University of Kentucky
UKnowledge

Theses and Dissertations--Molecular and Cellular Biochemistry

Molecular and Cellular Biochemistry

2012

PROTEASOME REGULATION OF CASPASE-8: SIGNIFICANCE IN CANCER

Michael Vincent Fiandalo
University of Kentucky, mvfian2@uky.edu

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation

Fiandalo, Michael Vincent, "PROTEASOME REGULATION OF CASPASE-8: SIGNIFICANCE IN CANCER" (2012). *Theses and Dissertations--Molecular and Cellular Biochemistry*. 3.
https://uknowledge.uky.edu/biochem_etds/3

This Doctoral Dissertation is brought to you for free and open access by the Molecular and Cellular Biochemistry at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Molecular and Cellular Biochemistry by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Michael Vincent Fiandalo, Student

Dr. Natasha Kyprianou, Major Professor

Dr. Kevin Sarge, Director of Graduate Studies

PROTEASOME REGULATION OF CASPASE-8: SIGNIFICANCE IN CANCER

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
Department of Molecular and Cellular Biochemistry
at the University of Kentucky

By

Michael Vincent Fiandalo

Lexington, Kentucky

Director: Dr. Natasha Kyprianou, Professor of Urology, Molecular and Cellular
Biochemistry, and Toxicology

Lexington, Kentucky

Copyright © Michael V. Fiandalo 2012

Abstract of Dissertation

PROTEASOME REGULATION OF CASPASE-8: SIGNIFICANCE IN CANCER

Anti-tumor therapeutic strategies based on combinations of chemotherapeutic agents with a death inducing ligand such as TNF-α Related Apoptosis Inducing Ligand (TRAIL), are directed towards selective and effective cancer cell apoptosis and enhanced therapeutic response. We previously demonstrated that proteasome inhibition sensitizes TRAIL resistant prostate cancer cells to TRAIL-mediated apoptosis via stabilization of the active p18 subunit of initiator caspase-8. The present study investigated the functional link between caspase-8 and the proteasome, by analyzing the impact of caspase-8 ubiquitination and proteasomal degradation on the outcomes of the extrinsic apoptosis pathway in cancer cells. Caspase-8 ubiquitination status was assessed by polyubiquitin immunoprecipitation (IP) and fluorescent microscopy. Apoptosis induction in response to death receptor stimuli or proteasome inhibitor was evaluated using the Annexin V/Propidium iodide staining (PI). To determine the consequences of proteasome inhibition on caspase-8 stability, trafficking, and activity following death receptor activation, we used the TRAIL-resistant human prostate cancer LNCaP cells, and the caspase-8 deficient Neuroblastoma 7 (NB7) cells, as cellular models for reconstituting the non-cleavable mutant forms of caspase-8. Our findings demonstrate that the non-cleavable forms of caspase-8 are capable of inducing apoptosis comparably to wild-type caspase-8 upon treatment with proteasome inhibitor and GST-TRAIL. Furthermore, caspase-8 processing into its active subunits preceded caspase-8 polyubiquitination, implicating caspase-8 processing as a potential regulatory mechanism, rather than a requirement for caspase-8 activation in apoptosis induction. The mechanistic control of caspase-8 by ubiquitination in cancer cells may have significant significance in bypassing mechanisms of therapeutic resistance in human tumors and optimization of anti-cancer treatment strategies in human tumors and optimization of anti-cancer treatment strategies.

Keywords: Caspase-8, Proteasome inhibitor, TRAIL, Velcade, Apoptosis.

Michael V. Fiandalo
Student Signature

5-10-2012
Date

PROTEASOME REGULATION OF CASPASE-8 IN CANCER

By

Michael V. Fiandalo

Dr. Kevin Sarge
Director of Graduate Studies

Dr. Natasha Kyprianou
Director of Dissertation

To my parents who have always believed in me and have stood by me through out every step of this journey towards attaining my Doctorate in Biochemistry, thank you Mom and Dad for all of your love and support. I love you both.

Acknowledgements

The dissertation and the works described within are the result of five years worth of acquired experience, knowledge, and insight provided by the brilliant minds and expertise of the following.

First and foremost I would like to extend a heartfelt thank you to Dr. Natasha Kyprianou (Biochemistry). During the course of my graduate career I had several unfortunate events occur and through the chaos and uncertainty, Dr. Kyprianou was there and provided me with unlimited amounts of support. Although she has been my mentor for a short period of time during my graduate training she has provided guidance and opened my eyes to the bigger picture always reminding me that science is about much more than just pathways. Words cannot express the gratitude and immense respect I have for Dr. Kyprianou as a mentor and as a person. Thank you for all your help.

I would like to thank my former mentor Dr. Steven Schwarze (Biochemistry), for taking me on as his graduate student. Dr. Schwarze pushed me, from experimental design right down to running the actual experiments and although he pushed hard, in the end this has made me a much stronger and more independent scientist. The lessons I learned under Dr. Schwarze's tutelage are invaluable.

My committee members, Dr. Vivek Rangnekar (Radiology), Dr. Haining Zhu (Biochemistry), and Dr. Tianyan Gao (Biochemistry) for sharing their wisdom and experimental insights. Throughout my training my committee members always provided novel and exciting perspectives to my project. It has been a pleasure presenting and having scientific discussions with all of you, thank you. I would like to thank Dr. Chang-Gao Zhan (Pharmacy) for serving as my outside examiner. I would also like to thank Dr. Kevin Sarge (Biochemistry) for all of his guidance.

To my esteemed colleagues and lab mates, thank you for being there through the good and tough times. Thank you for having the patience of sitting throughout the relentless assault of presentations concerning my project, your patience and feedback were most appreciated. I would like to especially thank Sarah Martin for her help with statistical analysis, providing scientific insight, and overall being my friend, through the thick of it. I wish nothing but the best for Sarah in her career and look forward to the day when I can call her colleague again.

Lastly, I would like to thank my colleagues and friends who have shared this journey. Shaun Carlson for our weekly science coffee breaks where we shared our triumphs as well as our failures. Valerie Reeves for providing me with a good starting point for membrane fractionations, sharing the pain of writing our dissertations and defending, and most of all being a good friend who always listened. Thank you, Marie, Wehenkel, Robin and Josh Webb for their perspectives and for being good friends.

Table of Contents

Acknowledgements iii

List of Figures vi

Chapter 1 Background and Significance 1

1.1. Signaling Pathways in Cancer Development 1

1.2 Prostate Cancer 5

1.3 Mechanisms of Apoptosis Regulation 7

1.4 Caspases Key Apoptosis Regulators.....16

1.5. Caspase-8 in Death Receptor Independent and Dependant Pathways 19

1.6 Proteasome Regulation of Cellular Proteins. 22

1.7 Proteasome Inhibition. 23

Chapter 2: Materials and Methods 53

2.1. Cell Culture and Reagents 53

2.2 Cell Viability and Apoptosis Evaluation.....54

2.3. Half-life Evaluation 54

2.4. Polyubiquitin Immunoprecipitation (IP) and Western Blot Analysis 55

2.5. Subcellular Fractionation 55

2.6. Caspase-8 Activity Evaluation 56

2.7. Immunocytochemical Analysis 56

2.8. Statistical Analysis.....57

Chapter 3 Experimetnal Outcomes.....58

3.1a. Caspase-8 Activation is Independent of Caspase-8 Processing.....58

3.1b. Caspase-8 Processing not Required for Apoptosis Induction. 59

3.2a. Proteasome Inhibition Stabilizes Caspase-8 p18 Subunit. 59

3.2b. Processed Caspase-8 is Polyubiquitinated. 60

3.3a. Proteasome Inhibition Increases Caspase-8 Accumulation in the Cytosol and Plasma Membrane. 60

3.3b. Caspase-8 Co-localizes with Na^+/K^+ ATPase. 62

Chapter 4 Discussion 75

Chapter 5 Future Directions 82

5.1 Caspase-8 Polyubiquitination 82

5.2 Identification of Ubiquitinated Amino Acids in Caspase-8 and Determination of the Apoptotic Action of Stabilized Caspase-8 Mutants. 83

5.3 Role of E3 ligase POSH and Siah2 in Caspase-8 Activity Regulation.....88

Appendix.....103

References.....104

Vita 119

List of Figures

Figure 1.1.1 Mechanisms of Cancer 27
Figure 1.1.2 The Actions of p53 in Cell Cycle Arrest 29
Figure 1.1.3 AKT Pathway 31
Figure 1.3.1 Anoikis Pathway. 33
Figure 1.3.2 Intrinsic and ER Pathways of Apoptosis. 35
Figure 1.3.3 TRAIL Mediated Extrinsic Pathway of Apoptosis 37
Figure 1.3.4 TNF-α Mediated NFκB or Apoptosis Activation. 39
Figure 1.3.5 Ripoptosome Formation. 41
Figure 1.4.1 General Cleavage Events for Critical Apoptotic Related Caspases. 43
Figure 1.4.2 Unfolded Protein Response and Intrinsic Pathway Activation. 45
Figure 1.6.1 Ubiquitin Proteasome System. 47
Figure 1.6.2 26S Proteasome. 49
Figure 1.7.1Proteasome Inhibition Stabilizes Caspase-8 p18 Catalytically Active Subunit 51
Figure 3.3.1a Caspase-8 Processing is not Required for Activation or Apoptosis. 63
Figure 3.1.1b MTT Viability and Annexin V/PI Studies 65
Figure 3.2.1a Proteasome Inhibition Stabilizes Caspase-8 p18 Subunit and Extends the Half-life. 67
Figure 3.3.1a Proteasome inhibition leads to increased cleaved caspase-8 in plasma membrane. 71
Figure 3.3.1b Co-localization of caspase-8 with Na^+K^+ ATPase. 73
Figure 4.1.1 Translational significance of Caspase-8 Studies 80
Figure 5.1.1 Experimental Flow Chart and Construct Design. 95
Figure 5.1.2 Caspase-8 Autoprocessing Confers Dependence on Proteasome Inhibition for Expression and Suggests that the p18 Subunit is Polyubiquitinated. 97
Figure 5.2.1 Caspase-8 re-expression in caspase-8 deficient I9.2 Jurkat cells. 99

Chapter 1 Background and Significance

1.1. Signaling Pathways in Cancer Development

Cancer is a disease where normal healthy a cell undergoes oncogenesis through the accumulation of a series of genetic and epigenetic mutations that results in the deregulation of critical regulatory pathways and ultimately giving rise to tumorgenesis (Hanahan and Weinberg, 2000). As a result of uncontrolled signaling pathways, tumor cells acquire new characteristics (Fig. 1.1.1) such as the ability to invade surrounding tissues, recruit their own vasculature, (termed angiogenesis) and metastasize. Several major pathways are altered, including the proliferation, cell cycle progression, and pro-survival pathways which are induced while the apoptotic pathways are down-regulated. These pathways intersect one another at some point in their respective signaling cascades. Cell cycle consists of several phases; G0, G1, S, G2, and Mitosis. These phases are regulated by various cyclins and CDK (cyclin dependant kinases) and progression from one phase to another is dependent on specific checkpoints (Fig 1.1.2). One of the most heavily studied checkpoints is the G1/S checkpoint which allows a cell to enter S phase (DNA replication) (Vermeulen et al., 2003). A very critical player at this checkpoint is p53 (notoriously known as the Guardian of the Genome) due to its role in rescuing damaged DNA, via up-regulation of downstream effectors, such as p21 (induces cell cycle arrest) and PUMA which blocks anti-apoptotic players leading to apoptosis induction (Fig 1.1.2) (Haupt et al., 2003; Malumbres and Barbacid, 2009). Cell cycle regulation via p53 can primarily be abrogated by loss-of-function mutations, losing p53 activity allows for the cell to replicate regardless of DNA integrity and increases

apoptosis resistance (Wang et al., 2011). Additional mechanisms of p53 down-regulation involve the over-expression of MDM2, an E3 ligase that mediates the polyubiquitination of p53 resulting in its degradation. Over-expression of MDM2 ensures rapid degradation of p53 leading to diminished if not abolished p53 activity and unregulated cell cycle progression (Wasylyk et al., 1999). Proteasome inhibitors, agents that block protein degradation mediated by 26S proteasome, have been shown to stabilize p53 and restore p53 mediated apoptosis (Williams and McConkey, 2003). Established chemotherapeutic agents such as Mitomycin C or Doxorubicin are chemotherapeutic agents that are used to induce cell cycle arrest in a variety of epithelial cancers. These agents work to either cross link DNA (Mitomycin C) or bind directly to the DNA intercalating into the double-helix strands causing the DNA to become rigid and break (Doxorubicin) (Celli and Jaiswal, 2003). Although these agents are effective they aren't specific to cancer cells and damage surrounding healthy normal cells, tissues, and organs such as kidney (Mitomycin C) or the heart (Doxorubicin) (Bugger et al., 2011; Kumari et al., 2009). Treatment of MCF-7 breast cancer xenographs with a combination of Mitomycin C with curcumin significantly decreased Mitomycin C related side effects (Zhou et al., 2009).

Strategies involved with overcoming the toxic side effects of Doxorubicin by changing the mode of Doxorubicin delivery by encapsulating the drug in titanium nanoparticals which showed promising results (Chen et al., 2011). Circumventing the caveats associated with systemic toxicities of these chemotherapeutics involved examining other pro-survival pathways, such as the AKT signaling pathway which can also impact cell cycle regulation and growth arrest.

The AKT pathway is activated through binding of growth factors to their cognate tyrosine kinase receptors which then carry out the signal transduction through the interplay between SRC, Phosphatidylinositol-3 Kinase (PI3K) and Phosphotidylinositol-4, 5-bisphosphate (PIP_2), and Phosphotidylinositol-3, 4, 5-triphosphate (PIP_3) and a critical regulatory molecule, PTEN (Fig. 1.1.3) (Fruman et al., 1998; Hennessy et al., 2005; Hiles et al., 1992). PTEN, a phosphatase, regulates AKT activation because it dephosphorylates and converts PIP_3 to PIP_2 thus preventing PIP_3 -- AKT interaction (Sansal and Sellers, 2004). AKT is a kinase that phosphorylates several different targets such as mTOR, IKK (an inhibitory binding protein that prevents NFκB activation), and cell cycle inhibitors, (p21, p27). In cancer activation of the AKT pathway can become aberrant because of a variety of mutations that can occur within PI3K, AKT, and PTEN (Skeen et al., 2006). One of the most detrimental and oncogenic potential promoting mutations are those that render these molecules constitutively active. Constitutively active PI3K can lead to the increase in the conversion rate of PIP_2 to PIP_3 favoring PIP_3 production leading to increased AKT activation (Kang et al., 2005).

A specific AKT mutation, E17K in either AKT1 or AKT3 leads to constitutive activation and promotes increased trafficking to the plasma membrane (Carpten et al., 2007; Davies et al., 2008). PI3K and AKT activating mutations are deleterious for the cell; however, another mechanism that can elevate AKT activity to supra-physiological levels and contribute to oncogenesis is the loss of PTEN, a critical regulator of AKT activation. Interrogation of the signaling events dictated by AKT, mTOR, and PI3K has lead to the development of a powerful class of pharmacologic inhibitors. The most promising class of AKT inhibitors are the lipid based inhibitors which essentially inhibit

AKT binding to the plasma membrane. Perifosine has emerged as one of the most promising AKT inhibitors and has been through several phase II clinical trials (Brachmann et al., 2009; Dubrovska et al., 2009; Ernst et al., 2005; Rahmani et al., 2005). The mTOR inhibitors include Rapamycin and its derivatives, such as CCI-779, blocks mTOR function through similar mechanisms that primarily involve binding to the co-factor FKBP and together, Rapamycin and FBKP bind to mTOR and inhibit activity (Faivre et al., 2006). PI3K inhibitors like Wortmannin or its derivative, LY294002 bind covalently to PI3K to inhibit its kinase activity (Maira et al., 2009b). These agents however, lack specificity and new carefully designed inhibitors such as CAL-101 (Calistoga) have shown promising results in clinical trials (Maira et al., 2009a; Maira et al., 2009b; Markman et al., 2010) which are on-going at Clearview Cancer institute (2012). PI3K inhibitors exhibit higher efficacy combination with existing chemotherapeutic agents such as, an AKT or mTOR inhibitors because these combinations block two pathways, eliminating individual pathway activity as well as preventing activation of alternate or redundant non-AKT mediated pathways activated through PI3K (Courtney et al., 2010).

PTEN is the direct inhibitor of AKT because it converts AKT activating PIP_3 into PIP_2, which does not activate AKT. PTEN studies have revealed that PTEN may be down-regulated either through inactivating mutations, gene deletions, and phosphorylation of PTEN by CK2 have the same result, persistent AKT signaling that contributes to tumor formation (Vazquez et al., 2001; Vazquez et al., 2000) PTEN loss has been associated with several cancers at the advanced stage of disease, including prostate cancer(Stahl et al., 2003). PTEN mutations have been linked with aggressive

androgen dependant or androgen independent (termed castration recurrent) prostate cancer (Bismar et al., 2012; Yoshimoto et al., 2012) and evidence suggests that oncogenesis results due to the loss of AKT and cell cycle regulation (Faivre et al., 2006; Sircar et al., 2009; Squire, 2009; Suzuki et al., 1998; Vlietstra et al., 1998).

1.2 Prostate Cancer

Prostate cancer is one of the most prevalent causes of cancer releated death in males with several risk factors, such as age, race, and diet contributing towards prostate cancer development and progression (Fedewa et al., 2010; Jemal et al., 2010). Regulation of androgen signaling via the androgen receptor (AR) is critical to maintaining prostate homeostasis. The androgen axis involves conversion of testosterone, synthesized by the prostate, which is then converted into 5α-dihydrotestosterone (DHT) by 5α-reductase. Once testosterone is converted into DHT it can then bind with AR and both ligand and receptor translocate to the nucleus to activate subsequent signaling pathways (Heinlein and Chang, 2002). When prostate cells undergo tumorgenesis they take on different molecular characteristics, one of the more prominent changes is the up-regulation of androgen receptor either through gene amplification or through other processes leading to over-expression of the receptor (George and Moul, 2012). Up-regulation of the AR leads to increased activation of the AR pathways ultimately activating proliferation pathways, inhibiting apoptosis, or further sensitizing prostate cancer cells to growth factors stimuli such as EGFR (Niraula and Tannock, 2011; Stanbrough et al., 2006). Currently the best therapy for treating primary prostate cancer is castration induced androgen deprivation, which involves chemically depleting androgens in the prostate androgen inhibitors such

as abiraterone (Nelson et al., 2011). Aside from abiraterone, there are other agents being tested in clinical trials such as MDV3100 which is a competitive inhibitor blocking AR-androgen signaling (Scher et al., 2010).

Although androgen depletion strategies are effective at reducing tumor burden, there are unfortunate cases where prostate cancer can reoccur. What makes this recurrent prostate cancer difficult to treat is because these prostate cancer cells can become androgen independent rendering androgen ablation ineffective. One strategy to treat recurrent prostate cancer involves using a class of microtubule targeting chemotherapeutic agents called taxanes. These agents target microtubules, a component of the cellular cytoskeleton, and stabilize microtubules thereby preventing microtubule reorganization, and disrupting kineticore formation during mitosis (Giannakakou et al., 2000; Jordan et al., 1993). Taxanes have limited efficacy as prostate cancer can be very resistant to taxane therapies. Proposed mechanisms conferring taxane resistance involve either microtubule mutations that prevent drug binding or the cell itself pumps out the taxane through p-glycoprotein pumps at the cellular membrane (Madan et al., 2011). In addition to prostate cancer, taxanes have been used against other solid tumors such as breast, lung, ovarian, and esophageal cancers (Ehrlichova et al., 2005a; Ehrlichova et al., 2005b; Ramalingam and Belani, 2002).

Although some of the agents described above have proven effective against specific cancers they have their own caveats such as, adverse side-effects but more importantly, tumors, either primary or refractory, possess either innate or acquired resistance to these agents rendering them ineffective. Therefore investigators have sought to over-come tumor resistance by interrogating different combinations of therapeutic

strategies. Alternative approaches involve inducing or restoring the apoptotic pathways through a variety of other agents such as staurosporin, etoposide, and a new emerging class of apoptosis inducing agents, the death ligands such as TNF-α Related Apoptosis Inducing Ligand (TRAIL) an agent that will be discussed further in subsequent sections.

1.3 Mechanisms of Apoptosis Regulation

Apoptosis (programmed cell death) plays a critical role in regulating cell growth and tissue development. Therefore one of the tumor survival mechanisms is to deactivate the cell's ability to undergo any form of apoptosis mediated through the up-regulation of anti-apoptotic players as well as the down-regulation pro-apoptotic players. Since loss of apoptosis leads to tumor initiation, growth, and progression (Lowe and Lin, 2000), exploitation of apoptosis mechanisms can lead to developing new anticancer strategies, that can effectively impair the tumorgenic process.

Each pathway of apoptosis is activated by different triggers such as cell-detachment (Anoikis Fig 1.3.1), mitochondrial signals (intrinsic pathway), or death ligands (extrinsic pathway), (Fig 1.3.1-1.3.3). A mechanism designed to protect against cellular metastasis is anoikis, which is an apoptosis program that is induced upon the loss of critical protein interactions between the cell and the extracellular matrix. The major players and pathways involved with anoikis are integrin, focal adhesion, and growth factors (IGF-1) interactions as well as the JNK pathway, and caspase activation signaling events (Zhan et al., 2004). Anoikis plays a role in all tissue and cell types preventing the detachment and migration of epithelial cells (Frisch and Screaton, 2001; Zhan et al., 2004). Examples include some shedding of colon epithelial cells (Bullen et al., 2006;

Strater et al., 1996) and mammary gland reduction (Haenssen et al., 2010; Lund et al., 1996). Anoikis is initiated when adherent cells detach from the basement membrane, more specifically, the loss of integrin (either α5 or β3) signaling with the focal adhesion points (Frisch and Ruoslahti, 1997; Vachon, 2011). Upon cell detachment and loss of integrin signaling the apoptotic pathways are activated (Fig. 1.2.2). Cancer cells can inhibit anoikis through a variety of mechanisms such as over-coming the loss of FAK (focal adhesion kinase), acquiring mutations in FAK that induce anoikis inhibitory mechanisms (Zouq et al., 2009) or influence from the external cellular environment may provide signals that result in shutting down the apoptotic pathways (Rennebeck et al., 2005).

There are two pathways of classical apoptosis, the intrinsic and extrinsic pathway. The intrinsic pathway is under heavy regulation by several different types of molecules that can be separated into two main classes, anti-apoptotic proteins such as the XIAP (inhibitors of apoptosis), BCL family proteins such as BCL_2, BCL_{xl} or the pro-apoptotic proteins which include, BCL family members; BAX, BAD, BID, SMAC, and Diablo are activated through signaling events that lead to mitochondrial outer membrane permeabilization (MOMP). Cytochrome C is released, binds with APAF-1 and caspase-9 to form the apoptosome (Siu et al., 2008). Upon apoptosome formation, caspase-9 becomes catalytically active and acts on downstream targets such as executioner caspases 3 and 7 (Fig. 1.3.2) (Ohtsuka et al., 2003).

Tumor cells can inactivate cell death signaling programs, by engaging anti-apoptotic mechanisms involving the up-regulation of apoptotic suppressors, (Bcl_2, Bcl_{xl}) and/or through the down-regulation of critical apoptosis inducing players such as the

caspase family (2, 4, 6, 8, 9, 10, 12) (Elmore, 2007). Mechanisms that inhibit the intrinsic pathway of apoptosis are interconnected with activities of the AKT (Fig 1.1.3) and NFκB (figure 1.3.4) pathways. Therefore, should the AKT pathway be up-regulated through any means previously discussed, then ultimately this would lead to an inhibition of the intrinsic pathway of apoptosis as AKT signaling promotes BCL_2 and BCL_{xl} activity while inhibiting BAX and BAD players involved with inducing apoptosis (Tait and Green, 2010) (Fig. 1.3.2). Blocking BAX and BAD activity can prevent MOMP from opening, thus preventing Cytochrome C release, and consequentially inhibiting apoptosome formation. Another family of anti-apoptotic proteins that can inhibit both the intrinsic and extrinsic pathways is the Inhibitors of Apoptosis (IAP) which have two arms, the cIAP or X-linked IAP (XIAP). The IAP family consists of E3 ligases that can block apoptosome formation through binding directly to APAF-1 or caspase-9, thus inhibiting caspase-9 activation (Deveraux et al., 1998; Sun et al., 2009). XIAP can also bind directly to caspase-3 preventing its activation, and in-addition to blocking activation XIAP can facilitate the transfer of ubiquitin, thereby tagging the caspases for degradation by the 26S proteasome (Schile et al., 2008).

In addition to BCL and IAP family involvement with apoptosis inhibition there are also mutations acquired in the pro-apoptotic machinery itself and the most notable mutations are those that occur within the caspase family. For example, Srinvisula et al, identified caspase-9b, a caspase-9 mutant that lacks the large active subunit and established that caspase-9b acts in a dominant negative fashion preventing caspase-3 activation (Srinivasula et al., 1999). Moreover, Park et al; identified several gene polymorphisms that give rise to altered forms of caspase-9 that impair caspase-9 activity

and thereby block apoptosis induction (Park et al., 2006). Post-translational modification of caspase-9 phosphorylation at Thr 129 mediated as a result of CDK-1 and cyclin B in cell cycle (Allan and Clarke, 2009) also prevents caspase-9 recruitment to the apoptosome blocking caspase-9 activation. Regardless of how caspase-9 is impacted, if this caspase fails to become active then the subsequently executioner caspases 3, 7 activation is blocked and so is apoptosis activation through the intrinsic pathway (Bratton and Salvesen, 2010; Janssen et al., 2007).

The extrinsic pathway (also referred to as the death receptor pathway) involves the induction of apoptosis through the activation of death receptors via death ligands such as Tumor Necrosis Factor-α (TNF-α), FAS, and TNF Related Apoptosis Inducing Ligand (TRAIL)(Wallach et al., 2008). While FAS and TRAIL strictly activate the extrinsic pathway mediated apoptosis, TNF-α can play two different roles, although this molecule is capable of inducing apoptosis, TNF-α is also capable of activating pro-survival pathway. TNF-α, is a pro-inflammatory cytokine that is a part of the TNF superfamily. TNF-activation impacts several critical cellular pathways some of which include cellular proliferation, differentiation, and apoptosis (Naude et al., 2011). More specifically, TNF-α binding to its cognate receptor can lead to the formation of two separate complexes, Complex 1 which can lead to the induction of either the Nuclear factor of kappa B (NFκB) pathway (pro-survival) (Wajant and Scheurich, 2011) or Complex 2 which activates the apoptotic pathway mediated primarily through FADD and caspase-8 activation (Tran et al., 2009; Wang et al., 2008) (Fig. 1.2.4).

Complex 1 mediated NFκB induction is initiated through the binding of TNF-α to its cognate receptor TNFR-1 which then leads to the recruitment of two adaptor proteins

TNF Receptor-Associated protein with a Death Domain (TRADD) and Receptor-interacting protein 1 (RIP1) (Chen, 2005). Upon binding of TRADD another adaptor molecule, TNF Associated factor-2 (TRAF), followed by the recruitment of cIAP (cellular inhibitors of apoptosis), as well as Ubc6 and Ubc13 (E2 ubiquitin conjugating enzymes) (Fukushima et al., 2007; Wu et al., 2005) to form Complex 1. Once Complex 1 is formed, key signaling events are activated and TRAF2 is capable of being either K63 or K48 linked polyubiquitinated. TRAF2 is phosphorylation by PKC results in K63 link polyubiquitination (Li et al., 2006; Li et al., 2009) and once K63 linked polyubiquitinated, TRAF 2 can mediate NFκB activation through signaling cascades involving the IKK complex. Should TRAF2 become K48 linked polyubiquitinated this leads to degradation by the proteasome (Habelhah et al., 2004; Shi and Kehrl, 2003).

In addition to TRAF2 posttranslational modifications, RIP-1 is also subjected to K48 or K63 polyubiquitination. TRAF2, cIAP, and Ubc13 function in concert to facilitate K63 linked polyubiquitination of RIP1 (Wicovsky et al., 2009). K63 linked polyubiquitination of RIP-1 leads to activation of Tak1/TAB complex to activate the IKK complex, which interestingly enough can also be activated by K63 modified TRAF2 (Habelhah, 2010). The IKK complex consists of several components, IKK α, IKK β, and IKK γ, this kinase complex phosphorylates the Inhibitor of Kappa B molecule (IκB-α) (Ea et al., 2006). IkB α, is a bound inhibitor of NFκB that functions to prevent nuclear import of NFκB into the nucleus. However, once IκB-α is phosphorylated, it is susceptible to K48 linked polyubiquitination and is degraded by the 26S proteasome thus releasing NFκB inhibition. Once released, NFκB can translocate into the nucleus and binds to its respective DNA binding sites leading to gene activation (Mathes et al., 2008).

NFκB up-regulates several different gene types, such as inflammatory response (Bond et al., 2001) pro-survival genes, BCL family, caspase-8 inhibitor c-FLIP, cIAP and angiogenesis players and proliferation genes, cyclin D1 and MYC (Papa et al., 2006; Puszynski et al., 2009)(Nishikori, 2005). Interestingly enough, proteasome inhibition can impede the NFκB pathway because, it prevents the proteasome degradation of IκB-α thus leading to decreased activation of NFκB (Li et al., 2001; Markovina et al., 2008).

The induction of apoptosis through Complex 2 of the TNF-α pathway is induced through several different events, however, the critical events for apoptosis induction involve the depletion of c-Flip and/or c-IAP expression levels, as well RIP1 kinase phosphorylation (Biton and Ashkenazi, 2011; Rangamani and Sirovich, 2007). Once phosphorylated, RIP1, Fas Associated Death Domain (FADD) and initiator caspase-8 are recruited thus assembling complex 2. Once Complex 2 is formed, caspase-8 is processed and can then cleave its downstream targets, executioner caspases 3, 7 which can then carry the apoptosis program (1.3.4)(Yuan and Kroemer, 2010).

