8-2014

THE ROLE OF TRAF6 PHOSPHORYLATION IN Src/TRAF6-MEDIATED IKK, JNK, Akt ACTIVATION AND TUMORIGENESIS

Yun Seong Jeong

THE ROLE OF TRAF6 PHOSPHORYLATION IN Src/TRAF6-MEDIATED IKK, JNK, Akt ACTIVATION AND TUMORIGENESIS

by

Yun Seong Jeong, M.S.

APPROVED:

_____

Hui-Kuan Lin, Ph.D. Supervisory Professor

_____

Xin Lin, Ph.D.

_____

Mong-Hong Lee, Ph.D.

_____

Angabin Matin, Ph.D.

_____

Dos Sarbassov, Ph.D.

APPROVED:

_____

Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

THE ROLE OF TRAF6 PHOSPHORYLATION IN Src/TRAF6-MEDIATED IKK, JNK, Akt ACTIVATION AND TUMORIGENESIS

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

And

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Yun Seong Jeong, M.S.

Houston, Texas

August, 2014

Dedication

I dedicate this dissertation to my dearest family

Ji-Hyun Shin

Alice Heejin Jeong

Ryan Seokho Jeong

And my parents and sisters

The late Pyo-Young Jeong

Keun-Nam Lee

Sang-Ok Jeong

Ji-Yeon Jeong

Hee-Jung Jeong

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my supervisor, Dr. Hui-Kuan Lin. Dr. Lin was willing to accept and support me when I had a hard time in finding a new lab. His support and guidance throughout my graduate training has helped me mature as a scientist. I am also very grateful to have such a great academic committee, Dr. Xin Lin, Dr. Mong-Hong Lee, Dr. Angabin Matin, and Dr. Dos Sarbassov. They continued to encourage me to not lose my interest and motivation in my science career. Without their valuable discussions, suggestions and guidance, the completion of my thesis study would not have been possible. I want to particularly express my gratitude to Dr. Victoria Knutson and Dr. William Mattox. Without their help, I could not make it through the hardest time of my graduate school career. I would like to thank the former and present lab mates for their technical help and critical discussions. Especially, I want Wei-Lei Yang, Abdol-Hossein Rezaeian, Yuan Gao, and Szu-Wei Lee to know how valuable your help and friendships are to me over the past 4 years.

My very special gratitude also goes to my parents and sisters. My mother, Keun-Nam Lee has shown endless love and support to me throughout my life time, I believe my father, Pyo-Young Jeong is also looking at his son's achievements in heaven with a smile. I want to thank my sisters, Sang-Ok Jeong, Ji-Yeon Jeong, and Hee-Jung Jeong for their love, encouragement and supports. Finally, I must express my sincere gratitude to my dearest wife, Ji-Hyun Shin for her ceaseless love, support, and encouragement. She shared my hardship and the happiest moment thoughout my Ph.D. study. Without her, the completion of my Ph.D. study would not have been possible. I have also been fortunate to have such wonderful kids, my adorable daughter Alice Heejin Jeong, and cute son, Ryan Seokho Jeong. Together, my family has made my life happy and perfect. Thank you all and I will always love you forever.

THE ROLE OF TRAF6 PHOSPHORYLATION IN Src/TRAF6-MEDIATED IKK, JNK, Akt ACTIVATION AND TUMORIGENESIS

Yun Seong Jeong, Ph.D.

Supervisory Professor: Hui-Kuan Lin, Ph.D.

TRAF6 E3 ligase regulates numerous essential biological processes such as innate immune response, cell survival and osteoclast differentiation. Upon activation, it mediates activation of IKK/NF-κB and JNK signaling in response to engagement of toll-like receptor 4 (TLR4), interleukin-1 receptor (IL-1R), and receptor activator of NF-κB (RANK) to their cognate ligands, including lipopolysaccharide (LPS), IL-1, and RANK ligand (RANKL). Recently, TRAF6 has also been shown to be involved in Akt signaling activation upon activation of insulin-like growth factor-1 receptor (IGF-1R), in turn orchestrating cell survival and tumorigenesis. Therefore, TRAF6 is a key player for the activation of IKK, JNK and Akt by diverse receptor signaling. Although TRAF6 activity is induced by these receptors, the mechanism by which TRAF6 is activated under these stimulating conditions remains largely unclear.

Here, we show that TRAF6 Y473 phosphorylation is commonly induced and serves as an essential mode for TRAF6 activation by multiple signaling stimuli including IGF-1, IL-1, LPS, and RANKL. We identify Src tyrosine kinase as a direct kinase for TRAF6 and a mediator of TRAF6 Y473 phosphorylation. Notably, we find that Src or Traf6 deficiency impairs IKK, Akt, and JNK activation, which can be rescued by phosphomimetic TRAF6, but not by phosphorylation-dead TRAF6 mutant.

Mechanistically, we show that TRAF6 phosphorylation is imperative for TRAF6 dimerization/oligomerization and its binding to E2 enzyme, UBC13. Significantly, we find that TRAF6 phosphorylation is critical for TRAF6-mediated cancer progression and represents a biomarker for poor survival outcome. Our study therefore resolves the long-standing puzzle of achievement of TRAF6 activation and elucidates TRAF6 phosphorylation as a central mode for Src/TRAF6-mediated downstream kinase activation and tumorigenesis

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AP-1   Activator protein -1

ASK-1   Apoptosis singal activating kinase-1

ATF   Activating transcription factor

ATP   Adenosine triphosphate

AWP1   Associated with PRK1

BAFF   B-cell activating factor

BMH   Bismaleimidohexane

CHIP   Carboxyl terminus of Hsc-70-interacting protein

c-IAP1/2   Cellular inhibitor of apoptosis protein ½

CSK   C-terminal Src Kinase

CYLD   Cylindromatosis

DUBA   Deubiquitinating enzyme A

EGFR   Epidermal growth factor receptor

ERK1   Extracellular signal-regulated protein kinase 1

GCK   Glucokinase

GSK3-β Glycogen synthase kinase-β

GST   Glutathione S-transferase

HA   Hemagglutinin

HSP90   Heat-shock protein 90

HECT   Homologous to the E6-AP carboxyl terminus

HectH9   Homologous to E6AP carboxyl terminus homologous protein 9

HGF-R	Hepatocyte growth factor receptor
IFN	Interferon
IGF-1R	Insulin-like growth factor-1 receptor
IκB	Inhibitor of NF-κB
IL-1	Interleukin-1
IKK	Inhibitor of nuclear factor kappa-B kinase
IRAK1	IL-1 receptor-associated kinase 1
JAK2	Janus Kinase2
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LTβ	Lymphotoxin-β
MAPK	Mitogen-activated protein kinase
MEF	Mouse Embryonic Fibroblast
MEKK1	Mitogen-activated protein kinase kinase kinase 1
MyD88	Myeloid differentiation primary response 88
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappa B
N-HTT	N-terminal fragment of mutant huntingtin
NIK	NF-κB inducing kinae
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NUMBL	Num homolog like
OUT	Ovarian tumor-like protease
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDGF-R	Platelet derived growth factor receptor
PDK-1	Phosphoinositide-dependent kinase-1
PKC	Protein Kinase C
PKN1	Protein Kinase N1
PTP	Protein tyrosine phosphatase
RANKL	Receptor activator of NF-κB ligand
RHO	REL-homology domain
RING	A really interesting new gene
RNF8	Ring finger protein 8
RTK	Receptor tyrosine kinase
SCF-R	Stem cell factor receptor
SHP1	SH2 domain-containing protein tyrosine phosphatases 1
Smad2/3	SMAD family member 2/3
SOCS2	Suppressor of cytokine signaling2
TACE	TNF-α converting enzyme
TCPTP	T-cell protein tyrosine phosphatase
TAK1	TGF-β activating kinase-1
TBK1	TANK-binding kinase
TβRI	Type I serine/threonine kinase TGF-β receptor
TGF-β	Tumor growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor

TRAF6	TNF receptor-associated factor 6
UBC13	Ubiquitin-conjugating enzyme 13
UBCH5	Ubiquitin-conjugating enzyme H5
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1

CHAPTER 1

INTRODUCTION

1-1.   Ubiquitination pathways

Protein ubiquitination is involved in regulating numerous essential biological processes [1,2,3]. Initially, protein ubiquitination is considered as a posttranslational modification for protein degradation through 26S proteasome pathway [4]. Recently, it has also been found that protein ubiquitination has non-proteolytic functions such as receptor endocytosis, DNA damage repair, protein trafficking, and regulation of kinase signaling activation (Figure 1-1) [1,5,6].

Ubiquitin is a highly conserved 76 amino acid protein [7]. Ubiquitination is a three - step enzymatic process that covalently attaches the ubiquitin(s) to lysine (K) residues of target proteins[7]. There are three classes of enzymes, E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase), which participate in the ubiquitination process.   Initially, ubiquitin is activated by E1 enzyme in an ATP-dependent manner and the activated ubiquitin is then transferred to E2 enzyme. Next, the E3 ubiquitin ligase recognizes its specific target proteins, and then the activated ubiquitin is transferred from E2 to target proteins (Figure 1-1)[8]. During the ubiquitination process E2 enzyme determines the formation of ubiquitin chains. Ubiquitin has seven lysine residues, K6, K11, K27, K29, K33, K48 and K63, which are all involved in the formation of ubiquitination chains [9]. So far, K48-linked and K63-linked ubiquitination have been well studied. The K48-linked ubiquitination chain is recognized by the 26S proteasome for protein degradation [7]. Although it is known that most E2s trigger the K48-linked ubiquitination, resulting in protein degradation through 26S proteasome, the roles of ubiquitination are not restricted to protein degradation. Mono and multiubiquitination formation play roles in endocytosis, apoptosis, regulation of

transcription, and DNA repair. The K63-linked ubiquitination chain has non-proteolytic functions such as receptor endocytosis, DNA damage repair, protein trafficking and kinase signaling activation [1,2,3,10,11] (Figure 1-1). Although other types of ubiquitin-linked chains are also observed, the exact physiological relevance of these linkages is still unclear [8,12,13]. Interestingly, it has been recently reported that TRAF6 is implicated in pathogenesis of neurodegenerative diseases including Huntington diseases and Parkinson's disease by promoting atypical ubiquitination of misfolded mutant DJ-1, α-synuclein and N-HTT, proteins related to Parkinson's disease and Huntington disease pathogenesis. Instead of the typical K63–linked ubiquitination of target proteins, TRAF6 was shown to promote K6-, K27-, and K29-linked ubiquitination of misfolded mutant DJ-1, α-synuclein and N-HTT resulting in aggregation of mutant DJ-1, α-synuclein and N-HTT in neurodegenerative disease [14,15].

