CHARACTERIZATION OF A NOVEL REGULATOR AND PREDICTORS OF SENSITIVITY TO TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

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by

JENNIFER L. DINE, RN

Dr. Jane Armer, Dissertation Supervisor

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

CHARACTERIZATION OF A NOVEL REGULATOR AND PREDICTORS OF SENSITIVITY TO TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

presented by Jennifer L. Dine, a candidate for the degree of Doctor of Philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

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Dr. Jane Armer

_____

Dr. Tina Bloom

_____

Dr. Elizabeth Bryda

_____

Dr. Stanley Lipkowitz

_____

Dr. Deidre Wipke-Tevis

DEDICATION

This work is dedicated to my parents, with thanks.

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Abbreviation	Definition
ACADVL	Acyl-coA dehydrogenase, very long chain
ACTN4	Alpha-actinin 4
AKT	Protein kinase B
AMFR	Autocrine motility factor receptor
APAF-1	Apoptotic peptidase activating factor 1
ATF6	Activating transcription factor 6
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha
BAD	BCL2-associated agonist of cell death
BAK	BCL2-antagonist
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2 like 1
BCL2L2	BCL-w
BCL-w,	BCL2-like 2
BCL-XL	BCL2-like 1
BCR	Breakpoint cluster region
BID	BH3 interacting-domain death agonist
BIM	BCL2-like 11
Bip	Heat shock 70 kilodalton protein
BIRC2	Baculoviral IAP repeating containing 2
BIRC3	c-IAP2
BLK	Proto-oncogene, Src family tyrosine kinase
BRCA1	Breast cancer 1,early onset
CDK9	Cyclin-dependent kinase 9
CHOP	CCAAT/enhancer-binding protein homologous protein
CNKSR1	Connector enhancer of kinase suppressor of Ras 1
CR	Complete response
CUE	Coupling of ubiquitin conjugation to ER degradation
DcR	Decoy receptor
DcR1	Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain
DcR2	Tumor necrosis factor receptor superfamily, member 10d,decoy with truncated death domain
DD	Death domain
DED	Death effector domain
DFS	Disease free survival
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DR	Death receptor
DR4	Death receptor 4
DR5	Death receptor 5

EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ER+	Estrogen receptor positive
ERAD	ER-associated protein degradation
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERK	Mitogen-activated protein kinase 1
Fab	Fragment antigen-binding
FADD	Fas-associated death domain
Fas	Fas cell surface death receptor
FcγR	Fcγ receptor
FGFR2	Fibroblast growth factor receptor 2
FGFR4	Fibroblast growth factor receptor 4
FLIP	FLICE-like inhibitory protein
FUT3	Fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, lewis blood group)
FUT6	Fucosyltransferase 6 (alpha (1,3) fucosyltransferase)
G2BR	Ubiquitin-conjugating enzyme E2G 2 binding
GAK,	Cyclin G associated kinase
GALNT14	Polypeptide N-acetylgalactosaminyltransferase 14
GALNT3	Polypeptide N-acetylgalactosaminyltransferase 3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gp78	Autocrine motility factor receptor
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIPK1	Homeodomain interacting protein kinase 1
HIPK2	Homeodomain interacting protein kinase 2
HK1	Hexokinase 1
HK2	Hexokinase 2
IAP	Inhibitor of apoptosis
IC50	Half maximal inhibitory concentration
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IRE1α	Inositol-requiring enzyme 1 α
KAI1	Cluster of differentiation 82
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia 1
MEK	Mitogen-activated protein kinase kinase
MKNK1	MAP kinase interacting serine/threonine kinase 1
MOMP	Mitochondrial outer membrane permeabilization
nab-PAC	Albumin bound paclitaxel
NAGK	N-acetylglucosamine kinase

NEK6	NIMA-related kinase 6
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NSCLC	Non-small-cell lung carcinoma
OS	Overall survival
p53	Tumor protein p53
p97	Valosin containing protein
PARP	Poly (ADP-ribose) polymerase 1
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor, beta polypeptide
PDPK1	3-Phosphoinositide dependent protein kinase 1
PERK,	Eukaryotic translation initiation factor 2-alpha kinase 3
PFKL	Phosphofructokinase, liver
PFS	Progression free survival
PIP5KIC	Phosphatidylinositol-4-phosphate 5-kinase, type I
PKFL	Phosphofructose kinase liver
PKLR	Pyruvate kinase, liver and red blood cells
PKN1	Protein kinase N1
PLK3	Polo-like kinase 3
PR	Progesterone receptor
PR	Partial response
PRKC1	Protein kinase c, iota
PrP	Prion protein
PUMA	BCL2 binding component 3
qPCR	Quantitative polymerase chain reaction
Raf	Proto-oncogene, serine/threonine kinase
RANKL	Receptor activator of nuclear factor kappa-B Ligand
RING	Really interesting new gene
RIOK3	RIO kinase 3
RIP1	Receptor interacting protein 1
RIP3	Receptor interacting protein 3
RISC	RNA induced signaling complex
SD	Stable disease
SMAC	Diablo, IAP-binding mitochondrial protein
SPHK2	Sphingosine kinase 2
SRC	Proto-oncogene, non-receptor tyrosine kinase
tBid,	Truncated BID
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TP53	Tumor protein P53
TRAF2	TNF receptor-associated factor 2
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand

TRAIL-R1	TRAIL-receptor 1
TRAILR2	TRAIL-receptor 2
Ube2G2	Ubiquitin-conjugating enzyme E2G 2
UPR	Unfolded protein response
UTR	Untranslated region
VEGFR	Vascular endothelial growth factor receptor
VIM	Valosin interacting motif
WEE1	WEE1 G2 checkpoint kinase
XBP1	X-box binding protein 1
XIAP	X-linked inhibitor of apoptosis
ZC3HC1	Poly (ADP-ribose) polymerase family, member 12

Characterization of a Novel Regulator and Predictors of Sensitivity to TRAIL-Induced Apoptosis in Breast Cancer Cells

Jennifer Dine, RN

Dr. Jane Armer, Dissertation Supervisor

Abstract

Mesenchymal triple negative breast cancer (TNBC) cells are very sensitive to tumor necrosis factor-related apoptosis inducing ligand (TRAIL) for reasons that are poorly understood. The purpose of this study was to characterize a negative regulator of TRAIL sensitivity in TNBC cells and potentially predictive biomarkers of TRAIL sensitivity in human TNBC. First, gp78, an ubiquitin ligase that facilitates endoplasmic reticulum-associated protein degradation, was found to negatively regulate TRAIL-induced caspases-3/7 activity and loss in viability independently of the unfolded protein response in the TNBC mesenchymal cell line MB231. Second, TNBC cell lines sensitive to drozitumab, a TRAIL pathway agonist, were found to express the mesenchymal markers vimentin and Axl. Vimentin and Axl were determined to be co-expressed in a publically available cDNA microarray dataset and by immunohistochemistry in human TNBC. These findings provide insight into a novel gp78-associated mechanism that governs sensitivity to TRAIL-induced apoptosis in breast cancer cells and demonstrate that the proteins vimentin and Axl may predict sensitivity to a TRAIL pathway agonist in TNBC and are identifiable in human TNBC, which reflects their potential utility in identifying patients who may benefit from a TRAIL pathway agonist. Collectively, these findings may aid in the selection of appropriate combinatorial therapies and the identification of TNBC-affected patients for treatment with a TRAIL pathway agonist.

CHAPTER ONE

GENERAL INTRODUCTION

Since the early 1990's, cancer-related mortality has declined in both American men and women (Kohler et al., 2015). Despite progress in improving the overall cancer survivorship rate, breast cancer persists as the second-leading cause of cancer-related deaths in American women and is estimated to impact ~12% of all American women at some point in their lifetimes (American Cancer Society, 2015; Kohler et al., 2015). Moreover, breast cancer accounts for ~3% of all deaths in women in the United States and is projected to be the cause of death in >40,000 American women in 2015 (American Cancer Society, 2015). These findings demonstrate a significant burden of breast cancer related-disease in American women and necessitate the further development of treatment strategies to improve survival outcomes for patients with breast cancer.

Treatment for breast cancer is directed, in part, by the presentation of distinct molecular markers that stratify breast cancer into different subtypes of disease (Brenton, Carey, Ahmed, & Caldas, 2005; Prat & Perou, 2011). Estrogen receptor (ER) or progesterone receptor (PR) positive breast cancer accounts for ~60-70% of diagnoses and has the best prognosis of all subtypes (Brenton et al., 2005; Kohler et al., 2015). Amplified human epidermal growth factor receptor 2 (HER2) protein expression characterizes ~15-30% of cases, and the remaining 10-15% of breast cancers lack expression of ER or PR and amplification of HER2 (Brenton et al., 2005). These latter tumors lacking the three aforementioned molecular markers are called triple negative breast cancer (TNBC) (Brenton et al., 2005; Irvin & Carey, 2008).

TNBC is characterized by an aggressive presentation, poor survival outcomes, and is over represented in young African American women (Carey et al., 2006; Kohler et al., 2015; Millikan et al., 2008; Morris et al., 2007). Unlike ER or PR positive or HER2 amplified breast cancer, targeted therapies have yet to be developed for TNBC. Chemotherapy is the standard of care for those affected by TNBC (Perou, 2011). The development of targeted therapies for TNBC is warranted to address resistance to available treatments.

Interestingly, TNBC cells with a mesenchymal phenotype have been found to be exquisitely sensitive to cell death induced by the cytokine tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) while cells representative of the other subtypes of disease have been resistant (Rahman, Davis, et al., 2009; Rahman, Pumphrey, & Lipkowitz, 2009). TRAIL is a cytokine that has been found to selectively activate the extrinsic (and in some cells, intrinsic) pathways to induce apoptosis in cancer cells *in vitro* and *in vivo* (Ashkenazi, 2008). TRAIL pathway activation has been explored extensively in clinical trials; despite being well-tolerated, TRAIL pathway activating agents have not been effective in treating an array of adult and pediatric solid and hematological malignancies (Holland, 2014; Johnstone, Frew, & Smyth, 2008). Understanding the molecular underpinnings that govern sensitivity to TRAIL and characterizing potential biomarkers that predict sensitivity to TRAIL could aid in selecting patients who would most likely respond to a TRAIL pathway activating agent and in selecting appropriate combinatorial therapies to help sensitize cancer cells to TRAIL.

The purpose of this dissertation project was to characterize potential regulators and biomarkers of TRAIL sensitivity that were identified in TRAIL-sensitive TNBC cells *in vitro* and in patients affected by TNBC. The following chapters of this manuscript reflect distinct elements necessary for meeting the overarching goals of this dissertation project. Chapter 2 is a

literature review manuscript concerning the components and signaling mechanisms of the TRAIL pathway. The effectiveness of all TRAIL pathway agonists tested in clinical trial is discussed and includes strategies for improving their effectiveness in patients. Chapter 3 is the proposal originally submitted to the dissertation committee that describes the goals of this project. Chapter 4 is a research report manuscript concerning the characterization of autocrine motility factor receptor (gp78), a negative regulator of TRAIL-induced apoptosis in the mesenchymal TNBC cell line MB231. Chapter 5 is a second research report manuscript exploring breast cancer cell line sensitivity to drozitumab, a TRAIL pathway activating agent, and expression of potential biomarkers of TRAIL sensitivity, vimentin and Axl, in patients with TNBC. Chapter 6 is the final chapter of the dissertation manuscript and discusses the overall findings and implications of this work.

Finally, this study is relevant to nursing science with respect to providing effective, quality health care that takes into account patient data that is associated with genomic function (Calzone et al., 2010). Nurses are critical to health care delivery, and as the incorporation of genomic information is increasingly utilized in the clinical setting to improve care delivery strategies, including diagnosis and treatment, in a non-specialty capacity, nurses across the practice spectrum must also become versed in how genomic information can shape patient care (Genomic Nursing State of the Science Advisory et al., 2013).This dissertation project demonstrates the evaluation of potentially clinically-relevant information in terms of understanding the underlying biological characteristics that may predict responsiveness to a treatment strategy. Thus, this dissertation project helps forward the inclusion of information that is a product of genomic function within the realm of nursing science.

CHAPTER TWO

TRAIL PATHWAY SIGNALING IN THE TREATMENT OF CANCER

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Abstract

The tumor necrosis factor (TNF) superfamily member TNF-related apoptosis inducing ligand (TRAIL) induces apoptosis in cancer cells via death receptor (DR) activation and does so with little toxicity to normal cells or tissues. The selectivity for facilitating cancer cell death confers an ideal therapeutic characteristic to TRAIL, which has led to the development and clinical testing of many TRAIL DR agonists. While well-tolerated, these therapies have been widely ineffective in the treatment of cancer for reasons that remain poorly characterized. In this paper, the signaling components and mechanisms governing TRAIL pathway activation and clinical trial findings are reviewed. Challenges and potential solutions for using TRAIL DR agonists in the clinic are also identified and discussed, including consideration of the pharmacokinetic and pharmacodynamic properties of TRAIL DR agonists, improving the selection of potential candidates for treatment by the identification of biomarkers that may predict sensitivity to TRAIL DR agonist therapy, and by combining TRAIL DR agonists with other agents.

TRAIL PATHWAY SIGNALING IN THE TREATMENT OF CANCER

Apoptosis is a physiological process that eliminates unnecessary or abnormal cells in metazoans (Baehrecke, 2002; Danial & Korsmeyer, 2004). Hallmarks of apoptosis include cell membrane blebbing; chromatin condensation; shrinking of the nucleus, mitochondria, and cytoplasm; and eventual phagocytosis of the cell corpse (Czabotar, Lessene, Strasser, & Adams, 2014; Kroemer et al., 2009). The intrinsic and extrinsic death pathways mediate apoptosis and are activated by numerous stimuli, including DNA damage, cytokine deprivation, infection, stress, and death receptor (DR) ligands (Czabotar et al., 2014; Danial & Korsmeyer, 2004). The intrinsic death pathway is primarily regulated by anti- and pro-apoptotic B cell/CLL lymphoma 2 (BCL2) family members that inhibit or promote mitochondrial outer membrane permeabilization (MOMP) and subsequent activation of initiator caspase-9 (Czabotar et al., 2014; Danial & Korsmeyer, 2004).The extrinsic pathway is activated by ligand binding to DRs on the cell surface to promote initiator caspases-8 and-10 activation (Gonzalvez & Ashkenazi, 2010). The activation of the downstream executioner caspases by initiator caspases is a point of convergence between the intrinsic and extrinsic pathways that ultimately culminates in apoptosis (Gonzalvez & Ashkenazi, 2010).

Cancer is characterized by resistance to apoptotic cell death (Hanahan & Weinberg, 2000). Consequently, overcoming resistance to apoptosis by activating the intrinsic or extrinsic death pathways has been a major focus in developing therapeutic strategies for the treatment of cancer. Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a promising therapeutic candidate because it activates the extrinsic – and in some cells, the intrinsic – death pathway in cancer cells with little

normal cell cytotoxicity *in vitro* and *in vivo* (Gonzalvez & Ashkenazi, 2010). Phase 1 and 2 clinical trials utilizing recombinant human TRAIL and agonistic antibodies directed at death receptors 4 (DR4) and 5 (DR5) have been well-tolerated but generally ineffective in improving patient outcomes, raising questions about the pharmacokinetic and pharmacodynamic properties of TRAIL DR agonists and criteria for patient selection. In this review, we will discuss the structure and function of components of the TRAIL pathway; TRAIL-based therapeutic strategies and their performance in clinical trial; and barriers to using TRAIL DR agonists in the clinic with proposed strategies to address these challenges.

TRAIL and its Receptors

TRAIL (*a.k.a.* Apo-2L, gene symbol TNFSF10) is a type II transmembrane protein that undergoes proteolytic cleavage to produce an extracellular ligand (Cha et al., 1999). TRAIL shares structural homology with other TNF superfamily members, including TNF and Fas cell surface death receptor (Fas) ligand (~30% homology), and is able to potently induce apoptosis in its soluble and membrane-bound forms (Pennati et al., 2015; Pitti et al., 1996; Wiley et al., 1995). Like other members of the TNF family, TRAIL is comprised of three monomers and is further characterized by a zinc-binding site located near the trimerization interface that is necessary for maintaining the stability and activity of the protein (Hymowitz et al., 2000). Zinc depletion by chelation induces TRAIL dimerization that results in dramatically decreased apoptotic function (Hymowitz et al., 2000).

The TNF super family members have been well described as important modulators of immune cell function. TRAIL mRNA is expressed in many tissues types

(Pitti et al., 1996; Wiley et al., 1995) and has been found to play particularly important roles in a wide variety of innate and adaptive immune cells (Falschlehner, Schaefer, & Walczak, 2009; Screaton & Xu, 2000). Stimulated natural killer (NK) cells, T cells, and dendritic cells demonstrate upregulation of TRAIL (Falschlehner et al., 2009; Screaton & Xu). Importantly, TRAIL is upregulated in response to IFN-γ in NK cells and serves as an effector of NK cell function *in vitro* and *in vivo* (Falschlehner et al., 2009). TRAIL upregulation in response to IFN-γ suppressed primary experimental tumors and metastasis and has also been found on IFN-γ-producing dendritic cells, demonstrating an association between innate and adaptive immune functions (Falschlehner et al., 2009). Loss or inhibition of TRAIL in mice results in defects in thymocyte apoptosis and increased autoimmune responses (Cretney et al., 2005; Lamhamedi-Cherradi, Zheng, Maguschak, Peschon, & Chen, 2003). Moreover, TRAIL depletion in mice resulted in enhanced experimental and spontaneous metastasis occurrence in an NK cell-mediated manner (Cretney et al., 2002). These findings demonstrate that TRAIL provides critical immune surveillance and regulatory functions, including the suppression of autoimmunity and inhibiting tumor growth and metastasis.

TRAIL is able to bind to five different receptors, including DR4 (*a.k.a.* TRAIL-R1; gene symbol TNFRSF10A), DR5 (*a.k.a.* TRAIL-R2, TRICK2, and KILLER; gene symbol TNFRSF10B), decoy receptor (DcR) 1 (*a.k.a.* TRAIL-R3, TRID, and LIT; gene symbol TNFRSF10C), DcR2 (*a.k.a.* TRAIL-R4 and TRUNND; gene symbol TNFRSF10D), and osteoprotegerin (*a.k.a.* TRAIL-R5; gene symbol TNFRSF11B) (Degli-Esposti, Dougall, et al., 1997; Degli-Esposti, Smolak, et al., 1997; Emery et al., 1998; MacFarlane et al., 1997; Marsters et al., 1997; Pan, Ni, Yu, Wei, & Dixit, 1998;

Pan, O'Rourke, et al., 1997; Pan, Ni, et al., 1997; Schneider, Bodmer, et al., 1997; Screaton et al., 1997; Sheridan et al., 1997) (Figure 1). DR4 and DR5 possess a death domain (DD) that allows for the transduction of the apoptotic signal through the recruitment of adaptor proteins and caspases-8 and -10 to facilitate formation of the death inducing signaling complex (DISC) (Ashkenazi & Dixit, 1999; Gonzalvez & Ashkenazi, 2010). DcR1 lacks a DD altogether, and DcR2 has a truncated DD and thus is unable to initiate DISC formation, thereby preventing transduction of an apoptotic signal (Pan et al., 1998; Pan, Ni, et al., 1997). DcR1 and DcR2 antagonize DR-mediated apoptotic function by competitively binding the TRAIL ligand. Overexpression of DcR1 and DcR2 has been found to inhibit apoptosis, and inhibition of DcR1 and DcR2 can promote TRAIL-mediated apoptotic signaling (Ashkenazi & Dixit, 1999; Pan et al., 1998; Pan, Ni, et al., 1997). Osteoprotegerin (also lacking a DD and the ability to facilitate apoptosis) is a soluble protein that also binds to TRAIL and inhibits activation of DR4 and DR5 in experimental models (Emery et al., 1998; Shipman & Croucher, 2003). Osteoprotegerin competes with receptor activator of nuclear factor kappa-B Ligand (RANKL) for binding to the receptor receptor activator of nuclear factor kappa-B (RANK); osteoprotegerin/RANK binding inhibits osteoclast differentiation and bone resorption (Gonzalvez & Ashkenazi, 2010). Findings from studies have been mixed regarding whether TRAIL interferes with the anti-osteoclast activities of osteoprotegerin (Colucci et al., 2004; Labrinidis et al., 2008; Sedger et al., 2002; Vitovski, Phillips, Sayers, & Croucher, 2007; Zauli, Rimondi, & Secchiero, 2008). However, the loss of TRAIL was not found to affect bone density *in vivo*, suggesting that the interaction between TRAIL and osteoprotegerin may not impact RANK signaling with respect to bone development

or turn-over (Labrinidis et al., 2008). Whether osteoprotegerin affects TRAIL signaling under physiological conditions is unclear (Holland, 2014).

TRAIL Signaling Pathways

The canonical TRAIL apoptotic signaling pathway is an example of apoptosis mediated through the extrinsic death pathway, which entails activation of cell-surface receptors by a ligand to induce activation of downstream caspases (Figure 2) (Rahman, Pumphrey, & Lipkowitz, 2009). Activation of DR4 and DR5 by trimeric TRAIL promotes receptor clustering and formation of the DISC (Ashkenazi, 2002). The adaptor protein fas-associated death domain (FADD), and pro-caspases-8 and -10 are subsequently recruited (Ashkenazi, 2002). The forced proximity of the pro-caspases-8 and-10 at the DISC leads to autoprocessing of the pro-caspases, resulting in a tetramer of two large and small subunits that are more active than the pro-forms of the proteins (Ashkenazi, 2002). FLICE-like inhibitory protein (FLIP), a negative regulator of the TRAIL pathway, is structurally related to pro-caspases-8 and -10 with N terminal death effector domains (DED) and a C-terminal caspase-like domain. However, the catalytic cysteine is replaced by a tyrosine, thereby rendering it catalytically inactive (Irmler et al., 1997). FLIP may also be recruited to the DISC, prevent caspases-8 and -10 from interacting with FADD, and thus attenuate the apoptotic signal (Ashkenazi, 2002; Irmler et al., 1997). In some cells, activated caspases -8 and-10 are able to cleave the pro-apoptotic Bcl-2 homologous 3 (BH3) only BCL2 family member BH3 interacting-domain death agonist (Bid) protein into truncated Bid (tBid), which translocates to the mitochondria to induce intrinsic death pathway signaling (Czabotar et al., 2014; Danial & Korsmeyer, 2004; Li., Zhu, Xu, & Yuan, 1998). The BCL2 protein family regulates the

intrinsic death pathway via anti- and pro-apoptotic family members (Czabotar et al., 2014). When the intrinsic death pathway is activated, pro-apoptotic BCL2 family members, such as BCL2-associated agonist of cell death (BAD), BID, BLC2-like 11 (BIM), and BLC2 binding component 3 (PUMA), antagonize anti-apoptotic family members, including BLC2-like 1 (BCL-XL), BCL2, BCL2-like 2 (BCL-w), and myeloid cell leukemia 1 (MCL-1) (Cragg, Harris, Strasser, & Scott, 2009; Czabotar et al., 2014). tBid directly and indirectly activates pro-apoptotic proteins BCL2-antagonist (BAK) and BCL2-associated X protein (BAX), causing mitochondrial outer membrane permeabilization and cytochrome *c* release (Czabotar et al., 2014; Danial & Korsmeyer, 2004; Luo, Budihardjo, Zou, Slaughter, & Wang, 1998; Westphal, Dewson, Czabotar, & Kluck, 2011). The scaffold protein apoptotic peptidase activating factor 1 (APAF-1) binds to cytochrome *c*, and the initiator caspase-9 is subsequently activated on APAF-1 (Czabotar et al., 2014; Danial & Korsmeyer, 2004). IAP-binding mitochondrial protein (SMAC), an inhibitor of the inhibitor of apoptosis (IAP) proteins that suppress caspase function, is also released during MOMP to help facilitate apoptosis (Du, Fang, Li, Li, & Wang, 2000). At this point, the extrinsic and intrinsic death pathways converge; caspases-8, -10, and -9 are able to proteolytically process the executioner caspases-3 and -7 which carry out the final steps of apoptosis by cleaving numerous substrates (Czabotar et al., 2014; Danial & Korsmeyer, 2004). Caspase-3 is also able to process caspase-8 in a feedback-loop, thus amplifying the apoptotic signal (Slee et al., 1999).

Like TNF, TRAIL-induced DR4 and DR5 activation has also been associated with the induction of NF-κB and mitogen-activated protein kinase (MAPK) signaling (Chaudhary et al., 1997; Keane et al., 2000; Schneider, Thome, et al., 1997; Varfolomeev

et al., 2005). Interestingly, TNF signaling is a more potent activator of these pathways than TRAIL (Varfolomeev et al., 2005). After TRAIL-induced caspase activation, receptor interacting protein 1 (RIP1), FADD, TNF receptor-associated factor 2 (TRAF2)rec, and caspase-8 form a ligand and receptor-independent secondary complex that facilitates kinase signaling in a RIP1-dependent manner (Varfolomeev et al., 2005). However, findings have been mixed concerning the anti- and pro-survival mechanisms induced by TRAIL-mediated NF-κB and MAPK activation (Azijli, Weyhenmeyer, Peters, de Jong, & Kruyt, 2013; Holland, 2014). Inhibition of components of the NF-κB and MAPK signaling pathways has produced examples of both enhanced and attenuated sensitivity to TRAIL-induced apoptosis, demonstrating that the outcomes associated with TRAIL-mediated activation of NF-κB and MAPK will vary depending on context and requires further characterization (Azijli et al., 2013; Holland, 2014). TRAIL signaling has also been associated with non-apoptotic cell death mechanisms, including the caspase-independent, cell-regulated form of necrosis, necroptosis (Azijli et al., 2013; Jouan-Lanhouet et al., 2012; Kemp, Kim, Crist, & Griffith, 2003; Meurette et al., 2005; Meurette et al., 2007). TRAIL has been found to induce necroptosis regulators RIP1 and receptor interacting protein 3 (RIP3) in an acidic pH-dependent manner, and NF-κB inhibition has been found to enhance sensitivity to TRAIL-induced necroptosis (Azijli et al., 2013; Jouan-Lanhouet et al., 2012; Meurette et al., 2005; Meurette et al., 2007). Further elucidation of the mechanisms and conditions under which TRAIL activates necroptotic signaling is needed.

Interestingly, TRAIL and DR expression have also been found to support cell growth under certain conditions. Specifically, TRAIL signaling has been found to

promote proliferation and INF-γ production on pre-activated T cells (Chou et al., 2001). DR5 and TRAIL/murine TRAIL receptor (mTRAIL-R) signaling have been associated with enhanced TNBC-associated bone and Kirsten rat sarcoma viral oncogene homolog (KRAS)-driven cancer metastasis, respectively (Fritsche et al., 2015; von Karstedt et al., 2015). The pro-metastatic phenotypes were reversed with DR5 and TRAIL/mTRAIL receptor inhibition, suggesting that TRAIL DR agonist therapy may promote metastasis under certain conditions. Additional characterization of the conditions under which TRAIL signaling is pro-proliferative is necessary to determine the contexts wherein TRAIL DR agonist therapy may be an inappropriate therapeutic strategy.

Clinical Results with TRAIL Death Receptor Agonists

DR4 and DR5 agonists have been developed for clinical use. Dulanermin is a form of recombinant human TRAIL that activates both DR4 and DR5 (Ashkenazi et al., 1999). The majority of TRAIL DR agonists are antibodies, including the DR4-specific agonist mapatumumab (Pukac et al., 2005) and the DR5-specific agonists conatumumab (Kaplan-Lefko et al., 2010) drozitumab (Adams et al., 2008), lexatumumab (Zeng et al., 2006), and tigatuzumab (Yada et al., 2008). TAS266, a DR5 agonist tetravalent Nanobody®, has also been developed for use in clinical trials (Huet et al., 2014).

Phase 1, 1b, and 2 clinical trials using these TRAIL DR agonists have been conducted (Table 1). Phase 1 dose-escalating monotherapeutic studies have been carried out in children and adults with advanced solid tumors and adults with non-Hodgkin's lymphoma (NHL) (Camidge et al., 2010; Doi et al., 2011; Forero-Torres et al., 2010; Herbst, Eckhardt, et al., 2010; Herbst, Kurzrock, et al., 2010; Hotte et al., 2008; Merchant et al., 2012; Papadopoulos et al., 2015; Plummer et al., 2007; Tolcher et al., 2007;

Wakelee et al., 2010). Of the TRAIL DR agonists, dulanermin and antibodies were well-tolerated. Dose limiting toxicities varied, and the maximum tolerated dose was never reached while using these TRAIL DR agonists (Camidge et al., 2010; Doi et al., 2011; Forero-Torres et al., 2010; Herbst, Eckhardt, et al., 2010; Herbst, Kurzrock, et al., 2010; Hotte et al., 2008; Merchant et al., 2012; Plummer et al., 2007; Tolcher et al., 2007; Wakelee et al., 2010; Younes et al., 2010). However, TRAIL DR agonist therapy has demonstrated only modest therapeutic benefit. The majority of patients treated with a TRAIL DR agonist progressed on treatment or had stable disease (SD). Very few patients experienced sustained partial responses (PR). Two patients with chondrosarcoma achieved a PR while treated with dulanermin and continued to receive treatment ~3 and ~4 years from the initiation of treatment (Herbst, Eckhardt, et al., 2010). In another study, one patient with non-small cell lung cancer (NSCLC) maintained a PR ~4 years after the initiation of conatumumab treatment (Herbst, Kurzrock, et al., 2010). Although biomarkers to assess for drug pharmacodynamic activity were assessed, including activated caspase-3 from tumor samples (Herbst, Eckhardt, et al., 2010; Herbst, Kurzrock, et al., 2010), caspase-3 activation was not associated with the prolonged response observed in the three aforementioned study participants. Surprisingly, hepatotoxicity (grade ≥3 elevations in aspartate aminotransferase and/or alanine aminotransferase levels) and immunogenicity to the highly potent TRAIL DR superagonist TAS266 were observed in 3 of 4 patients, resulting in early termination of the clinical trial (Papadopoulos et al., 2015). The presence of preexisting antibodies to TAS266 in the patients who experienced hepatotoxicity, possible elevated expression of hepatic DR5, and high potency of the TAS266 Nanobody® have been proposed as the

potential basis for the hepatotoxicity observed in patients (Papadopoulos et al., 2015). This was the only TRAIL DR agonist that failed at the phase 1 stage.

The combination of TRAIL DR agonists with other agents has been explored in phase 1b and 2 studies. Dulanermin and conatumumab were each separately combined with FOLFOX6 and bevacizumab for the treatment of metastatic colorectal cancer (Fuchs et al., 2013; Wainberg et al., 2013), and dulanermin was combined with paclitaxel, carboplatin, and bevacizumab for the treatment of NSCLC (Soria et al., 2010). Conatumumab was also combined with another investigational drug, ganitumab (a human monoclonal antibody against type 1 insulin-like growth factor receptor), for the treatment of advanced solid tumors (Tabernero et al., 2015). The addition of TRAIL DR agonists to the treatment regimens was well-tolerated, and drug-drug interactions were not reported. However, the addition of a TRAIL DR agonist did not significantly improve outcomes.

TRAIL DR agonists have also been studied in phase 2 studies. A single complete response (CR) was observed in the first-line treatment of NSCLC with the combination of the DR 5 agonist conatumumab, paclitaxel, and carboplatin (Paz-Ares et al., 2013). Interestingly, this response occurred in the arm of the study in which the lowest dose of conatumumab was tested (3 mg/kg vs. 15 mg/kg every 3 weeks). Another CR was achieved using the combination of the DR4 agonist mapatumumab, paclitaxel, and carboplatin for NSCLC (von Pawel et al., 2014), and a CR was observed in a single-agent trial using mapatumumb for the treatment of NHL (Younes et al., 2010). Three CRs out of 39 in the treatment arm were observed in triple negative breast cancer (TNBC) patients who received tigatuzumab and albumin bound paclitaxel (nab-PAC) (Forero-Torres et al., 2015). No CRs were observed in any of the 21 patients who received nab-PAC alone, but

the overall response rate and progression free survival (PFS) were similar in both treatment and control arms. Despite rare instances wherein a CR was achieved, the findings from these phase 2 studies indicate that the addition of a TRAIL DR agonist did not add any significant clinical benefit to unselected patients (Cohn et al., 2013; Demetri et al., 2012; Forero-Torres et al., 2013; Forero-Torres et al., 2015; Fuchs et al., 2013; Kindler et al., 2012; Paz-Ares et al., 2013; Reck et al., 2013; Soria et al., 2011; Tabernero et al., 2015; Trarbach et al., 2010; von Pawel et al., 2014; Younes et al., 2010).

Evaluating Pharmacokinetic and Pharmacodyanmic Characteristics of TRAIL DR Agonists

Despite *in vitro* and *in vivo* preclinical studies demonstrating efficacy of TRAIL DR agonists alone or in combination with other agents to selectively induce apoptosis in cancer cells (Ashkenazi, 2008), results from clinical trials have been largely disappointing in demonstrating significant TRAIL DR agonist efficacy. Evaluating the pharmacokinetic and pharmacodynamic properties of TRAIL DR agonists may provide some insight into the lack of clinical activity.

Dulanermin, a form of the human TRAIL protein (Ashkenazi et al., 1999), has a short half-life of ~30 minutes to 1 hour and was administered at 0.5 to 30 mg/kg/day for 3 to 5 consecutive days every 2 to 3 weeks (Herbst, Eckhardt, et al., 2010; Soria et al., 2011; Soria et al., 2010; Wainberg et al., 2013). Its short half-life may partially explain the observed lack of effectiveness when using dulanermin in advanced tumors. The TRAIL DR antibody agonists have demonstrated a considerably longer half-life of approximately 10 days to 2 weeks (Camidge et al., 2010; Doi et al., 2011; Forero-Torres et al., 2010; Herbst, Kurzrock, et al., 2010; Hotte et al., 2008; Plummer et al., 2007;

Tolcher et al., 2007; Wakelee et al., 2010), which may improve tissue penetrance. However, TRAIL DR antibody agonists again had little activity; prolonged half-life may not address all of the issues that led to poor clinical activity.

Cross linking of drozitumab (a DR5 selective agonist antibody) by both activating and inhibitory Fcγ receptors (FcγR) on leukocytes was found to enhance apoptosis *in vitro* and *in vivo*, although in other targeted antibody agents, such as rituximab and trastuzumab, engagement specifically with activating FcγR was necessary (Wilson et al., 2011). Similarly, conatumumab requires crosslinking to facilitate apoptosis (Kaplan-Lefko et al., 2010). Thus, the presence or absence of infiltrating immune cells that express FcγR may impact the effectiveness of TRAIL DR antibody agonists in humans. Moreover, variants in FcγR, specifically FcγRIIA^{131H} and FcγRIIIA^{158V}, have been associated with the enhanced therapeutic efficacy of rituximab, trastuzumab, and cetuximab in humans (Bibeau et al., 2009; Cartron et al., 2002; Musolino et al., 2008; Weng, Czerwinski, Timmerman, Hsu, & Levy, 2004; Weng & Levy, 2003; Wilson et al., 2011; Zhang et al., 2007). FcγR polymohrphisms have been characterized in patient tumor samples from clinical trials using conatumumab for the treatment of soft tissue sarcoma (Demetri et al., 2012), colorectal cancer (Cohn et al., 2013; Fuchs et al., 2013), and NSCLC (Paz-Ares et al., 2013) and tigatuzumab for the treatment of NSCLC (Reck et al., 2013) in an effort to determine if FcγR polymorphisms segregated with outcomes. None of the FcγR polymorphisms were found to be significantly associated with PFS or overall survival (OS), but patients with F158V polymorphisms that were homozygous for the high affinity binding alleles (V/V) who received conatumumab in combination with paclitaxel and carboplatin for NSCLC trended toward longer OS (Paz-Ares et al., 2013).

Further, there was a trend towards significance for improved PFS in patients with F158V polymorphisms when conatumumab was combined with bevacizumab and FOLFOX6 for the treatment of colorectal cancer (Fuchs et al., 2013). Patients with high affinity binding alleles of FcγR may have experienced marginally enhanced benefit from conatumumab and tigatuzumab. Modifying the Fc region to increase the affinity for the FcγR has been proposed as one strategy to enhance the efficacy of TRAIL DR antibody agonists in the clinical setting (Carter, 2006; Jefferis, 2009; Lazar et al., 2006; Presta, Shields, Namenuk, Hong, & Meng, 2002; Satoh, Iida, & Shitara, 2006; Wilson et al., 2011).

Determining whether the TRAIL DR agonist has reached the tumor and initiated apoptosis has been challenging to assess in the clinic. DR4 and DR5 levels were assessed by immunohistochemistry (IHC) in tumor tissues (Doi et al., 2011; Greco et al., 2008; Leong et al., 2009; Merchant et al., 2012; Mom et al., 2009; Plummer et al., 2007; Rocha Lima et al., 2012; Tolcher et al., 2007; Trarbach et al., 2010; von Pawel et al., 2014; Wakelee et al., 2010; Younes et al., 2010), although the levels of DR4 and DR5 expression have not always correlated with response in the preclinical setting (Rahman, Davis, et al., 2009). Expression levels varied tremendously within and between tumors and did not correlate with response to treatment (Doi et al., 2011; Greco et al., 2008; Leong et al., 2009; Merchant et al., 2012; Mom et al., 2009; Plummer et al., 2007; Rocha Lima et al., 2012; Tolcher et al., 2007; Trarbach et al., 2010; von Pawel et al., 2014; Wakelee et al., 2010; Younes et al., 2010). Circulating DNA, active caspase-3, active caspase-8, and full-length and caspase-cleaved forms of the intermediate filament protein, cytokeratin 18 (Weng, Cui, & Fang, 2012), are commonly utilized biomarkers of apoptosis (Ward et al., 2008) and have been measured in clinical trials to evaluate the

effectiveness of TRAIL DR agonists (Fuchs et al., 2013; Herbst, Kurzrock, et al., 2010; Pan et al., 2011; Soria et al., 2011; von Pawel et al., 2014). The levels of these biomarkers of apoptosis have varied among tumors and have not correlated with response. However, a comprehensive assessment of apoptosis in tumors is difficult in the clinical setting. Apoptosis is a rapidly occurring event; specimens that are available for assaying may not be representative of TRAIL DR agonist-induced tumor cell death as apoptotic cells are rapidly engulfed and destroyed by phagocytes in the surrounding microenvironment (Ward et al., 2008). In light of the difficulty of obtaining solid tumor biopsies, acquiring serial samples of easily accessed tissues, such as blood, for the evaluation of tumor cell apoptosis by validated assays may provide the most helpful insights into TRAIL DR agonist activity in tumors (Duffy et al., 2015; Ward et al., 2008). The M30 and M65 sandwich enzyme-linked immunosorbent assay (ELISA) systems, for example, allow for the evaluation of cleaved and uncleaved cytokeratin 18 in serum (Cummings et al., 2008). Serum samples are generally more easily attainable than serial tumor tissue for the assessment of apoptotic biomarkers. Thus, the M30 and M65 assay systems could potentially provide more information about tumor cell death in real time than the IHC of apoptotic biomarkers in tumor tissues that are infrequently acquired. Furthermore, given the plasticity of the tumor microenvironment that can facilitate the development of drug resistance (Singh & Settleman, 2010), biomarkers of apoptosis that provide real-time information about the effectiveness of TRAIL DR agonists – and therapeutic agents in general – hold the potential to more expediently help identify insensitivity to treatment.

Strategies to Improve TRAIL DR Agonist Activity

The utilization of patient selection criteria that will help identify who may benefit from TRAIL DR agonist therapy may provide the most critical starting point for enhancing TRAIL DR agonist performance in the clinical setting. To date, patients who have received TRAIL DR agonists have been unselected (Holland, 2014; Lemke, von Karstedt, Zinngrebe, & Walczak, 2014). The identification of a predictive biomarker of response to TRAIL DR agonists may aid in the selection of patients who will most likely benefit from treatment.

Several predictive biomarkers have already been characterized. First, O-glycosylation is necessary for full activity of TRAIL DRs (Wagner et al., 2007). Higher expression of O-glycosylation genes, specifically polypeptide N-acetylgalactosaminyl transferase 14 (*GALNT14*) in NSCLC, melanoma, and pancreatic cancer cell lines and polypeptide N-acetylgalactosaminyl transferase 3 (*GALNT3*), fucosyltransferase 3 (*FUT3*), and fucosyltransferase 6 *(FUT6*) in colon cancer cell lines, were associated with sensitivity to TRAIL-induced apoptosis (Wagner et al., 2007). Expression levels of O-glycosylation genes served as better predictors of response to TRAIL than the anti-apoptotic proteins BCL2, X-linked inhibitor of apoptosis (XIAP), or FLIP. DR5 is O-glycosylated by GALNT14, and its modification enhances ligand-receptor clustering to facilitate more efficient downstream signaling (Wagner et al., 2007). Overexpression and inhibition of specific O-glycosylation genes enhanced and attenuated TRAIL-induced apoptosis, respectively. Evaluation of expression of O-glycosylation genes has been proposed as a strategy to predict sensitivity to TRAIL, and efforts have been directed toward the development of an IHC assay for the evaluation of the expression levels of

these genes in the clinical setting (Stern, Padilla, Wagner, Amler, & Ashkenazi, 2010). Inhibition of syndecan-1 was associated with enhanced sensitivity to TRAIL due to TRAIL DR O-glycosylation in myeloma cells (Wu, Yang, Chien, Lin, & Lai, 2012). In a phase 2 study combining dulanermin with FOLFOX6 and bevacizumab for colorectal cancer, high expression of GALNT14 evaluated by IHC has been found to significantly associate with PFS and OS in the treatment over control arm, suggesting GALNT14 expression served as a predictor of sensitivity to cytotoxic agents (Soria et al., 2011). However, expression of O-glycosylation genes has inconsistently been associated with TRAIL sensitivity. In TRAIL-sensitive TNBC cells, elevated expression of O-glycosylation genes was not observed (Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). These findings suggest that biomarkers for TRAIL resistance and sensitivity may need to be determined on a tissue-specific basis.