Recent exciting new insights into the mechanism of apoptosis induction through the formation of the Ripoptosome, a 2mD apoptosis signaling complex composed of RIP-1, FADD, and caspase-8 that forms independent of both intrinsic and extrinsic pathway activation (Fig. 1.3.5) (Tenev et al., 2011). This complex assembles when cIAP expression levels are depleted, either through up-regulation of SMAC a direct inhibitor of cIAP or through SMAC mimetics or other chemotherapeutics such as etoposide, which is a topoisomerase II inhibitor used to treat a variety of solid tumor cancers. In-addition to inducing DNA breaks etoposide has been shown to down-regulate cIAPs (Bertrand and Vandenabeele, 2011; Feoktistova et al., 2011; Imre et al., 2011; Tenev et al., 2011). The

ability to form this apoptosis inducing complex, can serve as a powerful tool for developing anti-cancer strategies because an agent (or combination of agents) could induce apoptosis, while bypassing the normal requirements for apoptosis induction. Bypassing the "traditional" requirements, would provide opportunities to circumvent the anti-apoptotic approaches tumor cells up regulate to ensure its survival. Additional studies have provided evidence suggesting that the Ripoptosome is also capable of inducing necroptopsis (programmed necrosis) (Declercq et al., 2001; Oberst et al., 2011) mediated through RIP3 signaling events as opposed to RIP1 (Feoktistova et al., 2012).

The extrinsic pathway of apoptosis is abrogated through several mechanisms, including the up-regulation of the Inhibitors of Apoptosis proteins such as cIAP or XIAP. Up-regulation of these inhibitors of apoptosis molecules will drive the TNF-α pathway towards NFκB activation in the same manner as described above. Apart from inhibition by the IAP family, recent studies published data showing that IL-6/STAT3 signaling can override apoptotic signals by activating pro-survival, (BCL_2, BCL_{xl}) as well as cyclin D (Li et al., 2012). TRAIL and FAS mediated apoptosis pathways are very similar to one another in that these trimeric ligands bind their specific cognate receptors to induce apoptosis. TRAIL binds to the DR 4/DR5 receptors which leads to receptor oligomerization in the plasma membrane, some groups suggest that these receptors oligomerize in the lipid rafts of the plasma membrane (Mollinedo and Gajate, 2006; Song et al., 2007). Once the receptors oligomerize there is recruitment of adaptor protein Fas Associated Death Domain (FADD). FADD binding to the TRAIL receptor leads to initiator caspase-8 recruitment to form the death inducing signaling complex (DISC). Following DISC formation procaspase-8 becomes autocatalytically active, once active

caspase-8 is processed into the active p18 and p10 subunits via two cleavage events. Once processed the p18 and p10 dimers can oligomerize with other p18/p10 dimers to form active heterotetramers, that cleaves specific targets such as HDAC7 (Scott et al., 2008), and executioner caspases (3 and 7) which fully induce the apoptotic response (Fig. 1.3.3) (Diessenbacher et al., 2008; Zhang et al., 2007).

During onocogenesis, tumor cells utilize a variety of mechanisms to inactivate the extrinsic pathway of apoptosis; one mechanism in particular involves the down-regulation of death receptor expression or up-regulation of decoy receptors (Matsuda et al., 2005). It could be argued that loss of receptor expression would have a more profound effect on FAS and TRAIL compared to TNF-α. A tumorgenic cell prefers, that TNF-α receptor expression because this enables the activation of the NFκB and other pro-survival pathways. Tumors down regulate the FAS and TRAIL receptors to inhibit the extrinsic pathway activation. With respect to TRAIL, its cognate receptors consist of Death Receptor 4 or 5 (DR4, 5), as a protective measure, the cell also expresses decoy receptors Dcr1 and Dcr2 as an effort to prevent unintended apoptosis induction through TRAIL binding to the death receptors (Almasan and Ashkenazi, 2003; LeBlanc and Ashkenazi, 2003). In addition to receptor or decoy mediated inhibition, the extrinsic pathway can also be inhibited through similar mechanisms that inhibit the intrinsic pathway, such as over-expression of BCL_2, BCL_{xl}, cIAP and XIAP anti-apoptotic proteins (Fulda et al., 2002; Hinz et al., 2000; Newsom-Davis et al., 2009)

TRAIL enables an attractive therapeutic platform for development as a single chemotherapeutic agent or in combination with existing chemotherapeutics. Several investigators have studied TRAIL using different mouse models which have

demonstrated that TRAIL preferentially targets cancer cells and not healthy non-neoplastic cells (Mitsiades et al., 2001; Plasilova et al., 2002). This specificity renders TRAIL a valuable chemotherapeutic agent due to the limited side effects and TRAIL protein can be synthesized via standard protein purification methods. However, TRAIL is very unstable in solution therefore, in order to use TRAIL for lab studies, investigators have conjugated the TRAIL protein with protein tags, for example, our lab conjugated GST-tag to the C-terminal domain of TRAIL. TRAIL C-terminal conjugation can extend TRAIL half-life by 11 hours allowing it to be used for *in-vitro* and *in-vivo* experiments (Thorpe et al., 2008b). For clinical trial and treatment applications companies, like Human Genome Sciences (Rockville MD, USA) have generated TRAIL receptor activating antibodies with the intent to extend TRAIL half-life. These companies were successful in generating TRAIL receptor antibodies as evidenced by several *in-vitro* and *in-vivo* studies that analyzed TRAIL antibodies, Mapatumumab and Lexatumumab indicating these antibodies are functionally capable of inducing apoptosis (Belyanskaya et al., 2007; Huang and Sheikh, 2007; Luster et al., 2009).

Additional studies have combined TRAIL with existing chemotherapeutic agents in pre-clinical studies, some of these agents include phytoshingosine (impacts sphingolipid metabolism) (Choi et al., 2007), Doxorubicin (Guo et al., 2011; Wu et al., 2002), docetaxel (Yoo et al., 2008) and paclitaxel (Mielgo et al., 2009) all of which have shown promising results suggesting that TRAIL should be investigated further as a chemotherapeutic strategy. In-progress is a clinical trial studying the combination of TRAIL and VEGF inhibitor, Bevacizumab, (Clinical trial identifier, NCT00508625).

Emerging involve testing TRAIL in combination with Proteasome inhibitor Velcade in both the pre-clinical and clinical settings[113, 114].

1.4 Caspases Key Apoptosis Regulators

Caspases are a family of cysteine proteases that play roles in several different pathways, including, inflammatory, development, and apoptotic pathways. Although each caspase serves a different purpose there are several similarities in cleavage, the proform is cleaved into a large catalytically active subunit and a small subunit as shown for the critical apoptotic caspases (1.4.1). Caspases 1 and 5 play a role in inflammation (Bian et al., 2011; Yu and Finlay, 2008). ER stress response pathways, Unfolded protein response (UPR), or ER associated degradation (ERAD) are mediated through caspase-4, eventually leading apoptosis induction through the intrinsic pathway.

The endoplasmic reticulum (ER) is a critical cellular organelle whose primary function is to ensure proteins are properly folded before export into the golgi apparatus and respective regions of the cell (Anelli and Sitia, 2008). The protein folding machinery consists of several components that work in concert to ensure proper protein folding. However, when the ER is overwhelmed with polypeptides that are incapable of being folded correctly, three sensors, IRE-1, PERK, and ATF6 trigger the unfolded protein response (UPR) (Shuda et al., 2003). If these pathways are incapable of clearing the ER of these misfolded proteins and the high level of ER stress persists, CHOP expression is markedly increased. An increase in CHOP expression leads to a down-regulation in pro-survival BCL family members, BCL_2 and BCL_{xl} allowing for BAX and BAD expression and activity to increase and thereby leading to intrinsic pathway activation (Fig. 1.4.2)

(Lai et al., 2007). Alternative intrinsic pathway induction mechanisms can involve the activation of caspases 2, 4, and 12 (Bian et al., 2009; Hitomi et al., 2004) which then contributes to the processing of caspase-9 either through disrupting the mitochondria(Jing et al., 2012) or through APAF-1 independent mechanisms (Fig 1.3.2) (Rao et al., 2002). Caspase-2 has been implicated to induce intrinsic pathway apoptosis either through Bid cleavage thus leading to intrinsic pathway activation via the opening of the MOMP releasing Cytochrome C facilitating apoptosome formation (Upton et al., 2008) or through caspase-2, PIDD, and adaptor protein RAIDD bind and form piddosome (Tinel and Tschopp, 2004) Compared to healthy cells, tumor cells have a marked increase in protein synthesis as well as an over-production of misfolded or unfolded protein in the ER, resulting in extremely high ER stress levels. Therefore investigators have developed strategies to induce apoptosis by further elevating ER stress using chemotherapeutics such as Velcade (Verfaillie et al., 2010). Velcade is designed to block the 26S proteasome and once inhibited, UPR response mechanisms become impaired leading to apoptosis induction (Dong et al., 2009; Obeng et al., 2006). To circumvent proteasome inhibition, cells activate lysosomal pathways as an alternate mechanism for protein clearance. Exploitation of this mechanism as an anti-cancer strategy involves the use of Velcade in combination with lysosomal inhibitor, Tubacin (an HDAC6 inhibitor that block aggresome formation) and reports have demonstrated that together these agents are capable of apoptosis induction (Bolhuis and Richter-Landsberg, 2010; Hideshima et al., 2005; Rodriguez-Gonzalez et al., 2008).

The intrinsic pathway is a mitochondrial mediated pathway of apoptosis that can be activated through several different types of signals. The major players involved with

apoptosis induction, most notably, Cytochrome C, APAF-1, and caspase-9 which associate with one another to form the apoptosome. Once the apoptosome is formed, caspase-9 is then processed into its active p37 and p19 subunits (Rodriguez and Lazebnik, 1999) and capable of cleaving executioner caspases 3/7 (Figs. 1.3.2, 1.4.1). Cancer circumvents intrinsic pathway activation by a variety of mechanisms, one of them involves the down-regulation of caspase-9 activation which is mediated with the involvement of the BCL family members such as BCL_2 and BCL_{xl} or XIAP binding as described previously above, or through the prevention of apoptosome formation either through pro-survival signals that prevent the opening of the mitochondria thus preventing the release of Cytochrome C into the cytosol (Hu et al., 1998; Shiozaki et al., 2003). Tumors transected from patients ranging from a variety of cancer types including, colorectal, lung, and gastric tumors, revealed several different point mutations in caspase-9 that render this caspase inactive and incapable of inducing apoptosis (Soung et al., 2006).

Both the intrinsic and extrinsic pathways converge at the same point, the cleavage of executioner caspases 3 and 7 (Figs. 1.3.2, 1.3.3). Executioner caspases 3, 7 (also termed effector caspases) are processed into active subunits (Fig. 1.3.1) and responsible for the execution of the apoptosis program through the cleavage of ICAD (Caspase-activated DNase) which then translocates to the nucleus and cleaves DNA (Larsen et al., 2010). Caspase-3 propagates and amplifies the apoptosis signal through a loop that leads to caspase-9 cleavage thus further propagating the apoptotic cascade (Blanc et al., 2000; Fujita et al., 2001). Loss of caspase-3 expression has been suggested to play a role in promoting oncogenesis (Devarajan et al., 2002) Caspase-7 is down regulated in cancer

and investigators have suggested to use this lack of caspase-7 expression as a marker for colon cancer (Palmerini et al., 2001).

Caspase-10 is an initiator caspase that is recruited to the DISC like caspase-8. Caspase-10 has been extensively studied and the data from these studies have drawn mixed conclusions regarding its apoptosis inducing capabilities. Reports have provided evidence that show caspase-10 is capable of inducing apoptosis in caspase-8 deficient cells (Kischkel et al., 2001; Krug, 2012). In particular, pediatric neuroblastoma tumors are caspase-8 deficient however caspase-10 is sufficient to induce apoptosis (Lafont et al., 2010). However, other studies have reported that caspase-10 lacks the capacity to induce apoptosis in the absence of caspase-8 in other cancer types; caspase-8 remains the critical initiator caspase responsible for extrinsic pathway activation (Sprick et al., 2002).

1.5. Caspase-8 in Death Receptor Independent and Dependant Pathways

Of mechanistic significance is growing evidence implicating caspase-8 having a role in non-apoptotic signaling pathways. Stupack et al, (2009) reported that caspase-8 in neuroblastoma cell lines plays a role in mediating focal adhesion complex formation and cellular migration (Barbero et al., 2009). Earlier studies described a pathway that was nearly identical to the anoikis pathway, termed Intergin Mediated Death (IMD), although interestingly enough anoikis was not identified as the main form of apoptosis in this study (Stupack et al., 2006).

Further studies by the group revealed that caspase-8 is phosphorylated on Tyrosine residue 380 via SRC kinase activity (Barbero et al., 2008); moreover it was demonstrated that caspase-8 is associated with Focal Adhesion Kinase (FAK) and CNB2

and upon its recruitment and association with these focal adhesion players, leads to the activation of the calpain family of proteases which then cleave Talin. As indicated by the authors the N-terminal cleavage product of Talin is termed the FERM, an integrin binding domain which facilitates cell migration (Barbero et al., 2009) and thus implicating that caspase-8 serves a role in mediating metastasis through its involvement with the focal adhesion complex (Barbero et al., 2009). Subsequent evidence identified Rab5, as a critical modulator of caspase-8 action in cell migration (Torres et al., 2010; Torres et al., 2008). Rab5 itself plays several roles with migration, either through lamellipodia formation (Spaargaren and Bos, 1999), β1 integrin binding, or through actin cytoskeletal rearrangement (Pellinen and Ivaska, 2006; Pellinen et al., 2008).

Several lines of evidence have demonstrated the involvement of caspase-8 in EGF signaling pathways inducing ERK activation through the incorporation of caspase-8 in SRC containing complexes. This work identified through a RXDLL motif found within the DED of caspase-8 pro-domain, this allows caspase-8 to associate with SRC although the data did not show any evidence that caspase-8 phosphorylation was required for SRC association and EGF pathway activation (Finlay et al., 2009; Finlay and Vuori, 2007).

Initiator caspase-8 is a critical player for extrinsic pathway activation, and because of its importance caspase-8 has been considered a tumor suppressor molecule. Caspase-8 deficient cells are insensitive to death ligand stimulus and cannot induce apoptosis through the extrinsic pathway and this provides the cell an opportunity to undergo cellular tumorgenesis (Krelin et al., 2008). Human cancer cells regulate caspase-8 activity through a variety of mechanisms, one mechanism is caspase-8 partial or whole gene deletion, (Teitz et al., 2000) or gene methylation. For example, Medulloblastoma

Pediatric Neuroblastoma tumors down-regulate caspase-8 expression through methylation of the caspase-8 promoter thereby inhibiting caspase-8 transcription thus preventing protein translation and expression (Gonzalez-Gomez et al., 2004; Muhlethaler-Mottet et al., 2003; Pingoud-Meier et al., 2003).

Several caspase-8 mutations have been identified in various tumor types. An interesting study by Soung et al, involved a screen across multiple cancer types to uncover common caspase-8 mutations among these cancer types (Soung et al., 2005). This screen identified frame shift and missense mutations which altered amino acid compositions in the DED domain, a domain which is absolutely critical for caspase-8 recruitment to the DISC and initiating subsequence cleavage events (Soung et al., 2005). Moreover, mutations were found in the p18 catalytically active subunit and the p10 regions validation of the screen results revealed that most of the mutants severely diminished apoptosis induction in gastric carcinomas (Soung et al., 2005).

One unique feature of caspase-8 and caspase-10 is that these the only caspases processed at the DISC localized at the plasma membrane. Upon recruitment to the DISC caspase-8 undergoes two cleavage events, the first cleavage event occurs at aspartic acid residue 384 in the p10 subunit, giving rise to the p43/41 intermediate which is bound at the DISC. This cleavage event is followed by a second cleavage at aspartic acid residues 210, 216 which then release caspase-8 from the DISC into the cytosol (Fig. 1.3.3). Caspase-8 binding has been extensively studied throughout the years by several investigators, most notably; the pioneer work by Dr. Marcus Peter defined how the DISC components were assembled at the plasma membrane through TRAIL and/or FAS receptor and FADD palmitylation (Feig et al., 2007; Medema et al., 1997a; Medema et

al., 1997b). Moreover studies by Walczak et al, and Lenardo et al, provided evidence towards DISC mediated caspase-8 processing (Ganten et al., 2004; Martin et al., 1998) Additional studies focusing on DISC formation by Marcus Peter's lab identified that c-flip was a specific inhibitor of caspase-8 DISC recruitment and activation (Scaffidi et al., 1999). Subsequent work identified cFlip isoforms that block gene induction as well as processing of caspase-8 (Kavuri et al., 2011). Aside from cFlip, investigators have shown that either XIAP or cIAP are also capable of blocking caspase-8 activation (Kruidering and Evan, 2000).

More recent studies by Peng et al and Jin, et al(Jin et al., 2009; Peng et al., 2011), have shown that caspase-8 is polyubiquitinated in HEK293 cells under EGR activated conditions (Peng et al., 2011) or in lung cancer cell line H460 (Jin et al., 2009). Previously our lab has shown that proteasome inhibition and TRAIL can induce TRAIL mediated apoptosis in TRAIL resistant prostate cancer cell lines and through these studies we acquired data that further suggested caspase-8 was under proteasome regulation (Christian et al., 2009b; Thorpe et al., 2008b).

1.6 Proteasome Regulation of Cellular Proteins.

Protein degradation is an important process that affects a myriad of pathways. The proteasome has a role in regulating protein expression and in maintaining protein integrity (clearing misfolded proteins)(Davies, 2001; Leverkus et al., 2003). Protein degradation is mediated primarily through the ubiquitin proteasome system (David et al., 2002), which involves the covalent attachment of ubiquitin to the lysine residues of the target protein or other ubiquitin residues (K48-G76 linkages) to result in the formation of

a ubiquitin chain (Shabek et al., 2009). The ubiquitination process is carried out by three key enzymes, the E1 ubiquitin activating enzyme, E2 conjugating enzyme, and E3 ligase which transfers the ubiquitin to the lysine residue of the protein (Soucy et al., 2009b). There are two classes of E3 ligases, the HECT class, which directly transfers ubiquitin from the E2 conjugating enzyme to the substrate, and RING finger class, which acts as a scaffold bringing the protein substrate in contact with ubiquitin containing E2 enzyme (Joazeiro and Weissman, 2000; Soucy et al., 2009a). Once polyubiquitinated, the protein is then translocated to the 26S proteasome, which in an ATP dependant manner, degrades the protein (Fig. 1.6.1). The 26S proteasome (Fig. 1.6.2) has a barrel like structure and is composed of 19S and 20S particles. The 19s is a regulatory particle and identifies ubiquitin bound proteins, and removes ubiquitin as the protein enters the catalytically active 20S particle. The 20S particle consists of two α and two β domains and contains several different proteases that degrade the protein (Orlowski and Wilk, 2000).

1.7 Proteasome Inhibition.

Investigators have shown that proteasome inhibitors can induce apoptosis and thus have been used as an anti-cancer strategy (Shah et al., 2001; Vu et al., 2008). There are two types of proteasome inhibitors, natural inhibitors (lactacystin and epoxomicin) and synthetic inhibitors such as MG132 and Velcade (Almond and Cohen, 2002). MG132 inhibits the chymotrypsin like activity of the 26S proteasome (Li et al., 2007b). Velcade, (PS-341/bortizomib) is an FDA approved proteasome inhibitor used in treating multiple myeloma (Crawford et al., 2006). Velcade is a dipeptide boronic acid small molecule that blocks the chymotrypsin-like activity of the 20S particle (Berkers et al., 2005).

Investigators have reported that Velcade has an impact on several key cellular processes such as, inhibiting cell cycle and NF-κB activation (Yu et al., 2004). Velcade has been shown to sensitize cancer cells to apoptosis through several mechanisms, such as the down-regulation of c-Flip, which inhibits caspase-8 activation at the DISC (Fukazawa et al., 2001; Sayers et al., 2003). Several in vitro and in vivo studies have shown that Velcade is successful in inducing apoptosis in multiple myeloma cells (Balsas et al., 2009).

Although Velcade is effective against multiple myeloma and evidence is growing supporting Velcade as an effective anti-cancer strategy in other cancer cell lines, Velcade has drawbacks, such as cell resistance and lack of specificity. Some studies have shown that multiple myeloma patients were either initially resistant or acquired resistance to Velcade during the course of treatment with this drug (Nencioni et al., 2005). Attempts to overcome Velcade resistance have led to development of various combinations of Velcade with different chemotherapeutic agents such as, PCI-24781 (an HDAC inhibitor) which was found to synergize with Velcade to induce reactive oxygen species damage as well as caspase-8 activation in Non-Hodgkins lymphoma (Bhalla et al., 2009).

Significantly enough, Mitsiades and colleagus (Mitsiades et al., 2003), showed that Velcade in combination with Doxorubicin, can effectively overcome Velcade resistance in multiple myeloma. Another anti-cancer strategy involved using Velcade in combination TNF-α Related Apoptosis Inducing Ligand (TRAIL). Recent studies by Christian et al suggests the ability of Velcade to sensitize TRAIL resistant prostate cancer cell lines *in vitro* and *in vivo* to TRAIL mediated apoptosis and together TRAIL and Velcade stabilize caspase-8 p18 subunit (Christian et al., 2009a; Thorpe et al., 2008a).

A major caveat for using Velcade as an anti-cancer strategy is the lack of cell type and cell signaling specificity. Velcade was designed to block the proteasome and not to discriminate between a malignant or a healthy cell therefore, all cells are impacted by Velcade treatment. Additionally, although Velcade specifically targets the proteasome evidence shows that multiple pathways are impacted and as a result of the non-specific pathway modulation, Velcade treatment can lead to side effects such as thrombocytopenia and peripheral neuropathy (Badros et al., 2007; Richardson et al., 2009). To bypass the caveats associated with Velcade, while achieving apoptosis induction investigators have taken an interest in studying the activity of E3 ligases in an attempt to achieve and their potential involvement with the extrinsic pathway of apoptosis.

Emerging evidence provided by two different groups, Jin et al, (2009) and Peng et al, (2011), provided new insights regarding caspase-8 polyubiquitination. Jin et al, provided evidence that showed E3 ligase CUL3 mediated polyubiquitination lead to caspase-8 incorporation into an aggresome. Moreover functional activity analysis established that caspase-8 was catalytically active within the aggresome. The authors concluded CUL3 mediated polyubiquitination of caspase-8 promotes p62 mediated aggresome formation and that both events play a role in caspase-8 activation. Although caspase-8 ubiquitination may initially result in enzymatic activation, Jin et al only studied very early time points. Our data suggests that caspase-8 ubiquitination ultimately results in its degradation by the 26S proteasome (p18 subunit) and, thereby, would limit caspase-8 activity and block apoptosis. A second group investigated caspase-8 polyubiquitination

while studying EGR signaling, more specifically looking at the phosphorylation of caspase-8 through Thr 263 mediated through RSK6 activity (Peng et al., 2011).

Studies from our lab also discovered two E3 ligases, Siah2 and POSH that play a role in modulating caspase-8 activity (Christian et al., 2011). These E3 ligases were discovered using a siRNA screen from Dharmacon which knocked down roughly 230 of the known E3 ligases at that time. By reverse transfecting DU145 prostate cancer cells and assessing caspase-8 activity following TRAIL and proteasome inhibitor stimulation, the data revealed that knocking down Siah2 and POSH led to dramatic increases in caspase-8 activity. Furthermore Siah2 and POSH can functionally regulate caspase-8 activity down-stream of the DISC and caspase-8 processing(Christian et al., 2011).

Combination treatment of proteasome inhibition with TNF-α related apoptosis inducing ligand (TRAIL), induces apoptosis in TRAIL resistant prostate cancer cells *in-vitro* and *in-vivo*(Christian et al., 2009b). Moreover combination of TRAIL and Velcade led to caspase-8 p18 subunit stabilization(Brooks et al., 2010; Thorpe et al., 2008b), evidence implicating caspase-8 degradation under the control of the 26S proteasome (Fig. 1.6.1). This study was designed to determine the functional consequences of proteasome inhibition on caspase-8 trafficking, ubiquitination, and activity in cancer cells.

Figure 1.1.1 Mechanisms of Cancer

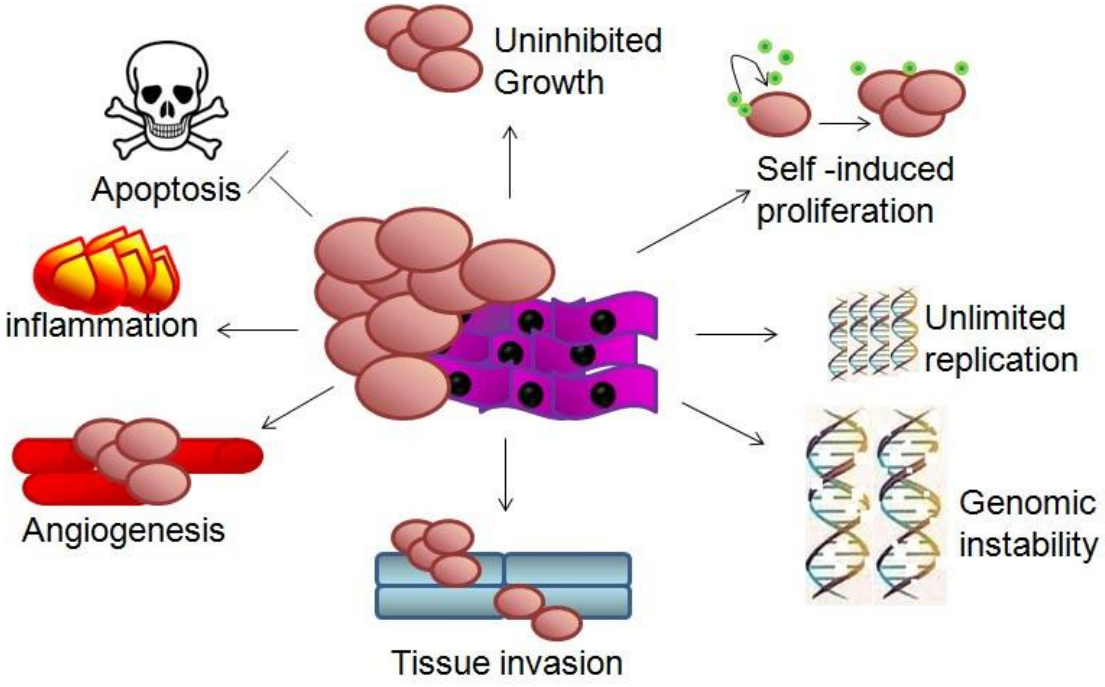

Figure 1.1.1 Mechanisms of Cancer.

As cells undergo tumorgenesis critical regulatory mechanisms are inhibited. Through inhibition of these mechanisms cell proliferation becomes unregulated, cells are capable of autocrine/paracrine stimulation of growth pathways. Increased proliferation leads to increased DNA replication resulting in accumulation of mutations that causes genomic instability. Genome mutations ultimately lead to acquisition of new characteristics such as tissue/organ invasion and angiogenesis. Apoptotic pathways are down-regulated.

Figure 1.1.2 The Actions of p53 in Cell Cycle Arrest

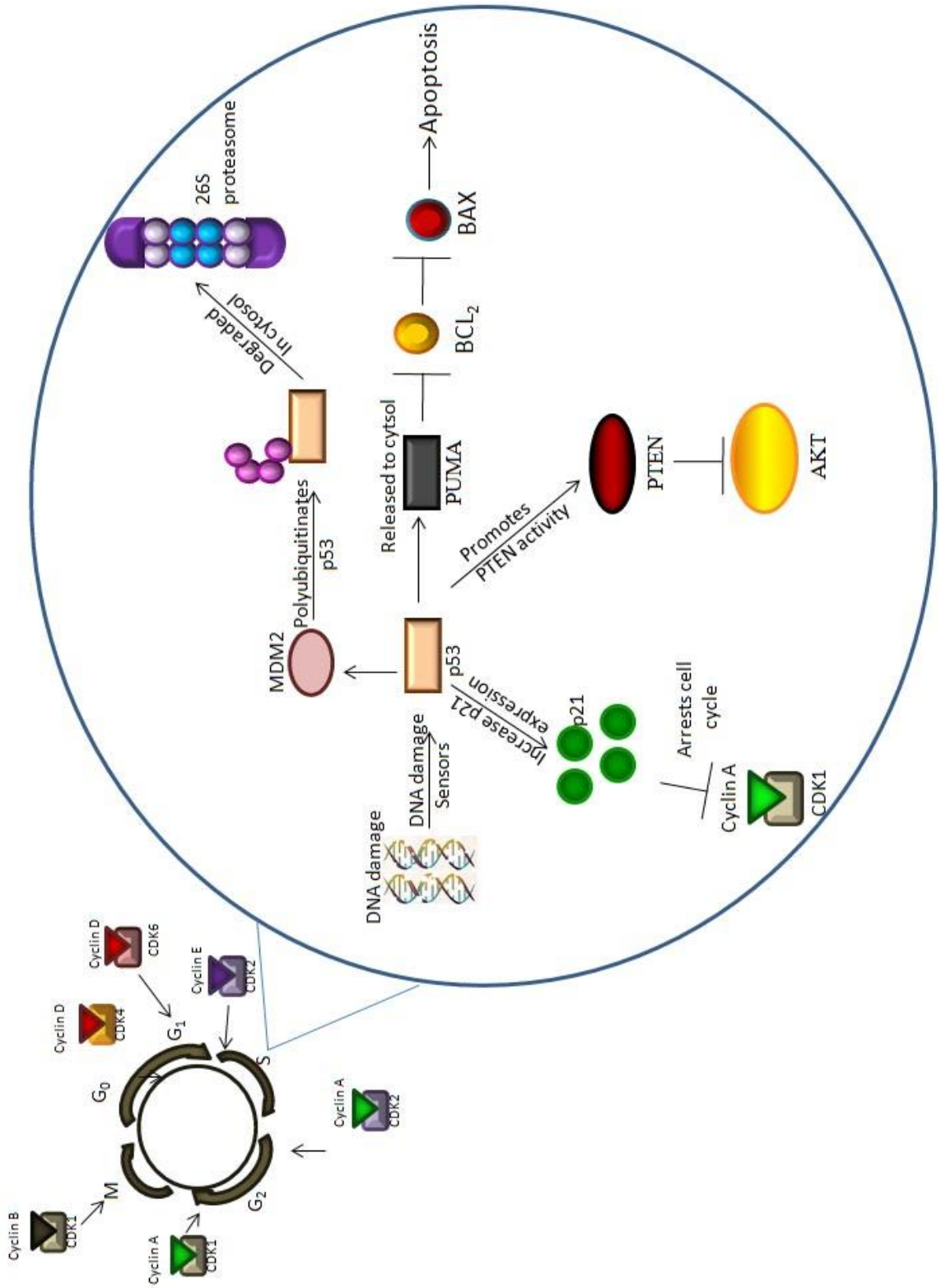

Figure 1.1.2 The Actions of p53 in Cell Cycle Arrest.

