Calpain 9 functions in TNF receptor mediated apoptosis

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Calpain 9 functions in TNF receptor mediated apoptosis

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Dedication

I would like to dedicate my thesis to my parents, Thomas and Elizabeth Kunkel, and my sister, Kristi Kunkel.

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The say it takes a village to raise a child, but it takes a community to train a scientist. I am truly blessed to have so many wonderful influences in my scientific development and would like to express my gratitude for all your help.

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One area I have been particularly blessed in is friendship. When I moved to Dallas in 2005, the nearest person I knew was 800 miles away. Today I am overwhelmed by the love and support I receive from my friendships. To my Dallas peeps, Thank you.

Last I wish to thank my family; my mom who taught me strength, my sister who taught me compassion, and my dad who taught me what it truly means to be a man. This accomplishment is shared between all of us.

Calpain 9 functions in TNF receptor mediated apoptosis

Gregory Thomas Kunkel

The University of Texas Southwestern Medical Center at Dallas, 2011

Xiaodong Wang, Ph.D.

Evasion of apoptosis is a hallmark of cancer development. The Inhibitor of Apoptosis Proteins, IAPs, block Caspase activity and cell death. Release of the Second Mitochondria-Derived Activator of Caspases, Smac, from the mitochondria relieves IAP Caspase inhibition, activating apoptosis. Our lab has developed a small molecule Smac mimetic. Surprisingly, approximately 25% of cell lines show single agent Smac mimetic sensitivity through activation of autocrine TNF-α secretion and TNF dependent apoptosis. Using Smac mimetic sensitivity as a model system, I performed a genome-wide high-throughput siRNA screen and identified Calpain 9, CAPN9, as a novel component of TNF-

alpha induced apoptosis. CAPN9 knockdown does not affect TNF-α secretion, yet is essential for downstream activation. Two splice variants are reported for CAPN9. The smaller splice, CAPN9-SP2, is required for effective TNF-α induced apoptosis. CAPN9 is essential for RIPK1 recruitment and ubiquitination at the TNFR1 upon activation with TNF-alpha. CAPN9 knockdown demonstrates previously unreported association of ubiquitinated proteins, and actin binding proteins with TNFR1 in the absence of stimulus. This interaction is CAPN9 dependent and correlates with CAPN9 regulation of TNF-α induced apoptosis.

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Prior Publications

Kunkel GT, Wang X. “Sam68 Guest STARs in TNF-α Signaling.” Mol Cell. 2011 Jul 22;43(2):157-8

Bao YP, Wei TF, Lefebvre PA, An H, He L, Kunkel GT, Müller UR. “Detection of protein analytes via nanoparticle-based bio bar code technology.” Anal Chem. 2006 Mar 15;78(6):2055-9.

Ramakrishnan R, Buckingham W, Domanus M, Gieser L, Klein K, Kunkel G, Prokhorova A, Riccelli PV. “Sensitive assay for identification of methicillin-resistant Staphylococcus aureus, based on direct detection of genomic DNA by use of gold nanoparticle probes.” Clin Chem. 2004 Oct;50(10):1949-52.

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Definitions

TNF – Tumor Necrosis Factor

AMACR – Alpha Methyl Acyl-CoA Racemase

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

IκB – Inhibitor of kappa B

Chapter One

Introduction

1.1 Development of a small molecule Smac mimetic

Apoptosis, programmed cell death, is a tightly regulated cellular process necessary for development(Kerr, Wyllie et al. 1972). Apoptosis is required for homeostasis of multicellular organisms, and is morphologically defined by nuclear membrane breakdown, chromosome condensation, DNA fragmentation and membrane blebbing (Hengartner 2000). Neighboring cells engulf packaged cellular contents from apoptotic cells, preventing inflammation and damage from cellular components being freely released. Too much apoptosis can result in multiple diseases, such as neurodegeneration, while apoptotic inhibition is a hallmark of cancer development (Thompson 1995). Apoptosis is divided into two pathways, defined by the source of the initiating signal. Signals coming from outside the cell through death receptors initiate the extrinsic apoptotic program. Signals from the inside of the cell, such as DNA damage, cause release of mitochondrial components and intrinsic apoptotic activation. Apoptosis is carried out by caspases, a family of cysteine proteases (Yuan, Shaham et al. 1993).

Caspases, cysteine-dependent, aspartic-specific proteases, cleave targets to trigger and execute the apoptotic program. Caspases are zymogens, requiring

cleavage to activate their enzymatic activity, and can be divided into two subcategories, the initiator and effector caspases. Initiator caspases are the first responders to the apoptotic stimulus. The initiator for intrinsic apoptosis is Caspase 9, while the extrinsic pathway is activated by Caspase 8 and 10. Extrinsic and intrinsic apoptosis both activate effector caspases 3, 6, and 7. The effector caspases are the driving force behind apoptosis, and a marker of programmed cell death(Thornberry 1998).

Apoptotic evasion is a hallmark of cancer development(Hanahan and Weinberg 2011). Both extrinsic and intrinsic apoptosis have multiple layers of regulation that cancer can exploit to block cell death. The intrinsic apoptotic pathway is governed by the release of cytochrome c from the mitochondria (Liu, Kim et al. 1996; Li, Nijhawan et al. 1997). This regulation is achieved by the BCL-2 family of proteins. Two proteins in this family, Bax and Bak, oligomerize to form pores on the mitochondria allowing for cytochrome c release (Danial and Korsmeyer 2004; Youle and Strasser 2008). BCL-2 and BCL-xL are anti-apoptotic proteins that block pore formation and cytochrome c release. This can be overcome by BH3 only domain proteins, such as Bad, Bid and Bim. Cytochrome c, once released, interacts with APAF-1 to form a heptameric complex known as the apoptosome (Liu, Kim et al. 1996; Zou, Henzel et al. 1997; Zou, Li et al. 1999; Wang 2001). The apoptosome serves as a platform for Caspase 9 activation.

Extrinsic apoptosis is controlled by the TNF superfamily of receptors. This family includes TNFR1, the TRAIL receptors DR4 and DR5, and the FAS receptor (Tartaglia, Rothe et al. 1993; Smith, Farrah et al. 1994; Ashkenazi and Dixit 1998; Ashkenazi 2002). All death receptors contain a cytoplasmic death domain (Reed, Doctor et al. 2004). Upon binding with their cognate ligand, death receptors trimerize, allowing interaction with multiple components culminating in FADD and Caspase 8 interaction (Figure 1.1). FADD contains both a death domain and death effector domain allowing it to serve as a bridge interacting between death domain containing proteins and the death effector domain of Caspase 8 to form the Death Inducing Signaling Complex (DISC). This leads to activation of Caspase 8. Caspase 8 cleaves the BH3 only protein, Bid (Luo, Budihardjo et al. 1998). Truncated Bid then translocates to the mitochondria and activates the intrinsic apoptotic program. In some cells, Caspase 8 activity is sufficient to activate effector caspases and drive cell death; however some cells require activation of the intrinsic program to achieve full activation of effector caspases and subsequent cell death.

Control of Caspase activation is critical for cell survival. The Inhibitor of

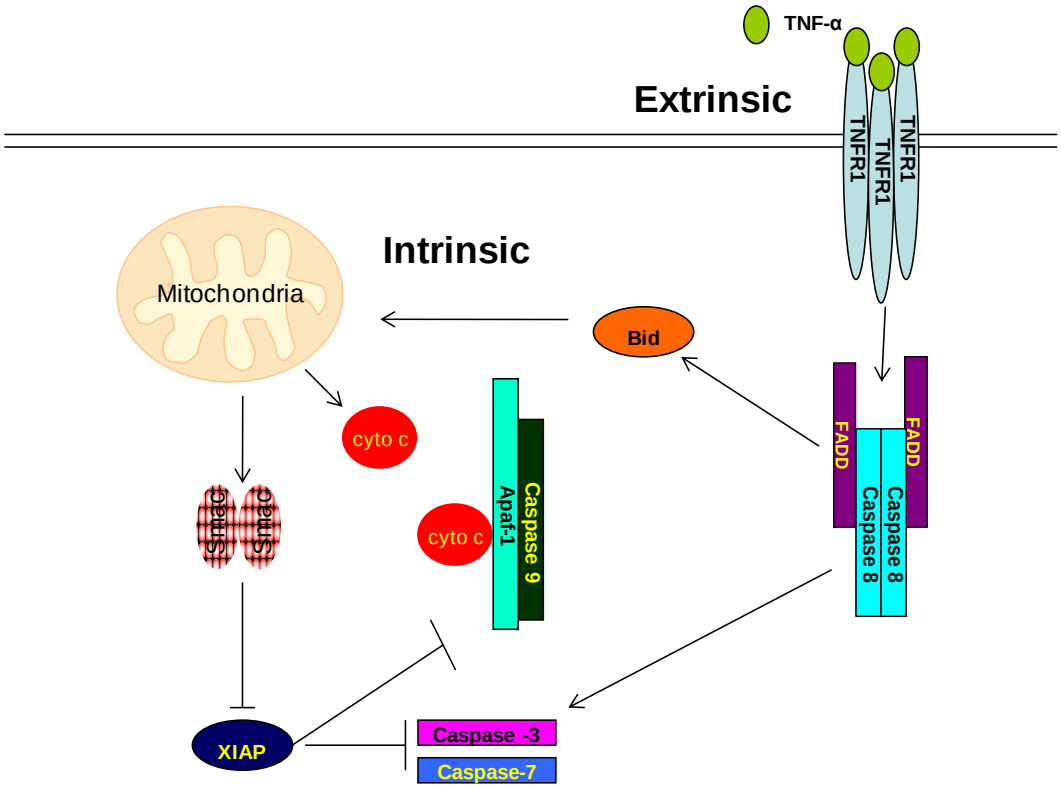


Figure 1.1 Schematic representations of extrinsic and intrinsic apoptosis

Intrinsic apoptosis is driven by release of factors from the mitochondria. Cytochrome C is released to the cytosol, and binds to Apaf-1, which oligomerizes and binds Caspase 9 to activate. Caspase 9 then activates Caspase 3/7 to initiate apoptosis. The extrinsic pathway signals through death receptors like TNFR1. Ligand binds to the receptor signaling to Caspase 8. Active Caspase 8 can cleave effector Caspases 3/7. Caspase 8 can also activate the intrinsic pathway through cleavage of Bid. XIAP inhibits cell death by blocking active Caspase 3/7/9. XIAP is inhibited by Smac, released from the mitochondria, allowing apoptosis to occur.

Apoptosis Proteins (IAPs) are regulators of caspase activity. IAPs are characterized by the inclusion of approximately 70 amino acids comprising the BIR, Baclovirus IAP Repeat, domain (Birnbaum, Clem et al. 1994; Hinds, Norton et al. 1999). This domain allows for protein-protein interactions. Of the eight mammalian IAPs, only XIAP, X-linked inhibitor of apoptosis, is directly capable of binding to caspase. XIAP binds initiator Caspase 9 and effector Caspases 3 and 7, inhibiting their activity (Deveraux, Takahashi et al. 1997; Chai, Shiozaki et al. 2001). Over-expression of XIAP prevents cell death from both intrinsic and extrinsic apoptotic programs (Deveraux, Takahashi et al. 1997). IAP over-expression has been detected in multiple cancer cell lines, making them attractive targets for therapeutic intervention (LaCasse, Baird et al. 1998).

IAP regulation is necessary for cell death. One method of regulation is through interaction with Smac, Second Mitochondria-derived Activator of Caspases (Du, Fang et al. 2000; Verhagen, Ekert et al. 2000). Smac is a 25kDa mitochondrial protein released to the cytosol upon cell death stimuli. The N-terminal residues are exposed when Smac is imported into the mitochondria and the localization signal is cleaved. The new N-terminal Ala-Val-Pro-Ile, AVPI, residues of the mature Smac interact with the BIR domain of IAPs (Chai, Du et al. 2000). Binding of Smac to the BIR domain requires an N-terminal Ala, so Smac requires cleavage to interact with the IAPs (Chai, Du et al. 2000; Liu, Sun et al.

2000; Wu, Chai et al. 2000). This prevents nuclear encoded Smac from interacting with the IAPs in the absence of apoptotic stimuli (Figure 1.2).

The relatively small recognition sequence and powerful effect of Smac makes it an attractive lead for drug development. Our lab, in collaboration with the laboratories of Dr. Patrick Harran and Dr. Jef Debrabander, has developed a small molecule Smac mimetic. The compound was designed to mimic the amino acid sequence Ala-Val-Pro-Phe (AVPF), which was found to have higher affinity for XIAP than AVPI. The compound is a dimer, like the endogenous Smac protein, and strongly interacts with XIAP, CIAP1 and CIAP2 (Figure 1.3). The Smac mimetic synergized with TRAIL and TNF-α to induce apoptosis (Li, Thomas et al. 2004).

Smac mimetics function to relieve inhibition from the IAPs, thus removing an apoptotic block, allowing for Caspase activation and cell death. In theory, IAP removal should not induce apoptosis on its own, since there must first be an active cell death signal for the IAPs to inhibit. To our surprise, approximately one quarter of cancer cell lines showed single agent Smac mimetic sensitivity (Figure 1.4) (Petersen, Wang et al. 2007). z-VAD, a pan Caspase inhibitor, blocks apoptosis in these cell lines indicating that something is driving the activation of Caspases. The large percentage of single agent sensitive cell lines suggests a generic means of Caspase activation. siRNA knockdown of the

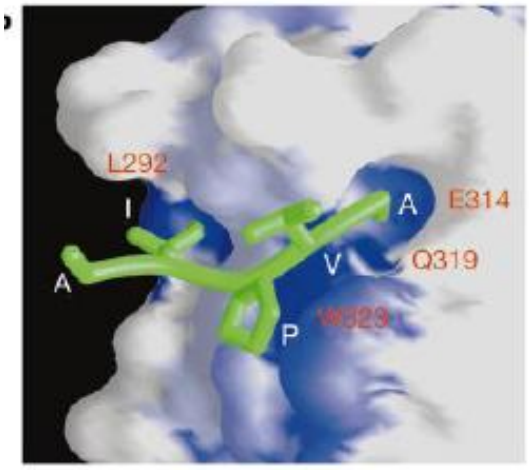


Figure 1.2 Smac N-terminal interaction with XIAP

This is a representation of the interaction between the N-terminal AVPI residues of Smac and XIAP-BIR3 domain. (Wu, Chai et al. 2000)

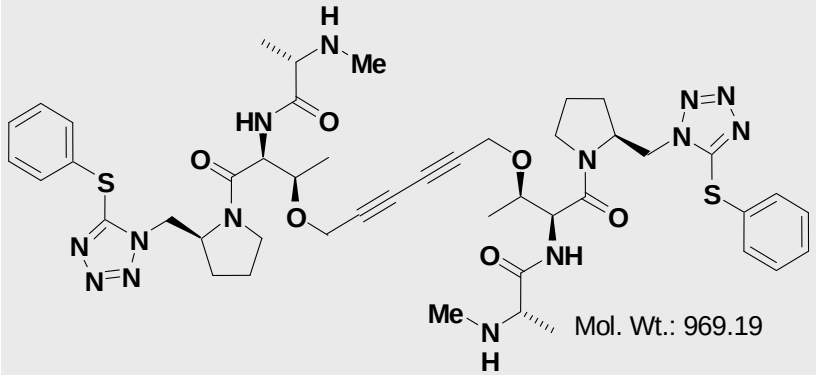
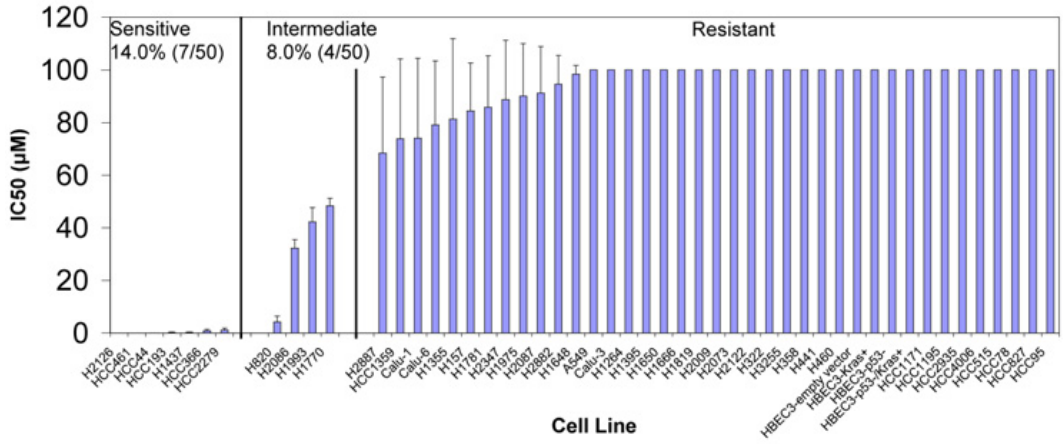


Figure 1.3 Stucture of the Smac mimetic

Chemical structure of the Smac mimetic. (Li, Thomas et al. 2004)



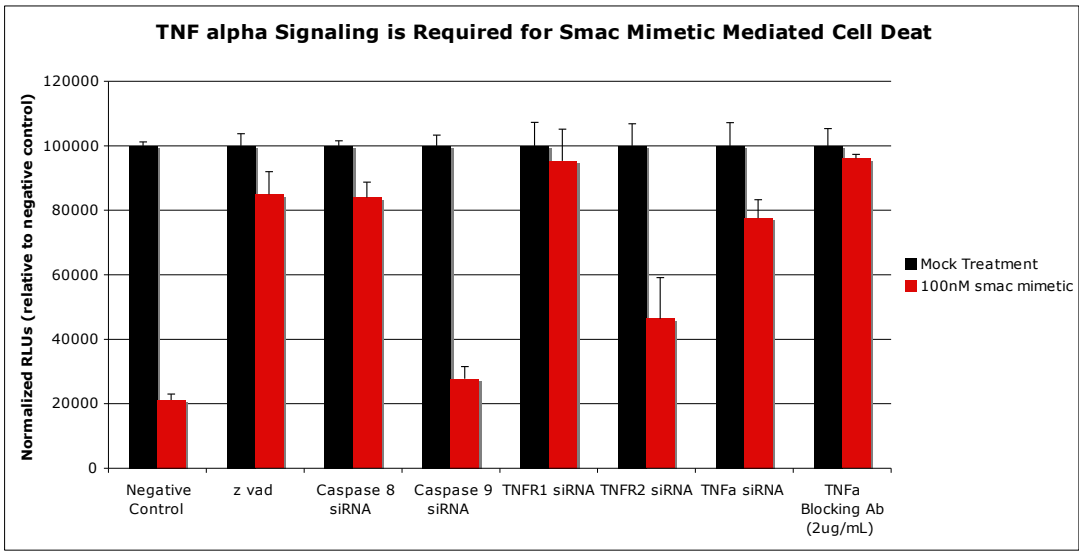


Figure 1.4 Single agent Smac mimetic sensitivity is TNF-alpha dependent

Upper panel, IC50 was calculated for single agent Smac mimetic sensitivity in multiple cell lines. In the lower panel, HCC44 cells were transfected with siRNA pools against known target genes, and treated with 100nM Smac Mimetic. Cell viability was measured by Cell TiterGlo. Data from (Petersen, Wang et al. 2007)

extrinsic, Caspase 8, and intrinsic, Caspase 9, pathways revealed single agent Smac sensitivity is conveyed through the extrinsic apoptotic pathway. Further delineation revealed single agent Smac mimetic sensitivity to be dependent on TNF receptor signaling. Smac mimetic inspired IAP agonists induce autocrine secretion of TNF-α in these cell lines, and cell death is dependent on TNF-α signaling (Petersen, Wang et al. 2007; Varfolomeev, Blankenship et al. 2007; Vince, Wong et al. 2007).

TNF-α is a target of NF-κB transcription, making NF-κB a strong candidate for determining the secretion of TNF-α (Figure 1.5). There are two main NF-κB activation pathways. The canonical NF-κB occurs through TNFR1 induced degradation of IκB. The noncanonical NF-κB is controlled by Nik, NF-κB inducing kinase, stabilization, reviewed in (Hayden and Ghosh 2008; Vallabhapurapu and Karin 2009). Ubiquitination and degradation controls the basal levels of Nik. CIAP1 and CIAP2 ubiquitinate Nik, targeting Nik for proteasomal degradation. Smac mimetics induce degradation of CIAP1 and CIAP2, leading to Nik stabilization (Varfolomeev, Blankenship et al. 2007; Vince, Wong et al. 2007). Nik phosphorylates P100, leading to ubquitination and partial degradation to P52 (Xiao, Harhaj et al. 2001). P52 associates with RELB,

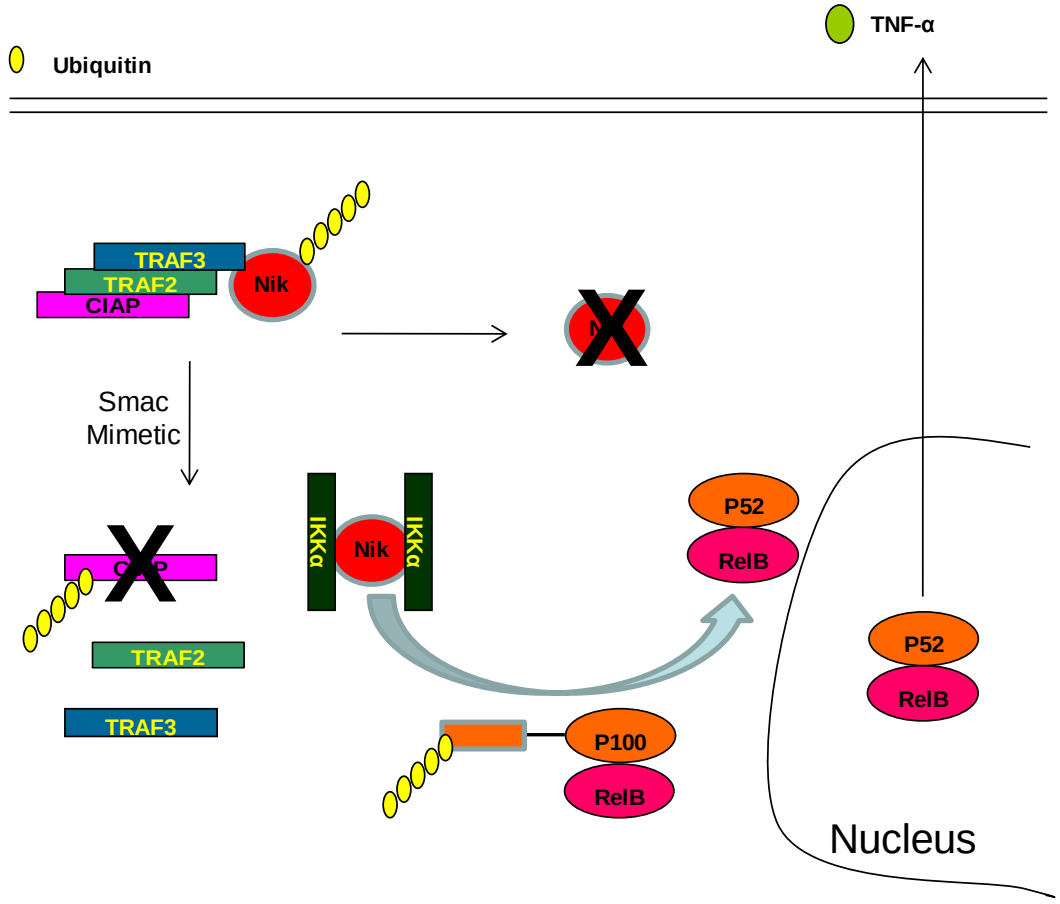


Figure 1.5 Smac mimetic triggers Nik stabilization and autocrine secretion of TNF-alpha

Under normal conditions Nik is ubiquitinated by CIAP1/2 targeting it for proteasomal degradation. Smac mimetic treatment causes degradation of CIAP1/2. Nik is stabilized, associates with IKKα, and phosphorylates P100. P100 is ubiquitinated and partially degraded to the 52kDa P52. P52 translocates to the nucleus and initiates transcription of NF-κB target genes including TNF-alpha.

and translocates to the nucleus, activating NF-κB target genes including TNF-α.

1.2 TNF-α signaling

Tumor Necrosis Factor, TNF-α, is a pleiotropic cytokine capable of inducing cell survival, inflammation, cell proliferation and cell death (Ashkenazi and Dixit 1998) (Varfolomeev and Ashkenazi 2004). Improper TNF signaling has been implicated in multiple diseases from diabetes to cancer, making TNF signaling a high priority for biological dissection. Multiple therapeutic strategies are currently being employed. Detailed biochemical analysis has revealed a complex signaling network with multiple nodes of regulation. However, current therapeutic strategies largely focus on soluble receptors or blocking antibodies to neutralize the signal (Feldmann and Maini 2003). Further dissection of the signaling network and regulation will lead to novel drug targets and therapeutic strategies.

TNF-α engages two distinct, sequential, protein complexes to signal cell survival and cell death through TNFR1 (Figure 1.6). Upon TNF-α binding to the receptor, multiple components are recruited to the membrane bound TNF receptor, Complex I, including TRADD (TNFRSF1A-associated via death

domain), TRAF2 (TNF receptor-associated factor 2), cIAP1 (Cellular inhibitor of apoptosis-1), and RIPK1 (receptor (TNFRSF)-interacting serine-threonine kinase 1) (Micheau and Tschopp 2003). These components are stabilized by the association of SAM68 (Ramakrishnan and Baltimore 2011). RIPK1 undergoes K63-linked polyubiquitylation, allowing recruitment and stabilization of the IKK (IκB kinase) complex consisting of IKKα/β/γ through the polyubiquitin chain (Ea, Deng et al. 2006). RIPK1 polyubiquitination was originally thought to be essential for NF-κB signaling. However, new evidence shows that RIPK1 knockout MEF cells are still capable of activating NF-κB when treated with TNF-α (Wong, Gentle et al. 2010). This challenges a long held view, and further analysis is required to properly decipher the conflicting data.