TNBC cell lines with a mesenchymal (*a.k.a.* basal B) phenotype are extremely sensitive to apoptosis induced by TRAIL, whereas cell lines representative of the other subtypes (ER+/luminal, HER2 amplified, and basal A TNBC) of breast cancer are comparatively resistant (Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). The mechanistic basis for the differential sensitivity observed in the different subtypes of breast cancer cell lines is not known. The basal B TNBC cell lines highly express the mesenchymal marker vimentin and express very little of the epithelial marker E-cadherin (Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). Interestingly, E-cadherin was found to play an important role in promoting DR4 and DR5 clustering and formation of the DISC (Lu et al., 2014). Higher levels of E-cadherin expression were characterized with lower half maximal inhibitory concentration (IC50)) levels of TRAIL in pancreatic,

lung, and colon cancer cell lines (Lu et al., 2014). These contrasting findings also suggest that tissue-specific characteristics may need to be taken into account when identifying a biomarker for TRAIL DR agonists that is relevant to a particular tumor type.

In head and neck cancer cell lines, caspase-8 and Bid expression has been associated with sensitivity to TRAIL (Raulf et al., 2014). Interestingly, low levels of caspase-8 resulted in insensitivity to TRAIL but sensitivity to a SMAC mimetic. Genotyping to identify caspase-8 mutations in this particular population of patients may be beneficial in predicting TRAIL DR agonist efficacy. Caspase mutations occur in ~9% of head and neck cancers and have been found to confer resistance to TRAIL-induced apoptosis in head and neck cancer cell lines, as well as promote cancer cell migration, invasion, and tumor proliferation (Li, Egloff, Sen, Grandis, & Johnson, 2014). Caspase-8 has been evaluated by IHC of tumor sections in a TRAIL DR agonist clinical trial (Merchant et al., 2012), but expression has not been associated with disease response. However, others have shown that caspase-8 expression does not necessarily predict sensitivity to TRAIL (Rahman, Davis, et al., 2009), suggesting that caspase-8 expression may not be a suitable biomarker for predicting TRAIL sensitivity across tumor types.

Interestingly, even in cells sensitive to TRAIL-mediated apoptosis, the cells may cycle through sensitive and resistant states. Spencer et al. (2009) demonstrated the responsiveness of HeLa cells to TRAIL-induced apoptosis was governed by the variation in expression levels of proteins prior to the initiation of treatment (Spencer, Gaudet, Albeck, Burke, & Sorger, 2009). Specifically, variation in Bid expression was found to be predictive of the time to TRAIL-induced apoptosis (Spencer et al., 2009).

The identification of synergistic combinations with TRAIL DR agonists has been proposed as a strategy to overcome resistance to TRAIL and has already been widely explored in preclinical contexts (Ashkenazi, 2008; Bullenkamp et al., 2014; Cuello et al., 2001; Keane, Ettenberg, Nau, Russell, & Lipkowitz, 1999; Koschny et al., 2014; Nojiri et al., 2013; Zinonos et al., 2014). The combining of drugs has been proposed to overcome resistance to cancer therapies by reducing variability among cells with respect to timing and susceptibility to an apoptosis inducer (Flusberg & Sorger, 2013; Spencer et al., 2009). Moreover, TRAIL DR agonists are able to activate apoptosis independently of tumor protein 53 (p53) – which is commonly mutated in cancer – and this characteristic provides another rationale for using TRAIL DR agonists in combination with other therapeutic agents to overcome resistance to cell death induction (Ashkenazi, 2002).

Chemotherapy has been found in preclinical studies to synergize with TRAIL, including 5FU, bortezomib, gemcitabine, and irinotecan (Lemke, von Karstedt, Zinngrebe, et al., 2014). The mechanisms proposed to sensitize cells to TRAIL include enhanced DISC formation, upregulation of pro-apoptotic regulators, including TRAIL DR expression, and downregulation of anti-apoptotic regulators. However, the results from clinical trials to date do not demonstrate that the addition of a TRAIL DR agonist to a chemotherapeutic regimen improves patient outcomes (Holland, 2014; Lemke, von Karstedt, Zinngrebe, et al., 2014). Targeted inhibition of anti-apoptotic components of the intrinsic pathway, including BCL2 family members (Cristofanon & Fulda, 2012; Garimella et al., 2014) and IAPs (Allensworth, Sauer, Lyerly, Morse, & Devi, 2013; Finlay et al., 2014), have also sensitized cells to TRAIL. ABT-199, an inhibitor of anti-apoptotic members of the BCL2 family, is safe in humans (Souers et al., 2013) and may

potentially be used in combination with TRAIL DR agonists in the future. Preclinical data suggest that IAP-inhibiting SMAC mimetics can enhance TRAIL mediated apoptosis (Allensworth et al., 2013; Cristofanon et al., 2015; Zhang et al., 2012). Currently, the SMAC mimetic birinipant is being tested in combination with the DR5 agonist, conatumumab, for ovarian cancer treatment (Lemke, von Karstedt, Zinngrebe, et al., 2014). Results from this clinical trial may support the combination of SMAC mimetics with conatumumab as a novel therapeutic strategy for ovarian cancer.

Histone deacetylase (HDAC) inhibitors, which prevent the remodeling of chromatin through the removal of acetyl groups from histone tails, have been found to synergize with TRAIL (Fulda, 2012). FLIP downregulation and DR5 upregulation have been reported as HDAC inhibitor-mediated mechanisms of TRAIL sensitization (Jazirehi, Kurdistani, & Economou, 2014; Venza, Visalli, Oteri, Teti, & Venza, 2014). Further study is warranted to fully clarify HDAC inhibition and TRAIL sensitization in cancer cells. Finally, inhibition of cell cycle regulators, including cyclin-dependent kinase 9 (CDK9) and WEE1 G2 checkpoint kinase (WEE1), has sensitized cells to TRAIL (Garimella, Rocca, & Lipkowitz, 2012; Lemke, von Karstedt, Abd El Hay, et al., 2014). Interestingly, CDK9 inhibition downregulated the anti-apoptotic regulators MCL-1 and FLIP, indicating that inhibition of CDK9 sensitizes cells to TRAIL through regulation of components of the extrinsic and intrinsic pathway. Additional investigation is warranted to determine the optimal drug combinations requisite to sensitize cells to DR agonists for different types of cancers.

More recently, the enhancement of endogenous TRAIL and DR expression has emerged as another TRAIL pathway activation strategy. The small molecule ONC201

(*a.k.a.* TIC10 and NSC350625) was identified in a National Cancer Institute chemical library screen as an inducer of TRAIL expression in a colon cancer cell line (Allen et al., 2013; Allen, Krigsfeld, et al., 2015). ONC201 inhibits protein kinase B (Akt) and mitogen-activated protein kinase kinase (MEK) activity, resulting in de-phosphorylation of the FOXO3a transcription factor and subsequent transcriptional activation of TRAIL (Allen et al., 2013; Allen, Krigsfeld, et al., 2015; Jacob, Lockner, Kravchenko, & Janda, 2014). DR5 upregulation is also observed (Allen et al., 2013; Allen, Krigsfeld, et al., 2015), which may help further potentiate TRAIL pathway activity. Cytotoxic effects associated with ONC201 have been found specifically in solid and hematological cancer cell lines *in vitro* (Allen et al., 2013; Allen, Krigsfeld, et al., 2015; Allen, Prabhu, et al., 2015; Prabhu, Allen, Dicker, & El-Deiry, 2015; Talekar, Allen, Dicker, & El-Deiry, 2015) and in xenograft models of hepatocellular carcinoma and colon, breast, and brain cancers (Allen et al., 2013; Allen, Krigsfeld, et al., 2015). The specificity, potency, stability, oral availability, and ability to cross the blood brain barrier confer ideal characteristics to ONC201 for cancer treatment, thus warranting further study (Allen et al., 2013; Greer & Lipkowitz, 2015). Moreover, ONC201 has been found to synergize with chemotherapeutic and targeted agents, including sorafenib, a multikinase inhibitor of vascular endothelial growth factor receptor (VEGFR), plate-derived growth factor receptor (PDGFR), and rapidly accelerating fibrosarcoma (Raf kinases in *in vitro* and *in vivo* hepatocellular carcinoma models and with cytarabine, a DNA damaging agent, in pediatric NHL cell lines (Allen, Prabhu, et al., 2015; Talekar et al., 2015). The combination of ONC201 and additional agents may provide another strategy for overcoming resistance to TRAIL pathway activation and apoptosis in general.

Conclusions and Future Directions

The TRAIL apoptotic pathway has been an enticing therapeutic target for the treatment of cancer. Because TRAIL has been well-characterized as an inducer of apoptosis selectively in cancer cells with minimal toxicity to normal cells, TRAIL DR agonists have been under development and tested in phase 1 and 2 clinical trials. TRAIL DR agonists have included recombinant human TRAIL and numerous TRAIL-mimetic antibodies and a Nanobody® designed to activate either DR4 or DR5. Despite a robust and selective killing effect *in vitro* and *in vivo,* TRAIL DR agonist therapy, though well-tolerated, has failed to demonstrate clinical efficacy in adult and pediatric patients with advanced solid tumors and hematological malignancies. This conundrum poses several challenges to improving the effectiveness of TRAIL DR agonists in the clinic.

First, improving the assessment of the pharmacodynamic effects of TRAIL DR agonists would provide better insight into whether the drugs are able to induce apoptosis in tumors. Frequent assessment of pharmacodynamics may provide the most comprehensive evaluation of TRAIL DR effectiveness. IHC of tumor tissues for biomarkers of apoptosis, such as cleaved caspase-3, may not be representative over time, as tumor samples are not frequently acquired. Using a more easily attainable tissue specimen, such as serum or circulating tumor cells, to evaluate pharmacodynamic characteristics may allow for more frequent monitoring and thus facilitate a more detailed understanding of drug effectiveness. Optimization of the structure of the TRAIL DR agonists, particularly the antibody-based agonists, may also help to improve therapeutic efficacy.

Identifying biomarkers that predict response may aid in selecting patients who will benefit the most from TRAIL DR therapy. However, biomarkers may be disease-specific, as findings have produced conflicting results concerning biomarkers of TRAIL sensitivity. Mesenchymal markers have been associated with enhanced sensitivity to TRAIL in the TNBC setting, whereas such markers have been associated with resistance in pancreatic, colon, and lung cancer cells (Lu et al., 2014; Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). Similarly, O-glycosylation gene upregulation has been associated with enhanced sensitivity to TRAIL in colon, NSCLC, lung, and myeloma cells and was associated with improved survival outcomes in a DR agonist clinical trial for the treatment of colorectal cancer (Soria et al., 2011; Wagner et al., 2007; Wu et al., 2012). However, O-glycosylation has not been determined to be a predictor of sensitivity to TRAIL in breast cancer cells (Rahman, Davis, et al., 2009). Thus, biomarkers need to be evaluated based on disease type. Moreover, monitoring for fluctuations in biomarkers that predict sensitivity to a therapeutic agent may be informative in light of the plasticity of the tumor environment.

Finally, combining TRAIL DR agonist therapies with other chemotherapeutic and targeted agents may most effectively enhance treatment by limiting the inherent variability observed among cancer cells (Flusberg & Sorger, 2013; Spencer et al., 2009). Although efforts toward combining TRAIL DR agonists and other agents have been carried out in clinical trials, the results from those studies have not identified a combinatorial therapeutic strategy that was optimized by the addition of a TRAIL DR agonist. Efforts toward further characterizing regulation of the TRAIL pathway may provide crucial insights into understanding the molecular underpinnings that control

sensitivity to TRAIL (Garimella et al., 2014) and guide the development of novel therapeutic agents or drug combinations to enhance TRAIL DR agonist effectiveness in humans. TRAIL DR agonists provide a promising therapeutic strategy for inducing apoptosis in cancer cells. Improving strategies to promote effectiveness of TRAIL DR agonists in the clinical setting are warranted.

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Figure 2.1

TRAIL and its Receptors

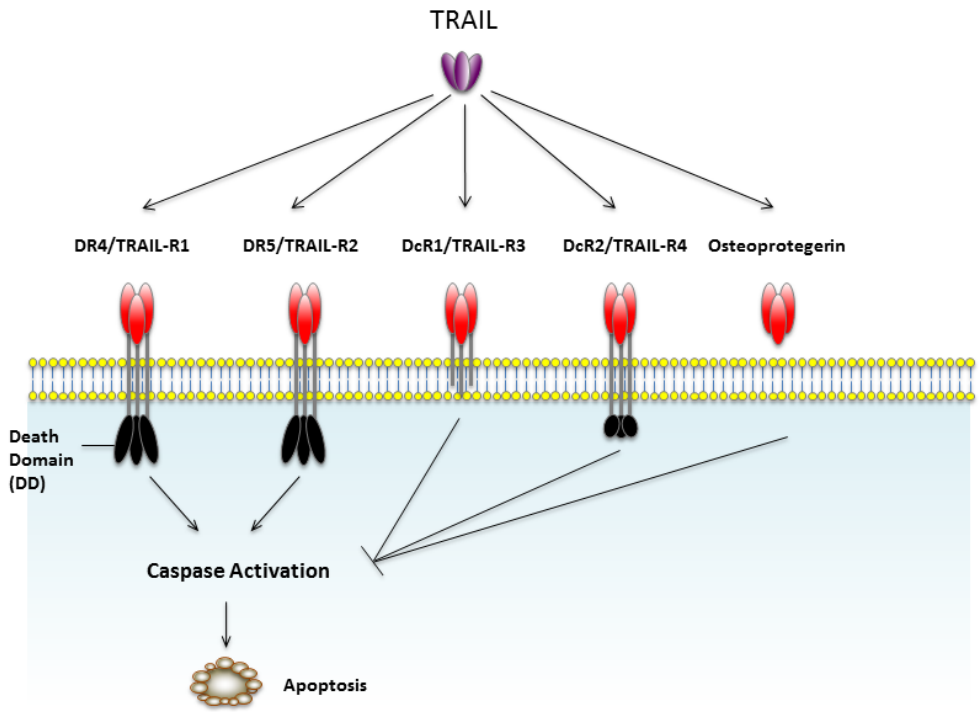


Figure 2.1. TRAIL binds to DR4, DR5, DcR1, DcR2, and osteoprotegerin. DR4 and DR5 possess complete DDs that activate the apoptotic signal via recruitment and activation of initiator caspases. DcR2 possesses a truncated DD, and DcR1 and osteoprotegerin lack DDs altogether and thus they cannot activate caspases. DcR1, DcR2, and osteoprotegerin have been found to attenuate the apoptotic signal by binding to TRAIL and thus preventing its interaction with DR4 and DR5.

Figure 2.2

The Extrinsic and Intrinsic Apoptotic Pathways

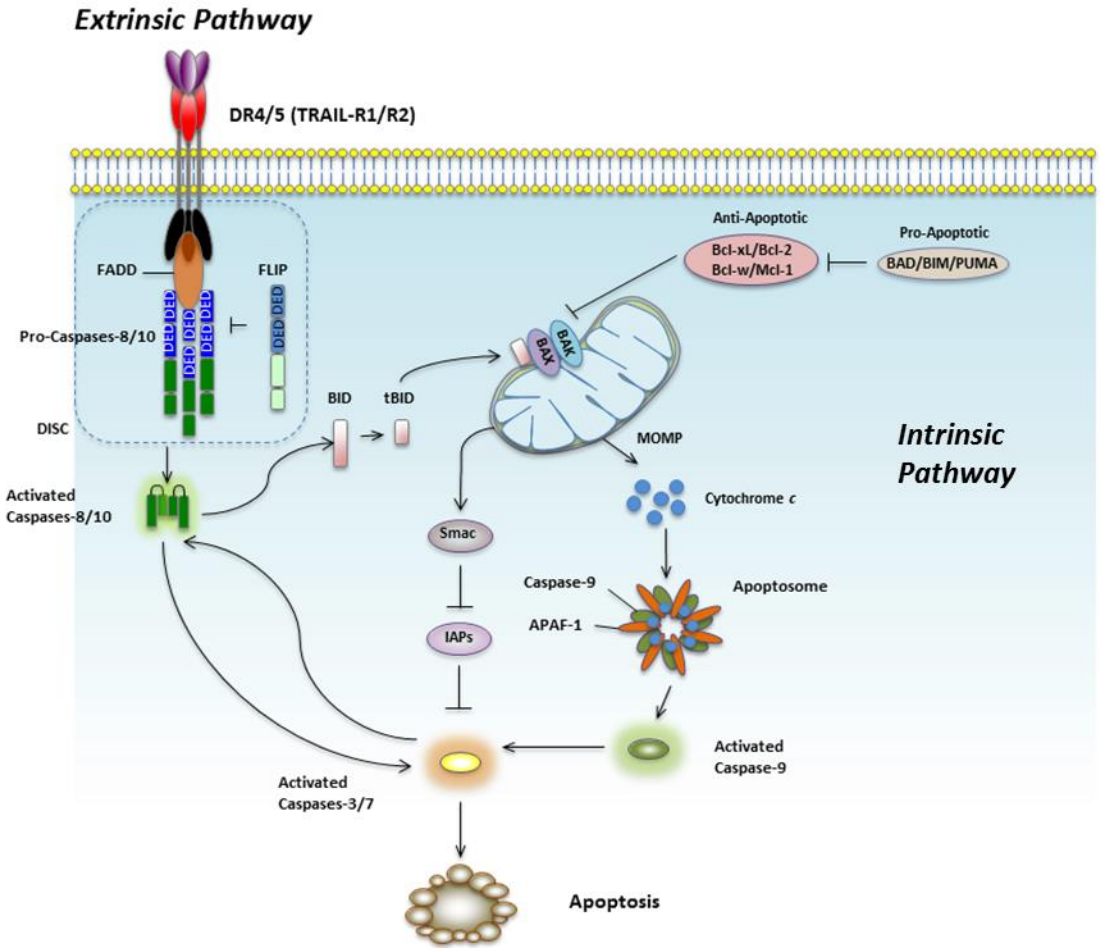


Figure 2.2. TRAIL activates the extrinsic apoptotic pathway by binding to DR4 and DR5 on the cell surface. Adaptor molecules, including FADD, and initiator caspases-8/10 are recruited to form the DISC. Caspases-8/10 subsequently self-activate. The anti-apoptotic protein FLIP can inhibit apoptosis by preventing caspases-8/10 from interacting with FADD. Caspases-8/10 activate the downstream executioner caspases-3/7, which can then reciprocally activate caspase-8. In some cells, caspases-8/10 can activate the intrinsic pathway by cleaving Bid into tBid, which helps activate BAK and BAX-mediated MOMP. Pro-apoptotic BAD, BIM, and PUMA inhibit anti-apoptotic BCL-XL, BCL2, BCL-w, and MCL-1 proteins. Cytochrome *c* is released from the mitochondria, and the apoptosome is formed when cytochrome *c*, APAF-1, and caspase-9 interact, forming a scaffold on which caspase-9 is able to activate. Caspase-9 activates caspases-3/7. Caspases-3/7 cleave other substrates, culminating in apoptosis.

CHAPTER THREE

METHOD

This project proposal was submitted to the dissertation committee as a description of the proposed aims for the dissertation project.

Approximately 40,000 American women (American Cancer Society, 2015) and more than 450,000 women worldwide (Jemal et al., 2011) are estimated to die of breast cancer each year, demonstrating a need for continued research in developing innovative treatments for improving survival in this population. Currently, breast cancer is categorized into different subtypes based on expression of estrogen receptor (ER), progesterone receptor (PR), and amplification of human epithelial growth factor receptor 2 (*Her-2*) (Brenton, Carey, Ahmed, & Caldas, 2005). Researchers have capitalized on these molecular markers by developing targeting therapeutics. While 80-85% of breast cancers are characterized by the expression or amplification of these markers, around 15-20% do not express ER, PR, or have HER-2 amplification This particular subtype of breast cancer, termed triple negative breast cancer (TNBC), is especially challenging to treat in the absence of a distinct molecular target, leaving chemotherapy as a sole treatment option. In part due to the absence of targeted therapies, TNBC is associated with poorer prognosis, higher rates of metastasis, and lower rates of disease-free and overall survival (Bauer, Brown, Cress, Parise, & Caggiano, 2007; Liedtke et al., 2008; Lin et al., 2012). Research aimed at developing novel therapies for TNBC is necessary in order to address the limited therapeutic options available to treat this aggressive and difficult-to-treat subtype of breast disease.

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) has been studied in preclinical models of cancer and has been shown to induce apoptotic cell death in a wide variety of cancer cells, including TNBC. TRAIL is a member of the tumor necrosis factor superfamily and initiates a caspase-mediated signaling cascade that ultimately results in apoptosis. TRAIL initiates apoptosis by binding to its cognate receptors, TRAIL receptors 1 and 2 (TRAIL-R1 and R2). The death inducing signaling complex (DISC) is then formed via recruitment of the fas-associated death domain (FADD) adaptor protein to the cytoplasmic death domains of the receptors. The inactive form of the pro-apoptotic initiator caspase, caspase-8, is subsequently recruited to the DISC where it is cleaved and becomes active (Sprick et al., 2000). Activated caspase-8 cleaves and activates the effector caspases, caspases-3/7. Effector caspases cleave multiple substrates, which leads to cell death. Active caspase-8 also cleaves the pro-apoptotic protein BH3-interacting domain death agonist (BID). BID translocates to the mitochondria and induces cytochrome *c* release (Li, Zhu, Xu, & Yuan, 1998; Luo, Budihardjo, Zou, Slaughter, & Wang, 1998). Cytochrome *c* forms a complex with and activates the initiator caspase, caspase-9, which further feeds into the caspase cascade by activating caspases-3/7 (Li et al., 1997). Preclinical studies have found that TRAIL ligands are not toxic to healthy tissues (Tian et al., 2011) and appear to be selective for transformed cells (Ashkenazi et al., 1999; Walczak et al., 1999). TRAIL-induced apoptosis has been proposed as a therapeutic strategy for cancer treatment, and phase 1 and phase 2 trials of TRAIL agonists are ongoing (Yerbes, Palacios, & Lopez-Rivas, 2011).

We and others have previously shown that TNBC cell lines are sensitive to TRAIL-mediated apoptosis while cell lines from other breast cancer subtypes (*e.g.*, ER positive and HER-2 amplified) are resistant to TRAIL-mediated apoptosis. In particular, TNBC cell lines that have mesenchymal features (classified as basal B cells) are the most sensitive to TRAIL-mediated apoptosis (Brenton et al., 2005; Rahman et al., 2009). While these studies identify a subset of TNBC that are sensitive to TRAIL-

mediated apoptosis, the mechanisms governing sensitivity to TRAIL-induced apoptosis in breast cancer remain elusive.

In order to identify potential regulators of the TRAIL pathway, our laboratory utilized RNAi technology to carry out a high-throughput screen of the kinome, phosphatome, and other candidates from the druggable genome in the mesenchymal TNBC cell line, MB-MDA-231 (MB231). One hundred fifty negative regulators of the TRAIL signaling pathway were identified by this screen (Garimella et al., 2014). The initial objective of this study was to select potential candidates for further investigation based on a review of the literature, and findings from replication of the caspases-3/7 and viability assays.

Of these 150 negative regulators of TRAIL-mediated apoptosis, the endoplasmic reticulum (ER) membrane-bound ubiquitin ligase (*a.k.a.*, E3), autocrine motility factor receptor (gp78), was selected for further characterization. gp78 is a really interesting gene (RING) finger E3 that facilitates ubiquitylation of proteins by binding E2s and transferring ubiquitin to the target substrate. gp78 also retrotranslocates ubiquitylated proteins across the ER membrane for degradation by the proteasome in a process known as ER-associated degradation (Fang et al., 2001). gp78 has been show to ubiquitinate and promote degradation of mutant huntingtin, alpha-1 antitrypsin, CFTRDeltaF508 (Chen, Du, & Fang, 2012), and the cluster of differentiation (KAI1) tumor suppressor protein in a mouse model of metastasis (Tsai et al., 2007). These findings demonstrate the diverse repertoire of disease linked to gp78, including cancer. However, gp78 has not yet been studied in the context of TRAIL-induced apoptosis in TNBC. Here, we have identified gp78 as a novel negative regulator of the TRAIL pathway and have initiated exploration into its functional relationship with TRAIL-induced apoptosis in the TNBC cell line MB231.

Background

In order to identify regulators of the TRAIL-induced apoptotic pathway in breast cancer cells, our laboratory carried out a high-throughput screen of the kinome, phosphatome, and others using RNAi technology to transcriptionally knockdown ~1300 candidate genes using the Qiagen Human Druggable Genome Version 2.0 library (Qiagen Inc.; Germantown, MD) (Garimella et al., 2014). Four independent siRNAs were used to transcriptionally silence each candidate gene in the mesenchymal TNBC cell line MB231. Regulators of the TRAIL pathway were selected if at least three of the four siRNAs were found to enhance or diminish TRAIL-induced caspases-3/7 activity at least two standard deviations above or below, respectively, the non-targeting siRNA and TRAIL treated control.

One hundred fifty negative regulators of the TRAIL signaling pathway were identified using these selection criteria (Garimella et al., 2014) (Figure 3.1). The screen identified several known negative regulators of apoptosis as negative regulators of TRAIL-induced caspases-3/7 activation including BCL2-like 1 (*BCL2L1*; BCL-XL), BCL2-like 2 (*BCL2L2*; BCL-w), baculoviral IAP repeating 2 containing (*BIRC2*; c-IAP1), and baculoviral repeating containing 3 (*BIRC3*; c-IAP2) (Deveraux & Reed, 1999; Gross, McDonnell, & Korsmeyer, 1999). These findings lend credence to the screen. We focused our subsequent analysis on those genes identified as negative regulators of TRAIL-induced caspases-3/7 activation rather than positive regulators because of the number of genes identified and because they may be potential targets that when inhibited will enhance TRAIL-mediated apoptosis. Given the relatively large number of putative negative regulators of TRAIL-mediated apoptosis we subjected the 150 genes to network gene analysis in order to aid in identification of common regulatory networks in which these genes function. Of the 147 genes with curated interaction data, the largest network identified connected 79 genes. Of these 79 genes, 42 were connected principally *via* four genes with seven or more interactions (Figure 3.2). The genes situated at these nodes *BCL2L1*, inhibitor of

kappa light polypeptide gene enhancer in B-cells, kinase beta (*IKBKB*), 3-phosphoinositide dependent protein kinase 1 (*PDPK1)* and proto-oncogene tyrosine-protein kinase Src (*SRC*) (indicated by the blue circles in Figure 3.2).

Three or more siRNAs against 26 of the 79 genes that enhanced TRAIL-mediated activation of caspases-3/7 also enhanced TRAIL cytotoxicity by greater than two standard deviations from the mean viability seen in non-targeting siRNA (siNeg)-transfected cells plus TRAIL (Garimella et al., 2014). As indicated by the red rectangles in Figure 3.1B, 14 of these 26 genes map to the direct interaction network. The silencing of *BCL2L1* and two genes directly linked to it, ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 (*ATP5A1*) and homeodomain interacting protein kinase 2 (*HIPK2)*, by multiple siRNAs increased TRAIL-induced caspases-3/7 activation and cytotoxicity. In addition, the RNAi induced loss of several genes linked to *SRC* enhanced TRAIL-induced cytotoxicity including *PDPK1*, connector enhancer of kinase suppressor of Ras 1(*CNKSR1*), phosphatidylinositol-4-phosphate 5-kinase, type I, gamma (*PIP5K1C*), fibroblast growth factor receptor 4 (*FGFR4*), breakpoint cluster region (*BCR*), RIO kinase 3 (*RIOK3)* and (MAP kinase interacting serine/threonine kinase 1) *MKNK1*. All three of the siRNAs corresponding to *SRC* that activated caspases-3/7 in the presence of TRAIL induced an enhancement in cytotoxicity but only using a the relaxed criteria of greater than one standard deviation from the mean viability seen in siNeg-transfected cells plus TRAIL. Multiple siRNAs corresponding to *PDPK1* and several genes linked to *PDPK1* also increased TRAIL-mediated caspases-3/7 activation and cytotoxicity. These include protein kinase c, iota (*PRKC1*), the known apoptosis inhibitor *BIRC2 (a.k.a.*, cIAP-1), polo-like kinase 3 (*PLK3*), protein kinase N1 (*PKN1*), and actinin 4 (*ACTN4*). Silencing by 2 of 4 siRNAs of many of the remaining genes mapping to the direct interaction network induced a decrease in cell viability greater than 2 standard deviations from that seen in siNeg-transfected cells plus TRAIL with at least 1 further siRNA inducing a decrease in viability at least 1

standard deviation from that seen in siNeg-transfected cells plus TRAIL. This included silencing of *IKBKB*, BLK proto oncogene, src family kinase (*BLK)*, Erb-B2 receptor tyrosine kinase 2 (*ERBB2*), *FGFR2*, N-acetylglucosamine kinase (*NAGK*), and poly (ADP-Ribose) polymerase family, member 12 (*ZC3HC1*, PARP12). This work has been published (Garimella et al., 2014).

Research Strategy

Aim 1: Select a Candidate TRAIL Pathway Regulator for Further Characterization in the Triple Negative Breast Cancer Cell Line MB-MDA-231

Candidates were selected for further investigation based on a review of the literature and the robustness of the findings from the caspases-3/7 and viability assays from the initial screen. Eight candidates were initially selected for further validation. They included acyl-coA dehydrogenase, very long chain (ACADVL); autocrine motility factor receptor (AMFR, a.k.a gp78); connector enhancer of kinase suppressor of Ras 1 (CNKSR1); hexokinase 1 and 2 (HK1 and HK2, respectively); homeodomain interacting protein kinase 2 (HIPK2); phosphofructokinase, liver (PFKL); and pyruvate kinase, liver and red blood cells (PKLR).

Unfortunately, few candidates were detectable on immunoblot. Those detectable on immunoblot included HK1 and gp78, which prompted further investigation into their effects on TRAIL-induced apoptosis. Efforts were initially directed toward exploring the effects of HK1 knockdown on TRAIL-induced apoptosis in MB231. HK1, along with HK2, is part of the initial irreversible step of the glycolysis pathway. PFKL and PKLR similarly mediate subsequent irreversible steps in the glycolysis pathway, emphasizing the potential significance of glycolysis inhibition in enhancing TRAIL-induced apoptosis. HK2, PFKL, and PKLR were undetectable on immunoblot, but HK1 was detectable (Figure 3.3B). Knockdown of HK1 did not significantly enhance TRAIL-induced apoptosis (Figure 3.3A). Furthermore, others have published that inhibition of glycolysis promoted TRAIL-induced apoptosis by

promoting formation of the DISC and activation of caspase 8 (Munoz-Pinedo, Ruiz-Ruiz, Ruiz de Almodovar, Palacios, & Lopez-Rivas, 2003). Thus we chose not to study these genes further.

We next evaluated gp78 as a regulator of TRAIL-mediated apoptosis. gp78 is a ubiquitin ligase which localizes to the ER membrane and is involved in protein retrotranslocation and degradation from the ER membrane (Fang et al., 2001; Zhong et al., 2004). gp78 is involved in degradation of unfolded or misfolded proteins and has also been implicated as a prometastasis protein by degradation of the KAI1 metastasis suppressor protein in a mouse model of metastasis (Tsai et al., 2007). gp78 has not been studied in the context of TRAIL-induced apoptosis. The published findings reflect a relationship to cancer without detracting from the novelty of the candidate in the context of TRAIL-mediated apoptosis. Therefore, gp78 was selected as the primary candidate for further characterization in this study.

In order to explore its effects on TRAIL-induced apoptosis, a cell viability assay was used to assess the effect of gp78 knockdown on TRAIL sensitization in MB231. Cells were first reversed transfected with 50 nM of a gp78-targeting pool of 4 siRNAs or non-targeting siRNA (Qiagen, Inc.; Germantonwn, MD) and seeded at 5000 cells per well in the inner 60 wells of a 96-well plate. Additional reverse transfected cells were plated for protein harvesting to assess knockdown. After 24 hours, TRAIL added to the cells. After another 24 hours, cells were incubated with CellTiter 96® AQueous One Solution Cell Proliferation (MTS) reagent (Promega; Madison, WI) to assess viability. The half maximal inhibitory concentration (IC50) for TRAIL in cells with gp78 knockdown was <4ng/mL, whereas the IC50 for TRAIL in the cells incubated with non-targeting siRNA was > 500ng/mL TRAIL (Figure 3.4A). Immunoblots of the gp78 protein show efficient knockdown by the gp78-targeting siRNA pool compared to the non-targeting siRNA did not knock down gp78 (Figure 3.4B). These findings indicate that gp78 knockdown sensitized MB231 to TRAIL-induced decrease in viability.

To confirm that the effects of gp78 knockdown on TRAIL sensitivity was caspase-mediated, caspases-3/7 activity was assessed in MB231 transfected gp78 siRNA or a non-targeting siRNA in the presence of absence of TRAIL. Cells were reverse transfected as described above. Forty-eight hours post-transfection, MB231 cells were treated with 0, 10, 30, 100, and 300 ng/mL TRAIL and incubated for 2 hours. Cells were then incubated with Caspase 3/7 Glo (Promega; Madison, WI) reagent to evaluate caspases-3/7 activity. Cells undergoing caspase-mediated cell death were predicted to show higher levels of caspases-3/7 activity, which become activated when TRAIL binds to its receptor. There was a TRAIL dose dependent increase in caspase activity in the gp78 knockdown cells and the control cells. However gp78 knockdown resulted in significantly higher level of caspases-3/7 activity compared to cells treated with the non-targeting siRNA (Figure 3.5A). Immunoblots of the gp78 protein show efficient knockdown by the gp78-targeting siRNAs compared to the non-targeting siRNA (Figure 3.5B). These findings indicate that gp78 knockdown sensitized MB231 to TRAIL-induced caspase activation.

In order to verify that knockdown of gp78 was specific and that subsequent sensitization to TRAIL was not the outcome of an off-target effect from the siRNA pool, each of the individual siRNAs in the gp78-targeting siRNA pool was tested separately to evaluate the effects on TRAIL-induced apoptosis. Cells were reverse transfected as described above, and after 24 hours, cells were treated with 0, 30, or 1000 ng/mL TRAIL, incubated for 2 hours, and then incubated with Caspase 3/7 Glo (Promega; Madison, WI) reagent. Interestingly, 3 of the 4 independent gp78-targeting siRNAs (siRNAs 1,3, and 4) significantly induced caspases-3/7 activity above the non-targeting siRNA in the presence of TRAIL, but one of the gp78-targeting siRNA (siRNA 2) did not (Figure 3.6A). All of the individual siRNAs including siRNA 2 efficiently reduced expression of gp78 (Figure 3.6B), These findings, while overall supportive of the original hypothesis that gp78 knockdown enhances TRAIL-induced apoptosis,

raise concerns due to the lack of biological effect produced in the siRNA 2-treated sample despite efficient knockdown of the protein.

To further confirm the specificity of knockdown of gp78 on TRAIL mediated apoptosis, we used an independent siRNA pool from Dharmacon and created stable cell lines expressing a shRNA for gp78. The stably transfected MB231 cells were then transfected with wild type shRNA resistant gp78 to reconstitute gp78. This latter experiment was the most rigorous test of the specificity of gp78 knockdown on TRAIL-mediated apoptosis.

Aim 2: Elucidate the Functional Relationship between gp78 and TRAIL-induced Apoptosis

The gp78 protein consists of five transmembrane domains; a really interesting new gene (RING) domain that confers E3 catalytic activity; a coupling of ubiquitin conjugation to ER degradation (CUE) domain that binds to ubiquitin; an ubiquitin-conjugating enzyme E2G 2 (Ube2g2) binding region (G2BR) domain that interacts with Ube2G2; and a valosin interacting motif (VIM) domain that interacts with the valosin containing protein (p97) AAA ATP-ase to facilitate retrotranslocation of substrates across the ER membrane (Chen et al., 2012). To determine the functional relationship between gp78 and TRAIL-induced apoptosis, MB231 cells stable clones expressing a gp78-targeting shRNA were generated (see above). The stably transfected MB231 cells were then transfected with wild type gp78 and RING finger domain mutant-encoding plasmids with silent mutations that confer resistance to the gp78 targeting shRNA (Chen et al., 2006). Cell viability, caspases-3/7 activity, and effects on components of the TRAIL pathway were then planned to be assessed to determine the effects of gp78 on TRAIL-induced apoptosis. These experiments will also allow us to determine the structural domains of gp78 necessary for the regulation of TRAIL-mediated apoptosis. All plasmids described in this aim were a generous gift from Allan Weissman, Frederick, MD (Chen et al., 2006). Unfortunately, the stably knocked-down cells failed to reconstitute and will need to be re-attempted at a later time.

To further elucidate the mechanism by which gp78 regulates TRAIL-mediated pathway we will explore the effects of gp78 loss on TRAIL pathway component expression and on ER stress. Since gp78 is an ubiquitin ligase which can mediated ubiquitination and degradation of proteins, it is conceivable that TRAIL pathway proteins are direct targets of gp78. The protein levels of the TRAIL receptors were assessed in cells with gp78 knockdown compared to controls. The ER stress response has also been implicated in sensitizing cancer cells, including ovarian and gastric cancer cells, to TRAIL-induced apoptosis. ER stress results in transcriptional upregulation of TRAIL-R2 by CCAAT/enhancer-binding protein homologous protein (CHOP), an ER stress-induced transcription factor (He et al., 2013; Moon et al., 2013; Tian et al., 2011). Inhibition of gp78 activity may enhance the ER stress response, potentially by disrupting the ubiquitination and retrotranslocation of substrates. Increased ER stress may prompt CHOP-mediated TRAIL-R2 expression or other ER stress-related events that enhance TRAIL-induced apoptosis. To assess the effects knockdown of gp78 on ER stress expression of markers of ER stress, including CHOP, caspase 2, X-box binding protein 1 (XBP1), and eukaryotic translation initiation factor 2-alpha kinase 3 (PERK), were measured by quantitative real-time polymerase chain reaction in MB231 cells with and without gp78 knockdown in the presence and absence of TRAIL.

Figure 3.1

Negative Regulators of the TRAIL Pathway

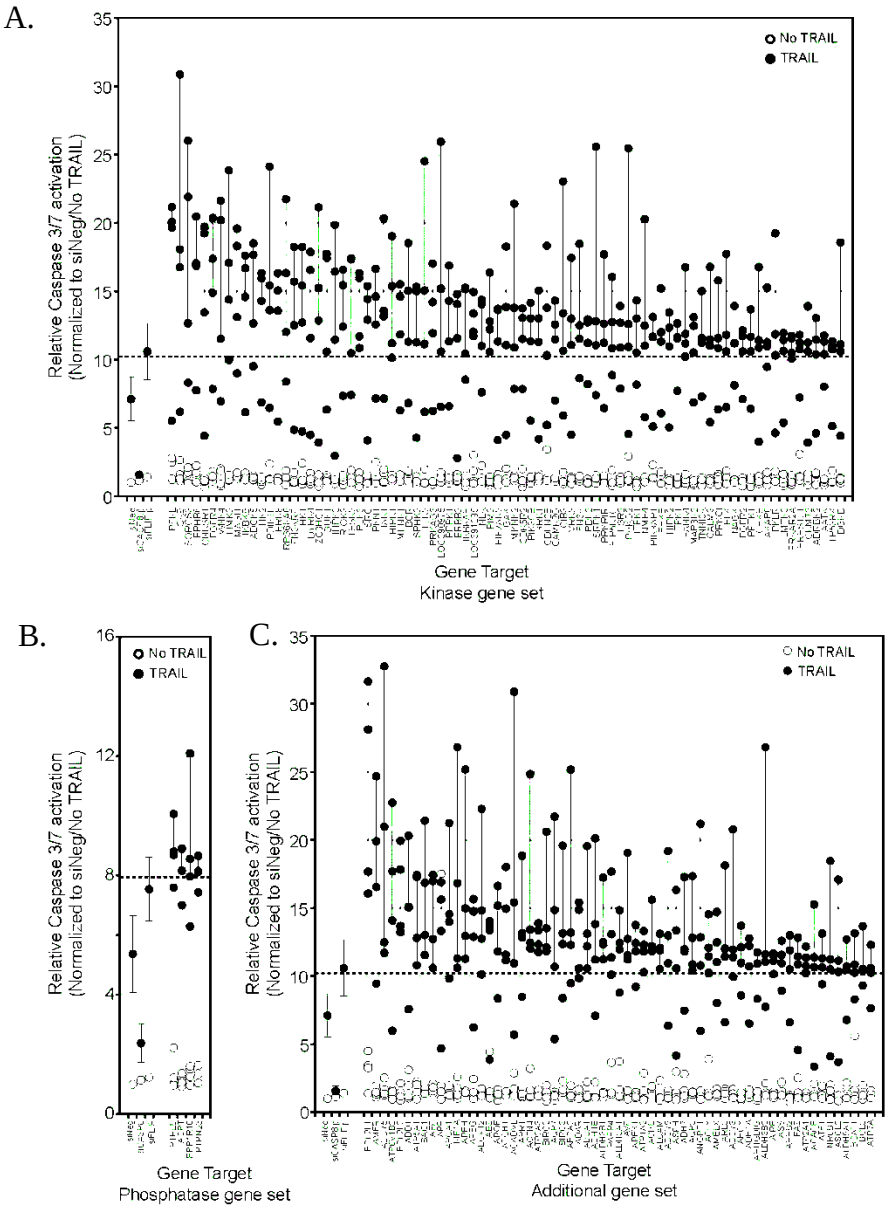


Figure 3.1. A. Caspases-3/7 activity in significant negative regulators from the kinome.*B.* Caspases-3/7 activity in significant negative regulators from the phosphatome. *C.* Caspases-3/7 activity in significant negative regulators from non-kinome and phosphatome-associated genes (Garimella et al., 2014).

