NORTHWESTERN UNIVERSITY

Caspase Cleavage of HER-2/*neu* Releases a BH3-like Cell Death Effector

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By

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It's hard to know where to begin thanking everyone who's been in my life for the past six years. No one tells you that graduate school will fundamentally change you and the way you look at the world. On 'good days' when you come out of the darkroom with a great result, science is one of the most gratifying and exciting enterprises I can think of. But anyone who's spent time on the bench can tell you those moments are all too rare. The reality is that the day to day execution of research is challenging both mentally and physically. You strive to understand complex problems, and design experiments to rigorously test your hypotheses. True growth comes when you begin to realize that the models in the literature are simply that, models, which may or may not fit your experimental data. I was fortunate enough to be a graduate student studying cell death during a period when the established models of apoptosis were actively being revised. It was then that I saw how exciting the practice of science can be: watching the battle between two competing models play out in the literature, and realizing that this kind of competition is what good science is all about.

First and foremost I need to thank my mentor Vince for letting me join his lab, even though I didn't want to work on a certain small heat shock protein close to his heart. From the beginning, Vince was open to finding a question and a project that I would be interested in. My follow up on some preliminary data about HER-2 as a caspase substrate is the subject of this dissertation. Vince gave me a great deal of freedom in the design and execution of this project, which I'll always be grateful for. Although challenging at the time, knowing that I can successfully tackle a complex problem and design experiments to tease out the underlying biology has given me a

confidence in my scientific abilities that will serve me well as a postdoc and in my subsequent scientific career. Vince was also good at making me think about what my experiments meant, and our discussions together often uncovered untested areas of the project . His door was always open, and like any good PI, Vince loved seeing new data. Crucially, he believed in the project at times when I did not, and was able to give me pep talks when confusing data emerged, enabling me to go back to the bench. As a mentor, Vince encouraged me to have confidence in myself and my data, and to think about what I wanted for the next stage of my career. I'm proud to say I completed my Ph.D. in his lab. Thanks for everything, Vince.

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ABSTRACT

Caspase cleavage of HER-2/*neu* releases a BH3-like cell death effector

Anne Marie Strohecker

Breast cancer cells acquire many genetic alterations in apoptotic signaling pathways rendering them resistant to apoptosis. Human Epidermal growth factor Receptor-2 (HER-2/ErbB2/*neu*) is amplified or overexpressed in approximately 30% of breast and ovarian tumors and correlates with poor prognosis. Although HER-2 is an orphan receptor, it forms potent signaling heterodimers with other family members (HER-1/EGFR, HER-3 or HER-4) and plays a central role in both the establishment and progression of breast tumors.

Using a novel expression cloning strategy, we identified HER-2 as a substrate of multiple caspases. HER-2 is cleaved by caspases at four aspartic acid residues (Asp^{1016}, Asp^{1019}, Asp^{1087}, and Asp^{1125}) in its cytoplasmic tail *in vitro* and in breast cancer cells during the induction of apoptosis. HER-2 is initially cleaved by caspases at Asp^{1016}/Asp^{1019}, releasing a 47-kDa product from the cell membrane. This product is subsequently processed after Asp^{1125} into a predicted 25 kDa product and an unstable 22-kDa fragment that is rapidly degraded by the proteasome.

We demonstrate that expression of either a caspase cleavage-resistant or a truncated HER-2 conferred greater protection against apoptosis than wild-type HER-2 in breast cancer cells

stably expressing these proteins, suggesting a pro-apoptotic function for one or more of the HER-2 cleavage products. Indeed, transfection of cDNAs encoding the 25 or 47-kDa HER-2 caspase cleavage products into breast cancer cells was sufficient to induce apoptosis in a caspase-dependent manner. Biochemical fractionation and confocal analyses documented the presence of the HER-2 cleavage products at the mitochondria after ectopic expression and in dying cells which endogenously express HER-2. Once in the mitochondria, these fragments .0 induce the release cytochrome c in a Bcl-X_L-inhibitable manner. Further, we identify a novel BH3-like domain in a sequence shared by the 47 and 25 kDa HER-2 products which is required for cell death. Recombinant peptides containing the HER-2 BH3 domain, but not a mutant (2XE) domain, were sufficient to release cytochrome c from isolated mitochondria. Collectively, our results indicate that caspases activate a previously unrecognized pro-apoptotic function of HER-2 by releasing a BH3 domain containing product, which translocates to the mitochondria , triggers cytochrome c release, and induces apoptosis.

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ABBREVIATIONS

7-AAD	7-amino-actinomycin D
AIF	Apoptosis Inducing Factor
APAF-1	Apoptosis Protease Activating Factor-1
BAD	Bcl-2 Associated Death promoter
BAK	Bcl-2 Antagonist Killer
BAX	Bcl-2 Antagonist X protein
Bcl-2	B-cell lymphoma-2
BID	BH3 Interacting Domain death agonist
BH3	Bcl-2 homology domain 3
BMH	Bis-Maleimidohexane
C. elegans	*Caenorhabditis elegans*
CARD	Caspase Recruitment Domain
CrmA	Cytokine response modifer A
Ced	Cell Death Abnormal
Cyto c	Cytochrome c
DAPI	4’, 6’-diamidino-2-phenylindole, dihydrochloride
DcR	Decoy Receptor
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DKO	Double Knock Out

DN	Dominant Negative
DR4/5	Death Receptor 4 or 5
DTT	Dithiothreitol
ECD	Extracellular Domain
egl-1	Egg laying defective-1
Endo G	Endonuclease G
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immuno-Sorbent Assay
ErbB2	Epidermal growth factor receptor 2 (also referred to as HER-2)
Epo	Epoxomicin
Etop	Etoposide
FADD	Fas Associated protein with Death Domain
FLIP	Flice Inhibitory Protein
GSK-3	Glycogen Synthase Kinase-3
GFP	Green Fluorescence Protein
HER-2	Human Epidermal growth factor Receptor 2 (also referred to as ErbB2)
Hrg1β	Heregulin-1 beta
LCK	Lymphocyte cell-specific protein-tyrosine kinase
IL-3	Interleukin -3
Mcl-1	Myeloid cell leukemia-1
MEF	Mouse Embryonic Fibroblast

MMP	Matrix Metalloproteinase
MOMP	Mitochondrial Outer Membrane Permeabilization
MT	Mitochondria
MT 633	Mitotracker Deep Red 633
PBS	Phosphate buffered saline
PI	Proteasome Inhibitor
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulphonylfluoride
SH2	Src Homology 2
SPEC	Small Pool Expression Cloning
qVD-OPH	quinoline-Val-Asp-CH2-difluorophenoxy
RIPA	Radio-ImmunoPrecipitation Assay buffer
RT	Room Temperature
tBid	Truncated Bid
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
TRAIL	Tumor necrosis factor Related Apoptosis Inducing Ligand
WT	Wild type
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluromethyl ketone

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CHAPTER 1

BACKGROUND

1. Overview of Apoptosis

Apoptosis, or programmed cell death, is a carefully orchestrated series of events culminating in the removal of damaged or unneeded cells from the body in the absence of an inflammatory response. Dying cells exhibit marked blebbing of the plasma membrane, possess a dense cytoplasm, condensed chromatin (resulting from both pyknosis and karyorrhexis), and are ultimately phagocytosed by circulating macrophages which recognize the externalized phosphatidylserine (PS) on the plasma membrane of the apoptotic cell (Kerr et al., 1972). Apoptosis is morphologically and biochemically distinct from necrosis.

Seminal work in *C. elegans* by Horvitz and coworkers established that cell death is genetically programmed and that a hierarchical relationship exists between the core components of the apoptotic machinery. CED-9 is the nematode homologue of the anti-apoptotic protein Bcl-2. Consistent with its role in cell survival, loss of function mutants in *ced-9* were lethal due to profound apoptosis. Importantly, this cell death could be suppressed by a combined loss of function in *ced-3 and ced-4*, the homologues of executioner caspase-3 and the mammalian apoptosome proteins APAF-1/ cytochrome c respectively, locating CED-9 upstream of CED-3 and CED-4. Further studies identified EGL-1, the nematode homologue of the BH3 only class of Bcl-2 proteins, as a negative regulator of CED-9 (Hengartner et al., 1992; Metzstein et al., 1998; Yuan and Horvitz,

1990). This framework has proved to be highly conserved from nematode to mammals, although the latter program is more complex (Figure 1.1).

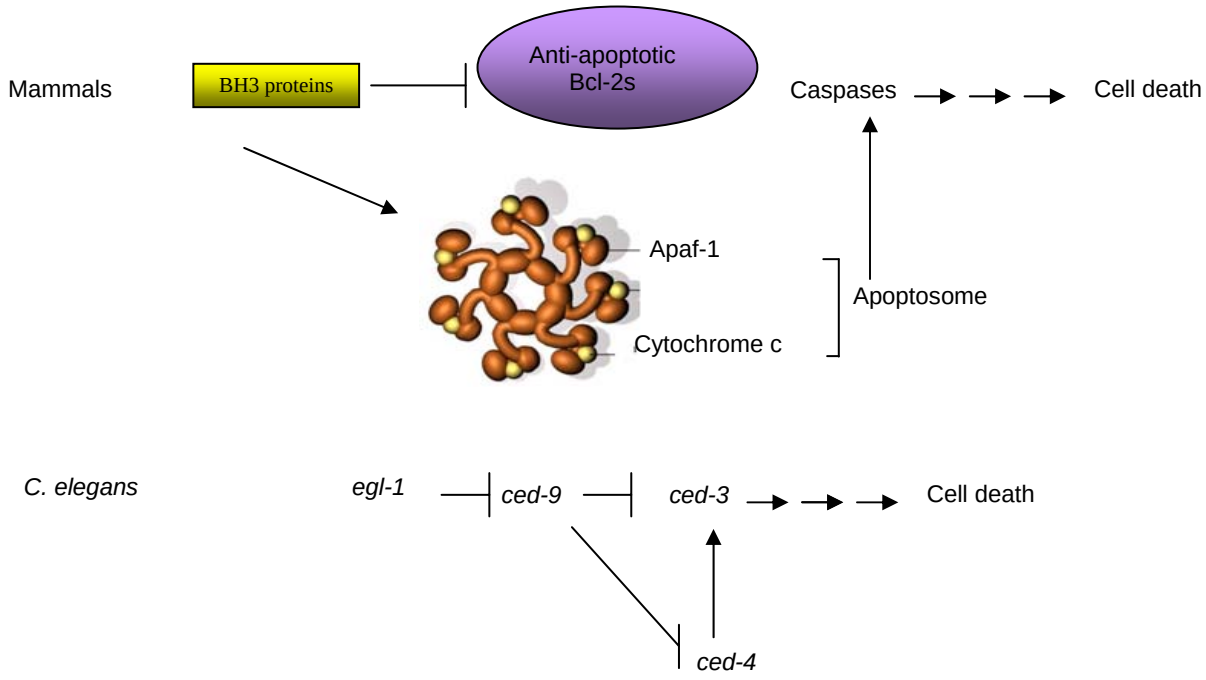


Figure 1.1 Parallels between apoptotic regulatory programs in *C. elegans* and mammals.

This simplified schematic illustrates the parallels between the mammalian intrinsic pathway and apoptosis regulation in *C. elegans.* Note that mammalian cells possess multiple BH3 proteins, anti-apoptotic Bcl-2 family members, as well as a compliment of caspases in contrast to the worm.

In addition to its role as a mechanism of clearance of virally infected or damaged cells, apoptosis plays a key role in normal physiology (e.g. involution following lactation, and menstrual cycling)(Cryns and Yuan, 1998) . Developmentally, programmed cell death plays a central role in limb sculpting (e.g. interdigital regression, resorption of the tadpole tail) and removal of auto-reactive lymphocytes cells during immune maturation. Moreover, apoptosis is essential for maintenance of normal tissue homeostasis. As such, defects in the apoptotic pathway have important pathological consequences. Excessive or inappropriate cell death results in tissue damage, as seen in ischemia or stoke, as well as in the neurodegenerative pathologies Alzheimer's and Huntington's disease. Conversely, insufficient cell death results in developmental defects, autoimmune disease, and malignancy.

2. Apoptosis and Cancer

Cancer is thought to arise, at least in part, as a consequence of uncontrolled cell proliferation in combination with evasion of the cell's apoptotic program (Cory and Adams, 2002; Hanahan and Weinberg, 2000). Cancer cells frequently possess defects in key components of the apoptotic machinery itself or possess aberrant expression of anti-apoptotic proteins such as Bcl-2 and Mcl-1, tipping the scales in favor of cell survival. Similarly, cancer cells may have acquired mutations in cell signaling molecules resulting in constitutive signaling to cell survival pathways such as Akt or NF-κB (e.g. HER-2) antagonizing convention therapeutic modalities(Reed, 2003).

3. The Apoptotic Machinery as a Molecular Target in Cancer

Although programmed cell death occurs in response to conventional cytotoxic therapies for cancer, a more sophisticated approach, and one that promises to generate fewer side effects for patients, is to directly engage the apoptotic machinery within the tumor cell itself. Several emerging therapies are based on this idea. One of the most promising of these therapeutic agents is the Tumor Necrosis Factor Receptor Apoptosis Inducing Ligand (TRAIL). TRAIL is a ubiquitously expressed type II transmembrane protein (the amino terminus of the protein is cytoplasmic) originally isolated in a screen of a human heart cDNA library due to the presence of a conserved β-pleated sheet common among Tumor Necrosis Factor (TNF) family members (Wiley et al., 1995).

TRAIL binds to five distinct receptors on the cell surface: the Death Receptors DR4 and DR5 containing a cytoplasmic death domain (DD) needed for activation of the extrinsic cell death pathway; the so-called Decoy Receptors which lack a death domain (DcR1) or possess a non-functional truncated death domain (DcR2); and a related TNF family member, osteoprotegerin (OPG), which binds TRAIL with substantially lower affinity than the other receptors (reviewed in (Kelley and Ashkenazi, 2004)). Early studies by several groups established that TRAIL preferentially targets transformed or virally infected cells for cell death while leaving healthy and non-transformed cells virtually unharmed. The precise mechanisms underlying this specificity are not completely understood. Unlike Fas or TNFα, TRAIL does not elicit a significant immune response upon systemic administration, and is sufficient to partially delay tumor progression and lead to tumor shrinkage either as a single agent or in combination with conventional

chemotherapies (Kelley and Ashkenazi, 2004). Moreover, TRAIL's actions via the extrinsic cell death pathway are independent of p53 status, an important caveat as more than half of all tumors possess mutant or non-functional p53 proteins.

Soluble TRAIL (amino acids 114-281 in human) is homotrimeric in structure and requires an internal zinc atom to be bound by each of the subunits; this coordination is essential for both the biologic activity and stability of the ligand. Epitope tagged versions of soluble TRAIL vary in their ability to properly coordinate this atom leading to significant toxicity in cell culture studies with primary human hepatocytes (Jo et al., 2000). However, optimization of zinc binding and stability resulted in production of a recombinant ligand which is non-toxic to human hepatocytes (Lawrence et al., 2001). Since this study, optimized TRAIL has been shown to be well tolerated in non-human primates and is in Phase I clinical trials with Genentech (Kelley and Ashkenazi, 2004) (Reed and Pellecchia, 2005).

Alternative strategies targeting the apoptotic pathway in cancer take advantage of the fact that certain proteins are overexpressed in a subset of human tumors. One such strategy couples an antibody which recognizes a specific protein overexpressed in the tumor to an activated caspase, thereby selectively inducing death of the tumor cell and leaving the non-transformed cells, which fail to overexpress the protein targeted by the antibody, unharmed. Thus, Immunocasp-3 combines a single chain HER-2 antibody to an active caspase-3 subunit which is cleaved in the lysosome, translocates to the cytosol and induces apoptosis in HER-2/*neu* overexpressing cells (Jia et al., 2003). Similarly, coupling an activated form of caspase-6 to a HER-2 antibody has had

success in preclinical trials. Importantly, this study demonstrated that the chimeric molecule was sufficient to prolong survival and slow progression of xenograft tumors from the HER-2 overexpressing cells line SKBR3, suggesting the clinical feasibility of this approach (Xu et al., 2004).

More recent approaches have focused on modulating the Bcl-2 family of proteins directly. The anti-apoptotic members of this family antagonize standard chemotherapeutics by preserving mitochondrial integrity and ultimately preventing apoptosis. In contrast, the pro-apoptotic members of this family facilitate cell death. Ongoing drug development programs aim to manipulate one or both arms of this family to selectively induce tumor cell death. Genasense (generic name: oblimersen) is an anti-sense oligonucleotide to Bcl-2 currently in Phase III trials for solid (colon, lung, and prostate) as well as hematopoetic tumors (myeloma, leukemia, lymphoma). Although this agent appeared promising in early trials, sustained downregulation of Bcl-2 remains problematic and while the agent delayed time to progression, it had no impact on overall survival. (Kim et al., 2007). Simultaneous downregulation of Bcl-2 and Bcl-x_L via anti-sense technologies effectively enhanced cell death in combination with standard therapies in cancer cell lines, although these agents have not been tested in the clinic (Fulda and Debatin, 2006).

A subset of the pro-apoptotic components of the Bcl-2 family, the BH3-only proteins, bind to a hydrophobic cleft in the anti-apoptotic Bcl-2 family member and through mechanisms which are not entirely clear, lead to induction of cell death (Sattler et al., 1997; Wang et al., 1998; Wang et

al., 1996). Proof of concept experiments demonstrated that a peptide containing only a conserved 16 amino acid motif called a BH3 domain was sufficient to displace Bax from its anti-apoptotic binding partner Bcl-x_L and induce apoptosis (Moreau et al., 2003). BH3 peptides alone are cell impermeable, preventing their use therapeutically. Conjugation of a polymer of eight arginines to the peptides (so called R8-BH3 peptides) renders them cell permeable, and these modified BH3 peptides have been used successfully by multiple groups in cancer cell lines and xenograft models, demonstrating the feasibility using these agents clinically (Goldsmith et al., 2006; Letai et al., 2002; Rothbard et al., 2000). Inclusion of chemically modified amino acids within the BH3 domain, so called hydrocarbon stapling, stabilizes the critical alpha-helical backbone of the BH3 peptides by several fold, and enhances their resistance to cellular proteases, resulting in maximal induction of cell death in leukemic cell cultures and xenograft models (Walensky et al., 2006). These modified BH3 peptides, or SAHBs are in preclinical development at Harvard University.

Synthetic inhibitors which mimic the function of the BH3 proteins (BH3 mimetics) by tightly binding to the hydrophobic cleft in the anti-apoptotic Bcl-2 proteins have shown success in solid tumors either alone or in combination with traditional agents. Perhaps the best known of these agents is orally available compound ABT-737 in preclinical development at Abbott laboratories. Discovered by NMR guided structure based design, ABT-737 is designed to fit into the hydrophobic pocket of Bcl-2, Bcl-x_L and the related Bcl-w and inactivate the survival function of these family members. ABT-737 has no activity against Mcl-1(Oltersdorf et al., 2005; van Delft et al., 2006). Other related mimetics in preclinical development include BH3 I-1 and -2, the

natural product epigallechatechin (EGCG) found in green teas, and TW-37 which has high affinity for Bcl-2, Bcl-x_L and Mcl-1(Degterev et al., 2001; Leone et al., 2003; Reed and Pellecchia, 2005; Verhaegen et al., 2006).

The tumor selective nature of BH3 therapies has yet to be molecularly proven, although several lines of evidence lend support to the notion that cancer cells may be 'primed for death' rendering cancer cells more sensitive to these agents than non-transformed cells (Certo et al., 2006). The excitement surrounding these agents, whether targeted to the intrinsic or extrinsic pathways, is their potential to induce apoptosis in a p53 independent manner.

4. Mechanics of Cell Death

Mammalian cells employ two distinct but convergent pathways for the induction of programmed cell death, the extrinsic (death receptor mediated) and the intrinsic (mitochondrial) pathways (Figure 1.2). In both, key proteins are cleaved by activated executioner caspases ultimately leading to the demise of the cell (Cryns and Yuan, 1998; Lavrik et al., 2005; Reed, 2003).

4.1 Extrinsic Pathway

In the extrinsic pathway, members of the TNF superfamily such as TNFα or TRAIL, bind to their cognate death receptors on the cell surface and induce receptor trimerization. Next, the adapter molecule FADD binds to the receptors via its Death Domain (DD). Initiator caspases-8 and -10 are recruited to this complex, called the Death Inducing Signaling Complex (DISC), by virtue of their Death Effector Domains (DED). Once in the DISC, the pro-domain of the initiator

caspase is removed, thereby activating the caspase. Caspase-8 processes the executioner caspase-3 which, in turn, cleaves the majority of downstream caspase targets. In type II cells, caspase-8 activation of caspase-3 is inefficient and cell death occurs largely via the intrinsic pathway by efficient caspase-8 processing of the Bcl-2 family member Bid, which translocates to the mitochondria and activates Bax and Bak. In these cells, the intrinsic pathway serves as an amplification loop for caspase activation (Scaffidi et al., 1998). The concept of type I and II cells has also been validated in a *Xenopus* model, in which addition of active recombinant caspase-8 was insufficient to trigger robust cell death in extracts depleted of mitochondria, underscoring the need for signaling from this organelle to amplify the death signal (Kuwana et al., 1998).

4.2 Intrinsic Pathway

In the intrinsic pathway, cellular stress agents (such as γ irradiation, cytokine and/or growth factor withdrawal, reactive oxygen species, hypoxia, microtubule damaging agents, and topoisomerase II inhibitors such as etoposide) trigger depolarization of the outer mitochondrial membrane and lead to release of cytochrome c and other apoptogenic agents (e.g. AIF, Endonuclease G, Smac/Diablo) from the mitochondria. Upon release, cytochrome c binds to and facilitates the oligomerization of the scaffold protein Apaf-1. Caspase-9 is processed to its active form (a dimer) and its affinity for procaspase-3 is enhanced in a complex referred to as the apoptosome (Pop et al., 2006; Yin et al., 2006). Activated caspase-9 processes caspase-3 to its active form and cell death ensues, largely as a result of proteolysis of key cellular proteins by activated caspases.

4.2.1 Cytochrome c

Seminal work by Wang and coworkers established cytochrome c as a potent initiator of cell death in a cell free system (Liu et al., 1996). This same approach was subsequently utilized to identify the two additional components of the mammalian apoptosome: APAF-1 and pro-caspase-9 (Li et al., 1997). The central importance of cytochrome c in apoptosis was solidified by the findings that Bcl-2 and Bcl-x_L function to prevent release of this protein from the mitochondria (Johnson et al., 2000; Yang et al., 1997), providing an important parallel between the stress induced apoptotic signaling pathway and the cell death program in *C. elegans*. It is now known that upon release from the inner mitochondrial membrane space holo-cytochrome c (cytochrome c complexed with a heme group) binds to the scaffold protein APAF-1 via its carboxy terminal WD40 repeats and, in the presence of dATP, induces a conformational change in APAF-1 which facilitates its oligomerization. Procaspase-9 is recruited to this complex via its caspase recruitment domain (CARD domain) and is proteolytically activated. In its active state, caspase-9 activates the executioner caspase-3 (and to a lesser extent caspases-6 and -7).Thus, release of mitochondrial proteins is controlled by regulating the induction of mitochondrial outer membrane permeability (MOMP). While the precise events leading up to MOMP remain controversial, it is hypothesized to be initiated at least two distinct mechanisms: opening of a putative permeability transition pore (the details of which are beyond the scope of this work) or by modulation of the outer mitochondrial membrane protein members of the Bcl-2 family.

It is hypothesized that cytochrome c is released in two distinct waves which correspond to cytochrome c that is either loosely or tightly attached to inner membrane proteins. In the latter,

larger pool of cytochrome c is released only after cristae remodeling. The differential release of cytochrome c implies that transient MOMP is insufficient to trigger the demise of the cell. Rather, it is the *irreversible* MOMP from which the cell cannot recover. In the presence of activated caspases, apoptosis will ensue. However, when confronted with caspase inhibition, mammalian cells eventually die by necrosis or autophagy, at least in part as a consequence of mitochondrial collapse but also due to release of caspase independent apoptogenic factors from the inner membrane space such as Apoptosis Inducting Factor (AIF) or Endonuclease G (Endo G)(Garrido et al., 2006). Given the fatal consequences for the cell subsequent to irreversible MOMP induction, it is not surprising that cancer cells are exquisitely resistant to this process.

Normally residing between the inner and outer mitochondrial membranes in close contact with cardiolipin, cytochrome c displays a punctate staining pattern in healthy cells. Multiple apoptotic stimuli trigger cytochrome c release, resulting in a continuum of staining from diffuse to complete absence/loss of staining when monitored by immunofluoresence. Cytochrome c release is independent of caspase activation, and the broad spectrum caspase inhibitor zVAD-fmk is widely used in microscopy studies to prevent dying cells from detaching from the plate (Bossy-Wetzel et al., 1998; Goldstein et al., 2000; Kennedy et al., 1999).

The absolute requirement of cytochrome c in oxidative phosphorylation hampered initial efforts to characterize the apoptotic function of the protein, resulting in embryonic lethality at E8.5. However, MEFs isolated from the knockout animals were resistant to cell death induced by UV radiation, staurosporin, or serum starvation but retained sensitivity to TNFα (Li et al., 2000). In

a more recent study, the lysine 72 of cytochrome c, which is required for binding to Apaf-1, was mutated to alanine (K72A) and this allele was used to generate transgenic animals. Importantly, this variant of cytochrome c (KA allele) functions normally in oxidative phoshorylation and is released from the mitochondria with similar kinetics to normal littermates, but is defective in activation of caspase-9 and -3 allowing a specific investigation of the apoptotic role of

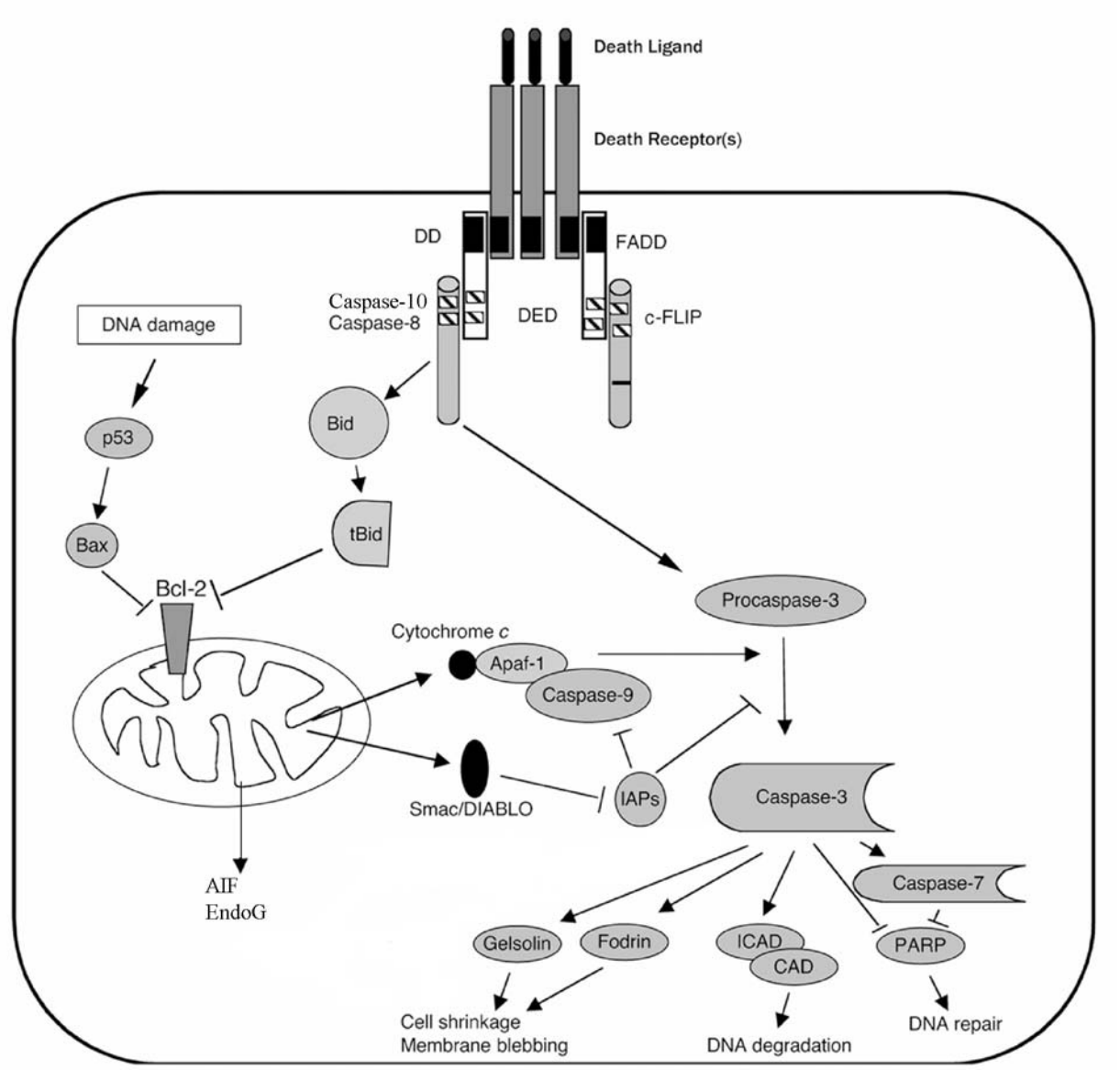


Figure 1.2 Apoptosis occurs via the extrinsic or intrinsic cell death pathways in mammalian cells.

Figure adapted from Budd and coworkers *JCI (*2002) 109:437-442.

cytochrome c. As in previous studies, MEFs from these animals were resistant to cell death induced by UV radiation or staurosporin, underscoring the importance of this protein in mitochondrial cell death. However, a tissue type specificity emerged from studies of these animals, as thymocytes from the KA/KA animals remained sensitive to a variety of apoptotic stimuli (dexamethasone, etoposide, UV and γ-radiation, or Fas) the significance of which has yet to be elucidated (Hao et al., 2005).

5. Apoptotic Regulation by the Bcl-2 Family

The Bcl-2 family of proteins regulates cell death by the intrinsic pathway (Cory and Adams, 2002; Danial and Korsmeyer, 2004). Ectopic Bcl-2 expression is sufficient to protect cells treated with radiation or serum withdrawal but not Fas induced cell death (Strasser et al., 1995).

The Bcl-2 family includes more than 20 members consisting of both pro and anti apoptotic proteins defined by the repertoire of conserved Bcl-2 homology (BH) domains contained within each protein. The BH domains correspond loosely to four highly conserved alpha helical domains found in Bcl-2 (Figure 1.3). The anti-apoptotic members (Bcl-2, Bcl-x_L, and Mcl-1 among others) possess all four BH domains, although the BH3 domain is often buried. The pro-apoptotic members of the family can be separated into those with multiple BH domains (such as Bax and Bak) or those whose only similarity to the superfamily lies in the small BH3 domain, referred to as 'BH3-only' proteins (Bid, Bim, Bad and others).

Cannonically, the BH3 domain is an amphipathic α helical region which binds within the hydrophobic cleft on anti-apoptotic Bcl-2 family members (*e.g.* Bcl-2, Mcl-1, Bcl-x_L) and is sufficient to induce MOMP and cell death (Chou et al., 1999; Willis and Adams, 2005) (Sattler et al., 1997). The BH3 domain is believed to constitute a minimal death inducing unit. Recent studies indicate that the BH3 only proteins bind and inactivate distinct subsets of anti-apoptotic Bcl-2 proteins (Chen et al., 2005; Kim et al., 2006; Letai et al., 2002). Bid, Bim, and Puma bind to all known anti-apoptotic Bcl-2 family members, while Bad and Noxa interact with distinct but complimentary subsets; Bad with Bcl-2 and Bcl-x_L, Noxa with Mcl-1. The precise mechanism through which the BH3 proteins induce activation of Bax and Bak activation, and consequently apoptosis, is controversial (discussed further below).

Individual Bcl-2 family members are discussed in greater detail below.

5.1 Anti-apoptotic Multidomain Members

5.1.1 Bcl-2

Cloned from the region flanking the t(14;18)(q32;p21) translocation in B cell lymphomas by multiple groups (Bakhshi et al., 1985; Tsujimoto et al., 1985), Bcl-2 was initially proposed to play a critical role in cellular transformation. However, its role as a promoter of cell survival (as opposed to enhancing the proliferative capacity of the cell) was uncovered by its ability to prevent cell death in the absence of IL-3 in myeloid and lymphoid cell lines that are dependent on this cytokine for survival (Vaux et al., 1988). Bcl-2 is the mammalian homologue of the

nematode CED-9 (Hengartner and Horvitz, 1994). Subsequent studies with spleenocytes isolated from Bcl-2 transgenic mice demonstrated a profound survival advantage when cultured in low serum compared to those isolated from normal littermates, providing a functional role for Bcl-2 in the regulation of cell death (McDonnell et al., 1989). Targeted disruption of Bcl-2 resulted in viable animals, possessing abnormal kidneys marked by numerous cysts and 'proliferative lesions', and abnormal hair pigmentation. This was accompanied by profound apoptosis in the thymus and spleen of these animals. Thymocytes from the *bcl-2* $^{-/-}$ animals displayed enhanced sensitivity to dexamethasone and γ- irradiation compared to their normal littermates, although they possessed no inherent difference in basal cell death rate (Veis et al., 1993).

Bcl-2 is primarily localized to the outer mitochondrial membrane, although it is less frequently observed in the nucleus and endoplasmic reticulum. Trafficking to these sites is thought to be mediated by a carboxy terminal hydrophobic domain within the protein.

Caspase-3 cleaves Bcl-2 after Asp^{34} of the protein. Cleavage releases a pro-apoptotic fragment which translocates to mitochondria, releases cytochrome c, and accelerates cell death in response to diverse stimuli. A cleavage resistant Bcl-2 conferred enhanced protection against intrinsic pathway apoptotic stimuli when compared to wild type Bcl-2 implicating its role in the amplification of the cell death signal (Cheng et al., 1997; Kirsch et al., 1999).

Bcl-2 potently inhibits release of cytochrome c from mitochondria (Yang et al., 1997).

