Characterization of the Role of Akt Kinase in Necroptosis

A thesis

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Abstract

Necroptosis in a novel form of programmed cell death that has been implicated in many disease states including ischemia-reperfusion injuries, Huntington's disease, pancreatitis, and Crohn's disease. It has also been shown to play an important role in inflammatory diseases such as Systemic Inflammatory Response Syndrome (SIRS). Work done in recent years has shown that necroptosis is a highly regulated process. Many signaling proteins, most importantly RIP1 and RIP3 kinases, have been discovered to be involved in the regulation of necroptosis. However, a complete understanding of molecular mechanisms involved in necroptosis remains to be elucidated.

The goal of this work was to characterize the role of Akt kinase in necroptosis by gaining an understanding of how it becomes activated in response to pro-necroptotic stimuli and elucidating the downstream consequences of its activation in the process of cell death. We also sought to identify novel Akt interacting proteins and to characterize the role that these proteins play in necroptosis.

In this work we show that Akt kinase activity is critical for necroptosis in L929 cells and that it plays a key role in TNFα production. During necroptosis, Akt is activated in a RIP1 dependent fashion through its phosphorylation specifically on Thr308. In L929 cells, this activation requires independent signaling inputs from both growth factors and RIP1. Akt controls necroptosis through downstream targeting of mammalian Target of Rapamycin complex 1

(mTORC1). Akt activity, mediated in part through mTORC1, links RIP1 to JNK activation and autocrine production of TNFα. In other cell types, such as mouse lung fibroblasts and macrophages, Akt exhibited control over necroptosis-associated TNFα production without contributing to cell death. We discovered several putative Akt interacting proteins, including Arf1, Nur77, and GAPDH, and began to describe the role that they play in cell death. Overall, our results provide new insights into the mechanism of necroptosis and the role of Akt kinase in both cell death and inflammatory regulation.

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This work is dedicated to my father, Joseph Patrick McNamara.

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List of Abbreviations

4EBP1	Eukaryotic initiation factor 4E binding protein 1
aa	Amino Acid
Act	Actinomycin D
AIF	Apoptosis-inducing factor
ANT	Adenine Nucleotide Translocase
Arf1	ADP-ribosylation Factor 1
Bcl-2	B-cell Lymphoma 2
BFA	Brefeldin A
CHX	Cycloheximide
cIAP1/2	cellular Inhibitor of Apoptosis Proteins 1 and 2
CrmA	Cytokine Response Modifier Protein A
DD	Death Domain
DMSO	Dimethyl Sulfoxide
EGF	Epidermal Growth Factor
eIF	Eukaryotic Translation Initiation Factor
ELISA	Enzyme-linked Immunosorbant Assay
ERK	Extracellular Signal-Regulated Kinase
FADD	FAS-Associated protein with Death Domain
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR1	Fibroblast Growth Factor Receptor 1

GAP	GTPase-Activating Protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GEF	Guanine nucleotide-Exchange Factor
GLUD1	Glutamate Dehydrogenase 1
GLUL	Glutamate-Ammonia Ligase
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine-5'-triphosphate
IGF	Insulin-like Growth Factor
JNK	Jun N-terminal Kinase
KD	Kinase Domain
LMP	Lysosome Membrane Permeabilization
LPS	Lipopolysaccharide
LUBAC	Linear Ubiquitin Chain Assembly Complex
MLKL	Mixed Lineage Kinase domain-like protein
MPT	Mitochondrial Permeability Transition
MS	Mass Spectrometry
mTOR	Mammalian Target of Rapamycin
N-WASP	Neural Wiskott-Aldrich Syndrome Protein
Nec-1	Necrostatin-1
NF-kB	Nuclear Factor kappa-B
PAGE	Polyacrylamide Gel Electrophoresis
PARP1	Poly (ADP-ribose) Polymerase 1
PDGF	Platelet-derived Growth Factor

PDK1	Phosphoinositide-dependent Kinase-1
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
PRAS40	Proline-rich Akt substrate of 40 kDa
PTPC	Permeability Transition Pore Complex
PYGL	Glycogen Phosphorylase
RHIM	RIP Homotypic Interaction Motif
RIP1	Receptor-Interacting Protein 1
ROS	Reactive Oxygen Species
S6K1	Ribosomal p70S6 kinase
SILAC	Stable Isotope Labeling with Amino Acids in Culture
TAB2/3	TAK1 Binding Protein 2/3
TAK1	Transforming Growth Factor-activated Kinase
TBS-T	Tris-Buffered Saline - Tween 20
TCA	Trichloroacetic acid
TCN	Triceribine
TNF	Tumor Necrosis Factor
TRAF2/5	TNFR-Associated Factor 2/5
TRAILR	TNF-related apoptosis-inducing ligand receptor
TSC	Tuberous sclerosis complex
Ub	Ubiquitin

zVAD.fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

Characterization of the Role of Akt Kinase in Necroptosis

Chapter I: Introduction to Necroptosis

1.1 Necroptosis is a Novel Form of Programmed Cell Death

i. Necroptosis is a Form of Programmed Cell Death

Cellular necrosis has historically been thought of as an unregulated form of death that occurs in response to extreme stress inflicted upon the cell. This manner of death has typically been regarded in stark contrast to the highly regulated programmed cell death mode known as apoptosis. On the surface, these two types of death are morphologically distinct. Unlike apoptosis, which involves caspase activation, chromosome condensation, cell shrinkage, and apoptotic body formation; necroptosis is characterized by the absence of caspase activation, cellular and organelle swelling, and rupture of the plasma membrane (Vandenabeele et al., 2010). The initiation and execution of apoptotic cell death is known to require the coordination of multiple proteins complexes in addition to the activation of caspases, cysteine proteases, which eventually lead to disassembly of the cell and death. Necrosis, on the other hand, has been represented as a disorganized, passive death that occurs after overwhelming cellular damage.

Approximately twenty-five years ago, in 1988, the perception of necrosis as being wholly unregulated began to be challenged. In that year it was discovered that cells could die with either apoptotic or necrotic morphology in

response to the same stimulus, Tumor Necrosis Factor (TNF), depending on the cell type (Laster et al., 1988). It was later found that L929 fibrosarcoma cells, which die with necrotic morphology in response to TNF, require Reactive Oxygen Species (ROS) formation for death to occur (Goossens et al., 1995; Schulze-Osthoff et al., 1992). Over the intervening years, evidence of the regulated nature of necrotic death has continued to accumulate (Reviewed in (Galluzzi et al., 2011)). Importantly, in 2000, Holler et. al. reported that T cells could die both with an apoptotic morphology in a caspase-8 dependent way and with a necrotic morphology in a Receptor-Interacting Protein 1 (RIP1) dependent fashion (Holler et al., 2000). In 2005 Degterev et. al., in a random unbiased cell based screen of U937 monocytes treated with TNFα and zVAD.fmk, identified the first small-molecule, Necrostatin-1 (Nec-1) that could prevent TNFα-induced caspase-independent necrotic cell death, necroptosis, from occurring (Degterev et al., 2005). The authors later found that the mechanism of action of Nec-1, and two structurally distinct classes of necrostatins, specifically inhibit RIP1 kinase (Degterev et al., 2008). Taken together, these data cemented necroptosis as a regulated from of cell death. Since this time significant advances toward appreciating the physiologic role of necroptosis have been made. A substantial amount of research has gone in to understanding both the molecular mechanisms of necroptosis and the significance it holds in health and disease.

ii. Initiation of Necroptosis

A great deal of research has been done in order to better understand the programmed, or regulated, nature of necrosis. Programmed necrosis, or necroptosis, can be split in to three major components initiation, execution, and downstream consequences. While there are many known initiators of necroptosis, TNFα activation of the TNF receptor 1 (TNFR1) death is the best-defined mechanism (Figure 1.1). Ligation and activation of several other death domain containing receptors including Fas (Holler et al., 2000; Vercammen et al., 1998b), TNF-related apoptosis-inducing ligand receptors (TRAILR) (Laster et al., 1988), and TNFR2 (Chan et al., 2003) can also induce necroptosis. Notably, activation of these receptors often induces caspase-dependent apoptosis. However, in cases where caspase activity is inhibited the existence of caspase-independent necrotic death has been revealed.

Depending on the cellular context, TNFR1 activation can lead to several possible cellular outcomes (Figure 1.1). These possibilities include Nuclear Factor kappa-B (NF-κB) activation and cell survival, apoptosis, or necroptosis. TNFR1 trimerization and activation by TNFα leads to the formation of an intracellular membrane associated complex called TNFR1 Complex I (Micheau and Tschopp, 2003). This complex contains TNFR-Associated Death Domain protein (TRADD), TNFR-Associated Factor 2 (TRAF2), TRAF5, cellular Inhibitor of Apoptosis Proteins 1 and 2 (cIAP1/2), and RIP1. The TRAF proteins recruit the cIAP E3 ubiquitin ligases to the complex and stabilize them by inhibiting their ability to auto-polyubiquitinate (Csomos et al., 2009; Rothe et al., 1995). In this complex, the K63-linked polyubiquitination of RIP1 on K377 is mediated by

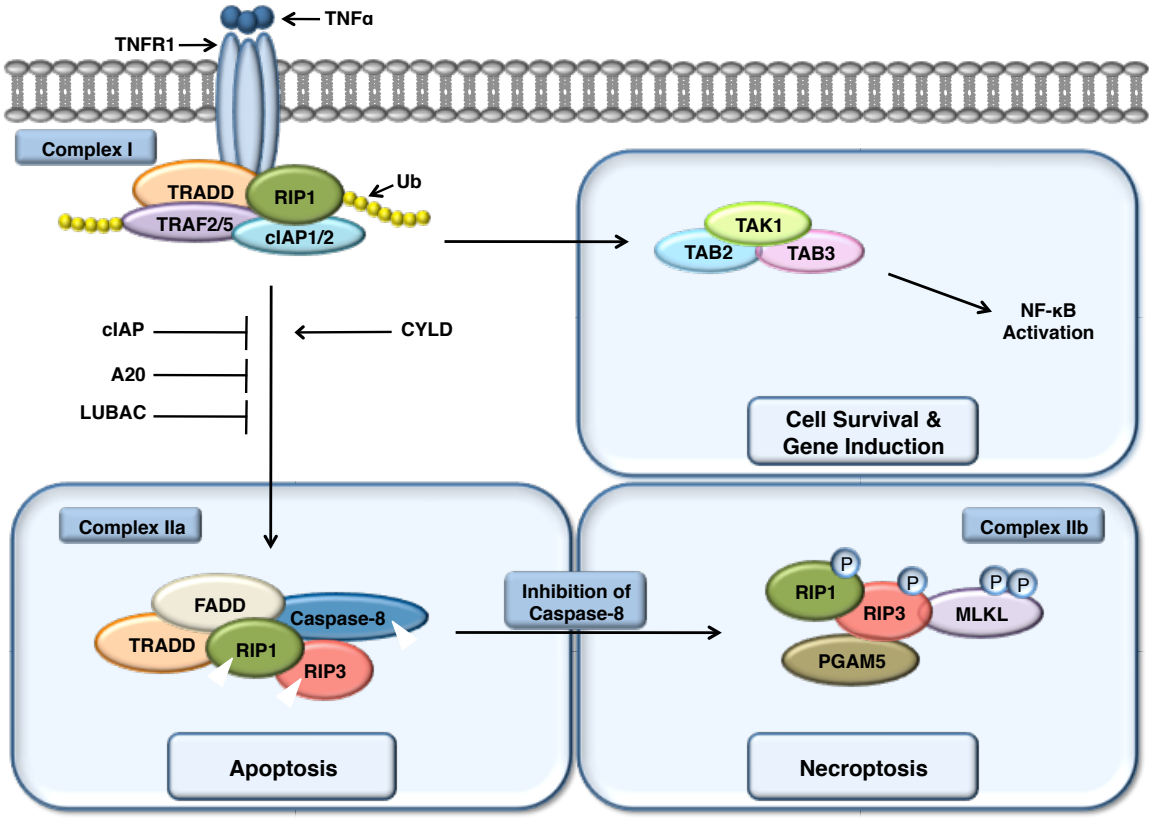

Figure 1.1 TNFα-induced necroptosis pathway.

Figure 1.1 TNFα-induced necroptosis pathway. TNFR1 activation leads to the formation of Complex I. Complex I contains TRADD, TRAF2/5, RIP1, cIAP1/2. Complex I, when RIP1 is ubiquitinated by cIAPs, leads to recruitment and activation of the TAK1-TAB2-TAB2, NF-κB activation, and survival. When RIP1 is deubiquitinated by CYLD, Complex II is formed. Complex IIa leads to apoptosis and contains activated Caspase-8, RIP1, RIP3, FADD, and TRADD. In cases when Caspase-8 is inactivated, the necrosome containing RIP1, RIP3, MLKL, and PGAM5 is formed and necrosis occurs.

cIAP1/2 (Bertrand et al., 2008). Polyubiquitinated RIP1 leads to the activation of the Transforming growth factor-Activated Kinase (TAK1)-TAK1-Binding Protein 2 (TAB2)-TAB3 complex and activation of the NF-κB pathway (Hacker and Karin, 2006). Activation of NF-κB transcription factors leads to cell survival and inflammatory signaling.

Endocytosis of the TNFR1 leads to the rearrangement of Complex I and formation of Complex II, or Death-Inducing Signaling Complex (DISC) (Micheau and Tschopp, 2003). Complex II contains RIP1, RIP3, TRADD, FAS-Associated protein with Death Domain (FADD), and caspase-8 (Cho et al., 2009a; He et al., 2009; Micheau and Tschopp, 2003). Several proteins and complexes that modify the RIP1 ubiquitination state influence the formation of Complex II. For example, cIAP1/2 and the Linear Ubiquitin Chain Assembly Complex (LUBAC) (Haas et al., 2009) mediated ubiquitination of RIP1 leads to the recruitment and activation of the TAK1-TAB2-TAB3 complex and pushes the cell towards activation of the NF-κB pathway (Bertrand et al., 2008; Ea et al., 2006; Wu et al., 2006). Knockdown of cIAPs, or inhibition with smac mimetics, sensitizes cells to cell death (Wang et al., 2008). Likewise, A20, a ubiquitin editing enzyme, negatively regulates necroptosis induced in L929 cells by TNFα (Vanlangenakker et al., 2011). On the other hand, the RIP1 deubiquitylating enzyme cylindromatosis (CYLD) positively regulates Complex II formation, as deubiquitinated RIP1 is required for formation of this complex. Importantly, CYLD knockdown inhibits TNFα induced necroptosis (Hitomi et al., 2008).

The make-up of Complex II determines whether the cell will die from apoptosis or necroptosis. Activated Caspase-8 in Complex II leads to the cleavage and inactivation of both RIP1 and RIP3 (Feng et al., 2007; Lin et al., 1999) in addition to the cleavage of other substrates and the execution of caspase-dependent apoptosis. Caspase-independent necroptosis occurs when caspase-8 is inhibited in either a genetic or pharmacologic fashion (Holler et al., 2000; Vercammen et al., 1998a). Genetic inhibition of caspase-8 can be achieved through siRNA knockdown or expression of the viral protein Cytokine Response Modifier Protein A (CrmA), a caspase-8 inhibitory protein. Pharmacologic inhibition can be achieved through the use of a peptide inhibitor of caspases such as zVAD.fmk.

The formation of the necrosome, containing RIP1 and RIP3 (Figure 1.2), leads to necroptosis when caspase-8 activity is inhibited. The importance of RIP1 in necroptosis was confirmed by its identification as the target of Nec-1 (Degterev et al., 2008). In 2009 three laboratories independently discovered RIP3 as another kinase that plays an important role in necroptosis. The labs used a combination of siRNA screening and microarray analysis to identify RIP3 as a key component of the necroptotic pathway (Cho et al., 2009a; He et al., 2009; Zhang et al., 2009). It was found that RIP3 expression levels correlate with the cellular ability to undergo necroptosis (He et al., 2009). RIP1 and RIP3 form a complex with each other through their RHIM domains (Sun et al., 2002) in a Nec-1 dependent fashion (Cho et al., 2009a).

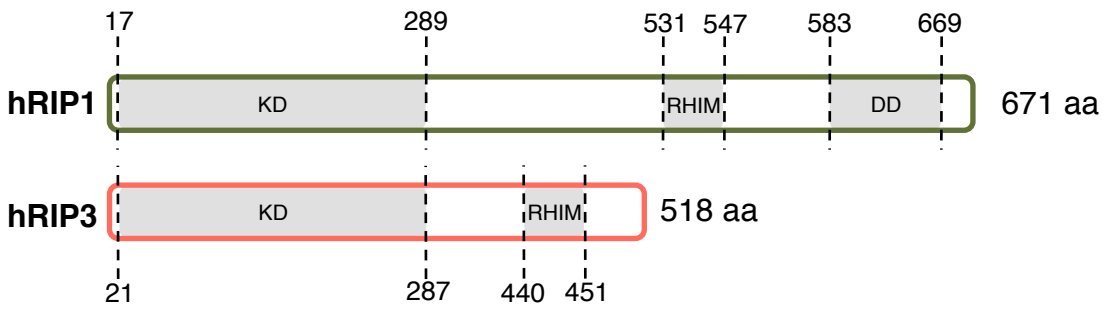

Figure 1.2 Domain structures of human RIP1 and RIP3.

Figure 1.2 Domain structures of human RIP1 and RIP3. A schematic depicting the domains of human RIP1 and RIP3. Both proteins contain an amino terminal kinase domain (KD) and a RIP homotypic interaction motif (RHIM) domain that facilitates their interaction. In addition, RIP1 contains a death domain (DD) that allows it to interact with other DD containing proteins such as TNFR1.

RIP1 and RIP3 can auto-phosphorylate themselves, on Ser161 and Ser199 respectively (Degterev et al., 2008), and it has been shown that RIP3 can also phosphorylate RIP1 (Cho et al., 2009a; He et al., 2009). In some situations such as during murine cytomegalovirus infections, RIP1-independent RIP3-dependent necroptosis can occur (Upton et al., 2010) indicating that RIP1 is not always necessary for necroptosis.

Two new proteins have recently been discovered to be part of the necrosome complex, MLKL and PGAM5. MLKL, or Mixed Lineage Kinase domain-Like protein, was discovered by two independent groups to be involved in necroptosis (Sun et al., 2012; Zhao et al., 2012). Xiadong Wang's group screened a chemical library and found that a compound called necrosulfonamide (NSA) inhibits necroptosis. They went on to identify MLKL as the molecular target of NSA (Sun et al., 2012). Zhao et al used an shRNA library to identify MLKL as a critical molecule involved in necroptosis (Zhao et al., 2012). The RIP3/MLKL interaction requires phosphorylation of RIP3 on Ser227 and results in the phosphorylation of MLKL on Thr357 and Thr358 by RIP3. However, the exact role of MLKL in necroptosis remains to be seen. PGAM5, a mitochondrial phosphoglyceratemutase, was also recently proposed to a be a component of the necrosome in a study that analyzed cellular extracts containing RIP3 (Wang et al., 2012). PGAM5 regulates Drp1 by dephosphorylation. In turn, dephosphorylated Drp1 promotes mitochondrial fission and necroptosis. The roles of both MLKL and PGAM5 in necroptosis need to be studied further in order to fully understand their function in necroptosis.

iii. Execution of Necroptosis

There are several known mechanisms that contribute to the execution of TNFR1 activated necroptosis. These can be broken down into bioenergetic processes, ROS generation, and Lysosome Membrane Permeabilization (LMP) (reviewed in (Vandenabeele et al., 2010)).

Bioenergetics. ATP-consuming processes such as translation (Saelens et al., 2005), proteosome dependent protein degradation (Sun et al., 2004), and poly (ADP-ribose) polymerase 1 (PARP1) activity continue during necroptosis (Soldani and Scovassi, 2002). This continuance of ATP-consuming processes differs from apoptosis where the utilization of ATP decreases. The fact that these cellular processes continue to occur during necroptosis contribute to the rapid, dangerous decline in ATP levels in the cells (Leist et al., 1997). For example, over-activation of PARP1 in response to damaged DNA in L929 cells in response to both TNFα and zVAD.fmk contributes to death (Los et al., 2002; Wu et al., 2011b). PARP1 activation leads to the accumulation of PAR and NAD depletion that together result in translocation of apoptosis-inducing factor (AIF) from the mitochondrial membrane into the cytosol. AIF then localizes to the nucleus where it contributes to DNA fragementation (Kroemer et al., 2007). Interestingly, activated PARP1 leads to JNK activation which also plays an important role in necroptosis (Xu et al., 2006).

Adenine nucleotide translocase (ANT) is an inner mitochondrial

membrane protein that imports ADP into the mitochondrial matrix and exports ATP into the cytosol. RIP1 inhibits ANT leading to reduced ADP levels in the matrix and reversal of the F_1-F_0 ATP synthase. This causes an increase in ADP levels and a transient increase in mitochondrial membrane potential (Goossens et al., 1999; Kroemer et al., 2007; Temkin et al., 2006). In addition, ANT is thought to interact with other mitochondrial proteins such as VDAC and CYPD to form the permeability transition pore complex (PTPC). The PTPC typically regulates the movement of solutes between the mitochondrial matrix and the cytosol. In response to some stimuli the PTPC becomes deregulated and allows the unregulated passage of water and solutes into the mitochondrial membrane. The transition of the PTPC to this high conductance conformation is called the mitochondrial permeability transition (MPT). Both genetic and pharmacological inhibitions of the PTPC mediate cytoprotective effects during necroptosis (Kroemer et al., 2007) (Galluzzi et al., 2009). These results suggest that bioenergetics and mitochondrial activities play a key role in the execution of necroptosis.

Reactive Oxygen Species. Mitochondrial energy has been linked to the execution of programmed necrosis since 1992 when it was shown that ROS generation is a requirement for necrotic death in L929 cells (Schulze-Osthoff et al., 1992). Mechanistically, it seems as though RIP3 kinase activity links TNFR signaling to mitochondrial bioenergetics and ROS production. RIP3 physically interacts with several metabolic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate dehydrogenase 1

(GLUD1) leading to their activation (Zhang et al., 2009). Knockdown of these enzymes protects cells from necroptosis and inhibits ROS production. PYGL activation can lead to the activation of glycolysis and generation of advanced glycation end products, or AGEs, both of which contribute to ROS generation. Both GLUL and GLUD1 are required for glutaminolysis, a process that makes α-ketoglutarate and leads to localized accumulation of ammonia and ROS generation. Overall these data indicate that there are several ways RIP3 activation can lead to ROS generation during necroptosis (Galluzzi et al., 2011).

There are several non-mitochondria-dependent mechanisms in which cells undergoing necroptosis can induce ROS production. These include ROS generation at the plasma membrane by the NADPH oxidase NOX1 (Kim et al., 2007). The ROS generated at the plasma membrane trigger mitochondrial ROS production. Knockdown of NOX1 down regulates ROS production and provides some protection from necroptosis in L929 cells. Additionally, JNK1 activation leads to degradation of ferritin and an increase in the labile iron pool and ROS generation.

Lysosome Membrane Permeabilization. ROS generate reactive aldehydes such as 4-hydroxynonenal that can attack the integrity of membranes through lipid peroxidation (Benedetti et al., 1980). Lipid peroxidation occurs when free radicals such as ROS take electrons from lipids in cellular membranes resulting in damage. Lipid peroxidation causes breakdown of plasma, lysosomal, and ER membranes causing leakage of proteases and calcium that contribute to cell death (reviewed in (Vandenabeele et al., 2010)).

iv. Necroptosis in Development and Disease.

Development and Adult Homeostasis. Necroptosis has recently been shown to play a very interesting role in development. RIP1 knockout mice die in the first few days post birth while RIP3 knockout mice are viable and healthy. Caspase-8 knockout mice die *in utero* at approximately embryonic day 10.5 (E10.5). When the caspase-8 knockout mice are crossed with the RIP3 knockout mouse, the double knockouts are protected from death. The mice survive normally but have increased lymphoaccumulation as adults. Additionally, FADD deficient mice also die at approximately day E10.5. The double knockout of FADD and RIP1 rescues the embryonic defects associated with FADD knockout. These mice, however, still die very soon post-birth. Indicating that FADD deficiency does not rescue the RIP1 phenotype (Oberst et al., 2011; Welz et al., 2011; Zhang et al., 2011). Based on these reports, it is likely that the pro-apoptotic proteins caspase-8 and FADD work to silence necroptosis during development. Additionally, it has been shown that necrosis plays a role in bone growth plate development and chondrocyte death (Roach and Clarke, 2000). Necroptosis can also serve as a backup mechanism when apoptosis is disabled. For example, interdigital cells in Apaf -/- mice die by necrosis to a similar extent to which they would normally die by apoptosis (Chautan et al., 1999).

Disease. Necroptosis seems to play a significant role in health and disease (reviewed in (Galluzzi et al., 2011) and occurs in several experimental conditions: ischemia-reperfusion injury (Degterev et al., 2005), cerulein-induced

acute pancreatitis (He et al., 2009; Zhang et al., 2009), viral infection (Cho et al., 2009a), retinal detachment-induced photoreceptor necrosis (Trichonas et al., 2010), and skin (Bonnet et al., 2011) and intestinal inflammation (Welz et al., 2011). Conceptually, necroptosis could cause a physiological problem if it occurs at either undesirably high or undesirably low rates. For example, during brain or myocardial ischemia-reperfusion injuries cells die at a high rate and inhibition of this death could produce a favorable outcome. On the other hand, in cases such as in viral infections or tumorigenesis it might be beneficial to induce necroptotic cell death (Galluzzi et al., 2011).

Necroptosis plays a key role in intestinal inflammation. Intestinal epithelial cell specific knockout of either caspase-8 or FADD leads to the spontaneous development of inflammatory lesions in the intestine. These lesions do not develop in the double knockout of RIP3 with either caspase-8 or FADD. This suggests that inhibition of necroptosis could potentially treat chronic intestinal inflammation states such as inflammatory bowel disease or Crohn's disease (Gunther et al., 2011; Kaiser et al., 2011; Welz et al., 2011).

The role of necroptosis in inflammation was recently highlighted by Duprez et. al. while investigating what factors drive lethal Systemic Inflammatory Response Syndrome (SIRS). Deletion of apoptotic executioner caspase-3 and caspase-7 or inflammatory caspase-1 did not affect lethal SIRS induced by TNFα. However, deletion of RIPK3 or inhibition of RIP1 with Nec-1 caused complete protection against lethal SIRS and reduced the amounts of circulating damage-associated molecular pattern molecules (DAMPs) (Duprez et al., 2011).

