, caspase-8, and cFLIP expression. Similar findings have also been shown in colorectal cancer. Marini and co workers use both colorectal tumors and cell lines to analyze the effects of the HGS-ETR2 agonistic antibody on apoptosis (Marini et al., 2006). They demonstrated that the antibody was able to effectively induce apoptosis alone, and this effect was increased in combination with radiation therapy. In both of these studies, the HGS-ETR1 agonistic antibody was also analyzed and similar results were found in this case. In addition, the two antibodies were tested in combination with each other, although this did not yield significant changes in apoptosis compared to the individual antibodies. Given these results, the agonistic TRAIL antibody has excellent potential as a therapeutic agent used alone or even more promising, to be used in conjunction with chemotherapy.

1.4 The Intrinsic Pathway and the Bcl-2 Family

Whereas the extrinsic pathway is dependent on external signals in order to function, the intrinsic apoptotic pathway is induced by DNA damage or other cellular stresses, such as heat stress or hypoxia. These stressors induce the translocation of pro-apoptotic Bcl-2 family members, Bax and Bak, to the mitochondrial membrane through an unknown mechanism (Scorrano and Korsmeyer, 2003). Once in the membrane, Bax and Bak cause a release of mitochondrial proteins such as cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low pl), Omi/HtrA2, and AIF (apoptosis

inducing factors) through a complex mechanism which remains poorly understood (Du et al., 2000; Liu et al., 1996; Lorenzo et al., 1999; Susin et al., 1999; Susin et al., 1996; Verhagen et al., 2000; Yang et al., 2003).

The Bcl-2 family of proteins are fundamental regulators of apoptosis. This family is divided into three groups based on the number of the Bcl-2 (B-cell leukemia/lymphoma protein 2) homology (BH) domains: the anti-apoptotic, the pro-apoptotic, and the BH3-only groups (Fig 3) (Danial and Korsmeyer, 2004). The most well studied anti-apoptotic members, Bcl-2, Bcl-x_L, and Mcl-1, contain all four Bcl-2 homology domains. These proteins inhibit apoptosis through the intrinsic pathway by interfering with the pro-apoptotic Bcl-2 family members, Bax and Bak, thereby preventing the release of cytochrome c from the mitochondria.

In contrast to the anti-apoptotic Bcl-2 family members, the pro-apoptotic proteins function to aid in the release of cytochrome c and other mitochondrial proteins subsequently leading to cell death. The pro-apoptotic members of the family consist of two groups: the multidomain proteins (MDP) and the BH3-only proteins. The multidomain proteins, Bax and Bak, contain three of the four BH domains, BH1-BH3. The MDP are monomers which upon activation form homo- or hetero-oligomers that directly cause mitochondrial disruption and cytochrome c release (Antonsson et al., 2001; Reed, 2006; Wei et al., 2000). Early studies proposed that Bax and Bak form a pore in the mitochondrial membrane, thus disrupting the stability of the mitochondria and releasing its contents into the cell. A new theory states that the pro-apoptotic MDP members do not form an organized pore in the mitochondrial membrane, but

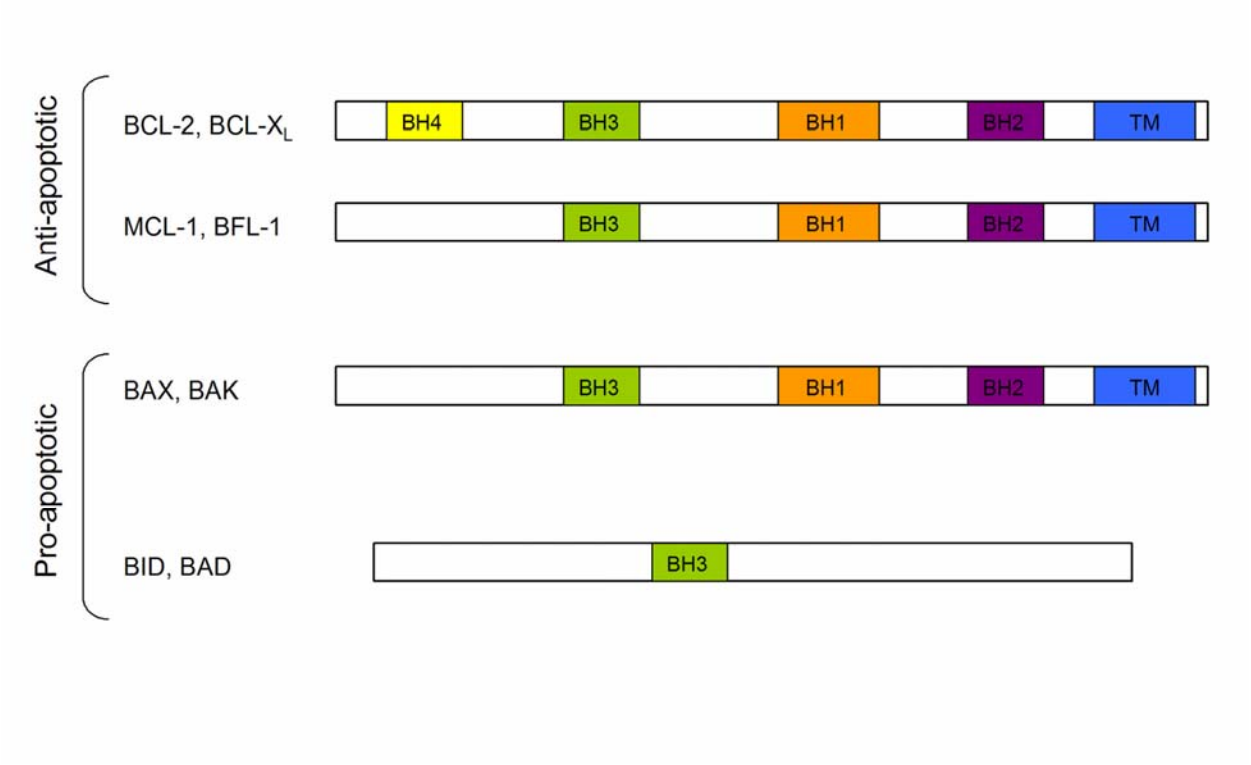


Figure 3: Bcl-2 Family Proteins

Bcl-2 homology (BH) domain map of anti- & pro-apoptotic proteins.

rather form polymer chains that can cause a random disruption and breakdown of the membrane (Kuwana et al., 2002). In accordance with this theory, anti-apoptotic proteins Bcl-2 and Bcl-XL can bind to these polymers and terminate chain formation. The BH3-only domain members are so named since they contain only the BH3 domain. The exact mechanism for how these proteins regulate the multidomain proteins has yet to be defined. One view divides this group into activators and sensitizers/derepressors. In this scenario, the cell's default action is to survive. The activators Bid, Bim, and in some cases Puma have the ability to directly activate the MDP protein Bax and Bak (Certo et al., 2006; Kim et al., 2006; Kuwana et al., 2005). Peptides corresponding to the BH3-only regions of activator and derepressor proteins showed that activator peptides could induce Bax oligomerization and cytochrome c release in vitro (Kuwana et al., 2002). Peptides to Bad could only induce Bax oligomerization in the presence of Bid (Kuwana et al., 2002). Taking these findings further, Certo and colleagues demonstrated that BH3-only proteins interact with specific anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Mcl-1 (Certo et al., 2006). There are different sensitivity levels of peptide binding thus allowing displacement of the activator BH3-only proteins Bid and Bim from their anti-apoptotic counterparts. According to this data, cells can be rendered sensitive to the effects of anti-apoptotic Bcl-2 family members through selective binding of specific Bcl-2 proteins by their corresponding BH3-only proteins (Certo et al., 2006). Additional work using in vitro transcribed and translated BH3 proteins were incubated together showed that Bid, Bim, and Puma could directly activate mitochondrial release of cytochrome c whereas the derepressors Bad, Noxa, Bmf and others could not (Kim et al., 2006). Similar to the other studies, this group found that

these derepressor BH3 proteins could displace activator BH3s that were bound to anti-apoptotic proteins (Kim et al., 2006).

Alternatively, it is thought that BH3-only proteins do not bind Bax and Bak at all. In this model, anti-apoptotic proteins are bound to Bax and Bak and BH3-only proteins bind anti-apoptotic Bcl-2 thereby releasing Bax and Bak to oligomerize and ultimately induce apoptosis. Utilizing BH3 domain peptides, Willis and colleagues demonstrated that Bid, Bim, and Puma peptides either do not bind Bax at all, or weakly bind to Bax after a conformational change, while the interactions of these peptides with anti-apoptotic Bcl-2 are readily detected. Moreover, Bid and Bim induce apoptosis in the absence of Bax or Bak binding (Willis et al., 2007). They also showed that Noxa and Bad could cause apoptosis in the absence of Bid and Bim which contradicts the previous model. In further support, another group demonstrated that both activator and derepressor BH3-only peptides could trigger cytochrome c release equally (Uren et al., 2007). Mutations of the BH3 domain could attenuate the apoptotic activities of these peptides whereas combinations of Mcl-1 and Noxa or Bcl-X_L and Bad could induce apoptosis. Moreover, association of Bak with Mcl-1 and Bcl-X_L could be disrupted with the introduction of BH3 peptides (Uren et al., 2007). Although the first model is the favored mechanism for BH3-only proteins, the second is gaining momentum, and together these studies enforce that this is a highly contested topic and that BH3-only proteins play a pivotal role in intrinsic pathway apoptosis.

While the mechanism of release of mitochondrial proteins is still being disputed, the end result remains that pro-apoptotic cytochrome c, Smac/DIABLO, Omi/HtrA2, and AIFs are released into the cytosol. Electron transport chain member, cytochrome c, is one of the major proteins released. The accumulation of cytochrome c in the cytoplasm attracts the scaffolding protein Apaf-1 (apoptotic protease-activating factor-1) through its WD40 domain (Zou et al., 1999). This causes an ATP-dependent conformational change resulting in the oligomerization of Apaf-1. Apaf-1 has a CARD domain which interacts with procaspase-9. This complex is known as the apoptosome and is essential for the enhanced activation of caspase-9. Activated caspase-9 cleaves caspase-3 into its active form. Aside from cytochrome c, other pro-apoptotic proteins released from the mitochondria are Smac/DIABLO and Omi/HtrA2 (Du et al., 2000; Verhagen et al., 2000; Yang et al., 2003). These proteins enhance apoptosis by sequestering IAPs (inhibitor of apoptosis proteins). Omi/HtrA2 translocates from the mitochondria to the nucleus to induces chromatin condensation (Lorenzo et al., 1999). Thus, all of these proteins contribute to the manifestation of apoptosis.

Although, the extrinsic and intrinsic pathways can function efficiently independent of one another, they are also closely linked by BH3-only protein, Bid. Although the two pathways are stimulated by very different mechanisms, the extrinsic pathway is able to activate the intrinsic pathway and amplify the effects of the apoptotic caspase cascade. Upon ligand binding to its death receptor, the DISC forms and caspase-8 is activated. Active caspase-8 cleaves and activates caspase-3; however, it can also cleave another Bcl-2 family member, Bid, into tBid (truncated Bid) causing its translocation to the mitochondrial membrane where it induces Bax

and Bak activity (Li et al., 1998). Once these proteins are activated, Smac/DIABLO and cytochrome c are released. Cytochrome c can then trigger apoptosome formation and subsequent activation of caspase-3.

1.5 Inhibitors of Apoptosis

Apoptosis is a tightly regulated process both by positive and negative regulators. There are several negative regulators of cell death. In the extrinsic pathway, c-FLIP (Flice inhibitory protein) binds the adaptor protein through a death effector domain and prevents DISC assembly and caspase activation. c-FLIP has three known isoforms all of which contain death effector domains; the full length, c-$FLIP_L$, and two shorter forms, c-$FLIP_S$ and c-$FLIP_R$ (Djerbi et al., 2001; Golks et al., 2005; Rasper et al., 1998). Both of the shorter isoforms of c-FLIP lack C-terminal signaling regions and therefore cannot activate apoptosis. c-$FLIP_L$ has been shown to have both pro- and anti-apoptotic functions (Jin et al., 2004; Micheau et al., 2002; Sharp et al., 2005). Downstream of the mitochondria there are inhibitors of apoptosis (IAPs), specifically, XIAP (X-linked inhibitor of apoptosis protein) (Deveraux et al., 1997). XIAP acts to inhibit the activation of caspases-9, -3, and -7 by preventing cytochrome c release (Deveraux et al., 1999; Deveraux et al., 1998; Deveraux et al., 1997). Smac/DIABLO, which is released from the mitochondria upon membrane disruption binds to XIAP via its BIR (baculoviral IAP repeat) domain (Liu et al., 2000; Wu et al., 2000). This binding prevents XIAP from binding caspases and inhibiting their activation.

1.6 HEAT SHOCK PROTEINS

Heat shock proteins are involved in many aspects of physiology from cell growth to cell death. In *E. coli*, heat shock proteins DnaK, and Hsp70 homolog, releases λP to initiate DNA replication and in mice and HeLa cells, heat shock protein is increased prior to cellular proliferation (Pechan, 1991). Additionally, mammalian heat shock proteins Hsp27, Hsp70, and Hsp90 have been found to be active at different stages of the cell cycle and in promoting cell growth through association with proteins such as MAPK, Cdk4, and p53 (Helmbrecht et al., 2000). The more well known function of heat shock proteins is to act as chaperones by providing protection from cellular stresses such as heat and hypoxia. Hsp90 can sequester misfolded proteins until they are refolded by other heat shock proteins such as Hsp70 or destroyed. Heat shock proteins 70 and 90 have also been shown to be involved in cellular metabolism control by increasing kinase activity of other binding partners. There are six families of heat shock proteins which are categorized according to their molecular size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small heat shock proteins. Many of the larger heat shock proteins are dependent on their N-terminal ATPase domains for chaperone function (Prodromou et al., 1997). ATP hydrolysis is required for heat shock proteins to bind and release co-chaperones and target misfolded proteins. In addition to their roles physiological role of maintaining protein structure, members of the Hsp family such as Hsp90 and Hsp70 have been implicated in disease states such as neurological diseases, myopathy, muscular dystrophy, and most notably, cancer.

1.6.1 Hsp90

Hsp90 has been extensively studied in cancer. One of its primary functions in cancer is to aid in self sufficient cancer cell growth by acting as a chaperone. Commonly, Hsp90 forms large molecular weight complexes in order to carry out this function such as the structure of Hsp90, Hsp70-40, Hop, and p23 (Pratt et al., 2004). Through these large complexes, Hsp90 acts as a chaperone to stabilize proteins thereby allowing continuous signaling to cell growth proteins like survival protein, Akt. Akt is activated in response to growth signals in the cell are held in their active conformation by Hsp90 (Neckers and Ivy, 2003; Pratt and Toft, 2003). In cancer cells, there is a higher affinity for Hsp90 to form complexes and signal to Akt than in normal cells (Kamal et al., 2003; Vilenchik et al., 2004). Hsp90 interacts with Akt in its phosphorylated form. It holds Akt and prevents its dephosphorylation and subsequent inactivation by phosphatase 2A (Sato et al., 2000). The requirement of Hsp90 for stability of protein kinases Akt, c-Src, and Raf-1 has linked Hsp90 to the stabilization of HER-2, a protein that has been linked to breast cancer (Neckers and Ivy, 2003). HER-2, a receptor tyrosine kinase, is expressed in 20-30% of breast cancers and its overexpression is indicative of poor prognosis (Press et al., 1997). It has also been shown that Hsp90 contributes to cancer by stabilizing the mutant forms of other proteins such as v-Src, Bcr-Abl, and p53 by stabilizing their mutant state (Neckers, 2002; Nimmanapalli et al., 2001). In addition to preventing protein degradation and stabilizing kinase activity in cancer cells, Hsp90 also promotes cancer cell progression by inhibiting apoptosis. Pandey and colleagues demonstrated that Hsp90 inhibits the intrinsic apoptotic pathway by sequestering Apaf-1 and preventing apoptosome formation resulting in failure to

activate downstream caspases (Pandey et al., 2000b). In tumor necrosis factor (TNF)-induced apoptosis, Hsp90 binds to and stabilizes RIP (receptor-interacting protein) thereby promoting activity of NFκB (nuclear factor κB) and cell survival (Lewis et al., 2000). Hsp90 also chaperones survivin, which has been shown to promote proliferation in cancer cells and prevent apoptosis (Fortugno et al., 2003). Caspase-9 and Bad, a Bcl-2 family member becomes inactive due to phosphorylation by Akt which is kept from degradation by Hsp90 (Cardone et al., 1998; Neckers and Ivy, 2003; Pratt and Toft, 2003). Hsp90 is also found to inhibit Bid cleavage and release of cytochrome c when NIH3T3 cells are stimulated with TNFα (Zhao and Wang, 2004).

In light of its role in cellular survival, Hsp90 is a promising drug target. Although there are many ways to inhibit Hsp90, the most promising inhibitors are those that bind its N-terminal ATP pocket (Chiosis, 2006). It is speculated that these inhibitors maintain their heat shock protein specificity due to the unique folding of an ATP pocket known as the Bergerat fold. This fold is not found in Hsp70 or other kinases (Chene, 2002). Ansamycin antibiotics (geldamycin (GM) and its derivatives 17AAG & 17DMAG) and Radicicols (KF55823, cycloproparadicicol) are the main Hsp90 N-terminal ATP pocket binding potential therapeutics (Isaacs et al., 2003; Neckers et al., 1999; Schulte et al., 1998; Stebbins et al., 1997). The derivatives of GM are currently in Phase I & II clinical trials. 17DMAG has the benefit of being water soluble and has potential to be used for oral administration (Kaur et al., 2004). However, 17AAG is being favored over GM due to its decreased hepatotoxicity (Solit et al., 2002). Hsp90 isolated from tumors has a higher affinity for 17AAG and another inhibitor PU24FC1 compared to Hsp90 from normal tissue (Kamal et al., 2003; Vilenchik et al., 2004). Additionally, tumor isolated

Hsp90 is already complexed to co-chaperones compared to solitary Hsp90 in normal tissues. Therefore, inhibitors can interfere with the formation of functional Hsp90 complexes, thus sensitizing cancer cells to cell death (Kamal et al., 2003; Vilenchik et al., 2004). Hsp90 inhibitors can induce expression of other heat shock proteins such as Hsp72 and Hsp27 (Zaarur et al., 2006). Zaarur and colleagues have found that using Emunin and NZ28 which inhibit both Hsp72 and 27 provide an additive effect to Hsp90 inhibitors' abilities to treat cancer (Zaarur et al., 2006).

Although Hsp90 is one of the main heat shock proteins targeted for potential therapeutics, it does not act alone. As stated earlier, Hsp90 forms large complexes with other heat shock proteins such as Hsp70, thus making Hsp70 an attractive drug target as well. Hsp90 and Hsp70 both contain a highly conserved C-terminal regulatory sequence, EEVD, which is imperative for binding in complex formations (Chen et al., 1998; Freeman et al., 1995). This conserved sequence is the binding site for the adaptor protein, Hop. Hop can bind the C-terminus of both Hsp90 and Hsp70 therefore hastening complex formation and chaperone activities (Scheufler et al., 2000). Hsp70 enters the complex already bound by Hsp40 family members. Hsp40 is involved in the regulation of the ATPase activity of Hsp70 (Fan et al., 2003). Together, these proteins make up a large chaperone complex that contributes jointly to the survival of cancer cells.

1.6.2 Hsp70

Similar to Hsp90, Hsp70 family members have a major role in the regulation of protein folding and in inhibition of apoptosis. Hsp70 binds to BAG-1 which binds to the anti-apoptotic protein Bcl-2 thereby inhibiting apoptosis (Takayama et al., 1997; Takayama et al., 1995). Although Hsp70 does not inhibit the release of cytochrome c, it prevents activation of caspase-3 by binding Apaf-1 (Beere et al., 2000; Li et al., 2000; Saleh et al., 2000). Furthermore, Hsp70 prevents cell death in the absence of Apaf-1 by binding to apoptosis inducing factor (AIF) in the caspase-independent death pathway (Ravagnan et al., 2001). All of these studies showed that the C-terminal peptide binding domain is essential for the anti-apoptotic actions of Hsp70. Another family member, Hsp72 inhibits apoptosis independent of the C-terminal EEVD domain by interacting with JNK and prevents Bid activation and downstream apoptotic events in human lung and murine embryonic fibroblasts (Gabai et al., 2002). Similarly, Hsp70 inhibits JNK activated translocation of Bax to the mitochondria in human acute lymphoblastic T cells thus inhibiting apoptosis (Stankiewicz et al., 2005). However, in these studies there was no apoptotic inhibition downstream of cytochrome c release. It is therefore evident that the Hsp70 family acts at different levels of inhibition of apoptosis.

1.7 Small Heat Shock Proteins

Small heat shock proteins are so named due to their small molecular weights ranging from 15 kDa to 30 kDa (Taylor and Benjamin, 2005). Most small heat shock proteins have

chaperone functions that are carried out in large oligomeric complexes and this oligomerization occurs via the N-terminal end of the proteins (de Jong et al., 1998). Toward the C-terminal end, these small heat shock proteins contain a highly conserved domain known as the α-crystallin domain, thus these proteins are also classified as the α-crystallin family of heat shock proteins. The carboxy terminal domain, which mediates substrate binding, varies in length amongst the different family members. Recently it was determined by chimeric small heat shock proteins that both the N-terminal portion of small heat shock protein structure can contribute to substrate binding as well as chaperone function (Basha et al., 2006). To date there are 10 mammalian members of this family and are termed HspB1-10 (Kappe et al., 2003; Taylor and Benjamin, 2005). Similar to Hsp90 and Hsp70, some members of this family can regulate cell death. Two of the most extensively studied are the small heat shock protein family members Hsp27 (HspB1) and αB-crystallin (HspB5).

1.7.1 Hsp27

Hsp27 has the capability to inhibit apoptosis in the intrinsic pathway. Different studies show that Hsp27 inhibits the apoptotic pathway at various places by diverse mechanisms. Bruey and colleagues found that Hsp27 inhibits apoptosis in a cell free system as well as in response to etoposide treatment in U937 leukemia cells (Bruey et al., 2000). This inhibition is due to binding of cytochrome c following release from the mitochondria preventing apoptosome formation and subsequent activation of downstream caspases. In contrast, it was reported that in human U 937 leukemia cells, HEK 293 fibroblasts and L929 murine fibroblasts, Hsp27 has no

effect of apoptosome formation and inhibits apoptosis by binding to caspase-3 (Pandey et al., 2000a). Another level of apoptotic inhibition by Hsp27 is through Bid. It is postulated that Hsp27 binds to and stabilizes F-actin filaments thereby preventing translocation of Bid to the mitochondrial membrane (Paul et al., 2002).