Although most E2 enzymes facilitate the K48-linked ubiquitination, one of the E2 enzymes, UBC13, mostly mediates the K63-linked ubiquitination with its cofactor UEV1A [9]. The E3 ubiquitin ligase family members fall into two groups, which comprise of homologous to the E6-AP carboxyl terminus (HECT) domain group and a really interesting new gene (RING) domain group [1]. Whereas most E3 ligases recognize substrates for K48-linked ubiquitination, it is known that TRAF6, HectH9 (homologous to E6-AP carboxyl terminus homologous protein 9), c-IAP1/2 (cellular inhibitor of apoptosis protein 1/2), and RNF8 (ring finger protein 8) recognize target protein for K63-linked ubiquitination [1,16,17,18,19].
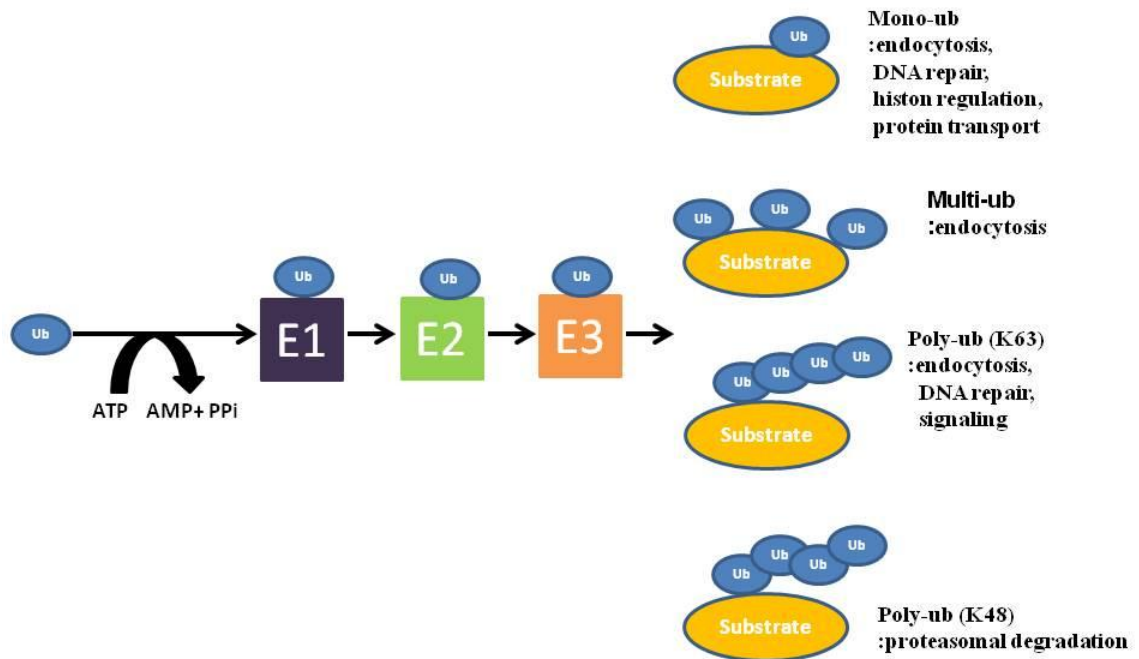
Figure1-1.Ubiquitination regulates protein degradation or activation.

Ubiquitination is a three step enzyme reaction. Ubiquitin is activated by the E1, ubiquitin-activating enzyme, and is then transferred to an E2, ubiquitin-conjugating enzyme. The ubiquitin will be finally transferred from the E2 to a lysine residue of target protein by E3 ubiquitin ligase. Ubiquitination regulates the fate of proteins. The K48– linked ubiquitination of target protein leads to degradation through the 26S proteasome pathway. Whereas the K63-linked ubiquitination regulates signaling activation involved in various biological functions including DNA damage, receptor endocytosis and protein trafficking.

1-2.   The tumor necrosis factor receptor (TNF-R)-associated factors (TRAFs)

TRAFs were first identified as cytoplasmic adaptor proteins that are directly associated with numerous TNF-R superfamily [20,21,22]. There are seven TRAF family members identified (Figure 1-2) in mammals. Among TRAFs, TRAF6 also mediates signaling other than TNF-R superfamily. For example, TRAF6 is participated in IL-1R, IL-18R, TLR and Akt signaling [22,23].

The TRAF proteins consist of N-terminal RING-finger, which facilitates E3 ligase functions, a zinc-finger motif and C-terminal TRAF-C domain, which mediate TRAF oligomerization and interactions with upstream regulators including receptors and downstream effector proteins [21,22,24] (Figure 1-2). Among TRAFs, TRAF2, TRAF3, TRAF5 and TRAF6 have been demonstrated to have E3 ligase activity, which mediate K63-linked ubiquitination [22,25,26].

The TRAFs have distinct expression patterns. TRAF2, 3 and 6 are expressed ubiquitously. However, TRAF1, 4, and 5 are expressed more distinctly. TRAF1 is expressed in tonsil, spleen, lung and testis [27], TRAF4 is expressed during embryogenesis and in neural tissues [28], and TRAF5 expression has been observed in spleen, lung, and thymus [29,30].
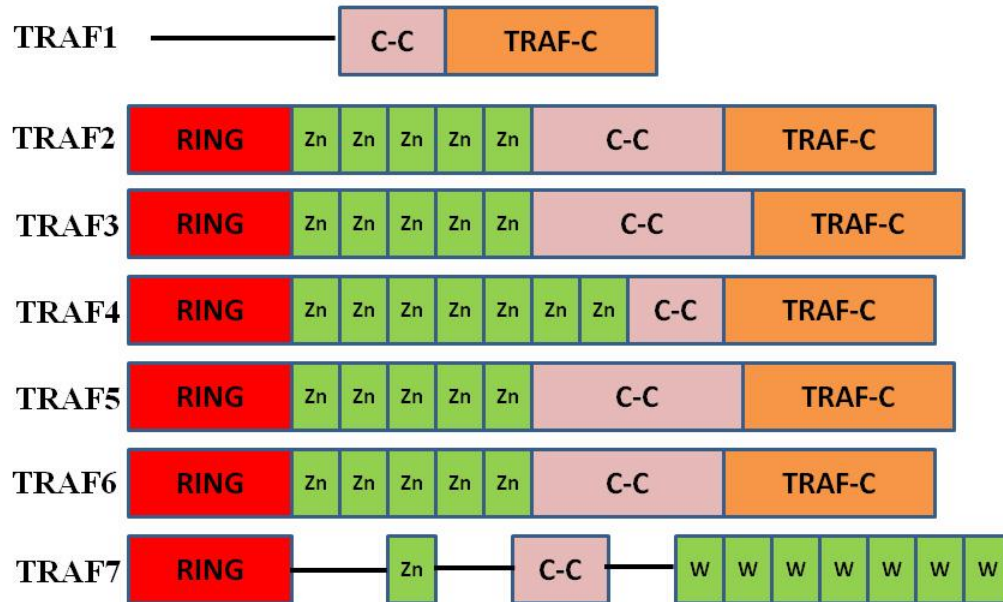
Figure 1-2. Characteristics of TRAF protein domain.

The TRAF family member consists of seven proteins. Except TRAF1, TRAFs have a RING domain which facilitates E3 ligase function and zinc-finger motif. TRAF-C domain is highly conserved among the TRAFs and mediate TRAF oligomerization and associations with upstream regulators including receptors and downstream effector proteins. TRAF7 has seven WD40 domains instead of TRAF-C. TRAF7 interacts with MEKK3 using WD40 domain.

1-3. TRAF proteins in activating NF-κB and AP-1 transcription factors

Association of TRAFs to TNF-R typically leads to activation of NF-κB and AP-1 transcription factors. NF-κB is a dimeric transcription factor consisting of c-Rel, RelA (p65), RelB, p50, and p52. NF- κB has a highly conserved REL-homology domain (RHD) that is required for DNA binding, dimerization, nuclear translocation and interaction with IκB protein [31,32,33]. NF-κB activation pathways can be divided by either canonical or the non-canonical pathway depending on involving IκB degradation or p100 processing [33,34]. In the canonical pathway, stimulation with TNF-R, IL-1R and TLRs induce activation of IκB kinase (IKK) complex consisting of IKKα, IKKβ, and NEMO (IKKγ). The active IKK complex phosphorylates IκB, resulting in its degradation. The NF-κB is then released from IκB and translocates into the nucleus to regulate target genes that control cytokines, adhesion molecules, transcription factors, and survival factors. In the non-canonical pathway, stimulation with BAFF, Lymphotoxin-β(LTβ) and CD40 ligand induce activation of the protein kinase NIK and IKKα, leading to p100 phosphorylation, ubiquitination, and production of p52. The p52 and RelB then translocate into the nucleus to regulate target gene expression.

AP-1 transcription factor is a dimeric complex, which consists of Jun, Fos or activating transcription factor proteins including ATF2, ATF3, or B-ATF. It is known that a stress-related MAP kinase, Jun N-terminal kinase (JNK) activates c-Jun to regulate target genes that control cell cycle, cell survival, and apoptosis, metalloproteinases, and nuclear hormone receptors [35]. TRAF proteins activate JNK by activating upstream MAP3Ks such as TGF-β activating kinase 1(TAK1)[36], ASK1[37,38], MEKK1[39], and MAP4K GCK[40].

1-4.   Regulation of TRAF proteins in signaling

Ubiquitination is a key regulatory mechanism of TRAFs in signaling. As mentioned in **1-1**, K63-linked ubiquitination of TRAFs mediates non-degradation functions such as promoting protein interaction and signal transduction. For example, in IL-17R signaling, Act1 catalyzes K63-linked ubiquitination of TRAF6 leading to NF-κB activation [41], while Pellino3 ubiquitinates TRAF6 at Lys124 leading to limited ability of TRAF6 to induce IRF7 ubiquitination. This results in inhibiting production of type I interferon (IFN) in TLR3 signaling pathway [42]. In contrast to K63-linked ubiquitination, K48-linked ubiquitination of TRAF leads to degradation of TRAFs through 26S proteasome pathway. For example, cIAP1/2, Triad3A, AWP1, SOCS2, Siva-1, Numbl and CHIP have been shown as K48-linked E3 ligases of TRAFs [43] (Table 1-1).