Undesirable High Rates	**Ischemia-reperfusion**	Necrostatin-mediated inhibition of RIP1K activity was shown to protect from adult brain ischemia, neonatal hypoxia-ischemia, and myocardial infarction.
	Neuro-degeneration	Typical necroptotic events are associated with neurodegenerative cell loss during stroke, AD, PD, or HD. Inhibition of oxidative stress-induced necrosis was shown to delay the manifestations of such pathologies.
Deregulated Rates	**Defective Adult tissue homeostasis**	Necrosis can function as a backup mechanism when apoptosis is blocked and thus contribute to tissue homeostasis and development. Reinstatement of healthy rates of necroptosis could in turn help to combat pathophysiological disregulation of normal cell death.
	Developmental defects	
Undesirable Low Rates	**Infection**	Studies suggest that necroptosis can act as an antiviral mechanism. Induction of necroptosis might therefore be beneficial for the treatment of certain types of viral infection.
	Tumorigenesis	As a back-up mechanism of impaired apoptosis, necroptosis potentially has similar antitumorigenic capacities. Induction of necroptosis specifically in tumor cells could assist conventional apoptosis-inducing chemotherapeutic approaches.

Figure 1.3 Pathological and therapeutic implications of necroptosis.

(Adapted from (Galluzzi et al., 2011))

Figure 1.3 Pathological and therapeutic implications of necroptosis. Review of rates of necroptosis in disease and potential pathological interventions. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD). (Adapted from (Galluzzi et al., 2011))

1.2 Akt Signaling Pathway

Portions of this section of the text are excerpts from (McNamara and Degterev, 2011): McNamara, C.R., and Degterev, A. (2011). Small-molecule inhibitors of the PI3K signaling network. Future Med Chem *3*, 549-565.

i. Introduction

Akt, or Protein Kinase B, is a serine/threonine kinase with three family members Akt1, Akt2, and Akt3. It can be activated by the PI3K signaling pathway in response to growth-factor stimulation of various receptor tyrosine kinases. This includes the EGF, FGF, PDGF, and IGF-1 receptors among many others (Stephens et al., 1993). Akt is an important signaling hub that plays a role in the regulation of many cellular processes including protein synthesis, cell metabolism, cell proliferation, and cell death and survival (Chan et al., 1999; Manning and Cantley, 2007).

ii. Phosphorylation and Activation of Akt

Activation of receptor tyrosine kinases, such as the FGFR, leads to the formation of secondary adaptor complexes and recruitment of PI3K to the plasma membrane (Figure 1.4). This results in the synthesis of PtdIns-3–4–5-P_3 (PIP_3) by PI3K from PIP_2. PIP_3 is a critical phospholipid second messenger (Rameh and Cantley, 1999). This message is relayed through proteins that contain PIP3-binding domains such as pleckstrin homology (PH) domains. PIP_3 binding to PH domains affects the localization, conformation and activity of PH domain-

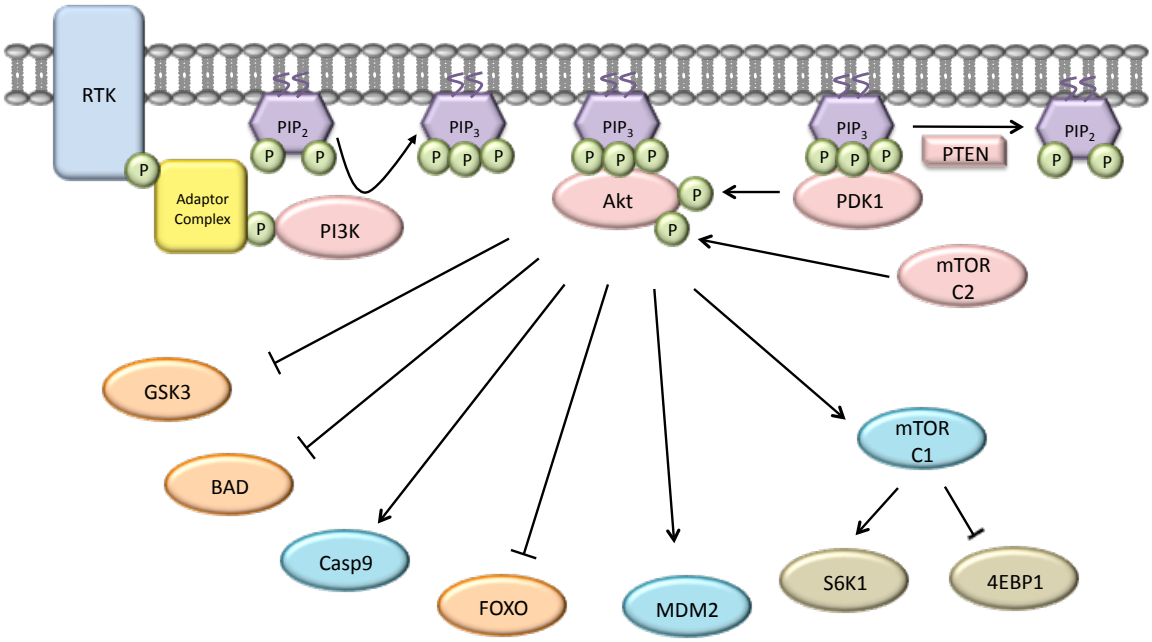

Figure 1.4 The Akt signaling pathway.

Figure 1.4 The Akt signaling pathway. RTKs, activated in response to growth factor signaling, initiate PI3K signaling. Activated PI3K phosphorylates PIP_2 to generate PIP_3. Akt and PDK1 then bind to PIP3 via their PH domains and are localized to the plasma membrane. Akt is activated by phosphorylation of Thr308 by PDK1 and Ser473 by mTORC2. Activated Akt controls cell death and survival, cell cycle regulation, regulation of protein synthesis, angiogenesis and cell metabolism through activating or inhibitory phosphorylations of many downstream substrates. Signaling is terminated when enzymes, such as PTEN, dephosphorylate PIP_3.

containing proteins and PDK1 (Kutateladze, 2010). Two of the most well known PH domain containing proteins are Akt and PDK1 (Brazil et al., 2004; Rameh and Cantley, 1999). Both of these proteins accumulate at the plasma membrane in response to PIP_3 formation. Akt is phosphorylated by PDK1 at Thr308 (Alessi et al., 1997). Akt is also phosphorylated at Ser473 by mTORC2 (Sarbassov et al., 2005) leading to fully activated Akt protein.

iii. Signaling Downstream from Akt

Akt plays a role in the regulation of many cellular processes including protein synthesis, cell metabolism, cell proliferation, and cell death and survival (Figure 1.4) (reviewed in (Manning and Cantley, 2007; McNamara and Degterev, 2011)). Akt regulates protein synthesis and cell growth through the activation of mTORC1 and the subsequent phosphorylation of ribosomal p70S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1). Similarly to mTORC2, mTORC1 contains the proteins mTOR, mLST8 and deptor. Unlike mTORC2, mTORC1 contains raptor, a scaffolding protein that allows mTOR interaction with its substrates and proline-rich Akt substrate of 40 kDa (PRAS40), a protein that inhibits mTORC1 activity. Akt activates mTORC1 through phosphorylation and inactivation of tuberous sclerosis complex (TSC) 2, which forms a heterodimer with TSC1. TSC2 is a GAP that controls the activation of the small G-protein rheb. When TSC2 is inactivated, rheb promotes mTORC1 activation. Activated mTORC1 in turn phosphorylates and activates S6K1. S6K1

substrates include many proteins involved in translational control, including the S6 ribosomal protein. Activation of mTORC1 also leads to the phosphorylation of 4EBP1 and inhibition of its binding to eukaryotic translation initiation factor (eIF) 4E, thus allowing for formation of the translation initiation complex eIF4F. The eIF4F complex is composed of eIF4E, eIF4G and eIF4A, and initiates cap-dependent translation. In addition to TSC2 phosphorylation, Akt also promotes the activity of mTORC1 by phosphorylating and inactivating PRAS40 (Foster and Fingar, 2010).

In addition to promoting cell survival and growth, Akt signaling also promotes cellular proliferation. Phosphorylation of the cyclin-dependent kinase inhibitors, $p27^{Kip1}$ and $p21^{Cip1/Waf1}$, results in their translocation from the nucleus to the cytoplasm where they are unable to inhibit cell cycle progression. In addition, GSK-3β, which phosphorylates and destabilizes several proteins involved in cell cycle progression, such as cyclin D, cyclin E and the transcription factors c-Jun and c-Myc, is inactivated by Akt-mediated phosphorylation, thus increasing the stability and levels of proteins that promote cell-cycle progression (Liang et al., 2007).

Along with cell growth and proliferation, Akt signaling regulates cellular metabolism and nutrient uptake in a variety of ways. Akt2, for example, stimulates glucose uptake in cells. Its activation leads to the translocation of glucose transporter (Glut) 4 to the plasma membrane where it can mediate the uptake of glucose. The Rab-GAP AS160, a known Akt substrate, is likely to promote Glut4 vesicle translocation. Glut1, the major glucose transporter in most

cells, is also influenced by Akt signaling. Its expression levels are controlled through HIF-1α-induced transcription downstream of mTORC1 activation. Furthermore, Akt stimulates glycogen synthesis by inhibiting GSK-3β and promotes glycolysis by increasing the expression of glycolytic enzymes. Finally, phosphorylation of BAD on Ser-136 by Akt promotes its further phosphorylation on Ser-112 and Ser-155. Tri-phosphorylated BAD nucleates the formation of a mitochondria-associated complex containing glucokinase and other proteins, which increases glucose-driven mitochondrial respiration and insulin secretion by pancreatic β cells (Danial et al., 2008).

iv. Akt and Cell Death

Akt plays a critical role in the regulation of cell death and survival. Multiple Akt substrates mediate this effect. For example, the B-cell lymphoma (Bcl)-2 homology domain (BH3)-only pro-apoptotic Bcl-2 family member, BAD associates with the anti-apoptotic proteins Bcl-2 and Bcl-XL and causes disruption of outer mitochondrial membrane integrity and cytochrome c release. Phosphorylation of BAD by Akt releases it from the Bcl-2/Bcl-XL complex and increases cell survival (Datta et al., 1997). Caspase-9, on the other hand, is an important part of the apoptosis signaling cascade and is inhibited by Akt phosphorylation, which, therefore, also promotes cell survival (Manning and Cantley, 2007). In addition, Akt activation can repress the transcription of apoptosis-related genes by phosphorylating members of the forkhead family of

transcription factors (FOXOs). Upon phosphorylation, FOXOs are sequestered in the cytosol resulting in inhibition of transcription of target genes that contribute to apoptosis, such as BIM and FasL (Van Der Heide et al., 2004). Furthermore, Mdm2 phosphorylation by Akt allows Mdm2-dependent ubiquitination and degradation of p53 (Marine and Lozano, 2010). Finally, direct phosphorylation and inhibition of glycogen synthase kinase (GSK)-3 by Akt has also been found to contribute to increased cell survival (Nair and Olanow, 2008).

v. Small-molecule Modulation of the PI3K-Akt-mTOR Signaling Network

There are many small-molecule modulators of the pathway (McNamara and Degterev, 2011). Here, I will discuss some that we used in this research. We used LY294002, the first synthetic inhibitor of PI3K, which was discovered by Eli Lilly through the analysis of several chromone-containing compounds that resembled a nonspecific PI3K inhibitor, the flavonoid quercetin (Vlahos et al., 1994). LY294002 is a useful research tool to delineate PI3K signaling in cells, but its poor pharmacological properties, such as limited stability, have precluded clinical development of this molecule (Cleary and Shapiro, 2010; Garlich et al., 2008).

PI-103 is a PI3K inhibitor that also inhibits mTOR kinase activity in mTORC1 and mTORC2 (Raynaud et al., 2007; Workman et al., 2010). Analysis of PI-103, revealed that it effectively blocks Akt activation and inhibits

proliferation of glioblastoma cells (Fan et al., 2006). This potent effect of PI-103 was linked to the ability of this molecule not only to inhibit PI3K, but also mTOR. This has led to the development of multiple classes of dual PI3K-mTOR inhibitors. PI-103 itself, despite its potent activity, is rapidly metabolized and has a low solubility.

Multiple studies have pursued the development of Akt kinase inhibitors (reviewed in (Crowell et al., 2007; Garcia-Echeverria and Sellers, 2008; Liu et al., 2009; LoPiccolo et al., 2007)). A high-throughput screen identified an Akt inhibitor, AKTi-1/2 (commercially known as Akt inhibitor VIII), which displays selectivity towards Akt1 and Akt2 relative to Akt3 (Lindsley et al., 2005). A recently published crystal structure revealed that the binding site for Akt inhibitor VIII is on the interface of the interacting PH and kinase domains, locking Akt1 into the inactive conformation in which both PIP_3 binding via the PH domain and ATP binding by the kinase domain are inhibited (Wu et al., 2010). MK-2206 (Merck) is another allosteric inhibitor that targets all three Akt isoforms (displaying ~fivefold lower activity towards Akt3) (Hirai et al., 2010). Triciribine (TCN) phosphate, the active metabolite of the clinical candidate TCN, works by binding the PH domain of Akt. This molecule was found to bind Akt PH domain with sub-micromolar affinity, and to inhibit plasma membrane translocation and Akt phosphorylation at Thr308 and Ser473, resulting in the inhibition of proliferation and induction of apoptosis (Berndt et al., 2010).

Rapamycin (Wyeth) is an mTORC1 inhibitor that interacts with FKBP12 and binds specifically to mTOR (Faivre et al., 2006). While mTORC1 primarily

acts to regulate translation through p70S6K and 4EBP1 pathways, rapamycin-resistant mTORC2 regulates survival through phosphorylation of Akt on Ser473 and other AGC kinases. Since mTORC1 mediates only a subset of mTOR-dependent functions and because even some of the mTORC1-dependent pathways, for example 4EBP1 phosphorylation, are relatively resistant to rapamycin, significant effort has been made to target the kinase activity of mTOR (Feldman et al., 2009; Thoreen et al., 2009). Small molecules that inhibit the kinase activity of mTOR present in both mTORC1 and mTORC2 are expected to be better inhibitors of the PI3K signaling pathway. A low-nanomolar ATP-competitive mTOR kinase inhibitor, Torin1, has recently been described and was shown to efficiently inhibit both mTOR complexes, resulting in promising activity in U87MG glioblastoma xenografts (Liu et al., 2010).

1.3 Significance and Aims

Necroptosis is a relatively new mechanism of cell death and the full extent of its roles in normal physiology and disease state are not well understood. A recent genome wide siRNA screen for mediators of necroptosis induced by the pan-caspase inhibitor zVAD.fmk in mouse fibrosarcoma L929 cells, revealed a broad and diverse cellular network of 432 genes that may regulate this process (Hitomi et al., 2008). Approximately 32 of these genes acted as regulators or downstream if RIP1 kinase and 7 additional genes that are required for both apoptosis and necroptosis. These data provided important confirmation of the

highly regulated nature of necroptosis and revealed the first insight into the full repertoire of mediators of this form of cell death. A more complete understanding of the molecular mechanisms involved in necroptosis is needed.

In this work, we will examine the hypothesis that Akt is vital component of signal transduction in necroptosis. A clear understanding of the molecular pathway of necroptosis will give a better understanding of when necroptosis might be modulated and how we might be able to do that. The goal of this work is to characterize the role of Akt kinase in necroptosis by gaining an understanding of how it becomes activated in response to pro-necrotic stimuli and elucidating the downstream consequences of its activation in the process of cell death. We also seek to identify novel Akt interacting proteins and to characterize the role that these proteins play in necroptosis.

Chapter II: Materials and Methods

Antibodies

The following antibodies were used: phospho-Akt (Thr308) (clone C31E5E) rabbit mAb, phospho-Akt (Ser473) (clone D9E) XP rabbit mAb, Akt (pan) (clone C67E7) rabbit mAb, Akt1 (clone C73H10) rabbit mAb, Akt2 (clone D6G4) rabbit mAb, Akt3 (clone 62A8) rabbit mAb, phospho-JNK (Thr183/Tyr185) (81E11) rabbit mAb, SAPK/JNK rabbit pAb, phospho-c-Jun (Ser63) II rabbit pAb, c-Jun (60A8) rabbit mAb, α-tubulin (clone DM1A) mouse mAb, phospho-FoxO1 (Thr24)/FoxO3a (Thr32) rabbit pAb, FoxO1 (L27) rabbit pAb, phospho-FoxO4 (Ser193) rabbit pAb, FoxO4 rabbit pAb, phospho-MDM2 (Ser166) rabbit pAb, phospho-GSK-3α/β (Ser21/9) rabbit pAb, phospho-p70 S6 Kinase (Thr389) (clone 108D2) rabbit mAb, phospho-S6 Ribosomal Protein (Ser235/236) (clone D57.2.2E) XP rabbit mAb, S6 Ribosomal Protein (clone 54D2) mouse mAb, phospho-4E-BP1 (Thr37/46) rabbit pAb, mTOR (clone 7C10) rabbit mAb, PDK1 rabbit pAb, GAPDH (clone D16H11) rabbit mAB, phospho-Nur77 (Ser351) (D22G5) rabbit mAB (all Cell Signaling), MDM2 rabbit pAb (Bioworlde) GFP rabbit pAB (Sigma), GAPDH rabbit pAB (Abcam), GAPDH mouse pAb (Genetex), Nur77 (ab13851) (Abcam), Nur77 (Santa cruz), Arf1 (Pierce) .

Reagents and Chemicals

Necrostatin analogs were synthesized as previously described. The following reagents and final concentrations (unless otherwise specified in the

text/figures) were used in the experiments: Akt inhibitor VIII (10 μM, Calbiochem), MK-2206 (10 μM, Selleck Chem), Triciribine (100 μM, National Cancer Institute), SP600125 (10 μM, Calbiochem), JNK inhibitor VIII (10 μM, Calbiochem), UO126 (10 mM, Cayman Chem), PD169316 (10 μM, Calbiochem), LiCl (10 mM, Sigma), SB216763 (10 μM, Calbiochem), BX912 (10 μM, Axon Med Chem), PF-4706871 (Sigma), rapamycin (100nM, Santa Cruz), PI-103 (10 μM, Calbiochem), Torin-1 (500 nM, gift of Dr. Nathanael Grey (Harvard Medical School), LY249002 (10 μM, Cell Signaling), PD173074 (2 μM, Cayman Chem), PD166866 (20 μM, Calbiochem), 4EGI-1 (50 μM, Calbiochem). Pan-caspase inhibitor zVAD.fmk (20-30 μM) was purchased from Bachem. Human and mouse TNFα (10 ng/ml), human bFGF (25 ng/ml), EGF (50 ng/ml), PDGF-BB (20 ng/ml), and IGF-1 (50 ng/ml) were from Cell Sciences or Peprotech. All other reagents were from Sigma.

DNA

Cloning of Myr-Akt1, containing C-terminal FLAG tag, has been described (Miao et al., 2010). Myr-Akt1-FLAG was amplified by PCR and subcloned into the BglII and EcoRI sites of pMSCV-puro retroviral vector (Invitrogen). Mutant versions of Myr-Akt1 were generated using the same strategy. hGAPDH clone was purchased from the DNASU plasmid repository. hGAPDH was FLAG tagged using the forward primer 5' - CCAAGATCTGGATCCGCCACCATGGACTACAAG

GACGACGACGACAAGGGCGGCATGGGGAAGGTGAAGGTC -3' and the reverse primer 5'- CCAGAATTCCTCGAGTTACTCCTTGGAGGCCAT-3'. GFP tagged hGAPDH used the reverse primer 5' - CCAGAATTCGCTCCTTGGAG GCCAT -3' to move hGAPDH into peGFP-N1 (clonetech).

Cell lines

L929 and FADD-deficient Jurkat cells were obtained from ATCC. Lung fibroblasts were a generous gift of Dr. Philip Tsichlis (Tufts University). J774A.1 (ATCC) cells and RAW264.7 (ATCC) cells were generous gifts of Dr. Junying Yuan (Harvard University) and Alexander Poltorak (Tufts University), respectively. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture (Invitrogen). The mouse lung fibroblast media was additionally supplemented with L-glutamine, non-essential amino acids, and sodium pyruvate. Jurkat cells were maintained in RPMI1640, supplemented with 10% FetalPlex (Gemini) and 1% antibiotic-antimycotic.

Stable infection of Myr-Akt1, Arf1 shRNA, and Nur77 shRNA constructs

To generate MSCV retroviruses, HEK293FT cells (Invitrogen) were transfected with 2 μg of viral DNA and 1 μg of gal/pol and VSV-G accessory plasmids in 6 well plates using GenJet transfection reagent (Signagen Labs). To generate pLKO lentivirus, HEK293FT cells were transfected with 2ug DNA, LP1, LP2, and LP3 accessory plasmids. Virus-containing media was collected 72 hr later, filtered through 0.45 μm filter and applied to L929 cells with 8 μg/ml polybrene.

Cells were infected three times before selection and maintenance in 10 μg/ml puromycin.

Cell viability experiments

Cells were seeded into white clear bottom 96 well plates at the density of $1X10^4$ cells/well and treated as described for western blot experiments. Cell viability was determined using CellTiter-Glo Cell Viability Assay (Promega). Experiments were performed in duplicate or triplicate. Viability of the control untreated cells was set as 100%. Relative viability of cells, induced to undergo necroptosis and treated with the compound relative to the control compound-treated cells, was determined and plotted to exclude the possible effects of non-specific toxicity of the small molecules.

QPCR primers

mouse TNFα:	forward 5'-CCCTCACACTCAGATCATCTTCT-3'
	reverse 5'-GCTACGACGTGGGCTACAG-3'
mouse 18S:	forward 5-' ATAACAGGTCTGTGATGCCCTTAG-3
	reverse 5'-CTAAACCATCCAATCGGTAGTAGC-3'
human TNFα:	forward 5'- ATGAGCACTGAAAGCATGATCC-3'
	reverse 5'-GAGGGCTGATTAGAGAGAGGTC-3'
human 18S:	forward 5'- CAGCCACCCGAGATTGAGCA -3
	reverse 5'-TAGTAGCGACGGGCGGTGTG-3'
mouse GAPDH:	forward 5'- AAGGTGAAGGTCGGAGTCAAC-3'

reverse 5'- GGGGTCATTGATGGCAACAATA-3'

human GAPDH: forward 5'- AAGGTGAAGGTCGGAGTCAAC-3'

reverse 5'- GGGGTCATTGATGGCAACAAT-'3

mouse Nur77: forward 5'- TTGAGTTCGGCAAGCCTACC -3'

reverse 5'- GTGTACCCGTCCATGAAGGTG -3'

mouse Arf1: forward 5'- TGGGCGAAATTGTGACCACC -3'

reverse 5'- TCCACTACGAAGATCAAGCCT -3'

qRT-PCR

Cells were treated as described for Western blots. Total RNA was isolated using ZR Miniprep kit (Zymo Research). 1 μg of RNA was converted to cDNA using random primers (M-MuLV cDNA kit, New England Biolabs). 1 μL of cDNA was used with 500 pM primers in qPCR reactions. Reactions were performs using SYBRGreen 2XMaster mix (SABiosciences) in a LightCycler480 (Roche).

siRNA knockdown

siRNAs were purchased from Dharmacon. Mouse ribosomal S6 protein (L-040893-00 and L-045791-00), mouse Akt1 (L-040709-00), mouse Akt2 (L-040782-00), mouse Akt3 (L-040891-00), mouse mTOR (L-065427-00), mouse PDK1 (L-040658-00), non-coding control (D-001810-10-05), mouse Mapk8 (J-040128-05), mouse Mapk9 (J-040134-05), mouse Jun (L-043776-00), mouse GAPDH (L-040917-00). siRNA were transfected using RNAiMAX reagent (Invitrogen), according to the manufacturer's recommendations. After 72 hr, cells

were treated with zVAD.fmk or TNFα for 9 hr (RNA or Western blot) or 24 hr (cell viability).

shRNA knockdown

MISSION shRNA lentiviral plasmid (pLKO.1-puro) bacterial stock panels were ordered from Sigma for mouse Nur77 (SHCLND-NM_010444) and Arf1 (SHCLNG-NM_007477).

Western blot

For Western blot, $4X10^5$ adherent cells ($1X10^6$ Jurkat cells) were seeded into 35 mm^2 dishes. After 24-48 hr, cells were stimulated with 30 μM zVAD.fmk or 10 ng/ml mouse TNFα. For treatments under serum free conditions, cells were serum starved for 24 hr prior to the addition of growth factors, 20 μM zVAD.fmk or 10 ng/ml mouse TNFα. Cells were harvested in 1XRIPA buffer (Cell Signaling) supplemented with 50 μg/ml phenylmethanesulfonylfluoride. After brief sonication, cell lysates were spun down for 15 min at 14,000Xrpm. Protein concentrations were measured using the Pierce 660nm Assay Reagent (Pierce). Equal amounts of proteins were boiled for 5 min at 95^0C. Western blotting was performed according to standard protocols. Briefly, SDS-PAGE gels were transferred to PVDF membrane, blocked in 3% milk or 5% bovine serum albumin (BSA) in Tris-buffered Saline – Tween 20 (TBS-T) buffer for 30 min at room temperature. Primary antibodies were incubated in 5%BSA/TBS-T overnight at 4^0C. Secondary antibodies were incubated in TBS-T for 30 min at room

temperature. Luminata (Millipore) ECL reagents were used to develop the signals. In some cases, membranes were stripped using OneMinute stripping buffer (GM Biosciences) and reprobed with new antibodies.

ELISAOne assay

ELISAOne assays (TGRBio, Hindmarsh, Australia) were performed according to manufacturer's protocol with the following modifications. Cell lysates were prepared in RIPA buffer as described for Western blots. Five microliters of samples were diluted in 45 μL of ELISAOne lysis buffer prior to analysis. Primary antibodies to phospho-Thr308 and phospho-Ser473 were incubated with the samples for 2 hr at room temperature. Primary antibody to pan-Akt was incubated overnight at 4^0C. Signals for phospho-antibodies were normalized based on pan-Akt values.

TNFα ELISA

Mouse TNFα Quantikine ELISA assays (R&D Systems) were performed according to manufacturer's descriptions. Cell lysates were prepared from $3X10^6$ cells plated and treated in a $10cm^2$ dish.