Hsp27 also displays involvement in an alternative apoptotic pathway according to its phosphorylation status. Early work determined Hsp27 has three phosphorylation sites controlled by MAP kinase-activated protein kinase-2 (MAPKAP kinase-2) that regulate its functions (Gaestel et al., 1991; Lambert et al., 1999; Landry et al., 1991; Ludwig et al., 1996; Rogalla et al., 1999; Stokoe et al., 1992). For example, in receptor mediated signaling, there are two divergent pathways. One pathway is caspase dependent and requires Fas ligand-receptor binding and subsequent recruitment of adaptor protein, FADD, and caspase-8. There is an alternate caspase independent pathway where Fas binds to Daxx and activates the p38 and JNK kinase cascade through Ask-1 (apoptosis signal regulating kinase-1) (Chang et al., 1998; Khelifi et al., 2005; Yang et al., 1997). When phosphorylated, Hsp27 binds to Daxx thereby preventing activation of JNK (Chang et al., 1998; Charette et al., 2000). Phosphorylated Hsp27 dissociates from Akt leading to increases apoptosis (Rane et al., 2003). In contrast, unphosphorylated Hsp27 binds Akt and promotes its activation and cell survival. Furthermore, when RNAi against Hsp27 is used, there is an enhanced release of cytochrome c and induction of VP-16-mediated apoptosis in TF-1 leukemia cells (Schepers et al., 2005). Taken together, this data shows that Hsp27 functions at various points along the apoptotic pathways to affect both cell death and survival.

1.7.2 αB-crystallin

αB-crystallin is another small heat shock protein that displays anti-apoptotic functions. αB-crystallin is homologous to Hsp27 in the highly conserved α-crystallin domain. Like Hsp27, αB-crystallin is widely expressed in various tissues and exhibits high levels of expression in the lens. αB-crystallin also contains phosphorylation sites in its N-terminal domain, which, similarly to Hsp27, are phosphorylated in response to heat stress (Ito et al., 1997). αB-crystallin and Hsp27 form large oligomeric complexes to trap misfolded proteins with Hsp20 and upon heat stress, the complex is dissolved (Kato et al., 1994; Zantema et al., 1992). Regarding its role in apoptosis, αB-crystallin acts at the junction of the extrinsic and intrinsic pathways in response to death receptor ligands and etoposide. In response to both TNFα and etoposide treatment, αB-crystallin over expression resulted in a marked reduction in apoptosis. Unlike Hsp27 and Hsp70, which inhibit caspase-9 cleavage, αB-crystallin did not. Instead, αB-crystallin inhibits caspase-3 cleavage by binding to the zymogen and intermediate p24 form of caspase-3 and preventing its maturation to the active state (Kamradt et al., 2001). This inhibitory function was also demonstrated in MDA-MB-231 breast cancer cells. Interestingly, when mutants of αB-crystallin were overexpressed in C2C12 myoblasts, apoptotic inhibition was eliminated (Kamradt et al., 2005). These results showed that the two mutants, R120G, which is thought to cause desmin-related myopathy, and a pseudophosphorylation form 3XSE, are necessary for the anti-apoptotic function of αB-crystallin (Kamradt et al., 2002). Furthermore, αB-crystallin was able to inhibit apoptosis induced by TRAIL in MDA-MB-231 and -435 breast cancer cells via the same

mechanism (Kamradt et al., 2005). Taken together, studies of αB-crystallin and Hsp27 highlight how small heat shock proteins regulate apoptosis.

1.8 HSPB2 - MKBP

HspB2 is a relatively new member of the small heat shock protein family. Like most proteins in this family, it is expressed at high levels in muscle tissue (Iwaki et al., 1997; Suzuki et al., 1998). It also forms large oligomeric structures with other small heat shock proteins (Sugiyama et al., 2000). HspB2 shares a high level of homology with Hsp27 and αB-crystallin, although this homology is mostly within the α-crystallin domain. Iwaki et al first cloned HspB2 while analyzing an area upstream region of αB-crystallin (Iwaki et al., 1997). In glial cells, analysis of two DNase I hypersensitivity sites located upstream of the transcription initiation sites of αB-crystallin revealed a new member of the α-crystallin family. This gene did not contain a TATA box, but did have a GC box. Two HSE were identified, along with two E boxes for binding to MyoD family transcription factors. Through Northern blot analysis, expression of HspB2 mRNA was found in skeletal and smooth muscles, but not in the lens (Iwaki et al., 1997). Using a yeast two hybrid system, Suzuki et al found a protein that bound to DMPK (myotonic dystrophy protein kinase) in skeletal muscle tissue thereby stabilizing its expression (Suzuki et al., 1998). Sequencing analysis revealed a protein of identical sequence to the one found by Iwaki. This protein was termed MKBP (myotonic dystrophy protein kinase binding protein). HspB2 binds directly to DMPK making it thermoresistant thereby allowing activation of its kinase activity when the body is subjected to heat stress such as fever. Through this function,

HspB2 advances disease progression of some myotonic dystrophy patients (Suzuki et al., 1998). This may be a replenishing mechanism in response to the decrease or absence of DMPK in myotonic dystrophy patients. To date, nothing else is known about the link between HspB2 and DMPK.

1.8.1 Myotonic Dystrophy

Myotonic dystrophy (DM) is a very prevalent form of muscular dystrophy. An estimated 1 in 8000 people suffer from myotonic dystrophy (www.myotonicdystrophy.org). People afflicted with myotonic dystrophy exhibit symptoms of progressive muscle weakness and myotonia. Myotonia occurs when there is an activation of the muscle followed by hyperexicitation caused by repetitive action potentials. This results in the inability of the muscle to relax. In addition to myotonia, myotonic dystrophy has a number of other symptoms including insulin resistance and cardiac defects. There are two types of myotonic dystrophy, DM1 and DM2. DM1 effects the distal parts of the body such as limbs and has two subtypes, one can affect patients from birth, congenital DM1 (CDM1), and the other presents during adulthood (DM1) (Mankodi and Thornton, 2002). CDM1 causes mental retardation and impaired muscle development. The other subtype of myotonic dystrophy is DM2 or PROMM (proximal myotonic myopathy) which mainly effects the proximal or trunk of the body. Both types are caused by untranslated nucleotide expansion. DM2 has a CCTG expansion in the ZNF9 gene on chromosome 3 (Liquori et al., 2001). DM1 is caused by a CTG expansion located in the 3'-untranslated region of DMPK on chromosome 19 (Brook et al., 1992). Several

mechanisms have been proposed for how the trinucleotide expansion causes myotonic dystrophy (Gatchel and Zoghbi, 2005).

The first is haploinsufficiency of DMPK, which theorizes that the 3' mutation may cause a change in the expression of DMPK and that the altered expression level could be the cause of the effects (Fu et al., 1992; Hofmann-Radvanyi et al., 1993; Novelli et al., 1993). However, DMPK knockout mice failed to exhibit the multi-symptom phenotype therefore arguing against the role of expression of DMPK in the disease (Jansen et al., 1996; Reddy et al., 1996). These studies only explain the existence of cardiac defects, but fail to show responsibility for the other clinical symptoms.

In order to explain the other symptoms, the second theory states that the expansion of the trinucleotides effects not only DMPK, but also neighboring genes. The CTG expansion is located at the 3' end of DMPK and can vary in the number of repeats and the longest expansions seen in some CDM1 cases are greater than 1000 repeats. Longer repeats can overlap onto the promoters of the neighboring genes, specifically, the gene SIX5 (Klesert et al., 1997; Thornton et al., 1997). Mice with SIX5 knocked down develop cataracts (Klesert et al., 2000; Sarkar et al., 2000). Assuming that the CTG expansion can affect a number of neighboring genes, this suggests a possible theory for the multiple symptoms of DM.

The third and most favored hypothesis is that the expansion causes RNA pathogenesis. According to this theory, there is a nucleic accumulation of transcripts made up of the CUG

repeats that congregate into foci and interfere with cellular functions such as gene splicing. Support of this model was found by Mankodi et al using transgenic mice with mutant DMPK messenger RNA that contained multiple CUG repeats. They found that retention of the repeats in the cellular nuclei of these mice and that their accumulation cause the phenotype of myotonic dystrophy (Mankodi et al., 2000). Since none of these models explains all of the symptoms of myotonic dystrophy, another theory combines the three models to explain the various clinical manifestations. This model is called the additive model, and as the name implies, each of the aforementioned theories contributes a small part in explaining the larger more encompassing symptoms of myotonic dystrophy. Supporting data is still needed in order to prove or disprove this theory. Regardless of the mechanism, what is known is that DMPK activity results in myotonic dystrophy. Thus, it was postulated that the increase of MKBP seen in these patients is a negative feedback response in the body's attempt to replenish DMPK expression and activity (Suzuki et al., 1998).

1.8.2 HspB2 Binding Partners

Due to the fact that heat shock proteins tend to function as large oligomeric complexes, it is likely that HspB2 has many binding partners. While only a few have been discovered so far, they have very different functions. As stated earlier, DMPK binds to HspB2 (MKBP) to hasten the development of muscular dystrophy. In *Psammomys obesus*, the beacon protein interacts with HspB2 and CLK4 as shown by a yeast two hybrid assay (Kantham et al., 2003). Beacon is expressed in the hypothalamus and has been linked to obesity. Increased food consumption and

weight gain occurred in *Psammomys obesus* upon increasing levels of Beacon (Collier et al., 2000). CLK4 (CDC2/CDC28-like 4) is a serine/arginine rich protein which can autophosphorylate as well as phosphorylated serine, threonine, and tyrosine residues on other proteins (Schultz et al., 2001). Given these findings, it is possible that HspB2 may be involved in stabilizing the Beacon/CLK4 complex and promote obesity. Aside from DMPK and Beacon, other known HspB2 binding partners are heat shock proteins.

It was speculated that since HspB2 shared an enhancer region with αB-crystallin, it was possible that they could bind to each other. However, results from a yeast two hybrid system using a human skeletal muscle cDNA library conclude that HspB2/MKBP does not interact with Hsp27 or αB-crystallin (Suzuki et al., 1998). Since αB-crystallin is expressed in the lens where there is no HspB2 expression and HspB2 does not bind to either αB-crystallin or Hsp27, Suzuki speculated that HspB2 is forming an independent oligomeric complex (Suzuki et al., 1998). This was later confirmed through a discovery of sequencing errors, when a former member of the HspL27 family was correctly reclassified as HspB3 (Sugiyama et al., 2000). HspB3 is a 17kDa novel member of the small heat shock protein family that exhibits mRNA expression patterns similar to those of HspB2 in adult human tissues (Sugiyama et al., 2000). Like all of the members of the α-crystallin family, HspB3 is expressed in the human heart tissue and has low expression in human skeletal muscle. HspB2 and HspB3, but not Hsp27 or Hsp20 have induced expression levels during differentiation in mouse C2C12 myoblasts (Sugiyama et al., 2000).

While HspB2 does not interact with Hsp27 or αB-crystallin, it does interact with Hsp20 in rat heart tissue demonstrating another hetero-oligomeric state of small heat shock proteins. In contrast to Hsp27 and αB-crystallin, HspB2, HspB3, and Hsp20 are not heat inducible and do not form the same oligomeric complexes. Upon the discovery of a new small heat shock protein, Hsp22 (HspB8), Sun and coworkers showed that it is a binding partner of HspB2. The N-terminal portion of Hsp22 binds to the full length MKBP but not the single N- or C-terminal portions using two hybrid and immunoprecipitation in both human 293T epithelial and COS-7 monkey kidney cells. Interestingly, follow-up work performed by Fontaine and colleagues showed that the binding of Hsp22 to HspB2 and HspB3 was not always consistent (Fontaine et al., 2005) . Hsp22 did show an interaction with HspB3 by a yeast two hybrid assay, but failed to show an interaction by FRET (fluorescence resonance energy transfer). It was reasoned that due to the smaller size requirement for the yeast two hybrid system, potential binding proteins would encounter less interference than in the FRET. Nevertheless, HspB2 does function independently of αB-crystallin and thus is likely forming a separate heterogeneous complex.

1.8.3 HspB2 Localization

Unlike other members of the α-crystallin family, heat shock does not induce an accumulation of HspB2 mRNA, but rather causes a translocation of HspB2 to an insoluble fraction and to the Z-membranes of skeletal muscle (Sugiyama et al., 2000). This study also reported that HspB2 does localize to actin bundles like αB-crystallin and Hsp27. In contrast,

both HspB2 and Hsp20 are partially extracted from the myocardium following treatment with 1M NaSCN and actin filament can also be extracted with the same treatment demonstrating that these small heat shock proteins may have an affinity for actin-related proteins (Golenhofen et al., 2004). However, they provided no direct evidence of an interaction between actin and HspB2. Another localization study provides evidence that HspB2 localizes to the mitochondria in both mouse C2C12 myoblasts and human KNS-81 glioma cells under normal conditions by immunofluorescence (Nakagawa et al., 2001). HspB2 localizes to the outer membrane of the mitochondria, but not to the matrix. In heat stress, an increase in expression of HspB2 increases the survival of C2C12 myoblasts under heat stress and that this enhances the association of HspB2 with the mitochondrial membrane (Nakagawa et al., 2001). In addition, the number of mitochondria are higher in slow-twitch (cardiac and soleus) versus fast-twitch (femoral and gastrocnemius) muscle. mRNA staining of samples of these tissues were compared with NIH3T3 cells constitutively expressing HspB2. There were higher levels of HspB2 mRNA in the slow-twitch muscle enforcing the claim that HspB2 associates with the mitochondrial outer membrane under heat stress conditions. Other members of the small heat shock protein family have been shown to localize to the mitochondria. In *Neurospora crassa*, small heat shock protein, Hsp30 was found to localize to the mitochondrial membrane in response to cellular heat shock (Plesofsky-Vig and Brambl, 1990). In plant cells from *Chenopodium rubrum* L. and potato, Hsp23 localizes to the mitochondrial matrix under heat shock conditions (Debel et al., 1997). Similarly, *Pisum sativium L.* contain a 22-kD small heat shock protein that translocates to the mitochondrial matrix following heat shock exposure (Lenne et al., 1995). A corresponding molecular weight protein was also discovered in *Drosophila melanogaster*. DM-Hsp22 also

localizes to the mitochondrial matrix under heat stress conditions (Morrow et al., 2000). Since all of these small heat shock proteins localize to the mitochondria in response to cellular stress, it is possible that they, along with HspB2, do so in order to protect the mitochondria and its contents from degradation induced by these stresses.

1.9.4 HspB2 Regulation

Since data suggests that HspB2 both localizes and functions in a different manner than other small heat shock proteins, the question of how it is regulated arises. As stated earlier, αB-crystallin and HspB2 share promoter regions, thus it is possible that HspB2 is regulated in a similar manner as αB-crystallin (Iwaki et al., 1997). Through deletions and inversions of the enhancer region between αB-crystallin and HspB2 transiently transfected into C2C12 myoblasts and α-TN4 epithelial cells dual-reporter luciferase results provide evidence that the enhancer favors the αB-crystallin promoter and its native enhancing effects are directionally dependent (Swamynathan and Piatigorsky, 2002). However, these experiments were performed in artificial settings in cell lines. In vivo expression of the enhancer region deletions and inversions in transgenic mice caused decreased HspB2 promoter activity (Swamynathan and Piatigorsky, 2002). This indicated that the shared promoter region controls both genes significantly in vivo in contrast to in vitro experiments. This intergenic region is conserved in all mammalian species and in some avian species and suggests that transcription factors and other elements within this region are interdependent, thus very resistant to evolutionary separation of the genes (Doerwald et al., 2004). Since this region selectively enhances the αB-crystallin promoter in the lens yet

enhances the promoter activities in both the αB-crystallin and HspB2 genes in muscle tissue, there are elements within this region that are specific to αB-crystallin yet the genes have retained their head-to-head orientation over time in many species. What the exact reasons are for conserving this region over time can only be speculated; however it is clear that this region is required for the function of both αB-crystallin and HspB2 (Doerwald et al., 2004; Swamynathan and Piatigorsky, 2002). A 2001 study by Brady and co-workers sought to determine the effects of knocking out the αB-crystallin gene; however, through targeted deletions of the αB-crystallin gene in embryonic stem cells, they inadvertently eliminated HspB2 in the process due to its close proximity and shared promoter region (Brady et al., 2001). This double knockout mouse displays normal development and fertility. Surprisingly, even with the absence of αB-crystallin the lenses of the mice are normal. Although initial development is normal in the absence of both heat shock proteins, upon 40 weeks of age, the mice began to show postural problems which are attributed to the musculo-protective roles of both proteins. This same system utilized by another group analyzed the effects of ischemia-reperfusion on the mouse myocardium (Morrison et al., 2004). The αB-crystallin/HspB2 knockout mice display normal heart morphology and function. Upon induction of ischemia, the wild type and knockout mice appear to have similar reactions. The apparent differences are seen when reperfusion occurs. The mice without αB-crystallin and HspB2 have a higher level of apoptosis and necrosis compared to wild type mice. This study provides evidence that together or separate, αB-crystallin and HspB2 provide protective effects against cell death upon recovery from ischemia. In further support of this data, Kadono and colleagues examine the role of Ca^{2+} uptake in the mitochondria of wild type and knockout mice (Kadono et al., 2006). They hypothesize that the increase in Ca^{2+} levels induce the

mitochondrial pore transition (MPT) in knockout mice since the MPT contributes greatly to myocyte injury from ischemia-reperfusion (Joseph et al., 1997). αB-crystallin/HspB2 knockout mice show an increase in mitochondrial Ca^{2+} uptake and this increase rendered the mice more susceptible to MPT. These findings support the previous work by Morrison where they demonstrated an increase in cell death (Morrison et al., 2004). The observation that HspB2 associates with the mitochondrial membrane may also support this hypothesis (Nakagawa et al., 2001). In myocytes, HspB2 may act to prevent Ca^{2+} uptake by associating with the outer mitochondrial membrane thus preventing MPT.

As illustrated above, the protective capacity of heat shock proteins in normal physiology and apoptosis is a growing field. The role they play in regulating apoptosis is of particular interest to the study of cancer progression and prevention. To date, Hsp90, Hsp70, Hsp27, and αB-crystallin have been shown to exert inhibitory effects through pro-survival pathways as well as stabilization of pro-apoptotic proteins downstream of the mitochondria. Understanding how these proteins function to maintain cell survival in disease settings is vital to potential therapeutic development. Hsp90 is a prime example of how knowledge of protein function can aid in therapy. Described earlier, Hsp90 has a wide variety of functions in cancer progression from stabilizing pro-survival proteins such as mutant p53 and RIP to inhibiting apoptosis through Apaf-1 (Lewis et al., 2000; Neckers, 2002; Nimmanapalli et al., 2001; Pandey et al., 2000b). Inhibitors of Hsp90 are in clinical trials and are showing promising potential in cancer treatment (Isaacs et al., 2003; Neckers et al., 1999; Schulte et al., 1998; Stebbins et al., 1997). Since only some of the heat shock proteins have been studied in cancer and many of these proteins have

similar structures and work together in large hetero-oligomeric complexes, it is easy to speculate that there are other members of the heat shock protein family that similar functions in both cellular survival and death. Of the small heat shock proteins, only αB-crystallin and Hsp27 have been studied extensively. There are eight other members of this family that have potential roles in cancer progression. HspB2 is a newly discovered member of this family that has only been shown to promote myotonic dystrophy (Iwaki et al., 1997; Suzuki et al., 1998). This work explores a new functional role for HspB2 in apoptosis.

2. MATERIALS AND METHODS

2.1 CELL CULTURE

Human MDA-MB-231 cells were grown in DMEM (Gibco - Invitrogen #11965-118, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS #16140-071, Gibco – Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin-glutamine (# 10378-016, Gibco - Invitrogen, Carlsbad, CA). Human T47D breast carcinoma cells were obtained from ATCC and grown in RPMI 1640 (#11875093, Gibco - Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, #16140-071, Gibco - Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (#30-002-CI, Cell Grow – Mediatech, Herndon, VA). Phoenix-Ampho packaging cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS (#16140-071, Gibco – Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin-glutamine (# 10378-016, Gibco - Invitrogen, Carlsbad, CA). 2LMP, LCC6, DY36T2, A549, NCIH226, NCIH358, NCIH1299, NCIH2122, MIA PaCa2, PANC1, PC3, BxPC3, SW948, U87MG, D54MG, LG11, & BT474 cancer cell line lysates were generously provided by Dr. Patsy Oliver (University of Alabama, Birmingham, Alabama). U373 & DiNTC (Rat glioma) were provided by Dr. Linda Van Eldyck (Northwestern University, Chicago, IL) and U251 cells were obtained from Dr. Olga Volpert (Northwestern University, Chicago, IL). All three cell lines were cultured in DMEM/F12 (#11320033, Gibco – Invitrogen, Carlsbad, CA), containing 10% FBS (#16140-071, Gibco – Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (#30-002-CI, Cell Grow – Mediatech, Herndon, VA).

2.2 Antibodies and Reagents

Mouse monoclonal antibodies against caspase-3 (#610323), -7 (#551241), MLH-1 (#551091), MKBP (#611298), FADD (# 556402), and cytochrome c (#556433) were purchased from BD Biosciences (San Jose, CA). Monoclonal mouse antibody against caspase-2 (#2224) and polyclonal antibody against caspase-10 (#9752) were purchased from Cell Signaling (Danvers, MA). Polyclonal antibody against caspase-9 was purchased from Cayman Chemical (#160790, Ann Arbor, MI). Mouse monoclonal α-Tubulin (#T6074), mouse monoclonal FLAG-M2 (#F1804), and Rabbit polyclonal FLAG (# F7425) antibodies were purchased from Sigma Aldrich (St. Louis, MO). Rabbit polyclonal antibody against TRAIL was purchased from Abcam (#2435, Cambridge, MA). Mouse monoclonal antibody against caspase-8 was kindly provided by Dr. Marcus E. Peter (University of Chicago, Chicago, IL). Rat polyclonal antibody against Bid was kindly provided by Dr. Honglin Li (Northwestern University, Chicago, IL). Goat polyclonal antibodies against DR4 and DR5 were purchased from Santa Cruz Biotech (DR4 #sc-6824, DR5 #sc-7192, Santa Cruz, CA). Mouse monoclonal antibody against CoxIV was purchased from Molecular Probes, Inc (#A-21348, Eugene, OR). The horseradish peroxidase-conjugated goat anti mouse (#1010-5), rabbit (4010-5), rat (#3010-5) were purchased from Southern Biotechnology (Birmingham, AL). Horseradish peroxidase-conjugated donkey anti goat secondary was purchased from Santa Cruz Biotechnology (#sc-2020, Santa Cruz, CA). Rabbit IgG control Santa Cruz Biotechnology (#sc-2027, Santa Cruz, CA). TNFα was purchased from R&D Systems (#210-TA, Minneapolis, MN). Etoposide (#E1383) and protease inhibitor cocktail (#P8340) were purchased from Sigma (St. Louis, MO).

2.3 Expression and Purification of TRAIL

Recombinant soluble His-tagged TRAIL (amino acids 95-281) was expressed in E. coli by transforming BL-21 cells (#69449, EMD Biosciences, San Diego, CA) with pET15b plasmid (#69661-3, EMD Biosciences, San Diego, CA) containing a partial TRAIL cDNA (Pan et al., 1997). A single clone was isolated and grown to log phase, at which time 1mM IPTG was added to induce protein expression. Following cell growth for and additional 2hr, bacteria were lysed and the His-tagged protein was purified under native conditions using the QIAexpress Type IV kit (QIAGEN #32149, Valencia, CA). Briefly, bacteria were suspended in lysis buffer (50mM NaH_2PO_4, 300mM NaCl, and 10mM imidazole (pH 8.0)) with 100 μg/mL PMSF and 10 μg/mL lysozyme. The lysate was them incubated on ice for 30 minutes, sonicated, and centrifuged at 10,000 x g for 20 minutes at 4°C to pellet cellular debris. Next, the lysate was incubated with Ni-NTA resin (QIAGEN #30230, Valencia, CA) for 90 minutes at 4°C with gentle shaking. The lysate-Ni-NTA mixture was then loaded onto a column, washed twice (50mM NaH_2PO_4, 300mM NaCl, and 20mM imidazole (pH 8.0)), and His-tagged TRAIL was eluted in 250mM imidazole. Following elution, fractions which contained high concentration of purified TRAIL (as determined by SDS-PAGE Western analysis and protein assay (BioRad, Hercules, CA)) were pooled and stored in aliquots containing 10% glycerol at -80°C.