Upon binding the polyubiquitin chains, the IKK complex is phosphorylated and activated by TAK1/TAB2/TAB3 (TGF-beta activated kinase 1, TGF-beta activated kinase 1/MAP3K7 binding protein 2, 3) complex at the receptor (Kanayama, Seth et al. 2004). Subsequently, the IKK complex dissociates from TNFR1 and phosphorylates IκB to allow IκB degradation. Once released from IκB inhibition, NF-κB subunits are free to translocate to the nucleus where they

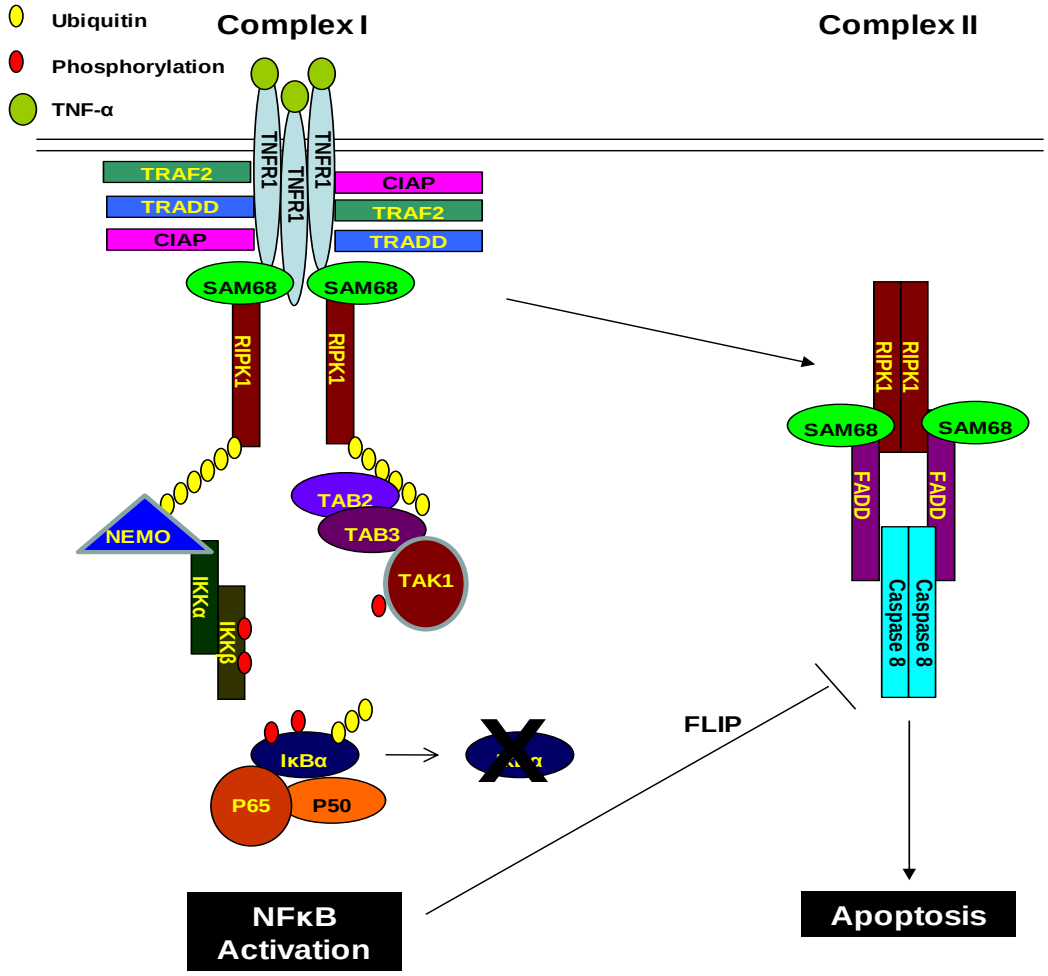


Figure 1.6 TNF-alpha induced NF-κB and apoptosis

CIAP1/2, TRAF2, TRADD, SAM68, and RIPK1 are recruited to TNFR1 (Complex I) upon binding of TNF-alpha. RIPK1 is ubiquitinated allowing recruitment of factors leading to IκB degradation and NF-κB activation. TNFR1 is internalized and RIPK1/SAM68 dissociates from TNFR1 and forms a cytoplasmic complex with FADD and Caspase 8 (Complex II). Caspase 8 activates and induces apoptosis. Caspase 8 activation can be inhibited by induction of FLIP.

activate transcription of cytokines and pro-survival genes including cIAP1/2 and FLIP (FLICE-inhibitory protein) (Irmler, Thome et al. 1997).

TNF-α induction of NF-κB happens within minutes of ligand binding to the receptor. On a scale of hours, in a mechanism that has yet to be defined, TNFR1 internalizes and components dissociate from the receptor. Both A20 and CYLD have been implicated in the deubiquitination of RIPK1 (Song, Rothe et al. 1996; Ferran, Stroka et al. 1998; He and Ting 2002; Wang, Du et al. 2008) . The precise mechanism of TNFR1 endocytosis has not been clearly defined, but deletion of the internalizationn domain from TNFR1 demonstrates that recruitment of receptor components happens prior to endocytosis (Schneider-Brachert, Tchikov et al. 2004). The released RIPK1 associates with FADD (Fas-associated death domain-containing protein) and caspase 8 in the cytoplasm to form Complex II within which caspase 8 is activated and initiates a caspase cascade resulting in apoptosis (Micheau and Tschopp 2003).

TNF-α induced Caspase 8 activation can be induced by co-treatment with the protein synthesis inhibitor cycloheximide. Cycloheximide inhibits NF-κB driven production of FLIP, an inhibitor of Caspase 8. Interestingly, co-treatment with Smac mimetic induces a different signaling complex than co-treatment with CHX. Smac mimetic induced apoptosis is completely dependent on RIPK1, unlike cycloheximide (Wang, Du et al. 2008). Treatment with a Smac mimetic

causes degradation of CIAP1 and CIAP2, proteins associated with TNFR1 (Figure 1.7). When the IAPs are degraded, RIPK1 is deubiquitinated and forms a cytosolic complex with FADD and Caspase 8. This complex is not regulated by endogenous c-FLIP.

While a significant amount of work has been devoted to deciphering this pathway, many lingering questions remain. Such as, how is the TNF receptor internalized upon activation and what role does that have in proper conduction of the cell death signal? TNFR1 also localizes to lipid raft regions, essential for function (Legler, Micheau et al. 2003). It is unknown how TNFR1 gets to these regions, and why localization is required for proper signaling. The combination of Smac and TNF-α induces a different cytoplasmic Caspase 8 activation complex than alternative TNF-α co-treatments, but the upstream signaling events are largely unchanged (Wang, Du et al. 2008). Therefore, TNF-α and Smac mimetic co-treatment serves as a tool for dissection of TNF-α signaling. For my thesis, I used a high-throughput siRNA screen to identify new components of Smac mimetic induced apoptosis. Extensive secondary screening validated CAPN9 as a

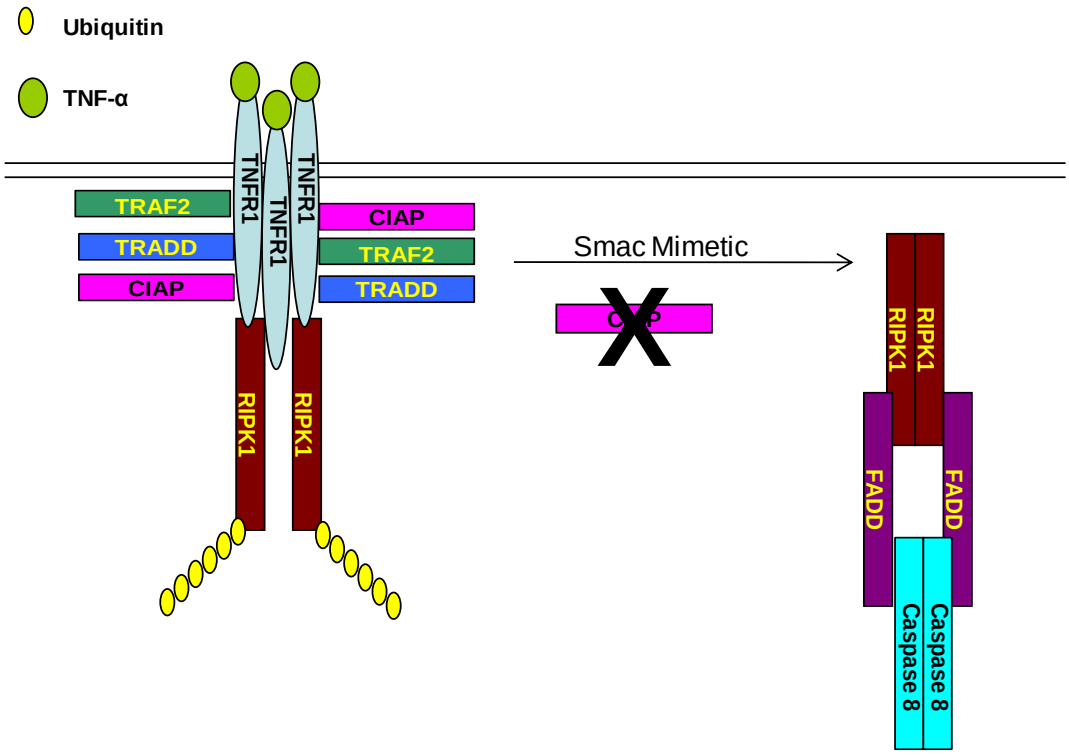


Figure 1.7 TNF-alpha and Smac mimetic induced apoptosis

TNF-alpha engages the recruitment of multiple factors to TNFR1, including RIPK1 which is heavily ubiquitinated. Smac mimetic binds to and induces degradation of CIAP1 and CIAP2. Through a mechanism not clearly understood, RIPK1 is released from the receptor to form a cytoplasmic complex with FADD and Caspase 8. This leads to Caspase 8 activation and cell death.

novel modulator of TNF-α induced cell death.

1.3 Calpain family proteins

Calpains are calcium activated cysteine proteases involved in a variety of functions. The ubiquitously expressed calpains, CAPN1 (μ-Calpain) and CAPN2 (m-Calpain), are the best studied. μ-Calpain and m-Calpain are named based off the calcium concentration required to activate them in vitro, reviewed in (Storr, Carragher et al. 2011). CAPN1 and CAPN2 both require a small regulatory subunit, CAPNS1, to be stably expressed and active. Calpain activation results in limited proteolysis of the protein, but proteolysis of calpains is not required for enzymatic activity (Cong, Goll et al. 1989; Cottin, Thompson et al. 2001; Reverter, Strobl et al. 2001; Goll, Thompson et al. 2003). The protein undergoes some level of autolysis during activation at physiological conditions.

Calpain activity is linked to multiple diseases. CAPN1 activity has been observed in the brains of Alzheimer's patients and is linked to cleavage of the beta-amyloid precursor protein (Saito, Elce et al. 1993; Nixon 2003). CAPN2 has been implicated in cataract formation (Huang and Wang 2001) . Mutations in CAPN3 are associated with limb girdle muscular dystrophy type 2A (Richard, Broux et al. 1995; Jia, Petrounevitch et al. 2001), while CAPN10 has been

associated with type 2 diabetes (Horikawa, Oda et al. 2000; Paul, Harmon et al. 2003). Calpains functions to cleave multiple protein targets, however there is no clear consensus sequence for proteolytic cleavage, making this family an attractive, yet difficult, biological system to study.

CAPN9 is of particular interest because of its potential role as a tumor suppressor. CAPN9 is predominately expressed in the stomach and small intestine (Lee, Sorimachi et al. 1998). Comparisons between normal gastric tissue and stomach cancer cell lines revealed CAPN9 expression is decreased (Yoshikawa, Mukai et al. 2000). In this study, a shorter splice variant corresponding to an in frame exclusion of an 81 nucleotide exon, CAPN9 splice variant 2, was isolated from normal gastric tissue. All further experiments focus on the longer splice variant, CAPN9 splice variant 1. Expression of antisense mRNA against CAPN9 increased transformation and tumorigenesis of NIH3T3 fibroblasts (Liu, Li et al. 2000). These studies suggest that CAPN9 is a tumor suppressor regulating the development of gastric carcinoma.

CAPN9 has been implicated in tumor development, but its function is still unknown. In vitro expression of recombinant CAPN9 requires co-expression of CAPNS1, indicating a dependence on the small subunit. The recombinant CAPN9 Splice Variant 1 was expressed and purified from SF-9 cells. The recombinant CAPN9 50% maximal activity concentration of calcium was

0.125mM, higher than μ-Calpain, but greater than 10 fold lower than m-Calpain. CAPN9 underwent autoproteolysis similar to CAPN1 and CAPN2. The in vitro purified CAPN9 shows many similar properties to CAPN1 and CAPN2 (Lee, Tomioka et al. 1999).

One recent study found CAPN9 can form a complex with CAPN8 in stomach cells. CAPN9 knockout mice show higher susceptibility to ethanol induced stomach lesions, indicating a protective role for CAPN9 (Hata, Abe et al. 2010). Another study also found CAPN9 to be involved in lumen formation of breast cancer cells grown in 3D culture. CAPN9 activity correlated with cleavage of PKC-δ, Protein Kinase C delta (Chen, Nguyen et al. 2010). Both reports correlate CAPN9 to a function, but do not offer an indication of the mechanism. In my thesis I demonstrate CAPN9 is novel modulator of TNF-α induced cell death. CAPN9 splice variant two is required for TNFR1 associated ubiquitination and basal association with cytoskeleton components, thus allowing recruitment and ubiquitination of RIPK1 upon binding of TNF.

Chapter Two:

Fishing the Genome for Determinants of Smac Sensitivity

2.1 High-Throughput siRNA Screen Development

Forward genetic screening is a powerful technique for discovery of biologically important signaling pathways. For decades, scientists have employed different mutagenesis strategies to evoke change in their model organism of choice; fly, worm, yeast etc. Selective mating of these organisms reveals the mutated gene causing the phenotype, which is then related to a human homologue. Aside from the obvious moral and ethical reasons, human beings are logistically and temporally intractable genetic models for screening. To this purpose, human cell lines have been developed which allow direct study of signaling networks. However, mutational screens are still improbably due to the inability to mate human cell lines for positional cloning. The advent of RNA interference technology has allowed researchers to overcome this issue. siRNAs target specific genes, eliminating the need for positional cloning. Several companies have developed libraries of siRNAs targeting known open reading

frames allowing for forward genetics screens, in a high-throughput format, on human cell lines.

Successful forward genetic screens require several factors. The screen must first have a clear definable goal. Several mechanistic elements of TNF-α induced apoptosis have been identified, as described in chapter one, yet many questions still remain. The identification of single agent, TNF-α dependent, sensitivity to the Smac mimetic opened a window to exploring the signaling pathway. Given this opportunity, I performed a forward genetic screen to identify novel components of TNF-α induced apoptosis, that when knocked down blocked Smac mimetic induced apoptosis.

A forward genetic screen must also have a wide coverage. Dharmacon has developed several siRNA libraries for this purpose. At the start of my screen, the Dharmacon siGenome library was at my disposal. This library consists of 21,125 open reading frame targets. For each gene, four siRNA oligos are pooled together. These oligos were designed using predictive software to maximize specific target gene knockdown efficiency while minimizing affects on alternative genes. However, this library was developed *in silico*, without exhaustive validation of every oligo. Pooling four siRNA oligos increased the probability of knocking down the target gene, with the assumption at least one of the four oligos would show efficacy. Also, pooling oligos allows for using each individual oligo

at a lower concentration. This decreases the chances of off-target effects brought on by partial sequence recognition and nonspecific binding of the oligo to genes other than they are designed to. The siGenome library provided exhaustive coverage of known open reading frames, making it an excellent resource for forward genetic screening.

Data collection is another key component in successful forward genetic screens. The final readout must be rapid to allow for high-throughput processing of multiple samples, robust to clearly reflect any phenotype, and repeatable to decrease the number of false positives. For this screen, I chose the Promega Cell TiterGlo assay, a luciferase assay measuring intercellular ATP, which correlates to the number of viable cells in the well. This assay provides a quantitative assessment of cell survival, allowing for easy processing of the final data. The protocol requires addition of reagent directly to a 96 well, followed by luminescence reading on a plate reader. During optimization, I determined decreasing the amount of reagent greatly increased the signal to noise ratio of the assay, without affecting the linear correlation between signal intensity and cell number (Figure 2.1). Results from this assay are stable for longer than an hour, allowing ample processing time without greatly affecting results.

Choosing the appropriate system is another essential decision for successful forward genetic screening. I sought to identify novel components in

TNF-α induced apoptosis. One optional approach would be to take a cell line resistant to apoptotic stimuli, and screen for siRNAs that sensitive the cells. However, several siRNA conditions decrease cell viability, making the cells more

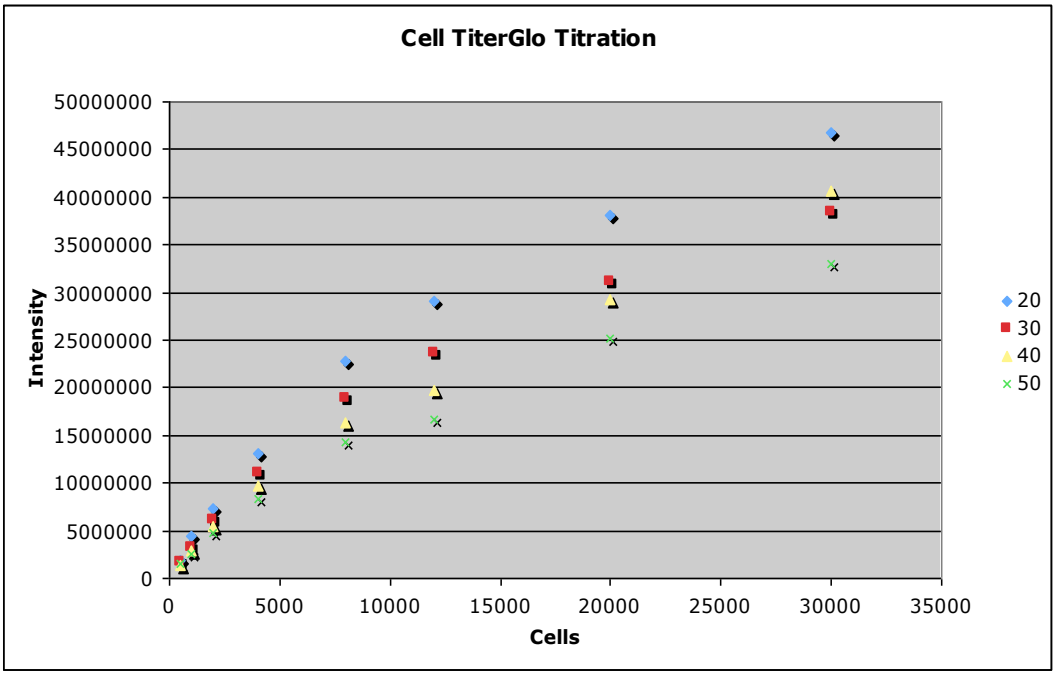


Figure 2.1 Optimization of Cell TiterGlo concentration

HCC44 cells were seeded in 96 well plates at the indicated cell number. Cells were allowed to adhere overnight. Cell TiterGlo reagent was added directly to the well in the indicated amounts (µL). Luminescence intensity was measured using the EnVision Multilabel Plate Reader (Perkin Elmer)

sensitive to a variety of stimuli. Also, knockdown of specific pathway signaling components would not increase cell sensitivity. Therefore, I chose to use a sensitive cell line, and identify siRNA knockdowns that made the cells resistant.

There are many factors to consider when choosing a cell line for High-Throughput siRNA Screening. The cell line must have a robust reaction to the stimulus being tested. Also, the cell line needs to have efficient RNA knockdown. I chose HCC44 for my screen. HCC44 showed strong sensitivity to the Smac mimetic, and efficient siRNA knockdown of target genes with multiple transfection agents. HCC44 cells also grow rapidly, doubling time of ~30hours, allowing for rapid generation of material for the screen. It is important to note, HCC44 cells do not efficiently express plasmid DNA. This made follow-up experiments difficult, and should be a considered when picking a cell line for any future high-throughput siRNA screens.

Every step of an siRNA screen has the potential to add variability to the system, making the final data analysis more difficult. A successful screen has a minimum of processing steps. One potential source of variability is the siRNA transfection. There are two options, first is a direct transfection, where cells are seeded in the well, allowed to adhere, then the next day media is removed and transfection reagent and siRNA are added to the cells. The alternative, reverse transfection, is seeding the cells in a well containing the transfection complexes.

Reverse transfections provide the advantage of less overall hands on processing and allow for easier control of the cell number during transfection. I chose a reverse transfection for my screen, and optimized transfection reagent conditions to yield the highest transfection efficiency.

There are many sources of variability in a high-throughput siRNA screen. Thus, proper controls must be in place to insure high quality data. One important consideration is the affect of siRNA knockdown on cell viability. This is critical for a screen looking for oligos that increase cell survival in response to a drug treatment, because a decrease in cell growth could give a false negative result. To overcome this issue a two condition screen was employed, where cells were transfected with siRNAs, then either treated with Smac mimetic, or a vehicle control. The cell viability, measured by Cell TiterGlo, of the Smac mimetic treated was normalized to the non-treated control. This also compensated for any variation due to positional effects. Different wells within a 96 well plate can have an effect on growth rate and cell viability, based off location. The treated and non treated, transfected cells were in the same location within the 96 well plate. Non-treated normalization of the treated cells compensated for this effect. It is important to note that the non-treated control does not distinguish between cytotoxic siRNAs and siRNAs that block cell growth.

Another source of variation occurs from processing the assay across multiple days. The Dharmacon siRNA library is arrayed in sets of 80, in 96 well plates. The first and last columns are left open for on plate controls. It is logistically unrealistic to process the entire siRNA library in one day. To overcome this variation, an average of signal from the first column of cells in the 96 well plate were used to normalize all the data within the plate (Figure 2.2). This normalization compensated for any variation introduced by the condition of the cells.

Human cell lines, grown in culture, are notorious for mutating and adapting during the course of cell line maintenance. Prolonged culture can affect the cell lines response to treatment with the Smac mimetic. The final column of every 96 well plate was treated with the smac mimetic (figure 2.2). The first 5 rows were averaged to verify the cells in every plate were still capable of responding to compound treatment, thus providing a negative control. Cells were carefully cultured, and not allowed to reach confluency. Frozen stocks of the cell line were thawed approximately once a month to ensure the most comparable data.

Cell transfection is another source of variation in a siRNA screen. The last 3 rows of the last column of the 96 well plate were transfected with an siRNA to RIPK1. RIPK1 is a critical transducer of the cell death signal triggered by the

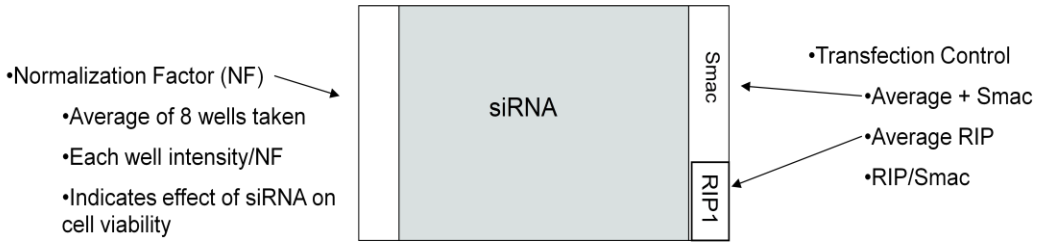


Figure 2.2 Schematic representation of screen controls

The first and last columns of the 96 well plate did not contain test siRNA and were used for controls. Luminescence intensity from wells in the first column were averaged giving a normalization factor for the entire plate. All luminescence intensities were divided by this factor.

The final column was treated with Smac mimetic and used as an assay control. The first five rows were averaged to indicate the amount of cell death of a negative control. The last three rows were transfected with a positive control oligo, RIPK1, known to completely block Smac mimetic induced apoptosis.

Smac mimetic. siRNA knockdown of RIPK1 completely blocks cell death with a minimal effect on cell viability (see introduction). The RIPK1 transfected cells were treated with the Smac mimetic, normalized by the plate control, and compared to the negative control. This served as a positive control; any plate where RIPK1 did not completely block cell death would be excluded from the final data analysis.

A successful siRNA screen requires stability of every processing step. Transfection, drug treatment, and data collection were all independently optimized to yield the most robust and repeatable results (Figure 2.3). The final assay was next tested against 16 known siRNA (Figure 2.4). Positive controls, Caspase 8 and RIPK1, were clearly distinguishable from other siRNAs. The assay was next applied to the entire Dharmacon siRNA library.

2.2 Results of a high-throughput siRNA screen for factors inhibiting Smac mimetic induced apoptosis

Data was collected for 21,125 siRNA transfections, treated with or without the Smac mimetic. Each data point was normalized to the plate control. The average of three replicates was calculated, as well as the standard deviation, and percent coefficient of variation (standard deviation/mean*100). The average of the treated sample was divided by the average of the untreated sample to give a

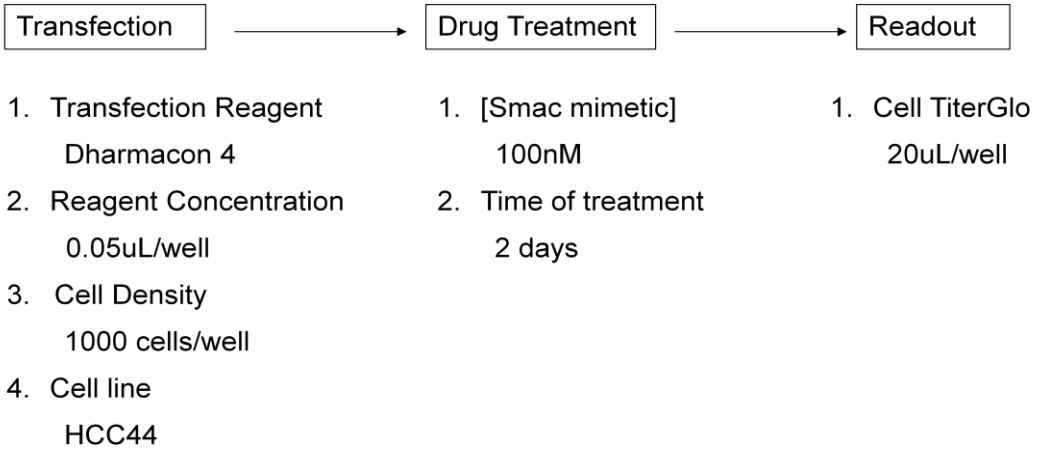


Figure 2.3 Variables optimized for siRNA Screen

All variables for the screen were independently optimized to yield the most robust and reliable results. Final assay conditions are listed above. Note: partway through the screen it was observed that liquid was spilling out of the well into the plate reader. Protocol was changed so media was removed from the plate and100µL of a 1:5 dilution of Cell TiterGlo in 1X DPBS and 1%Triton were added.

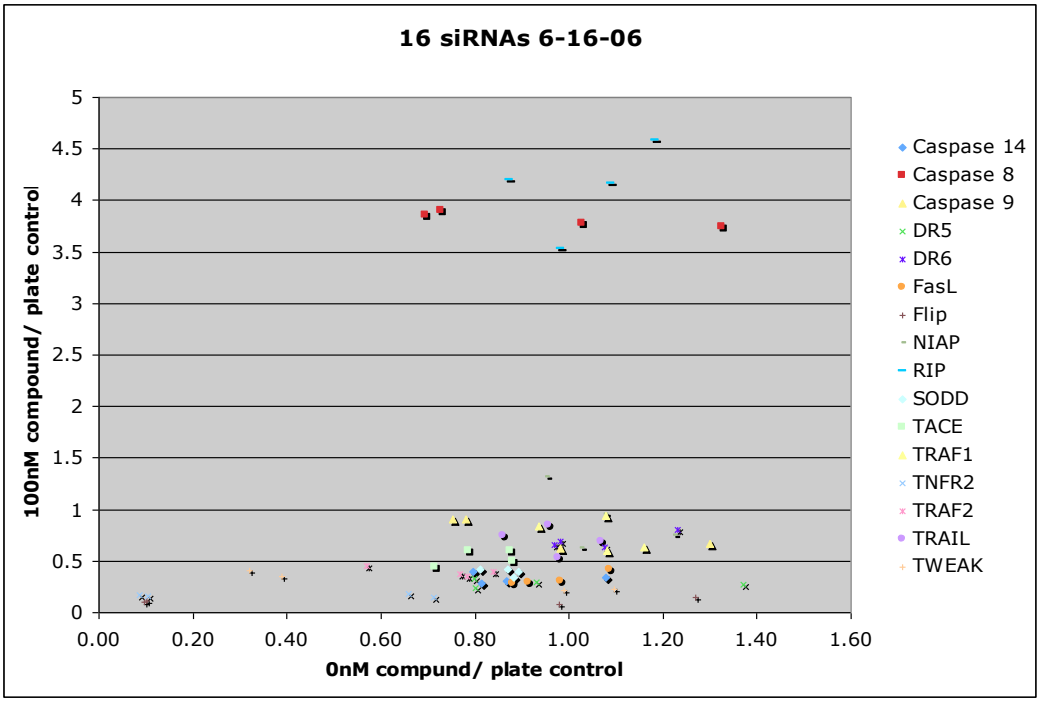


Figure 2.4 Baseline for High-Throughput siRNA Screen

HCC44 cells were transfected with known siRNAs using the screen assay format. The X-axis indicates the effect of the siRNA on cell viability. The Y-axis represents the fold change in cell survivability when treated with 100nM Smac mimetic. Positive controls were Caspase 8 and RIPK1.

survival ratio. A ratio of one indicates that the siRNA completely blocked cell death caused by the Smac mimetic.