Deubiquitinases also regulate TRAFs (Table 1-2). A NF-κB target gene, CYLD has negative impacts on NF-κB and JNK activation by removing K63-linked ubiquitin chains of TRAF2 and TRAF6 [44,45]. Up-regulation of DUBA expression is observed in TLR and IL-1R stimulated cells [46,47,48]. DUBA removes the K63-linked ubiquitin chains of TRAF3, leading to TBK1-IKKε separation from TRAF3 and reducing production of Type I IFN in TLRs [46,47,48]. Another interesting example of deubiquinase, A20, contains both ubiquitinating and deubiquitinating activities. A20 negatively regulates NF-κB activation in TNF-R and TLR signaling. A20 is also an NF- κB target gene and removes K63-linked ubiquitin chains of TRAF6 to inhibit NF-κB activation. Interestingly, A20 is able to block E3 ligase activities of TRAF2, TRAF6, cIAP1 by catalyzing K48-linked ubiquitination and degradation of E2 enzymes, UBC13 and UBCH5 [25,46,49]. In addition, A20 is able to target TRAF2 for lysosomal degradation.

In addition to ubiquitination and deubiquitination, it is also reported that phosphorylation and glutathionylation regulate TRAFs in signaling. PKN1-mediated TRAF1 S146 phosphorylation negatively regulates TNF-R2-dependent NF-κB and JNK signaling activation in HeLa cells and T cells [50,51]. Phosphorylation of TRAF2 by PKCζ, IKKε, PKCδ, or PKCε promotes K63-linked ubiquitination of TRAF2 and NF-κB activation in TNF-R signaling and transformed cells [52,53,54,55]. IKKα-mediated TRAF4 S426 phosphorylation upon NOD2 (nucleotide binding-oligomerization domain 2) activation inhibits NF-κB activation, cytokine production, and antibacterial activity in macrophage [56]. Interestingly, it is reported that under normal conditions TRAF6 is S-glutathionylated. Upon IL-1 stimulation, glutathionylation of TRAF6 is removed by glutaredoxin-1 and this deglutathionylation is required for TRAF6 auto-ubiquitination and TRAF6-mediated NF-κB activation [57].

Table 1-1. E3 ligases that promote ubiquitination of TRAFs

E3 ligases	Targets	Receptor signaling	Ubiquitin-linkage
Act1	TRAF6 (Lys124)	IL-17R	K63
	TRAF5	IL-17R	K63
cIAP1/2	TRAF3 and TRAF6	RIG-1	K63
	TRAF2	TNF-R2	K48
	TRAF2	M-CSFR	K48
	TRAF2 and TRAF3	CD40 and TLR4	K48
	TRAF3 and TRAF6	RIG-I	K48
Pellino3	TRAF6 (Lys124)	TLR3	K63
Triad3A	TRAF3	RIG-I	K48
AWP1	TRAF2	TNF-R1/2	K48
SOCS2	TRAF6	AhR	K48
Siva-1	TRAF2	TCR	K48
Numbl	TRAF6 and TRAF5	IL-1R	K48
CHIP	TRAF2	Cancer cell invasion	K48

Table 1-2. Deubiquiting enzymes of TRAFs

DUBs	TRAFs	Signaling	Reference
A20	TRAF6	TLR2, TLR4	[25,46,49]
CYLD	TRAF2,TRAF6	CD40,XEDAR,EDAR,RANK IL-1R, TNF-α	[44,45]
DUBA (OUTD5)	TRAF3	IL-1R,TLR9,TLR3,TLR4, TLR7, RIG-I, MDA-5	[46,47,48]
MCPIP1	TRAF2,TRAF3,TRAF6	IL-1R, TLR4	[58]
OTUB1	TRAF3,TRAF6	RIG-I	[59]
OTUB2	TRAF3,TRAF6	RIG-I	[59]
USP2a	TRAF2	TNFR1	[60]
	TRAF6	IL-1R and RIG-1	[61]
USP4	TRAF2,TRAF6	TNF-α	[62]
	TRAF6	IL-1R,TLR4	
USP20	TRAF6	IL-1R	[63]
USP25	TRAF5,TRAF6	IL-17R	[64]

1-5. TRAF6 regulates various signaling pathways

TRAF6 is a unique TRAF family member involved in TLR-4/IL-1- and TNFR-mediated NF-κB and AP-1 activation. Upon activation with cognate ligands of TLR4/IL-1R such as lipopolysaccharide (LPS) or IL-1, MyD88, IRAK1 and IRAK4 are associated with the cognate receptor. After IRAK4 phosphorylates IRAK1, phosphorylated IRAK1 then interacts with TRAF6, leading to activated E3 ligase function and K63-linked ubiquitination. Even though this model is recently challenged, these K63-linked ubiquitin chains of TRAF6 have been reported to serve as a scaffold to recruit to TAK1 and IKK complexes [1,65]. TAK1 phosphorylate IKK complex and MAP kinase, which result in activation of NF-κB and AP-1[1,65,66] (Figure 1-3).

In addition to LPS and IL-1, TRAF6 also mediates CD40 ligand (CD40L), receptor activator of NF-κB ligand (RANKL)-induced NF-κB activation [67]. TRAF6 is also participated in TGF-β-mediated activation of JNK and p38, but not canonical activation of Smad signaling pathway [68,69,70,71,72]. Whereas the kinase activity of type I serine/threonine kinase TGF-β receptor (TβRI) is required for activation of Smad signaling pathway, TRAF6 activates and regulates TAK1 activation. Upon TGF-β stimulation, TRAF6 is recruited to TβRI leading to TRAF6 autoubiquitination and activation. This enhances TRAF6 mediated K63-linked ubiquitination and activation of TAK1, resulting in phosphorylation and activation of p38 and JNK to regulate cell apoptosis in a receptor independent manner [68,69,70,71,72]. Interestingly, in cancer cells, it is reported that TRAF6 is also involved in TGF-β-mediated cancer cell invasion [73]. Upon stimulation with TGF-β, TRAF6 promotes K63-linked ubiquitination of TβRI resulting in TβRI cleavage by TNF-α converting enzyme (TACE) in a PKCζ-dependent

manner [73]. The intracellular domain of TβRI then interacts with the p300 to regulate genes controlling invasion of cancer cells. In addition, TRAF6 also mediates inhibitory impact of LPS or IL-1β on TGF-β-mediated signaling pathway. Co-stimulation with TGF-β and LPS or IL-1β induces TRAF6 ubiquitination and association with phosphorylated TβRIII. This releases TβRIII from TβRII/TβRI complex, leading to inhibition of Smad2/3 activation [74]. These suggest that TRAF6 may have multiple functions in TGF-β-receptor -mediated signaling.

Recently, TRAF6 has also been shown to be involved in Akt signaling activation upon activation of insulin-like growth factor-1 receptor (IGF-1R), in turn orchestrating cell survival and tumorigenesis [23]. We showed that TRAF6 is an E3 ligase for Akt and induces its K63-linked ubiquitination, leading to Akt membrane recruitment, phosphorylation, and activation upon stimulation by growth factor IGF-1[23]. Therefore, TRAF6 is a key player for the activation of IKK, JNK and Akt by diverse receptor signaling.
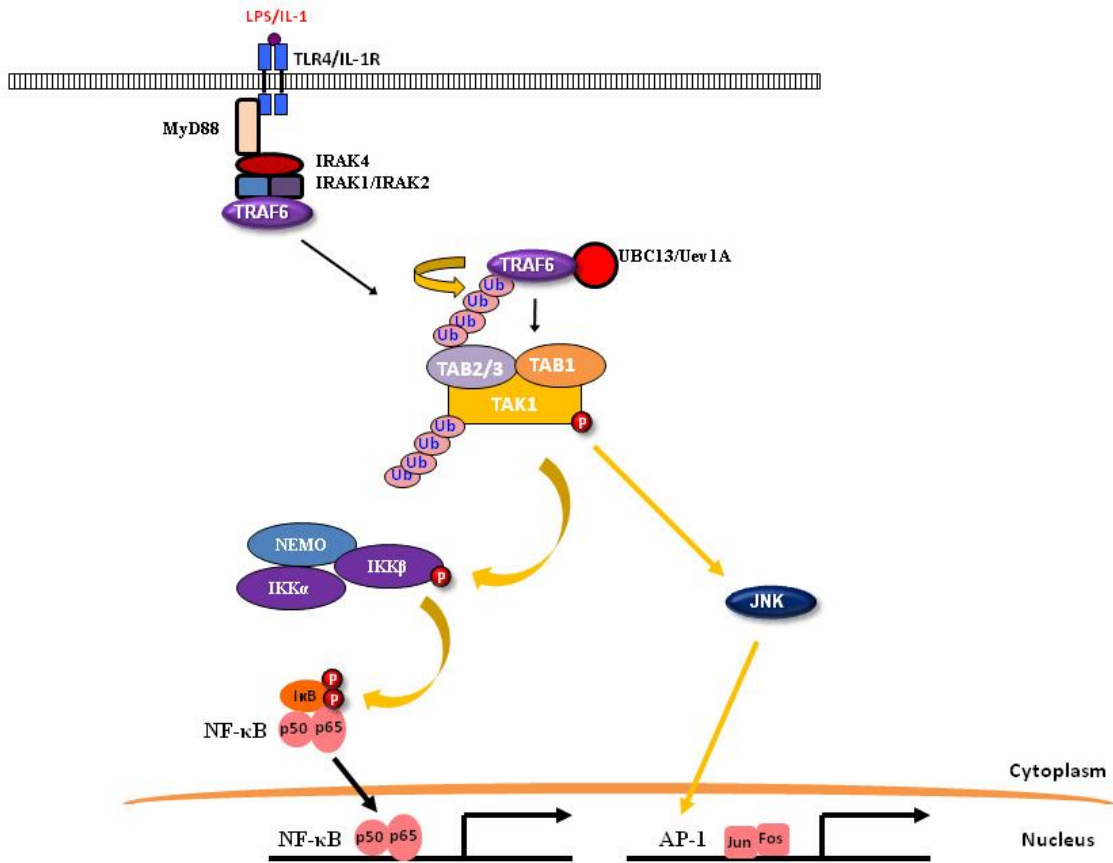
Figure 1-3. TRAF6-mediated activation of NF-κB and AP-1 in the TLR4 and IL-1R

Ligand-stimulated TLR4 and IL-1R recruit the adaptor MyD88, which in turn recruits

IRAK1 and IRAK4. IRAK4 is activated and phosphorylates IRAK1. Phosphorylated

IRAK1 then associates with TRAF6. This association leads to TRAF6 activation and then

TRAF6 promotes the synthesis of K63-linked polyubiquitin chains. These chains serve as

a scaffold to recruit both TAK1 and IKK complexes. TAK1 phosphorylate IKK complex

and MAP kinase, which result in activation of NF-κB and AP-1.

1-6. Src family tyrosine kinases and their structures

The Src tyrosine family kinases (SFKs) are implicated in regulating multiple signal transduction pathways that regulate cell differentiation, migration, invasion, proliferation, and cell survival [75]. They are non-receptor type tyrosine kinases consisting of 9 kinase members, Src, Fyn, Yes, Frk, Blk, Fgr, Hck, Lck and Lyn. Their distribution and expression patterns are distinct in normal tissues. For examples, Src, Fyn, Yes, and Frk are ubiquitously found, whereas Blk, Fgr, Hck, Lck, and Lyn are expressed in specific cells [76,77] (Table 1-3).