In vitro Akt kinase assay

Akt kinase activity was measured using the Akt kinase assay kit (nonradioactive) from Cell Signaling Technology. In brief, Myr-Akt was immunoprecipitated from L929 cells using anti-FLAG M2 magnetic beads (Sigma). The *in vitro* assay was

performed in the presence of a GSK fusion protein substrate. Phosphorylation of the GSK fusion protein was visualized by western blot.

Arf1 Activity Assay

The Active Arf1 Pull-Down and Detection Kit (Pierce) was used according to the manufacturer's instructions. In short, L929 cells treated for 9hrs with zVAD.fmk were lysed in the kit buffer. GST-GGA3-PBD bound to glutathione resin was used to precipitate active Arf1 from the lysates. The amount of active Arf1 was detected by western blot.

Gel Filtration

L929 cells treated with zVAD.fmk for 9hrs were lysed in buffering containing 150mM NaCl and 1M Tris pH 7.4 and (.22um) filtered. The samples were run on an AktaPurifier and separated using a Superdex200 10/300 GL column. Fractions were collected and the presence of GAPDH in each fraction was analyzed by western blot.

Native PAGE

The NativePAGE Novex Bis-Tris Gel System (Invitrogen) was used. L929 cells treated with zVAD.fmk for 9hrs were lysed in buffering containing 150mM NaCl and 1M Tris pH 7.4. Samples were prepared in 4X NativePAGE Sample buffer with NativePAGE 5% G-250 Sample Additive. The samples were loaded onto a

NativePAGE Novex Bis-Tris gel and subjected to electrophoresis. GAPDH was detected by western blot.

Chapter III: Akt is Activated in the L929 Mouse Fibrosarcoma Cell Line During Necroptosis

The data from this chapter are published in (McNamara et al., 2013):
McNamara, C.R., Ahuja, R., Osafo-Addo, A.D., Barrows, D., Kettenbach, A., Skidan, I., Teng, X., Cuny, G.D., Gerber, S., and Degterev, A. (2013). Akt Regulates TNFalpha synthesis downstream of RIP1 kinase activation during necroptosis. PLoS One 8, e56576.

3.1 Introduction

In this chapter we investigated the importance of Akt activation in necroptosis. Akt activation was recently linked to necroptosis in L929 cells (Wu et al., 2009). In this paper Akt activation by insulin was suggested to promote insulin-dependent activation of necroptosis by suppressing autophagy. However, several labs have reported that autophagy acts to promote zVAD.fmk induced necroptosis not to suppress it (Chen et al., 2011; Degterev et al., 2005; Yu et al., 2004). This raised the possibility that Akt controls more general mechanisms in the execution of necroptosis and raised the question of whether or not Akt is an intrinsic component of RIP1 signaling.

In this study, we found a serum dependence for cell death in L929 cells and identify growth factors, in particular bFGF and IGF, as the particular serum factors that allow zVAD.fmk dependent necroptosis to occur. Growth factors are known to activate receptor tyrosine kinases that lead to activation of the PI3K-Akt signaling pathway. Our data show that Akt is activated in a RIP1 dependent fashion during necroptosis by phosphorylation specifically at its Thr308 site. This site is phosphorylated by PDK1, and inhibition and knockdown of PDK1

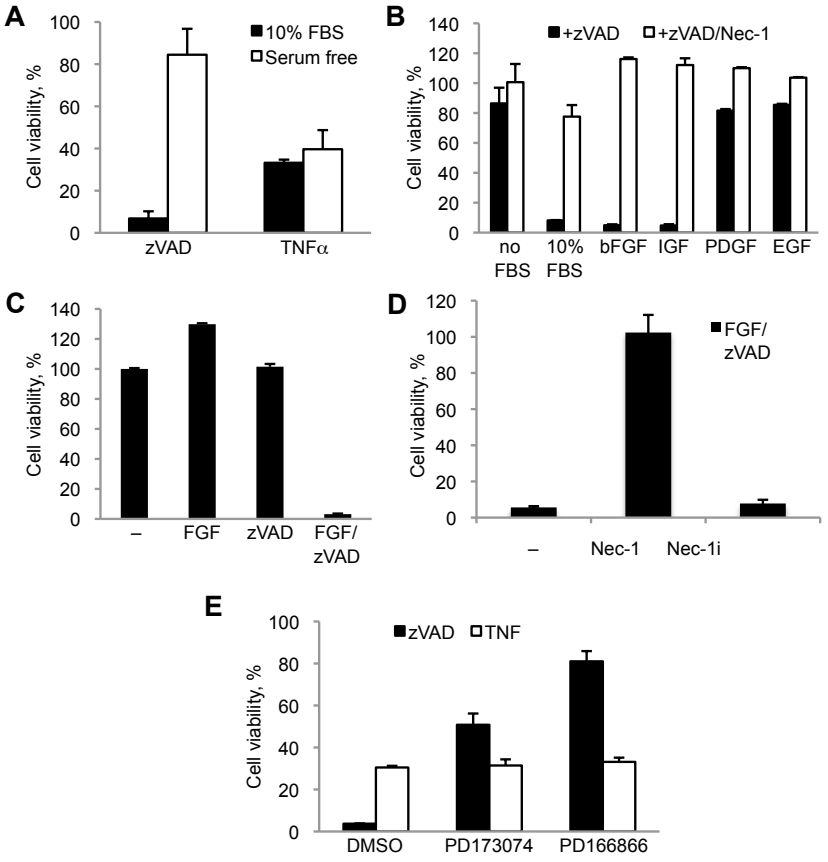

Figure 3.1 bFGF and IGF-1 promote necroptosis in concert with zVAD.fmk.

Figure 3.1 bFGF and IGF-1 promote necroptosis in concert with zVAD.fmk. (A) L929 cells were treated with TNFα or zVAD.fmk under normal serum (10% FBS) or serum free conditions. Cell viability was determined after 24 hr using the CellTiter-Glo Viability assay. The concentrations of all necroptosis-inducing agents are listed in the *Materials and Methods* section or indicated in the figures. (B) Cells were treated with zVAD.fmk, the indicated growth factors, and Nec-1 under serum free conditions for 24 hrs followed by measurement of cell viability. (C) Cells under serum free conditions were treated with FGF, zVAD.fmk, or both for 24hrs followed by viability assay. (D) Cells under serum free conditions were treated with FGF and zVAD.fmk with either Nec-1 or Nec-1i for 24hrs followed by viability assay. (E) Cell death was induced by zVAD.fmk or TNFα under full serum condition in the presence of 2 μM PD173074 and 20 μM PD166866. In all graphs, average±SD was plotted.

protect these cells from death indicating that the preferential Thr308 phosphorylation of Akt during necroptosis is PDK1 dependent. It is important to note that two inputs were required for Akt activation during necroptosis. First an input from growth factors was required. This requirement could be overcome by the expression of a constitutively active Akt construct, Myr-Akt, which was active even in the absence of serum. The second required input was RIP1 kinase dependent. Inhibition of RIP1 kinase activity completely prevents Akt phosphorylation and cell death from occurring. Overall, these data suggest that active, Thr308 phosphorylated Akt is generated during necroptosis in a RIP1 kinase dependent manner.

3.2 Results

Basic Fibroblast Growth Factor Promotes Necroptosis in L929 Cells. We used an L929 fibrosarcoma cellular model of necroptosis. It is well established that these cells die by necroptosis when treated either with TNFα or zVAD.fmk (Degterev et al., 2005; Hitomi et al., 2008; Yu et al., 2004). We compared cell viability in cells treated in regular 10% Fetal Bovine Serum (FBS) containing media or media that was serum free and found that serum starvation protected the zVAD.fmk treated cells but not the TNFα treated cells from death indicating that something present in the FBS was necessary for cell death to occur (Figure 3.1 A). This is consistent with previous reports that serum starvation protects L929 cells from necroptosis (Wu et al., 2009). FBS is a highly

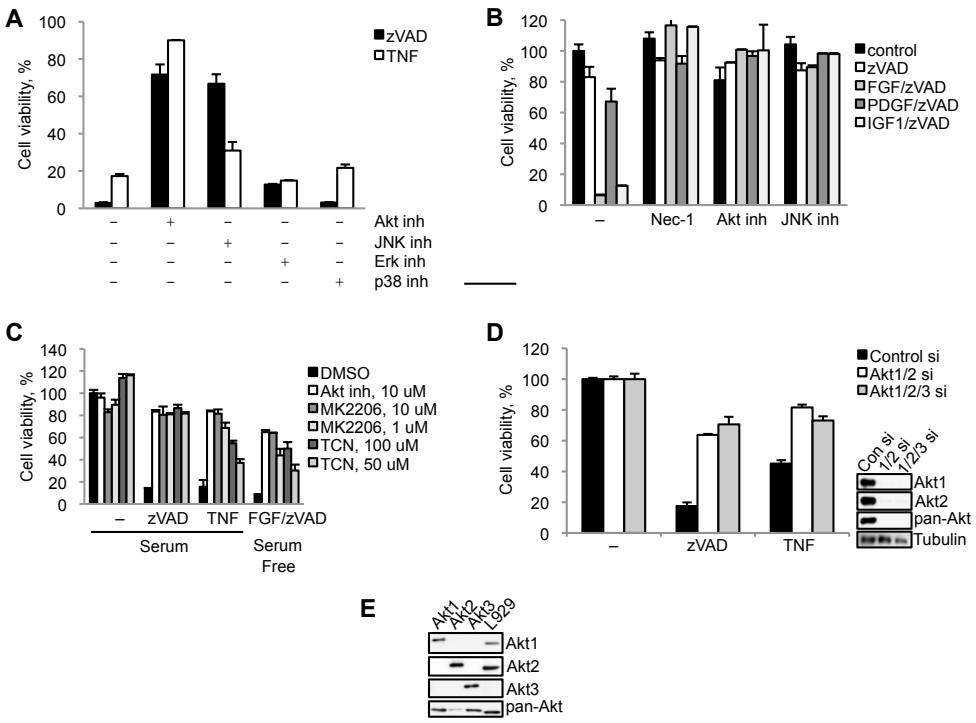

Figure 3.2 Akt contributes to necroptosis induced by zVAD.fmk and TNFα.

Figure 3.2 Akt contributes to necroptosis induced by zVAD.fmk and TNFα. (A,B) Necroptosis was induced by zVAD.fmk or TNFα (full serum, A) or growth factors/zVAD.fmk (serum free, B) in the presence of inhibitors of Akt (Akt inhibitor VIII), JNK (SP600125), p38 (PD169316), and Erk (UO126). Cell viability was determined after 24hrs. (C) L929 cells were treated with zVAD.fmk or TNFα or bFGF/zVAD.fmk (serum free conditions) in the presence of the Akt inhibitors (Akt inhibitor VIII 10 μM, MK2206 10 μM or Triceribine (TCN) 100 μM) and cell viability was measured 24 hrs post-treatment. (D) L929 cells transfected with Akt1, Akt2, and Akt3 siRNAs for 72 hrs were treated with zVAD.fmk or TNFα for 9hrs. Cell viability and Akt expression levels were determined after 24hrs. (E) Mouse lung fibroblasts expressing either Akt1, Akt2, or Akt3 and L929 lysates were harvested and western blotted using the indicated antibodies. In all graphs, average±SD was plotted

complex mixture of growth factors, vitamins, and many other nutrients. We evaluated a panel of growth factors including basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF), and Platelet-derived Growth Factor (PDGF) to determine whether each could rescue the effect of serum starvation in zVAD.fmk treated L929 cells (Figure 3.1 B). It is important to note that some of the growth factors, bFGF and IGF, but not others such as PDGF and EGF were able to facilitate cell death in the presence of zVAD.fmk under serum starved conditions indicating that this was not a generic requirement for growth factor signaling. It is important to note that under serum free conditions neither bFGF nor zVAD.fmk alone was sufficient to induce necroptosis but that both FGF and zVAD.fmk were necessary to induce death (Figure 3.1 C). Additionally, this FGF/zVAD.fmk induced death was inhibited by Nec-1 but not its inactive analogue Nec-1i (Figure 3.1 D), indicating that this death is RIP1 kinase dependent. Two FGF receptor 1 (FGFR1) tyrosine kinase antagonists, PD173074 (Mohammadi et al., 1998) and PD166866 (Panek et al., 1998), protected L929 cells from zVAD.fmk induced cell death under normal serum conditions (Figure 3.1 E). It is important to note that serum starvation did not protect L929 cells from TNFα induced necroptosis (Figure 3.1 A). Overall these data indicate the bFGF contributes to zVAD.fmk induce necroptosis in L929 cells and this represents a specific signaling event since all growth factors are not able to mediate death in this way.

RIP1 Kinase-dependent Activation of Akt Contributes to Necroptosis. We next determined whether pathways downstream of FGFR activation contribute to

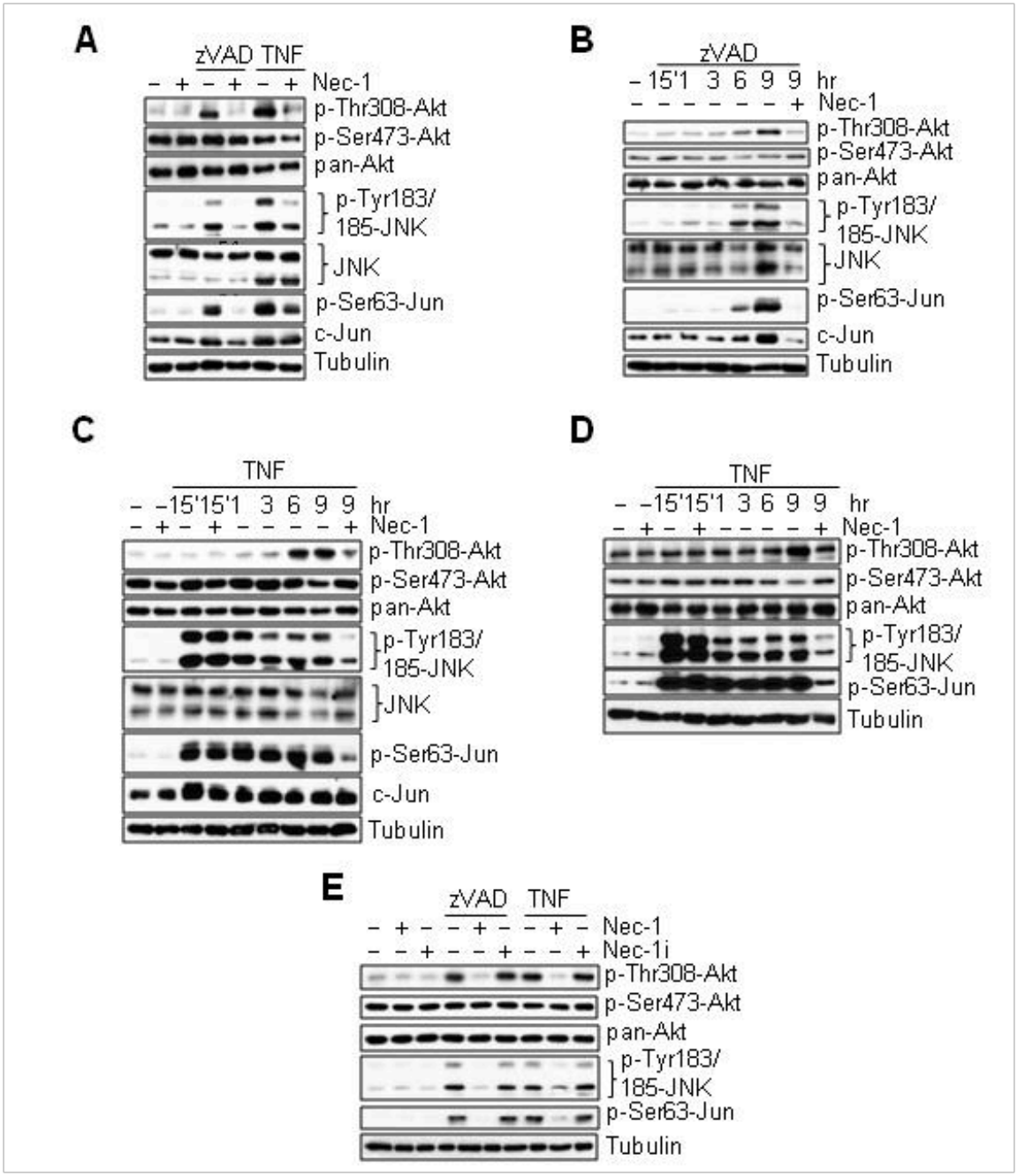

Figure 3.3 RIP1 kinase-dependent phosphorylation of Akt and JNK during necroptosis.

Figure 3.3 RIP1 kinase-dependent phosphorylation of Akt and JNK during necroptosis. (A) L929 cells were treated with zVAD.fmk or TNFα (serum) for 9 hr, followed by western blotting with indicated antibodies. (B) L929 cells were treated with zVAD.fmk (serum) and samples were collected at the indicated time points for western blot. (C-D) Cells were treated with TNFα under normal serum (C) or serum free conditions (D) and samples were collected at the indicated time points for western blot. (E) Nec-1 or Nec-1i was added to the cells stimulated with zVAD.fmk or TNFα for 9 hr followed by western blot with the indicated antibodies.

necroptosis in these cells. We chose to inhibit Akt, (Akt inhibitor VIII), JNK (SP600125), p38 (PD169316), and ERK (UO126) in order to examine whether these downstream kinases inhibit zVAD.fmk or TNFα induced necroptosis. Both inhibition of JNK and Akt protected the cells from death (Figure 3.2 A) (Wu et al., 2009). Inhibition of Akt and JNK also protected L929 cells from necroptosis under serum free conditions in cells treated with the growth factors FGF and IGF in the presence of zVAD.fmk (Figure 3.2 B). Protection by the JNK inhibitor SP600125 is consistent with previous reports (Kim et al., 2007; Yu et al., 2004). We used a combination of two Akt inhibitors in addition to Akt inhibitor VIII and siRNA knockdown in order to verify these results. Cells were treated either under serum conditions in the presence of zVAD.fmk or under serum free conditions in the presence of bFGF plus zVAD.fmk with MK2206 (Hirai et al., 2010) or Triceribine (TCN) (Figure 3.2 C) (Berndt et al., 2010). The cells were protected from death by each Akt inhibitor. We additionally used siRNAs against Akt to verify these results (Figure 3.2 D). Knockdown of Akt isoforms 1 and 2 significantly protected L929 cells from both zVAD.fmk and TNFα induced necroptosis. Additional siRNA against the Akt3 isoform did not change the viability data results. This is most likely because L929 cell do not significantly express the AKT3 isoform (Figure 3.2 E).

We next investigated the activation state of Akt during cell death given that inhibition or knockdown of Akt kinase protected L929 cells from necroptosis induced by multiple stimuli including zVAD.fmk (serum), TNFα (serum), and bFGF/zVAD.fmk under serum free conditions. Akt has two phosphorylation sites

that are indicative of its activation, Thr308 that is phosphorylated by PDK1 and Ser473 that is phosphorylated by mTORC2. As seen in Figure 3.3 A, Akt is phosphorylated on Thr308 in response to stimulation by either zVAD.fmk or TNFα in a RIP1 kinase dependent fashion. It is important to note that the JNK and c-Jun phosphorylation is present in this system as previously reported. It is also important to note changes in Ser473 phosphorylation were not obvious. The phosphorylation of Thr308 occurred in a time dependent fashion (Figure 3.3 B). There was a slow increase in phosphorylation that correlated with the increase in cell death induced by zVAD.fmk. The increased Thr308 phosphorylation was also seen in cells treated with TNFα regardless of whether they were growing under serum (Figure 3.3 C) or serum free (Figure 3.3 D) conditions. This, in concert with the data from Figure 3.1 A where cells treated with TNFα were not protected from death indicate that while death induced by zVAD.fmk seems to be growth factor dependent and TNFα was not, signifies that Akt activation in TNFα treated cells is also growth factor independent. Phosphorylation of JNK and Akt at Thr308 was RIP1 kinase activity dependent in the case of treatment with either zVAD.fmk or TNFα as treatment with Nec-1 inhibited the phosphorylations while treatment with Nec-1i, the inactive analogue that does not protect from cell death, did not inhibit phosphorylation (Figure 3.3 E). Overall, these data indicate that there is an increase in Akt phosphorylation on Thr308, but not Ser473, during zVAD.fmk and TNFα induced necroptosis in L929 cells.

Serum starvation of L929 cells and treatment with bFGF with or without zVAD.fmk allowed us to investigate the nature of this phosphorylation at Thr308

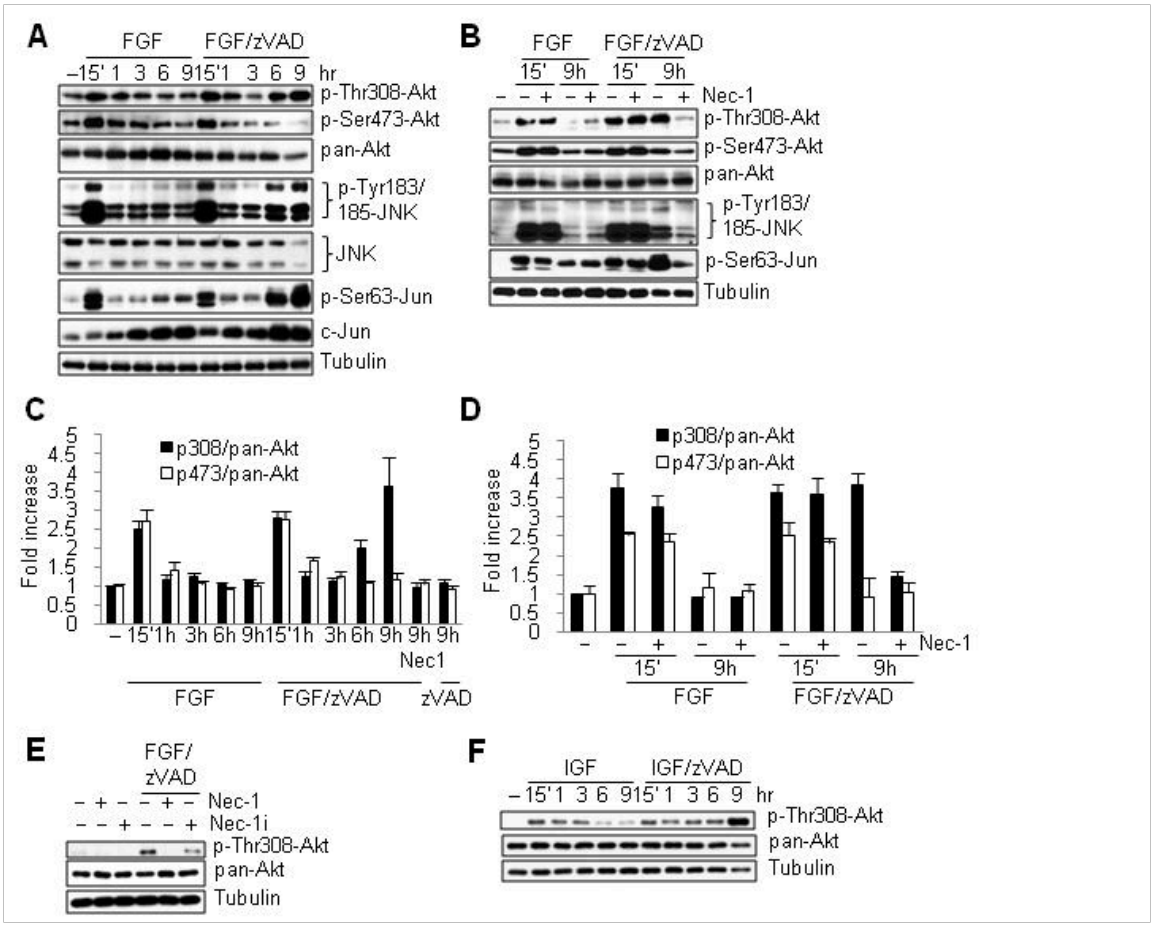

Figure 3.4 Phosphorylation of Akt during necroptosis requires both growth factors and zVAD.fmk.

Figure 3.4 Phosphorylation of Akt during necroptosis requires both growth factors and zVAD.fmk. (A-D) Serum starved L929 cells were stimulated with bFGF and/or zVAD.fmk and Nec-1 (N1) for the indicated periods of time. Samples were analyzed by western blot (A,B) or by using phospho-Thr308, phospho-Ser473 and pan-Akt ELISAOne assays (C,D). Phospho-signals were normalized to pan-Akt. Fold induction over control cells is plotted. (E) Serum starved cells were treated with bFGF/zVAD.fmk (9hrs) in the presence of Nec-1 or Nec-1i and collected for western blot. (F) Serum starved cells were treated with IGF/zVAD.fmk and collected at the indicated time points for western blot In all graphs, average±SD was plotted.

even further. Treatment of serum starved cells with bFGF alone caused a transient increase in Akt phosphorylation at both Thr308 and Ser473 and JNK phosphorylation as expected (Figure 3.4 A-D) at approximately 15 minutes post treatment. In the bFGF alone treatment, phosphorylation of these sites gradually decreased over the course of 9 hours. This initial transient increase in Akt and JNK phosphorylation was also seen when the cells were treated with both bFGF and zVAD.fmk. However, there was an additional delayed increase (between 6-9 hours post-induction) in the Thr308 Akt phosphorylation and in JNK phosphorylation when cells were treated with bFGF/zVAD.fmk. It is important to note that only the delayed Akt and JNK phosphorylation caused by bFGF/zVAD and not the early transient increase by bFGF alone or bFGF/zVAD.fmk was RIP1 kinase dependent (Figure 3.4 B,D). The kinetics of the Akt phosphorylation changes were confirmed using a quantitative ELISAOne assay (Figure 3.4 C,D). The early increases in both bFGF and bFGF/zVAD.fmk induced Akt and JNK phosphorylation were not effected by the addition of Nec-1. RIP1 kinase inhibition by Nec-1 did, however, inhibit the delayed Thr308 Akt and JNK phosphorylation in the bFGF/zVAD.fmk treated cells. Nec-1i, on the other hand, was unable to inhibit these phosphorylations. This Thr308 specific phosphorylation of Akt was also seen in response to other growth factors that can induce necroptosis in L929 cells. Serum starved L929 cells treated with either IGF or IGF/zVAD showed a similar time course of Thr308 phosphorylation with an early transient increase under both treatment conditions followed by a delayed increase in only the IGF/zVAD.fmk treated samples (Figure 3.4 F).