Figure 3.2

Pathway Analysis of Regulators of TRAIL-induced Apoptosis

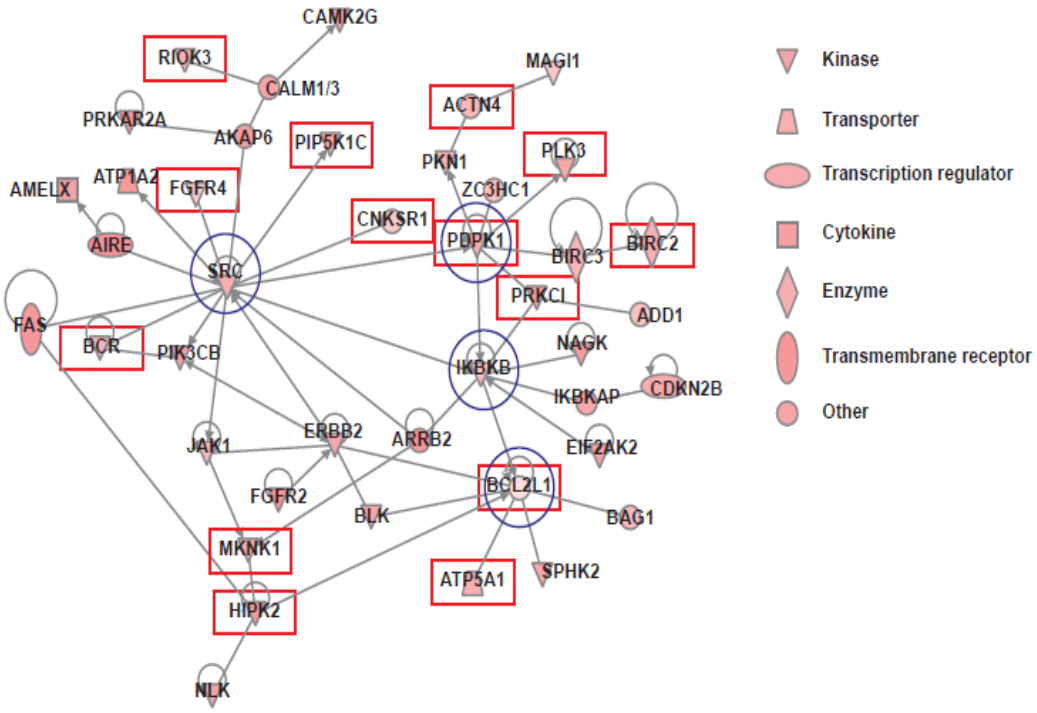
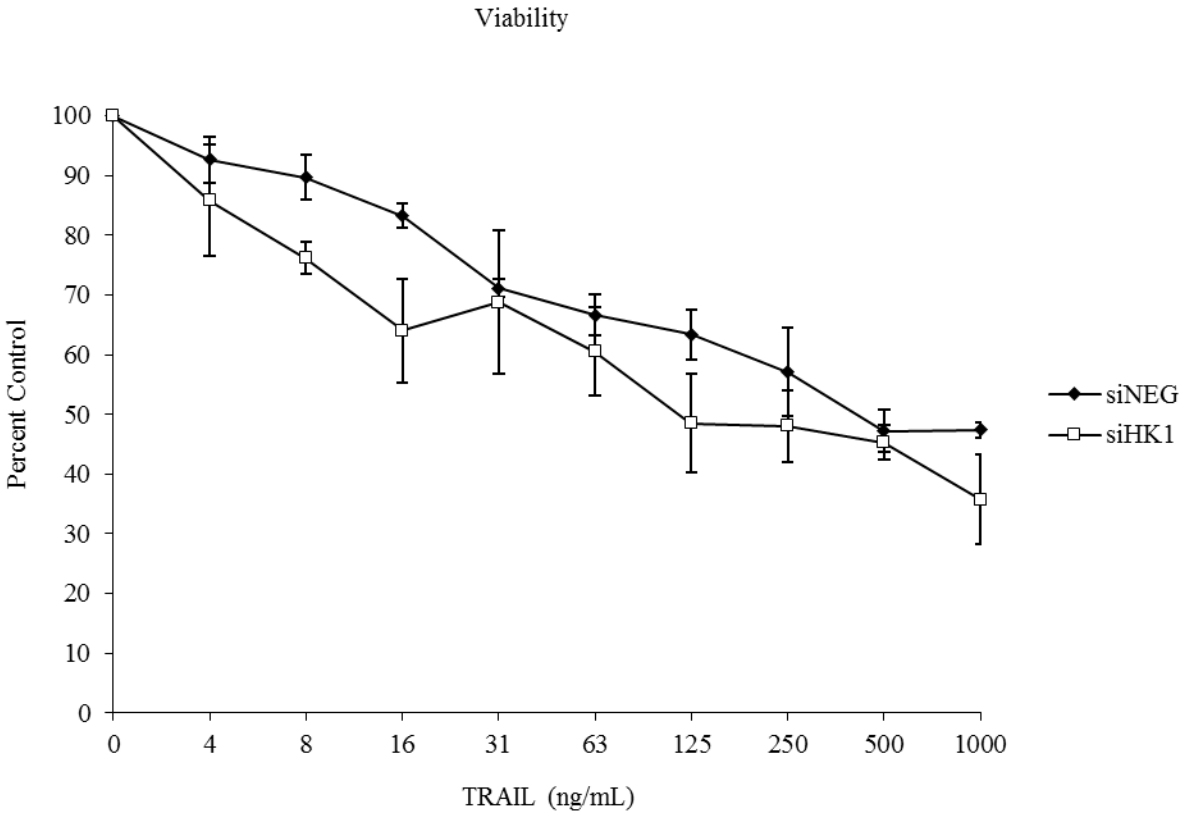


Figure 3.2. Gene interaction networks of significant candidates identified in the high-throughput screen (Garimella et al., 2014).

Figure 3.3

HK1 Knockdown by siRNA did not enhance TRAIL-induced Apoptosis

A.



B.

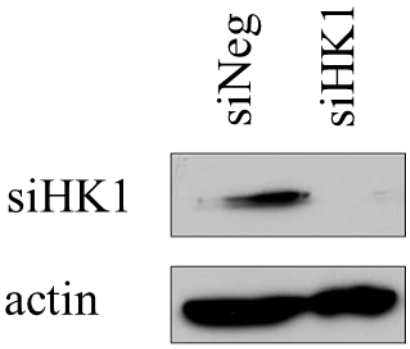
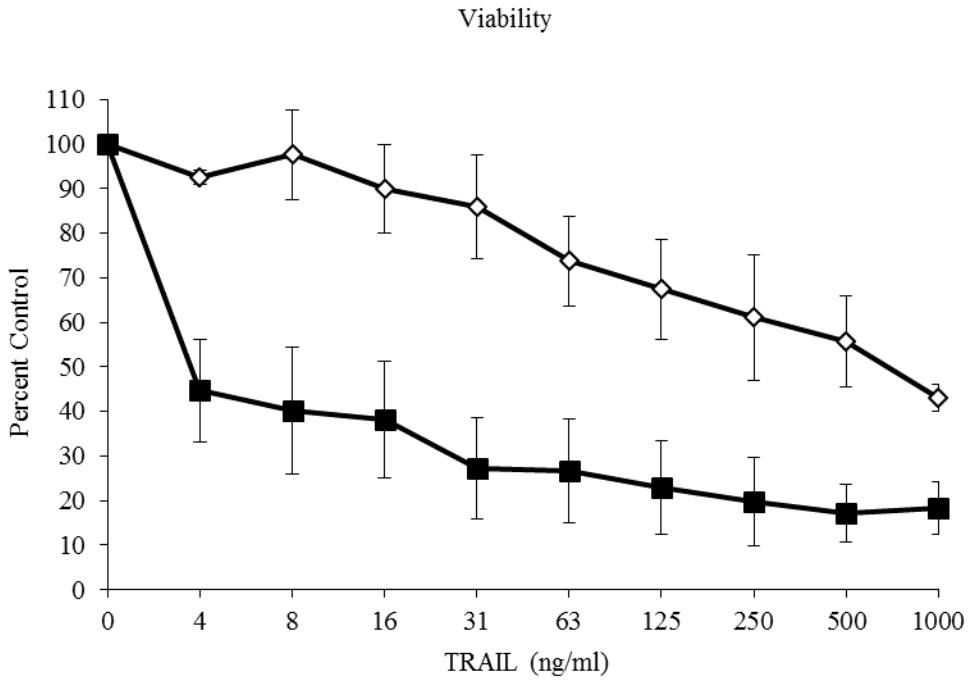


Figure 3.3. A. Viability curve of MB231 cells reverse transfected with HK1-targeted siRNA or non-targeting siRNA and treated with TRAIL at the indicated concentrations. The results represent the mean +/- SE for 2 experiments. *B.* Immunoblot of protein harvested from MB231 cells reverse transfected with HK1-targeted siRNA or non-targeting siRNA.

Figure 3.4

gp78 Knockdown Enhances TRAIL-induced Apoptosis

A.



B.

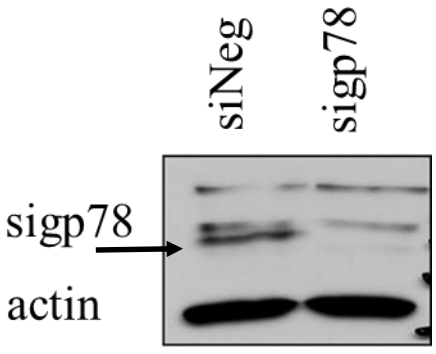
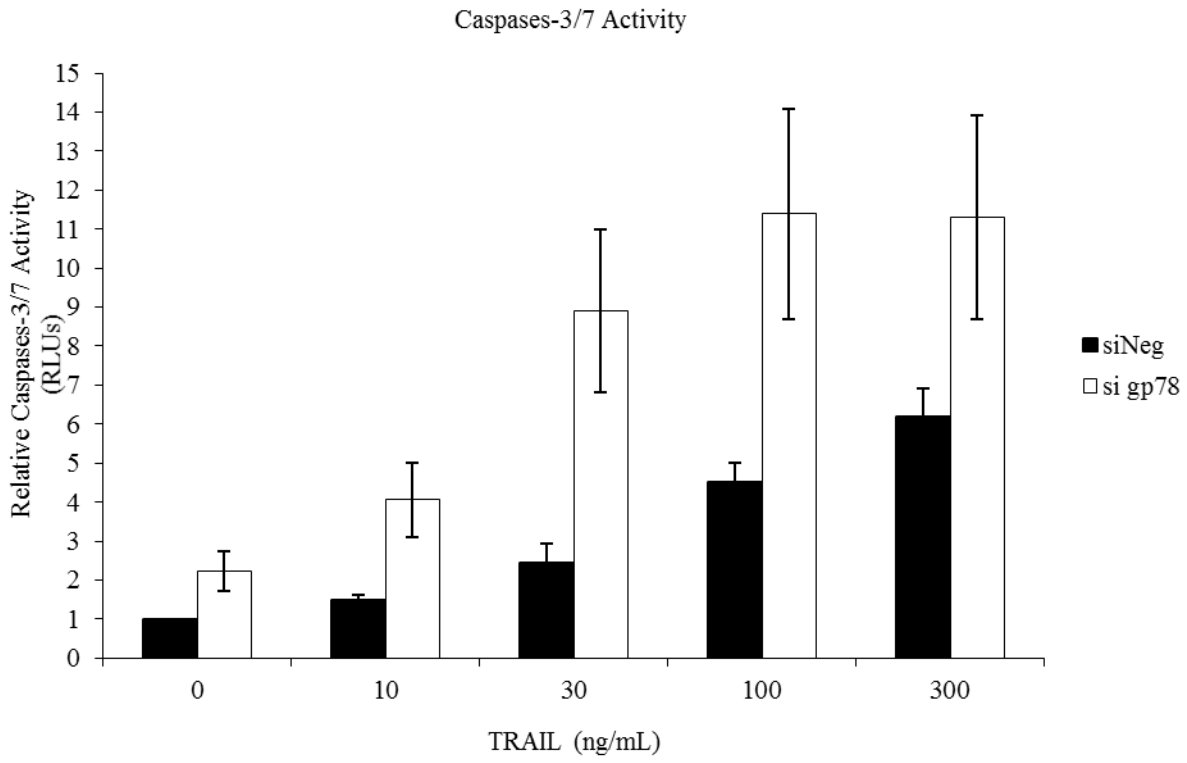


Figure 3.4. A. Viability curve of MB231 cells reverse transfected with gp78-targeted siRNA or non-targeting siRNA and treated with TRAIL at the indicated concentrations. The results represent the mean +/- SE for 3 experiments. *B.* Immunoblot of protein harvested from MB231 cells reverse transfected with gp78-targeted siRNA or non-targeting siRNA.

Figure 3.5

gp78 Knockdown Enhances TRAIL-induced Caspases-3/7 Activity

A.



B.

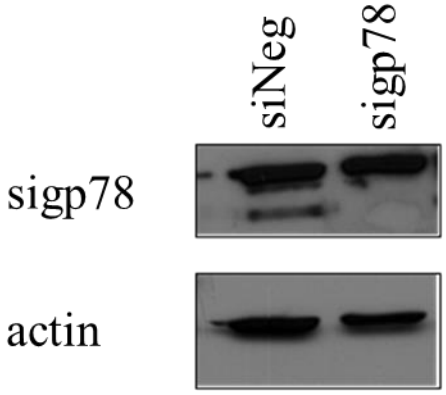
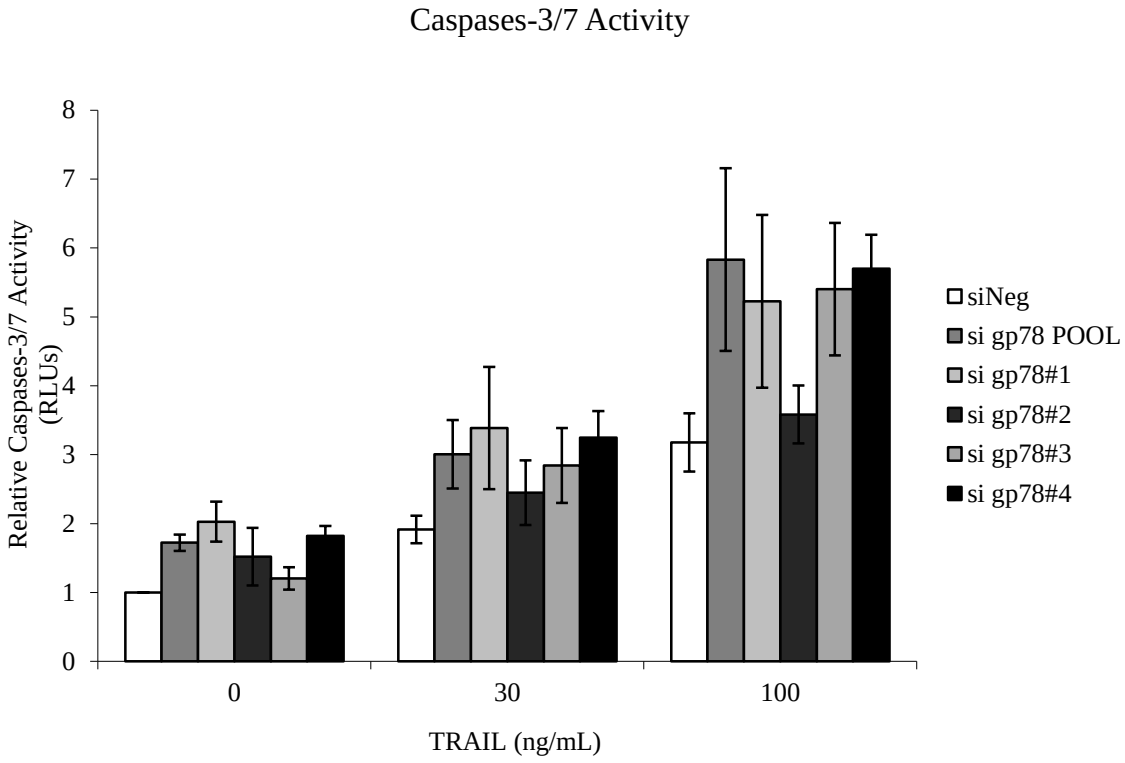


Figure 3.5. A. Caspases-3/7 activity of MB231 cells reverse transfected with gp78-targeted siRNA or non-targeting siRNA and treated with TRAIL at the indicated concentrations. The results represent the mean +/- SE for 3 experiments. *B.* Immunoblot of protein harvested from MB231 cells reverse transfected with gp78-targeted siRNA or non-targeting siRNA.

Figure 3.6

gp78 Knockdown by 3 of 4 Independent siRNAs enhances TRAIL-induced Caspases-3/7 Activity

A.



B.

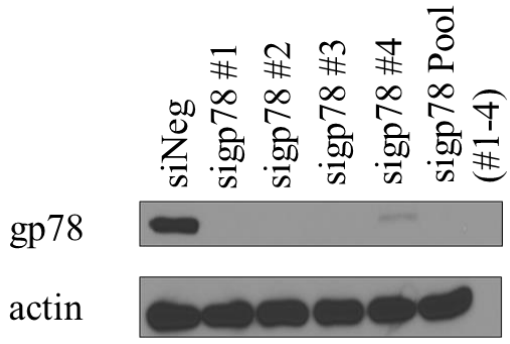


Figure 3.6. A. Caspases-3/7 activity of MB231 cells reverse transfected with individual and pooled gp78 targeting siRNAs or non-targeting siRNA and treated TRAIL at the indicated concentrations. The results represent the mean +/- SE for 3 experiments. *B.* Immunoblot of protein harvested from MB231 cells reverse transfected with gp78 targeting siRNA or non-targeting siRNA.

CHAPTER FOUR

GP78 NEGATIVELY REGULATES TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

Dine, J.L., & Lipkowitz, S. (2015). gp78 negatively regulates TRAIL-induced apoptosis in breast cancer cells. Manuscript in preparation.

Abstract

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a type II transmembrane protein found to selectively induce apoptosis in cancer cells. TRAIL activates a caspase-driven cell death program by binding to and activating its cognate cell surface receptors, death receptor 4 and death receptor 5. In light of the specificity for inducing apoptosis in cancer cells, TRAIL pathway activating agents have been widely explored in clinical trials. Although TRAIL pathway activating agents have been well tolerated, they have been only modestly effective in humans for reasons that have been poorly characterized. Understanding regulators of sensitivity to TRAIL may provide insight into identifying mechanisms to help sensitize cancer cells to TRAIL, aid in the selection of combinatorial therapies to enhance TRAIL effectiveness, and identify patients who may benefit the most from a TRAIL pathway activating agent. Previously, our laboratory identified the endoplasmic reticulum-associated protein degradation (ERAD) pathway facilitator and ubiquitin ligase autocrine motility factor receptor (gp78) as a potential negative regulator of TRAIL-induced apoptosis. Here, we further characterize gp78 as a negative regulator of TRAIL sensitivity in the triple negative breast cancer cell line MB231. gp78 knockdown sensitizes MB231 to TRAIL-induced

caspases-3/7 activity and loss in viability. Previous work has demonstrated that activation of the unfolded protein response (UPR) causes DR5 upregulation (and subsequent TRAIL sensitization) through a CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP)-mediated mechanism. In light of the interrelatedness of ERAD and UPR pathways, the CHOP signaling axis was initially interrogated but was not found to play a role in gp78 knockdown-related TRAIL-induced caspases-3/7 activity and loss in viability. Thus, these findings demonstrate a novel regulator of TRAIL sensitivity in MB231. Further investigation is warranted to clarify the mechanisms governing gp78-associated TRAIL resistance.

GP78 NEGATIVELY REGULATES TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is a cytokine found to activate apoptosis by binding to its cognate cell surface receptors, death receptor 4 (DR4) and death receptor 5 (DR5) (Ashkenazi, 2008). Adaptor proteins and the initiator caspase, caspase-8, are recruited, and the apoptotic program is facilitated. TRAIL selectively induces apoptosis in cancer cells in a non-tumor protein 53 (p53) dependent manner *in vitro* and *in vivo* (Gonzalvez & Ashkenazi, 2010). In light of the high frequency of p53 mutation in cancer (Gurpinar & Vousden, 2015), identifying cancer cell-specific inducers of apoptosis that are not reliant on canonical p53 function have been widely investigated, including DR4 and DR5 agonists (Ashkenazi, 2008; Gonzalvez & Ashkenazi, 2010). Although numerous DR4 and DR5 agonists have been tested in clinical trials and were well tolerated, modest effectiveness was observed in patients (Holland, 2014; Lemke, von Karstedt, Zinngrebe, & Walczak, 2014). Unfortunately, the reasons contributing to the observed modest effectiveness in humans have so far been poorly characterized. Better understanding of the regulatory mechanisms that govern sensitivity to TRAIL may provide crucial insights into the molecular underpinnings that may be addressed to sensitize cancer cells in humans to DR4 and DR5 agonists.

Previously, our laboratory determined triple negative breast cancer (TNBC) cells with a mesenchymal phenotype were especially sensitive to TRAIL-induced apoptosis while cell lines representative of the other subtypes of breast cancer were resistant (Rahman, Davis, et al., 2009; Rahman, Pumphrey, & Lipkowitz, 2009). TNBC derives its

name from the absence of three molecular markers (estrogen receptor and progesterone receptor expression and human epidermal growth factor receptor 2, or HER2, amplification) that are used to classify the other subtypes of breast cancer (Brenton, Carey, Ahmed, & Caldas, 2005). The standard of care for TNBC is chemotherapy, and, unlike the other breast cancer subtypes, TNBC lacks targeted therapies (Perou, 2011). The development of targeted therapies for TNBC is warranted because of its aggressive presentation and poor survival outcomes, especially in cases of resistance or metastasis (Carey et al., 2006; Millikan et al., 2008; Morris et al., 2007).

In light of the exquisite sensitivity of mesenchymal TNBC cells to TRAIL (Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009), our laboratory previously carried out a high-throughput siRNA-mediated screen of the kinome, phosophatome, and other targets in the TRAIL sensitive, mesenchymal TNBC cell line MB231 to attempt to identify novel positive and negative regulators of TRAIL-induced apoptosis (Garimella et al., 2014). The ubiquitin ligase gp78 (*a.k.a.* AMFR) was identified among the 150 negative regulators that were characterized from the screen. First discovered in murine B16-F1 melanoma cells (Silletti, Watanabe, Hogan, Nabi, & Raz, 1991), gp78 ubiquitinates and facilitates the retrotranslocation of substrates from the inner lumen of the endoplasmic reticulum (ER) to the cytosol for 26S proteasomal degradation as part of the ER-associated protein degradation (ERAD) pathway (Chen et al., 2006; Fang et al., 2001).

ERAD is a critical quality control mechanism that eliminates misfolded proteins and helps maintain cellular homeostasis (Ruggiano, Foresti, & Carvalho, 2014). Substrates of gp78 include cytochrome P450 CA4 (Kim, Acharya, Engel, & Correia,

2010), prion protein PrP (Shao et al., 2014), and mutant huntingtin (Yang et al., 2010), among others, and reflects its role in regulating diverse cellular functions (Chen, Du, & Fang, 2012). gp78 has also been linked to promoting metastasis by targeting the cluster of differentiation 82 (KAI1) metastasis suppressor protein for degradation in a mouse model of sarcoma (Tsai et al., 2007). In the same study, transient knockdown of gp78 resulted in the restoration of KAI1, which led to enhanced caspase activity in an experimental metastasis model. This suggests that gp78 may play a role in promoting metastasis by inhibiting caspase activation induced by KAI1. However, gp78 inhibition was not associated with a decrease in primary tumor burden in mice. Our observations from the siRNA screen (Garimella et al., 2014) extended these findings in the context of breast cancer cells and warranted additional investigation.

Interestingly, TRAIL sensitivity may be enhanced, in part, by upregulation of DR5 through activity of the unfolded protein response (UPR)-associated transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP) (Martin-Perez et al., 2014; Pennati et al., 2015; Shiraishi et al., 2005; Yamaguchi & Wang, 2004). The UPR and ERAD pathways are closely related; when perturbations in normal cellular function lead to the accumulation of unfolded or misfolded proteins in the ER (a state termed "ER stress"), the UPR is activated to help the cell re-establish homeostasis or, in situations where the cell cannot recover, facilitate apoptosis (Hetz, Chevet, & Harding, 2013). As a component of the ERAD machinery, we hypothesized loss of gp78 may play a role in inducing ER stress and promoting activation of the UPR, thereby sensitizing cells to TRAIL-induced caspases-3/7 activity and loss in viability. Consequently, the UPR – specifically, CHOP – signaling axis and DR4 and DR5 expression levels were

first interrogated in light of the established relationship between ER stress, the UPR, and TRAIL sensitivity. Surprisingly, gp78 inhibition was not associated with activation of the UPR or enhancement in DR4 and DR5 expression, despite sensitizing MB231 cells to TRAIL-induced caspases-3/7 activity and loss in viability.

The purpose of this study was to further elucidate the relationship between gp78 and TRAIL resistance in the mesenchymal TNBC cell line MB231. gp78 knockdown-related TRAIL sensitivity was determined to be caspase-mediated but not dependent on UPR signaling. Further analysis is needed to clarify the functional relationship between gp78 and sensitivity to TRAIL.

Materials and Methods

Cell Culture

The MB231 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The cell line was grown in media consisting of RPMI 1640 plus 10% fetal calf serum, 100 units/ml of penicillin, and 100 units/ml of streptomycin.

siRNA Transfection

MB231 cells were plated in T-75 flasks 24-48 hours prior to transfection. Twenty-five µl Lipofectamine RNAiMax Transfection Reagent (Cat #13778150, Invitrogen, Grand Island, NY) and 50 µl from a 10 µM stock of non-targeting siRNA, siRNA targeting gp78, or seed control siRNA (designed to evaluate potential off-target effects associated with Qiagen siRNA #1) (Table 1), and Optimem Reduced Serum media were combined in a total volume of 5 mL and allowed to incubate for 20 minutes at room temperature. Five mL of MB231 cells at a stock concentration of $2x10^5$ cells/mL in RPMI1640 plus 10% fetal calf serum were then combined with the transfection reagent

and siRNA mix for a final volume of 10 mL and final siRNA concentration of 50 nM. Cells were then plated at $1x10^5$ cells/mL in a final volume of 3mL in 35 mm dishes and 50 µl per well in 96-well dishes. Twenty-four hours after transfection, the cells were ready for evaluation of caspase activation, viability, and protein expression as indicated in the figures.

Plasmid and Generation of MB231 Stably Expressing +/- shRNA Targeting gp78

The gp78-targeting shRNA (sequence AATGCACACCTTGGCTTTCAT) encoded in the p.Super Puro (Oligoengine, Seattle, WA) plasmid was previously described (generously provided by Allan Weissman) (Fang et al., 2001). Using Lipofectamine 2000 Transfection Reagent (Cat # 11668-019 Life Technologies, Grand Island, NY), cells were reverse transfected with the transfection reagent alone, empty vector, or plasmid expressing the gp78 shRNA. Forty-eight hours after transfection, the old media was removed, and 10 mL of fresh base media plus 2 µg/mL puromycin (Cat # A1113803, Life Technologies, Grand Island, NY) were added. When all non-DNA transfected cells were dead, the cells transfected with DNA were monitored for colony growth. Colonies were subsequently isolated using cloning discs (Cat # Z374431-100EA, Sigma, St. Louis, MO) and expanded and maintained in the base media plus 2 µg/mL puromycin.

Caspases-3/7 Activity

Cells were treated with GST-TRAIL, and caspases-3/7 activity was assessed using the Caspase-Glo 3/7 assay system (Cat # G80943, Promega Corporation, Madison, WI) as previously described (Murrow, Garimella, Jones, Caplen, & Lipkowitz, 2010). Caspases-3/7 activity was evaluated in relative light units (RLUs), and the data were

normalized to untreated cells. Three independent experiments normalized to control +/- SE were performed. A two-tailed paired Student's t-test was utilized to evaluate statistical significance between non-targeting siRNA-transfected MB232 cells and gp78-targeting siRNA-transfected MB231.

Viability Assay

Five thousand cells per well were plated in 96 well plates and then treated under the experimental conditions described in the body of the text. Viability was subsequently determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Cat # G3582, Promega Corporation, Madison, WI) as previously described (Rahman, Davis, et al., 2009). At least three independent experiments were carried out and included six replicates per experiment. Results are provided as the mean +/- SE of at least three independent experiments.

Caspase Inhibitor Experiments

Z-VAD-FMK, a pan-caspase inhibitor (Cat # P416, Biomol International, Plymouth Meeting, PA), was reconstituted in DMSO. Cells were plated overnight at 5000 cells per well in 96 well plates, treated with 50 μM final concentration ZVAD-FMK or an equivalent volume of DMSO for one hour, and then treated with GST-TRAIL for 2 hours if assessing caspases-3/7 activity or overnight if assessing viability.

Transcript Quantitation

RNA was isolated from cells following the directions included in the RNeasy kit (Cat # 74104, Qiagen, Valencia, CA). cDNA was synthesized following the directions included in the RT^2 First Strand kit (Cat # 330401, Qiagen, Valencia, CA). Quantitative polymerase chain reaction (qPCR) was used to assess the relative expression levels of

transcriptional targets. Samples were prepared for analysis on an ABI 7900HT (384 well standard block) Fast Real Time PCR System (Life Technologies, Grand Island, NY) using the RT^2 SYBR ROX qPCR Mastermix with included instructions. The RT^2 Profiler PCR Array Human Unfolded Protein Response (Cat # PAHS-089Z, Qiagen, Valencia, CA) was used to assess the differential expression of UPR-associated target genes across experimental conditions. C_T values were normalized to the internal GAPDH control. The relative expression levels of target transcripts were evaluated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Lysate Preparation and Immunoblotting

Cell lysate preparation and immunoblotting were performed as previously described (Rahman, Davis, et al., 2009). The rabbit polyclonal antibodies DR4 (Cat # GTX28414, GeneTex, Irvine, CA), DR5 (Cat # 2019, ProSci, Poway, CA), gp78 (Cat # 8302-889A, Bethyl Laboratories, Inc., Montgomery, TX) and mouse monoclonal antibodies to actin (Cat # A5316-.2ML, Sigma, St. Louis, MO) and HSP70 (Cat # 7298, Santa Cruz Biotechnology, Dallas, TX) were used for immunoblotting. The affinity purified gp78 rabbit antibody used in Figure 4.2A was generously provided by Allan Weissman.

DR5 Cell Surface Expression

Cells were plated overnight and harvested to assess DR5 cell-surface expression by flow cytometry using reagents and equipment previously described (Garimella, Rocca, & Lipkowitz, 2012). Three independent experiments were carried out +/- SE. A two-tailed paired Student's t-test was utilized to evaluate statistical significance.

Sub-G1 Analysis

MB231 cells with and without gp78 stable knockdown were plated overnight and subsequently treated +/- 63 ng/ml GST-TRAIL. Cell harvesting, propidium iodide (Cat # P4864-10ML, Sigma, St. Louis, MO) staining, flow cytometry method, and data analysis technique were described previously (Garimella et al., 2012). Three independent experiments +/- SE were performed.

Results

gp78 Knockdown Sensitizes MB231 to TRAIL

In order to further investigate our initial observations from the high-throughput siRNA-mediated screen (Garimella et al., 2014), gp78 was knocked down in MB231 using a pool of the siRNAs used in the screen and then evaluated for TRAIL-induced caspases-3/7 activity (Figure 4.1A) and loss in viability (Figure 4.1B). gp78 knockdown enhanced TRAIL-induced caspases-3/7 activity by ~2 fold relative to the siRNA control. Moreover, the IC50 with respect to cell viability was reached with ~4 ng/mL TRAIL when gp78 was knocked down, whereas the IC50 was approached with ~1000 ng/mL TRAIL treatment in the control siRNA-treated cells. Thus, gp78 knockdown sensitized MB231 to TRAIL-induced loss in viability by ~250 fold. These findings demonstrated that gp78 knockdown dramatically increased the potency of TRAIL-induced apoptosis in MB231 and lent support to our initial observations from the high-throughput screen.

However, off-target siRNA effects are a widely recognized problem in which a non-specific target of the siRNA contributes to the phenotype (Sledz & Williams, 2004; Stephan, 2014). In order to evaluate the possibility of off-target siRNA effects contributing to the observed phenotype of the pooled siRNAs (Table 1A), gp78 protein

expression and caspases-3/7 activity were assessed using each of the individual siRNAs that make up the siRNA pool (Figure 4.2A). The pooled siRNAs were included as a positive control. Relative to the non-targeting siRNA control-treated sample, gp78 protein knockdown was apparent with all gp78-targeting siRNA treatments. siRNA #4 was associated with less efficient gp78 knockdown than any of the other gp78-targeting siRNAs, but siRNA #2 was the only siRNA that did not sensitize cells to TRAIL-induced caspases-3/7 activity. These results raised immediate concern regarding the possibility of an off-target siRNA effect and warranted additional investigation.

A C911 seed control siRNA designed to address potential off-target effects by Qiagen siRNA #1 was utilized (Table 1A). C911 seed controls are siRNAs that preserve the seed region of a target siRNA sequence (bases 2-8 on the 5' end of the siRNA loaded into the RNA induced signaling complex [RISC]) but lack the critical sequences necessary to cleave the specific mRNA (Buehler, Chen, & Martin, 2012). Thus, to retain the guide (bases 2-8) and passenger strand (bases 12-17) sequences of the siRNA and eliminate on-target function while maintaining off-target function, the C911 siRNA control preserves the sequence of the siRNA in question, except for bases 9-11. The bases of the original target siRNA sequence are replaced with their complementary bases. TRAIL-induced caspases-3/7 activity and gp78 protein levels were assessed in cells that were transfected with the non-targeting siRNA control, gp78-targeting siRNA #1, or the C911 seed control designed to assess off-target function associated with siRNA #1 (Figure 4.2B). Unfortunately, the C911 is not failsafe and may preserve the on-target function (Buehler et al., 2012). The results demonstrate that the C911 control was indeed able to maintain on-target function by knocking down gp78 protein and sensitizing cells

to TRAIL-induced caspases-3/7 activity by >2 fold. These findings are inconclusive regarding the identification of a potential off-target effect associated with a gp78-targeting siRNA.

Qiagen siRNAs #1-4 all target the coding sequence of gp78. To further confirm the specificity of the knockdown, we designed two new siRNAs (Qiagen siRNAs #5 and #6) that targeted the 3' untranslated region of gp78 (Table 1A). Qiagen siRNA #1 was included in the experiment as a positive knockdown control (Figure 4.2C). All targeting siRNAs were able to inhibit gp78 protein expression, but siRNAs #1 and #6 were the most efficient and appeared to have knocked down gp78 to a comparable degree. Cells transfected with siRNAs #1 and #6 were also the most sensitized to TRAIL-induced caspases-3/7 activity, while cells transfected with siRNA #5 were more sensitized to TRAIL-induced caspases-3/7 activity than the control but less so than siRNA #1 and #6-transfected cells. These findings corroborate each other and support the hypothesis that gp78 knockdown sensitizes cells to TRAIL-induced caspases-3/7 activity, potentially in a dose-dependent manner.

A Dharmacon set of siRNAs targeting gp78 was also utilized to characterize potential off-target effects (Table 1B). Caspases-3/7 activity and gp78 protein expression were again interrogated with and without gp78 knockdown by each individual siRNA and by a pool of the siRNAs (Figure 4.2D). Like the Qiagen siRNAs, each targeting siRNA was able to reduce gp78 protein expression. siRNAs #7 and #8 were most efficient in knocking down gp78 protein. siRNA #6 was the least efficient. In inspecting TRAIL-induced caspases-3/7 activity, cells transfected with siRNAs #5 and #6 were less sensitive to TRAIL than the control, while siRNAs #7 and #8 sensitized cells to TRAIL.

siRNA #2 from the Qiagen siRNA set also shares a similar sequence homology with Dharmacon siRNA #5 and likewise did not sensitize MB231 to TRAIL. However, Dharmacon siRNAs #5 and #6 mediated less knockdown of gp78 than siRNAs #7 and #8. These findings suggest that a threshold in gp78 protein reduction must be met in order sensitize cells to TRAIL-induced caspases-3/7 activity.

MB231 stably transfected with a puromycin-resistant empty vector or plasmid encoded with a gp78-targeting shRNA were generated to evaluate the effects of chronic gp78 reduction on TRAIL sensitivity. gp78 protein expression and TRAIL-induced caspases-3/7 activity (Figure 4.3A) and loss in cell viability (Figure 4.3B) were evaluated. Apoptosis was assessed by subG1 DNA content. Stable gp78 knockdown was able to increase TRAIL-induced caspases-3/7 activity and apoptosis relative to the empty vector control. This further supports the role of gp78 as an inhibitor of TRAIL-mediated apoptosis

In summary, 7 of 10 gp78-targeting siRNAs, the C911 seed control, and the stable knockdown of gp78 with shRNA resulted in concomitant gp78 knockdown and enhanced TRAIL-induced caspases-3/7 activity. Dharmacon gp78-targeting siRNA #6 did not knockdown gp78 as well as the other Dharmacon siRNAs and was unable to sensitize cells to TRAIL. Two gp78-targeting siRNAs, Qiagen siRNA #2 and Dharmacon siRNA #5, had overlapping oligonucleotide sequences, knocked down gp78, but did not enhance sensitivity to TRAIL. These findings support the observation that the loss of gp78 enhances TRAIL-induced apoptosis.

Knockdown of gp78 Sensitizes MB231 to TRAIL in a Caspase-Dependent Manner

In order to confirm that knockdown of gp78 sensitizes cells to TRAIL by a caspase-dependent apoptotic mechanism, TRAIL-induced caspases-3/7 activity (Figure 4.4A) and loss in viability (Figure 4.4B) were assessed in MB231 with and without gp78 knockdown in the presence of DMSO or Z-VAD-FMK, a pan-caspase inhibitor. Z-VAD-FMK abrogated TRAIL-induced caspases-3/7 activity and prevented TRAIL-induced loss of viability in both non-targeting siRNA transfected and gp78-targeting siRNA transfected cells. These findings demonstrate that gp78 knockdown sensitizes cells to TRAIL-induced apoptosis in a caspase-dependent manner.

Knockdown of gp78 Sensitizes MB231 to TRAIL in a non UPR-Dependent Manner

Several papers have shown that activation of the UPR upregulates CHOP, which in turn upregulates DR5 protein expression (Martin-Perez et al., 2014; Pennati et al., 2015; Yamaguchi & Wang, 2004). As a component of the ERAD machinery, gp78 (Chen et al., 2006; Fang et al., 2001) knockdown was hypothesized to sensitize cells to TRAIL-induced apoptosis through a CHOP/DR5 regulatory mechanism. In order to evaluate the effects of gp78 on CHOP activity, the relative differential regulation of UPR-associated genes in MB231 cells with and without gp78 knockdown was evaluated (Figure 4.5) using a qPCR array. The results from two experiments were averaged together. Gene transcriptional regulation assessed on the array encompassed those involved in canonical UPR signaling, including activating transcription factor 6 (ATF6, gene name *ATF6*), binding immunoglobulin protein (Bip, gene name *HSP5A*), CHOP (gene name *DDIT3*), inositol-requiring enzyme 1 (IRE1α, gene name *ERN1*), and protein kinase RNA-like endoplasmic reticulum kinase (PERK, gene name *EIF2AK3*), among others, including

those implicated in non-canonical signaling. The differential transcriptional regulation of ATF6, Bip, IRE1α, and PERK is especially significant in that they are the initiating components that activate the UPR (Hetz et al., 2013), and CHOP is consistently upregulated in the context of CHOP/DR5-mediated TRAIL sensitization. gp78 (gene name *AMFR*) mRNA knockdown was dramatic (>10 fold) and was the most differentially regulated gene on the array. However, none of the other UPR-associated genes demonstrated at least 10-fold differential regulation (most showed less than a 2 fold change), which is reported wherein UPR-associated TRAIL sensitization is observed (Do et al., 2014; He et al., 2013; Martin-Perez et al., 2014). Thus, none of the other targets were explored. The differential transcriptional regulation of CHOP or any of the other UPR signaling genes was not identified with gp78 knockdown. This suggests that UPR signaling pathways are not involved in the increase in TRAIL sensitivity observed when gp78 is knocked down.

Next, DR4 and DR5 total protein levels were evaluated. Changes in total protein levels of DR4 and DR5 upon knockdown of gp78 was not observed (Figure 4.6A), suggesting that alterations in total DR4 and DR5 levels were not affecting gp78 knockdown-mediated TRAIL-induced apoptosis. However, canonical TRAIL-induced apoptotic signaling involves TRAIL activation of DR4 and DR5 on the cell surface (Ashkenazi, 2008; Pan, Ni, et al., 1997; Pan, O'Rourke, et al., 1997). Thus, DR5 cell surface expression was investigated (Figure 4.6B). Interestingly, DR5 cell surface expression levels did not differ between samples with and without gp78 knockdown, demonstrating that changes in cell surface DR5 expression was not associated with gp78 knockdown-mediated TRAIL sensitivity in MB231. Cell surface DR4 expression was not

performed due to the absence of an antibody that specifically detected surface DR4. However, previous work has demonstrated that DR5, but not DR4, mediates TRAIL-induced apoptosis in MB231 cells (Rahman, Davis, et al., 2009).

Discussion

The purpose of this study was to further characterize gp78, an ubiquitin ligase and ERAD signaling component, as a negative regulator of TRAIL-induced apoptosis in breast cancer cells. Others have found that gp78 promotes murine metastasis by targeting the tumor suppressor protein KAI1 for degradation (Tsai et al., 2007). Previously, our laboratory identified gp78 as a candidate negative regulator of TRAIL-induced apoptosis in a high-throughput siRNA-mediated screen in the mesenchymal TNBC cell line MB231 (Garimella et al., 2014). In this study, we have further characterized these findings by demonstrating that with 7 of 10 gp78-targeting siRNAs and one MB231 clone with stable knockdown of gp78, we are able to observe both protein knockdown of gp78 and enhanced sensitivity to TRAIL-induced apoptosis.

Interestingly, Qiagen siRNA #5 was able to knock down gp78 relative to the non-targeting control but was less efficient in gp78 knockdown relative to siRNA #6 and the positive knockdown control siRNA #1. TRAIL-induced aspases-3/7 activity was evaluated in parallel with the immunoblots and demonstrated that Qiagen siRNA #5 sensitized cells to TRAIL but less so that siRNAs #6 and #1. These findings indicate that gp78 knockdown-mediated TRAIL-induced caspases-3/7 activity is dose-dependent, and our laboratory has observed similar findings with respect to other negative regulators of TRAIL-induced apoptosis, including the anti-apoptotic protein BCL-XL (Garimella et al., 2014).

The Dharmacon gp78-targeting siRNAs provided additional information concerning gp78-mediated TRAIL sensitivity in breast cancer cells. Dharmacon siRNAs #5 and #6 were unable to sensitize cells to TRAIL, despite knocking down gp78 relative to the control. However, siRNAs #7 and #8 more efficiently knocked down gp78 protein and sensitized cells to TRAIL-induced caspases-3/7 activity. Therefore, in order to enhance TRAIL-induced caspases-3/7 activity via gp78, gp78 knockdown may be required to meet a threshold in protein reduction to have an effect. The cell cycle regulator WEE1 was also previously found to negatively regulate TRAIL-induced apoptosis in breast cancer cells in which a threshold in protein reduction was necessary to sensitize cells to TRAIL (Garimella et al., 2012). Additional characterization is necessary to determine the extent of gp78 knockdown required to meet the threshold for increasing TRAIL-induced apoptosis in breast cancer cells.