Plate controls were analyzed as described in section 2.1. All plates had acceptable positive and negative controls. Spontaneous contamination of wells was observed. Bacteria and mold contamination was visible to the naked eye. All contaminated wells were eliminated from final analysis. Part way through the screen it was observed that the final well volume was too high and liquid was spilling into the plate reader. To overcome this issue, media was emptied from the plates prior to addition of 100µL of cell TiterGlo diluted 1:5 in 1 X DPBS plus 1% Triton X-100. Experiments were conducted to show little variation between the two methods (data not shown).

The non-treated siRNA transfections represent the effects of knockdown on cell growth and viability. Analysis of the data revealed two populations of response to the siRNA transfection (Figure 2.5). Conditions which resulted in cell death may create false positives because the ratio of treated to non-treated may be raised due to the influence of background signal. To avoid this, an arbitrary cut off of 0.4 was set. All siRNAs that decreased cell viability to 40%, or lower, were eliminated from further analysis. Other labs are currently mining this data set, and comparing the data to other cell lines.

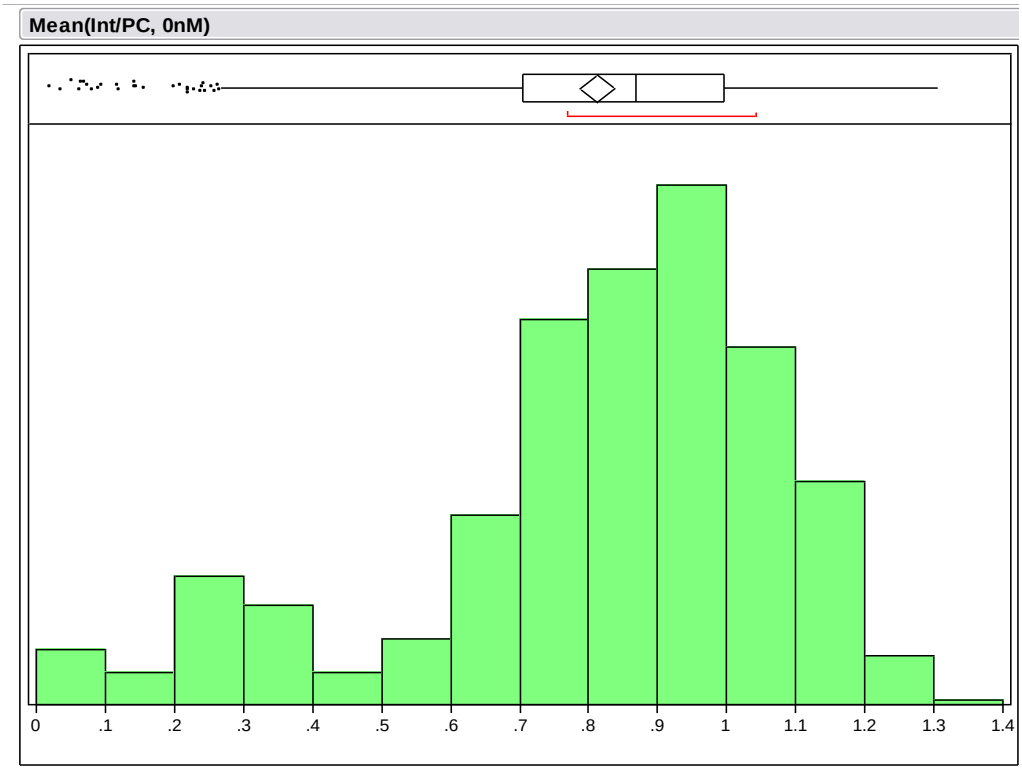


Figure 2.5 Effects of siRNA transfection on cell viability

This is a distribution of normalized signal data from each siRNA transfection of the siRNA screen. The X-axis represents the affect on cell viability, with 1 being no affect, 0.5 being 50% viability.

Despite all the controls, there were still siRNA treatments that had a high degree of variation. These results are not reliable and were eliminated from further analysis. A distribution of %CVs was generated (Figure 2.6). Arbitrary cutoffs were placed at 30% and 20% for Smac mimetic treated and non-treated respectively. All samples with larger %CVs were not analyzed further. It is possible that some of these siRNAs could block cell death from the Smac mimetic, but the primary data is not reliable due to the high degree of variation.

The survival ratio, Smac treated divided by non-treated, was calculated for each siRNA condition. The average survival ratio of every siRNA screen was 0.17, with a standard deviation of 0.11. Small z scores ((Sample mean – Population mean)/(Population standard deviation) were calculated for every hit, representing the number of "standard deviations" from the average of all survival ratios. A small z score of three or higher was considered a positive hit. Several known members of the TNF-α signaling pathway were identified, including caspase 8 and RIPK1, validating the screens ability to identify components of the signaling network (Figure 2.7).

394 siRNA conditions had z scores of three or greater. A literature search was conducted for each gene. Several siRNAs corresponding to open reading frames that no longer correlated to expressed genes. These discontinued genes were eliminated from further analysis. Several genes corresponding to olfactory

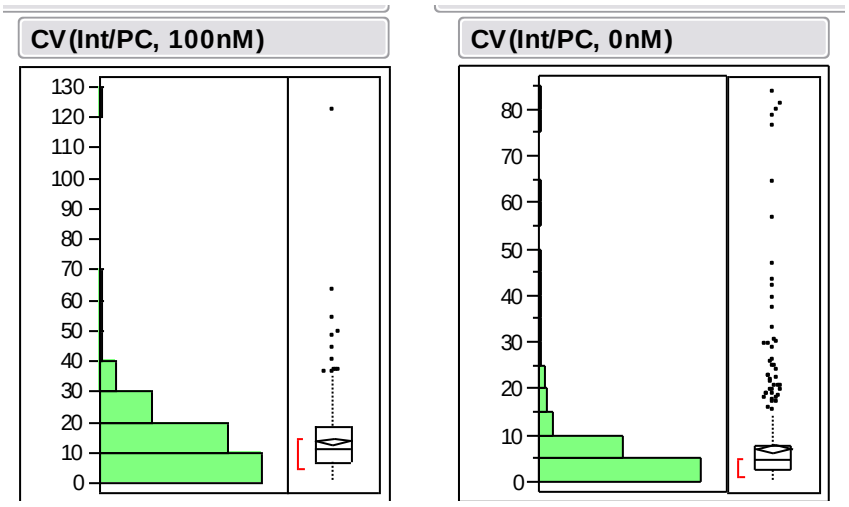


Figure 2.6 Variability of siRNA screen

This is a distribution representing the coefficient of variation for both the Smac mimetic treated(100nM) and non-treated(0nM) samples for all data points of the siRNA screen. Majority of CVs for the non-treated samples were under 10%. The treated samples had a higher degree of variation.

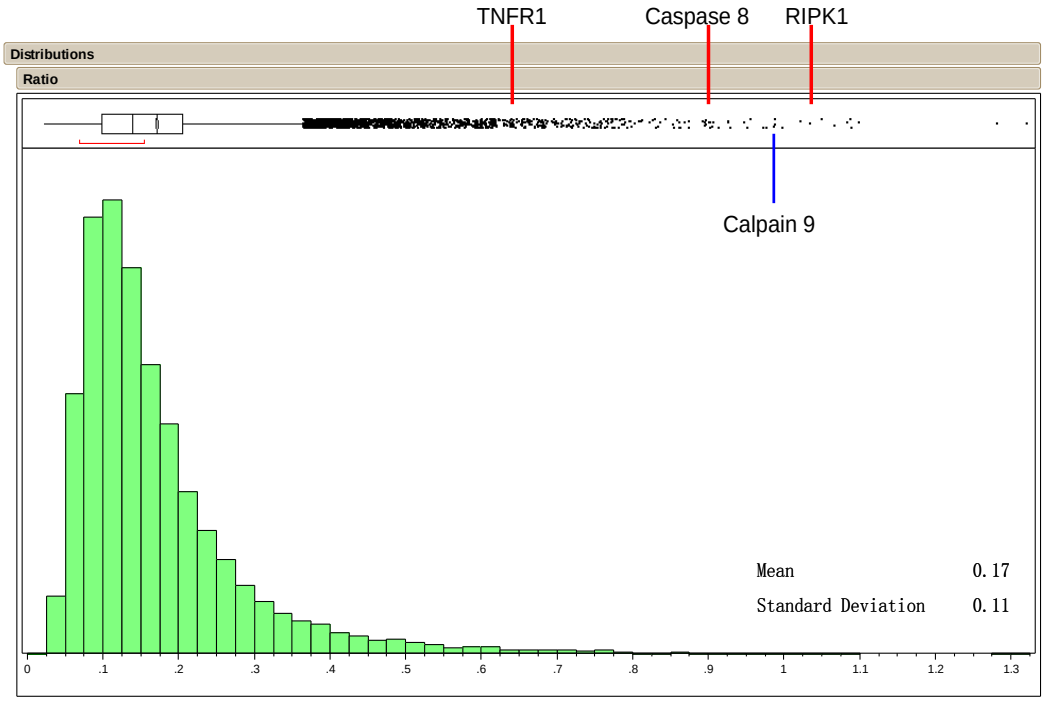


Figure 2.7 High-Throughput siRNA screen results

This is a distribution of survival ratios from all 21,125 genes screened. Survival ratio was calculated as the mean normalized signal of compound treated/mean normalized signal of non-treated. The majority of siRNAs had little effect on cell survival after Smac mimetic treatment. The mean ratio was 0.17 with a standard deviation of 0.11. Landmark genes, Caspase 8, RIPK1 and TNFR1 are indicated as well one novel positive hit from the screen, CAPN9.

transmembrane receptors were also eliminated due to a high probability that they were off-target effects. This reduced the positive hit list to 320 siRNAs. This data set was tested using siRNA from a Qiagen whole genome siRNA library. In parallel, siRNAs with a z score of five or higher were reordered. Pools of four oligos for each siRNA were split and retested individually (Figure 2.8).

2.3 Qiagen siRNA library followup

The Dharmacon siRNA library was designed using a specific algorithm to predict siRNA sequences with high affinity and specificity for their specific target genes. A rapid way to screen for off-target effects is to use an siRNA library designed with a different algorithm, thus increasing the likelihood of different sequences. The assumption is that both libraries will have at least one oligo in their pools that can efficiently knockdown the target gene, and any phenotypes seen from off-target effects will not occur in both sets of oligos. To this end, the top 320 hits were chosen for rescreening using the Qiagen whole genome library (Table 2.1).

Prevention of cell death was not nearly as strong in the Qiagen library as it was in the Dharmacon siRNA libray. An average and standard deviation for the negative control across all assay plates was used to create a baseline to determine a positive hit. siRNA data was controlled and processed the same as the primary screen (Figure 2.9). Positive hits were determined as any survival ratio greater

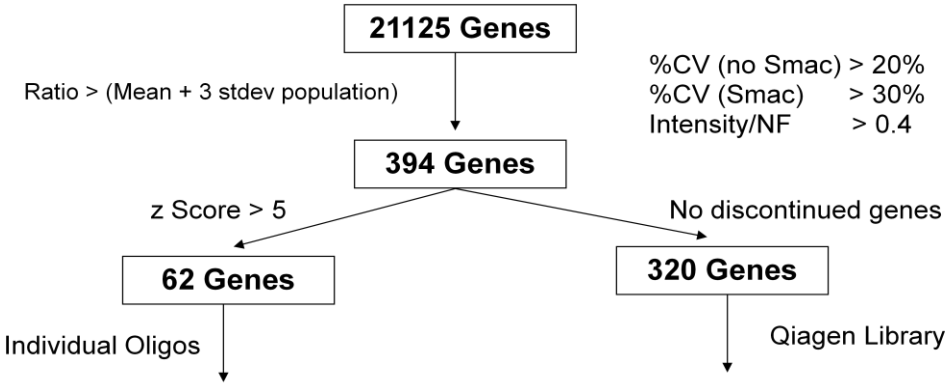


Figure 2.8 Schematic representation of sorting of hits from the siRNA screen

Two independent follow-up approaches were pursued for narrowing the list of positive hits from the siRNA screen. First, hits with high variability, or strong effects on cell viability were eliminated from further analysis. Next, all genes representing greater than 3 z score subject to literature survey. All non-discontinued genes were rescreened using the Qiagen siRNA library. All siRNA with greater than 5 z score were reordered from Dharmacon and individual oligos for each were tested.

Gene Symbol	Gene Name	Class	z score
YAP	101 associated protein 1	Transcription factor	7.75
LOC125958		OTR	7.69
TFDP1	Transcription factor Dp-1	Transcription factor	7.66
C13ORF17	Chromosome 13 open reading frame 17	Enzyme	7.64
FBXL6	F-box and leucine-rich repeat protein 6	Enzymes	7.62
NPIP	Nuclear pore complex interacting protein	Signaling	7.21
LOC393076		Unknown	7.20
OR9G4	Olfactory receptor, family 9, subfamily G, member 4	OTR	7.10
CUL3	Cullin 3	Enzymes	6.82
NPHP1	Nephronophthisis 1 (juvenile)	Signaling	6.82
GALR2	Galanin receptor 2	Signaling	6.81
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	Enzyme	6.71
LOC400720	Similar to hypothetical protein FLJ23506	Unknown	6.69
ANP32E	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	Signaling	6.55
LOC283585	Hypothetical protein LOC283585	Unknown	6.51
HIAN2	GTPase, IMAP family member 6	Enzyme	6.48
MYBPC3	Myosin binding protein C, cardiac	Structural	6.33
BID	BH3 interacting domain death agonist	Signaling	6.30
SRP46	Splicing factor, arginine/serine-rich, 46kD	Enzyme	6.19
ELMO3	Engulfment and cell motility 3 (ced-12 homolog, C. elegans)	Signaling	6.14
ADAMTS18	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 18	Enzyme	6.13
PCDH10	Protocadherin 10	Signaling	6.10
AMACR	Alpha-methylacyl-CoA racemase	Enzyme	6.06
FOXP2	Forkhead box P2	Transcription factor	6.06
LPL	Lipoprotein lipase	Enzyme	6.05
PRV1	Polycythemia rubra vera 1	Signaling	6.04
BTBD2	BTB (POZ) domain containing 2	Enzymes	6.03
KIAA1036	KIAA1036(vasohibin)	Signaling	5.87
NQO3A2	NAD(P)H:quinone oxidoreductase type 3, polypeptide A2(PHAP)	Signaling	5.87
SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	Secretion	5.82
PHYHIPL	Family with sequence similarity 13, member C1	Unknown	5.80
FLJ20811	Armadillo repeat containing, X-linked 6	Unknown	5.78
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	Signaling	5.77
FTO	Fatso	Unknown	5.76
LOC90835		Unknown	5.71
SYN2	Synapsin II	Signaling	5.70
GPP34R	Golgi phosphoprotein 3-like	Unknown	5.62
ITGAX	Integrin, alpha X (antigen CD11C (p150), alpha polypeptide)	Secretion	5.56
CAPN9	Calpain 9	Enzyme	5.53
UNG2	Uracil-DNA glycosylase 2	Enzyme	5.47
CHIA	Eosinophil chemotactic cytokine	Signaling	5.34
PPM1H		Enzyme	5.32
COPS7A	COP9 constitutive photomorphogenic homolog subunit 7A (Arabidopsis)	Enzyme	5.28
BCCIP	BRCA2 and CDKN1A interacting protein	Signaling	5.24
CXORF6	Chromosome X open reading frame 6	Unknown	5.23
MORC	Microrchidia homolog (mouse)	Unknown	5.22
NR5A1	Nuclear receptor subfamily 5, group A, member 1	Signaling	5.19
LGP1	Homolog of mouse LGP1	Signaling	5.18
USP8	Ubiquitin specific protease 8	Enzyme	5.16
IL4	Interleukin 4	Signaling	5.16
ZNRD1	Zinc ribbon domain containing, 1	Transcription factor	5.14
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	Signaling	5.13
ZNF501	Zinc finger protein 501	Transcription factor	5.13
PNLIPRP1	Pancreatic lipase-related protein 1	Enzyme	5.07
NS5ATP13TP2	NS5ATP13TP2 protein	Unknown	5.06
SPN	Sialophorin (gpL115, leukosialin, CD43)	Signaling	5.03
CASP8	Caspase 8, apoptosis-related cysteine protease	Enzyme	5.03
CPEB1	Cytoplasmic polyadenylation element binding protein 1	Structural	5.03
C7ORF21	Chromosome 7 open reading frame 21(TMUB1)	Structural	5.02
SEMG1	Semenogelin I	Unknown	5.02

Gene Symbol	Gene Name	Class	z score
JPH2	Junctophilin 2	Structural	5.02
FLJ21511	Hypothetical protein FLJ21511	Unknown	4.98
C1QB	Complement component 1, q subcomponent, beta polypeptide	Secretion	4.98
RETNLB	Resistin like beta	Signaling	4.97
GPC6	Glypican 6	Signaling	4.95
NGLY1	N-glycanase 1	Enzyme	4.95
APOA4	Apolipoprotein A-IV	Secretion	4.94
KRAS2	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Enzyme	4.91
SNX15	Sorting nexin 15	Secretion	4.90
GGT1	Gamma-glutamyltransferase 1	Enzyme	4.90
RAB34	RAB34, member RAS oncogene family	Secretion	4.90
CHRNA1	Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	Signaling	4.90
OR5K4	Olfactory receptor, family 5, subfamily K, member 4	OTR	4.88
C17ORF39	Chromosome 17 open reading frame 39	Transcription factor	4.85
CD68	CD68 antigen	Signaling	4.85
TCIRG1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein a isoform 3	Secretion	4.83
S100A9	S100 calcium binding protein A9 (calgranulin B)	Signaling	4.83
TRIM8	Tripartite motif-containing 8	Enzymes	4.81
TAGLN2	Transgelin 2	Unknown	4.79
C19ORF27	Chromosome 19 open reading frame 27	Unknown	4.77
CTEN	C-terminal tensin-like	Signaling	4.77
DBI	Diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	Signaling	4.75
DKFZP434N1923	Shank-interacting protein-like 1	Enzyme	4.74
CKAP4	Cytoskeleton-associated protein 4	Structural	4.73
LOC90353	LOC90353	Unknown	4.72
EZI		Transcription factor	4.69
LRRTM3	Leucine rich repeat transmembrane neuronal 3	Signaling	4.67
MGC12966	Hypothetical protein MGC12966	Unknown	4.66
PHACS	1-aminocyclopropane-1-carboxylate synthase	Enzyme	4.66
CTRC	Chymotrypsin C (caldecrin)	Enzyme	4.64
PTK7	PTK7 protein tyrosine kinase 7	Enzyme	4.64
NCB5OR	NADPH cytochrome B5 oxidoreductase	Enzyme	4.63
GRP58	Protein disulfide isomerase-associated 3	Enzyme	4.62
CD2BP2	CD2 antigen (cytoplasmic tail) binding protein 2	Unknown	4.59
BAL	Poly (ADP-ribose) polymerase family, member 9	Enzyme	4.59
WISP3	WNT1 inducible signaling pathway protein 3	Signaling	4.58
TRIM10	Tripartite motif-containing 10	Enzymes	4.58
SEC61A1	Sec61 alpha 1 subunit (S. cerevisiae)	Secretion	4.56
LOC255275	Similar to myeloid-associated differentiation marker	Unknown	4.55
CCIN	Calicin	Structural	4.52
TP53I3	Tumor protein p53 inducible protein 3	Enzyme	4.52
SLC9A7	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 7	Secretion	4.52
PLAG1	Pleiomorphic adenoma gene 1	Transcription factor	4.51
ITM2C	Integral membrane protein 2C	Secretion	4.49
CABC1	Chaperone, ABC1 activity of bc1 complex like (S. pombe)	Signaling	4.49
ULBP3	UL16 binding protein 3	Signaling	4.48
DMN	Desmuslin	Structural	4.48
MGC12972	Hypothetical protein MGC12972	Unknown	4.47
DKKL1-PENDING	Dickkopf-like 1 (soggy)	Signaling	4.43
C14ORF1	Chromosome 14 open reading frame 1	Unknown	4.42
PHF5A	PHD finger protein 5A	Transcription factor	4.42
TLOC1	Translocation protein 1	Secretion	4.42
FLJ13386	Centrosome protein Cep63	Unknown	4.41
TTC10	Tetratricopeptide repeat domain 10	Structural	4.41
RNF150		Enzymes	4.40
SDS3	Likely ortholog of mouse Sds3	Transcription factor	4.39
RASL11A	RAS-like, family 11, member A	Signaling	4.37
C19ORF24	Chromosome 19 open reading frame 24	Unknown	4.32
EVI2A	Ecotropic viral integration site 2A	Signaling	4.31
ANGPT1	Angiopoietin 1	Signaling	4.30

Gene Symbol	Gene Name	Class	z score
EZH2	Enhancer of zeste homolog 2 (Drosophila)	Transcription factor	4.29
FLJ14957	Cingulin-like 1	Unknown	4.28
OSBP2	Oxysterol binding protein 2	Unknown	4.27
LANCL1	LanC lantibiotic synthetase component C-like 1 (bacterial)	Signaling	4.27
SLC39A10		Secretion	4.26
MPDU1	Mannose-P-dolichol utilization defect 1	Signaling	4.26
DKFZP547G0215	Zinc finger protein 663	Transcription factor	4.25
FLJ21415	Hypothetical protein FLJ21415	Unknown	4.25
KIAA1573	KIAA1573 protein	Unknown	4.25
C14ORF58	Chromosome 14 open reading frame 58	Unknown	4.24
GZMM	Granzyme M (lymphocyte met-ase 1)	Enzyme	4.24
LILRB3	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 6	Signaling	4.22
MGC32065	Hypothetical protein MGC32065	Signaling	4.21
ZZZ3	Zinc finger, ZZ domain containing 3	Transcription factor	4.19
DSPG3	Dermatan sulfate proteoglycan 3	Structural	4.18
OGG1	8-oxoguanine DNA glycosylase	Enzyme	4.16
DAB1	Disabled homolog 1 (Drosophila)	Signaling	4.16
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	Enzyme	4.14
LOC339521		Unknown	4.14
CSNK1A1L	Casein kinase 1, alpha 1-like	Enzyme	4.13
FLJ10815	Amino acid transporter	Secretion	4.12
TBX20	T-box 20	Transcription factor	4.11
SRGAP1	SLIT-ROBO Rho GTPase activating protein 1	Signaling	4.09
FLJ21934	Hypothetical protein FLJ21934 UGT2A3	Enzyme	4.09
MGC3047	Limitrin	Structural	4.07
MGC10561	Chromobox homolog 2 (Pc class homolog, Drosophila)	Transcription factor	4.07
FBXL20	F-box and leucine-rich repeat protein 20	Enzymes	4.06
CYP4F3	Cytochrome P450, family 4, subfamily F, polypeptide 3	Enzyme	4.04
FLJ40869	Hypothetical protein FLJ40869	Unknown	4.04
SLC38A3	Solute carrier family 38, member 3	Secretion	4.03
FLJ43860	FLJ43860 protein	Unknown	4.03
C21ORF67	Chromosome 21 open reading frame 67	Unknown	4.00
IFNAR2	Interferon (alpha, beta and omega) receptor 2	Signaling	4.00
MYO5C	Myosin VC	Structural	3.97
FLJ20321	Hypothetical protein FLJ20321 CASZ1	Transcription factor	3.95
PRDX3	Peroxiredoxin 3	Enzyme	3.94
DKFZP434K046	Chromosome 16 open reading frame 49	Unknown	3.94
SEC5L1	SEC5-like 1 (S. cerevisiae)	Secretion	3.94
IL15RA	Interleukin 15 receptor, alpha	Signaling	3.93
SLC22A1LS	Solute carrier family 22 (organic cation transporter), member 1-like antisense	Secretion	3.92
ARPC1A	Actin related protein 2/3 complex, subunit 1A, 41kDa	Structural	3.91
TPM2	Tropomyosin 2 (beta)	Structural	3.91
SSR4	Signal sequence receptor, delta (translocon-associated protein delta)	Secretion	3.91
GPT2	Glutamic pyruvate transaminase (alanine aminotransferase) 2	Enzyme	3.91
BTN2A1	Butyrophilin, subfamily 2, member A1	Structural	3.88
KLHL1	Kelch-like 1 (Drosophila)	Structural	3.87
C9ORF111	Chromosome 9 open reading frame 111	Enzyme	3.86
GPR15	G protein-coupled receptor 15	Signaling	3.84
PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	Signaling	3.84
CLTA	Clathrin, light polypeptide (Lca)	Secretion	3.82
PECI	Peroxisomal D3,D2-enoyl-CoA isomerase	Enzyme	3.78
CENTG1	Centaurin, gamma 1	Signaling	3.78
SYNJ1	Synaptojanin 1	Enzyme	3.78
FLJ20211	Hypothetical protein FLJ20211	Unknown	3.76
LOC255411	Hypothetical LOC255411	Unknown	3.75
KIF2	Kinesin heavy chain member 2	Structural	3.74
LOC390345	Similar to 60S ribosomal protein L10 (QM protein) (Tumor suppressor QM) (Laminin receptor homolog)	Unknown	3.74
DNAJC5	DnaJ (Hsp40) homolog, subfamily C, member 5	Structural	3.74
SIN3A	SIN3 homolog A, transcription regulator (yeast)	Transcription factor	3.73
ZNF346	Zinc finger protein 346	Transcription factor	3.73
ZNF541	Zinc finger protein 541	Transcription factor	3.72
DPPA4	Developmental pluripotency associated 4	Signaling	3.71
ZNF600	Zinc finger protein 600	Transcription factor	3.71
CSF3R	Colony stimulating factor 3 receptor (granulocyte)	Signaling	3.71
PWP2H	PWP2 periodic tryptophan protein homolog (yeast)	Signaling	3.71
KCNV1	Potassium channel, subfamily V, member 1	Secretion	3.71
NYD-SP14	NYD-SP14 protein	Unknown	3.70
SLC9A8		Secretion	3.69
C1QL1	Complement component 1, q subcomponent-like 1	Secretion	3.69
TM4SF6	Tetraspanin 6	Signaling	3.67