2.4 CLONING OF CONSTRUCTS

2.4.1 Flag-tagged cDNAs

To generate cDNA encoding wild type HspB2, human skeletal muscle cDNA was subjected to PCR amplification with the following primers. The restriction enzyme sites for EcoRI and XhoI are underlined:

MKBPECO (forward): 5'–GGCCGAATTCATGTCGGGCCGCTCAGTG-3'

MKBPXHO (reverse): 5'–GGCCCTCGAGTCAGGGCTCAACTATGGC-3'

Following amplification, the product was subjected to 1% agarose gel electrophoresis. The product was then extracted from the gel using a QIAGEN gel extraction kit (#328704, Valencia, CA), digested with EcoR1 (#R6017) and Xho1 (#R6165, Promega, Madison, WI) and purified with a QIAGEN PCR purification kit (#28104, Valencia, CA). The pcDNA3-FLAG vector was double digested with EcoR1 and Xho1 and purified with a QIAGEN PCR purification kit. For size confirmation, an aliquot of undigested and digested vector and the digested PCR product were run on a 1% agarose gel. Ligation of the digested vector and PCR product was performed using T4 ligase (New England Biolabs # M0202S, Ipswich, MA). The ligation was transformed using *E. coli* HB101 competent cells (Invitrogen #18925-014, Carlsbad, CA). Positive clones were confirmed by sequencing using HspB2 Gene Bank Accession Number NM_001541.

2.4.2 pSUPER.RETRO.PURO.GFP vector plasmid

To generate the pSuper.retro.puro.GFP vector plasmid, the GFP was isolated from the pSIREN-RetroQ-ZsGreen vector (Clontech #632455, Mountain View, CA). PCR amplification of the GFP was performed using the following primers:

GFP HindIII Forward:

5'- CCGGAAGCTTGGATCCTAGTTATTAATAGTAATCAA-3'

GFP HindIII Reverse: 5'-CCGGAAGCTTCCCCCTTTTTCTGGAGAC-3'

The GFP product was digested with HindIII (underlined) (New England Biolabs #R0104S, Ipswich, MA) and DNA was isolated using a QIAGEN gel extraction kit. The pSUPER.RETRO.PURO vector was purchased from OligoEngine (#VEC-PRT-0001, Seattle, WA). The pSUPER.RETRO.PURO vector was linearized by digestion with HindIII ((New England Biolabs #R0104S, Ipswich, MA) and treated with Antarctic phosphatase to remove 5' phosphate groups (New England Biolabs # M0289L, Ipswich, MA). The digested GFP product was ligated into the digested pSUPER.RETRO.PURO vector using T4 ligase (New England Biolabs # M0202S, Ipswich, MA). The ligation was transformed using subcloning efficiency DH5α competent cells (Invitrogen #18265-017, Carlsbad, CA). Orientation was determined by digestion with SalI (New England Biolabs #R0138S, Ipswich, MA). The plasmid was purified using a QIAGEN endonuclease free maxi kit (#12362, Valencia, CA) and confirmed by sequencing.

2.4.3 HspB2 shRNAs

HspB2 shRNAs were designed using a base template sequence from OligoEngine (see supplemental material for #VEC-PRT-0001, Seattle, WA) and HspB2 target sequences were substituted for the target sequence in the template. HspB2 target sequences were obtained from Openbiosystems (# RHS3979-9572505 and RHS3979-9572506, Huntsville, AL). The resulting oligo sequences were ordered through Integrated DNA Technologies (Coralville, IA):

HspB2 3814 forward: 5'-GATCCCCGAGGTGACTGTGAGGACTGTTTCAAGA GAACAGTCCTCACAGTCACCTCTTTTTA-3'

HspB2 3814 reverse: 5'- GATCCAAAAAGAGGTGACTGTGAGGACTGTTCTC TTGAAACAGTCCTCACAGTCACCTCGGG-3'

HspB2 3815 forward: 5'- GATCCCCGACTGTGAGGACTGTGGATAACTTCAAG AGAGTTATCCACAGTCCTCACAGTCTTTTTA-3'

HspB2 3815 reverse: 5'- GATCCAAAAAGACTGTGAGGACTGTGGATAACTCTCT TGAAGTTATCCACAGTCCTCACAGTCGGG-3'

HspB2 scrambled forward: 5'- GATCCCCGAAGACCAGTTACGGTGTTGGATTCAA GAGATCCAACACCGTAACTGGTCTTCTTTTTA-3'

HspB2 scrambled reverse: 5'- GATCCAAAAAGAAGACCAGTTACGGTGTTGGA TCTCTTGAATCCAACACCGTAACTGGTCTTCGGG-3'

Each oligo primer was resuspended in TE to a concentration of 3 μg/μL. 20 μg (6.7 μL) of the forward and reverse oligo primers for each construct was added to 2 μL 5M NaCl and water to a total volume of 20 μL in microfuge tubes. Tubes were placed in a heat block at 100°C for 5

minutes then the block was removed and placed on the bench top overnight to allow the tubes to cool slowly. The resulting annealed constructs are diluted 1:500 in 500mM TRIS in TE. Each of the shRNA constructs was ligated into the digested pSUPER.RETRO.PURO.GFP vector. The p.SUPER.RETRO.PURO.GFP vector was digested with BamHI and BglII and treated with Antarctic phosphatase and ligations were performed using T4 DNA ligase. Post ligation, constructs were transformed into subcloning efficiency DH5α competent cells (Invitrogen #18265-017, Carlsbad, CA) and plasmid purification was performed using a QIAGEN endonuclease free maxi kit (#12362, Valencia, CA) and confirmed by sequencing.

2.5 STABLE TRANSFECTIONS

2.5.1 MDA-MB-231 Flag-tagged cell lines

MDA-MB-231 breast carcinoma cells were plated at 30% confluency and transfected with 1ug of pcDNA3-FLAG control vector or pcDNA3-FLAG-HspB2 using LipofectAMINE and Plus reagent according to manufacture instructions (Invitrogen, Carlsbad, CA) in serum free DMEM and allowed to recover for 48 hours. Cells were cultured in DMEM supplemented with 10 % FBS, 1% penicillin-streptomycin, and 800 ug/mL G418 (Invitrogen, Carlsbad, CA) for three weeks to selected for clones expressing these vectors. Expression was confirmed by Western blotting.

2.5.2 T47D HspB2 null cell lines

The pSUPER.RETRO.PURO.GFP vector alone, shRNA HspB2 3814, shRNA HspB2 3815, or shRNA Scrambled constructs were transfected into Phoenix-Ampho cells in DMEM using calcium chloride. Transfected cells were placed in a 32°C incubator for 24 hours to enhance viral particle production. T47D target cells were plated at 25% confluency. The virus particle containing media was filtered (0.45 μM filter) and polybrene (Hexadimethrine bromide, Sigma #H9268, St. Louis, MO) was added at 4 μg/mL. This mixture was overlayed onto the target cells and incubated at 37°C for 5 hours. 24 hours after adding the virus, the media was changed again. Following the media change, the cells were collected and sorted for GFP positive cells using the DakoCytomation MoFlo machine and the 48 laser (Dako United States of America, Carpinteria, CA). Each cell line yielded 10-20% positive cells that were expanded for use in experiments.

2.6 TIMECOURSE AND FACS ANALYSIS OF APOPTOSIS

2.6.1 TRAIL & TNFα

Cells stably expressing pcDNA3-FLAG empty vector, αB-crystallin, or HspB2 were plated onto 6-well plates at 0.4 x 10^6 cells/well. These cells were treated with DMEM alone for control or DMEM with 500 ng/mL TRAIL from 0-24 hours or 10ng/mL TNFα (R&D systems #210-TA, Minneapolis, MN) and 1μg/mL Cycloheximide (Sigma, St. Louis, MO) from 0-24 hours. Both floating and attached cells were collected by 0.05% Trypsin (Invitrogen #23500-112, Carlsbad,

CA) and fixed with glutaraldehyde (Sigma #G6257, St. Louis, MO) for 15 minutes. Cell pellets were washed 1X in PBS (phosphate buffered saline) (Invitrogen, Carlsbad, CA) and cell nuclei were stained with 10 μg/mL bisBenzimide (DAPI) (Sigma #H 33258, St. Louis, MO) for 30 minutes at 37°C. Percent apoptosis was determined by scoring cells alive or dead based on their apoptotic nuclei as determined under fluorescent microscopy (NIKON). A total of 200 cells were counted for each condition and experiments were performed in triplicate.

As a confirmation of apoptosis, Annexin V-PE apoptosis detection kit I (BD Biosciences #559763, San Jose, CA) was used for the TRAIL and TNFα treatments. MDA-MB-231 cells over expressing empty pcDNA3 vector, αB-crystallin, or HspB2 were left untreated or treated with either 500 ng/mL TRAIL or 10 ng/mL TNFα and 1 μg/mL cycloheximide for four hours. After four hours, both adherent and floating cells were collected by 0.05% Trypsin. Cells were then washed twice with cold PBS and resuspended in the 1X Binding Buffer as directed by the manufacturer. Annexin V-PE and 7-AAD were added too 100uL aliquots followed by a 15 minute incubation at room temperature and analysis by flow cytometry (Beckman Coulter Epics XL-MCL, Northwestern University Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility).

2.6.2 Etoposide, doxorubicin, and staurosporine

Cells stably expressing pcDNA3-FLAG empty vector or HspB2 were plated onto 6-well plates at 0.4 x 10^6 cells/well. These cells were treated with DMEM alone for control or DMEM plus

50 μM Etoposide (Sigma #E1383, St. Louis, MO) from 0-72 hours, 750 nM Doxorubicin (Sigma #D1515, St. Louis, MO) from 0-72 hours, or 750 nM Staurosporine (Sigma #S4400, St. Louis, MO) from 0-24 hours. Both floating and attached cells were collected by 0.05% Trypsin (Invitrogen #23500-112, Carlsbad, CA) and fixed with glutaraldehyde (Sigma #G6257, St. Louis, MO) for 15 minutes. Cell pellets were washed 1X in PBS (phosphate buffered saline) (Invitrogen, Carlsbad, CA) and cell nuclei were stained with 10 μg/mL bisBenzimide (DAPI) (Sigma #H 33258, St. Louis, MO) for 30 minutes at 37°C. Percent apoptosis was determined by scoring cells alive or dead based on their apoptotic nuclei as determined under fluorescent microscopy (NIKON). A total of 200 cells were counted for each condition and experiments were performed in triplicate.

2.7 Analysis of xenograft tumor growth in response to TRAIL

MDA-MB-231 cells stably expressing FLAG-empty vector or HspB2 were injected into the mammary fat pads of 4-5 week old female athymic nude mice and tumor development was monitored weekly. After two weeks, tumors were excised and dissected into 1 mm^3 pieces. These pieces were transplanted subcutaneously into both mammary fat pads of 4–5-week old female athymic nude mice (Harlan Sprague-Dawley) 5 mice per group. Two weeks later, mice with established tumors (expressing vector or HspB2) were treated with PBS (vehicle) or TRAIL 5 mg/kg/day by intraperitoneal injection. Tumor volume was measured weekly for a 5 weeks.

2.8 MEASUREMENT OF CASPASE ACTIVATION WITH IETD AND DEVD FLUOROGENIC ASSAYS

MDA-MB-231 cells were plated at a concentration of 2 x 10^6 in 10 cm dishes and grown overnight. Cells were then treated with TRAIL 200 (DEVD)-500 ng/mL (IETD) for 0-4 hours at 37°C. Following treatment the cells were collected using 0.05% Trypsin (Invitrogen, Carlsbad, CA) and the assay was performed as directed by the manufacturer for DEVD or IETD Fluorogenic assay kits (R&D Systems DEVD # BF1100, IETD # BF2100, Minneapolis, MN). Percent fluorescence over control was determined by spectrophotometer (IBB, Atlanta, GA – Spectra Max Gemini Microplate Spectrofluorimeter) excite at 405nm read at 500nm.

2.9 CASPASE CLEAVAGE ANALYSIS BY WESTERN BLOTTING

MDA-MB-231 cells stably expressing pcDNA3 FLAG vector or HspB2 were plated at 2 x 10^6 in 10 cm plates and grown overnight. Cells were left untreated or treated with 500 ng/mL TRAIL for 0, 1, 2, 4, 6, 8 hours. After 8 hours, both floating and adherent cells were collected by 0.05% Trypsin (Invitrogen #23500-112, Carlsbad, CA) and pelleted by microcentrifugation. Cells were washed 1X with ice cold 1X PBS supplemented with 1mM PMSF and pelleted by microcentrifugation. The cell pellets were resuspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.5% Deoxycholic acid, 150 mM NaCl, 1% NP40, 1mM PMSF, 1X protease inhibitor cocktail) and incubated on ice and at 10 minute intervals samples were vortexed. At the end of 30 minutes, the samples were spun in a microcentrifuge at 13,200 rpm for 15 minutes. The supernatants were transferred to new tubes. Protein quantification was performed using

Pierce BCA assay kit (Pierce Biotechnology, Inc. #23225, Rockford, IL) and analysis was read on plate reader at 595 nm (Biorad). Following quantification, an equal volume of 2X sample buffer (100 mM Tris pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.01% Bromophenol blue) was added. Samples were then boiled for 5 minutes in a 100°C heat block and subjected to SDS-PAGE analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on 13.3% acrylimide gels, semidry transfer 1 hour, block in 5% milk in TBS-Tween 1 hour, incubated with primary antibodies as directed, 3-10'washes, secondary incubation 1:2000 1 hour, 4 washes of 10 minutes each, and developed with Perkin Elmer Western Lightning Chemiluminescence (#NEL 101, Boston, MA).

2.10 Measurement of Cytochrome c release

2.10.1 Western blotting

MDA-MB-231 cells stably expressing pcDNA3 FLAG vector of HspB2 were plated at 5 x 10^6 cells per 15 cm dish. Cells were left untreated or treated with 500ng/mL TRAIL for 1, 2, 4, & 6 hours. Following the timecourse, both adherent and floating cells were collected using 0.05% Trypsin. Cells were washed with cold PBS with protease inhibitor added (Sigma #P8340,) and resuspended in cold lysis buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1X protease inhibitor cocktail, 4 mM DTT) and incubated on ice for 1 hour with intermittent vortexing. Samples were then spun in a microcentrifuge for 15 minutes at 13,200 rpm. Supernatant was placed into new tubes and the pellets were

resuspended with RIPA (50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.5% Deoxycholic acid, 150 mM NaCl, 1% NP40, 1mM PMSF, 1X protease inhibitor cocktail) and protein quantification was performed using Pierce BCA assay kit (Pierce Biotechnology, Inc. #23225, Rockford, IL) and analysis was read on plate reader at 595 nm (Biorad). Samples were resuspended in 2X sample buffer (100 mM Tris pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.01% Bromophenol blue) and equal amounts of protein were loaded onto 15% acrylimide gels and analyzed by SDS-PAGE analysis for cytochrome c, Cox IV, and Smac/DIABLO.

2.10.2 ELISA

MDA-MB-231 cells stably expressing pcDNA3 FLAG vector or HspB2 were plated at 5 x 10^6 cells per 15cm dish. Cells were left untreated or treated with 500 ng/mL TRAIL for two hours. Following the timecourse, both adherent and floating cells were collected using 0.05% Trypsin. Cells were washed with cold PBS with protease inhibitor added (Sigma #P8340, St. Louis, MO) and resuspended in 5 volumes of cold isolation buffer pH 7.4 (0.3 M mannitol, 0.1% BSA, 0.2 mM EDTA, 10 mM HEPES, 1X protease inhibitor cocktail). Samples were homogenized with a 2 mL glass homogenizer and dounced 5 times loose and 7 times tight. Whole cells and nuclei were removed by microcentrifugation for 10 minutes at 1000g. The supernatant was subjected to further microcentrifugation for 15 minutes at 14,000g to separate the cytosolic and mitochondrial fractions. Mitochondrial fractions were washed twice with cold isolation buffer and resuspended in RIPA buffer and protein concentration was determined using Pierce BCA assay kit (Pierce Biotechnology, Inc. #23225, Rockford, IL) on a plate reader at 595 nm (Biorad). Using these

fractions, relative levels of cytochrome c were determined using the Quantikine Human Cytochrome c ELISA kit as directed by the manufacturer (R&D Systems # DCTC0, Minneapolis, MN).

2.11 Analysis of apoptosis induced by tBid transient transfection

MDA-MB-231 cells stably expressing pcDNA3 FLAG vector or HspB2 were transiently transfected with pEGFP-N1 (Clontech Laboratories #6085-1, Mountain View, CA) and either pCMV-5a empty vector or pCMV-5a-tBid (kindly provided by Honglin Li, Northwestern University). Both adherent and floating cells were collected using 0.05% Trypsin and fixed with 4% paraformaldehyde. Following fixation, the cells were stained with 10 μg/mL bisBenzimide (DAPI) (Sigma #H 33258, St. Louis, MO). Apoptosis was determined by scoring GFP positive cells for condensed nuclear morphology.

2.12 Immunoprecipitation of the TRAIL DISC

In order to isolate the TRAIL DISC, a protocol for isolating the Fas DISC was kindly provided by Christine Feig of the Marcus Peter Lab at the University of Chicago and modified for our purposes. 25 x 10^6 cells per condition of MDA-MB-231 cells stably over expressing HspB2 or pcDNA3 FLAG vector were cultured. Both cell lines were used for treatment with 500 ng/mL TRAIL for 10, 30, & 60 minutes. An untreated condition as well as untreated IgG controls were used. Prior to TRAIL treatment, cells were trypsinized and resuspended in 5 mL of complete

DMEM for each condition to which TRAIL was added. Following the end of the time course, enough ice cold PBS was added to fill each tube and centrifuged at 1500rpm for 5 minutes at 4°C. Cell pellets were washed once with cold PBS and transferred to microfuge tubes. Cells were microcentrifuged for 5 minutes at 2000rpm at 4°C. Washed cells were resuspended and lysed for 10 minutes in 1mL TritonX-100 lysis buffer (For 1L: 10% glycerol, 30 mM Tris pH 7.5, 160 mM NaCl, 2 mM EDTA pH 7.5, 1% Triton X-100) supplemented with 1mM PMSF and a 1:100 dilution of protease inhibitor cocktail. Cell membranes and debris were removed by microcentrifugation for 10 minutes at 13,200rpm at 4°C. A 50 uL input lysate aliquot was taken, 2X sample buffer added, and boiled for 5 minutes. 20 μg polyclonal TRAIL antibody was added to each 1mL sample and samples were rotated at 4°C overnight. 90uL Immobilized Protein A beads (Pierce Biotechnology, Inc. # 20333, Rockford, IL) were added to each sample and rotated at 4°C for 2 hours. Beads were washed 4 times with cold Triton X-100 lysis buffer. Following the last wash, the beads were suspended in 90 μL 2X sample buffer and boiled for 5 minutes. 40 μL of each sample was loaded onto 13.3% acrylimide gels and analyzed by SDS-PAGE with antibodies against DR4, DR5, FADD, caspase-8, and HspB2.

2.13 Measurement of HspB2-induced protein expression by BD PowerBlot

MDA-MB-231 cells stably expressing pcDNA3 FLAG vector, αB-crystallin, or HspB2 were cultured in 2-300 cm² flasks per cell line. Cells were collected using 0.05% Trypsin and washed with cold PBS with protease inhibitor added (Sigma #P8340, St. Louis, MO), resuspended in cold lysis buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM

EGTA, 1 mM PMSF, 1X protease inhibitor cocktail, 4 mM DTT) and incubated on ice for 1 hour with intermittent vortexing. Samples were then spun in a microcentrifuge for 15 minutes at 13,200 rpm. Supernatant was placed into new tubes and the pellets were resuspended with RIPA (50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.5% Deoxycholic acid, 150 mM NaCl, 1% NP40, 1 mM PMSF, 1X protease inhibitor cocktail) and protein quantification was performed using Pierce BCA assay kit (Pierce Biotechnology, Inc. #23225, Rockford, IL) and analysis was read on plate reader at 595 nm (Biorad). 6mg samples were aliquoted for each cell line and frozen at -80°C overnight. Samples were shipped to BD Biosciences for PowerBlot analysis.

2.14 Expression of HspB2 in a panel of cancer cell lines

U373, DiNTC, and U251 cells were cultured and collected with 0.05% Trypsin (Invitrogen, Carlsbad, CA) and pelleted by microcentrifugation. Cells were washed 1X with ice cold 1X PBS supplemented with 1mM PMSF and pelleted by microcentrifugation. The cell pellets were resuspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.5% Deoxycholic acid, 150 mM NaCl, 1% NP40, 1M PMSF, 1X protease cocktail inhibitors) and incubated on ice and at 10 minute intervals samples were vortexed. At the end of 30 minutes, the samples were spun in a microcentrifuge at 13,200 rpm for 15 minutes. The supernatants were transferred to new tubes. Protein quantification was performed using Pierce BCA assay kit (Pierce Biotechnology, Inc. #23225, Rockford, IL) and analysis was read on plate reader at 595 nm (Biorad). Following quantification, an equal volume of 2X sample buffer (100 mM Tris pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.01% Bromophenol blue) was added. In addition, 2LMP, LCC6, DY36T2,

A549, NCIH226, NCIH358, NCIH1299, NCIH2122, MIA PaCa2, PANC1, PC3, BxPC3, SW948, U87MG, D54MG, LG11, & BT474 cancer cell line lysates were included. All cell line samples were boiled for 5 minutes in a 100°C heat block and subjected to SDS-PAGE analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on 13.3% acrylimide gels, semidry transfer 1 hour, block in 5% milk in TBS-Tween 1 hour, incubated with primary antibodies as directed, 3-10'washes, secondary incubation 1:2000 1 hour, 4-10'washes, developed with Perkin Elmer Western Lightning Chemiluminescence (#NEL 101, Boston, MA).

2.15 Expression of HspB2 in T47D cells transfected with HspB2 shRNA

T47D cells stably expressing HspB2 shRNA or scrambled sequence control were plated onto 6-well plates at 0.4 x 10^6 cells/well. Cells were collected by 0.05% Trypsin (Invitrogen #23500-112, Carlsbad, CA), washed 1X with ice cold 1X PBS supplemented with 1mM PMSF, and pelleted by microcentrifugation. The cell pellets were resuspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.5% Deoxycholic acid, 150 mM NaCl, 1% NP40, 1 mM PMSF, 1X protease inhibitor cocktail) and incubated on ice and at 10 minute intervals samples were vortexed. At the end of 30 minutes, the samples were spun in a microcentrifuge at 13,200 rpm for 15 minutes. The supernatants were transferred to new tubes. Protein quantification was performed using Pierce BCA assay kit (Pierce Biotechnology, Inc. #23225, Rockford, IL) and analysis was read on plate reader at 595 nm (Biorad). Following quantification, an equal volume of 2X sample buffer (100 mM Tris pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.01% Bromophenol blue) was added. Samples were then boiled for 5 minutes in a 100°C heat block

and subjected to SDS-PAGE analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on 13.3% acrylimide gels, semidry transfer 1 hour, block in 5% milk in TBS-Tween 1 hour, incubated with primary antibodies as directed, 3-10'washes, secondary incubation 1:2000 1 hour, 4 washes of 10 minutes each, and developed with Perkin Elmer Western Lightning Chemiluminescence (#NEL 101, Boston, MA).