SFKs share well conserved distinct protein domains including Src homology 4 (SH4) domain, Src homology 3(SH3) domain, Src homology 2 (SH2) domain, kinase domain (SH1), and a short C-terminal tail (Figure.1-4). The N-terminal SH4 domain facilitates myristoylation and palmitoylation. It is well reported that myristoylation and palmitoylation regulate membrane recruitment, kinase activity and stability [77,78,79]. SH3 and SH2 domains play important roles in binding adaptor proteins and phosphotyrosine residue on itself. The SH1 Kinase domain regulates enzymatic activities and substrate binding. A C-terminal domain contains negative regulatory site that interacts with the SH2 domain (Figure 1-4).

Table1-3.Known functions and expression pattern of Src family kinases

SFKs	Expression	Functions	Reference
Src	Ubiquitous	Cell proliferation,adhesion,migration, chemotaxis,phagocytosis and survival	[80]
Fyn	Ubiquitous	T-cell inhibition and Rac and stress kinase activation	[81]
Lyn	Platelet, granulocyte Monocyte, macrophage, B cell	Netrophil recruitment Inhibition of platelet aggregation	[82]
Lck	T cell, B cell	Regulation of signaling and B-1 cell fate	[83]
Hck	Granulocyte,monocyte, macrophage	Inhibition of Hck and Fgr kinase activity	[84]
Fgr	Granulocyte,monocyte, macrophage	Inhibition of Hck and Fgr kinase activity Inhibition of β2integrin receptor and Syk kinase signaling	[84,85]
Blk	Granulocyte,monocyte, macrophage, B cell	Control of b cell proliferation	[86]
Frk	Ubiquitous	Unclear	
Yes	Ubiquitous	LTB4-mediated degranulation	[87]
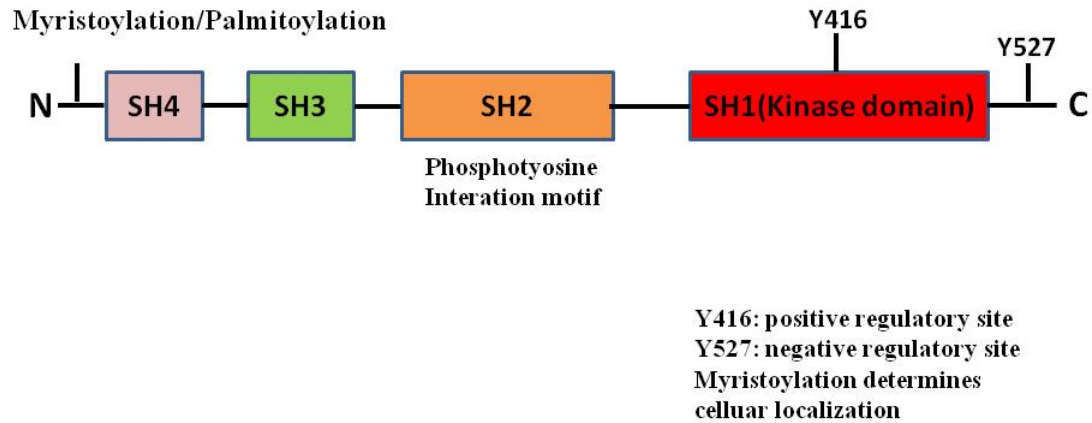
Figure1-4. The structure of Src kinase.

The Src is consists of an N-terminal myrisotylation or palmitoylation sequence attached to the SH4 domain followed by SH3 and SH2 domains, a kinase domain (SH1) which contains positive regulatory site Y416, and C-terminal regulatory domain which contains Y527.

1-7. The regulation of SFK activity

The regulation of SFK activity is achieved through both intramolecular and intermolecular interactions. SFK kinase activity is majorly regulated by conformational changes by phosphorylation and dephosphorylation of its own two major tyrosine residues, Tyr 527 and Tyr 416. Particularly, Tyr 527 phosphorylation is critical regulatory site for Src activity regulation. SFK activity is negatively regulated when Tyr 527 is phosphorylated, interacts with the SH2 domain, and at the same time, the SH3 domain interacts with polyproline motifs. In this closed conformation, ATP and substrate binding are prevented. Contrarily, dephosphorylation of Tyr 527 leads to conformational change from closed configuration to open configuration by disrupting the interaction of the SH2 or SH3 domain with C-terminal tail and proline motifis. This allows the SFK kinase to access substrate proteins and lead to autophosphorylation of Tyr 416, which is critical for full kinase activity [79,88,89,90] (Figure 1-5). This inhibitory phosphorylation of Tyr 527 in SFK can also be achieved by two other tyrosine kinases, C-terminal Src kinase (Csk)[91] and Csk homology kinase [92].

Interestingly, different regulatory phosphorylation site has been reported recently. It has been shown that human epidermal growth factor receptor2 (HER2) or platelet-derived growth factor receptor (PDGFR)-mediated phosphorylation of Src Y212 inhibit interaction with C-terminal regulatory sequence, leading to Src activation[93].

It is also known that protein phosphatases are involved in regulating SFK activity by dephosphorylating Tyr 527 in SFKs. Multiple protein tyrosine phosphatases (PTPs) including T-cell protein tyrosine phosphatase (TCPTP), the SH2 domain-containing protein tyrosine phosphatases 1 and 2 (SHP1 and SHP2), PTP1B, PTPα, PTPε, PTPκ,

and receptor-PTPα (R-PTPα) dephosphorylate Tyr 527 and induce active formation of

SFKs[94,95].
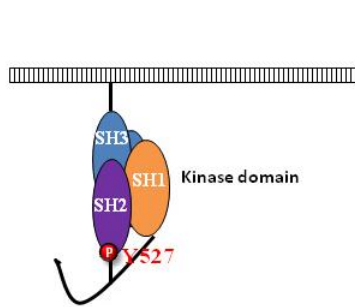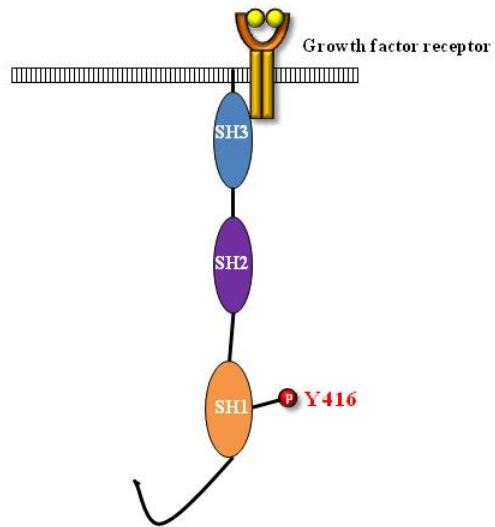
Figure 1-5. Activation of Src tyrosine kinase.

Phosphorylation of Y527 is a key negative regulatory event associated with closed

conformation. SFKs are activated when extracellular ligands such as growth factors bind

to cognate receptors. Phosphorylation of Y416 is a key positive molecular event for full

kinase function.

CHAPTER 2

MATERIALS

AND

METHODS

2-1. Cell culture

Wild-type and *Traf6$^{-/-}$* MEFs were prepared as described previously [23]. *Src$^{-/-}$* MEF cells were kind gift from Dr. Zhimin Lu (MD Anderson cancer center, Houston, TX). HEK 293, HEK 293T, PC-3, Hep3B, MDA-MB-231 and MEF cells were grown in DMEM containing 10% FBS, and Raw264.7 cells were grown in RPMI medium containing 10% FBS.

2-2. Reagents

Recombinant active Src and RANKL were purchased from Millipore. Src inhibitor, PP2, was purchased from Sigma. IGF-1 was purchased from Calbiochem and Sigma. LPS, murine and human IL-1β was purchased from PeproTech. BMH cross-linker was obtained from Thermo Scientific. Glutathione-agarose beads were purchased from Invitrogen. 8-µm polyethylene terephthalate membrane inserts were purchased from Becton-Dickinson. Matrigel was purchased from BD sciences.

2-3. Plasmids

To create pWZL-HA-TRAF6 construct, the Traf6 gene was amplified from pCR3-Flag-TRAF6 by PCR and then subcloned into pWZL-Hygro vector with a HA-tagged sequence. TRAF6 mutant constructs ,pCR3.1-Flag-TRAF6 Y56F, pCR3.1-Flag-TRAF6 Y56E, pCR3.1-Flag-TRAF6 Y68F, pCR3.1-Flag-TRAF6 Y68E, pCR3.1-Flag-TRAF6 Y217F, pCR3.1-Flag-TRAF6 Y217E, pCR3.1-Flag-TRAF6 Y231F, pCR3.1-Flag-TRAF6 Y231E, pCR3.1-Flag-TRAF6 Y326F, pCR3.1-Flag-TRAF6 Y326E, pCR3.1-Flag-TRAF6 Y353F pCR3.1-Flag-TRAF6 Y353E, pCR3.1-Flag-TRAF6 Y406F,

pCR3.1-Flag-TRAF6 Y406E, pCR3.1-Flag-TRAF6 Y418F, pCR3.1-Flag-TRAF6 Y418E, pCR3.1-Flag-TRAF6 Y473F, pCR3.1-Flag-TRAF6 Y473E, pMX-Flag-TRAF6 Y473F, pMX-Flag-TRAF6 Y473E, pMX-Flag-TRAF6 Y473D, pGEX-4X1-TRAF6 Y473F, pWZL-HA-TRAF6 Y473E, pWZL-HA-TRAF6 Y473F were generated by standard mutagenesis protocol using a site-directed mutagenesis kit (Stratagene). Src Y527F and Src K295R plasmids were gifts from Dr. Dihua Yu (MD Anderson cancer center, Houston, TX).

2-4. Immunoprecipitation (IP) and Immunoblottng (IB).