Cell cycle activation involves a series of checkpoints through interphase which are governed by the interplay between CDK and cyclin families of proteins and the regulatory molecules that modulate their expression. A critical regulatory molecule is p53 which assesses DNA integrity prior to entry into S phase. Should DNA integrity be irreparable p5 will signal a series of signaling pathways that inhibit cell cycle, induce apoptosis, and block survival pathways. MDM2 facilitates the polyubiquitination of p53 leading to its degradation.

Figure 1.1.3 AKT Pathway

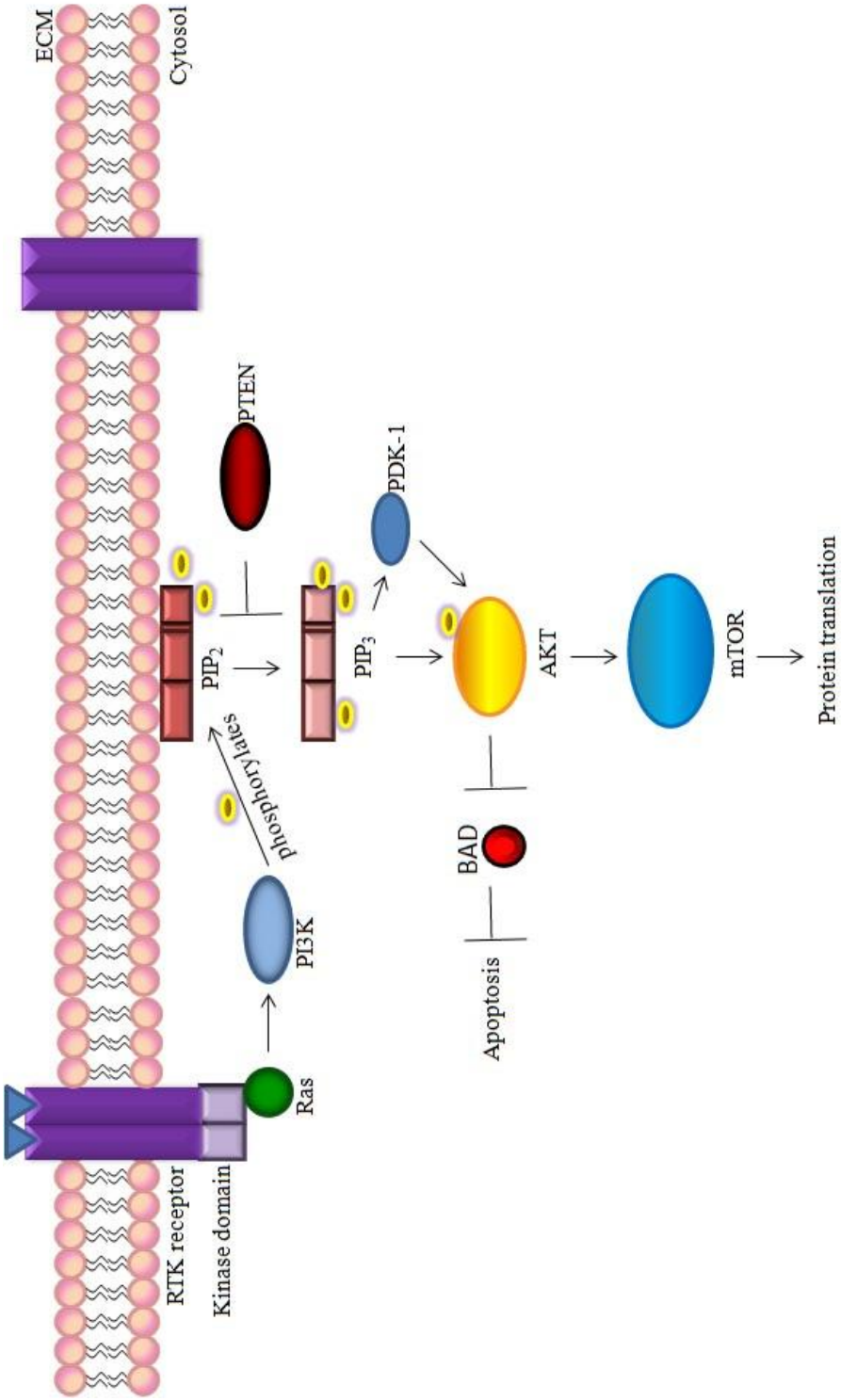

Figure 1.1.3 AKT Pathway.

Mediated through a variety of cell signaling events within the cell. Once activated, PI3K phosphorylates PIP2 thus generating PIP3 which can then activate PIDK, leading to AKT activation.

Figure 1.3.1. Anoikis Pathway.

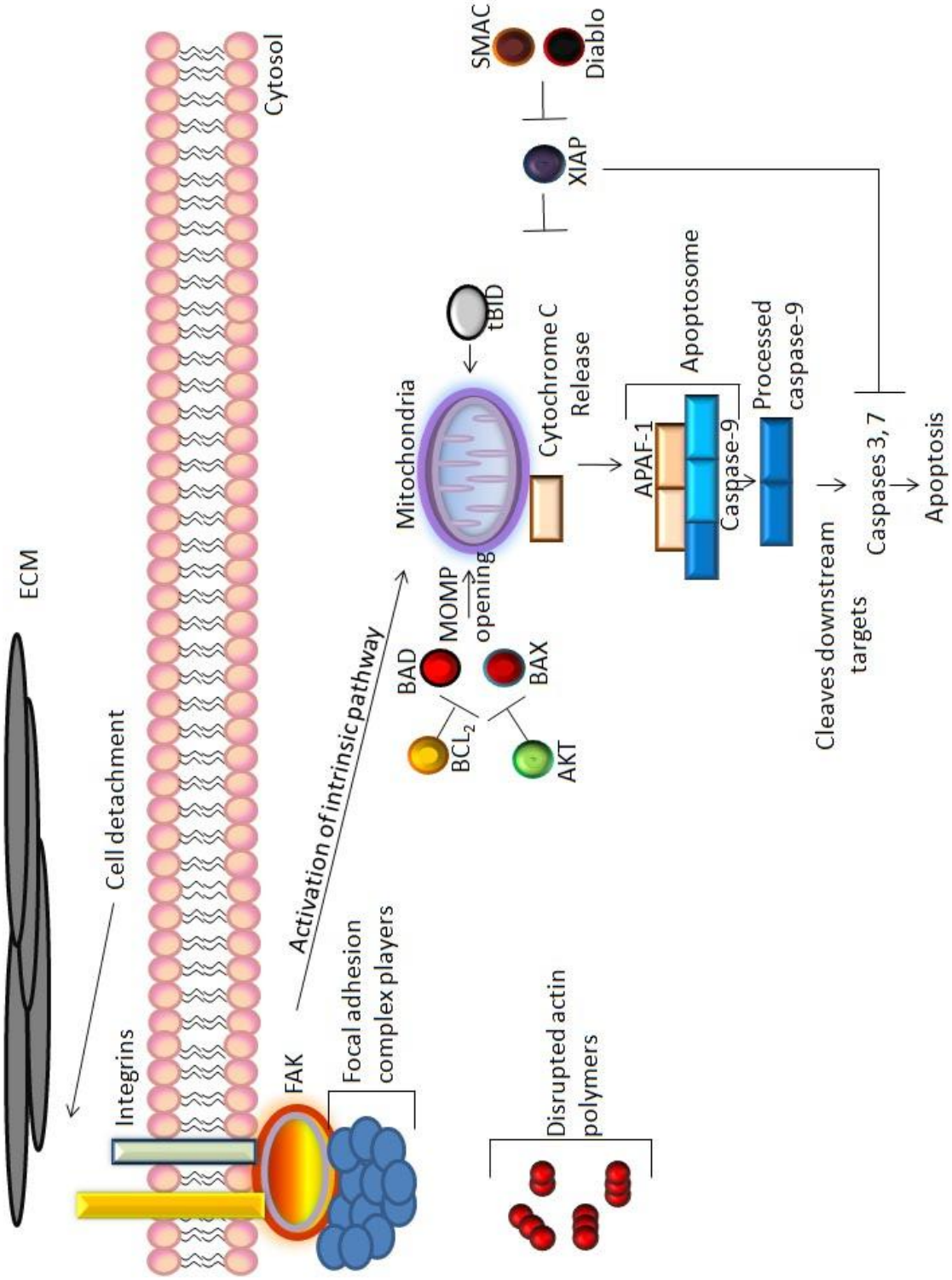

Figure 1.3.1. Anoikis Pathway.

Integrin and extracellular matrix protein signaling is interrupted, the cell detaches from the CM, this leads to signals throughout the cell to induce in intrinsic pathway of apoptosis

Figure 1.3.2 Intrinsic and ER Pathways of Apoptosis.

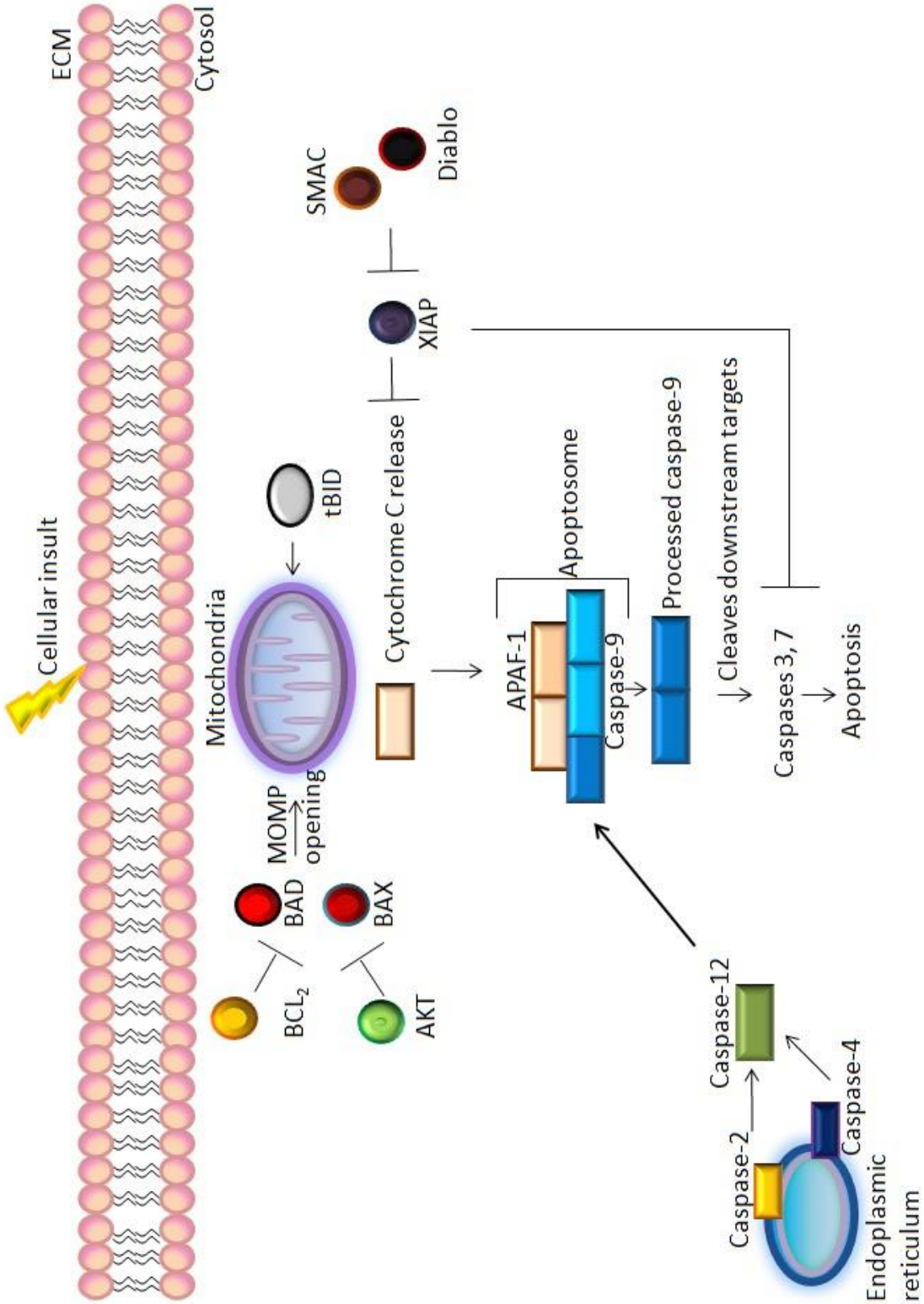

Figure 1.3.2 Intrinsic and ER Pathways of Apoptosis.

The intrinsic pathway of apoptosis is activated through a variety of signals. This leads to a down-regulation of anti-apoptotic BCL family members, allowing pro-apoptotic members to perturb the mitochondria. Cytochrome C release from the mitochondria leads to APAF-1 and pro-caspase-9 recruitment forming the Apoptosome. Upon formation caspase-9 is processed and cleaves downstream targets, such as executioner caspases 3, 7. The ER stress pathway induces the activation of caspases 2, or 4, leading to 12 activation which then activations the intrinsic pathway of apoptosis.

Figure 1.3.3. TRAIL Mediated Extrinsic Pathway of Apoptosis .

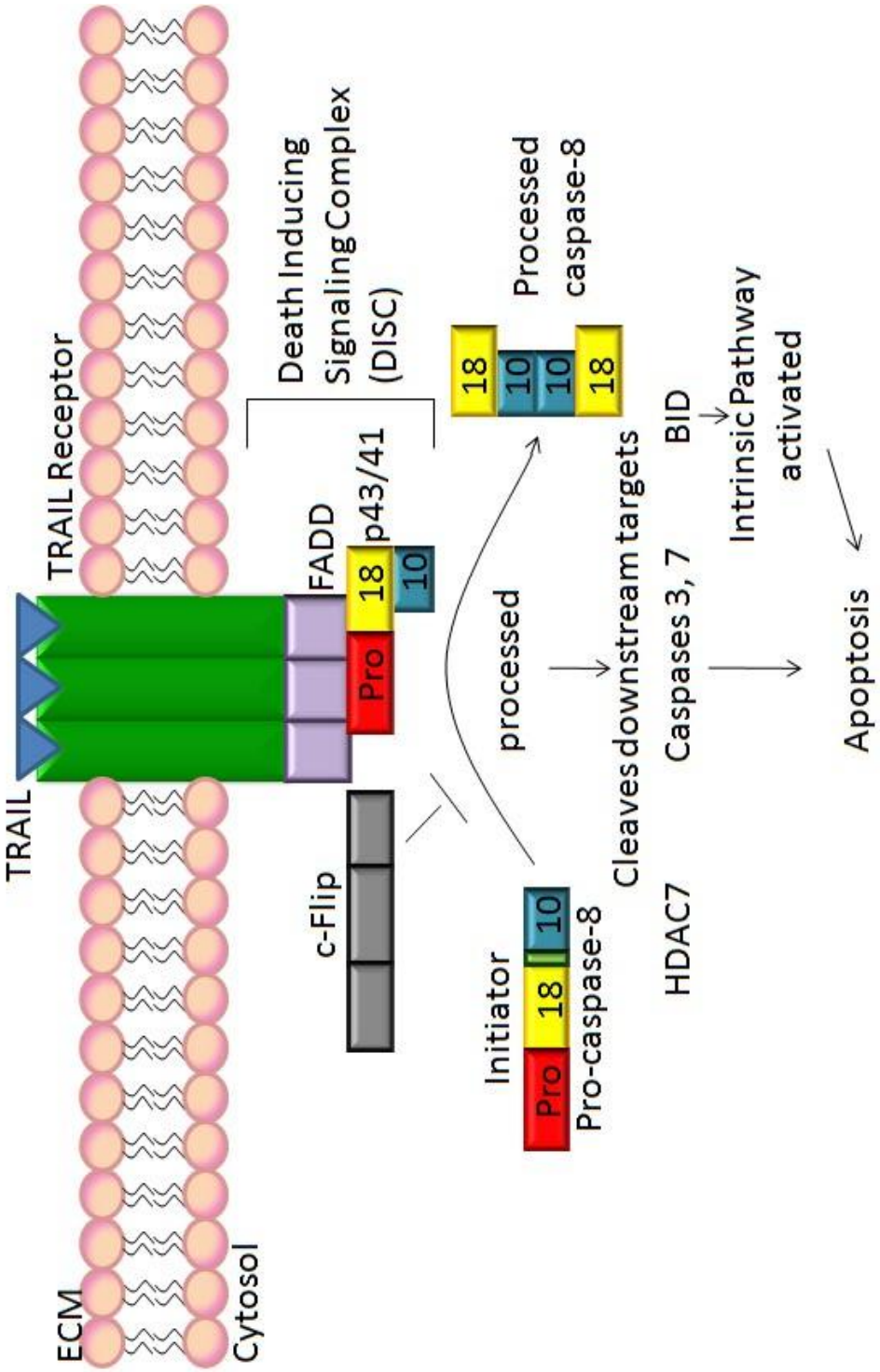

Figure 1.3.3. TRAIL Mediated Extrinsic Pathway of Apoptosis .

Mediated through the binding of TRAIL to its cognate receptor, upon binding the receptors oligomerize within the membrane. Fas associated Death Domain (FADD) is recruited, followed by pro-caspase-8 which is then processed into its active p18 and p10 subunits which then can oligomerize into a heterotetramer

Figure 1.3.4. TNF-α Mediated NFκB or Apoptosis Activation.

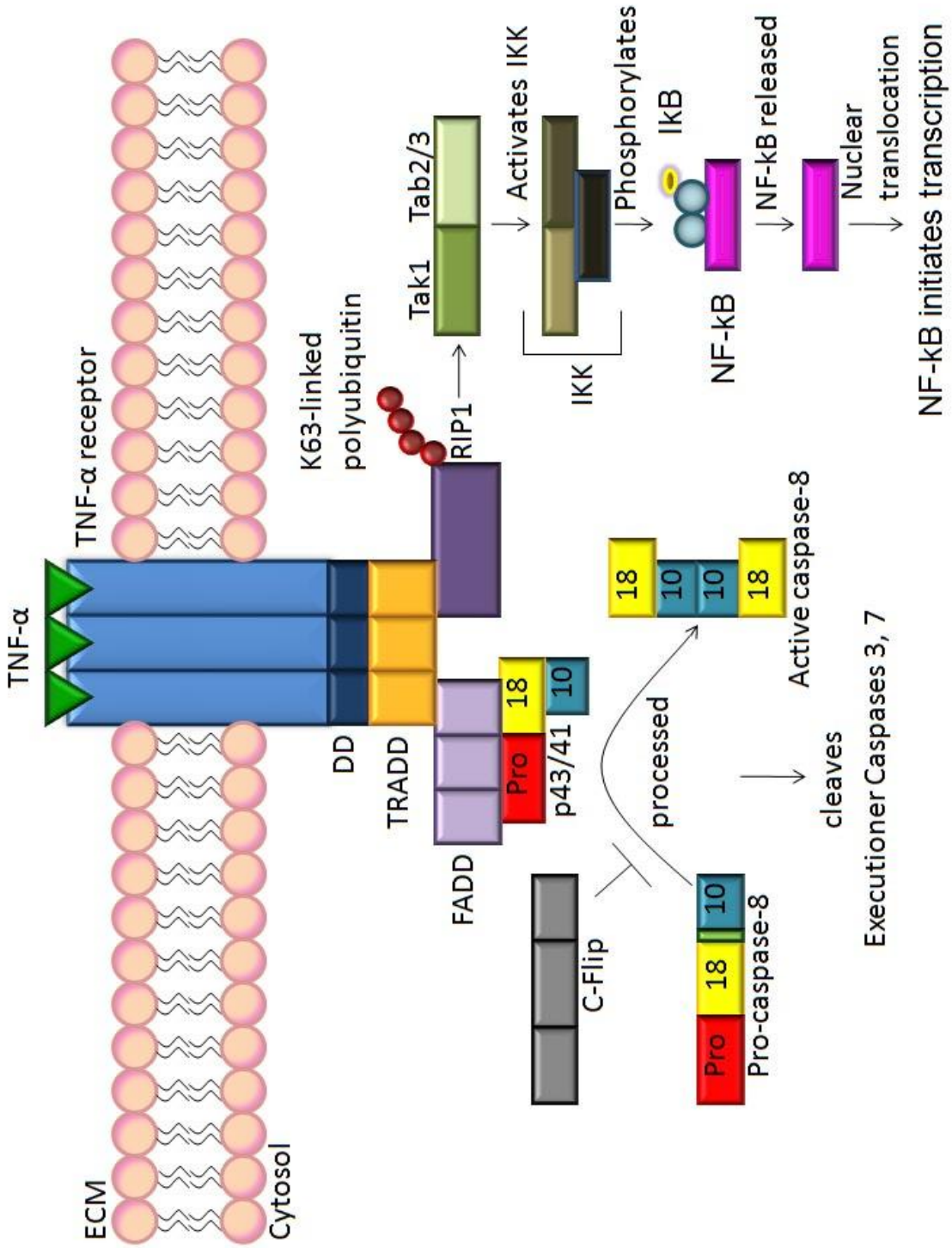

Figure 1.3.4. TNF-α Mediated

NFκB or apoptosis activation. TNF-alpha activation can elicit its signaling events through the formation of two complexes. Complex 1 mediates the NFκB activation through the K3 ubiquitin linkage of RIP1. This leads IKK activation via TAK1/TAB2/3, IKK then goes on to phosphorylates IkB, causing its polyubiquitination and degradation, thus releasing NFκB to translocate to the nucleus.

Figure 1.3.5 Ripoptosome Formation.

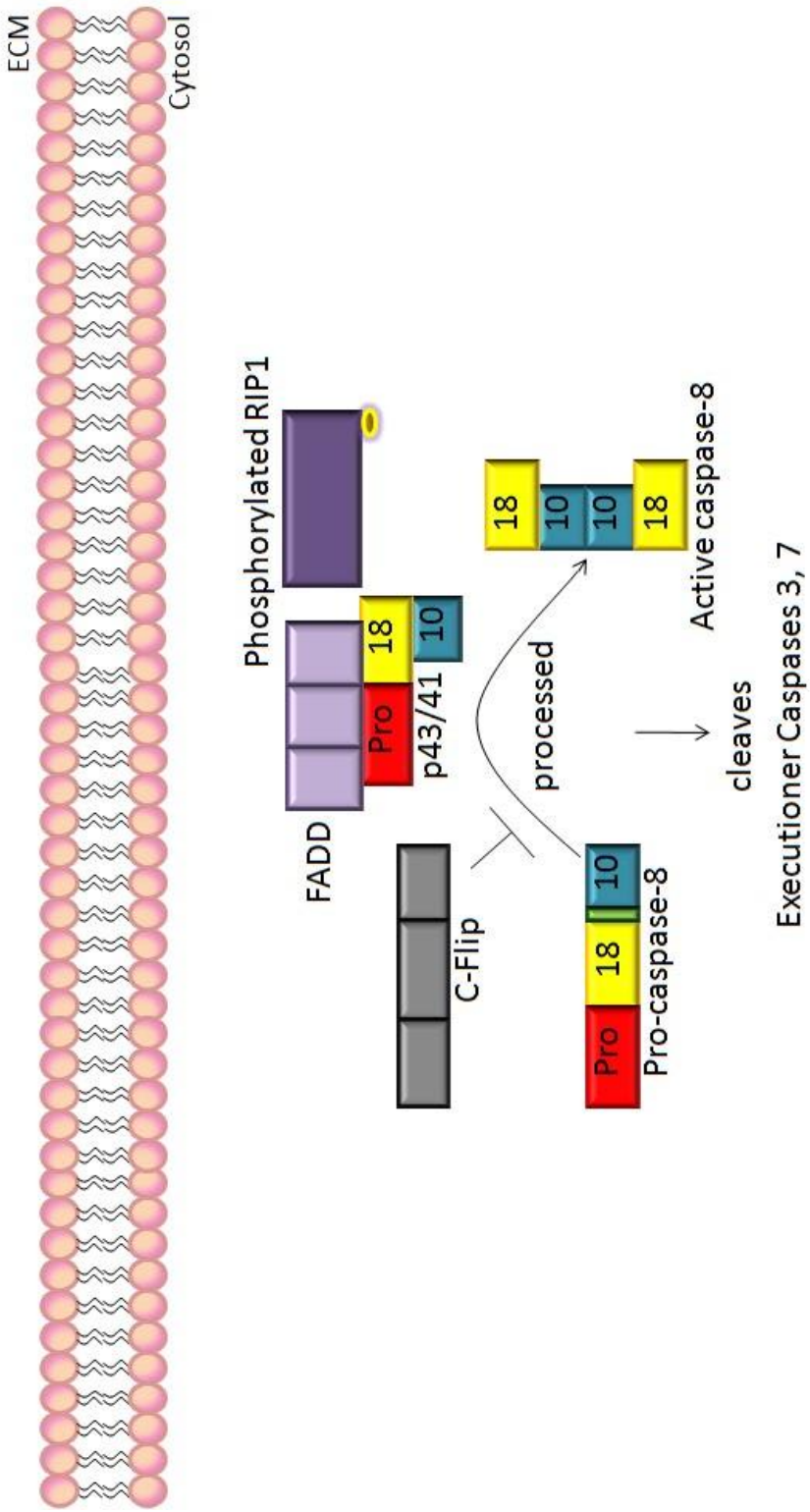

Figure 1.3.5 Ripoptosome Formation.

A 2mD complex discovered to form independent of receptor or mitochondrial activation. The ripoptosome forms upon depletion of cIAP either through etoposide, SMAC over-expression or through SMAC mimetics. The major components are phosphorylated RIP1, FADD and caspase-8. This complex has been shown to induce apoptosis through caspase-8 processing.

Figure 1.4.1. General Cleavage Events for Critical Apoptotic Related Caspases.

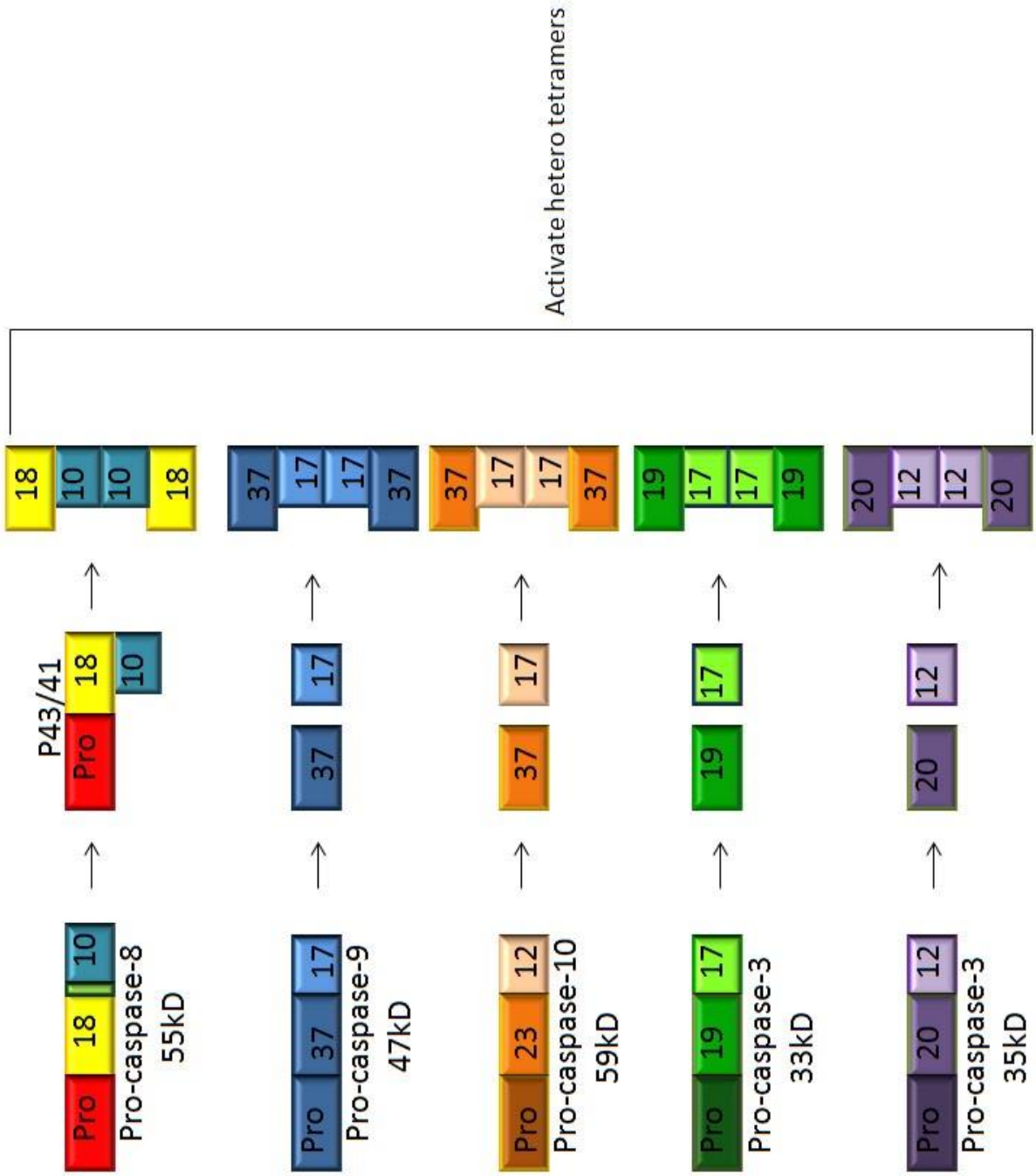

Figure 1.4.1. General Cleavage Events for Critical Apoptotic Related Caspases. Caspases 8 and 10 are larger than other caspases because they contain DED domains responsible for binding to the DISC complex.