2.16 Statistical Analysis

Unless otherwise stated, all statistical analysis of the data was analyzed by two-way anova followed by a Bonferroni post test to compare experimental results to vector controls. Analysis was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

3. RESULTS

3.1 HspB2 is not expressed in TRAIL-resistant breast cancer cell lines

The role of HspB2 in apoptosis has not been defined. Previously, a variety of breast carcinoma cell lines were analyzed for their sensitivity to TRAIL (Kamradt et al., 2005). It was found that of the tested cell lines, MDA-MB-435 and -468, MCF-7 and T47D were resistant to TRAIL-induced apoptosis. MDA-MB-231 cells, however, were sensitive to TRAIL-induced apoptosis. Further analysis showed that there were similar expression levels of DR4, DR5, FADD, caspase-8, Bax, Bcl-XL, and Hsp27 and 70. Given these findings, HspB2 expression levels were determined in the above mentioned cell lines. RIPA lysates from these cell lines were subjected to SDS-PAGE analysis and probed for HspB2 expression by western blot. Low levels of HspB2 were expressed in both the MDA-MB-435 and -468 cell lines and high levels were expressed in the MCF-7 and T47D cell lines (Fig 4). Interestingly, HspB2 was not expressed in MDA-MB-231 cells, the only TRAIL sensitive line that was tested. Thus HspB2 expression correlated with resistance to TRAIL-induced apoptosis in a similar manner to what had been previously reported for αB-crystallin (Kamradt et al., 2005).

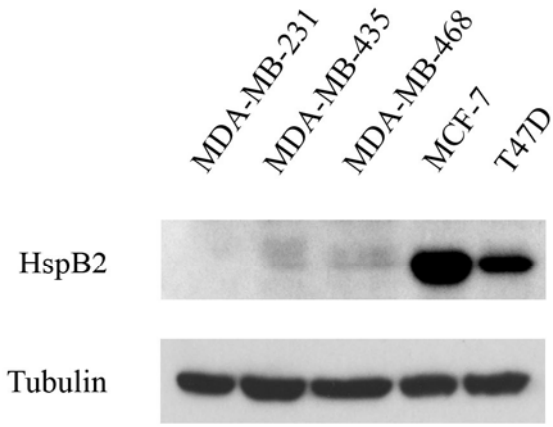


Figure 4: HspB2 is not expressed in TRAIL-resistant breast cancer cell lines.

Immunoblot of MDA-MB-231, MDA-MB-435, MDA-MB-468, MCF-7, and T47D breast cancer cell lines were probed for HspB2 expression. Tubulin was used as a loading control.

3.2 Ectopic overexpression of HspB2 in MDA-MB-231 cells confers protection against TRAIL-induced apoptosis

3.2.1 HspB2 inhibition against TRAIL-induced apoptosis by timecourse analysis

To determine if overexpression of HspB2 expression in MDA-MB-231 cells would render these cells resistant to TRAIL-induced apoptosis, FLAG-tagged HspB2 cDNA was constructed and stably transfected into MDA-MB-231 cells (Fig 5A). It had been previously shown that αB-crystallin expression in these cells rendered them resistant to apoptosis. Therefore, cell lines expressing αB-crystallin were used as a positive control in these assays. Empty vector, two HspB2 clones, or αB-crystallin were individually plated in 6-well plates and left untreated or treated for various time points with 500 ng/mL TRAIL over the course of 24 hours. Samples were subsequently scored for apoptosis by nuclear morphology as described in the methods. In the cells overexpressing HspB2, there was a decrease in the level of apoptosis compared to the vector control (Fig 5). After 24 hours, the vector cells had 46% ± 14% SEM apoptosis while cells expressing HspB2 A2, HspB2 A3, or HspB2 A4 had 10% ± 5% SEM, 7% ± 1% SEM, and 18% ± 11% SEM apoptosis respectively. HspB2 expression protected from apoptosis to similar levels as what was observed in cells overexpressing αB-crystallin.

3.2.2 HspB2 inhibition against TRAIL-induced apoptosis by FACS analysis

To further confirm these results, FACS analysis was used to quantitate the levels of apoptosis in MDA-MB-231 FLAG-tagged cell lines. Empty vector, αB-crystallin,

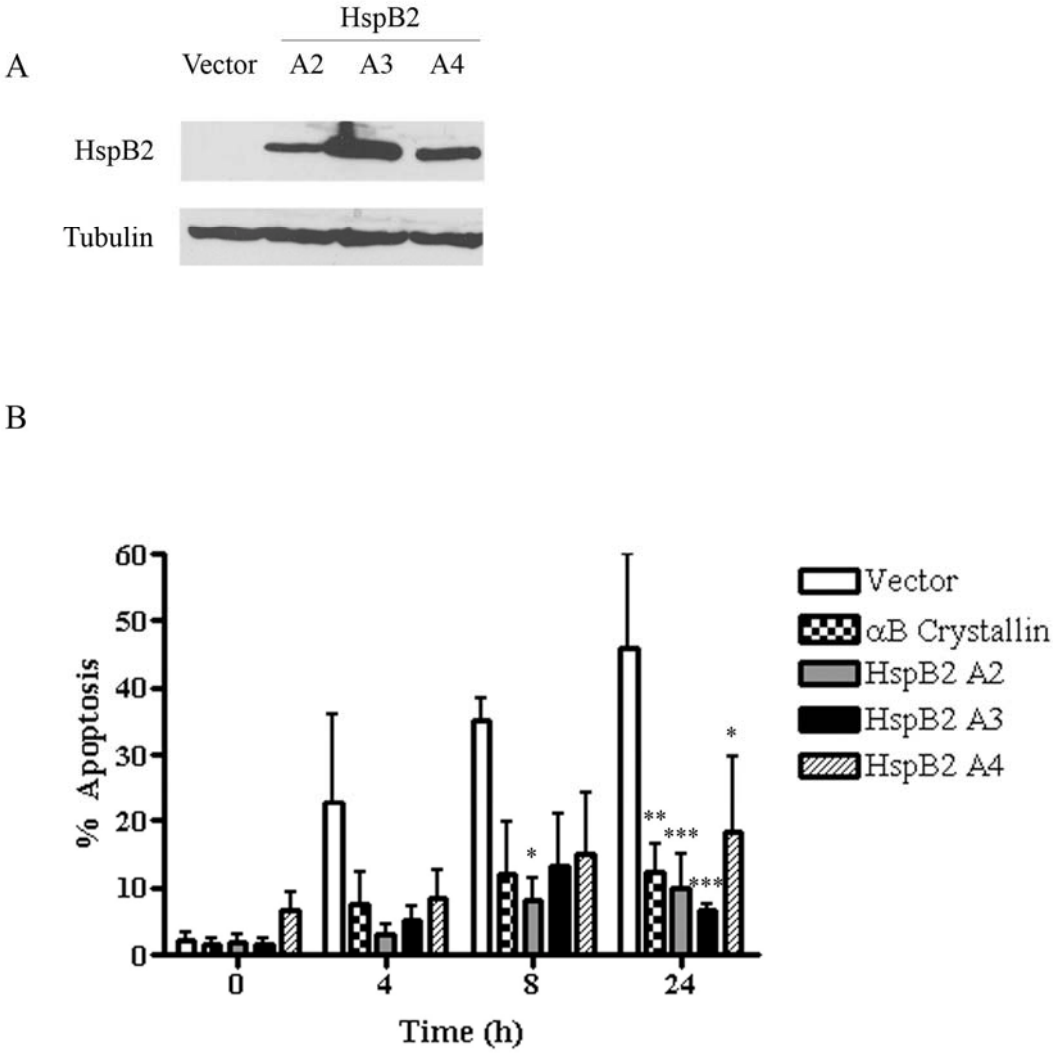


Figure 5: HspB2 inhibits TRAIL-induced apoptosis measured by timecourse analysis.

A Immunoblot of MDA-MB-231 cells stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. The ectopically expressed proteins were detected using the Flag M2 monoclonal antibody.

B MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. αB-crystallin was used as a positive control. Cells were treated with 500 ng/mL TRAIL from 0-24h. Both floating and adherent cells were collected, Hoecsht stained, and scored for apoptotic nuclei. The data represents the mean +/- SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vector control for each time point).

or HspB2 overexpressing cells were plated and left untreated or treated with 500ng/mL TRAIL for 4 hours. Cells were collected, stained with Annexin-V-PE and 7AAD, and analyzed by FACS. When cells are undergoing apoptosis, an early event is the translocation of phosphatidyl serine phospholipids from the inner plasma member to the outer side. Annexin-V binds to the phosphatidyl serines and is conjugated to PE, a fluorochrome that can be detected by flow cytometry. Therefore, cells that are negative for Annexin-V-PE are alive and as they become positive, they are undergoing apoptosis. 7AAD is a vital dye that is used in conjunction with Annexin V-PE in order to distinguish early and late apoptosis. When cells are negative for both Annexin V-PE and 7AAD, they are counted as live cells and appear in the bottom left quadrant. Early apoptotic cells that are Annexin-V positive and 7AAD negative appear in the bottom right quadrant. Late stage apoptotic cells are positive for both Annexin V-PE and 7AAD and appear in the top right quadrant. The two right quadrants are added together for the total amount of apoptotic cells. In the untreated condition, 87% of the cells expressing vector were alive (Fig 6). When these cells were treated with TRAIL, the amount of viable cells decreased to 64%. In comparison to vector control cells, untreated αB-crystallin expressing cells showed similar levels of viability at 82%, but that changed only marginally to 78% upon treatment with TRAIL due to the protection conferred by αB-crystallin. HspB2 cells were protected in a similar manner from TRAIL treatment. Cells expressing HspB2 declined only 5% in cell viability when treated with TRAIL. These results confirm that ectopic overexpression of HspB2 in MDA-MB-231 cells inhibits of TRAIL-induced apoptosis.

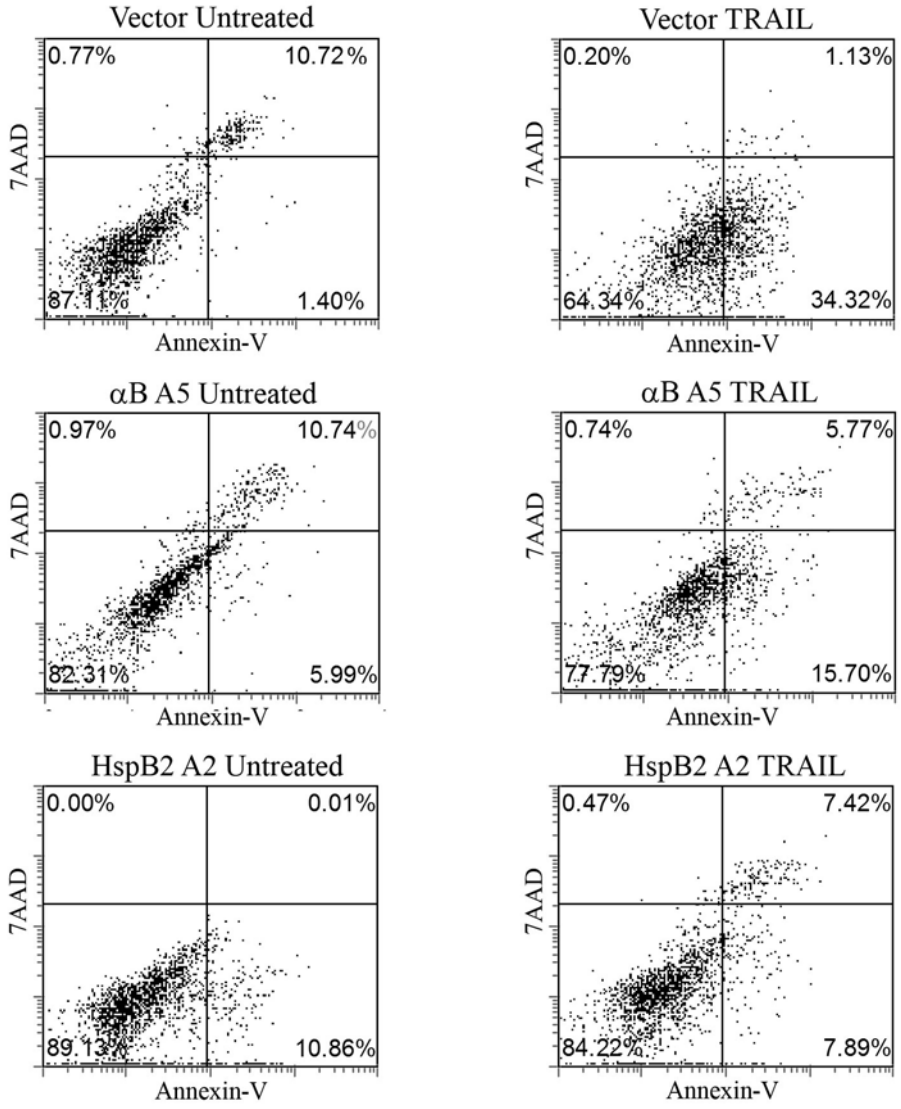


Figure 6: HspB2 inhibits TRAIL-induced apoptosis measured by FACS analysis.

MDA-MB-231 overexpressing empty vector, αB-crystallin, or HspB2 were left untreated or treated with 500ng/mL TRAIL for 4 hours. After 4 hours, both adherent and floating cells were collected and stained with AnnexinV-PE and 7AAD and analyzed by flow cytometry.

3.3 EXPRESSION OF HSPB2 DELAYS REGRESSION OF TUMOR GROWTH IN VIVO

If HspB2 plays a physiological role in apoptosis, the anti-apoptotic in vitro effects of HspB2 should also be apparent in vivo. To demonstrate this, a xenograft experiment using nude mice was designed. MDA-MB-231 cells stably expressing either empty vector or wild type HspB2 were cultured for subsequent transplant into the mammary fat pads of female nude mice in order to establish start-up tumors. These tumors were then excised and dissected into $1mm^3$ pieces implanted into both mammary fat pads of new nude mice. These new mice were divided into four treatment groups of 5 mice per group. In the control groups, there were those injected with either tumors established from empty vector expressing cells or from HspB2 expressing cells. These mice were given PBS over the length of the study. The experimental groups were injected with tumors from the same groups as the controls; however, these mice were given injections of TRAIL at 5 mg/kg/day instead of PBS. The mice were subjected to treatment for five weeks. Tumor size was measured weekly. After five weeks, the data was analyzed and the results were as follows (Fig 7). Mice injected with empty vector tumors showed a steady increase in tumor growth when treated with PBS as a control. Similarly, when mice injected with HspB2 expressing tumors and treated with PBS also showed an increase in tumor growth over five weeks. When treated with TRAIL, the vector tumors immediately decreased in size and were almost unapparent throughout the experiment. In contrast, when mice with HspB2 expressing tumors were given TRAIL, these tumors showed an initial decrease similar to empty vector tumors following treatment with TRAIL. Between 2 and 3 weeks there was an increase in tumor growth, and this increase continued until the end of five weeks. However, these tumors

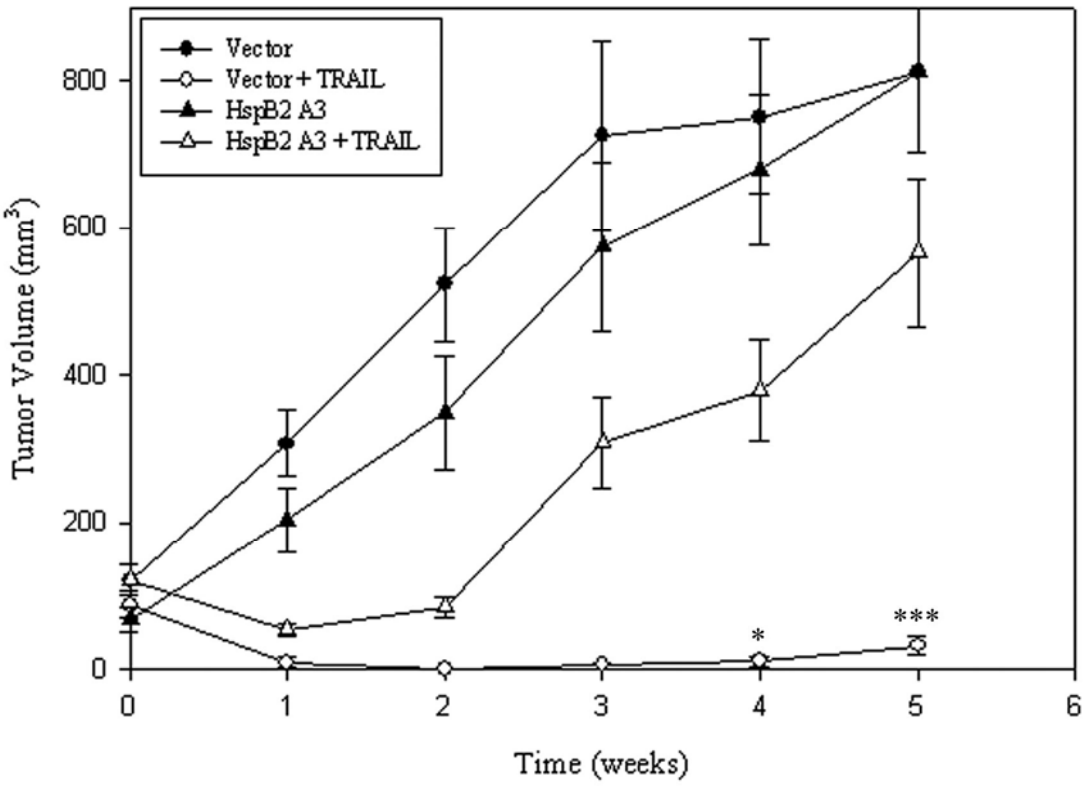


Figure 7: Expression of HspB2 delays regression of tumor growth in vivo.

One mm^3 pieces of MDA-MB-231 xenograft breast carcinomas expressing vector or HspB2 were transplanted subcutaneously into both mammary fat pads of 4–5-week old female athymic nude mice (Harlan Sprague-Dawley). Two weeks later, mice with established tumors (expressing vector or HspB2) were treated with PBS (vehicle) or TRAIL 5 mg/kg/day by intraperitoneal injection (4 groups of 5 mice each). Tumor volume was measured weekly. Data represents the mean +/- SEM of 10 tumors/group (* $p < 0.05$, *** $p < 0.001$ versus HspB2 TRAIL condition).

never reached the size of the tumors treated with PBS. Overall, HspB2 expression conferred resistance to TRAIL-induced apoptosis in vivo.

3.4 Ectopic overexpression of HspB2 in MDA-MB-231 cells confers protection TNFα-induced Apoptosis

3.4.1 HspB2 inhibition against TNFα-induced apoptosis by timecourse analysis

Since the ectopic overexpression of HspB2 was able to inhibit TRAIL-induced apoptosis both in vitro and in vivo, it was postulated that HspB2 would also be effective against other extrinsic pathway apoptotic inducers such as TNFα. As mentioned earlier, TNFα binds to the TNF receptor (TNFR) and initiates DISC formation and subsequent activation of apoptosis. MDA-MB-231 cells were stably transfected with empty vector, αB-crystallin, or HspB2 and left untreated or treated with 10 ng/mL TNFα and 1μg/mL cycloheximide over the course of 24 hours (Fig 8). Following each time point, the cells were collected and stained with Hoecsht. Apoptosis was determined by scoring nuclear morphology. Following this, the percent apoptosis was calculated. Similar to TRAIL, the vector cells treated with TNFα had an increased percentage of apoptosis and plateau at 42% ± 1% SEM apoptosis after 24 hours. αB-crystallin expressing cells showed 25% ± 5% SEM apoptosis and both HspB2 cell lines reached only 17% ± 6% SEM apoptosis after 24 hours. This demonstrated the ability of HspB2 to confer protection against TNFα-induced apoptosis as well as apoptosis induced by TRAIL.

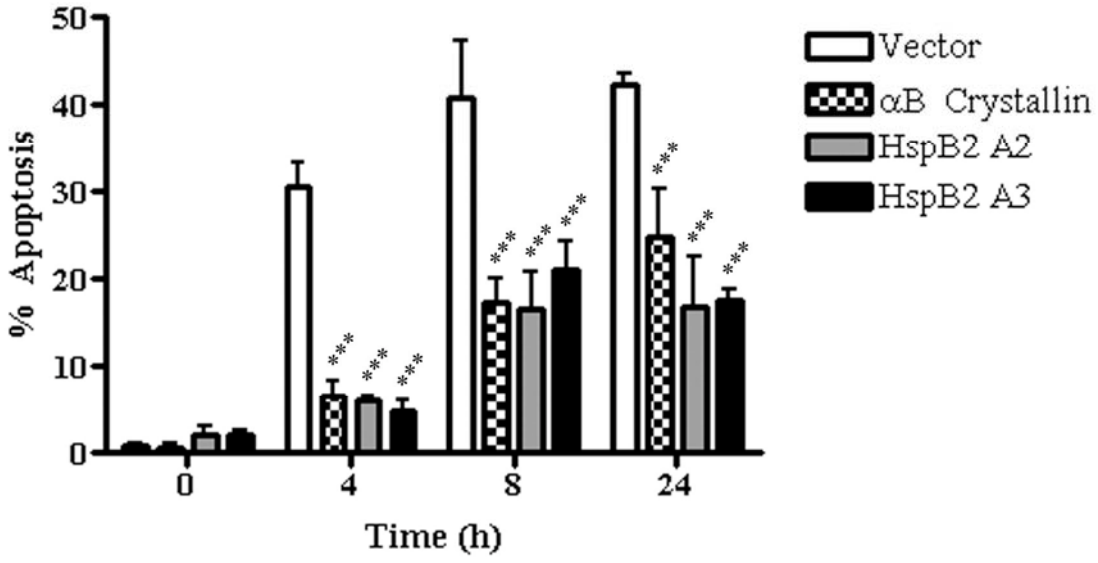


Figure 8: HspB2 inhibits TNFα-induced apoptosis measured by timecourse analysis.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. aB crystallin was used as a positive control. Cells were treated with 10ug/mL TNFα and 1ug/mL cycloheximide from 0-24h. Both adherent and floating cells were collected, Hoecsht stained, and scored for apoptotic nuclei. The data represents the mean +/- SEM of three independent experiments (*** $p < 0.001$ versus control at each time point).

3.4.2 HspB2 inhibition against TNFα-induced apoptosis by FACS analysis

To further characterize this phenotype, FACS analysis was performed to quantitate apoptosis. Empty vector, αB-crystallin, or HspB2 expressing MDA-MB-231 cells were left untreated or treated with TNFα and cycloheximide for 4 hours. Cells were then collected and stained with Annexin V-PE and 7AAD and submitted for FACS analysis. In the vector untreated cells, 85% of the cells were viable (Fig 9). When these cells were treated with TNFα, viability decreased to 61%. αB-crystallin maintained 70% viability with and without treatment and in HspB2 expressing cells, untreated the cells were 80% viable and when treated with TNFα, viability only decreased to 73%. Therefore, HspB2 expression in these cells also conferred apoptotic protection against TNFα. This provides further evidence that HspB2 is able to protect against death receptor induced apoptosis.