Concerns surrounding the potential off-target effects raised by Qiagen siRNA #2, which knocked down the protein as efficiently as the other siRNAs but did not sensitize the cells to TRAIL, may be addressed by rescue experiments to confirm that restoration of gp78 protein function protects cells from TRAIL-induced apoptosis. Additional siRNA seed controls may also be used as an orthogonal method to assess off-target siRNA effects. Nevertheless, findings associated with 7 of 10 gp78-targeting siRNAs indicate that knockdown of gp78 sensitizes MB231 to TRAIL-induced apoptosis.

The UPR is closely related to the ERAD protein quality control mechanism and can induce apoptosis (Hetz et al., 2013). Others have demonstrated that UPR-mediated upregulation of the CHOP transcription factor increases DR5 levels and thus sensitizes cells to TRAIL (Martin-Perez et al., 2014; Pennati et al., 2015; Yamaguchi & Wang,

2004). gp78 is a component of ERAD, and consequently, loss in gp78 was hypothesized to sensitize cells to TRAIL in a CHOP/DR5-dependent manner. Examination of the differential transcriptional regulation of UPR-associated genes by qPCR array, total DR4 and DR5 protein expression, and cell surface DR5 protein expression did not reveal upregulation of CHOP, DR4, or DR5 or any of the genes on the array associated with UPR signaling in cells with and without gp78 knockdown. These findings suggest that gp78 knockdown-associated TRAIL-induced apoptosis in MB231 is not UPR-dependent or associated with changes in DR4 or DR5 protein expression. Further study is warranted to determine the functional relationship between gp78 knockdown and TRAIL-induced apoptosis. Exploration of gp78-mediated TRAIL resistance in breast cancer cell lines representative of the other subtypes of breast cancer is also warranted to assess the generalizability of these results.

Conclusions

In this study, we have characterized the ERAD-associated ubiquitin ligase gp78 as a negative regulator of TRAIL-induced apoptosis in the mesenchymal TNBC cell line MB231. Knockdown of gp78 is able to sensitize MB231 to TRAIL-induced apoptosis without activation of CHOP, upregulation of total DR4 and DR5, or upregulation of cell surface DR5. These findings indicate that gp78 negatively regulates TRAIL-induced apoptosis through a novel mechanism that has not yet been identified in the scope of this study. Further work is necessary to clarify the functional relationship between gp78 knockdown and TRAIL-induced apoptosis. Understanding this mechanism may aid in the development or selection of agents to use in combination with TRAIL pathway agonists for the treatment of cancer.

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Yang, H., Liu, C., Zhong, Y., Luo, S., Monteiro, M. J., & Fang, S. (2010). Huntingtin interacts with the CUE domain of gp78 and inhibits gp78 binding to ubiquitin and p97/vcp. *PloS One, 5*(1), e8905. doi: 10.1371/journal.pone.0008905

Table 4.1

siRNA Target Sequences

A.

Qiagen Product Information	Catalog Number	Target Sequence
AllStars Negative Control siRNA	1027281	Proprietary
gp78 siRNA #1	S100022533	CACGCTCAGTTGAAATAACAA
gp78 siRNA #2	S100022540	AAGGATCGATTTGAATATCTT
gp78 siRNA #3	S100022547	TCGCAAACGTTTCTTGAACAA
gp78 siRNA #4	S100022554	CCGGCGAGCCGGACCAGCTAA
gp78 siRNA #5 (custom seq)	1027423	AAGGTGTTGACTGGACTGCTT
gp78 siRNA #6 (custom seq)	1027423	AAGGGTTTGGTCCTTGAACAA
gp78 siRNA#1 seed control (custom seq)	1027423	CACGCTCAGTACTAATAACAA

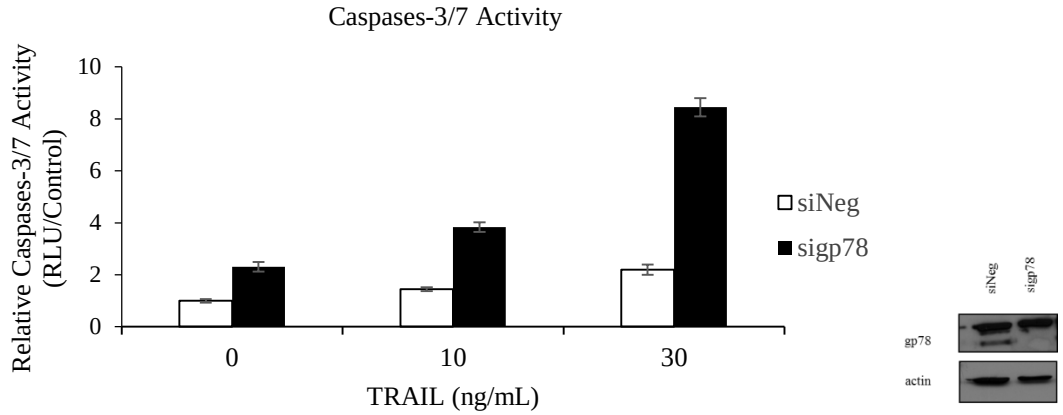
B.

Dharmacon Product Information	Catalog Number	Target Sequence
ON-TARGET Plus Non-Targeting siRNA #2	D-001810-02-05	TGGTTTACATGTTGTGTGA
gp78 siRNA #5	J-006522-05	GCAAGGATCGATTTGAATA
gp78 siRNA #6	J-006522-06	GGACGTATGTCTATTACAC
gp78 siRNA #7	J-006522-07	GAATTCGTCGGCACAAGAA
gp78 siRNA #8	J-006522-08	GTAAATACCGCTTGCTGTG

Figure 4.1

Knockdown of gp78 Sensitizes Cells to TRAIL-induced Caspases-3/7 Activity and Cell Death

A.



B.

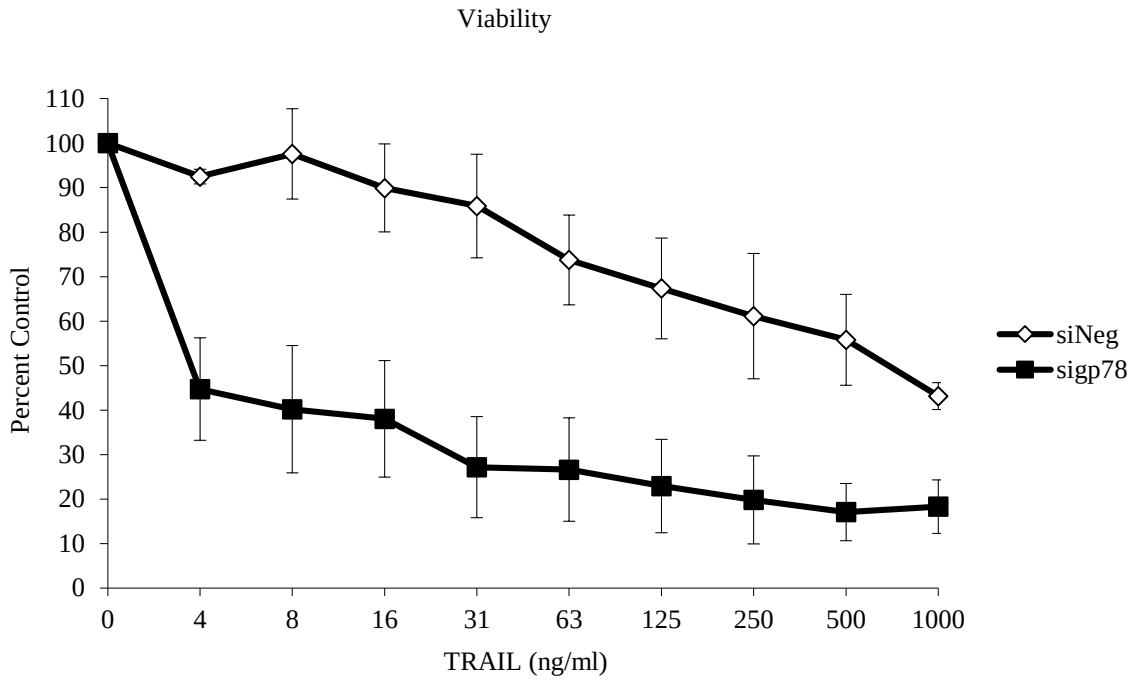


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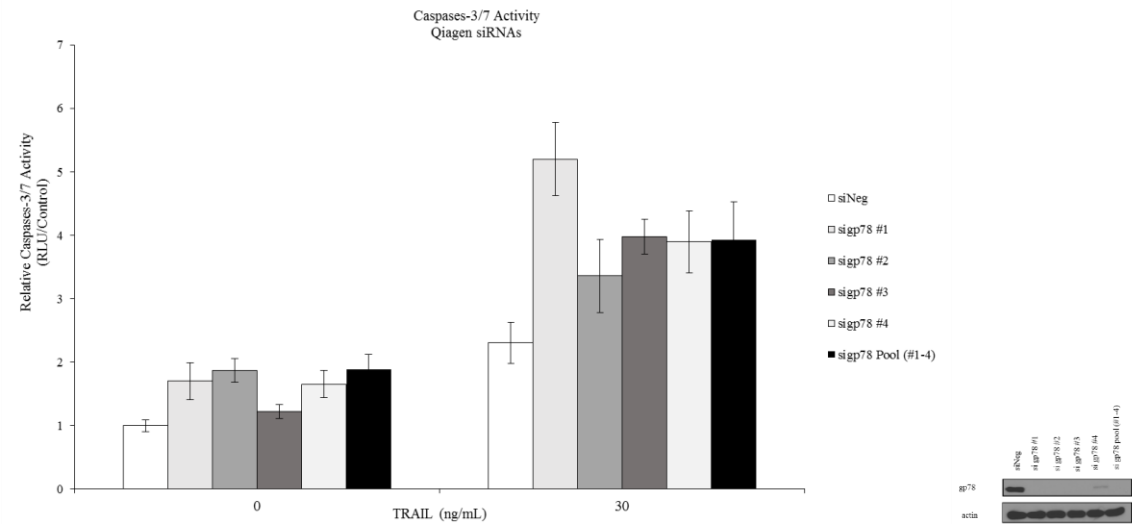
Figure 4.1. A. MB231 cells were transfected with a Qiagen non-targeting siRNA or pool of gp78-targeting siRNAs. Twenty-four hours after transfection, cells were treated with TRAIL for two hours. TRAIL-induced caspases-3/7 activity was then measured using a luminescent assay system. gp78 knockdown significantly increased TRAIL-induced caspase activation inMB231 cells relative to the TRAIL-treated control ($p \leq 0.0001$). *Inset:* Immunoblot of gp78 expression in cells with and without gp78 knockdown. Actin is used as a loading control. *B.* MB231 cells were transfected with a Qiagen non-targeting siRNA or gp78-targeting siRNA pool. Twenty-four hours after transfection, cells were treated with TRAIL for twenty-four hours. Cell viability was then measured using the MTS assay system. Experiments described in A and B were carried out in triplicate and in parallel. Three independent experiments normalized to control +/- SE were performed.

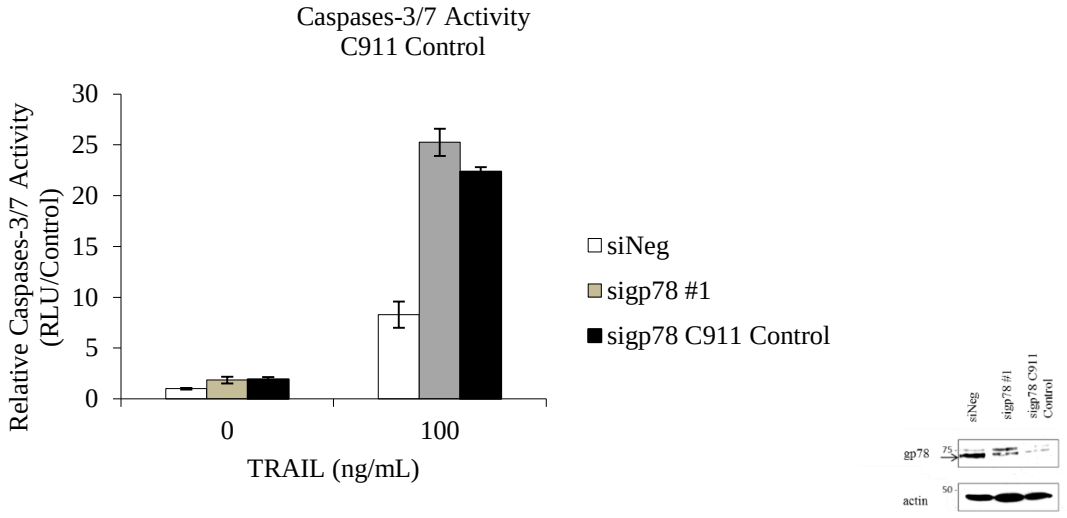
Figure 4.2

Knockdown of gp78 with Individual siRNAs Sensitizes Cells to TRAIL-induced Caspases-3/7 Activity

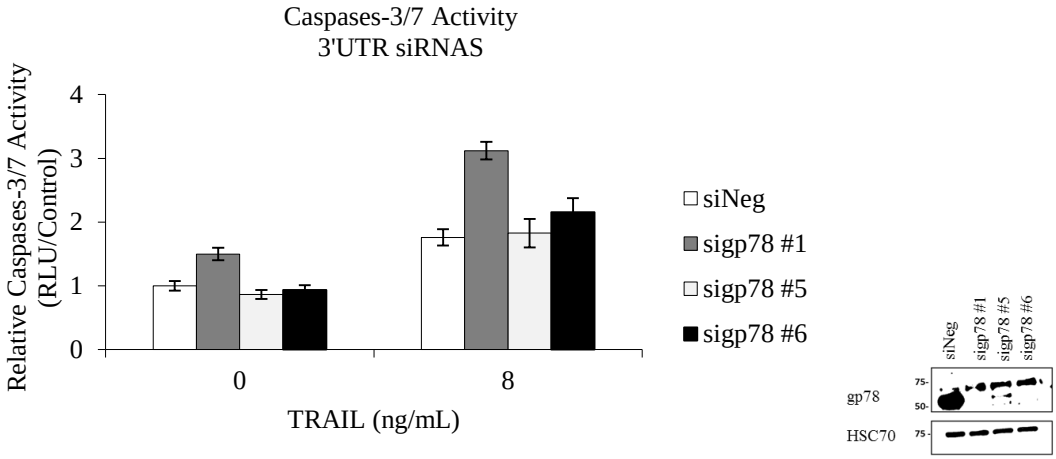
A.



B.



C.


Caspases-3/7 Activity
3'UTR siRNAS
Relative Caspases-3/7 Activity (RLU/Control)
0
1
2
3
4
0
8
TRAIL (ng/mL)
siNeg
sigp78 #1
sigp78 #5
sigp78 #6
siNeg
sigp78 #1
sigp78 #5
sigp78 #6
gp78
HSC70
75-
50-
75-

D.

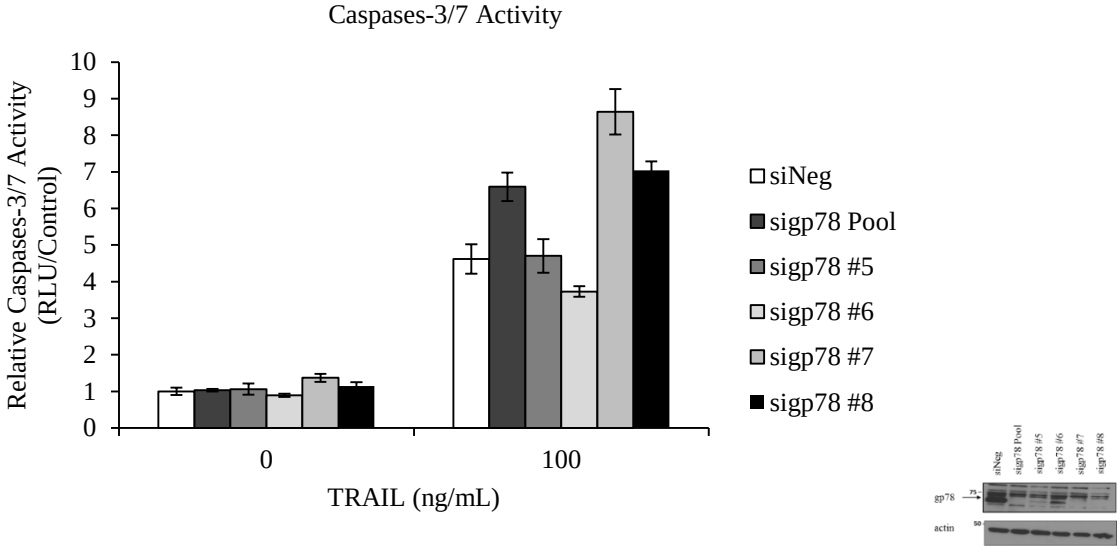
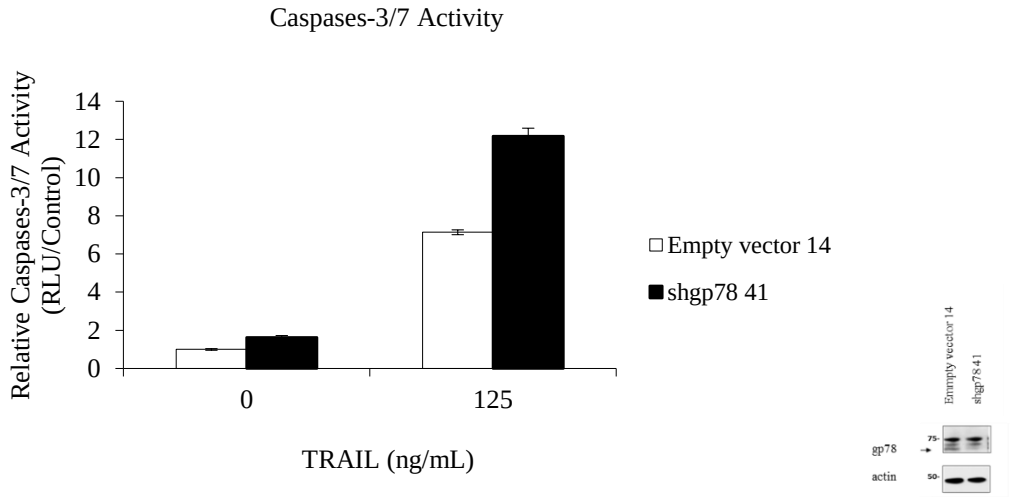

Caspases-3/7 Activity
Relative Caspases-3/7 Activity (RLU/Control)
0
1
2
3
4
5
6
7
8
9
10
0
100
TRAIL (ng/mL)
siNeg
sigp78 Pool
sigp78 #5
sigp78 #6
sigp78 #7
sigp78 #8
gp78
actin

Figure 4.2. A. MB231 cells were transfected with a Qiagen non-targeting siRNA, pool of gp78-targeting siRNAs, or individual siRNAs (siRNAs #1-4). Caspases-3/7 activity was subsequently measured. Knockdown of gp78 with siRNAs #1,3,4, and the siRNA pool significantly increased TRAIL-induced caspase activation in MB231 cells relative to the TRAIL-treated control ($p \leq 0.001$). gp78 knockdown with siRNA #2 did not as robustly sensitize cells to TRAIL as knockdown with the other siRNAs ($p=0.01$). *B.* MB231 cells were transfected with a Qiagen non-targeting siRNA, gp78-targeting siRNA #1, or a C911 seed control siRNA designed to assess the off-target effects of siRNA #1. MB231 cells transfected with siRNA #1 or the C911 seed control were both comparably sensitized to TRAIL relative to the TRAIL-treated non-targeting siRNA control ($p<0.01$). *C.* MB231 cells were transfected with a Qiagen non-targeting siRNA or individual gp78-targeting siRNAs (siRNAs #5 and #6). MB231 cells transfected with siRNA #5 or #6 showed significantly more TRAIL-induced caspase activation relative to the control ($p<0.001$). *D.* MB231 cells were transfected with a Dharmacon non-targeting siRNA, gp78-targeting pool of siRNAs, or individual siRNAs (siRNAs #5-8). MB231 cells transfected with siRNAs #7 and 8 showed significantly more TRAIL-induced caspase activation relative to the control ($p< 0.0001$), whereas MB231 cells transfected with siRNAs #5 were not significantly sensitized to TRAIL ($p=0.5$) and cells transfected with siRNA #6 were not as sensitized as those transfected with siRNA #7 and #8 ($p=0.001$). *A-D.* Cells were treated with TRAIL for two hours, and caspases-3/7 activity was evaluated using a luminescent assay system. *Inset:* Immunoblot of gp78 expression in cells with and without gp78 knockdown. *A,B,D. Inset:* Actin is used as a loading control. *C. Inset:* HSC70 is used as a loading control. Experiments represented in A, C, and D are single representative experiments normalized to control +/-SD. Figure B is representative of three independent experiments normalized to control +/- SE.

Figure 4.3

Stable Knockdown of gp78 Sensitizes Cells to TRAIL-induced Caspases-3/7 Activity and Cell Death

A.



B.

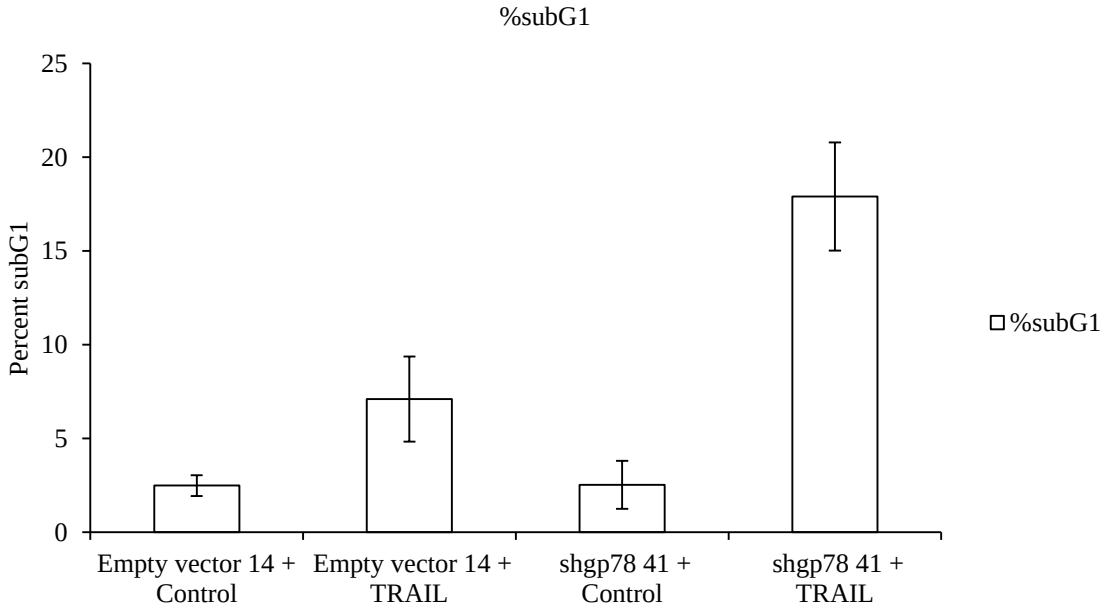
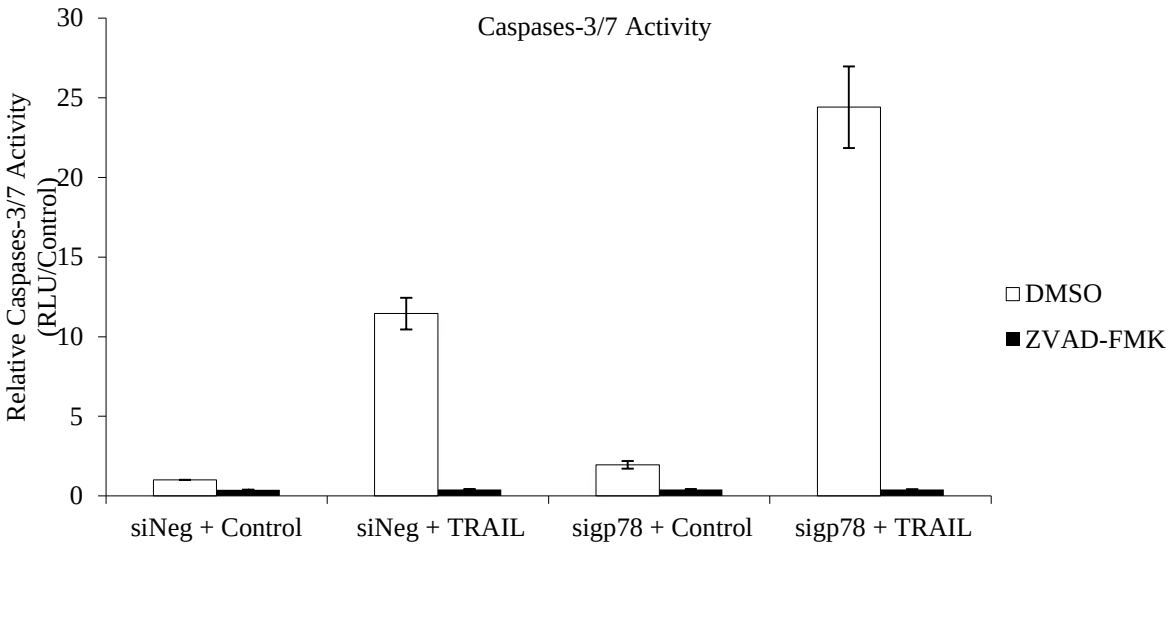


Figure 4.3. A. MB231 cells stably transfected with empty vector (clone #14) or gp78 targeting shRNA (clone #41) were plated overnight and then treated with TRAIL for 2 hours. Caspases-3/7 activity was then evaluated using a luminescent assay system. MB231 cells with stable knockdown of gp78 showed greater TRAIL-induced casapase activation compared to the TRAIL-treated control ($p<0.01$). *Inset* Immunoblot of gp78 expression in cells with and without gp78 knockdown. Actin is used as a loading control. *B.* MB231 cells stably transfected with empty vector (clone #14) or gp78-targeting shRNA (clone #41) were plated overnight, then treated with 63 ng/mL TRAIL. Twenty-four hours after TRAIL-treatment, cells were harvested for propidium iodide staining and percent subG1 DNA content was measured. Knockdown of gp78 significantly increased the fraction of cells with sub-G1 DNA content in MB231 cells treated with TRAIL-induced relative to the control ($p<0.01$). All experiments were done in triplicate +/- SE.

Figure 4.4

gp78 Knockdown Sensitizes Cells to TRAIL-induced Caspases-3/7 Activity and Cell Death in a Caspase-Dependent Manner

A.



B.

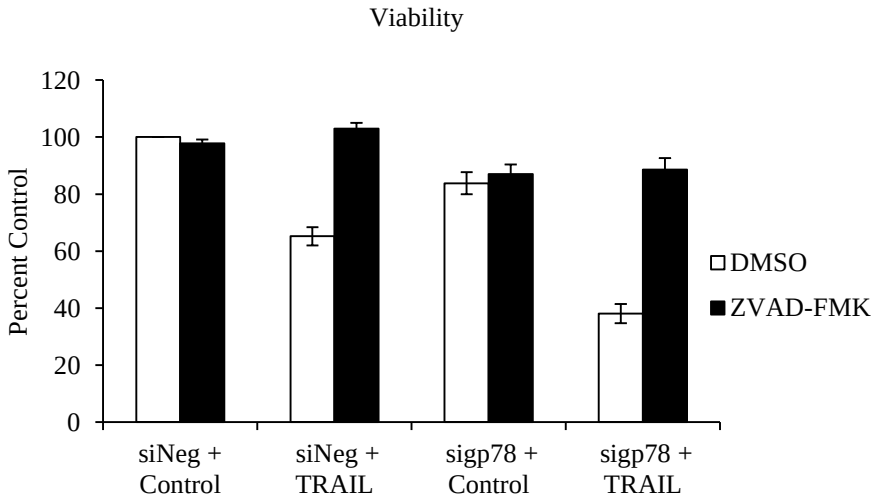


Figure 4.4. A-B. MB231 cells were reverse transfected with a Qiagen non-targeting siRNA or gp78-targeting siRNA #1. Twenty-four hours after transfection, cells were treated with 50 µM Z-VAD-FMK pan-caspase inhibitor or an equivalent volume of DMSO for 1 hour. *A.* After Z-VAD-FMK or DMSO treatment, cells were treated with TRAIL for 2 hours. TRAIL-induced caspases-3/7 activity was then measured using a luminescent assay system. *B.* After Z-VAD-FMK or DMSO treatment, cells were treated with TRAIL for 24 hours. Cell viability was then measured using the MTS assay system. Both experiments were carried out in triplicate, in parallel, and normalized to the control +/- SE.

Figure 4.5

Knockdown of gp78 Sensitizes Cells to TRAIL in a non UPR-Dependent Manner

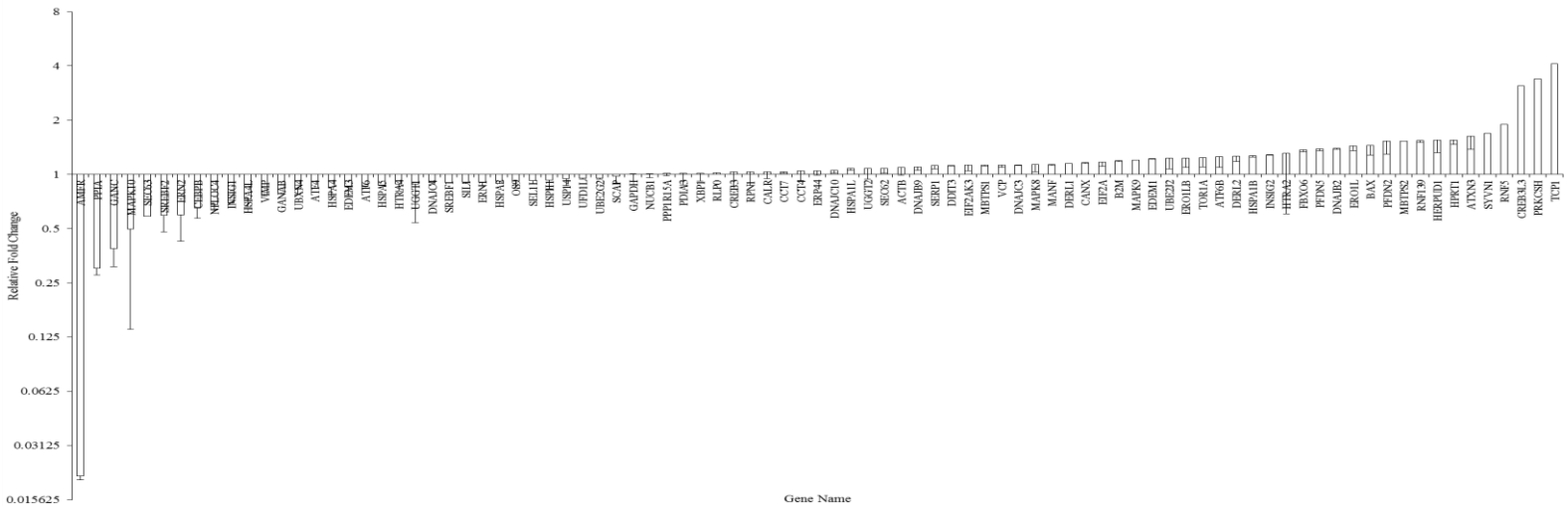


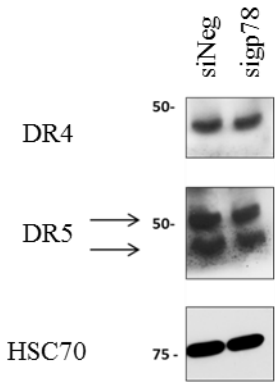
Figure 4.5. MB231 cells were trasnfected with a Qiagen non-targeting siRNA or gp78-targeting siRNA #1. Twenty-four hours after transfection, cells were harvested for RNA isolation, and cDNA was subsequently synthesized. The differential transcriptional regulation of UPR-associated genes was evaluated using a qPCR array. The data shown are the averages of two experiments normalized to GAPDH and relative to the non-targeting siRNA-treated control +/- SD.

Figure 4.6

gp78 Knockdown is Not Associated with Upregulation of DR4 or DR5 Protein Expression

A.

Immunoblot:



B.

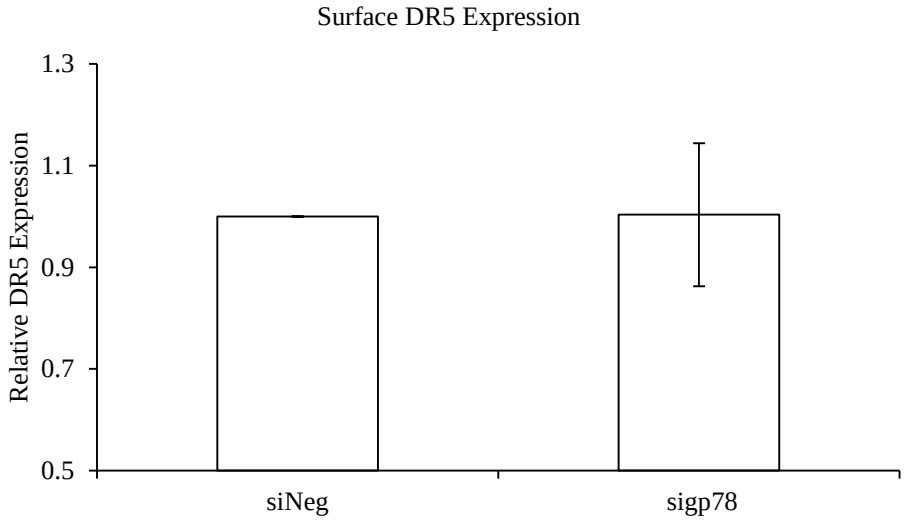


Figure 4.6. A. MB231 cells were transfected with a Qiagen non-targeting siRNA or gp78-targeting siRNA #1. Twenty-four hours after transfection, the cells were harvested for protein isolation and subsequent immunoblotting. DR4 and DR5 expression with and without gp78 knockdown is shown. HSC70 is used as a loading control. *B.* MB231 cells were transfected with a Qiagen non-targeting siRNA or gp78-targeting siRNA #1. Twenty-four hours after transfection, cells were harvested and live-stained for DR5 cell surface expression. The experiment was carried out in triplicate. The DR5 cell surface expression in siNeg and sigp78 cells was not statistically different ($p=0.9$). The data are shown +/- SE.

CHAPTER FIVE

THE TRAIL RECEPTOR AGONIST DROZITUMAB TARGETS BASAL B TRIPLE NEGATIVE BREAST CANCER CELLS THAT EXPRESS VIMENTIN AND AXL

Dine, J.L.*, O'Sullivan, C.C.*, Chavez, K.J., Conway, C.M., Sinclair, S., Stone, B., Amiri-Kordestani, L., Merchant, A.S., Hewitt, S.M., Steinberg, S.M., Swain, S.M., & Lipkowitz, S. (2015). The TRAIL receptor agonist drozitumab targets basal B triple negative breast cancer cells that express vimentin and Axl. Manuscript submitted for publication.

*indicates equal contribution

Abstract

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) binds and activates the death receptors (DRs) 4 and 5 on the cell surface, thereby inducing a caspase-driven death program. The selectivity of TRAIL-receptor agonists for killing cancer cells has made the TRAIL pathway a widely explored therapeutic target. To date, evaluation of these agents in clinical trials has demonstrated that they are well-tolerated, but modestly effective in unselected patient populations. As death receptor targeted therapy is an attractive concept in oncology, research efforts should focus on identifying subsets of patients who may benefit from this approach. Previous findings demonstrated that triple negative breast cancer (TNBC) cells with a basal B phenotype are sensitive to TRAIL, while cell lines representative of the other subtypes of breast cancer (*i.e.*, estrogen receptor (ER) positive, human epidermal growth factor 2 (HER2) amplified, and

basal A TNBC) are relatively resistant. In this study, drozitumab, a clinically tested agonistic monoclonal anti-DR5 antibody, was used to treat cell lines representing the previously described subtypes of breast cancer. Only the basal B TNBC cells were sensitive to drozitumab induced apoptosis. These cells express the mesenchymal markers vimentin and Axl. We next evaluated expression levels of vimentin and Axl in publically available cDNA microarray data sets and by immunohistochemistry (IHC) to explore the potential clinical utility in using vimentin and Axl as biomarkers for drozitumab sensitivity in breast cancer. Our findings demonstrate that a subset of TNBC express vimentin and Axl. In conclusion, the TRAIL receptor agonist drozitumab selectively kills basal B TNBC cells that express vimentin and Axl, and IHC can identify TNBC tumors that express these potential biomarkers for drozitumab therapy. Therefore, drozitumab, either as a single-agent or as combination therapy, may potentially be effective for the treatment of basal B TNBC. Further clinical studies are warranted.

THE TRAIL RECEPTOR AGONIST DROZITUMAB TARGETS BASAL B TRIPLE NEGATIVE BREAST CANCER CELLS THAT EXPRESS VIMENTIN AND AXL

Breast cancer is a heterogeneous group of diseases that may be stratified into subtypes based on the presence of distinct molecular markers (Prat & Perou, 2011). Approximately 60-70% of breast cancers are estrogen receptor (ER) or progesterone receptor (PR) positive, and 15-30% of cases have amplification and overexpression of the human epidermal growth factor receptor 2 (HER2) protein (Brenton, Carey, Ahmed, & Caldas, 2005). Additionally, 10-15 % of breast cancers are termed "triple negative" due to the absence of ER and PR expression and *HER2* amplification (Brenton et al., 2005). Most triple negative breast cancers (TNBC) are characterized by an aggressive presentation and inferior survival outcomes, especially in the relapsed or metastatic setting (Carey et al., 2006; Millikan et al., 2008; Morris et al., 2007). The TNBC subset is over represented in African American women and accounts, in part, for the worse outcomes in this group (Carey et al., 2006; Morris et al., 2007). Unlike the treatment strategies available for ER and/or PR expressing or *HER2* amplified subsets of breast cancer, effective targeted therapies have yet to be developed for TNBC. In the absence of a targeted therapy with which it may be combined, chemotherapy is currently the standard of care for this patient population (Perou, 2011). There is a clear need to develop effective, targeted therapies for TNBC.

Extensive characterization has revealed remarkable diversity in the molecular attributes of TNBC (Cancer Genome Atlas, 2012; Lehmann et al., 2011; Neve et al., 2006; Perou et al., 2000). The majority of TNBC is basal-like, which is characterized by

elevated expression of keratins 5/6 and 17, *TP53* mutation, aberrations in DNA repair pathways (e.g., *BRCA1* loss), and pro-proliferative gene expression (Cancer Genome Atlas, 2012). Preclinically, basal-like TNBC cell lines have been further segregated into basal A (epithelial) and basal B (mesenchymal) subtypes (Neve et al., 2006). While the basal A subtype retains a more epithelial phenotype, the basal B subtype possesses stem cell-like characteristics and also preferentially expresses specific markers, including the intermediate filament protein vimentin and receptor tyrosine kinase Axl (Neve et al., 2006). Subsequent studies have explored using vimentin (Livasy et al., 2006; Rodriguez-Pinilla et al., 2007; Sousa et al., 2010; Tsang et al., 2013) or Axl (D'Alfonso et al., 2014) as TNBC/basal-like biomarkers in human breast tumors. Vimentin has been a particularly robust biomarker for TNBC, and vimentin and Axl have been associated with poor prognosis (D'Alfonso et al., 2014; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007; Sousa et al., 2010; Tsang et al., 2013).

Previously, we determined that basal B TNBC cell lines were the most sensitive breast cancer subtype to tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) while breast cancer cell lines representative of the other subtypes of the disease remained comparatively resistant (Rahman, Davis, et al., 2009). TRAIL induces apoptosis via ligand binding to the death receptors, DR4 and 5 (*a.k.a.* TRAIL receptor 1 and TRAIL receptor 2, respectively), which results in the formation of the death inducing signaling complex (DISC) and recruitment and activation of caspase-8 (Ashkenazi, 2002). In some cells, the mitochondrially-mediated apoptotic pathway may also be activated downstream of DR activation by caspase-8 mediated cleavage of BH3 interacting-domain death agonist (Bid), resulting in mitochondrial outer membrane

permeabilization, apoptosome formation, and caspase-9 activation (Ashkenazi, 2002). Both caspases-8 and -9 are then able to directly activate the executioner caspases-3/7, culminating in apoptosis (Ashkenazi, 2002). Interestingly, TRAIL has been found to be highly specific in selecting for transformed cells, resulting in little or no toxicity to normal cells *in vitro* and little toxicity *in vivo* (Ashkenazi et al., 1999; Walczak et al., 1999). The available clinical evidence suggests that TRAIL receptor agonists, either as monotherapy or in combination with other agents, are generally well-tolerated but exert limited efficacy in unselected patient populations (Camidge et al., 2010; Herbst et al., 2010; Holland, 2014; Trarbach et al., 2010; von Pawel et al., 2014; Younes et al., 2010). Thus, further drug development of TRAIL receptor agonists will require predictive biomarkers to identify subsets of patients with tumors most likely to respond to these agents.

Given the preclinical observation that basal B TNBC cells are sensitive to TRAIL (Rahman, Davis, et al., 2009), we have sought to specifically investigate the sensitivity of breast cancer cell lines to drozitumab (Adams et al., 2008), a monoclonal DR5-specific TRAIL-receptor agonist antibody, and to explore the expression levels of biomarkers that may correlate with sensitivity, including vimentin and Axl. We then aimed to investigate the expression levels of those biomarkers in publically available cDNA microarray data sets and by immunohistochemistry (IHC) in breast cancer tissues derived from African-American women. The goal of this study is to evaluate sensitivity to drozitumab in cell lines representative of the different subtypes of breast cancer (*i.e.*, ER positive, HER2 amplified, and TNBC), identify biomarkers of sensitivity to drozitumab, and characterize expression of those biomarkers in human breast cancer.