5.1.2 Bcl-x_L

Identified due to its ability to hybridize with Bcl-2 cDNA probes, Bcl-x_L is a broadly anti-apoptotic protein containing all four Bcl-2 homology domains; BH domains 1-3 form a hydrophobic pocket required for interaction with other family members. Mutation of a conserved amino acid within this pocket abrogates the anti-apoptotic function of Bcl-x_L (Muchmore et al., 1996). A smaller isoform, produced by alternative mRNA splicing, Bcl-x_s is pro-apoptotic as a consequence of deletion of the BH4 domain (Boise et al., 1993). The processes regulating splicing of Bcl-x are poorly understood.

Expression of Bcl-x_L in IL-3 deprived FL5.12 cells conferred significantly greater protection against cell death than observed with Bcl-2 expression, indicating that in this experimental system Bcl-x_L is more potently anti-apoptotic than Bcl-2.

In contrast to Bcl-2, Bcl-x_L is cytoplasmic in healthy cells, and translocates to the outer mitochondrial membrane in response to stress. It has also been reported in association with the endoplasmic reticulum (Hsu et al., 1997).

Genetic ablation of Bcl-x in mice results in embryonic lethality. Death occurs by E13 due to extensive apoptosis in the central nervous system. Further examination of these animals revealed

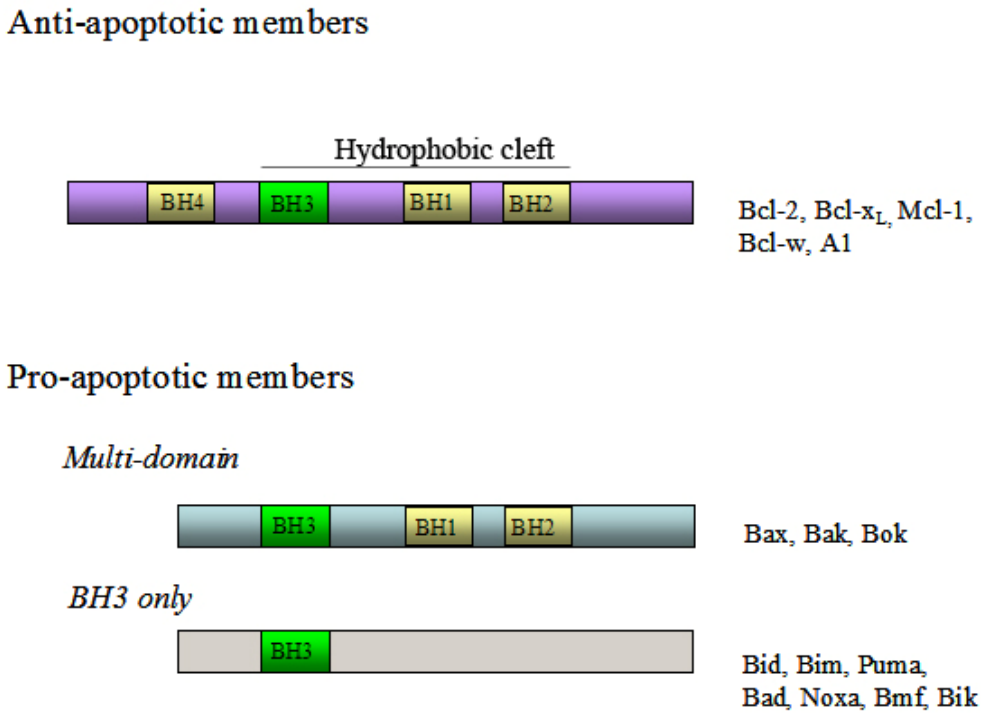


Figure 1.3 The Bcl-2 family consists of both pro and anti-apoptotic members.

Schematic representation of the Bcl-2 family. Conserved Bcl-2 homology (BH) regions are indicated. Anti-apoptotic Bcl-2 family members possess all four homology domains; the BH4 domain is believed to be essential for the survival function of these proteins. In contrast, the pro-apoptotic family members contain a subset of these domains and may be stratified into BH3-only or multidomain containing proteins.

a marked defect in lymphocyte maturation, providing genetic evidence for the importance of Bcl-x in the developing nervous and immune systems (Motoyama et al., 1995). Bcl-x_L is constitutively expressed in adult neural tissue (Boise et al., 1993).

Bcl-x_L potently inhibits the release of cytochrome c from mitochondria (Johnson et al., 2000). Overexpression of Bcl-x_L under the control of the LCK promoter protected thymocytes from γ irradiation and dexamethasone induced apoptosis, similar to Bcl-2. Moreover, the Bcl-x_L transgene was sufficient to rescue the embryonic lethality Bcl-2 animals, molecularly proving the functional redundancy of the two proteins (Chao et al., 1995).

Caspases cleavage of Bcl-x_L after Asp^{61} removes the anti-apoptotic BH4 domain, releasing a multidomain containing pro-apoptotic fragment similar in structure to Bcl-x_s (Clem et al., 1998).

5.1.3 Mcl-1

Myeloid cell leukemia-1 (Mcl-1), identified as a gene expressed during chemically induced differentiation of the myeloid leukemia cell line ML-1, possess Bcl-2 homology domains 1-4 and confers protection against diverse DNA damaging agents and growth factor withdrawal (Kozopas et al., 1993; Zhou et al., 1997)(reviewed in (Zhuang and Brady, 2006)). Two isoforms of Mcl-1 exist as a consequence of alternative mRNA splicing: isoform 1 is anti-apoptotic, while isoform 2 (which lacks Bcl-2 homology (BH) domains 1 and 2 domains, retaining only the BH3

domain) induces apoptosis (Bae et al., 2000). Details below attend to the characterization of protein isoform 1.

Genetic ablation of Mcl-1 in mice leads to embryonic lethality due to an implantation defect suggesting that the protein has roles outside of the cell death arena (Rinkenberger et al., 2000). Conditional deletion of Mcl-1 revealed a requirement of this protein for both the development and maintenance of T and B lymphocytes (Opferman et al., 2003). Moreover, Mcl-1 expression has been shown to be absolutely required for survival of hematopoetic stem cells (Opferman et al., 2005).

Given its similarity to Bcl-2 and its importance in differentiation, it is not surprising that Mcl-1 is frequently involved in cancer. Mice overexpressing Mcl-1 develop B cell lymphomas at a greater rate than their control littermates reminiscent of the phenotype of Bcl-2 transgenic animals (Zhou et al., 2001b). Similarly, high Mcl-1 protein levels have been reported in human B cell chronic lymphocytic leukemia (Kitada et al., 1998) and in acute myelogenous leukemic patients at the time of relapse, leading some to argue that chemotherapeutic regimens may select for cells expressing Mcl-1(Kaufmann et al., 1998).

Mcl-1 has a short half life (30 minutes to several hours) despite being induced by multiple cytokines (Yang et al., 1996). Interestingly, apoptosis cannot occur while intact Mcl-1 is present in the cytosol (Willis et al., 2005). Mcl-1 is targeted for destruction via the 26S proteasome by its E3 ligase, a BH3 containing protein called Mule. Depletion of Mule resulted in stabilization

of Mcl-1 and enhanced protection against DNA damage induced apoptosis (Zhong et al., 2005). Interaction with the BH3 protein Noxa also leads to degradation of Mcl-1. Although the mechanism of Noxa induced degradation of Mcl-1 is unknown, Czabotar and coworkers report that this process is mediated by residues found in the carboxy terminal portion Noxa's BH3 domain (Czabotar et al., 2007). Granzyme B and Glycogen Synthase Kinase 3 (GSK-3) also mediate the destruction of Mcl-1, although the mechanisms though which this occurs are poorly understood (Han et al., 2004; Maurer et al., 2006).

Mcl-1 shares the characteristic mitochondrial localization with other Bcl-2 family members which is thought to be mediated by a stretch of 20 hydrophobic amino acids in the carboxy terminus of the protein (Yang et al., 1995). It has recently been reported that an internal EELD motif recognized by the mitochondrial import receptor Tom70 is required in addition to the carboxy terminal tail for efficient mitochondrial targeting (Chou et al., 2006).

Capase-3 cleaves Mcl-1 after Asp^{127} (which is the aspartic acid residue recognized by Tom70) and Asp^{157}, removing the amino terminus of the protein which is thought to mediate its anti-apoptotic function. Ectopic expression of the carboxy terminal cleavage fragment of Mcl-1 induced apoptosis in Jurkat and NIH 3T3 cells (Michels et al., 2004; Weng et al., 2005). Truncated Mcl-1 was less effective in preventing Bim or tBid induced apoptosis than the wild type protein in HeLa cells, although the interactions with these two proteins remained intact (Zhuang and Brady, 2006).

Mcl-1's preferred binding partners are Bim, Bid, Puma, and Noxa, partially explaining why ablation of this gene has such profound consequences for the lymphoid and hematopoetic compartments.

The redundancy and complexity of the anti-apoptotic components of the Bcl-2 family stands in marked contrast to *C. elegans*, in which a single gene, *ced-9,* is sufficient to block cell death.

5.2 Multidomain Bcl-2 Family Members

Induction of mitochondrial outer membrane permeability during apoptosis depends of the presence of one or more of the multidomain pro-apoptotic Bcl-2 family members, Bax and Bak at the mitochondria. MEFs from $Bax^{-/-}$ $Bak^{-/-}$ cells were resistant to multiple apoptotic stimuli including staurosporine, etoposide, UV radiation, endoplasmic reticulum stressors, and growth factor withdrawal (Wei et al., 2001) although ablation of either gene alone had modest effects on cell death. However, the DKO animals retained sensitivity to Fas induced cell death (Knudson et al., 1995; Lindsten et al., 2000). Importantly, sensitivity to various apoptotic stimuli was restored to the $Bax^{-/-}$ $Bak^{-/-}$ MEFs when Bax was reintroduced into the cells. Similarly, co-introduction of Bax and a BH3 protein led to cell death at levels comparable to that observed with wild type MEFs (reviewed in (Lindsten and Thompson, 2006)).

The precise mechanism by which Bax and Bak are activated by BH3-only proteins is poorly understood. Two current models are discussed below.

5.2.1 Bax

Bcl-2 antagonist X protein (Bax) contains Bcl-2 homology domains 1-3, and readily forms heterodimeric complexes with Bcl-2 *in vivo* (Oltvai et al., 1993).

Bax is cytoplasmic in unstressed cells where it exists in complex with 14-3-3 proteins. In non stressed cells, the carboxyl terminal α helix (helix 9) of Bax rests in the hydrophobic pocket that BH3-only proteins interact with, thereby preventing activation of the protein (Suzuki et al., 2000). However, in response to apoptotic stimuli Bax undergoes a profound conformational change involving both the carboxyl and amino termini of the protein (exposing the hydrophobic pocket targeted by BH3-only proteins) which results in its translocation to the outer mitochondrial membrane and induction of MOMP (Hsu et al., 1997). Deletion mapping studies identified a mitochondrial anchor sequence in the carboxyl terminus of Bax (Goping et al., 1998).

Studies with synthetic liposomes mimicking the outer mitochondrial membrane demonstrated that Bax assembles into homo or hetero dimeric complexes (the latter involving the multidomain protein Bak) which are structurally reminiscent of the pore forming regions of bacterial toxins. Importantly, these higher order structures were sufficient to permeabilize the lipid membrane and this function was dependent on the presence of the Bax BH3 domain (Kuwana et al., 2005; Wang et al., 1998). Activation of Bax can be induced by tBid, or by p53 (both transcriptionally and via direct interaction)(Chipuk et al., 2004; Desagher et al., 1999; Miyashita and Reed, 1995; Nechushtan et al., 1999; Nechushtan et al., 2001).

Bax deficient animals are viable, but exhibit deficits in the development of neuronal lymphoid systems, as a consequence of insufficient apoptosis induction (Knudson et al., 1995).

Bax null cells are resistant to TRAIL induced apoptosis even in the presence of Bak (LeBlanc et al., 2002; Theodorakis et al., 2002). Similarly, HCT-116 colorectal cancer cell lines containing naturally occurring Bax mutations (Bax $^{-/-}$) were less sensitive to 5-florouracil (5-FU) treatment than those with wild type Bax, suggesting that Bax plays a critical role in chemotherapy induced apoptosis (Zhang et al., 2000).

The contribution of Bax to p53 induced apoptosis *in vivo* was shown in a p53 dependent model of mouse glioma. Crossing these animals with Bax deficient mice accelerated tumor formation and suppressed apoptosis by 50%, suggesting that Bax is required for the complete tumor suppressive function of p53 (Yin et al., 1997). In a related set of experiments, Knudson and coworkers reported that Bax expression in a p53 deficient background accelerated tumor progression (Knudson et al., 2001).

5.2.2 Bak

In contrast to Bax, Bcl-2 antagonist killer (Bak) is a resident integral mitochondrial outer membrane protein which is maintained in an inactive state in association with the outer mitochondrial membrane protein VDAC2 (Cheng et al., 2003) or by Mcl-1 and Bcl-x_L (Cuconati et al., 2003; Willis et al., 2005).

In response to apoptotic stimuli, Bak dissociates from the aforementioned complexes and undergoes a conformational change exposing its amino terminus and subsequently forms homo or hetero-oligomers which are sufficient to permeabilize the outer mitochondrial membrane *in vitro* and *in vivo* (Griffiths et al., 1999; Wei et al., 2000).

Ectopic expression of Bak induced apoptotsis in fibroblasts (Chittenden et al., 1995). Genetic ablation of Bak in the mouse had a modest phenotype with no overt apoptotic defects in these animals, suggesting a functional redundancy with Bax (Lindsten et al., 2000). Of note, Lindenboim and coworkers recently reported that cell death induced by the pro-apoptotic Bcl-x_s in SV40 immortalized mouse embryonic fibroblasts was dependent on the presence of Bak, but not Bax, suggesting that these proteins may in fact possess non-redundant functions (Lindenboim et al., 2005). Similarly, Bak null Jurkat cells were markedly resistant to a variety of chemotherapeutic agents and this phenotype was readily reversed by reconstitution of Bak into the system, although this finding may merely reflect the absence of Bax in this experimental system (Wang et al., 2001).

The crystal structures of a wild type Bak BH3 peptide and several BH3 mutant peptides in complex with Bcl-x_L have been solved and reveal that the BH3 domain is indeed an amiphipathic α helix which binds to the hydrophobic cleft on Bcl-x_L. Moreover, peptides containing single mutations at position 78 (Leu →Ala) or 83 (Asp→ Ala) exhibited significantly decreased

binding affinity for Bcl-xL, underscoring the importance of these residues for the function of Bak (Sattler et al., 1997).

5.3 BH3 only proteins

The pro-apoptotic activity of the BH3 proteins is tightly regulated to prevent unwanted apoptosis. For example, Bax and Noxa are transcriptionally upregulated by p53 activation, while posttranslational modifications such as phosphorylation or caspase cleavage control the subcellular localization of Bad and Bid sequestering them from the mitochondria.
There appears to be stimulus specificity entrenched within the BH3 proteins, potentially explaining the existence of multiple BH3 proteins in mammalian cells (reviewed in (Cory and Adams, 2002)). This multiplicity of genes is in striking contrast to the worm in which *egl-1* is the sole BH3 protein. Importantly, *egl-1* is essential for apoptosis in *C. elegans.*

Individual BH3 proteins employed in these studies are discussed in greater detail below.

5.3.1 Bid

BH3 interacting domain death agonist (Bid) was identified as an interacting partner with Bcl-2 and Bax that induced caspase mediated cell death. The potent pro-apoptotic nature of Bid, which possessed no homology to other members of the Bcl-2 outside of the BH3 motif, solidified the notion that the BH3 domain constituted a minimal killing unit (Wang et al., 1996).

Subsequent work established that Bid was cleaved by caspase-8, and that removal of the amino terminus of the protein was required for efficient targeting of the p15 Bid to mitochondria, cytochrome c efflux, and ultimately induction of cell death. Importantly, these effects were inhibitable by co-expression of Bcl-2 or Bcl-x_L (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). The amino terminal glycine of truncated Bid (tBid) is myristolayted following caspase cleavage, targeting the protein to the outer mitochondrial membrane where it proceeds to induce Bax and/or Bak oligomerization and trigger MOMP(Korsmeyer et al., 2000; Wei et al., 2000; Zha et al., 2000).

Bid deficient mice are viable, and develop normally. However, these animals are markedly resistant to Fas induced cell death at doses which were fatal to normal wild type littermates due to profound hepatocellular apoptosis. Closer examination of hepatocytes and thymocytes from the Bid $^{-/-}$ animals revealed a striking defect in the activation of the effector caspases-3 and -7, as well as a failure to release cytochrome c from the mitochondria. Similarly, Bid deficient animals were less sensitive than normal littermates to the DNA damaging agent adriamycin (Sax et al., 2002). Taken together, these data suggest that Bid activation of the intrinsic pathway is critical for amplification of the caspase cascade required for efficient activation of executioner caspases (Yin et al., 1999). It was later reported that given sufficient time, Bid deficient mice spontaneous develop tumors which closely resemble chronic myelomonocytic leukemia suggesting a role in tumor surveillance/suppression for Bid (Zinkel et al., 2003).

Bid can bind to all known anti-apoptotic Bcl-2 family members.

5.3.2 Bad

Bcl-2-associated death promoter (Bad) was identified as an interacting partner with Bcl-2 in a yeast two hybrid assay. Compellingly, this report detailed what we now appreciate to be the key function of the BH3-only proteins: the ability to displace multidomain Bcl-2 family members (Bax or Bak) from anti-apoptotic members (Bcl-2 or Bcl-x_L) and induce apoptosis (Yang et al., 1995).

Ectopic expression of Bad induces in modest cell death. However, co-expression of Bad with Bcl-x_L was sufficient to abrogate protection conferred by this protein and this activity was dependent on the presence of an intact BH3 domain in Bad (Kelekar et al., 1997; Zha et al., 1997) .

Unlike some members of the Bcl-2 family, Bad does not contain a fatty acid tail which anchors it to the outer mitochondrial membrane. Rather, its subcellular localization is controlled by phosphorylation by survival signaling proteins (such as components of the Akt/PI-3K pathway) on three regulatory serines (Ser-112, -136, and -155) within the protein. When hyper-phosphorylated, Bad is sequestered in the cytoplasmic compartment, complexed with 14-3-3 proteins, thereby preventing its interaction with Bcl-x_L (Zha et al., 1996). However, this interaction is disrupted in the absence of nutrients or trophic factors, leading to translocation of (dephosphorylated) Bad to the outer mitochondrial membrane where it interacts with Bcl-2 and Bcl-x_L via its BH3 domain, and contributes to the induction of MOMP.

Bad deficient animals are born phenotypic ally normal, but frequently develop B cell lymphomas at a greater frequency than normal littermates given sufficient time. In agreement with the regulation of Bad by trophic factors, mammary epithelial cultures (but not thymocytes) from Bad $^{-/-}$ animals were markedly protected from EGF withdrawal compared to wild type controls. Similarly, MEFs from the Bad $^{-/-}$ animals displayed enhanced protection from Fas induced apoptosis (Ranger et al., 2003). In a related set of experiments, Datta and coworkers demonstrated that expression of a constitutively active Bad, in which the regulatory serines have been mutated to alanines, resulted in enhanced susceptibility to various apoptotic stimuli at doses previously established to be sub-lethal (Datta et al., 2002).

Two isoforms of murine Bad exist due to alternative splicing, known as Bad_L or Bad_S for the long and short isoforms respectively. In humans, only Bad-s exists and can be cleaved by caspases after Asp^{57}. Expression of a caspase cleavage resistant Bad mutant (D57A) protected cells from apoptosis following growth factor withdrawal supporting a role for Bad as a metabolic sensor in cells (Seo et al., 2003). Expression of a truncated human Bad, which lacked its amino terminal 28 residues, in Jurkat cells led to greater sensitivity to Fas induced apoptosis than observed with cells containing the full length protein, suggesting a potential feed forward mechanism involving caspase activation of Bad (Condorelli et al., 2001).

Bad binds to Bcl-x_L with greater affinity than to Bcl-2 (Yang et al., 1995). It is reported not to interact with Mcl-1.

5.3.3 Noxa

Latin for damage, Noxa was identified by a differential mRNA screen designed to isolate p53 targets genes by comparing the mRNA profiles of *p53/IRF-1* wild type to double knock out MEFs following x-ray irradiation. Five-fold induction of Noxa mRNA was seen subsequent to irradiation of WT MEFs but not p53 $^{-/-}$ MEFs, demonstrating that Noxa was indeed a p53 responsive protein. A similar result was observed in thymocytes isolated from wild type or double knock out animals. Induction of Noxa mRNA was rapid (reaching maximum expression at 3 hours post irradiation) and occurred concomitantly with accumulation of *Mdm2* mRNA. Murine Noxa contains two BH3 domains referred to as Noxa A and Noxa B (located between amino acids 27-35, and 78-86 respectively). In contrast, human Noxa possess a single BH3 domain (located between amino acids 29-37) which is highly conserved, although not identical to murine Noxa B (mouse B: QLRRIGDKVN, human B QLRRFGDKLN). Interestingly, human Noxa was cloned ten years earlier from an adult T cell leukemia cell line as a phorbol-12-myrstate-13-acetate (PMA) responsive gene (Hijikata et al., 1990). Ectopic expression of wild type murine Noxa, but not a BH3 mutant variant (in which the conserved leucine in both BH3 domains was mutated to alanine) in HeLa cells was sufficient to lead to mitochondrial targeting of the protein and cytochrome c efflux, culminating in profound apoptosis (Oda et al., 2000).

Deletion mapping identified a mitochondrial targeting sequence in human Noxa (amino acids 40-50: KLLNLISKLF). Mutants lacking this domain appeared diffusely cytoplasmic, and failed to induce apoptosis presumably as a result of their altered subcellular localization (Seo et al., 2003).

Noxa expression is not required for normal development, as *Noxa* $^{-/-}$ animals were phenotypically normal and survived to adulthood. However, MEFs from these animals (but not thymocytes or lymphocytes) were markedly protected from etoposide induced apoptosis(Villunger et al., 2003).

Noxa is involved in apoptosis induced by the proteasome inhibitors lactacystin, MG-132, and bortezomib in melanoma cells. Significantly, proliferating melanocytes were less sensitive to proteasome inhibitor induced apoptosis and failed to induce robust levels of Noxa mRNA or protein. To formally test the requirement of Noxa expression for proteasome inhibitor induced cell death, antisense oligonucleotides were used to knock down Noxa in melanoma cells previously shown to be sensitive to these agents, and the cells were analyzed for sensitivity to the proteasome inhibitors. In agreement with the authors' hypothesis, depletion of Noxa led to an approximately 50% reduction in effectiveness of these agents (Qin et al., 2005).

Noxa's preferred binding partner among the anti-apoptotic Bcl-2 family members is Mcl-1, although it interacts with Bcl-2 weakly (Certo et al., 2006; Kim et al., 2006; Oda et al., 2000). Ectopic expression of Noxa results in modest cell death, although robust death ensues when Noxa and Bad are co-expressed (Willis et al., 2005)suggesting that inactivation of both classes of anti-apoptotic Bcl-2 family members (Bcl-2/Bcl-x_L/Bcl-w and Mcl-1) is required for efficient activation of Bax and Bak.

5.3.4 Mechanism of BH3-induced Bax/Bak Activation

The precise role of the BH3 only proteins in the induction of cell death remains controversial (reviewed in (Willis and Adams, 2005)) (Figure 1.4). Letai and co-workers have proposed a two tier classification scheme: naming those proteins which can directly release cytochrome c from isolated mitochondria and induce oligomerization of Bax and Bak activators, and designating those which merely bind to the anti-apoptotic members of the Bcl-2 family sensitizers. The latter proteins function by binding to anti-apoptotic multidomain Bcl-2 family members and displacing activator BH3 proteins which propagate the death signal (Certo et al., 2006; Letai et al., 2002). This model is supported by several lines of evidence, the most compelling of which is the finding that the so-called activators, but not the sensitizers, are sufficient to oligomerize Bax and Bak when added to isolated mitochondria from normal mouse livers in the presence of the crosslinker BMH. Similar work by Kuwana and coworkers demonstrated that only Bid and Bim were sufficient to release cytochrome c from artificial liposomes (Kuwana et al., 2005). A key criticism of these studies remains the absence of detectable Bax/Bak-activator complexes *in vivo* while interactions between Bax and/or Bak and anti-apoptotic Bcl-2 proteins have been reported by multiple groups (Cuconati et al., 2003; Leu et al., 2004; Sato et al., 1994; Willis et al., 2005; Yin et al., 1994). However, Walensky and coworkers recently characterized an interaction between Bax and Bid in Jurkat leukemia cells using hydrocarbon stapled BH3 peptides, supporting the hypothesis that these interactions occur *in vivo* but are difficult to capture with current experimental systems (Walensky et al., 2006) . A related concern with the direct activator model is that the Bid $^{-/-}$ Bim $^{-/-}$ DKO mouse did not have the striking phenotype possessed by the Bax/ Bak DKO mice, suggesting the existence of other ways to activate Bax and Bak in the

absence of Bid and Bim. Depletion of the (modest) activator Puma from the Bid/Bim DKO cells by siRNA failed to prevent induction of apoptosis (Willis et al., 2007). These findings have led to the suggestion that the central role of the BH3 proteins may be to bind and neutralize anti-apoptotic proteins such as Mcl-1 and Bcl-x_L and that interactions with Bax and Bak, if occurring at all, are of little consequence in the activation of Bax and Bak (Willis et al., 2005). In this model, referred to as the indirect binding model, it is proposed that the binding specificity of the BH3 proteins determines their ability to induce MOMP: Bid, Bim, and Puma, activators in the direct model, are bind both classes of multidomain anti-apoptotic proteins while Bad, Noxa, and the remaining sensitizers in the direct model only interact with a subset of the anti-apoptotic proteins and as such are incapable of inducing MOMP. Clearly, additional studies are needed to elucidate the precise mechanisms by which BH3 only proteins induce Bax and Bak activation, and ultimately cell death.

Regardless of the mechanism employed to activate Bax and Bak, upon cytochrome c release into the cytosol, the caspase cascade is maximally activated and results in cleavage of proteins that (i) are required for essential cell functions or (ii) contribute to the execution of cell death. We turn now to a closer examination of the caspases and their role in the execution of cell death.

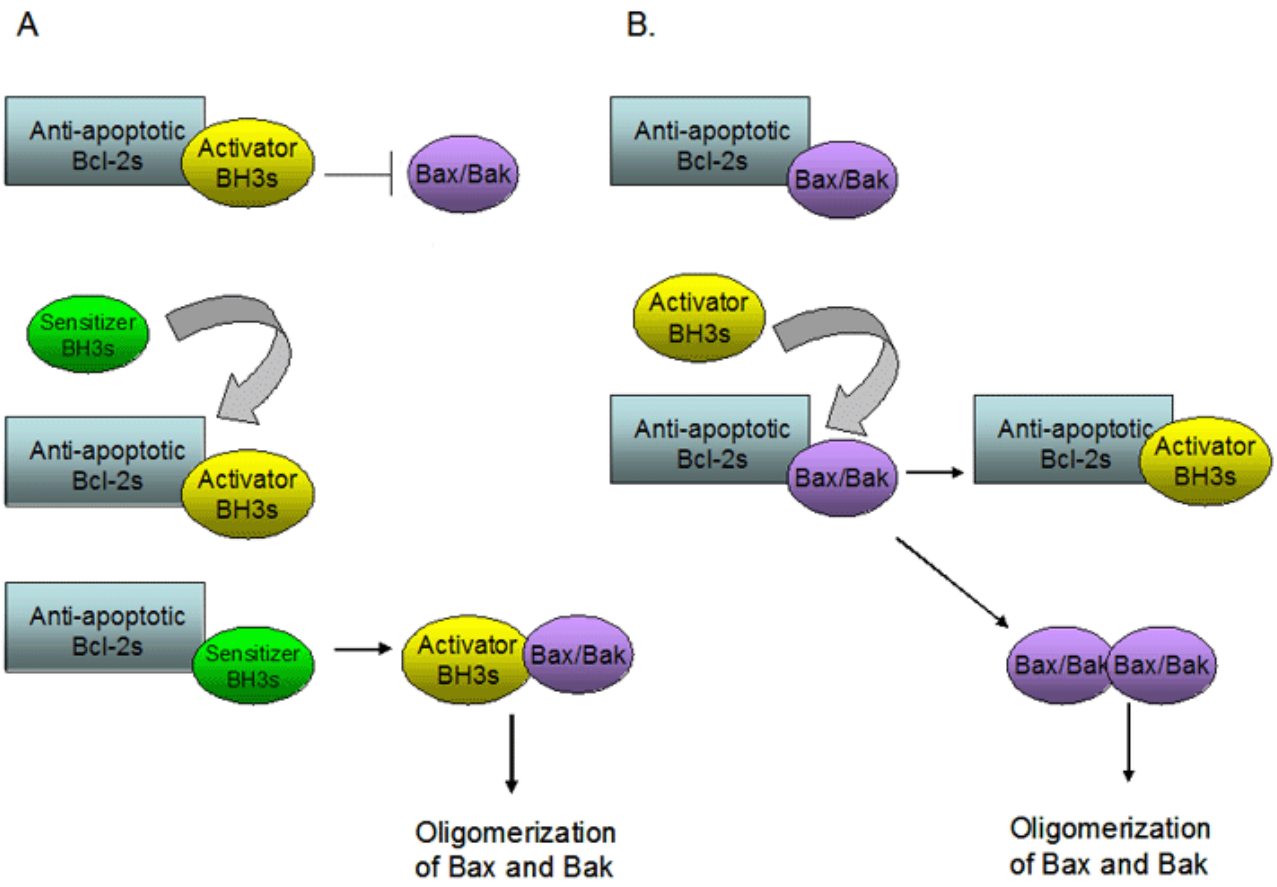


Figure 1.4 The means of activation of Bax and Bak by BH3 proteins is controversial.

A. The direct binding model is shown. In this model, Bax and Bak are activated via interaction with a subset of the BH3 proteins termed activators. Sensitizer BH3 proteins may displace activators from anti-apoptotic Bcl-2 family members, enabling their binding to Bax and Bak. Bak and Bak oliogomerize subsequent to interaction with activator BH3 proteins. B. The indirect model is shown. Here, Bax and Bak are held in check by anti-apoptotic Bcl-2 family members. BH3 proteins may disrupt this complex based on their binding affinity for the various anti-apoptotic Bcl-2 proteins. Once liberated from the anti-apoptotic Bcl-2s, Bax and Bak oligomerize. Adapted from Willis et al. (2005) Curr Opinion in Cell Biol 17(6):615-625.

6. Caspases

Horvitz and Yuan established a central role for caspases in the execution of cell death by demonstrating that loss of function mutants of *ced-3* suppressed cell death and overexpression of its protein product, CED-3, was sufficient to induce extensive apoptosis in *C. elegans* (Metzstein et al., 1998; Yuan and Horvitz, 1990). However, the finding that CED-3 was highly related to human and murine interleukin-1β converting enzyme (ICE-like) raised the intriguing possibility (which was subsequently proven) that both mouse and man executed cell death with the same enzyme used in the worm (Yuan et al., 1993).

6.1 Structure and Means of Activation

Mammalian caspases, a family of cystinyl aspartate specifice proteases, can be grouped into three distinct categories: group I consists of the inflammatory caspases which play little or no role in cell death, while groups II/III are CED-3 homologues which play an active role in apoptosis and can be further divided into initiator or executioner caspases based on structure of their pro-domains. Initiator caspases possess large amino terminal pro-domains which mediate protein: protein interactions such as the death effector domain (DED) in caspase-8 and the caspase recruitment domain (CARD) in caspase-9. Class II caspases are primarily thought to be initiators and class III executioners, although there are exceptions to these rules (Thornberry et al., 1997) (Figure 1.5).

Caspases are synthesized as inactive zymogens that are proteolytically cleaved and activated via autocatalysis, cleavage by another active caspase, or cleavage by a related protease. Cleavage

results in the separation of the large (p20) and small (p10) subunits of the caspase. The active caspase protease is a tetramer containing two p20 and two p10 subunits.

Caspases proteolyze their substrates by cleaving on the carboxyl side of a conserved aspartic acid located within a tetrapeptide recognition motif. Screening combinatorial libraries of synthetic peptides with recombinant caspases elucidated the cleavage preferences for individual caspases: caspase-8 preferentially cleaves substrates containing an I/L ETD motif, while the executioner caspases-3,-6-, 7 recognize and cleave adjacent to a DEVD motif (Lavrik et al., 2005; Thornberry et al., 1997). Standard nomenclature labels the conserved aspartic acid residue P1, with the P4 residue being the carboxy terminal residue in the motif. The residue in the P4 position is the most important determinant in caspase specificity. The residue occupying the P3 position is invariably glutamic acid.

Pharmacologic inhibition of caspases antagonizes cell death by preventing cleavage of key substrates which trigger cell demise. First generation caspase inhibitors were designed based on the tetrapeptide specificity discussed above. The pan caspase inhibitor zVAD-fmk contains a valine-alanine-aspartic acid motif predicted to inhibit most caspases, although this agent is only weakly active against caspase-2. Functionally, zVAD-fmk works as an irreversible pseudosubstrate, thereby preventing activation of the caspase cascade and its downstream consequences. The compound is only weakly cell permeable, and chemical moieties added to compensate for this defect are often toxic. Newer generation inhibitors such as qVD-OPH are cell permeable, and are superior chemically, allowing *in vivo* studies not previously possible, as

they are considerably less toxic and more effective at previous generation inhibitors (Callus and Vaux, 2007).

6. 2 Caspase knock out animals

Targeted deletion of caspases in murine models has extended our knowledge of the specific roles played by these enzymes in cell death. Pertinent details of the more striking models are discussed below.

Deletion of caspase-8 in the mouse was embryonically lethal at E12. MEFs obtained from these animals were resistant to cell death induced by TNF or Fas, consistent with a key role of this caspase in the death receptor pathway. Apoptosis proceeding via the intrinsic pathway was unaffected (Varfolomeev et al., 1998). Interestingly, humans with a mutation in caspase-8 are immunodeficient as a result of inability to robustly activate lymphocytes, supporting the role of caspase-8 signaling in immune function (Chun et al., 2002; Siegel, 2006).