3.5 Ectopic overexpression of HspB2 inhibits fluorogenic caspase activation

3.5.1 HspB2 inhibits caspase-8 activity (IETD)

Given the ability of HspB2 to protect against both TRAIL- and TNFα-induced apoptosis, identification of the site of action for HspB2 was ascertained by caspase activity assays. MDA-MB-231 cells expressing empty vector or HspB2 were plated and were left untreated or treated with 50 ng/mL TRAIL for various time points over 4 hours. Cells were then incubated with a fluorogenic substrate, AFC, which contains a caspase-8 cleavage site, IETD. When caspase-8 is

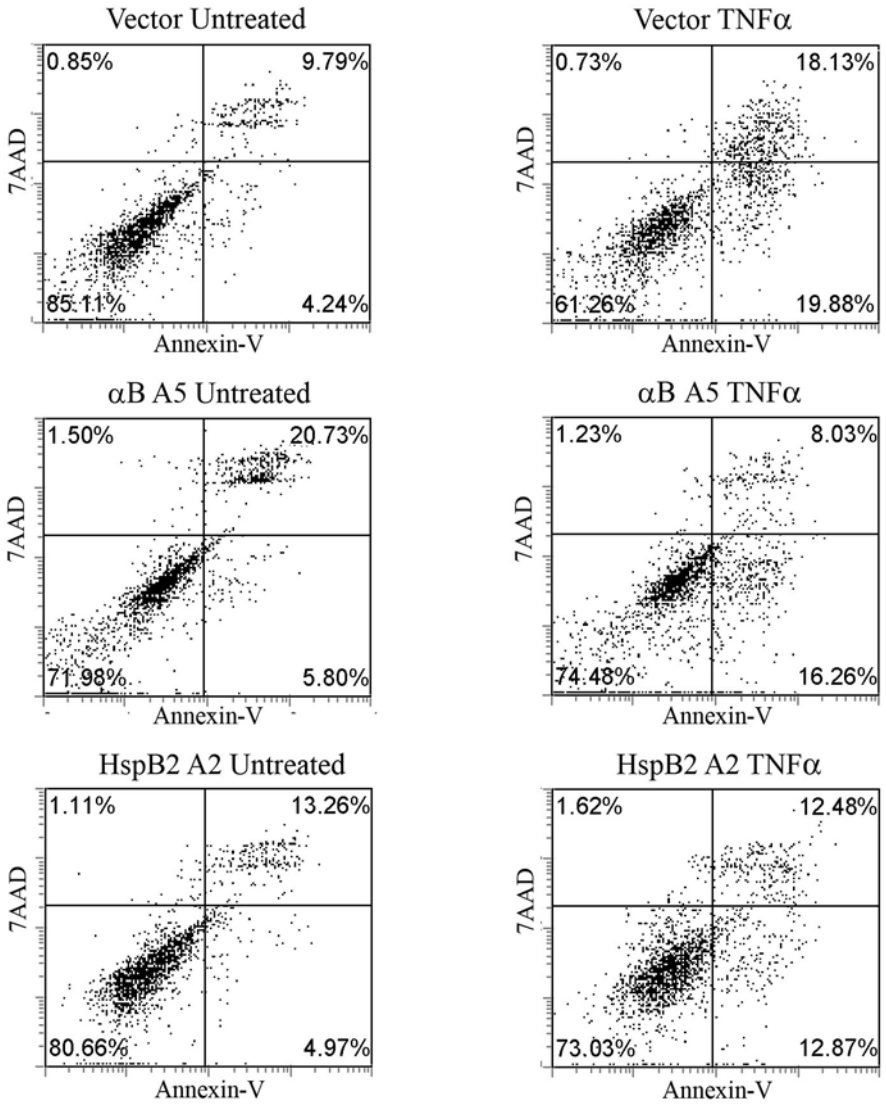


Figure 9: HspB2 inhibits TNFα-induced apoptosis measured by FACS analysis.

MDA-MB-231overexpressing empty vector, αB-crystallin, or HspB2 were left untreated or treated with 10ng/mL TNFα and 1μg/mL cycloheximide for 4 hours. After 4 hours, both adherent and floating cells were collected and stained with AnnexinV-PE and 7AAD and analyzed by flow cytometry.

active, it is cleaved after the last aspartic acid residue, releasing the fluorogenic AFC. This fluorescence was measured via spectrophotometer and the relative amounts over control are calculated (Fig 10). In the vector line, there was an increase in relative fluorescence of about 2-fold over the course of 4 hours. In contrast, the HspB2 line showed a minimal increase in fluorescence, reflecting low amounts of caspase-8 activation. This result showed that when overexpressed, HspB2 is capable of inhibiting caspase-8 cleavage in response to TRAIL.

3.5.2 HspB2 inhibits caspase-3 activity (DEVD)

The next step was to examine the cleavage of downstream executioner caspase-3. For this assay, the same protocol was used except the fluorogenic AFC tag was conjugated to a DEVD target site which is specific for caspase-3. MDA-MB-231 cells expressing empty vector or HspB2 were plated and were left untreated or treated with 200 ng/mL TRAIL for various time points over 3 hours. Cells are then incubated with a fluorogenic substrate, AFC, which contains a caspase-3 cleavage site, DEVD (Fig 11). When caspase-3 is active, the fluorogenic AFC is released and the relative amounts of this fluorescence over control are measured and calculated. In the vector line, there was a noticeable increase in relative fluorescence of approximately 6 - fold immediately at the 30 minute time point. In contrast, the HspB2 line again showed a minimal increase in fluorescence, reflecting low amounts of caspase-3 activation. These results illustrated that overexpression of HspB2 prevented activation of both caspase-8 and caspase-3 in response to TRAIL treatment.

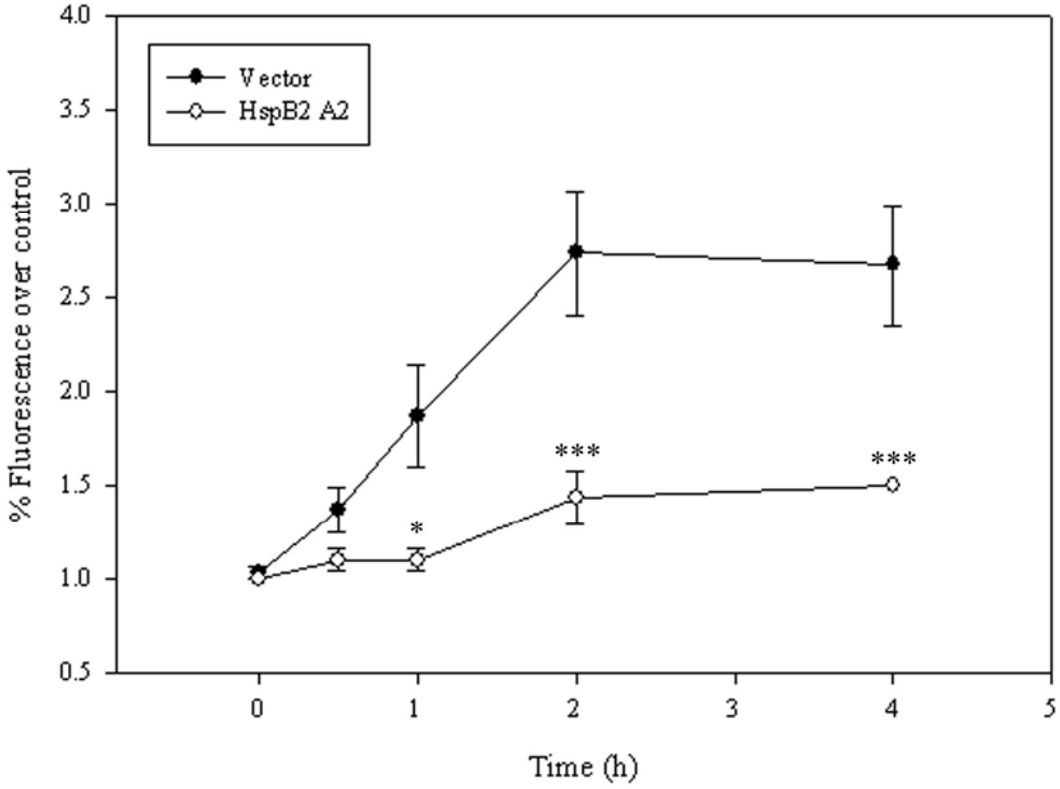


Figure 10: Ectopically expressed HspB2 prevents activity of caspase-8.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 50 ng/mL TRAIL for 0-3h. After treatment, both adherent and floating cells were collected and incubated with a fluorogenically tagged caspase-8 cleavage sequence, IETD. Active caspase-8 levels were measured using a spectrophotometer as percent fluorescence over control. The data represents the mean +/- SEM of three independent experiments (* $p < 0.05$, *** $p < 0.001$ versus control at each time point).

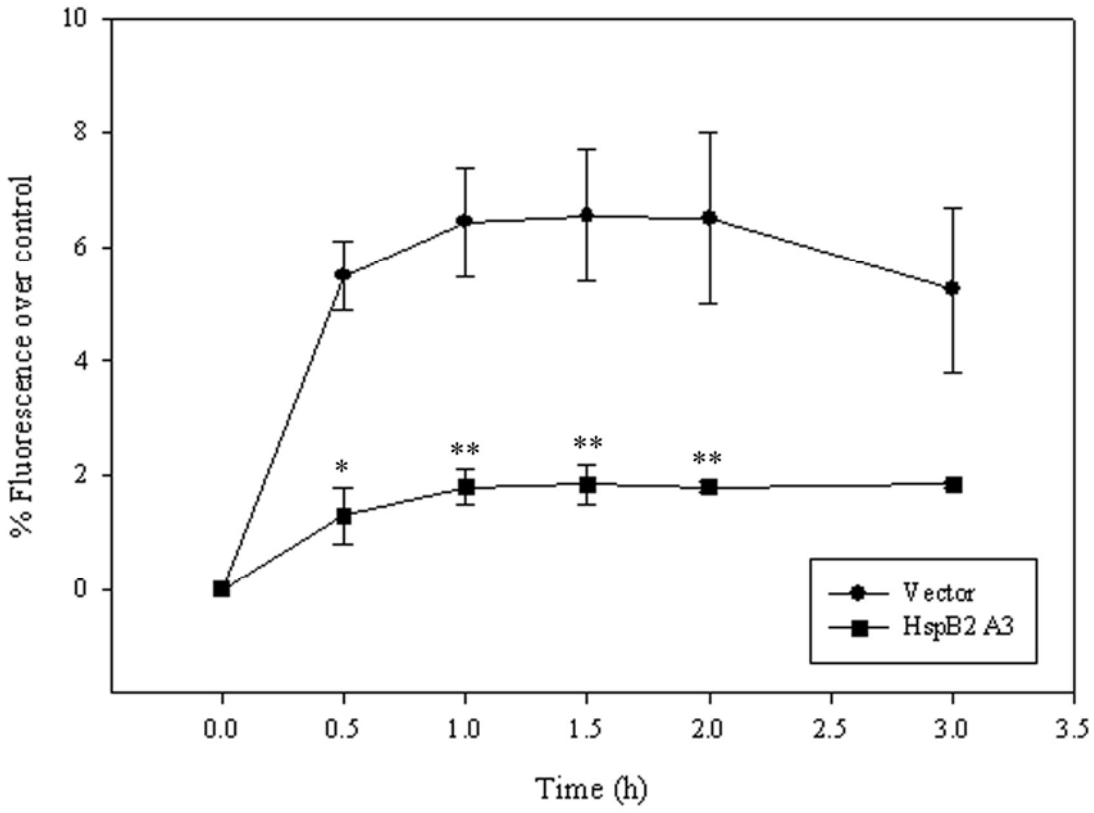


Figure 11: Ectopically expressed HspB2 prevents caspase-3 activity.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 200 ng/mL TRAIL for 0-3h. After treatment, both floating and adherent cells were collected and incubated with a fluorogenically tagged caspase-3 cleavage sequence, DEVD. Active caspase-3 levels were measured using a spectrophotometer as percent fluorescence over control. The data represents the mean +/- SEM of two independent experiments (* $p < 0.05$, ** $p < 0.01$ versus control at each time point).

3.6 WESTERN BLOTTING ANALYSIS SHOW DELAY OF BROAD CASPASE CLEAVAGE BY HSPB2

To further map HspB2 activity in the apoptotic pathway the major members of programmed cell death, the caspases, was analyzed. SDS-PAGE was used for analysis. MDA-MB-231 cells stably expressing empty vector or HspB2 were plated and left untreated or treated with 500 ng/mL over the course of 8 hours. Cells were collected at each time point and RIPA lysates were made. Representative cells were simultaneously treated with TRAIL and collected for Hoecsht staining. The percentage of cells undergoing apoptosis at each time point was calculated by nuclear morphology. Blots were probed with various antibodies against several caspases and regulatory proteins in the caspase cascade (Fig 12). Vector expressing cells showed cleavage of apical caspase-8 as early as one hour after treatment. By 8 hours, most of the full length caspase-8 had been cleaved. In contrast, the cells expressing HspB2 show limited caspase-8 cleavage over 8 hours consistent with the IETD fluorogenic assay results. Apical caspase-10 also showed a similar cleavage pattern. By 6 hours, the vector expressing cells showed no full length caspase-10 while the cells expressing HspB2 maintained the level of caspase-10 throughout the 8 hours. Since active caspase-8 can cleave pro-apoptotic Bcl-2 family member, Bid, we tested the kinetics of Bid cleavage. While vector expressing cells lost full length Bid levels by 6 hours, HspB2 expressing cells exhibited full length Bid, albeit at a low level. Once cleaved, Bid induces cytochrome c and Smac/DIABLO release from the mitochondria. Release of cytochrome c triggers apoptosome formation and activation of caspase-9. When vector transfected cells were treated with TRAIL, there was a disappearance of the pro-form of caspase-9 by 4 hours. In contrast, HspB2 expressing cells showed a different pattern. Full length caspase-9 was maintained over the course of 8 hours. As stated earlier,

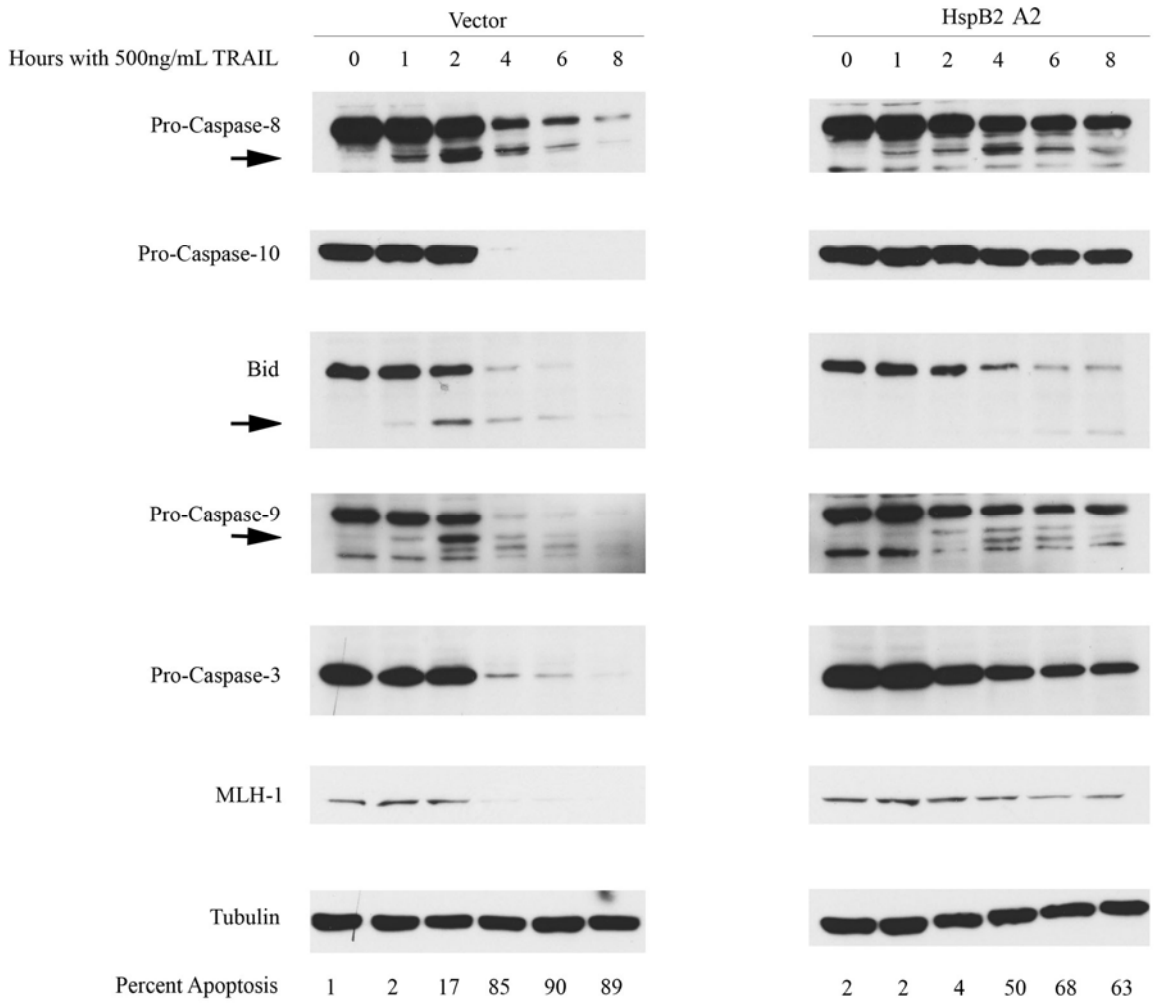


Figure 12: HspB2 inhibits broad caspase cleavage in response to TRAIL.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 500 ng/mL TRAIL for 0-8h. At each time point both floating and adherent cells were collected and subjected to immunoblotting and scoring of apoptotic nuclei by DAPI. Immunoblots were probed with antibodies against the following: caspase-8, caspase-10, Bid, caspase-9, caspase-3, and MLH-1. Tubulin was used as a loading control. Arrows indicate cleavage fragments. Data is representative of three independent experiments.

HspB2 expression inhibited caspase-3 activation by DEVD fluorogenic assay. Inhibition of caspase-3 cleavage by western blot supported this data. In accordance with this kinetic profile, the proform of caspase-3 disappeared in vector transfected cells by 4 hours. Additionally, MLH-1, a protein involved in DNA mismatch repair and a caspase-3 substrate, disappeared in vector transfected cells, but was maintained in cells expressing Hspb2 (Chen et al., 2004). Moreover, overall levels of percent apoptosis were 20% lower in cells expressing HspB2 as compared to vector alone. These observations demonstrate that HspB2 delays apoptosis throughout the entire caspase cascade beginning at the apical caspase level as well as providing further support of the previous data.

3.7 HSPB2 HAS NO EFFECT ON CHEMOTHERAPY-INDUCED APOPTOSIS

We have shown that ectopic expression of HspB2 in a TRAIL sensitive cell line can render cells resistant to TRAIL-induced apoptosis. To explore the mechanism of action of HspB2, the next step was determine if inhibition was restricted to drugs that stimulate the extrinsic pathway, or if HspB2 expression can protect cells from intrinsic pathway stimulation by chemotherapy agents etoposide, doxorubicin, and staurosporine. MDA-MB-231 transfected with either empty vector or HspB2 and treated with each of the indicated drugs (Fig 13A).

3.7.1 Etoposide-induced apoptosis

MDA-MB-231 transfected with either empty vector or HspB2 were examined via a 0-48 hour timecourse analysis for their ability to protect against 50 μM etoposide, a topoisomerase II inhibitor. Apoptosis was visible after 24 hours with vector cells showing 54% ± 12% SEM and both HspB2 A2 and HspB2 A4 clones displaying 65% ± 15% SEM (Fig 13B). No significant difference in apoptosis levels compared to vector even 48 hours post treatment.

3.7.2 Doxorubicin-induced apoptosis

A similar experiment was carried out studying apoptosis following treatment with doxorubicin. Using the same cell lines and time points, we treated these cells with 750 nM doxorubicin over 48 hours (Fig 14). Following the timecourse, cells were collected, stained, and analyzed for apoptosis by nuclear morphology as above. Similar results were obtained showing that HspB2 was unable to protect against doxorubicin-induced apoptosis. Interestingly, the cells which expressed HspB2 had higher levels of apoptosis than the vector alone. Most notable was the HspB2 A2 clone which showed 54% ± 4% SEM apoptosis after 48 hours compared to vector and HspB2 A4 cell lines which exhibited 19% ± 4% SEM and 37% ± 2% SEM apoptosis respectively.

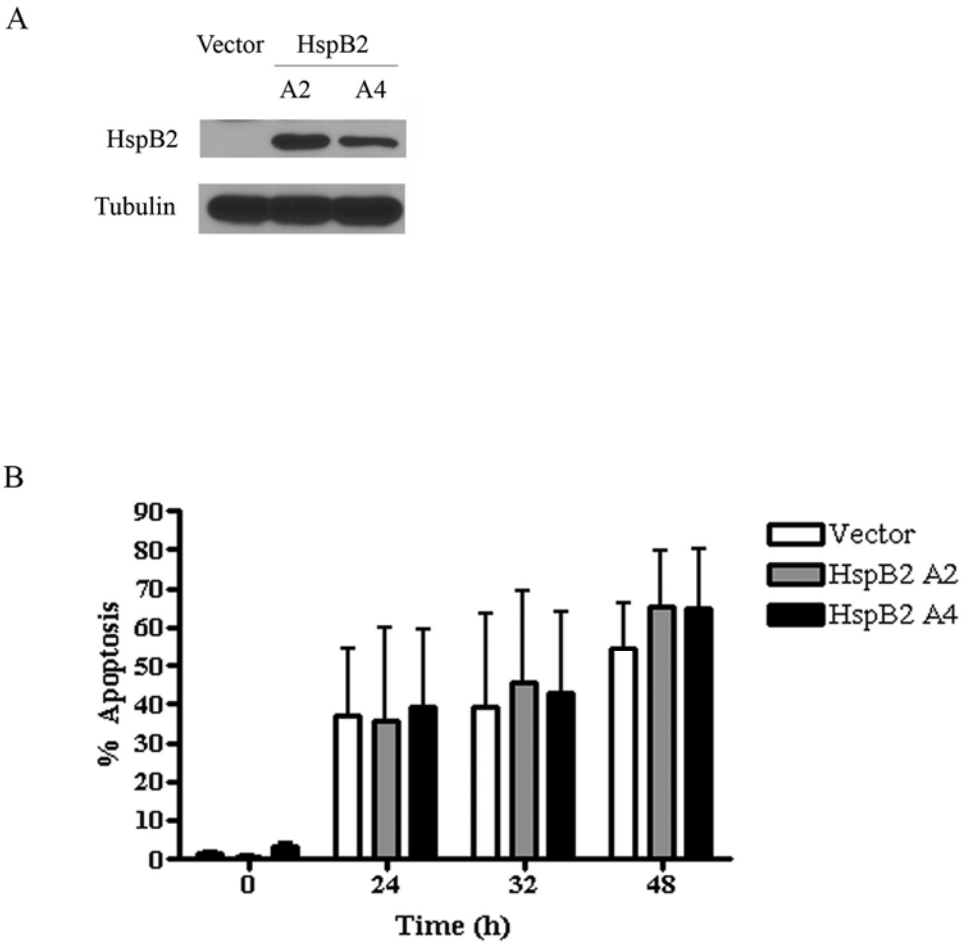


Figure 13: HspB2 does not inhibit apoptosis induced by etoposide.