Figure 1.4.2 Unfolded Protein Response and Intrinsic Pathway Activation.

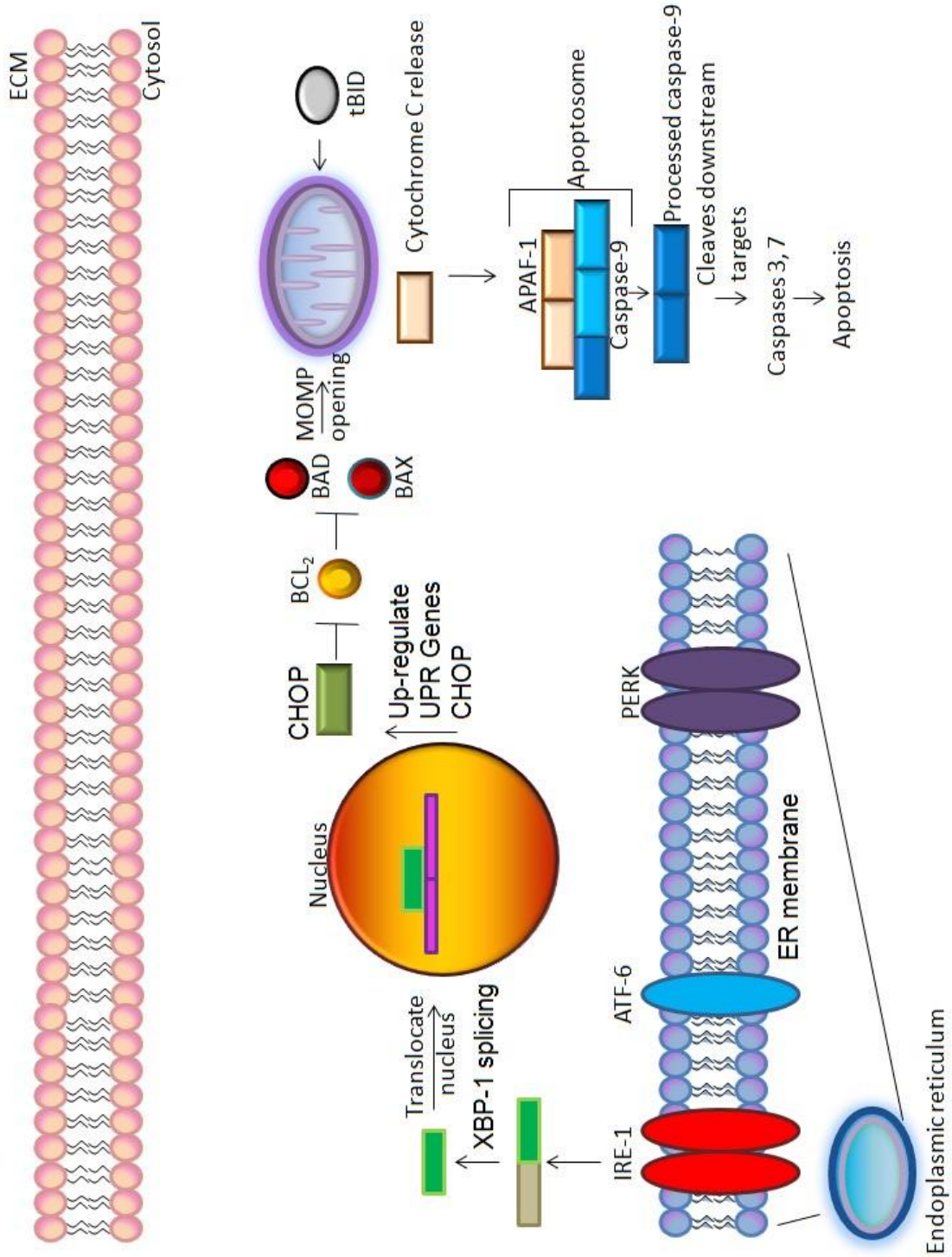

Figure 1.4.2 Unfolded Protein Response and Intrinsic Pathway Activation.

Sensor proteins IRE-1 ATF-6, and PERK detect an overload of misfolded or unfolded proteins and up-regulate protein folding chaperones and machinery to clear ER load. Extended periods of ER stress results in the up-regulation of CHOP which inhibits the anti-apoptotic BCL family members allowing BAD/BAX to induce the intrinsic pathway of apoptosis induction.

Figure 1.6.1. Ubiquitin Proteasome System.

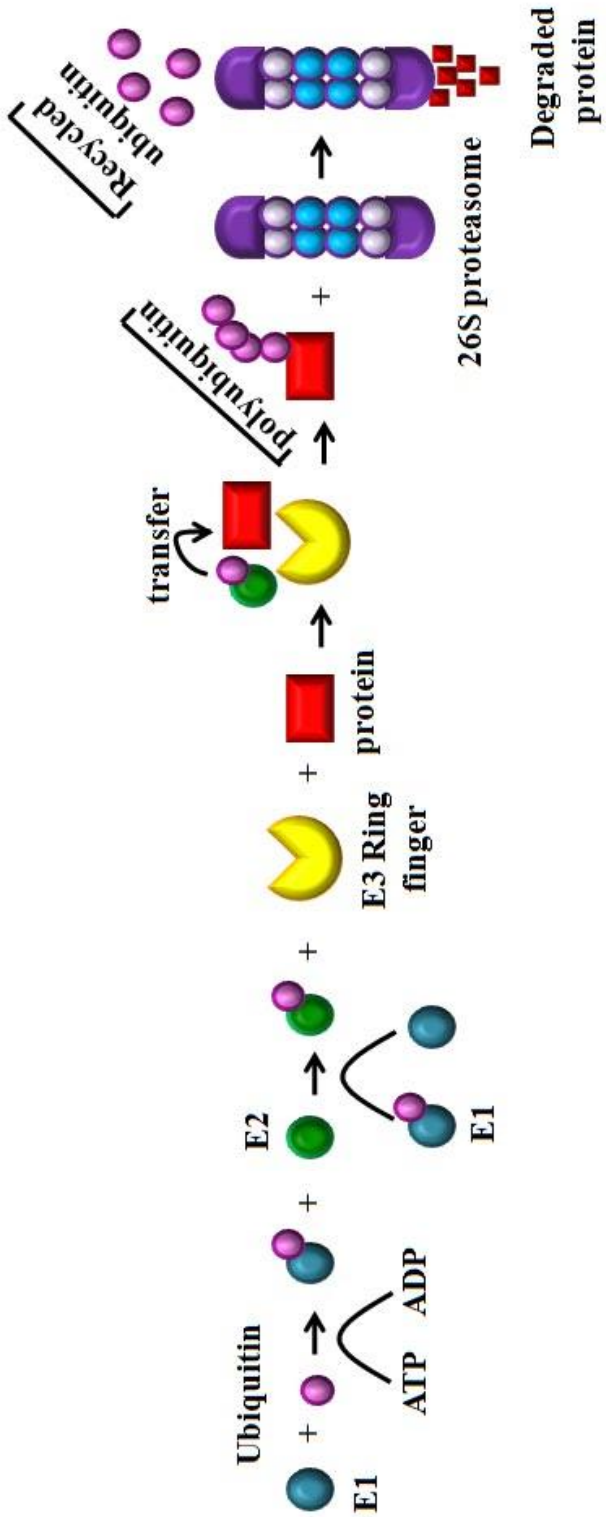

Figure 1.6.1. Ubiquitin Proteasome System.

Pathway involved with mediating the co-valent attachment of ubiquitin to proteins marked for degradation. Ubiquitin tagging is mediated through three classes of enzymes E1 (ubiquitin activating) E2 (ubiquitin conjugated) and E3 (Ubiquitin Ligase). E3 ligases facilitate the transfer of ubiquitin to the target substrate.

Figure 1.6.2. The 26S Proteasome.

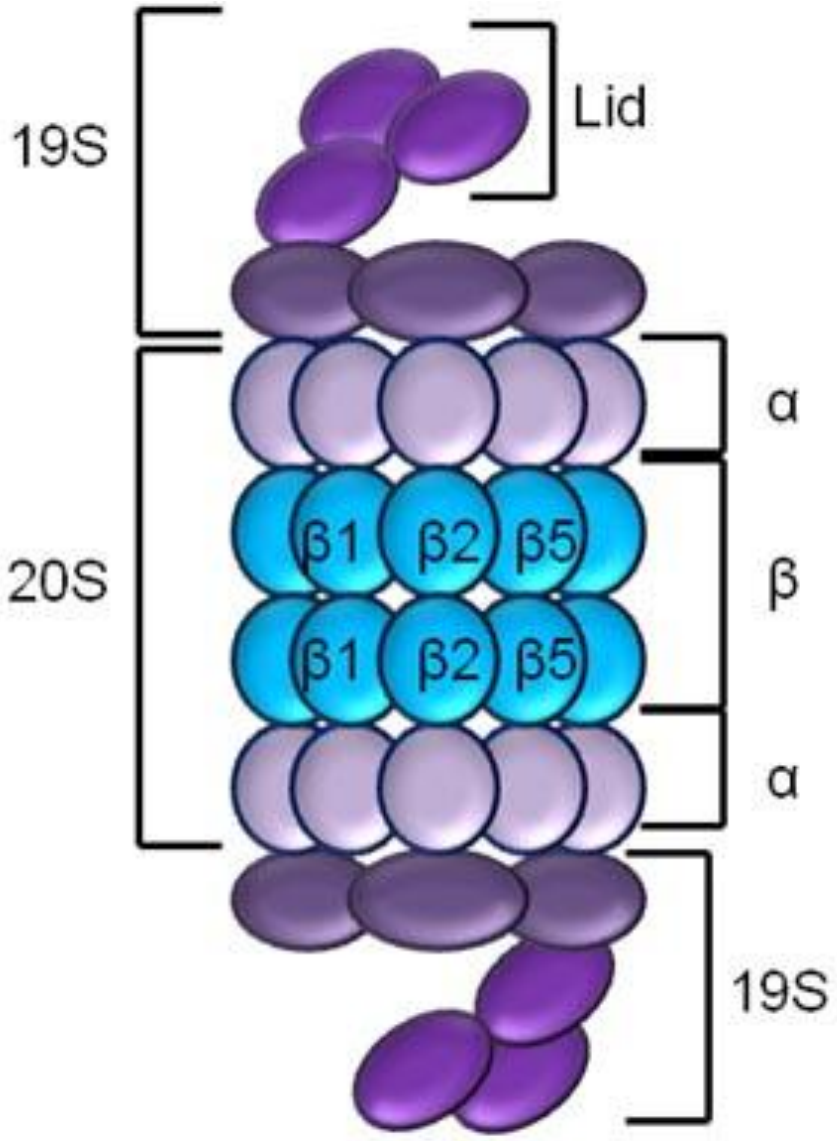

Figure 1.6.2. 26S Proteasome: Composed of 19s regulatory subunit that recognizes polyubiquitinated proteins and recycles Ubiquitin and the 20s particle which contains the catalytic activity of the proteasome.

Figure 1.7.1.Proteasome Inhibition Stabilizes Caspase-8 p18 Catalytically Active Subunit

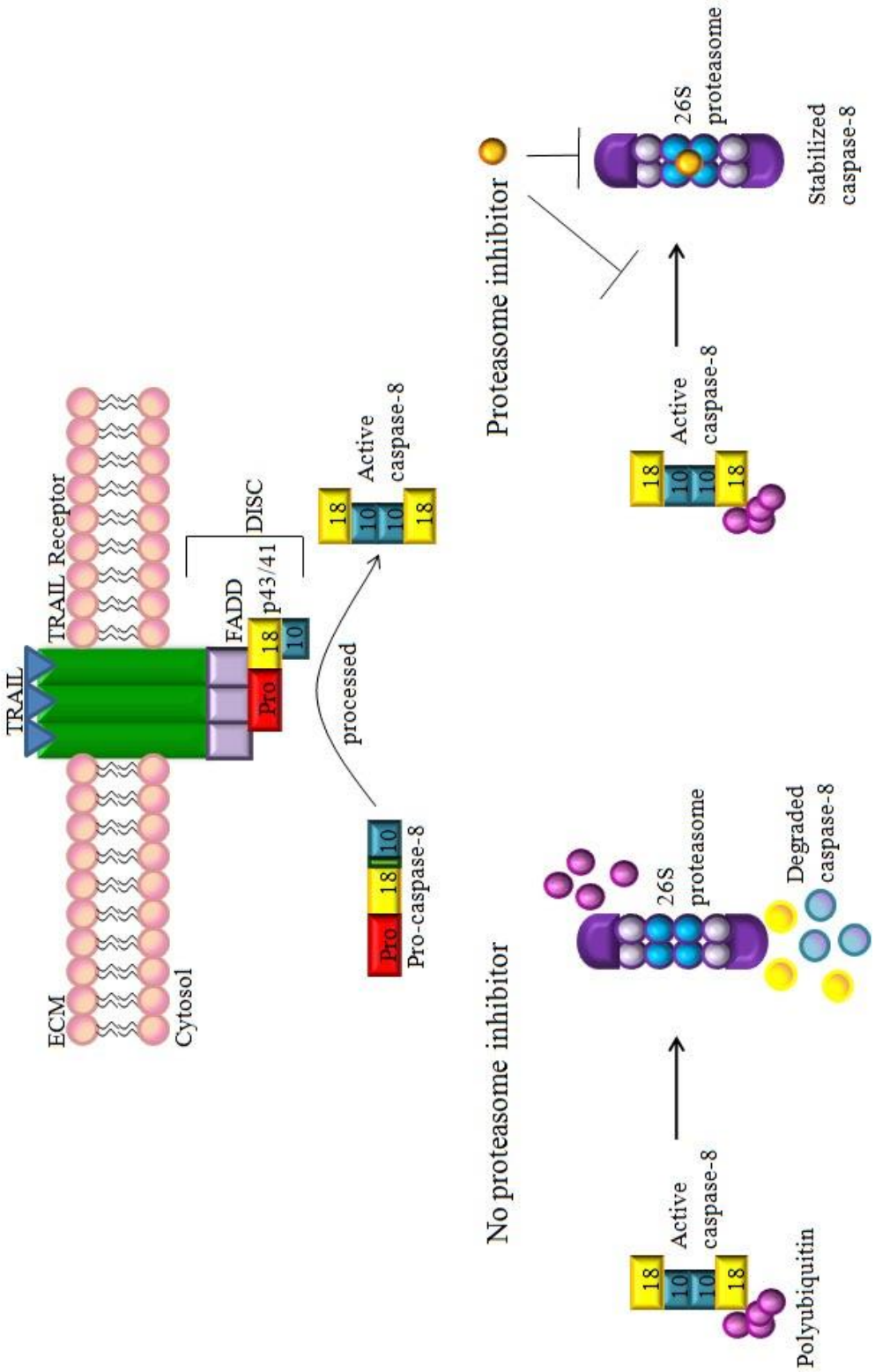

Figure 1.7.1. Proteasome Inhibition Stabilizes Caspase-8 p18 Catalytically Active Subunit

We hypothesized that caspase-8 was degraded by the 26S proteasome. Proteasome inhibition can stabilize the catalytically active caspase-8 p18 subunit by preventing its degradation by the 26S proteasome.

Copyright © Michael V. Fiandalo 2012

Chapter 2: Materials and Methods

2.1. Cell Culture and Reagents

Human prostate cancer lines, LNCaP and PC3 were cultured in Dulbecco's Modified Eagles Media (DMEM) (Media Tech Inc, Manassas, VA), or F12K media (Media Tech Inc, Manassas, VA). Lung cancer cell line H460 (a kind gift from Dr. John Yanneli at University of Kentucky, Lexington KY), Jurkat T cell line was purchased from the American Type Culture Collection (ATCC) and Neuroblastoma cell line (NB7) caspase-8 deficient cell line (Caspase-8 constructs and NB7 cell line were a very generous gift from Drs. Andrew Oberst and Doug Green (St. Jude Children's Research Hospital, Memphis, TN) were cultured in RPMI (Mediatech). Media was supplemented with 10% FBS (Cellagro), 1% Penicillin (10,000 I.U/ml) / Streptomicin (10,000ug/ml). Epoxomicin was a generous gift provided by Dr. Kyung Bo Kim (College of Pharmacy, University of Kentucky). Velcade (Millennium Pharmaceuticals, Cambridge, MA) was donated by the University of Kentucky, Markey Cancer Center Pharmacy. The GST-TRAIL fusion protein as purified using affinity chromatography as described (Christian et al., 2009b; Thorpe et al., 2008b). Caspase-8 reconstitution was carried out as described in (Oberst et al., 2010). NB7 cells were transduced with RT virus encoding either wild-type or non-cleavable mutant caspase-8 constructs (Caspase-8 expression was standardized via flow cytometry (Beckman Coulter MoFlo, Ft.Collins, CO) at Flow Cytometry Core Facility, University of Kentucky.

2.2 Cell Viability and Apoptosis Evaluation

Cell viability was assessed using MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)), after treatment with GST-TRAIL or Epoxomicin. Results were read using a spectrophotometer at 570 nm minus 690 nm. For evaluation of apoptosis induction we employed Annexin V, APC/PI (Invitrogen, Eugene, OR) staining. Cells were seeded in 24-well plates and treated with GST-TRAIL or Epoxomicin separately or in combination. After treatment, cells were harvested, washed with Annexin V binding buffer, resuspended in Annexin binding buffer with PI, and Annexin V APC was added to samples as recommended by manufacturer. Staining was analyzed by flow cytometry (FACSCalibur, BD Immunocytometry Systems, San Jose, CA) at the Flow Cytometry Core Facility, University of Kentucky. Annexin V positive and viable cells were quantified using CellQuest Pro software (BD Immunocytometry Systems, San Jose, CA) in 20,000 gated events. All MTT and Annexin V/PI experiments were performed in triplicate.

2.3. Half-life Evaluation

Cells ($2x10^6$) were plated into 6 well plates, were either pre-treated with proteasome inhibitor or not, followed by 100ng GST-TRAIL stimulation for two hrs. After two hrs cells were treated with cycloheximide or caspase inhibitor Z-VAD-FMK and harvested at indicated time points. Caspase-8 expression was analyzed using Western blot with the monoclonal IC12 caspase-8 antibody (Cell-Signal Technology, Danvers, MA).

2.4. Polyubiquitin Immunoprecipitation (IP) and Western Blot Analysis

LNCaP cells ($6x10^6$) were treated either separately or in combination with GST-TRAIL (400ng/ml) or (1μM) Epoxomicin for 8hrs. Cells were subsequently harvested in (1%) NP-40 in PBS and protein content was determined using BCA assay (Pierce). Prior to immunoprecipitation, lysates (10% of total lysate) were used as input. Polyubiquitin immunoprecipitation was performed using Ubiquitin Enrichment Kit (Thermo Fisher Scientific, Rockford, IL) and caspase-8 expression was determined using Western blot probing with the monoclonal IC12 anti-caspase-8 antibody (Cell Signaling Technology, Danvers, MA).

2.5. Subcellular Fractionation

LNCaP ($1.2x10^7$) or NB7 (1.6×10^7) cells were plated into 100mm tissue culture plates and were treated with GST-TRAIL (400ng/ml) and/or (1μM) Epoxomicin. All steps were performed at 4^oC. After treatment cells were harvested in 1x sucrose buffer A, subjected to dounce homogenization and centrifugation (1000xg; 10mins). Lysates were overlayed onto a 30% Percoll gradient in 2x buffer A and subjected to ultracentrifugation in a Beckman Coulter Optima L-90K Ultracentrifuge (1hr, 26,000 RPM (61,000 x g) at 4^oC). Following ultracentrifugation, cytosol and plasma membrane fractions were collected; inner membrane fractions were collected by adding 1x buffer to 30% Percoll (GE Healthcare)/2x sucrose buffer A followed by ultracentrifugation (1hr, 200,000xg at 4^oC). Cellular fractions were subjected to Western blot analysis and/or caspase activity assays.

2.6. Caspase-8 Activity Evaluation

NB7 cells ($1.6x10^7$), parental or caspase-8 expressing clones were treated either separately or in combination with GST-TRAIL (100ng/ml) or (1μM) Epoxomicin for 4hrs, caspase-8 activity was assessed using the Caspase-8-glo luminescence kit, (Promega, Madison, Wisconsin) according to the manufacturer's protocol; luminescence was measured by using Veritas™ Microplate luminometer (Promega Corp., Sunnyvale, CA). NB7 cells were subjected to caspase-8 immunoprecipitation either in whole cell lysate or cytosol and plasma membranes fractions. Caspase-8 activity assay was performed on the beads containing antibody-caspase-8 complexes. All experiments were performed in triplicate.

2.7. Immunocytochemical Analysis

EZslide (Millipore, Temecula, CA) were coated with 400ng Fibronectin (Sigma, St. Louis, MO) for 2hrs. LNCaP cells ($8x10^4$) were plated into each well and treated with 100ng GST-TRAIL or 1uM Epoxomicin separately or in combination. Cells were fixed with 4% paraformaldehyde, permiabilized with SAP buffer, blocked with BSA and probed with respective primary antibodies, (Affinity purified polyclonal caspase-8 (R&D, Minneapolis, MN), and Na^+K^+ ATPase (Millipore Temecula CA) and 26S Proteasome 20S particle (Calbiochem, San Diego, CA). Cells were washed with 1X PBST and probed with either 488 Alexa fluor goat-anti-mouse or 594 Alexa Fluor goat-anti rabbit, (Invitrogen, Eugene, OR) conjugated secondary antibodies. Following secondary antibody incubation and wash steps, slides were mounted using Vectashield (Vector

laboratories Inc, Burlingame, CA). Images were photographed using Leica TSP SP5 Confocal microscope at University of Kentucky Imaging Facility.

2.8. Statistical Analysis

MTT and Annexin V/PI experiments were performed in triplicate and data were standardized to the non-treated results. Caspase-8 activity assay results acquired from either whole cell lysates or fractions were normalized to protein concentrations based on BCA protein quantification. Half-life Western blots were quantified by densitometry using the Image J software. All experiments were performed in triplicate and the data were analyzed using unpaired Student t-Test analysis. Statistical significance of values among different treatment groups was set at $p < 0.01$

Copyright © Michael V. Fiandalo 2012

Chapter 3 Experimetnal Outcomes

3.1a. Caspase-8 Activation is Independent of Caspase-8 Processing

We previously demonstrated that the combination of TRAIL and proteasome inhibitor not only restored TRAIL sensitivity in TRAIL resistant prostate cancer (LNCaP) in both *in-vitro* and *in-vivo* models (Christian et al., 2009b) but also leads to stabilization of caspase-8 p18 subunit (Thorpe et al., 2008b). Our goal was to further examine the impact of proteasome inhibition on caspase-8 using non-cleavable caspase-8 constructs (Fig. 3.1.1a, Panel A). After successful reconstitution of wild-type and non-cleavable caspase-8 mutant constructs in NB7 caspase-8 deficient cells (Fig. 3.1.1a, panel B), caspase-8 activity was comparatively analyzed between the wild-type and non-cleavable constructs in response to treatment. Caspase-8 activity was analyzed in either whole cell lysates or cytosolic and plasma membrane fractions (Fig. 3.1.1a, panels C and D respectively) subjected to caspase-8 immunoprecipitation. Low levels of caspase-8 activity were observed in cells treated with GST-TRAIL alone and activity increased when epoxomicin was used with TRAIL in combination. The caspase-8 non-cleavable mutants possess higher levels activity than the wild-type caspase-8 (Fig. 3.1.1a panel C) ($P = 0.017, 0.031, 0.003$). This prompted us to analyze Analysis of caspase-8 activity in the subcellular fractions; the results indicate that the wild-type caspase-8 had the highest level of activity in the cytosol and the lowest at the membrane, while the non-cleavable constructs had significantly low activity in the cytosol and higher at the plasma membrane (Fig. 3.1.1a, panel D).

3.1b. Caspase-8 Processing not Required for Apoptosis Induction.

We subsequently compared cell viability and apoptosis induction between the wild-type and non-cleavable caspase-8 constructs. Treatment with either GST-TRAIL or Epoxomicin separately or in combination, led to significant decrease in cell viability in NB7 caspase-8 expressing cell lines (Fig. 3.1.1b, panel A). Treatment with a lower dose of GST-TRAIL revealed that the non-cleavable mutants were more effective at inducing apoptosis than wild-type caspase-8 (Fig. 3.1.1b, panel B). The combination of GST-TRAIL and Epoxomicin resulted in a significant induction of apoptosis (Fig. 3.1.1, panel C, P = 0.0001, 0.0001, 0.0001).

3.2a. Proteasome Inhibition Stabilizes Caspase-8 p18 Subunit.

Proteasome inhibition confers caspase-8 stabilization in different cancer cell lines; PC3 (prostate cancer), Jurkat (T cell), and H460 (lung cancer) cell lines (Fig. 3.2.1a, panels A, B, C). Either simultaneous addition of GST-TRAIL and proteasome inhibitor or proteasome inhibitor pretreatment followed by GST-TRAIL stimulation leads to caspase-8 p18 subunit accumulation (Fig 3.2.1a, panels A, B, C). To determine whether accumulation of the caspase-8 p18 subunit is a result of proteasome inhibition (and not due to increased processing), we evaluated the half-life of p18 and p10 subunits in the presence and/or absence of proteasome inhibitor. Proteasome inhibition stabilizes caspase-8 p18 subunit, thereby extending its half-life (Fig. 3.2.1, panels D and E). Thus p10 expression persisted over time in the presence or absence of proteasome inhibitor (Fig. 3.2.1, panel F).

3.2b. Processed Caspase-8 is Polyubiquitinated.

The next sequence of experiments set to determine whether pro-caspase-8 or cleaved caspase-8 was polyubiquitinated. We used the LNCaP human prostate cancer cell line and performed caspase-8 immunoprecipitation followed by Western blot to probe for ubiquitin. LNCaP cells were treated with different combinations of proteasome inhibitors (Velcade or Epoxomicin) separately or in combination with GST-TRAIL. Input was analyzed by Western blotting and caspase-8 IP followed by Western blot analysis probing for polyubiquitin (Figure 3.2.1b, panels A and B, respectively). We observed polyubiquitin smears in the GST-TRAIL and GST-TRAIL/Velcade treated lanes and no trace of polyubiquitin in the non-treated or Velcade only lanes. These results were further supported by immunoprecipitation (IP) analysis using the Ubiquitin Enrichment kit to pull-down ubiquitinated proteins and Western blotting for caspase-8 (Fig. 3.2.1b, panel D). Probing for the caspase-8 p10 subunit, revealed that the p10 is not polyubiquitinated (data not shown).

3.3a. Proteasome Inhibition Increases Caspase-8 Accumulation in the Cytosol and Plasma Membrane.

To determine the impact of proteasome inhibition on caspase-8 trafficking, I employed subcellular fractionation which yielded cytosol, plasma membrane, and internal membrane fractions. To assess if proteasome inhibition modulated caspase-8 trafficking, LNCaP cells were treated with Epoxomicin and GST-TRAIL separately or in combination followed by subcellular fractionation and caspase-8 expression was analyzed via Western blot (Fig. 3.3.1a, panel A). Caspase-8 expression was not detected

in the internal membrane fractions however, there was caspase-8 expression (full-length and cleaved forms) in cytosol and plasma membrane fractions (Fig. 3.3.1a, panel B). The p43/41 intermediate and p18 subunit expression was predominantly elevated in the plasma membrane than cytosol. This accumulation of the p43/41 intermediate and p18 subunit in the plasma membrane and cytosol fractions was enhanced in the presence of the proteasome inhibitor (Fig. 3.3.1a, panel B).

Next I pursued to examine the consequences of caspase-8 activity in LNCaP cells. As shown in Figure 3.3.1a caspase-8 p43/41 expression with epoxomicin and GST-TRAIL treatment was higher than when cancer cells were exposed to GST-TRAIL alone (Fig. 3.3.1a, panel C). There was however an increase in plasma membrane associated caspase-8 activity with GST-TRAIL treatment alone and a marked increase in caspase-8 activity with the combination of GST-TRAIL and epoxomicin (Fig. 5, panel C)($P = 0.001$ and $P = 0.0006$). Time course analysis of the consequences of proteasome inhibition on caspase-8 trafficking and activity in LNCaP prostate cancer cells, revealed that p43/41 intermediate expression increased in the plasma membrane (Fig. 3.3.1a, panel D). There was a temporal increase in the activity in both fractions followed by a steep decline observed in the plasma membrane fraction (Fig. 3.3.1, panel E) (2hr fractions $P = 0.0001$, 4hr fractions, cytosol $P = 0.0003$, pm $P = 0.0001$, 6hr fractions $P = 0.0001$, 8hr fractions $P = 0.0001$, 12hr = 0.0001).

3.3b. Caspase-8 Co-localizes with Na^+/K^+ ATPase.

To confirm the fractionation data immunocytochemistry analysis was conducted, I used confocal microscopy to determine whether caspase-8 co-localizes with Na^+/K^+ ATPase (Fig. 3.3.1b). LNCaP cells were treated and subjected to fluorescence staining as described above, to determine the cellular localization of the 20S particle of the 26S proteasome and Na^+/K^+ ATPase. The representative images shown on figure 3.3.6 clearly indicate the lack of co-localization between the 26S proteasome and the Na^+/K^+ ATPase, while there is significant co-localization detected between caspase-8 and Na^+/K^+ ATPase, in untreated and treated prostate cancer cells.

Figure 3.3.1a: Caspase-8 Processing is not Required for Activation of Apoptosis.