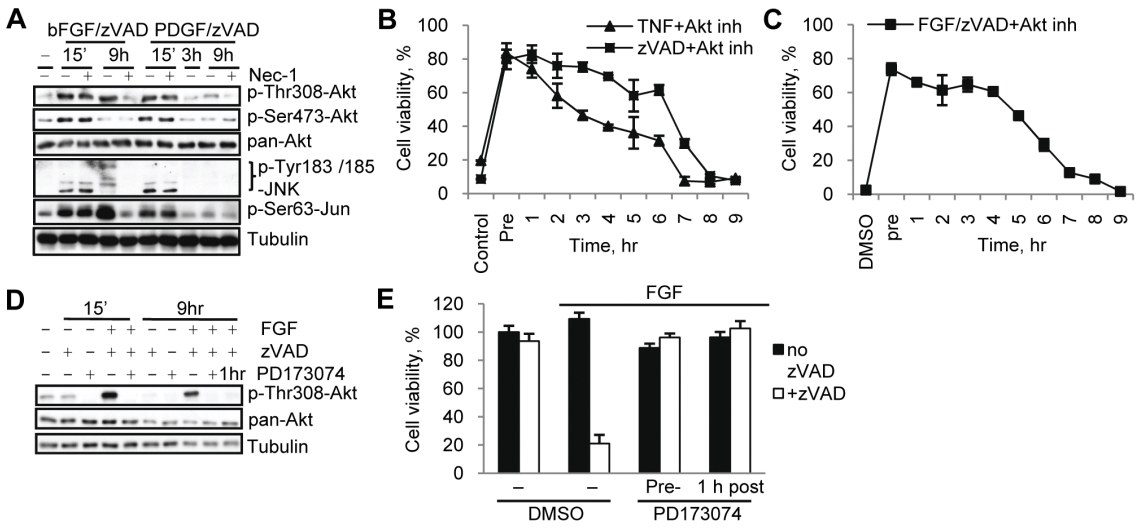

Figure 3.5 Late Thr308 phosphorylation of Akt contributes to necroptosis.

Figure 3.5 Late Thr308 phosphorylation of Akt contributes to necroptosis. (A) L929 cells were treated with zVAD.fmk and bFGF or PDGF, with or without Nec-1, for the indicated periods of time. (B, C) L929 cells were stimulated by zVAD.fmk or TNFα (B) or bFGF/zVAD.fmk under serum free conditions (C). Akt inh. VIII was added 15 min before necroptotic stimulation (Pre) or at the indicated times after stimulation. Viability was measured 24 hr after activation of necroptosis. (D) L929 cells were stimulated with bFGF/zVAD under serum free conditions. PD173074 was added 15 min before or 1 hr after FGF/zVAD. Samples for western blot were collected at 15 min and 9hr time points. (E) Cells were pretreated with PD173074 or it was added 1 hr after bFGF/zVAD.fmk, followed by viability assessment at 24 hr. In all graphs, average±SD was plotted.

Late Increase in Akt Thr308 Phosphorylation Contributes to the Induction of Necroptotic Cell Death. In contrast to the multiple growth factors that help facilitate cell death under serum free conditions (bFGF and IGF) (Figure 3.2), PDGF, which does not facilitate death (Figure 3.1 B), does not lead to a delayed increase in Thr308 phosphorylation. However, the early transient increase in phosphorylation is still intact (Figure 3.5 A) indicating the PDGFR signaling does occur in L929 cells. Additionally, we noted that addition of an Akt inhibitor (Akt inhibitor VIII) after the early, transient increase in Thr308 phosphorylation has already occurred but before the late increase in Thr308 phosphorylation begins protects L929 cells from death induced by both zVAD.fmk or TNFα (serum) and bFGF/zVAD.fmk (serum free) induced necroptosis (Figure 3.5 B, C). Moreover, pre-treatment of serum starved cells with the FGFR1 inhibitor PD173074 before bFGF/zVAD.fmk treatment protected the cells from death. Cells treated with PD173074 one hour post bFGF/zVAD.fmk treatment when the early transient Akt phosphorylation has already subsided also protects the cells from death (Figure 3.5 D, E). Taken together, these data indicate the importance of the late increase in Thr308 phosphorylation on Akt in the induction of necroptosis.

PI3-kinase and PDK1 Mediate the Increase in Akt Thr308 Phosphorylation Under Necroptotic Conditions. We used a combination of small-molecule inhibitors and transient siRNA mediated knockdown to investigate what factors mediate this delayed increase in Thr308 phosphorylation. Typical Akt regulation involves recruitment of Akt to the plasma membrane, which is mediated by the PH domain of Akt and PIP_3 generated by PI3K. Akt is then phosphorylated by

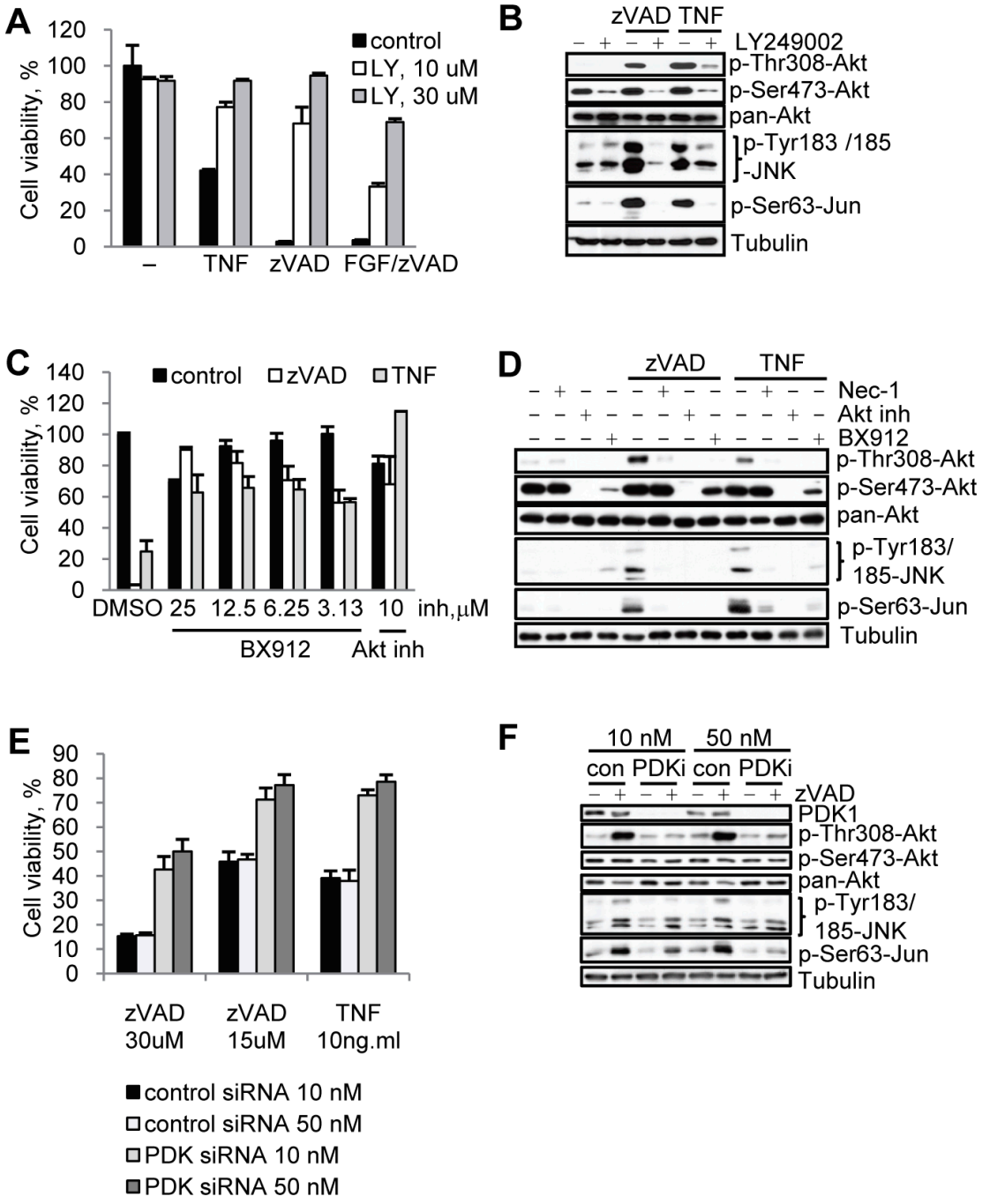

Figure 3.6 PI3-Kinase and PDK1 mediate the increase in Akt Thr308 phosphorylation under necroptotic conditions.

Figure 3.6 PI3-Kinase and PDK1 mediate the increase in Akt Thr308 phosphorylation under necroptotic conditions. (A-D) L929 cells were stimulated by zVAD.fmk and TNFα in the presence of LY249002 (A,B) or BX912 (C,D). Viability assays were performed after 24 hr (A,C). Western blot samples were collected after 9 hr. (B,D). (E,F) L929 cells were transfected with PDK1 siRNAs (PDKi). After 72 hr, necroptosis was induced by TNFα or zVAD.fmk. Viability assays were performed after 24 hr (E). Western blot samples were collected after 9 hr (F). In all graphs, average±SD was plotted.

PDK1 at Thr308 and mTORC2 at Ser473. First, inhibition of PI3K with the inhibitor LY249002 (Vlahos et al., 1994) protected cells from death (Figure 3.6 A) and inhibited phosphorylation of Akt (Figure 3.6 B). Likewise, inhibition of PDK1 with BX-912 (Feldman et al., 2005) (Figure 3.6 C, D) or siRNA knockdown (Figure 3.6 E, F) of PDK1 protected L929 cells from death (Figure 3.6 C, E) and inhibited Thr308 phosphorylation on Akt (Figure 3.5 D, F) indicating that PI3K and PDK1 activity are required for Akt activation during necroptosis.

Expression of Constitutively Active Akt Rescues Necroptosis Under Serum Free Conditions. We used a constitutively active Akt construct in order to further evaluate the role of Akt in L929 cell necroptosis. This construct "Myr-Akt" is a FLAG tagged Akt that has had its PH domain removed and had a myristoylation site added which provides localization to the plasma membrane (Figure 3.7 A) (Kohn et al., 1996). Myr-Akt and the catalytically inactive Myr-Akt K179M mutants were stably expressed in L929 cells. Under serum free conditions, where cell death is normally inhibited, the cells expressing the active Myr-Akt were able to die in the presence of zVAD.fmk alone whereas the cells expressing the catalytically inactive mutant were still protected from death (Figure 3.7 A). Under normal serum conditions the Myr-Akt expressing cells died similarly to wild-type L929 cells, however under serum free conditions only the Myr-Akt expressing cells died in the presence of zVAD.fmk (Figure 3.7 B). This serum independent death in the presence of zVAD.fmk was inhibited by Nec-1 and was therefore RIP1 dependent (Figure 3.7B). FLAG-tagged full length Akt and PH domain mutants that lacked the myristoylation tag to localize Akt to the

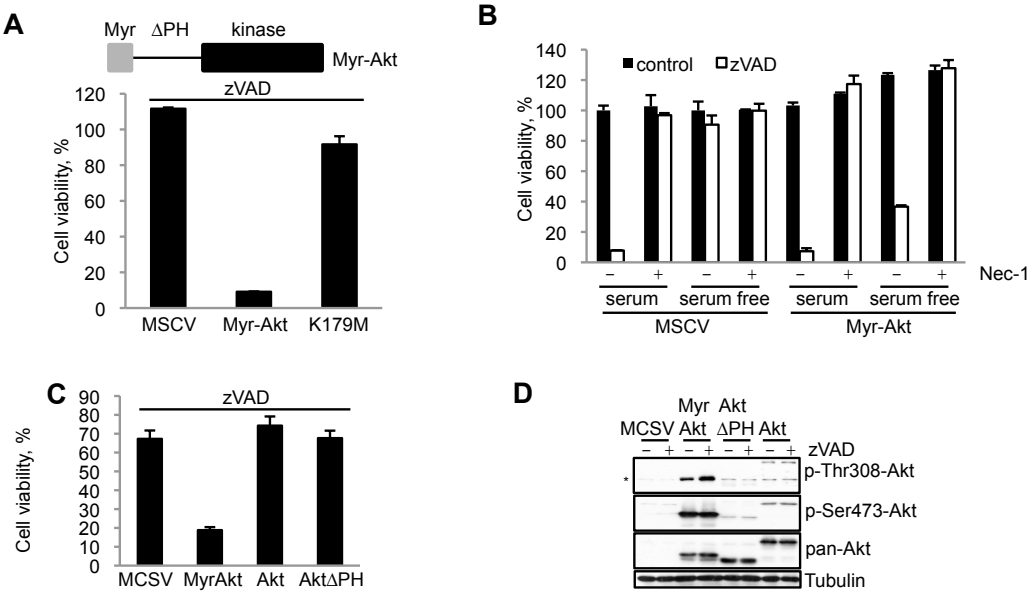

Figure 3.7 Over-expression of constitutively active Akt restores necroptosis under serum free conditions.

Figure 3.7 Over-expression of constitutively active Akt restores necroptosis under serum free conditions. (A,B) L929 cells were stably infected with empty MSCV retrovirus or viruses encoding Myr-Akt or the catalytically inactive Myr-Akt K179M. Necroptosis was induced by the addition of zVAD.fmk under serum free conditions (A) or serum or serum free conditions with Nec-1 (B). Viability assays were performed after 24 hr. (C,D) L929 cells expressing Myr-Akt, full length Akt and a mutant lacking the PH domain were treated with zVAD.fmk under serum free conditions, followed by viability measurement at 24 hr (C) or western blot at 9 hr (D). * - non-specific band, coinciding with the migration of Myr-Akt was detected by some lots of the p308 antibody. In all graphs, average±SD was plotted.

membrane were unable to rescue cell death under serum free conditions regardless of whether or not they contained a PH domain (Figure 3.7 C). Importantly, only cells expressing the Myr-Akt construct were phosphorylated on Thr308 in response to zVAD.fmk under serum free conditions (Figure 3.7 D).

We next explored the necessity of phosphorylation at Thr308 and Ser473 in the Myr-Akt construct using cells expressing the Alanine (non-phosphorylatable) or Aspartate (phospho-mimetic) mutant versions of Myr-Akt. It has been reported that Ala mutations of Thr308 and Ser473 decrease catalytic activity, while the Asp mutants increase activity (Alessi et al., 1996). In our hands, both Asp mutants displayed activity comparable to wild type Akt, while both Ala mutants displayed comparable decreases in activity (Figure 3.8 A). Despite similar catalytic activities, Thr308 and Ser473 mutants displayed major differences in their ability to promote necroptotic changes (Figure 3.8 A, B, C). As expected, the S473D mutant, which was phosphorylated on Thr308 after the addition of zVAD, displayed only slightly reduced activity, while S473A was significantly less active in all aspects of necroptosis. S473A was unable to be efficiently phosphorylated on Thr308 possibly due to the inability of the Ala mutated 473 site to be phosphorylated and provide a docking site for PDK1 to phosphorylate Thr308. Strikingly, both Ala and Asp mutants of Thr308 were significantly less active in promoting cell death and phosphorylation of JNK and c-Jun. This suggests that T308D, in spite of being an active Akt construct, may not be a perfect mimic of phosphorylation

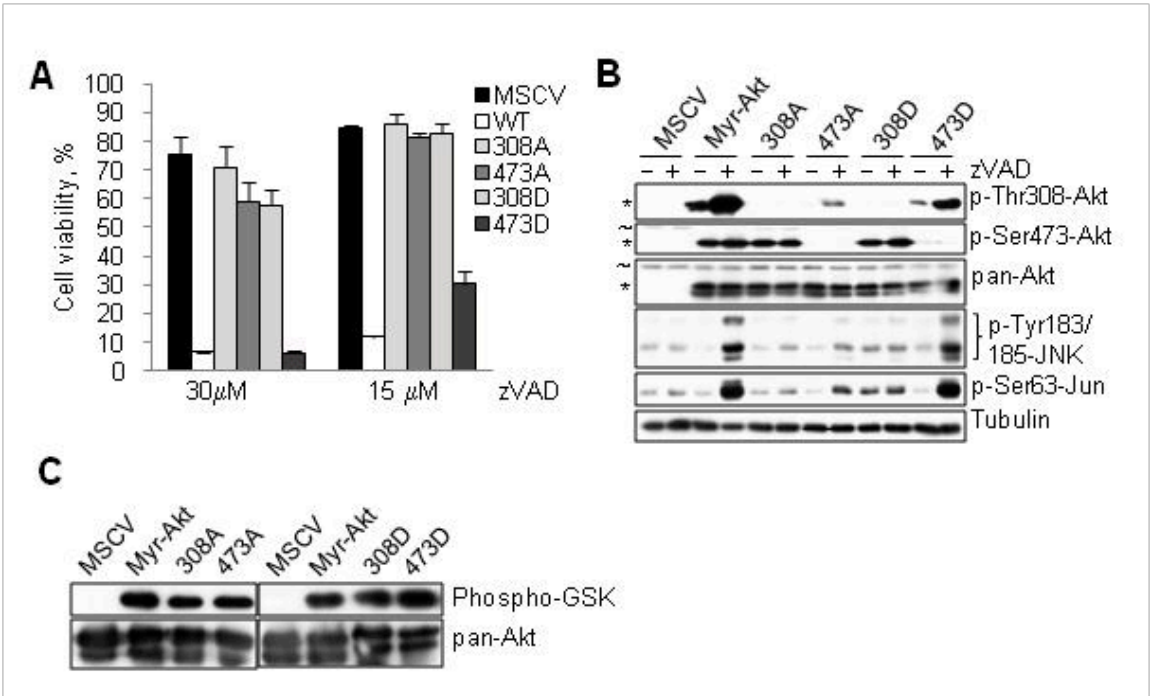

Figure 3.8 Phosphorylation of Thr308 is required for necroptosis.

Figure 3.8 Phosphorylation of Thr308 is required for necroptosis. (A-C) L929 cells expressing Myr-Akt and Ala and Asp mutants of Thr308 and Ser473 were treated with zVAD.fmk under serum free conditions, followed by viability assay at 24 hr (A) or western blot at 9 hr (B). (C) The Myr-Akt and Ala and Asp mutants of Thr308 and Ser473 were immunoprecipitated from L929 cells and their in vitro catalytic activity towards GSK-3β peptide was determined.

3.3 Discussion

Overall, these experiments show that Akt is phosphorylated at Thr308 during necroptosis and this phosphorylation contributes to the induction of necroptosis in L929 cells. Importantly, two inputs are required for necroptotic death to occur in L929 cells. First, a growth factor signal to Akt that results in delayed Akt phosphorylation must be present. This signal can be overcome by the presence of constitutively active Myr-Akt and requires both Akt kinase activity and that Akt is localized to the plasma membrane. However, a second input is also required. This second input is through RIP1 kinase and inhibition of this kinase inhibits death. Neither the growth factor nor zVAD.fmk alone are sufficient. The inputs act in concert to allow death to occur.

These data are important for several reasons. First, they show that Akt, a protein that is typically thought of as being a pro-survival protein, can act as a pro-death molecule depending on cellular circumstances. The uniquely pro-survival role for Akt has already begun to be questioned in the literature (Andrabi et al., 2007; Jin et al., 2007). Andrabi et. al. showed that Akt can act as either a pro-survival or pro-death molecule depending on the protein phosphatase 2A (PP2A) activity and growth factors. The authors demonstrated that small T increases Akt phosphorylation in the cell by inhibiting PP2A and this leads to cell survival or apoptosis depending on whether or not growth factors are present in the serum. It is important to note that the PP2A inhibitor, okadaic acid, did not effect Thr308 phosphorylation or the activation of necroptosis in L929 cells (data

not shown). It is therefore not completely surprising that we have a found a pro-death function for Akt in L929 cell necroptosis.

Second, they show that this delayed phosphorylation of Akt on Thr308 is regulated in the expected way, meaning that the phosphorylation is mediated by PI3K and PDK1. This indicated to us that the canonical Akt signaling pathway is being utilized in the expected fashion and a more in depth analysis of the Akt signaling pathway in necroptosis is warranted.

Third, they raise the interesting question as to why some growth factors, such as bFGF and IGF, but not others, such as PDGF and EGF, were able to mediate cell death. We showed that PDGF signaling is intact in these cells by showing that PDGF stimulated Akt phosphorylation at 15 minutes of treatment. It remains to be discovered then why PDGF could not mediate cell death. One possibility is that a signaling complex is forming in response to bFGF or IGF that is not forming in response to PDGF or EGF.

Chapter IV: Regulation in necroptosis downstream of Akt

The data from this chapter are published in:
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4.1 Introduction

Given that necroptosis in L929 cells requires inputs from both growth factors and RIP1 kinase to generate Thr308 phosphorylation of Akt and necroptosis, we decided to further investigate the role of Akt in these cells. The Akt signaling pathway is very well defined (Manning and Cantley, 2007) (Figure 1.4). Akt is typically thought of as a pro-survival protein that asserts this power through a combination of controlling protein synthesis, cell metabolism, cell proliferation, and inhibiting apoptotic proteins. In this work we examine whether the proteins in the canonical Akt signaling pathway also function in necroptosis.

The role of JNK and c-Jun in necroptosis and TNFα synthesis has been described by several groups (Christofferson et al., 2012; Hitomi et al., 2008; Kim et al., 2007; Wu et al., 2011b; Yu et al., 2004). Yu et. al. show JNK phosphorylation during caspase-independent cell death and use a small molecule inhibitor of JNK (SP6001250) and c-Jun RNAi to protect cells from death. Wu et. al. demonstrates both JNK and c-Jun phosphorylation and use SP600125 and c-Jun RNAi to protect L929 cells from death and inhibit TNFα transcription. Finally, Christofferson et. al. also establishes JNK phosphorylation

during necroptosis and use SP600125 to inhibit cell death and TNFα transcription. Each of these examples demonstrates that JNK and c-Jun play a key role in necroptosis. Our previous data show that JNK and c-Jun are phosphorylated in our model system in a RIP1 kinase dependent fashion.

We investigated whether canonical Akt signaling pathways were activated during necroptosis, what role these pathways play in necroptosis, and where Akt fits into what is already known about necroptosis signaling pathway including JNK and c-JUN activation and TNFα production.

4.2 Results

The Akt Signaling Pathway Contributes to the Regulation of Necroptosis. Given the requirement for Akt activation and Thr308 phosphorylation during necroptosis we chose to look at several downstream proteins in the PI3K-Akt signaling pathway to determine whether they play a role in necroptosis. L929 cells treated with zVAD.fmk or TNFα showed activation of several downstream molecules including FoxO1, FoxO4, MDM2, p70S6k, and S6 (Figure 4.1 A). These phosphorylation changes occurred on a time scale similar to the increase in Thr308 phosphorylation for both zVAD.fmk (Figure 4.1 C) and TNFα (Figure 4.1 D). These downstream phosphorylations also occurred in response to bFGF/zVAD under serum free conditions (Figure 4.1 B). These signaling molecules were also phosphorylated in cell lines stably expressing the Myr-Akt

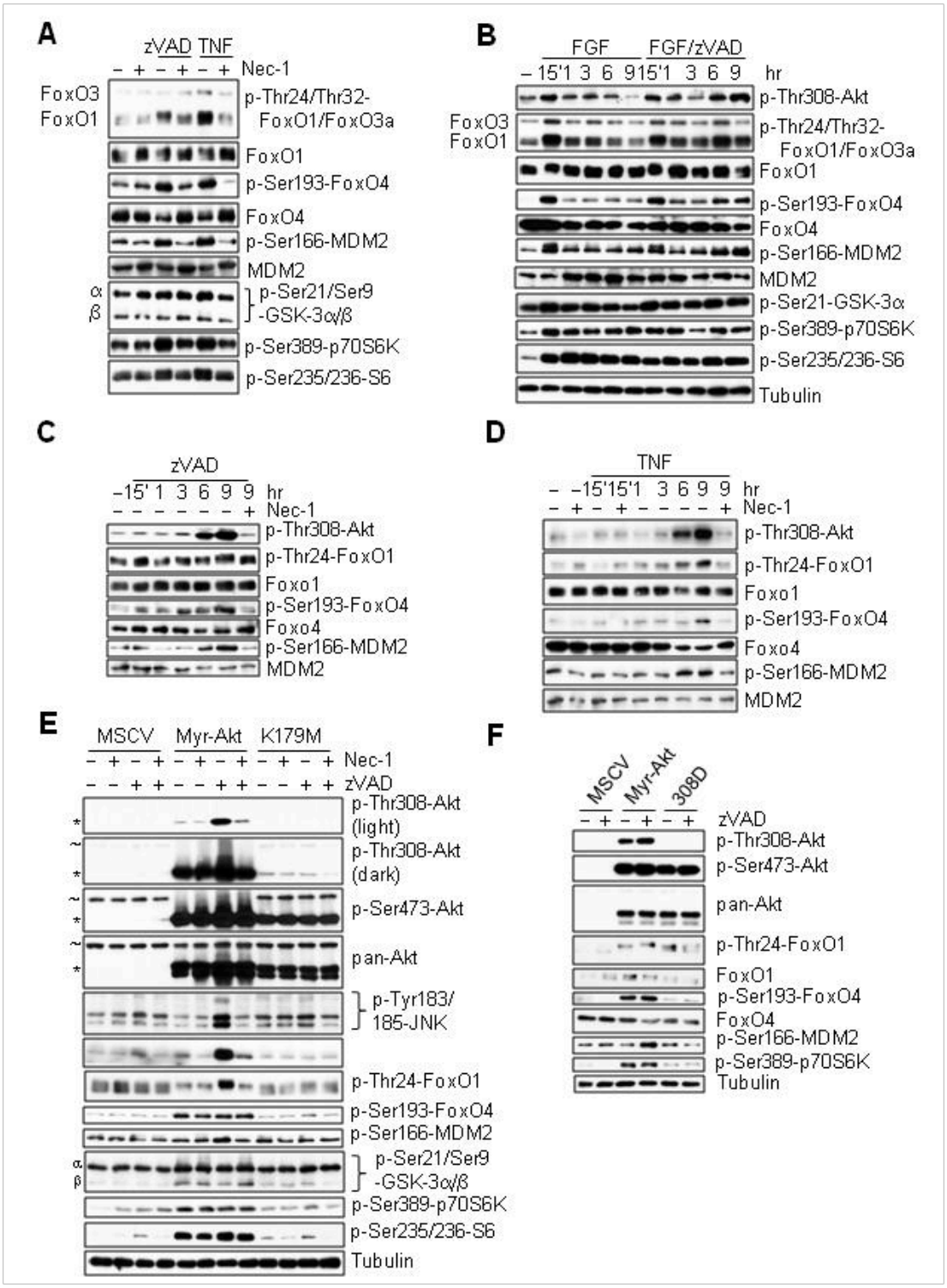

Figure 4.1 Downstream Akt signaling pathways are activated during necroptosis.