Caspase-9 is widely believed to be apical caspase in the mitochondrial pathway, but accumulating evidence from a variety of transgenic animals has called this into question. Initial reports of caspase-9 knock out mice (Kuida et al., 1998)supported this hypothesis, with the

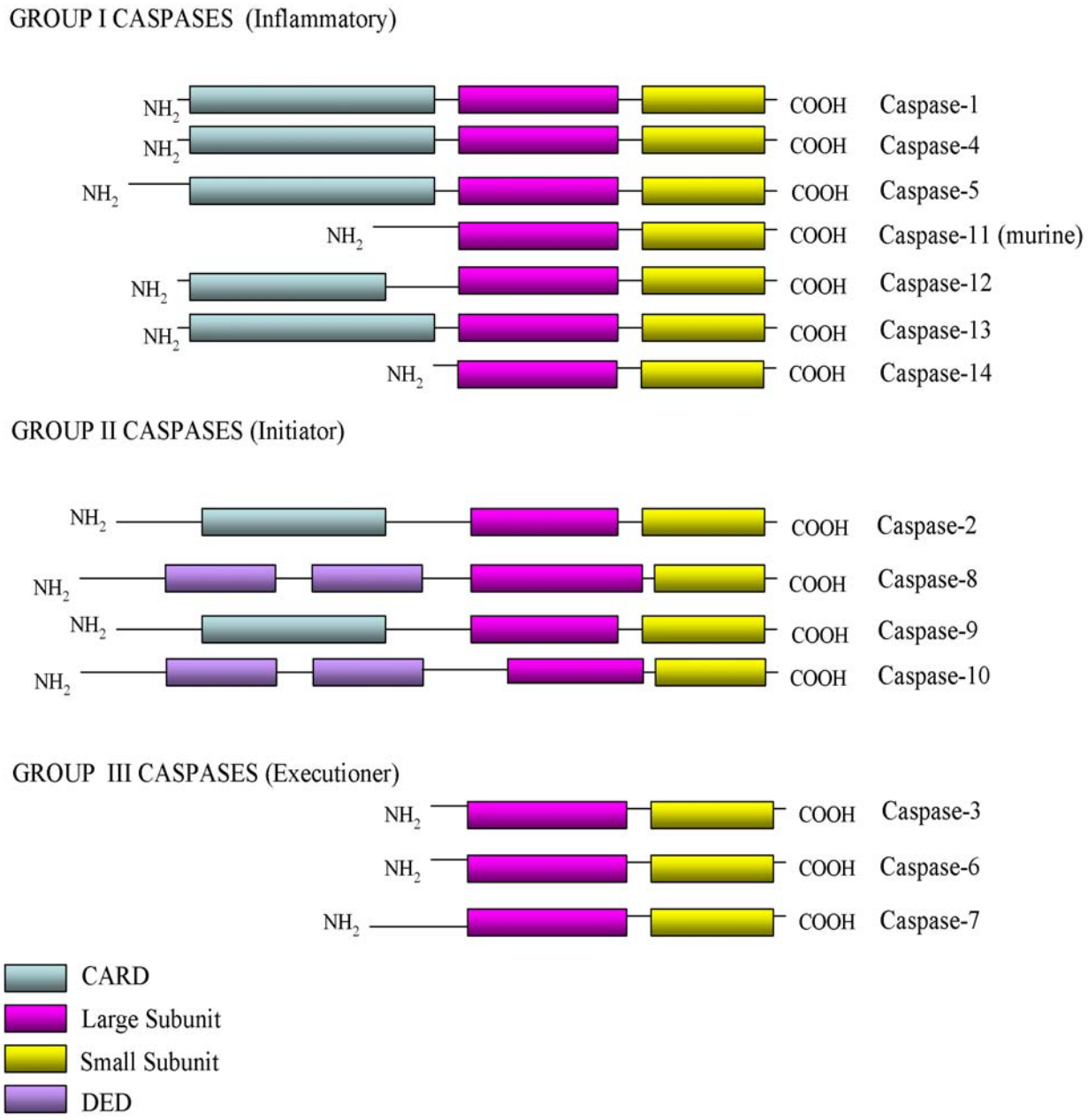


Figure 1.5 Caspases can be separated into three distinct groups on the basis of structure and function.

Figure adapted from (Lavrik et al., 2005).

animals possessing massive brain abnormalities due to one or more defects in neuronal apoptosis, absence of cytochrome c release and lack of cleavage of caspase-3 in cell free extracts. However, work by Marsden and coworkers demonstrated that cells lacking Apaf-1 or caspase-9 were able to undergo caspase dependent apoptosis in response to a variety of stress stimuli, suggesting a role for one or more upstream caspases to activate the mitochondrial pathway (Marsden et al., 2002). The identity of these caspase(s) is unclear to date, as a combined caspase-2/caspase-9 knockout mouse displayed robust caspase-dependent death, and released cytochrome c from the mitochondria in response to diverse stimuli formally ruling out caspase-2 as the apical caspase in the mitochondrial pathway (Marsden et al., 2004). Although the identity of the intiatior caspase in the mitochondrial pathway remains unclear, caspase-9 is widely accepted to be centrally important in the amplification of the caspase casade downstream of mitochondria.

Caspase-3 deficient animals were reported to die perinatally as a consequence of diminished neuronal apoptosis and brain abnormalities (Kuida et al., 1996). However, this striking phenotype was not observed when caspase-3 was deleted on the C57BL/6J background or when the Caspase-3-deficient 129X1/SvJ animals were backcrossed onto the C57BL/6J background, calling the prior report into question (Leonard et al., 2002). C57BL/6J mice deficient in either of the executioner caspases-3 and -7 are viable and grossly normal. However, the combined knock out (DKO caspase-3/-7) animals died perinatally as a result of cardiac defects. Consistent with a central role of these enzymes in cell death, fibroblasts and thymocytes isolated from these DKO

animals were markedly resistant to a variety of apoptotic stimuli. Intriguingly, data from these animals suggest a critical role for caspase-3 and -7 in mitochondrial induced cell death. Specifically, the DKO animals failed to undergo significant loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) following UV radiation treatment. Moreover, the DKO cells exhibited delayed cytochrome c release and Bax translocation to the mitochondria. Importantly, caspase-9 processing was unchanged in the DKO cells, leading the authors to "suggest a pathway from caspase-9 through caspases-3 and -7 that promotes cytochrome c release at early time points", that is, locating their actions upstream of the mitochondria (Lakhani et al., 2006).

7. Caspase Substrate Discovery

Given the importance of caspases in the execution of cell death, many groups have focused their efforts on the identification of the targets of these proteases. As of February 2007, 390 caspase substrates have been reported, although the evidence supporting these findings varies greatly. Careful examination of the consequences of the destruction of caspase substrates may be instructive for our elucidation of the mechanics of cell death and may ultimately aid in the design and implementation of new therapeutic modalities.

To facilitate the cell death researcher, Martin and colleagues have assembled CASBAH (www.casbah.ie), a searchable database summarizing the known literature on caspase substrates. Information for each substrate includes a listing of caspase cleavage sites (both putative and validated), species tested in, and the functional consequences of cleavage of the substrate (both known and putative) (Luthi and Martin, 2007).

Surprisingly, only a handful of caspase substrates have been conclusively linked to the physical execution of the cell in a rigorous manner. These include the inhibitor of CAD nuclease (ICAD), which when degraded, allows CAD to cleave nuclear DNA (Enari et al., 1998). Cleavage of the actin depolymerizing factor, gelsolin, results in membrane blebbing and animal deficient in this protein exhibit delayed membrane blebbing in response to apoptotic stimuli (Kothakota et al., 1997). Similarly, cleavage of the p75 subunit of Complex I of the mitochondrial electron transport chain ensures that cells will undergo apoptosis as a consequence of mitochondrial compromise. Expression of a non-cleavable p75 retarded production of reactive oxygen species and depletion of ATP levels and loss of membrane potential (Ricci et al., 2004).

More subtly, caspase cleavage can control the magnitude of signaling from substrates by either inhibiting signaling from the substrate or by unmasking a new function for the protein such that cell death will be enhanced. For example, caspase-9 cleaves itself, exposing an amino terminus that serves as a recognition/binding motif for the anti-apoptotic protein XIAP, thereby minimizing unwanted activation of downstream caspases. However, during robust caspase activation, caspase-3 cleaves caspase-9 again, removing the XIAP recognition motif and allowing recruitment to the apoptosome and subsequent activation of executioner caspases (reviewed in (Timmer and Salvesen, 2007)). Caspase cleaved Bid, tBid, serves as a damage sensor and a means of activation of the intrinsic cell death program (and in type II cells, as amplification loop for caspase activation) by inducing MOMP. However, prior to caspase activation, full length Bid is cytoplasmic and has no apoptogenic activity. Caspase cleavage of

the anti-apoptotic members of the Bcl-2 family not only disrupts the survival function of these proteins, but also gives rise to pro-apoptotic BH3-containing fragments which contribute to the execution of cell death (Cheng et al., 1997; Clem et al., 1998; Weng et al., 2005).

Several approaches have been utilized to identify caspase substrates. Computer based strategies trained on a subset of known caspase substrates have been employed with some success, although this approach is limited by the quality of the training set utilized. Yeast two hybrid approaches have also been used, but are limited by the bait used in the experiment. Given the inherent problems with these approaches, we and others have turned to unbiased screens, such as the one utilized in this dissertation, small pool expression cloning (SPEC), as a means to identify novel caspase substrates (Lustig et al., 1997). In this screen, protein pools (obtained via *in vitro* transcription and translation of individual cDNA clones) are incubated with a panel of recombinant caspases and examined for cleavage. This system facilitates discovery of novel substrates, and allows rapid screening of hundreds of proteins which can readily be identified by searching the sequence against nucleotide databases. Using this approach, our group has successfully identified diverse caspase substrates including the intermediate filament protein vimentin (Byun et al., 2001), the DNA repair protein MLH1 (Chen et al., 2004), the cohesion component Rad 21(Chen et al., 2002), the pathogenic protein in Alzheimer's disease Tau (Gamblin et al., 2003; Horowitz et al., 2004), integrin β4 (Werner et al., 2007) and the desmosomal cadherin desmoglein 1(Dusek et al., 2006).

In this work, we report the identification of HER-2 as a substrate of multiple caspases *in vitro* and in human breast cancer cells. We identify the cleavage sites *in vitro* and *in vivo*, and identify a pro-apoptotic activity associated several of the cleavage products. This substrate is discussed in greater detail below.

8. HER-2/neu

8.1 Identification and Structure of the Receptor

HER-2/*neu* is a member of the Epidermal Growth Factor Receptor (EFGR) family, located on chromosome 17q21 in humans. Members of the EGFR family share several conserved cysteine rich domains in subdomains II and IV of their extracellular region which mediate dimerization with other family members, a single pass transmembrane region, and a carboxy terminal tail containing a highly conserved tyrosine kinase domain as well as the major autophosphorylation and substrate binding sites which link the receptor to multiple and diverse signaling pathways within the cell (Kumagai et al., 2003; Qian et al., 1999; Yarden and Sliwkowski, 2001).

HER-2 was simultaneously identified in a screen of salivary adenocarcinoma for EFGR related genes and by virtue of its overexpression in human mammary carcinoma. It was designated HER-2/*neu* due to its similarity to the rat oncogene *neu* (isolated from neuroblastomas of ENU treated rats) (Coussens et al., 1985; King et al., 1985; Semba et al., 1985; Yamamoto et al., 1986).

8.2 Clinical Significance of HER-2 Expression in Breast and Ovarian Cancers

Transfection of NIH 3T3 cells with HER-2 cDNA (under the control of the MMTV promoter) or rat *neu* led to transformation of NIH 3T3 cells, classifying HER-2/*neu* as an oncoprotein (Di Fiore et al., 1987; Hudziak et al., 1987). It was later discovered that a single valine to gluatamic acid mutation at position 664 mediated the transformation in the NIH 3T3 system (Bargmann et al., 1986) although this mutation was not commonly found in human breast cancers. Expression of the wild type form of the rat *neu* gene containing a valine at position 654, which mimicked the protein contained in mammary tumors, resulted in mammary tumors which metastasized to lung. However, these tumors arose much later than those driven by the activated form of the receptor (Guy et al., 1992).

HER-2/*neu* is overexpressed in 30% of human breast and ovarian tumors and is correlated with poor prognosis (Slamon et al., 1987; Slamon et al., 1989) (Yarden and Sliwkowski, 2001). HER-2 is highly expressed in ductal carcinoma *in situ* (DCIS), particularly the comedo subtype, although the significance of this observation remains unclear. The receptor is also amplified or overexpressed in bladder (Swanson et al., 1992), pancreatic (Okada et al., 1995), and non-small cell lung cancers (Shi et al., 1992).

Several studies have linked HER-2 overexpression to resistance to conventional chemotherapeutic agents including Taxol (Tan et al., 2002; Yu et al., 1998), tamoxifen (Chung et al., 2002), TRAIL (Cuello et al., 2001), and retinoic acid (Tari et al., 2002), and to multidrug resistance (MDR) in the breast (Knuefermann et al., 2003). The relative resistance or enhanced

sensitivity of HER-2 overexpressing tumors to anthracyclins (e.g. etoposide) may be explained by co-amplification or deletion (which occur with equal frequencies in breast cancer) of the molecular target of these agents, the *topo IIα* gene, with HER-2 on chromosome 17 (Jarvinen and Liu, 2006).

8.3 Signal Transduction from the receptor

Although an orphan receptor itself, HER-2 is the preferred dimerization partner for other EGFR family members and signals to pathways controlling cell growth, migration, adhesion, differentiation, apoptosis, and tumorigensis (Yarden and Sliwkowski, 2001). Importantly, HER-2 can be activated by its own overexpression (Muthuswamy et al., 2001) or transactivated in heterodimeric complexes by the heregulins.

While HER-2 is able to complex with all of the EGFR family members, the HER-2/HER-3 heterodimer appears to be particularly important for transformation and tumor progression. Co-expression of these receptors transformed NIH 3T3 cells under experimental conditions in which neither receptor alone was transforming, suggesting cooperation between the receptors (Alimandi et al., 1995). Further, downregulation of the HER-3 co-receptor markedly slowed proliferation of HER-2 expressing breast cancer cells, and led to loss of activated Akt signal (Holbro et al., 2003). Recent work by Sergina and coworkers provides further evidence for the importance of HER-3 in signaling from the HER-2 receptor. They demonstrate that siRNA knockdown of HER-3 converted cytostatic tyrosine kinase inhibitors to apoptotic agents,

indicating that downregulation of HER-3 may enhance the effectiveness of these agents in the clinic (Sergina et al., 2007).

Enhanced signaling to the PI-3K/Akt pathway is believed to be the underlying mechanism of the chemoresistance discussed above. HER-2 signaling to this pathway is largely mediated by HER-3, which contains a docking site for the p85 subunit of PI3K in its cytoplasmic domain. However, studies with synthetically induced HER-2 homodimers demonstrated that these complexes were sufficient to activate the Akt pathway, although the mechanism through which this occurs is poorly understood (Muthuswamy et al., 1999). Phosphoinositol binding to the plextrin homology (PH) domain of Akt recruits the kinase to the plasma membrane where it is fully activated. The serine/threonine kinase Akt antagonizes cell death in several ways. By phosphorylating the BH3 only protein BAD at Ser 136, a docking site for 14-3-3 proteins is created disrupting the interaction between BAD and the potent anti-apoptotic molecules Bcl-2 and its related Bcl-X_L. Similarly, phosphorylation of a Forkhead transcription factor (FKHRL1) promotes interaction with 14-3-3 proteins thereby preventing nuclear localization, effectively shutting down transcription of its target gene Fas(Brunet et al., 1999). HER-2 induced phosphorylation of the Mdm2 by Akt leads to enhanced ubiquitination of p53, thereby preventing p53 dependent apoptosis (Zhou et al., 2001a). More directly, NF-κB can be activated via HER-2 signaling in an Akt/PI3K dependant manner leading to cell survival (Zhou et al., 2000).

8.4 HER-2 as a Molecular Target in Cancer

8.4.1 Antibody Based Therapies: Herceptin and 2C4

Slamon and coworkers reported a correlation between HER-2 overexpression in breast and ovarian cancer and poor clinical outcome in their landmark paper in 1987 (Slamon et al., 1987). Builiding on this work, *in vitro* and *in vivo* studies demonstrated that monoclonal antibodies targeted to the extracellular domain of HER-2 had significant anti-proliferative effects (Carter et al., 1992; Hudziak et al., 1989). Humanized versions of two of the antibodies generated in this work, 4D5 and 2C4, are being studied as therapeutic agents for women with HER-2 positive breast cancer.

Herceptin (Trastuzumab) is a humanized monoclonal antibody carrying the antigen binding loops from the murine 4D5 identified in earlier studies (Carter et al., 1992). Although the precise mechanisms underlying Herceptin's effectiveness are poorly understood, the agent has become a standard of care for women with locally advanced or metastatic HER-2 positive breast tumors alone or in combination with conventional chemotherapeutics (Cobleigh et al., 1999; Slamon et al., 2001). Herceptin has long been thought to only be effective in tumors which overexpress the receptor at high levels, although recent data by Menendez and coworkers has called this into question (Menendez et al., 2006).

The related humanized monoclonal antibody 2C4 (Pertuzumab) inhibits HER-2 heterodimerization, independent of the overexpression level of the receptor (Agus et al., 2002).

8.4.2 Anasamycin Antibiotics: Geldanamycin and 17-AAG

The benzoquinoid anasamycin class of antibiotics are well known inhibitors of HSP 90, a molecular chaperone for many receptor tyrosine kinases, including HER-2. Binding of these agents disrupts the chaperone:kinase complex by interfering with the ATPase function of HSP 90, ultimately leading to degradation of the client proteins via the ubiquitin proteasome pathway (Sharp and Workman, 2006). Geldanamycin is hepatotoxic precluding its use in cancer therapy, however its derivative, 17-demethoxygeldanamycin (17-AAG), is in ongoing Phase II trials for the treatment of locally advanced or metastatic breast cancers alone or in combination with Herceptin (Miller et al., 1994; Schulte and Neckers, 1998; Supko et al., 1995). Pre-clinical studies demonstrated that 17-AAG could sensitize HER-2 expressing breast cancer xenografts to Taxol therapy, possibly as a consequence of inhibition of signaling to the Akt pathway (Solit et al., 2003).

8.5 Subcellular Localization of the Receptor

Similar to other type I transmembrane receptor tyrosine kinases, HER-2 is expressed on the cell surface and may cluster into lipid rafts upon activation. More recently, endosomal trafficking of full length HER-2 to the nucleus has been reported, mediated by a canonical nuclear localization sequence adjacent to the transmembrane domain (amino acids 645-657). Once in the nucleus, HER-2 stimulates COX-2 transcription (Hsu and Hung, 2007) (Giri et al., 2005; Wang et al., 2004).

8.6 Known Truncations of the Receptor

8.6.1 Splice Isoforms

Kwong and coworkers identified a splice isoform of HER-2 they call ΔHER-2 which contains a sixteen amino acid deletion in the extracellular domain of the receptor. This isoform displayed higher basal levels of (auto)phosphorylation than wild type HER-2, and was more transforming than wild type HER-2 in focus formation assays in NIH 3T3 cells(Kwong and Hung, 1998). These data indicate that truncations of the receptor have important biological consequences for downstream signaling.

8.6.2 Posttranslational Modifications

8.6.2a Cleavage by Matrix Metalloproteinases (MMPs)

Studies with cell lines and tumors overexpressing HER-2 indicate that the extracellular domain (ECD) of HER-2 is released from the cell surface, generating a soluble ~100 kDa fragment and ~95 kda (p95) fragment corresponding to the membrane bound cytoplasmic portion of the receptor (Pupa et al., 1993; Zabrecky et al., 1991). This process has been attributed as yet unidentified matrix metalloproteinases, as addition of any of several MMP inhibitors prevented this shedding, and shedding of the ECD was observed subsequent to activation of MMPs (Codony-Servat et al., 1999; Molina et al., 2001).

Expression of the p95 fragment in NIH 3T3 cells was more transforming in focus formation assays and more tumorigenic in xenograft models than the wild type receptor (Di Fiore et al., 1987). Similar experiments carried out in MCF-7 cells demonstrated constitutive tyrosine kinase

activity, sustained ERK levels and a loss of classic epithelial morphology, providing a molecular basis for the enhanced transforming ability of this product (Egeblad et al., 2001).

8.6.3 Engineered Truncations of the Receptor

To study the role of the carboxy terminal residues in HER-2 mediated transformation, Mikami and coworkers introduced a Y1253F HER-2 mutant and carboxy terminal deletion mutant (corresponding to amino acids 1-1132) into NIH 3T3 cells and examined the transformative capacity of the mutants in focus formation assays and xenograft models. Significantly, both mutants displayed diminished transforming activity *in vitro* and *in vivo* when compared to wild type HER-2, underscoring the importance of the carboxyl terminal residues of the receptor for both the establishment and progression of HER-2 driven tumors (Mikami et al., 1992).

A truncated HER-2 lacking the majority of the cytoplasmic domain (containing amino acids 1-691) functions as a dominant negative when co-expressed with the wild type receptor (Qian et al., 1996). Similarly, the naturally occurring HER-2 inhibitor Herstatin, which contains the first 340 amino acids of HER-2, binds tightly to HER-2 and EGFR and inhibits cell growth (Doherty et al., 1999).

8.7 Means of degradation of the receptor

8.7.1 CHIP

The E3 ubiquitin ligase Carboxy terminus of HSC 70 interacting protein (CHIP) disrupts the HSC 70 or HSP 90 chaperone function, resulting in proteasomal destruction of HSP 70/90 client

proteins. Thus HSP 90 inhibition leads to ubiquitination and degradation of its client protein HER-2 (Xu et al., 2002; Zhou et al., 2003). This process is strongly activated by treatment with ansamycin antibiotics as discussed above.

8.7.2 Cbl

Ligand mediated degradation of growth factor receptors typically involves the E3 ubiquitin ligase, Cbl, which recognizes tyrosine phosphorylated substrates. In contrast to EGFR which is a well established Cbl target, HER-2 only weakly associates with this ligase and as such is often referred to as an internalization resistant receptor. Moreover, in complex with HER-2, EGFR is internalization resistant and actively signals to downstream targets. Of note, treatment of HER-2 overexpressing breast cancer cells with Herceptin strengthened the interaction with cbl (at residue tyrosine 1112 of HER-2) potentially explaining the mechanism of Herceptin mediated disappearance of HER-2 from the cell surface (Klapper et al., 2000; Levkowitz et al., 2000).

9. SUMMARY AND RESEARCH FOCUS

Cancer cells subvert the apoptotic cascade at many levels, undermining conventional strategies for the treatment of cancer. A more complete understanding of the components of the apoptotic machinery and its regulation will aid in the design of more effective therapies.

Caspases are the central executers of cell death in mammalian cells. Caspase deficent animals as well as pharmacologic inhibition of these enzymes in animal models exhibit profound apoptotic defects, underscoring the funcational importance of these enzymes in the execution of cell death.

Caspase cleavage of substrates proteins results in diverse outcomes: in some cases the subcellular localization of the target is altered or the predominant function of the protein is inhibited. Alternatively, cleavage may unmask new functions for the protein. The receptor tyrosince kinase HER-2/*neu* plays a central role in the establishment and progression of many solid tumors including breast, ovarian, bladder, and lung. Heterodimeric complexes with other EGFR family members link HER-2 to multiple cell survival pathways which confer resistance to apoptosis. The importance of the HER-2 kinase domain in signaling and in the stability of the receptor is also well established. However, the fate of HER-2 in cells undergoing apoptosis is unclear.

In this disseratation, I identify HER-2/*neu* as a substrate for multiple caspases *in vitro* and *in vivo* and detail the consequences of this cleavage for the execution of cell death in breast cancer cells. Specifically, my work (i) maps the the cleavage site(s) within the receptor *in vitro* and *in vivo* and (ii) identifies the individual caspases responsible for this cleavage. Moreover, the consequences of cleavage for cell death are investigated via comparative studies employing the wild type (WT) receptor, a caspase truncated receptor (Trun), or a caspase cleavage resistant receptor, employing morphologic identification of apoptosis or by disturbances in the normal distribution of membrane phosphatdyl-serine as endpoints for the study. Finally, (iii) the mechanisms underlying the observed cell death phenotype are described. Using a combination of site directed mutatgenesis, cytotoxicity, and ELISA based assays the pro-apoptotic activity of the HER-2 cleavage product was mapped to two amino acids in the receptor which together constitute a functional BH3-like domain.

CHAPTER 2

METHODOLOGY

1. Small Pool Expression Cloning and Caspase Cleavage Assays

Small pool expression cloning to identify cDNAs encoding caspase substrates was performed as described previously (Byun et al., 2001; Chen et al., 2004; Chen et al., 2002; Lustig et al., 1997) with the following modifications. Small pools (48 cDNAs/pool) of a human prostate adenocarcinoma cDNA library (Invitrogen, Fredrick, MD catalogue# 11597010) were used to generate ^{35}S-labeled protein pools with the TNT SP6-coupled transcription/translation system (Promega, Madison WI catalogue # L2080, Redivue ^{35}S-methonine, cell labeling grade was from Amersham Biosciences, Piscataway, NJ catalogue # AG1094). ^{35}S-Labeled protein pools were incubated with freshly made 1X Caspase Cleavage Buffer (50 µl 10X Caspase Cleavage Buffer, 448.75 µl MilliQ water, and 1.25 µl 1M DTT. 10X Cleavage Buffer may be stored at 4ºC for up to six months: 200 mM Hepes, 1M NaCl adjusted to pH 7.4 in MilliQ water) alone or in combination with 2.5 or 25.0 ng of recombinant caspases-1 -2, -3, -5, -6, -7, -8 or -9 as indicated (Biomol, Plymouth Meeting PA catalogue # SE-168, SE-175, SE-169, SE-171, SE-170, SE-177, SE-172, SE-173 respectively or from RV Talanian to VLC) for 1 hour at 37 °C. Cleavage reactions were terminated by boiling the reaction in the presence of 10 µl 2X Laemmli sample buffer (100 mM Tris pH 6.8; 2% SDS; 20% glycerol; 4% β-mercaptoethanol freshly added) at 100º C for 3 minutes. Cleavage reaction products were run on SDS-PAGE gels and fixed in 5% glacial acetic acid in MilliQ water for 10 minutes. The ^{35}S signal was routinely enhanced by incubation with the enhancing solution Enlightening (PerkinElmer, formerly New

England Nuclear, Waltham MA catalogue # NEF974) for 15 minutes at RT with gentle shaking prior to gel drying and exposure to film. Single cDNAs encoding putative caspase substrates were isolated by systematically subdividing small cDNA pools and retesting the corresponding ^{35}S-labeled protein pools until a single cDNA representing the caspase substrate was identified. Final substrate identification was achieved by sequencing the individual cDNA with an SP6 primer and comparing it to sequences in GENEBANK using nucleotide-nucleotide BLAST (BLAST). The analysis revealed that clone 158-5E encodes amino acids 762-1254 of human HER-2 (GENEBANK accession # NM-004448).

2. Cell lines and Media

MDA-MB-231 and MDA-MB-453 cells were obtained from ATCC (Manassas, VA) and maintained in Dubecco's modified Eagle medium (DMEM) (Invitrogen catalogue # 11965-092) supplemented with 10% heat inactivated FBS (Invitrogen catalogue # 16140-071) and 1% penicillin/streptomycin/L-glutamine (Invitrogen catalogue # 15140-122). Jurkat, SKBR3, and T47D cells (ATCC) were maintained in RPMI 1640 (Invitrogen catalogue # 11875-093) supplemented with 2 mM L-glutamine (Invitrogen 25030-081), 10% FBS, and 1% penicillin/streptomycin. SV40 large T antigen immortalized Bax/Bak WT and Bax/Bak DKO MEFs, kindly provided by Dr. Craig Thompson (Lindsten et al., 2000; Wei et al., 2001), were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin.

Recombinant human EGF (catalogue #E 9644), etoposide (catalogue # E 1383), cycloheximide (catalogue # C 4859) bisBenzimide/Hoescht #33258 (catalogue # B1155) and DAPI (catalogue #

32670) were obtained from Sigma Chemical Company (St. Louis, MO). Recombinant soluble TRAIL (amino acids 95-281) was produced in *E. Coli* from a pET15b plasmid containing truncated TRAIL cDNA and the His tagged protein purified under native conditions as described previously (Kamradt et al., 2001). Epoxomicin (Calbiochem La Jolla, CA catalogue # 324800) was obtained obtained from and was used at a final concentration of 100nM.

3. Transfection conditions

Transfections were performed with Lipofectamine/ PLUS reagent system (Invitrogen, Frederick MD catalogue # 18324-012 and # 11514-015 respectively) according to the manufacturer's instructions. To minimize toxicity to target cells, transfected DNA amounts did not exceed 1.2 μg per well of a 6 well plate or 2.2 μg per 60 mm dish.

Calcium phosphate transfections were conducted as described below. Note: this transfection method was exclusively used with phoenix cells. 2.0-2.5 x 10^6 phoenix cells were plated into a T25 and allowed to attach overnight. One hour prior to transfection, 4 mls of fresh DMEM containing 10% FBS supplemented with 25 nM chloroquine were added. For the transfection: 6-10 μg of retroviral plasmid DNA was incubated with 450 μl MilliQ water and 50 μl 2.5 M $CaCl_2$ and mixed gently. Next, 500 μl of 2X Hepes Buffered Saline (2X-HBS 50 mM Hepes, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM $NaPO_4$ pH 7.10 or pH 7.05) was added dropwise and mixed. This mixture was added in its entirety to phoenix cells. Media was removed 18 hours post transfection, cells were washed once with PBS and allowed to recover in 4 mLs full media at 37º C. Twenty four hours post transfection, the flasks were transferred to 32º C

incubator for an additional 24 hours and the resulting supernatant was collected, filtered through a 0.45μm PVDF syringe filter, and used to infect target cells (1 ml virus/T25 flask) five hours in the presence of 4mg/mL polybrene. Duplicate transfections were set up, and used to create duplicate pools for each construct.

3.1 Modification of Transfection Protocol for Caspase-Dependence Assays

For experiments elucidating the caspase dependent nature of killing by the HER-2 fragments or tBid, cells were incubated with 100 μM zVAD-fmk 1 hour prior to transfection, transiently transfected for four or five hours according to the manufacturer's instructions, rinsed once with PBS prior to addition of 100 μM zVAD-fmk to the recovery media.

3.2 Modification of Transfection Protocol for Annexin-V Analysis

It is well established that the cell membrane is disrupted during lipid mediated transient transfection assays. Thus for transfection experiments in which samples will be analyzed by Annexin-V externalization, cells should be allowed 36-48 hours between transfection and Annexin-V analysis to prevent false positive readings. This modification is based on the observation that analysis of MDA-MB-231 cells 24 hours after transfection resulted in all cells (treated or untreated) being read as Annexin-V positive, despite appearing normal by eye.

4. Western Blotting Conditions

SDS-PAGE gels were transferred to Immobilon-P PVDF membrane (Milipore, catalogue # IPVH 00010) by semi-dry method for 45 minutes (or 75 minutes for blots with full length HER-

2) at 15V with a Biorad transfer apparatus. Non-specific binding was blocked by incubation with 5% (w/v) non-fat dry milk in TBST (0.1% Tween-20) for a minimum of 1 hour. Primary antibodies were diluted as indicated in the antibody section, and incubated for 3 hours at room temperature (or overnight at 4C if recommended by manufacturer). Secondary antibodies were diluted 1:2000 in 5% milk/TBST and incubated for 1 hour. Western blots were analyzed with the Perkin Elmer ECL reagent (catalogue # NEL101).

4.1 Protocol for Stripping Western Blots

Probed membranes were wrapped in saran wrap and stored at –20C until re-probed. The membrane was placed in a dedicated stripping blot box in the presence of 20 mls of western blot strip solution (50 mls 20 % SDS, 62.5 mls 0.5 ml Tris-Cl pH 6.8, BME added immediately before stripping 694 μl/100 mls solution) and placed in a 65C shaking water bath for 30 minutes. Following three 10 minute washes with TBST (discarded into a chemical waste recepticle in a fume hood due to the presence of BME in the solution),the membrane was re-blocked with 5% milk/TBST solution and probed with the desired antibodies.

In general, probe with the weakest antibody first. For example, I would probe for HER-2 cleavage, then strip the blot and probe for tubulin.

5. Generation of Stable Pools by Retroviral Transduction

5.1 Generation of MDA-MB-231 pools stably overexpressing HER-2 varients.

pLXSN-vector, WT, Caspase Truncated, and Cleavage Resistant (4X) HER-2 described above were transiently transfected into phoenix cells (generously provided by Dr. Nolan /ATCC) by calcium phosphate method as described under transfection conditions. 24 hours post transfection, fresh media was added and the flask was transferred to a 32ºC incubator, during which time active viral particles accumulated into the supernatant for 24 hours prior to collection and infection of MDA-MB-231 target cells. Following infection of MDA-MB-231 cells with these viruses cells were selected with G418 (800μg/ml), and the resulting pools were screened for expression of specific HER-2 varients by western blotting.

5.2 Generation of MDA-MB-231 pools stably overexpressing Bcl-x_L

Phoenix cells were transfected with 10 μg pBABE vector or pBABE- Bcl-x_L by calcium phosphate method as described under transfection conditions. Stably expressing pools were established after growth in 1μg/ml puromycin for 7 days. Expression was confirmed by western blotting with the appropriate antibodies.

6. HER-2 Plasmid Construction and Site Directed Mutagenesis

The Xho I fragment of wild type HER-2 cDNA, a gift from Dr. Gibbes Johnson (Wong et al., 1999), was ligated into pcDNA3 and employed as template for all mutagenesis work. Cleavage resistant HER-2 was created by site directed mutatagenesis of four caspase cleavage sites in the carboxy terminus (D1016E, D1019E, D1087E, D1125E) using the Stratagene (La Jolla, CA). Quik-change site directed mutagenesis kit (catalogue # 200519) according to manufacturer's instructions. Primer sequences are listed below. Construction of the truncated (amino acids 1-

1016) and 4X (full length cleavage resistant) HER-2 required destruction of two internal EcoRI sites by silent mutation (nucleotides 1450 and 3072), with no change in the coding sequence. Primer sequences are listed below. The 25 kDa HER-2 (amino acids 1017 to 1125, WT D1019) and 47 kDa HER-2 (amino acids 1017-1255, WT 1019) products were amplified from wild type HER-2 and cloned into FLAG-pcDNA3. Site directed mutagenesis was used to make L1120E, D1125E mutations (alone or in combination) in the HER-2 BH3 domain. Primer sequences are listed below.