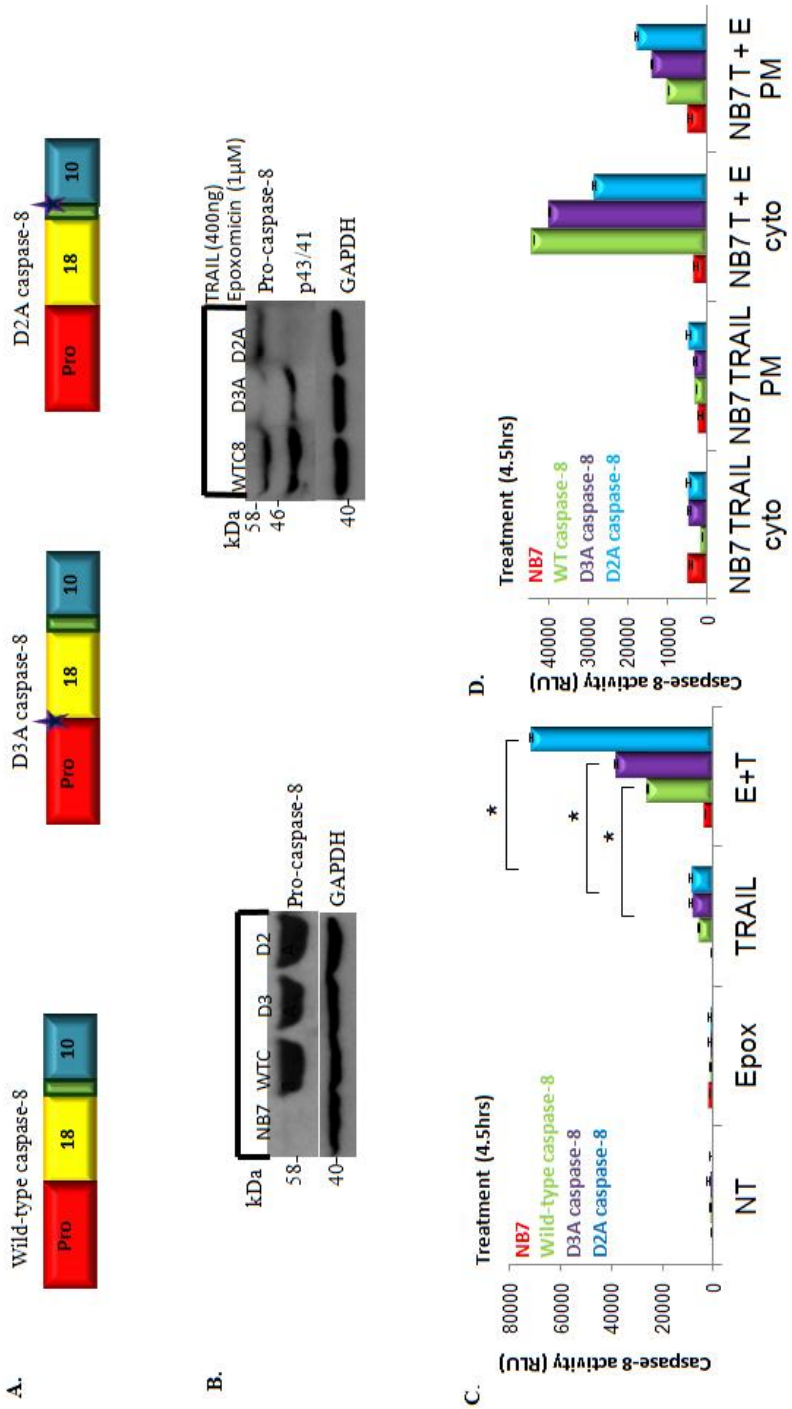

Figure 3.3.1a: Caspase-8 processing is not required for activation or apoptosis. A) Schematic showing caspase-8 non-cleavable mutant constructs. B). Western blots demonstrating successful reconstitution and respective cleavage events of wild-type and mutant caspase-8 in NB7 cells. C) MTT viability studies NB7 cells treated with a combination of epoxomicin and GST-TRAIL (50ng or 100ng as indicated) following treatment MTT was performed. D) Apoptosis induction was measured in NB7 cells that were treated as indicated and stained with Annexin V/ PI followed by analysis by FACS analysis. Figure E) Caspase-8 activity was evaluated in NB7 cell lines treated as indicated for 4 hours. Whole lysates were subjected to IP and activity was measured using the caspase-8 containing beads. F) Caspase-8 activity was measured in cytosol and plasma membrane factions using the same treatment conditions described in E), followed by subcellular fractionation and evaluating caspase-8 activity as described in materials and methods (F).

Figure 3.1.1b. MTT Viability and Annexin V/PI Studies

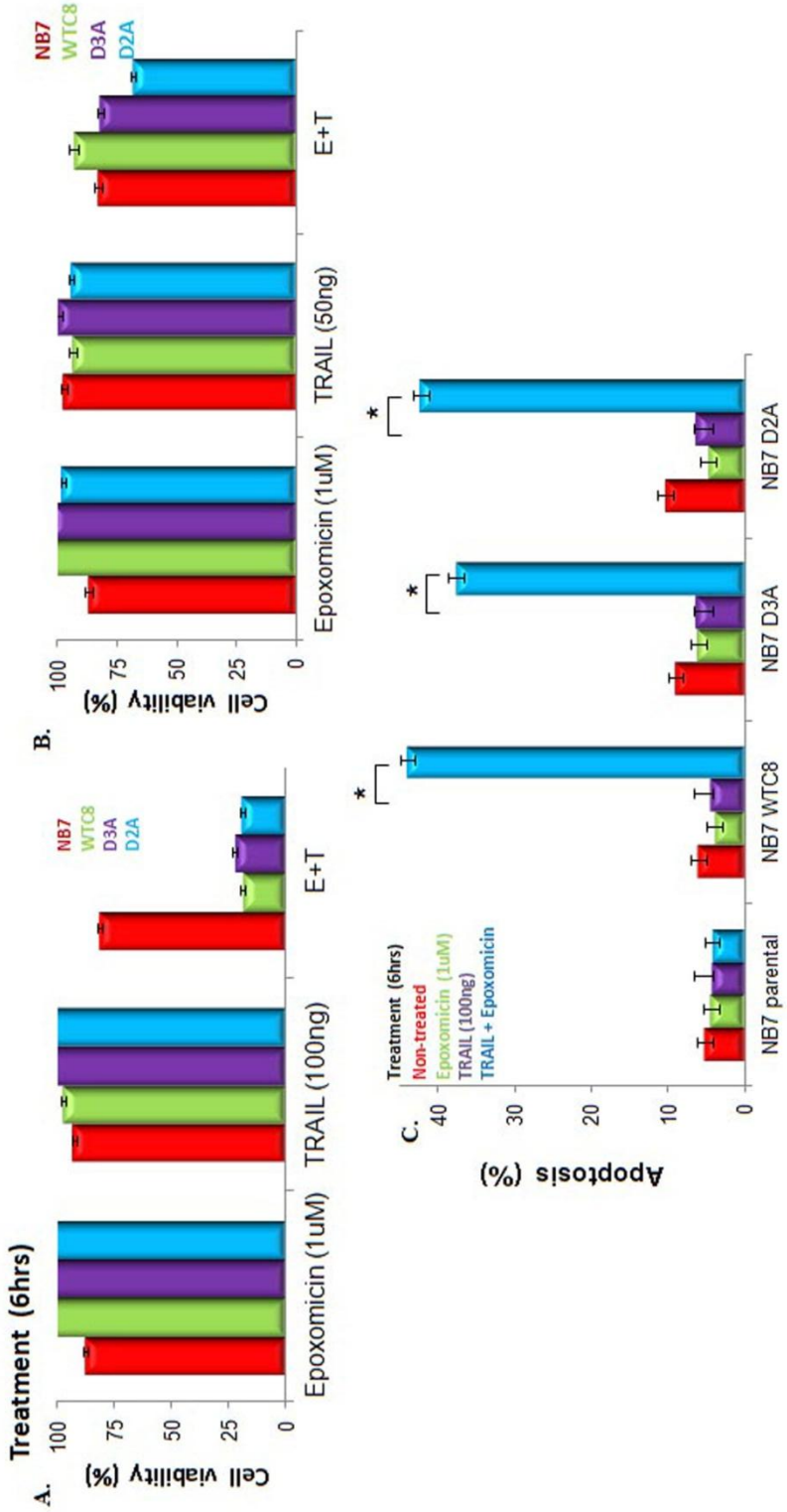

Figure 3.1.1b. MTT Viability and Annexin V/PI Studies.

NB7 cells treated with a combination of epoxomicin and GST-TRAIL [50ng (7.5 hrs) or 100ng (5.5 hrs) as indicated] following treatment MTT was performed (A, B). Apoptosis induction was measured in NB7 cells that were treated as indicated and stained with Annexin V/ PI followed by analysis by FACS analysis (C).

Figure 3.2.1a Proteasome Inhibition Stabilizes Caspase-8 p18 Subunit and Extends the Half-life.

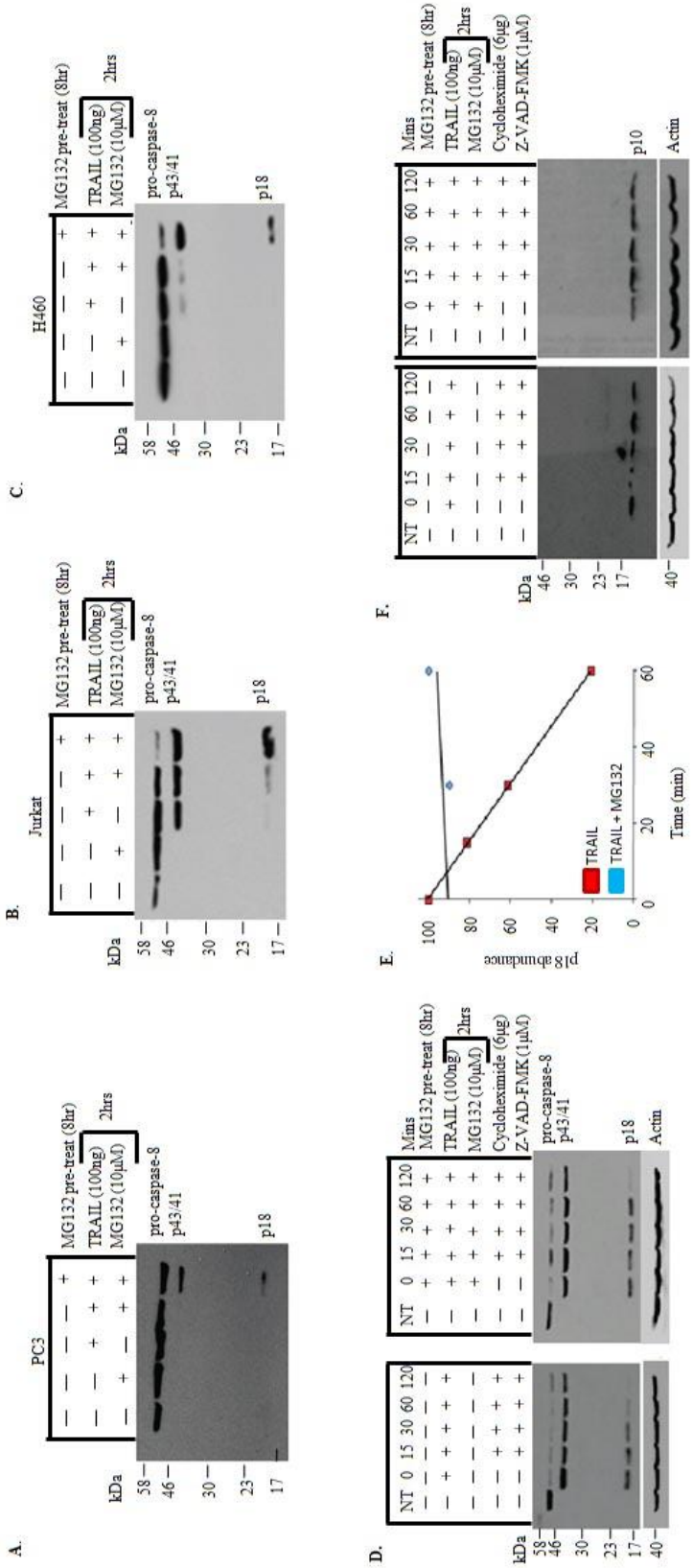

Figure 3.2.1a Proteasome Inhibition Stabilizes Caspase-8 p18 Subunit and Extends the Half-life.

Panels A, B and C; PC-3, Jurkat, H460 cells were plated into 6-well dishes, treated with proteasome inhibitor and GST-TRAIL. Treated and untreated control cells were harvested and caspase-8 expression was analyzed by Western blot. Proteasome inhibition leads to caspase-8 stabilization. Panels D, E; caspase-8 p18 subunit half-life studied. Panel F; Quantification of p18 half-life data on panel E.

Figure 3.2.1b: Processed Caspase-8 is Polyubiquitinated.

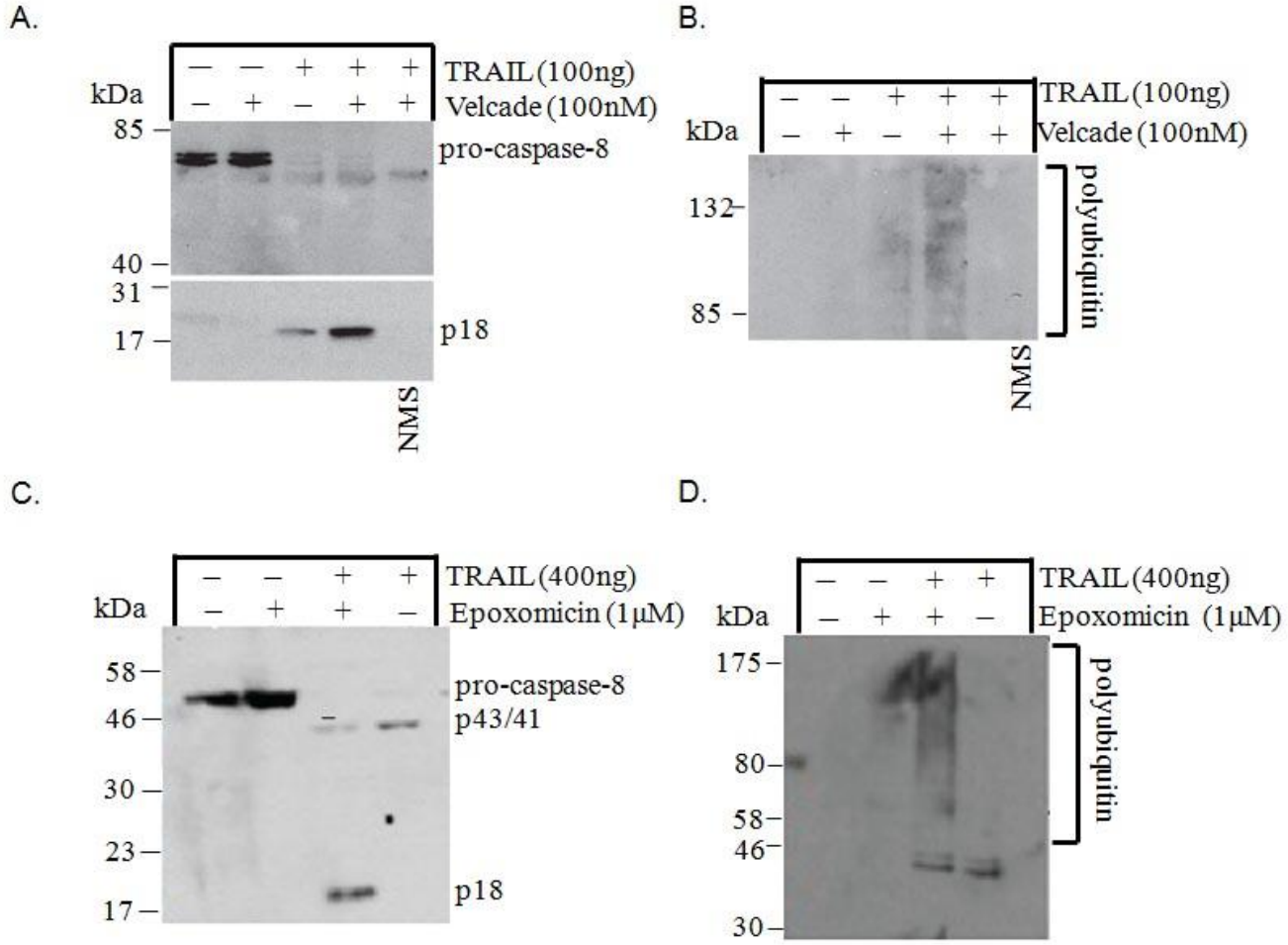

Figure 3.2.1b: Processed Caspase-8 is Polyubiquitinated.

Polyubiquitin immunoprecipitation was carried out using the protocol for Ubiquitin Enrichment kit. LNCaP cells were treated with GST-TRAIL or Epoxomicin separately or in combination for 8 hrs. A). Western blot analysis of caspase-8 expression in response to Epoxomicin and GST-TRAIL treatments prior to Ubiquitin pull down. Panel B, Western blot showing caspase-8 polyubiquitination following Ubiquitin IP.

Figure 3.3.1a: Proteasome inhibition leads to increased cleaved caspase-8 in plasma membrane.

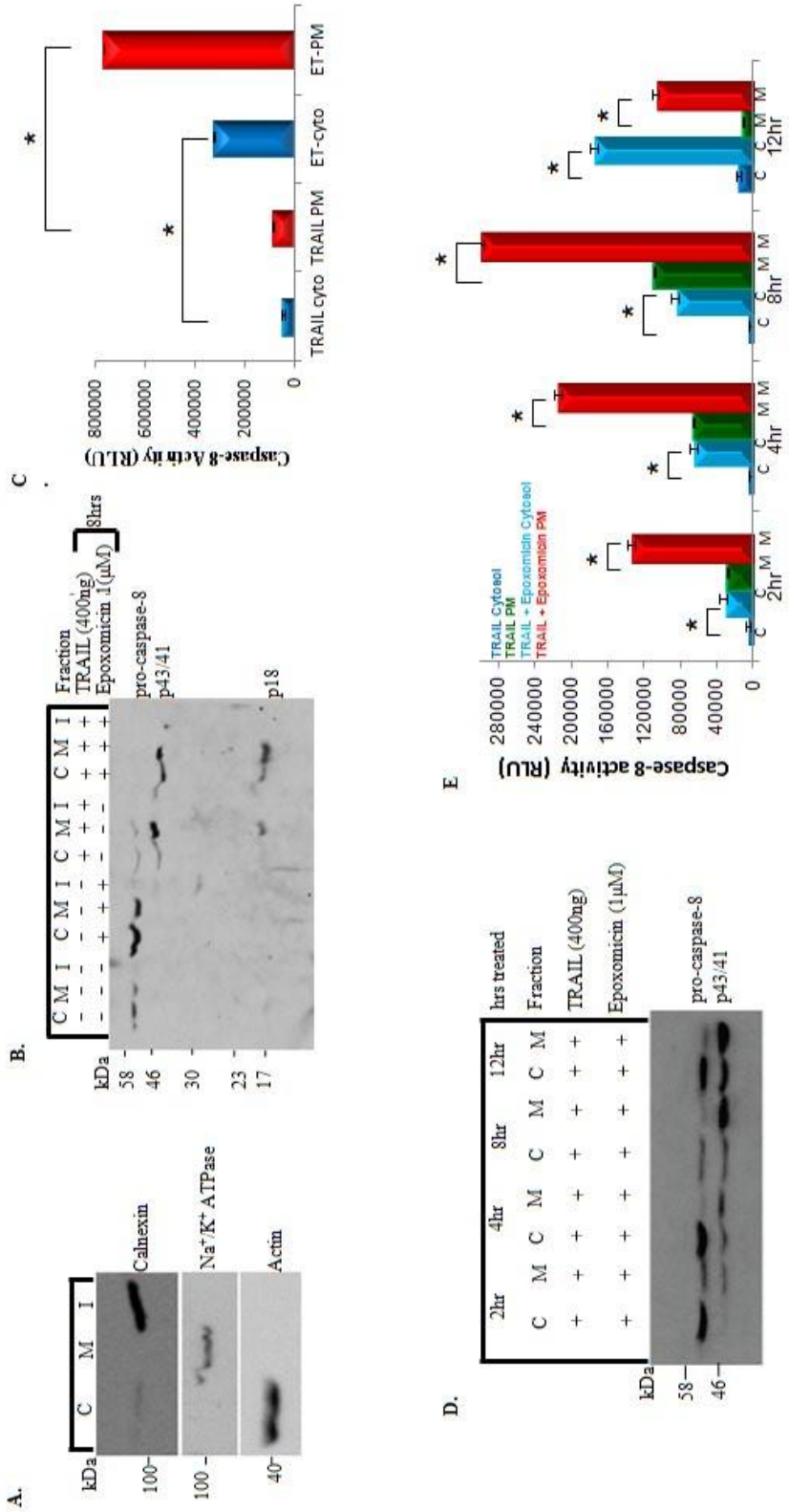

Figure 3.3.1a: Proteasome Inhibition Leads to Increased Cleaved Caspase-8 in Plasma Membrane.

Panel A, Markers indicating integrity of fractions. B. Western blot showing caspase-8 expression only in cytosol and plasma membrane fractions. C. Western blot showing impact of proteasome inhibition on caspase-8 expression in cytosol and plasma membrane fractions.

Figure 3.3.1b: Co-localization of caspase-8 with Na^+K^+ ATPase.

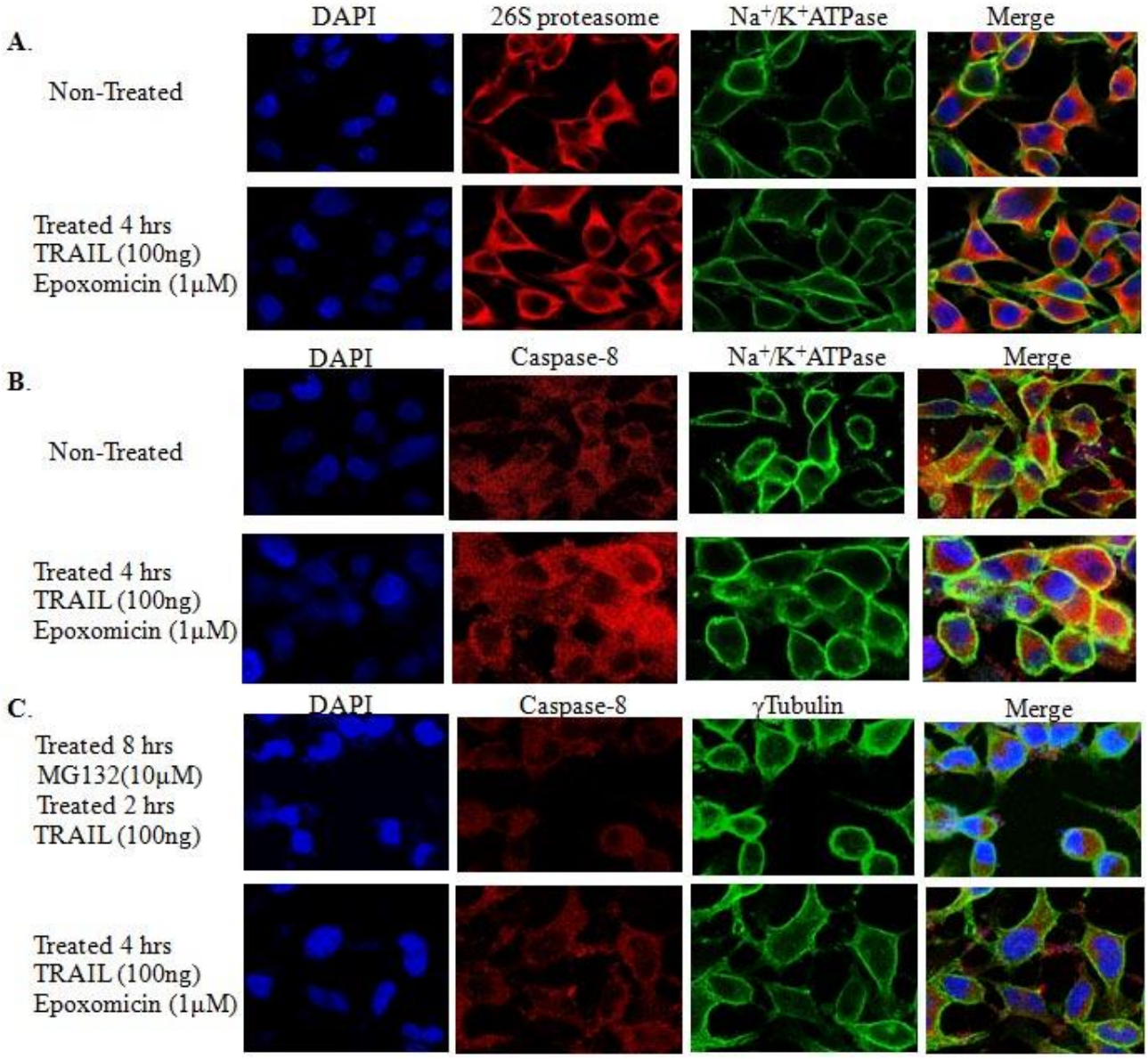

Figure 3.3.1a: Proteasome Inhibition Leads to Increased Cleaved Caspase-8 in Plasma Membrane. Panel A, Markers indicating integrity of fractions. B. Western blot showing caspase-8 expression only in cytosol and plasma membrane fractions. C. Western blot showing impact of proteasome inhibition on caspase-8 expression in cytosol and plasma membrane fractions.

Copyright © Michael V. Fiandalo 2012

Chapter 4 Discussion

Growing evidence supports a dynamic functional interaction between proteasome inhibition and caspase-8 mediated apoptosis in cancer cells from different cell types (Brooks et al., 2010; Jane et al., 2011; Koschny et al., 2007). The combination of TRAIL and Velcade has become a promising chemotherapeutic strategy because of their efficacy of inducing apoptosis in tumors (Christian et al., 2009b; Mitsiades et al., 2001; Sayers et al., 2003). Another advantage to using TRAIL as a chemotherapeutic agent is its specificity in inducing apoptosis in tumor cells and not healthy cells (Plasilova et al., 2002; Zhang et al., 2010). The present study establishes that proteasome inhibition increases cleaved caspase-8 stability as well as caspase-8 activity and protein accumulation at the plasma membrane and in the cytosol. Using appropriate markers we verified our fractions and further interrogation revealed caspase-8 p43/41 and p18 cleavage products in both the cytosolic and plasma membrane fractions, with p43/41 and p18 subunit expression were predominately in the plasma membrane.

One may argue that it is still unclear on whether polyubiquitination occurs at the DISC causing its release into the cytosol or if polyubiquitination occurs in the cytosol following caspase-8 release from the DISC. It would indeed be of major significance to address this point and determine if caspase-8 is polyubiquitinated at the DISC or in the cytosol would provide a platform for further studying caspase-8 regulation. Identifying whether polyubiquitination is the trigger for releasing caspase-8 into the cytosol as well as identifying the specific E3 ligase responsible for caspase-8 K48 linked polyubiquitination. Evidence suggests that caspase-8 polyubiquitination can be either

K63 linked on the p10 subunit which leads to caspase-8 activation or K48 linkage leading towards caspase-8 degradation (Jin et al., 2009; Peng et al., 2011). Our results indicate that processed caspase-8 is polyubiquitinated on the p18 subunit rather than the p10 subunit as previously shown (Jin et al., 2009). This evidence that supports p18 polyubiquitination, is in the fact that the IC12 caspase-8 antibody targets an epitope in the p18 region. Therefore any caspase-8 polyubiquitination observed with this antibody implies that the p18 also a target site for polyubiquitination in addition to the p10 subunit as suggested by Jin et al (Jin et al., 2009). A key consideration is that we are investigating endogenous caspase-8 in a physiologically relevant tumor microenvironment, rather than non-cleavable constructs or caspase-8 fragments that may be characterized as artifacts. Indeed we focused on caspase-8 polyubiquitination under the activation of the extrinsic pathway of apoptosis, rather than through activation of non-apoptotic pathways, such as EGF signaling pathway (Peng et al., 2011). It could be argued that the conditions involved analyzing caspase-8 ubiquitination following EGF treatment are distinct from those due to death receptor activation. The cellular platform hosting the kinetics for caspase-8 processing, polyubiquitination, and 26S proteasome degradation is cell type-dependent and another compounding factor is that the entire pool of pro-caspase-8 is not completely processed into cleavage products.

To address these issues we performed a combination time-course and dose response experiments to determine the conditions that yielded the maximal amount of caspase-8 p18 product available for polyubiquitination. Velcade or Epoxomicin treatment revealed that caspase-8 polyubiquitination wasn't the consequence of the actions of one specific inhibitor, but rather a specific result from proteasome inhibition. Epoxomicin

serves as a control indicates that caspase-8 p18 stabilization is a result of proteasome inhibition rather than through the potential manipulation of c-FLIP expression using Velcade or MG132 treatment (Li et al., 2007a; Liu et al., 2007). Ongoing studies seek to determine the cellular localization hosting the occurrence of caspase-8 polyubiquitination, cytosol vs. plasma membrane.

To further explore the mechanism of caspase-8 regulation, we used non-cleavable forms of caspase-8 and examined whether caspase-8 processing is required for caspase-8 activation and apoptosis induction. We unexpectedly observed a decrease in cell viability with the D2A non-cleavable construct which was comparable to the D3A and wild-type caspase-8 constructs. Lower doses of GST-TRAIL (in combination with Epoxomicin), revealed that the D2A and D3A non-cleavable mutants had larger decreases in cell viability compared to wild-type caspase-8. Annexin V/Pi staining complemented our MTT data as apoptosis induction was only observed upon combination treatment with Epoxomicin and GST-TRAIL. One may argue that the non-cleavable mutant forms of caspase-8 may be more effective in apoptosis induction due to caspase-8 forms being highly resistant to proteasome regulation.