Figure 4.1 Downstream Akt signaling pathways are activated during necroptosis. (A) L929 cells were treated with zVAD.fmk or TNFα for 9 hr and harvested for western blot. (B) Cells under serum free condition were treated with bFGF or bFGF/zVAD.fmk for the indicated amounts of time, followed by western blotting using the indicated antibodies. (C-D) L292 cells were treated with zVAD.fmk (C) or TNFα (D) for the indicated periods of time and collected for western blot. (E) Myr-Akt and Myr-Akt K179M cells were treated with zVAD.fmk and/or Nec-1 under serum free conditions for 9 hr, followed by western blot using the indicated antibodies. Endogenous Akt (~) and Myr-Akt (*) bands are indicated. (F) L929 cells expressing Myr-Akt or the T308D mutant were treated with zVAD.fmk for 9 hrs under serum free conditions followed by western blot analysis.

construct but not the catalytically inactive K179M mutant (Figure 4.1 E) or the Thr308D mutant (Figure 4.1 F) under serum free conditions in response to zVAD.fmk.

We further explored the role of mTOR pathway in these cells since the pro-survival role of Akt is in part mediated through activation of mTOR. We wanted to determine whether these molecules reverse their pro-survival roles during necroptosis. L929 cells treated with the inhibitors rapamycin (Rapa), Torin-1 (Torin), or PI-103 were protected from both zVAD.fmk and TNFα induced death (Figure 4.2 A). Rapamycin is an mTORC1 inhibitor. It inhibits Raptor, a specific component of that complex. Torin-1 and PI-103 are direct mTOR kinase inhibitors and inhibit mTOR in both complex I and complex II. PI-103 inhibits PI3K in addition to mTOR. Similarly, siRNA knockdown of mTOR protected cells from necroptosis (Figure 4.2 B). The combination of the mTOR inhibitor and knockdown data confirms that mTOR activation in response to Akt activation during necroptosis does indeed play a role in cell death.

Furthermore, inhibition of p70 S6 kinase, downstream from mTOR activation, with PF-4708671 also protects cells from death (Figure 4.2 C, D). Knockdown of S6 itself also inhibits death, although it protects from zVAD.fmk induced death better that TNFα induced death (Figure 4.2 E). mTOR regulates translation through p70S6 kinase and S6 (Martin and Blenis, 2002) suggesting that translational control by p70S6K/S6 may play a role in necroptosis. Overall, the complete role of Akt effector proteins in necroptosis remains to be fully

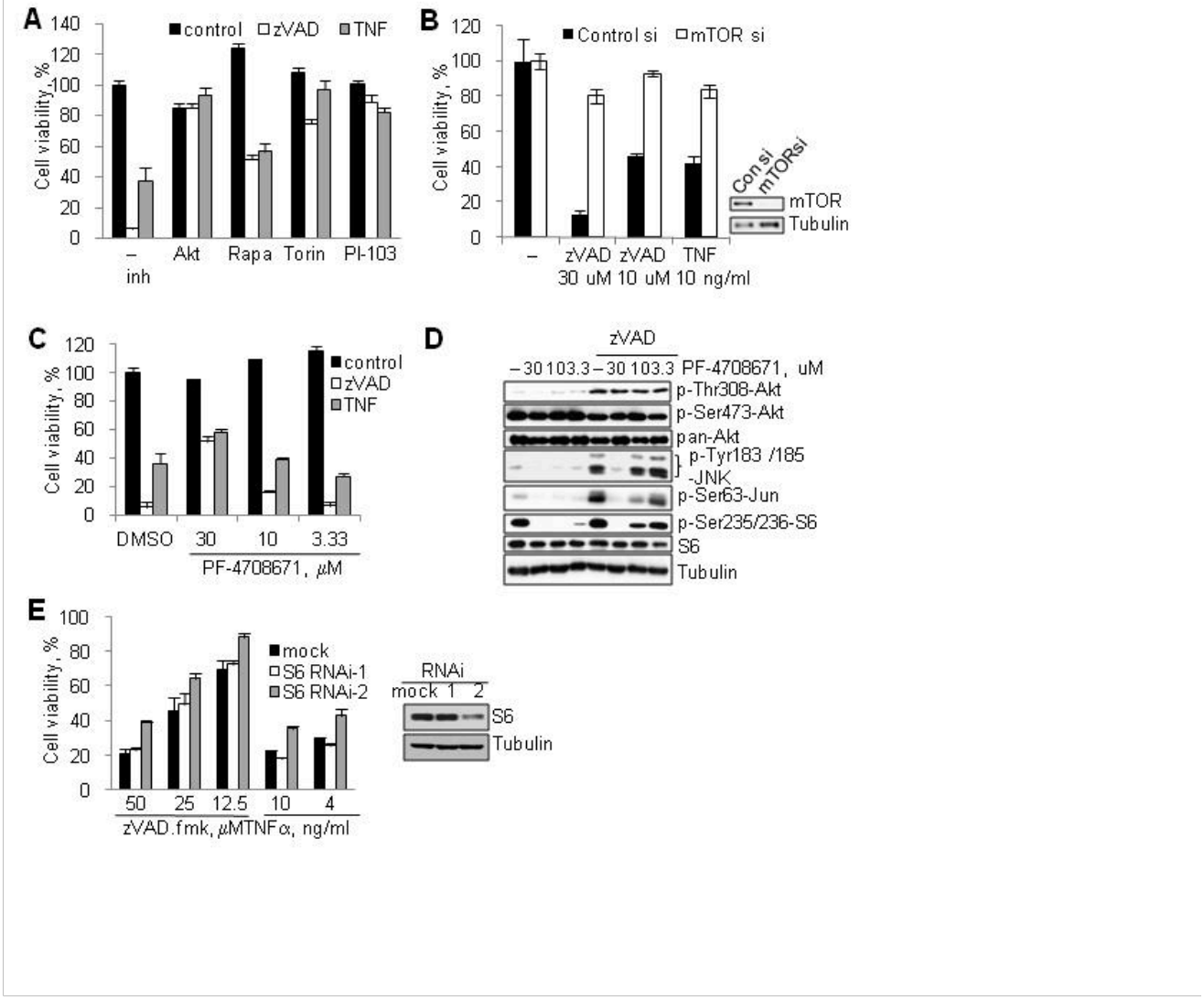

Figure 4.2 mTORC1 and downstream signaling contribute to the regulation of necroptosis.

Figure 4.2 mTORC1 and downstream signaling contribute to the regulation of necroptosis. (A) Necroptosis was induced by zVAD.fmk or TNFα in L929 cells in the presence of inhibitors of Akt (Akt inh. VIII) or mTOR (rapamycin, Torin-1 and PI-103). (B) L929 cells with mTOR siRNA knockdown were harvested for western blot or treated with zVAD.fmk or TNFα for 24 hrs. Cell viability was determined 24 hr after activation of necroptosis. (C,D) L929 were stimulated with TNFα or zVAD.fmk in the presence of the indicated concentrations of the p70 S6 kinase inhibitor PF-4706871. Viability was determined after 24 hr (C). Western blot samples were collected after 9 hr (D). (E) L929 cells were transfected with S6 siRNAs. After 48 hr, necroptosis was induced by TNFα and zVAD.fmk for 24 hr. Inset, levels of S6 were determined 48 hr after transfection. In all graphs, average±SD was plotted.

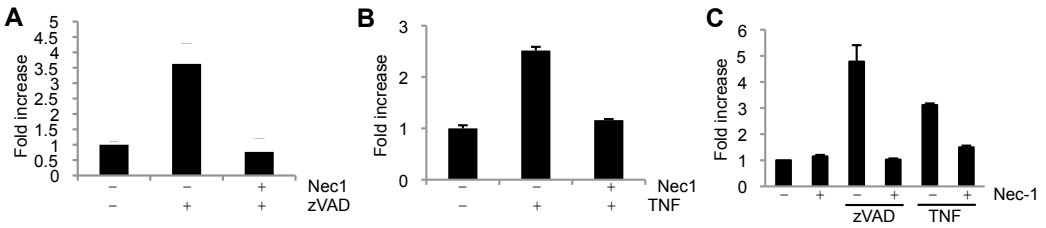

Figure 4.3 TNFα protein and mRNA levels increase during necroptosis.

Figure 4.3 TNFα protein and mRNA levels increase during necroptosis.

(A,B) L929 cells were stimulated by zVAD.fmk (A) and human TNFα (B) for 9 hr. Cell lysates were subjected to mouse TNFα ELISA. (C) L929 cells were stimulated by zVAD.fmk and TNFα in the presence of Nec-1 followed by measurement of TNFα mRNA levels by qRT-PCR at 9 hr.

elucidated. However, it is clear that multiple proteins that play anti-apoptotic roles elsewhere play a pro-necroptotic roles in L929 cell necroptosis.

Akt and mTORC1 Control TNFα Synthesis and the Activation of JNK during Necroptosis. We next explored how Akt and mTOR signaling fit into the upregulation of TNFα during necroptosis. Increases in TNFα protein (Figure 4.3 A, B) and mRNA (Figure 4.3 C) levels during necroptosis are well documented. It is also known that the autocrine production of TNFα is critical for necroptosis (Hitomi et al., 2008) and addition of an anti-TNFα neutralizing antibody effectively protects L929 cells from necroptosis. In order to understand the role Akt plays in TNFα production we looked at serum starved cells treated with either bFGF or PDGF in the presence or absence of zVAD.fmk (Figure 4.4 A). bFGF alone caused increase in TNFα mRNA levels that were further increased by the addition of zVAD.fmk. Only the increase in TNFα mRNA that was made by the addition of zVAD.fmk was inhibited by Nec-1. TNFα mRNA levels in response to PDGF and PDGF/zVAD.fmk, a combination that was not able to induce necroptosis, caused only a very modest change in TNFα mRNA levels.

Cells stably expressing the Myr-Akt constitutively active construct (Figure 4.4 B), but not the catalytically inactive mutant K179M, were able to induce TNFα in response to zVAD.fmk under serum free conditions. Likewise, most of the Myr-Akt Thr308 and Ser473 phospho-mutants were unable to support TNFα mRNA production in response to zVAD.fmk under serum free conditions. The Thr308A, Thr308D, and Ser473A mutants were unable to support cell death, Thr308 phosphorylation (Figure 3.8), or TNFα mRNA production (Figure 4.4 C) under

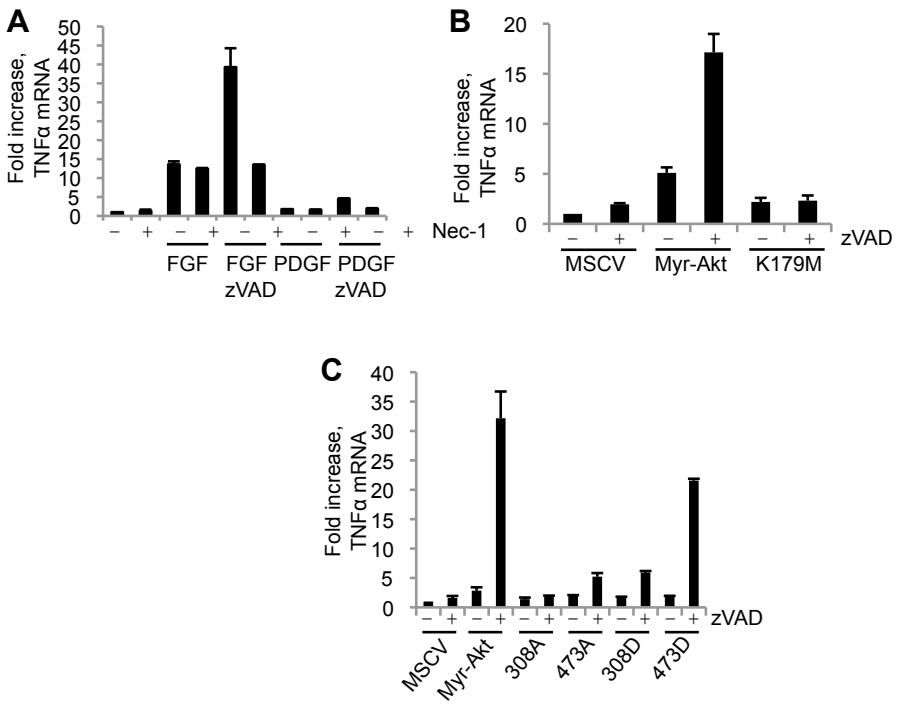

Figure 4.4 Growth factors and active Akt contribute to increase in TNFα mRNA.

Figure 4.4 Growth factors and active Akt contribute to increase in TNFα mRNA. (A) Cells were treated under serum free conditions with bFGF or PDGF with or without zVAD.fmk for 9 hr, followed by qRT-PCR analysis of mTNFα. Data was normalized to mouse 18S RNA. (B) L929 cells, stably infected with Myr-Akt and Myr-Akt K179KM, were stimulated with zVAD.fmk for 9 hr under serum free conditions. TNFα mRNA levels were determined by qRT-PCR and normalized using mouse 18S RNA. (C) L929 cells expressing Myr-Akt and Ala and Asp mutants of Thr308 and Ser473 were treated with zVAD.fmk under serum free conditions, followed by evaluation of TNFα mRNA levels by qRT-PCR at 9 hrs (G). In all graphs, average±SD was plotted.

serum free conditions plus zVAD.fmk. However, the S473D mutant, which did allow some cell death to occur, did exhibit increased TNFα mRNA levels (Figure 4.4 C). Taken together, these data indicate that active Akt plays a role in regulating TNFα mRNA levels during necroptosis.

Small molecule inhibition of Akt and mTOR, with Akt inhibitor VIII and rapamycin respectively, attenuated TNFα production (Figure 4.5 A) and inhibited JNK and c-Jun phosphorylation in response to both zVAD.fmk (Figure 4.5 B) and TNFα (Figure 4.5 C). The Akt and mTOR inhibitors acted similarly to siRNA knockdown. TNFα mRNA levels were attenuated after zVAD.fmk and TNFα treatment by knockdown of both Akt1/2 (Figure 4.6 A) and mTOR (Figure 4.6B). Likewise, in this instance both JNK and c-Jun phosphorylation was reduced (Figure 4.6 B, D). Data previously shown with the p70S6K inhibitor, PF-4708671, shows that p70S6K inhibition also reduces JNK and c-Jun phosphorylation (Figure 4.2 D). These data suggest that the Akt-mTORC1-S6K pathway acts downstream of RIP1 and is required for the increased JNK phosphorylation and TNFα production during necroptosis.

c-Jun is a critical for TNFα production and necroptosis. We wanted to further examine the role of activation of JNK and c-Jun in necroptosis. As mentioned previously, sustained JNK activation leading to TNFα production is a hallmark of necroptosis. Our model based on our data places Akt activation downstream of RIP1 activation but upstream of JNK activation. Inhibition of JNK by SP600125 is commonly used to show that inhibition of JNK protects from necroptosis (Christofferson et al., 2012; Wu et al., 2011b; Yu et al., 2004). Here

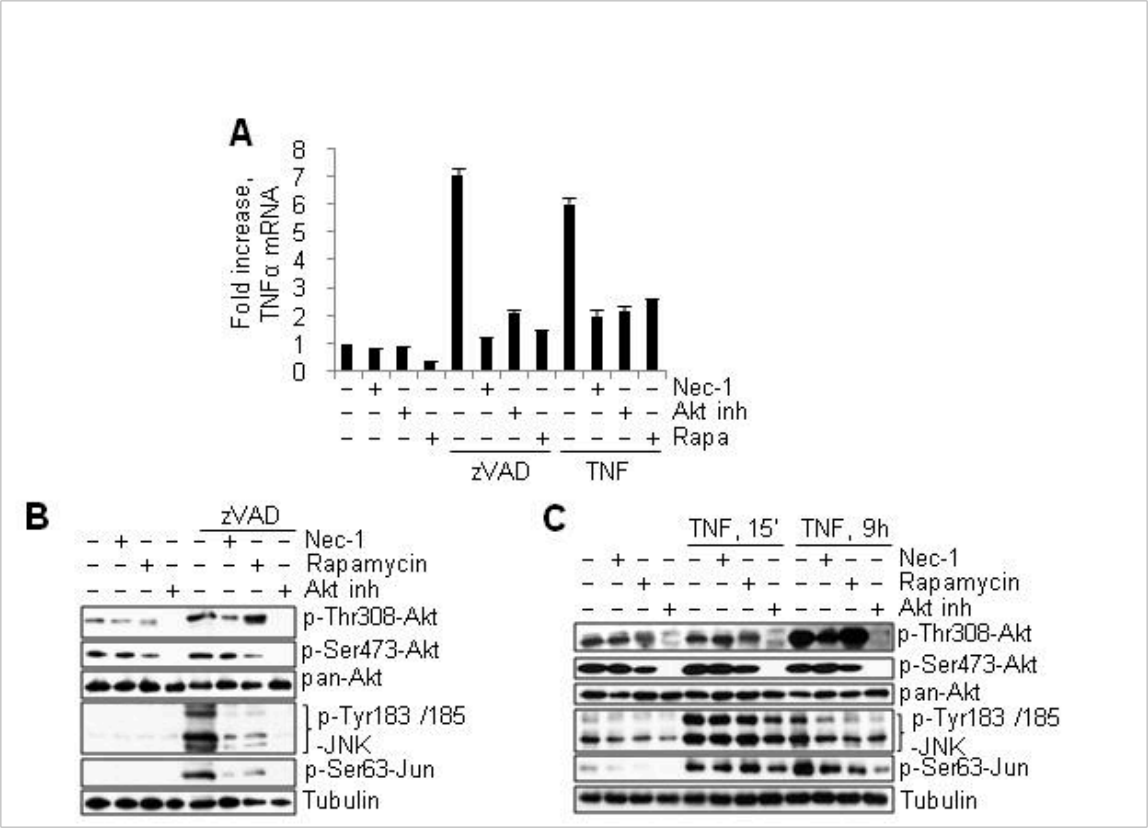

Figure 4.5 Inhibition of Akt and mTOR attenuate TNFα mRNA production and JNK phosphorylation.

Figure 4.5 Inhibition of Akt and mTOR attenuate TNFα mRNA production and JNK phosphorylation. L929 cells were treated with Akt inhibitor VIII or rapamycin and zVAD.fmk or TNFα for 9 hrs hours followed by qRT-PCR analysis (A) of mTNFα. Data was normalized to mouse 18S RNA or western blot (B, C).

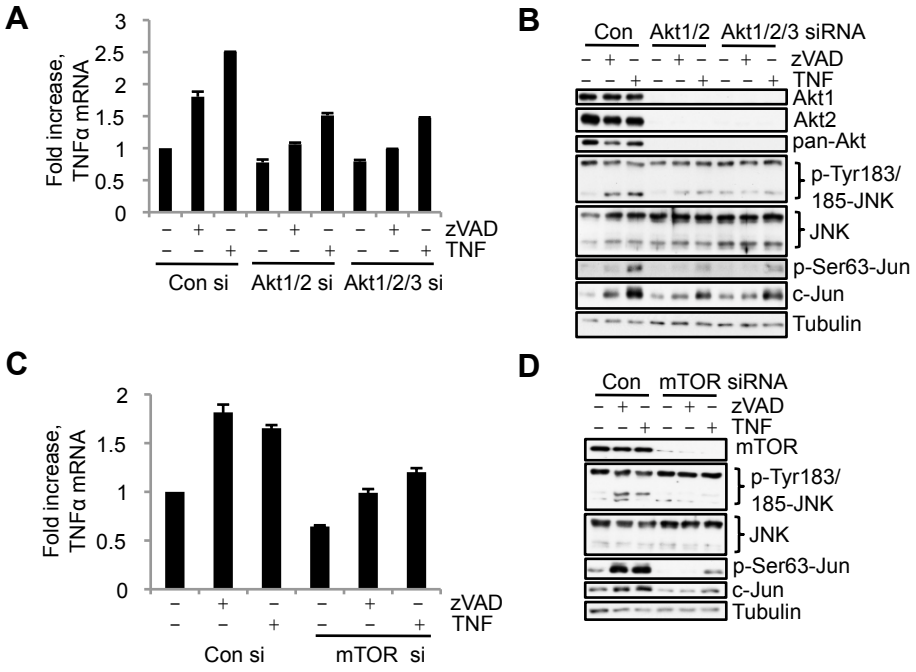

Figure 4.6 Akt and mTOR contribute to TNFα mRNA production and JNK activation.

Figure 4.6 Akt and mTOR contribute to TNFα mRNA production and JNK activation. (A-B) L929 cells with siRNA knockdown of Akt isoforms were stimulated with zVAD.fmk or TNFα for 9 hr, followed by qRT-PCR analysis of mTNFα (A) or western blot (B). (C-D) L929 cells with siRNA knockdown of mTOR were stimulated with zVAD.fmk or TNFα for 9 hr, followed by qRT-PCR analysis of mTNFα (C) or western blot (D). In all graphs, average±SD was plotted.

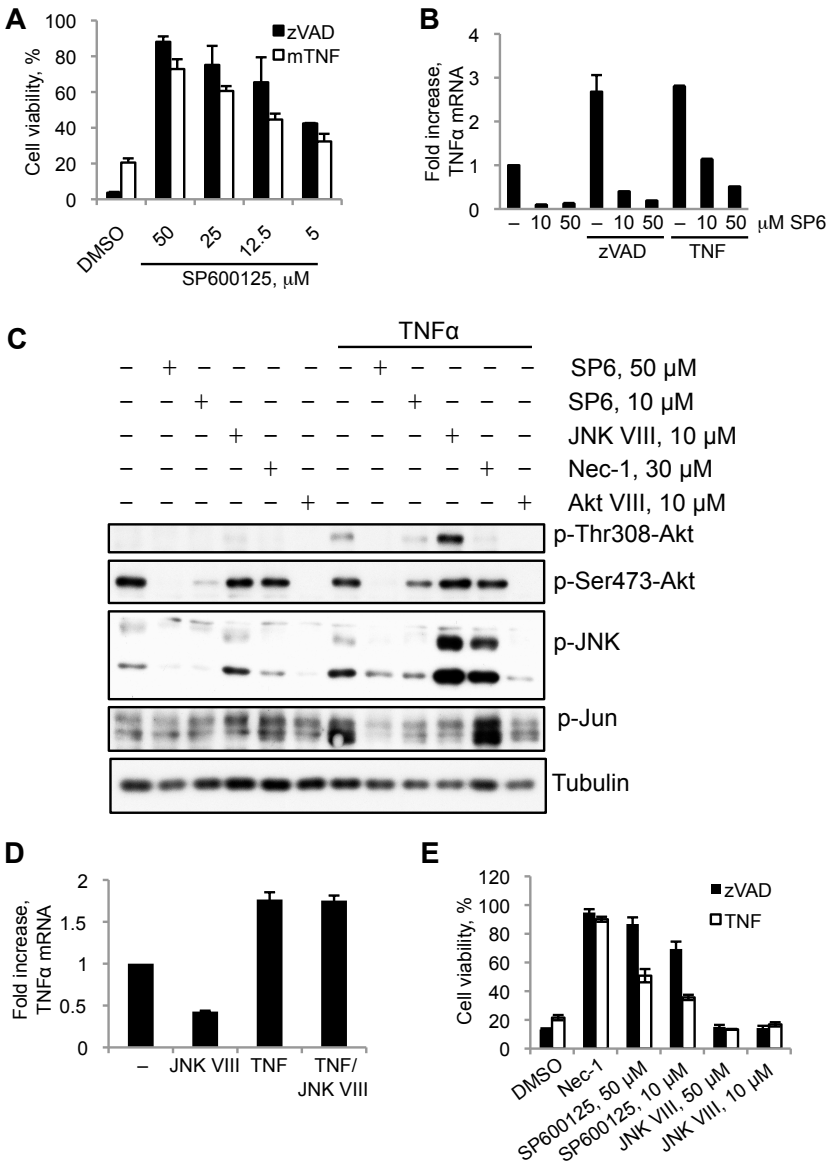

Figure 4.7 Differential inhibition of necroptosis by small molecule JNK inhibitors.

Figure 4.7 Differential inhibition of necroptosis by small molecule JNK inhibitors. (A-B) L929 cells treated with zVAD.fmk or TNFα in the presence of the indicated concentrations of the JNK inhibitor, SP600125. Cells were analyzed for cell viability 24 hrs post-treatment (A) and qRT-PCR of mTNFα at 9 hrs post-treatment (B). (C-D) L929 cells were treated with zVAD.fmk or TNFα in the presence of the indicated concentrations of the JNK inhibitor, SP600125 or JNK inhibitor VIII. Cells were analyzed for western blot (C) and qRT-PCR of mTNFα at 9 hrs post-treatment (D) and cell viability at 24 hrs post-treatment.

we show that SP600125 protects L929 cells from zVAD.fmk and TNFα induced necroptosis (Figure 4.7 A) and inhibits TNFα mRNA production (Figure 4.7 B). However, we observed that SP600125 is able to inhibit the basal phosphorylation levels of Akt in untreated cells at both the Thr308 and Ser473 sites (Figure 4.7 C). This indicated to us that the protective effect that SP600125 has on L929 cells might actually be from its inhibition of Akt phosphorylation, not from decreased JNK or c-Jun phosphorylation. Indeed, it has been published that SP600125 is a somewhat non-specific JNK inhibitor and it may inhibit both PI3K (Tanemura et al., 2009) and PDK1 (Bain et al., 2007). The off target effects of SP600125 on either PI3K or PDK1 could inhibit basal Akt phosphorylation levels and precluded us from using this molecule to test our model pathway.

In order to test this further we used a more specific inhibitor of JNK, JNK inhibitor VIII (Anastassiadis et al., 2011). Importantly, this small molecule did not inhibit basal Akt phosphorylation. When added to cells treated with TNFα, it inhibited c-Jun phosphorylation as expected (Figure 4.7 C). It did not inhibit Akt Thr308 phosphorylation indicating that JNK is in fact downstream of Akt activation as predicted in our model. However, JNK inhibitor VIII did not decrease the amount of TNFα produced (Figure 4.7 D) or protect L929 cells from necroptosis (Figure 4.7 E).