To generate pLXSN-HER-2 cDNAs, the HER-2 inserts from pcDNA3 (WT or 4X) or pCDNA3 FLAG (truncated) plasmids were released by restriction digest, and ligated into pLXSN digested with the appropriate restriction enzymes as follows. Specifically, the XhoI/EcoRI fragment of Truncated HER-2 was ligated into the retroviral vector pLXSN. Cloning of the WT, or 4X HER-2 constructs into pLXSN utilized the Xho I exclusively.

Amino terminal FLAG tagged 47, 25 and 22 kDa HER-2 fragments were constructed by ligation into N FLAG tagged pcDNA3. Primer sequences are listed below.

A bicistronic GFP-fragment virus was created by cloning the EcoRI (with an artificial ATG adjacent to amino acid 1017)/Xho I fragment of 25 or 47 kDa HER-2 into the retroviral vector pBMN-IRES-GFP (a gift from Hiro Kiyokawa, Northwestern University). Primer sequences are listed below.

All cDNAs were verified by sequencing.

6.1 Primer Sequences

The candidate caspase cleavage sites in HER-2 were mutated using the following primers:

D1016E

5’-TGACATGGGGGAACTGGTGGATGCTGAGGAGTATC-3’

5’-GATACTCCTCAGCATCCACCAGTTCCCCCATGTCA-3’

D1019E

5’-GACCTGGTGGAGGCTGAGGAGTATCTGGTA CCC-3’

5’-ATACTCCTCAGCCTCCACCAGGTGCCCCATGTC-3’

D1087E

5’-GATGTATTTGAGGGTGACCTGGGAATGGG-3’

5’-CCCCCATTCCCAGGTCACCCTCAAATACATC-3’

D1125E

5’-CCCTCTGAGACTGAGGGCTACGTTGCCCCCCTGACC-3’

5’-GGGCAACGTAGCCCTCAGTCTCAGAGGG-3’

D1016E/ D1019E (for 4X HER-2)

5’-TGACATGGGGGAACTGGTGGAGGGCTGAGGAGTATCTGGTAC-3’

The primer sequences used to PCR amplify each of the HER-2 cDNA constructs are listed below:

Truncated HER-2 (amino acids 1-1016)

5’-GGCCGAATTCATGGAGCTGGCGGCCTT -3’

5’-GGTTCACTCGAGTCAGTCCCCCATGTCATCGTCGTCCTCC-3’

25 kDa HER-2 (amino acids 1017-1125)

5’-GGCCGAATTCCTGGTGGATGCTGAGGAGTATC-3’

5’-GGCCCTCGAGTCAATCAGTCTCAGAGGGCAGGGGTACT-3’

22 kDa HER-2 (amino acids 1126-1254)

5’-GGCCGAATTCGGCTACGTTGCCCCCCTGACCTGCAG-3’

5’-GAGAGCCCTCGAGTCACACTGGCACGTCCAGACCCAG-3’

47 kDa HER-2 (amino acids 1017-1254)

5’-GGCCGAATTCCTGGTGGATGCTGAGGAGTATC-3’

5’-GAGAGCCCTCGAGTCACACTGGCACGTCCAGACCCAG-3’

Two internal EcoRI sites in the wild-type full length HER-2 cDNA were mutated without altering the coding sequence using the following primers:

EcoRI Site Ablation by Mutagenesis of Nucleotide 1450

5’-TCCGGGGACGAATACTGCACAATGGCGCC-3’

5’-GGCGCCATTGTGCAGTATTCGTCCCCGGA-3’

EcoRI Site Ablation by Mutagenesis of Nucleotide 3072

5’-GGGAGTTGGTGTCTGAGTTCTCCCGCATG-3’

5’-CATGCGGGCGAACTCAGACACCAACTCCC-3’

The conserved L and D residues in the HER-2 BH3 domain were mutated in the 25 and 47 kDa HER-2 constructs using the following primers:

L1120E

5’-CCACAGTACCCGAGCCCTCTGAG-3’

5’-CTCAGAGGGCTCGGGTACTGTG-3’

D1125E (25 kDa HER-2 construct)

5’-CCCTCTGAGACTGAGTGACTCGAGCCGG-3’

5’-CCGGCTCGAGTCACTCAGTCTCAGAGGG-3’

D1125E (47 kDa HER-2 construct)

5’-CCCTCTGAGACTGAGGGCTACGTTGCCCCCCTGACC-3’

5’-GGGCAACGTAGCCCTCAGTCTCAGAGGG-3’

7. Miscellaneous Plasmids

Construction of pBABE-puro-Mcl-1 (murine): pEF-FLAG-Mcl-1 (murine) , pMIG (MSCV IRES GFP bicistronic retroviral plasmids)-HA Noxa (murine) and pMIG-HA Bad (murine) were generously provided by Dr. David Huang, WEHI, Australia. pcDNA3/FADD DN, pBABE-puro CrmA, pBABE-puro Bcl-2, pBABE-puro-Bcl-x_L , pBABE-puro p35 have been described previously (Byun et al., 2001).

8. Antibodies

Antibodies directed against HER-2 (C terminal epitope) (catalogue # 554299) and amino terminal epitope (catalogue # 19420) antibodies were purchased from BD Biosciences (San

Diego, CA). Alpha tubulin (catalogue # T5168), monoclonal M2 FLAG(catalogue # F3165) and FITC conjugated M2 FLAG (catalogue # F 4049) antibodies were purchased from Sigma Chemical Company (St. Loius, MO). Total and phospho (Ser473 and Thr 308) specific Akt antibodies (catalogue #s 9272, 9271, and 9275) , as well as polyclonal Bcl-x_L for immunoprecipitation (catalogue # 7262) and polyclonal phospho-tyrosine 1248 HER-2 (catalogue #2244) antibodies were purchased from Cell Signaling (Danvers, MA). HER-2 Ab2 (clone 9G6.10), possessing an epitope in the extracellular domain of HER-2, was used for FACS analysis (Neomarkers, catalogue # MS-229-P). Cox-IV antibody (catalogue # A-21348) was purchased from Molecular Probes (Eugene, OR). The native conformation cytochrome C antibody used in immunofluoresence studies was from BD/Pharmagen (catalogue # 65971A). Mouse Bcl-x (recognizing both short and long isoforms) was purchased from Biosource (catalogue # AHO0222).

9. Cell Surface Labeling by FACS

1 x 10^6 MDA-MB-231 cells were resuspended in 1ml of PBS and incubated with 1μg of Neomarkers HER-2 (Clone 9G6.20)Ab-2 (LabVision, formerly Neomarkers, Fremont CA catalogue # MS- 229-Pabx) for 45min at room temperature with gentle shaking. Cells were washed twice prior to incubation with 1:50 FITC tagged anti-mouse antibody (MP Biomedicals, formerly ICN, Irvine CA catalogue # 55514) for 30 minutes at RT. Cells were washed twice with PBS, fixed in PBS containing 0.5% paraformaldehyde, and analyzed by a Beckman Epics XL

flow cytometer (Robert H. Lurie Comprehensive Cancer Center, NWU). Samples were read immediately but may be stored at 4ºC overnight prior to analysis.

10. EGF Treatment of MDA-MB-231 Stable Pools

MDA-MB-231 cells were plated at a density of 0.5 $x10^6$ per well in a 6 well plate and allowed to attach overnight. Sixteen hours later, the cells were washed PBS twice and serum starved in DMEM containing 0.1 % FBS for 18 hours prior to treatment with 100 ng/mL EGF for 10 minutes. Media, cells, and PBS washes were combined and RIPA lysates were made in the presence of phosphatase and protease inhibitor cocktails. 20 μg of each lysate was analyzed by immunoblot with the indicated antibodies.

11. Apoptosis assessment

MDA-MB-231 HER-2 stable pools were plated at a density of $0.5x10^6$ per well in a 6 well plate. Cells were treated with PBS or 0-5.0μg of TRAIL (prepared as described previously) for 24 hours. Media, PBS washes, and trypsinized cells were combined and analyzed for apoptosis by the FACS analysis of Annexin-V externalization or nuclear morphology (see below).

11.1 Apoptosis assessment by Annexin-V analysis

Annexin-V PE staining was carried out according to manufacturer's instructions (BD Pharmagen San Diego, CA catalogue # 559763). Briefly, media, PBS washes, and trypsinized cells were collected and pelleted by centrifugation at 2300 x g for 5 minutes at RT, resuspended in freshly made 1X binding buffer (BB) (1:10 dilution of stock supplied with kit), and adjusted to a final

concentration of 1 x10^6 cells/ mL in 1X binding buffer. 100,000 cells (100 µl) were aliquoted per condition into 5 mL polystyrene round bottom tubes (Becton Dickenson, Franklin Lakes NJ catalogue #35-2008), incubated with 5µl Annexin-V-conjugated to PE and 5 µl of the vital dye 7-AAD, gently mixed, and incubated in the dark for 15 minutes at RT. Finally an additional 400 µl 1X Binding Buffer was added to each tube (V_f = 500µl) and samples were analyzed on a Beckman Epics XL flow cytometer within 1 hour (Robert H. Lurie Comprehensive Cancer Center, NWU). When conducting these experiments with a new cell line, the following controls should be added: (i) untreated cells in Binding Buffer, (ii) untreated cells incubated with 7-AAD, and (iii) untreated cells incubated with Annexin-V-PE.

For co-transfection experiments with eGFP-N1, analysis was delayed by 36-48 hours post transfection to allow the plasma membrane to recover from transfection, and a Cy-5 conjugated annexin (Becton Dickenson, Franklin Lakes NJ catalogue # 559933) was utilized in combination with 1:1000 dilution of DAPI to minimize bleed through of annexin signal with GFP.

11. 2 Apoptosis Assessment by Nuclear Morphology

Apoptotic cells were characterized by condensed chromatin and/or presence of significantly fragmented nuclei. A minimum of 200 cells per condition were scored to ensure statistical significance.

Media, PBS washes, and trypsinized cells were collected in 2ml tubes and fixed by incubation with 50 µl/ mL of grade II 25% gluteraldehyde (Sigma Chemicals, St. Louis MO catalogue

G6257) for 15 minutes at RT in a chemical fume hood. The fixed cells were pelleted by centrifugation at 2300 *x g* for 5 minutes at RT, and washed twice with 1 mL PBS prior to addition of 10 μg/ mL Bisbensimide (a 1:1000 dilution of the 10mg/ml stock) to stain the nuclei for 15 minutes at RT. The cells were washed twice with PBS and stored at 4ºC in the dark until ready to score. Samples may be stored for up to several weeks in this manner.

11.2.1 Modification of Apoptosis Assessment Fixation Protocol for Samples Containing GFP

Media, PBS washes, and trypsinized cells were collected and pelleted by centrifugation at 2300 *x g* for 5 minutes at RT. The cell pellet was resuspended 500 μl PBS and an equal volume of 8% paraformaldehyde added, resulting in a 4% paraformaldehyde/ PBS solution. Cells were fixed in this solution for 10 minutes at RT, and washed twice with PBS prior to incubation 10 μg/ml Bisbenzimide (a 1:1000 dilution of the 10mg/ml stock) for 15 minutes, and subsequent washings as described above. Samples should be scored immediately to minimize loss of GFP staining. Glutaraldehyde fixation is too harsh, and results in complete loss of GFP flouresence.

12. Apoptosis Induction

In cases where cells were treated with the pan caspase inhibitor zVAD-fmk in combination with a proteasome inhibitor, cells were first incubated with zVAD for 1 hour, then the proteasome inhibitor for 1 hour prior to addition of death stimulus. zVAD-fmk (MP Biomedical, Irvine CA catalogue # FK009) was routinely utilized at 50-100 μM and required 1 hour pretreatment.

12.1 TRAIL

T47D and SKBR3 cells were routinely treated with 2.0 μg/ml TRAIL in the presence of 1μg/ml cycloheximide. Following transient transfection experiments, MDA-MB-231 cells were routinely treated with 0.5 μg/ml TRAIL. In dose response studies with MDA-MB-231/ HER-2 stable pools doses up to 5 μg/ml of TRAIL were used.

12.2 Etoposide

SKBR3 and MDA-MB-453 cells were treated with 200 μM etoposide as indicated.

13. Analysis of Mitochondrial Localization by Immunofluorescence

0.25x 10^6 MDA-MB-231 cells plated on coverslips were transfected with 1.0 μg each FLAG vector, FLAG-17 or FLAG-40 kDa HER-2 constructs and allowed to recover ON. Note: significant numbers of detached (presumably dead) cells were observed in wells transfected with the 47 and 25 kDa fragments. Twenty four hours later, 100 nM Mitotracker Deep Red 633 (Molecular Probes, Eugene OR catalogue #22426) was added to full media and incubated for 30 minutes at 37ºC. Cells were washed with PBS three times, and allowed to destain in fresh media containing 10% FBS for an additional 30 minutes at 37ºC. Cells were fixed in 4% paraformaldehyde/ PBS for 10 minutes at RT, permeabilized with 0.01% Triton for 5 minutes at RT and blocked for 1 hour in blocking buffer (TBS supplemented with 5% goat serum and 1% BSA). Cells were stained with pre-absorbed mouse FITC conjugated M2 FLAG antibody (10 μg/mL) for 1 hour at 37ºC, washed three times in PBS, stained with 1 μg/ml DAPI and mounted

using Prolong Anti-fade Gold (Molecular Probes, Eugene OR catalogue # P36930) . Samples were analyzed with a Zeiss LSM Confocal Microscope. Co-localization of mitochondrial DNA (visualized by DAPI), FITC, and Mitotracker was required for a cell to be scored as positive for mitochondrial co-localization. Quantitation is the result of 3 independent experiments in which a minimum of 200 GFP positive cells were scored. Confocal settings were adjusted to vector transfected cells or untransfected control cells prior to analysis.

14. Analysis of Cytochrome c Release by Immunofloresence

A modified version of the protocol described by Kennedy and coworkers (Kennedy et al., 1999) was utilized. 0.25 $x10^6$ MDA-MB-231/pBABE or 231/Bcl-x_L cells were plated on coverslips, treated with 100 μM zVAD-fmk 1 hour prior to transfection, co-transfected with eGFP-N1 and either the 25, 47 kDa HER-2 products or tBid and allowed to recover ON in the presence of zVAD-fmk. Cells were collected 24 hours after transfection, fixed in freshly made 4% paraformaldehyde / PBS for 10 minutes at RT. Cells were permeabilized with 0.01% Triton-X100 for 5 minutes at RT and washed three times in PBS. Cells were incubated in blocking buffer (10% goat serum, 1% BSA in PBS) for 1 hour at RT. Cells were stained with a native conformation cytochrome c antibody (BD Biosciences, formerly Pharmingen San Jose CA catalogue #65971A) diluted 1:250 in TBS supplemented with 1% BSA for 30 minutes at 37ºC. Cells were washed three times in PBS with gentle shaking, followed by incubation with anti-mouse TRITC (Jackson Immunoresearch, West Grove PA catalogue #715-025-150) diluted 1:50 in 1% BSA/TBS for 30 minutes at 37ºC. Following three additional washes with PBS, nuclei were stained with 10 μg/ mL bis-benzimide (Hoescht #33258) for 15 minutes at RT, and washed

again with PBS to remove non-specific staining. Coverslips were mounted using Prolong Anti-fade Gold (Molecular Probes, Eugene OR catalogue # P36930) and analyzed with a Zeiss LSM Confocal Microscope. Cells which had released their cytochrome c were characterized by absent or diffuse staining (i.e. absence of punctuate, perinuclear staining characteristic of intact cytochrome c). Note: it is not unusual to see only a small percentage of cells with released cytochrome c. Quantitation is the result of 5 independent experiments in which a minimum of 200 GFP positive cells were scored. Confocal settings were adjusted to vector control levels prior to analysis.

15. Mitochondrial Isolation From Jurkat Acute T Cell Leukemia Cells

A modified version of the protocol described by Gross and coworkers (Gross et al., 1999) was utilized. 20 x10^6 Jurkat cells were pelleted by centrifugation at 2300 *x g* and resuspended in 18 mLs of ice cold HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes pH 7.5) supplemented with PMSF and protease inhibitors, and lysed with 20 strokes of a prechilled dounce homogenizer. Nuclei and unlysed cells were pelleted by spinning at 120 *x g* for 10 minutes in a pre-chilled SS34 rotor at 4ºC. The supernatant was transferred to a new tube and spun 7500 *x g* for 15 minutes in an SS34 rotor. The pellet, containing the mitochondria, was washed twice in cold HIM and resuspended in 1-2 mL of freshly made MRM buffer (250mM sucrose, 10 mM Hepes, 1mM ATP, 5 mM sodium succinate, 0.8 mM ADP, 2 mM K_2HPO_4). Mitochondria were used within 4 hours of isolation.

16. Isolation of Cytosolic and Mitochondria Enriched/Heavy Membrane Fractions by Subcellular Fractionation

For immunoblot analysis, 0.22 x 10^6 SKBR3 cells were treated with 2.5 μg/mL TRAIL and 1 μg/mL cycloheximide for 0-4 hours. After washing in cold PBS, cells were pelleted by centrifugation at 120 x g, resuspended in freshly made Buffer A (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, 1 mM PMSF, 4 mM DTT and 1X protease inhibitor cocktail I), and allowed to sit on ice for 1 hour. Lysates were centrifuges at 16,000 x g to separate the supernatant (Sup) and heavy membrane (HM) fractions. The HM pellet was solubilized with RIPA buffer for 30 minutes on ice, prior to centrifugation at 16,000 x g for 10 minutes to remove cellular debris (pellet). The protein concentrations of these lysates were determined with the Pierce BCA kit (catalogue #23225) and 20 μg was analyzed via SDS-PAGE electrophoresis and immunoblotting with antibodies to the carboxy terminal HER-2, Cox IV, or α tubulin.

17. Immunoprecipitations

MDA-MB-231 cells overexpressing Bcl-x_L were transiently transfected with FLAG tagged vector, 25 kDa HER-2 (amino acids 1017-1125), the BH3 mutant 25 kDa 2XE (L1120E, D1125E) and allowed to recover ON. Eighteen hours later, RIPA lysates were made and 600 μg of each lysate was incubated ON with 1:50 polyclonal Bcl-x_L (Cell Signaling, Danvers MA catalogue # 2762), or normal rabbit IgG (Santa Cruz Biotech, Santa Cruz CA catalogue # sc 2027) antibody. Mixtures were then incubated with protein A agarose beads (Pierce Chemical, Rockford IL catalogue # 20333) for 1 hour at 4º C. Samples were eluted into 2X Lamelli buffer

and analyzed on 15% SDS-PAGE gels. Immunoprecipitated complexes were detected by blotting with mouse M2 FLAG (to detect 25 and 25 2XE constructs), and mouse Bcl-x for quality control of the pull down. 20 μg of each lysate was collected prior to immunoprecipitation and analyzed as 'IP input'.

18. Analysis of Cytochrome c release by ELISA

Mitochondria were harvested from 20 $x10^6$ Jurkat acute T cell leukemia cells as described above and incubated in MRM buffer (250mM sucrose, 10mM Hepes, 1mM ATP, 5mM sodium succinate, 0.8mM ADP, 2 mM K_2HPO_4) with DMSO (vehicle) or 10-100μM peptides and incubated for 3 hrs at RT with gentle shaking. Mitochondria were pelleted by centrifugation 16,000 x g for 15 mins in an Eppendorf 5415 centrifuge at RT and the resulting supernatant was transferred to a new tube, (placed at -80) and analyzed for cytochrome c content by ELISA (R&D Systems, Minneapolis, MN catalogue # DCTC0) according to the manufacturer's instructions. Data was normalized to cytochrome c released during vehicle control treatment of mitochondria in each experiment.

18.1 HER-2 BH3 Peptide design

Custom recombinant peptides encoding WT HER-2 BH3 (SEDPTVP**L**PSET**D**GYVAPLT) and its 2XE BH3 mutant (SEDPTVP**E**PSET**E**GYVAPLT) were purchased from Abgent (San Diego, CA).Bid BH3 (CIRNIARH**LAQVGDSMDR**SIPP) was purchased from Abgent as a positive control for these studies. Peptides were HPLC purified and 10mM stock solutions were made in DMSO and stored in -80° C prior to useage.

CHAPTER 3

RESULTS

1. SUBSTRATE IDENTIFICATION

1. 1 HER-2 is a substrate for multiple caspases in vitro

In order to identify novel caspase substrates, a prostate adenocarcinoma library was screened via small pool expression cloning {KDA Lustig, 1997 #12; Byun, 2001 #38; VL Cryns, 1997 #13; Chen, 2002 #40; F Chen, 2003 #23; Chen, 2004 #43}. In this system, cDNA pools (of approximately 48 individual cDNAs) were *in vitro* transcribed and translated in the presence of ^{35}S methionine. These radio-labeled pools were then incubated with caspase cleavage buffer, purified recombinant caspases, or with the combination of recombinant caspases and the broad spectrum caspase inhibitor zVAD-fmk and subsequently analyzed for cleavage via SDS-PAGE autoradiography. When cleavage was observed, the pools were subdivided and reanalyzed until a single cDNA was identified. This cDNA was sequenced and analyzed by BLAST (Figure 3.1)

Pool 158 contained a band of approximately 76 kDa which was cleaved by caspases-3, and -8, but not caspase-2 or buffer control (Figure 3.2A). Subdivision and reanalysis of the pool led to the identification of the clone 158-5E as the cDNA encoding the ~76 kDa protein, which was cleaved into multiple fragments by caspase-3 (Figure 3.2B). Sequence analysis revealed that clone 158-5E (hereafter 158-5E/WT HER-2) was a partial HER-2 cDNA encoding amino acids 762-1254 of the receptor (Fig. 3.2C). Importantly, this fragment contains the majority of the

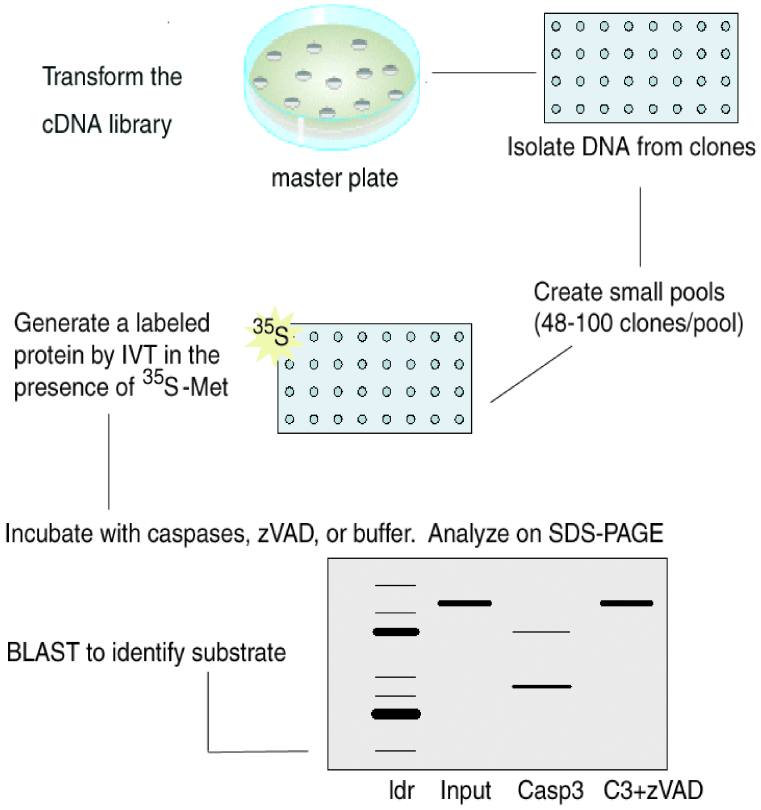


Figure 3.1 Small pool expression cloning is a powerful tool for caspase substrate

Schematic of the screen used to identify HER-2 as a caspase substrate. A cDNA library was transformed into bacteria and plasmid DNA was isolated from individual clones. Pools were created by grouping multiple cDNAs, and radiolabeled proteins were obtained by *in vitro* transcription and translation in the presence of ^{35}S methionine. These protein pools were then incubated with recombinant caspases, caspases and the broad spectrum caspase inhibitor zVAD-fmk, or buffer control and analyzed by audioradiography. If caspase cleavage of a substrate was observed, the corresponding cDNA pool was re-examined until the single cDNA of interest was identified. This cDNA was subsequently sequenced and identified via BLAST.

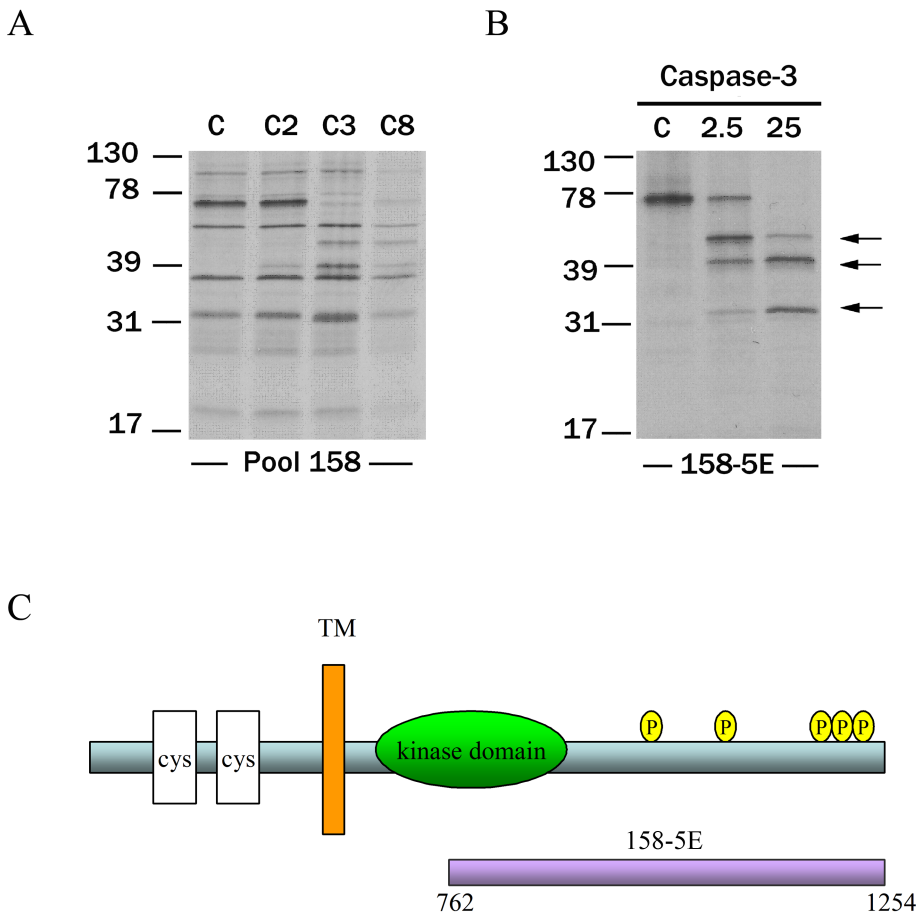


Figure 3.2 **Identification of a caspase substrate via small pool expression cloning of**

A. Small pool 158 contained a ~76 kDa polypeptide which was cleaved by caspases-3 (C3) and -8 (C8) but not caspase-2 (C2). IVT proteins were incubated with buffer (C) or 25 ng of recombinant caspases for 1 hour and cleavage products were analyzed by SDS-PAGE gel electrophoresis followed by audioradiography. B. Subdivision and reanalysis of the pool led to the identification of 158-5E as the cDNA encoding the ~76 kDa protein. 158-5E was cleaved into multiple fragments by caspase-3 when incubated with 2.5 or 25.0 ng of recombinant caspases. C. Sequence analysis showed that 158-5E is a partial HER-2 cDNA (GENEBANK accession # NM_004448), encoding amino acids 762-1254.

kinase domain as well as multiple tyrosine residues known to play a critical role in signaling (Mikami et al., 1992).

An *in vitro* caspase panel demonstrated that this partial HER-2 cDNA could be effectively cleaved by the executioner caspases-3, -6, and -7 as well as the initiator caspase-8 and to a lesser extent by caspase-2 (Figure 3.3). Despite having distinct canonical cleavage patterns *(e.g*. DxxD for caspase-3, and IETD for caspase-8), an identical fragmentation pattern was observed when any of several caspases were incubated with 158-5E/WT HER-2, indicating that multiple caspases, at various locations in the apoptotic cascade, utilize the same site(s) to cleave the cytoplasmic tail of HER-2.

1.2 Endogenous HER-2 is cleaved by caspases in response to apoptotic stimuli

To determine whether HER-2 is cleaved by caspases during apoptosis *in vivo,* SKBR3 human breast cancer cells, which overexpress HER-2 by gene amplification and overexpression, were treated with the pro-apoptotic cytokine TRAIL or with the DNA damaging agent etoposide, and were examined the cells for cleavage of HER-2 by western blot. Treatment with either agent led to disappearance of full length HER-2, and the rapid but transient production of a 47 kDa fragment, suggesting the existence of a third labile fragment. This process can be attributed to caspases, as addition of the caspase inhibitor zVAD-fmk suppressed cleavage of HER-2 (Figure 3.4A).

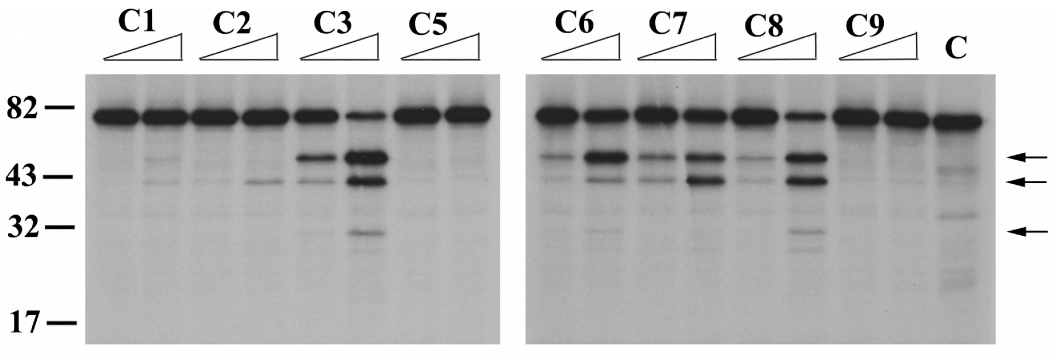


Figure 3.3 **The partial HER-2 cDNA 158-5E is cleaved by multiple caspases *in vitro*.**

To define the repetoire of caspases which cleave 158-5E, the IVT substrate was incubated with a panel of caspases as previously described. The ~76 kDa 158-5E was efficiently cleaved by the executioner caspases-3, -6 and -7 (C3, C6, C7 respectively) as well as the initiator caspases-8 (C8) and to a lesser extent by caspase-2 (C2). The production of identical fragmentation patterns indicate that the caspases utilize the same site(s) to proteolyze HER-2.

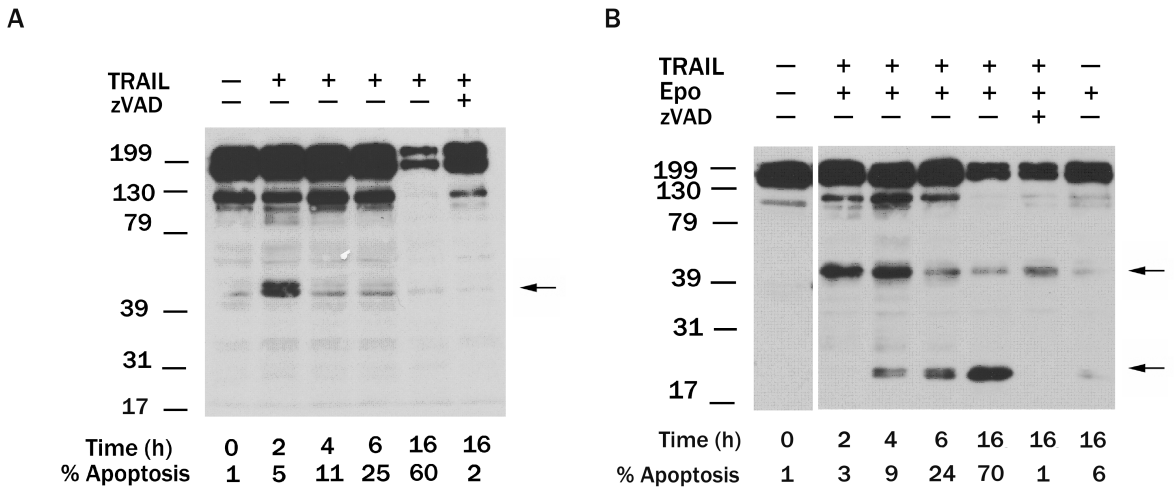


Figure 3.4 **Endogenous HER-2 is cleaved by caspases in response to TRAIL and results in two observable cleavage fragments.**

A. SKBR3 cells were pretreated with 50 μM zVAD-fmk, and treated with 2.0 μg/mL TRAIL in the presence of 1 μg/mL cycloheximide for the time indicated. Cleavage of HER-2 was assessed by western blotting with a HER-2 antibody directed against a C terminal epitope. Caspase cleavage products are indicated by arrows. Apoptosis was assessed at each time point by analysis of nuclear morphology. B. SKBR3 cells were pretreated with 50 μM zVAD-fmk and 100 nM epoxomicin, followed by 2.0 μg/mL TRAIL for the time indicated.

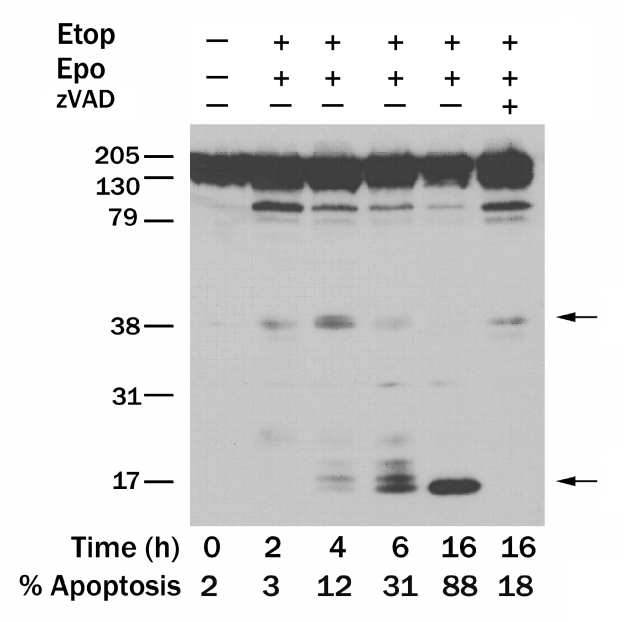


Figure 3.5 **Endogenous HER-2 is cleaved by caspases in response to etoposide treatment and results in the production of two observable fragments.**

SKBR3 cells were pretreated with 50 μM zVAD-fmk alone or in combination with 100 nM epoxomicin followed by treatment with 200 μM etoposide for the times indicated. HER-2 cleavage products are indicated by arrows. Apoptosis was assessed at each point by nuclear mophology analysis.