A Lysates of MDA-MB-231 cells stably expressing empty vector or HspB2 were subjected to western analysis and probed for Tubulin or HspB2 expression.

B MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 50 μM etoposide or vehicle control (not shown) over 48 hours. Both adherent and floating cells were collected. Apoptosis was measured by scoring for nuclear morphology. The data represents the mean +/- SEM of three independent experiments. Statistical analysis revealed no significant differences between vector and HspB2 expressing cells.

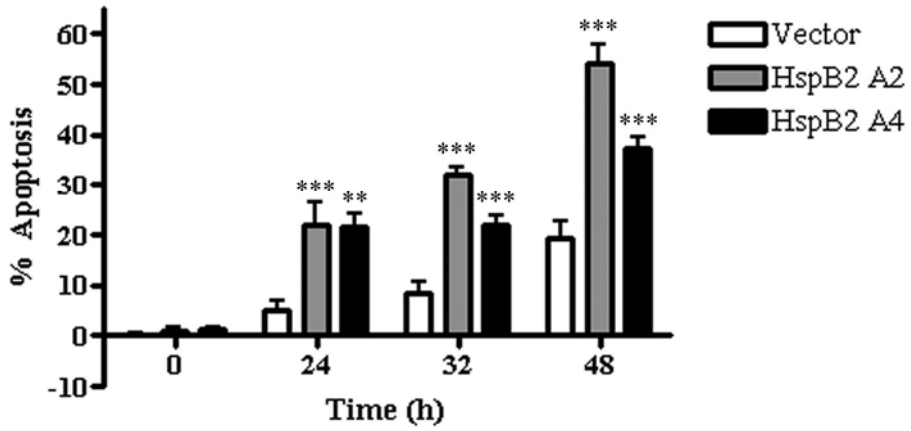


Figure 14: HspB2 does not inhibit apoptosis induced by doxorubicin.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 750 nM doxorubicin or vehicle control (not shown) for up to 48 hours. Both adherent and floating cells were collected. Apoptosis was measured by scoring for nuclear morphology. The data represents the mean +/- SEM of three independent experiments (** $p < 0.01$, *** $p < 0.001$ versus control at each time point).

3.7.3 Staurosporine-induced apoptosis

To provide further support with another potent inhibitor, an experiment was performed using staurosporine. A 24-hour timecourse with 750 nM staurosporine revealed that HspB2 expression did not inhibit apoptosis (Fig 15). At 8 and 16 hours both clones of HspB2 displayed no apoptotic inhibition. By 24 hours, cells expressing vector had 44% ± 4% SEM apoptosis, and HspB2 A2 and HspB2 A4 showed 60% ± 13% SEM and 57% ± 13% SEM apoptosis respectively. Together, these experiments show that in response to chemotherapy drugs, HspB2 is unable to inhibit apoptosis.

3.8 HSPB2 FAILS TO PREVENT MITOCHONDRIAL RELEASE OF CYTOCHROME C

3.8.1 Cytochrome c release measured by western blotting

Since HspB2 expression was unable to prevent apoptosis induced by chemotherapy drugs, the effect of HspB2 on cytochrome c release was investigated to further map its anti-apoptotic mechanism. MDA-MB-231 cells ectopically over expressing HspB2 or empty vector were treated with 500 ng/mL TRAIL for 0, 1, 2, 4, & 6 hours. Both floating and adherent cells were collected, lysed, and separated into cytosolic and mitochondrial fractions by centrifugation. Cytochrome c release was analyzed by SDS-PAGE analysis with antibodies against cytochrome c, Smac/DIABLO. CoxIV and tubulin were used as controls. Further observation

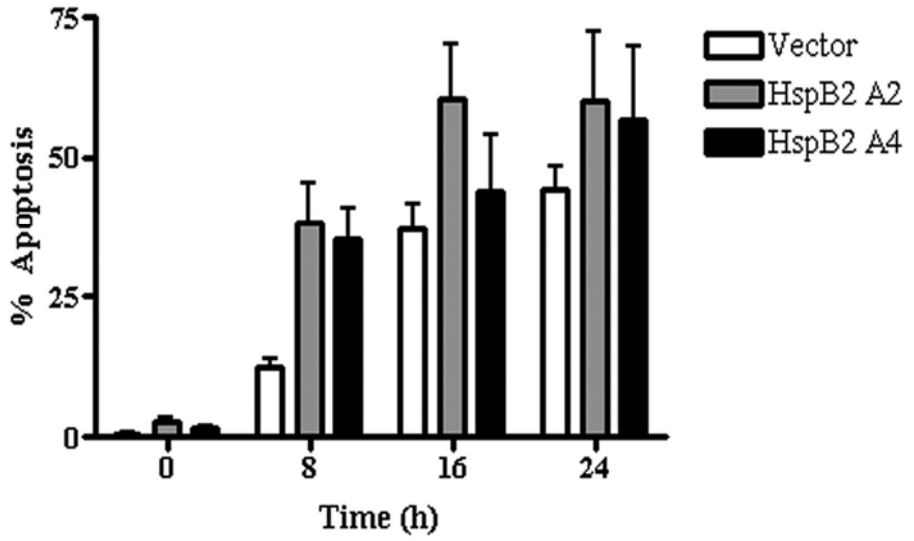


Figure 15: HspB2 does not inhibit apoptosis induced by staurosporine.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 750 nM staurosporine or vehicle control (not shown) over 24 hours. Both adherent and floating cells were collected. Apoptosis was measured by scoring for nuclear morphology. The data represents the mean +/- SEM of three independent experiments. Statistical analysis revealed no significant differences between vector and HspB2 expressing cells.

showed an increasing release of both cytochrome c and Smac/DIABLO over the time in the empty vector cells (Fig 16). TRAIL treatment of HspB2 transfected cells resulted in release of cytochrome c and Smac/DIABLO with comparable kinetics to vector expressing cells treated with TRAIL. These results would suggest that HspB2 inhibits apoptosis downstream of the mitochondria, perhaps at caspase-3.

3.8.2 Cytochrome c release measured by ELISA

To confirm this result, further analysis was carried out by a more sensitive assay, the cytochrome c ELISA. MDA-MB-231 cells expressing either empty vector or HspB2 were treated with 500 ng/mL TRAIL for 2 hours (Fig 17A). Following the timecourse, both floating and adherent cells were collected and mitochondrial isolation was performed by fractionation to obtain a supernant representative of the released cytochrome c, and a pellet representative of the non-released cytochrome c. The amount of released cytochrome c was determined by ELISA (Fig 17B). Vector transfected cells treated with TRAIL had an average of 3 fold increase in cytochrome c release as compared to control treated cells. HspB2 A2 transfected cells had an average of 2.5 fold and HspB2 A4 averaged 1.5 fold increase in cytochrome c release as compared to their respective untreated conditions. Although there was less cytochrome c release in HspB2 A4 corresponding with a higher expression of HspB2 protein, the results were not significant when compared to vector. Since this experiment, in conjunction with the western blot data, demonstrates that HspB2 does not inhibit the release of cytochrome c upon TRAIL stimulation, HspB2 may inhibit apoptosis downstream of the mitochondria.

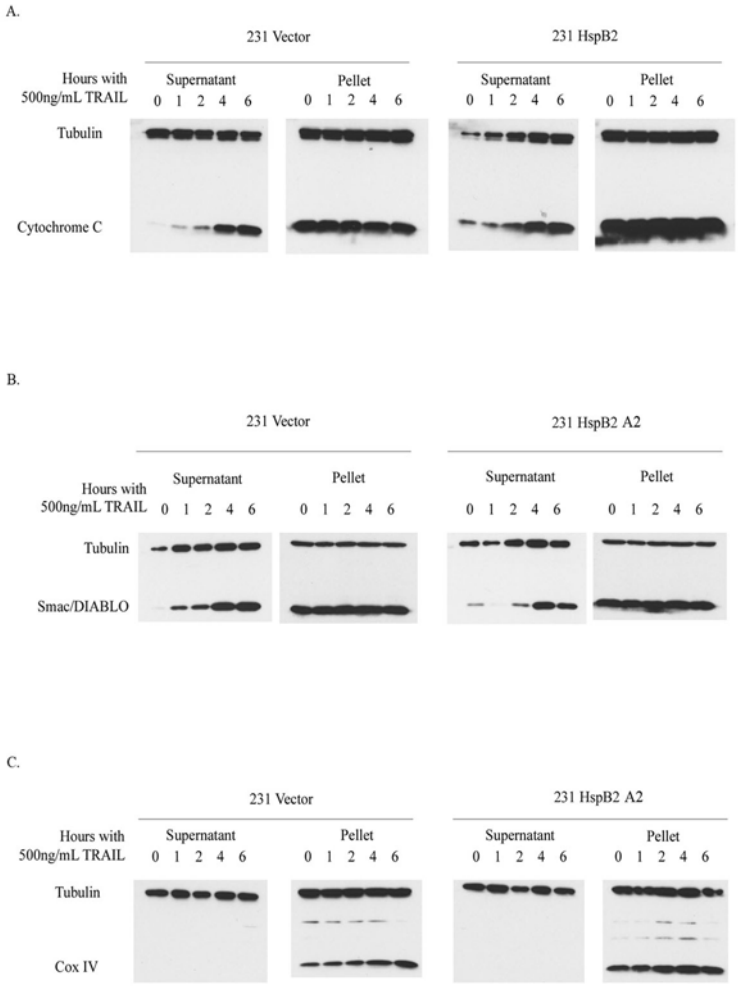


Figure 16: HspB2 expression does not prevent release of mitochondrial proteins by western blotting.

MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. Cells were treated with 500 ng/mL TRAIL for 0-6h. Following treatment, both adherent and floating cells were collected and separated into mitochondrial (pellet) and cytoplasmic (supernatant) fractions by centrifugation. These fractions were then subjected to immunoblotting antibodies against monoclonal antibodies against cytochrome c (A), Smac/DIABLO (B), and cox IV (C). Tubulin was used as a loading control. Data is representative of three independent experiments.

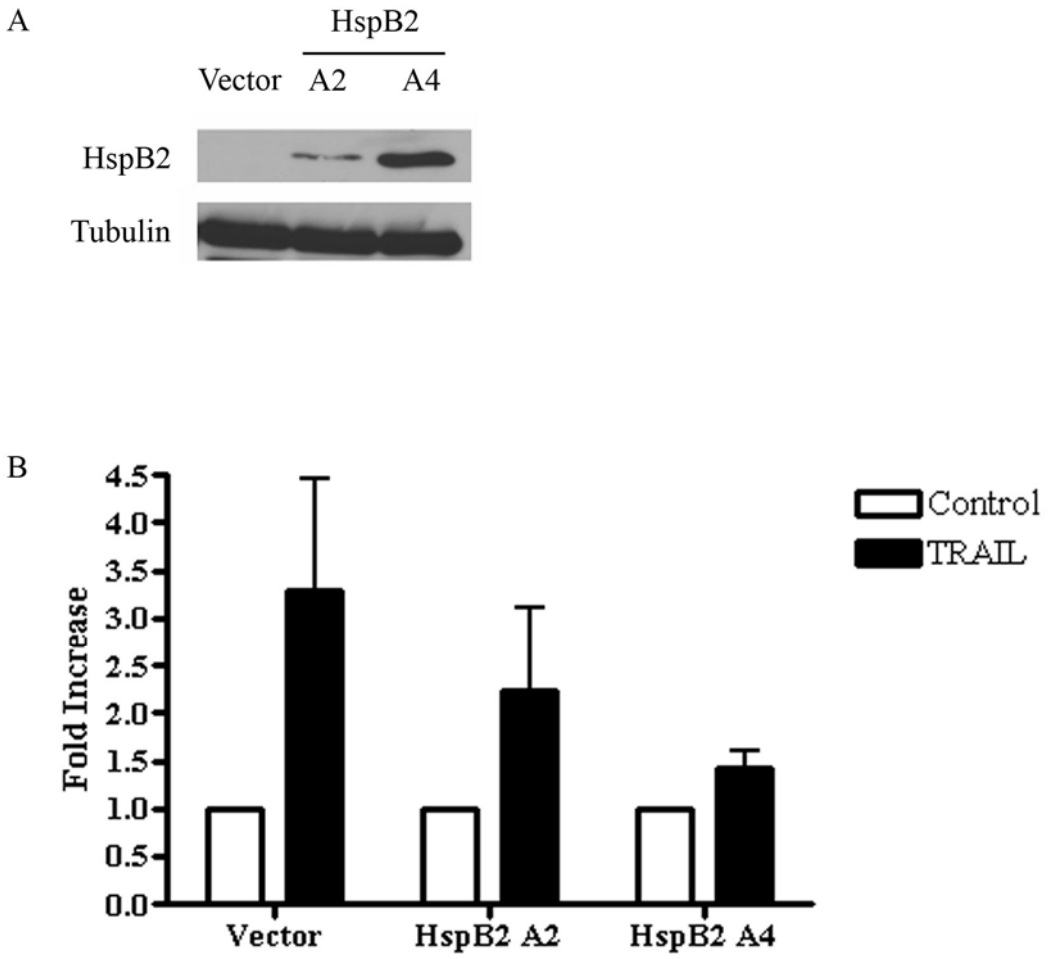


Figure 17: HspB2 does not prevent cytochrome c release by ELISA.

A Lysates of MDA-MB-231 cells stably expressing empty vector or HspB2 were subjected to western analysis and probed for Tubulin or HspB2 expression.

B MDA-MB-231 cells stably overexpressing empty vector or HspB2 were treated with TRAIL for 2 hours. Following treatment both adherent and floating cells were collected and fractionated by centrifugation. Cytosolic fractions were analyzed by cytochrome c ELISA and results displayed as relative fold increase of cytochrome c levels. Data represents the mean +/- SEM of 5 independent experiments. Statistical analysis revealed no significant difference between TRAIL-treated vector and both clones of HspB2.

3.9 HspB2 inhibits tBid-induced apoptosis

To further investigate the mechanism of HspB2 in apoptosis, the ability of HspB2 to confer protection downstream of the mitochondria was examined. MDA-MB-231 cells stably expressing either empty vector or two individual clones of HspB2 were co-transfected with GFP and increasing amounts of tBid, and cell were examined for apoptosis by nuclear morphological analysis. A clonal variance in apoptotic protection was observed (Fig 18). HspB2 A2 clones transfected with tBid showed no inhibition of apoptosis with levels equaling vector levels. In contrast, HspB2 A4 clones transfected with pCMV5a-tBid exhibited decreased apoptosis compared to both vector and HspB2 A2 cell lines. This discrepancy in apoptotic protection may be explained by the higher levels of HspB2 expression in the HspB2 A4 clone. Protection from tBid induced cell death was seen in the clone expressing high levels of HspB2, thus perhaps the expression level was not high enough in the HspB2 A2 clone to have an effect against such a strong apoptosis inducer such as tBid. These results support the cytochrome c release assays and suggest a mechanism for HspB2 downstream of the mitochondria, perhaps at the level of caspase-3 activation.

3.10 TRAIL-DISC immunoprecipitation

Hypothesizing that HspB2 inhibited apoptosis in MDA-MB-231 breast cancer cells upstream in the caspase cascade, the next course of action was to see if HspB2 was physically associating with components of the DISC. MDA-MB-231 cells over expressing either HspB2 or

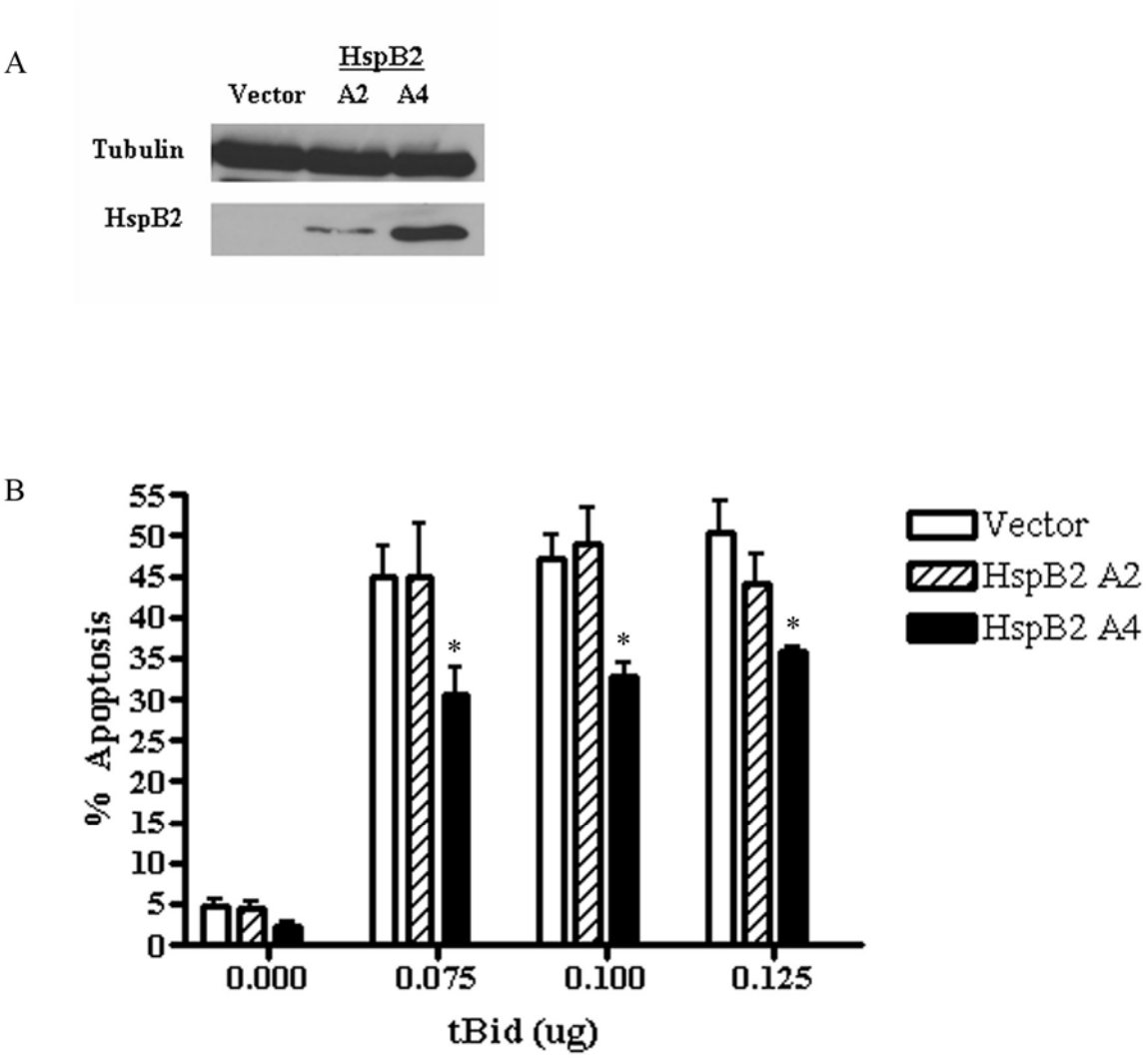


Figure 18: HspB2 inhibits apoptosis induced by transient transfection of active Bid.

A Lysates of MDA-MB-231 cells stably expressing empty vector or HspB2 were subjected to western analysis and probed for Tubulin or HspB2 expression.

B MDA-MB-231 breast cancer cells were stably transfected with empty vector or Flag-tagged wild type HspB2 plasmids. These cells were transiently co-transfected with GFP and increasing amounts of tBid or empty vector. Transfected cells were allowed to recover for 12-14 hours. Cells were then fixed with paraformaldehyde and stained with DAPI. GFP positive cells were scored for apoptosis by nuclear morphology. Data represents the +/- SEM of three independent experiments (* $p < 0.05$ versus control at each time point).

empty vector were treated for 0, 5, 15, 30, and 60 minutes with 500 ng/mL TRAIL and subjected to immunoprecipitation with a TRAIL antibody to isolate the DISC. Immunoprecipitated complexes were probed for TRAIL receptors DR4 and DR5, FADD, caspase-8, and HspB2. Immunoprecipitations using antibodies for DISC components or HspB2 were also performed. Due to many technical difficulties, there was no clear evidence of HspB2 association with the proteins in question. Multiple problems were encountered with the immunoprecipitation itself, but those obstacles were over come; however, background interference by controls prevented a clear conclusion. Although preliminary data showed no association of HspB2 with the tested proteins, results provided consistent proof could not be obtained.

4. DISCUSSION

4.1 A NEW ROLE FOR HSPB2 IN APOPTOSIS

To date, heat shock proteins play roles in many aspects of cellular death and survival. Hsp90 targets a number of apoptotic regulators such as Apaf-1, Bid, and RIP (Lewis et al., 2000; Pandey et al., 2000b; Zhao and Wang, 2004). Hsp70 also inhibits apoptosis through Apaf-1, AIF, JNK signaling, and by stabilizing Bcl-2 through BAG-1 (Beere et al., 2000; Gabai et al., 2002; Ravagnan et al., 2001; Saleh et al., 2000; Stankiewicz et al., 2005; Takayama et al., 1997). Hsp27, a member of the small heat shock protein family, inhibits apoptosis in many ways such as interacting with caspase-3, preventing Bid translocation to the mitochondria, and regulation of Akt (Pandey et al., 2000a; Paul et al., 2002; Rane et al., 2003). It has been previously shown that αB-crystallin suppresses apoptosis by preventing processing and subsequent maturation of caspase-3 (Kamradt et al., 2002). These studies clearly portray the intricate roles that heat shock proteins have in the regulation of apoptosis. Of the small heat shock proteins, only αB-crystallin and Hsp27 have been extensively studied in apoptosis. Therefore determining the functions of other small heat shock proteins may uncover new roles for this family in cell fate. HspB2 is a relatively unstudied small heat shock protein. Its only known role to date is to act as a chaperone of DMPK in muscular dystrophy. A putative role for HspB2 in cancer and cell death regulation has been, until now, left unexamined.

4.2 HspB2 confers protection against apoptosis by extrinsic pathway stimuli

The main goal of this study was to analyze the role of HspB2 in apoptosis. MDA-MB-231 breast cancer cells have been previously shown to be sensitive to TRAIL-induced cell death (Kamradt et al., 2005). TRAIL is a promising therapeutic for certain types of cancer; however breast cancer is one type that remains insensitive to its effects. Most breast cancer cell lines are resistant to TRAIL-induced apoptosis ((Kamradt et al., 2005)and unpublished data). Breast cancer is second most common cancer and one of the leading causes of death (Ries, 2007). The four main treatment options are surgery, radiation therapy, chemotherapy, and hormone therapy. However, these are not always effective and many breast cancers are resistant to chemotherapy causing an urgent need for different treatment options. One of these options is to target the programmed cell death pathway. Interestingly, HspB2 is not expressed in MDA-MB-231 cells; therefore ectopic overexpression HspB2 in MDA-MB-231 was used to determine if HspB2 expression protects breast cancer cells from TRAIL-induced apoptosis (Fig 4). Following treatment, HspB2 expression reduced sensitivity to TRAIL (Fig 5 and Fig 6). Thus, MDA-MB-231 cells acquire apoptotic resistance with the ectopic overexpression of HspB2. This finding was also demonstrated in an in vivo xenograft experiment where athymic nude mice were injected with cells over expressing HspB2 or vector and given TRAIL to see how tumor growth was affected (Fig 7). Tumors from the HspB2 group were more resistant to TRAIL-induced apoptosis than the vector group where the tumors were almost eliminated. Significantly, this demonstrated that HspB2 overexpression in breast cancer cells not only reduces TRAIL-induce apoptosis in vitro, but also has physiological relevance in vivo.