The present results establish that non-cleavable mutants D3A and D2A were capable of becoming activated in the presence of GST-TRAIL alone and the activity of these caspase-8 mutants were markedly increased with the combination of GST-TRAIL and Epoxomicin. Interestingly enough, the data revealed two activity patterns, in the cytosol caspase-8 activity decreased depending on the cleavage mutation; while in the plasma membrane the wild-type caspase-8 had the least amount of activity while the non-cleavable D2A and D3A had higher activity. These findings in combination with the

LNCaP suggest that caspase-8 is processed at the DISC and is cycled back into the cytosol. These data are interesting in their own right because the accepted dogma for caspase-8 activation and apoptosis induction (Martin et al., 1998; Medema et al., 1997a; Oberst et al., 2010) states that caspase-8 processing is required for activation and apoptosis induction; however, the D2A mutant is fully capable of doing both and this form of caspase-8 is not cleaved. This mechanistic insight challenges the existing caspase-8 dogma. Future studies will focus on examining the impact of proteasome inhibition on apoptosis induction with the D2A non-cleavable mutant. Proteasome inhibition may potentially act to facilitate caspase-8 dimerization or stabilizes the p43/41 intermediate form at the DISC. Further elucidation of caspase-8 regulation could lead to the development of agents that stabilize the DISC with either full-length caspase-8 or p43/41 intermediate. Stabilizing either form at the DISC would slow down or prevent rapid caspase-8 degradation and increase caspase-8 activity for apoptosis induction. Thus caspase-8 stabilizing agents would be highly selective for apoptosis induction in cancer cells. Such agents will potentially circumvent issues associated with conventional chemotherapy i.e. normal vs. cancer cell type-specificity, and natural or acquired resistance in cancer cells as associated with existing chemotherapeutics such as Doxorubicin or Velcade.

Taken together this evidence provides a strong platform for exploiting new potential molecular targets such as, E3 ligases responsible for caspase-8 degradation. Studies by Jin et al, provided evidence identifying CUL3 as an E3 ligase that modulates caspase-8 activation (Jin et al., 2009), and this is in accord with our previous studies indicating that E3 ligases Siah2 and furthermore, our previous studies revealed E3

ligases, Siah2 and POSH (Christian et al., 2011) have a role in modulating caspase-8 activity. It is important to point out that in the Jin et al, studies caspase-8 degradation was not investigated and while our study provided the first evidence which showed SIAH2 or POSH had no impact caspase-8 processing or stability.

Together, these studies suggest there is another E3 ligase capable of mediating caspase-8 polyubiquitination and degradation. Identifying this E3 ligase may reveal the trigger for caspase-8 release from the DISC, such as polyubiquitination which may occur following initial cleavage event at aspartic acid residue 384. This could also imply that perhaps there is a preferred cleavage intermediate (likely the p43/41) that is optimal for crossing the caspase-8 activity threshold required to induce apoptosis rather than complete processing of caspase-8 into the p18/10 heterodimer.

Elucidating the mechanisms behind caspase-8 regulation can contribute to the generation of new effective caspase-8 driven anti-cancer approaches that activate or restore apoptosis in chemotherapeutic resistant cancer. By identifying molecules critical for caspase-8 regulation, this could lead to the development of specific inhibitors designed to, stabilize caspase-8 at the DISC, inhibit E3 ligases, to effectively inhibit cancer growth and induce apoptosis, DISC stabilizing molecules or even caspase-8 dimer inducing molecules (Figure 4.1.1).

Figure 4.1.1 Translational significance of Caspase-8 Studies

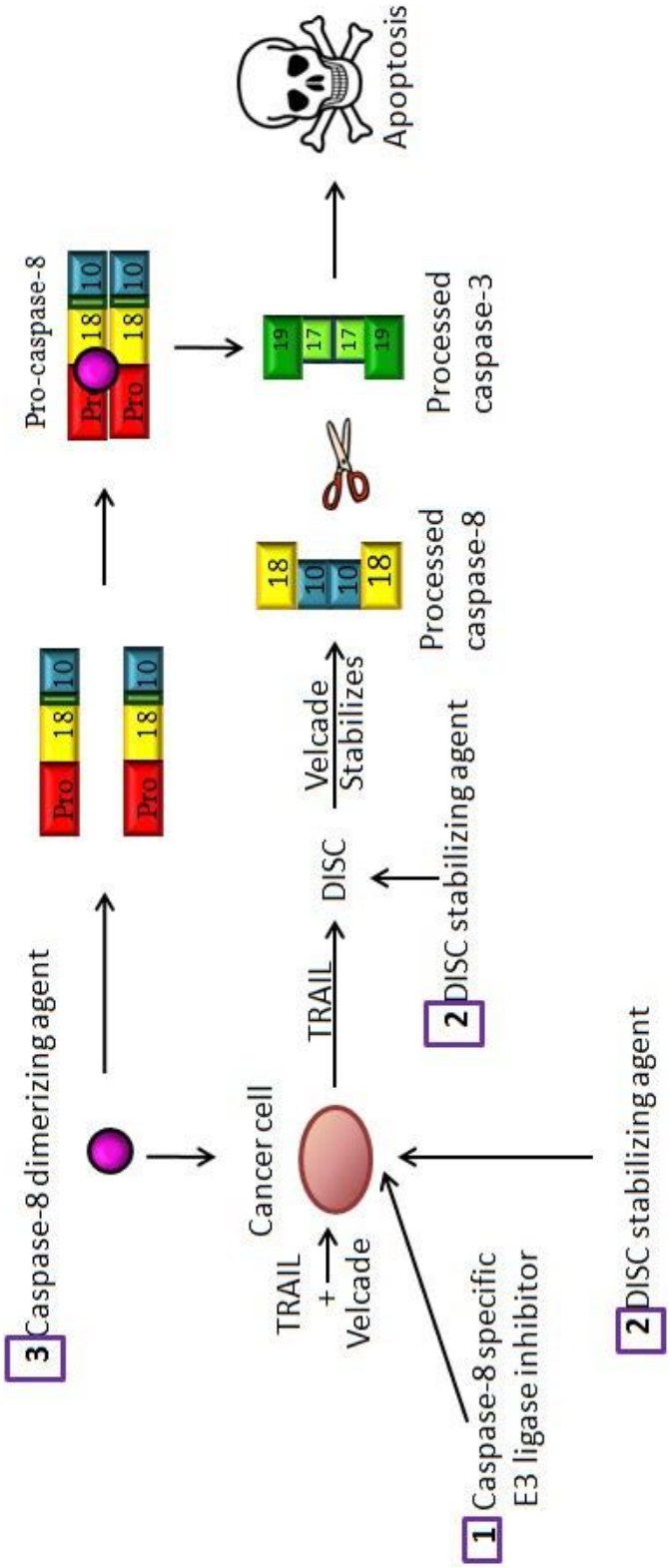

Figure 4.1.1: Translational Significance of Caspase-8 Studies

This schematic diagram illustrates the potential mechanism of action regarding the three possible clinically significance therapies as a result of further defining caspase-8 regulation. These strategies can be used to over-come Velcade resistance by eliciting the same effect observed when Velcade and TRAIL are used in combination for treatment of cancer. ., mnnn

Copyright © Michael V. Fiandalo 2012

Chapter 5 Future Directions

5.1 Caspase-8 Polyubiquitination

One of my early tasks in my doctoral studies was to analyze HA-tagged caspase-8 fragments and determine which region of caspase-8 was polyubiquintated. This task entailed generating an HA-tagged version of each of the caspase-8 subunits (Fig. 5.1 panel A). Once generated, these constructs were transfected into PC3 cells which were treated with or without Velcade and expression was evaluated through Western blot. The p20, and p18 fragments demonstrated a banding / smear pattern that was indicative of polyubiquitination. Originally we thought maybe the p2 is conferring some sort of stabilization to the p18, however, the data showed that the p20 (p18+p2) possessed a banding/smearing pattern indicative of polyubiquitination Another interesting finding was that p30 (p18, p2, p10) was stable with or without proteasome inhibitor, providing another piece of evidence suggesting that caspase-8 processing is required for polyubiquitination.

One of the major caveats to this study is the fact that these are over-expressed caspase-8 fragments, granted they provide insight into ubiquitination but the physiological relevance comes into question. However, this data provided us with a better understanding and prompted us to evaluate cleaved vs. intact caspase-8 polyubiquitination status in endogenous caspase-8 using our prostate cancer cell models. These experiments also set us up for the next component of the project which was to identify the amino acid residues within the p18 subunit that were polyubiquitinated.

5.2 Identification of Ubiquitinated Amino Acids in Caspase-8 and Determination of the Apoptotic Action of Stabilized Caspase-8 Mutants.

Our goal was to further interrogate caspase-8 ubiquitination and attempt to define which lysine residues were being covalently linked to ubiquitin. To accomplish this goal our strategy involved first identifying the polyubiquitinated lysine residue(s) within the p18 subunit. Once identified, the residue will be mutated, reintroduced into caspase-8 deficient cells and then effect on apoptosis examined. The first objective is to identify the amino acids of caspase-8 that control ubiquitination by mass spectrometry (MS). To obtain enough protein for analysis we devised a strategy to tag caspase-8 with a hemagglutinin (HA) epitope. A p18HA construct (in addition various other fragments as controls) was expressed in LNCaP cells. Cells were treated with or without proteasome inhibition and examined by Western blotting. The pro-domain and p30 fragment (containing the uncleaved C-terminus) were readily expressed without proteasome inhibition (Fig. 21A), however the p18 and p10 fragments required proteasome inhibition for expression.

Furthermore, a high molecular weight smear is a characteristic of polyubiquitination, and this was only observed with the p18HA and p20HA fragments. Therefore, we felt confident that the overexpressed p18HA construct could be purified and the polyubiquitinated residues could be identified using MS. The strategy to identify these residues involved using overexpressed p18HA in cells in the presence of epoxomicin. Two steps will be used to purify ubiquitinated p18HA, the first, total p18 was to be isolated using an anti-HA-agarose conjugated antibody (Santa Cruz industries) and the second step involved using the polyubiquitin enrichment kit (Pierce) to pull down

polyubiquitinated p18HA. This second pull down ensured only polyubiquitinated caspase-8 p18-HA species was processed and prepared for mass spectrometry analysis.

The polyubiquitinated p18HA protein mass spectrometric analysis will be performed at the University of Kentucky Center for Structural Biology Protein Core Facility in our Department. Briefly, the sample will be treated with trypsin to create peptide fragments. Trypsin cleaves after Arg and Lys residues. Studies have shown that ubiquitination sites can be identified using MS, by taking advantage of the fact that isopeptide-linked ubiquitin is cleaved by trypsin at between Arg 74 and Gly 75, producing a GG signature peptide with 114.04 Da of extra mass (29). The peptides will be desalted and analyzed by MALDI TOF. Peptide mass data will be compared to the predicted 18HA peptide masses. As each peptide will contain one lysine residue, we will conclude any peptide with an additional 114.04 will be a polyubiquitination site.

Access to the mass spectrometry facilities and the guidance and expertise of Dr. Haining Zhu, Director of the Structural Biology Protein Core in the Department of Biochemistry, facilitated the successful completion of these studies. The issue is that the p18HA subunit is not physiologic and may yield non-physiologic polyubiquitination of residues not normally polyubiquitinated, in a physiologically-relevant setting. An alternative strategy involves performing the described IP steps using LNCaP cells and scaling up until we are able to pull down enough polyubiquitinated caspase-8 required for mass-spectrometry (MS).

A second important objective was to select an appropriate system to study mutant caspase-8 constructs for stability, activity, and apoptosis induction. We sought to use a caspase-8 deficient cell line as an expression system because this characteristic offers the

advantage of collecting and evaluating expression and functional data of our constructs without having to take endogenous caspase-8 into consideration. Unfortunately, caspase-8 deficient prostate cancer cell lines do not exist. However, I9.2 caspase-8 deficient Jurkat-derived cell line and NB7 neuroblastoma cell lines are perfect candidates as both can induce apoptosis via TRAIL stimulation upon caspase-8 reconstitution. Evidence from unpublished data indicates that the caspase-8 expressing Jurkat cell line behaved similarly to PC-3 human prostate cancer cells, in terms of caspase-8 cleavage, p18 stability, and the apoptotic response to proteasome inhibitor and TRAIL treatment. To confirm the ability of I9.2 cells to undergo TRAIL-mediated apoptosis upon restoring caspase-8 these cells were transduced with a pBABE-puro encoding wild-type caspase-8. Following puromycin selection, comparisons were made between I9.2 reconstituted caspase-8 (I9.2 RC8), I9.2 puro against the wild-type Jurkat, and I9.2 cell lines to assess caspase-8 expression, cleavage and TRAIL induced death via Western blot and MTT assay (Figure 23C). The results showed the I9.2 puro and I9.2 RC8 behaved identically to wild-type cell lines. Therefore, we felt that this model could be used as a tool to study the effect of ubiquitination-defective caspase-8 constructs on apoptosis.

The goal was to reconstitute ubiquitination-defective caspase-8 mutant constructs into the caspase-8 deficient I9.2 cells to generate stable caspase-8 expressing cell lines. Once we have identified the ubiquitinated lysine residue(s) then we can proceed with site directed mutagenesis, converting the lysine residue to arginine (K→R). The pGEM shuttle vector encoded with wild-type caspase-8 will be used to generate the mutant forms of caspase-8 and once mutations have been verified by restriction digest, the mutant caspase-8 construct will then be subcloned into pBABE expression vector. After

confirming the sequence is correct, the caspase-8 mutant construct will then be introduced into the Jurkat I9.2 system. We transduced I9.2 caspase-8 deficient cells with the pBABE puro caspase-8 mutant expressing vectors by following the same procedures used to generate the I9.2 wild-type caspase-8 expressing cell line as explained in the preliminary data. After having reconstituted and selected for the I9.2 cells expressing our mutant caspase-8 construct, using Western blot to confirm caspase-8 expression. Annexin V staining will be performed following the treatment of the I9.2 K→R caspase-8, RC-8 wild-type caspase-8, and I9.2 puro cell lines with TRAIL to assess apoptosis. The University of Kentucky Flow Cytometry Facility will analyze the staining. All assays will be carried out in triplicate and statistical comparisons between the K→R caspase-8 and RC8 control cells carried out with a pair-wise Student's *t* test. Additionally, we will use immunoprecipitation/Western blotting to assess association between ubiquitin and caspase-8 K→R mutants. It is anticipated that the K→R mutations will reduce and/or abolish polyubiquitination of the p18 fragment. One of the potential difficulties may be that regardless of the K→R mutations, the p18 may still be subjected to ubiquitination (as a result of site shifting) and proteasome degradation. To address this problem we could subject the constructs to the same IP/Mass Spectrometry conditions to determine which amino acid is being ubiquitinated. After identifying the ubiquitinated lysine residues we can then carry out site directed mutagenesis and test the new construct for caspase-8 as outlined above. Additionally, there is the potential that the K→R mutations may confer p18 stability but may result in catalytically inactive form of caspase-8. Studies may involve comparing caspase-8 stability via the half-life assay caspase-8 activity between wild-type Jurkat and I9.2 RC8 using the caspase-8 GLO activity kit. After reconstituting

caspase-8 in the I9.2 caspase-8 deficient cells, and performing a series of experiments with those clones their maintenance presented serious challenges. Several attempts were made to generate these caspase-8 expressing I9.2 lines again, the problem encountered generating these cell lines, was the large number of false positive cells. The pLSG vector is an excellent vector to use with retroviral transduction strategies because these vectors have EFGP that are linked to LTR while the promoter for the protein of interest is an SV40. However, the issue arises that EGFP expression and the protein of interest expression were not linked in some fashion, leading to strong EGFP expression but not caspase-8 expression. One potential solution to overcome these difficulties is to use an inducible promoter system, however, this meant that multiple constructs would have to be introduced via transduction methods. My goal at this point was to identify a caspase-8 deficient cell line that were responsive to either TNF-α or TRAIL and of the many caspase-8 deficient cell lines, only the NB7 and the I9.2 showed apoptosis induction with either ligand.

In our pursuit for a suitable caspase-8 deficient cellular tool, we continued using LNCaP cells to determine if it would be possible to acquire enough caspase-8 p18 to use for mass spec. This goal was addressed by performing several caspase-8 experiments using several different conditions that maximized caspase-8 processing and p18 yield with the pull down. Unfortunately, through our efforts we were incapable of pulling down sufficient amounts of processed p18 subunit. Another alternative was to perform a set of IP experiments pulling down the p18-HA construct as this construct had good expression and revealed polyubiquitin smears on a normal Western blot (Fig.1 panel b B). Although the pull-down was successful and we could see the p18 subunit, we were

unfortunate and failed to observe any polyubiquitin smears (data not shown). It was critical to use the free standing p18 subunit rather than rely on the p43/41 subunit at the membrane because with the free standing p18 we were looking at one specific component. Using the p43/41, this would require removing the DISC components which would complicate the mass spec results. Having acquired the NB7 cells and the caspase-8 mutant constructs from Dr. Doug Green's, provided promise for picking up and completing these experiments at a later time once we resolve the issue with having enough polyubiquitinated p18 to analyze via mass spec. Despite these caveats this remains an important pursuit towards understanding caspase-8 regulation because, specifically uncovering if can uncover which of the lysine molecules are regulatory, then this would allow for the development of a specific molecular inhibitor that would block polyubiquitination of these residues (provided there is no polyubiquitin site shifting), thus stabling caspase-8.

5.3 Role of E3 ligase POSH and Siah2 in Caspase-8 Activity Regulation

from our lab first identified a role for Siah2 and POSH as E3 ligases that played a role in regulating caspase-8 activity, however, the mechanism by which caspase-8 activity was being influenced was not defined(Christian et al., 2011). There were still several avenues to pursue looking at both POSH and Siah2, as I previously mentioned the data suggests that although neither E3 ligase impacts caspase-8 processing(Christian et al., 2011), I hypothesize that these E3 ligases are impacting caspase-8 trafficking through the polyubiquitination of caspase-8 at the DISC triggering the second cleavage event of caspase-8 releasing it into the DISC.

The rationale for this hypothesis comes from the activity data from both NB7 and LNCaP studies. Both studies showed that were significant caspase-8 activity at the plasma membrane, in the LNCAP cell lines plasma membrane activity was markedly increased over the cytosol while the NB7 cells showed that while the plasma membrane had much less activity than the cytosol. Time course analysis in the LNCaP cancer cells clearly indicates a decreased activity at the plasma membrane after 12 hours of TRAIL/Epoxomicin treatment, paralleled by an increase in activity in the cytosol. My studies support the notion that the change in activity is attributed to the release of caspase-8 from the membrane into the cytosol thus suggesting that the over-all activity isn't changing, just the location. Once the cytosolic pool of processed caspase-8 is degraded, then one would observe the decrease in caspase-8 activity however, with the addition of proteasome inhibitor, caspase-8 degradation is blocked and this may account for the consistent cytosolic increase in caspase-8 activity over time. The NB7 data showed an increase in caspase-8 at the plasma membrane with the D3A and D2A mutants compared to wild-type caspase-8 because these constructs are not being released from the DISC.

Therefore, my goal was to further interrogate POSH using LNCaP cells expressing either MYC tagged wild-type or ring mutant POSH. The initial experiments involved using confocal microscopy showed strong co-localization between caspase-8 and POSH (Fig. 5.3.1) A. Unfortunately, subsequent repeat experiments failed to show any co-localization at all. Next, we examined whether silencing POSH or Siah2 had any impact on c-FLIP levels. This is a logical relationship to examine because c-FLIP is an inhibitor of caspase-8 processing and can lead to a decrease in caspase-8 activity.

Therefore, we hypothesized that c-FLIP levels may be decreased as a result of silencing POSH and Siah2. Siah2 or POSH were silenced and cells were harvested and FLIP expression was analyzed via Western blot. There was no real change in expression of c-FLIP regardless of Siah2 or POSH expression. We then used yeast two hybrid experiments to determine whether c-FLIP, POSH, and Siah2 interacted with one another. The positive control was POSH and Siah2 as these two E3 ligases have already been shown to interact with one another(Christian et al., 2011). Colonies transformed with cFLIP and POSH failed to grow on selection medium which indicated that there was no interaction between cFLIP and POSH.

I subsequently conducted a series of immunoprecipitation experiments to determine the association between caspase-8 and MYC-POSH. Unfortunately the outcomes were negative in terms of revealing association. Next I employed various immunoprecipitation techniques to reveal look an association between POSH and potential following TRAIL / proteasome inhibitor stimulation. To ensure clean pull-downs and circumvent antibody contamination, we used the uMACS epitope tag isolation kit that was specific for MYC tags. Although we were successful in pulling down POSH using MYC antibodies and with the uMACS pull-down kit we were unsuccessful in finding any binding partners in either non-treated or TRAIL/Epoxomicin treated samples.

This specific project was put on hold for several reasons (including effort and time constraints), many critical avenues beg exploration of the contribution by POSH and Siah2 in the apoptotic signaling. Considering the evidence that neither POSH nor Siah2 impacts caspase-8 processing (Christian et al., 2011). The fractionation activity data provided the rationale that these E3 ligases modulate caspase-8 activity by triggering

caspase-8 release from the DISC by polyubiquitination. The data showed that caspase-8 activity accumulated over time up till 8 hours after which there was a sharp decrease in the plasma membrane and an increase in cytosolic caspase-8 activity at 12 hours. This activity ties into the polyubiquitination pull-downs because caspase-8 was shown to be polyubiquintated at 8 hours. Therefore, this suggests that following the initial cleavage event, caspase-8 may be polyubiquitinated thus, triggering the second cleavage event that releases caspase-8 into the cytosol to be degraded.

To address this hypothesis, subcellular fractionation assays can be performed following POSH and Siah2 silencing to evaluate caspase-8 processing and observe any changes in caspase-8 remaining at the DISC and if its release is delayed as a result of knocking down POSH and Siah2. POSH and Siah2 may be polyubiquitinating caspase-8 at the DISC and while processing isn't altered in the respect of degradation, caspase-8 trafficking from the DISC to the cytosol is what's being affected. Therefore, when Siah2 or POSH were knocked down, the increase in activity could have been the result of caspase-8 remaining at the DISC and its released was delayed by the lack of POSH and Siah2 expression. To test this hypothesis, the experiment would involve using a set of LNCaP cells that stably over-expressed either POSH and Siah2, as well as a set of cells that were transfected with siRNA targeting either POSH or Siah2. Cells would be either non-treated or with TRAIL and epoxomicin (separately or in combination) and evaluate caspase-8 expression via Western blot and activity via Caspase-8 GLO kit. I would expect that the cells over-expressing POSH and Siah2 would have elevated caspase-8 activity levels and expression in the cytosol compared to the plasma membrane while the reverse should hold true for the POSH and Siah2 knockdown lines. Additional functional

studies in LNCaP human prostate cancer cells and/or the NB7 caspase-8 expressing cells, would involve silencing or over expressing of Siah2, and POSH via transfections, and treatment of stable clones with either TRAIL and/or Epoxomicin. Ubiquitin pull-down experiments would subsequently identify the functional involvement of these E3 ligases in the regulation of caspase-8 polyubiquitination.

It would be critical to determine if there is any change at all caspase-8 activity by polyubiquitination, regardless of the expression status of POSH and Siah2, thus defining the role of E3 ligases as major control players in the process. Other potential players besides these E3 ligases may mediate caspase-8 polyubiquitination. Identifying this E3 ligase could be pursued by using a method similar to the reverse siRNA library approach we employed, the difference is that Dharma-con has new all-encompassing E3-ligase screens that go beyond the 239 E3 ligase limit with the prior screen.

Chemotherapeutic strategies involving caspase activating or stabilizing molecules have been emerging in the last few years. Caspase-9 studies have revealed that FKBP-catalytic caspase-9 subunit conjugates are capable of dimerizing to one another resulting in an active caspase-9 (iCasp9). This fusion, iCasp9 is capable of inducing apoptosis as demonstrated with in-vitro and in-vivo prostate cancer models (Xie et al., 2001). Further studies have shown iCasp9 is capable of inducing apoptosis in chemotherapeutic resistant colorectal cancer stem cells (Kemper et al., 2012). Recent evidence indicates that small molecules like PAC-1, are capable of inducing caspase-3 activation independent of mitochondrial or extrinsic pathway stimulation using *in-vitro* and *in-vivo* models (Putt et al., 2006).

Despite the exciting promise surrounding these emerging strategies, they too are limited to those cancer types capable of having functional apoptotic machinery. Identifying caspase-8 regulatory molecules would provide the capability of over-coming some of the barriers mediating apoptosis resistance such over-expression of the pro-survival players that would inhibit caspase-9 activation. Additionally, it may be possible to use the caspase-8 stabilizing agents with TRAIL to produce the same cancer cell specific apoptosis induction as observed with TRAIL and Velcade, without having the associated side effects associated with Velcade and general proteasome inhibition. The rationale for this concept is based on the fact that caspase-8 is only processed upon stimulation with death receptor ligands, and TRAIL has been demonstrated to induce apoptosis in cancer cells.

The significance of this work surrounds the identification of new caspase-8 regulatory molecules that will provide new attractive targets for a development of a molecular therapeutics platform exploiting key players in the pathway. This ultimately would lead to generation of effective inhibitors against caspase-8 stability, leading to apoptosis induction in cancer cells, while circumventing the caveats associated with current chemotherapeutic strategies in the management of cancer patients.

Figure 5.1.1: Experimental Flow Chart and Construct Design.

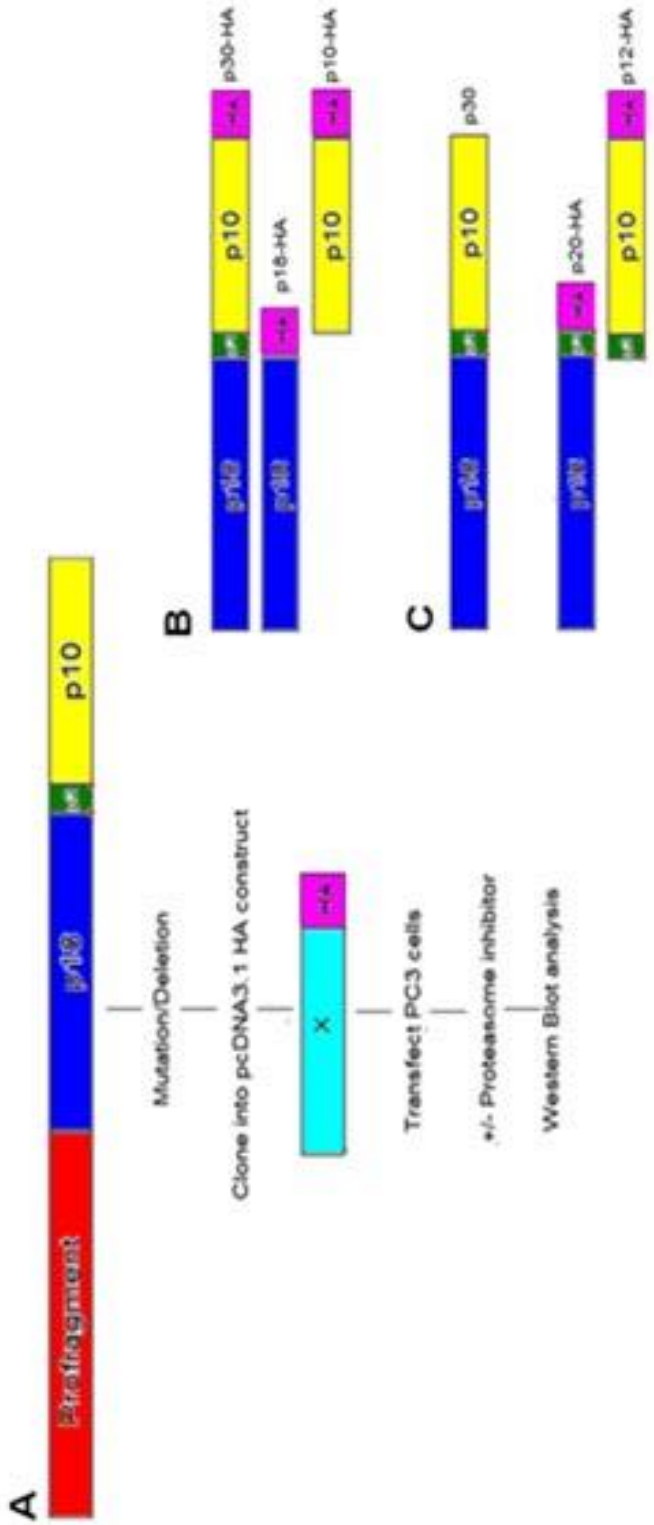

Figure 5.1.1: Experimental flow chart and construct design.

HA-tagged fragments of caspase-8 were to be subcloned into pBABE expression vector, used for transient transfection into PC3 cells. Cells were treated with or without Velcade and expression was evaluated via Western blot.

Figure 5.1.2. Caspase-8 Autoprocessing Confers Dependence on Proteasome Inhibition for Expression and Suggests that the p18 Subunit is Polyubiquitinated.