IP and IB were carried out as described previously [23,83]. For IP experiment, PBS washed cells were harvested, and lysed with E1A lysis buffer [250mM NaCl, 50mM HEPES, (pH7.5), 0.1% NP40, 5 mM EDTA, 5 mM NaV, 1 mM NaF , 1 mM PMSF, protease inhibitor cocktail (Roche)]. 1-2 mg Protein lysates were then incubated with specific antibody at 4ºC for 2hrs to overnight. Protein A/G beads (Santa Cruz) were added and incubated at 4ºC for 1-3 hrs to pull down antibody-protein complex. Following antibodies were used for IP and IB experiments: anti-phospho-tyrosine (IB: 1:1000; Cell Signaling), anti-phospho-tyrosine (IB: 1:500; Santa Cruz), anti-TRAF6 (IP: 1:100; IB: 1: 10000; LifeSpan Biosciences), anti-TRAF6 (IP: 1:200; IB: 10000; Calbiochem), anti-TRAF6 (IP: 1:100; IB: 1:1000; Santa Cruz), anti-phospho (Y473)-TRAF6 (IB: 1000; New England Peptide), anti-Src (IB: 1:1000, Cell signaling), anti-phospho (Y416)-Src (IB: 1: 1000; Cell signaling), anti-phospho (Y527)-Src (IB: 1:1000; Cell signaling), anti-ubiquitin (IB: 1:1000; Santa Cruz), anti-panAkt (IB: 1:5000; Cell Signaling), anti-phospho (T308)-Akt (IB: 1: 1000; Cell Signaling), anti-phospho (S473)-Akt (IB: 1:1000; Cell Signaling), anti-phospho-Foxo1 (T24)/Foxo3a (T32) (IB: 1:1000;

Cell Signaling), anti-Foxo3a (IB: 1:1000; Millipore), anti-phospho (S176/180)-IKKα/β (IB: 1: 1000; Cell Signaling), anti-phospho (S32/36)-IκBα (IB: 1: 1000; Cell Signaling), anti-phospho (T187)-TAK-1(IB: 1: 1000; Cell Signaling), anti-phospho (T183/Y185)-JNK(IB: 1:1000; Cell Signaling), anti-Flag (M2, IP: 1:200; IB: 1: 3000, Sigma), anti-HA (IB: 1: 10000, Covance, Upstate), anti-β-actin (IB: 1:10000;Sigma), and anti-UBC13 (IB: 1:1000; Invitrogen).

2-5. *In Vitro* Src kinase assay.

Purified recombinant GST-TRAF6 and GST-TRAF6 Y473F proteins were used for substrates of Src kinase. BL-21 bacterial cells were transformed with pGEX-4X-1-TRAF6 and pGEX-4X-1-TRAF6 Y473F plasmids. GST-TRAF6 and GST-TRAF6 Y473F proteins were then purified by using glutathione-agarose beads according to the manufacturer's manual. Purified GST-TRAF6 and GST-TRAF6 Y473F proteins were incubated with recombinant active Src protein for 15min at room temperature in the 1×kinase buffer (25mM Tris-HCl pH7.5, 5mM β-glycerophosphate, 2mM dithiothreitol, 0.1mM Na_3VO_4, 10mM $MgCl_2$, and 0.5mM ATP). Reactions were terminated by boiling in 2× SDS loading buffer. IB analysis was then carried out with the indicated antibodies.

2-6. *In Vivo* Ubiquitination Assay.

In vivo ubiquitination assays were carried out as previously described [23,83]. HEK 293T cells were co-transfected with the indicated plasmids for 24-48hr and PBS washed cells were harvested, and lysed in the denaturing buffer (6M guanidine-HCl, 0.1M Na2HPO4/NaH2PO4, 10 mM imidazole). The cell extracts were then incubated with

nickel beads for 3hr at room temperature. IB analysis was then carried out with the indicated antibodies.

2-7. Generation of phospho-TRAF6 (Y473) antibody.

The phospho-TRAF6 (Y473) peptide (CRNPKGFGY^{P}VTFMH) was synthesized and injected into the rabbit three times. Double affinity purification was then carried out according to the manufacturer's standard procedures (New England Peptide). For immunoblotting, the phospho-TRAF6 (Y473) antibody was blocking with the TRAF6 non-phospho-peptide (CRNPKGFGYVTFMH) [5 (peptide): 1 (antibody); w/w] at 4˚C overnight before performing IB experiment.

2-8. Protein cross-linking.

To evaluate TRAF6 oligomerization, protein cross-linking was carried out according to the manufacturer's standard procedure. In brief, cytokine or growth factor stimulated cells were washed with ice cold PBS and then lysed and incubated with 0.2mM BMH at room temperature for 1hr. The reaction was terminated by adding and boiling with 2x SDS loading buffer, followed by IB analysis with the indicated antibody.

2-9. Cell growth assay.

For the cell growth assay, 5×10^3 cells were seeded in 12 well plates, collected, and stained with trypan blue on the indicated days. Viable cells were counted under the microscope.

2-10. Cell migration and invasion assays.

The cell migration assay was carried out in 24-well transwell plate with 8-µm polyethylene terephthalate membrane filter inserts (Falcon cell culture insert; Becton-Dickinson). 10^5 PC-3, Hep3B, or MDA-MB-231 cells with Mock, TRAF6 wild-type, Y473D, and Y473F stable overexpression were plated in the upper chambers with serum free DMEM. DMEM media containing 10% FBS was put in the bottom chamber. After 18 hr incubation for PC-3 and Hep3B or 5hr incubation for MDA-MB-231, cells in the top of the filter were wiped out and migrating cells on the lower filter were fixed with 4% formaldehyde for 20min, and stained with Crystal violet for 20min. Three to five random fields were then counted. The cell invasion assay was carried out similarly to cell migration assay, but using matrigel-coated filter inserts (Matrigel: serum free DMEM, 1:5).

2-11. Viral infection and in vivo tumorigenesis assay

Viral infection and *in vivo* tumorigenesis assay were performed as described previously [23]. Phoenix packaging cells were transfected with retroviral plasmid PMX, PMX-TRAF6, PMX-TRAF6 Y473D, and PMX-TRAF6 Y473F for were 2 days, and the virus-containing media were collected, filtered and used to infect target cells. For lentiviral shRNA production, TRAF6-lentiviral shRNA (5'-GCCACGGGAAATATGTAATATCT-3') and GFP control shRNA(5'-gcaagctgaccctgaagttc-3') were used. TRAF6 or GFP control shRNA plasmids along with packaging plasmids (delta VPR8.9) and envelop plasmid (VSV-G) were co-transfected into HEK 293T cells using Lipofectamine 2000 reagent according to the manufacturer's

standard manual for 2days. Filtered virus particles were then used to infect 293 cells. All infected cells were selected in media with 2 μg/ml puromycin for 1 week. For *in vivo* tumorigenesis assay, Mock, TRAF6 WT, TRAF6 Y473D, TRAF6 Y473F stably overexpressing PC-3 cells (4×10^5) were mixed with matrigel (1:1) and subcutaneously injected into 6-week-old female nude mice. The tumor size was then measured every week with a caliper, and tumor volume was calculated by the following formula: $L \times W^2 \times 0.52$. *L* is the longest diameter and *W* is the shortest diameter.

2-12. Patients and human materials

The institutional review board of Chi-Mei Medical Center had approved procurement of formalin-fixed tissues and acquisition of clinical information for this study (IRB10210004). Immunoexpression was assessed on 212 consecutively treated primary breast carcinomas that had undergone modified radical mastectomy between 1997 and 2002. No adjuvant chemotherapy was given in these patients. Pathological staging was assigned based on the 7th edition of American Joint Committee on Cancer (AJCC) system.

2-13. Immunohistochemistry and scoring

The procedures of immunohistochemical studies were performed as previously described [96]. In brief, sections were cut onto an adhesive-coated glass slides at 3-μm thickness. For staining in human samples, the slides were incubated with primary antibodies targeting $pSrc^{Tyr416}$ (Cell Signaling, polyclonal, 1:50), $pTRAF6^{Tyr473}$ (New England Peptide, 1:50), $pAkt^{Ser473}$ (Cell Signaling, clone D9E, 1:25), and pIkkα/β (Cell Signaling, clone 16A6, 1:20), respectively. Primary antibodies were detected using the

ChemMate DAKO EnVision kit (DAKO, K5001). The slides were incubated with the secondary antibody for 30 minutes and developed with 3, 3-diaminobenzidine for 5 minutes. Incubation without the primary antibody was used as a negative control. Immunoexpression was scored using a multiheaded microscope to reach a consensus for each case on the H-score, defined by using the following equation: H-score = $\Sigma Pi \, (i + 1)$, where i is the intensity of the stained tumor cells (0 to 4 +), and Pi is the percentage of stained tumor cells for each intensity.

2-14. Statistical analysis

Statistical analyses were performed using the SPSS 14 software package. The Kruskal-Wallis H test and Mann-Whitney U test were used to assess the differential expression level of $pSrc^{Tyr416}$, $pTRAF6^{Tyr473}$, $pAkt^{Ser473}$, and $pIkk\alpha/\beta$ expression in relation to important clinicopathologic variables. The Pearson's correlation coefficient was used to clarify the association between $pSrc^{Tyr416}$, $pTRAF6^{Tyr473}$, $pAkt^{Ser473}$, and $pIkk\alpha/\beta$ expression levels. The endpoint analyzed was disease-specific (DSS) and distal metastasis-free survivals (MeFS), which were calculated from the starting date of surgery to the date disease-related death or metastatic events. The median period of follow-up was 103.7 months (range, 6 to 144). Univariate survival analysis was performed using the Cox proportional hazards model. Survival curves were plotted using the Kaplan-Meier method, and log-rank tests were performed to evaluate prognostic differences between groups for categorical variables. A multivariate model was performed using Cox proportional hazards regression, including parameters with univariate p<0.05. Since primary tumor status (pT) and nodal status (pN) are component

28

factors of tumor stage, only stage was enrolled into multivariate analysis. For all analyses, two-sided tests of significance were used with p=< 0.05 considered significant.

CHAPTER 3

The Role of TRAF6 Phosphorylation in Src/TRAF6-mediated IKK, JNK, Akt Activation and Tumorigenesis

A.RATIONALE

TRAF6 is a critical E3 ubiquitin ligase that regulates diverse biological processes, such as innate immunity, osteoclast differentiation, and cell survival. Upon activation, it mediates activation of IKK/NF-κB and JNK signaling activation in response to engagement of toll-like receptor 4 (TLR4), interleukin-1 receptor (IL-1R), and receptor activator of NF-κB (RANK) to their cognate ligands, including lipopolysaccharide (LPS), IL-1, and RANK ligand (RANKL) [1,66,97,98,99]. Recently, TRAF6 has also been shown to be involved in Akt signaling activation upon activation of insulin-like growth factor-1 receptor (IGF-1R), in turn orchestrating cell survival and tumorigenesis [23]. We showed that TRAF6 is an E3 ligase for Akt and induces its K63-linked ubiquitination, leading to Akt membrane recruitment, phosphorylation, and activation upon stimulation by growth factor IGF-1[23]. Therefore, TRAF6 is a key player for the activation of IKK, JNK and Akt by diverse receptor signaling. Although TRAF6 activity is induced by these receptors, the mechanism by which TRAF6 is activated under these stimulating conditions remains largely unclear. Understanding how TRAF6 is activated by these stimuli has important clinical implications for autoimmune disease, osteoporosis, and cancer.