We next used siRNA knockdown in order to further establish the roles of JNK and c-Jun in necroptosis. Knockdown of JNK isoforms 1 and 2 resulted in a decrease in c-Jun phosphorylation in response to TNFα and zVAD.fmk (Figure 4.8 A) but did not decrease Akt Thr308 levels as was seen in the JNK inhibitor

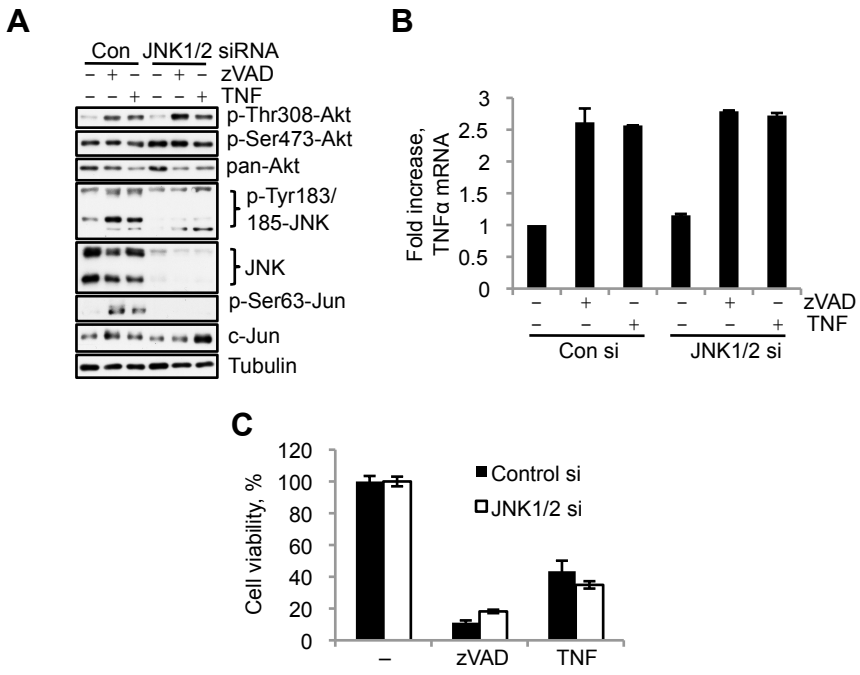

Figure 4.8 JNK1/2 knockdown does not protect L929 cells from necroptosis.

Figure 4.8 JNK1/2 knockdown does not protect L929 cells from necroptosis. (A-C) L929 cells transfected with JNK1 and JNK2 siRNAs for 72 hrs were treated with zVAD.fmk or TNFα followed by western blot at 9 hr (A), evaluation of TNFα mRNA levels by qRT-PCR at 9 hrs (B), or viability assay at 24 hr (C).

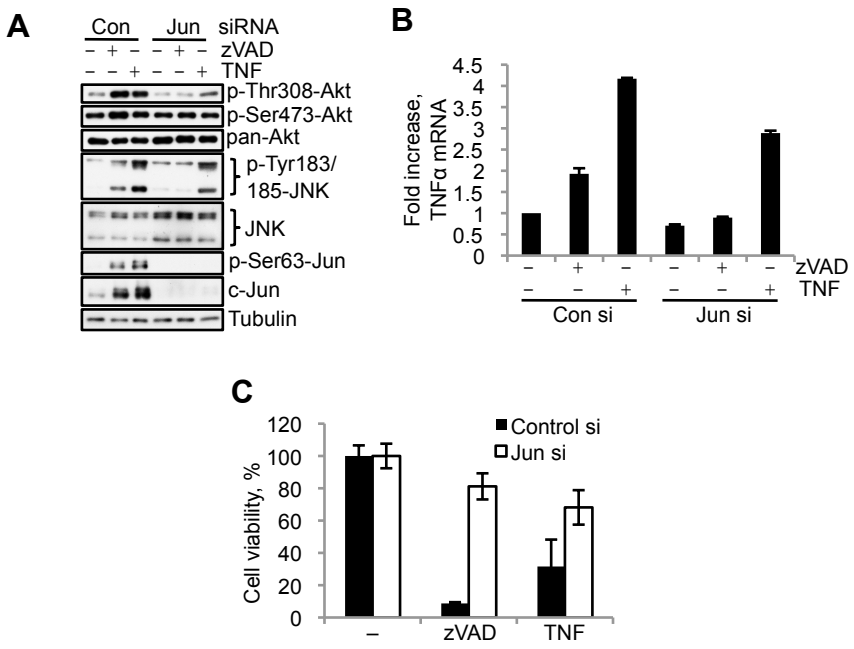

Figure 4.9 c-Jun knockdown protects L929 cells from necroptosis.

Figure 4.9 c-Jun knockdown protects L929 cells from necroptosis. (A-C) L929 cells transfected with c-Jun siRNAs for 72 hrs were treated with zVAD.fmk or TNFα followed by western blot at 9 hr (A), evaluation of TNFα mRNA levels by qRT-PCR at 9 hrs (B), or viability assay at 24 hr (C).

VIII studies. Likewise, these cells produced similar amounts of TNFα mRNA (Figure 4.8 B) and were not protected from cell death (Figure 4.8 C). These data correlate very well with the JNK inhibitor VIII data but not the SP600125 data. However, previously published data indicate strongly that the JNK-c-Jun signaling axis is critical for autocrine TNFα production (Christofferson et al., 2012; Wu et al., 2011b; Yu et al., 2004). As previously published, siRNA knockdown of c-Jun both protected L929 cells from necroptosis (Figure 4.9 C) and attenuated TNFα mRNA production (Figure 4.9 B). Interestingly, c-Jun knockdown did in fact reduce Thr308 phosphorylation on Akt. Indicating that autocrine TNFα production, dependent on c-Jun, may create a feedback loop that contributes to the delayed activation of Akt.

Akt Controls TNFα Production in Other Cell Types. We next wanted to expand our model of RIP1 kinase dependent Akt activation to other cell types undergoing necroptotic cell death. We used FADD deficient Jurkat cells treated with TNFα (Figure 4.10 A-C), RAW 264.7 macrophages treated with zVAD.fmk (Figure 4.10 D-F), and J774A.1 macrophages treated with zVAD.fmk (Figure 4.10 G-I). In each of these cell types, we show that Akt gets phosphorylated at Thr308 in response to necroptotic stimuli and that this phosphorylation is RIP1 kinase dependent as it is inhibited by Nec-1 (Figure 4.10 B,E,H). Additionally, inhibition of Akt using Akt inhibitor VIII does inhibit TNFα mRNA production in each case (Figure 4.10 A, D, E). Inhibition of Akt, however, does not protect these cell types from death (Figure 4.10 C,F,I).

We next chose to examine the role of Akt in necroptosis in mouse lung fibroblasts. Mouse lungs fibroblasts that contain only endogenous Akt1 or Akt2 also demonstrated a RIP1 dependent increase in TNFα mRNA production as well as Thr308 phosphorylation in response to treatment with TNFα and zVAD.fmk (Figure 4.11 A,C). Lung fibroblasts selected to survive after deletion of all three Akt isoforms (Maroulakou et al., 2007) were resistant to necroptosis. However, expression of the catalytically active Myr-Akt construct restored TNFα mRNA production in these cells but did restore cell death in response to TNFα and zVAD.fmk treatment (Figure 4.11 B,D). These data indicate that in cell types beyond L929 cells, Akt may play a bigger role in the regulation of TNFα synthesis and inflammatory signaling than in cell death.

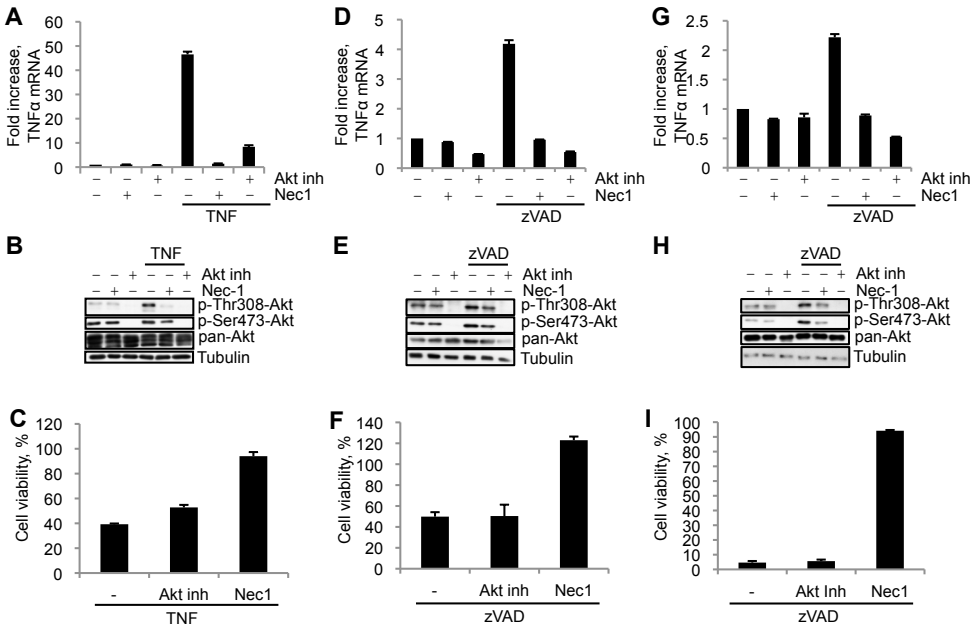

Figure 4.10 Akt signaling contributes to autocrine TNFα production in multiple cell types.

Figure 4.10 Akt signaling contributes to autocrine TNFα production in multiple cell types. FADD deficient Jurkat cells were treated with TNFα followed by measurement of (A) human TNFα mRNA levels by qRT-PCR and normalized using human 18S RNA and (B) western blot at 9 hr and (C) cell viability at 24 hrs. RAW 264.7 or J774A.1 cells were treated with zVAD.fmk (100 uM or 50 uM respectively) followed by (D,G) measurement of TNFα mRNA levels by qRT-PCR or (F,H) western blot at 9 hr and cell viability (F,I) at 24 hrs..

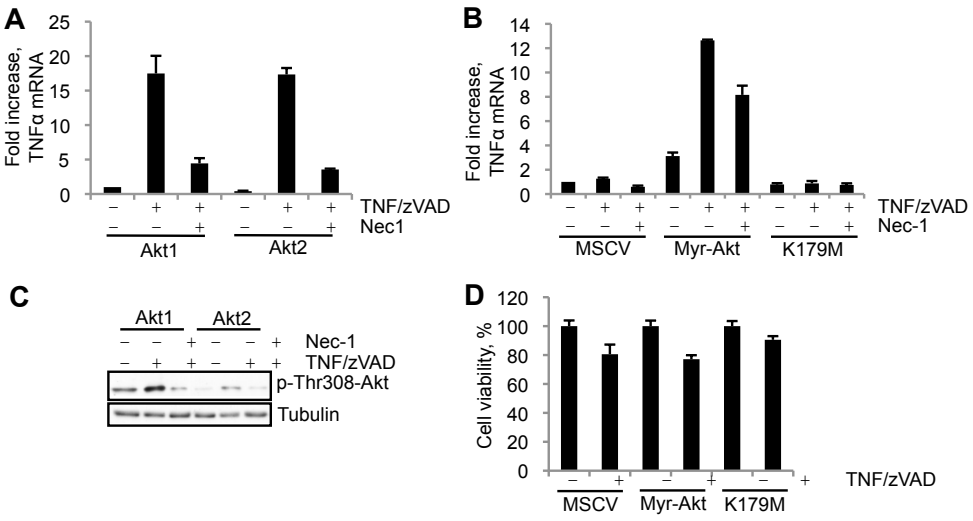

Figure 4.11 Akt expression correlates with TNFα mRNA expression.

Figure 4.11 Akt expression correlates with TNFα mRNA expression. (A,C) Mouse lung fibroblasts expressing only the endogenous Akt1 or Akt2 isoforms were treated with zVAD.fmk and TNFα followed by measurement of TNFα mRNA levels by qRT-PCR (A) and western blot (C) at 9 hr. (B,D) Akt null mouse lung fibroblasts expressing Myr-Akt or K179M were treated with zVAD.fmk and TNFα followed by measurement of TNFα mRNA levels by qRT-PCR (B) and western blot (D) at 9 hr.

4.3 Discussion

We set out to investigate canonical Akt signaling pathway activation during necroptosis, what role these pathways play in necroptosis, and where Akt fits into what is already known about necroptosis signaling pathway including JNK and c-JUN activation and TNFα production. We found that multiple canonical Akt effector proteins are activated during necroptosis and that these proteins also play a role in necroptosis. Akt pathway molecules including FoxO1, FoxO4, Mdm2, p70S6K, and S6 were all phosphorylated in a RIP1 kinase dependent manner during necroptosis. Some of the proteins were also shown to play a role in necroptosis. For example, inhibition or knockdown of two proteins activated downstream of mTORC1, p70S6K and S6, protected L929 cells from death. We went on to show that mTOR also plays a critical role in necroptosis as inhibition and knockdown protects cells from death. Akt and mTOR both play an important role in the upregulation of TNFα during necroptosis. We confirmed that Akt activation occurs downstream of RIP1 kinase activation and upstream of JNK activation based on JNK inhibitor and knockdown data. TNFα production however, may be more critically controlled by c-Jun rather than JNK. In multiple other models of necroptosis, Akt kinase was phosphorylated on Thr308 in response necroptotic stimuli and TNFα was produced in an Akt dependent fashion. However, Akt kinase activity did not control cell death in the alternative models of necroptosis indicating an important role for Akt in inflammatory signaling.

Chapter V: Identification of Novel Akt interacting proteins

5.1 Introduction

We previously demonstrated that several canonical Akt pathway proteins are involved in necroptosis (McNamara et al., 2013). We next wanted to further evaluate the role of Akt kinase in necroptosis by identifying proteins that interact with Akt under necroptotic conditions. The goals for this project were to 1) identify potential known Akt interacting proteins during necroptosis 2) identify potential novel Akt interacting proteins during necroptosis and 3) understand the role that these interacting proteins play in necroptosis. We identified three proteins, Arf1, Nur77, and GAPDH, to investigate further.

5.2 SILAC MS

We chose to use a method called Stable Isotope Labeling with Amino Acids in Culture (SILAC) mass spectrometry (MS) in order to identify Akt interacting proteins (Ong and Mann, 2007). This is a quantitative proteomics method in which the relative differences in levels of proteins between control and experimental treatments can be distinguished. An overview of the approach is provided in Figure 5.1. In brief, the process is split into two phases. The first phase is the adaptation phase in which either heavy ($^{13}C_6$ Arginine and $^{13}C_6$

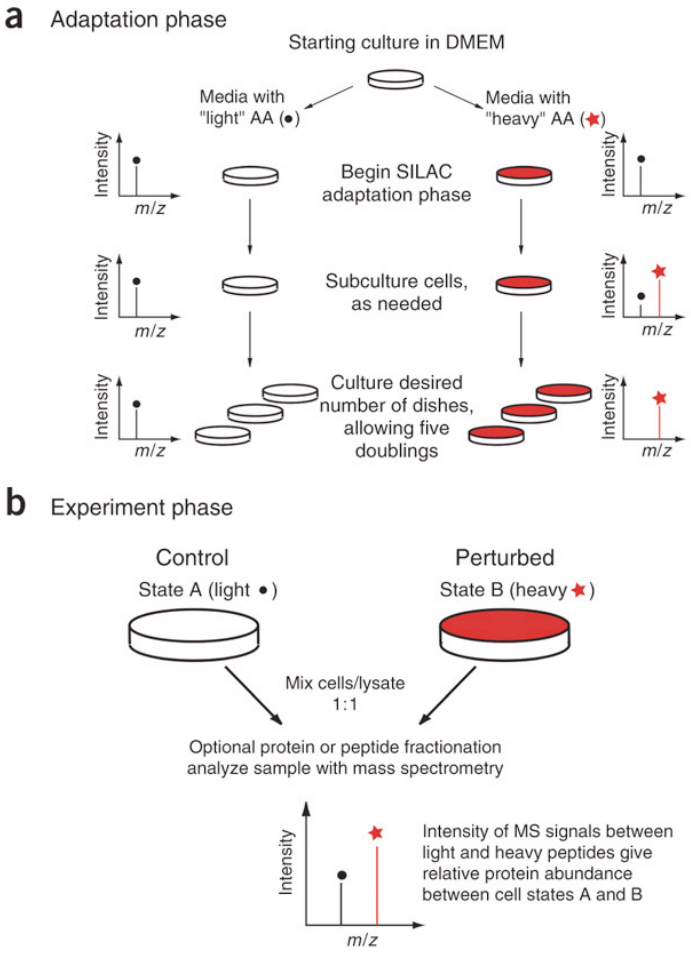

Figure 5.1 Overview of Stable Isotope Labeling with Amino Acids in Culture (SILAC) Mass Spectrometry.

Figure 5.1 Overview of Stable Isotope Labeling with Amino Acids in Culture (SILAC) Mass Spectrometry. During the Adaptation Phase cells are passaged for at least five doubling time in growth media containing either “Heavy” ($^{13}C_6$ Arginine and $^{13}C_6$ Lysine) or “Light” (($^{12}C_6$ Arginine and $^{12}C_6$ Lysine) amino acids in order to allow full incorporation of the amino acids into the proteome. During the Experimental Phase the “Heavy” and “Light” cells are treated under different experimental conditions, for example Control and Perturbed. The cell lysates are then mixed at a 1:1 ratio and subjected to MS. The relative protein abundance between the two samples is then analyzed (Ong and Mann, 2007).

Lysine) or light ($^{12}C_6$ Arginine and $^{12}C_6$ Lysine) amino acids are incorporated into the proteome of the cells through normal metabolic processes by growing the cells in media containing either heavy or light amino acids for at least five double times. In the second experimental phase, one set of cells is treated as control (for example the “light aa cells”) and the other set (“heavy cells”) is treated experimentally, in this case treated to induce necroptosis with zVAD.fmk. The cells are then mixed, experimentally processed, and then relative protein abundance is evaluated by mass spectrometry. The SILAC method provides a simple and robust way to compare protein abundance between experimental conditions. In this set of experiments we identified three potential interacting proteins, Arf1, Nur77, and GAPDH, to investigate further.

i. Arf1

ADP-ribosylation factor 1 (ARF1) is a member of the Ras superfamily of small GTPases (D'Souza-Schorey and Chavrier, 2006). Arfs, in general, are ubiquitously expressed and highly conserved proteins. Arf1 is a Class I Arf meaning that it regulates the assembly of coat complexes for budding vesicles in the secretory pathway (Kahn et al., 2006). Arf1 regulates secretory membrane transport between the Golgi and endoplasmic reticulum (ER) by recruiting coat protein complex I (COPI) proteins to budding vesicles. It is also involved in the recruitment of clathrin to the Golgi and ER.

Arf activation is necessary in order for it to participate in coat assembly. Arfs switch between their active GTP-bound and inactive GDP-bound form with the help of GTPase Activating Proteins (GAPs) and Guanine nucleotide-Exchange Factors (GEFs) (Jackson and Casanova, 2000; Randazzo and Hirsch, 2004). There are several known Arf1-GEFS including Golgi-associated brefeldin A (GBF1), BIG1, BIG2, cytohesin-1 (PSCD1), ARNO (PSCD2), and GRP1 (PSCD3). GBF1, BIG1, and BIG2 are each sensitive to Brefeldin A while others such as PSCD1-3 are Brefeldin A resistant (D'Souza-Schorey and Chavrier, 2006). Brefeldin A works to inhibit Arf1 by stabilizing the Arf1-GDP complex with a GEF and inhibiting GDP displacement. GTP hydrolysis on Arf1 is mediated by Arf-GAPs such as ARFGAP1 and is required for the dissociation of COPI from transport vesicles (Tanigawa et al., 1993).

ii. Nur77

Nur77 is an orphan nuclear receptor (also known as NR4A1, NGFIB, TR3, TIS1, NAK-1, or N10) that acts as a transcription factor (To et al., 2012). It belongs to the NR4A subgroup of the nuclear receptor superfamily, which also includes Nurr1 (NR4A2) and Nor-1 (NR4A3). Nur77 contains an N-terminal transactivation domain (TAD), a DNA-binding domain (DBD), and a C-terminal ligand binding domain (LBD). However, no physiological ligands of Nur77 have been found.

Importantly, it has been shown that Nur77 plays a role in apoptosis. It is induced during T cell receptor signaling and disruption of Nur77 blocks apoptosis in T cells. Nur77's role in apoptosis is accomplished through both its transcriptional activity (Kuang et al., 1999) and re-localization from the nucleus to the mitochondria (Zhang, 2007). In contrast to its role in apoptosis, Nur77 has also been shown to act as a pro-survival and proliferation molecule. Notably, Nur77 has been shown to be a survival factor in response to TNF signaling (Suzuki et al., 2003). Both protein localization between the nucleus and mitochondria and protein expression levels are thought to play a key role in the pro-survival versus pro-death effects of Nur77 (Moran et al., 2011).

Nur77 is regulated on both the transcriptional and post-translational levels. Histone deacetylases, for example, negatively regulate Nur77 expression levels (Youn and Liu, 2000). On the other hand, the AP-1 complex of c-FOS and c-Jun can enhance Nur77 transcription (Wu et al., 2011a). There are several post-translational phosphorylations that are known to modulate Nur77 function (To et al., 2012). Two of the most relevant include JNK phosphorylation of Nur77 at Ser95 that promotes its degradation (Han et al., 2006) and Akt phosphorylation of cytoplasmic Nur77. This phosphorylation event is proposed to prevent the localization of Akt to the mitochondria and apoptosis (Chen et al., 2008). Additionally, Akt phosphorylation of Nur77 while it is localized to the nucleus prevents DNA binding and transcription factor activities.

iii. GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is best known as a key enzyme in glycolysis that catalyses the conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate in the presence of NAD+ and inorganic phosphate (Sirover, 1999). It is commonly referred to and used as a "housekeeping" gene. However, it has recently begun to be appreciated as a protein with diverse cellular functions. GAPDH can bind to both DNA and RNA to regulate both mRNA stability and gene expression. For example, binding of Colony-stimulating factor 1 (CSF1) mRNA by GAPDH leads to mRNA stability and an increase in CSF-1 protein levels. This cytokine is positively associated with tumor progression (Zhou et al., 2008). On the other hand, GAPDH binding to endothelin-1 mRNA brings about degradation of the mRNA (Rodriguez-Pascual et al., 2008). GAPDH has also been shown to play roles in telomere protection and act as a transcription factor (Nicholls et al., 2012).

Importantly, GAPDH has been shown to play a role in apoptosis (Chuang et al., 2005; Hara and Snyder, 2006; Sirover, 2005). In brief, S-Nitrosylation of GAPDH allows its interaction with Siah1, a ubiquitin ligase, and nuclear localization of the GAPDH-Siah1 complex. The complex then ubiquitinates nuclear proteins and leads to their degradation (Hara et al., 2005). GAPDH translocation to the nucleus is blocked by a protein called GAPDH's competitor of Siah protein enhances life (GOSPEL) by binding GAPDH and preventing its interaction with Siah1 (Sen et al., 2009). Additionally, GAPDH overexpression has also been shown to protect cells from caspase-independent cell death

following mitochondrial membrane permeabilization by maintaining ATP levels (Colell et al., 2007)

4.2 Results

Arf1, Nur77, and GAPDH are potential Akt interacting proteins. We used SILAC MS in order to identify Akt interacting proteins during necroptosis (Figure 5.2). Using L929 cells that stably express the constitutively active FLAG-tagged (C-terminal) Myr-Akt (Myr-Akt), we generated cell lines suitable for use in SILAC experiments by passaging them for at least five generations in either heavy or light amino acids. The cells were serum starved overnight before being treated for nine hours with either DMSO or zVAD.fmk to induce cell death. The cell lysates were mixed in a 1:1 ratio and subjected to overnight FLAG immunoprecipitation using anti-FLAG M2 magnetic beads (Sigma) and the Myr-Akt complexes were then eluted from the beads using a 3X FLAG peptide (Sigma). The samples were then processed and subjected to mass spectrometry.

Using this method we identified three proteins to investigate further. We used two basic criteria to determine which proteins to pursue. The first was using the relative ratio of peptide abundance comparing the zVAD.fmk treated samples to the untreated controls (Table 5.1). The second was completing a literature search to see if any of the identified interacting proteins are known to play a role in cell death or interact with Akt. We identified Arf1, Nur77, and GAPDH as potential Akt interacting proteins to investigate further.

1. FLAG-WT-AKT L929
   Cultured in Heavy or Light media
2. Serum Starve – ON
   DMSO or zVAD treated – 9 hrs
3. Mix lysates
4. FLAG IP overnight
   Elute from beads
5. Coomassie gel or TCA precipitation
6. Send for MS

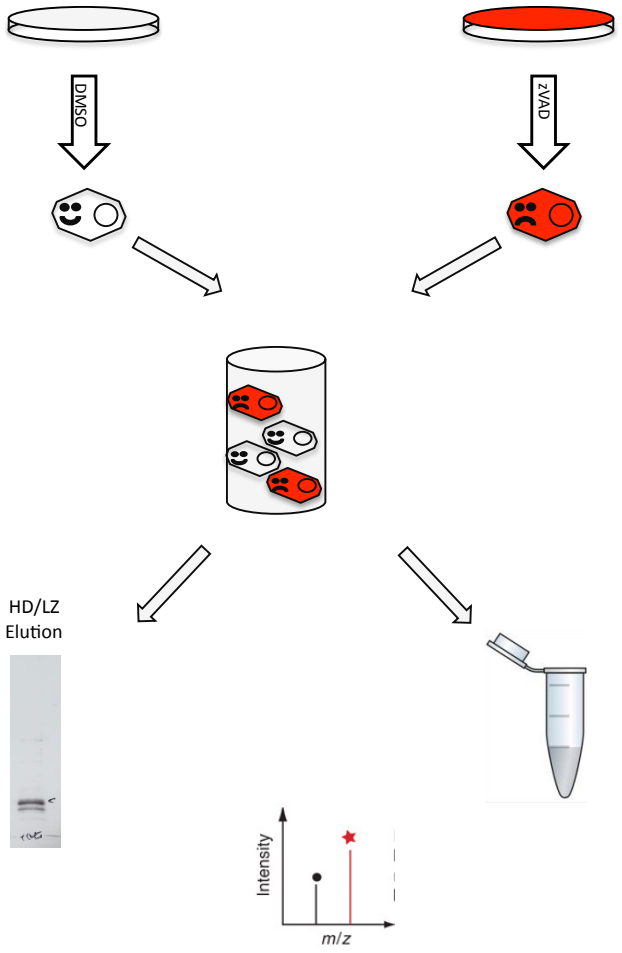

Figure 5.2 Workflow for SILAC MS of FLAG-WT-AKT expressing L929 cells.