Gene Symbol	Gene Name	Class	z score
MASP2	Mannan-binding lectin serine protease 2	Enzyme	3.67
CLDN2	Claudin 2	Structural	3.67
PRRG2	Proline rich Gla (G-carboxyglutamic acid) 2	Signaling	3.66
C20ORF23		Structural	3.64
PAX2	Paired box gene 2	Transcription factor	3.64
KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Signaling	3.64
MMP11	Matrix metalloproteinase 11 (stromelysin 3)	Enzyme	3.64
LOC387750	Similar to mFLJ00251 protein	Enzyme	3.64
CTTN	Cortactin	Signaling	3.63
OLFM1	Olfactomedin 1	Signaling	3.63
MGC3040	Hypothetical protein MGC3040	Unknown	3.62
MGC3200	Ankyrin repeat domain 34	Transcription factor	3.62
FGFRL1	Fibroblast growth factor receptor-like 1	Signaling	3.62
ABI1	Abl-interactor 1	Enzyme	3.62
LOC390790	ADP-ribosylation factor-like 12	Unknown	3.61
SENP7	SUMO1/sentrin specific protease 7	Enzyme	3.60
COL4A1	Collagen, type IV, alpha 1	Structural	3.60
PTBP1	Polypyrimidine tract binding protein 1	Transcription factor	3.59
PPP2CZ	Protein phosphatase 1J (PP2C domain containing)	Enzyme	3.59
MGC14288	Hypothetical protein MGC14288	Unknown	3.58
LOC389308	Similar to ribosomal protein L10a	Unknown	3.58
ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12	Signaling	3.58
RPL36	Ribosomal protein L36	Structural	3.57
SAV1	Salvador homolog 1 (Drosophila)	Signaling	3.56
TARP	T cell receptor gamma variable 9	Signaling	3.54
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	Transcription factor	3.54
CNTNAP2	Contactin associated protein-like 2	Structural	3.54
NIP30	NEFA-interacting nuclear protein NIP30	Unknown	3.54
SCUBE1	Signal peptide, CUB domain, EGF-like 1	Signaling	3.54
GGT2		Enzyme	3.53
IL17B	Interleukin 17B	Signaling	3.53
MPRP-1	OMA1 homolog, zinc metallopeptidase (S. cerevisiae)	Enzyme	3.52
LAIR1	Leukocyte-associated Ig-like receptor 1	Signaling	3.52
KIAA0738	KIAA0738 gene product	Unknown	3.51
AMY2B	Amylase, alpha 2B; pancreatic	Enzyme	3.50
LAMC3	Laminin, gamma 3	Structural	3.50
RNF6	Ring finger protein (C3H2C3 type) 6	Enzymes	3.48
C21ORF69	Chromosome 21 open reading frame 69	Unknown	3.47
LOC388538	Sprouty-related, EVH1 domain containing 3	Signaling	3.47
FLJ10349	Hypothetical protein FLJ10349	Unknown	3.47
CADPS	Ca2+-dependent secretion activator	Secretion	3.45
TAC3	Tachykinin 3 (neuromedin K, neurokinin beta)	Signaling	3.45
KCNE4	Potassium voltage-gated channel, Isk-related family, member 4	Secretion	3.45
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H	Enzyme	3.44
EED	Embryonic ectoderm development	Transcription factor	3.44
ZNF339	Zinc finger protein 339	Transcription factor	3.44
FLJ11280	Family with sequence similarity 63, member A	Unknown	3.43
LEPREL1	Leprecan-like 1	Enzyme	3.42
ACAA1	Acetyl-Coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-Coenzyme A thiolase)	Enzyme	3.42
LOC405753	Similar to Numb-interacting homolog gene	Secretion	3.41
BASE	Breast cancer and salivary gland expression gene	Unknown	3.41
HIAN6	GTPase, IMAP family member 8	Enzyme	3.41
LOC389105	Hypothetical LOC389105	Unknown	3.41
RPL36AL	Ribosomal protein L36a-like	Transcription factor	3.41
APG4B	APG4 autophagy 4 homolog B (S. cerevisiae)	Enzyme	3.40
NES	Nestin	Structural	3.40
CCL2	Chemokine (C-C motif) ligand 2	Signaling	3.37
GORASP2	Golgi reassembly stacking protein 2, 55kDa	Structural	3.36
OPN4	Opsin 4 (melanopsin)	Signaling	3.36
ATP1B4	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	Secretion	3.35
CCL24	Chemokine (C-C motif) ligand 24	Signaling	3.35
LOC390669	Similar to ATP-binding cassette, sub-family A member 3; ATP-binding cassette 3; ABC transporter 3	Unknown	3.35
OR10H1	Olfactory receptor, family 10, subfamily H, member 1	OTR	3.34
FLJ10707	Hypothetical protein FLJ10707	Enzyme	3.33
FLJ10490	Hypothetical protein FLJ10490	Unknown	3.33
CCL13	Chemokine (C-C motif) ligand 13	Signaling	3.32
LOC285697		Transcription factor	3.32
LOC341412	Similar to ribosomal protein L31	Unknown	3.32
LOC391248	Similar to LPIN3	Unknown	3.32
ZMYND12	Zinc finger, MYND domain containing 12	Transcription factor	3.32

Gene Symbol	Gene Name	Class	z score
BRIX	Brix domain containing 2	Transcription factor	3.32
HOM-TES-103	HOM-TES-103 tumor antigen-like	Structural	3.31
RILP	Rab interacting lysosomal protein	Secretion	3.30
BHLHB3	Basic helix-loop-helix domain containing, class B, 3	Transcription factor	3.30
SIAT4A	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	Enzyme	3.30
LU	Lutheran blood group (Auberger b antigen included)	Structural	3.30
EXO1	Exonuclease 1	Enzyme	3.29
CT120	Family with sequence similarity 57, member A	Signaling	3.29
MGC15429	Hypothetical protein MGC15429(ABHD14B)	Enzyme	3.28
ZNF154	Zinc finger protein 154 (pHZ-92)	Transcription factor	3.28
LRRTM4	Leucine rich repeat transmembrane neuronal 4	Unknown	3.26
LY6D	Lymphocyte antigen 6 complex, locus D	Structural	3.22
LOC284393	Similar to 60S ribosomal protein L10 (QM protein homolog)	Structural	3.22
NIFIE14	Seven transmembrane domain protein	Unknown	3.21
MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	Enzyme	3.21
UPF3B	UPF3 regulator of nonsense transcripts homolog B (yeast)	Transcription factor	3.19
HBZ	Hemoglobin, zeta	Signaling	3.19
SH3GLB2	SH3-domain GRB2-like endophilin B2	Transcription factor	3.19
ITGAL	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	Signaling	3.18
HSA272196	Hypothetical protein, clone 2746033	Unknown	3.17
C21ORF61	Melanocortin 2 receptor accessory protein	Secretion	3.16
KRT3	Keratin 3	Structural	3.16
FLG		Structural	3.16
KIAA0363	Similar to KIAA0363	Unknown	3.15
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	Signaling	3.14
SPG7	Spastic paraplegia 7, paraplegin (pure and complicated autosomal recessive)	Enzyme	3.14
LOC402643		Unknown	3.14
ASS	Argininosuccinate synthetase	Enzyme	3.14
TENC1	Tensin like C1 domain containing phosphatase	Enzyme	3.14
KIAA1128	KIAA1128	Unknown	3.13
ARRDC2	Arrestin domain containing 2	Unknown	3.13
PTCD2	Pentatricopeptide repeat domain 2	Unknown	3.13
SBLF	TFIIA-alpha/beta-like factor	Transcription factor	3.12
C2ORF32	Chromosome 2 open reading frame 32	Unknown	3.10
KIAA0870		Unknown	3.10
THAP11	THAP domain containing 11	Transcription factor	3.09
NYD-SP12	Spermatogenesis associated 16	Unknown	3.08
LIMR	Lipocalin-interacting membrane receptor	Secretion	3.08
HSPB2	Heat shock 27kDa protein 2	Signaling	3.08
FNDC3	Fibronectin type III domain containing 3A	Structural	3.08
RKHD3	Ring finger and KH domain containing 3	Enzymes	3.07
PHEMX	Pan-hematopoietic expression(tetraspanin)	Signaling	3.07
RNF126	Ring finger protein 126	Enzymes	3.06
NEUROD6	Neurogenic differentiation 6	Transcription factor	3.06
GK001	GK001 protein	Unknown	3.05
UPK1A	Uroplakin 1A(tetraspanin family)	Signaling	3.05
EFNA3	Ephrin-A3	Signaling	3.04
FLJ46257	FLJ46257 protein	Unknown	3.04
KIAA1724		Enzyme	3.04
APOM	Apolipoprotein M	Secretion	3.04
PLEC1	Plectin 1, intermediate filament binding protein 500kDa	Structural	3.03
C20ORF36	Chromosome 20 open reading frame 36	Transcription factor	3.03
MCM8	MCM8 minichromosome maintenance deficient 8 (S. cerevisiae)	Transcription factor	3.02
LOC388554	Hypothetical BC282485_1	Unknown	3.02
ARHI	DIRAS family, GTP-binding RAS-like 3	Enzyme	3.01
TRAF3IP1	TNF receptor-associated factor 3 interacting protein 1	Signaling	3.01
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Signaling	1.66
TNF	Tumor necrosis factor (TNF superfamily, member 2)	Signaling	1.40
CASP9	Caspase 9, apoptosis-related cysteine protease	Enzyme	-0.32
CAPNS1	Calpain, small subunit 1	Enzyme	-0.69

Table 2.1 siRNA hits repeated in the Qiagen library

List of positive hits rescreened in the Qiagen library. z-score is from the primary screen. Class indicates the type of protein based off a literature survey: transcription factor, enzyme, unknown, signaling – involved in a known signaling pathway, secretion, structural – involved in cytoskeleton, OTR – olfactory transmembrane receptor.

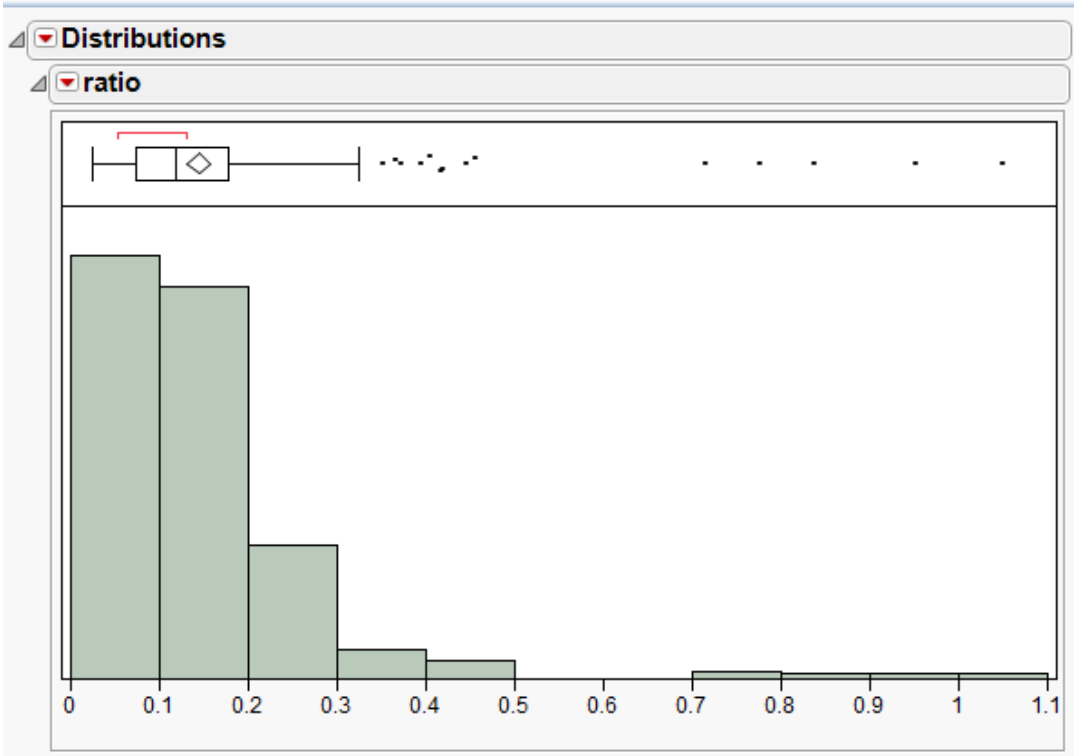


Figure 2.9

This is a histogram of distributions from the Qiagen cherry pick library screen. X-axis is the survival ratio of Smac treated/ non treated. Negative control samples averaged to 0.11 with a standard deviation of 0.007.

than 0.2, representing greater than thirteen standard deviations from the negative control mean. Sixty-two siRNAs were considered positive hits from the secondary screen.

2.4 Individual oligo follow-up

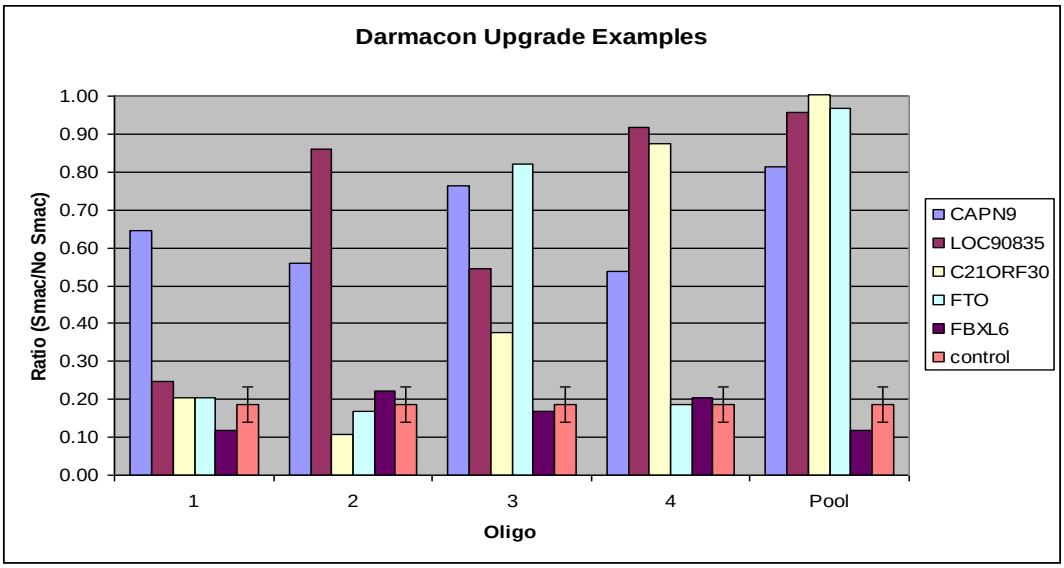
The Dharmacon siRNA library contains a pool of four siRNA oligos for each specific gene. One method to confirm siRNA affects are specific to gene knockdown is to test each oligo individually. Individual oligo screening serves as a rapid and robust method to narrow down positive hits. A positive hit will have multiple oligos conferring the same phenotype. This is based off the assumptions that more than one siRNA in the pool is capable of efficiently knocking down expression of the target gene, and that off-target effects are a rare event, unlikely to occur from more than one oligo designed to a gene. This method was done in parallel with the Qiagen library followup.

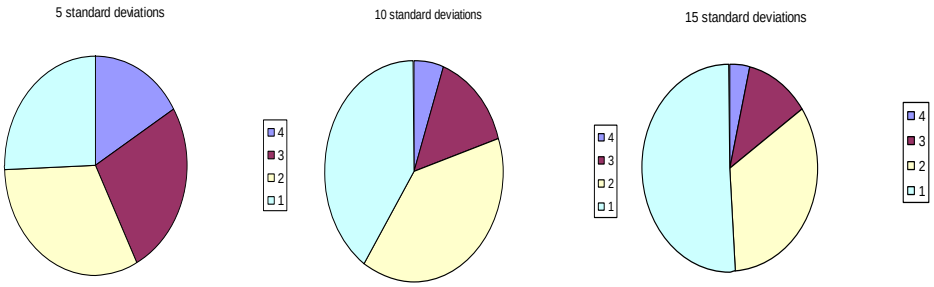
Logistical and financial constraints limited the number of hits followed up with individual oligo screening. siRNA knockdowns with a z score of greater than five were used (Table 2.1). Individual oligos and pools for sixty-two genes, as well as the positive controls RIPK1 and Caspase 8, were arrayed in 96 well plates and assayed following the same protocol as the initial screen. Negative control wells were used to establish a baseline. Mean and standard deviation were calculated for the negative controls. siRNAs with a survival ratio greater than

fifteen standard deviations above the mean of the negative control were considered positive hits. Fifty-four genes repeated prevention of cell death from the Smac mimetic. Twenty-eight of the fifty-four genes had only one oligo corresponding to prevention of cell death (Figure 2.10). Genes with more than one working siRNA were considered for further study (Table 2.2)

Bid is a known participant in TNF alpha induced apoptosis. When activated, Caspase 8 cleaves Bid and the truncated form translocates to the mitochondria, releasing Cytochrome C, activating the intrinsic apoptotic program. Some cells require activation of the intrinsic pathway to achieve complete cell death. siRNA of Caspase 9, the intrinsic apoptosis activating caspase, did not prevent cell death in HCC44 cells. However, it is possible for the HCC44 cells to require activation of caspase 9, yet the knockdown is not efficient enough to reflect this. Western blot analysis revealed a direct correlation between siRNA knockdown efficiency and prevention of cell death for BID (Figure 2.11). This indicates that elimination of genes based off the number of individual oligos might rule out positive hits. Alternative methods of secondary screening are required to further refine the list of positive hits.

siRNA transfection can have a variety of effects on a given cell line, potentially not specific to the TNF-α signaling pathway. Gene knockdowns





#oligos rescue	5 stdev	10 stdev	15 stdev
1	14	22	28
2	17	21	18
3	14	8	6
4	9	3	2

Figure 2.10 Individual oligo rescue

A. Individual oligos were transfected for 62 siRNA in HCC44 cells following screen transfection protocol. Examples of each type of result are graphed here. CAPN9 had four oligos rescue, LOC90835 had three, two oligos for C21ORF30, one oligo for FTO and no oligos for FBXL6.

B. 54 of the 62 siRNA had at least one oligo repeat, as measured by the number of standard deviations the survival ratio was from the control.

Gene Symbol	Gene Name	15stdev
CAPN9	Calpain 9	4
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	4
C13ORF17	Chromosome 13 open reading frame 17(DCN1 cullin neddylation)	3
CPEB1	Cytoplasmic polyadenylation element binding protein 1	3
LOC90835		3
MORC	Microrchidia homolog (mouse)	3
NS5ATP13TP2	NS5ATP13TP2 protein(OAF Homolouge)	3
SYN2	Synapsin II	3
AMACR	Alpha-methylacyl-CoA racemase	2
BTBD2	BTB (POZ) domain containing 2	2
C1QB	Complement component 1, q subcomponent, beta polypeptide	2
C21ORF30		2
COPS7A	COP9 constitutive photomorphogenic homolog subunit 7A (Arabidopsis)	2
CXORF6	Chromosome X open reading frame 6	2
ELMO3	Engulfment and cell motility 3 (ced-12 homolog, C. elegans)	2
FLJ21511	Hypothetical protein FLJ21511	2
FOXP2	Forkhead box P2	2
JPH2	Junctophilin 2	2
KIAA1036	KIAA1036(vasohibin 1)	2
LOC393076		2
NPHP1	Nephronophthisis 1 (juvenile)	2
SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	2
SEMG1	Semenogelin I	2
ZNF501	Zinc finger protein 501	2
ZNRD1	Zinc ribbon domain containing, 1	2

Table 2.2 siRNA conditions where two or more oligos prevented cell death

Names of siRNAs with more than two oligos preventing cell death from the Smac mimetic.

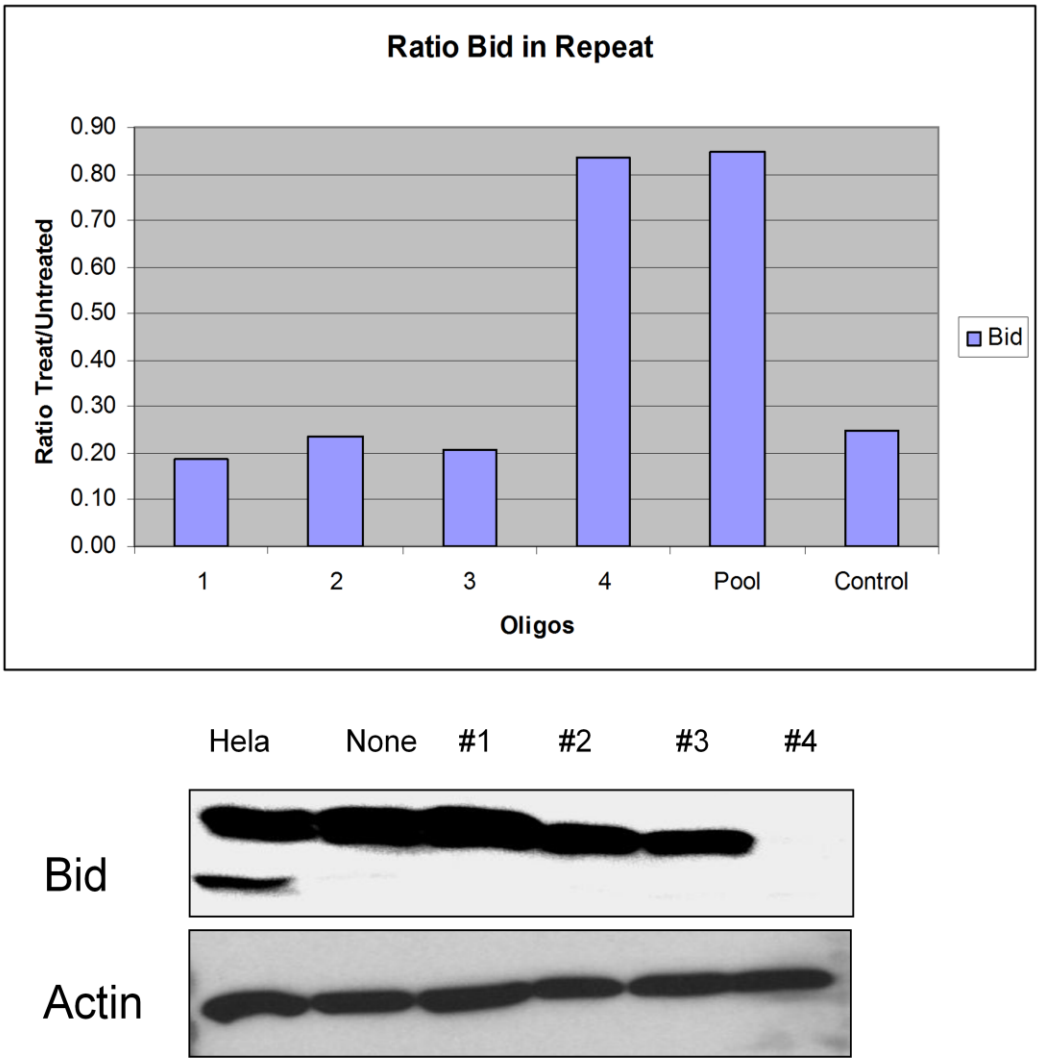


Figure 2.11 Efficacy of elimation of hits by number of individual oligos

HCC44 were tranfected with siRNA to Bid and treated with 100nM Smac mimetic. Survival ratio was calculated as treated signal/ non-treated. Oligo 4 was able to prevent Smac mimetic induced cell death. Lower panel is a western blot analysis using antibodies against Bid and Actin.

conferring resistance to the Smac mimetic in multiple cell lines would have a higher potential of being specific to the signaling pathway. The top fifty-four repeating siRNAs were first retested in HCC461. Both the primary screening cell line and HCC461 are non-small cell lung carcinoma cell lines. All oligos repeated between the two lines except BID. This is consistent with previous data that some cell lines require activation of the extrinsic apoptotic program, while others do not. In an effort to further narrow down the number of positive hits, siRNAs were screened in the breast cancer cell line, MDA-MB231. Twenty-nine of the 54 hits repeated (Figure 2.12).

2.5 Results of Secondary Screening

Each secondary screening method relies on several assumptions. Combining the methods increases the likelihood that each hit is not the product of an off-target effect, and has a strong impact on the signaling pathway. The three most promising hits from the screen were CAPN9, AMACR, and NS5ATP13TP2 (OAF). These genes had multiple siRNAs confer resistance to the Smac mimetic in multiple cell lines (Table 2.3). siRNA from Qiagen, designed with a different algorithm, also block Smac mimetic induced apoptosis. While this data was compelling, further experiments were required to verify these targets as regulators of Smac mimetic induced apoptosis.

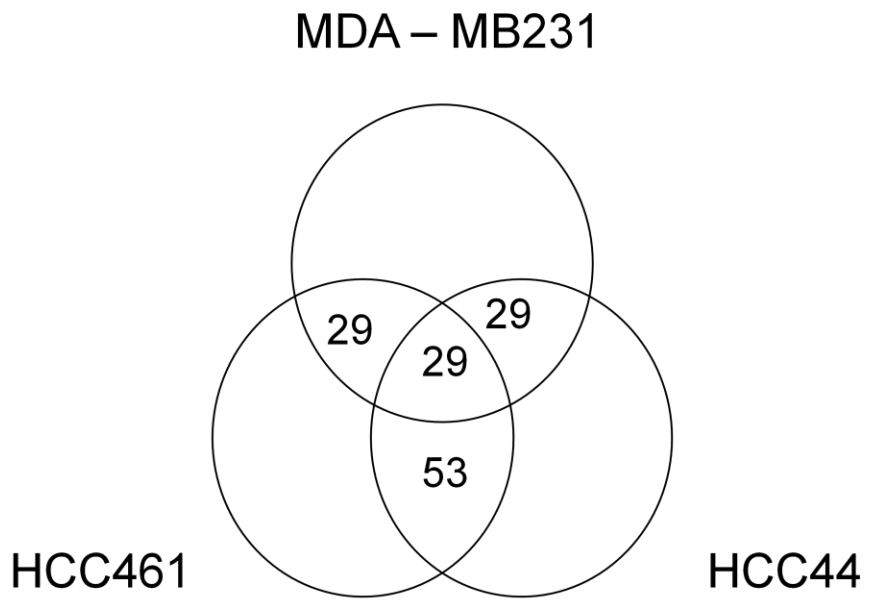


Figure 2.12 Number of positive in hits overlapping between cell lines

Pools of four oligos to the 54 repeating siRNA hits were transfected into each of the following cell lines. Number of overlapping hits resulting in a z score of 5 or higher above a negative control is demonstrated here.

siRNA	Description	HCC461	MDA-MB231	Qiagen	#oligos
RIPK1	Kinase	X	X	X	4
Caspase 8	Extrinsic Caspase	X	X	X	4
CAPN9	Protease	X	X	X	4
NS5ATP13TP2	Out at First Homologue	X	X	X	3
AMACR	racemase	X	X	X	2

Table 2.3 Results of Secondary Screening

This is a summary of results from all secondary screening. Cells were transfected with the following siRNA and treated with 100nM Smac mimetic. Cell viability scores corresponding to a prevention of cell death with a z score greater than 5 are marked with an X.