It is well known that LPS and IL-1 act through TLR4 and IL-IR to induce TRAF6 autoubiquitination and oligomerization, which are considered as readouts for TRAF6 activation[33]. Previously our lab showed that IGF-1 also induces TRAF6 autoubiquitination and tyrosine phosphorylation. Additionally, phosphorylation is a critical regulatory mechanism for other TRAF member proteins. These suggest that TRAF6 function may also be regulated by phosphorylation.

The goal of my study is to determine the mechanism by which TRAF6 activation is achieved through multiple physiological stimuli.

B. RESULT

3-1. TRAF6 tyrosine phosphorylation is commonly induced by IGF-1R, TLR4/IL-1R, and RANK

Since TRAF6 is activated by various receptor signaling pathways, we rationalized that these stimuli drive TRAF6 activation through a common mode. Our previous studies provided us with a clue by showing that TRAF6 undergoes tyrosine phosphorylation by IGF-1[23,96], although the functional significance of this phosphorylation remains unknown. I confirmed in this study that TRAF6 tyrosine phosphorylation was induced by IGF-1 stimulation (Figure 3-1A). Next, in order to determine whether TRAF6 tyrosine phosphorylation is commonly induced by various stimuli, I examined whether other stimuli like LPS, IL-1, and RANKL can also induce TRAF6 tyrosine phosphorylation. Notably, I found that LPS, IL-1, and RANKL could readily induce TRAF6 tyrosine phosphorylation (Figures 3-1B-D). My data therefore suggest that TRAF6 tyrosine phosphorylation can be commonly induced by IGF-1R, TLR4/IL-1R, and RANK.
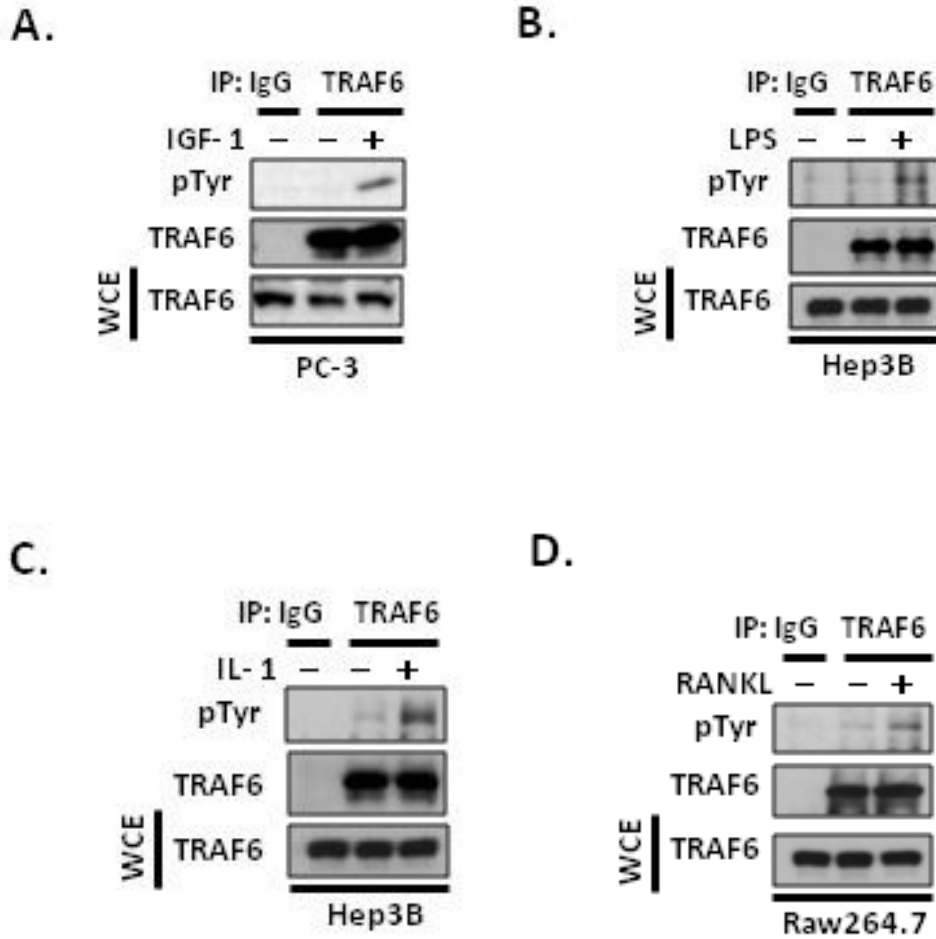
Figure 3-1. TRAF6 undergoes tyrosine phosphorylation upon IGF-1, LPS, IL-1 and RANKL stimulation

(A) PC-3 cells were serum starved for 1 day, treated with or without IGF-1(50 ng/ml) for 15min, and harvested for immunoprecipitation (IP), followed by immunoblotting (IB) analysis. WCE indicates whole-cell extracts; and pTyr, phospho-tyrosine antibody. (B) Hep3B cells were treated with or without LPS (5μg/ml) for 5min, and harvested for IP and IB analysis.

3-2. Src tyrosine kinase is responsible for TRAF6 phosphorylation

To investigate the physiological role of TRAF6 tyrosine phosphorylation, I sought to identify the tyrosine kinase(s) that mediates this phosphorylation. Since TRAF6 phosphorylation is elicited by various receptors (Figures 3-1A-D), we reasoned that the kinase(s) triggering TRAF6 tyrosine phosphorylation would be downstream of these receptors and involved in Akt and NF-κB signaling pathways. To identify potential TRAF6 kinase(s) I analyzed the TRAF6 protein sequence using Phosphomotif finder program. Examining the TRAF6 sequence revealed that several putative kinase motifs are within the TRAF6 sequence including EGFR, JAK2, and Src kinase (Figure 3-2A). Among these candidate kinases, we suspected that Src tyrosine kinase may be a kinase for TRAF6, as it has been implicated in Akt and NF-κB signaling activation [67,100,101,102,103,104,105,106]. To test this hypothesis, I first examined if there is an inducible interaction between TRAF6 and Src in response to IGF-1 stimulation. I found that indeed the interaction between Src and TRAF6 was induced within 15 min of IGF-1 stimulation and gradually declined after 30 min of treatment (Figure 3-2B). I next performed in vitro and in vivo kinase assays to examine whether Src is a kinase for TRAF6. Expression of constitutive active form of Src (Src Y527F) increased Src autophosphorylation on Y416, an active mark for Src kinase activation, and TRAF6 tyrosine phosphorylation, whereas expression of kinase-dead form of Src (K295R) failed to do so (Figure 3-2C). In vitro kinase assay revealed that recombinant active Src kinase readily induced TRAF6 tyrosine phosphorylation (Figure 3-2D). Furthermore, notably, *Src*$^{-/-}$ mouse embryonic fibroblasts (MEFs) failed to induce TRAF6 phosphorylation compared to wild-type (*WT*) MEFs upon IGF-1 stimulation (Figure 3-2E). Accordingly,

these results suggest that Src is a direct kinase that mediates TRAF6 tyrosine phosphorylation.

A.

PhosphoMotif

	Position in query protein	Sequence in query protein	Corresponding motif described in the literature (phosphorylated residues in red)	Features of motif described in the literature	Link to original article describing the motif
1	300 - 303	DPNY	[E/D]XXpY	ALK kinase substrate motif	PubMed
2	65 - 68	ESKY	[E/D]XXpY	ALK kinase substrate motif	PubMed
3	215 - 218	CEYC	X[E/D]pYX	EGFR kinase substrate motif	PubMed
4	424 - 427	GEYD	X[E/D]pYX	EGFR kinase substrate motif	PubMed
5	414 - 417	YISL	pYXX[L/I/V]	JAK2 kinase substrate motif	PubMed
6	56 - 57	YD	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
7	231 - 232	YD	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
8	426 - 427	YD	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
9	68 - 69	YE	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
10	198 - 199	YE	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
11	303 - 304	YE	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
12	389 - 390	YT	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed

Figure 3-2. Src tyrosine kinase is responsible for TRAF6 phosphorylation

(A)Potential kinase motifs within the TRAF6 protein sequence using the Phosphomotif finder program. Motif scan results from Human Protein Reference Database at http://www.hprd.org.
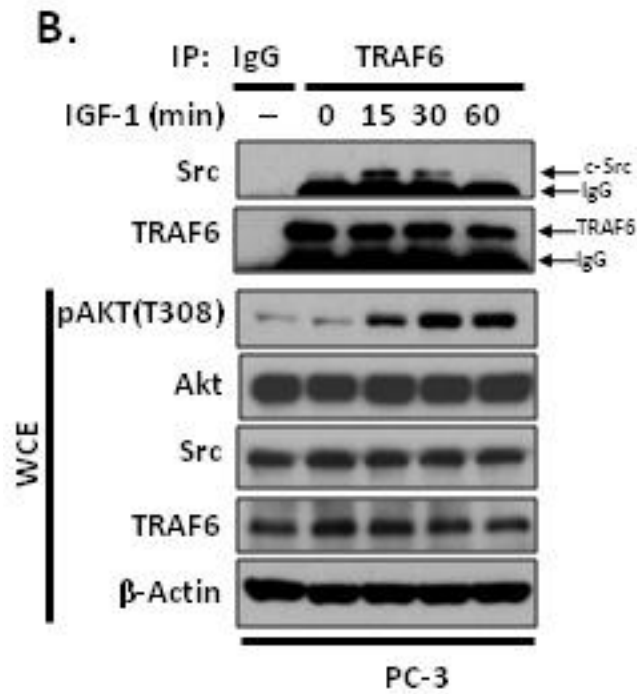
Figure 3-2. Src tyrosine kinase is responsible for TRAF6 phosphorylation

(B) Association of TRAF6 with Src is induced upon IGF-1 treatment. PC-3 cells were serum-starved for 1 day, treated with IGF-1 for indicated time points, and collected for co-immunoprecipitation assay, followed by IB analysis.
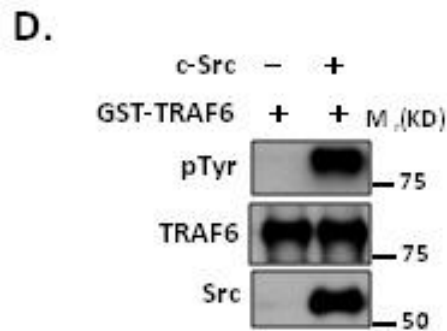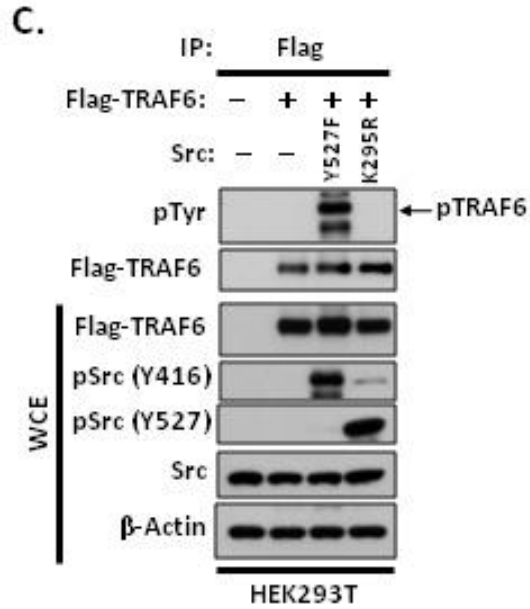
Figure 3-2. Src tyrosine kinase is responsible for TRAF6 phosphorylation