Figure 5.2 Workflow for SILAC MS of FLAG-WT-AKT expressing L929 cells. L929 cells the stably express constitutively active FLAG-tagged WT Myr-Akt (Myr-Akt) were passaged for five doubling times in the presence of media containing either “Heavy” or “Light” amino acids. The cells were then plated, serum starved overnight, and then treated either with DMSO (Control) or zVAD.fmk for nine hours. The cell lysates were collected and mixed at a 1:1 ratio. Overnight FLAG immunoprecipitations were then performed and the proteins were eluted off the beads with 3X FLAG peptide. The resulting samples were TCA precipitated then subjected to analysis by mass spectrometry.

Exp. #	1	2	3	4	5	Ave	St Dev
Arf1	7.811	4.192	1.574		0.71	3.572	3.19
GAPDH	2.681	2.986	1.909		1.351	2.232	0.742
Nur77				5.726		5.726	
Akt1	1	1	1	1	1	1	

Table 5.1 Ratios of relative peptide abundance normalized to Akt. The table shows the relative change in peptide abundance of Arf1, GAPDH, and Nur77 normalized to Akt in 5 separate SILAC experiments. An empty box indicates that peptides from that protein were not identified in that particular experiment.

Arf1 interacts with Akt. We first looked at ADP-ribosylation factor 1 (Arf1). Arf1 is a member of the Ras superfamily of small GTPases. It is localized to the Golgi and ER and plays a role in membrane trafficking (D'Souza-Schorey and Chavrier, 2006). We performed co-IP assays in order to validate our SILAC results. In these experiments Arf1 co-IP'd with Myr-Akt under all three tested conditions DMSO treated, zVAD.fmk treated, and zVAD.fmk plus Nec-1 treated (Figure 5.3 A). However, there was no increase in the zVAD.fmk treated sample as we had predicted based on the SILAC MS data. We next used lentiviral expression of shRNAs to ARF1 to create stable ARF1 knockdown cell lines and evaluate the role of Arf1 in necroptosis. Stable knockdown of Arf1 protein and mRNA levels were seen via western blot (Figure 5.3 D) and qPCR analysis (Figure 5.3 C). Three of the four hairpins showed protection of cell viability after treatment with zVAD.fmk compared to untransfected cells. However, the empty vector control transfection was also protected from death, rendering the data inconclusive (Figure 5.3 B). Additionally, the amount of cell death did not correlate with the amount of Arf1 protein present on the western blot. Further analysis of Arf1 knockdown in L929 cell necroptosis is needed.

Inhibition of Arf1 protects L929 cells from death. Brefeldin A (BFA) is a fungal metabolite that is thought to interfere with transport from the endoplasmic reticulum to the golgi apparatus by inhibiting Arf1 activity. Addition of BFA to L929 cells undergoing necroptosis induced by either zVAD.fmk or TNFα were protected from death (Figure 5.4A). We hypothesized that BFA and Arf1 may play a role in TNFα transport within the cell. Using an Enzyme-Linked

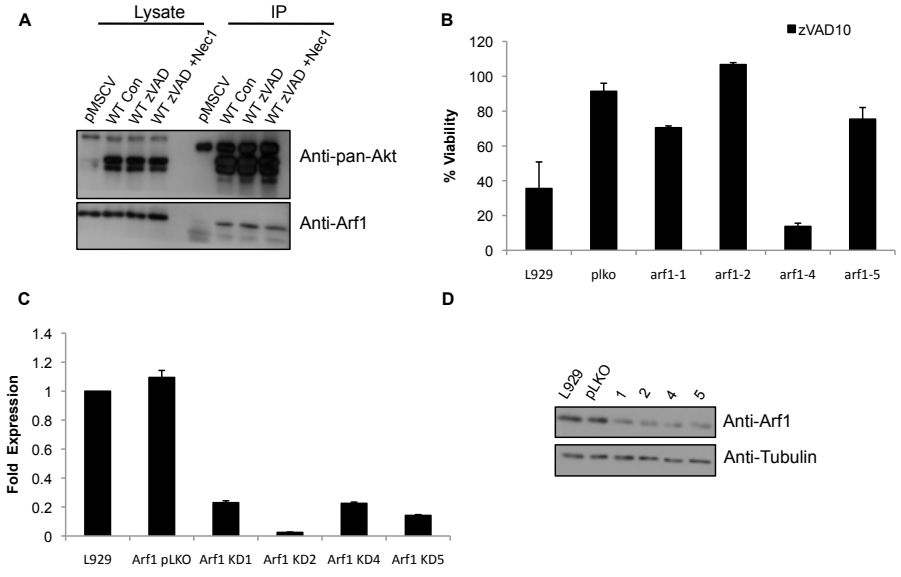

Figure 5.3 Arf1 interacts with FLAG-WT-AKT.

Figure 5.3 Arf1 interacts with FLAG-WT-AKT. (A) FLAG-WT-Akt (Myr-Akt) was immunoprecipitated from stable expression cells. The cells were serum starved overnight and treated with zVAD.fmk for 9hrs. The co-immunoprecipitation of Arf1 was detected via western blot. (B-D) L929 cells stably expressing shRNAs to Arf1. (B) Cellular viability was measured after 24 hrs of treatment with zVAD.fmk. (C) Arf1 mRNA and (D) protein levels were detected by qPCR and western blot, respectively.

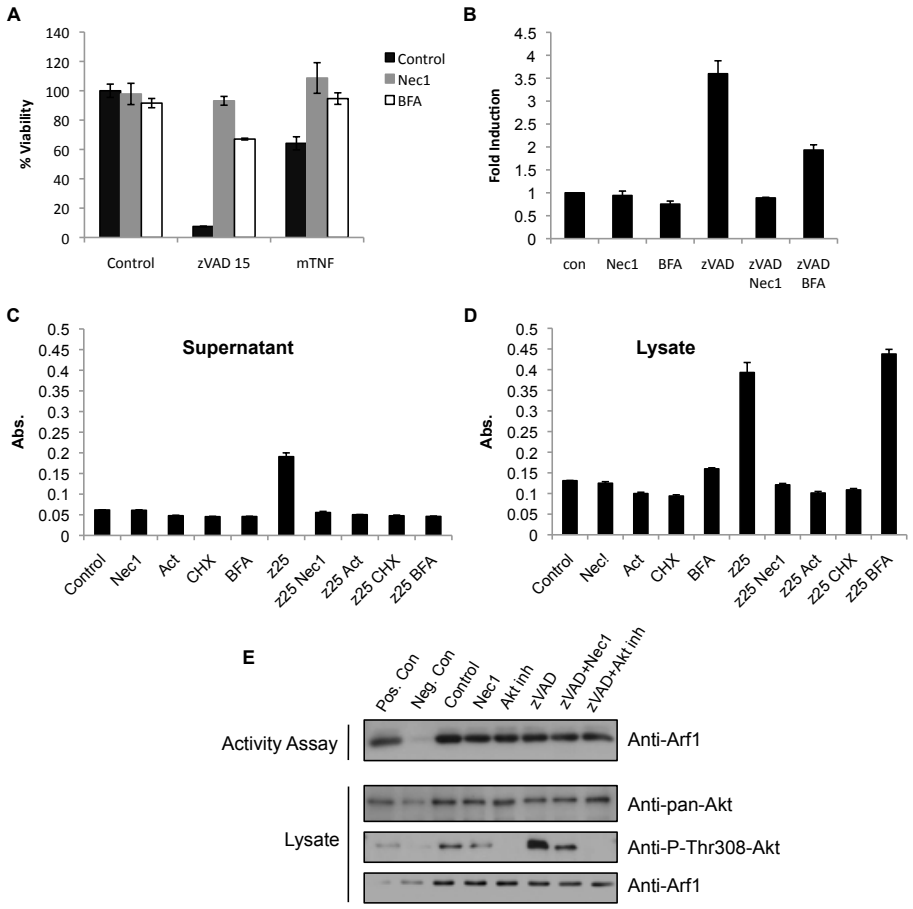

Figure 5.4 Inhibition of Arf1 with Brefeldin A protects L929 cells from necroptosis.

Figure 5.4 Inhibition of Arf1 with Brefeldin A protects L929 cells from necroptosis. (A-D) L929 cells were treated with Nec-1 or BFA in the presence of either zVAD.fmk or TNFα. Cell viability was measured at 24 hrs (A) and TNFα mRNA levels were detected by qPCR at 9hrs (B). ELISA on supernatants and cell lysates were performed after 9hrs of treatment (C-D). (E) Cells were treated with Nec-1 or Akt inhibitor VIII in the presence or absence of zVAD.fmk for 9hrs followed by lysis and Arf1 activity assay (Pierce). GST-GGA3-PBD bound to glutathione resin was used to precipitate active Arf1 from the lysates. The amount of active Arf1 was detected by western blot. Actinomycin (Act), Cycloheximide (CHX), Brefeldin A (BFA).

Immunosorbant Assay (ELISA) and treating the cells with actinomycin D (Act) to inhibit transcription, cycloheximide (CHX) to inhibit translation, or BFA we found that BFA did not inhibit TNFα levels in the cellular lysates (Figure 5.4 D). However, it did inhibit the secretion of TNFα into the supernatant (Figure 5.4 C). This indicates a possible role for Arf1 in transport of TNFα. It should also be noted that BFA seemed to reduce overall mRNA levels of TNFα (Figure 5.4 B). We next noted, using an Arf1 activity assay, that neither the induction of necroptosis nor the inhibition of Akt using Akt inhibitor VIII changed the activity of Arf1 in L929 cells (Figure 5.4 E). BFA did inhibit Arf1 activity in the activity assay (data not shown). This assay works by precipitation of only active Arf1 from cell lysates using GST-GGA3-PBD bound to glutathione resin. The amount of active Arf1 in each sample is then detected by western blot.

Nur77 interacts with Akt. We next chose to evaluate the role of Nur77 in necroptosis. Nur77 is an orphan nuclear receptor that acts as a transcription factor. It has a known Akt phosphorylation site at Ser351 that is thought to decrease the transcriptional activity and/or localization to the mitochondria of Nur77 when phosphorylated. We again validated our SILAC results by co-immunoprecipitation of Nur77 with Myr-Akt under all three tested conditions DMSO treated, zVAD.fmk treated, and zVAD.fmk plus Nec-1 treatment (Figure 5.5 A). As per our hypothesis, Nur77 interacted more strongly with Akt under necroptosis inducing conditions and this interaction was attenuated by Nec-1. As with the Arf1 stable lentiviral knockdown, knockdown of Nur77 did not correlate

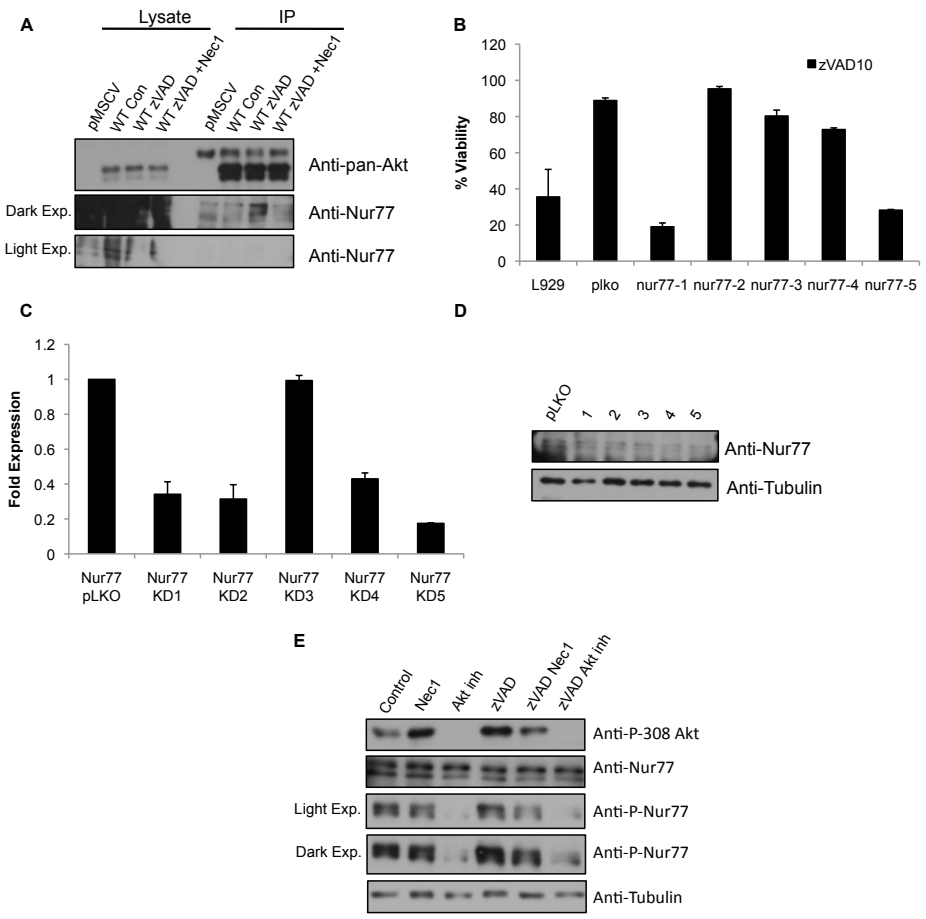

Figure 5.5 Nur77 interacts with FLAG-WT-AKT during necroptosis.

Figure 5.5 Nur77 interacts with FLAG-WT-AKT during necroptosis. (A) FLAG-WT-Akt (Myr-Akt) was immunoprecipitated from stable expression cells. The cells were serum starved overnight and treated with zVAD.fmk for 9hrs. The co-immunoprecipitation of Nur77 was detected via western blot. (B-D) L929 cells stably expressing shRNAs to Nur77. (B) Cellular viability was measured after 24 hrs of treatment with zVAD.fmk. (C) Nur77 mRNA and (D) protein levels were detected by qPCR and western blot, respectively. (E) L929 cells treated with Nec-1 and Akt inhibitor VIII were in the presence or absence of zVAD.fmk for 9hrs. The cell lysates were subjected to western blot.

well with cellular survival (Figure 5.5 B-D). We noted that inhibition of Akt by Akt inhibitor VIII is able to inhibit basal phosphorylation levels of Nur77 at S351. There is also an increase in phosphorylation of the Ser351 site under necroptotic conditions that is inhibited to basal levels by Nec-1 (Figure 5.5 E).

GAPDH interacts with Akt and contributes to cell death in L929 cells. The final protein that we chose to evaluate was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). While this enzyme is most widely known to catalyze the conversion of glyceraldehyde-3-phosphate to 1-3-diphosphoglycerate, it has several known non-glycolytic functions as well. These roles include DNA repair, tRNA export, membrane fusion and vesicle transport, cytoskeletal dynamics, and cell death (Seidler, 2013). We again validated our results by co-immunoprecipitation of GAPDH with Myr-Akt under all three conditions: DMSO treated, zVAD.fmk treated, and zVAD.fmk plus Nec-1 treatment. As per our hypothesis, GAPDH interacted more strongly with Akt under necroptosis inducing conditions and this interaction was attenuated by Nec-1 (Figure 5.6 A). Both stable shRNA (KD1) (Figure 5.6 B,D) and transient siRNA knockdown (Figure 5.6 C,D) of GAPDH were able to protect L929 cells from zVAD.fmk induced cell death. We next want to rescue the GAPDH knockdown phenotype by re-expressing GAPDH in the L929 cells with knockdown. To do this we created FLAG-hGAPDH (Figure 5.7 A) and GFP-hGAPDH (Figure 5.7 B) constructs. While we were able to see expression of the construct in HEK 293T cells, expression of GAPDH in the L929 cells was weak even when compared to the eGFP expression alone. Overexpression of GAPDH needs to be improved so

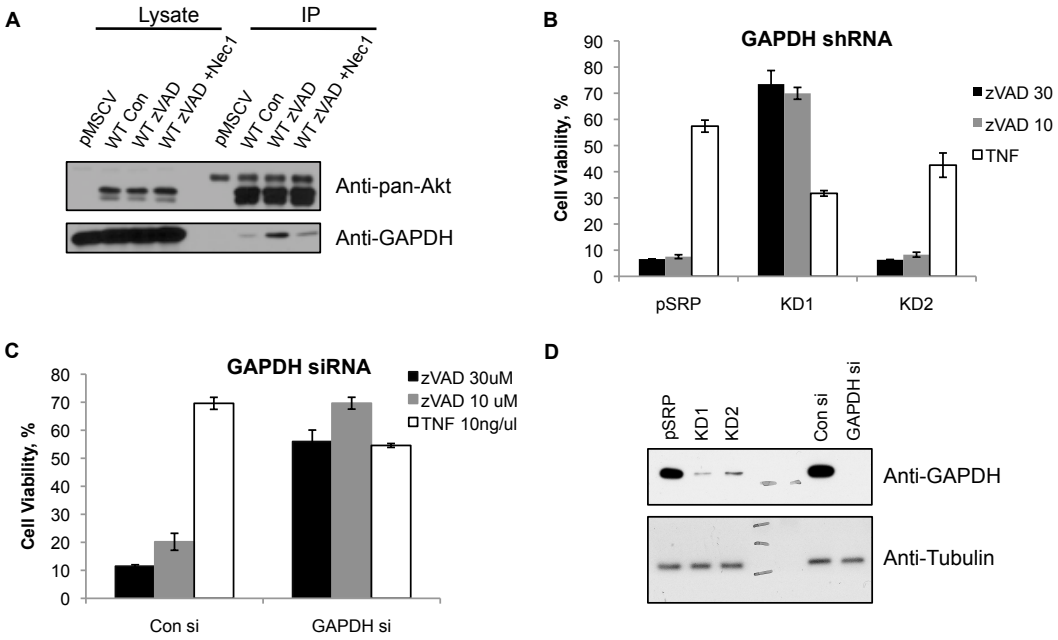

Figure 5.6 GAPDH interacts with FLAG-WT-AKT during necroptosis.

Figure 5.6 GAPDH interacts with FLAG-WT-AKT during necroptosis. (A) FLAG-WT-Akt (Myr-Akt) was immunoprecipitated from the stable expression cell line. The cells were serum starved overnight and treated with zVAD.fmk for 9hrs. The co-immunoprecipitation of GAPDH was detected via western blot. (B-D) L929 cells stably expressing shRNAs (B) or transient transfection of siRNA (72 hrs post transfection) to GAPDH (C) were made. (B-C) Cellular viability was measured after 24 hrs of treatment with zVAD.fmk. (D) Protein levels were detected by western blot, respectively.

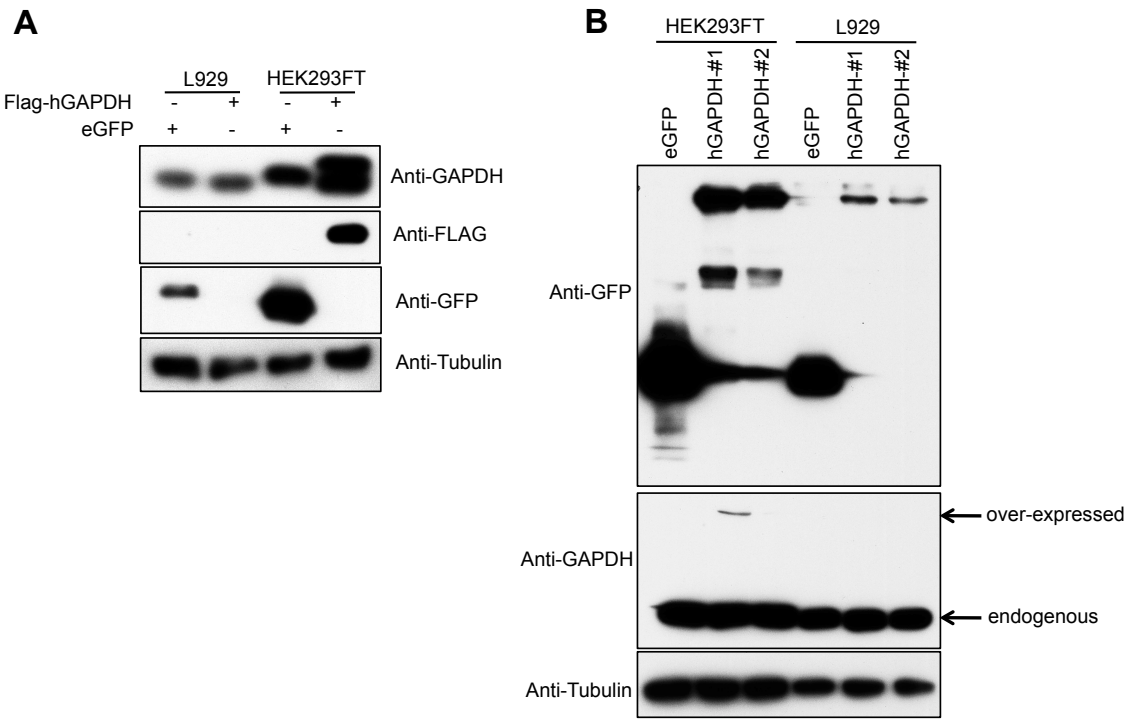

Figure 5.7 GAPDH does not over-express well in L929 cells.

Figure 5.7 GAPDH does not over-express well in L929 cells. (A) Transient transfection of L929 or HEK293FT cells with either Flag-tagged hGAPDH or peGFP. Lysates were collected 48hrs post transfection and subjected to western blot (B). Transient transfection of L929 or HEK293FT cells with either peGFP-hGAPDH (construct #1 and construct #2) or peGFP. Lysates were collected 48hrs post transfection and subjected to western blot.

that protection conferred by the siRNA and shRNA knockdown experiments can be fully attributed to GAPDH.

GAPDH knockdown did not protect all models of necroptosis. However, GAPDH siRNA knockdown was not able to protect several alternative necroptosis models from death including a recently transformed B6 bone marrow macrophage cell line treated with Lipopolysaccharide (LPS) and zVAD.fmk (Figure 5.8 A,C) and WT MEFS treated with TNFα and zVAD.fmk (Figure 5.8 B,C). Similarly, the RAW264.7 macrophage cell line treated with GAPDH siRNA was not protected from cell death nor was there a reduction in TNFα mRNA production. These data indicate that the role of GAPDH necroptosis may be limited to L929 cells.

Further evaluation of the GAPDH/Akt interaction. We next attempted to co-immunoprecipitate endogenous GAPDH and Akt in L929 cells. The results were ambiguous. Immunoprecipitation of Akt was not able to co-IP GAPDH (Figure 5.10 A). Two separate GAPDH antibodies gave different results. One (Abcam) was able to weakly IP GAPDH but strongly co-IP Akt under both control and necroptotic conditions (Figure 5.10 B). The other was only able to IP GAPDH under necroptotic conditions and it did not co-IP Akt (Figure 5.10 C). Thus the endogenous interaction of Akt and GAPDH was not validated and remains to be further studied.

Formation of high molecular weight GAPDH complexes. We were intrigued by the observation that one GAPDH antibody could only immunoprecipitate GAPDH in under necroptotic conditions (Figure 5.10 C). It is

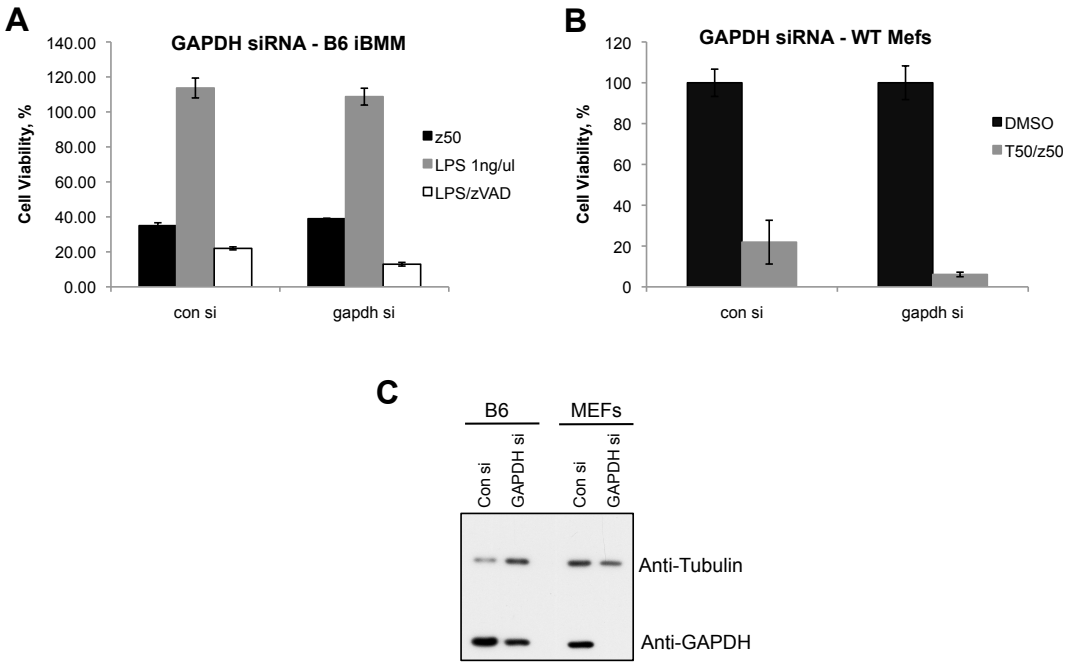

Figure 5.8 GAPDH knockdown does not protect macrophage or MEFs cells from necroptosis.

Figure 5.8 GAPDH knockdown does not protect macrophage or MEFs cells from necroptosis. (A-C) Transformed B6 Bone Marrow Macrophages or MEFS were treated for 72hrs with GAPDH siRNA. The B6 BMM (A) were treated with zVAD.fmk (50uM) LPS (1ng/ul) or LPS plus zVAD.fmk while the MEFs (B) were treated with TNFα (50ng/ml) plus zVAD.fmk (50uM) for 24 hrs followed by measurement of cellular viability. GAPDH protein levels were monitored by western blot (C).