In addition to TRAIL, it was found that HspB2 was able to inhibit apoptosis in response to TNFα, another activator of the extrinsic pathway (Fig 8 and Fig 9). This demonstrated that HspB2 was able to attenuate the apoptotic effects of both TRAIL and TNFα. Since both drugs activate apoptosis via similar DISC components, the results from these experiments give consistent support that HspB2 can inhibit the extrinsic pathway. MDA-MB-231 cells also lack expression of αB-crystallin and its introduction into these cells resulted in apoptotic inhibition that occurred through preventing the full maturation of caspase-3 (Kamradt et al., 2005). In addition to αB-crystallin, the small heat shock protein Hsp27 has also been shown to prevent apoptosis by inhibiting caspase-3 (Pandey et al., 2000a). Given this information, it was possible that HspB2 also prevented apoptosis through a similar mechanism. To further explore this possibility, a fluorogenic caspase-3 activity assay was performed to determine if HspB2 over expression would inhibit apoptosis at the level of caspase-3. As expected, HspB2 overexpression yielded a strong inhibition of caspase-3 activity (Fig 11). Thus, HspB2 could inhibit activation of caspase-3 similar to what has been shown with αB-crystallin and Hsp27.

Although heat shock proteins function with a certain level of redundancy, they may also have process-specific roles. To seek out what specific function HspB2 might have in apoptosis, analysis of apical caspase-8 activity was performed since this is the first caspase activated in the extrinsic pathway of apoptosis. Expression of HspB2 in MDA-MB-231 cells treated with TRAIL concurred with prevention of caspase-8 activity although to a lesser degree than was shown by caspase-3 (Fig 10 and Fig 11). This experiment showed that HspB2 inhibited

activation of caspase-8 where αB-crystallin has been shown to be ineffective (Kamradt et al., 2005).

These results revealed potential for a unique anti-apoptotic mechanism for HspB2. In order to look more closely at the caspase inhibition by HspB2, western analysis was used to examine the major cell death proteins throughout the apoptotic caspase cascade (Fig 12). In confirmation of the results seen by IETD assay, HspB2 was able to mediate cleavage of initiator caspases -8 and -10 and inhibit their activation. Interestingly, Bid, a target of caspase-8, also exhibited delayed activation by cleavage. Active Bid translocates to the mitochondria and releases cytochrome c. Cytochrome c associates with Apaf-1 to activate caspase-9 and form the apoptosome. Consequently, Bid can activate caspase-9 and as expected, attenuation of Bid cleavage also affected caspase-9. As demonstrated by DEVD assay, executioner caspase-3 maturation was also prevented. Cleavage of MLH-1, which is a substrate of caspase-3, was also delayed. These experiments suggested that HspB2 expression in MDA-MB-231 cells delayed TRAIL-induced apoptosis throughout the entire caspase cascade. This pattern of caspase inhibition has not been previously demonstrated, thus providing evidence for a new role for HspB2 in apoptosis.

So far, the data has shown that HspB2 inhibits apoptosis from the level of apical caspases through the effector caspases. This insinuates that HspB2 could hinder apoptosis at the apical caspase level prior to mitochondrial activation and cytochrome c release. In order to map the activity of HspB2 in apoptosis, release of cytochrome c and Smac/DIABLO from the

mitochondria was analyzed after cells expressing HspB2 or empty vector were treated with TRAIL. HspB2 expressing cells showed a basal level of both cytochrome c and Smac/DIABLO released in the untreated samples as well as an increase in the release of both proteins over time (Fig 16). This result suggested that HspB2 does not inhibit activation of apical caspases since it was unable to prevent release of cytochrome c and Smac/DIABLO. For further confirmation of this result, a more sensitive measurement of TRAIL-induced cytochrome c release by ELISA was used. Cytochrome c release measurements by ELISA indicated that there was no significant difference between the vector expressing cells and either HspB2 clone; however, there seemed to be a correlation between the amount of HspB2 expressed and the level of cytochrome c release (Fig 17). Taken together, the results of two independent methods of cytochrome c release indicate that expression of HspB2 does not inhibit apoptosis prior to mitochondrial disruption and cytochrome c release. This would imply that apoptotic inhibition of HspB2 occurred downstream of the mitochondria.

4.3 INHIBITION OF CASPASE-3 BY HSPB2

The Human Interactome Map (HiMap) is a web browser that allows the user to find protein-protein interactions that are both known and predicted through the use of a database of literature-confirmed interactions and Bayesian analysis (Qin et al., 2003; Rhodes et al., 2005). Using this browser, predictions showed a hypothetical protein-protein interaction between HspB2 and caspase-3 giving evidence that the structures of these proteins are compatible (Fig 19) (Rhodes et al., 2005). Taking this into consideration, if HspB2 inhibits caspase-3

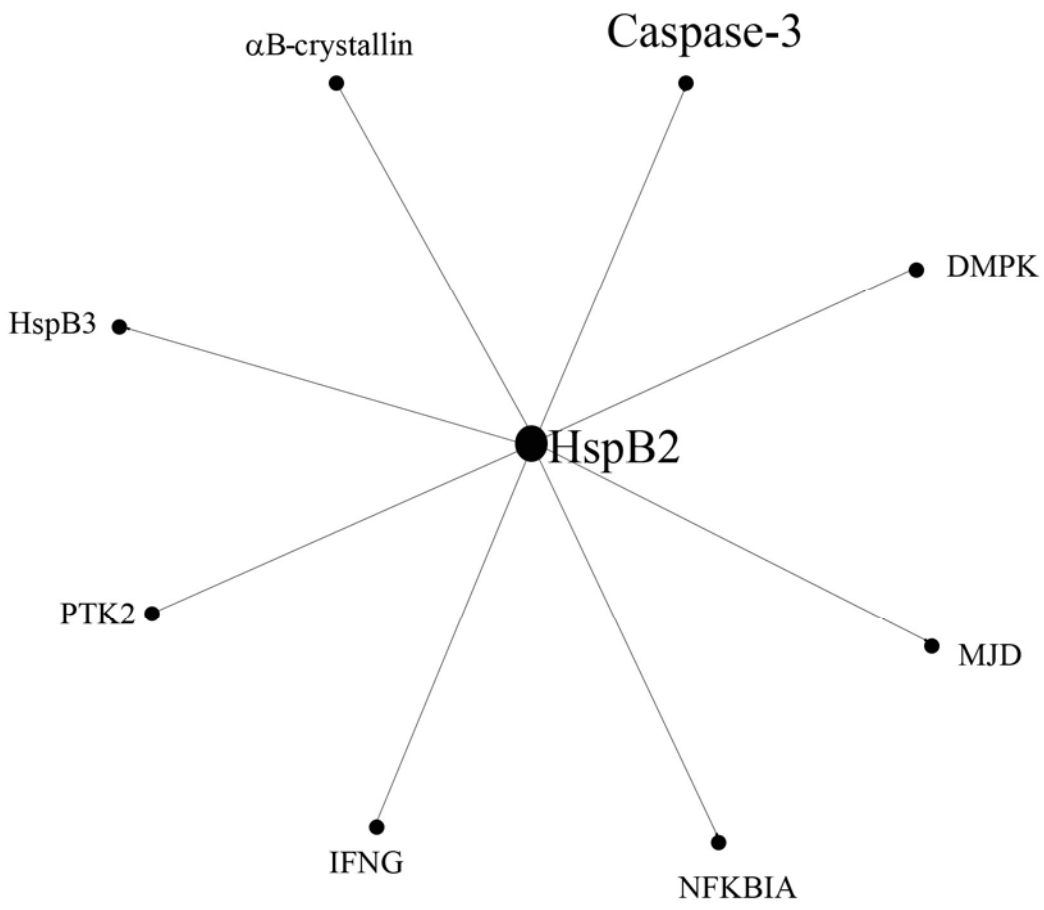


Figure 19: A Hypothetical Interaction of HspB2 with Caspase-3.

The predicted protein-protein interaction between HspB2 and caspase-3 as well as other proteins produced using HiMAP.

maturation, then the inhibition of caspases upstream of caspase-3 needs to be explained. There is evidence for a feedback loop in the apoptotic pathway whereby caspase-3 can activate caspase-8 to amplify apoptosis (Cha et al., 2001; Slee et al., 1999; Tang et al., 2000; Viswanath et al., 2001; Wajant, 2003; Yang et al., 2006). This being said, if HspB2 inhibits caspase-3, then any caspase that caspase-3 induces would also be inhibited, such as caspase-8. Cleavage of Bid, which is instigated by caspase-8, would also be inhibited and that would in turn hinder caspase-9 activation. This could also explain why the inhibition of caspase-3 shown by DEVD assay was so much stronger than caspase-8 inhibition shown by IETD assay. HspB2 expression would correlate with stronger inhibition of the caspase that it directly affected. Given that caspase-8 would be inhibited after its initial activation, its inhibition would be to a lesser degree. In the caspase cleavage western blot, caspase-8 and caspase-3 displayed the same activation pattern (Fig 12). Thus, it is likely that the time difference in activation was not seen due to the time points chosen. TRAIL DISC formation occurs within minutes after stimulation, so in order to properly visualize the kinetics occurring, the assay would need to be performed using much earlier time points (Varfolomeev et al., 2005). An additional method to test this theory would be to use cell free extracts from the MDA-MB-231 vector and HspB2 expressing cell lines and analyze them for caspase activation following cytochrome c/dATP administration over time. In the vector cell extracts, there should be an activation of caspase-9, caspase-3, and caspase-7 followed by caspase-8. In extracts from HspB2 expressing cells, caspase-9 would show activation; however, caspase-3 and caspase-8 would not. Alternatively, a caspase-3 inhibitor could be administered to vector expressing cells treated with TRAIL. The resulting analysis of caspase cleavage should reflect the same pattern as TRAIL treated HspB2 expressing cells.

4.4 HspB2 Inhibits Downstream Caspase Activation

To support the hypothesis that HspB2 inhibits caspase-3 activation, the next step was to activate the mitochondria directly and look for inhibition of apoptosis. Active Bid (tBid) has the ability to induce apoptosis via Bax/Bak dependent release of cytochrome c from the mitochondria. Three concentrations of tBid or empty vector were transiently co-transfected with GFP into cells expressing or not expressing HspB2. Through measurement of apoptosis, inhibition by HspB2 was revealed, albeit by only one of the two clones (Fig 18). However, the clone that yielded apoptotic inhibition was also the one that expressed high levels of HspB2. There was a very low level of protein expression in the other clone suggesting that there was not enough HspB2 present to significantly affect apoptosis. This effect could be confirmed through the use of an inducible HspB2 system to demonstrate a dose dependent effect on tBid-induced apoptosis.

In an effort to provide more evidence for a possible caspase-3 inhibition of apoptosis by HspB2, chemotherapy drug-induced stimulation of the intrinsic pathway was used. Protein kinase C inhibitor, staurosporine, and topoisomerase II inhibitors etoposide and doxorubicin all activate the intrinsic pathway of apoptosis. HspB2 overexpressing MDA-MB-231 cells were subjected to treatment with etoposide and the levels of apoptosis compared with vector expressing cells was determined by nuclear morphology. HspB2 overexpression was unable to prevent apoptosis in response to etoposide showing both vector and HspB2 cell lines dying at the same rate (Fig 13). When doxorubicin was used to initiate apoptosis in the same cell lines, no

apoptotic protection was found (Fig 14). To provide further confirmation, MDA-MB-231 cells overexpressing HspB2 or vector alone were treated with staurosporine and apoptosis levels were measured. Again, HspB2 expression did not cause resistance to staurosporine-induced apoptosis (Fig 15).

These results are the only evidence that contradict the hypothesis that HspB2 inhibits apoptosis downstream of mitochondrial activation. Conceivably, this contradictory phenomenon had to do with the cells used for the experiment. It is formally possible that the HspB2 stable cell lines used for the chemotherapy experiments had lost their resistance to apoptosis while still maintaining expression of the protein. These experiments were performed quite a bit later than those in the rest of the study and the cells were not tested for apoptotic resistance to TRAIL prior to use. A similar occurrence was observed when an effort to create new stable pools of MDA-MB-231 HspB2 expressing cells by retroviral infection resulted in cells that expressed the HspB2 protein, but were unable to protect against TRAIL-induced apoptosis. In addition, this could explain why HspB2 expressing cells had higher levels of apoptosis compared to vector when treated with doxorubicin. Perhaps the phenotype had changed in these cells making them more sensitive to doxorubicin-induced apoptosis. Given these explanations, the fact that HspB2 could not confer apoptotic protection against chemotherapeutic agents does not discount the hypothesis of caspase-3 inhibition by this protein.

Another explanation for these findings is that studies have revealed the ability of chemotherapy agents to induce apoptosis in the absence of caspase-3 (Benjamin et al., 1998;

Boehrer et al., 2002; Clarke et al., 2005; Cuvillier et al., 2001; Mooney et al., 2002; Xue et al., 2003). These studies used MCF-7 cells, a breast cancer cell line that lacks endogenous expression of caspase-3. Using protease activity assays and analysis of PARP cleavage in etoposide-induced MCF-7 cells, it was shown that caspases were active and apoptosis was still induced in the absence of caspase-3 (Benjamin et al., 1998). Additionally, use of zVAD-FMK, a broad caspase inhibitor, abrogated apoptosis, suggesting that this apoptosis was indeed caspase dependent. Experiments in HeLa cells produced similar results and showed that caspase-7 was being activated following treatment with etoposide in the absence caspase-3 (Clarke et al., 2005). Identical results were shown by another group following treatment with doxorubicin (Cuvillier et al., 2001). Boehrer et al. provided evidence of caspase-7 activation by doxorubicin in Jurkat cells treated with a caspase-3 inhibitor (Boehrer et al., 2002). In the previous studies the reconstitution of caspase-3 in the MCF-7 line restored the normal pathway of apoptosis; however, this gives evidence that apoptosis can occur through caspase-7 when caspase-3 is unavailable. Staurosporine has also been found to activate apoptosis in the absence of caspase-3 (Mooney et al., 2002). This group demonstrated that breast cancer cells MCF-7 and T47D, which express endogenous caspase-3, showed a release of cytochrome c as well as apoptosis in the absence of caspase activity. Another study implicated staurosporine in caspase independent apoptosis in melanoma cells treated with TRAIL (Zhang et al., 2004). Caspase independent apoptosis was also seen in response to staurosporine by Annexin-V and nuclear morphology assays in renal proximal tubule cells (RPTC) (Cummings et al., 2004). Finally, staurosporine and etoposide demonstrated caspase independent cell death in acute lymphoid leukemia cells (Belmokhtar et al., 2001). Together these studies show that etoposide, doxorubicin, and

staurosporine, all have the ability to activate apoptosis in the absence of caspase-3. This can be through caspase-7 or in a caspase independent manner; thus providing another explanation for the conflicting results seen in the HspB2 expressing MDA-MB-231 cell experiments.

Given the controversial data obtained by chemotherapy drug-induced apoptosis, an alternative method of apoptotic induction could be utilized. Recombinant active caspase-9 could be used to activate caspase-3 apoptosis. The DEVD-AFC caspase-3 activity assay could be used along with apoptotic nuclear morphology analysis to determine the effects of HspB2 on apoptosis without using chemotherapy drugs. Recently, Lakhani and colleagues have published results from a new caspase-3/caspase-7 double knockout mouse (DKO) (Lakhani et al., 2006). Surprisingly, they have found that both caspase-3 and caspase-7 are necessary for mitochondrial apoptosis. Deletion of caspase-7 in addition to HspB2 inhibition of caspase-3 should prevent apoptosis induced by chemotherapy drugs in these cells. They should yield a similar pattern of apoptotic inhibition as the MEFs (murine embryonic fibroblasts) from the caspase-3/caspase-7 null mice. These MEFs also showed only a delay in Bax translocation and cytochrome c release given that caspase-9 activity was still functional (Lakhani et al., 2006). Therefore, if HspB2 inhibits caspase-3 activity, its concomitant expression in caspase-7 null MEFs should exhibit a similar pattern of caspase activation when compared to the DKO MEFs in response to UV irradiation or chemotherapy drugs.

Validating the HiMap prediction of a physical interaction between HspB2 and caspase-3 is a key next step. Using the MDA-MB-231 system established here, immunoprecipitation of

HspB2 could be utilized to look for association with caspase-3. One factor to consider is the previous lack of success with the immunoprecipitation experiments performed in this study. It is important to note that successful demonstration of interactions between heat shock proteins and other proteins, such as those between Hsp90 with Akt and Hsp27 with caspase-3, have been shown (Pandey et al., 2000a; Sato et al., 2000). The only commercially available antibody for HspB2 cannot be used for immunoprecipitation; therefore previous efforts were performed using a Flag antibody and Flag-tagged constructs. The creation of an antibody against HspB2 that can be used for immunoprecipitation would greatly enable efforts to establish a physiological interaction both in artificial and endogenous expressing systems. Efforts to establish an interaction by immunofluorescence were also unsuccessful as the antibody for HspB2 did not yield a clear signal and had very high background which made it impossible to determine localization. If the transient nature of chaperone protein-protein interactions is hindering experiments, a chemical crosslinker, such as dimethylsuberimidate (DMS) which as been shown to be effective for Hsp20, could be used (Chernik et al., 2007). In addition, a yeast two hybrid assay could be performed as this helped identify Beacon as a binding partner of HspB2 (Kantham et al., 2003). An artificial system of recombinant HspB2 or wild type caspase-3 in a GST-pull down could be used and protein-protein interactions could be seen by immunoblotting. An added control would be an NH_2 terminal truncation of caspase-3 that has no binding capabilities.

4.5 HSPB2 AND αB-CRYSTALLIN

One factor to consider is that αB-crystallin binds to caspase-3 to exert its anti-apoptotic effects and when αB-crystallin expressing cells are treated with etoposide they are resistant to apoptosis (Kamradt et al., 2001). If αB-crystallin and HspB2 have similar mechanisms of apoptosis inhibition, then HspB2 should be able to inhibit apoptosis induced by etoposide. As stated earlier, it is likely that the HspB2 expressing MDA-MB-231 cells had lost their phenotype in these experiments. However, it is possible that αB-crystallin and HspB2 while having a similar mechanism of apoptotic inhibition also have specialized functions. Injection of αB-crystallin expressing MDA-MB-231 cells caused a significantly increased rate of tumor growth over vector control cells (Kamradt et al., 2005). Conversely, untreated HspB2 tumors grew at the same rate as untreated vector tumors (Fig 7). This suggests that αB-crystallin may have stronger anti-apoptotic effects than HspB2 and a different mechanism. To elucidate this possibility, a side by side comparison of αB-crystallin and HspB2 would provide helpful information. MDA-MB-231 cells made null for caspase-3 by shRNA or immunodepletion and wild type cells would be transfected with either αB-crystallin or HspB2. These cells would be treated with etoposide and the activation of caspases and other apoptotic regulators could be analyzed. Naturally it would be ideal to use MCF-7 cells since they already lack caspase-3; however, these cells also express both αB-crystallin and HspB2 so both would either have to be silenced or another cell line lacking both proteins and caspase-3 would need to be found. If both proteins function only to inhibit caspase-3 activity, then experiments with either protein would

yield similar findings. However, if the results are different, then these cells could be further analyzed to define their specialized functions.

To examine the expression levels of HspB2 in other types of cancer cells lines, lysates or cell lines from various sources were obtained and subjected to western analysis on SDS-PAGE gels by probing for either tubulin as a loading control, or HspB2 antibody (Fig 20). There was no evidence of HspB2 protein expression in breast cancer cell lines BT474, DY36T2, LCC6, or 2LMP. In addition, there was no expression of HspB2 in colon cancer cell lines COLO205, HT29, or SW948; pancreatic cancer cell lines BxPC3, MIAPaCa2, or PANC-1; prostate cancer cell line PC3; lung cancer cell line NCI H35; or glioma line LG11. There was HspB2 protein expression in a number of lung cancer cell lines A549, NCI H226, NCI 1299, and NCI 2122; gliomas U87MG, D54MG, U373, U251, and DiNTC. Expression levels were high in the majority of these lines with the exception of gliomas U251 and DiNTC which had very low levels of expression.

Unpublished data determined both αB-crystallin expression status and TRAIL sensitivity in these lines. HspB2 and αB-crystallin are simultaneously expressed in TRAIL-resistant cancer cell lines A549, U87MG, U373, U251, and DiNTC. They are also differentially expressed in intermediately resistant lines such as BT474, DY36T2, NCI H226, NCI 1299, and DU145. Analysis of the caspase cleavage in cell lines expressing either or both of the proteins, in addition to study of the protein complexes that HspB2 and αB-crystallin form, may shed some light on possible compensatory actions of these proteins. Although, they have been shown to form

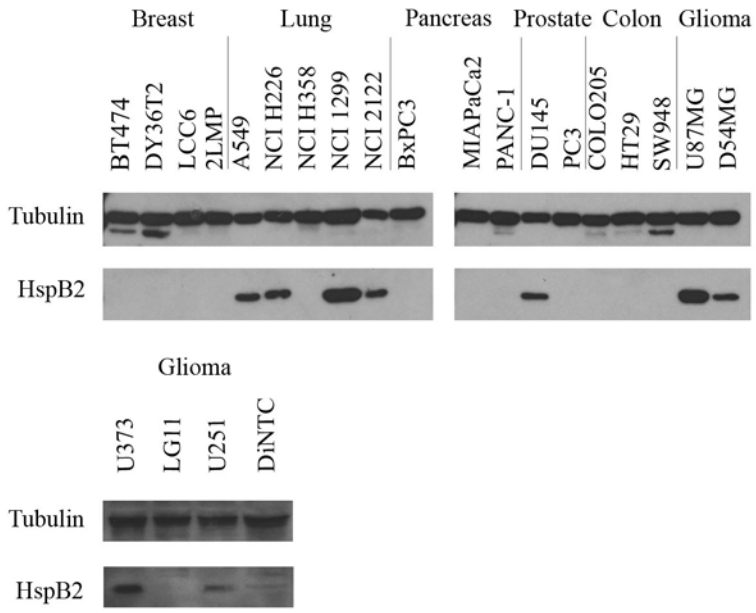


Figure 20: Endogenous expression of HspB2 in cancer cell lines.

The endogenous expression levels of HspB2 were analyzed in various cancer cell lines. Equal concentration of whole cell lysates were subjected to SDS-PAGE analysis and probed for HspB2 and Tubulin expression.

separate complexes, HiMap did show a hypothetical interaction (Fig 19) (Suzuki et al., 1998). The lack of a HspB2- αB-crystallin complex was only shown in muscle cells, therefore it is possible that in a cancer cell, an entirely different complex is formed. The two proteins may function cooperatively to inhibit apoptosis when they are both expressed, and in situations where only one protein is available, they may compensate for each other.