Materials and Methods

Cell Culture

The HCC1937, BT20, and MB157 cell lines were obtained from Reinhard Ebner (Avalon Pharmaceuticals, Germantown, MD). The MCF7, ZR75-1, T47D, MB453, SKBR3, HCC1953, MB468, HCC38, HS578t, and MB231 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were grown in RPMI 1640 base media plus 10% fetal calf serum, 100 units/ml of penicillin, and 100 units/ml of streptomycin.

Inhibitors

Z-VAD-FMK, a pan-caspase inhibitor (Cat # P416, Biomol International, Plymouth Meeting, PA), was reconstituted in DMSO and used at 100 μM concentration. Drozitumab, a monoclonal DR5 agonist antibody, and the vehicle control [0.5 M arginine succinate, 20 mM Tris, 0.02% Tween 20 (pH 7.2)], were kindly provided by Dr. Avi Ashkenazi (Genentech, Inc., South San Francisco, CA).

Viability Assay

Cells were plated overnight in 96 well plates and then treated under the experimental conditions described in the body of the text. Viability was subsequently determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Cat # G3582, Promega Corporation, Madison, WI) as previously described (Rahman, Davis, et al., 2009). At least three independent experiments were carried out and included six replicates per experiment. Results are provided as the mean +/- SE of at least three independent experiments.

Lysate Preparation and Immunoblotting

Cell lysate preparation and immunoblotting were performed as previously described (Rahman, Davis, et al., 2009). Rabbit monoclonal antibodies to Axl (Cat # 4566, Cell Signaling, Beverly, MA) and ER alpha (Cat # 8644, Cell Signaling, Beverly, MA); rabbit polyclonal antibodies to ERBB2 (Cat # Rb103P0, Thermo Scientific, Pittsburgh, PA) and PARP (Cat # 7150, Santa Cruz Biotechnology, Dallas, TX); and mouse monoclonal antibodies to caspase-8 (Cat # 9746, Cell Signaling, Beverly, MA), HSP70 (Cat # 7298, Santa Cruz Biotechnology, Dallas, TX), E-cadherin (Cat # 610181, BD Biosciences Pharmingen, San Jose, CA), tubulin (Cat #T9026, Sigma, St. Louis, MO), and vimentin (Cat # 550513, BD Biosciences Pharmingen, San Jose, CA) were used for immunoblotting.

Caspase-3/7 Glo

Cells were treated with 100 µM ZVAD-FMK or dimethyl sulfoxide (DMSO) for 2 hours and subsequently incubated overnight with 2500 ng/mL drozitumab or 2500 ng/mL drozitumab plus 10 µg/mL Fab. Caspases-3/7 activity was assessed using the Caspase-Glo 3/7 assay system (Cat # G80943, Promega Corporation, Madison, WI) as previously described (Murrow, Garimella, Jones, Caplen, & Lipkowitz, 2010). Three independent experiments normalized to the DMSO-treated control +/- SE were performed.

Sub-G1 Analysis

MB231 cells were plated overnight and subsequently treated with 2500 ng/ml drozitumab, 2500 ng/ml drozitumab plus 10 µg/ml Fab, or equal volumes of drozitumab vehicle or drozitumab vehicle plus Fab. Cell harvesting, propidium iodide staining, flow

cytometry method, and data analysis technique were described previously (Garimella, Rocca, & Lipkowitz, 2012). Three independent experiments normalized to the vehicle-treated control +/- SE were performed.

Analysis of Publically Available Microarray Data

A single merged superset of 696 samples was created from collating publicly available breast cancer datasets (GSE2034, GSE3494 and GSE 1456) that were conducted on the same microarray platform (Affymetrix Human Genome HGU133A). Based on available clinical metadata, this superset comprised of 484 ER positive, 177 ER negative (or TNBC), and 15 ERBB2/Her2 positive patients. Twenty patients did not have information (NA). Normalized log-transformed expression values for vimentin and Axl genes were extracted for the 177 TNBC samples, and median centering along with euclidean clustering was applied to generate the heatmap (Figure 5.4A). Spearman's correlation analysis was used to statistically assess the correlation between expression of vimentin and Axl in this subset of TNBC samples.

Immunohistochemistry

Immunohistochemistry (IHC) stains for vimentin and Axl were performed on slides prepared from tumor blocks from patients with TNBC diagnosed at MedStar Washington Hospital Center, Washington, D.C. between February 2003 and February 2009. The study at MedStar Washington Hospital Center was approved by the appropriate Institutional Review Board. Tumor samples analyzed by IHC at NIH were unlinked from patient identifiers and the protocol was reviewed by the NIH Office of Human Subjects Research and determined to be exempt from IRB approval.

Slides were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was performed in a pressure cooker for 20 minutes with citrate buffer (pH 9). Primary antibody was incubated at room temperature for 2 hours, and antibody-antigen reaction detected with Dako Envision+ secondary and DAB (Dako, Carpinteria CA). Primary antibodies were vimentin (mouse monoclonal, clone V9, Dako, Carpinteria, CA) at 1:4000 dilution and Axl (rabbit polyclonal, Cat # ab72069, Abcam, Cambridge MA) at 1:100 dilution. Slides were dehydrated in grade alcohols, cleared in xylene and coverslipped.

Slides were imaged with a Hamamatsu Nanozoomer HT (Hamamatsu, Bridgewater , NJ) at 20X resolution. Images were uploaded to SlidePath DIH (Leica, Dublin, Ireland) for annotation and image quantification. Tumor cells were identified and annotated for quantification by inspection. Quantification was performed with TIA, within SlidePath DIH, using a cytoplasmic staining algorithm to quantify DAB intensity. For Axl the average intensity was normalized for the final output. For vimentin, a threshold intensity was defined, and the number of positive pixels/total number of pixels was used as a surrogate for percent cells staining as the final output. The correlation between the expression of vimentin and Axl protein was determined by Spearman correlation.

Statistical Methods for Analysis of Clinical Parameters

This was a retrospective cohort study. A retrospective search was carried out in the database of all women diagnosed with invasive breast cancer at Washington Hospital Center between February 2003 and February 2009. Eligible patients were females aged ≥ 18 years, of African-American ethnicity, with a confirmed pathological diagnosis of stage

I- IV TNBC. Patient characteristics included age at diagnosis, date of diagnosis, stage, and laterality of disease. Treatment characteristics included the type of surgery (breast conserving vs. mastectomy), chemotherapy (yes/no), radiotherapy (yes/no), and whether or not patients declined or did not complete chemotherapy and/or radiotherapy. The primary outcomes were disease free survival (DFS) and overall survival (OS). Cause of death was determined from the electronic patient record. The potential observation time was from the date of diagnosis until the date of death or end of available follow-up information, as recently as April 16, 2014. All information was collected on Excel spreadsheets and stored in password-protected files in accordance with the Health Insurance Portability and Accountability Act (HIPAA) guidelines. The data were cleaned and de-identified. Analyses were performed by a statistician (SMS) in the Biostatistics and Data Management Section of the National Cancer Institute (NCI). The study was approved by the IRB at Georgetown University prior to its initiation.

Fifty-three patients with TNBC were included in analyses to determine the association of Axl and vimentin with clinical outcomes. OS was calculated from date of surgery until date of death or last follow-up. DFS was calculated from the date the patient was identified as being free of disease (at the first or subsequent surgery) until the date of recurrence or date last followed without a recurrence. Patients without a known date of surgery were excluded from analyses, as were patients who were not able to be identified as having a definite date in which they were found to be disease free. Patients for whom it was not able to be determined if they had a recurrence were excluded from the DFS analyses.

The significance of the difference among Kaplan-Meier curves was determined by a log-rank test. Axl, vimentin, and age at diagnosis values were divided approximately into quartiles based on data from all available TNBC patients before being used in actuarial analyses. The groupings thus result in approximate effects and, to the extent they suggest trends, may be refined in subsequent confirmatory analyses.

In general, p-values are reported unadjusted for multiple comparisons because they are all considered to be exploratory. However, when patients were analyzed initially by grouped Axl, vimentin, or age values and the results suggested that a preferred, dichotomous division in the groupings would indicate a better prognostic association with the outcome, the resulting p-values were adjusted by multiplying by the implicit number of such comparisons performed to identify the final grouping. A Cox proportional hazards model analysis was also performed to determine if Axl or vimentin retained prognostic value after adjusting for other factors which were jointly associated with outcome. All p-values are two-tailed.

Results

Drozitumab Induces Apoptosis in Breast Cancer Cells

In our prior work using a recombinant GST-TRAIL fusion protein, we found that the basal B TNBC cells are sensitive to TRAIL-induced apoptosis while other subtypes of breast cancer cells were relatively resistant (Rahman, Davis, et al., 2009). Also, we demonstrated that DR5 activation is required for the induction of apoptosis whereas DR4 activation is dispensable (Rahman, Davis, et al., 2009). To confirm the findings that the basal B TNBC are most sensitive to TRAIL receptor agonists that activate DR5, we evaluated the ability of drozitumab (a fully human anti-DR5 specific TRAIL receptor

agonist) (Adams et al., 2008) to kill breast cancer cells. We first tested drozitubmab for activity on the TRAIL sensitive, basal B TNBC MB231 cell line. MB231 was treated for 1 and 5 days with vehicle; 10 ug/mL of anti-human IgG cross-linking fragment antigen binding region (Fab); drozitumab alone; vehicle plus10 µg/mL of Fab; and drozitumab plus 10 µg/mL of Fab. MTS assays were then used to assess cell viability (Figure 5.1A and B). Crosslinking drozitumab with Fab has been reported to potentiate DISC formation, caspases-8 and -3/7 activation, and cell death compared to drozitumab alone (Adams et al., 2008). We found that drozitumab +/-Fab killed cells in a dose-dependent manner but that crosslinking drozitumab with Fab decreased viability more rapidly and potently than drozitumab alone. The half maximal inhibitory concentration (IC50) of drozitumab plus Fab after 1 day was ~10 µg/mL, and the IC50 after 5 days was ~300 ng/mL. By contrast, drozitumab alone did not result in any loss in viability at 1 day post treatment, and the IC50 was ~625 ng/mL after 5 days.

Cleavage of pro-caspase-8 into a mature and active product (Cohen, 1997) is one of the earliest steps of TRAIL-stimulated DR5 signaling. Once activated, caspase-8 will cleave and activate other caspases, including the executioner caspases-3/7, to carry out the apoptotic program (Ashkenazi, 2002). To investigate the effects of drozitumab on DR5-mediated caspase activation, we measured the cleavage of pro-caspase-8 over time after treatment with drozitumab +/-Fab. We also measured in parallel the cleavage of PARP, a caspases-3/7 substrate whose cleavage is a hallmark of apoptosis (Figure 5.2A) (Duriez & Shah, 1997). The loss of the precursors and the appearance of cleaved product for both caspase-8 and PARP were more rapid and greater for drozitumab + Fab than for drozitumab alone. Drozitumab + Fab was able to robustly induce cleavage of caspase-8

and PARP after 30 minutes of treatment while drozitumab alone required 1 hour of treatment to attain comparable levels of cleavage (Figure 5.2A). At later time points (*i.e.*, 2-8 hours) the loss of the precursors was greater in cells treated with drozitumab + Fab than it was for drozitumab alone, consistent with greater activation of the DR5 receptor by the Fab-crosslinked drozitumab (Figure 5.2A). These findings are consistent with earlier studies indicating that the addition of Fab to drozitumab more rapidly induced caspase activation than drozitumab alone (Adams et al., 2008). The dependence of drozitumab mediated toxicity on caspase activation was further explored by measuring caspases-3/7 activity and viability in MB231 cells in the presence or absence of pre-treatment with the pan-caspase inhibitor ZVAD-FMK (Figure 5.2B and C respectively). Caspase activation was measured using a luminescent caspases-3/7 assay (Figure 5.2B). Drozitumab induced an ~2.5 fold increase in caspases-3/7 activity, compared to vehicle alone (Figure 5.2B). Fab crosslinked drozitumab induced an ~3 fold increase in caspases -3/7 activity compared to vehicle treated cells (Figure 5.2B). Fab alone did not induce any caspases-3/7 activity compared to vehicle treated cells (data not shown). The addition of the pan-caspase inhibitor ZVAD-FMK completely blocked the increase in caspases-3/7 activity induced by drozitumab or Fab crosslinked drozitumab (Figure 5.2B). In parallel experiments, pretreatment of the cells with ZVAD-FMK completely rescued the viability of the cells treated with either drozitumab or Fab crosslinked drozitumab (Figure 5.2C). Together these findings demonstrate that drozibumab or Fab crosslinked drozitumab promotes caspase-dependent loss of viability in the cells.

To confirm that drozitumab induces apoptotic cell death, sub-G1 DNA content was measured in MB231 cells treated with drozitumab or drozitumab + Fab for 48 hours.

Drozitumab and Fab crosslinked drozitumab substantially increased the percentage of cells with sub-G1 DNA content by 4-5 fold compared to their respective controls (Figure 5.2D). Fab crosslinked drozitumab did not induce significantly different levels of sub-G1 cells and in fact the percentages were overall slightly lower. This could reflect a more rapid cell death kinetic in the cells treated with Fab crosslinked drozitumab, resulting in fewer cells with sub-G1 content at 48 hours.

In summary, drozitumab induces caspase-dependent apoptotic cell death. Activation of the apoptotic machinery is more rapidly and potently activated with the addition of the Fab cross-linking agent to drozitumab.

Drozitumab Preferentially Kills Basal B TNBC cells

We have previously shown that cell lines that represent TNBC with mesenchymal features (basal B TNBC cells) are the most sensitive to GST-TRAIL-induced apoptosis (Rahman, Davis, et al., 2009). To explore the sensitivity of breast cancer cells to drozitumab a panel of 14 breast cancer cell lines representative of the different subtypes of disease were treated with drozitumab +/- Fab for 5 days. Viability was then measured by MTS assay (Figure 5.3). Drozitumab induced loss of viability in every basal B TNBC cell line tested (HCC38, HS578t, MB157, and MB231), and Fab crosslinking of dozitumab increased the loss of viability (Figure 5.3). Drozitumab with or without crosslinking did not induce loss of viability in the ER positive, HER2 amplified, and the basal A TNBC cell lines (Figure 5.3). In parallel, we assessed the expression of protein markers to confirm the phenotype of the cells (Figure 5.3, immunoblot shown below bar graph). Expression of ER and HER2 confirmed the identification of cells as ER positive, HER2 amplified, or TNBC. To further characterize the basal A and basal B subsets of

the TNBC, expression of E-cadherin, vimentin, and Axl were measured. E-cadherin is expressed in epithelial cells and vimentin is expressed in mesenchymal cells (Guarino, Rubino, & Ballabio, 2007). Axl is highly expressed in mesenchymal breast cancer cells (Meric et al., 2002; Rahman, Davis, et al., 2009). The basal B TNBC all expressed vimentin (Figure 5.3, lanes 11-14), whereas none of the other cell lines expressed vimentin. Axl was expressed in all of the basal B TNBC and in the basal A TNBC HCC1937 (Figure 5.3, lanes 11-14). E-cadherin expression was found in the basal B TNBC HCC38 cell line but not in any of the other basal B TNBC cell lines. E-cadherin was expressed in all of the basal A TNBC and ER positive cell lines (Figure 5.3 lanes 1-3 and 8-10). The only HER2 amplified cell line that expressed E-cadherin is the HCC1954 cell line. The viability data and the immunoblots suggest that a subset of TNBC that express the mesenchymal markers vimentin and Axl are the most sensitive to drozitumab-induced loss of viability.

Vimentin and Axl are Expressed in Human TNBC

In order to characterize vimentin and Axl expression in TNBC tumors, we investigated transcriptional and protein expression levels from human TNBC tumors. mRNA expression of vimentin and Axl was evaluated in TNBC tumor biopsies from 177 patients derived from three merged publically available cDNA microarray data sets that utilized the same microarray platform for analysis (Figure 5.4A) (Miller et al., 2005; Pawitan et al., 2005; Wang et al., 2005). Of the 177 TNBC samples, ~43% and ~41% samples had high mRNA expression of vimentin and Axl, respectively. Approximately 39% of TNBC samples expressed high levels of both vimentin and Axl mRNA. The expression of vimentin and Axl showed strong correlation in the TNBC samples from

these merged data sets ($r= 0.6$; $p<0.0001$). These findings confirm that vimentin and Axl are transcriptionally expressed in a subset of human TNBC breast tumors.

The mRNA expression of vimentin and Axl in tumors described above could be in either the tumor cells or the stroma. In order to demonstrate that expression of vimentin and/or Axl was in the tumor cells, we performed IHC in TNBC samples from a cohort of 53 African-American women that were treated over a 6 year period (February 2003-February 2009) at Washington Hospital Center in Washington, D.C. (Table 5.1). The median age at diagnosis was 56 years (range: 31-72). The majority of patients had either stage II or III disease at diagnosis (n=46; 86.8%), and most patients received chemotherapy (n=50; 94.3 %). A substantial proportion of patients received neoadjuvant chemotherapy (n=21; 39.6 %). Approximately one-third of participants (16/53=30.1%) declined or did not complete chemotherapy, a finding which is likely related to complex cultural and socio-economic factors in this patient demographic.

We assessed staining of vimentin and Axl in the tumor cells by IHC. Vimentin was quantified based on the percentage of tumor cells positive for vimentin staining. The range of staining was from 0-49% of cells staining positive for vimentin. The top quartile samples showed vimentin staining in 15-49% of tumor cells. Axl was quantified based on the intensity of staining with the top quartile of samples showing a Axl score of 0.575-1.00 in the tumor. Thus a subset of TNBC can be identified that express vimentin, Axl, or both. We were able to identify vimentin and Axl expression in the tumor cells and differentiate between expression in tumor and stroma. We observed combinations of low Axl and low vimentin , high Axl and low vimentin, low Axl and high vimentin , and high Axl and high vimentin in the tumor cells (Figure 5.4B). The expression of vimentin and

Axl protein was only weakly correlated in the IHC data set (r=0.29; p=0.035) (Figure 5.5). However, approximately 15% of tumors expressed high levels of both vimentin and Axl (as defined by expression in the top quartile for both). These findings demonstrate that a subset of TNBC with high vimentin and Axl protein are identifiable using IHC.

We explored DFS and OS in this cohort of patients with TNBC. DFS and OS were superior in patients with stage I or II disease compared to that of patients with stage III or IV disease. The 5 year DFS probabilities were 79.0% (95% Confidence Interval (CI): 61.0-90.0%) for stage I and II vs. 26.7% (95% CI: 8.4-59.0%) for stage III and IV (p=0.0006). The 5 year OS probabilities were 75.4% (95% CI: 57.2-87.0%) for stage I and II vs. 60.2 % (95% CI: 35.7-80.5%) for stage III and IV (p=0.024). Of the 21 patients who received neoadjuvant chemotherapy, two achieved a pathological complete response (pCR), 16 responded, and three progressed on therapy. Those patients that achieved a pCR or responded to neoadjuvant chemotherapy had a significantly better OS (5 year OS probability of 74.7%, with 95% CI: 49.8-89.7%) compared to those who progressed on therapy (all three died, at 15.1, 18.5, and 22.3 months; adjusted p=0.01).

Following exploratory analyses, good separation of Kaplan-Meier curves for DFS and OS was obtained by splitting the data into the top quartile vs. the lower 3 quartiles of both vimentin (values >0.1455 vs. <=0.1455 and Axl staining (values >0.669 vs. <=0.669). These results are shown in Figure 5.6A and B for vimentin and Axl respectively. High vimentin expression (defined as the top quartile) showed a trend towards an association with improved DFS and a significantly better OS by univariate analysis than those whose tumors had lower vimentin expression (Figure 5.6A). High

Axl expression showed weak association with DFS or OS by univariate analysis (Figure 5.6B).

Among these patients with TNBC, the following parameters were associated with at least a trend towards significance with respect to DFS in univariate analyses: Axl (<0.669 vs. >0.669); vimentin (<=0.1455 vs. >0.1455); age (29-55 vs. >55); stage (I,II vs. III, IV); neoadjuvant treatment (none, not applicable vs. yes); and response to neo-adjuvant therapy (progression vs. anything else: PCR, residual, not applicable). Response to neo-adjuvant therapy was excluded from further consideration for models. Since the best division only consisted of three patients with progression with the rest in a combined category, the generalizability of the parameter was limited. Using backward elimination, Cox multivariable analysis led to a model showing that vimentin >0.1455 was associated with superior DFS (p=0.046) when adjusting for age and stage (Table 5.2A). For OS, Axl (<0.330 vs. >=0.330—the median value, selected for the Cox model because it had slightly better univariate prognostic value than did 0.669), vimentin (<=0.1455 vs. >0.1455), stage (I,II vs. III, IV), and response to neo-adjuvant therapy (progression vs. anything else: PCR, residual, not applicable) were factors found to be potentially associated with OS in univariate analyses. By backward elimination, Cox model analysis showed that vimentin >0.1455 was associated with lower probability of dying (p=0.026) when adjusting for stage, the only other potential prognostic factor remaining in the model (Table 5.2B).

Discussion

The results presented here demonstrate that the DR5 rececptor agonist drozitumab induces caspase dependent apoptosis in basal B TNBC but not in cells from other

subtypes of breast cancer (Figures 5.1, 5.2 and 5.3). The addition of cross-linking Fab to drozitumab more rapidly and potently induced loss in viability than drozitumab treatment alone, consistent with previous findings (Adams et al., 2008). Crosslinking of drozitumab, mediated by leukocyte Fc gamma receptor expression, is required for activity of drozitumab *in vivo* (Wilson et al., 2011). The killing of basal B TNBC by drozitumab is concordant with our previous data demonstrating that a recombinant form of the natural ligand, GST-TRAIL, selectively induced apoptosis in basal B TNBC through activation of DR5 (Rahman, Davis, et al., 2009). Thus, both recombinant TRAIL and an agonist antibody to DR5 induced apoptosis in this subset of breast cancer cells. While both DR4 and DR5 can be activated by the GST-TRAIL ligand used in our prior study, only DR5 was found to be required for the induction of apoptosis by GST-TRAIL in breast cancer cells (Rahman, Davis, et al., 2009). The basis for the DR5 selectivity of GST-TRAIL is unclear but could be due to either absolute expression levels of DR5 vs. DR4 or the relatively higher affinity of DR5 for TRAIL (Truneh et al., 2000). The results using drozitumab, a specific DR5 agonist, are consistent with our previous observation that TRAIL receptor agonists which activate DR5 induce apoptosis in the basal B TNBC.

TRAIL receptor agonists, including drozitumab, have been tested either alone or in combination with other agents in phase I and II clinical trials. Little clinical benefit has been observed to date (Holland, 2014). This has led to discontinuation of the development of these agents in many cases. However, none of the clinical trials stratified patients based on potential predictive biomarkers of response, and none specifically evaluated activity in TNBC. In our work, all of the cell lines express either DR4 and

DR5, and we did not find any correlation between TRAIL sensitivity and DR4 or DR5 total or surface protein level expression (Rahman, Davis, et al., 2009; Rahman, Pumphrey, & Lipkowitz, 2009). One study of pancreatic cancer, colorectal cancer, non-small-cell lung cancer, and melanoma cell lines identified low expression of O-glycosylation genes as a potential mechanism of TRAIL-resistance (Wagner et al., 2007). In our previous analysis of the breast cancer cell lines we did not find a correlation between the expression of genes for O-glycosylation and TRAIL sensitivity (Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). Lu *et al.* demonstrated that epithelial to mesenchymal transition in non-small cell lung cancer cells attenuated DR4 and DR5 apoptotic signaling and further that E-cadherin directly interacts with DR4 and DR5 to facilitates DISC assembly and caspase-8 activation in response to TRAIL in lung, colon, and pancreatic cancer cell lines (Lu et al., 2014). However, our findings indicate that the majority of basal B TNBC cell lines express low levels of E-cadherin, express mesenchymal markers such as vimentin and Axl, and are most sensitive to TRAIL (Figure 5.3) (Rahman, Davis, et al., 2009). These findings suggest there may be tissue-specific differences regarding sensitivity to TRAIL, requiring the identification of biomarkers relevant to each tissue type (Ashkenazi, 2015; Wilson et al., 2011).

Transcriptional profiling of the breast cancer cell lines has identified two subsets of basal TNBC, so called basal A and basal B cells (Neve et al., 2006). These cell types differed in expression of either epithelial (basal A) or mesenchymal (basal B) genes. Both our previous work and the current data found that the basal B TNBC cells were selectively killed by TRAIL receptor agonists. These cell lines all expressed the mesenchymal markers vimentin and Axl. The majority of the basal B TNBC cell lines

that we tested in this work (Figure 5.3) and in our previous work [14] cluster with molecular subtypes of TNBC identified by expression analyses that are characterized by mesenchymal features, including the claudin-low TNBC (Prat et al., 2010) and mesenchymal stem like TNBC (Lehmann et al., 2011). Mesenchymal markers, including high vimentin and low E-cadherin expression, are included in the definition of these subtypes and so our findings are consistent with these classifications (Lehmann et al., 2011; Prat et al., 2010). The claudin low TNBC represent ~25-39% of TNBC, and the mesenchymal stem like TNBC represent ~15% of TNBC. However, not all of the basal B cell lines are classified as claudin low or mesenchymal stem cell like. The HCC38 cell line, for example, clusters with the basal TNBC in both of these analyses, and the BT549 characterized in our previous study clusters with mesenchymal like cells in the analysis by Lehmann *et al.* (2011). Thus the expression of vimentin and Axl identify a broader group of TNBC that may be sensitive to TRAIL receptor agonists than is identified as claudin low or as mesenchymal stem like.

mRNA and protein expression of vimentin and Axl was found in TNBC tumors and approximately 15% of the TNBC have high levels of both proteins by IHC (Figure 5.4A, B and 5.5). While the molecular mechanisms that regulate TRAIL sensitivity in breast cancer remain elusive (Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009), vimentin and Axl expression identified a subset of TNBC that express one or the other of these mesenchymal markers (Figure 5.3, 5.4A and B). Although vimentin expression has been found to consistently be associated with TNBC (Livasy et al., 2006; Rodriguez-Pinilla et al., 2007; Sousa et al., 2010; Tsang et al., 2013), one study found that Axl expression, while associated with aggressive disease, did not segregate with

TNBC but was found across ER negative and ER positive subtypes (D'Alfonso et al., 2014). Thus, vimentin may be a more selective marker for the basal B TNBC. Since only a small fraction of the TNBC would be identified by co-expression of vimentin and Axl (~15% with high levels of both), an unstratified clinical trial would have a majority of the TNBC that are predicted to be resistant to TRAIL receptor agonists and thus more likely to show no benefit to these agents. Thus, vimentin and Axl expression could be used to stratify patients in a clinical trial to test whether our preclinical observations also predict responses to TRAIL receptor agonists in patients.

In an exploratory analysis of the relationship of vimentin and Axl expression to outcomes in 53 African American women, Axl, vimentin, stage, and response to neoadjuvant chemotherapy were factors found to be potentially associated with OS in univariate analyses, while Axl, vimentin, age, neoadjuvant chemotherapy, response to neoadjuvant chemotherapy, and stage were associated with at least trends towards significance with respect to DFS in univariate analyses.

Improved outcomes were associated with early stage and response to neoadjuvant chemotherapy. This latter finding reflects chemosensitivity in TNBC tumors (Carey et al., 2007). In the evaluated cohort, high expression of vimentin was associated with improved DFS and OS (Figure 5.6 and Table 5.2). This finding contradicts published data showing either association of vimentin with worse outcomes with DFS and OS outcomes in this setting. Published data have generally found vimentin expression to be associated with basal and TNBC phenotype, and generally most publications have found worse outcomes or no association with outcome associated with vimentin (D'Alfonso et al., 2014; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007; Sousa et al., 2010; Tsang et al., 2013;

Willipinski-Stapelfeldt et al., 2005). Our finding of improved outcomes could be due to the small sample size or unknown or unmeasured patient characteristics. Interestingly, the majority of the patients received chemotherapy, many in the neoadjuvant setting. Thus, our findings could indicate that the TNBC that express vimentin are particularly sensititive to chemotherapy compared to other TNBC. Our lab previously demonstrated that combining TRAIL and chemotherapy results in increased cytotoxicity in breast cancer cells (Keane, Ettenberg, Nau, Russell, & Lipkowitz, 1999). Thus, combining TRAIL receptor agonists with chemotherapy may be useful in patients with TNBC and particularly in those TNBC which express vimentin and/or Axl.

Our data, combined with the existing evidence, demonstrates that the TNBC cell lines which express mesenchymal markers (*i.e.*, vimentin and Axl) are most sensitive to TRAIL receptor agonists. IHC identifies TNBC tumors with mesenchymal features, and therefore this strategy could be used in clinical trials to select patients with TNBC who may derive benefit from TRAIL receptor agonists. Together, these data suggest that TRAIL receptor agonists targeted to basal B TNBC should be explored further in clinical trials.

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Table 5.1

Patient Characteristics

Stage at Diagnosis (n=53)	Number and Percentage
Stage 1	6 (11.3%)
Stage 2	30 (56.6%)
Stage 3	16 (30.1%)
Stage 4	1 (1.8%)
Type of Surgery	**Number and Percentage**
Breast Conserving	33 (62.2%)
Mastectomy	19 (36.8%)
Unknown	1 (0.01%)
Chemotherapy	**Number and Percentage**
Yes	50 (94.3%)
No	3 (5.6%)
Type of Chemotherapy	
Neoadjuvant	21 (42.0%)
Adjuvant	29 (58.0%)

Response to Neoadjuvant Chemotherapy (n=21)	Number and Percentage
Pathological Complete Response (PCR)	2 (9.5%)
Residual Disease	16 (7.8%)
Progressed	3 (14.2%)
Completed Chemotherapy	Number and Percentage
Yes	33 (66.0%)
No	16 (32.0%)
Unknown	1 (2.0%)
Disease Recurrence	Number and Percentage
Yes	35 (66.0%)
No	18 (33.9%)

Table 5.2

Vimentin and Axl Correlate with DFS and OS, Respectively

A.

Multivariable Cox proportional hazards model for DFS

Parameter	p-value	Hazard Ratio	95% Hazard Ratio Confidence Limits
Vim >0.1455	0.046	0.210	0.046-.970
Age >55	0.016	0.234	0.072-.764
Stage	0.0004	7.555	2.486-22.960

Vimentin>0.1455 was shown to be associated with superior DFS when adjusting for age and stage.

B.

Multivariable Cox proportional hazards model for OS

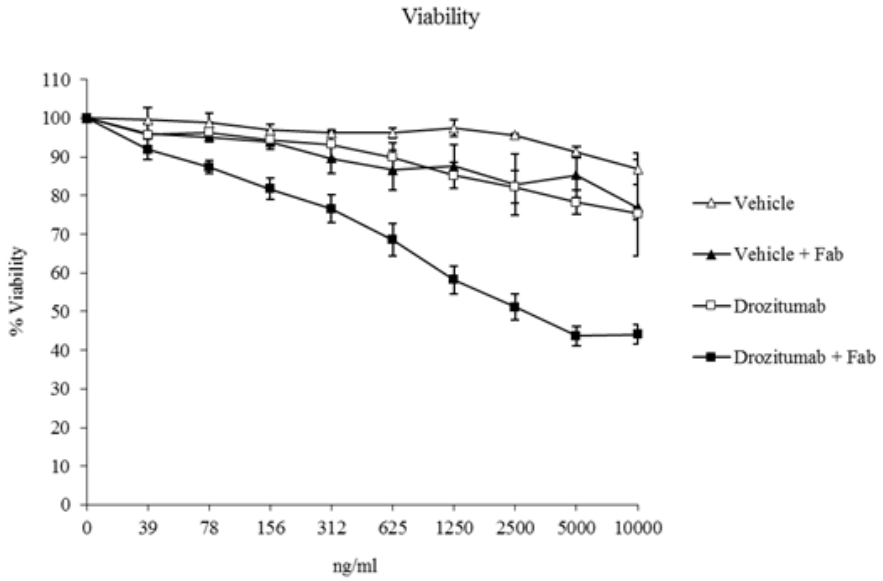
Parameter	p-value	Hazard Ratio	95% Hazard Ratio Confidence Limits
Vim >0.1455	0.026	0.101	0.013-.762
Stage	0.016	3.192	1.244-8.191

Vimentin>0.1455 was associated with lower probability of dying when adjusting for other potential prognostic factors.

Figure 5.1

Drozitumab Induces Loss in Viability

A.



B.

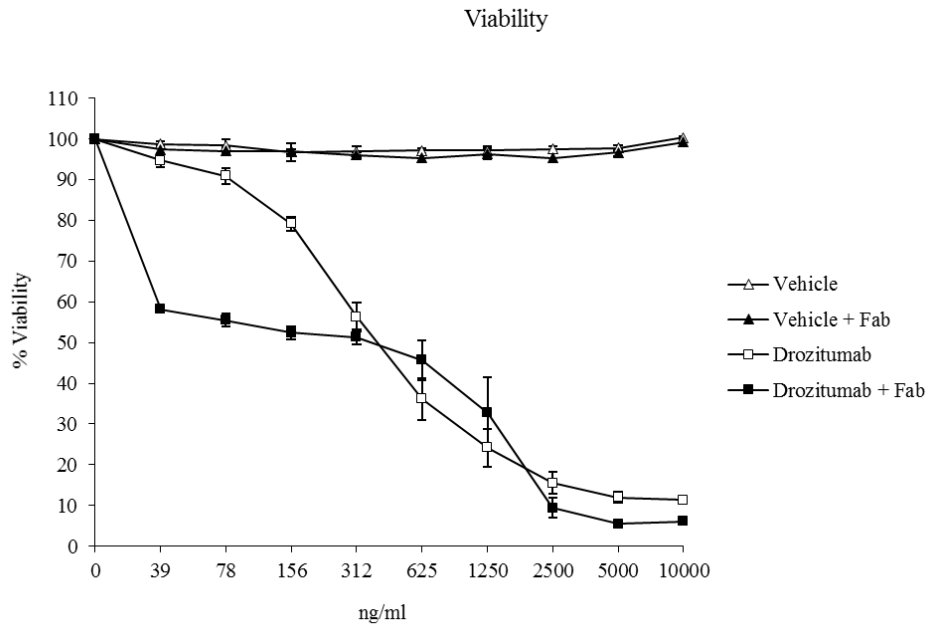
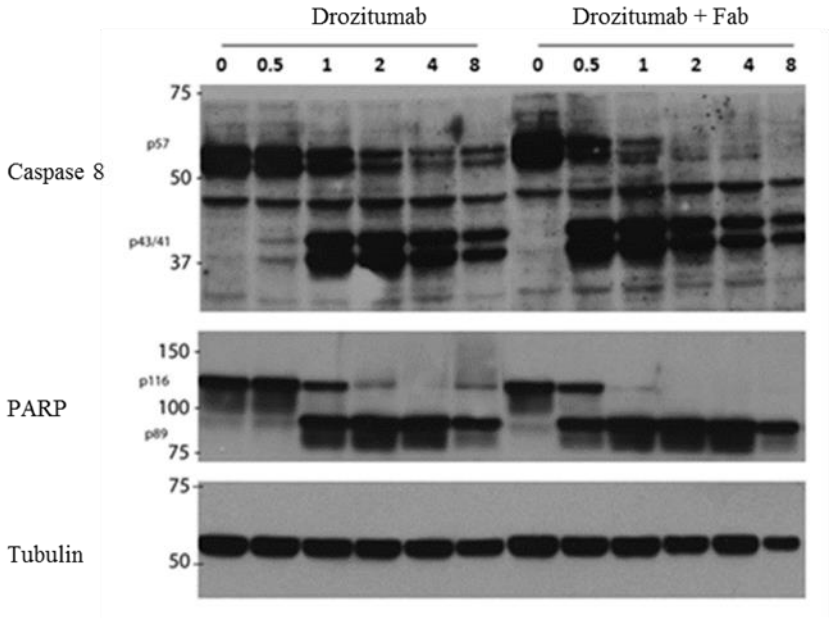


Figure 5.1. A-B. MB231 cells were incubated with indicated doses of drozitumab vehicle (open square), drozitumab vehicle plus 10 µg/mL Fab (open triangle), drozitumab (black square), or drozitumab plus 10 µg/mL Fab (black triangle) for 1 and 3 days, respectively. Viability was assessed using the MTS assay system. Data were normalized to the treatment control. Percent viability is representative of three experiments +/- SE.

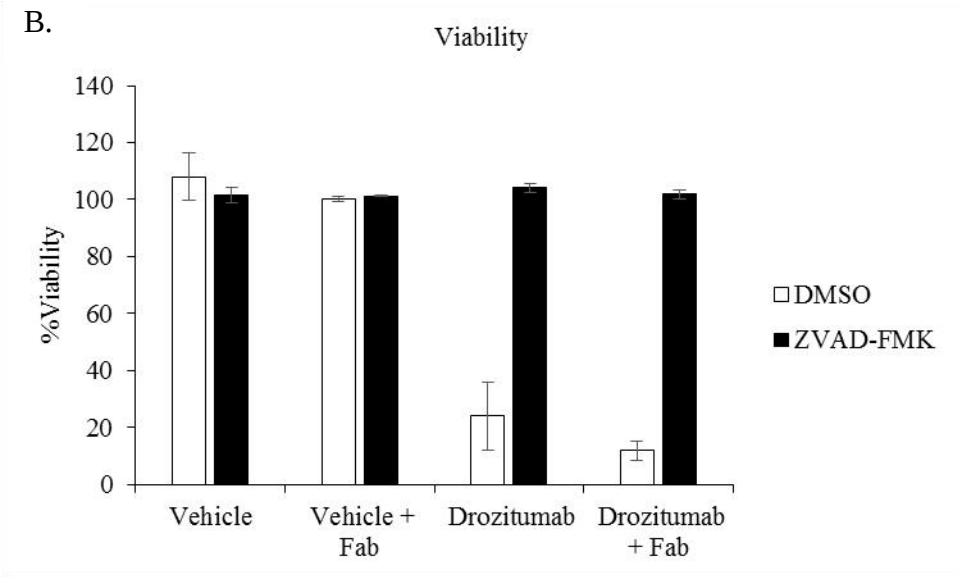
Figure 5.2

Drozitumab-induced Cell Death is Caspase Dependent Apoptosis

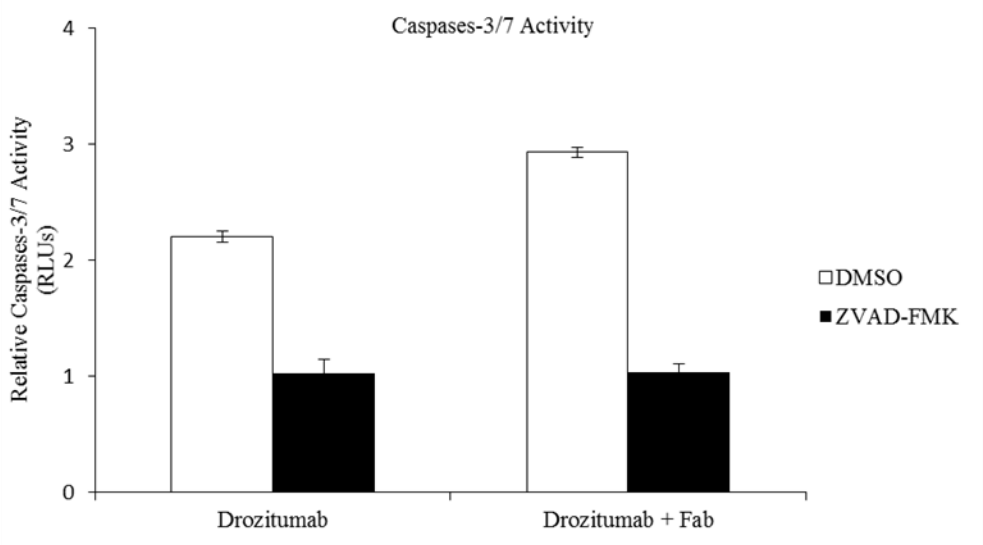
A.



B.



C.


Caspases-3/7 Activity
Relative Caspases-3/7 Activity (RLUs)
4
3
2
1
0
Drozitumab
Drozitumab + Fab
DMSO
ZVAD-FMK

SubG1

D.

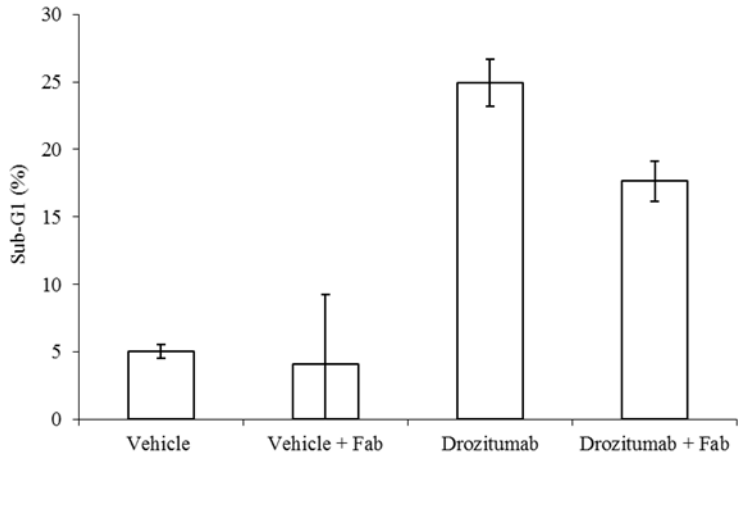

Sub-G1 (%)
30
25
20
15
10
5
0
Vehicle
Vehicle + Fab
Drozitumab
Drozitumab + Fab

Figure 5.2. A. MB231 cells were incubated with drozitumab or drozitumab plus 10 µg/mL Fab until cells were harvested for protein isolation at the indicated time points (hours). Immunoblot analysis was used to characterize caspase-8 and PARP cleavage. Tubulin was used as a loading control. *B.* MB231 cells were pre-treated with vehicle or the pan-caspase inhibitor ZVAD-FMK for 2 hours and subsequently treated overnight with drozitumab or drozitumab plus Fab. Caspases-3/7 activity was subsequently measured using a luminescent assay system. *C.* Cells were treated as in 2B, with the addition of drozitumab vehicle and drozitumab vehicle plus Fab. Cells were subsequently incubated for 5 days before viability was assessed via MTS assay. *D.* MB231 cells were treated with 10 µg/mL drozitumab vehicle, drozitumab vehicle plus Fab, drozitumab, and drozitumab plus Fab for two days. Cells were stained with propidium iodide and underwent FACS analysis to assess sub-G1 DNA content. All data were normalized to the treatment control for each experiment and are the average of three experiments +/- SE.