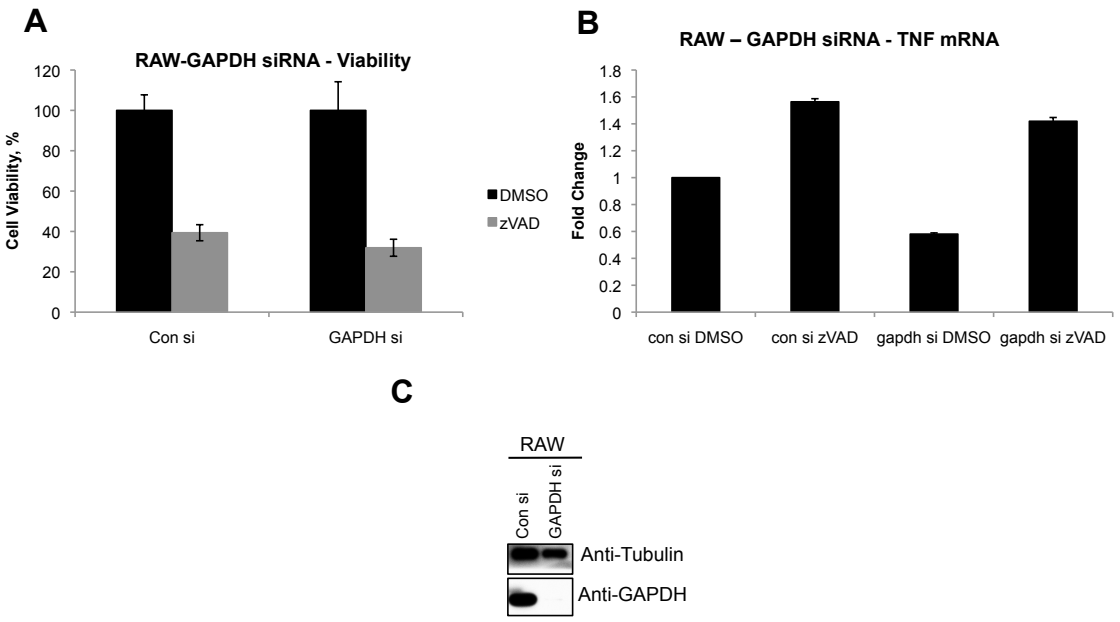

Figure 5.9 GAPDH knockdown does not protect RAW macrophages from necroptosis.

Figure 5.9 GAPDH knockdown does not protect RAW macrophages from necroptosis. (A-C) RAW264.7 macrophages or were treated for 72hrs with GAPDH siRNA followed by treated with zVAD.fmk (50uM). Cell viability was measured 24 hrs after treatment (A). TNFα mRNA levels were measured by qPCR 9hrs after treatment (B). GAPDH protein levels were monitored by western blot (C).

known that GAPDH can form high molecular weight complexes (Mazzola and Sirover, 2002) in some situations and breakdown from its glycolytically active tetrameric state to dimers or monomers in other situations (Seidler, 2013). We evaluated the possibility that GAPDH complex formation might be changing during necroptosis using a combination of Native-PAGE and Gel Filtration. We treated L929 cells with zVAD.fmk and subjected the subsequent cell lysates to analysis by Native-PAGE (Figure 5.11 B) or separation by gel filtration (Figure 5.11 C). On the Native-PAGE gel the band that we think corresponds to the tetramer GAPDH disappeared. By gel filtration, we saw a leftward shift of GAPDH elution. This indicated that the complex that GAPDH is associated is getting larger during necroptosis. These two results indicated that GAPDH was not breaking down into monomers during necroptosis but was in fact forming higher molecular weight complexes.

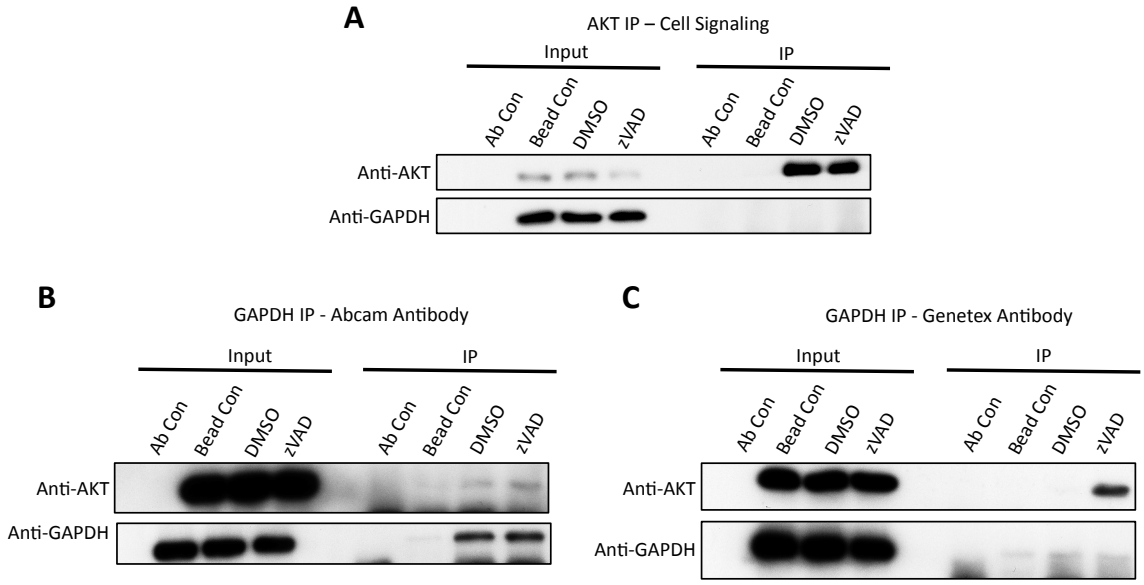

Figure 5.10 Co-immunoprecipitation of endogenous GAPDH and Akt is inconclusive.

Figure 5.10 Co-immunoprecipitation of endogenous GAPDH and Akt is inconclusive. (A-C) L929 cells were treated with zVAD.fmk for 9hrs. The lysates were incubated overnight with the Akt – Cell Signaling antibody (A), GAPDH – Abcam antibody (B), or GAPDH – Genetex antibody (C) at 4°C. Lysates and antibodies were incubated with Protein G beads for one hr at 4°C followed by washes and western blot analysis.

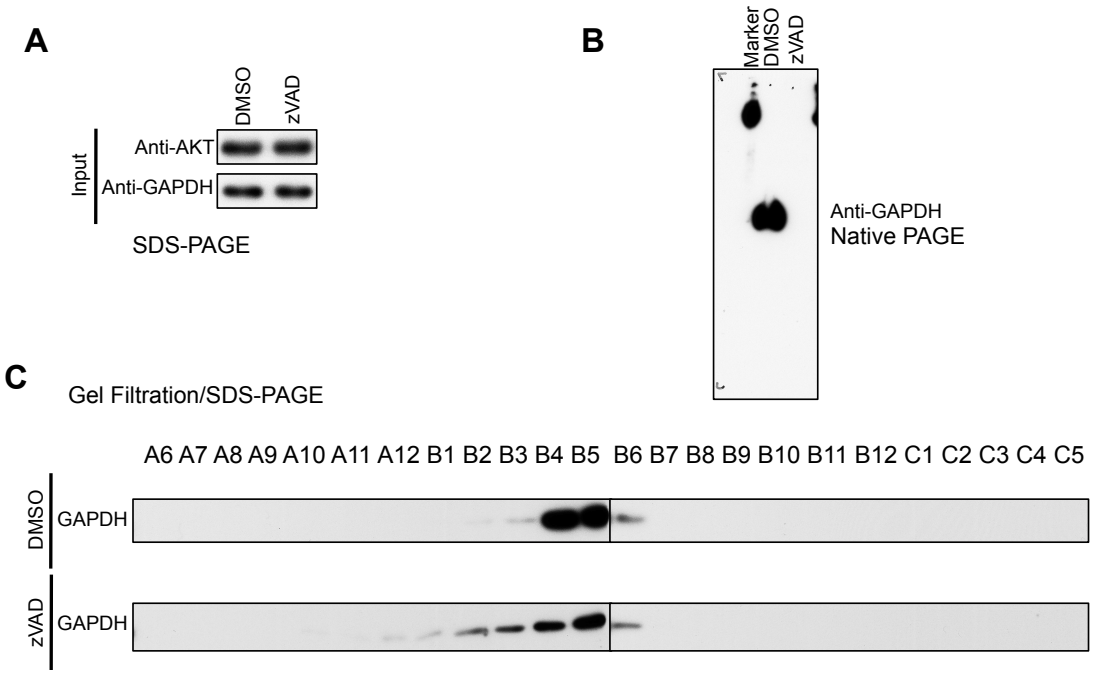

Figure 5.11 GAPDH may form a higher molecular weight complex during necroptosis.

Figure 5.11 GAPDH may form a higher molecular weight complex during necroptosis. (A-C) L929 cells were treated with or without zVAD.fmk for 9hrs. Lysates were run on SDS-PAGE for western blot (A), Native-PAGE (B), and separated on a Superdex 200 gel filtration column followed by SDS-PAGE and western blot.

4.3 Discussion

We demonstrated in Chapter IV that several canonical Akt pathway proteins are involved in necroptosis. We wanted to further evaluate the role of Akt kinase in necroptosis by identifying Akt interacting proteins that interact with Akt under necroptotic conditions. The goals for this project were to 1) identify potential known Akt interacting proteins during necroptosis 2) identify potential novel Akt interacting proteins during necroptosis and 3) understand the role that these interacting proteins play in necroptosis. We identified three proteins, Arf1, Nur77, and GAPDH, to investigate further.

We found that Arf1 interacted with Myr-Akt in L929 cells when were serum starved. However, this interaction was not changed by the addition of zVAD.fmk or Nec-1. Using shRNA knockdown of Arf1 to evaluate its role in cell death was inconclusive. However, the Arf1 inhibitor, BFA, was able to protect the cells from death indicating that Arf1 may indeed play a role in necroptosis in L929 cells. Additionally, the BFA data indicated that transport and secretion of TNFα may be attenuated by inhibition of Arf1 in necroptosis in L929 cells.

Nur77 interacts with Myr-Akt in L929 cells more readily during the induction of necroptosis. Knockdown of Nur77 was inconclusive and further analysis needs to be undertaken. One interesting point is that Nur77 was phosphorylated at the Akt phosphorylation S351 site during necroptosis in an AKT and RIP1 kinase dependent fashion. Indicating that this interaction may be important during necroptosis and should be evaluated further.

The necroptosis specific interaction of GAPDH with Myr-Akt was confirmed and a role for GAPDH in necroptosis in L929 cells was revealed. Knockdown of GAPDH significantly protected zVAD.fmk treated L929 cells from death. This protective effect of GAPDH knockdown was not seen in other cell types. The endogenous interaction of GAPDH and Akt remains to be proven. Interestingly, changes in GAPDH complexes may play a role in cell death. We observed the formation of high molecular weight GAPDH complexes during zVAD.fmk induced death in L929 cells.

Chapter VI: Discussion and Future Directions

6.1 Introduction

Our objective was to characterize the role of Akt kinase in necroptosis by understanding of how it becomes activated in response to pro-necrotic stimuli and elucidating the downstream consequences of its activation in the process of cell death. We also sought to identify novel Akt interacting proteins and to characterize the role that these proteins play in necroptosis.

In this study we investigated the role of RIP1 kinase-dependent signaling pathways in mouse fibrosarcoma L929 cells zVAD.fmk-induced necroptosis. Altogether, our results suggest that Akt kinase is specifically engaged in signaling downstream from RIP1 kinase, which leads to a selective increase in its phosphorylation on Thr308, but not Ser473. According to our model (Figure 6.1), necroptosis-associated phosphorylation of Akt requires two distinct signals. The first input, which is induced by growth factors, leads to the plasma membrane localization of Akt. Expression of a constitutively membrane-targeted Akt construct, Myr-Akt, overcomes the requirement for growth factors. At the same time, expression of Myr-Akt alone is not sufficient for the induction of necroptosis. A second, RIP1 kinase-dependent input is required for Thr308 phosphorylation of Akt in response to caspase inhibition and is essential for the propagation of the necroptotic signal.

Using Akt inhibitors, knockdown of Akt isoforms, and the expression of Akt mutants, we showed that necroptotic activation of Akt is indispensable for this form of cell death in L929 cells. We also investigated downstream Akt-dependent pathways that contribute to necroptosis. First, we demonstrated that selective necroptotic phosphorylation of Thr308 of Akt is sufficient to increase its activity towards a number of known substrates and Akt effector pathways such as the mTORC1 pathway, which, in turn, contributes to cell death. Second, our data suggested that Akt activation provides a pivotal link connecting RIP1 kinase to known downstream signaling and execution events in necroptotic L929 cells, namely, JNK activation and autocrine TNFα synthesis, a critical event in necroptosis in L929 cells. We went on to discover several putative Akt interacting proteins, including Arf1, Nur77, and GAPDH, and began to describe the role that they play in cell death. Overall, our results provide new insights into the mechanism of necroptosis and the role of Akt kinase in both cell death and inflammatory regulation.

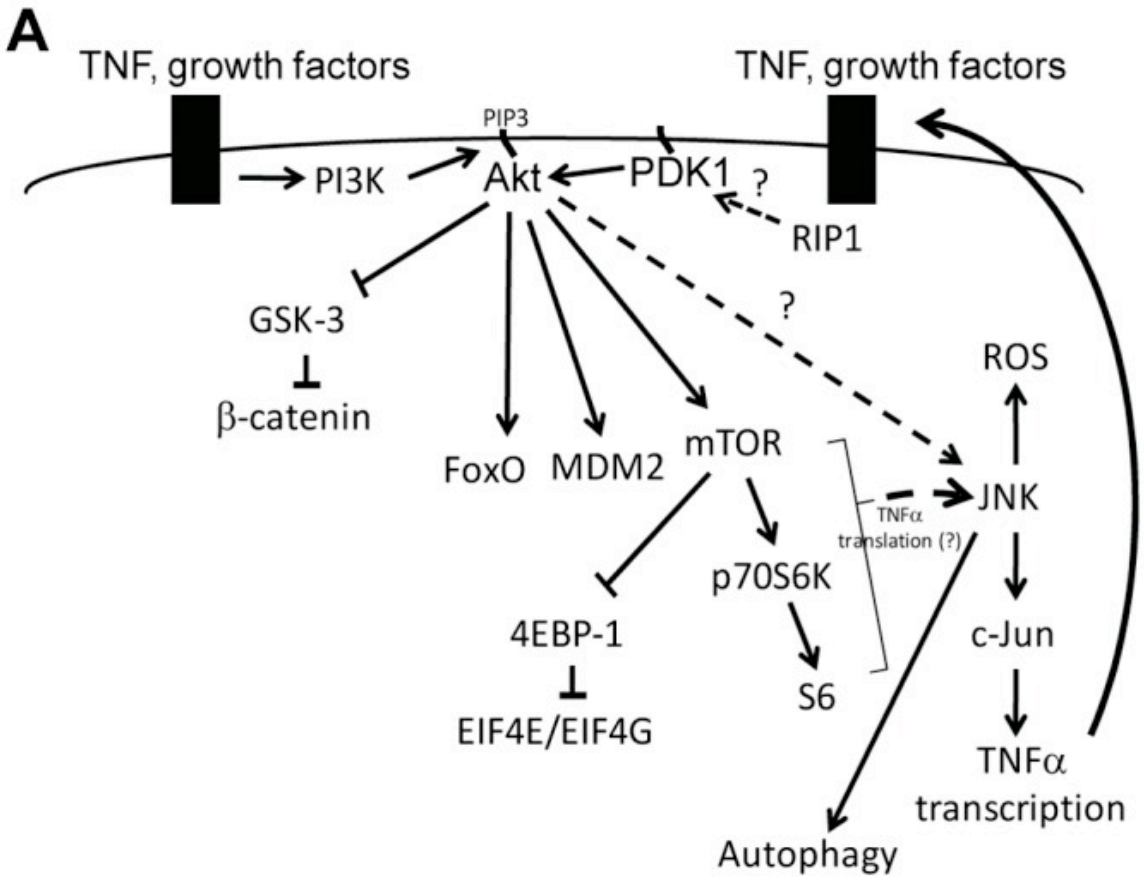

Figure 6.1 Model of RIP1, Akt and JNK dependent signaling in necroptotic L929 cells.

Figure 6.1 Model of RIP1, Akt and JNK dependent signaling in necroptotic L929 cells. Akt phosphorylation at Thr308 during necroptosis requires inputs from both growth factors and RIP1 kinase. Downstream from Akt, JNK activation leads to TNFα synthesis. Activation of Akt during necroptosis also leads the phosphorylation of several known Akt substrates, such as mTOR, which contribute to the execution of necroptotic death in L929 cells.

6.2 Akt is Activated in the L929 Mouse Fibrosarcoma Cell Line During Necroptosis

In Chapter III we demonstrated that Akt is phosphorylated at Thr308 during necroptosis and that this phosphorylation contributes to the induction of necroptosis in L929 cells. The necroptosis-associated phosphorylation of Akt requires two distinct signals, one from growth factors and the other from RIP1 kinase. We also demonstrated that phosphorylated and activated Akt contributes to cell death. This illustrated to us that Akt, a protein that is typically thought of as being a pro-survival protein, can also act as a pro-death molecule depending on cellular circumstances. However, two main questions still need to be addressed including 1) Why and how is Akt getting phosphorylated in a delayed manner only at Thr308 and 2) Why do some growth factors and not others facilitate necroptosis?

These data show us that the delayed phosphorylation of Akt on Thr308 is regulated in the predicted way, meaning that the phosphorylation is mediated by PI3K and PDK1. This indicated that the canonical Akt signaling pathway is being utilized in the expected fashion but a more thorough understanding of how and why Akt is actually getting phosphorylated during necroptosis remains to be seen. As, discussed earlier, inhibition of PP2A, the phosphatase that dephosphorylates Akt at Th308, by okadaic acid did not effect Thr308 phosphorylation or the activation of necroptosis in L929 cells (data not shown). It would be interesting to see whether a PP2A agonist such as forskolin effected cell death. It would be expected that increased PP2A activity would decrease

Thr308 phosphorylation and inhibit necroptosis. Additionally, it was recently shown that RIP1 could lead to the down regulation of PTEN (Park et al., 2009). PTEN is a phosphatase that "turns off" PIP_3 dependent signaling pathways, such as the Akt pathway, by converting PIP_3 to PIP_2. PTEN expression and activation remains to be explored in our model system. However, we might expect that PTEN levels/activation would be decreased in our system allowing for increased Akt signaling. Importantly, our lab did test whether RIP1 can directly phosphorylate Akt. Results *in vitro* suggested Akt can be phosphorylated by RIP1, however we were unable to confirm that this phosphorylation occurs endogenously (data not shown). Another possibility is that a novel complex between Akt and PDK1 is being formed and allowing PDK1 specific phosphorylation of Akt during necroptosis.

These data also raise the interesting question as to why some growth factors, such as bFGF and IGF, but not others, such as PDGF and EGF, were able to mediate cell death. We showed that PDGF signaling is intact in these cells by showing that PDGF stimulated Akt phosphorylation at 15 minutes after treatment. It remains to be discovered then why PDGF could not mediate cell death while bFGF was able to do so. It is possible that understanding these questions better will help us understand why Akt can act as both a pro-survival and a pro-death molecule.

6.3 Regulation in Necroptosis Downstream of Akt

In Chapter IV we found that multiple canonical Akt effector proteins are activated during necroptosis and that these proteins also play a role in necroptosis. Akt pathway molecules including FoxO1, FoxO4, Mdm2, p70S6K, and S6 were all phosphorylated in a RIP1 kinase dependent manner during necroptosis. Some of the proteins, such as mTORC1, p70S6K and S6 were also shown to play a role in necroptosis. Akt and mTOR both play an important role in the upregulation of TNFα during necroptosis. Finally, we confirmed that Akt activation occurs downstream of RIP1 kinase activation and upstream of JNK activation based on JNK inhibitor and knockdown data. TNFα production however, may be more critically controlled by c-Jun rather than JNK. In multiple other models of necroptosis, Akt kinase was phosphorylated on Thr308 in response to necroptotic stimuli and TNFα was produced in an Akt dependent fashion. However, Akt kinase activity did not control cell death in the alternative models of necroptosis indicating an important role for Akt in inflammatory signaling.

Three main issues remain to be further investigated. First, we identified that many canonical Akt pathway proteins are activated during necroptosis. However, in this work we only began to investigate a few of theses molecules such as mTOR and S6. The roles, if any, that the other proteins, such as FoxO4 and Mdm2, play in necroptosis remains to be seen.

Second, we identified that both Akt and mTOR play a role in necroptosis. One consideration in conjunction with the regulation of cell death by Akt is autophagy. Akt activation leads to the inhibition of autophagy through activation

of mTOR (Meijer and Codogno, 2004). The role of autophagy in cell death in general is very complex and it can both promote and inhibit necroptosis in various situations. Several studies suggested that activation of autophagy promotes necroptosis induced by zVAD.fmk in L929 cells (Chen et al., 2011; Yu et al., 2004). However, our group and others (unpublished data), found that inhibition of autophagy promotes necroptosis by TNFα (Ye et al., 2011). This suggests that the inhibition of autophagy by Akt or mTOR in our system may contribute to necroptosis induced by TNFα, however, it is more difficult to reconcile with the positive role of these proteins in zVAD-induced death. Further identification of the factors differentiating between pro-death and pro-survival autophagy in mammalian cells is required to better understand its role in the regulation necroptosis by Akt.

Third, we arrived at the unexpected, but important conclusion that c-Jun, rather than JNK, is critical for necroptosis using inhibitor and knockdown data. JNK activation, while serving a useful marker of pathway activation, may be functionally either redundant or dispensable. In addition, researchers need to use caution when using SP600125 as it may be less accurate than using JNK knockdown due to off target effects. The role of JNK and Jun activation in necroptosis remains to be fully elucidated. Knockdown of JNK does not seem to be sufficient to protect L929 cells from death or inhibit TNF mRNA production. It remains to be seen whether c-Jun is being phosphorylated at a different site by a different kinase in response to necroptosis in the absence of JNK activation (e.g. phosphorylation of c-Jun on a site other than Ser63 (Cho et al., 2009b) thereby

allowing for TNFα transcription to occur. Alternatively, in absence of c-Jun activation, other components of the AP-1 complex may mediate TNFα transcription. One important next step is to utilize Tam67, a c-Jun dominant-negative construct, to determine whether AP-1 is the critical factor for TNFα transcription in necroptosis in L929 cells. These important questions remain to be explored.

6.4 Identification of Novel Akt Interacting Proteins

We wanted to further evaluate the role of Akt kinase in necroptosis by identifying proteins that interact with Akt under necroptotic conditions. The goals for this project were to identify potential known and novel Akt interacting proteins during necroptosis and to understand the role that these interacting proteins play in necroptosis. We identified three proteins, Arf1, Nur77, and GAPDH, to investigate further.

Arf1 is a small GTPase that is localized to the Golgi and ER that plays a role in membrane trafficking. To the best of our knowledge an interaction between Arf1 and Akt had not previously been described. We found that Arf1 interacts with Myr-Akt but that this interaction did not change during the induction of necroptosis. Therefore it is possible that Arf1 plays a more general role in Akt trafficking. Knockdown of Arf1 expression needs to be more thoroughly explored in order verify whether Arf1 plays a role in necroptosis. We found that inhibition of Arf1 with Brefeldin A was able to inhibit cell death and potentially TNFα

trafficking. Brefeldin A works to inhibit Arf1 by stabilizing the Arf1-GDP complex with a GEF and inhibiting GDP displacement. There are several known Arf1-GEFs that are sensitive to Brefeldin A, including GBF1, BIG1, and BIG2. While we did not observe a significant dependence for Arf1 activity on necroptosis or Akt activation, understanding the role that each of these Arf-GEFs plays may be interesting.

Nur77 is an orphan nuclear receptor that can act as a transcription factor. It is previously known to interact with Akt and has a well-characterized Akt phosphorylation site. We found that Nur77 interacts with Myr-Akt in L929 cells more readily during the induction of necroptosis. Knockdown of Nur77 was inconclusive and further analysis needs to be undertaken. One interesting point is that Nur77 was phosphorylated at the Akt phosphorylation S351 site during necroptosis in an AKT and RIP1 kinase dependent fashion indicating that this interaction may be important during necroptosis and should be evaluated further. Additionally, because Nur77 belongs to the NR4A subgroup of the nuclear hormone receptors and members of this family, such as Nurr1 (NR4A2) and Nor-1 (NR4A3) have been reported to be function redundantly (Martinez-Gonzalez and Badimon, 2005), it will be interesting to investigate the potential role of these other family members in necroptosis. Finally, cellular localization of Nur77 at the mitochondria or in the nucleus plays a significant role in its cellular functions. Localization changes of Nur77 during necroptosis should be evaluated. Knowledge of its localization could give a hint as to its possible function in necroptosis.

GAPDH is best known as a key enzyme in glycolysis. However, it has recently begun to be appreciated as a protein with diverse cellular functions. We found a necroptosis specific interaction of GAPDH with Myr-Akt and a role for GAPDH in necroptosis in L929 cells. Importantly, interactions between Akt and GAPDH have recently been reported (Baba et al., 2010; Huang et al., 2011; Jacquin et al., 2013). In particular, Akt phosphorylation of GAPDH at Thr237 was shown. It would be very interesting to understand the role that this phosphorylation site plays in necroptosis in L929 cells. We showed that knockdown of GAPDH in L929 cells protects cell from death but that this protective effect was not seen in other cell types. It could be possible that L929 cells depend on GAPDH for energy production more than other cell types and we need to understand whether the protection from death that we show is purely from inhibition of glycolysis rather than from non-glycolytic functions of GAPDH. Finally, GAPDH has also been shown to play a role in apoptosis through interactions with a protein call Siah1. Upon interaction with Siah1, GAPDH localizes to the nucleus. The Siah1/GAPDH interaction is impeded by a protein called GOSPEL. The potential roles that Siah1/GOSPEL and cellular localization of GAPDH might play in L929 cell induced necroptosis should be evaluated in the future.

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