Addition of the proteasome inhibitor, epoxomicin (Meng et al., 1999) led to the stabilization of a previously undetected 22 kDa fragment that was accompanied by disappearance of both the 47 kDa fragment and the full length protein, indicating that the 22 kDa fragment is degraded by the proteasome. Significantly, proteolysis of HER-2 into the 47 kDa product occurred when only 5% of SKBR cells were apoptotic, indicating that HER-2 is initially cleaved during the induction of apoptosis and could contribute to its execution. (Figure 3.4B-3.5). Of note, the unstable 22 kDa HER-2 cleavage product, but not the 47 kDa fragment, was reported recently {O Tikhomirov, 2001 #19}.

To examine how universal caspase cleavage of HER-2 was in breast cancer, I repeated these experiments with T47D and MDA-MB-453 breast cancer cells. Importantly, caspase cleavage of HER-2 was not dependent on level of expression of the receptor, as a similar cleavage pattern was observed in the low expressing, but amplified cell line T47D (Figure 3.6). Sequential cleavage of the receptor was also observed in the HER-2 overexpressing breast cancer cell line MDA-MB-453, although the cleavage products were markedly less abundant than those seen in the SKBR3 system (Figure 3.7).

2. MAPPING THE HER-2 CASPASE CLEAVAGE SITES *IN VITRO* AND IN BREAST CANCER CELLS

2.1 The cytoplasmic tail of HER-2 contains multiple caspase cleavage sites (in vitro analysis)

Having demonstrated that stimuli which exert their effects via either the mitochondrial (etoposide) or death receptor pathway (TRAIL) are sufficient to induce caspase cleavage of

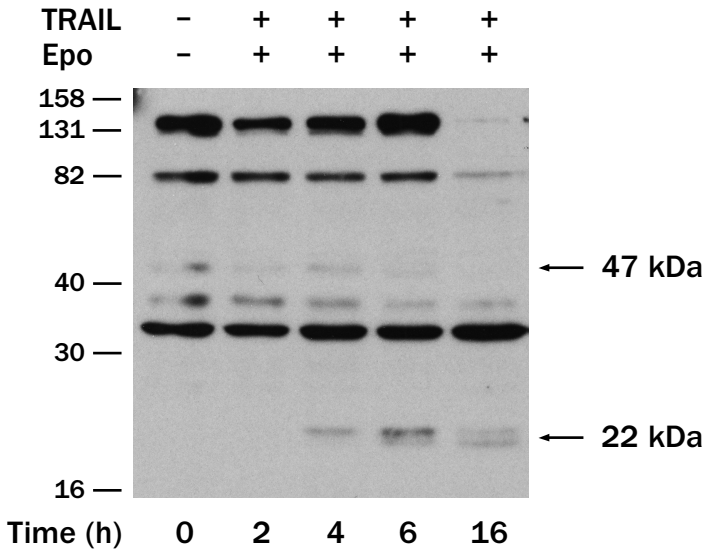


Figure 3.6 **TRAIL induced cleavage of HER-2 occurs in the moderate HER-2 expressing cell line T47D.**

T47D cells were treated with 2.0 μg/mL TRAIL and 1 μg/mL cycloheximide in the presence of 50 μM zVAD-fmk and 100 nM epoxomicin for 0-16 hours. 20 μg of protein lysate was analyzed by western blot, using a HER-2 antibody directed against a C terminal epitope.

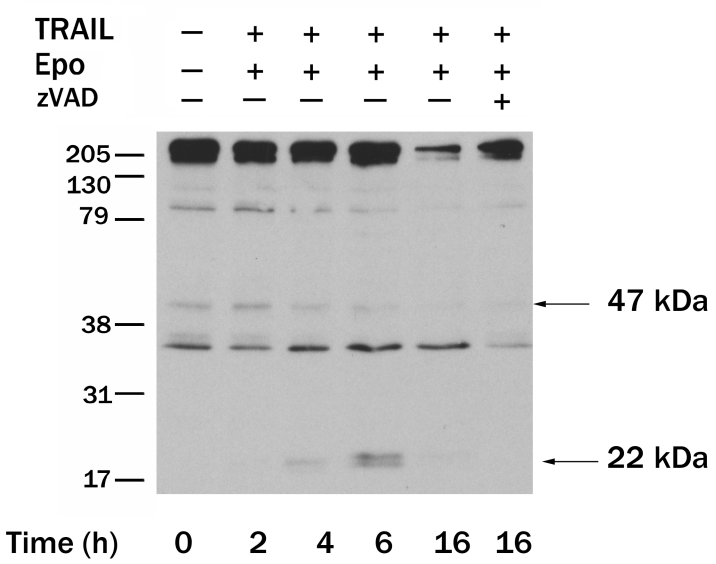


Figure 3.7 **TRAIL induced cleavage of HER-2 occurs in the HER-2 overexpressing cell line MDA-MB-453.**

MDA-MB-453 cells were treated with 2.0 μg/mL TRAIL and 1 μg/mL cycloheximide in the presence of 50 μM zVAD-fmk and 100 nM epoxomicin for 0-16 hours. 20 μg of protein was analyzed via immunoblotting with a HER-2 antibody containing a C terminal epitope.

endogenous HER-2, I began to map the caspase cleavage sites within HER-2. Examination of the amino acid sequence of 158-5E/WT HER-2 identified four putative caspase cleavage sites in the carboxy tail of the receptor which would yield fragments of similar sizes as observed in SKBR3 cells: ^{1016}DMGD, ^{1019}DLVD, DVF^{1087}D, and SET^{1125}D (Figure 3.8). These aspartic acids were changed to glutamate residues by site directed mutagenesis either alone or in combination in the partial HER-2 cDNA isolated in the small pool screen, 158-5E. The resulting plasmids were *in vitro* transcribed and translated in the presence of ^{35}S methionine and then incubated with buffer or increasing amounts of recombinant caspases and analyzed by SDS-PAGE autoradiography. Following incubation with recombinant caspases, the 158-5E/WT HER-2 migrated as an approximately ~76 kDa band which was cleaved into three major products (with apparent molecular weights of ~48, ~38, and a smaller band which migrated faster than the 32 kDa standard) following incubation with recombinant caspase-3 (Figure 3.9). As shown in the schematic (Figure 3.9B), the 48 kDa product could be generated by cleavage after aspartic acid 1087. Subsequent processing of this fragment at amino acids 1016/1019 could generate the ~38 kDa product observed. However, the cleavage events that give rise to the smallest fragment are less clear. This band likely corresponds to the predicted 25 kDa product containing amino acids 1088-1254, mimicking cleavage after aspartic acid 1087. However, the predicted 16 kDa product corresponding to the region between amino acids 1016/1019 and amino acid 1087 was not observed in this study.

Armed with the above understanding of caspase cleavage of 158-5E/WT HER-2 *in vitro*, I mutated the putative caspase cleavage sites in HER-2 alone or in combination and examined the

721 rkvkvlgsga fgtvykgiwi pdgenvkipv aikvlrents pkankeilde ayvmagvgsp

781 yvsrllgicl tstvqlvtql mpygclldhv renrgrlgsq dllnwcmqia kgmsyledvr

841 lvhrdlaarn vlvkspnhvk itdfglarll dideteyhad ggkvpikwma lesilrrrft

901 hqsdvwsygv tvwelmtfga kpydgipare ipdllekger lpqppictid vymimvkcwm

961 idsecrprfr elvsefsrma rdpqrfvviq nedlgpaspl dstfyrslle ddDMGDLVDa

1021 eeylvpqqgf fcpdpapgag gmvhhrhrss strsgggdlt lglepseeea prsplapseg

1081 agsDVFDgdl gmgaakglqs lpthdpsplq rysedptvpl pSETDgyvap ltcspqpeyv

1141 nqpdvrpqpp spregplpaa rpagatlerp ktlspgkngv vkdvfafgga venpeyltpq

1201 ggaapqphpp pafspafdnl yywdqdpper gappstfkgt ptaenpeylg ldvpv

Figure 3.8 **The cytoplasmic tail of HER-2 contains four putative caspase cleavage sites.**

The amino acid sequence of human HER-2 (Protein Accession # NP_ 004439) corresponding to 158-5E was examined for caspase cleavage sites which could be efficiently cleaved by caspases-3, -6, -7, and -8. Four sites, underscored above, were chosen for further analysis, in which the aspartic acid (D) in the P1 position was mutated to the non-cleavable glutamic acid (E) by site directed mutagenesis.

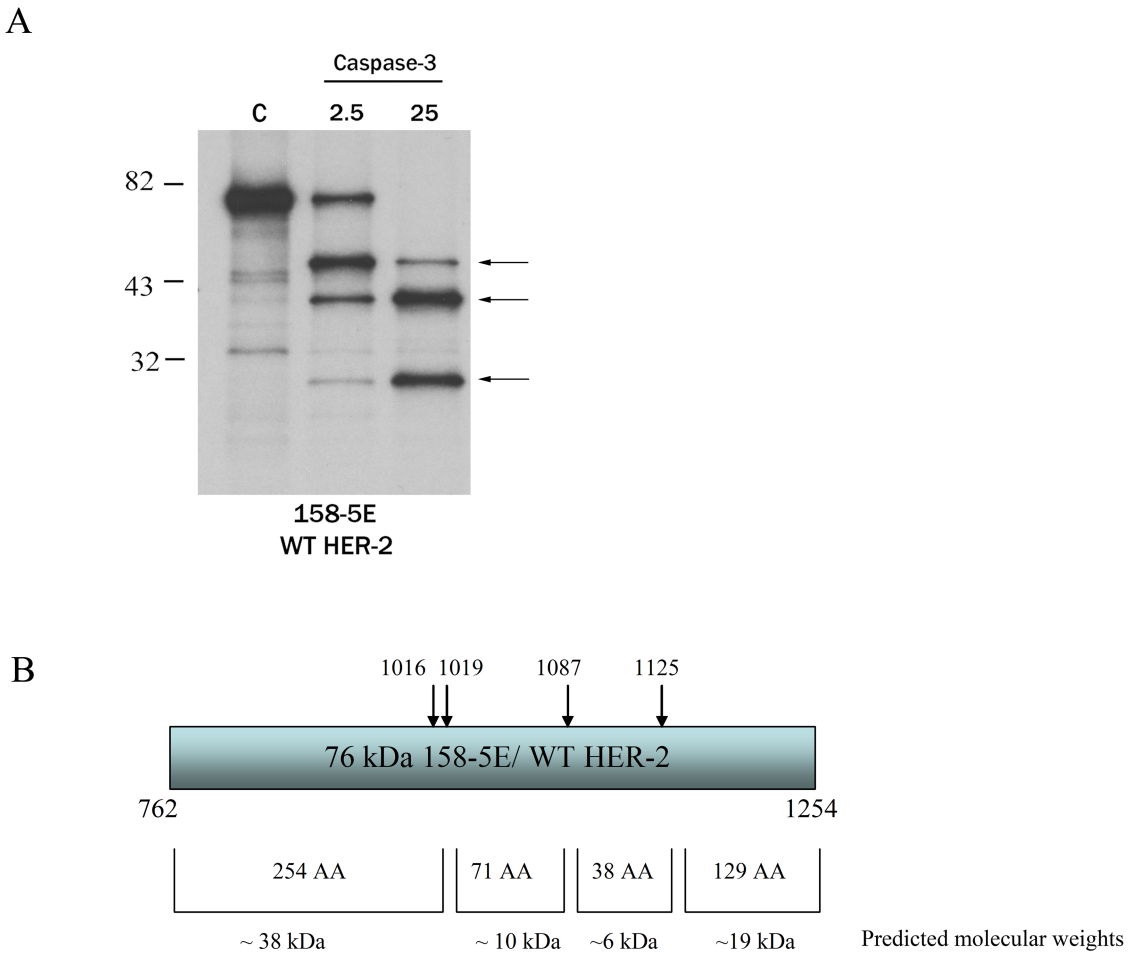


Figure 3.9 **Caspase cleavage of 158-5E/WT HER-2 generates three observable products *in vitro*.**

A. A representative autoradiograph of 158-5E/WT HER-2 after incubation with buffer (C) or 2.5 or 25 ng of recombinant caspase-3 is shown. Cleavage products are indicated by arrows. B. Diagram of the portion of the receptor identified in the SPEC screen (amino acids 762-1254) showing the locations of the putative caspase cleavage sites (marked by arrows) and predicted molecular weights of the hypothetical products.

resulting plasmids for sensitivity to caspase-3 cleavage *in vitro* as above with the goal of generating a caspase cleavage resistant HER-2 protein. Mutation of D1016 resulted in disappearance of the smallest cleavage fragment while destruction of the D1125 site prevented the appearance of the ~48 kDa product. However, the absence of the latter product in the D1125E mutant could be attributed to processing by caspases into the smaller proteolytic products (Figure 3.10A). Compound mutations of D1087E and D1125E cleavage sites prevented the production of both the ~48 and ~38 kDa products, although the third product was still generated. In contrast, mutation of the D1016E/D1019E cleavage sites prevented the appearance of the third fragment. Of note, the ~48 kDa product was not observed in this experiment, although its absence may be attributed to efficient caspase proteolysis into smaller products. (Figure 3.10B). In contrast to these studies, the quadruple mutant (D1016E/D1019E/D10187E/D1125E) designated 158-5E/4X HER-2, was completely resistant to cleavage by caspases-3 and -8 (Figure 3.10C). A schematic based on the ^{35}S mutagenesis studies is presented in Figure 3.10D. The hypothetical product generated by caspase cleavage after aspartate 1125 was not visualized in my analysis due to an absence of methionine residues in the product. To ensure that all possible fragments were visualized in our radio-labeled studies, 158-5E/WT HER-2 or the 158-5E/4X mutant HER-2 were *in vitro* transcribed and translated in the presence of ^{14}C-labeled cysteine and analyzed in the caspase assay once more. Consistent with the data obtained with ^{35}S labeled proteins, 158-5E/WT HER-2 resulted in the generation of three products of approximately ~48, ~38, and ~25. However, ^{14}C analysis detected a smaller molecular weight product migrating at approximately ~22 kDa which was not observed in prior

studies. A schematic summarizing the data obtained with ^{35}S and ^{14}C mutagenesis studies is presented in Figure 3.11.

2.2 The cytoplasmic tail of HER-2 contains multiple caspase cleavage sites (in vivo analysis)

Similar studies conducted with full length wild type or mutant HER-2 cDNAs demonstrated that caspases cleave four distinct sites within the cytoplasmic tail of HER-2 *in vivo*. Wild type or mutant HER-2 cDNAs were transiently transfected into the breast cancer cell line MDA-MB-231, which expresses low endogenous levels of HER-2, and were examined for their ability to resist cleavage by TRAIL treatment by western blot. Consistent with our prior observations, WT HER-2 was cleaved into a 22 kDa product which was stabilized by the proteosome inhibitor epoxomicin. The D1125E mutant generated a cleavage product of approximately 30 kDa, while both the double (D1125E/D1087E) and triple (D1125E/D1087E/D1016E) mutants resulted in appearance of a ~ 47 kDa fragment, presumably due to cleavage after D1016 or D1019. In contrast, the quadruple HER-2 mutant (D1125E/D1087E/D1016E/D1019E) was completely resistant to caspase proteolysis induced by TRAIL treatment (Figure 3.12A). These results are presented schematically in Figure 3.12B. Taken together, these data indicate that destruction of four distinct caspase cleavage sites is required to generate a cleavage resistant HER-2 construct *in vitro* and *in vivo.*

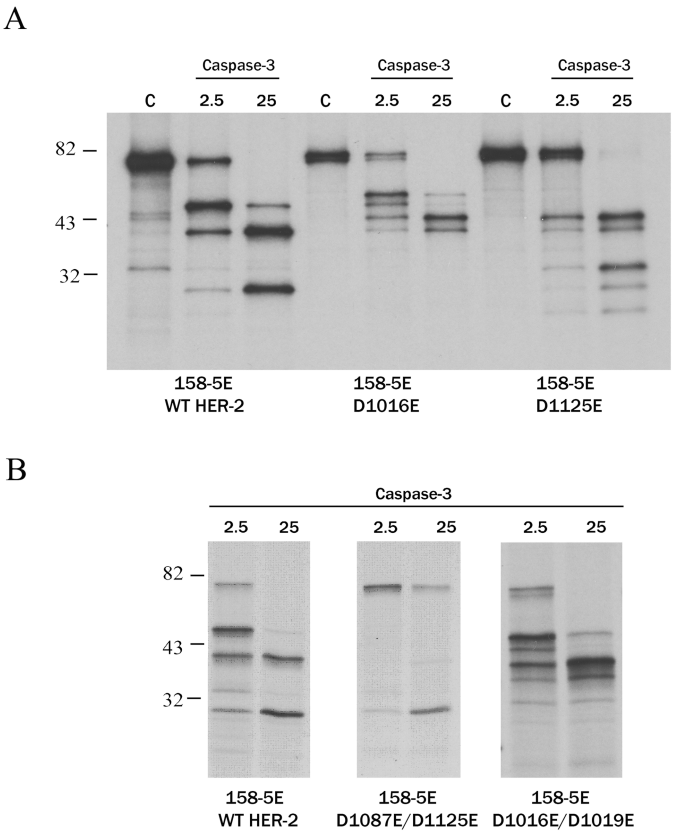
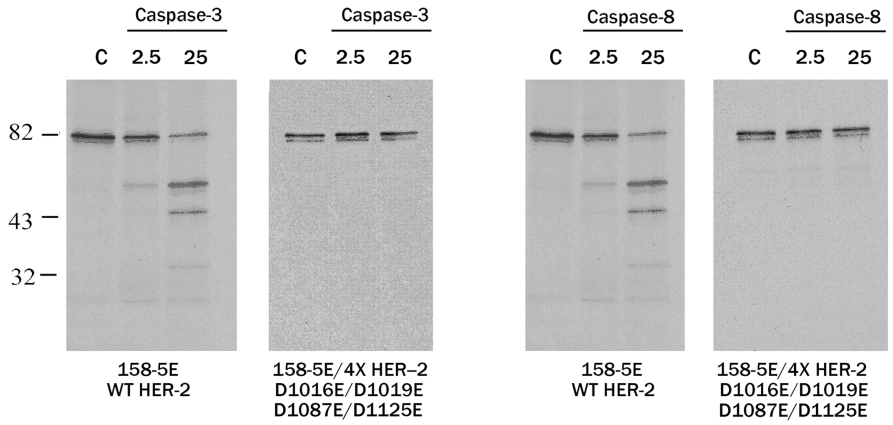


Figure 3.10 **Caspases cleave the carboxyl terminus of HER-2 at four distinct sites *in vitro* (^{35}S analysis).**

Site directed mutagenesis was used to mutate the conserved aspartic acid (D) residues to glutamate (E) in four putative caspase cleavage sites in 158-5E/WT HER-2 either alone or in combination. The resulting cDNAs were in vitro transcribed and translated in the presence of ^{35}S methionine prior to incubation with recombinant caspases as described previously. A-B. Results from 158-5E/WT HER-2 or single, double caspase cleavage site mutants are shown. Figure continues on the next page.

C



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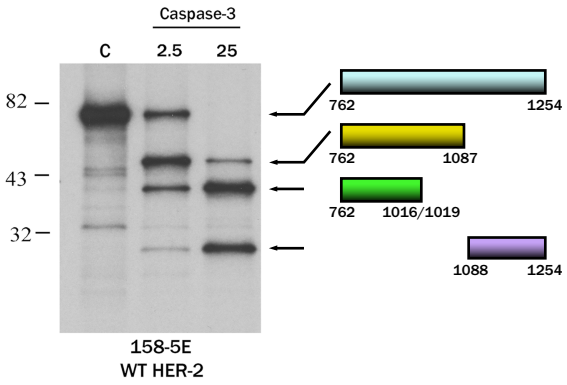


Figure 3.10 **Caspases cleave the carboxyl terminus of HER-2 at four distinct sites *vitro* (^{35}S analysis).**

C. The quadruple mutant 158-5E is resistant to cleavage by recombinant caspases-3 and -8. D. A schematic summarizing the ^{35}S mutagenesis data is shown.

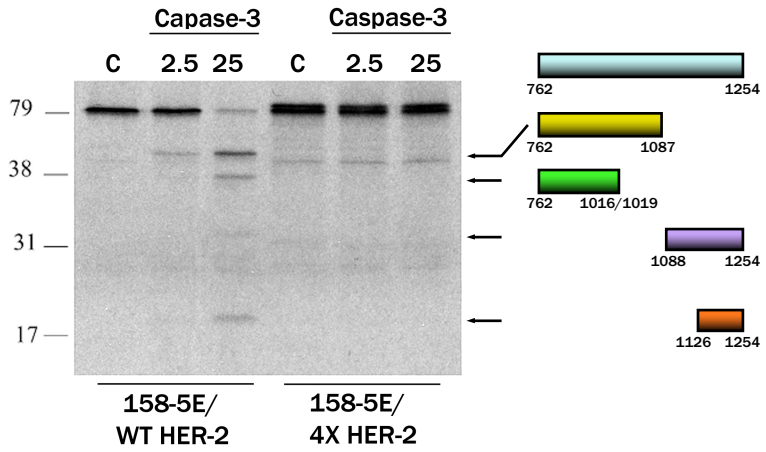


Figure 3.11 **Caspases cleave the carboxyl terminus of HER-2 at four distinct sites *in vitro* (^{14}C analysis).**

The hypothetical product generated by caspase cleavage after D1125 in 158-5E was not observed in the previous analysis due to an absence of methionine residues in this fragment. However 158-5E made by IVT in the presence of ^{14}C –labeled cysteine allows detection of four proteolytic products generated by caspase cleavage. ^{14}C –labeled 158-5E/WT HER-2 or 158-5E/4X HER-2 proteins were incubated with buffer (C) or 2.5 or 25 ng of recombinant caspase-3. The quadruple mutant is resistant to cleavage by recombinant caspase-3.

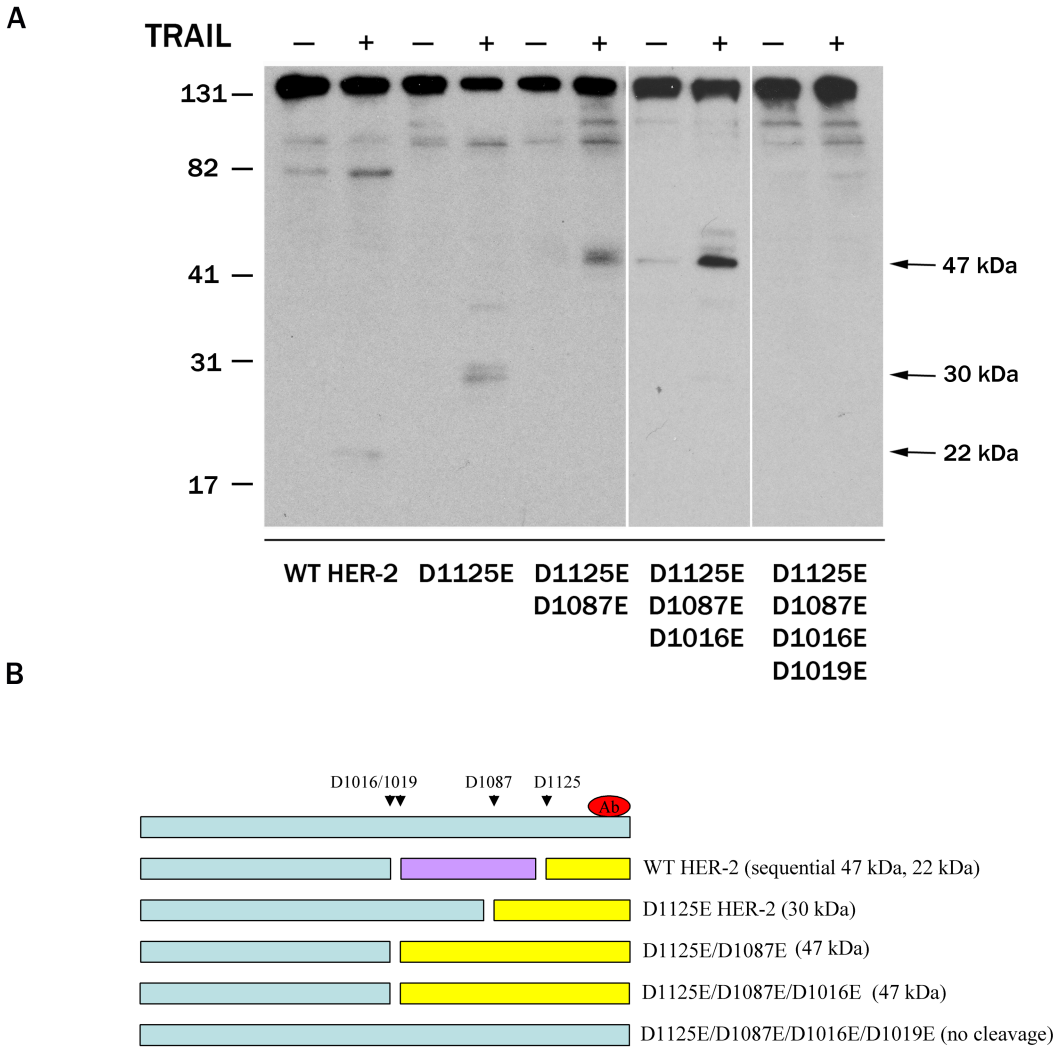


Figure 3.12 **Caspases cleave the cytoplasmic tail of HER-2 at four sites *in vivo*.**

Site directed mutagenesis was used to mutate the conserved aspartic acid (D) residues to glutamic acid (E) in four putative caspase cleavage sites within full length human HER-2. A. Mutation of four distinct sites is required to generate a caspase cleavage resistant HER-2. MDA-MB-231 cells overexpressing wild type, single, double, triple, or quadruple mutant HER-2 proteins were treated with 500 ng/mL TRAIL in the presence of 100 nM epoxomicin for 6 hours and analyzed for cleavage by western blot with a HER-2 antibody with a C terminal epitope. Cleavage products are indicated by arrows. B. Schematic of the HER-2 cleavage products shown in A.

3. FUNCTIONAL RELEVANCE OF HER-2 CLEAVAGE IN CELL DEATH

3. 1 Stable Cell Line Generation

To determine if caspase cleavage of HER-2 played a role in cell death, we generated stable pools of MDA-MB-231 which express vector, wild type, caspase truncated HER-2 (which lacked the 47kDa fragment) or the cleavage resistant 4X HER-2 by retroviral transduction. Western blot analysis demonstrated that expression levels of the HER-2 constructs were similar. Tubulin is shown as a loading control (Figure 3.13). Moreover, we confirmed that the cells expressing the wild type and cleavage resistant (4X) HER-2 constructs were responsive to epidermal growth factor (EGF) treatment, using phosphorylation of tyrosine 1248 of HER-2 as an endpoint. Interestingly, both the wild type and 4X HER-2 constructs display low basal levels of phosphorylation at this residue, which could be enhanced by activation of the receptor in heterodimeric complexes (Figure 3.14). Due to the presence of multiple mutations in several of the constructs which could affect protein conformation, cell surface labeling experiments were conducted to ensure each of the HER-2 constructs was properly targeted to the cell surface. Surface expression is noted by the presence of a second/shifted peak to the right of the histogram (Fig. 3.15).

3.2 Caspase truncated or cleavage resistant HER-2 confer greater protection against TRAIL induced apoptosis than the wild type receptor

To directly assess the significance of HER-2 cleavage on cell survival, the MDA-MB-231 pools stably expressing vector, wild type (WT), truncated (Trun), or cleavage resistant (4X) were analyzed for their sensitivity to TRAIL induced cell death in dose response studies. Expression

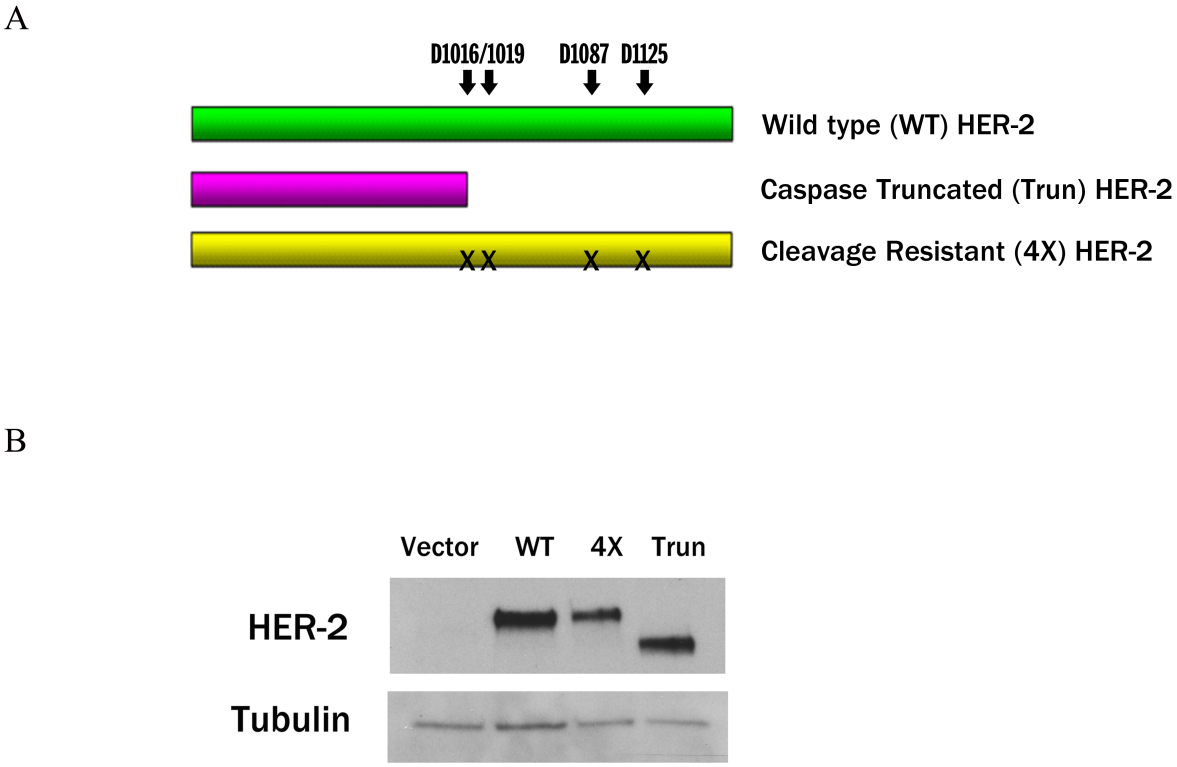


Figure 3.13 Immunoblot analysis of MDA-MB-231 pools retrovirally transduced with HER-2 cDNAs.

MDA-MB-231 pools expressing vector, full length wild type (WT), caspase truncated (Trun), or cleavage resistant (4X) HER-2 were generated by retroviral infection followed by G418 selection. A. Schematic of the various HER-2 constructs. Amino acids and caspase cleavage sites are indicated. WT and 4X HER-2 are full length proteins, while the caspase truncated (Trun) construct encodes amino acids 1-1016 of human HER-2. B. Expression levels were assessed by immunoblotting 20 µg of lysate from each pool with a HER-2 antibody possessing an epitope in the extracellular domain of the receptor. Tubulin was included as a loading control.

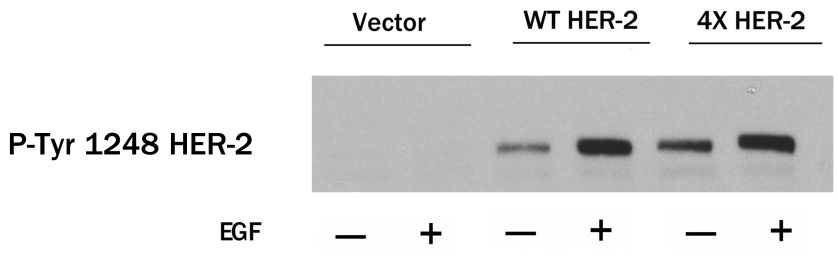


Figure 3.14 Phosphorylation of HER-2 tyrosine 1248 is enhanced by EGF treatment of MDA-MB-231 pools.

MDA-MB-231 pools expressing vector, wild type (WT), or cleavage resistant (4X) HER-2 were serum starved in DMEM containing 0.1% FBS for 24 hours prior to stimulation with 100 ng/mL EGF for 10 minutes at 37°C. 20 µg of protein from each condition was analyzed by western blot for phosphorylation of tyrosine 1248 in HER-2.

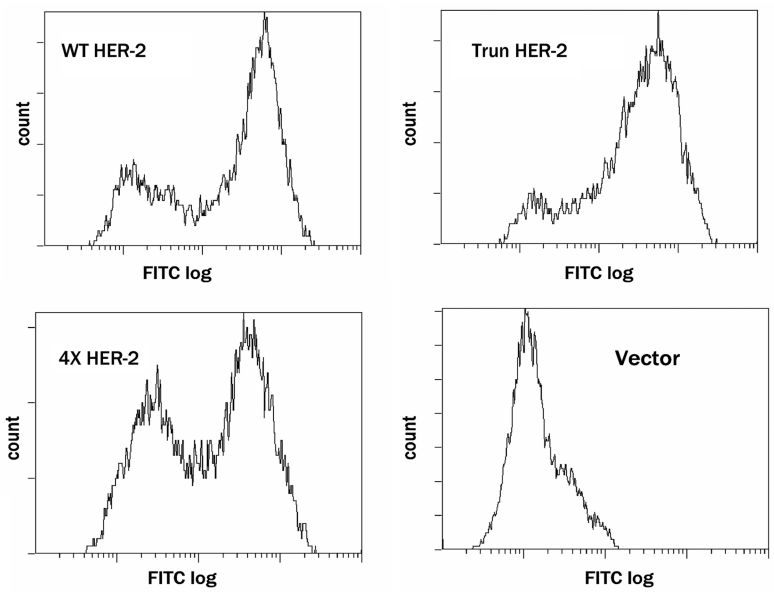


Figure 3.15 Cell surface expression of HER-2 proteins in MDA-MB-231 pools by FACS.