NS5ATP13TP2 is a homologue of the Drosophila protein OAF, out at first. Little is known about this protein, making followup assays difficult. The decision was made to focus on AMACR and CAPN9 because they are enzymes, thus more tractable for biochemical analysis. OAF may serve as an interesting target for future lab members.

AMACR is an enzyme involved in β-oxidation of fatty acids. AMACR was a high priority hit, with two oligos preventing apoptosis. AMACR was of particular interest because it also blocked TNF-α, Smac mimetic, and z-VAD induced necrosis in HT-29 cells (Sudan He, data not shown). To confirm AMACR was a specific hit, not an off-target effect, the gene was cloned from HCC44 cells by RT-PCR into a pcDNA 3.1+ vector with three N-terminal flag tags. 293T cells were co-transfected with the plasmid and individual siRNA from Dharmacon. Oligos one, two and four were able to efficiently knockdown expression of the AMACR construct. This did not correlate with the phenotype, where oligos two and three were able to completely block Smac mimetic induced apoptosis (Figure 2.13). This data clearly shows that AMACR is a false positive hit from the siRNA screen.

Despite rigorous secondary screening, AMACR proved to be an off-target effect. Correlation between phenotype and knockdown efficiency, while not

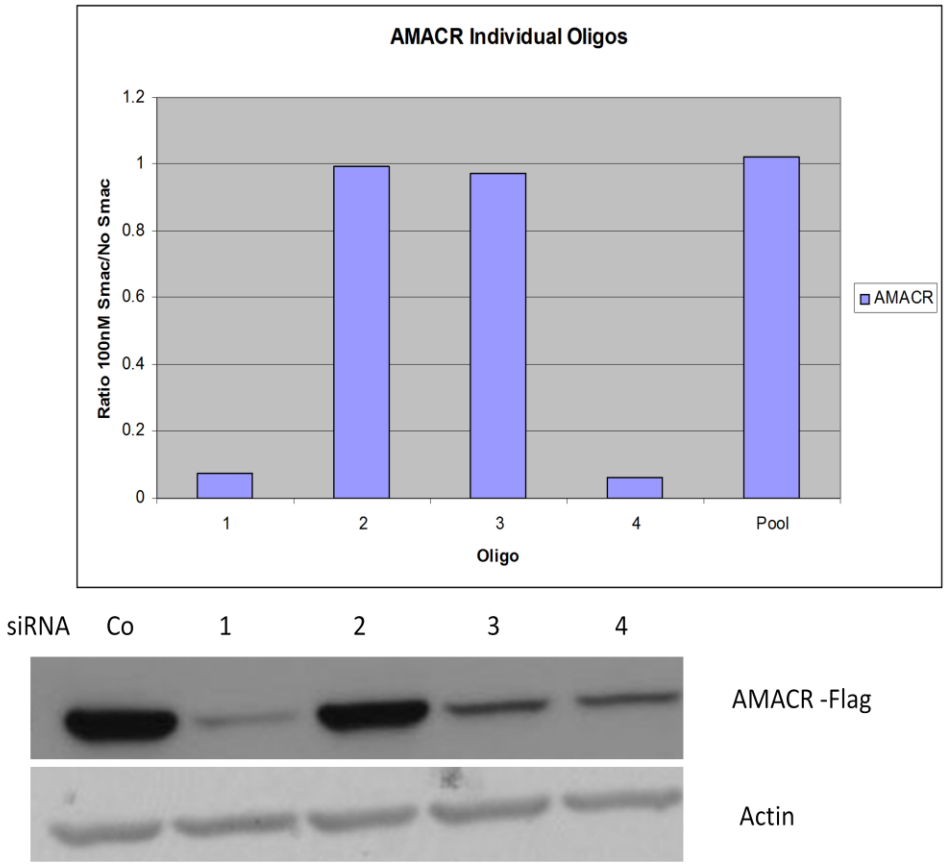


Figure 2.13 AMACR knockdown does not correlate with prevention of cell death

In the upper panel, HCC44 cells were transfected with corresponding siRNA to AMACR, and treated with or without 100nM Smac mimetic. Cell viability was measured using Cell TiterGlo and calculated as treated signal/ non-treated signal.

In the lower panel, a flag tagged AMACR expression construct was co-transfected into HEK293T cells with individual siRNA oligos to AMACR and analyzed by western blot with Flag and Actin antibodies.

logistically favorable, appears to be the best method for narrowing down a hit list from siRNA screening. Future screens should employ this method first. Elimination of AMACR leaves CAPN9 as the focus of future experiments

Chapter Three

Validation of Calpain 9

3.1 A specific CAPN9 splice variant is required for Smac mimetic sensitivity

CAPN9 was a promising hit from the high-throughput siRNA screen. Multiple oligos conferred resistance to the Smac mimetic, in multiple cell lines. CAPN9 is a calcium sensing cystiene protease with unknown function. CAPN9 is predominantly expressed in gastric tissue and was originally identified as a gene lost in the development of stomach cancer. Antisense CAPN9 mRNA increased transformation efficiency of cells, identifying CAPN9 as a putative tumor suppressor. However, CAPN9 function has not been identified, and no prior evidence has linked CAPN9 to TNF-α signaling.

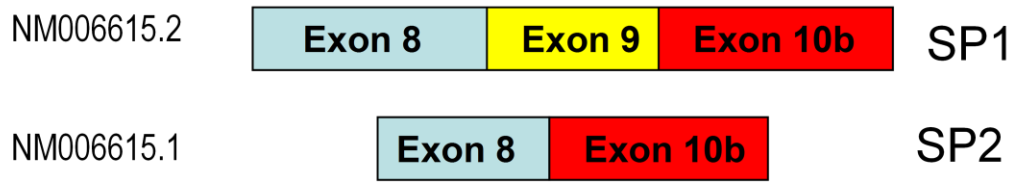
CAPN9 was first identified comparing different mRNA profiles of gastric cancer cells. This experiment identified NM006615.1 (Splice Variant 2, SP2), a CAPN9 splice variant lacking exon 9. Further experiments identified NM006615.2 (Splice Variant 1, SP1), a splice variant containing exon 9 (Figure 3.1A). All subsequent papers in the literature focus on this splice variant. Exon 9 is 81 nucleotides long. Deletion of exon 9 results in a protein 27 amino acids shorter. This deletion is within the catalytic domain, but does not directly affect

the catalytic triad. Currently there is no data directly comparing the function or catalytic activity of the two splice variants, so it was important to determine if one or both splice variants were responsible for sensitivity to the Smac mimetic.

RTPCR was performed, and CAPN9 SP1 and SP2 were cloned for HCC44 mRNA. Plasmids were sequenced confirming that both splice variants are expressed in HCC44. To determine relative expression levels, RTPCR primers were designed within exon 8 and exon 10b. RTPCR was performed using these primers on HCC44 mRNA. Results confirm that both splice variants are expressed and at relatively the same levels (figure 3.1B). This indicates that either splice variant, or both, could be responsible for Smac mimetic sensitivity.

AMACR was ruled an off-target siRNA effect because knockdown did not correlate to the phenotype. CAPN9 siRNA from the Qiagen library prevented cell death. Closer examination revealed an overlap in oligo design between the Qiagen and Dharmacon libraries, so I chose to design siRNAs targeting multiple regions of CAPN9. Knockdown efficiency was tested in HEK293T cells co-transfected with siRNA and flag-tagged CAPN9-SP1 expression plasmid (Figure 3.2). HCC44 cells were simultaneously transfected with siRNA, and treated with 100nM Smac mimetic. Four hours after treatment cells were harvested and incubated with a fluorescent Caspase 3 substrate. Caspase activation correlates to induction of cell death and is indicated by an increase in fluorescence intensity.

A.



B.

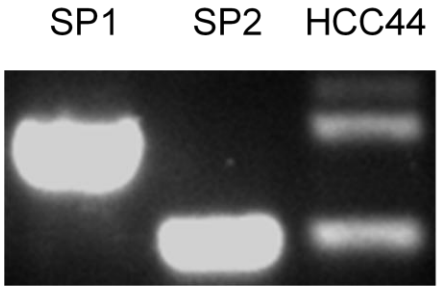


Figure 3.1 Expression of CAPN9 splice variants

A. Schematic representations of CAPN9 splice variants. SP2 lacks exon 9, resulting in a protein with twenty-seven less amino acids. B. RTPCR primers spanning from Exon 8 to Exon 10b were used on HCC44 mRNA. Plasmids expressing each splice variant were used as positive controls.

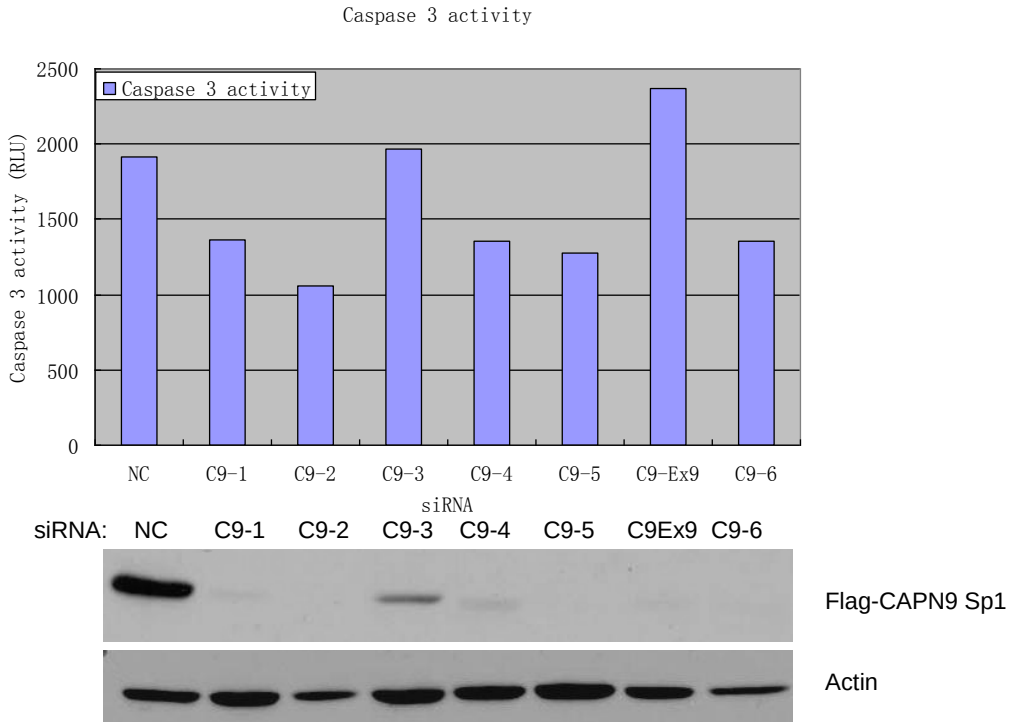


Figure 3.2 CAPN9 knockdown efficiency correlates with prevention of cell death

In the upper panel, HCC44 cells were transfected with siRNA to CAPN9, treated for 4 hours with 100nM Smac mimetic, lysed and analyzed by a Caspase 3 activity assay.

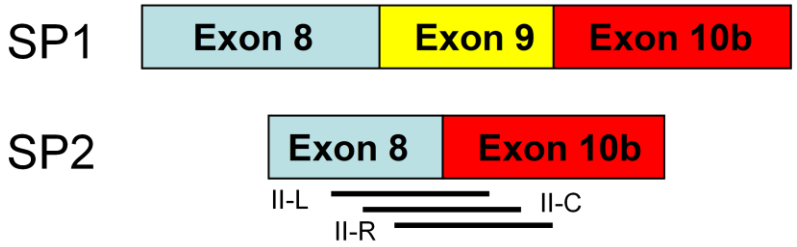
In the lower panel, HEK293T cells were co-transfected with a flag tagged CAPN9-SP1 construct and siRNA against CAPN9, and analyzed by western blot using Flag and Actin antibodies

CAPN9 oligos one, two, four, five, and six efficiently knocked down expression of CAPN9-SP1. CAPN9 oligo three did not efficiently knockdown CAPN9 expression, and also did not block Caspase activation. Reduction of protein directly correlates with inhibition of Caspase activation indicating the effect is specific and not due to non-specific interactions from the siRNA (Figure 3.2).

CAPN9 siRNA designed to exon 9 efficiently knocked down expression of CAPN9-SP1. This did not correlate with inhibition of Caspase 3 activation upon Smac mimetic treatment (figure 3.2). However, exon 9 is not present in the SP2, suggesting that CAPN9-SP2 might be required for Smac mimetic induced apoptosis. This data clearly demonstrates CAPN9-SP1 expression is not sufficient to confer sensitivity to the Smac mimetic.

CAPN9-SP1 specific siRNA was created by designing an oligo to exon 9, since SP2 does not contain this mRNA sequence. Development of an SP2 specific siRNA was more difficult due to the lack of a specific exon. siRNA was designed to the junction region of exon 8 and exon 10b. Three oligos were tested, designed to 3 nucleotides to the left, right, or directly overlapping the junction region. These oligos were tested for knockdown efficiency by co-transfection with flag-tagged expression constructs in HEK293T cells (Figure 3.3). siRNA designed directly to junction region of the two exons showed robust and specific knockdown of CAPN9-SP2.

A.



B.

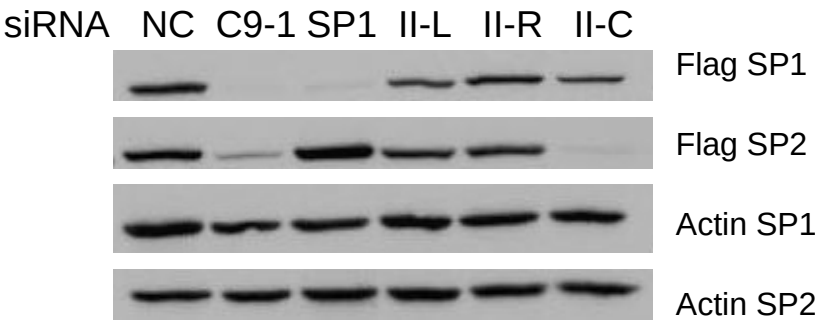


Figure 3.3 Development of a CAPN9 SP2 specific siRNA

A. CAPN9-SP2 specific siRNA oligos were designed to the junction region of the two splice variants

B. HEK293T cells were co-transfected with flag tagged CAPN9-SP1 or CAPN9-SP2 and CAPN9 specific siRNA oligos, and analyzed by western blot using flag and actin antibodies.

CAPN9-SP1 is not sufficient for the Smac sensitivity phenotype, raising the possibility that SP2 might be the specific splice variant causing drug sensitivity. CAPN9-SP2 siRNA was transfected into MDA-MB231 cells. Cells were treated with 100nM Smac mimetic for 48 hours, and cell viability was determined using Cell TiterGlo. Three CAPN9 siRNA oligos were used as positive controls, and successfully blocked apoptosis induced by the Smac mimetic. CAPN9-SP2 specific siRNA efficiently blocked cell death, while SP1 siRNA had no effect (Figure 3.4). This demonstrates CAPN9-SP2 is essential for Smac mimetic induced apoptosis. This also shows the affect is not cell line specific.

Typical Calpains function as heterodimers, where the protein is stabilized by another protein, CAPNS1. Knockdown of CAPNS1 is typical used to block activity of the ubiquitously expressed Calpains, μ-Calpain and m-Calpain. CAPN9 was recently demonstrated to function as a heterodimenr with CAPN8. siRNA against CAPNS1 and CAPN8 did not block Smac mimetic induced apoptosis (Figure 3.5). This indicates that CAPN9 function in Smac mimetic induced apoptosis might be independent of CAPN8 and CAPNS1. This does not rule out functional redundancy. Further experiments are required to determine if CAPN9 is capable of functioning independently, or requires associated proteins to form an active complex.

CAPN9 is a protease. ALLN is a known pan-Calpain inhibitor.

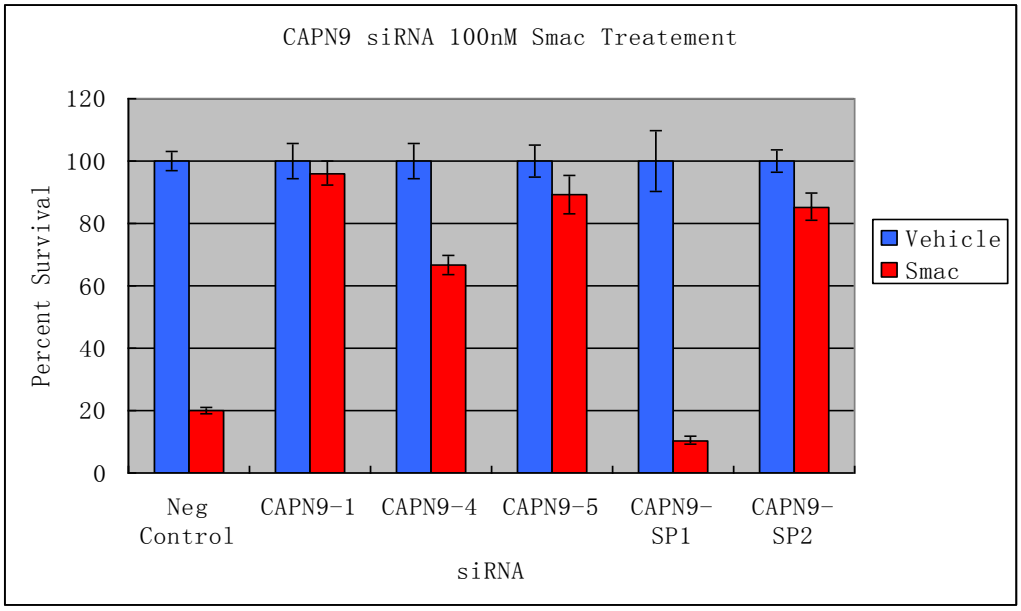


Figure 3.4 CAPN9 SP2 is required for Smac mimetic induced apoptosis

MDA-MB2381 cells were transfected with CAPN9 siRNA oligos and treated with or without 100nM Smac mimetic for 48 hours. Cell viability was measured using Cell TiterGlo.

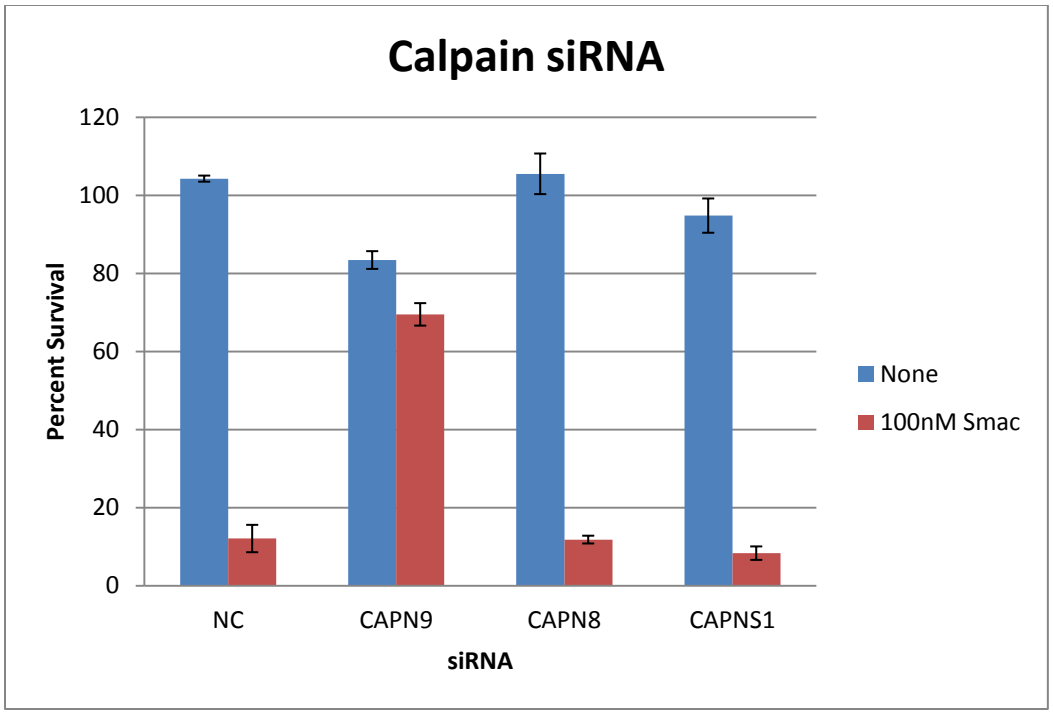


Figure 3.5 siRNA against CAPN8, CAPNS1, and CAPNS2 does not prevent apoptosis

HCC44 cells were transfected with Dharmacon pools of four siRNA oligos designed to each gene, and treated for 48 hours with 100nM Smac mimetic. Cell viability was determined using Cell TiteGlo.

Treatment of MDA-MB231 cells with 5μM ALLN was able to block apoptosis induced by the Smac mimetic. This suggests CAPN9 protease activity is required for Smac mimetic sensitivity (Figure 3.6).

3.2 CAPN9 does not function in autocrine secretion of TNF-α

CAPN9 blocks Smac mimetic induced cell death, but the function of the protein is unknown. CAPN9 was identified in a cell line with autocrine secretion of TNF-α. Upon treatment with the Smac mimetic, TNF-α secretion is increased. In MDA-MB231 cells, the TNF-α secretion has been deciphered. CIAP1/2 ubiquitinate Nik, targeting Nik for degradation by the proteasome. When cells are treated with the Smac mimetic, CIAP1/2 are degraded allowing stabilization of Nik. Nik then triggers NF-κB activation, allowing transcription and secretion of TNF-α, as outlined in the introduction.

Multiple siRNAs to CAPN9 were transfected into MDA-MB231 cells. Cells were treated for two hours with 100nM Smac mimetic. Protein was harvested and analyzed by western blot to determine the effect of CAPN9 knockdown Nik stabilization. Nik protein expression was unaffected by CAPN9 knockdown, and cleavage of p100 to p52 was unchanged. This indicates that CAPN9 does not affect the non-canonical NF-κB activation pathway (Figure 3.7).

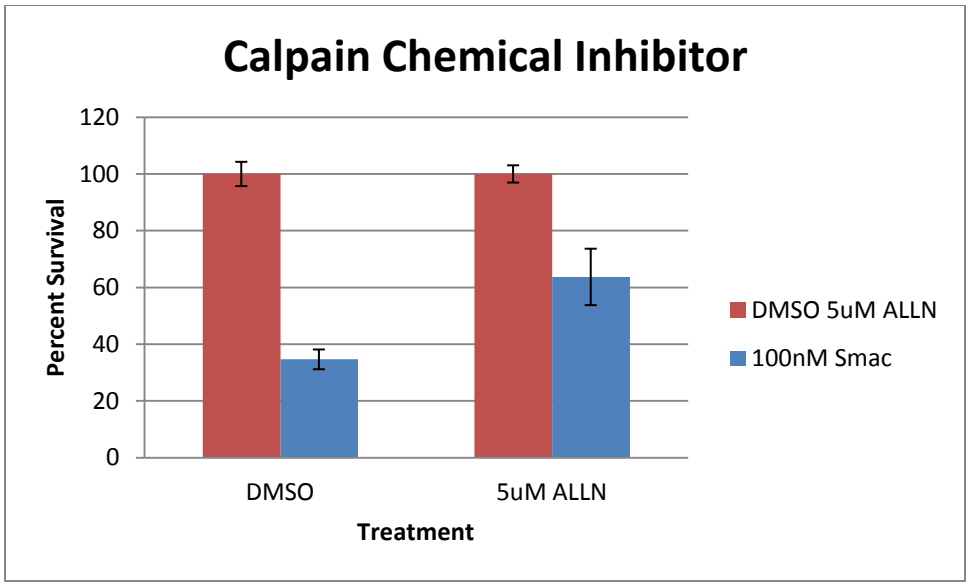


Figure 3.6 A Calpain chemical inhibitor blocks Smac mimetic induced apoptosis

MDA-MB231 cells were treated overnight with 5uM ALLN then treated with 100nM Smac Mimetic overnight. Cell viability was determined by Cell TiterGlo.

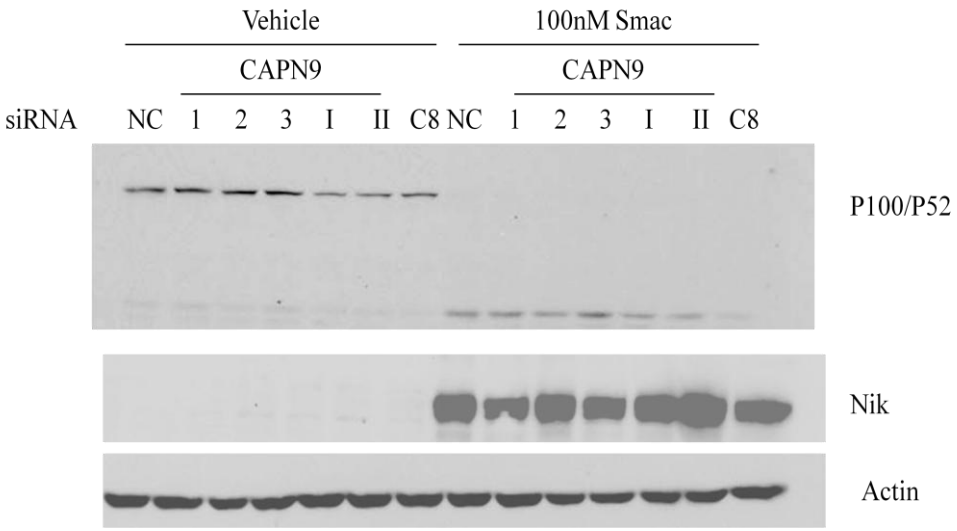


Figure 3.7 CAPN9 siRNA does not affect Non-Canonical NFκB activation by the Smac mimetic

MDA-MB231 cells were transfected with siRNA for CAPN9 and treated for four hours with 100nM Smac mimetic. Cells were analyzed by western blot for P100, Nik, and Actin antibodies.

CAPN9 does not affect NF-κB activation, but may still play a role in TNF-α secretion. TNF-α secretion in MDA-MB231 cells is near the limit of detection for most TNF-α ELISA detection kits. A simpler way to determine if CAPN9 affects TNF-α treatment is to add recombinant TNF-α directly to the media. If CAPN9 affects TNF-α secretion, knockdown of CAPN9 should not prevent cell death induced by the combination of exogenous TNF-α and the Smac mimetic. MDA-MB231 cells were treated with 20ng/mL GST-TNF-α and 100nM Smac mimetic, or Smac mimetic alone. Knockdown of TNFR1 prevented cell death from both stimuli, but siRNA against Nik was only capable of preventing single agent Smac mimetic treatment. CAPN9 siRNA completely blocked apoptosis induced by the Smac mimetic, and also inhibited TNF-α and Smac mimetic induced apoptosis (Figure 3.8). This indicates that Smac mimetic induced secretion of TNF-α is independent of CAPN9.