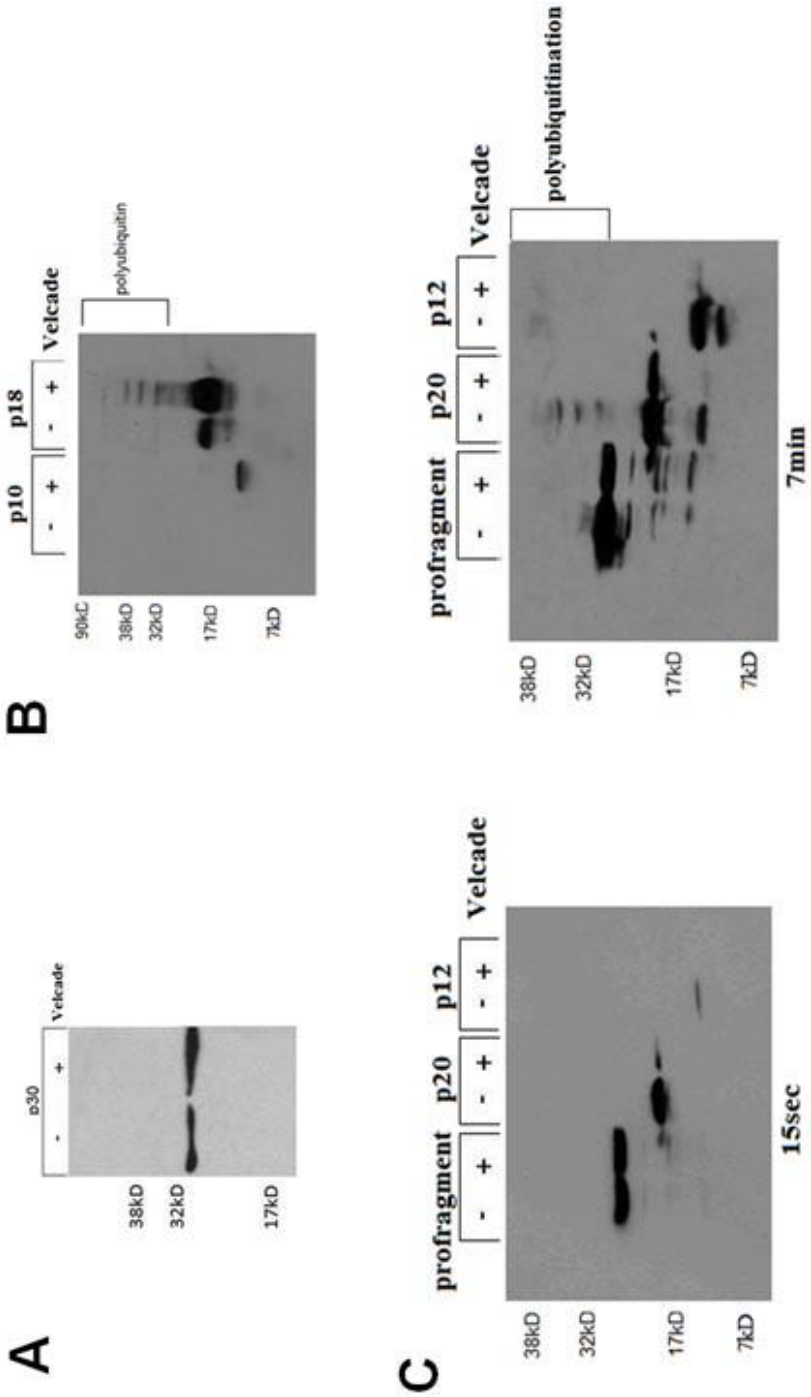

Figure 5.1.2. Caspase-8 autoprocessing confers dependence on proteasome inhibition for expression and suggests that the p18 subunit is polyubiquitinated. A Western blot showing the p30 fragment of caspase-8 can be expressed without proteasome inhibitor. B) Upon cleavage of the p30, the p10 and p18 fragments require proteasome inhibitor to see expression. C). Comparison of pro-fragment, p20, and p12 fragments for short(1min) and longer (7min) exposures

Figure 5.2.1. Caspase-8 re-expression in caspase-8 deficient I9.2 Jurkat cells.

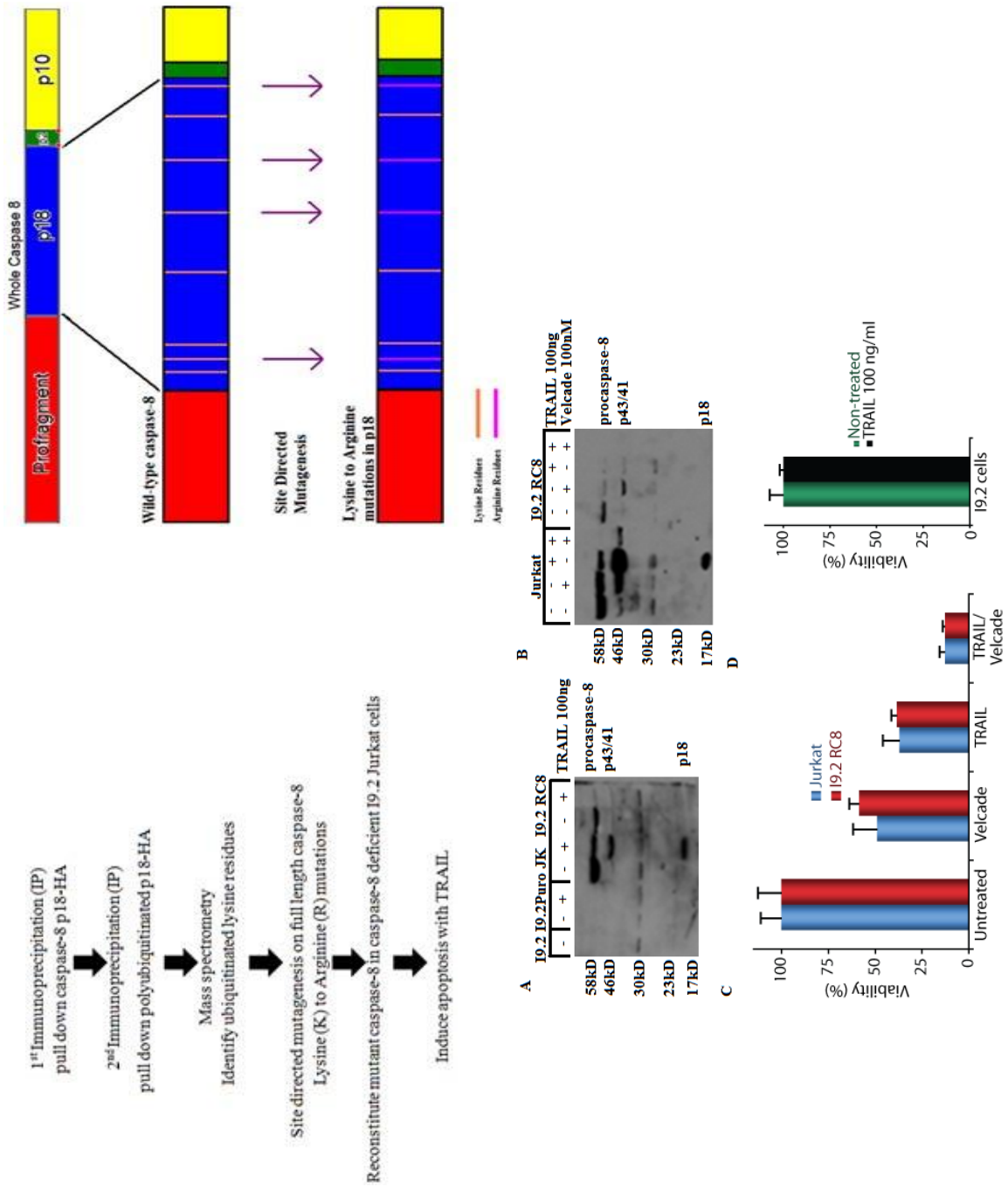

Figure 5.2.1. Caspase-8 re-expression in caspase-8 deficient I9.2 Jurkat cells. A.) Pull down caspase-8 polyubiquitinated p18 subunit, identify the polyubiquitinated lysine residues. B). Identified lysine residues within the p18 subunit were to be mutated to Arginine, followed by subcloning into pBABE or pLSG expression vectors to generate stably caspase-8 mutant expressing cell lines to evaluate the contribution of polyubiquitination to caspase-8 regulation. C). Reconstitution of caspase-8 in I9.2 caspase-8 deficient Jurkat cells.

.

Figure 5.3.1 Results of POSH studies.

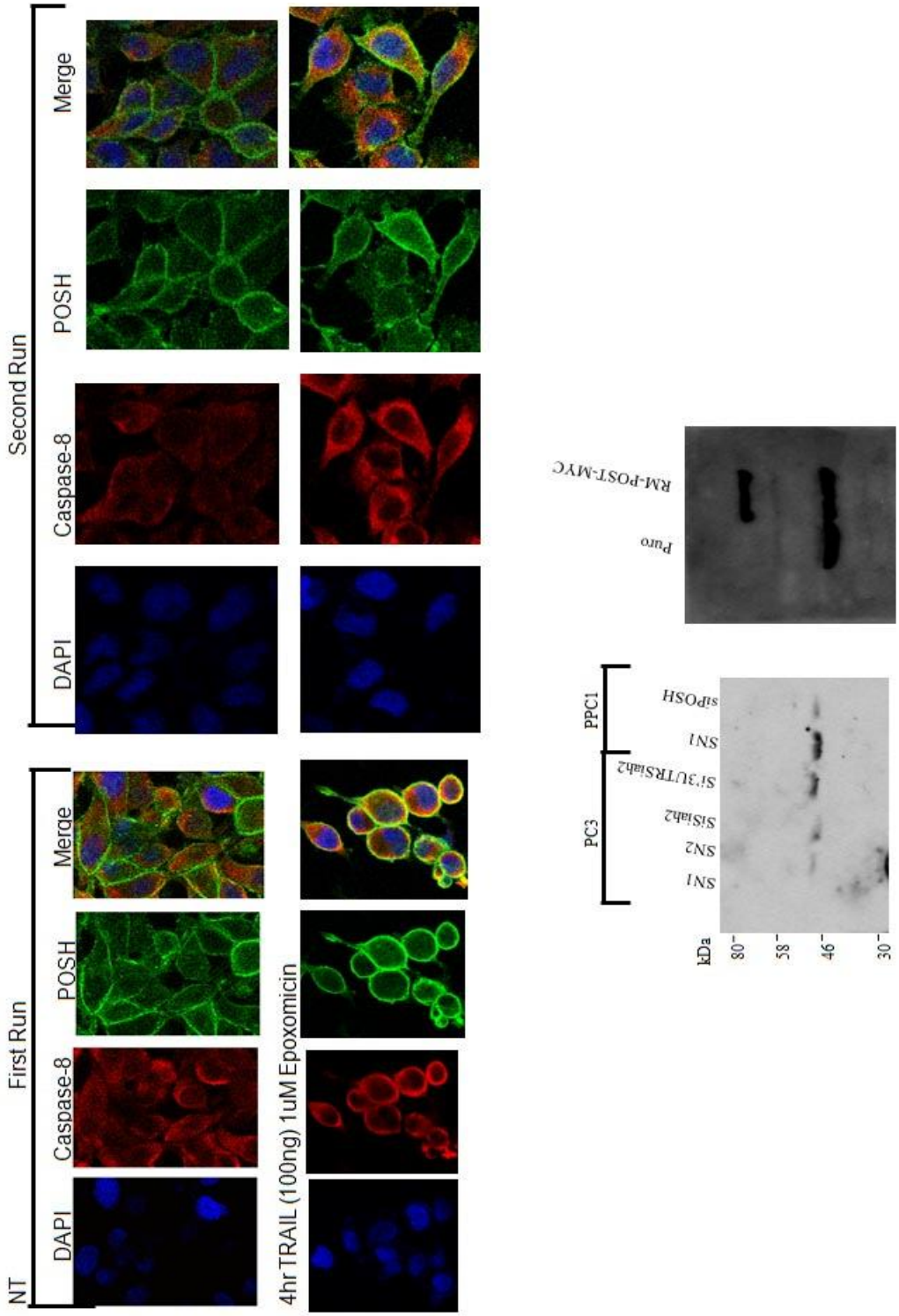

Figure 5.3.1 Posh Localization Studies

A. Co-localization studies showing co-localization status between Caspase-8 and POSH. B. Western blot demonstrating c-Flip expression as a result of POSH and SIAH2 knockdown. C. Western blot demonstrating successful expression of POSH-MYC.

Copyright © Michael V. Fiandalo 2012

Appendix

1. **Symbols**
    a. Alpha, α
    b. Beta, β
    c. Gamma, γ
    d. Kappa, κ
    e. Mu, μ

Abbreviations:
Immunoprecipitation (IP)
Propidium Iodide, (PI)
TNF-alpha Related Apoptosis Inducing Ligand (TRAIL)
Histone Deacetylase 7 (HDAC7)
Death Inducing Signaling Complex (DISC)
Fas associated Death Domain (FADD)
Neuroblastoma 7 (NB7)
Glutathione S-transferase GST
Tumor, Necrosis Factor-α (TNF-α),
American Type Culture Collection (ATCC),
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)),
Castration-recurrent prostate cancer (CRPC)
Allophycocyanin (APC)
Cell lines
Prostate Cancer Cell lines
LNCaP
DU145
PPC-1
PC-3
T Cell lines
Jurkat
I9.2 Jurket
Lung Cancer Cell line
H460
Breast Cancer Cell line
MCF-7

Copyright © Michael V. Fiandalo 2012

References

Allan, L.A., and Clarke, P.R. (2009). Apoptosis and autophagy: Regulation of caspase-9 by phosphorylation. The FEBS journal *276*, 6063-6073.

Almasan, A., and Ashkenazi, A. (2003). Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. Cytokine & growth factor reviews *14*, 337-348.

Almond, J.B., and Cohen, G.M. (2002). The proteasome: a novel target for cancer chemotherapy. Leukemia *16*, 433-443.

Anelli, T., and Sitia, R. (2008). Protein quality control in the early secretory pathway. Embo J *27*, 315-327.

Badros, A., Goloubeva, O., Dalal, J.S., Can, I., Thompson, J., Rapoport, A.P., Heyman, M., Akpek, G., and Fenton, R.G. (2007). Neurotoxicity of bortezomib therapy in multiple myeloma: a single-center experience and review of the literature. Cancer *110*, 1042-1049.

Balsas, P., Lopez-Royuela, N., Galan-Malo, P., Anel, A., Marzo, I., and Naval, J. (2009). Cooperation between Apo2L/TRAIL and bortezomib in multiple myeloma apoptosis. Biochem Pharmacol *77*, 804-812.

Barbero, S., Barila, D., Mielgo, A., Stagni, V., Clair, K., and Stupack, D. (2008). Identification of a critical tyrosine residue in caspase 8 that promotes cell migration. J Biol Chem *283*, 13031-13034.

Barbero, S., Mielgo, A., Torres, V., Teitz, T., Shields, D.J., Mikolon, D., Bogyo, M., Barila, D., Lahti, J.M., Schlaepfer, D., *et al.* (2009). Caspase-8 association with the focal adhesion complex promotes tumor cell migration and metastasis. Cancer Res *69*, 3755-3763.

Belyanskaya, L.L., Marti, T.M., Hopkins-Donaldson, S., Kurtz, S., Felley-Bosco, E., and Stahel, R.A. (2007). Human agonistic TRAIL receptor antibodies Mapatumumab and Lexatumumab induce apoptosis in malignant mesothelioma and act synergistically with cisplatin. Mol Cancer *6*, 66.

Berkers, C.R., Verdoes, M., Lichtman, E., Fiebiger, E., Kessler, B.M., Anderson, K.C., Ploegh, H.L., Ovaa, H., and Galardy, P.J. (2005). Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. Nat Methods *2*, 357-362.

Bertrand, M.J., and Vandenabeele, P. (2011). The Ripoptosome: death decision in the cytosol. Mol Cell *43*, 323-325.

Bhalla, S., Balasubramanian, S., David, K., Sirisawad, M., Buggy, J., Mauro, L., Prachand, S., Miller, R., Gordon, L.I., and Evens, A.M. (2009). PCI-24781 induces caspase and reactive oxygen species-dependent apoptosis through NF-kappaB mechanisms and is synergistic with bortezomib in lymphoma cells. Clin Cancer Res *15*, 3354-3365.

Bian, Z.M., Elner, S.G., and Elner, V.M. (2009). Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. Investigative ophthalmology & visual science *50*, 6006-6014.

Bian, Z.M., Elner, S.G., Khanna, H., Murga-Zamalloa, C.A., Patil, S., and Elner, V.M. (2011). Expression and functional roles of caspase-5 in inflammatory responses of human retinal pigment epithelial cells. Investigative ophthalmology & visual science *52*, 8646-8656.

Bismar, T.A., Yoshimoto, M., Duan, Q., Liu, S., Sircar, K., and Squire, J.A. (2012). Interactions and relationships of PTEN, ERG, SPINK1 and AR in castration-resistant prostate cancer. Histopathology.
Biton, S., and Ashkenazi, A. (2011). NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell *145*, 92-103.
Blanc, C., Deveraux, Q.L., Krajewski, S., Janicke, R.U., Porter, A.G., Reed, J.C., Jaggi, R., and Marti, A. (2000). Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res *60*, 4386-4390.
Bolhuis, S., and Richter-Landsberg, C. (2010). Effect of proteasome inhibition by MG-132 on HSP27 oligomerization, phosphorylation, and aggresome formation in the OLN-93 oligodendroglia cell line. J Neurochem *114*, 960-971.
Bond, M., Chase, A.J., Baker, A.H., and Newby, A.C. (2001). Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovascular research *50*, 556-565.
Brachmann, S., Fritsch, C., Maira, S.M., and Garcia-Echeverria, C. (2009). PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. Current opinion in cell biology *21*, 194-198.
Bratton, S.B., and Salvesen, G.S. (2010). Regulation of the Apaf-1-caspase-9 apoptosome. Journal of cell science *123*, 3209-3214.
Brooks, A.D., Jacobsen, K.M., Li, W., Shanker, A., and Sayers, T.J. (2010). Bortezomib sensitizes human renal cell carcinomas to TRAIL apoptosis through increased activation of caspase-8 in the death-inducing signaling complex. Mol Cancer Res *8*, 729-738.
Bugger, H., Guzman, C., Zechner, C., Palmeri, M., Russell, K.S., and Russell, R.R., 3rd (2011). Uncoupling protein downregulation in Doxorubicin-induced heart failure improves mitochondrial coupling but increases reactive oxygen species generation. Cancer Chemother Pharmacol *67*, 1381-1388.
Bullen, T.F., Forrest, S., Campbell, F., Dodson, A.R., Hershman, M.J., Pritchard, D.M., Turner, J.R., Montrose, M.H., and Watson, A.J. (2006). Characterization of epithelial cell shedding from human small intestine. Laboratory investigation; a journal of technical methods and pathology *86*, 1052-1063.
Carpten, J.D., Faber, A.L., Horn, C., Donoho, G.P., Briggs, S.L., Robbins, C.M., Hostetter, G., Boguslawski, S., Moses, T.Y., Savage, S., *et al.* (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature *448*, 439-444.
Celli, C.M., and Jaiswal, A.K. (2003). Role of GRP58 in mitomycin C-induced DNA cross-linking. Cancer Res *63*, 6016-6025.
Chen, Y., Wan, Y., Wang, Y., Zhang, H., and Jiao, Z. (2011). Anticancer efficacy enhancement and attenuation of side effects of Doxorubicin with titanium dioxide nanoparticles. International journal of nanomedicine *6*, 2321-2326.
Chen, Z.J. (2005). Ubiquitin signalling in the NF-kappaB pathway. Nature cell biology *7*, 758-765.
Choi, S.Y., Kim, M.J., Chung, H.Y., Lee, S.J., and Jang, Y.J. (2007). Phytosphingosine in combination with TRAIL sensitizes cancer cells to TRAIL through synergistic up-regulation of DR4 and DR5. Oncology reports *17*, 175-184.
Christian, P.A., Fiandalo, M.V., and Schwarze, S.R. (2011). Possible role of death receptor-mediated apoptosis by the E3 ubiquitin ligases Siah2 and POSH. Mol Cancer *10*, 57.

Christian, P.A., Thorpe, J.A., and Schwarze, S.R. (2009a). Velcade sensitizes prostate cancer cells to TRAIL induced apoptosis and suppresses tumor growth in vivo. Cancer Biol Ther *8*, 73-80.
Christian, P.A., Thorpe, J.A., and Schwarze, S.R. (2009b). Velcade sensitizes prostate cancer cells to TRAIL induced apoptosis and suppresses tumor growth in vivo. Cancer Biol Ther *8*, 73-80.
Courtney, K.D., Corcoran, R.B., and Engelman, J.A. (2010). The PI3K pathway as drug target in human cancer. J Clin Oncol *28*, 1075-1083.
Crawford, L.J., Walker, B., Ovaa, H., Chauhan, D., Anderson, K.C., Morris, T.C., and Irvine, A.E. (2006). Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132. Cancer Res *66*, 6379-6386.
David, D.C., Layfield, R., Serpell, L., Narain, Y., Goedert, M., and Spillantini, M.G. (2002). Proteasomal degradation of tau protein. J Neurochem *83*, 176-185.
Davies, K.J. (2001). Degradation of oxidized proteins by the 20S proteasome. Biochimie *83*, 301-310.
Davies, M.A., Stemke-Hale, K., Tellez, C., Calderone, T.L., Deng, W., Prieto, V.G., Lazar, A.J., Gershenwald, J.E., and Mills, G.B. (2008). A novel AKT3 mutation in melanoma tumours and cell lines. Br J Cancer *99*, 1265-1268.
Declercq, W., Van Herreweghe, F., Berghe, T.V., and Vandenabeele, P. (2001). Death Receptor-induced Necroptosis. In eLS (John Wiley & Sons, Ltd).
Devarajan, E., Sahin, A.A., Chen, J.S., Krishnamurthy, R.R., Aggarwal, N., Brun, A.M., Sapino, A., Zhang, F., Sharma, D., Yang, X.H., *et al.* (2002). Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. Oncogene *21*, 8843-8851.
Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S., and Reed, J.C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. Embo J *17*, 2215-2223.
Diessenbacher, P., Hupe, M., Sprick, M.R., Kerstan, A., Geserick, P., Haas, T.L., Wachter, T., Neumann, M., Walczak, H., Silke, J., *et al.* (2008). NF-kappaB inhibition reveals differential mechanisms of TNF versus TRAIL-induced apoptosis upstream or at the level of caspase-8 activation independent of cIAP2. J Invest Dermatol *128*, 1134-1147.
Dong, H., Chen, L., Chen, X., Gu, H., Gao, G., Gao, Y., and Dong, B. (2009). Dysregulation of unfolded protein response partially underlies proapoptotic activity of bortezomib in multiple myeloma cells. Leukemia & lymphoma *50*, 974-984.
Dubrovska, A., Kim, S., Salamone, R.J., Walker, J.R., Maira, S.M., Garcia-Echeverria, C., Schultz, P.G., and Reddy, V.A. (2009). The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. Proc Natl Acad Sci U S A *106*, 268-273.
Ea, C.K., Deng, L., Xia, Z.P., Pineda, G., and Chen, Z.J. (2006). Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell *22*, 245-257.
Ehrlichova, M., Koc, M., Truksa, J., Naldova, Z., Vaclavikova, R., and Kovarr, J. (2005a). Cell death induced by taxanes in breast cancer cells: cytochrome C is released in resistant but not in sensitive cells. Anticancer Res *25*, 4215-4224.

Ehrlichova, M., Vaclavikova, R., Ojima, I., Pepe, A., Kuznetsova, L.V., Chen, J., Truksa, J., Kovar, J., and Gut, I. (2005b). Transport and cytotoxicity of paclitaxel, docetaxel, and novel taxanes in human breast cancer cells. Naunyn Schmiedebergs Arch Pharmacol *372*, 95-105.
Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicologic pathology *35*, 495-516.
Ernst, D.S., Eisenhauer, E., Wainman, N., Davis, M., Lohmann, R., Baetz, T., Belanger, K., and Smylie, M. (2005). Phase II study of perifosine in previously untreated patients with metastatic melanoma. Investigational new drugs *23*, 569-575.
Faivre, S., Kroemer, G., and Raymond, E. (2006). Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov *5*, 671-688.
Fedewa, S.A., Etzioni, R., Flanders, W.D., Jemal, A., and Ward, E.M. (2010). Association of insurance and race/ethnicity with disease severity among men diagnosed with prostate cancer, National Cancer Database 2004-2006. Cancer Epidemiol Biomarkers Prev *19*, 2437-2444.
Feig, C., Tchikov, V., Schutze, S., and Peter, M.E. (2007). Palmitoylation of CD95 facilitates formation of SDS-stable receptor aggregates that initiate apoptosis signaling. Embo J *26*, 221-231.
Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D.P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Hacker, G., and Leverkus, M. (2011). cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell *43*, 449-463.
Feoktistova, M., Geserick, P., Panayotova-Dimitrova, D., and Leverkus, M. (2012). Pick your poison: The Ripoptosome, a cell death platform regulating apoptosis and necroptosis. Cell Cycle *11*, 460-467.
Finlay, D., Howes, A., and Vuori, K. (2009). Critical role for caspase-8 in epidermal growth factor signaling. Cancer Res *69*, 5023-5029.
Finlay, D., and Vuori, K. (2007). Novel noncatalytic role for caspase-8 in promoting SRC-mediated adhesion and Erk signaling in neuroblastoma cells. Cancer Res *67*, 11704-11711.
Frisch, S.M., and Ruoslahti, E. (1997). Integrins and anoikis. Current opinion in cell biology *9*, 701-706.
Frisch, S.M., and Screaton, R.A. (2001). Anoikis mechanisms. Current opinion in cell biology *13*, 555-562.
Fruman, D.A., Meyers, R.E., and Cantley, L.C. (1998). Phosphoinositide kinases. Annu Rev Biochem *67*, 481-507.
Fujita, E., Egashira, J., Urase, K., Kuida, K., and Momoi, T. (2001). Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. Cell Death Differ *8*, 335-344.
Fukazawa, T., Fujiwara, T., Uno, F., Teraishi, F., Kadowaki, Y., Itoshima, T., Takata, Y., Kagawa, S., Roth, J.A., Tschopp, J., *et al.* (2001). Accelerated degradation of cellular FLIP protein through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. Oncogene *20*, 5225-5231.
Fukushima, T., Matsuzawa, S., Kress, C.L., Bruey, J.M., Krajewska, M., Lefebvre, S., Zapata, J.M., Ronai, Z., and Reed, J.C. (2007). Ubiquitin-conjugating enzyme Ubc13 is a

critical component of TNF receptor-associated factor (TRAF)-mediated inflammatory responses. Proc Natl Acad Sci U S A *104*, 6371-6376.
Fulda, S., Meyer, E., and Debatin, K.M. (2002). Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene *21*, 2283-2294.
Ganten, T.M., Haas, T.L., Sykora, J., Stahl, H., Sprick, M.R., Fas, S.C., Krueger, A., Weigand, M.A., Grosse-Wilde, A., Stremmel, W., *et al.* (2004). Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. Cell Death Differ *11 Suppl 1*, S86-96.
George, D., and Moul, J.W. (2012). Emerging treatment options for patients with castration-resistant prostate cancer. Prostate *72*, 338-349.
Giannakakou, P., Gussio, R., Nogales, E., Downing, K.H., Zaharevitz, D., Bollbuck, B., Poy, G., Sackett, D., Nicolaou, K.C., and Fojo, T. (2000). A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells. Proc Natl Acad Sci U S A *97*, 2904-2909.
Gonzalez-Gomez, P., Bello, M.J., Inda, M.M., Alonso, M.E., Arjona, D., Aminoso, C., Lopez-Marin, I., de Campos, J.M., Sarasa, J.L., Castresana, J.S., *et al.* (2004). Deletion and aberrant CpG island methylation of Caspase 8 gene in medulloblastoma. Oncology reports *12*, 663-666.
Guo, L., Fan, L., Pang, Z., Ren, J., Ren, Y., Li, J., Chen, J., Wen, Z., and Jiang, X. (2011). TRAIL and Doxorubicin combination enhances anti-glioblastoma effect based on passive tumor targeting of liposomes. Journal of controlled release : official journal of the Controlled Release Society *154*, 93-102.
Habelhah, H. (2010). Emerging complexity of protein ubiquitination in the NF-kappaB pathway. Genes & cancer *1*, 735-747.
Habelhah, H., Takahashi, S., Cho, S.G., Kadoya, T., Watanabe, T., and Ronai, Z. (2004). Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. Embo J *23*, 322-332.
Haenssen, K.K., Caldwell, S.A., Shahriari, K.S., Jackson, S.R., Whelan, K.A., Klein-Szanto, A.J., and Reginato, M.J. (2010). ErbB2 requires integrin alpha5 for anoikis resistance via Src regulation of receptor activity in human mammary epithelial cells. Journal of cell science *123*, 1373-1382.
Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.
Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003). Apoptosis - the p53 network. Journal of cell science *116*, 4077-4085.
Heinlein, C.A., and Chang, C. (2002). The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol *16*, 2181-2187.
Hennessy, B.T., Smith, D.L., Ram, P.T., Lu, Y., and Mills, G.B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov *4*, 988-1004.
Hideshima, T., Bradner, J.E., Wong, J., Chauhan, D., Richardson, P., Schreiber, S.L., and Anderson, K.C. (2005). Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. Proc Natl Acad Sci U S A *102*, 8567-8572.
Hiles, I.D., Otsu, M., Volinia, S., Fry, M.J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N.F., *et al.* (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. Cell *70*, 419-429.

Hinz, S., Trauzold, A., Boenicke, L., Sandberg, C., Beckmann, S., Bayer, E., Walczak, H., Kalthoff, H., and Ungefroren, H. (2000). Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. Oncogene *19*, 5477-5486.
Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., Manabe, T., Yamagishi, S., Bando, Y., Imaizumi, K., *et al.* (2004). Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. The Journal of cell biology *165*, 347-356.
Hu, Y., Benedict, M.A., Wu, D., Inohara, N., and Nunez, G. (1998). Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl Acad Sci U S A *95*, 4386-4391.
Huang, Y., and Sheikh, M.S. (2007). TRAIL death receptors and cancer therapeutics. Toxicol Appl Pharmacol *224*, 284-289.
Imre, G., Larisch, S., and Rajalingam, K. (2011). Ripoptosome: a novel IAP-regulated cell death-signalling platform. Journal of molecular cell biology *3*, 324-326.
Jane, E.P., Premkumar, D.R., and Pollack, I.F. (2011). Bortezomib sensitizes malignant human glioma cells to TRAIL, mediated by inhibition of the NF-{kappa}B signaling pathway. Mol Cancer Ther *10*, 198-208.
Janssen, K., Pohlmann, S., Janicke, R.U., Schulze-Osthoff, K., and Fischer, U. (2007). Apaf-1 and caspase-9 deficiency prevents apoptosis in a Bax-controlled pathway and promotes clonogenic survival during paclitaxel treatment. Blood *110*, 3662-3672.
Jemal, A., Center, M.M., DeSantis, C., and Ward, E.M. (2010). Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev *19*, 1893-1907.
Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R., and Ashkenazi, A. (2009). Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell *137*, 721-735.
Jing, G., Wang, J.J., and Zhang, S.X. (2012). ER stress and apoptosis: a new mechanism for retinal cell death. Experimental diabetes research *2012*, 589589.
Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549-552.
Jordan, M.A., Toso, R.J., Thrower, D., and Wilson, L. (1993). Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci U S A *90*, 9552-9556.
Kang, S., Bader, A.G., and Vogt, P.K. (2005). Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc Natl Acad Sci U S A *102*, 802-807.
Kavuri, S.M., Geserick, P., Berg, D., Dimitrova, D.P., Feoktistova, M., Siegmund, D., Gollnick, H., Neumann, M., Wajant, H., and Leverkus, M. (2011). Cellular FLICE-inhibitory protein (cFLIP) isoforms block CD95- and TRAIL death receptor-induced gene induction irrespective of processing of caspase-8 or cFLIP in the death-inducing signaling complex. J Biol Chem *286*, 16631-16646.
Kemper, K., Rodermond, H., Colak, S., Grandela, C., and Medema, J.P. (2012). Targeting colorectal cancer stem cells with inducible caspase-9. Apoptosis.
Kischkel, F.C., Lawrence, D.A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., Gazdar, A., Blenis, J., Arnott, D., and Ashkenazi, A. (2001). Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J Biol Chem *276*, 46639-46646.