(C) In vivo Src kinase assay. HEK 293T cells were transfected with Flag-TRAF6, Src-Y527F (constitutive active form), and Src-Y295R (kinase dead), and collected for IP, followed by IB analysis. (D) In vitro Src kinase assay. GST-TRAF6 proteins were incubated with recombinant active Src for 15min, followed by IB analysis.
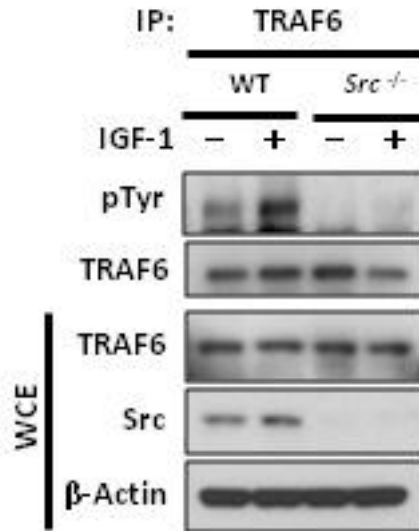
Figure 3-2. Src tyrosine kinase is responsible for TRAF6 phosphorylation

(E) Wild type (*WT*) and *Src$^{-/-}$* mouse embryonic fibroblasts (MEFs) were serum-starved for 24hrs, treated with or without 100ng/ml of IGF-1 for 15min, and harvested for IP, followed by IB analysis.

3-3. Src tyrosine kinase phosphorylates TRAF6 at Y473

In order to determine the function of TRAF6 tyrosine phosphorylation, I examined which tyrosine residue of TRAF6 is phosphorylated by Src kinase. For this aim I mutated all tyrosine residues within the TRAF6 sequence to non-phosphorylatable phenylalanine (F) or phosphomimetic glutamic acid (E) or aspartic acid (D), and subsequently carried out in vivo Src kinase assay with these TRAF6 mutants. Src-mediated TRAF6 phosphorylation was more significantly reduced on the Y473F mutant compared to TRAF6 WT and other TRAF6 mutants (Figure 3-3A). Interestingly, Y473 residue of TRAF6 is highly conserved among species and located within TRAF6 substrate binding motif (Figure 3-3B) [107]. Importantly, in vitro kinase assay confirmed that Src-mediated TRAF6 tyrosine phosphorylation was significantly reduced in the TRAF6 Y473F mutant (Figure 3-3C), suggesting that TRAF6 Y473 residue is a major phosphorylation site for Src kinase. To further validate these results, I generated a phospho-specific antibody against Y473 tyrosine phosphorylation of TRAF6. In vitro and in vivo kinase assays indeed confirmed that TRAF6 Y473 phosphorylation was induced by Src kinase (Figures 3-3D and 3E). I further showed that this phospho-TRAF6 antibody is specific for TRAF6 Y473 phosphorylation since this antibody detected the signal in TRAF6 WT, but not in TRAF6 Y473F mutant (Figures 3-3D and 3E).
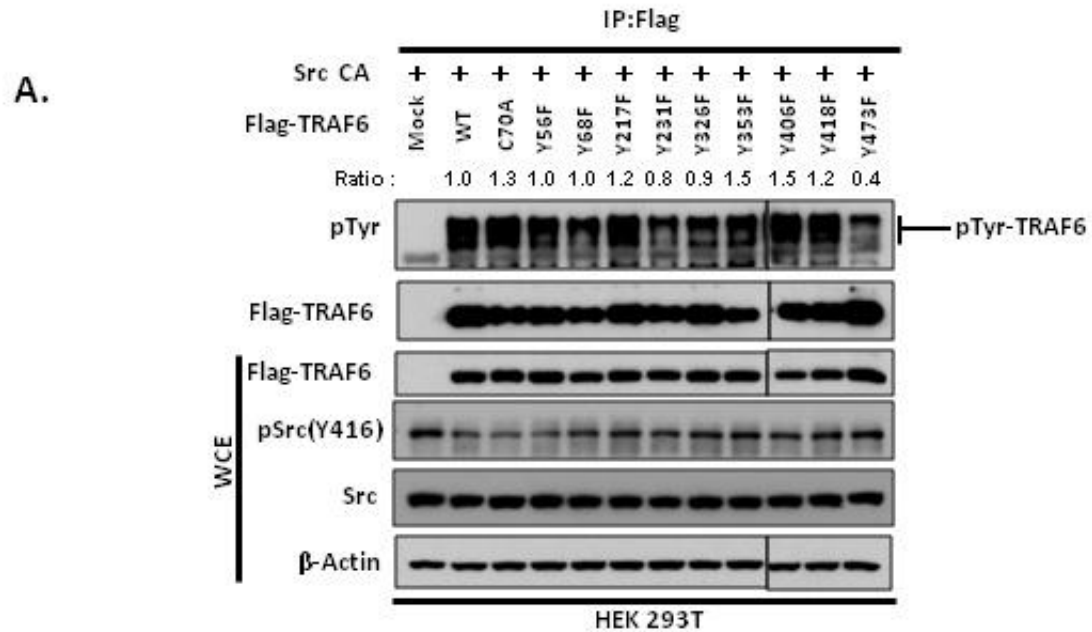
Figure 3-3. Src tyrosine kinase phosphorylates TRAF6 at Y473 residue.

(A) Identification of Src-mediated TRAF6 Phosphorylation Site. HEK 293T cells were transfected with indicated plasmids and harvested for IP, followed by IB analysis. C70A indicates TRAF6 C70A mutant, which loses E3 ligase activity and Src CA indicates Src constitutive active form (Src Y527F). (B) Alignment of TRAF6 proteins among species.
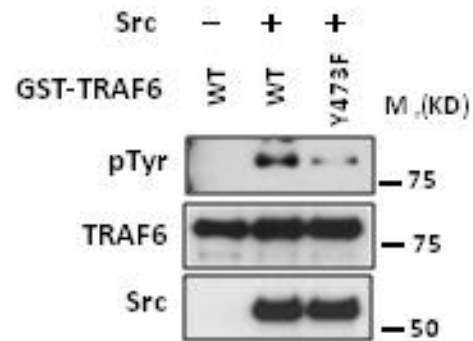
Figure 3-3. Src tyrosine kinase phosphorylates TRAF6 at Y473 residue

(C) In vitro Src kinase assay. GST-TRAF6 and GST-TRAF6 Y473F were incubated with recombinant active Src for 15 min, followed by IB analysis. (D) In vitro Src kinase assay with anti-pTRAF6 (Y473) antibody. Recombinant GST-TRAF6 proteins were incubated with recombinant active Src for 15min, followed by IB analysis with indicated antibody.
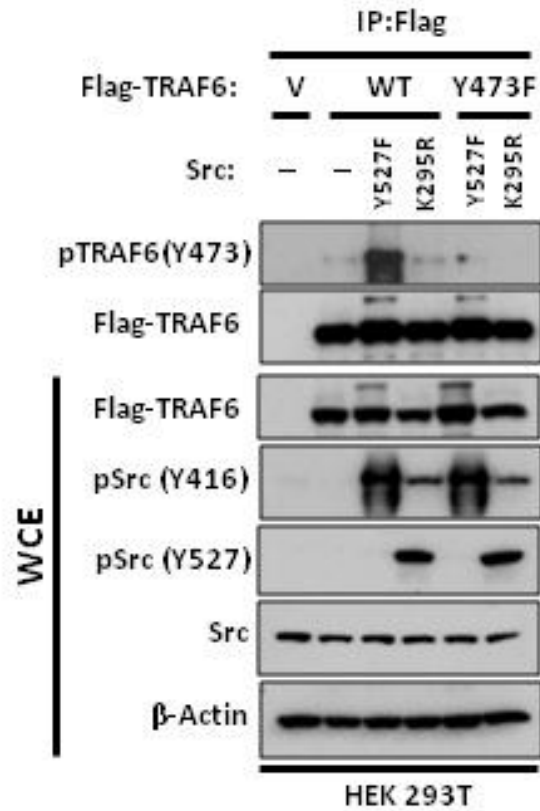
Figure 3-3. Src tyrosine kinase phosphorylates TRAF6 at Y473 residue

(E) In vivo Src kinase assay with anti-pTRAF6 (Y473) antibody. HEK 293T cells were transfected with Flag-TRAF6, Src-Y527F, and Src-Y295R, and harvested for IP, followed by IB analysis with indicated antibody.

3-4. TRAF6 Y473 phosphorylation is induced by IGF-1, LPS, IL-1 and RANKL stimulation in a Src tyrosine kinase dependent manner.

Since various receptors trigger TRAF6 tyrosine phosphorylation (Figures 3-1A-D), I determined whether TRAF6 Y473 phosphorylation can also be induced by these receptor signaling, and if so, it is Src-dependent. To this end, I stimulated *WT* MEFs and *Src*$^{-/-}$ MEFs with different ligands and preformed the immunoblotting assay. Notably, I found that Y473 phosphorylation of TRAF6 was induced by IGF-1, LPS, IL-1, and RANKL in *WT* MEFs, but not in *Src*$^{-/-}$ MEFs (Figures 3-4A-D). I further confirmed that TRAF6 Y473 phosphorylation induced by IGF-1, LPS, and IL-1 could be seen in other cell lines (Figures 3-4E-G). Moreover, I found that RANKL also induced TRAF6 Y473 phosphorylation in Raw 264.7 cells, which was significantly blocked by a Src inhibitor, PP2 (Figure 3-4H), suggesting that Src is involved in TRAF6 Y473 phosphorylation upon RANKL stimulation, although kinase(s) other than Src may be also involved. These results suggest that Y473 tyrosine phosphorylation is commonly induced by diverse stimuli in a Src-dependent manner.