4.6 Potential alternative mechanisms for HspB2 in apoptosis

4.6.1 Upstream apoptotic inhibition by HspB2

The majority of the data presented here suggests a downstream caspase inhibition by HspB2. Similar results have been shown using small heat shock proteins αB-crystallin and Hsp27 (Kamradt et al., 2001; Pandey et al., 2000a). Using DNA fragmentation and cell death dectection ELISA, Hansen and colleagues demonstrated an inhibition of doxorubicin-induced apoptosis in MDA-MB-231 cells stably expressing Hsp27 (Hansen et al., 1999). αB-crystallin confers apoptotic resistance to etoposide and staurosporine (Kamradt et al., 2001; Mao et al., 2004). In the presence of staurosporine treatment, αB-crystallin as well as αA-crystallin inhibit Bax and Bcl-X_s translocation to the mitochondria in retinal fibroblasts (Mao et al., 2004). In the traditional model, initiator caspases-8 and -10 activate either caspase-3 directly or activate Bid to induce mitochondrial activation of caspase-3. According to this, if HspB2 inhibited caspase-3 then stimulation of the intrinsic pathway by the aforementioned chemotherapy drugs would have resulted in a decrease in apoptosis in cells expressing HspB2. Instead, the results from this

experiment would suggest an inhibitory act upstream of the mitochondria even if most of the evidence points to the contrary. Still, this is easily rationalized by a phenotypic change in the MDA-MB-231 HspB2 system due to age. However, it is still possible that this data is valid, therefore advocating an upstream apoptotic inhibition.

Like αB-crystallin, HspB2 inhibited caspase-3 cleavage and activation; however, it also inhibited the cleavage of the initiator caspases and Bid (Fig. 3). As both caspase-8 and caspase-10 cleavage are inhibited, it is tempting to speculate that HspB2 inhibits the complete formation of the TRAIL DISC. Several efforts were exerted in order to determine if HspB2 associated with TRAIL DISC proteins to prevent full formation. Thorough attempts at immunoprecipitation of TRAIL ligand were unable to pull down the DISC. This protocol involved various amount of antibody and many different time points; however they unsuccessful. Attempts to pull down individual proteins such as HspB2, DR4, DR5, caspase-8, and FADD were also unsuccessful. Thus, as earlier stated, no definite conclusions could be drawn from this experiment and the above suggested experiments would need to be performed in order to delineate a binding partner for HspB2.

If in fact HspB2 inhibits apoptosis upstream in the extrinsic pathway and not at caspase-3, then the results from the cytochrome c release assays and tBid experiments disagree with this hypothesis. It could be that the time points chosen for the cytochrome c release western analysis and ELISA were too late to detect any inhibitory effects by HspB2. Therefore, a shorter time points should be used possibly along with a different method of analysis such as fluorescent

labeling and flow cytometry. It is also possible that HspB2 can inhibit apoptosis at different points in the caspase cascade depending on the stimulus. Perhaps TRAIL, chemotherapy drugs, or tBid incite different mechanisms of HspB2 inhibition.

4.6.2 Differential inhibition of apoptosis by HspB2 in response to various stimuli

Members of the heat shock protein family have a variety of functions instigated by different stimuli. They are active in cancer and other diseases, usually to maintain proteins necessary for disease progression. Some heat shock proteins inhibit the apoptotic pathway; however this is mostly at the level of the mitochondria. There is little evidence showing heat shock proteins inhibit apoptosis at the beginning of the extrinsic pathway. Hsp90 and Hsp70 can sequester Apaf-1 and preventing apoptosome formation resulting in failure to activate downstream caspases (Pandey et al., 2000b; Zhao and Wang, 2004). Hsp90 is also able to inhibit Bid cleavage and release of cytochrome c (Zhao and Wang, 2004). Hsp70 binds to BAG-1 which binds to the anti-apoptotic protein Bcl-2 thereby inhibiting apoptosis (Takayama et al., 1997; Takayama et al., 1995). Small heat shock protein, Hsp27, also inhibits the mitochondrial pathway in response to etoposide treatment by both cell free and leukemia and cervical cancer cell systems by binding cytochrome c following release from the mitochondria preventing apoptosome formation and subsequent activation of downstream caspases (Bruey et al., 2000). In contrast, in human leukemia and fibroblasts and murine fibroblasts, Hsp27 has no effect of apoptosome formation and inhibits apoptosis by binding to caspase-3 (Pandey et al., 2000a). It is also postulated that Hsp27 binds to and stabilizes F-actin filaments thereby preventing

translocation of Bid to the mitochondrial membrane (Paul et al., 2002). In response to both TNFα and etoposide treatment, αB-crystallin overexpression resulted in a marked reduction in apoptosis (Kamradt et al., 2001). Unlike Hsp27 and Hsp70 that inhibit caspase-9 cleavage, αB-crystallin did not. Instead, αB crystallin inhibits caspase-3 cleavage by binding to the intermediate p24 form of caspase-3 and preventing its maturation to the active state following treatment with TNFα or etoposide (Kamradt et al., 2001). Furthermore, αB-crystallin was able to inhibit apoptosis induced by TRAIL in breast cancer cells via the same mechanism (Kamradt et al., 2005).

HspB2 could have different anti-apoptotic functions in response to different stimuli and in different cell lines. To determine this, HspB2 overexpressing cells could be treated with various stimuli such as UV radiation, chemotherapy drugs, TRAIL, TNFα, and Fas and caspase activation could be studies as well as analysis of cytochrome c release. There may be differing patterns of caspase inhibition seen with each stimulus. Conversely, HspB2 could be acting at different locations in response to a single stimulus. Individual caspases and other apoptotic proteins would need to be deleted to determine if the anti-apoptotic effects of HspB2 are still visible.

4.6.3 HspB2 inhibition of caspase-2

If HspB2 does not inhibit caspase-3 or upstream caspase activation at the DISC, it is possible that HspB2 may also bind to another initiator caspase, caspase-2. It could also bind to

caspase-2 in addition to other anti-apoptotic functions. Caspase-2 forms a protein complex through its CARD domain similar to the apoptosome called the PIDDosome in apoptosis (Tinel and Tschopp, 2004). In the presence of caspase-8, caspase-2 associates with the Fas DISC to induce apoptosis. Through caspase activation analysis, Lavrik and colleagues have shown that caspase-2, in addition to caspase-8, is active in CD95 stimulated apoptosis (Lavrik et al., 2006). Furthermore, immunoprecipitation of the CD95 DISC showed recruitment of caspase-2; however caspase activation did not occur in the absence of caspase-8. Caspase-2 has also been implicated in TRAIL-induced apoptosis. It has been demonstrated that caspase-2 activity is dependent of levels of PKCK2 (protein kinase CK2) (Shin et al., 2005). High levels of PKCK2 inhibit the activity of caspase-2 in TRAIL treated cancer cells. When PKCK2 levels are lowered, caspase-2 processes caspase-8 thereby initiating apoptosis. In addition, caspase-2 has been linked to the activation of Bid in TRAIL-induced apoptosis. Using siRNA against caspase-2, Wagner and coworkers showed that optimal levels of Bid activity are dependent on caspase-2 activity in some cell types (Wagner et al., 2004). Interestingly, the activity of caspase-2 was found to be connected to caspase-8 and Bid activity where cleavage was absent upon caspase-8 knockdown. These studies provide evidence that caspase-2 functions upstream prior to caspase-8 activation. This would support HspB2 working to inhibit the initiator caspases; however, the majority of the data presented in this work supports inhibition of caspase-3. It has been shown that caspase-2 activation occurs after release of cytochrome c has taken place (Li et al., 1997; Mhaidat et al., 2007; O'Reilly et al., 2002; Paroni et al., 2001; Slee et al., 1999). Using MCF-7 cells with or without caspase-3, cell cycle analysis, caspase cleavage by western blotting, and recombinant caspase in vitro proteolytic assays, were used to analyze caspase-2 activation and apoptosis

induction following treatment with either UV-irradiation or TNFα (Paroni et al., 2001). Results from this study revealed that caspase-2 required caspase-9 and caspase-3 activation. They also demonstrated that caspase-7 had the ability to activate caspase-2. In Apaf-1 or caspase-9 null mouse thymocytes treated with γ-irradiation, caspase-2 activity was diminished compared to wild type cells (O'Reilly et al., 2002). Additionally, in caspase-3 immunodepleted Jurkat cells, caspase-2 activation was severely effected shown by [^{35}S]-labeled caspase activation assays (Slee et al., 1999). In these cases, caspase-2 inhibition could lead to inhibition of other caspases through a negative feedback loop. Consequently, experiments with melanoma cells treated with docetaxel showed that caspase-2 can activate caspase-3 and caspase-9 which induce caspase-8 activation, so inhibition of caspase-2 by HspB2 could directly affect caspase activation without a feed back loop (Mhaidat et al., 2007). Preliminary work demonstrated that HspB2 overexpression could inhibit cleavage of caspase-2 in response to TRAIL. Immunoprecipitation studies would need to be performed to determine if there is a physical interaction between HspB2 and caspase-2. Taken together, data exists to support the hypothesis that HspB2 could regulate apoptosis through interaction with caspase-2, and by inhibiting caspase-3 or caspase-8 cleavage.

4.6.4 PHOSPHORYLATION OF HSPB2 AND THE P38/JNK PATHWAY

It is also possible that HspB2 could have differing mechanisms of action depending on phosphorylation status. Both Hsp27 and αB-crystallin have altered functions depending on their phosphorylation state. It has been shown that Hsp27 can inhibit apoptosis upstream by binding

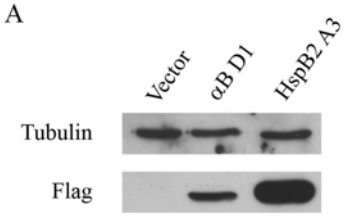
to Daxx which is a key protein in Fas-induced apoptosis (Yang et al., 1997). Fas binds to Daxx and activates the p38/JNK kinase cascade through apoptosis signal regulating kinase-1 (Ask-1) and induces apoptosis (Chang et al., 1998; Khelifi et al., 2005; Xia et al., 1995; Yang et al., 1997). When phosphorylated, Hsp27 binds to Daxx thereby preventing activation of JNK (Chang et al., 1998; Charette et al., 2000). αB-crystallin also has different functions depending on phosphorylation status. αB-crystallin displays inhibition of apoptosis in response to a variety of stimulants (Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005). Interestingly, when two mutant forms, R120G which is thought to cause desmin-related myopathy (DRM), and a pseudophosphorylation form 3XSE, were over expressed this apoptotic inhibition was eliminated (Kamradt et al., 2005).

Small heat shock protein, Hsp22/HspB8/H11, has many unique functions including plasma membrane association, disruption of αB-crystallin-amyloid aggregates DRM, as well as apoptotic activities (Chowdary et al., 2007; Gober et al., 2003; Sanbe et al., 2007). In contrast to other small heat shock proteins, expression of Hsp22 was found to induce apoptosis instead of inhibiting it (Gober et al., 2003). Although the actions of Hsp22 may be contrary to fellow family members, the pathways affected by it are not. Hsp22 induces apoptosis through caspases and the p38MAP kinase pathway (Gober et al., 2003). Hsp22 has been shown to have autophosphorylation capabilities and may have protein kinase activity, although this characteristic has been disputed (Gober et al., 2004; Kim et al., 2004a; Kim et al., 2004b). Additionally, another small heat shock protein, Hsp20 has been shown to inhibit apoptosis by a phosphorylation dependent block of caspase-3 activation and by stabilizing the actin

cytoskeleton in cardiomyocytes (Brophy et al., 1999; Fan et al., 2004). Hsp20 levels are increase upon beta adrenergic pathway activation and this increase protects against cardiac remodeling. Of late, this same group has linked the anti-apoptotic function of Hsp20 to Ask-1 and the p38/JNK pathway (Fan et al., 2006). This is the same pathway in which Hsp27 acts in leukemia and 293 cells (Chang et al., 1998; Khelifi et al., 2005; Schepers et al., 2005; Yang et al., 1997).

It is highly likely that HspB2 also has phosphorylation dependent functions. Mutation of the three serines in HspB2 corresponding to previous targeted serines in αB-crystallin have been designed as well one with the corresponding arginine mutation. It would be interesting to determine if these mutations in HspB2 affect its anti-apoptotic activities, and to what capacity this effect is seen. Interestingly, a PowerBlot analysis by BD Biosciences showed that the p38 pathway was also affected by HspB2 (Fig 21). The PowerBlot is a large scale western array analysis that screens, measures, and quantifies increasing and decreasing levels of proteins in submitted samples. Protein level analysis revealed that HspB2 expression in MDA-MB-231 cells caused a 3-fold increase in p38 expression compared to vector control. Furthermore, HspB2 expression yielded a 2-fold increase in p38 expression levels compared to αB-crystallin expressing cells. These results suggest that HspB2 could also affect the p38 pathway to induce apoptosis.

A



B

	Protein ID	Confidence Level	Sign of Change	Fold Change R1/R1	Fold Change R1/R2	Fold Change R1/R3	Fold Change R2/R1	Fold Change R2/R2	Fold Change R2/R3	Fold Change R3/R1	Fold Change R3/R2	Fold Change R3/R3
Changes in HspB2 A3 relative to Flag	p38a/SAPK2a	10	+	3.07	3.13	2.54	3.89	3.97	3.22	3.69	3.77	3.06
Changes in HspB2 A3 relative to AlphaB D1	p38a/SAPK2a	10	+	2.37	2.34	2.95	3.00	2.97	3.74	2.85	2.82	3.55

+ = represents an increase in protein level in the experimental sample relative to control
Confidence level = proteins are compared using a 3X3 matrix, R1 of control is compared to R1, R2 of Experimental, Run 2 of control is compared to R1, R2 of Experimental. Protein changes are listed in order of confidence, 10 being the highest confidence:
Level 10 - Changes greater than 2 fold in all 9 comparisons that are from good quality signals and pass a a visual inspection

Figure 21: Expression of HspB2 Increases Protein Expression of p38.

A Immunoblot for protein expression of MDA-MB-231 cells stably transfected with empty vector, aB crystallin, or HspB2.

B BD PowerBlot summary showing HspB2-induced protein expression of p38 compared to empty vector or aB crystallin.

4.6.5 HspB2 and a non-caspase protease

Recently, Milleron and Bratton provided evidence that there may be an additional apical protease that activates apoptosis. In response to heat shock, cells deficient in caspase-8, caspase-2, or both, were still able to activate and cleave caspase-3. Additionally, this occurred without the formation of the apoptosome (Milleron and Bratton, 2006). If efforts to establish a direct interaction with caspase-3, the TRAIL DISC, or caspase-2 fail, it is possible that there exists another apical protease similar to the one described by Milleron and Bratton. Our data strongly suggests that HspB2 renders cells resistant to TRAIL-induced apoptosis by associating with caspase-3.

4.7 Silencing HspB2

Aside from the issues and experiments proposed above, an important limitation of this study is that all of the data presented here was performed using MDA-MB-231 stably transfected cell lines. Supplementary support of the results should be performed in other cancer cell lines. It has been determined that the breast carcinoma cell lines used here, either endogenously express HspB2 or have ectopically over expressed HspB2, displayed resistance to TRAIL-induced apoptosis. Using one or more of the TRAIL sensitive cancer cell lines screened previously, cell lines that are negative for HspB2 expression should be used as a template for new stable lines to show that the results presented here are also valid in other cancer cell lines.

After solidifying the mechanism of HspB2 in apoptosis, it would be of interest to determine if deletion of HspB2 in an endogenously expressing cell line would create sensitivity to TRAIL treatment. The beginnings of this experiment have already been performed. High HspB2 expressing T47D breast carcinoma cell line was chosen and shRNA oligomers designed against HspB2 and scrambled target sequence oligomers were ligated into the pSUPER.RETRO.PURO.GFP vector. These constructs were retrovirally infected into T47D cells using the Phoenix packaging cell line. Following transfection, GFP positive cells were selected by flow cytometry and grown in the presence of puromycin to form two representative pools for each construct. HspB2 expression in these cell lines was determined by western blot analysis (Fig 22). Two shRNA oligos designed to inhibit HspB2 and surprisingly one scrambled control oligo successfully reduced expression of HspB2 protein levels. Unfortunately it was also discovered that although the sequences were specific for the HspB2 gene, they also knocked down αB-crystallin. As stated earlier, αB-crystallin and HspB2 are located adjacent to each other on the same chromosome. Since these cells could not be representative of the effects of HspB2 silencing alone, it was decided not to continue forward with subsequent experiments. A preliminary experiment was done where these cells were treated with increasing concentrations of TRAIL over the course of 4 hours. There was no increase in TRAIL sensitivity with the absence of both HspB2 and αB-crystallin. This result was unexpected, but other research has shown that T47D cells are resistant to TRAIL-induced apoptosis due to high expression levels of FLIP (Guseva et al., 2007; Hyer et al., 2005). Other shRNA constructs could be ordered through companies such as Dharmacon to further study the effects of inhibiting HspB2. Perhaps company made constructs would be able to knockdown HspB2 individually without affecting

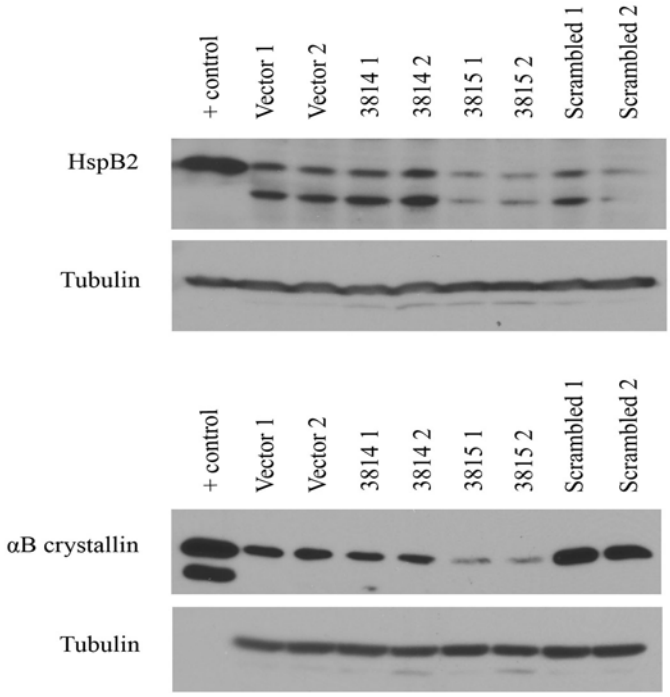


Figure 22: Silencing of HspB2 in T47D breast cancer cells also causes deletion of αB-crystallin.

T47D breast carcinoma cells were retrovirally infected with shRNA constructs designed against HspB2. Empty vector controls and a scrambled sequence were also used. Lysates of infected cells were subjected to SDS-PAGE analysis using antibodies against HspB2, αB-crystallin, and tubulin.

αB-crystallin expression. An additional approach could be immunodepletion of HspB2 from cell extracts to determine if its absence would have an effect on apoptosis.

4.8 Conclusion

In summary, the experiments presented here suggest an inhibitory role for HspB2 in apoptosis. Through analysis of apoptosis following treatment with TRAIL or TNFα through nuclear morphology assays and FACS, expression of HspB2 renders cells resistant to cell death in vivo confirmation of this effect was also shown through xenograft tumor growth analysis. Fluorogenic caspase activity assays show that both caspase-8 and caspase-3 activation are stunted by HspB2 expression. Furthermore, western blotting showed that there is a delay in cleavage of a wide spectrum of caspases in HspB2 transfected cells compared to vector controls. Cytochrome c release shown by two independent assays was not prevented in the presence of HspB2. Apoptosis induced by transfection of tBid was inhibited by HspB2. Taken together, these experiments propose a model whereby anti-apoptotic effects of HspB2 are due to inhibition of downstream caspases, most likely at the level of caspase-3 (Fig 23). Although contradicting evidence was also presented, proposed future studies will further delineate the mechanism. As a whole, HspB2 has an inhibitory role in apoptosis in breast cancer cells thus displaying a function separate from the role in muscular dystrophy, and shedding new light on the functions of small heat shock proteins.

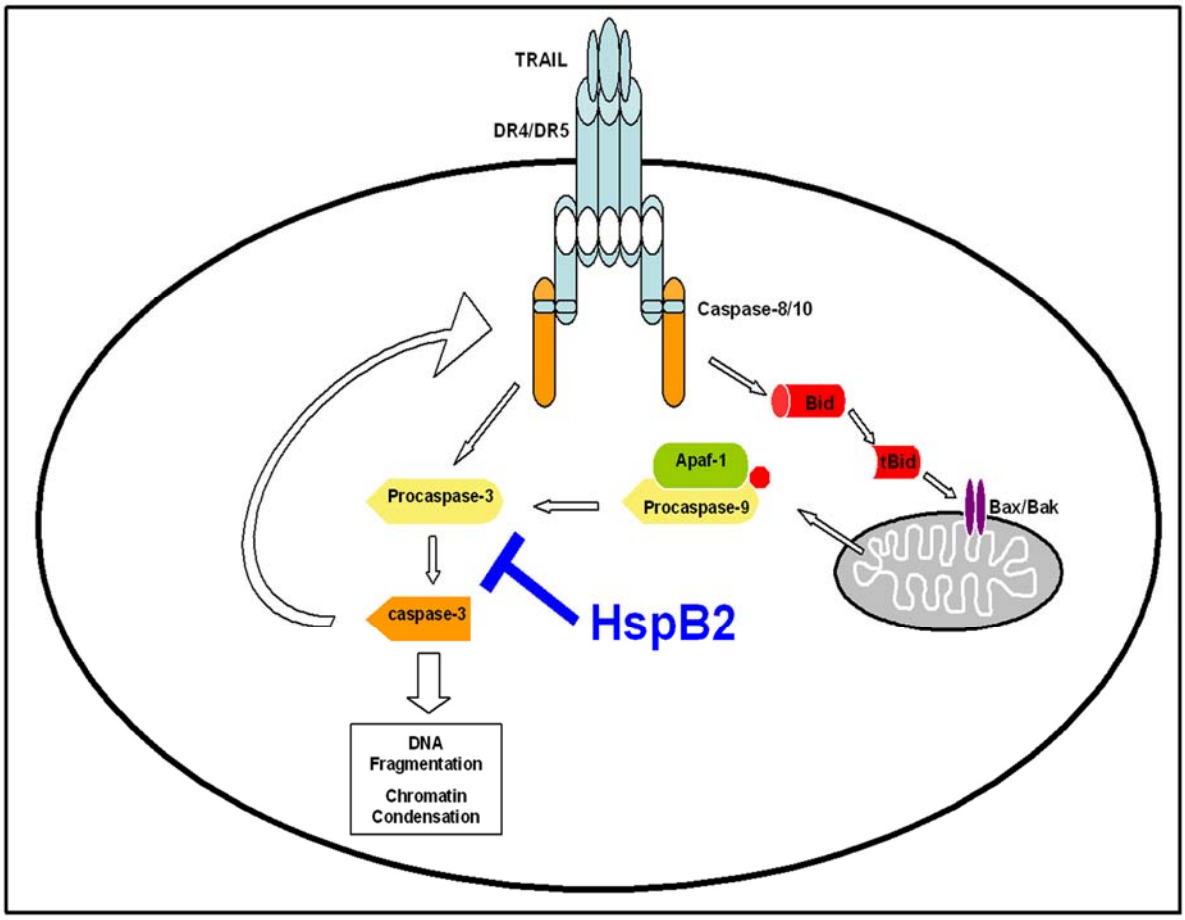


Figure 23: A Proposed Mechanism for the Anti-apoptotic Activity of HspB2.

HspB2 inhibits activation of caspase-3 which causes subsequent inhibition of both the extrinsic and intrinsic apoptotic pathways through a feedback loop.

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