Koschny, R., Holland, H., Sykora, J., Haas, T.L., Sprick, M.R., Ganten, T.M., Krupp, W., Bauer, M., Ahnert, P., Meixensberger, J., *et al.* (2007). Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Clin Cancer Res *13*, 3403-3412.
Krelin, Y., Zhang, L., Kang, T.B., Appel, E., Kovalenko, A., and Wallach, D. (2008). Caspase-8 deficiency facilitates cellular transformation in vitro. Cell Death Differ *15*, 1350-1355.
Krug, H.F. (2012). Caspase-10 is the key initiator caspase involved in tributyltin-mediated apoptosis in human immune cells. Journal of toxicology *2012*, 395482.
Kruidering, M., and Evan, G.I. (2000). Caspase-8 in apoptosis: the beginning of "the end"? IUBMB life *50*, 85-90.
Kumari, R., Sharma, A., Ajay, A.K., and Bhat, M.K. (2009). Mitomycin C induces bystander killing in homogeneous and heterogeneous hepatoma cellular models. Mol Cancer *8*, 87.
Lafont, E., Milhas, D., Teissie, J., Therville, N., Andrieu-Abadie, N., Levade, T., Benoist, H., and Segui, B. (2010). Caspase-10-dependent cell death in Fas/CD95 signalling is not abrogated by caspase inhibitor zVAD-fmk. PLoS One *5*, e13638.
Lai, E., Teodoro, T., and Volchuk, A. (2007). Endoplasmic reticulum stress: signaling the unfolded protein response. Physiology (Bethesda) *22*, 193-201.
Larsen, B.D., Rampalli, S., Burns, L.E., Brunette, S., Dilworth, F.J., and Megeney, L.A. (2010). Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. Proc Natl Acad Sci U S A *107*, 4230-4235.
LeBlanc, H.N., and Ashkenazi, A. (2003). Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ *10*, 66-75.
Leverkus, M., Sprick, M.R., Wachter, T., Mengling, T., Baumann, B., Serfling, E., Brocker, E.B., Goebeler, M., Neumann, M., and Walczak, H. (2003). Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation. Mol Cell Biol *23*, 777-790.
Li, S., Wang, L., Berman, M.A., Zhang, Y., and Dorf, M.E. (2006). RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. Mol Cell *24*, 497-509.
Li, S., Wang, L., and Dorf, M.E. (2009). PKC phosphorylation of TRAF2 mediates IKKalpha/beta recruitment and K63-linked polyubiquitination. Mol Cell *33*, 30-42.
Li, S., Wang, N., and Brodt, P. (2012). Metastatic Cells Can Escape the Proapoptotic Effects of TNF-alpha through Increased Autocrine IL-6/STAT3 Signaling. Cancer Res *72*, 865-875.
Li, W., Zhang, X., and Olumi, A.F. (2007a). MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating c-Fos/c-Jun heterodimers and repressing c-FLIP(L). Cancer Res *67*, 2247-2255.
Li, W., Zhang, X., and Olumi, A.F. (2007b). MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating c-Fos/c-Jun heterodimers and repressing c-FLIP(L). Cancer Res *67*, 2247-2255.
Li, X.H., Fang, X., and Gaynor, R.B. (2001). Role of IKKgamma/nemo in assembly of the Ikappa B kinase complex. J Biol Chem *276*, 4494-4500.
Liu, X., Yue, P., Chen, S., Hu, L., Lonial, S., Khuri, F.R., and Sun, S.Y. (2007). The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to

induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. Cancer Res *67*, 4981-4988.
Lowe, S.W., and Lin, A.W. (2000). Apoptosis in cancer. Carcinogenesis *21*, 485-495.
Lund, L.R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M.J., Dano, K., and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development *122*, 181-193.
Luster, T.A., Carrell, J.A., McCormick, K., Sun, D., and Humphreys, R. (2009). Mapatumumab and lexatumumab induce apoptosis in TRAIL-R1 and TRAIL-R2 antibody-resistant NSCLC cell lines when treated in combination with bortezomib. Mol Cancer Ther *8*, 292-302.
Madan, R.A., Pal, S.K., Sartor, O., and Dahut, W.L. (2011). Overcoming chemotherapy resistance in prostate cancer. Clin Cancer Res *17*, 3892-3902.
Maira, S.M., Furet, P., and Stauffer, F. (2009a). Discovery of novel anticancer therapeutics targeting the PI3K/Akt/mTOR pathway. Future medicinal chemistry *1*, 137-155.
Maira, S.M., Stauffer, F., Schnell, C., and Garcia-Echeverria, C. (2009b). PI3K inhibitors for cancer treatment: where do we stand? Biochemical Society transactions *37*, 265-272.
Malumbres, M., and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. Nature reviews. Cancer *9*, 153-166.
Markman, B., Atzori, F., Perez-Garcia, J., Tabernero, J., and Baselga, J. (2010). Status of PI3K inhibition and biomarker development in cancer therapeutics. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO *21*, 683-691.
Markovina, S., Callander, N.S., O'Connor, S.L., Kim, J., Werndli, J.E., Raschko, M., Leith, C.P., Kahl, B.S., Kim, K., and Miyamoto, S. (2008). Bortezomib-resistant nuclear factor-kappaB activity in multiple myeloma cells. Mol Cancer Res *6*, 1356-1364.
Martin, D.A., Siegel, R.M., Zheng, L., and Lenardo, M.J. (1998). Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. J Biol Chem *273*, 4345-4349.
Mathes, E., O'Dea, E.L., Hoffmann, A., and Ghosh, G. (2008). NF-kappaB dictates the degradation pathway of IkappaBalpha. Embo J *27*, 1357-1367.
Matsuda, T., Almasan, A., Tomita, M., Uchihara, J.N., Masuda, M., Ohshiro, K., Takasu, N., Yagita, H., Ohta, T., and Mori, N. (2005). Resistance to Apo2 ligand (Apo2L)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and constitutive expression of Apo2L/TRAIL in human T-cell leukemia virus type 1-infected T-cell lines. Journal of virology *79*, 1367-1378.
Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H., and Peter, M.E. (1997a). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). Embo J *16*, 2794-2804.
Medema, J.P., Toes, R.E., Scaffidi, C., Zheng, T.S., Flavell, R.A., Melief, C.J., Peter, M.E., Offringa, R., and Krammer, P.H. (1997b). Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. Eur J Immunol *27*, 3492-3498.
Mielgo, A., Torres, V.A., Clair, K., Barbero, S., and Stupack, D.G. (2009). Paclitaxel promotes a caspase 8-mediated apoptosis through death effector domain association with microtubules. Oncogene *28*, 3551-3562.

Mitsiades, C.S., Treon, S.P., Mitsiades, N., Shima, Y., Richardson, P., Schlossman, R., Hideshima, T., and Anderson, K.C. (2001). TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood *98*, 795-804.
Mitsiades, N., Mitsiades, C.S., Richardson, P.G., Poulaki, V., Tai, Y.T., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., *et al.* (2003). The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. Blood *101*, 2377-2380.
Mollinedo, F., and Gajate, C. (2006). Fas/CD95 death receptor and lipid rafts: new targets for apoptosis-directed cancer therapy. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy *9*, 51-73.
Muhlethaler-Mottet, A., Balmas, K., Auderset, K., Joseph, J.M., and Gross, N. (2003). Restoration of TRAIL-induced apoptosis in a caspase-8-deficient neuroblastoma cell line by stable re-expression of caspase-8. Ann N Y Acad Sci *1010*, 195-199.
Naude, P.J., den Boer, J.A., Luiten, P.G., and Eisel, U.L. (2011). Tumor necrosis factor receptor cross-talk. The FEBS journal *278*, 888-898.
Nelson, W.G., Haffner, M.C., and Yegnasubramanian, S. (2011). Beefing up prostate cancer therapy with performance-enhancing (anti-) steroids. Cancer Cell *20*, 7-9.
Nencioni, A., Hua, F., Dillon, C.P., Yokoo, R., Scheiermann, C., Cardone, M.H., Barbieri, E., Rocco, I., Garuti, A., Wesselborg, S., *et al.* (2005). Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. Blood *105*, 3255-3262.
Newsom-Davis, T., Prieske, S., and Walczak, H. (2009). Is TRAIL the holy grail of cancer therapy? Apoptosis *14*, 607-623.
Niraula, S., and Tannock, I.F. (2011). Broadening horizons in medical management of prostate cancer. Acta Oncol *50 Suppl 1*, 141-147.
Obeng, E.A., Carlson, L.M., Gutman, D.M., Harrington, W.J., Jr., Lee, K.P., and Boise, L.H. (2006). Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood *107*, 4907-4916.
Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., and Green, D.R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature *471*, 363-367.
Oberst, A., Pop, C., Tremblay, A.G., Blais, V., Denault, J.B., Salvesen, G.S., and Green, D.R. (2010). Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. J Biol Chem *285*, 16632-16642.
Ohtsuka, T., Buchsbaum, D., Oliver, P., Makhija, S., Kimberly, R., and Zhou, T. (2003). Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. Oncogene *22*, 2034-2044.
Orlowski, M., and Wilk, S. (2000). Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex. Arch Biochem Biophys *383*, 1-16.
Palmerini, F., Devilard, E., Jarry, A., Birg, F., and Xerri, L. (2001). Caspase 7 downregulation as an immunohistochemical marker of colonic carcinoma. Human pathology *32*, 461-467.
Papa, S., Bubici, C., Zazzeroni, F., Pham, C.G., Kuntzen, C., Knabb, J.R., Dean, K., and Franzoso, G. (2006). The NF-kappaB-mediated control of the JNK cascade in the

antagonism of programmed cell death in health and disease. Cell Death Differ *13*, 712-729.
Park, J.Y., Park, J.M., Jang, J.S., Choi, J.E., Kim, K.M., Cha, S.I., Kim, C.H., Kang, Y.M., Lee, W.K., Kam, S., *et al.* (2006). Caspase 9 promoter polymorphisms and risk of primary lung cancer. Human molecular genetics *15*, 1963-1971.
Pellinen, T., and Ivaska, J. (2006). Integrin traffic. Journal of cell science *119*, 3723-3731.
Pellinen, T., Tuomi, S., Arjonen, A., Wolf, M., Edgren, H., Meyer, H., Grosse, R., Kitzing, T., Rantala, J.K., Kallioniemi, O., *et al.* (2008). Integrin trafficking regulated by Rab21 is necessary for cytokinesis. Developmental cell *15*, 371-385.
Peng, C., Cho, Y.Y., Zhu, F., Zhang, J., Wen, W., Xu, Y., Yao, K., Ma, W.Y., Bode, A.M., and Dong, Z. (2011). Phosphorylation of caspase-8 (Thr-263) by ribosomal S6 kinase 2 (RSK2) mediates caspase-8 ubiquitination and stability. J Biol Chem *286*, 6946-6954.
Pingoud-Meier, C., Lang, D., Janss, A.J., Rorke, L.B., Phillips, P.C., Shalaby, T., and Grotzer, M.A. (2003). Loss of caspase-8 protein expression correlates with unfavorable survival outcome in childhood medulloblastoma. Clin Cancer Res *9*, 6401-6409.
Plasilova, M., Zivny, J., Jelinek, J., Neuwirtova, R., Cermak, J., Necas, E., Andera, L., and Stopka, T. (2002). TRAIL (Apo2L) suppresses growth of primary human leukemia and myelodysplasia progenitors. Leukemia *16*, 67-73.
Puszynski, K., Bertolusso, R., and Lipniacki, T. (2009). Crosstalk between p53 and nuclear factor-B systems: pro- and anti-apoptotic functions of NF-B. IET systems biology *3*, 356-367.
Putt, K.S., Chen, G.W., Pearson, J.M., Sandhorst, J.S., Hoagland, M.S., Kwon, J.T., Hwang, S.K., Jin, H., Churchwell, M.I., Cho, M.H., *et al.* (2006). Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. Nature chemical biology *2*, 543-550.
Rahmani, M., Reese, E., Dai, Y., Bauer, C., Payne, S.G., Dent, P., Spiegel, S., and Grant, S. (2005). Coadministration of histone deacetylase inhibitors and perifosine synergistically induces apoptosis in human leukemia cells through Akt and ERK1/2 inactivation and the generation of ceramide and reactive oxygen species. Cancer Res *65*, 2422-2432.
Ramalingam, S., and Belani, C.P. (2002). Taxanes for advanced non-small cell lung cancer. Expert Opin Pharmacother *3*, 1693-1709.
Rangamani, P., and Sirovich, L. (2007). Survival and apoptotic pathways initiated by TNF-alpha: modeling and predictions. Biotechnology and bioengineering *97*, 1216-1229.
Rao, R.V., Castro-Obregon, S., Frankowski, H., Schuler, M., Stoka, V., del Rio, G., Bredesen, D.E., and Ellerby, H.M. (2002). Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. J Biol Chem *277*, 21836-21842.
Rennebeck, G., Martelli, M., and Kyprianou, N. (2005). Anoikis and survival connections in the tumor microenvironment: is there a role in prostate cancer metastasis? Cancer Res *65*, 11230-11235.
Richardson, P.G., Xie, W., Mitsiades, C., Chanan-Khan, A.A., Lonial, S., Hassoun, H., Avigan, D.E., Oaklander, A.L., Kuter, D.J., Wen, P.Y., *et al.* (2009). Single-agent bortezomib in previously untreated multiple myeloma: efficacy, characterization of

peripheral neuropathy, and molecular correlations with response and neuropathy. J Clin Oncol *27*, 3518-3525.
Rodriguez-Gonzalez, A., Lin, T., Ikeda, A.K., Simms-Waldrip, T., Fu, C., and Sakamoto, K.M. (2008). Role of the aggresome pathway in cancer: targeting histone deacetylase 6-dependent protein degradation. Cancer Res *68*, 2557-2560.
Rodriguez, J., and Lazebnik, Y. (1999). Caspase-9 and APAF-1 form an active holoenzyme. Genes & development *13*, 3179-3184.
Sansal, I., and Sellers, W.R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol *22*, 2954-2963.
Sayers, T.J., Brooks, A.D., Koh, C.Y., Ma, W., Seki, N., Raziuddin, A., Blazar, B.R., Zhang, X., Elliott, P.J., and Murphy, W.J. (2003). The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. Blood *102*, 303-310.
Scaffidi, C., Schmitz, I., Krammer, P.H., and Peter, M.E. (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem *274*, 1541-1548.
Scher, H.I., Beer, T.M., Higano, C.S., Anand, A., Taplin, M.E., Efstathiou, E., Rathkopf, D., Shelkey, J., Yu, E.Y., Alumkal, J., *et al.* (2010). Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study. Lancet *375*, 1437-1446.
Schile, A.J., Garcia-Fernandez, M., and Steller, H. (2008). Regulation of apoptosis by XIAP ubiquitin-ligase activity. Genes & development *22*, 2256-2266.
Scott, F.L., Fuchs, G.J., Boyd, S.E., Denault, J.B., Hawkins, C.J., Dequiedt, F., and Salvesen, G.S. (2008). Caspase-8 cleaves histone deacetylase 7 and abolishes its transcription repressor function. J Biol Chem *283*, 19499-19510.
Shabek, N., Herman-Bachinsky, Y., and Ciechanover, A. (2009). Ubiquitin degradation with its substrate, or as a monomer in a ubiquitination-independent mode, provides clues to proteasome regulation. Proc Natl Acad Sci U S A *106*, 11907-11912.
Shah, S.A., Potter, M.W., McDade, T.P., Ricciardi, R., Perugini, R.A., Elliott, P.J., Adams, J., and Callery, M.P. (2001). 26S proteasome inhibition induces apoptosis and limits growth of human pancreatic cancer. J Cell Biochem *82*, 110-122.
Shi, C.S., and Kehrl, J.H. (2003). Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2). J Biol Chem *278*, 15429-15434.
Shiozaki, E.N., Chai, J., Rigotti, D.J., Riedl, S.J., Li, P., Srinivasula, S.M., Alnemri, E.S., Fairman, R., and Shi, Y. (2003). Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell *11*, 519-527.
Shuda, M., Kondoh, N., Imazeki, N., Tanaka, K., Okada, T., Mori, K., Hada, A., Arai, M., Wakatsuki, T., Matsubara, O., *et al.* (2003). Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. Journal of hepatology *38*, 605-614.
Sircar, K., Yoshimoto, M., Monzon, F.A., Koumakpayi, I.H., Katz, R.L., Khanna, A., Alvarez, K., Chen, G., Darnel, A.D., Aprikian, A.G., *et al.* (2009). PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. J Pathol *218*, 505-513.
Siu, W.P., Pun, P.B., Latchoumycandane, C., and Boelsterli, U.A. (2008). Bax-mediated mitochondrial outer membrane permeabilization (MOMP), distinct from the

mitochondrial permeability transition, is a key mechanism in diclofenac-induced hepatocyte injury: Multiple protective roles of cyclosporin A. Toxicol Appl Pharmacol *227*, 451-461.
Skeen, J.E., Bhaskar, P.T., Chen, C.C., Chen, W.S., Peng, X.D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H., and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell *10*, 269-280.
Song, J.H., Tse, M.C., Bellail, A., Phuphanich, S., Khuri, F., Kneteman, N.M., and Hao, C. (2007). Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. Cancer Res *67*, 6946-6955.
Soucy, T.A., Smith, P.G., Milhollen, M.A., Berger, A.J., Gavin, J.M., Adhikari, S., Brownell, J.E., Burke, K.E., Cardin, D.P., Critchley, S., *et al.* (2009a). An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature *458*, 732-736.
Soucy, T.A., Smith, P.G., and Rolfe, M. (2009b). Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer. Clin Cancer Res *15*, 3912-3916.
Soung, Y.H., Lee, J.W., Kim, S.Y., Jang, J., Park, Y.G., Park, W.S., Nam, S.W., Lee, J.Y., Yoo, N.J., and Lee, S.H. (2005). CASPASE-8 gene is inactivated by somatic mutations in gastric carcinomas. Cancer Res *65*, 815-821.
Soung, Y.H., Lee, J.W., Kim, S.Y., Park, W.S., Nam, S.W., Lee, J.Y., Yoo, N.J., and Lee, S.H. (2006). Mutational analysis of proapoptotic caspase-9 gene in common human carcinomas. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica *114*, 292-297.
Spaargaren, M., and Bos, J.L. (1999). Rab5 induces Rac-independent lamellipodia formation and cell migration. Molecular biology of the cell *10*, 3239-3250.
Sprick, M.R., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M.A., and Walczak, H. (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. Embo J *21*, 4520-4530.
Squire, J.A. (2009). TMPRSS2-ERG and PTEN loss in prostate cancer. Nat Genet *41*, 509-510.
Srinivasula, S.M., Ahmad, M., Guo, Y., Zhan, Y., Lazebnik, Y., Fernandes-Alnemri, T., and Alnemri, E.S. (1999). Identification of an endogenous dominant-negative short isoform of caspase-9 that can regulate apoptosis. Cancer Res *59*, 999-1002.
Stahl, J.M., Cheung, M., Sharma, A., Trivedi, N.R., Shanmugam, S., and Robertson, G.P. (2003). Loss of PTEN promotes tumor development in malignant melanoma. Cancer Res *63*, 2881-2890.
Stanbrough, M., Bubley, G.J., Ross, K., Golub, T.R., Rubin, M.A., Penning, T.M., Febbo, P.G., and Balk, S.P. (2006). Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Res *66*, 2815-2825.
Strater, J., Wedding, U., Barth, T.F., Koretz, K., Elsing, C., and Moller, P. (1996). Rapid onset of apoptosis in vitro follows disruption of beta 1-integrin/matrix interactions in human colonic crypt cells. Gastroenterology *110*, 1776-1784.

Stupack, D.G., Teitz, T., Potter, M.D., Mikolon, D., Houghton, P.J., Kidd, V.J., Lahti, J.M., and Cheresh, D.A. (2006). Potentiation of neuroblastoma metastasis by loss of caspase-8. Nature *439*, 95-99.

Sun, M., Meares, G., Song, L., and Jope, R.S. (2009). XIAP associates with GSK3 and inhibits the promotion of intrinsic apoptotic signaling by GSK3. Cellular signalling *21*, 1857-1865.

Suzuki, H., Freije, D., Nusskern, D.R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W.B., and Bova, G.S. (1998). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Res *58*, 204-209.

Tait, S.W., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nature reviews. Molecular cell biology *11*, 621-632.

Teitz, T., Wei, T., Valentine, M.B., Vanin, E.F., Grenet, J., Valentine, V.A., Behm, F.G., Look, A.T., Lahti, J.M., and Kidd, V.J. (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nature medicine *6*, 529-535.

Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K., *et al.* (2011). The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell *43*, 432-448.

Thorpe, J.A., Christian, P.A., and Schwarze, S.R. (2008a). Proteasome inhibition blocks caspase-8 degradation and sensitizes prostate cancer cells to death receptor-mediated apoptosis. Prostate *68*, 200-209.

Thorpe, J.A., Christian, P.A., and Schwarze, S.R. (2008b). Proteasome inhibition blocks caspase-8 degradation and sensitizes prostate cancer cells to death receptor-mediated apoptosis. Prostate *68*, 200-209.

Tinel, A., and Tschopp, J. (2004). The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. Science *304*, 843-846.

Torres, V.A., Mielgo, A., Barbero, S., Hsiao, R., Wilkins, J.A., and Stupack, D.G. (2010). Rab5 mediates caspase-8-promoted cell motility and metastasis. Molecular biology of the cell *21*, 369-376.

Torres, V.A., Mielgo, A., Barila, D., Anderson, D.H., and Stupack, D. (2008). Caspase 8 promotes peripheral localization and activation of Rab5. J Biol Chem *283*, 36280-36289.

Tran, T.M., Temkin, V., Shi, B., Pagliari, L., Daniel, S., Ferran, C., and Pope, R.M. (2009). TNFalpha-induced macrophage death via caspase-dependent and independent pathways. Apoptosis *14*, 320-332.

Upton, J.P., Austgen, K., Nishino, M., Coakley, K.M., Hagen, A., Han, D., Papa, F.R., and Oakes, S.A. (2008). Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. Mol Cell Biol *28*, 3943-3951.

Vachon, P.H. (2011). Integrin signaling, cell survival, and anoikis: distinctions, differences, and differentiation. Journal of signal transduction *2011*, 738137.

Vazquez, F., Grossman, S.R., Takahashi, Y., Rokas, M.V., Nakamura, N., and Sellers, W.R. (2001). Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J Biol Chem *276*, 48627-48630.

Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W.R. (2000). Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol *20*, 5010-5018.

Verfaillie, T., Salazar, M., Velasco, G., and Agostinis, P. (2010). Linking ER Stress to Autophagy: Potential Implications for Cancer Therapy. International journal of cell biology *2010*, 930509.
Vermeulen, K., Van Bockstaele, D.R., and Berneman, Z.N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell proliferation *36*, 131-149.
Vlietstra, R.J., van Alewijk, D.C., Hermans, K.G., van Steenbrugge, G.J., and Trapman, J. (1998). Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res *58*, 2720-2723.
Vu, H.Y., Juvekar, A., Ghosh, C., Ramaswami, S., Le, D.H., and Vancurova, I. (2008). Proteasome inhibitors induce apoptosis of prostate cancer cells by inducing nuclear translocation of IkappaBalpha. Arch Biochem Biophys *475*, 156-163.
Wajant, H., and Scheurich, P. (2011). TNFR1-induced activation of the classical NF-kappaB pathway. The FEBS journal *278*, 862-876.
Wallach, D., Kang, T.B., and Kovalenko, A. (2008). The extrinsic cell death pathway and the elan mortel. Cell Death Differ *15*, 1533-1541.
Wang, L., Du, F., and Wang, X. (2008). TNF-alpha induces two distinct caspase-8 activation pathways. Cell *133*, 693-703.
Wang, X., Di Pasqua, A.J., Govind, S., McCracken, E., Hong, C., Mi, L., Mao, Y., Wu, J.Y., Tomita, Y., Woodrick, J.C., *et al.* (2011). Selective Depletion of Mutant p53 by Cancer Chemopreventive Isothiocyanates and Their Structure-Activity Relationships. Journal of medicinal chemistry.
Wasylyk, C., Salvi, R., Argentini, M., Dureuil, C., Delumeau, I., Abecassis, J., Debussche, L., and Wasylyk, B. (1999). p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53. Oncogene *18*, 1921-1934.
Wicovsky, A., Henkler, F., Salzmann, S., Scheurich, P., Kneitz, C., and Wajant, H. (2009). Tumor necrosis factor receptor-associated factor-1 enhances proinflammatory TNF receptor-2 signaling and modifies TNFR1-TNFR2 cooperation. Oncogene *28*, 1769-1781.
Williams, S.A., and McConkey, D.J. (2003). The proteasome inhibitor bortezomib stabilizes a novel active form of p53 in human LNCaP-Pro5 prostate cancer cells. Cancer Res *63*, 7338-7344.
Wu, C.J., Conze, D.B., Li, X., Ying, S.X., Hanover, J.A., and Ashwell, J.D. (2005). TNF-alpha induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination. Embo J *24*, 1886-1898.
Wu, X.X., Kakehi, Y., Mizutani, Y., Kamoto, T., Kinoshita, H., Isogawa, Y., Terachi, T., and Ogawa, O. (2002). Doxorubicin enhances TRAIL-induced apoptosis in prostate cancer. International journal of oncology *20*, 949-954.
Xie, X., Zhao, X., Liu, Y., Zhang, J., Matusik, R.J., Slawin, K.M., and Spencer, D.M. (2001). Adenovirus-mediated tissue-targeted expression of a caspase-9-based artificial death switch for the treatment of prostate cancer. Cancer Res *61*, 6795-6804.
Yoo, J., Park, S.S., and Lee, Y.J. (2008). Pretreatment of docetaxel enhances TRAIL-mediated apoptosis in prostate cancer cells. J Cell Biochem *104*, 1636-1646.
Yoshimoto, M., Ludkovski, O., DeGrace, D., Williams, J.L., Evans, A., Sircar, K., Bismar, T.A., Nuin, P., and Squire, J.A. (2012). PTEN genomic deletions that

characterize aggressive prostate cancer originate close to segmental duplications. Genes Chromosomes Cancer *51*, 149-160.
Yu, C., Rahmani, M., Dent, P., and Grant, S. (2004). The hierarchical relationship between MAPK signaling and ROS generation in human leukemia cells undergoing apoptosis in response to the proteasome inhibitor Bortezomib. Exp Cell Res *295*, 555-566.
Yu, H.B., and Finlay, B.B. (2008). The caspase-1 inflammasome: a pilot of innate immune responses. Cell host & microbe *4*, 198-208.
Yuan, J., and Kroemer, G. (2010). Alternative cell death mechanisms in development and beyond. Genes & development *24*, 2592-2602.
Zhan, M., Zhao, H., and Han, Z.C. (2004). Signalling mechanisms of anoikis. Histology and histopathology *19*, 973-983.
Zhang, L., Ren, X., Alt, E., Bai, X., Huang, S., Xu, Z., Lynch, P.M., Moyer, M.P., Wen, X.F., and Wu, X. (2010). Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis. Nature *464*, 1058-1061.
Zhang, X., Zhang, L., Yang, H., Huang, X., Otu, H., Libermann, T.A., DeWolf, W.C., Khosravi-Far, R., and Olumi, A.F. (2007). c-Fos as a proapoptotic agent in TRAIL-induced apoptosis in prostate cancer cells. Cancer Res *67*, 9425-9434.
Zhou, Q.M., Zhang, H., Lu, Y.Y., Wang, X.F., and Su, S.B. (2009). Curcumin reduced the side effects of mitomycin C by inhibiting GRP58-mediated DNA cross-linking in MCF-7 breast cancer xenografts. Cancer science *100*, 2040-2045.
Zouq, N.K., Keeble, J.A., Lindsay, J., Valentijn, A.J., Zhang, L., Mills, D., Turner, C.E., Streuli, C.H., and Gilmore, A.P. (2009). FAK engages multiple pathways to maintain survival of fibroblasts and epithelia: differential roles for paxillin and p130Cas. Journal of cell science *122*, 357-367.

Copyright © Michael V. Fiandalo 2012

Vita

Michael Vincent Fiandalo

Born:
December 8th 1979 in Chicago Illinois.

Educational institutions attended:
Attended University of Illinois Chicago.
Attended Northeastern Illinois University and obtained a Bachelors Degree Of Science in Biology.
University of Kentucky.

Professional positions:
Pharmacy Technician, Resurrection Medical Center.

Professional Publications:
Fiandalo, M.V, Schwarze, S.R. , Kyprianou, K. Impact of Proteasome inhibition on Caspase-8 regulation in Cancer. (2012)

Christian, P.A., Fiandalo, M.V. & Schwarze, S.R. Possible role of death receptor-mediated apoptosis by the E3 ubiquitin ligases Siah2 and POSH. *Mol Cancer* **10**, 57 (2011).