H2009 cells do not secrete TNF-α when treated with the Smac mimetic, and serve as a good model to verify CAPN9 functions independently of TNF-α secretion. Four copies of CAPN9 oligo one were converted to shRNA sequences and cloned into a pSuperior vector each under a Tet repressor (see experimental procedures). This plasmid was transfected into H2009 cells expression the Tet repressor, and clonal stable cell lines were selected. These cell lines express CAPN9 shRNA when treated with Doxycycline. A negative control cell line was

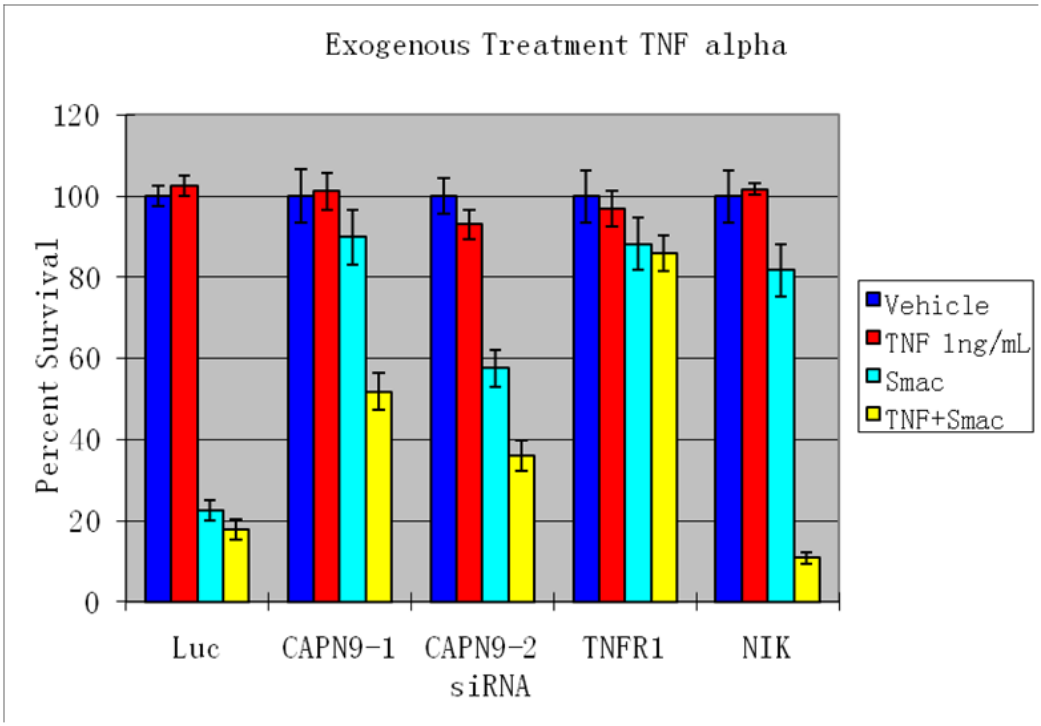


Figure 3.8 CAPN9 siRNA blocks TNF-alpha and Smac mimetic induced apoptosis

MDA-MB2381 cells were transfected with siRNA oligos and treated with or without 1ng/mL TNF- alpha and/ or 100nM Smac mimetic for 48 hours. Cell viability was measured using Cell TiterGlo.

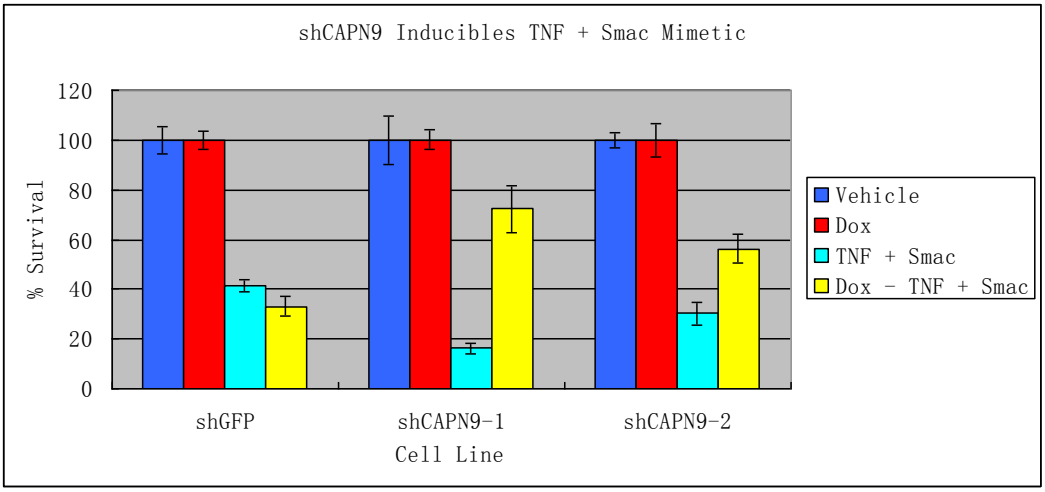


Figure 3.9 CAPN9 shRNA blocks TNF-alpha and Smac mimetic induced apoptosis

H2009 shGFP and shCAPN9-1 inducible cell lines were treated with 1ug/mL Dox twice every 3 days. Cells were then treated with or without 100ng/mL TNF-alpha and 100nM Smac mimetic. Cell viability was assessed using Cell TiterGlo.

also generated, expressing shRNA designed to GFP. These cell lines were treated with 100ng/mL TNF-α and 100nM Smac mimetic. Cell viability was measured using the Cell TiterGlo assay. Doxycycline (Dox) induced expression of CAPN9 shRNA block cell death, but shGFP did not, indicating that CAPN9 is necessary for cell death independent of TNF-α secretion (Figure 3.9).

CAPN9-SP2 siRNA blocks cell death induced by Smac mimetic independent of TNF-α secretion. This siRNA targets the junction region of exon 8 and exon 10b, which should be exclusive to splice variant two. CAPN9-SP2 was cloned into pcDNA 3.1 vector. CAPN9 siRNA oligo one target sequence was destroyed by mutating the wobble base pair nucleotides, rendering the plasmid resistant to CAPN9 siRNA oligo one without altering the overall protein sequence. Clonal stable cell lines were generated in H2009 cells expressing the siResistant CAPN9-SP2. Western blot analysis revealed protein expression was unaffected by CAPN9 oligo one, but significantly reduced by oligo two and the splice variant two specific siRNA. Overexpression and parental cells were transfected with siRNA and treated with TNF-α and the Smac mimetic. Cell viability was measured using Cell TiterGlo. Expression of a CAPN9 siRNA oligo one resistant plasmid reconstituted the cell death phenotype and had no effect on siRNA oligo two or the splice variant specific siRNA (Figure 3.10).

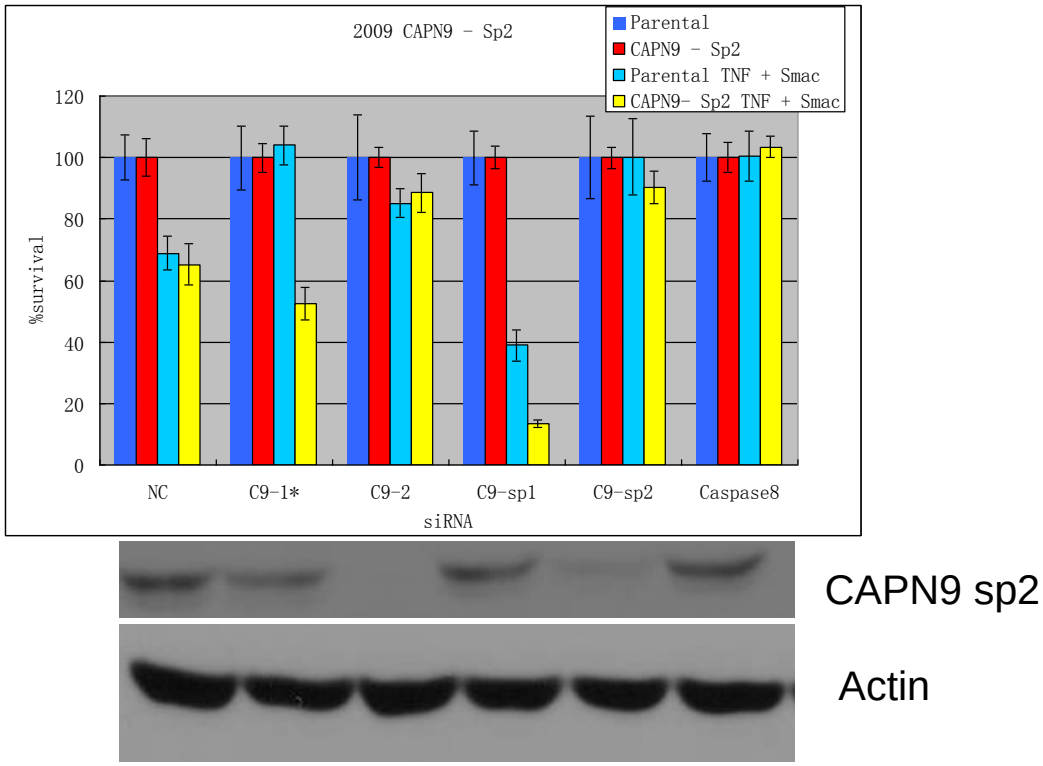


Figure 3.10 Expression of an siRNA resistant CAPN9-SP2 reconstitutes cell death

H2009 parental and CAPN9-SP2 over-expression cell lines were transfected with siRNA and treated with or without 100ng/mL TNF-alpha and 100nM Smac mimetic. Cell viability was assessed using Cell TiterGlo.

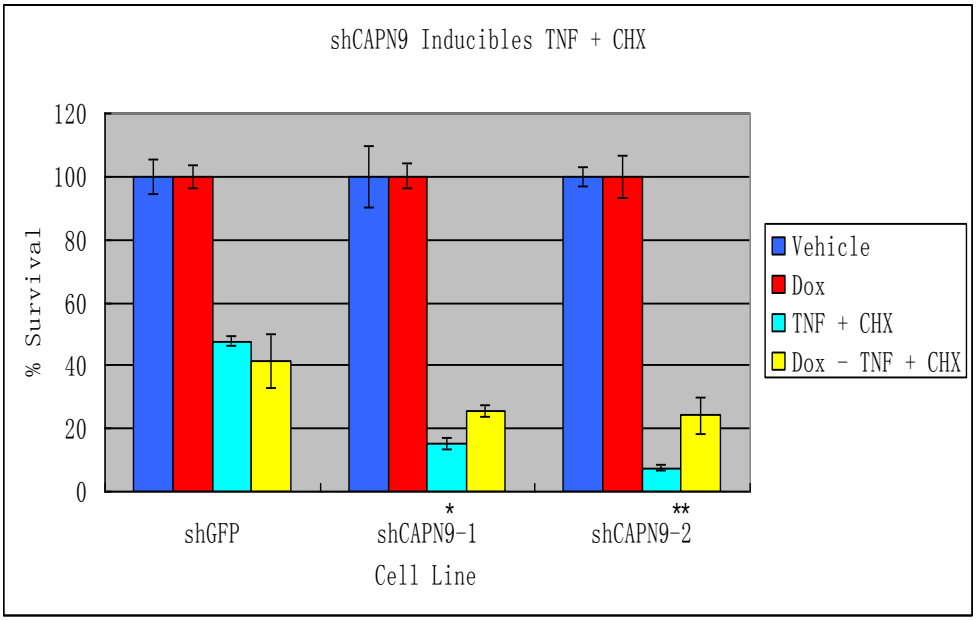
In the lower panel, H2009 parental and CAPN9-SP2 over-expression cell lines were transfected with siRNA and analyzed by western blot with antibodies to CAPN9 and Actin.

This clearly demonstrates CAPN9 splice variant two is necessary for cell death induced by TNF-α and the Smac mimetic.

3.3 CAPN9 is required for TNF-α induced apoptosis

TNF-α is capable of signaling for survival and cell death. TNF-α activates NF-κB, initiating transcription of FLIP, which blocks activation of Caspase 8 and prevents cell death. Co-treatment with the Smac mimetic is one method of triggering TNF-α dependent cell death. Co-treatment with the translation inhibitor, cycloheximide (CHX), blocks expression of FLIP, allowing for Caspase 8 activation and cell death. To determine if CAPN9 specifically functions in TNF-α and Smac mimetic induced apoptosis, shCAPN9 cells were treated with 100ng/mL TNF-α and 10ug/mL CHX overnight. Cell viability was measured using a Cell TiterGlo assay. CAPN9 shRNA blocked TNF-α and CHX induced cell death (Figure 3.11). This was repeatable over multiple experiments. The cell death prevention phenotype is more dramatic when co-treated with Smac mimetic, yet this indicates CAPN9 functions in a general mechanism to prevent cell death induced by TNF-α.

TNF-α binds to TNFR1 to trigger NF-κB through phosphorylation and degradation of IκB. This serves as an early marker of NF-κB activation. CAPN9 shRNA cells were treated with TNF-α for ten minutes, and cell lysates were prepare and analyzed by western blot. Dox induced expression of CAPN9



*p<.0001

** p<.001

Figure 3.11 CAPN9 shRNA blocks TNF-alpha and Cycloheximide induced cell death

H2009 shGFP and shCAPN9-1 inducible cell lines were treated with 1ug/mL Dox twice, every 3 days. Cells were then treated with or without 100ng/mL TNF-alpha and 10ug/mL CHX. Cell viability was assessed using Cell TiterGlo.

shRNA did not affect phosphorylation of degradation of IκB by TNF-α (Figure 3.12). This indicates that CAPN9 specifically affects TNF-α induced cell death, not cell survival.

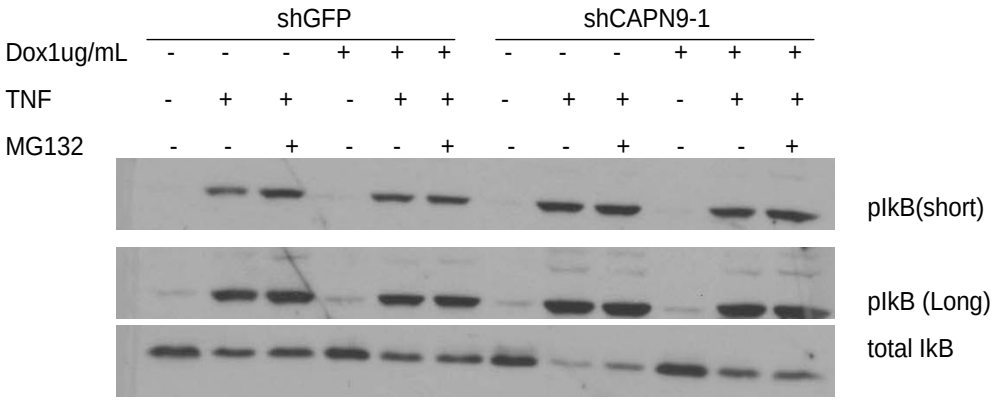


Figure 3.12 CAPN9 shRNA does not block TNF-alpha induced NF-κB activation

H2009 shGFP and shCAPN9-1 inducible cell lines were treated with 1ug/mL Dox twice every 3 days. Cells were then treated with or without 100ng/mL TNF-alpha and 10μM MG132 for 10 minutes, and analyzed by western blot using antibodies for phosphorylated - IκB and IκB.

Chapter Four

Exploring the Mechanism of CAPN9 in TNF-α Signaling

4.1 CAPN9 is required for RIPK1 recruitment to TNFR1

CAPN9 was verified as a novel protein regulating TNF-α induced apoptosis. Little is known about the function of CAPN9 making further studies of the protein difficult. However, detailed biochemical studies have revealed multiple sequential steps in TNF-α and Smac mimetic induced apoptosis. Upon treatment with ligand, TNF-α binds to TNFR1. RIPK1 is rapidly recruited and ubiquitinated at the TNF receptor complex. The Smac mimetic induces ubiquitination and degradation of CIAP1/2, allowing RIPK1 to disassociate from the receptor and form a complex with FADD and Caspase 8. The FADD-Caspase 8- RIPK1 complex allows cleavage and activation of Caspase 8 and triggers apoptosis. These clear, sequential steps allow for dissection of the role of CAPN9 within the signaling pathway.

Before Caspase 8 and RIPK1 interact, RIPK1 is recruited to TNFR1 and ubiquitinated. HCC44 cells were transfected with CAPN9 siRNA or a negative control oligo and treated with TNF-α, Smac mimetic and z-VAD for thirty minutes or one hour, lysed and TNFR1 was immunoprecipitated and analyzed by western blot. CAPN9 siRNA drastically reduced RIPK1 interaction with TNFR1

and subsequent ubiquitination. This indicates CAPN9 functions at or before RIPK1 recruitment to TNFR1, before Caspase 8 and RIPK1 interaction (Figure 4.1).

Total TNF-α binding to the receptor was also assessed. CAPN9 knockdown appeared to decrease the total amount of TNF-α interacting with the receptor (Figure 4.1). This indicates CAPN9 may be interrupting the TNF-α TNFR1 interaction. Total TNFR1 and RIPK1 protein levels are unchanged in the CAPN9 knockdown. CAPN9 could be function to affect TNFR1 presentation on the membrane, or knockdown could cause secretion of a factor that is blocking the receptor function. This data contradicts previous data that CAPN9 does not affect NFκB activation. However, the TNF receptor is internalized upon activation, and blockage of the internalization could be responsible for the phenotype seen.

Soluble TNFR1, cleaved from the membrane, can block TNF-α signaling. It is conceivable a secreted factor might be blocking TNF signaling in the CAPN9 knockdown. HCC44 cells were transfected with siRNA to CAPN9, RIPK1, and luciferase (Luc, a negative control). Conditioned media from two days of transfection was swapped between cells with each of the siRNA condition. Cells were then treated with 100nM Smac mimetic for four hours, and a Caspase 3 activity assay was performed. Conditioned media from CAPN9 and RIPK1 transfections were not able to block Caspase activation upon treatment with the

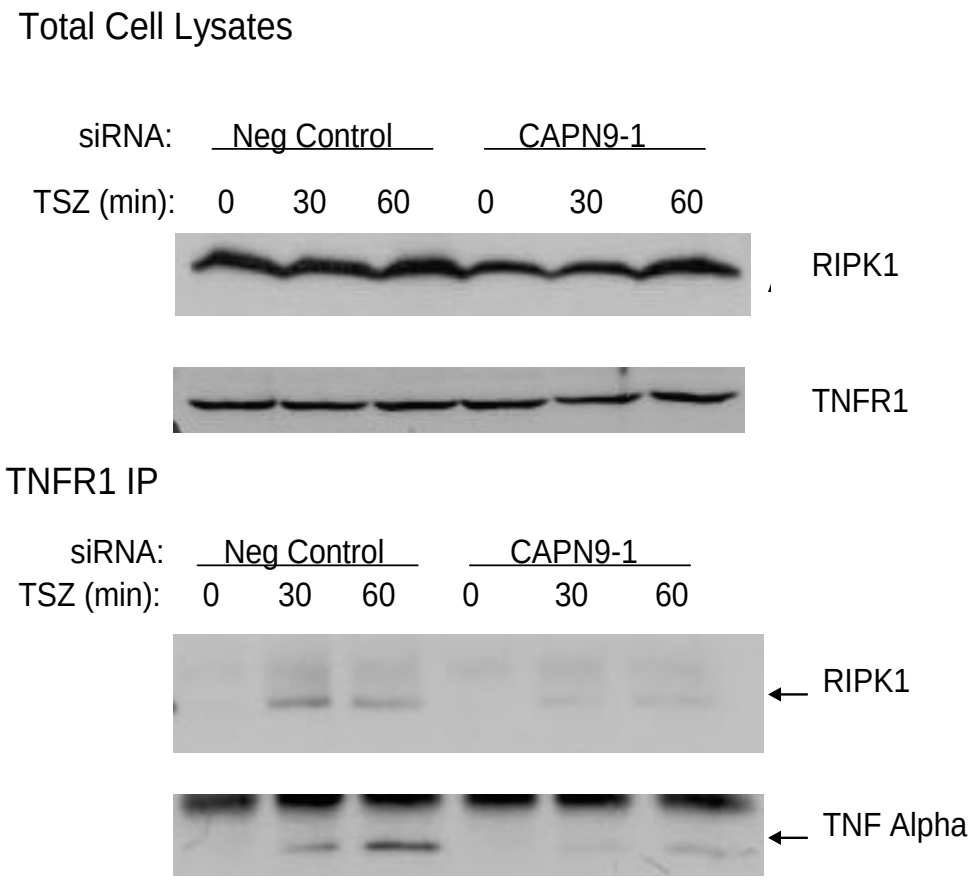


Figure 4.1 CAPN9 siRNA blocks RIPK1 recruitment to TNFR1

HCC44 cells were transfected with negative control siRNA, or siRNA against CAPN9, and treated with 100ng/mL TNF-alpha, 100nM Smac mimetic, and 20μM z-VAD for 30 or 60 minutes. In the upper panel lysates were analyzed by western blot for RIPK1 and TNFR1. Lower panel, lysates were immunoprecipitated for TNFR1 and analyzed by western blot for RIPK1 and TNF-alpha.

Smac mimetic (Figure 4.2). This indicates that CAPN9 siRNA does not induce a secreted factor that inhibits TNF-α interaction with the receptor.

CAPN9 siRNA does not affect the total expression levels of the TNF receptor. However total protein levels do not indicate if the receptor is properly presented on the cell membrane. CAPN9 may function in the localization of TNFR1. HCC44 cells were transfected with siRNA, lysed in Buffer A and 250mM sucrose, and sequentially centrifuged at 1000; 12,000; and 100,000 times gravity to produce p1, p12, and p100 fractions respectively. The p1 fraction represents the cell membrane, while the p12 fraction is the cell organelles, and the p100 is the light membrane franction. Pellets were resuspended and analyzed by western blot (Figure 4.3A). CAPN9 knockdown had no affect on the amount of TNFR1 present in the membrane fraction.

Cell surface proteins are affected by the environment they are exposed to. An alternative method for assaying total amount of cell surface proteins is to incubate the cells in trypsin. Enzymatic digestion of extracellular domains reveals the relative amount of total proteins on the cell surface. If a protein is internalized, it is protected from digestion. HCC44 cells were transfected with siRNA and incubated with Trypsin for fifteen minutes at 37C. Cells were washed in 1XDPBS and 100μM PMSF to stop the digestion, lysed, and analyzed by

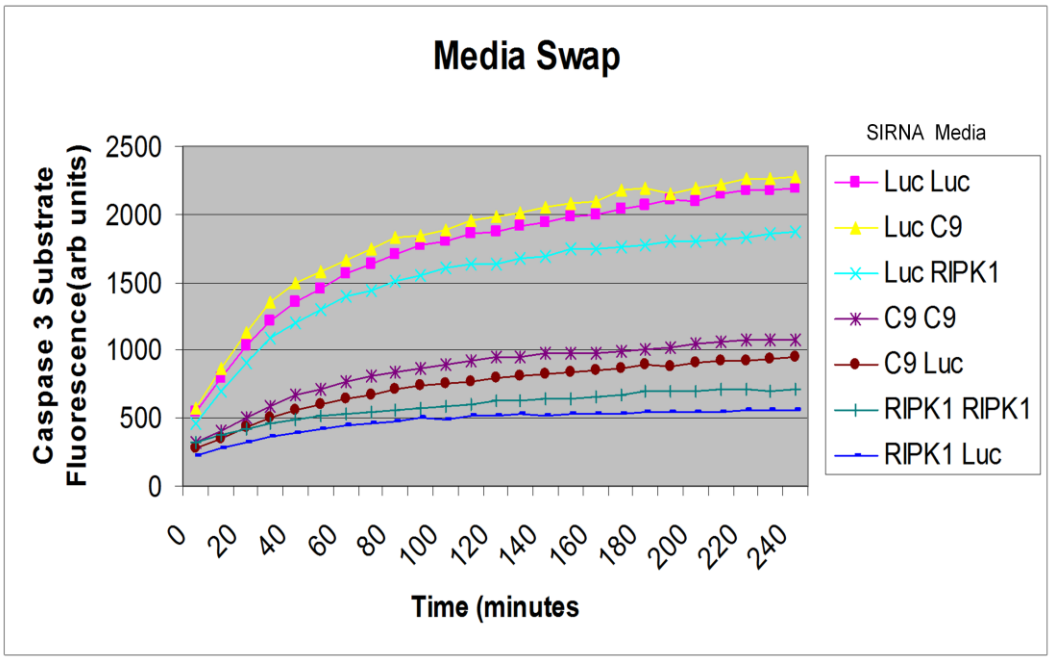


Figure 4.2 CAPN9 knockdown does not release a secreted factor affecting apoptosis

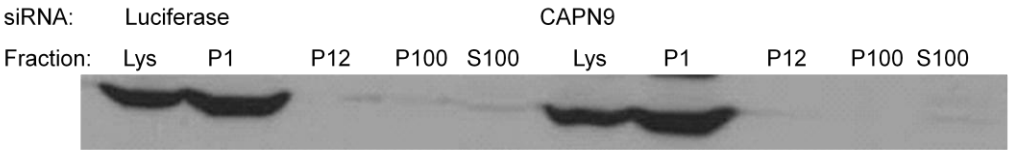
HCC44 cells were transfected with siRNA to RIPK1, CAPN9, and Luciferase as a negative control. After two days, conditioned media was removed from each well and exchanged as indicated. Cells were treated with 100nM Smac mimetic for 4 hours. Lysates were analyzed by a Caspase 3 activity assay.

western blot. CAPN9 knockdown did not affect TNFR1 digestion (Figure 4.3B) suggesting total receptor level at the membrane is unchanged. Cell fractionation, and cell surface protein degradation all indicate that CAPN9 does not affect TNFR1 presentation on the membrane. Thus CAPN9 must be affecting TNF signaling through an alternative mechanism.

4.2 Identification of novel TNFR1 interacting proteins

CAPN9 siRNA affects recruitment and ubiquitination of RIPK1 without affecting protein level, or cell surface presentation of TNFR1. The precise biochemical steps occurring after TNF-α binds to the receptor are not clearly defined. CAPN9 could affect TNFR1 specific interactions, which decreased the ability of RIPK1 to bind the receptor upon activation. Identification of these interactions can be difficult due to nonspecific interaction in an antibody IP. To get around this technical issue, TNFR1 IP was performed on fractions from HCC44 cells. Interestingly enough, three CAPN9 dependent bands were identified in the p100 fraction corresponding to 250, 100, 75Kda (Figure 4.4). These bands were excised and identified by mass spectrometry (Table 4.1). The 250kDa bands correspond to FLNA, FLNB, and SPTAN1. The 100kDa proteins were identified as DBN1 and MYH9, while the 75kDa protein was

A.



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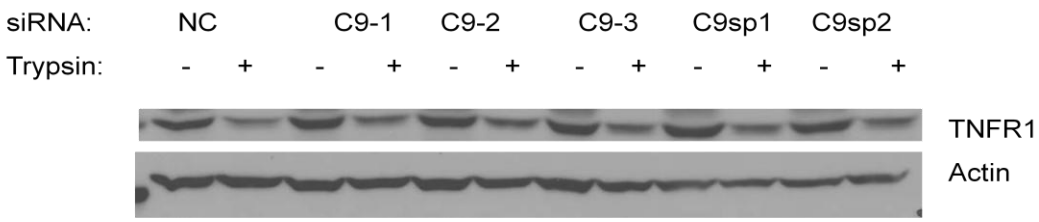


Figure 4.3 CAPN9 knockdown does not affect TNFR1 surface presentation

A. HCC44 cells were transfected with siRNA to luciferease or CAPN9. Cells were washed with 1XDPBS, removed from the plate surface, resuspended in Buffer A and 250mM sucrose, passed 22 times through a 22 gauge needle, and sequentially pelleted at 1K, 12K, and 100K RCF for 30 minutes at 4C. Equalivalent volumes for each fraction were added to the gel.