MDA-MB-231 pools stably expressing either vector, wild type (WT), caspase truncated (Trun), or cleavage resistant (4X) HER-2 were incubated with 1 μg of a HER-2 antibody with an extracellular epitope for 1 hour prior to addition of a FITC conjugated secondary antibody. Samples were washed in PBS, fixed in 0.5% paraformaldehyde and analyzed by flow cytometry. Cell surface expression in indicated by a FITC peak in the right of the histogram.

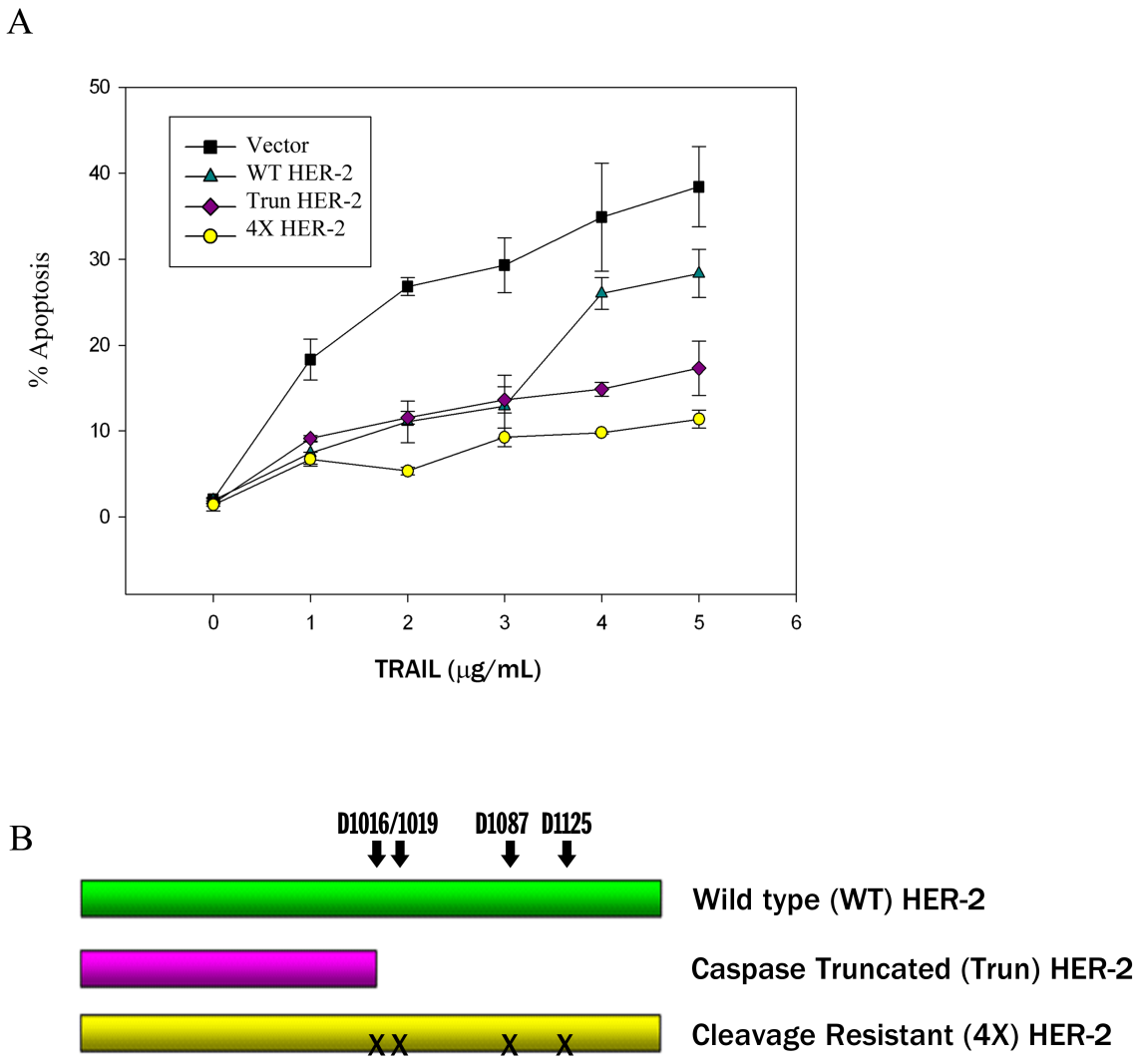


Figure 3.16 Cleavage resistant and truncated HER-2 confer greater protection against TRAIL induced apoptosis than wild type HER-2 (DAPI).

MDA-MB-231 pools stably expressing the various HER-2 constructs were treated with increasing doses of TRAIL to evaluate the contribution of caspase cleavage of HER-2 in the execution of cell death. A. Cells were treated with 0-5 μg/mL TRAIL for 24 hours and analyzed for apoptosis by nuclear morphology. Data are presented as mean ± SEM (n=3). B. Schematic of the HER-2 constructs used in this study. Caspase cleavage sites are indicated by arrows.

of the wild type HER-2 receptor conferred robust protection from TRAIL induced apoptosis at concentrations up to 3μg/ml. However, at doses of TRAIL higher than 3μg/mL this protection was lost, after which time the levels of cell death in the wild type expressing cells approached vector levels. In contrast, cells expressing either the caspase truncated receptor, containing amino acids 1-1016 of HER-2, or the cleavage resistant 4X HER-2 remained protected throughout the TRAIL treatment course (Figure 3.16). This led me to postulate that caspase cleavage of the wild type protein was releasing one or more pro-apoptotic fragments. To test the hypothesis that protection from TRAIL induced apoptosis was dependent on the expression of the un-cleaved full length receptor; I treated cells with vehicle or high dose TRAIL for 24 hours in duplicate and examined the cells for expression of HER-2 and apoptosis via FACS. Annexin-V analysis of the HER-2 stable cell lines confirmed the data obtained with nuclear morphologic scoring: The truncated (Trun) and cleavage resistant (4X) HER-2 conferred greater protection against 5 μg/mL TRAIL induced apoptosis than the wild type receptor (Figure 3.17). Western blot analysis revealed that the magnitude of protection against TRAIL induced apoptosis correlates with levels of un-cleaved full length HER-2 (Figure 3.18). Specifically, all of the endogenous HER-2 was cleaved in vector expressing MDA-MB-231 cells which were the most sensitive to TRAIL induced apoptosis. Similarly, a substantial reduction in the amount of full length HER-2 was observed in cells expressing WT HER-2 subsequent to TRAIL treatment. In contrast, TRAIL treatment had no effect on the abundance of HER-2 observed in cells overexpressing the caspase truncated (Trun) or cleavage resistant (4X) mutants. Moroever, western blot analysis revealed no significant differences in the ability of serum starved HER-2 stable pools to activate the Akt pathway, as measured by phosphorylation of serine 473, following treatment with 100 ng/mL

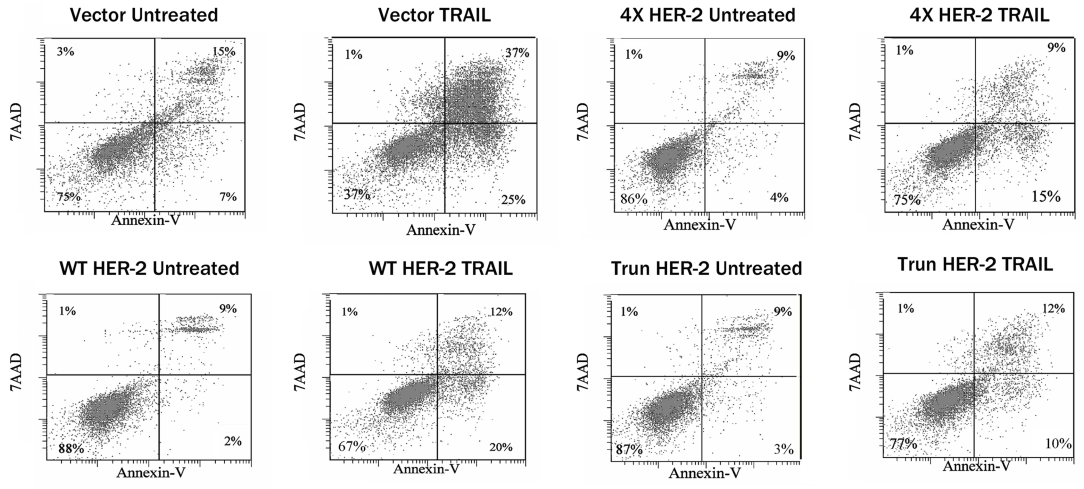


Figure 3.17 Cleavage resistant and truncated HER-2 confer greater protection against TRAIL induced apoptosis than wild type HER-2 (Annexin-V analysis).

MDA-MB-231 pools stably expressing vector, wild type (WT), caspase truncated (Trun), or cleavage resistant (4X) HER-2 were treated with 0 or 5 μg/mL TRAIL for 24 hours prior to assessment of apoptosis by Annexin-V externalization analysis. Apoptotic cells occupy the right two panels of each image, corresponding to early (Annexin-V positive/7AAD negative) and late apoptosis (Annexin-V positive/7AAD positive). The upper left panel contains very late apoptotic and/or necrotic cells (Annexin-V negative/7AAD positive). The percentage of cells in each quadrant is indicated. This experiment was conducted in parallel with the immunoblot shown in Figure 3.18.

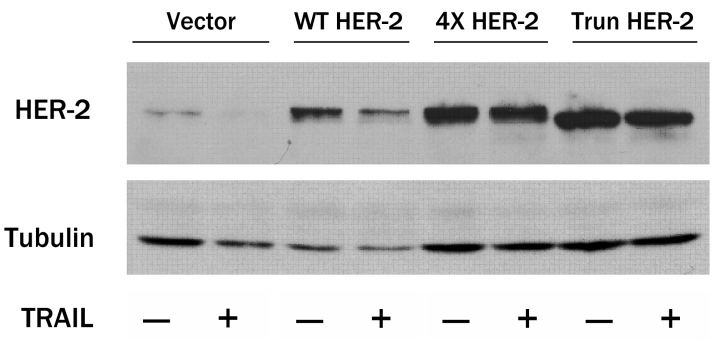


Figure 3.18 The magnitude of protection against TRAIL induced apoptosis correlates with levels of non-cleaved HER-2.

MDA-MB-231 pools stably expressing the various HER-2 constructs were treated with vehicle or 5 μg/mL TRAIL for 24 hours, collected, and analyzed by western blot using a HER-2 antibody possessing an amino terminal epitope. Tubulin was included as a loading control. This experiment was conducted in parallel with the Annexin-V experiment shown in Figure 3.17.

EGF for 10 minutes, indicating that the protective effect observed following TRAIL treatment is not due an enhanced ability to signal to this survival pathway (Figure 3.19). This result is consistent with the caspase truncated HER-2 being capable of forming heterodimeric complexes with HER-3, mediated by the cysteine rich domains in the extracellular domain of the receptor. However, HER-2 signals to the MAP kinase pathway via SH2 containing proteins which bind to phospho-tyrosine residues in the carboxy terminus of the receptor; many of which are absent in the caspase truncated HER-2. Therefore, we examined whether a differential activation of this pathway could be observed following 100 ng/mL EGF treatment for 10 minutes. As shown in Figure 3.19, activation of the MAP kinase pathway, using phosphorylated p42/p44 as an endpoint, was not affected by the cleavage status of HER-2 (although this does not rule out the possible contribution of heterodimeric signaling between the caspase truncated HER-2 and EGFR which is expressed at high levels within this cell line). Taken together, I hypothesized that the protective effect seen cells expressing the 4X and truncated HER-2 constructs is due to the inability to release pro-apoptotic fragment(s) either because the sites have been destroyed (as in the 4X HER-2) or because the construct was engineered to lack the domain (as in the case of the caspase truncated HER-2 construct).

3.3 Caspase proteolysis of HER-2 releases two pro-apoptotic cleavage products

To test this hypothesis directly, I turned my attention to the three cytoplasmic HER-2 caspase cleavage fragments, one or more of which I hypothesized to be pro-apoptotic. As shown in the schematic, HER-2 cleavage results in the production of two observable fragments seen *in vivo*: the 47 kDa fragment (amino acids 1017-1254) and two fragments which are generated by its

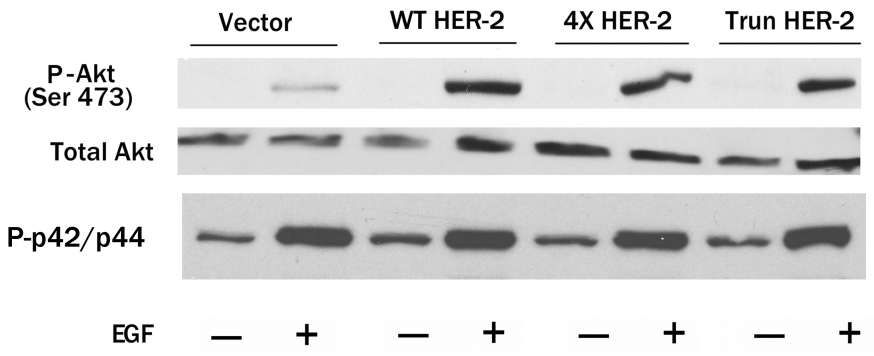


Figure 3.19 Activation of Akt and p42/p44 MAP kinase pathways in response to EGF treatment is unaffected by the cleavage status of the receptor.

MDA-MB-231 pools stably expressing vector, wild type (WT), cleavage resistant (4X), or caspase truncated (Trun) HER-2 were serum starved in DMEM containing 0.1% FBS for 18 hours prior to treatment with 100 ng/mL EGF for 10 minutes at 37° C. 20 μg of each lysate was probed for phosphorylated (Ser 473) and total Akt as well as phospho-specific p42/p44 MAP kinase expression. Both pathways are efficiently activated in cells expressing Truncated HER-2.

cleavage: 25 (amino acids 1017-1125) and 22 kDa (amino acids 1126-1254) respectively. The 25 kDa fragment cannot be visualized with commercially available antibodies (Figure 3.20).

To determine which of the HER-2 cleavage products were pro-apoptotic, MDA-MB-231 breast cancer cells were co-transfected with pEGFP-N1 and cDNAs encoding each of the three carboxyl-terminal HER-2 cleavage products (containing an amino terminal FLAG tag): the 47 kDa product (amino acids 1017-1254), and the 25 kDa (amino acids 1017-1125) and 22 kDa (amino acids 1126-1254) products generated by caspase proteolysis of the 47 kDa product after aspartic acid^{1125}. The 47 and 25 kDa HER-2 cleavage products induced apoptosis in 44% and 41% of transfected (GFP positive) cells, respectively, while the 22 kDa product did not induce apoptosis above levels observed in vector-transfected cells. Moreover, the apoptosis induced by the 47 kDa and 25 kDa products was suppressed by zVAD-fmk, indicating that the observed cell death was caspase-dependent. Truncated Bid (tBid), a positive control in these studies, induced apoptosis in 61% of transfected cells, and this apoptosis was also suppressed by zVAD-fmk, consistent with prior reports (Li et al., 1998; Luo et al., 1998). The lack of pro-apoptotic activity of the 22 kDa fragment was not due to its rapid degradation as the addition of an amino-terminal FLAG epitope tag stabilized the 22 kDa product (Figure 3.21).

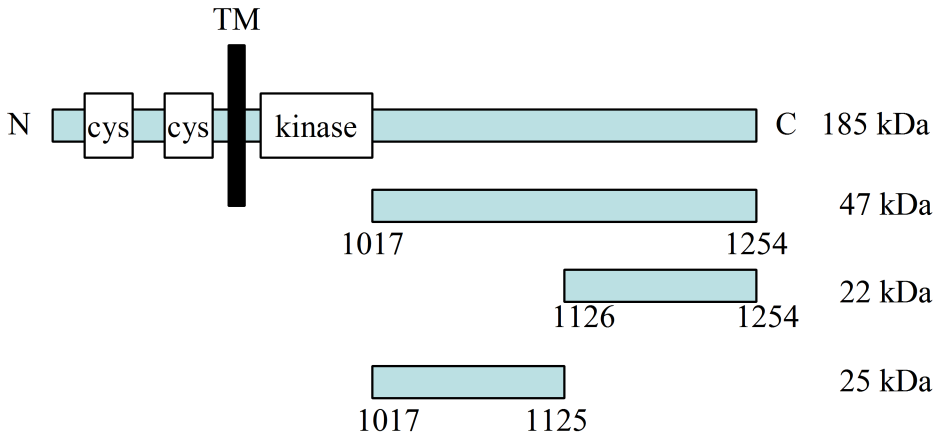


Figure 3.20 Capsase cleavage of HER-2 releases three cytoplasmic products.

Schematic of the HER-2 caspase cleavage products shown in relationship to the kinase domain and transmembrane portions of the receptor. The apparent molecular weights and amino acids encoded by each product are indicated.

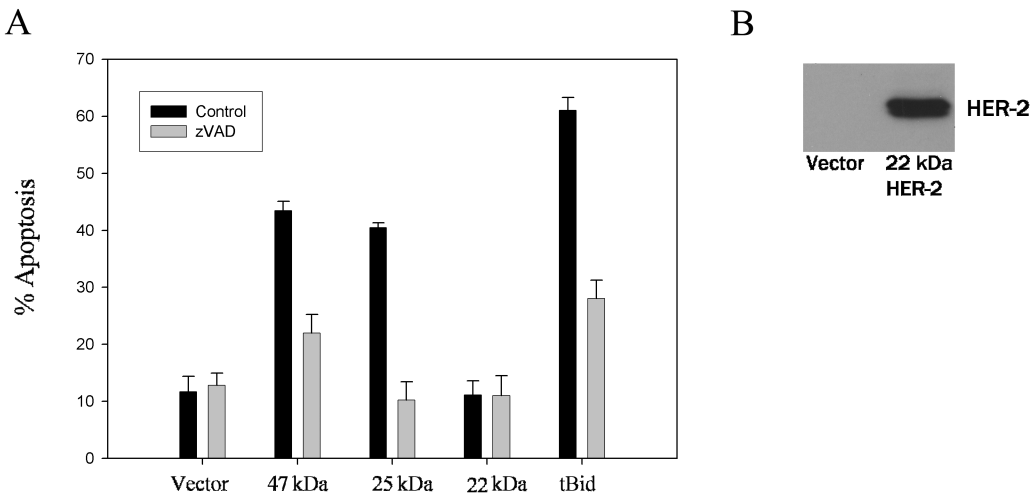


Figure 3.21 The 47 and 25 kDa HER-2 products induce apoptosis in a caspase-dependent manner.

Ectopically expressed 47 and 25 kDa HER-2 products, but not the 22 kDa product, induce apoptosis in breast cancer cells. A. MDA-MB-231 cells were co-transfected with 0.2 µg eGFP-N1 and 1.0 µg of either pCMV-tBid or an amino FLAG tagged HER-2 product. For caspase dependence analyses, cells were pretreated with 100 mM zVAD-fmk for 1 hour prior to transfection. Twenty-four hours post-transfection, cells were collected and apoptosis was scored in the GFP positive cells by nuclear morphology. Data is presented as mean ± SEM (n=3). B. Addition of an amino terminal FLAG tag stabilizes the 22 kDa HER-2 product. 20 µg of lysate from MDA-MB-231 cells transfected with the FLAG tagged 22 kDa HER-2 product were analyzed for expression by immunoblot with an anti-FLAG antibody.

4. MECHANISM OF CELL DEATH INDUCED BY THE PRO-APOPTOTIC HER-2 CLEAVAGE PRODUCTS

4.1 Mapping the site of action of the 25 and 47 kDa HER-2 cleavage products within the apoptotic pathway

I employed a genetic approach to delineate the mechanism(s) by which the 47 kDa and 25 kDa HER-2 products induce apoptosis. MDA-MB-231 cells were co-transfected with either the 47 or 25 kDa HER-2 product in combination eGFP-N1 and empty vector or one of several anti-apoptotic cDNAs known to act at distinct steps of the apoptotic pathway. Neither a FADD dominant negative mutant (DN) nor CrmA, which inhibit signaling from activated caspase-8 (Chinnaiyan et al., 1996; Muzio et al., 1996), suppressed apoptosis induced by the 25 kDa or 47 kDa products. In contrast, the bacloviral p35 protein, a broad spectrum caspase inhibitor, and Bcl-2, an anti-apoptotic protein which antagonizes cytochrome c release from mitochondria (Bump et al., 1995; Kluck et al., 1997), suppressed apoptosis induced by the 25 and 47 kDa products (Figure 3.22). These results provide genetic confirmation of the caspase dependent nature of the apoptosis induced by the HER-2 cleavage products and suggest that they act may act on mitochondria to trigger cytochrome c release.

4.2 Ectopically expressed HER-2 products co-localize with mitochondria

To directly test this hypothesis, I examined whether ectopically expressed HER-2 cleavage products would colocalize with mitochondria. Confocal immunofluoresence microscopy revealed that the FLAG-tagged 47 and 25 kDa products (green) colocalized with mitochondria visualized by Mitotracker staining (red): colocalization is shown in yellow in the merged image (Figure 3.23A). Colocalization of the 47 or 25 kDa products with mitochondria was observed in

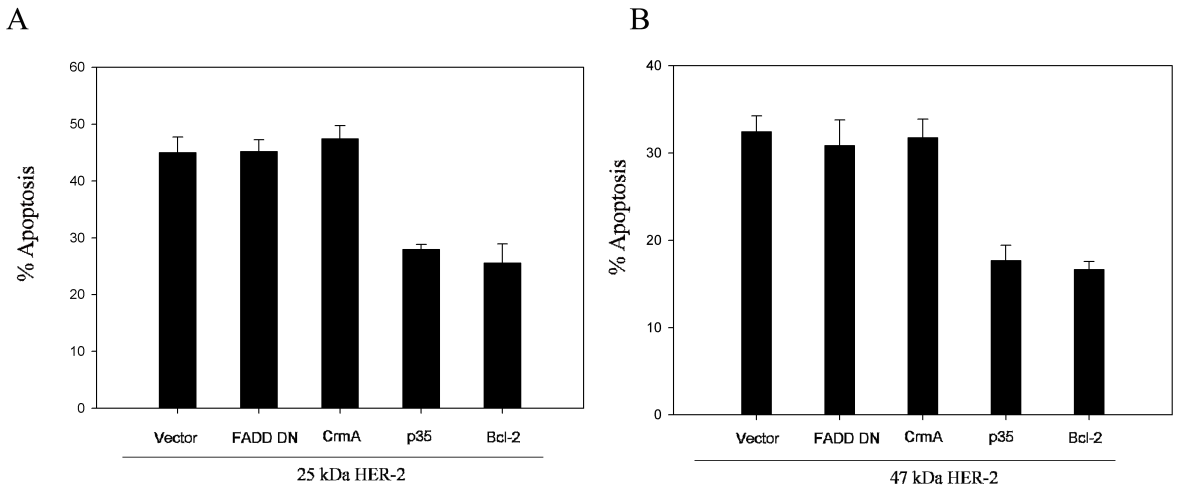
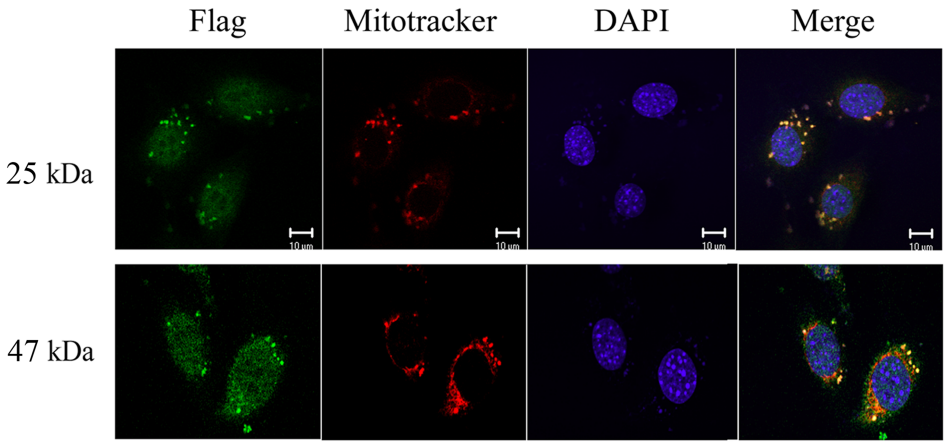


Figure 3.22 Cell death induced by the 25 or 47 kDa HER-2 products is suppressible by co-expression of Bcl-2 or p35.

To determine the mechanism(s) by which the HER-2 products induce apoptosis, MDA-MB-231 cells were transiently co-transfected with 0.2 µg eGFP-N1, 0.5 µg pCDNA3 FLAG 47 or 25 kDa HER-2 products, and 0.5 µg of empty vector or plasmid encoding FADD DN, CrmA, p35, or Bcl-2. 24 hours post-transfection, GFP positive cells were scored for apoptotic nuclei. Data is presented as the mean ± SEM (n=3).

A



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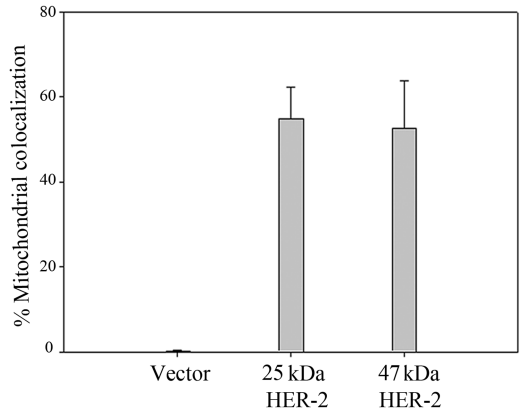

Figure 3.23 The pro-apoptotic HER-2 cleavage products co-localize with mitochondria.

MDA-MB-231 cells were transiently transfected with FLAG tagged vector the 25 or 47 kDa HER-2 cleavage products and allowed to recover overnight prior to labeling the mitochondria with Mitotracker. Note: detached cells were routinely observed in wells transfected with the HER-2 cleavage fragments. A. Representative confocal images demonstrating mitochondrial localization of the ectopically expressed HER-2 cleavage products. Cleavage products were stained with a FLAG-FITC antibody (green); mitochondria

Co-localization is shown in yellow in the merged image. Bar 10 µm. B. Quantitation of mitochondrial colocalization of the HER-2 products. Data is presented as mean ± SEM (n=3).

53% or 55% of transfected cells, respectively. Quantitation of these data is shown in Figure 3.23B.

4.3 Endogenous HER-2 traffics to the mitochondria in TRAIL treated breast cancer cells

To determine the subcellular localization of the endogenous 47 kDa caspase cleavage product, I treated SKBR3 cells with TRAIL and isolated cytosolic and mitochondria-enriched heavy membrane fractions by differential centrifugation. In agreement with our previous results with the 47 kDa fragment in SKBR3 cells, the fragment was only transiently detected in heavy membrane fractions. Cox IV was included as a positive control for mitochondrial components; tubulin as a marker of cytosolic proteins (Figure 3.24). Of note, an antibody directed against the amino terminal HER-2 epitope failed to detect the 47 kDa fragment observed in the heavy membrane fraction, consistent with my conclusion that the 47 kDa fragment corresponds to a cytoplasmic portion of the receptor. Moreover, the 22 kDa fragment was detected exclusively in the cytosolic fraction, suggesting that the 47 (and presumably 25) kDa HER-2 fragments are actively targeted to mitochondria. As noted previously, I was unable to detect the 25 kDa product with available antibodies and therefore cannot determine whether the endogenous 25kDa product is also present in the mitochondrial fraction.

4. 4 Ectopically expressed HER-2 cleavage products induce cytochrome c release in a Bcl-x_L inhibitable manner

Cytochrome c release from the mitochondria is a key event in apoptosis which is potently inhibited by Bcl-x_L (Johnson et al., 2000). MDA-MB-231 pools expressing pBABE vector or

pBABE Bcl-x_L were generated by retroviral transduction and used to examine whether the 25 and 47 kDa HER-2 products were sufficient to induce cytochrome c release by immunofluoresence (Figure 3.25). I took advantage of the fact that cytochrome c release is independent of caspase activity and pretreated the MDA-MB-231 cells with zVAD-fmk to prevent the released cells from detaching from the plate (Bossy-Wetzel et al., 1998). Cells were transiently co-transfected with eGFP-N1 and either the 25 or 47 kDa FLAG tagged HER-2 fragments, stained for cytochrome c, and examined by confocal microscopy. tBid was utilized as a positive control in these studies. Both the 47 and 25 kDa HER-2 products induced cytochrome c release in MDA-MB-231/pBABE cells, albeit less robustly than tBid (Figure 3.26A, left panel). Transfected cells with cytochrome c release (indicated by arrows in the GFP panels) exhibited diffuse or nearly absent cytochrome c staining. In the MDA-MB-231/pBABE-Bcl-x_L cells, Bcl-x_L antagonized cytochrome c release by the 47 and 25 kDa HER-2 products and tBid. Transfected cells with intact mitochondrial cytochrome c (indicated by arrows in the GFP panels) showed a punctate cytoplasmic pattern of staining and a characteristic sparing of the nucleus (Figure 3.26A, right panel). Ectopic expression of the HER-2 products (47 or 25 kDa HER-2) or tbid induced cytochrome c release in 27 %, 26 %, and 80% of MDA-MB-231/pBABE cells, respectively. These data are quantified in Figure 3.26B.

4.5 The sequence shared by the 47 and 25 kDa HER-2 products contains a functional BH3-like domain.

The mitochondrial localization and induction of cytochrome c release by the 25 and 47 kDa HER-2 fragments is reminiscent of the actions of pro-apoptotic BH3-only proteins. Thus, I

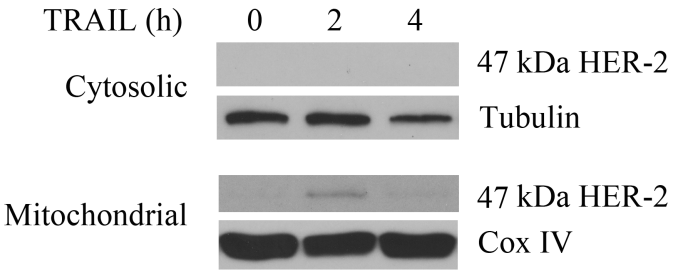

Figure 3.24 The endogenous 47 kDa HER-2 product is detected in mitochondrial fractions of TRAIL treated breast cancer cells.

SKBR3 cells were treated with 2.0 μg/mL TRAIL in the presence of 1 μg/mL cycloheximide for 0-4 hours and separated into cytoplasmic or mitochondria enriched/ heavy membrane fractions by subcellular fractionation. 20 μg from each time point and fraction was analyzed by immunoblot for the presence of the 47 kDa HER-2 product. Tubulin and Cox IV served as fractionation controls.

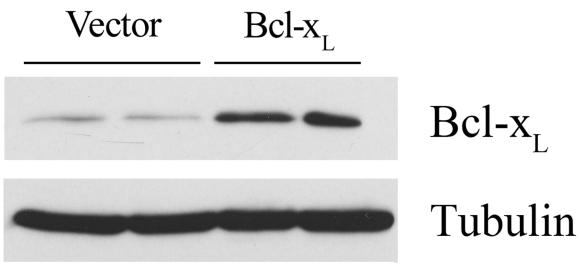

Figure 3.25 Immunoblot analysis of MDA-MB-231 pools stably expressing Bcl-x$_L$.

Retroviral transduction followed by selection in puromycin was used to generate pools of MDA-MB-231 breast cancer cells stably overexpressing pBABE vector or pBABE Bcl-x$_L$. 20 μg of each pool was analyzed by immunoblot with a monoclonal antibody against Bcl-x to confirm overexpression. Tubulin was included as a loading control.

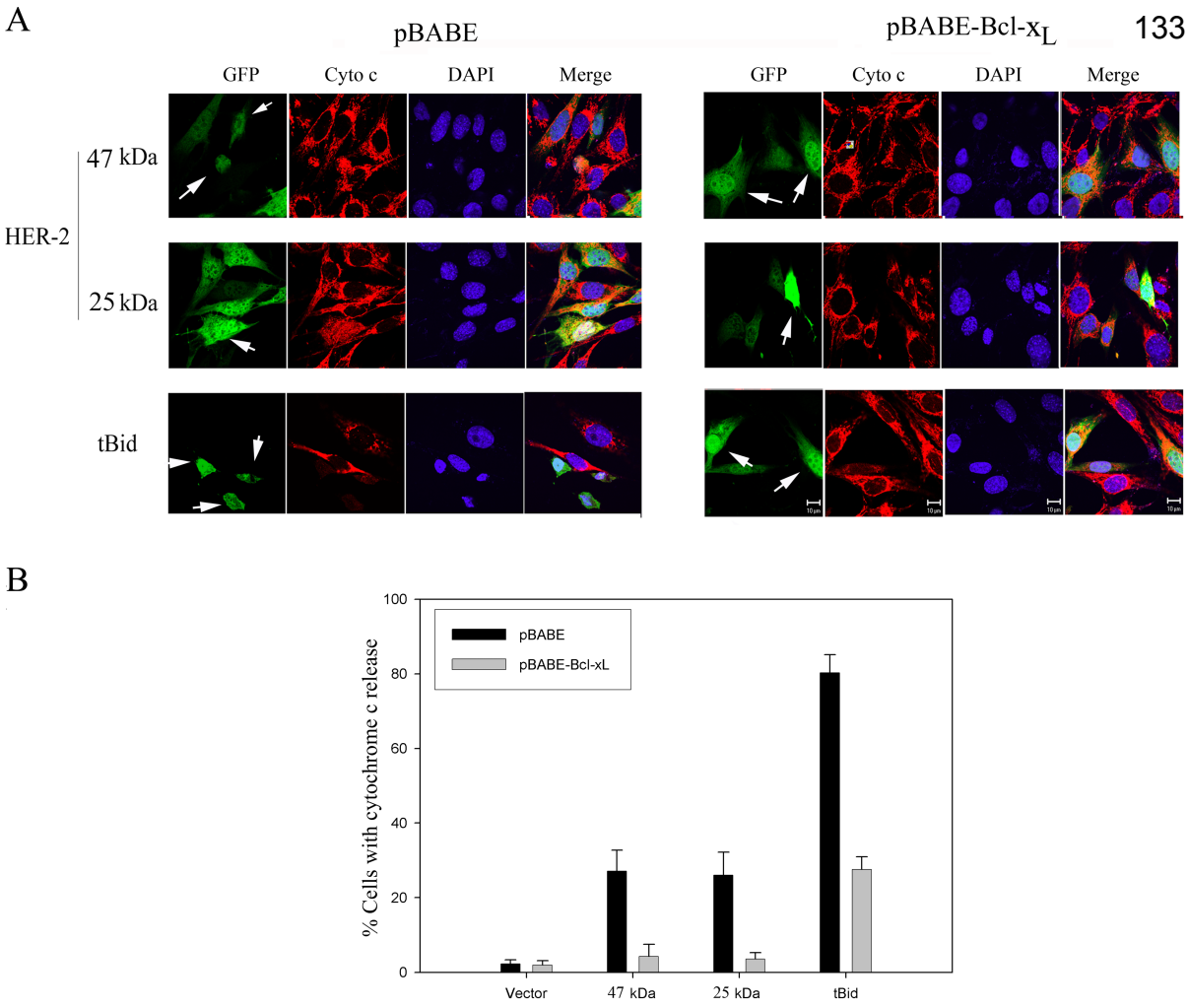

Figure 3.26 **The 47 and 25 kDa HER-2 products induce cytochrome c release from MDA-MB-231 cells in a Bcl-x_L-suppressible manner.**

MDA-MB-231 stable pools overexpressing pBABE vector or pBABE-Bcl-x_L were pretreated with 100 µM zVAD-fmk 1 hour prior to transfection with 0.2 µg eGFP-N1 and 1.0 µg each of pcDNA3 FLAG tagged 47 or 25 kDa HER-2

in 4% paraformaldehyde and permeabilized with 0.5% triton-x 100/PBS prior to staining with an antibody detecting native conformation cytochrome c. Slides were analyzed for the percentage of GFP positive (e.g. transfected) cells in which cytochrome c had been released from the mitochondria (indicated by diffuse or absent punctate staining). A. Representative confocal images of cytochrome c release induced by the HER-2 products. In the left panel, transfected cells with released cytochrome c are marked by arrows. In the right panel, Bcl-x_L overexpression prevents cytochrome c release induced by either the HER-2 products or tBid. B. Quantitation of the cytochrome c release by the HER-2 products. Data is presented as the mean ± SEM (n=5).

postulated that the HER-2 cleavage products may contain a BH3-like domain. Indeed, alignment of the protein sequence shared by the 47 and 25 kDa HER-2 products with the BH3 domains of well known Bcl-2 family members revealed conservation of leucine (L) and aspartic acid (D) residues (highlighted in yellow, Figure 3.27) which play a critical role in apoptosis induction (Letai et al., 2002; Wang et al., 1998).