Figure 5.3

TNBC Cells Express Vimentin and Axl

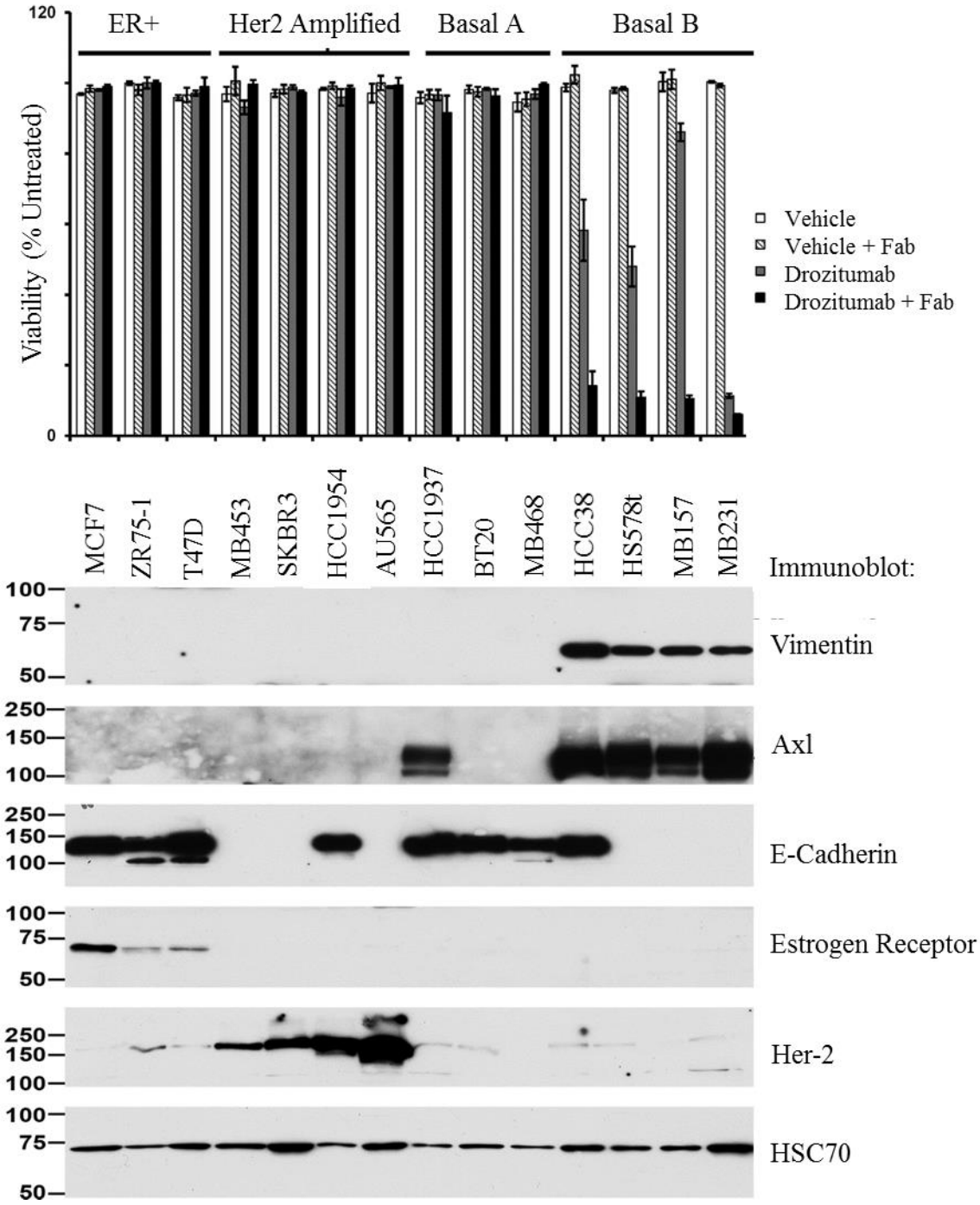
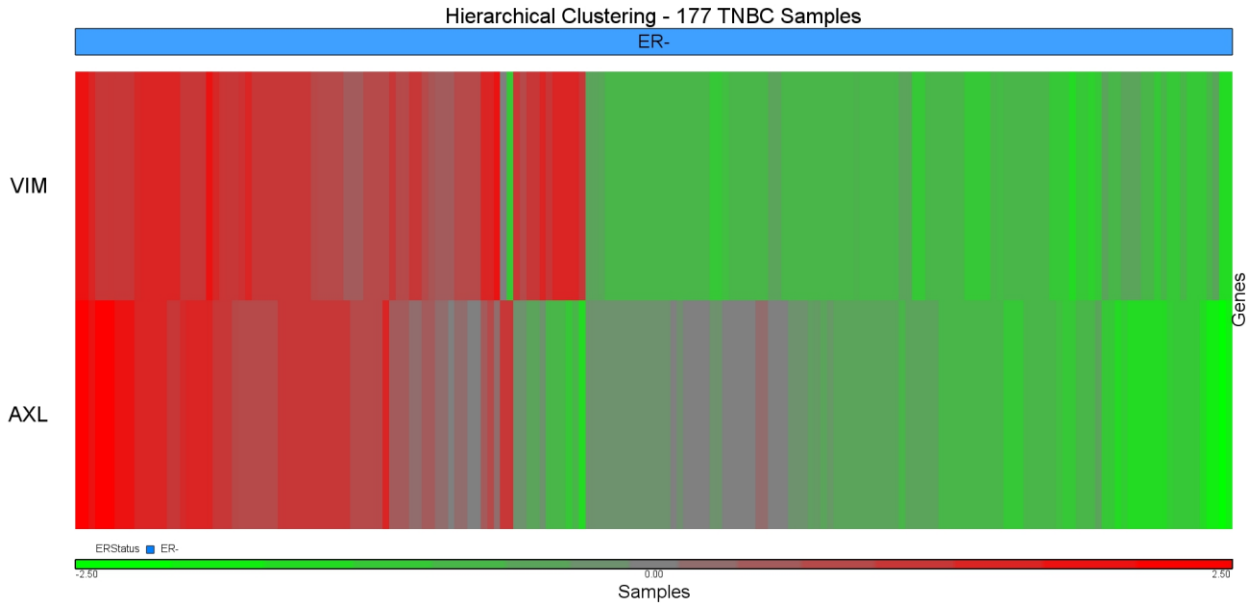


Figure 5.3. A panel of breast cancer cell lines representative of the different subtypes of disease were treated with drozitumab vehicle; drozitumab vehicle plus Fab; 10 µg/mL drozitumab; and 10µg/mL drozitumab plus Fab for 5 days. Viability was then measured by MTS assay, and cells were harvested for immunoblotting to assess the expression of subtype markers. All data were normalized to the treatment control for each experiment and are the average of three experiments +/- SE.

Figure 5.4

TNBC Tumors Express Vimentin and Axl

A.



B.

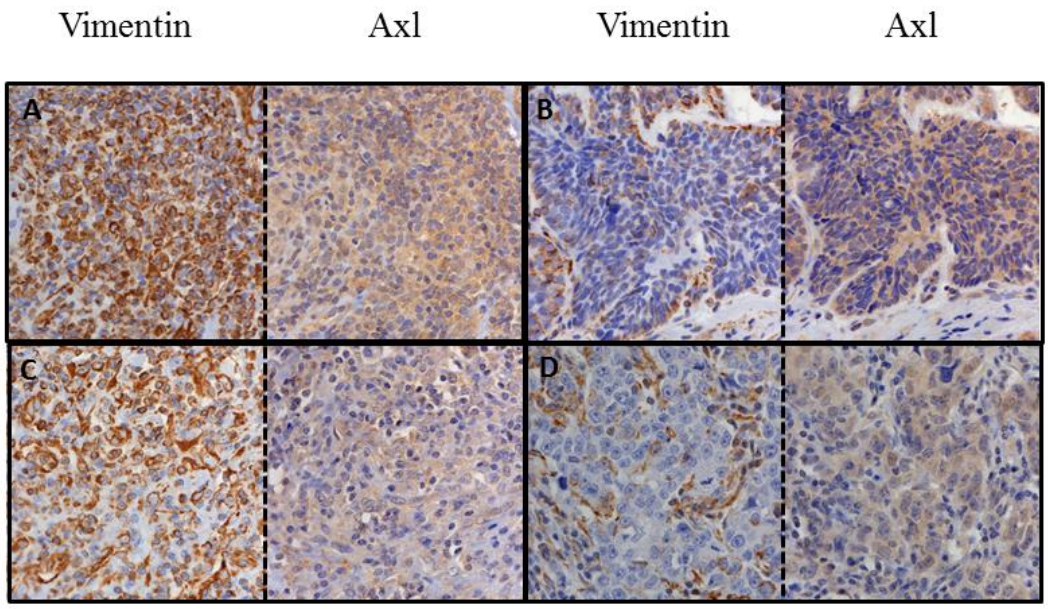


Figure 5.4. A. Heatmap displaying expression of vimentin and Axl for hierarchically clustered 177 TNBC samples. *B.* Representative IHC images of the four patterns of vimentin and Axl staining observed. A) High vimentin, high Axl, B) Low vimentin, high Axl, C) High vimentin, low Axl, and D) Low vimentin, low Axl. Vimentin image right, Axl image left. All images 450X magnification.

Figure 5.5

Vimentin and Axl are Co-expressed in TNBC

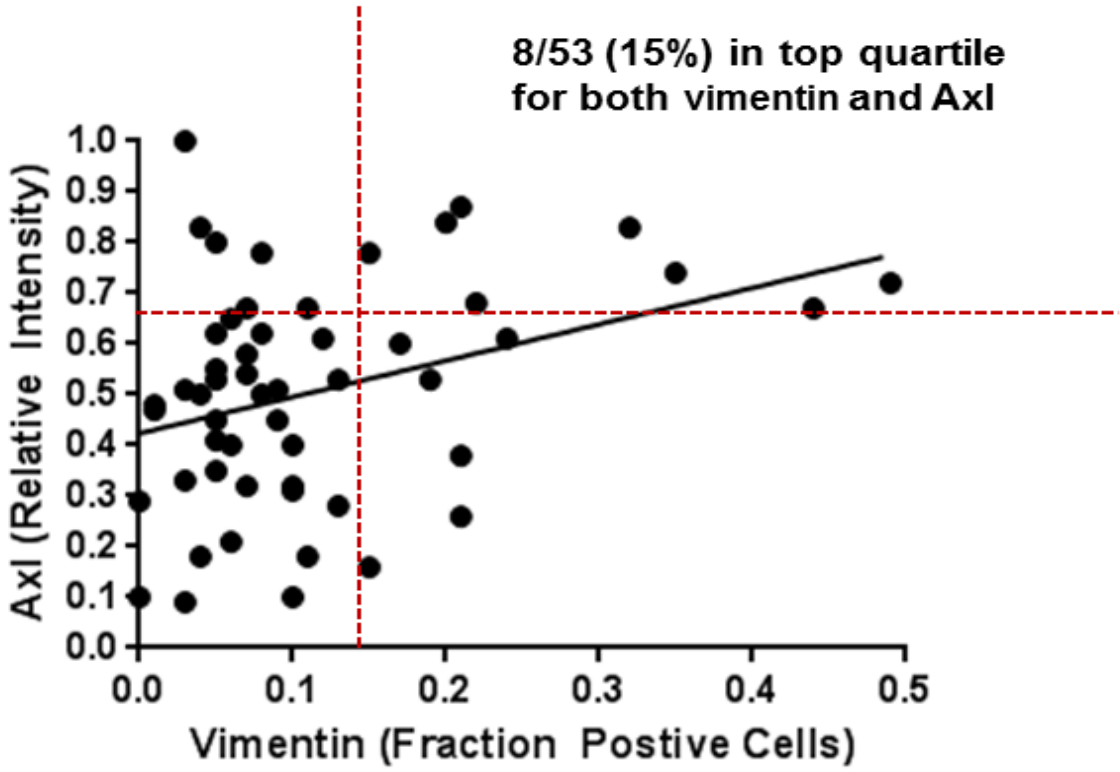
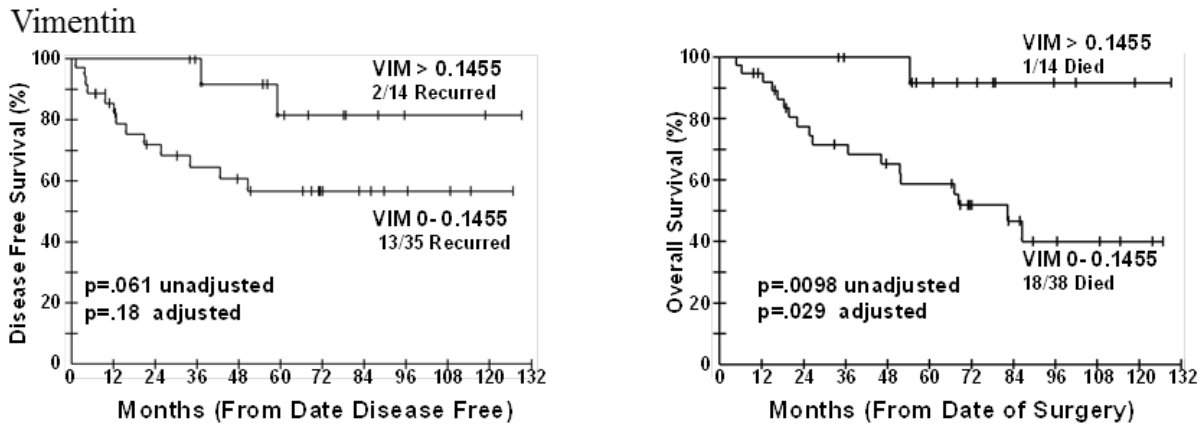


*Figure 5.5.*The vertical red dotted line indicates the top quartile for vimentin staining and the horizontal red dotted line indicates the top quartile for Axl staining.

Figure 5.6

Kaplan-Meier Curves for Vimentin and Axl Expression and DFS (Left Panels) and OS (Right Panels)

A.



B.

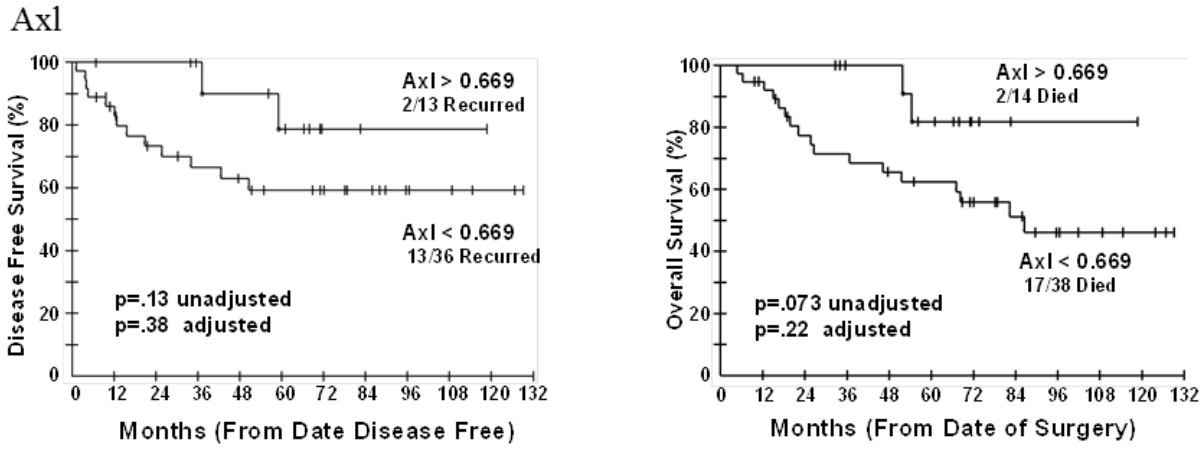


Figure 5.6. A. DFS and OS survival based on high vs. low vimentin expression by IHC. *B.* DFS and OS survival based on high vs. low Axl expression by IHC.

CHAPTER SIX

CONCLUSION

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a type II transmembrane protein able to activate the extrinsic apoptotic pathway by binding to its cognate receptors death receptor 4 (DR4) and death receptor 5 (DR5) on the cell surface (Ashkenazi, 2008). Adaptor molecules and the initiator caspase, caspase-8, form the death inducing signaling complex that mediates a caspase-driven apoptotic program. Interestingly, TRAIL induces apoptosis selectively in cancer cells, and this characteristic has been observed in normal cells *in vitro* and *in vivo.* In light of these characteristics, DR4 and DR5 agonists have been widely explored in clinical trials for the treatment of adult and pediatric advanced malignancies (Holland, 2014; Johnstone, Frew, & Smyth, 2008; Lemke, von Karstedt, Zinngrebe, & Walczak, 2014). Despite being well-tolerated, DR4 and DR5 agonists have not been effective in the treatment of unselected patients affected by cancer. These findings highlight the need for developing strategies that will help optimize DR4 and DR5 agonist activity, including identifying patients using biomarkers that may predict sensitivity to DR4 and DR5 agonists and understanding the regulatory mechanisms controlling TRAIL sensitivity to aid in the development and selection of drugs for combinatorial therapies.

Preclinically, breast cancer cells have demonstrated variations in sensitivity to TRAIL-induced apoptosis (Garimella et al., 2014; Rahman, Davis, et al., 2009; Rahman, Pumphrey, & Lipkowitz, 2009). Triple negative breast cancer (TNBC) cells (so-called because they lack expression of the molecular markers used to categorize the other

subtypes of breast cancer, including estrogen receptor and progesterone receptor expression and human epidermal growth factor receptor 2 amplification) (Brenton, Carey, Ahmed, & Caldas, 2005) with a mesenchymal phenotype are extremely sensitive to TRAIL-induced apoptosis while breast cancer cells representative of the other subtypes of breast cancer are comparatively resistant (Garimella et al., 2014; Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). TNBC is associated with an aggressive presentation, poor outcomes, overrepresentation in young African American women, and, unlike the other subtypes of breast cancer, lacks targeted therapies (Brenton et al., 2005; Carey et al., 2006; Irvin & Carey, 2008; Millikan et al., 2008). The development of a targeted therapy for TNBC is warranted in cases of resistance to chemotherapy, which is the standard of care for this population (Carey et al., 2007; Perou, 2011).

In this dissertation project, a regulator and two predictors of TRAIL sensitivity in TNBC cells have been characterized. The findings from this study have both preclinical and clinical implications. First, characterization of autocrine motility factor receptor (gp78), an ubiquitin ligase and regulator of protein quality control (Chen, Du, & Fang, 2012), demonstrates a novel mechanism for governing TRAIL sensitivity in breast cancer cells. Interestingly, signaling through the closely related unfolded protein response that results in canonical upregulation of DR5 was not identified in cells with gp78 knockdown. These findings point to a new signaling pathway for further preclinical exploration in the context of TRAIL sensitivity. These findings may also help identify a targetable component implicated in gp78 signaling and TRAIL resistance to enhance DR4 and DR5 agonist effectiveness in humans. Currently, pharmacologic inhibitors of gp78 do not exist. These results lend support toward developing a pharmacologic

inhibitor of gp78, which may potentially be utilized in other cancer-related contexts; gp78 has also been implicated in promoting metastasis *in vivo* (Tsai et al., 2007).

Second, the potentially predictive biomarkers of TRAIL sensitivity, the receptor tyrosine kinase Axl and intermediate filament protein vimentin, were identified in a panel of human breast cancer cell lines representative of the different subtypes of disease. All of the mesenchymal TNBC cell lines were sensitive to the DR5 agonist, drozitumab, which has been tested in clinical trial. Axl and vimentin were identified by immunoblot in the TRAIL-sensitive TNBC cell lines, suggesting that these proteins may be potentially predictive biomarkers of TRAIL pathway agonists in patients affected by TNBC. In order to extend these findings, Axl and vimentin expression in human TNBC was interrogated in 177 samples included in a publically available cDNA microarray dataset and by immunohistochemistry (IHC) of tumors from 53 African American women. In the cDNA dataset, ~39% of tumors highly co-expressed Axl and vimentin, and Axl and vimentin expression was highly correlated ($r=0.6$; $p<0.0001$). In the IHC dataset, Axl and vimentin were highly co-expressed in ~15% of tumors despite overall weak correlation ($r=0.29$; $p=0.035$). These findings indicate that Axl and vimentin expression can be identified in a subset of human TNBC tumors and thus may be used in the future as potentially predictive biomarkers of TRAIL sensitivity in TNBC.

However, several limitations must be acknowledged with regards to the findings of this dissertation project. With respect to characterizing gp78 as a negative regulator of TRAIL-induced apoptosis, questions arise regarding the potential off-target effects associated with siRNA and shRNA knockdown of gp78. Although efforts have been made to explore the possibility of off-target effects, including the utilization of 10 gp78-

targeting siRNAs, the generation of a stable clone expressing knockdown of gp78, and investigating a single siRNA-specific off-target effect by using a seed control siRNA, the possibility of an off-target effect remains a beleaguering concern. A rescue experiment, in which gp78 is re-expressed in cells and then interrogated along with cells with and without gp78 knockdown to assess for biological effects, would provide critical insights into the specificity of effects observed with gp78 knockdown.

Secondly, the generalizability of these findings needs to be assessed in breast cancer cell lines representative of the other subtypes. Knockdown of gp78 may only sensitize a subset of breast cancer cells to TRAIL. Knowing which subsets of breast cancer cells are sensitized to TRAIL with gp78 knockdown would help further refine the understanding of gp78-mediated TRAIL resistance and may aid in knowing which subsets of breast cancer may benefit from targeting a component of gp78 signaling in combination with DR4 and DR5 agonists.

Although caspase inhibitor experiments confirm that gp78 knockdown sensitizes cells to TRAIL in a caspase-dependent manner, the functional relationship between gp78 and TRAIL resistance is not yet clear. The proposed rescue experiment may provide insights into the relationship between gp78 and TRAIL resistance. More specifically, the identification of specific domains in the gp78 protein that are required for TRAIL resistance would supply important information about the functional relationship between TRAIL and gp78. Additional research is needed.

Finally, one major limitation was observed with respect to the identification of Axl and vimentin as potentially predictive biomarkers of DR4 and DR5 agonist sensitivity in patients with TNBC: the sample size of TNBC tumors sampled for IHC was

small (n=53). Interestingly, Axl and vimentin expression was associated with improved survival outcomes, which goes against the majority of findings that Axl and vimentin expression is not associated with improved survival outcomes (D'Alfonso et al., 2014; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007; Sousa et al., 2010; Tsang et al., 2013; Willipinski-Stapelfeldt et al., 2005). However, the results from this study demonstrated the point that Axl and vimentin can be identified in human breast tumors, thus lending support to using both as predictive biomarkers of DR4 and DR5 agonist sensitivity in TNBC. Larger sample sizes in future studies may be more reflective of the outcomes associated with Axl and vimentin expression in breast cancer that is described in the literature.

In conclusion, results from this dissertation project contribute important observations about TRAIL sensitivity in TNBC cells. The characterization of gp78 highlights a potentially novel regulatory mechanism that may reveal important insights into the molecular underpinnings that govern TRAIL sensitivity in TNBC and, with further investigation, may lead to the identification of other targetable components involved in gp78 signaling and TRAIL resistance in TNBC. Axl and vimentin may serve as predictive biomarkers of DR4 and DR5 agonist sensitivity in TNBC as both have been found to be expressed in TRAIL-sensitive TNBC cells and in human TNBC tumors. In its entirety, this dissertation project has linked together elements of the earliest phases of translational research to findings that are more immediately applicable to clinical science in the context of activating the TRAIL pathway for TNBC treatment. Further investigation is warranted.

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APPENDIX

IDENTIFICATION OF NOVEL MOLECULAR REGULATORS OF TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL)-INDUCED APOPTOSIS IN BREAST CANCER CELLS BY RNAI SCREENING

Garimella, V., Gehlhaus, K., Dine, J.L., Pitt, J.J., Grandin, M., Chakka, S., Nau, M.M., Caplen, N.J., & Lipkowitz, S. (2014). Identification of novel molecular regulators of tumor necrosis factor-related apoptosis inducing-ligand (TRAIL)-induced apoptosis in breast cancer cells by RNAi screening. *Breast Cancer Research*, *16*(2), R41. doi: 10.1186/bcr3645

Abstract

Introduction: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) binds to its receptors, TRAIL-receptor 1 (TRAIL-R1) and TRAIL-receptor 2 (TRAIL-R2), leading to apoptosis by activation of caspase-8 and the downstream executioner caspases, caspase-3 and caspase-7 (caspase-3/7). Triple-negative breast cancer (TNBC) cell lines with a mesenchymal phenotype are sensitive to TRAIL while other breast cancer cell lines are resistant. The underlying mechanisms that control TRAIL sensitivity in breast cancer cells are not well understood. Here, we performed small interfering RNA (siRNA) screens to identify molecular regulators of the TRAIL pathway in breast cancer cells.

Methods: We conducted siRNA screens of the human kinome (691 genes), phosphatome (320 genes), and ≈300 additional genes in the mesenchymal TNBC cell line MB231. Forty-eight hours post-transfection of siRNA, parallel screens measuring caspase-8 activity, caspase-3/7 activity, or cell viability were conducted in the absence or presence

of TRAIL for each siRNA relative to a negative control siRNA (siNeg). A subset of genes was screened in cell lines representing epithelial TNBC (MB468), HER2-amplified breast cancer (SKBR3), and estrogen receptor positive breast cancer (T47D). Selected putative negative regulators of the TRAIL pathway were studied using small molecule inhibitors.

Results: The primary screens in MB231 identified 150 genes, including 83 kinases, 4 phosphatases, and 63 non-kinases, as potential negative regulators of TRAIL. The identified genes are involved in many critical cell processes including apoptosis, growth factor receptor signaling, cell cycle regulation, transcriptional regulation, and DNA repair. Gene network analysis identified four genes (*PDPK1*, *IKBKB*, *SRC*, and *BCL2L1*) that formed key nodes within the interaction network of negative regulators. A secondary screen of a subset of the genes identified in additional cell lines representing different breast cancer subtypes and sensitivities to TRAIL validated and extended these findings. Further, we confirmed that small molecule inhibition of SRC or BCL2L1 in combination with TRAIL sensitizes breast cancer cells to TRAIL-induced apoptosis, including cell lines resistant to TRAIL-induced cytotoxicity.

Conclusions: These data identify novel molecular regulators of TRAIL-induced apoptosis in breast cancer cells and suggest strategies for the enhanced application of TRAIL as a therapy for breast cancer.

Keywords: TRAIL, RNAi screening, kinases, breast cancer, caspase, apoptosis

IDENTIFICATION OF NOVEL MOLECULAR REGULATORS OF TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL)-INDUCED APOPTOSIS IN BREAST CANCER CELLS BY RNAI SCREENING

TRAIL may have potential use in cancer therapy because of its ability to selectively kill cancer cells over normal cells (Ashkenazi et al., 1999; Pitti et al., 1996; Walczak et al., 1999). TRAIL binds to its receptors (TRAIL-R1 (DR4) or TRAIL-R2 (DR5)) on the cell surface leading to the recruitment of the adaptor molecule FADD and pro-caspase-8 (Ashkenazi, 2008). This forms the death inducing signaling complex (DISC). Pro-caspase-8 is cleaved to its active form at the DISC, which then cleaves and activates the downstream executioners caspase-3 and caspase-7 (caspase-3/7) resulting in apoptosis. Active caspase-8 also can cleave the BH3 protein BID resulting in activation of the intrinsic pathway of apoptosis by activating caspase-9 (Reviewed in (Rahman, Pumphrey, & Lipkowitz, 2009)). Studies in animals have shown that TRAIL mediates regression of cancer xenografts without affecting normal tissues and human phase I studies have demonstrated that TRAIL agonists are safe (Walczak et al., 1999; Yerbes, Palacios, & Lopez-Rivas, 2011). However the results published thus far have shown limited clinical efficacy suggesting the need to identify predictive biomarkers that will stratify cancers into those more likely to respond and/or to identify additional genes or pathways that can be targeted in combination with TRAIL to enhance the efficacy of TRAIL agonists (Camidge et al., 2010; Doi et al., 2011; Forero-Torres et al., 2010; Herbst et al., 2010; McGrath, 2011; Soria et al., 2011; Yerbes et al., 2011; Younes et al., 2010).

Preclinical studies have found that many cell lines of different cancer types are resistant to TRAIL. Initial studies of TRAIL-induced apoptosis in breast cancer cell lines demonstrated that while TRAIL could induce apoptosis in the MDA-MB-231 (MB231) breast cancer cell line, the majority of cell lines tested were very resistant to TRAIL-induced apoptosis (Ashkenazi et al., 1999; Buchsbaum et al., 2003; Keane, Ettenberg, Nau, Russell, & Lipkowitz, 1999; Singh, Shankar, Chen, Asim, & Srivastava, 2003). These studies established that TRAIL-induced caspase-mediated apoptosis in sensitive cell lines and that TRAIL activated caspases within minutes of addition to the cells (Keane et al., 1999; Keane et al., 2000). Data from three independent studies, including ours, demonstrated that ten of fourteen triple-negative breast cancer (TNBC) cell lines were sensitive to TRAIL-induced apoptosis while only two of eight HER-2 amplified cell lines, and none of seven estrogen receptor (ER) positive lines were sensitive to TRAIL-induced apoptosis (Buchsbaum et al., 2003; Chinnaiyan et al., 2000; Rahman, Davis, et al., 2009; Rahman, Pumphrey, et al., 2009). Amongst the TNBC subtype, cells with mesenchymal features are more sensitive to TRAIL than cells with epithelial features (Rahman, Davis, et al., 2009). However the underlying determinants of TRAIL sensitivity in the breast cancer cell lines have not been clearly established.

In this study, we took advantage of RNAi screening technology to identify novel molecular regulators of TRAIL-induced apoptosis in breast cancer cells. Using synthetic siRNA-mediated RNAi screens of the human kinome, phosphatome, and ≈300 additional genes, we identified a subset of ≈150 genes that, when silenced, enhance TRAIL-induced caspase-3/7 activation in MB231 cells. These genes can be grouped into cellular networks that modulate the sensitivity to TRAIL in breast cancer cells. Analysis of the

caspase-8 activation and cell viability RNAi screening data for those genes associated with these cellular networks corroborated a potential role for many of these proteins in regulating TRAIL-medicated apoptosis and cytotoxicity. RNAi screening of a subset of the identified genes in a panel of breast cancer cell lines representing different breast cancer subtypes (TNBC, HER2 amplified, and ER positive) identified potential targets that may have broad application in enhancing TRAIL activity in breast cancer cells. Importantly, pharmacological inhibition of two targets identified by RNAi screening, SRC or BCL2L1 (BCL-XL), sensitized cell lines known to be resistant to TRAIL-induced cell death confirming the utility of the RNAi screen.

Materials and Methods

Cell Culture

The MB231, HCC38, BT549, BT474, MCF7, Hs578T, and SKBR3 breast cancer cell lines were obtained from ATCC; BT20 and HCC1937 were obtained from Reinhard Ebner (Avalon Pharmaceuticals; Germantown, MD). All cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% Pen-Strep (R10). This research was performed with anonymized breast cancer cell lines and is exempt from ethics or IRB approval.

Inhibitors and Reagents

The GST-TRAIL construct and the isolation of recombinant GST-TRAIL fusion protein have been previously described (Keane et al., 1999). The inhibitors PP2 (529573) and PP3 (529574) were obtained from Calbiochem (La Jolla, CA), ABT-737 (S1002) was obtained from Selleck Chemicals (Houston, TX), and DEVD-CHO (P410) from Biomol International (Plymouth Meeting, PA). All inhibitors were dissolved in DMSO. Caspase-

Glo 8 assay (G8202) and Caspase-Glo 3/7 assay (G8092) and Caspase-Glo 9 (G8210) systems were purchased from Promega Corporation (Madison, WI).

Caspase Activation Assays, Cell Viability Assays, RNAi Screening, and Small Molecule Compound Analysis

Primary RNAi screens were conducted using siRNAs corresponding to 1135 genes arrayed from the Human Druggable Genome Version 2.0 library (Qiagen Inc.; Germantown, MD). The siRNAs target 691 genes annotated as associated with kinase activity, 206 genes associated with phosphatase activity, and 238 additional genes that include members of the ABC transporter family and several apoptosis associated genes. The majority of the genes within the kinase and phosphatase sets encode proteins with defined kinase or phosphatase activity respectively, though a limited number act as enzyme co-factors and a few have been re-annotated now as pseudogenes or withdrawn. See Additional file 1 (Table S1) for full details of genes targeted. The 16 genes (four siRNAs per gene) selected for secondary screening are detailed in Additional file 2 (Table S2).

For screening (primary and secondary), four siRNAs per gene were arrayed in 384 well plates, one siRNA per well. For each well, 2 pmol siRNA was complexed with 0.06 μl RNAiMax transfection reagent (Invitrogen; Grand Island, NY) in 20 μl RPMI for 15 minutes (m) at ambient temperature. Six hundred cells in 20 μl RPMI-1640/20% FBS were added to each well. Plates were maintained at room temperature for 15 min before incubation at 37°C/5% CO_2. Paired screens were conducted: 48 hours post siRNA transfection one screen received vehicle only (medium), while the other received 1000

ng/ml TRAIL (in medium) for one hour for the study of caspase-3/7 and caspase-8 activation or 100 ng/ml of TRAIL (in medium) for 24 hours for the study of cell viability. The activation of caspase-8 and caspase-3/7 were measured using Caspase Glo Assay systems following the manufacturer's instructions (Promega Corporation; Madison, WI) with modification of the caspase-8 assay to block caspase-3/7-induced activation of caspase-8 (see results for further details). Cell viability was measured using Cell Titer Glo assay following the manufacturer's instructions (G7572, Promega Corporation; Madison, WI). All assay plates were measured with a Victor luminometer (Perkin Elmer; Waltham, MA). The kinome and additional sets were screened together, while the phosphatase gene set was screened separately. As these screens were conducted at different times the data for each screen was initially analyzed independently. To validate each screen, untransfected cells (cells only) and wells transfected with negative (AllStars Negative Control siRNA (siNeg); Qiagen, Valencia, CA) and positive (AllStars Hs Celldeath siRNA (siCelldeath); Qiagen, Valencia, CA) control siRNAs were included on every plate as were siRNAs corresponding to *CASP8* and *FLIP* (siCASP8, L003466 and siFLIP, L003772; from Dharmacon, Thermo Fisher Scientific, Waltham, MA). A summary of the controls for the kinome/additional gene set screen are shown in Figure 2A and for the phosphatase gene set screen in Additional file 3 (Figure S1A). The summary of the controls also is included in Additional file 1 (Table S1) to ease comparison to the screening data. Assay specific Z-factors for each screen are shown in Additional file 4 (Table S3). The data for each experimental siRNA was normalized using the average value for siNeg-transfected cells without TRAIL for each plate. The data for all three screens is detailed in Additional file 1 (Table S1).

For assay development and treatment with the SRC or BCL-XL inhibitors (Figures 1, 6 and 7 respectively), cell viability was assessed using the Cell Titer 96AQueous One Solution Cell Proliferation Assay (G3582) from Promega Corporation (Madison, WI). All measurements were performed in replicates of six wells in a 96-well plate and each experiment was carried out at least three times. Results are presented as the mean ± the standard error of the mean (SE) of at least three independent experiments in Figures 1, 6, and 7.

Lysate Preparation and Immunoblotting

Cell lysates were made and immunoblotting was performed as described earlier (Rahman, Davis, et al., 2009). The following antibodies were used: anti-AKT (#4685), anti-phospho-AKT (T308; #4056), anti-caspase-8 (1C12; #9746), anti-ERK 1/2 (#9102), anti-phospho-ERK 1/2 (#9101), anti-GAPDH (#2118), anti-p70S6K (#2708), and anti-phospho-p70S6K (S371; #9205) from Cell Signaling Technology (Danvers, MA), anti-FLIP (#104) from Imgenex (San Diego, CA), anti-SRC (#OP07) from EMD Millipore (Billerica, MA), anti-phospho-SRC (#44-660G) from Life Technologies (Grand Island, NY), and anti-Tubulin (#T9026) from Sigma Aldrich (St. Louis, MO).

Statistics and Bioinformatics Analysis

Student's t-test (unequal variance) was used to determine statistical differences between siRNA control groups (calculated in Excel). A value of $p < 0.05$ was considered significant. A Pearson correlation coefficient was used to compare the relationship between screens and was calculated in Excel. Paired Student's t-tests were also performed to analyze the data shown in Figures 6C, 6E and 7C. To compare the effect of the combined treatment to the sum of the effects of the individual treatments, percent

inhibition was calculated for each condition as 100 percent viability. The inhibition of the combination was compared to the sum of the inhibition of TRAIL alone plus inhibitor alone. Knowledge-based gene networks were generated using Ingenuity Pathway Analysis (IPA) tools (Ingenuity Systems®; Redwood City, CA).

Results

The Development of Assays for RNAi Screens of TRAIL-induced Apoptosis

To identify regulators of TRAIL-induced apoptosis we established conditions compatible with siRNA-based RNAi screening for three assays that assess different steps in the TRAIL-induced apoptotic pathway in the MB231 breast cancer cell line. We chose to use the TRAIL-sensitive MB231 cell line and a concentration of TRAIL that induced approximately 50% maximum activity in each assay to enable identification of both positive and negative regulators of the TRAIL pathway. We used two assays that measured activation of caspases by TRAIL, one for activation of the initiator caspase-8 and one for the activation of the downstream effectors caspases-3 and 7 (caspases-3/7). We also utilized an assay of cell viability (Figure 1A).

Assays were optimized to detect measurable levels of caspase-8 and caspase-3/7 activity using substrates specific for each caspase. To identify an appropriate concentration of TRAIL to be used for identification of proteins that modulate early steps in TRAIL-induced apoptosis, MB231 breast cancer cells were treated with different concentrations of TRAIL and, after one hour, caspase activity was measured. A TRAIL concentration-dependent increase in activity was observed for both caspase-8 and caspase-3/7 (Figure 1B). At 1000ng/ml of TRAIL, we detected a six-fold change in caspase-3/7 activity and a 4.8-fold change in caspase-8 activity over untreated cells. 1000

ng/ml of TRAIL was used to induce robust caspase activation within the one hour caspase assays, and is a much higher concentration than that needed to induce loss of viability when cells were exposed to TRAIL for >17 hours to assess cytotoxicity (Figure 1D and discussed below). Caspase-8 is the first caspase to be activated upon TRAIL binding to its receptors. Also, caspase-8 can be activated in a retrograde fashion by active caspase-3/7 (Figure 1A) (Slee et al., 1999; Sun et al., 1999). To measure the caspase-8 activity triggered by the TRAIL receptors and not that produced from active caspase-3/7, we treated cells with a caspase-3/7 inhibitor, DEVD-CHO, one hour prior to TRAIL treatment (Figure 1C). In the presence of 0.03 μM DEVD-CHO, TRAIL-induced caspase-3/7 activity was inhibited to baseline levels in comparison to 5.5-fold activation over the untreated controls. By contrast, there was only slight loss in TRAIL-induced caspase-8 activation in the presence of DEVD-CHO compared to TRAIL-induced activation of caspase-8 in the absence of DEVD-CHO (3-fold versus 3.8-fold). Therefore to ensure direct measurement of TRAIL-receptor mediated caspase-8 activation we used 0.03 μM DEVD-CHO in our screening assay of caspase-8 activation.

To further develop the screening assays we utilized control siRNAs corresponding to a positive effector of TRAIL-induced apoptosis, caspase-8 (CASP8), and a known negative regulator of TRAIL-induced apoptosis, the FLICE-like inhibitory protein (FLIP, *a.k.a.* CFLAR; (Rahman, Pumphrey, et al., 2009)) (Figure 1A). Silencing of *CASP8* should lead to the suppression of apoptosis that can be assayed as an inhibition of caspase-8 and caspase-3/7 activation and a reduction in TRAIL-induced cytotoxicity. In contrast, silencing of FLIP should enhance the activation of caspase-8 and caspase-3/7 and further sensitize cells to TRAIL-induced cytotoxicity. We confirmed the effects of

silencing *CASP8* and *FLIP* by transfecting MB231 cells with specific siRNAs for these genes and 48 hours later treating with 100 ng/ml of TRAIL. Control cells were transfected with a control siRNA (siNeg). Seventeen hours after the addition of TRAIL, cell viability was measured by MTS assay and the values were plotted relative to untreated siNeg transfected cells (Figure 1D). In the siNeg-transfected control cells, treatment with TRAIL resulted in 49.0±9.5% cell death. Caspase-8 is a known positive regulator of the TRAIL-induced apoptotic pathway, and its silencing resulted in decreased caspase activation similar to untreated cells. Upon silencing of *CASP8* and treatment with TRAIL, viability was 92.7±10.45% and not statistically different from untreated *CASP8* silenced cells (104.97±12.73%). *FLIP* structurally resembles caspase-8, but lacks the proteolytic activity, and is a competitive inhibitor of the TRAIL pathway. The silencing of *FLIP* enhanced the sensitivity of MB231 cells to TRAIL as measured by increased loss of cell viability (68.0±2.0%) compared to siNeg-transfected cells. Thus, siRNAs corresponding to *CASP8* (siCASP8) and *FLIP* (siFLIP) were used as controls for positive and negative regulators of the TRAIL pathway respectively in all of the RNAi screens.

RNAi Screens of the TRAIL-induced Apoptotic Pathway in the Breast Cancer Cell Line MB231

RNAi screens designed to interrogate different aspects of the TRAIL-induced apoptotic pathway by measuring caspase-8 activation, caspase-3/7 activation, and cell viability were performed as described in the Materials and Methods. The kinome and additional gene sets were screened together using all three-assay end-points. The phosphatase gene set was screened separately using just the caspase-3/7 activation and

cell viability assays. The kinome and additional gene sets were screened and analyzed together while the phosphatase gene set was screened and analyzed separately. To validate each screen, wells of untransfected cells (cells only) and wells transfected with negative (siNeg) and positive (siCelldeath) control siRNAs were included on every plate, as were wells of siRNAs corresponding to *CASP8* and *FLIP*. A summary of the controls for the kinome/additional gene set screen is shown in Figure 2A and for the phosphatase gene set screen in Additional file 3 (Figure S1A). The Z-factor values for each assay are shown in in Additional file 4 (Table S3).