B. HCC44 cells were transfected with the siRNA oligos indicated. Cells were incubated for 15 minutes in trypsin at 37C, resuspended in 1XDPBS 100µM PMSF, lysed and analyzed by western blot for TNFR1 and actin.

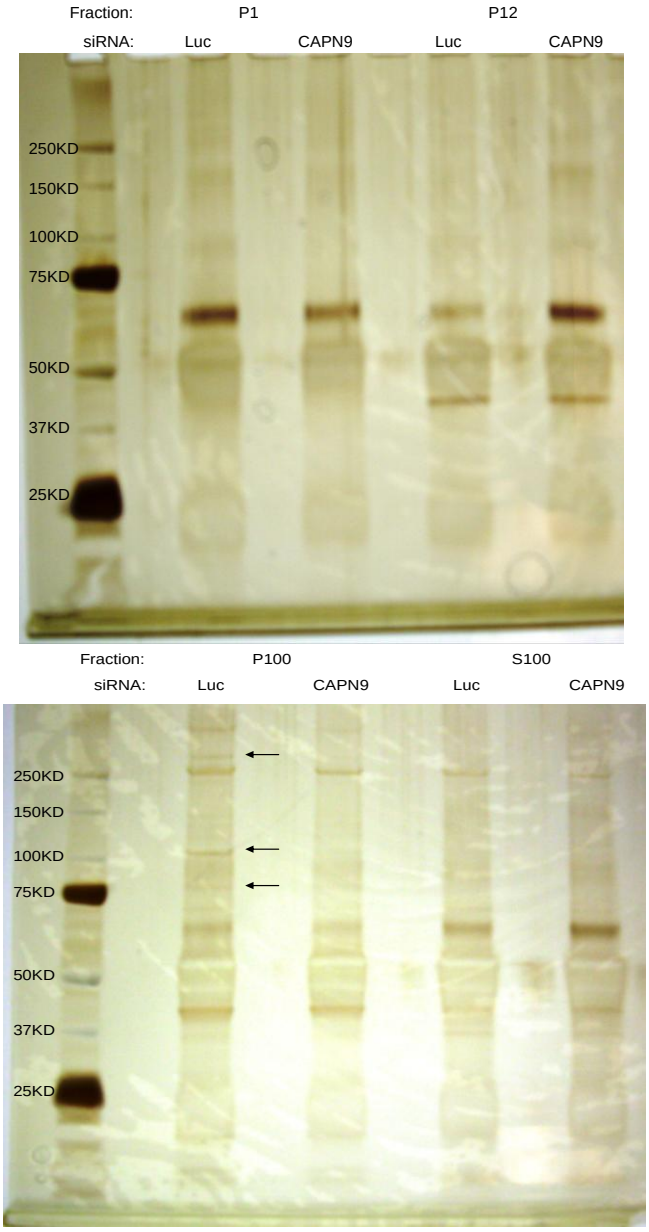


Figure 4.4 Identification of CAPN9 dependent TNFR1 interactions

HCC44 cells were fractionated as previously described. TNFR1 immunoprecipitation was performed and analyzed by SDS PAGE and subsequent silver stain.

Sample ID	GI	Protein Identified	Score	MW
P250	IPI00909642	- Filamin A	435	245713
	IPI00289334	FLNB Isoform 1 of Filamin-B	220	278021
	IPI00744706	SPTAN1 cDNA FLJ61399, highly similar to Spectrin alpha chain, bra	181	281973
P100	IPI00295624	DBN1 Isoform 2 of Drebrin	656	71555
	IPI00395772	MYH9 Isoform 2 of Myosin-9	643	159764
P75	IPI00796222	LIMA1 67 kDa protein	742	66950

Table 4.1 Identity of CAPN9 dependent p100 TNFR1 interacting proteins

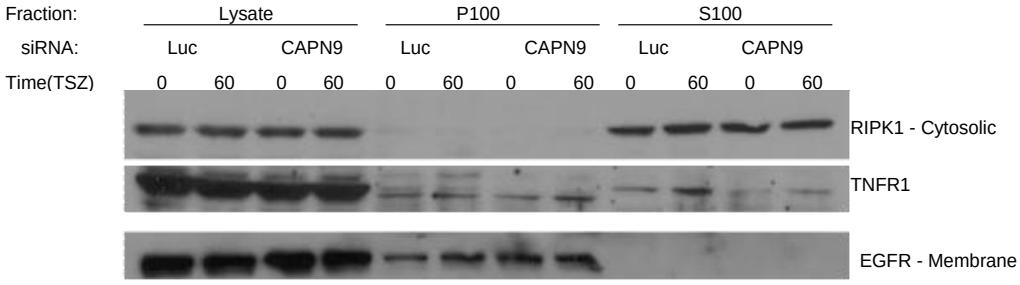
Individual bands were excised from the gel (Figure 4.5) and identified by Mass Spectrometry.

identified as LIMA1. All of these proteins are known to bind Actin and are involved in cytoskeleton remodeling.

The p100 represents the light membrane fraction. Western blot analysis reveals a portion of TNFR1 resident in the p100 fraction. This is consistent with the levels of EGFR. CAPN9 siRNA and treatment with TNF-α, Smac mimetic, and z-VAD does not affect the localization of the TNFR1 to this fraction (Figure 4.5). This suggests that CAPN9 is not affecting TNFR1 localization, but rather affecting the interaction between TNFR1 and the identified proteins. HCC44 cells were fractionated following the same protocol and probed for CAPN9. CAPN9 expression was enriched in the p100 fraction, correlating with the difference in TNFR1 interactions.

Proteins identified by mass spectrometry were confirmed using western blot analysis. HCC44 cells were transfected with siRNA against CAPN9, and TNFR1 was immunoprecipitated from total cell lysates. DBN1 was not detectable by western blot with multiple antibodies, and was not pursued further (Data not shown). LIMA1 and FLNB interaction with the TNF receptor did not repeat, but FLNA and SPTAN1 interactions did repeat. FLNA and SPTAN1 interaction with TNFR1 was lost after siRNA with two independent oligos against CAPN9 (Figure 4.6). This indicates that the FLNA and SPTAN1 interaction with TNFR1 is CAPN9 dependent. SPTAN1 is a known substrate of CAPN1, and may

A.



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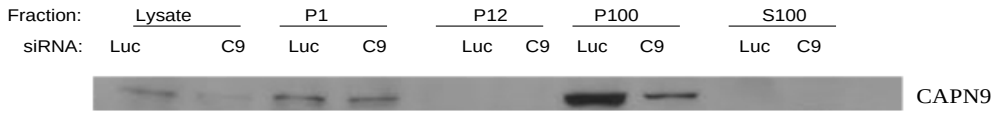


Figure 4.5 Isolation of TNFR1 and CAPN9 in the p100 fraction

HCC44 cells were transfected with siRNA oligos to CAPN9 or Luciferase and fractionated following the previously described protocol. A. HCC44 cells were treated with 100ng/mL TNF-alpha, 100nM Smac mimetic, and 20μM z-VAD. Protein amounts for each fraction were normailized and equal amounts were analyzed by western blot for RIPK1, TNFR1, and EGFR1.

B. Equivalent volumes of each fraction in comparison to total cell lysate were analyzed by western blot for CAPN9.

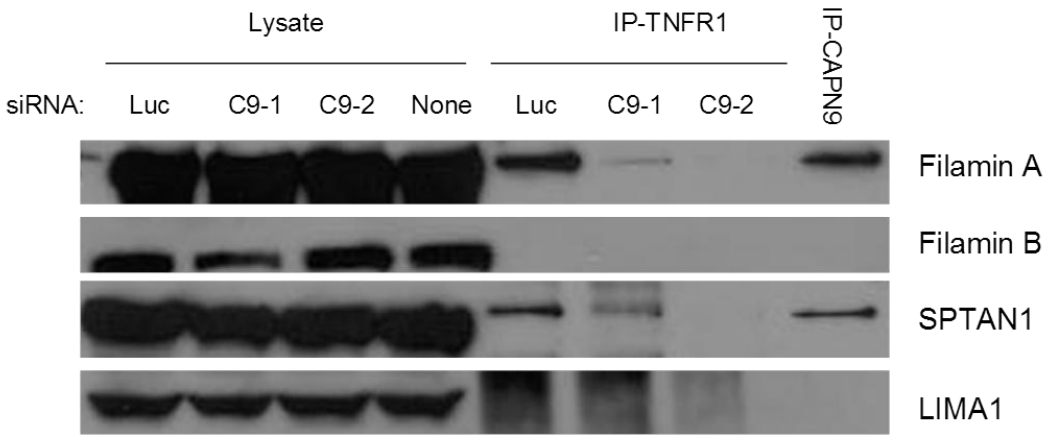


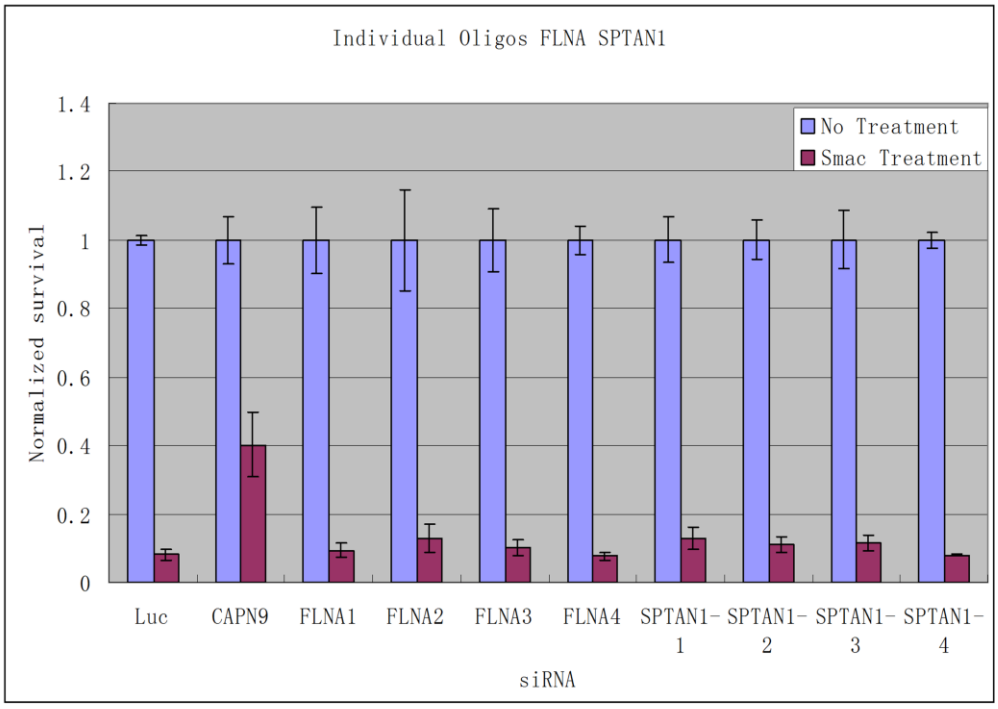
Figure 4.6 FLNA and SPTAN1 interact with TNFR1 dependent on CAPN9

HCC44 cells were transfected with the indicated siRNA and immunoprecipitated for TNFR1 or CAPN9, and analyzed by western blot for FLNA, FLNB, SPTAN1, and LIMA1.

be a target of CAPN9. CAPN9 was immunoprecipitated from HCC44 and analyzed by western blot. FLNA and SPTAN1 were found to interact with CAPN9, but FLNB and LIMA1 did not interact.

FLNA and SPTAN1 interact with TNFR1 and CAPN9. However, it is not clear if these proteins alone are required for TNF-α induced apoptosis. HCC44 cells were transfected with siRNA against FLNA and SPTAN1. Both proteins are highly abundant, so two rounds of transfection were completed to increase knockdown efficiency. Western blot analysis demonstrates multiple oligos were capable of decreasing the total protein levels (Figure 4.7). However, knockdown of these proteins did not prevent Smac mimetic induced apoptosis. This indicates that FLNA and SPTAN1 are not directly required for TNF-α and Smac mimetic induced apoptosis. However knockdown is not complete, and there could be sufficient protein to cause cell death sensitivity. SPTAN1 and FLNA both are involved in organization of the cytoskeleton and could affect recruitment of components to the receptor upon activation, possibly with redundant function.

MYH9 isoform two was identified as a TNFR1 interacting protein. MYH9 is an actin binding protein that has been shown to be involved in cytokinesis cell motility and maintenance of cell shape. Western blot analysis of TNFR1 immunoprecipitation revealed that MYH9 isoform one interacts with both TNFR1 independent of CAPN9. Isoform two was not clearly detected. However,



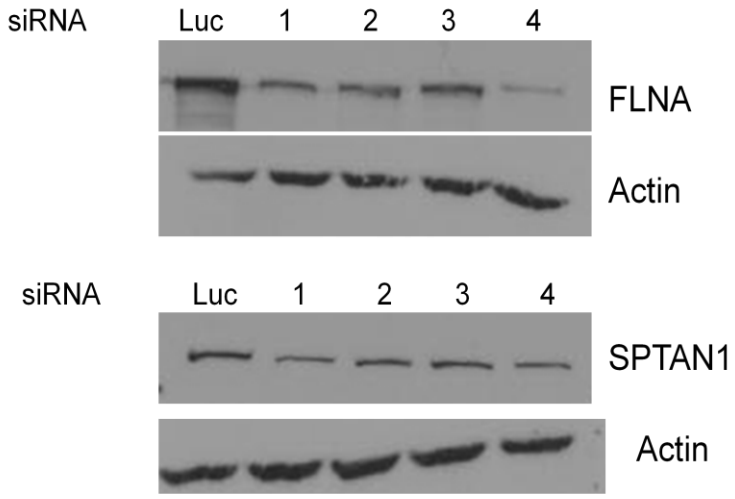


Figure 4.7 FLNA and SPTAN1 siRNA does not prevent Smac mimetic induced cell death

HCC44 cells were transfected with individual siRNA oligos for FLNA and SPTAN1 and treated with 100nM Smac mimetic for 48 hours. Cell viability was assessed using Cell TiterGlo. Western blot analysis was performed using antibodies against FLNA, SPTAN1 and Actin.

CAPN9 siRNA appears to decrease smaller interacting bands (Figure 4.8). Whether these are splice variants, or cleavage products remains to be determined.

MYH9 siRNA knockdown was compared to CAPN9 to determine if the generated the same phenotype. HCC44 cells were transfected with CAPN9 siRNA oligo one, and an oligo against MYH9 from a previous paper. MYH9 siRNA prevented TNF alpha and Smac mimetic induced apoptosis at similar levels to CAPN9 siRNA (Figure 4.9). TNFR1 was also immunoprecipitated from HCC44 treated with 100ng/mL TNF-α, 100nM Smac mimetic, and 20μM z-VAD for 45 minutes. RIPK1 recruitment to the receptor was analyzed by western blot analysis. RIPK1 interaction was blocked in both the CAPN9 and MYH9 knockdown. MYH9 siRNA results in the same phenotype as CAPN9 siRNA.

MYH9 siRNA demonstrated the same effect as CAPN9 siRNA making it a high priority target. MYH9 siRNA oligos were ordered from Dharmacon. siRNA knockdown using these oligos was compared to the siRNA oligo obtained from literature in HCC44 cells. Knockdown levels proved equivalent between the all the siRNA oligos (Figure 4.10). However, only the oligo obtained from literature was able to prevent cell death induced by the Smac mimetic in HCC44 cells. This indicates that the MYH9 phenotype is an off-target effect, and MYH9 knockdown alone is not sufficient to prevent cell death induced by the Smac mimetic. This suggest that apoptosis is not dependent on MYH9, but does not say

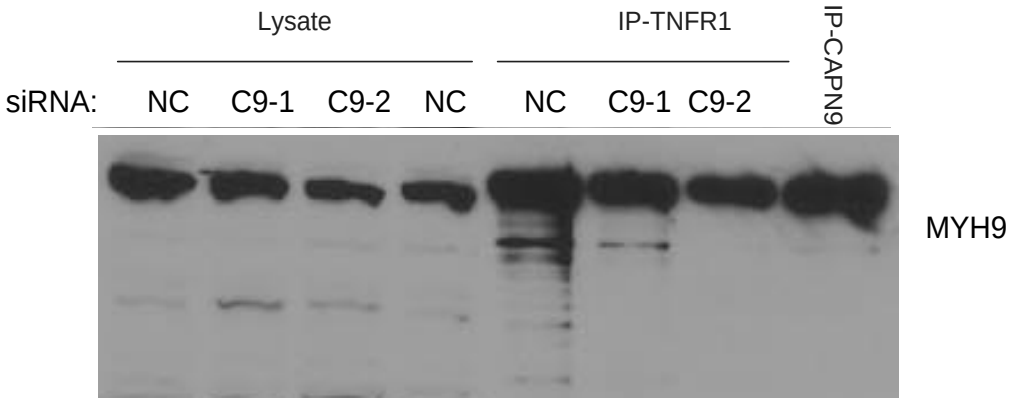
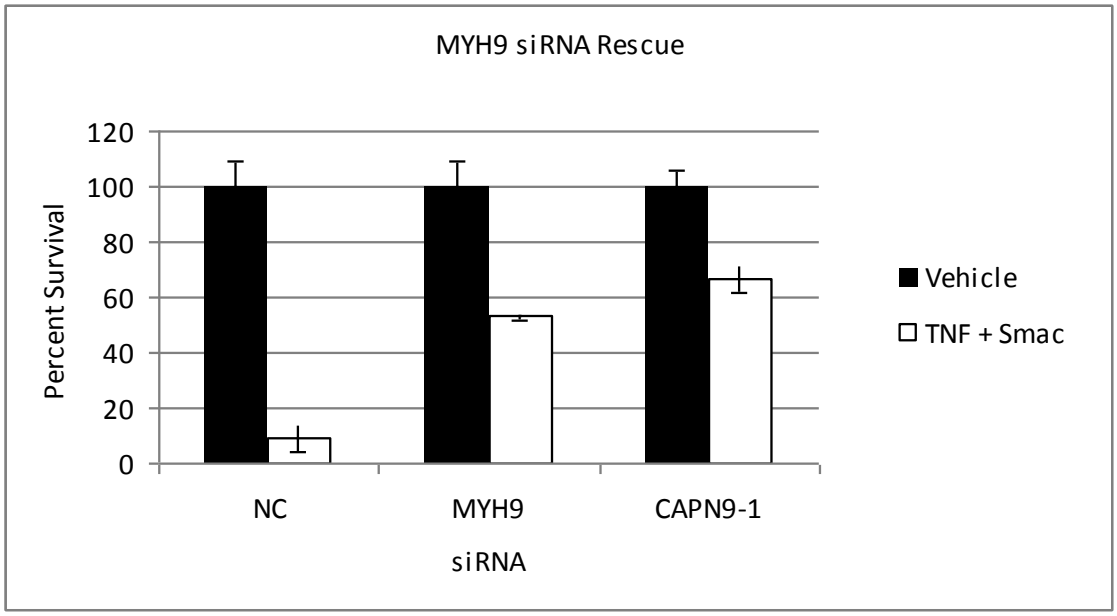


Figure 4.8 MYH9 interacts with TNFR1 and CAPN9

HCC44 cells were transfected with the indicated siRNA and immunoprecipitated for TNFR1 or CAPN9, and analyzed by western blot for MYH9.

A.



B.

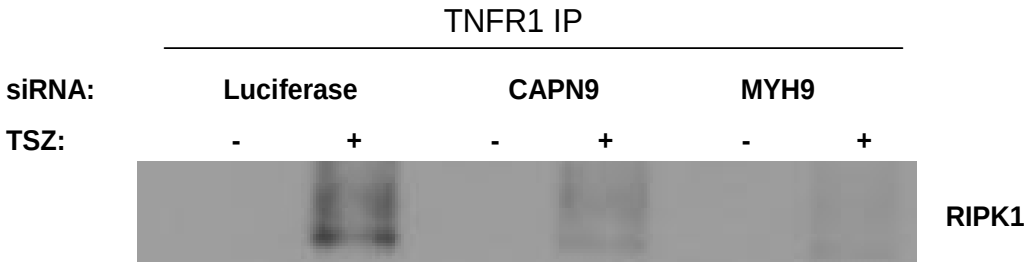
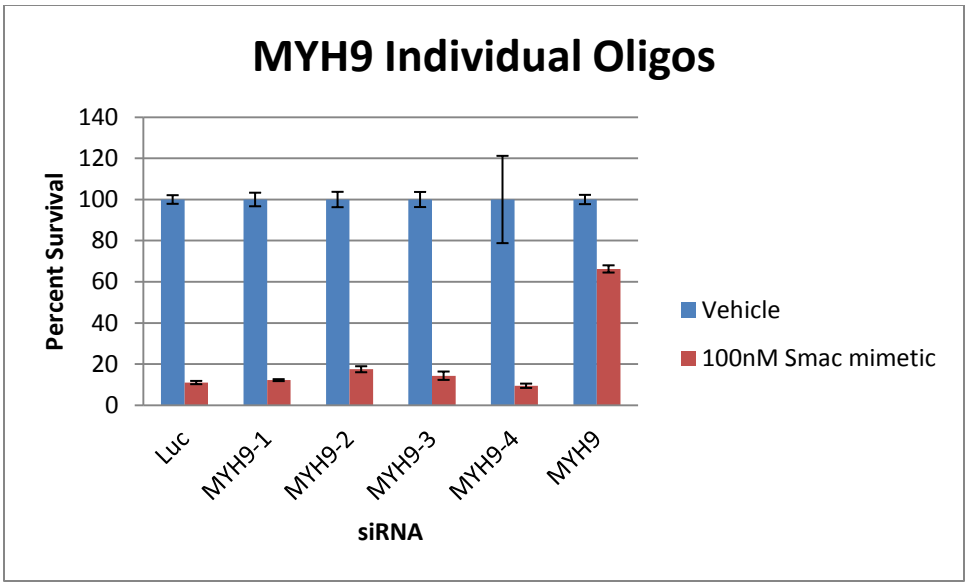


Figure 4.9 MYH9 siRNA phenocopies CAPN9 siRNA

A. HCC44 cells were transfected with siRNA against CAPN9 and MYH9, and Treated with 100ng/mL TNF-alpha and 100nM Smac mimetic for 48 hours. Cell viability was assessed using Cell TiterGlo.

B. HCC44 cells were transfected with the indicated siRNA, treated with 100ng/mL TNF-alpha, 100nM Smac mimetic, 20µM z-VAD. Samples were immunoprecipitated for TNFR1 and analyzed by western blot for RIPK1.



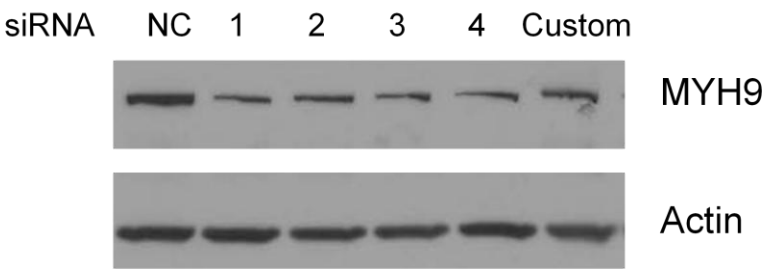


Figure 4.10 MYH9 knockdown does not correlate to phenotype

Dharmacon On-Target plus siRNA individual oligos were transfected into MDA-MB231 cell and treated with 100nM Smac Mimetic for 48 hours. Cell viability was measured by Cell TiterGlo.

Lower panel, MDA-MB231 cells were transfected with individual oligos to MYH9, lysed and analyzed by western blot for MYH9 and Actin.

that MYH9 could still possibly play a role in the cell death pathway.

CAPN9 is required for RIPK1 recruitment to TNFR1, and proper execution of TNF-α induced apoptosis. CAPN9 does not affect TNFR1 expression or presentation. This indicates CAPN9 functions to disrupt or destabilize RIPK1 binding to the receptor and ubiquitination. However, RIPK1 recruitment and ubiquitination is required for TNF-α induced activation of NF-κB activation. CAPN9 siRNA does not appear to affect NF-κB. CAPN9 siRNA may affect NF-κB at levels undetectable by western blot analysis of IκB activation, or cells may be signaling through TNFR2. Also, RIPK1 may require lower levels of RIPK1 recruitment, which are not detectable by western blot. Further investigation is required to determine why NF-κB activation is unaffected, but does not rule out that CAPN9 siRNA clearly affects RIPK1 recruitment to TNFR1 and is required for apoptosis.

CAPN9 knockdown has revealed three novel proteins interacting with TNFR1. Individual knockdown of each protein is unable to reproduce the effect of CAPN9 knockdown. FLNA, SPTAN1, and MYH9 are all actin binding proteins. Previous data suggests these proteins may be involved in regulating cytoskeleton and plasma membrane interactions. CAPN9 might be regulating

TNFR1 fluidity in the membrane, or accessibility of the receptor to RIPK1. Further dissection of the CAPN9 dependent interactions is needed to determine the mechanism of CAPN9 regulation of TNF-α induced apoptosis.

4.3 CAPN9 affects TNFR1 associated ubiquitination

Ubiquitin tagging of proteins is a common motif in TNF-α signaling. Ubiquitin serves as an anchor allowing proteins to interact. RIPK1 is rapidly ubiquitinated when recruited to TNFR1, allowing for recruitment of factors to activate NF-κB. CAPN9 knockdown blocks RIPK1 recruitment and ubiquitination. Since ubiquitin is integral to recruitment of factors to the receptor, total ubiquitin interactions with TNFR1 were assessed. TNFR1 was immunoprecipitated from H2009 CAPN9 shRNA stable cell lines and analyzed for total ubiquitination by western blot (Figure 4.11). A smear of bands, indicative of poly-ubiquitination was observed in the absence of stimulation with TNF-α, Smac mimetic, and z-VAD. This basal ubiquitination was unaffected by expression of a control shRNA to GFP, but disappeared with the expression of CAPN9 shRNA. This indicates that background ubiquitination associated with TNFR1 is dependent on CAPN9, and correlates with inhibition of RIPK1 recruitment and subsequent cell death stimulated by TNF-α.