To examine the functional relevance of these residues in cell death, L1120 and D1125 in human HER-2 were mutated to glutamic acid (E) residues by site-directed mutagenesis, either alone (L1120E or D1125E) or in combination (L1120E/D1125E designated 2XE) and examined for their apoptotic activity after transient transfection into MDA-MB-231 cells. Mutation of either L1120 or D11125 reduced the apoptosis induced by the 25 and 47kDa products, while the 2XE 25 kDa product was more impaired in its pro-apoptotic activity than either of the single BH3 domain mutants (Figure 3.28). Importantly, the reduction in the pro-apoptotic activity observed with the 2XE mutants can be attributed to destruction of the putative BH3 like domain, rather than a defect in caspase proteolysis after D1125, as the 47/D1125E and 17/D1125E which cannot be further processed by caspases were similarly impaired in their pro-apoptotic abilities (Figure 3.28).

4.5.1 Cell death induced by the pro-apoptotic HER-2 products is dependent on the presence of Bax and Bak

Both the 25 and 47 kDa products (and tBid) induced apoptosis in retro-virally infected WT MEFs, but not Bax/Bak double knock-out (DKO) MEFs (Figure 3.29) indicating that apoptosis

HER-2	1113	SEDPTVP**L**PSET**D**GYVAPLT	1132
Puma	141	**L**RRMA**D**DLNA	150
Bad	114	**L**RRMS**D**EFVD	123
Bid	90	**L**AQVG**D**SMDR	99
Mcl-1	213	**L**RRVG**D**GVQR	222
Noxa B	78	**L**RRIG**D**KVNF	87
Bak	78	**L**AIIG**D**DINR	87
Bok	72	**L**LRLG**D**ELEQ	81
Bax	63	**L**KRIG**D**ELDS	72
Bim	152	**L**RRIG**D**EFNA	161
Bcl-2	97	**L**RQAG**D**DFSR	106
Bcl-X_L	90	**L**REAG**D**EFEL	100

Figure 3.27 The sequence shared by the 47 and 25 kDa HER-2 products contains a putative BH3 domain.

Alignment of the sequence shared by the 47 and 25 kDa HER-2 cleavage products and the BH3 domains of established Bcl-2 family members. The conserved leucine (L) and aspartic acid (D) residues in the BH3 domain which are required for the pro-apoptotic activity of these proteins are highlighted in yellow.

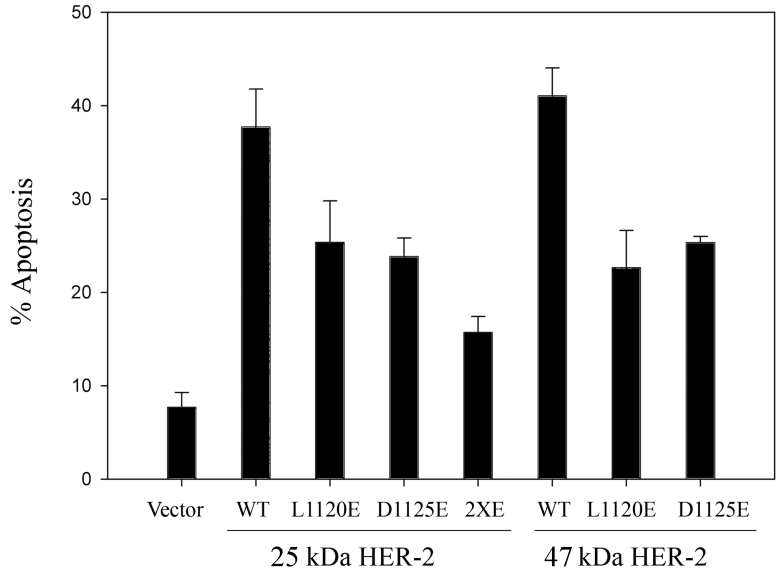

Figure 3.28 Mutagenesis of the putative HER-2 BH3 domain abrogates cell death induced by the HER-2 products.

The conserved L1120 and D1125 in human HER-2 were mutated to glutamic acid (E) either alone (L1120E, D1125E) or in combination (L1120E/D1125E designated 2XE). MDA-MB-231 cells were co-transfected with cDNAs encoding the 25 or 47 kDa product (WT or BH3 mutants) and eGFP-N1. GFP positive nuclei were scored 24 hours later. Data is presented as the mean ± SEM (n=3).

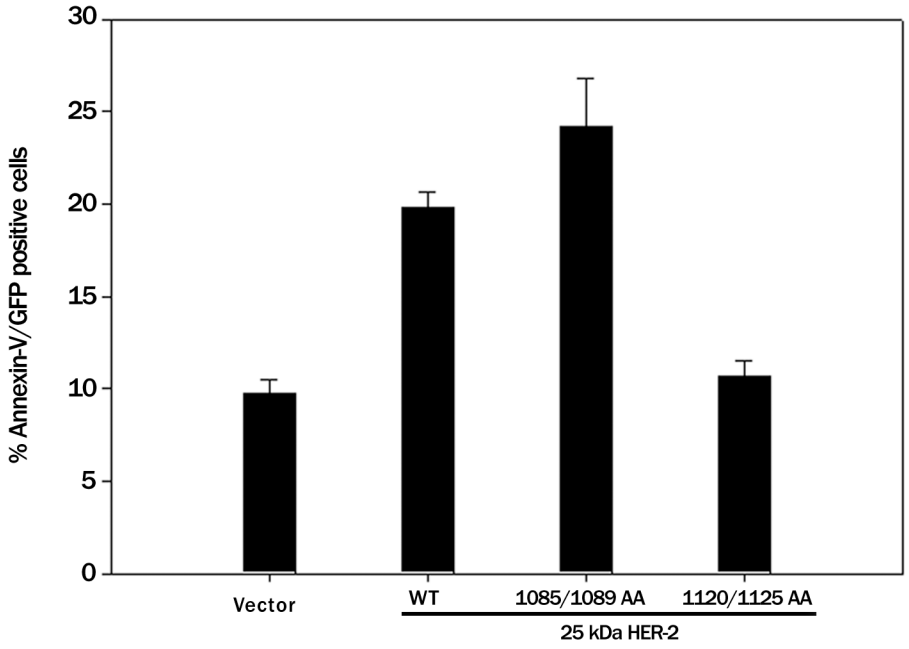

Figure 3.28b Mutagenesis of the putative HER-2 BH3 domain abrogates cell death induced by the HER-2 products.

The conserved BH3-like residues in HER-2, L1220 and D1125, and the irrelevant V1085 and D1089 residues were mutated to alanine by site directed mutatgenesis. MDA-MB-231 cells were co-transfected with cDNAs encoding the 25 kDa HER-2 (WT, irrelevant mutations, or BH3 mutants) and eGFP-N1. GFP positive cells were analyzed for Annexin-V externalization 48 hours post-transfection. Data is presented as the mean ± SEM (n=3).

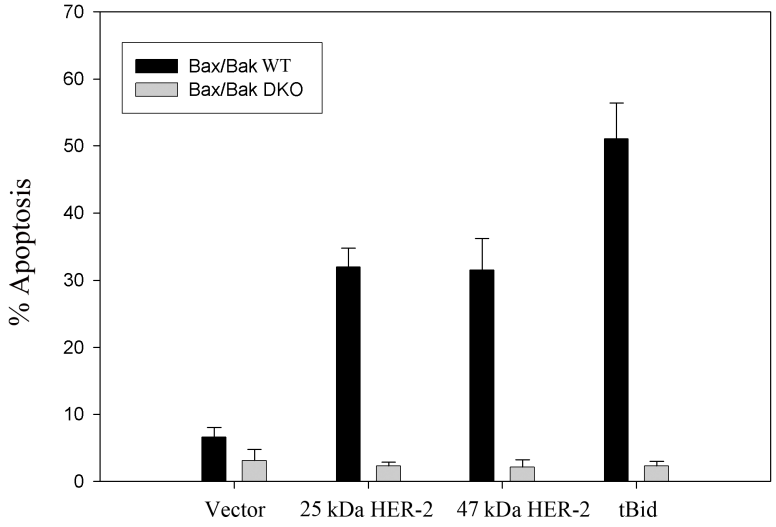

Figure 3.29 Cell death induced by the HER-2 products is dependent on the expression of Bax and Bak.

Apoptosis induced by the 25 or 47 kDa HER-2 products is Bax/Bak dependent. SV40 immortalized wild type (WT) or $Bax^{-/-}$ $Bak^{-/-}$ (DKO) MEFs were infected with a biscistronic retrovirus encoding GFP alone, GFP and a HER-2 product (either the 25 or 47 kDa), or GFP tagged tBid. 48 hours later, the percentage of GFP positive cells with apoptotic nuclei were scored. Data is presented as the mean ± SEM (n=3).

induction by these proteolytic products is Bax/Bak-dependent, a defining characteristic of cell death induced by BH3-only proteins (Wei et al., 2001).

4.5.2 A wild type HER-2 BH3 peptide induces cytochrome c release from isolated mitochondria

To determine whether the HER-2 BH3-like cleavage product was sufficient to induce cytochrome c release in a cell-free system, I added 20 mer peptides (amino acids 1113-1132 of human HER-2) containing the WT HER-2 BH3 sequence, a mutant 2XE HER-2 BH3 sequence, or a Bid BH3 sequence to mitochondria isolated from Jurkat human T cell leukemia cells and measured cytochrome c release by ELISA. The WT HER-2 BH3 peptide, but not the 2XE mutant peptide, induced modest cytochrome c release from these cancer cell mitochondria (Figure 3.30).

4.5.3 The 25 kDa HER-2 product (and its BH3 mutant) immunoprecipitate with Bcl-x_L

Cognizant of the fact that the BH3 motif binds to the hydrophobic pocket of multidomain anti-apoptotic Bcl-2 family members, we tested the ability of ectopically expressed 25 kDa HER-2 product or its BH3 mutant (2XE) to interact with Bcl-x_L by immunoprecipitation. (Figure 3.31) Surprisingly, both products interacted with Bcl-x_L.

4.5.4 The 25 kDa HER-2 product cooperates with tBid to induce cell death

Because the 25 kDa product and tBid bind to Bcl-x_L ((Certo et al., 2006) and Figure 3.31), I postulated that the 25 kDa product would inhibit Bcl-x_L and act in concert with tBid to induce

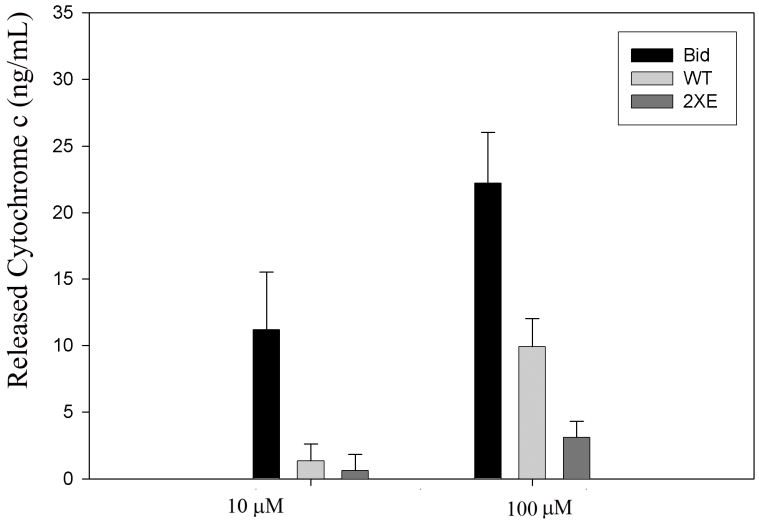

Figure 3.30 Peptides spanning the HER-2 BH3 domain are sufficient to induce cytochrome c release from isolated mitochondria.

20 mer peptides (amino acids 1113-1132) of human HER-2 containing either the wild type (WT) or mutant (2XE) HER-2 BH3 domain were incubated with isolated Jurkat cell mitochondria for 3 hours at RT. Mitochondria were pelleted, and the released cytochrome c in the supernatant was quantified by ELISA. Data was normalized to vehicle control and presented as mean ± SEM (n=3).

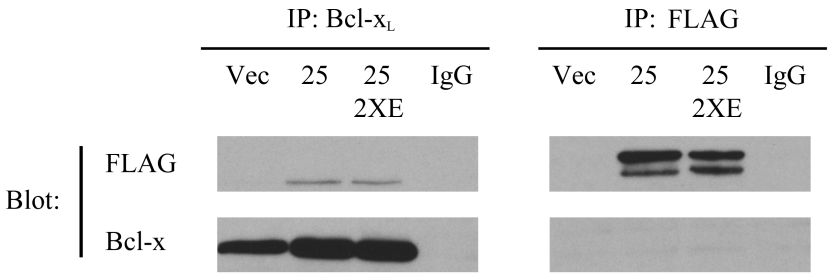

Figure 3.31 The 25 kDa HER-2 product (and its BH3 mutant) immunoprecipitate with Bcl-x$_L$.

MDA-MB-231 pools stably overexpressing Bcl-x$_L$ were transfected with pcDNA3 FLAG vector, 25 kDa HER-2, or its BH3 mutant 25 kDa HER-22XE and immunoprecipitated with either polyclonal Bcl-x$_L$ or anti-FLAG antibodies. Immunoprecipitated complexes were analyzed by western blot with mouse anti-FLAG or Bcl-x antibodies. Both the wild type and BH3 mutant 25 kDa HER-2 products interact with Bcl-x$_L$

apoptosis in MDA-MB-231 cells stably expressing Bcl-x_L. Indeed, these Bcl-x_L -overexpressing cells were resistant to tBid-induced apoptosis. Co-transfection of the 25 kDa HER-2 product or Bad, but not Noxa, sensitized these cells to tBid-induced apoptosis (Figure 3.32). These data are consistent with the binding of the 25 kDa product and Bad with Bcl-x_L, and the inability of Noxa to bind to Bcl-x_L (Figure 3.32 and data not shown).

4.5.5 The HER-2 BH3 domain is dispensable for mitochondrial localization of the protein.

BH3-only proteins are targeted to mitochondria by diverse mechanisms. These proteins are typically sequestered within distinct compartments of the cell and only translocate to mitochondria in response to cell stress, facilitated by post-translational modifications such as NH_2 –terminal myristolation, phosphorylation, or by exposure of a canonical mitochondrial targeting sequence. The BH3 only protein Noxa co-localizes with mitochondria in a BH3-dependent manner; murine Noxa carrying L→A mutations in the amino terminal leucine of the BH3 domains of Noxa A and Noxa B resulted in non-specific distribution of Noxa throughout the cell (Oda et al., 2000). In contrast, a Puma BH3 mutant lacking the LRR motif within the BH3 domain exhibited similar mitochondrial localization as the wild type protein (Nakano and Vousden, 2001). To investigate the contribution of the HER-2 BH3 domain in localization of the cleavage product, we examined the subcellular localization of ectopically expressed wild type or BH3 mutant (2XE) 25 or 47kDa HER-2 products in MDA-MB-231 cells by immunofluoresence (Figure 3.33). Both the wild type and BH3 mutant products efficiently colocalized with mitochondria, indicating that (i) the inability of the 2XE products to induce cell death is not a consequence of inappropriate subcellular targeting and (ii) that the BH3-like domain in HER-2 is

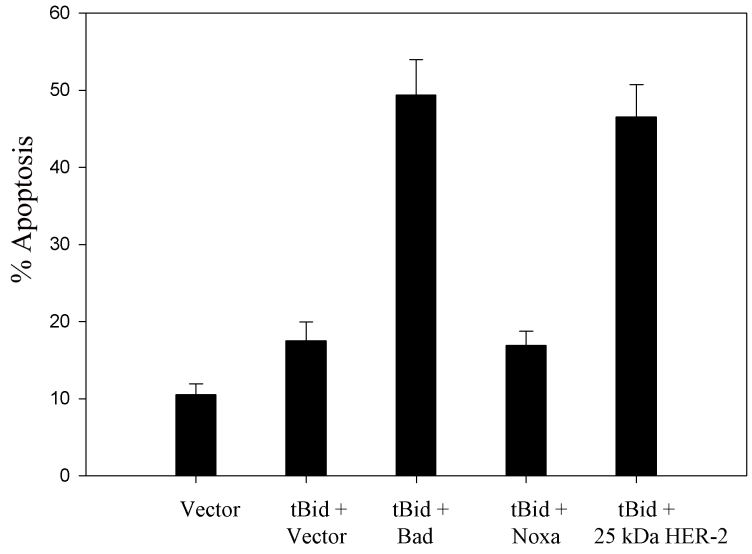

Figure 3.32 The 47 and 25 kDa HER-2 products cooperate with tBid to induce apoptosis in MDA-MB-231 cells stably overexpressing Bcl-x_L.

MDA-MB-231 cells stably expressing Bcl-x_L were transiently co-transfected with tBid and a bicistronic plasmid co-expressing GFP and vector, HA-Bad, HA-Noxa, or the 25 kDa HER-2 product. 24 hours later, GFP positive cells were scored for apoptotic nuclei. Data are presented as the mean ± SEM

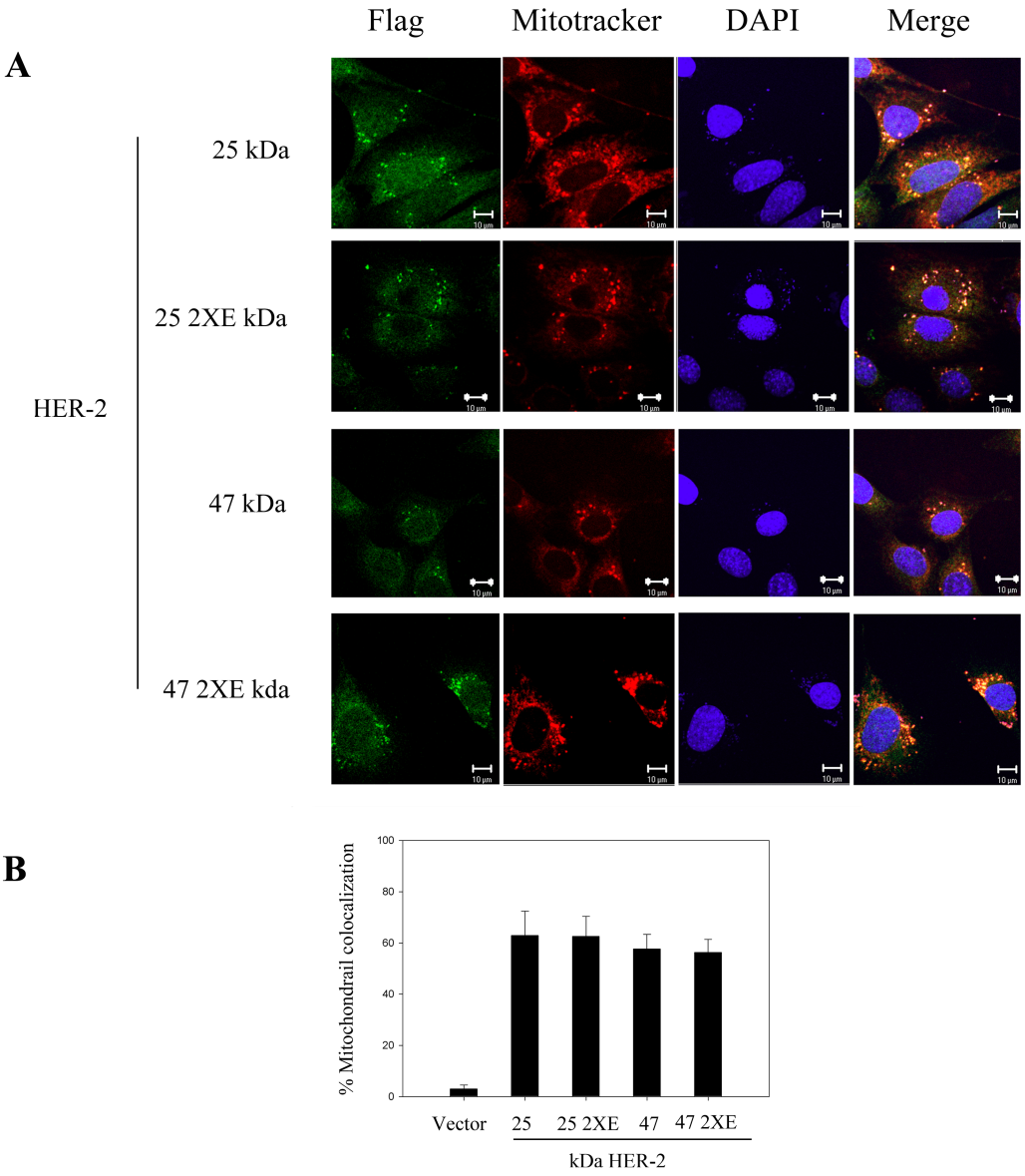

Figure 3.33 The BH3 domain is dispensable for mitochondrial localization of the pro-

A. Representative confocal images of MDA-MB-231 cells transiently transfected with wild type or BH3 mutant HER-2 (2XE) cDNAs. HER-2 products were stained with a FLAG antibody (green); mitochondria were visualized with Mitotracker Deep Red 633 (red); nuclei were stained with DAPI (blue). Colocalization is shown in yellow in the merged image. Bar 10 µm. B. Quantitation of mitochondrial colocalization observed with the various HER-2 products. Data is presented as the mean ± SEM (n=3).

dispensable for mitochondrial localization of the protein. Clearly further experiments are needed to define the manner in which the HER-2 products are targeted to this organelle.

4.5.6 The HER-2 BH3 mutant confers greater protection against TRAIL induced apoptosis than the wild type receptor.

To ascertain the functional relevance of the HER-2 BH3-like domain in the biology of the receptor, we examined MDA-MB-231 cells transiently expressing full length wild type or BH3 mutant HER-2 for their sensitivity to TRAIL induced apoptosis. Significantly, the mutant 2XE HER-2 conferred greater protection against apoptosis than WT HER-2 presumably due to the lack of a pro-apoptotic BH3 domain in the mutant protein. (Figure 3.34A). As expected, both the wild type and 2XE HER-2 released the 47 kDa product. A 30 kDa cleavage product was observed in cells transfected with the BH3 mutant, generated by cleavage after D1087 (recall that the BH3 mutant contains a D1125E mutation) (Figure 3.34B and Figure 3.12A-B). Taken together, these results indicate that caspases activate a previously unrecognized pro-apoptotic function of HER-2 by releasing a BH3-like cell death effector.

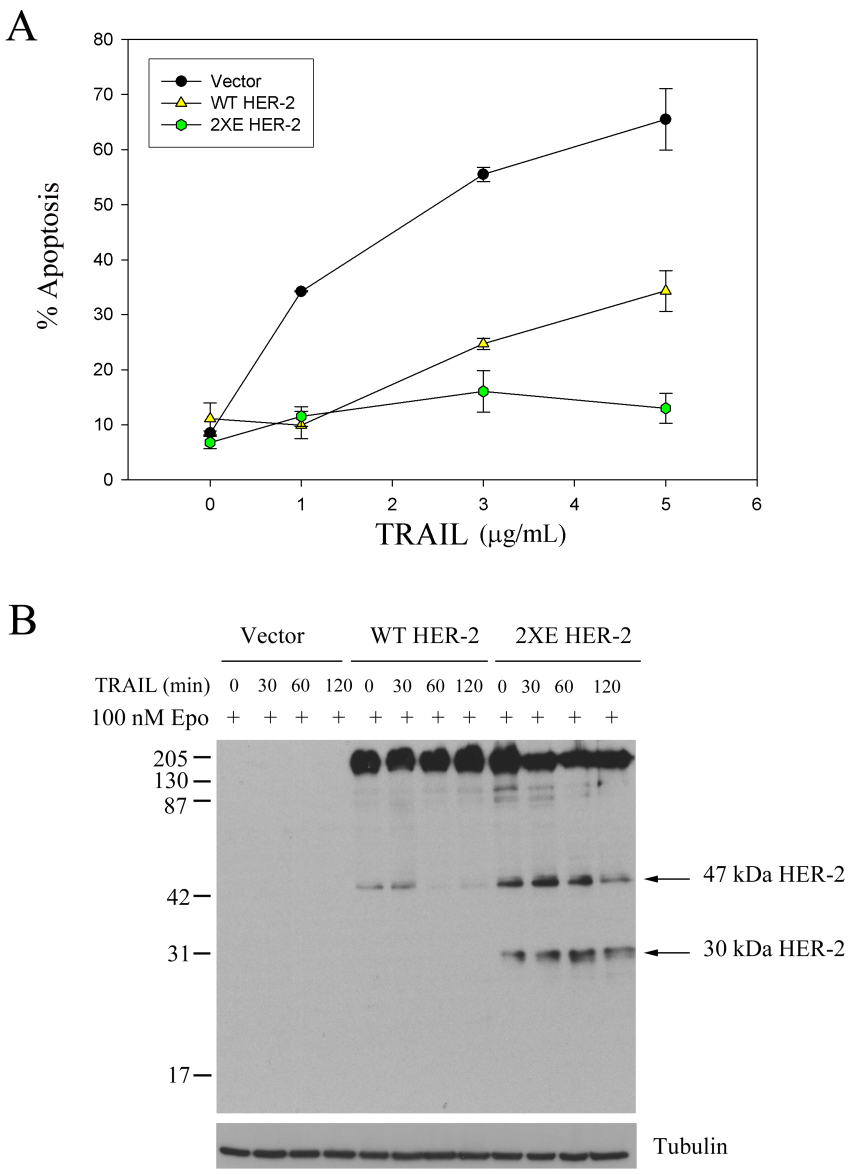

Figure 3.34 Destruction of the BH3 domain in full length HER-2 leads to enhanced protection from TRAIL induced apoptosis.

A. MDA-MB-231 cells were transiently co-transfected with eGFP-N1 and empty vector or cDNAs encoding full length wild type (WT) or BH3 mutant (2XE) HER-2 and examined for their sensitivity to 0-5 μg/mL TRAIL treatment. At the end of the 24 hour treatment, cells were collected and GFP positive cells were scored for apoptotic nuclei. Data is presented as the mean ± SEM (n=3). B. Both the wild type (WT) and BH3 mutant (2XE) HER-2 release a 47 kDa product in response to apoptotic stimuli.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Since the recognition of its pathogenic role in a subset of poor prognosis breast carcinomas**,** the receptor tyrosine kinase HER-2 has been one of the most intensely scrutinized molecules in cancer biology (Slamon et al., 1987). HER-2 has been directly linked to many of the hallmarks of cancer, including dysregulated proliferation, migration, invasion and suppression of apoptosis (Hanahan and Weinberg, 2000; Hynes and Lane, 2005). In this dissertation we describe an entirely new function for HER-2 as a cell death effector which is activated by caspases.

Specifically, we demonstrate that caspase proteolysis of HER-2 promotes apoptosis by releasing a BH3-like cell death effector. Several lines of evidence support this mechanism. First, our observation that stable expression of the 4X caspase cleavage-resistant HER-2 protein or the amino-terminal caspase cleavage product (Trun HER-2, encoding amino acids 1-1016) protects breast cancer cells against apoptosis more robustly than wild type HER-2, strongly suggests that caspase cleavage of HER-2 promotes apoptosis by releasing a pro-apoptotic carboxyl-terminal product, rather than by suppressing the anti-apoptotic function of the receptor (Figure 3.19). Second, we observed that ectopic expression of the 47 or 25 kDa HER-2 cleavage products (but not the 22 kDa fragment) was sufficient to induce apoptosis by a caspase-dependent, Bax/Bak-dependent, and Bcl-2-suppressible mechanism. Third, the 47 kDa caspase cleavage product was present in the mitochondrial fraction of SKBR3 cells undergoing apoptosis. Fourth, ectopic

expression of the 47 and 25 kDa products lead to their mitochondrial localization and Bcl-x_L-suppressible cytochrome c release. Fifth, both the 47 and 25 kDa products share a BH3-like domain which was required for apoptosis induction and cytochrome c release from isolated mitochondria: mutation of one or both highly conserved Leu or Asp residues in this BH3-like domain inhibited these activities. Finally, a full-length HER2 protein containing the 2XE BH3 domain mutant was cleaved by caspases but conferred greater protection against apoptosis than wild type HER-2 due to the lack of a pro-apoptotic BH3 domain in the mutant protein, thereby providing additional evidence supporting the functional relevance of the BH3 domain in HER-2. Collectively, these results strongly suggest that HER-2 contains a *bona fide* BH3-like domain, which is normally anchored at the cell membrane, and is released by caspase proteolysis, resulting in its mitochondrial translocation and induction of MOMP.

1. HER-2 IS A NOVEL CASPASE SUBSTRATE

1.1 HER-2 is cleaved by multiple caspases

We have shown that the cytoplasmic tail of HER-2 is cleaved at four sites (Asp^{1016}, Asp^{1019}, Asp^{1087} and Asp^{1125}) by the initiator caspase-8 and by the effector caspases-3, -6 and -7 *in vitro* and in multiple breast cancer cell lines treated with apoptotic stimuli which engage the intrinsic (etoposide) or extrinsic pathway (TRAIL). Caspases cleave HER-2 sequentially: cleavage at Asp^{1016}/Asp^{1019} releases a carboxyl terminal 47 kDa fragment which is subsequently processed by caspases into an unstable 22 kDa and a predicted 25 kDa product. Although we were unable to visualize the 25 kDa product (encoding amino acids 1017-1125) in breast cancer cells with existing antibodies, it is likely that this product is the third band observed in our *in vitro* studies

with the partial HER-2 cDNA 158-5E. Supporting this hypothesis is the fact that a D1016E mutant failed to generate this product (Figure 3.10A). Further, a cloned cDNA encoding amino acids 1017-1125 migrates with an apparent molecular weight of 25 kDa in breast cancer cells (data not shown). In breast cancer cells, mutation of Asp^{1125} caspase uncovered a fourth cleavage site, Asp^{1087}. Importantly, mutation of four distinct caspase cleavage sites (D1125E/ D1087E/ D1016E/D1019E, designated 4X HER-2) was necessary to generate a caspase cleavage resistant HER-2 protein.

1.2 Caspase cleavage of HER-2 is more complex than previously recognized

Although caspase proteolysis of HER-2 at Asp^{1087} and Asp^{1125} have been reported (Benoit et al., 2004; Tikhomirov and Carpenter, 2001), our findings indicate that the apoptotic proteolysis of HER-2 is more complex than previously recognized and has profound functional consequences for cell death regulation. Our data is in agreement with the work of Tikhomirov and others, who detailed the caspase dependent nature of a 23 kDa fragment (designated as the 22 kDa fragment in this work) which was generated following treatment with curcumin, geldanamycin, or staurosporin in transfected Cos-7 and SKBR3 cells. While the authors hypothesized the existence of a 40 kDa pro-apoptotic HER-2 cleavage product in their discussion, data to support this theory is not presented. The cleavage resistant HER-2 mutant in presented in the Benoit studies carries mutations in only two of the four caspase cleavage sites, Asp^{1087} and Asp^{1125}. This data is in marked contrast to our work, in which the D1087/D1125 mutant is readily cleaved by caspases *in vitro* and *in vivo* (Figures 3.10 and 3.12).

1.3 Consequences of caspase cleavage of HER-2

Caspase cleavage of HER-2 liberates cytoplasmic cleavage products from the plasma membrane which translocate to mitochondria and activate the intrinsic cell death pathway. Importantly, the amino terminal portion of the receptor (Trun HER-2) remains anchored in the plasma membrane, and retains the entirety of the tyrosine kinase domain as well as the cysteine rich regions in the extracellular domain which mediate interactions with other EGFR family members. Thus, caspase cleavage of HER-2 is not predicted to affect the kinase activity of the receptor, although this finding has not been experimentally confirmed. If true, the consequences of HER-2 cleavage stand in opposition to a body of literature in which caspase cleavage of receptor tyrosine kinases resulted in either constitutively active (Fischer et al., 2003) or inactivated kinase function (Bae et al., 2001).