In the absence of siRNA or in the siNeg treated cells, TRAIL induced a 2- to 2.5-fold increase in caspase-8 activity and 6- to 7-fold increase in caspase-3/7 activity (Figure 2Ai and 2Aii respectively). Silencing of *CASP8* resulted in a significant reduction of TRAIL-induced caspase-8 and -3/7 activities, similar to the level of untreated cells (Figure 2Ai and 2Aii respectively). Conversely, silencing of *FLIP* resulted in a statistically significant increase in caspase-8 or caspase-3/7 activity (Figure 2Ai and 2Aii respectively). TRAIL induced an approximately 50% reduction in cell viability in untreated or siNeg-transfected cells (Figure 2Aiii). Silencing *CASP8* completely blocked the TRAIL-induced loss of viability, whereas silencing *FLIP* resulted in a significantly greater TRAIL-induced loss of viability (Figure 2Aiii). Similar results for caspase-3/7 activation and viability were seen in the control samples for the siRNA screen of the phosphatase gene set (Additional file 3, Figure S1A). The data for each experimental siRNA was normalized using the average value for siNeg-transfected cells without TRAIL for each plate. The data for all three screens is detailed in Additional file 1 (Table S1).

We first evaluated the correlation between the results for each siRNA in the three screens, a total of over 4000 data points, (Figures 2B and C). In the absence of TRAIL few siRNAs affected caspase-8 or caspase-3/7 activation or the viability of MB231 cells (Figure 2B). Though, for example, we did observe that three of four siRNAs corresponding to PLK1 induced activation of caspase-8, caspase-3/7 and a decrease in viability in the absence of TRAIL (Additional file 3, Figure S1B), which is consistent with previous studies (Spankuch-Schmitt, Bereiter-Hahn, Kaufmann, & Strebhardt, 2002). In contrast, in the presence of TRAIL a substantial number of siRNAs increased activation of caspase-8 and caspase-3/7, and decreased the viability of MB231 cells in response to TRAIL (Figure 2C). Importantly, in the presence of TRAIL, a positive Pearson correlation of 0.47 was observed when levels of caspase-8 and caspase-3/7 were compared while negative correlations were observed when caspase-8 activation or caspase-3/7 activation were compared with cell viability (caspase-8 versus cell viability: $r = -0.23$; and caspase-3/7 versus cell viability: $r = -0.55$). These data demonstrated that the effects on caspase activation and cell viability were generally consistent for each of the individual siRNAs.

The Identification of Putative Regulators of the TRAIL Pathway

Of the three RNAi screening end-points, overall, the siRNA screens of TRAIL conducted using caspase-3/7 as an end-point showed the greatest range of fold-change in activation relative to controls (up to over 30 fold). Thus we chose to focus on the results of the caspase-3/7 screen to initially identify regulators of TRAIL and use the caspase-8 and cell viability screening data to corroborate our findings. We defined putative negative regulators of TRAIL as those genes for which at least three of the four siRNAs tested

caused an increase in TRAIL-induced caspase-3/7 activation two standard deviations or more from the TRAIL-induced caspase-3/7 activation seen with the control siNeg siRNA. This corresponded to a >10.28-fold change for the kinase and additional gene set screens and a >7.96-fold change for the phosphatase gene set. These fold changes were comparable with that seen following silencing of the negative regulator *FLIP* (10.58-fold change for the kinase and additional gene set screens and 7.54-fold for the phosphatase gene set). These criteria identified 83 kinases or kinase related genes (Figure 3A), four phosphatases (Figure 3B), and 63 genes from the additional gene set (Figure 3C) whose silencing augmented TRAIL-induced caspase-3/7 activity. The screen identified several known negative regulators of apoptosis as negative regulators of TRAIL-induced caspase-3/7 activation, including *BCL2L1* (BCL-XL), *BCL2L2* (BCL-w), *BIRC2* (c-IAP1), and *BIRC3* (c-IAP2) (Deveraux & Reed, 1999; Gross, McDonnell, & Korsmeyer, 1999).

Also we assessed whether any genes act as positive regulators of TRAIL activity. We defined positive regulators of TRAIL-induced caspase activation as those genes where at least three of four siRNAs resulted in TRAIL-induced caspase-3/7 activation that was two or more standard deviations less than that seen in cells treated with the siNeg control (a <4.06-fold change for the kinase and additional gene set screens and a <2.77-fold change for the phosphatase gene set). Interestingly, using these criteria no positive regulators of TRAIL-induced caspase-3/7 activation were identified. Silencing of *CASP8* clearly inhibited TRAIL-induced activation of caspase-3/7 by more than two standard deviations indicating that the screen was capable of identifying such genes (Figure 2Aii). Relaxing the criteria to siRNAs that resulted in more than a one standard

deviation reduction in TRAIL-induced caspase-3/7activation compared to the siNeg-control, identified eight genes as putative positive regulators of TRAIL-induced caspase-3/7 activation (*NEK6*, *ETNK1*, *NME5*, *PXK*, *CALM2*, *RPS6KB2*, *GK5*/MGC40579 and *AKR1B1;* Figure S1C).

Gene Network Analysis and Experimental Corroboration of Negative Regulators of TRAIL

We focused our subsequent analysis on putative negative regulators of TRAIL-induced caspase-3/7 activation rather than positive regulators because of the number of genes identified and because they may be potential targets that when inhibited will enhance TRAIL-induced apoptosis. Given the relatively large number of putative negative regulators of TRAIL-induced apoptosis we subjected the 150 genes to network gene analysis in order to aid in identification of common regulatory networks in which these genes function. Of the 147 genes with curated interaction data, the largest network identified connected 79 genes (see Additional file 5, Figure S2). Of these 79 genes, 42 were connected principally *via* four genes with seven or more interactions (Figure 4A). The genes situated at these nodes are *BCL2L1* (BCL-XL), *IKBKB*, *PDPK1* and *SRC* (indicated by the blue circles in Figure 4A). To further investigate the TRAIL-associated loss-of function (LOF) phenotype of the 79 genes that formed this regulatory network we examined the results of silencing these genes on caspase-8 activation and cell viability in the presence of TRAIL.

Three or more siRNAs against 26 of the 79 genes that enhanced TRAIL-induced activation of caspase-3/7 also enhanced TRAIL cytotoxicity by greater than two standard deviations from the mean viability seen in siNeg-transfected cells plus TRAIL. As

indicated by the red rectangles in Figure 4A, 14 of these 26 genes map to the direct interaction network. The silencing of *BCL2L1* and two genes directly linked to it, *ATP5A1* and *HIPK2*, by multiple siRNAs increased TRAIL-induced caspase-3/7 activation and cytotoxicity (Figure 4B and Additional file 6, Figure S3). In addition, the RNAi induced LOF of several genes linked to *SRC* enhanced TRAIL-induced cytotoxicity including *PDPK1*, *CNKSR1*, *PIP5K1C*, *FGFR4*, *BCR*, *RIOK3* and *MKNK1* (Figure 4B and Additional file 6, Figure S3). All three of the siRNAs corresponding to *SRC* that activated caspase-3/7 in the presence of TRAIL enhanced cytotoxicity, but only using a relaxed criterion of greater than one standard deviation from the mean viability seen in siNeg-transfected cells plus TRAIL (Additional file 6, Figure S3). Multiple siRNAs corresponding to *PDPK1* and several genes linked to *PDPK1* (Figure 4B) also increased TRAIL-induced caspase-3/7 activation and cytotoxicity. These included *PRKC1*, the known apoptosis inhibitor *BIRC2* (*a.k.a.*, *cIAP-1*), *PLK3*, *PKN1*, and *ACTN4* (Additional file 6, Figure S3). Silencing by two of four siRNAs of many of the remaining genes mapping to the direct interaction network induced a decrease in cell viability greater than two standard deviations from that seen in siNeg-transfected cells plus TRAIL with at least one further siRNA inducing a decrease in viability at least one standard deviation from that seen in siNeg-transfected cells plus TRAIL. This included silencing of *IKBKB* (Figure S3), *BLK*, *ERBB2*, *FGFR2*, *NAGK*, and *ZC3HC1 (PARP12)*.

The results for the activation of caspase-8 were more variable. Only one gene *BCL2L1* (BCL-XL) showed an increase in caspase-8 levels more than one standard deviation of that seen in siNeg-transfected cells for three or more siRNAs (Figure 4Bi). In several other cases two of four siRNAs corresponding to a specific gene mediated an

increase in caspase-8 levels more than one standard deviation of that seen in siNeg, including *CNKSR1*, *BCR*, and *PIP5KIC* which all linked to *SRC* (Figure 4Bi and Additional file 6, Figure S3), *ATP5A1* that links to *BCL2L1* (Additional file 6, Figure S3), and *PRKC1* that is linked to PDPK1 (Additional file 6, Figure S3). There were two genes for which three siRNAs activated caspase-3/7 and -8 but that did not map to the network based on direct interactions, *BCL2L2* and *APEX1*. Interestingly, all four siRNAs corresponding to *BCL2L2* (BCL-w) enhanced TRAIL-induced caspase-3/7 activation and three of these siRNAs also enhanced TRAIL-induced caspase-8 activation, but no effect on cell viability was observed (Additional file 6, Figure S3). Three siRNAs corresponding to the APEX nuclease (multifunctional DNA repair enzyme) 1 gene, *APEX1*, enhanced TRAIL-activated caspase-3/7 and caspase-8 and decreased cell viability, but the individual siRNAs that generated these phenotypic changes were inconsistent (Additional file 6, Figure S3).

Secondary RNAi Screen Validation in Additional Breast Cancer Cell Lines

To validate a subset of the genes identified by the primary siRNA screen in MB231 as putative negative regulators of TRAIL activity, we selected 16 genes for secondary screening in four breast cancer cell lines and assayed caspase-3/7 activation. Fifteen of the genes chosen (annotated by red boxes in Figure 4) were those that when silenced induced both an increase in activation of caspase-3/7 and a decrease in viability in response to TRAIL (Figures 4 and Additional file 6, Figure S3). We also included *IKBKB* as this formed a node in the interaction map with seven or more interactions, and when silenced, three of four siRNAs induced an increase in TRAIL-induced activation of caspase-3/7 and two of four siRNAs decreased viability in response to TRAIL (Figures 4

and Additional file 6, Figure S3). For the secondary screen we used MB231 and three additional breast cancer cell lines (MB468, SKBR3, and T47D) representing different subsets of breast cancer with different sensitivities to TRAIL. The MB231 cell line is a basal B/TNBC cell line, MB468 is a basal A/TNBC cell line, SKBR3 is a HER2 amplified cell line, and T47D is an ER positive cell line (Neve et al., 2006; Rahman, Davis, et al., 2009). Upon treatment with TRAIL, there is a robust activation of caspase-3/7 in the MB231 cell line, an intermediate activation of caspase-3/7 in the MB468 cell line, and little or no caspase-3/7 activation in the SKBR3 and T47D cell lines (see Additional file 7, Figure S4). The siRNAs used for the secondary screen are listed in Additional file 2 (Table S2). Some of the siRNAs used in the primary screen above were no longer available and substitutes were obtained.

The results for the secondary screen are detailed in Figure 5 and Additional file 8 (Table S4) and summarized in Table 1. Upon rescreening the 16 genes in MB231, the silencing of 13 of the 16 genes again showed a two standard deviation increase in TRAIL-induced caspase-3/7 activity by three or more of the siRNAs to each target (Figure 5A and Table 1 and Additional file 8, Table S4). We used two criteria to rank the degree of validation of a gene as a negative regulator of TRAIL-induced apoptosis based on three or more siRNAs corresponding to each gene enhancing TRAIL-induced caspase-3/7 activation by either (1) greater than two standard deviations (indicated in Table 1 as a +; high stringency) or (2) greater than one standard deviation (indicated in Table 1 as a (+); low stringency) from that observed in siNeg-transfected cells treated with TRAIL.

In MB231 cells, 13 of the 16 genes were validated at high stringency (based on criterion 1). LOF of two genes, *MNNK1* and *HIPK2*, only replicated when a more relaxed

stringency (criterion 2) was used (Figure 5A, Table 1 and Additional file 8, Table S4). Only LOF of IKBKB failed to replicate based on the lower stringency although two of the four siRNAs increased TRAIL-induced caspase-3/7 activation by more than one standard deviation. Overall, these results in MB231 confirmed the reliability of the primary screen results. Interestingly, LOF of all 16 genes enhanced TRAIL-induced caspase-3/7 activation in the TNBC/basal A cell line MB468 using the high stringency criterion of three or more siRNAs enhancing TRAIL-induced caspase-3/7 activation by more than two standard deviations. (Figure 5B, Table 1 and Additional file 8, Table S4).

The ER positive T47D cell line and the HER2 amplified cell line SKBR3 are resistant to TRAIL-induced cytotoxicity (Additional file 7, Figure S4). Any alteration in the sensitivity of these cells to TRAIL is likely to represent an important regulator of TRAIL and a potential target for enhancing its activity in breast cancer more broadly. In T47D cells, the LOF of 4 genes met the high stringency criterion 1 (*BCL2L1, BCR, ATP5A1, and ACTN4*), and six additional genes met the lower stringency criterion 2 (*BIRC2, PDPK1, PIP5K1C FGFR4, SRC, RIOK3*) (Figure 5C, Table 1 and Additional file 8, Table S4). In SKBR3, LOF of only two genes met the high stringency criterion (*BIRC2* and *ACTN4*) and LOF of one additional gene (BCL2L1) met the lower stringency criterion 2 (Figure 5D, Table 1 and Additional file 8, Table S4). Overall this suggests that *BCL2L1, BIRC2,* and *ACTN4* are potentially major regulators of TRAIL-induced caspase-3/7 in breast cancer and that their LOF has the potential to overcome resistance to TRAIL-induced cytotoxicity. Other genes including *ATP5A, BCR, FGFR4, PDPK1, PIP5K1C, RIOK3,* and *SRC,* also act to regulate TRAIL-induced apoptosis, but their

potential to overcome resistance to TRAIL-induced cytotoxicity when inhibited may be more context specific (*i.e.*, in a more restricted subset of breast cancer cells).

The Inhibition of SRC or BCL-XL Enhances TRAIL Sensitivity of TRAIL Resistant Breast Cancer Cell Lines

To translate the results of the RNAi screens by using a pharmacological approach, we chose to next focus on *SRC* and *BCL2L1* (BCL-XL) for which small molecule inhibitors are readily available. Based on our siRNA studies, LOF of SRC may potentially represent a context specific modulator of TRAIL activity while LOF of *BCL2L1* may modulate TRAIL activity in a broader range of breast cancer cell types.

In our screen of additional breast cancer cell lines, LOF of SRC enhanced TRAIL-induced caspase-3/7 activation by two or more standard deviations in the two TNBC cell lines MB231 and MB468, and by one standard deviation in the ER positive cell line T47D (Figure 5, Table 1 and Additional file 8, Table S4). Inhibition of SRC in MB231 cells by the SRC-kinase family small molecule inhibitor, PP2, resulted in decreased auto-phosphorylation of SRC compared to its non-functional structural analog, PP3 (Figure 6A) (Hanke et al., 1996). Prior work demonstrated that inhibition of SRC led to decreased activation of the PI3 Kinase/AKT pathway and this in turn resulted in increased TRAIL sensitivity (Phipps, Hino, & Muschel, 2011; Song et al., 2010). To test this we examined the effects of PP2 on downstream signaling pathways and demonstrated that inhibition of PP2 results in a decrease in activated AKT and activated p70 S6 Kinase as measured by phosphorylation of these proteins (Figure 6B). By contrast, no effect was seen in phosphorylation of ERK (Figure 6B).

Silencing of SRC by RNAi followed by TRAIL treatment enhanced caspase-3/7 activation by more than ten-fold over siNeg-treated cells (Figure 3A and Additional file 1, Table S1). To test if PP2 has similar effects, we treated MB231 cells with PP2 or PP3 for two hours followed by 1000 ng/ml TRAIL and measured caspase-3/7 activation (Figure 6C). Cells treated with TRAIL exhibited a six-fold increase in caspase-3/7 activity over untreated cells. Inhibition of SRC by PP2 followed by TRAIL treatment resulted in a 40% increase in the caspase-3/7 activity over control cells. Cells treated with PP3 and TRAIL showed no significant increase in caspase-3/7 activation compared to control cells.

The effect of inhibiting SRC on TRAIL-induced loss of viability was tested in MB231 cells pre-incubated with either PP2 or PP3 prior to the addition of TRAIL (Figure 6D). The IC_{50} of TRAIL in these cells was ≈125ng/ml; the SRC inhibitor, PP2, by itself did not affect cell viability at 10 μM (92.9±2.3%), but the sensitivity of the cell line to TRAIL was significantly enhanced in the presence of PP2 with an IC50 for TRAIL of approximately 32 ng/ml in the presence of PP2 ($p<0.05$). The inactive compound, PP3, had little or no effect alone or in combination with TRAIL.

Previously we have shown that TNBC cells are more sensitive to TRAIL than other subtypes of breast cancer (Rahman, Davis, et al., 2009). We next investigated if SRC inhibition would sensitize TRAIL-resistant cells to TRAIL by testing the combination of TRAIL +/- PP2 on a panel of breast cancer cell lines representing ER positive (T47D or MCF7), HER2 amplified (SKBR3, BT474, HCC1954, MB453), TNBC/basal A (HCC1937, BT20, MB468), and TNBC/basal B (MB231, MB157, Hs578t) subtypes (Figure 6E). The combination of TRAIL and PP2 was more effective

than TRAIL alone in all cell lines tested (*p≤0.05) and was more effective than PP2 alone in all cell lines except the HER2 amplified cell line BT474 (**p≤0.05). When the inhibition of viability by the combined treatment was compared to the sum of the inhibition seen with TRAIL alone and PP2 alone, a significant difference was seen in the basal B TNBC cell lines MB231 and Hs578t, and the basal A TNBC cell line HCC1937 (***p≤0.05). While the combination appeared more active than the sum of the two agents alone in the TNBC/basal B cell line MB157, these data did not reach statistical significance in part due to the high sensitivity to TRAIL alone in this cell line. In the other cell lines, while the combination was more toxic than either treatment alone, the effects were relatively modest and not greater than the sum of the individual treatments (Figure 6E).

Our primary screen identified *BCL2L1* (BCL-XL) and *BCL2L2* (BCL-w), known negative regulators of the mitochondrial (intrinsic) apoptosis pathway, as putative negative regulators of TRAIL-induced apoptosis in MB231 cells (Figure 3). Further, *BCL2L1* was identified as a node in the gene-interaction network generated using our RNAi screening data (Figure 4). Silencing of *BCL2L1* enhanced TRAIL-induced caspase activation in three of the four cell lines tested at high stringency (two standard deviation cutoff) and in all four lines if a lower stringency was used (Figure 5, Table 1 and Additional file 8, Table S4).

Expression of BCL2L1 (BCL-XL) protein was measured in the four cell lines assayed in the secondary screen. BCL-XL was expressed in the four cell lines tested but it was expressed at higher levels in the TRAIL-resistant T47D and SKBR3 cell lines (Additional file 9, Figure S5A). We confirmed the enhancement of TRAIL-induced

caspase-3/7 activity using five different *BCL2L1* siRNAs in the four cell lines used for in the secondary screen (siRNAs are listed in Additional file 2, Table S2). All five siRNAs enhanced TRAIL-induced caspase-3/7 activation by more than two standard deviations in the TNBC cell lines MB231 and MB468 and four of the five enhanced TRAIL-induced caspase-3/7 activation by more than two standard deviations in the ER positive cell line T47D and in the HER2 amplified cell line SKBR3 (Figure 7A). The RNAi screens above were performed in 384 well plates. To confirm that the enhancement of TRAIL activity correlated with the knockdown of BCL-XL protein, we tested two of the siRNAs (*siBCL2L1.3* and *siBCL2L1.5*) in a larger scale experiment on MB231 cells. In the plate experiments above, knockdown of BCL-XL with *siBCL2L1.3* consistently enhanced TRAIL-induced caspase-3/7 activity more than knockdown with *siBCL2L1.5* (Figure 7A). In the larger scale experiment, knockdown of BCL-XL by both siRNAs enhanced TRAIL-induced caspase-3/7 activity and again knockdown of BCL-XL with *siBCL2L1.3* was more effective than knockdown with *siBCL2L1.5* in enhancing TRAIL-induced caspase-3/7 activity across a wide range of TRAIL concentrations (see Additional file 9, Figure S5B). Concordant with the effects on TRAIL-induced caspase-3/7 activation, *siBCL2L1.3* resulted in a greater knockdown of the BCL-XL protein than *siBCL2L1.5* (Additional file 9, Figure S5B). Thus the degree of BCL-XL protein knockdown correlated with the effect on TRAIL-mediated caspase-3/7 activity (Additional file 9, Figure S5B). Together, these data suggest that loss or inhibition of BCL2L1 may be useful in combination with TRAIL in a broad spectrum of breast cancer subtypes.

ABT-737 is an inhibitor of BCL-XL (*BCL2L1*), BCL-w (*BCL2L2*), and BCL-2 that has been shown to enhance cell death, including in MCF7 breast cancer cells and

myeloma cells by binding and inhibiting the activity of anti-apoptotic BCL2 family members (Cragg, Harris, Strasser, & Scott, 2009; van Delft et al., 2006). Treatment of MB231 cells with ABT-737 resulted in increased TRAIL-induced activation of caspase-8, caspase-9, and caspase-3/7 (Figure 7B, compare striped bars to white bars). Unlike treatment of the cells with siRNA targeting BCL-XL, treatment of the MB231 cells with ABT-737 had no effect on the levels of BCL-XL protein (see Additional file 9, Figure S5C). There was little or no increase in caspase-8, -9 or -3 activation when ABT-737 was added to cells in the absence of TRAIL (Figure 7B). While an increase in caspase-9 and caspase-3 was expected by the inhibition of BCL2 family members by ABT-737, the increased TRAIL-induced activation of caspase-8 by ABT-737 was unexpected since ABT-737 works downstream of the initiator caspase-8. However, prior work has demonstrated that caspase-8 can be activated by caspase-3 in a retrograde fashion, thus making it both an initiator and executioner caspase (Engels et al., 2000; Fulda, Meyer, & Debatin, 2002; Pirnia, Schneider, Betticher, & Borner, 2002; Slee et al., 1999; Sun et al., 1999; Wieder et al., 2001). To test this we measured the activation of caspase-8, -9 and -3/7 in the presence of the caspase-3/7 inhibitor DEVD-CHO. A low submaximal concentration of DEVD-CHO was used (30 nM) as this concentration was found to significantly inhibit TRAIL-induced caspase-3 activity but not inhibit TRAIL-induced caspase-8 or caspase-9 activity directly (compare gray bars to white bars in Figure 7B). When cells were pre-incubated with the DEVD-CHO there was no effect on the TRAIL-induced activation of caspase-8 in the absence of ABT-737 but DEVD-CHO abrogated the ABT-737-induced increase in TRAIL-induced caspase-8 activation (Figure 7B, top panels). This is consistent with caspase-3/7 contributing to the increase in caspase-8

activation seen in the presence of ABT-737. Caspase-9 activation by TRAIL alone or by TRAIL plus ABT-737 was not affected by DEVD-CHO (Figure 7B, middle panels). DEVD-CHO significantly inhibited the TRAIL-induced activation of caspase-3 in both the presence or absence of ABT-737 (Figure 7B, lower panels). These data are consistent with ABT-737 causing increased caspase-9 activation by caspase-8. This in turn results in more caspase-3/7 activation and then retrograde activation of caspase-8 by caspase-3/7.

Treatment of a panel of breast cancer cell lines with 5 μM ABT-737 (Figure 7C) using sub-IC_{50} concentrations of TRAIL, enhanced TRAIL-induced toxicity in all of the breast cancer subtypes tested (TNBC/Basal B, TNBC/Basal A, HER2 amplified, and ER+ breast cancer) (Figure 7C). The combined treatment of TRAIL plus ABT-737 inhibited viability more than TRAIL alone (*$p<0.05$) or ABT-737 alone (**$p\leq0.05$) in all cells tested. The toxicity of the combined treatment was greater than the sum of the toxicities for the individual treatments for all cell lines (***≤0.05), except for MB157. Again the high sensitivity to TRAIL alone in this cell line probably accounts for the failure of ABT-737 to significantly enhance the toxicity by this analysis.

Discussion

TRAIL is a promising cancer therapeutic agent showing efficacy against tumor cells and not affecting normal cells. However, *in vitro* experiments have found that many cancer cell lines are resistant to TRAIL (Rahman, Pumphrey, et al., 2009). The underlying determinants of TRAIL sensitivity are not clearly understood. Investigations into the mechanisms in cells that regulate sensitivity to TRAIL have implicated several pathways and factors. Regulation of the TRAIL receptors at the level of expression, localization to the cell surface, and O-glycosylation of the receptor proteins partially, but

not fully, correlate with sensitivity (reviewed in (Rahman, Pumphrey, et al., 2009)). TRAIL-resistance is also associated with elevated expression of anti-apoptotic factors like c-FLIP (Irmler et al., 1997), IAP family proteins (Takeda, Stagg, Yagita, Okumura, & Smyth, 2007), and BCL-2 (Liston, Fong, & Korneluk, 2003). In ongoing clinical trials, responses to TRAIL have been rare, especially in solid tumors (Engels et al., 2000; Fulda et al., 2002; Pirnia et al., 2002; Slee et al., 1999; Sun et al., 1999; Wieder et al., 2001). Therefore there is a need to identify proteins that regulate the TRAIL pathway as they could potentially serve as predictive biomarkers of TRAIL-sensitivity and/or provide additional targets for enhancing the efficacy of TRAIL.

To this end, we performed primary siRNA screens of the human kinome, phosphatome and some additional genes to identify regulators of TRAIL-induced apoptosis in the MB231 breast cancer cell line. We identified 150 genes (83 kinases or kinase related genes, 4 phosphatases or phosphatase related genes, and 43 other genes) as putative negative regulators of TRAIL-induced caspase-3/7 activation. For this study we adapted commercially available assays of caspase-8, caspase-3/7, and cell viability for high-throughput siRNA screens including the identification of highly sensitive biologically relevant controls. There was good positive correlation between those siRNAs that enhanced TRAIL-induced caspase-3/7 and those that enhanced TRAIL-induced caspase-8 activation (Figure 2Ci-ii). There was also good inverse correlation between the TRAIL-induced enhancement of caspase activation and the viability of TRAIL-treated cells. Thus, the three assays together strengthen the likelihood that the identified genes are regulators of the TRAIL pathway. The identification of several established negative regulators of apoptosis as negative regulators of TRAIL-induced caspase-3/7 activation,

including *BCL2L1* (BCL-XL), *BCL2L2* (BCL-w), *BIRC2* (c-IAP1), and *BIRC3* (c-IAP2), lends further support to the validity of the screen results.

Interestingly, other candidate genes identified by our screens have been linked recently to TRAIL activity. For example, the expression of argininosuccinate synthase 1 (*ASS1*) has been described as a member of a predictive panel of 71 genes whose expression correlates with TRAIL sensitivity (Chen, Knudsen, Mazin, Dahlgaard, & Zhang, 2012). *ASS1* was the only gene in common between the 71-gene signature and the set of genes found in our screen. Based on our experiments, *ASS1* is a putative negative regulator of TRAIL sensitivity, and LOF induced an increase in caspase-3/7 activation (Figure 3). *ASS1* is the rate-limiting enzyme in arginine biosynthesis and interestingly two studies have demonstrated that loss of *ASS1* sensitizes lymphoma and glioblastoma cells to apoptosis induced by arginine deprivation (Delage et al., 2012; Syed et al., 2013). The LOF of *ASS1*, then, may result in arginine depletion and make cells more susceptible to TRAIL-induced apoptosis. Elucidating the mechanism by which *ASS1* negatively regulates TRAIL-induced apoptosis will require further study.

Amongst the ~1300 genes assessed at the higher stringency (that of a two standard deviation change in TRAIL-induced caspase-3/7 activity) these RNAi screens did not identify positive regulators of TRAIL. Several potential positive TRAIL-regulators were identified when the stringency was relaxed to a one standard deviation change in TRAIL-induced caspase-3/7 activation (Additional file 3, Figure S1). None of these putative positive regulators have been linked previously to the regulation of TRAIL-induced apoptosis or apoptosis in general, though one of the genes identified, *PXK*, has been recently shown to enhance degradation of the activated epidermal growth

factor receptor (EGFR) (Takeuchi, Takeuchi, Gao, Cantley, & Hirata, 2010). We and others have shown that EGFR activity can attenuate TRAIL-induced apoptosis and that inhibition of the EGFR enhances TRAIL-induced apoptosis (Bremer et al., 2005; Rahman, Davis, et al., 2009; Xu et al., 2011). Thus, PXK LOF may enhance TRAIL activity by the down-regulation of the EGFR, though this hypothesis will require further study.

The absence of strong positive regulators in our RNAi screens suggests that the primary regulation of TRAIL-induced apoptosis is *via* inhibition of the TRAIL pathway. However, our screen was focused on kinases and phosphatases and only included 300 additional genes from the druggable genome. Notably, caspase genes were not among the screened targets though, as shown by the caspase-8 controls in our screen, these would have been identified as positive regulators of TRAIL-induced caspase activation and apoptosis. To identify positive regulators of TRAIL-induced apoptosis, more comprehensive, genome-wide RNAi screens, using the assays developed for this study are quite likely to identify other positive regulators (and negative regulators) of TRAIL.

A previous RNAi-based screen of 510 genes conducted in HeLa cells identified both positive and negative regulators of the TRAIL pathway (Aza-Blanc et al., 2003). The reported screen included many kinases as candidate regulators of TRAIL, but there was little overlap between our results and the results reported by Aza-Blanc and co-workers. Of the top 20 negative TRAIL regulator genes identified in the previous study, only *PIP5K1C* was identified in our screens, and none of the top 20 positive TRAIL regulator genes described in the previous report were among the positive regulators our screen identified at relaxed stringency. The differing results are likely due to several

significant differences in our screens. First, we performed the screen in a TNBC breast cancer cell line while the prior study was performed in the cervical carcinoma HeLa cell line. It is likely that the predominant regulators of TRAIL-induced apoptosis are different in different cell types. Second, our primary selection of genes whose LOF altered TRAIL activity was based on caspase-3/7 activation one hour after the addition of TRAIL while the previous study measured viability 20 hours after the addition of TRAIL. Thus our screens were designed principally to identify regulators that affect early steps in TRAIL-induced apoptosis, contributing to the difference noted.

Review of the putative negative regulators identified in our primary RNAi screens in MB231 revealed genes involved in diverse cellular processes including growth factor receptor signaling (e.g., *BTK*, *ERBB-2*, *EPH6*, *ERK8*, *FGFR2*, *FGFR4*, *JAK1*, and *SRC*), cytoskeleton function (e.g., *ACTN4*, *KIF1A*, *LIMK2*, *MAGI1*, and *PKN1*), bioenergetics (e.g., *ACACB*, *ACLY*, *ATP5A1*, *CKB*, *CKMT2*, *FN3K*, *HK1*, *HK2*, *IHPK3*, *PDK2*, *PFKL*, and *PKLR*), cell cycle regulation (e.g., *CDK5R2*, *CDKN2B*, *GAK*, *PIK3*, *PFTK1*, and *ZC3HC*1), transcriptional regulation (e.g., *HIPK1*, *HIPK2*, *NLK*, and *PIM2*), and DNA repair (e.g., *APEX1*, *PARP4*, and *TLK*). Also of note several genes known to negatively regulate apoptosis were identified (e.g., *AATK*, *BCL2L1*, *BCL2L2*, *BIRC2*, *BIRC3*, *IKBKAP*, *IKBKB*, *PRKC*I, *PIM2*, and *SPHK2*).

The largest gene set in our RNAi library included the known kinases and kinase associated genes. Of the group of kinases that were identified as hits, the majority of them are serine/threonine kinases (33 of 83), while fewer belonged to the tyrosine kinase (10/83), lipid kinase (4/83), or sugar/metabolism kinase (12/83) families. Interestingly, four kinases were identified (hexokinase 1 (HK1), hexokinase 2 (HK2), pyruvate kinase

liver and red blood cells (PKLR), and phosphofructose kinase liver (PKFL)) which regulate irreversible steps of the glycolysis pathway (Figure 3). Several studies have previously found that inhibition of glycolysis enhances TRAIL-induced cell death (Liu et al., 2009; Munoz-Pinedo, Ruiz-Ruiz, Ruiz de Almodovar, Palacios, & Lopez-Rivas, 2003; Pradelli et al., 2010; Qin, Xin, & Nickoloff, 2010).

Based on the gene network analysis, four genes were identified that appear at central nodes of an interaction map generated using the caspase-3/7 screening dataset, *PDPK1*, *IKBKB*, *SRC*, and *BCL2L1* (BCL-XL) (Figure 4A). The caspase-8 and cell viability screening data confirmed these findings for BCL2L1 (BCL-XL) and PDPK1. PDPK1 phosphorylates and activates AKT. Constitutively active or overexpression of AKT has been shown to confer TRAIL resistance in several tumor types including breast (Keane et al., 2000), lung (Kandasamy & Srivastava, 2002), gastric (Plastaras et al., 2008), and prostate (Nesterov et al., 2001). Also, TRAIL can activate SRC leading to AKT activation and TRAIL resistance (Song et al., 2010). Inhibition of the PI3 kinase/AKT pathway has been found to enhance TRAIL-induced apoptosis (Bremer et al., 2005; Cuello et al., 2001; Gibson, Henson, Haney, Villanueva, & Gibson, 2002; Henson et al., 2003; Kandasamy & Srivastava, 2002; Plastaras et al., 2008; Shrader et al., 2007; Teraishi et al., 2005). Therefore identification of PDPK1 as one of the key nodes provides a rationale for pursuing studies on the combination of TRAIL with AKT inhibitors in treatment of TRAIL-resistant tumors.

NF-κB proteins are ubiquitously expressed proteins that can protect cells from apoptosis. The inhibitors of κB (IκB) family proteins regulate the activity of NF-κB. IκB proteins block nuclear localization signals of functional NF-κB dimers by binding to

dimerization domains and sequestering the dimers in the cytoplasm. IκB kinases (IκBK) phosphorylate IκB on a serine residue targeting them for proteasomal degradation, thereby activating NF-κB, which protects cells by increasing the expression of anti-apoptotic proteins (Ravi et al., 2001; Van Antwerp, Martin, Verma, & Green, 1998; Walczak et al., 1999). Previously, we have shown that inhibition of NF-κB increases TRAIL sensitivity in breast cancer cell lines (Keane et al., 2000). Similar results were reported in other cancer cell lines (Jeremias, Herr, Boehler, & Debatin, 1998; Jeremias, Kupatt, et al., 1998; Karacay, Sanlioglu, Griffith, Sandler, & Bonthius, 2004; Keane et al., 2000; Y. S. Kim, Schwabe, Qian, Lemasters, & Brenner, 2002). Again, our findings in this paper that silencing of IκBKB leads to enhanced TRAIL-induced caspase activation provide support for further studies of NF-κB inhibitors in combination with TRAIL.

To further confirm our primary screen results, we performed a secondary screen of 16 genes identified as negative regulators of TRAIL-induced caspase activation in four cell lines representing different subtypes of breast cancer (TNBC, ER positive, and HER2 amplified) (Figure 5, Table 1 and Additional file 8, Table S4). We selected 16 genes that were included in the network analysis in Figure 4 and that both increased TRAIL-induced caspase-3/7 activity and enhanced TRAIL-induced toxicity in a viability assay. In MB231, 13 of 16 genes scored positive in this assay (using the criterion of two standard deviation enhancement in TRAIL-induced caspase-3/7 activation) and 15 of 16 genes scored positive at a lower stringency cutoff (using the criterion of one standard deviation enhancement in TRAIL-induced caspase-3/7 activation). This high level of reproducibility between the primary and secondary screen in MB231 supports the validity

of the primary screen. All of the 16 genes scored positive using the high stringency criterion in MB468. The TNBC/basal A MB468 cell line is most closely related to the TNBC/basal B MB231 cell line by cDNA microarray expression analysis and thus the high degree of overlap between the two cell lines in this screen is not surprising (Kao et al., 2009; Neve et al., 2006). By contrast, fewer of the 16 genes were scored positive in T47D (four at high stringency and 10 at low stringency) and SKBR3 (two at high stringency and three at low stringency). The T47D cell line is an ER positive luminal breast cancer cell line and the SKBR3 cell line is a HER2 amplified luminal breast cancer cell line and thus are more distantly related to the MB231 cell line (Kao et al., 2009; Neve et al., 2006).

The only gene that scored positive in our screen at high stringency in all four cell lines is alpha-actinin 4 (*ACTN4*). ACTN4 is a cytoskeletal protein that has been found to interact with signaling molecules, chromatin remodeling factors, and transcription factors (reviewed in (Hsu & Kao, 2013)). Of note, ACTN4 can serve as a scaffold to promote AKT activation and it has been shown to interact with NFκB in breast cancer cells (although the significance of this latter interaction is not known) (Hsu & Kao, 2013). Thus it is plausible that by modulating activity though these two anti-apoptotic pathways, ACTN4 might serve as a negative regulator of TRAIL-induced apoptosis. The mechanisms by which ACTN4 regulate TRAIL-induced apoptosis in breast cancer cells will require further investigation. LOF of *BCL2L1* (BCL-XL) enhanced TRAIL-induced caspase-3/7 activation in three of the four cell lines at high stringency and in all four cell lines when a lower stringency was used. Expanded screening of five *BCL2L1* siRNAs confirmed that BCL2L1 LOF results in enhanced TRAIL activity in four breast cancer

cell lines (Figure 7A). A number of studies have directly or indirectly implicated BCL2 family as regulators of TRAIL-induced apoptosis in breast cancer cells (Fulda et al., 2002; S. Kim et al., 2008; Oh, Park, Pak, & Kim, 2012; Park et al., 2012; Shankar et al., 2009; Sung, Park, Yadav, & Aggarwal, 2010; Zhang et al., 2013). In this study we have identified *BCL2L1* (BCL-XL) as a key node in determining sensitivity (Figure 4) and have further shown that inhibition of the BCL-2 family by the small molecule inhibitor, ABT-737, enhances TRAIL-induced toxicity in breast cancer cell lines (Figure 7C). These results are in concordance with previous reports of the combined use of TRAIL and ABT-737 in renal, lung, prostate, and pancreatic cancer cell lines (Huang & Sinicrope, 2008; Song, An, Kwon, & Lee, 2007). ABT-737 is a BH3 mimetic inhibitor of BCL-XL, BCL-2, and BCL-w (Vogler, Dinsdale, Dyer, & Cohen, 2009). Interestingly, both BCL-XL (*BCL2L1*) and BCL-w (*BCL2L2*) were identified as negative regulators of TRAIL-induced caspase-3/7 activation in the breast cancer cells by our primary screen. This suggests that the effects of ABT-737 may be due to inhibition of multiple BCL2 family members. Most importantly, the concomitant treatment with ABT-737 and TRAIL resulted in significantly more cell death in both sensitive and resistant breast cancer cell lines of all phenotypes (Figure 7C). Thus this suggests that the BCL2 family may play a role more broadly in regulating TRAIL sensitivity in breast cancer cells and is worth further investigation.

SRC enhanced TRAIL-induced caspase-3/7 activation in the two TNBC cell lines at high stringency (MB231 and MB468) and in the T47D cell line at lower stringency. SRC is an important kinase regulating cell survival pathways (L. C. Kim, Song, & Haura, 2009). In our study, inhibition of SRC resulted in a decrease in the activity of the

PI3K/AKT/mTOR pathway, consistent with published findings that SRC regulates the activity of the PI3K/AKT/mTOR and that inhibition of this pathway increases TRAIL sensitivity (Figure 6B) (Panner, James, Berger, & Pieper, 2005; Phipps et al., 2011; Saturno et al., 2013; Song et al., 2010; Sridharan & Basu, 2011; Van Schaeybroeck et al., 2008). In the present study we show that SRC is a key node of TRAIL-induced apoptosis as illustrated in the pathway analysis map (Figure 4A) and that inhibition of SRC by PP2 increases the sensitivity of breast cancer cells to TRAIL (Figure 6) (Phipps et al., 2011; Song et al., 2010). The most significant effects of SRC inhibition on TRAIL-induced cell death were observed in the TNBC cells (both basal A and basal B). The TNBC/basal A breast cancer cell lines are relatively resistant to TRAIL compared to the TNBC/basal B cell lines (Rahman, Davis, et al., 2009). Our data raise the possibility that combinations of TRAIL and SRC inhibitors may be of use in TNBC. The effects of TRAIL plus PP2 in the HER2 amplified and ER positive cells were less dramatic. While the reason for this is not clear, the focus of further studies with SRC inhibitors combined with TRAIL should be in TNBC cells.

Conclusions

In this study we have successfully applied complementary siRNA screens using different end-point assays to identify negative regulators of TRAIL-induced apoptosis in breast cancer cells. The identification of *PDPK1*, *IKBKB*, *SRC* and *BCL2L1* as central nodes connecting the genes identified is consistent with previous studies. Importantly, this study demonstrates that phenocopying *SRC* and *BCL2L1* LOF by pharmacological inhibition can sensitize TRAIL-resistant breast cancer cell lines to TRAIL-induced apoptosis. In these screens we identified a large number of additional genes as potential

regulators of TRAIL-induced apoptosis whose role in the TRAIL pathway is heretofore unknown. It will require further study to elucidate how they regulate the TRAIL pathway. The genes identified by this screen are likely to include novel therapeutic targets that can be tested in combination with TRAIL in treating a variety of tumors, including breast cancer.

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Table 1

Summary of Secondary Screen of TRAIL-induced Caspase-3/7 Activation

Gene	MB231 (TNBC/Basal B)	MB468 (TNBC/Basal A)	T47D (ER+)	SKBR3 (HER2+)
ACTN4	+	+	+	+
BCL2L1	+	+	+	(+)
BIRC2	+	+	(+)	+
ATP5A	+	+	+	-
BCR	+	+	+	-
FGFR4	+	+	(+)	-
PDPK1	+	+	(+)	-
PIP5K1C	+	+	(+)	-
RIOK3	+	+	(+)	-
SRC	+	+	(+)	-
CNKSR1	+	+	-	-
HIPK2	(+)	+	-	-
MKNK1	(+)	+	-	-
PLK3	+	+	-	-
PRKC1	+	+	-	-
IKBKB	-	+	-	-

+ indicates that 3 or more siRNAs for a target enhanced TRAIL-induced caspase-3/7 activity by > 2 standard deviations (criterion 1)
(+) indicates 3 or more siRNAs for a target enhanced TRAIL-induced caspase-3/7 activity by > 1 standard deviation (criterion 2).