TNFR1 associated ubiquitination was observed in H2009 cells, and disappeared with expression of CAPN9 shRNA. To determine if this was cell line

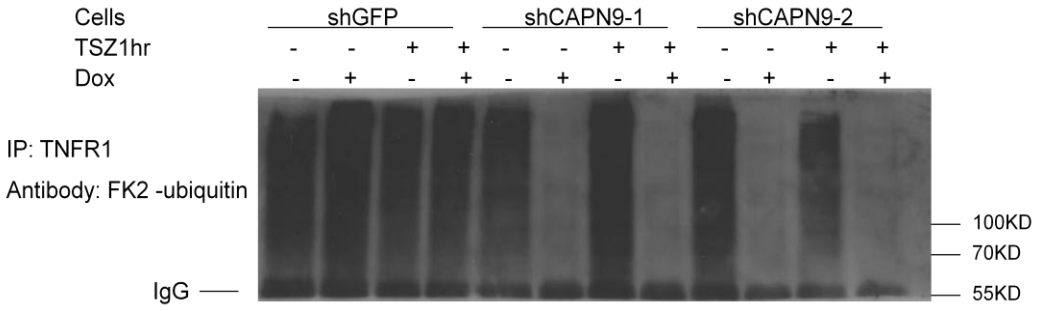


Figure 4.11 CAPN9 shRNA blocks basal TNFR1 associated ubiquitination

H2009 inducible shRNA expressing cells were treated with 1ug/mL Dox for 6 days. Next cells were treated with or without 100ng/mL TNF-alpha, 100nM Smac mimetic, and 20μM z-VAD for 1 hour. TNFR1 immunoprecipitations were analyzed by western blot for ubiquitin.

specific, MDA-MB231 cells were transfected with CAPN9 siRNA, lysed, immunoprecipitated for TNFR1, and analyzed by western blot. Basal TNFR1 ubiquitination was observed in MDA-MB231 cells (Figure 4.12). This ubiquitination was lost when cells were transfected with three different CAPN9 siRNAs. This indicates TNFR1 ubiquitination is not specific to H2009 cells. This data also shows multiple CAPN9 oligos are capable of preventing TNFR1 associated ubiquitination suggesting this is not an off-target artifact of CAPN9 siRNA.

TNFR1 associated ubiquitination is CAPN9 dependent, as well as FLNA and SPTAN1 interactions with the receptor. This observation allows for the identification of more components of the TNFR1 complex. TNFR1 was immunoprecipitated from H2009 CAPN9 shRNA cells, released from the antibody with a low pH elution buffer, and purified using a ubiquitin capture kit (Enzo Life Sciences). Elutions from the double immunoprecipitation were separated by SDS-PAGE and silver stained. CAPN9 knockdown eliminated most proteins from the elution (Figure 4.13). Total elutions were analyzed by mass spectrometry. Elution from CAPN9 shRNA cells induced with Dox were used to eliminate non specific interacting proteins. Several proteins were identified from the double immunoprecipitation (Table 4.2). Most notably, ubiquitin was identified.

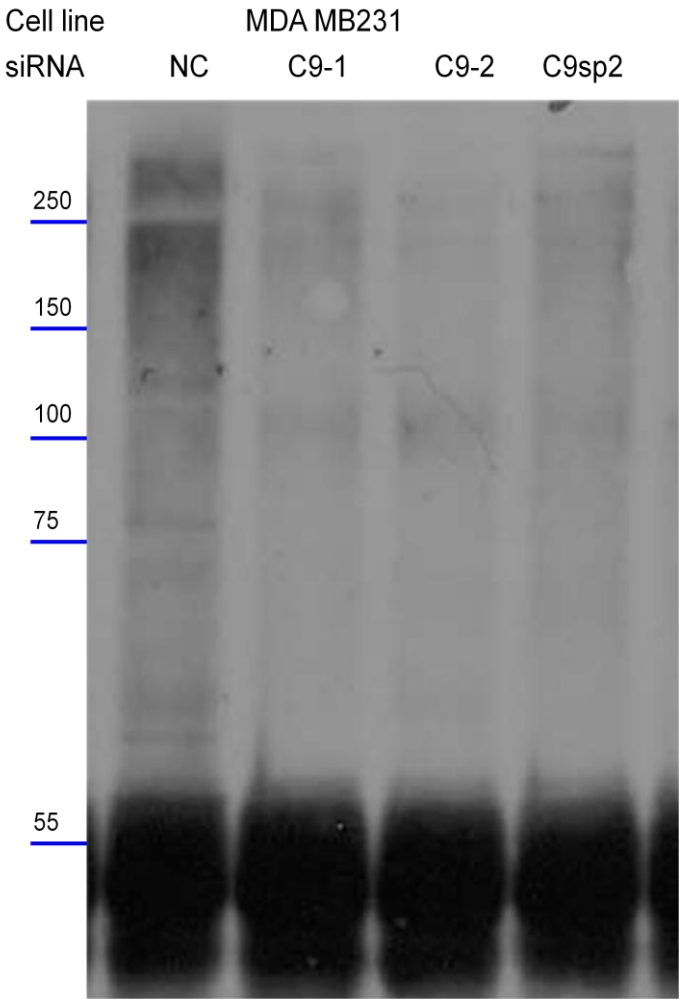


Figure 4.12 Multiple CAPN9 siRNA block TNFR1 associated ubiquitination

MDA-MB231 cells were transfected with CAPN9 siRNA oligos. TNFR1 immunoprecipitations were analyzed by western blot for ubiquitin.

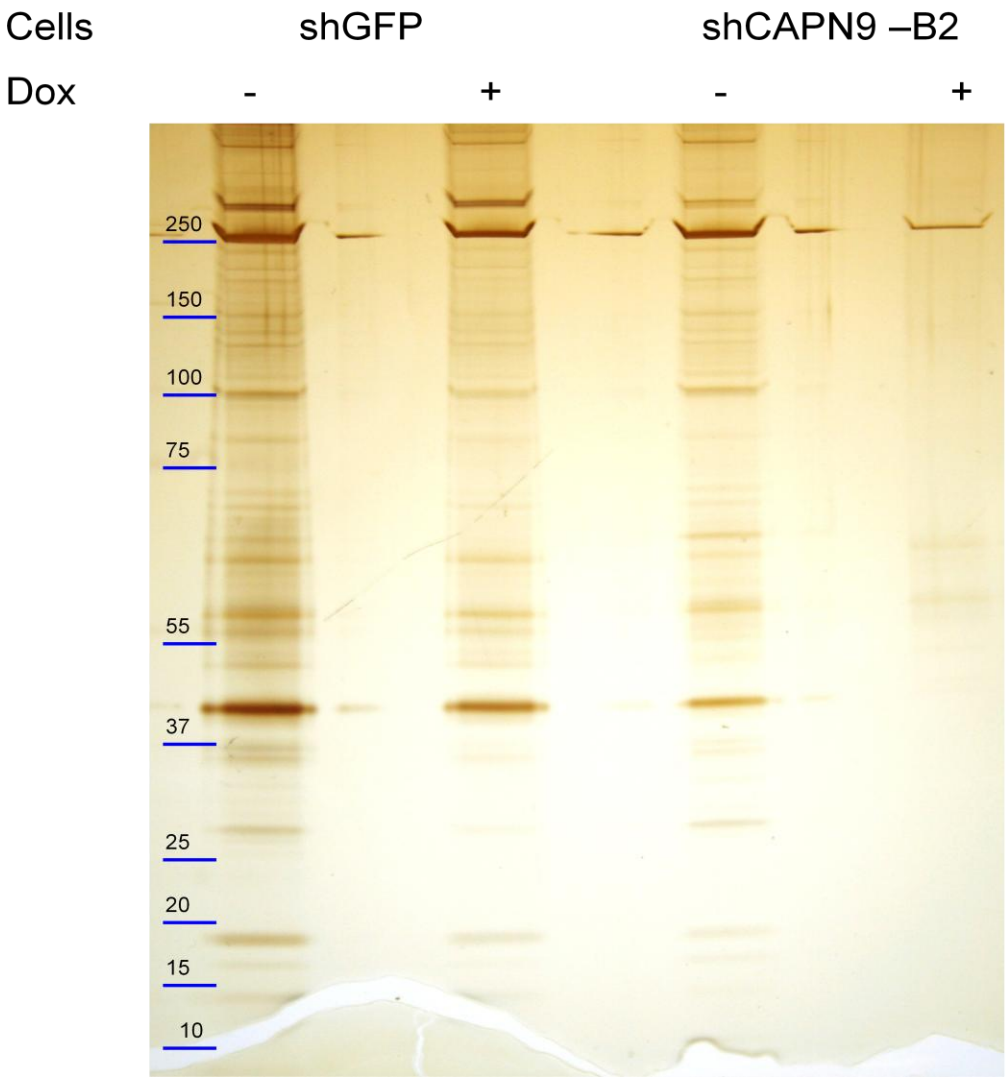


Figure 4.13 Double IP and silver stain identifies multiple CAPN9 dependent bands

H2009 inducible shRNA expressing cells were treated with 1ug/mL Dox for 6 days. Lysates were immunoprecipitated with a TNFR1 antibody, released with a low pH elution and ubiquitin was isolated using a ubiquitin capture kit (Enzo Life Sciences). Final elutions were separated by SDS-PAGE and silver stained.

Protein description	Mascot score	MW	Peptide hits
LMNA Isoform A of Lamin-A/C	1493	74095	63
HNRNPM Isoform 1 of Heterogeneous nuclear ribonucleoprotein M	381	77464	19
KRT19 Keratin, type I cytoskeletal 19	370	44065	22
MYH9 Isoform 1 of Myosin-9	353	226392	26
KRT4 keratin 4	322	63871	21
ACTB Actin, cytoplasmic 1	232	41710	15
LMNB1 Lamin-B1	228	66368	13
ACTA1 Actin, alpha skeletal muscle	167	42024	12
HNRPA1L-2 Similar to Heterogeneous nuclear ribonucleoprotein A1	162	34236	10
MYL12A Myosin regulatory light chain 12A	134	19781	5
PABPC1 Isoform 1 of Polyadenylate-binding protein 1	130	70626	8
TPM3 Isoform 2 of Tropomyosin alpha-3 chain	97	29015	7
SFRS1 Isoform ASF-1 of Splicing factor, arginine/serine-rich 1	87	27728	3
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	86	70854	3
RBMX Heterogeneous nuclear ribonucleoprotein G	85	42306	5
NCL cDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin	84	65922	3
HNRNPD Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	83	38410	4
HNRNPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	77	39571	6
HNRNPH2 Heterogeneous nuclear ribonucleoprotein H2	69	49232	3
RPA3 Replication protein A 14 kDa subunit	67	13560	3
TFAM Putative uncharacterized protein TFAM	67	25449	5
DDX17 Isoform 3 of Probable ATP-dependent RNA helicase DDX17	60	72511	1
SFRS4 Splicing factor, arginine/serine-rich 4	56	56645	1
TPM1 Isoform 3 of Tropomyosin alpha-1 chain	55	32856	5
TPM2 Isoform 3 of Tropomyosin beta chain	54	28666	3
RPS16 40S ribosomal protein S16	52	16435	2
BANF1 Barrier-to-autointegration factor	50	10052	1
JUP Junction plakoglobin	50	81693	3
UBC;RPS27A;UBB RPS27A protein	49	10776	3
SNRPA U1 small nuclear ribonucleoprotein A	45	31259	2
TPM4 Isoform 2 of Tropomyosin alpha-4 chain	43	32703	3
HNRNPL Heterogeneous nuclear ribonucleoprotein L	43	64092	1
RPLP0 60S acidic ribosomal protein P0	42	34252	1
HIST2H2BE Histone H2B type 2-E	37	13912	2
C1QBP Complement component 1 Q subcomponent-binding protein, mitochondrial	35	31343	1
H2AFV Histone H2A.V	33	13501	1
RALY RNA binding protein	33	32531	3
CAPZA1 F-actin-capping protein subunit alpha-1	33	32902	1
SERPINB4 Serpin B4	31	44825	1
Putative uncharacterized protein ENSP00000396059 (Fragment)	30	9306	1
HNRNPU HNRPU protein	30	79666	2
TOP2B Topoisomerase II beta	30	130398	4
FAM129C Isoform 1 of Niban-like protein 2	27	77352	1
RAN;RANP1 GTP-binding nuclear protein Ran	26	24408	1
TUBA4A Tubulin alpha-4A chain	22	49892	2
TPH1 Isoform 1 of Tryptophan 5-hydroxylase 1	22	50953	1
FAM35B Protein FAM35B	21	93616	1

Table 4.2 List of CAPN9 dependent TNFR1 and ubiquitin associated proteins

List of proteins identified by Mass spectrometry that were not identified after induction of CAPN9 shRNA.

Multiple proteins were found in the ubiquitin associated TNFR1 immunoprecipitation fraction. Previous results found three actin binding proteins. Actin was identified in the double immunoprecipitation. This was confirmed by western blot analysis. This is not a surprise since multiple proteins identified bind actin, and indicates CAPN9 may be affecting TNFR1 recruitment to signaling centers of the membrane. LMNB1 was also confirmed by western blot (Figure 4.14). Further analysis of candidate proteins from the double immunoprecipitation is required to completely understand the role of this ubiquitin associated protein.

CAPN9 is a novel protein involved in TNF-α signaling. CAPN9 regulates TNF-α induced apoptosis by regulating TNFR1 basal association with ubiquitinated proteins, thus allowing recruitment and ubiquitination of RIPK1.

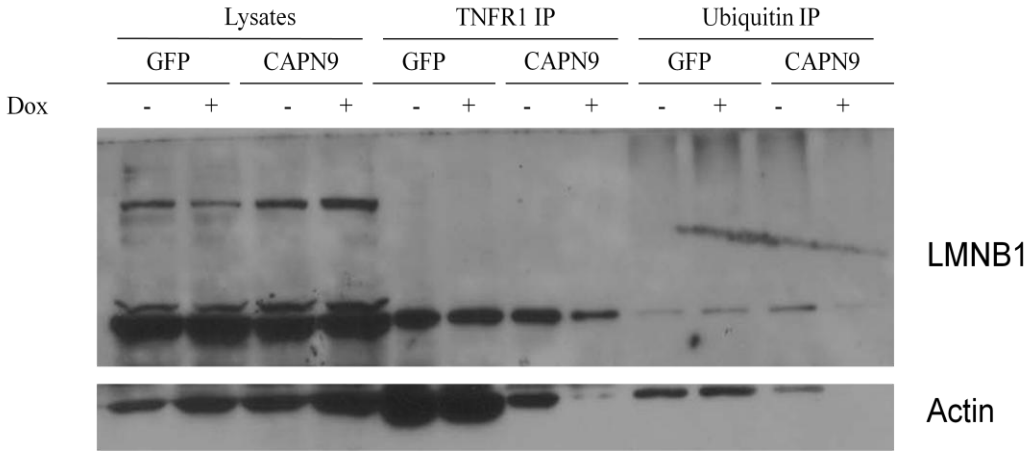


Figure 4.14 Antibody repeat of identified proteins

H2009 inducible shRNA cells were immunoprecipitated for TNFR1, eluted and immunoprecipitated for ubiquitin. Western blot analysis was performed with Actin and Lamin B1 antibodies.

Chapter Five

Conclusions

5.1 High-throughput siRNA screening for identification of novel signaling factors.

Forward genetic screening is a powerful tool for biological discovery. A high-throughput siRNA screen was conducted to identify novel components of single agent Smac mimetic induced apoptosis. TNFR1, RIPK1 and Caspase 8 were all positive hits, validating the efficacy of the screen. The advantage of such an experiment is also the drawback; the overabundance of data makes it difficult to separate the true hits from false positives. However, this experiment has generated a powerful database that is continually mined by our lab and others. Another siRNA screen was completed by Sudan He, looking for factors that prevented TNF-α, Smac mimetic, and z-VAD induced necrosis. My screen was used as a counter screen to filter out any potential siRNA that were not specific to necrosis, helping identify RIPK3 as a critical mediator of Necrosis (He, Wang et al. 2009). This data set has served as a valuable tool for all lab members working on the necrosis project. Other labs on campus are currently mining this data set for factors affect cell viability.

Secondary assays significantly narrowed down the number of positive hits. Each assay was designed to eliminate as many nonspecific affects and focus on siRNAs that truly affect TNF-α and Smac mimetic induced apoptosis. Two independent methods were used to isolate true "hits" from the screen. First, primary screen hits were retested using a different siRNA library. This was based off the assumption that a different siRNA library would have all different siRNA oligo sequences, all of which were capable of knocking down the gene with equal efficiency as the primary screen library. All siRNAs rescreened had a 50% survival or greater when treated with the Smac mimetic from the Dharmacon library. However, these 320 hits averaged a survival ratio of 17% with the Qiagen library. This suggests the Qiagen library was not nearly as efficient as the Dharmacon library, and many true hits were possibly eliminated during this secondary screen.

Calpain 9 was a positive hit in both siRNA libraries. Closer inspection of siRNA sequence reveals that one siRNA oligo is present in both libraries. Though CAPN9 was eventually validated as a true hit, this raises the question of the validity of using the Qiagen siRNA library. Future screens should look into using siRNA libraries from alternative sources.

Concurrent with the Qiagen library screen, individual oligos for the top 62 hits were ordered and retested. Under the most stringent conditions, the majority

of hits had only one oligo capable of preventing the cell death phenotype. In the case of BID, this one oligo showed directly correlation between phenotype and knockdown efficiency. However, AMACR had two oligos confer phenotype, but did not have a correlation to knockdown efficiency. This method of secondary screening appears to be useful as a means to validate a hit if three or more oligos are capable of producing the phenotype, and has low efficacy in ruling out off-target effects.

siRNA hits were also tested in multiple cell lines. This secondary screen was not intended to eliminate off-target effects. Cancer cell lines can have different responses to drugs. Screening multiple cell lines ensures the siRNA hits affect general pathways. This increases the probability the hit is a strong mediator of the signaling pathway. BID is a known effector of apoptotic signaling, yet was eliminated in screening multiple cell lines, because HCC461 is not dependent on concomitmant activation of the intrinsic apoptotic pathway.

The ultimate elimation of off-target effects is correlation between knockdown efficiency and phenotype. This can be logistically and financially difficult, but is necessary for identifying true hits from any siRNA screen. AMACR passed all secondary screening critera, while BID did not. AMACR was ultimately proven to be an off-target effect while BID was a true hit. High

priority should be given to correlation between knockdown efficiency and phenotype for future secondary screening.

5.2 CAPN9-SP2 is required for TNF-alpha induced apoptosis

Out of 394 possible candidate genes, CAPN9 was validated as a true hit from the primary siRNA screen. Multiple CAPN9 siRNA oligo knockdowns correlated perfectly with prevention of cell death. Interestingly, CAPN9-SP2 specific siRNA was capable of preventing cell death, while siRNA against SP1 had no effect. Expression of an siRNA resistant CAPN9-SP2 reconstitutes the cell death phenotype confirming this is the critical splice variant required for cell death.

The question remains, what are the differences between the two splice variants. Exclusion of exon 9 results in a loss of 27 amino acids in the proteolytic domain of CAPN9, directly adjacent to the N278, a member of the catalytic triad. This deletion could result in a change of activity or substrate recognition. Expression of recombinant CAPN9-SP1 and SP2 to compare in vitro catalytic activity is currently being explored.

The pan Calpain inhibitor, ALLN, blocks Smac mimetic induced apoptosis. This suggest CAPN9 catalytic activity is required for its function in the apoptotic program, and raises the question, what are the proteolytic targets of

CAPN9. Over-expression of tagged, catalytically dead, CAPN9 constructs were not able to isolate any clear potential substrates of CAPN9 (data not shown). Elucidation of the substrate is critical for determining how CAPN9 affects TNF-α induced apoptosis.

5.3 Possible mechanisms for CAPN9 mediation of TNF-α induced apoptosis.

CAPN9 affects RIPK1 recruitment to TNFR1 without changing total protein levels, or cell surface expression. Analysis into the CAPN9 has unveiled many new observations about the TNF receptor. CAPN9 knockdown demonstrates TNFR1 is associated with ubiquitinated proteins in the absence of stimulus. This association is CAPN9 dependent, and correlates with RIPK1 recruitment and apoptosis. This observation has allowed me to identify multiple proteins associating with receptor in a CAPN9 dependent manner. Deciphering this result will require multiple experiments.

CAPN9 siRNA also demonstrates novel interactions between actin binding proteins, FLNA, SPTAN1, and MYH9. It is not surprising actin interacts with the receptor in a CAPN9 dependent manner too. All of these experiments were done with a lysis buffer containing 1% Triton X-100. These proteins are known to be involved in cytoskeletal remodeling, suggesting that CAPN9 may be involved in the localization of TNFR1 to signaling centers such as lipid rafts. It has been demonstrated that lipid rafts are required for TNF-alpha signaling, and

lipid rafts are characterized as being Triton X-100 insoluble. Further experiments are required to examine this.

The data in this thesis definitively demonstrates CAPN9 is a novel mediator of TNF-alpha signaling. CAPN9 drives TNFR1 association with ubiquitinated and actin binding proteins enabling RIPK1 recruitment, ubiquitination, and activation of apoptosis in response to TNF-alpha.

Appendix A:

Materials and Methods

Reagents

Recombinant GST tagged TNF-α expression construct was a kind gift from Dr. Zhijian Chen (UT Southwestern Medical Center), and was generated as previously described (Ea, Deng et al. 2006). ALLN, CHX and MG-132 were purchased from Calbiochem. Z-VAD-fmk was purchased from Bachem. The following antibodies were used: CAPN9 Abnova H00010753-M02, TNFR1 Santa Cruz sc-7895, MYH9 Abcam ab24762, FLNB abcam ab53559, FLNA Novus H00002316, Pan cIAP R&D, MAB3400, RIPK1 BD 551041, Actin Sigma A2668, Flag-HRP Sigma A8592, LMNB1 Santa Cruz sc-6217, SPTAN1 Abnova MAB4911.

Plasmids and siRNA sequences

mRNA was extracted from HCC44 cells using Trizol(Invitrogen). Extracted mRNA was used for RT-PCR reactions using the Superscript One Step RTPCR kit(Invitrogen). CAPN9 and AMACR were amplified by RTPCR and cloned into pcDNA3.1 + with an N-terminal 3X flag tag using the following primers: CAPN9 ATG CCT TAC CTC TAC CGG G, and TC AGA TGT TCA TTG TCA AAT GGA TGA ACT C; AMACR ATG GCA CTG CAG GGC ATC,

and TTA GAG ACT AGC TTT TAC CTT ATT ACT TTC. Primers spanning exon 9 of CAPN9 were: GGG AAT TGA CCA GGT AAG CTT CC, and GTC TTC CTC CAG GGC ATC G. CAPN9 splice variants were cloned into pcDNA 3.1 vectors containing the Tet repressor sequence. Quick change mutagenesis was performed to create silent mutations in the target sequence of CAPN9 siRNA oligo 1.

All siRNA oligos were obtained from Dharmacon. On target plus pools were used for TNFR1, Nik, Caspase 8, FLNA, SPTAN1, MYH9. The siRNA sequence for luciferase is: CGTACGCGGAATACTTCGATT, for RIPK1 is: TCACCAATGTTGCAGGATATT. CAPN9 siRNA sequences are 1: ggAgTgTAgTTTCCTTgTA, 2: gCAgAAAgATAgAAggAAA, 3: ACAUCAUUUCCCUgAUggA, 4: ACAAGGAGTTCATTCATCT, CAPN9-SP1 specific siRNA is GCGUCUGUGUCACACUGCUUU, CAPN9-SP2 is GGAGCGACAgGATGGCATT. MYH9 custom designed oligo is aaggagcgttactactcaggg.

Cell culture and stable cell lines

HCC44, HCC461, MDA-MB231, and H2009 cells were cultured in RPMI (Hyclone) with 5% Fetal Bovine Serum, FBS (Gibco). HEK293T cells were cultured in DMEM and 10% FBS. H2009 cells were transfected with pcDNA6/TR plasmid from Invitrogen and selected with 10µg/ml Blasticidin.

H2009 TetR cells were used for generation of all stable cell lines. H2009 TetR cells were transfected with a CAPN9-SP2 expression construct under the tet repressor. Leaky gene expression caused constitutive expression of CAPN9. CAPN9 shRNA cells were generated as described in (Zhong, Gao et al. 2005). In brief, four copies of CAPN9 shRNA, designed using siRNA oligo 1 sequence, were cloned into pSuperior.puro (Oligoengine) vector under an H1 promoter. Stable cell lines were selected using 0.5µg/mL puromycin.

siRNA transfection

In general, plasmid DNA was mixed with Lipofectamine 2000 in Optimem for approximately 10 minutes to form DNA-transfection agent complexes. These complexes were incubated with pre-seeded cells in optimem for four hours. For siRNA; HCC44, HCC461, and H2009 Dharmafect 4 was used, while HEK293T cells were transfected with Dharmafect Duo. MDA-MB231 cells were transfected with Lipofectamine RNAi Max. All siRNA transfection complexes were prepared in Optimem, and cells were seeded into the transfection complexes.

Cell TiterGlo survival assay

Cell viability was measured using Cell TiterGlo from Promega in 96 well flat-bottom plates, with some minor alterations to the protocol. Cell TiterGlo was

diluted 1:5 in 1XDPBS, and 1% Triton X-100. Media was removed from the plate and 100µL Cell TiterGlo was added. Plates were placed on a rocker shaker for 1 minute, and then incubated at room temperature for 10 minutes. Luminescence measurements were completed using a Tecan SPECTRAFluor Plus reader. Results represent a minimum of four replicates, and the error bars are the standard deviation.

Caspase 3 activity assay

Cells were transfected as above in 6 well dishes, washed 2 times with 1XDPBS. Cells were lysed by the addition of cell lysis buffer (50mM Tris-HCl pH 7.7, 1mM EDTA, 150mM NaCl, 1% Triton X-100) directly to the well. Lysates were centrifuged for 10 minutes at 4C at 12,000xg. Protein concentrations were normalized for a final concentration of 1µg/mL. Caspase 3 Fluorogenic substrate II, cat #235425 Calbiochem, was added at a final concentration of 10µM to a 384 well black flat bottom plate. Assay was measured using a Tecan SPECTRAFluor Plus reader with excitation at 360nM and emission 465nM.

Cell lysis

Cell lysis was carried out in Flag lysis buffer unless otherwise indicated. Flag lysis buffer is 50mM Tris-HCl pH 8.0, 1mM EDTA, 137mM NaCl, 1%

Triton X-100, 10% Glycerol, 0.1mM PMSF, 10mM NaF. Rosche complete protease inhibitor cocktail was added to all lysis buffers. Cells were incubated in Flag lysis buffer for 30 minutes at 4C, then centrifuged for 10 minutes at 4C at 12,000xg. Protein concentrations were determined for protein normalization.

Immunoprecipitation protocol

Cell lysates were prepared as described above. Dynal Protein A magnetic beads were washed twice in 1XDPBS, then incubated for 2 hours, with rotation, with antibody (2ug of antibody for 20uL of magnetic beads) at RT in 1XPBS 3%BSA. Beads were washed in flag lysis buffer and incubated with lysates overnight at 4C while rotating. Samples were washed 3 times in flag lysis buffer and eluted using Pierce IgG pure elution buffer, a 2.5pH elution buffer.

For the ubiquitin IP, eluted samples from the TNFR1 IP were diluted in 1XPBS + 1mM Tris-HCl pH 7.4. Elutions were loaded onto ubiquitin capture beads (UbiCapture Kit, Enzo life sciences) following manufacturers protocol. Final elutions were sent for protein ID by mass spectrometry (National Institute of Biological Sciences – Beijing, China).

Cell Fractionation

Cells were lysed in buffer A (20mM Hepes,40mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1mM PMSF) with 250mM sucrose by

22 passages through a 22 gauge needle and incubated at 4C for 20 minutes. Cells were centrifuged at 1,000xg for 30 minutes at 4C to generate the P1 (resuspended in flag lysis buffer). Supernatant was centrifuged at 12,000xg for 30 minutes at 4C to generate the P12 (resuspended in flage lysis buffer). Next the supernatant was centrifuged at 100,000xg for one hour at 4C to generate the P100 and S100. Western blot analysis was performed with relative equal volumes of each fraction, and with protein level normalized for each fraction when comparing fractions from multiple samples.

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