2. STABILITY OF THE HER-2 CLEAVAGE PRODUCTS

2.1 Stability of the HER-2 cleavage products does not conform to N-end rule predictions

The stability of the observed HER-2 proteolytic products is unexpected. The N-end rule states that the half life of a peptide is determined by the identity of its amino terminal residue(Varshavsky, 1997). In this model, the 47 kDa HER-2 product is predicted to be unstable and rapidly degraded by the proteasome due to the presence of an amino terminal leucine (cleavage after D^{1016}) or alanine (cleavage after D^{1019}); both residues are designated as primary destabilizing residues in the N-end rule pathway. However, this product was only weakly stabilized by proteasome inhibitors (Figures 3.4-3.5). In contrast, the 22 kDa product which we and others have shown to be labile and rapidly by the 26S proteasome is predicted to be stable by

these guidelines due to the presence of an exposed amino terminal glycine residue. Thus, stability of the HER-2 proteolytic products does not conform to the predictions set forth by the N-end rule.

2.2 Observed stability of the HER-2 cleavage products

Addition of apoptotic stimuli led to a transient production of a 47 kDa HER-2 product, which was subsequently processed into a 25 kDa product and a 22 kDa product, the latter of which was rapidly degraded by the proteosome. The stability of the pro-apoptotic 25 kDa HER-2 product is unknown, although it is tempting to speculate that the predicted casein kinase II phosphorylation site at D1125 (which is the caspase cleavage site in the 47 kDa HER-2 product which, when cleaved, gives rise to the 22 and 25 kDa products) would enhance the stability of this pro-apoptotic product. Interestingly, phosphorylation of Bid by casein kinase II rendered it resistant to caspase-8 cleavage and protected type II cells from Fas induced apoptosis, demonstrating the plausibility of this mechanism (Desagher et al., 2001). If true, this would be the first report of inhibition of caspase action by casein kinase II resulting in apoptosis; typically, CKII's actions promote cell survival (Izeradjene et al., 2005; Litchfield, 2003). The generation of cleavage specific reagents (e.g. antibodies) would greatly facilitate these studies. However, in the absence of these reagents, pulse chase analysis could be used to determine the stability of the 25 kDa HER-2 product.

We provided evidence that the caspase truncated HER-2 was sufficient to signal to the Akt and MAP kinase pathways, raising the possibility that HER-2 may simultaneously signal to both pro

and anti-apoptotic pathways. Unfortunately, the truncated HER-2 was not visualized in our SKBR3 time courses when probed with a HER-2 antibody containing an amino terminal epitope (Figure 3.4-3.5, amino terminal antibody data not shown), thus simultaneous signaling from the receptor cannot be confirmed at this point in time. However, this issue clearly warrants further investigation.

Sensitivity to the pro-apoptotic HER-2 products may be determined by the expression level of the HER-3 coreceptor in the cell, explaining, in part, why ectopic expression of the 47 kDa HER-2 into SKBR3 cells (which possesses high HER-3 levels) was insufficient to induce apoptosis (data not shown); while the MDA-MB-231 cell line (which possesses modest amounts of HER-3) was sensitive to apoptosis induced by the HER-2 products. Recall that HER-3 links the HER-2 receptor to the Akt/PI3K signaling axis, thus lower expression of HER-3 may result in weaker signaling to this survival pathway. To test this hypothesis directly, the levels of HER-3 in a panel of cell lines known to be sensitive or resistant to HER-2 cleavage product induced apoptosis should be compared by western blot. If, in fact, a correlation between high HER-3 expression levels and resistance to HER-2 product induced apoptosis is observed, small interfering RNA (siRNA) or function blocking antibodies directed to HER-3 should be able to sensitize previously resistant cells to HER-2 product induced apoptosis. These studies may have important clinical ramifications. Specifically, downregulation of HER-3 may enable the HER-2 BH3 protein to induce apoptosis in cells in response to apoptotic stimuli which liberates the HER-2 BH3 domain from the plasma membrane.

The activity of the truncated HER-2 is also worthy of further study. Although we determined that ectopic expression of the truncated receptor in SKBR3 cells did not induce cell death, the more plausible scenario, in which the truncated HER-2 binds to HER-2 (and other family members) and acts as a dominant negative was not examined. Indeed, a naturally occurring HER-2 inhibitor, Herstatin contains the first 340 amino acids of HER-2 and is sufficient to impair signaling from the receptor (Doherty et al., 1999). Further, the transformative capacity of the truncated HER-2 should be investigated in colony formation assays and animal models. Previous studies in NIH 3T3 cells expressing a truncated HER-2 (amino acids 1-1132) was less transforming in soft agar and in xenograft studies, underscoring the importance of the carboxyl terminal tyrosine phosphorylation sites in HER-2 (Mikami et al., 1992). Data obtained with the caspase truncated HER-2 would identify the significance (if any) of residues 1017-1132 in HER-2 for transformation and tumor formation.

2.3 Importance of the activation state of the HER-2 kinase

The activation state of the HER-2 kinase was not studied in this work. However, it would be interesting to determine whether caspases preferentially target activated HER-2 for destruction. This would be analogous to the E3 ubquitin ligase c-Cbl, which only recognizes phosphorylated tyrosine residues and mediates ligand-dependent turnover of growth factor receptors. To test this hypothesis, wild type or a kinase dead HER-2 mutants would be expressed in breast cancer cells, serum starved, treated with ligand and observed for both stability of the receptor and kinetics of cleavage in response to apoptotic stimuli.

2.4 Coordinated destruction of HER-2 via caspases and the 26S proteosome

2.4.1 The 22 kDa HER-2 product is degraded by the proteosome

We and others have concluded that the 22 kDa HER-2 product is degraded by the 26S proteosome due to its stabilization by proteosome inhibitors (Figures 3. 4-3.7 (Tikhomirov and Carpenter, 2001)). However, the ubiquitin-22 kDa HER-2 complex has not been visualized directly. This could be achieved by transfection of an HA-tagged ubiquitin plasmid into breast cancer cells expressing wild type HER-2 and subsequently treated with proteosome inhibitors in combination with apoptotic stimuli. Lysates collected at various time points could then be immunoprecipitated with an HA antibody and immunoblotted with a HER-2 antibody containing a carboxyl terminal epitope. Direct interaction with ubiquitin would be indicated by the presence of higher order 22 kDa HER-2 products in cells transfected with HA-ubiquitin, but not HA-vector or IgG control.

2.4.2 The 22 kDa HER-2 product may be a substrate for N terminal ubiquitination

Intriguingly, addition of a FLAG epitope tag to the amino terminus of the 22 kDa HER-2 product prevented its degradation by the 26S proteosome, raising the possibility that this product may be an N-terminal ubiquitin substrate. In contrast to ubiquitination in which the ubiquitin moiety is attached to an internal lysine residue, N-terminal ubiquitination attaches the ubiquitin moiety to the exposed amino terminal residue of a protein (Ciechanover and Ben-Saadon, 2004). To determine whether the 22 kDa HER-2 product is an N-terminal ubiquitination substrate, the stability of a lysine-less (K1238R, K1171R) non-tagged 22 kDa HER-2 product should be observed *in vitro* and *in vivo.* Further, the stability of amino and carboxyl FLAG tagged 22 kDa

products should be compared. Efficient degradation of both the lysine-less and carboxyl FLAG tagged HER-2 products (but not the N FLAG tagged product) would indicate that HER-2 should be added to the growing list of N terminal ubiquitin substrates (Fajerman et al., 2004). The significance of this modification remains unclear.

3. CASPASE CLEAVAGE OF HER-2 RELEASES TWO PRO-APOPTOTIC PRODUCTS

3.1 Cleavage of HER-2 is cell line and stimulus independent

Endogenous HER-2 was cleaved with differing efficancies in the breast cancer cell lines SKBR3, MDA-MB-453 and T47D in response to the DNA damaging agent etoposide or the tumor specific cytokine TRAIL. However, neither of these agents are used in the management of HER-2 positive breast tumors. Thus, it will be important to determine whether clinically relevant agents such as Taxol and Doxorubicin similarly cleave HER-2. An intrieguing possibility is that the humanized monoclonal antibody Herceptin would also lead to cleavage of HER-2 as this agent has been reported to weakly activate caspases. (However, the mechanism by which an antibody induces caspase activation requires further investigation.) Our prediction, based on our own work and that of others is that any stimuli which activates caspases will lead to HER-2 proteolysis. Cleavage of the receptor would allow trafficking of the BH3-like product to the mitochondria where it contributes to the execution of cell death by displacing ‘activator’ BH3s from anti-apoptotic Bcl-2 family members.

3.2. Characterization of the pro-apoptotic function of the HER-2 products

3.2.1 Cell death function of the HER-2 products is cell line independent

The pro-apoptotic activity of the HER-2 products was shown in two cell lines, the MDA-MB-231 human breast cancer cell line and the SV40 large T antigen immortalized Bax/Bak wild type mouse mouse embryonic fibroblasts (Figures 3. 21 and 3.29). However, these data do not represent the full spectrum of breast cancer. Molecular profiling studies by Perou and coworkers has led to the classification of breast cancer tumors (and cell lines) into several distinct subtypes: luminal epithelial tumors which are estrogen receptor (ER) positive, 'normal' breast like, HER-2 positive/ER negative, and the so-called 'triple negative' basal cluster, characterized by the absence of HER-2 and the estrogen and progesterone receptors (Sorlie et al., 2001). The MDA-MB-231 cell line is basal-like and is characterized by low expression of the HER-2 receptor, thus it will be important to confirm the pro-apoptotic function of the HER-2 products in HER-2 positive (such as T47D) and ER positive, luminal epithelial (such as MCF-7) cancer cell lines.

3.2.2 Cell death induced by HER-2 products is caspase-dependent

Genetic (p35) and pharmacologic (zVAD-fmk) inhibition of caspases prevented the induction of HER-2 product induced cell death (Figure 3.21). These data indicate that apoptosis induced by the HER-2 products requires amplification of the cell death signal by the caspases, similar to tBid induced cell death (Li et al., 1998).

3.3 HER-2 products translocate to mitochondria

Caspase cleavage of HER-2 releases cytoplasmic fragments which translocate to, and exert their effects at the mitochondria (Figure 3.23). However, the observation that only 50 and 60 % of the ectopically expressed products co-localize with mitochondria is somewhat surprising. No diffuse cytoplasmic staining was observed in cells transfected with the wild type 25 or 47 kDa HER-2 products which would indicate non-specific localization, thus it is possible that the additional 40 to 50% of the cells may have undergone apoptosis. Indeed, significant numbers of detached cells were observed in wells transfected with the wild type products. In contrast, very few detached cells were observed in cells transfected with the BH3 mutants although diffuse cytoplasmic stained cells were noted. However, conservative scoring of co-localization, requiring the presence of three or more points per cell in which mitochondrial DNA (DAPI), FLAG tagged HER-2 products (FLAG), and mitochondria (Mitotracker) were co-localized, may underrepresent the subcellular localization of the HER-2 products. The comparable localization of the wild type or HER-2 BH3 mutants indicates that regions other than the BH3 domain are required for mitochondrial localization of these proteins (Figure 3.33). Similarly, deletion of the BH3 domain in the p53 responsive BH3 only protein Puma had no effect on its mitochondrial localization (Nakano and Vousden, 2001).

3.3.1 Mechanism of transport to mitochondria is unknown

The mechanism of transport of the HER-2 products to the mitochondria is unclear. Neither of the HER-2 products posess a mitochondrial targeting sequence as predicted by MITOP (data not shown) (Claros and Vincens, 1996).

N terminal glycosylation faciliates the translocation of tBid from the cytoplasm to the outer mitochondrial membrane (Zha et al., 2000). Two other caspase-truncated proteins which are targeted to mitochondria (actin and gelsolin) have also been shown to be N-myristoylated on amino terminal glycine residues (Sakurai and Utsumi, 2006; Utsumi et al., 2003). Thus, we examined whether mitochondrial localization of the HER-2 product could be mediated by N terminal glycosylation. However, neither the 47 nor 25 kDa HER-2 cleavage products contain an amino-terminal glycine residue, suggesting that mechanisms other than N-myristoylation are likely to be responsible for targeting the HER-2 products to mitochondria.

Small Ubiquitin-related Modifer -1 (SUMO-1) is a member of a ubiquitin-like protein family. In constrast to ubiquitination which targets substrates for destruction, sumoylation is a reversible posttranslational modification which alters the subcellular localization of its substrates (Muller et al., 2001). Although sumolyation has historically been associated with cytoplasmic to nuclear translocations, Harder and coworkers reported mitochondrial targeting of the dynamin related protein-1 (DRP-1) following Sumo-1 conjugation (Harder et al., 2004). Thus, we examined whether the HER-2 products might be similarly modified. However, examination of the sequence shared by the two pro-apoptotic products revealed an absence of the canonical sumoylation recognition motif (BK-x-D/E; where B is any hydrophobic amino acid, K is lysine, and D/E represent charged amino acids), indicating that the mitochondrial localization of the HER-2 products does not involve sumolyation. Similarly, the absence of lysine residues in the 25 kDa

HER-2 product rules out mono-ubiqutination as a means of trafficking to the mitochondria, the mechanism by which p53 is targeted to mitochondria (Marchenko et al., 2007).

Clearly further experiments are needed to define the residues in the HER-2 products which mediate the mitochondrial trafficking of this protein. Alanine scanning mutagenesis of the HER-2 sequence shared by the pro-apoptotic products (amino acids 1017-1125) is likely to identify the regions of HER-2 needed for targeting to the mitochondrial membrane. This approach is superior to traditional deletion mapping studies, which may result in altered conformation of such a small protein.

3.4 Apoptosis induced by the HER-2 products is Bax/Bak dependent

Naresh and coworkers report that the ErbB4 BH3 domain is dependent on the presence of Bak, but not Bax, for its pro-apoptotic function drawing attention to a non-redunant role for these proteins in apoptotic signaling (Naresh et al., 2006). While we demonstrated a requirement for Bax and Bak for induction of cell death by the HER-2 products, further investigation is needed to define the individual role of Bax and Bak in HER-2 product induced apoptosis.

4. IDENTIFICATION OF HER-2 AS A NOVEL BH3-LIKE PROTEIN

We identified a novel BH3-like domain in a sequence shared by the 47 and 25 kDa HER-2 products (^{1120}LPSETDGYVA^{1129}) which is required for induction of cell death. Based on the existing crystal structures of BH3 proteins complexed with Bcl-x_L, leucine 1120, aspartate 1125 and the charge conserved tyrosine 1127 are predicted to be buried within the hydrophobic pocket

of Bcl-x_L (Oberstein et al., 2007; Sattler et al., 1997). Moreover, the charge conserved tyrosine residue at 1127 is similar to the phenylalanine found in this position in the BH3 domains of Bad, Bim, Bcl-2 and Bcl-x_L (Figure 3.27). Finally, the HER-2 BH3-like domain contains several hydrophobic and two negatively charged residues, analogous to Bid.

Intriguingly, Asp^{1125} is also the residue in the 47 kDa HER-2 product which is cleaved by caspases to generate the amino-terminal 25 kDa fragment, therefore it was critical to confirm that the reduction in apoptosis induced by the 25 kDa/ D1125E HER-2 mutant was due to destruction of a conserved BH3 residue rather than as a result of defective caspase proteolysis. This confirmation came from the comparision of the 47 kDa/D1125E and the fully processed 25 kDa/D1125E mutants. The similar impairment of the pro-apoptotic function of the two proteins affirms that the impairment of apoptosis induced by these proteins is a consequence of destruction of a BH3 motif and not due to inhibition of caspase cleavage.

While we have demonstrated translocation to mitochondria, induction of cytochrome c release in cells and from (primed) isolated mitochondria, and inhibition of these effects by Bcl-2 and Bcl-$x_{L,}$ several pieces of data do not fit well with the identification of HER-2 as a BH3-only protein, thus cell death induced by the pro-apoptotic products must be attributed to a BH3-like mechanism. These data are discussed below.

4.1 Problematic data with BH3-like hypothesis

4.1.1 Structure

First and foremost, the BH3 domain is a structural motif which is characterized by the presence of between two and four hydrophobic residues and two charged residues which are important for interaction with the hydrophobic cleft formed by the BH1-3 domains of the multidomain anti-apoptotic members of the Bcl-2 family. Importantly, the domain is typically an amphipathic α helix. As shown in Figure 3.27, the sequence shared by the pro-apoptotic products is not predicted to be α helical. Particularly problematic in the sequence is the presence of a proline residue which is expected to disrupt helical domains.

4.1.2 Cleavage within the BH3 domain

Additionally, cleavage in the middle of the BH3 domain is unprecedented. Surprisingly, caspase cleavage did not alter the pro-apoptotic ability of these products (which were similar in all assays tested), given the importance of structure in this domain. Our cytochrome c ELISAs were conducted with peptides mimicking the 47 product (that is, containing flanking sequences not found in the 25 kDa HER-2 product). A side by side comparison of the ability of the 25 (BH3 truncated) and the 47 kDa HER-2 product to induce cytochrome c release in this assay could shed light on the structural requirement of the BH3 peptides.

4.1.3 Incomplete inhibition of cell death in BH3 mutants

We noted a diminution of cell death induced by the HER-2 pro-apoptotic fragments when or or both of the conserved residues in the putative BH3 domain were mutated to glutamic acid

consistent with these residues playing an important role in HER-2 mediated cell death (Figure 3.28). Mutation of both residues (2XE mutant) reduced cell death induced by the 25 kDa HER-2 product by 2.5 fold, however 15% cell death was still observed, suggesting that other factors are involved in HER-2 product induced cell death. However the modest reduction in cell death is inconsistent with results obtained with analogous BH3 mutants in Puma (Nakano and Vousden, 2001), and Noxa (Oda et al., 2000), although similar to the magnitude of impairment reported for non-canonical BH3 proteins (discussed below).

4.1.4 BH3 mutant interacts with Bcl-x_L

Moreover, the 25 kDa BH3 mutant (2XE) was able in interact with Bcl-x_L by immunopreciptation. This data stands in contrast to the work with the crystal structures of BH3 proteins/peptides in complex with antiapoptotic Bcl-2 family members. For example, mutation of the L in the BH3 domain of Bak reduced its interaction with Bcl-x_L by a magnitude of 800 (Sattler et al., 1997). However, mutagenesis studies of the Bid BH3 domain by Korsmeyer's group identified a mutant which retained binding to Bcl-2 (G94A; located within the GDE motif), but was impaired in its pro-apoptotic activities, uncoupling these properties and echoing what we have observed with the HER-2 BH3 mutant (Wang et al., 1996). One possible explanation for this preserved interaction may be the charge conserving mutation of D1125E, which may still be sufficient to support the interaction with asparagine 139 of Bcl-x_L. If this were true, a BH3 mutant carrying alanines in place of the L1120 and D1125 (2XA) may fail to interact with Bcl-x_L, although this has not yet been experimentally tested.

4.1.5 Alternative explanation of data

However, it is possible that the HER-2 products induce caspase dependent apoptosis in a BH3-independent manner. Indeed, cytoplasmic p53 translocates to mitochondria where it directly interacts with Bcl-x_L and Bcl-2 via the hydrophobic pocket occupied by BH3-only proteins and induces cell death despite the absence of a BH3-like domain (Mihara et al., 2003; Petros et al., 2004). Similarly, the protein SIVA-1 lacks a BH3 motif but possesses an α helical region which mediates its interaction with Bcl-xL and is suffient to sensitize cells to UV radiation induced apoptosis (Xue et al., 2002).

4.1.6 Experiments to confirm BH3-like mechanism

However, given the requirement of the L1120 and D1125 residues in HER-2 for cytochrome c release and induction of cell death, it is likely that the pro-apoptotic products induce apoptosis via a BH3-like mechanism. However, this hypothesis would be greatly strengthened by a crystal structure of the HER-2 BH3 peptide in complex with Bcl-x_L, as lack of a predicted alpha helical domain is the most inconsistent piece of data with this mechanism. Circular dichroism experiments with the HER-2 BH3 peptide would also be instructive. Further proof of concept experiments could involve replacement of the Bad BH3 domain with that the HER-2 BH3-like domain.

Nonetheless, several novel BH3 proteins have been reported which, like the HER-2 BH3-like product, are conserved in only a small fraction of residues within the BH3 motif, are weakly apoptotic and interact with anti-apoptotic multidomain family members. These candidate BH3

proteins include Beclin-1 (domain: LKVTGDLF) (Oberstein et al., 2007)(Maiuri *et al. EMBO epub*), MAP-1 (domain: LSRALGHE) (Tan et al., 2001), Spike (domain: LDALGDEL) (Mund et al., 2003), Tissue Transglutaminase-1 (domain: LKNAGRDCSR)(Rodolfo et al., 2004), and Sphingosine kinase 2 (domain: LNGLLDRPD)(Liu et al., 2003). Perhaps these loosely conserved BH3 candidate proteins represent more recently evolved BH3 proteins than the established BH3 proteins such as Bim and Bax (Aouacheria et al., 2005). As more and more functionally validated BH3-like proteins are identified, our notion of what constitutes a BH3 domain may require revision.

4.2 HER-2 is a Bad-like BH3 protein

We demonstrated that the 25 kDa HER-2 product acts in concert with tBid to induce apoptosis in breast cancer cells overexpressing Bcl-x_L, mimicking the actions of the BH3 protein Bad (which binds Bcl-x_L and Bcl-2) but not Noxa (which binds Mcl-1) (Chen et al., 2005; Kim et al., 2006; Letai et al., 2002). These findings are consistent with previous reports indicating an at least additive effect between tBid and Bad and suggest that the HER-2 BH3-only protein is more Bad-like than Bid-like, designating the HER-2 BH3-like protein as a sensitizer (Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002).

Closer examination of the experimental systems employed in these studies supports the designation of the HER-2 product as Bad-like. First, the pro-apoptotic function of the HER-2 cleavage products was demonstrated in two cell lines, MDA-MB-231 breast cancer cells and SV40 immortalized mouse embryonic fibroblasts. An expression panel of anti-apoptotic

Bcl-2 family members present in the MDA-MB-231 system reveals low Bcl-2 expression, moderate Bcl-x_L expression and no detectable Mcl-1 ((Zapata et al., 1998) and data not shown). Thus, we would predict that ectopic expression of Bad would be sufficient to induce apoptosis in this system, due to its ability to neutralize Bcl-2 and Bcl-x_L. Similarly, Zong and coworkers demonstrated that retroviral infection of a constitutively active Bad (3SA) mutant induced apoptosis in the Bax Bak wild type MEFs (Zong et al., 2001). Further, 'sensitizer' BH3 proteins including Bad have been reported to induce cytochrome c release from primed mitochondria isolated from leukemia cells(Certo et al., 2006). Again, this is consistent with the data obtained with the HER-2 pro-apoptotic products. However, true proof of concept experiments which define the panel of anti-apoptotic Bcl-2 proteins neutralized by the 25 and 47 kDa HER-2 have not be conducted. The immortalized but not transformed breast cancer cell line MCF10A may be an ideal setting for these experiments due to equivalent expression of Bcl-x_L and Mcl-1 (the latter is rarely expressed in breast cell lines). If cell death induced by the HER-2 products is augmented by co-infection with Bad or Noxa, HER-2 may be definitively classified as a 'sensitizer' BH3 (or in the terminology of model II, limited specificity binders). Alternatively, it is possible that no augmentation in cell death would be observed, indicating that the HER-2 products are weak activator BH3s (or BH3s with a broad specificity of interactions with the anti-apoptotic family members). Defining the panel of anti-apoptotic Bcl-2 proteins which the HER-2 BH3 protein can interact with will add to the evidence for classification of the HER-2 BH3 protein as an activator or sensitizer. Related experiments might examine (via immunoprecipitation) whether the cooperation observed between tBid and the HER-2 BH3-like product is indeed due to a displacement of tBid from Bcl-x_L. Clearly, these are important

experiments for the understanding of the mechanism of cell death induced by the HER-2 pro-apoptotic products. Similarly, the ability of the HER-2 BH3-like product to induce Bax/Bak oligomerization would aid in the designation of the protein as activator or sensitizer. Inclusion of the modest activator Puma in these studies would be particularly important. These experiments could be conducted with BH3 peptides, or radiolabeled full length peptides added to mitochondria isolated from healthy mouse livers. Cytochrome c release by these proteins/peptides could also be assayed. Importantly, in this 'non-primed' setting, senisitizer BH3s will be unable to oligomerize Bax and/or Bak or release cytochrome c.

Our results are consistent with both models of BH3 ligand-induced activation of Bax and Bak. Caspase cleaved HER-2 promotes tBid induced cytochrome c release by neutralizing Bcl-x_L, acting either as a Bad-like "sensitizer" BH3-only protein, which displaces the activator BH3-only protein tBid from the tBid-Bcl-x_L complex ("direct activation" model), or as a Bad-like inhibitor of Bcl-x_L that displaces Bax and Bak ("indirect activation" model) from the tBid-Bcl-x_L complex.

While this work was being conducted, Tikhomirov and coworkers demonstrated that cells expressing a HER-2 product containing amino acids 1-1226 of human HER-2 were sufficient to form colonies in soft agar when expressed in Cos-7 cells (Tikhomirov et al., 2005). Although this product encompasses the HER-2 BH3-like domain reported in our studies, it was likely sequestered at the plasma membrane, and no death stimuli was applied in these experiments to cleave the receptor allowing its translocation to the mitochondria and activation of the intrinsic

cell death pathway. Moreover, the pro-apoptotic fragments of HER-2 described in this paper required a death inducing motif (RLLGI resides 783-788) which was not part of the predicted HER-2 BH3 domain.

4.3 Potential role of the HER-2 BH3 domain

Recent studies suggest that activation of intracellular BH3 domains in transmembrane receptors may be a more generalized mechanism of caspase amplification than previously recognized. Indeed, HER-4/ErbB4 has recently been shown to contain a BH3-like intracellular domain (sequence: ^{986}LVIQGDDDRMK^{996}) which is released by γ-secretase and triggers Bax/Bak-dependent apoptosis (Naresh et al., 2006). Similarly, two putative BH3 domains in HER-1/EGFR (^{979}LVIQGDER^{986} , and the weaker ^{1001}LMDEED^{1006}) have been identified by sequence analysis, although they have not been experimentally validated. In constrast, no such motif was found in the HER-3 co-receptor, and it is tempting to think that the absence of this domain is linked to the kinase deficient nature of the receptor.

Perhaps the BH3 domain serves as a built in 'brake' or means of balance/degradation of a powerful oncogene. Indeed, several other potently anti-apoptotic proteins have been shown to be converted into pro-apoptotic molecules by caspases, including the multidomain anti-apoptotic Bcl-2 family members Bcl-2, Bcl-x_L and Mcl-1 (Cheng et al., 1997; Clem et al., 1998; Weng et al., 2005). Cleavage of these proteins removes the BH4 domain, and the resulting protein contributes to the execution of cell death. Together with the data presented here, these findings suggest that cell death machinery is elegantly parsimonious with some molecules performing

both anti-and pro-apoptotic functions. Indeed, this work adds HER-2 to the list of components of the apoptotic pathway having ‘day jobs’ in addition to their roles in cell fate: Bad (metabolism), Bcl-2 (autophagy), cytochrome c (electron transport) (Cheng et al., 2006; Garrido and Kroemer, 2004).

4.4 Clinical significance of the HER-2 BH3 domain

Our observation that a full length BH3 mutant HER-2 confered greater protection against TRAIL induced apoptosis than the wild type receptor underscores the importance of the HER-2 BH3 domain in the biology of the full length receptor. Ongoing work is studying this trend in xenograft models of breast cancer, examining the contribution of the HER-2 BH3 domain (by comparision of cells expressing the 4X cleavage resistant HER-2 , 2XE BH3 mutant, or wild type HER-2) to tumor establishment, progression, and response to conventional chemotherapeutics. Control studies will confirm that any differences observed between the cell lines are not the result of altered proliferation rates.

Clinically it is well accepted that women with estrogen receptor negative/ HER-2 positive tumors respond exceptionally well to chemotherapeutics. Although this response is typically attributed to the high proliferative rate of these tumors, our data suggest that release of a latent BH3-containing cell death effector in response to chemotherapy may be involved although has not been experimentally confirmed.

Several lines of evidence suggest that HER-2 and Bcl-x may be coordinately regulated *in vitro* and *in vivo.* Kumar and coworkers noted the overexpression of HER-2 in MCF-7 breast cancer cells resulted in marked upregulation of Bcl-2 and Bcl-x_L (Kumar et al., 1996). Similar observations were made during analysis of a panel of ductal carcinoma *in situ* (DCIS) cases immunstained for expression of HER-2, Bcl-2 and Bcl-xL. The authors of this study noted that HER-2 expression was 'highly coordinated with the expression of the apoptosis suppressing gene Bcl-xL, but not with…Bcl-2'(Siziopikou and Khan, 2005). Perhaps this coupled regulation is a survival mechanism for the cell, whereby the overexpression of Bcl-x_L ensures that the survival function of HER-2 predominates in a particular cellular context. Importantly, this regulation was not limited to mammary epithelial cells. Downregulation of HER-2 by pharmacologic or anti-sense means altered the splicing of Bcl-x, favoring production of the pro-apoptotic splice isoform Bcl-x_S, in cardiomyocytes. Interestingly, the antracyclin daunorubicin similarly affected splicing of Bcl-x. Taken together, these data may partially explain the toxicity observed following combination treatment with Herceptin and an antracyclin to cardiomyocytes (Grazette et al., 2004; Rohrbach et al., 2005).

Our data raise the intriguing possibility that mutations in either the caspase cleavage sites or the BH3 domain could determine response to some chemotherapeutic regimens. Although the current dogma maintains that HER-2 is not mutated in breast cancer, the cytoplasmic portion of the receptor in question has not to our knowledge been examined. Significantly, when the kinase domain of HER-2 was sequenced in the wake of the clinical failure of Iressa, multiple somatic mutations were uncovered in lung (Shigematsu et al., 2005; Stephens et al., 2004), liver (Bekaii-

Saab et al., 2006) and breast cancers (Stephens et al., 2005). Thus it would be interesting to screen for somatic mutations in the caspase cleavage sites and/or BH3-like residues in HER-2 positive tumors by genomic PCR/sequencing and examine whether the presence of these mutations affects treatment response.

5. SUMMARY

We study caspase targets to find unexpected substrates like HER-2, which extend and alter our understanding of the execution of mammalian cell death. This dissertation describes a novel role for HER-2 in the regulation of apoptosis. Full length HER-2 protects cells from undergoing apoptosis by activating cell survival pathways, such as the PI3-kinase/Akt pathway (Zhou et al., 2000; Zhou et al., 2001a). However, in response to apoptotic stimuli, a functional BH3 domain in the cytoplasmic tail of the receptor is exposed which contributes to the execution of cell death. Additionally, our data add the pro-apoptotic HER-2 cleavage products to a growing list of 'candidate BH3s'/BH3-like proteins which may eventually lead to a revision of what constitutes a BH3 motif.

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EDUCATION

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1998 Certificate in Life Sciences, University of Sussex, School of Biological Sciences (BIOLS), Brighton, UK

1995-1999 B.A. in Biology; Minor in Science, Technology and Society (STS) Scripps College, [The Claremont Colleges] Claremont, CA

PROFESSIONAL EXPERIENCE

9/98-5/99 Scripps College, Keck Science Center, Claremont, CA
Joint Science Department of the Claremont Colleges. Advisor: Dr. David Sadava
Senior thesis in Biology: Avoidance of Apoptosis as a Mechanism of Multi-Drug Resistance in Human Small Cell Lung Carcinoma (SCLC)

6. Demonstrated that multiple drug resistant SCLC cells undergo significantly reduced apoptosis compared to drug sensitive SCLC by *in situ* staining and ELISA. Maintained sterile tissue cultures. Conducted cytotoxicity assays.

7/99-8/01 University of Southern California School of Medicine, Department of Molecular Microbiology and Immunology, Los Angeles, CA
Research Technician in the laboratory of Dr. J-H James Ou

7. Expressed and purified a novel HCV protein, F, from *E. Coli* inclusion bodies through a combination of electrophoretic and denaturing/renaturing protocols. This product was used in HCV diagnostic studies in collaboration with Chiron Corporation and in the generation of a polyclonal antibody to the protein.

8. Demonstrated via immunoprecipitation that F is present in the sera of HCV but not HBV patients.

1/03-4/03 Northwestern University, Chicago IL
Teaching Assistant, Molecular Basis of Drug Action

- Contributed to exam questions, proctored and assisted with grading exams
- Provided one on one assistance to students concerning basic concepts in molecular pharmacology

9/03-5/04 Northwestern University, Chicago IL
Co-organizer of Tumor Cell Biology (TCB) Student Journal Club

2001- 2007 Northwestern University, Feinberg School of Medicine, Chicago IL
Ph.D. Candidate, Integrated Program in the Life Sciences Advisor: Dr Vincent Cryns

Dissertation: HER-2/neu releases a pro-apoptotic BH3-like cell death effector

- Identified multiple caspase cleavage sites in the carboxy terminus of HER-2 *in vitro* and *in vivo*
- Demonstrated that caspase cleavage of HER-2 releases pro-apoptotic products
- Identified a functional BH3 domain in HER-2 which contributes to cell death in breast cancer cells

PUBLICATIONS

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ACADEMIC HONORS

Stone Award for Excellence in Cancer Research, Northwestern University, 2007
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Associate Member of American Association for Cancer Research, 2007
Associate Member of Sigma Xi, the Scientific Research Society, Claremont Chapter, 1999
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PRESENTATIONS

Anne M. Strohecker, Feng Chen, Fruma Yehiely, and Vincent L. Cryns. "Caspase Cleavage of HER-2 Promotes Apoptosis by Releasing a BH3 Domain Containing Product" (Oral presentation at Apoptosis Minisymposium) AACR Annual Meeting, Los Angeles, CA (April 14-19, 2007)

Anne M. Strohecker, Fruma Yehiely, Vincent L Cryns. "Caspase Cleavage of HER-2 releases a BH3-Like Cell Death Effector" (Poster) Annual Lewis Landsberg Research Day, Chicago IL. (March 7, 2007)

Anne M. Strohecker, Feng Chen, Fruma Yehiely, and Vincent L. Cryns **"**Caspase Cleavage of HER-2 Subverts its Anti-Apoptotic Function by Releasing a Pro-Apoptotic Fragment" (Poster) AACR Special Conference "Regulation of Cell Death in Oncogenesis", Waikoloa, Hawaii. (January 26–30, 2005).