Figure 1

The Development of siRNA-based RNAi Screens for the Identification of Regulators of TRAIL-induced Apoptosis in the MB231 Breast Cancer Cell Line

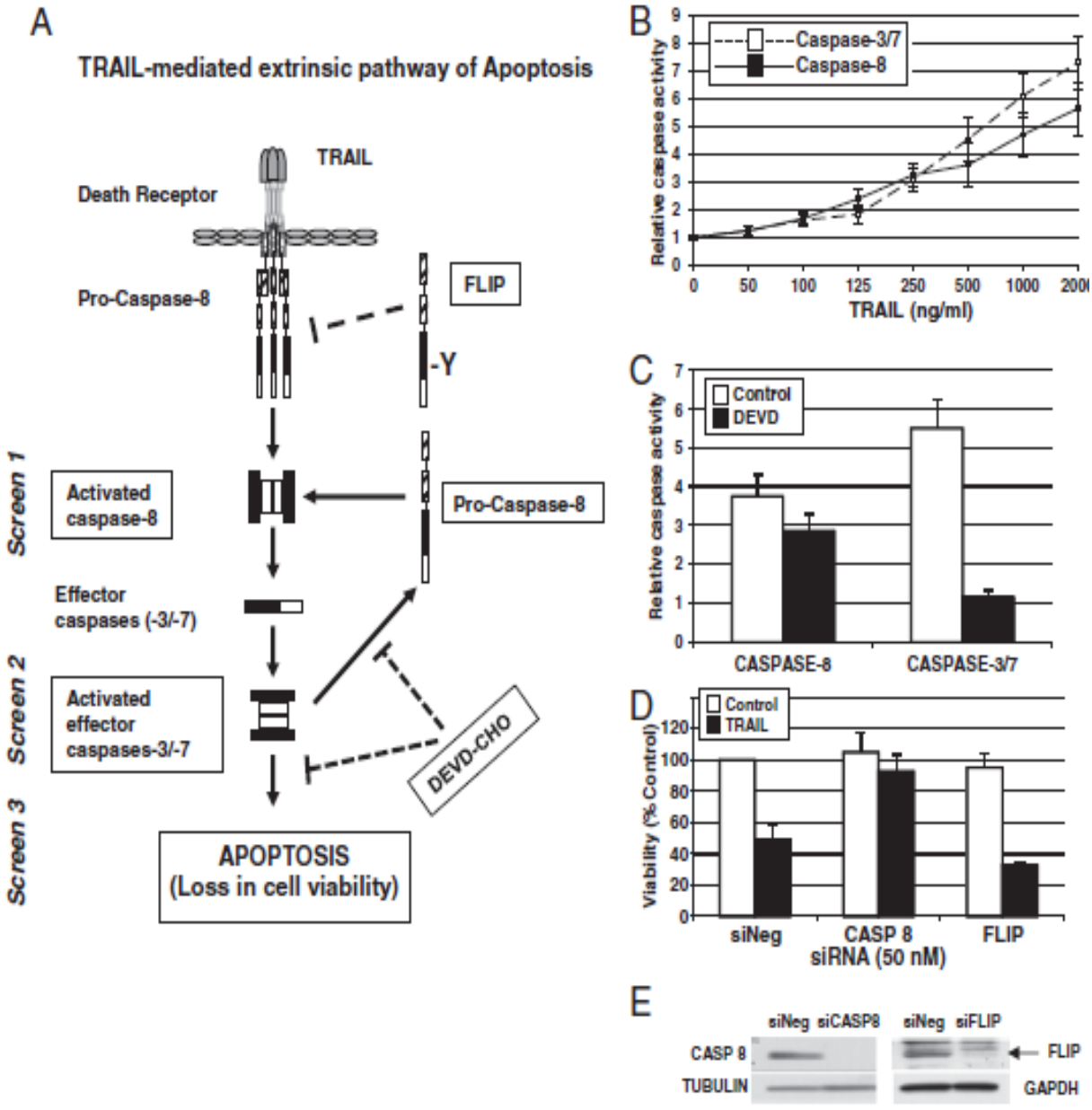

Figure 1. A. A diagrammatic representation of the extrinsic TRAIL-induced apoptotic pathway. RNAi screens were developed assaying caspase-8 activation (Screen 1), caspase-3/7 activation (Screen 2), and cell viability (Screen 3) in the absence and presence of TRAIL. Synthetic siRNAs corresponding to *CASP8* and *FLIP* were used as positive and negative regulator controls of the TRAIL pathway respectively. The caspase-3/7 DEVD-CHO inhibitor is shown on the diagram. *B.* Caspase-3/7 and caspase-8 activity was measured using caspase-3/7 and caspase-8-Glo assays. MB231 cells were treated with increasing concentrations of TRAIL (as indicated) or RPMI medium for one hour after which caspase activity was measured. Fold-increase in caspase activity is plotted relative to the untreated cells. Data is shown as the mean and standard error of three experiments. *C.* Caspase-3/7 and caspase-8 activity were measured following pre-treatment with or without 0.03 μM DEVD-CHO for one hour and then treatment with 1000 ng/ml TRAIL for one hour. Inhibition with DEVD-CHO blocked caspase-3/7 activity significantly compared to caspase-8 activity. Data is shown as the mean and standard error of three experiments. *D.* Viability of MB231 cells was measured by an MTS assay 48 hours following the transfection of the negative control siRNA (siNeg) or siRNAs corresponding to *CASP8* and *FLIP* respectively either in the absence or presence of 1000 ng/ml TRAIL for 17 hours. Data is shown as the mean and standard error of three experiments. *E.* Western blot analysis of cell lysates for CASP8 and FLIP expression, 48 hours following the transfection of the negative control siRNA (siNeg) or siRNAs corresponding to *CASP8* and *FLIP* respectively.

Figure 2

Caspase-8, Caspase-3/7, and Cell Viability RNAi screens of TRAIL-induced Apoptosis in MB231 Cells

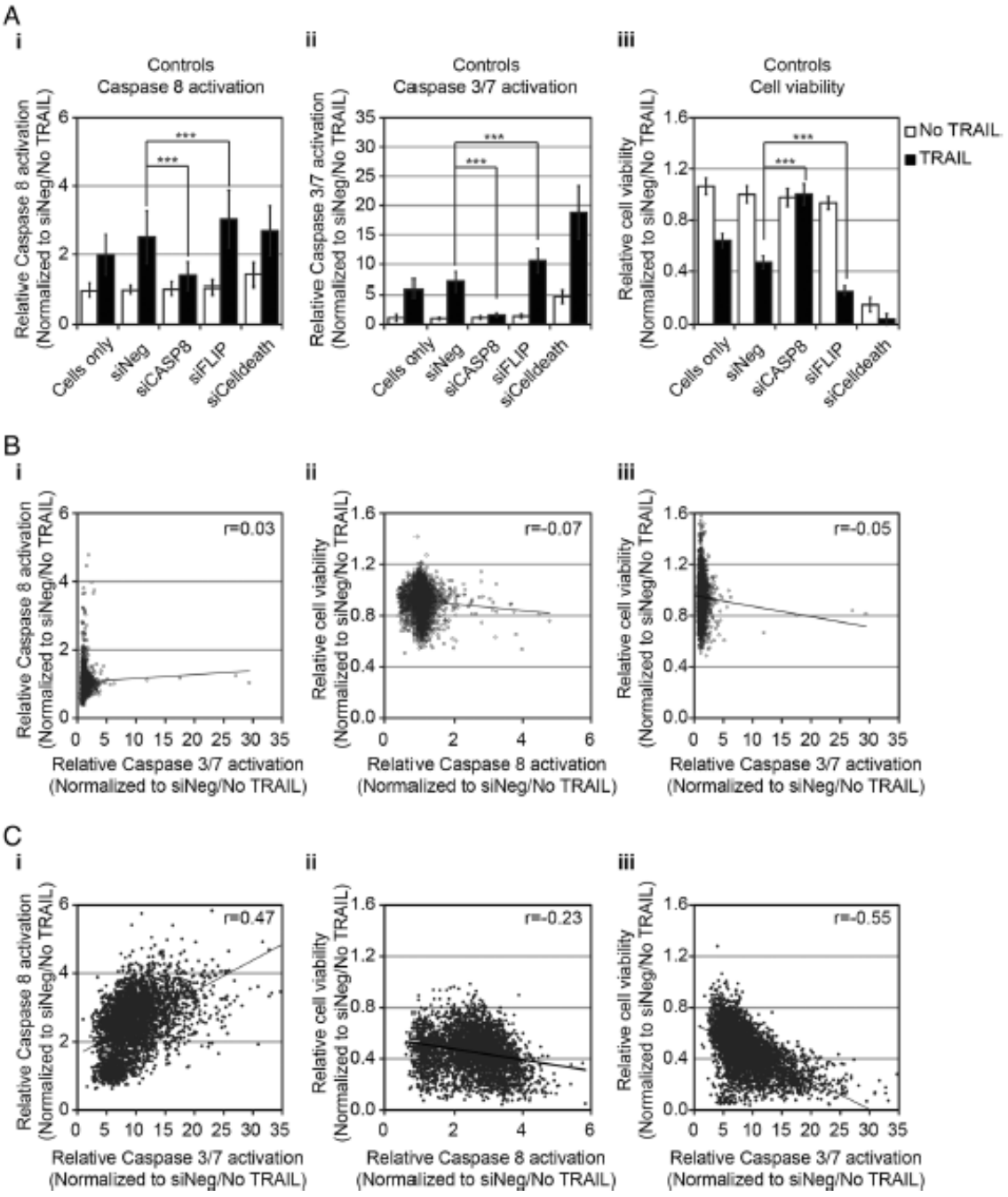

Figure 2. A. Summary of controls included in the RNAi screens of the kinome and additional gene sets in MB231 cells for (i) caspase-8 activation, (ii) caspase-3/7 activation, and (iii) cell viability in the absence (white bars) or presence (black bars) of TRAIL. For assessment of caspase-8 and caspase-3/7 activation, MB231 cells were transfected with the indicated siRNA and 48 hours later were treated with 1000 ng/ml TRAIL for one hour. For assessment of cell viability, MB231 cells were transfected with the indicated siRNA and 48 hours later were treated with 100 ng/ml TRAIL for 17 hours. Data are normalized to the mean value for cells transfected with the negative control siRNA (siNeg) in the absence of TRAIL and are shown as the mean and standard deviation for each treatment group obtained from the following number of wells: cells only, between 318 and 384 wells; and siRNA treatment groups, between 48 and 96 wells. Comparison of TRAIL-treated cells to untreated cells in the siNeg-transfected cells demonstrated a significant increase in caspase-8 and caspase-3/7 activation and a decrease in viability. siCASP8 reduced caspase-8 ($p=3x10^{-21}$) and caspase-3/7 ($p=2.5x10^{-22}$) activation and increased viability ($p=1.0x10^{-48}$) compared to siNeg treated cells. siFLIP significantly increased caspase-8 ($p=7.0x10^{-5}$) and caspase-3/7 ($p=2.3x10^{-58}$) activation and decreased viability ($1.0x10^{-67}$) compared to siNeg treated cells. *** = $p<0.001$. All comparisons were done using a two-tailed Student's t-test. *B.* Scatterplots comparing results for each siRNA (a total of 4540) in each screen in the absence of TRAIL: (i) compares the results of the activation of caspase-3/7 and caspase-8, (ii) compares the results of the activation of caspase-8 and cell viability, and (iii) compares the results of the activation of caspase-3/7 and cell viability. *C.* Scatterplots comparing results for each siRNA (a total of 4540) in each screen in the presence of TRAIL: (i)

compares the results of the activation of caspase-3/7 and caspase-8, (ii) compares the results of the activation of caspase-8 and cell viability, and (iii) compares the results of the activation of caspase-3/7 and cell viability. The trend line for each data comparison is shown as the Pearson correlation (r).

Figure 3

The Identification of Putative Negative Regulators of TRAIL-induced Apoptosis using siRNA-enhanced Activation of Caspase-3/7 in the Presence of TRAIL

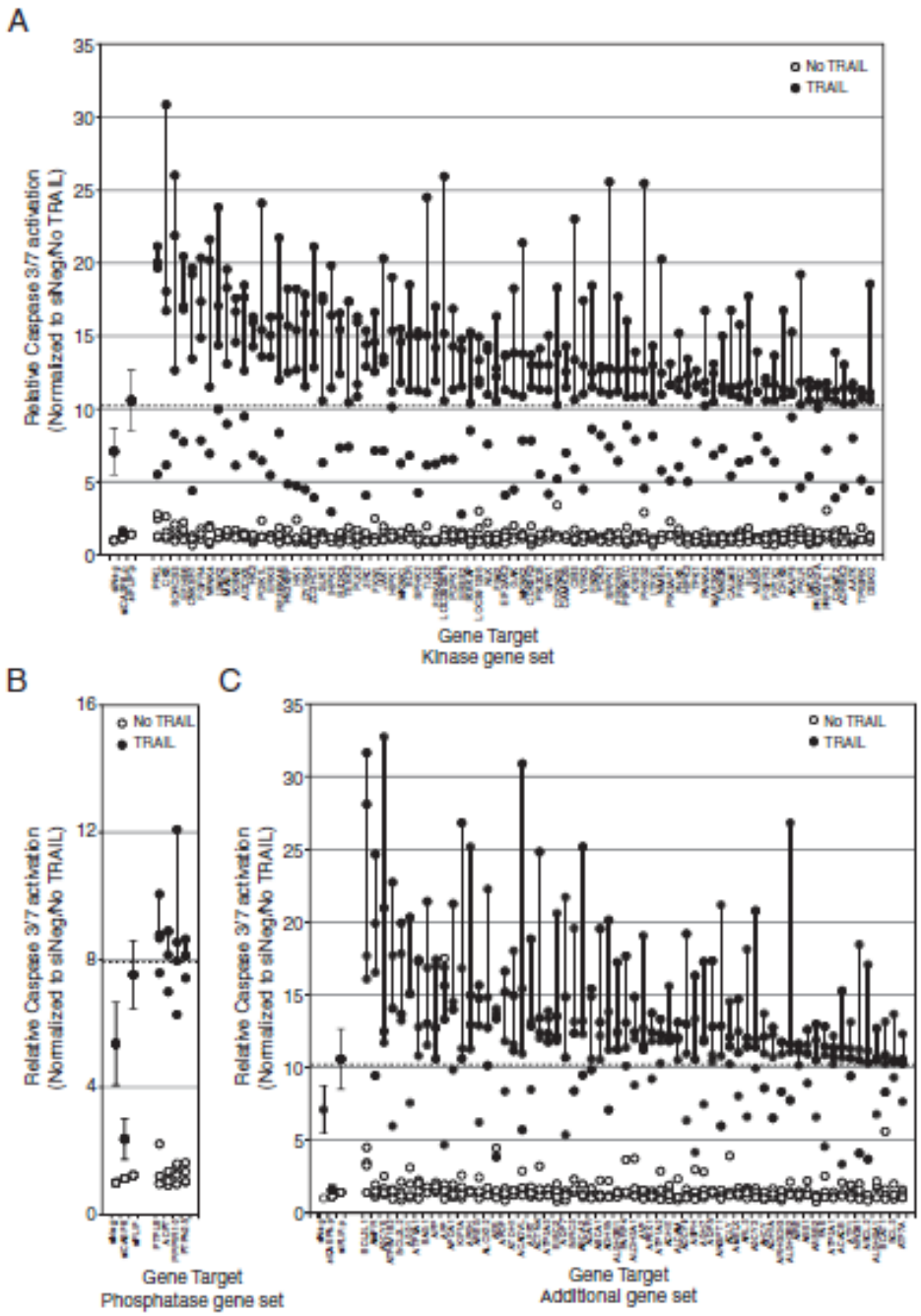

Figure 3. Genes for which three or four siRNAs mediated a fold change in the activation of caspase-3/7 in the presence of TRAIL of two or more standard deviations over that observed in control siRNA (siNeg) transfected cells plus TRAIL were considered putative negative regulators of TRAIL-induced apoptosis. The dashed line in each panel indicates the two standard deviation fold change, and a vertical line joins those siRNAs that induced at least this level of change for each gene. Data for the control siRNAs, siNeg, siCASP8, and siFLIP are included for reference and represent the same data for the siRNAs indicated as shown in Figure 2Aii and Figure S1Ai. *A.* Genes identified from the kinase gene set. *B.* Genes identified from the phosphatase gene set. *C.* Genes identified from the additional gene set. Genes are ranked in descending order based on the median value for each set of four siRNAs per gene.

Figure 4

Network Analysis of Putative Negative Regulators of TRAIL-induced Apoptosis

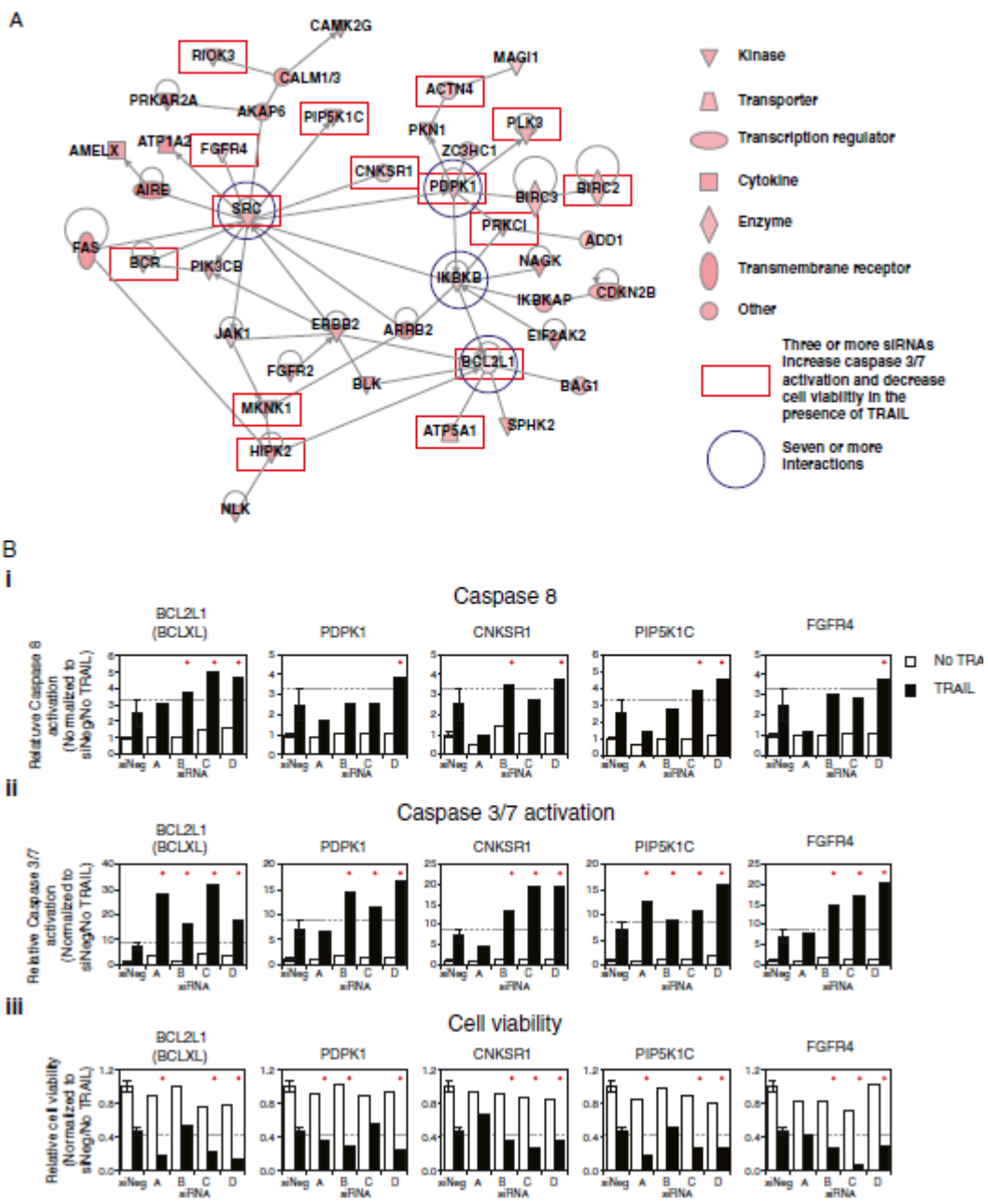

Figure 4. A. A gene interaction network generated by Ingenuity Pathway Analysis of the 150 genes for which three or more siRNAs induced increased TRAIL-induced activation of caspase-3/7 levels. The blue circles indicate those genes with seven or more interactions and the red rectangles genes for which three or more siRNAs caused both an increase in TRAIL-induced activation of caspase-3/7 levels and decreased viability in response to TRAIL. Legend shows the symbols indicated by the Ingenuity Pathway Analysis software. *B.* Assays of (i) caspase-8, (ii) caspase-3/7 activation, and (iii) cell viability in the absence (empty bars) and presence (black bars) of TRAIL for four siRNAs (*A-D*) corresponding to the genes shown. Average data (±1 standard deviation) for negative control siRNA (siNeg) transfected cells is shown in each graph. The grey dashed line indicates the relevant one standard deviation value for each assay, and the red dots indicate those siRNAs inducing fold-changes greater than one standard deviation.

Figure 5

A Secondary Screen of Putative Negative Regulators of TRAIL-induced Apoptosis

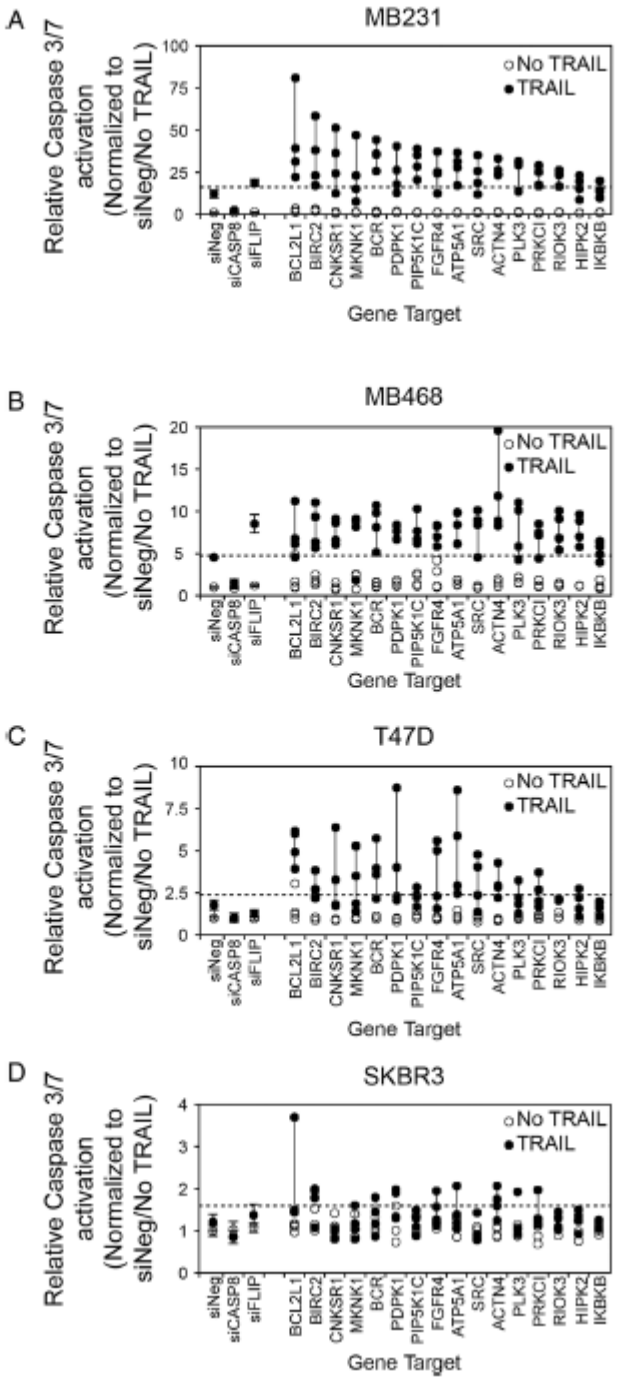

Figure 5. Four siRNAs corresponding to 16 genes were selected for additional analysis of TRAIL-induced caspase-3/7 activation in *A.* MB231, *B.* MB468, *C.* T47D, and *D.* SKBR3. Cells were siRNA transfected as for the primary screen and 48 hours later were treated with 1,000 ng/ml TRAIL for one hour before assessment of caspase-3/7 activation. The dashed line in each panel indicates the two standard deviation fold-increase in TRAIL-induced caspase-3/7 activation compared to TRAIL treated cells transfected with negative control siRNA (siNeg). The vertical line joins the siRNAs for each gene. Genes are ranked in descending order based on the median value for each set of four siRNAs per gene in the MB231 cell line. Data for the control siRNAs, siNeg, siCASP8 and siFLIP are included for reference.

Figure 6

Inhibition of SRC Sensitizes Breast Cancer Cells to TRAIL

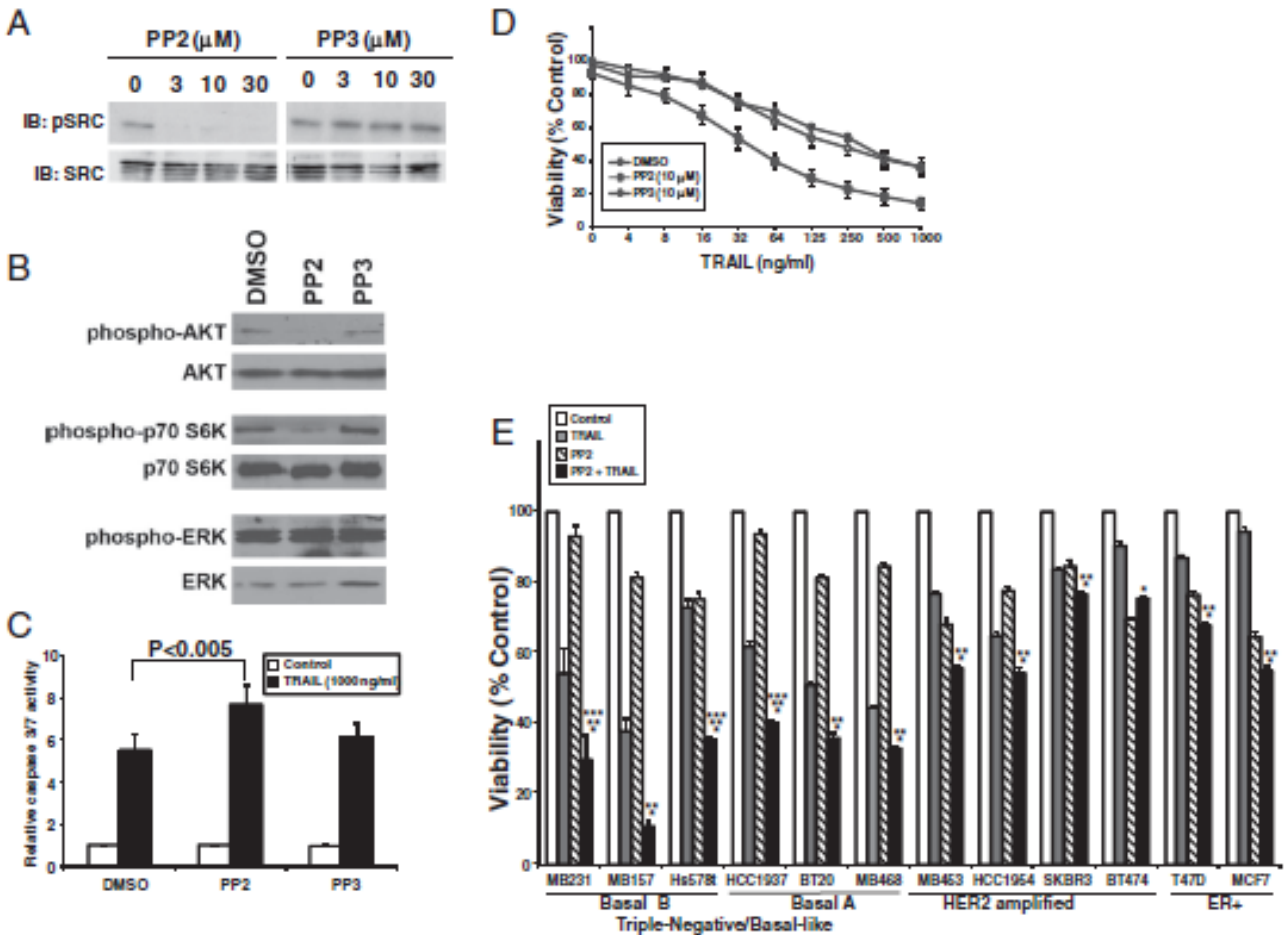

Figure 6. A. Western blot analysis of MB231 cell lysates treated with different concentrations of SRC inhibitor PP2 or its inactive analogue PP3 for one hour, probed for phospho- SRC (pSRC) and total SRC. *B.* Western blot analysis of MB231 cell lysates treated with 10 μM PP2, PP3, or DMSO for four hours and probed for the indicated proteins. *C.* Caspase-3/7 activation by pre-treatment with PP2 or PP3 for four hours followed by the addition of DMSO (white bars) or 1000 ng/ml of TRAIL (black bars) for one hour. Data are the mean +/- SE for three experiments. The comparison between PP2 treated and DMSO treated cells was performed using a paired, two-tailed Student's t-test. *D.* Cell viability assay of MB231 cells treated with 10 μM PP2 or PP3 or DMSO for one hour followed by different concentrations of TRAIL treatment for 17 hours. *E.* Cell viability of different breast cancer cells after pre-treatment with or without 10 μM PP2 for one hour followed by treatment with 125 ng/ml TRAIL for 17 hours as measured by MTS assay. Data are shown as the mean +/-SE for three experiments for each cell line, and the data were compared using a two-tailed Student's t-test. * represents $p < 0.05$ comparing the combination treatment to TRAIL alone; ** represents $p < 0.05$ comparing the combination treatment to PP2 alone; and *** represents $p < 0.05$ comparing the combination treatment to sum of the inhibition by TRAIL alone plus the inhibitor alone.

Figure 7

Inhibition of BCL2L1 (BCL-XL) Sensitizes Breast Cancer Cells to TRAIL

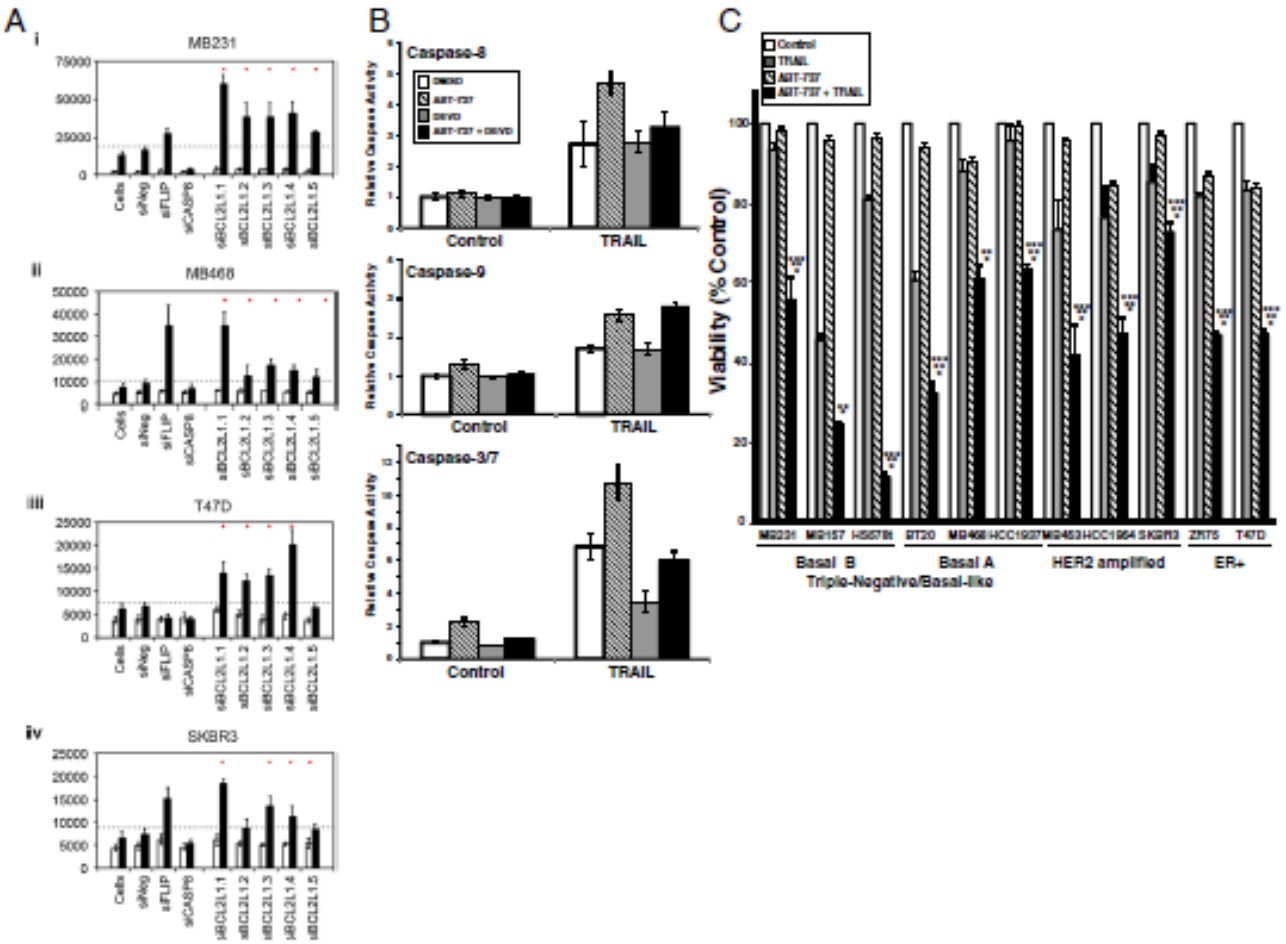

Figure 7. A. MB231, MB468, T47D and SKBR3 cells were incubated with five different targeting siRNAs for 48 hours, and caspase-3/7 activity was assayed one hour after addition of 1,000 ng/ml TRAIL. siNeg, siFLIP, and siCasp8 are included in each screen as controls. The grey dashed line represents one standard deviation increase in TRAIL-induced caspase-3/7 activation compared to TRAIL-treated cells transfected with the siNeg control. The red dots indicate those siRNAs that induced a fold change greater than one standard deviation. *B.* MB231 cells were incubated with TRAIL (1000 ng/ml), the BCL2 family inhibitor ABT-737 (5 μM), or both in the presence or absence of DEVD-CHO (30 nM). Activation of caspase-8, caspase-9, and caspase-3/7 as measured by Caspase-Glo assays one hour after TRAIL addition. Data show the mean activity +/- SD for one representative experiment. *C.* MTS assay to measure cell viability of different breast cancer cells after pre-treatment with or without 5 μM ABT-737 for one hour followed by treatment with 8 ng/ml (MB231, Hs578t) or 63 ng/ml (MB157, BT20, MB468, HCC1937, ZR75, T47D), 250 ng/ml (MB453, HCC1954) or 500 ng/ml (SKBR3) of TRAIL for 17 hours. Data shown are the mean +/-SE for three experiments for each cell line, and the data were compared using a two-tailed Student's t-test. * represents $p < 0.05$ comparing the combination treatment to TRAIL alone; ** represents $p < 0.05$ comparing the combination treatment to ABT-737 alone; and *** represents $p < 0.05$ comparing the combination treatment to sum of the inhibition by TRAIL alone plus the inhibitor alone.

Additional file 1: Table S1 Caspase-8, Caspase-3/7, and Cell Viability siRNA Primary Screening Data in MB-231 Cells in the Absence and Presence of TRAIL. Data are for four different siRNAs per gene (A, B, C, and D) shown as fold-change relative to siNeg-transfected cells in the absence of TRAIL.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s1.pdf

Additional file 2: Table S2 Genes and siRNA Sequences Selected for Secondary Screening of Putative Regulators of TRAIL-induced Apoptosis.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s2.pdf

Additional file 3: Figure S1 Caspase-3/7 and Cell Viability siRNA Screens of the Phosphatome and TRAIL-induced Apoptosis in MB231 Cells. *A.* Summary of controls included in the siRNA screens of the phosphatome gene set in MB231 cells for (i) caspase-3/7 activation and (ii) cell viability in the absence (white bars) or presence (black bars) of TRAIL. For assessment of caspase-3/7 activation MB231 cells were transfected with the indicated siRNA and 48 hours later were treated with 1000 ng/ml TRAIL for one hour. For assessment of cell viability MB231 cells were transfected with the indicated siRNA and 48 hours later were treated with 100 ng/ml TRAIL for 24 hours. Data are normalized to the mean value for cells transfected with the control siRNA (siNeg) in the absence of TRAIL and is shown as the mean and standard deviation for each treatment group. Comparison of TRAIL treated cells to untreated cells in the siNeg-

transfected cells demonstrated a significant increase in caspase-3/7 activation and a significant decrease in viability. siCASP8 reduced caspase-3/7 activation ($p=1.0x10^{-14}$) and increased viability ($p=1.5x10^{-14}$) compared to siNeg treated cells. siFLIP increased caspase-3/7 ($p=1x10^{-8}$) activation and decreased viability ($p=7.0x10^{-7}$) compared to siNeg treated cells. *** = $p<0.001$. *B.* Silencing of PLK1 activates caspase-8, caspase-3/7 and mediates a decrease in cell viability in the absence of TRAIL. To further confirm the sensitivity of our assays we confirmed that the LOF of the mitosis-associated kinase Polo-like kinase 1 (PLK1), an established essential protein in many cell lines, activated caspase-8, caspase-3/7, and decreased cell viability. The dashed line indicates the relevant one standard deviation value for each assay, and the red dots indicate those siRNAs inducing fold-changes greater than one standard deviation. *C.* The identification of putative positive regulators of TRAIL-induced apoptosis using siRNA decreased activation of caspase-3/7 in the presence of TRAIL. Genes for which three or four siRNAs mediated a decrease in the activation of caspase-3/7 in the presence of TRAIL of one or more standard deviations over that observed in control siNeg-transfected cells plus TRAIL were considered as putative positive regulators of TRAIL-induced apoptosis. The dashed line indicates the one standard deviation fold-change, and a vertical line joins those siRNAs that induced at least this level of change for each gene. Data for the control siRNAs, siNeg, siCASP8, and siFLIP are included for reference and represent the same data for the siRNAs indicated as shown in Figure 2Aii and Figure S1Ai.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s3.pdf

Additional file 4: Table S3 Primary Screen Z-factors Calculated for the Viability, Caspase-3/7 and Caspase-8 Assay Plates.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s4.pdf

Additional file 5: Figure S2 Interaction Network Analysis of Putative Negative Regulators of TRAIL-induced Apoptosis. An interaction network generated by analysis of the 150 genes for which three or more siRNAs induced increased TRAIL-induced activation of caspase-3/7 levels. All symbols are presented as depicted by the Ingenuity Pathway Analysis software. Gene names in black and linked by solid lines indicate evidence for a mechanistic relationship between the proteins indicated. Gene names in blue and linked by dashed lines indicate correlative relationships between the proteins indicated, but no mechanistic relationship has been established.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s5.pdf

Additional file 6: Figure S3 Validation of Genes Identified by Interaction Analysis. Caspase-8 and caspase-3/7 activation, and cell viability in the absence (empty bars) and presence (black bars) of TRAIL for four siRNAs (*A*-*D*) corresponding to the genes shown. Mean data (±one standard deviation) for control siRNA (siNeg) transfected cells are shown in each graph. The dashed line indicates the relevant one standard deviation value for each assay, and the red dots indicate those siRNAs inducing fold-changes greater than one standard deviation.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s6.pdf

Additional file 7: Figure S4 Caspase-3/7 Activation by TRAIL in Breast Cancer Cell Lines. Cell lines were treated with increasing concentrations of TRAIL as indicated along the X axis for one hour and caspase-3/7 activation was measured by Caspase-Glo-3/7 assay as described above.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s7.pdf

Additional file 8: Table S4 Caspase-3/7 siRNA Secondary Screen in a Panel of Breast Cancer Cell Lines in the Absence and Presence of TRAIL. Data is for four different siRNAs per gene shown as fold-change relative to siNeg-transfected cells in the absence of TRAIL. Values indicated in red are >2 standard deviations higher than TRAIL-induced caspase-3/7 in siNeg treated cells. Values indicated in blue are >1 standard deviations higher than TRAIL-induced caspase-3/7 in control siRNA (siNeg) treated cells.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s8.pdf

Additional file 9: Figure S5 BCL-XL Protein Expression in the Breast Cancer Cells. *A.* Expression of BCL-XL was measured by immunoblotting in the four cell lines used in the secondary RNAi screen. *B.* Two BCL-XL specific siRNAs (*siBCL2L1.3* and

siBCL2L1.5) were compared to a negative control siRNA (siNEG) for their ability to enhance TRAIL-induced caspase-3/7 activity as described above and in parallel for their knock down of BCL-XL protein. *C.* Levels of BCL-XL protein were measure by immunoblotting in cells treated with ABT-737 (5 μM) for the times indicated. In all of the blots, HSC70 is shown as a loading control and MW in kDa is shown to the left of the panels.

Accessible at: http://www.breast-cancer-research.com/content/supplementary/bcr3645-s9.pdf

VITA

Jennifer Dine was born to registered nurses George and Daisy Dine on October 2, 1985, in Philadelphia, Pennsylvania. She also became a registered nurse and has spent her professional career in the oncology research setting. In 2009, she obtained a National Institute of Nursing Research Graduate Partnerships Program Fellowship to pursue her doctoral dissertation research within the National Institutes of Health intramural program in conjunction with the Sinclair School of Nursing at the University of Missouri. She currently resides in Rockville, Maryland with her partner, Aaron, and practices Ashtanga yoga as an additional pursuit of knowledge.