Intriguingly, I found that Src was activated not only by IGF-1 and RANKL, but also LPS and IL-1, which is correlated to Akt, IKK-NF-κB, and JNK activation (Figures 3-4A-H). Notably, *Src* deficiency or inhibition impaired activation of Akt, IKK-NF-kB, and JNK in response to IGF-1, LPS, IL-1, and RANKL (Figures3-4A-H), consistent with previous reports showing that Src kinase inhibitor, PP1 and PP2 inhibited LPS- or IL-1 induced NF-κB activation [102,103,104]. Accordingly, these results suggest that Src is activated by distinct stimuli and is required for TRAF6 Y473 phosphorylation, Akt, IKK-NF-κB, and JNK activation.
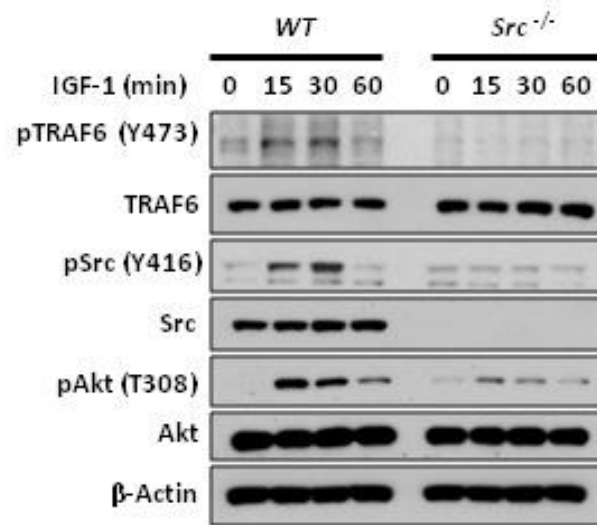
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(A) IGF-1 induces TRAF6 Y473 phosphorylation in a Src tyrosine kinase dependent manner. *WT* and *Src*$^{-/-}$ MEFs were serum-starved, stimulated with IGF-1 for indicated time points, and harvested for IB analysis.
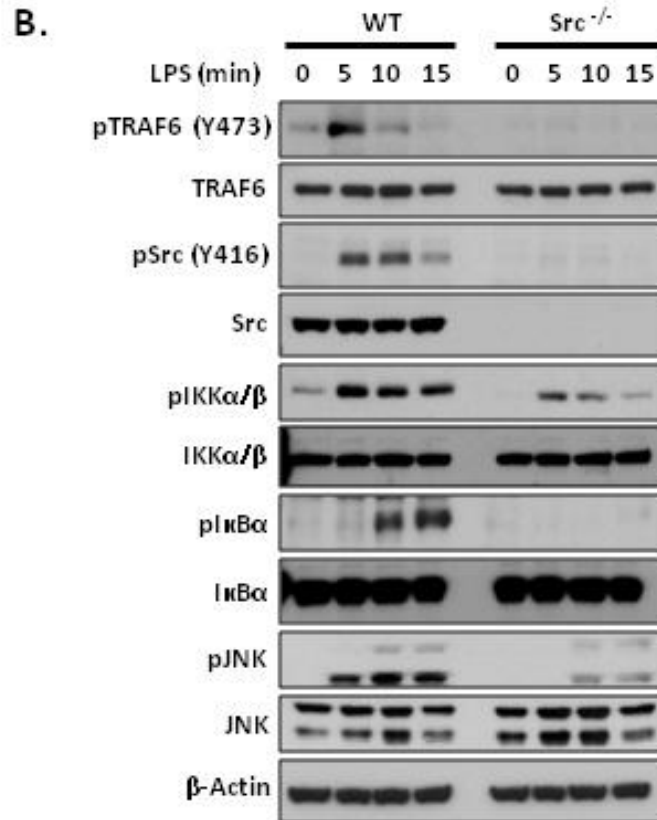
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(B) LPS induces TRAF6 Y473 phosphorylation in a Src tyrosine kinase dependent manner. *WT* and *Src$^{-/-}$* MEFs were stimulated with LPS (5µg/ml) for indicated times, and collected for IB analysis.
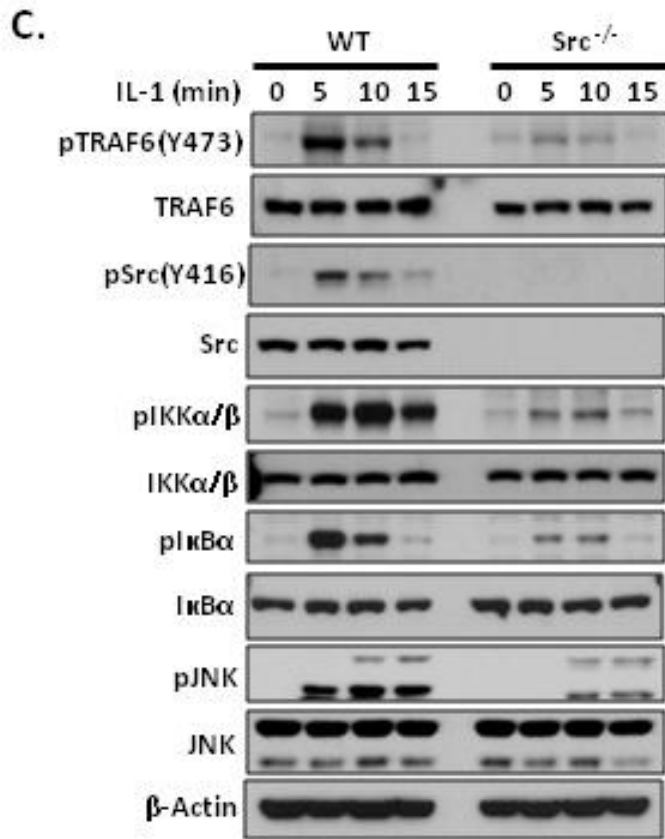
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(C) IL-1 induces TRAF6 Y473 phosphorylation in a Src tyrosine kinase dependent manner. *WT* and *Src*^{-/-} MEFs were stimulated with murine IL-1 (10ng/ml) and collected for IB analysis.

D.
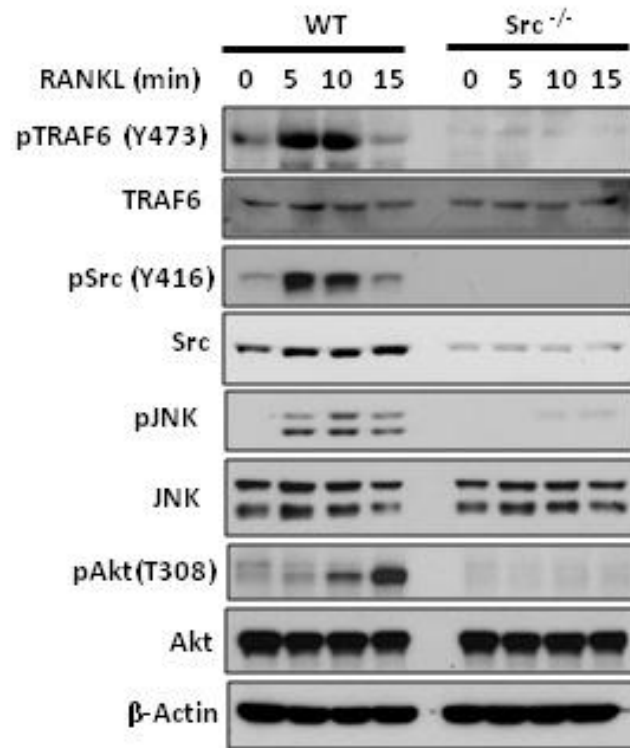
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(D) RANKL induces TRAF6 Y473 phosphorylation in a Src tyrosine kinase dependent manner. *WT* and *Src$^{-/-}$* MEFs were stimulated with RANKL (100ng/ml) for indicated times, and collected for IB analysis.
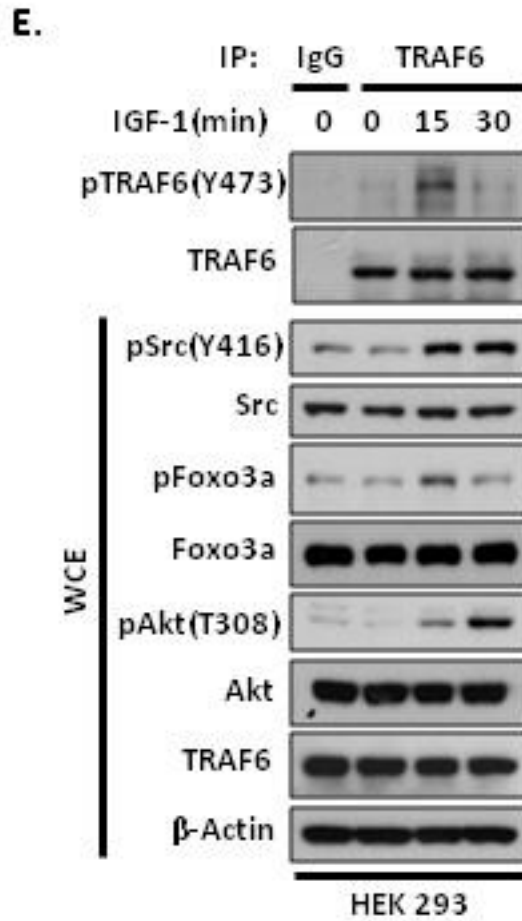
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(E) HEK 293 cells were serum-starved for 1 day, treated with IGF-1 for indicated time points, collected for IP, and followed by IB analysis.
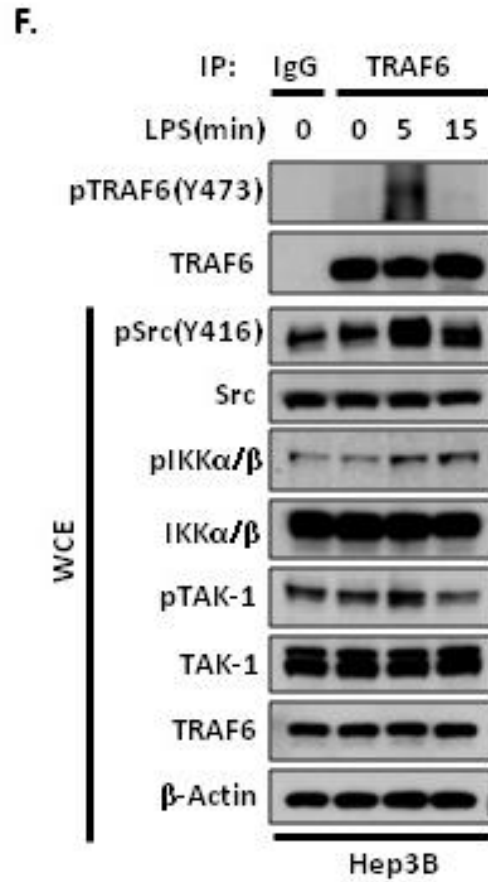
F.

Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(F) Hep3B cells were treated with LPS for indicated time points, harvested for IP, and followed by IB analysis.
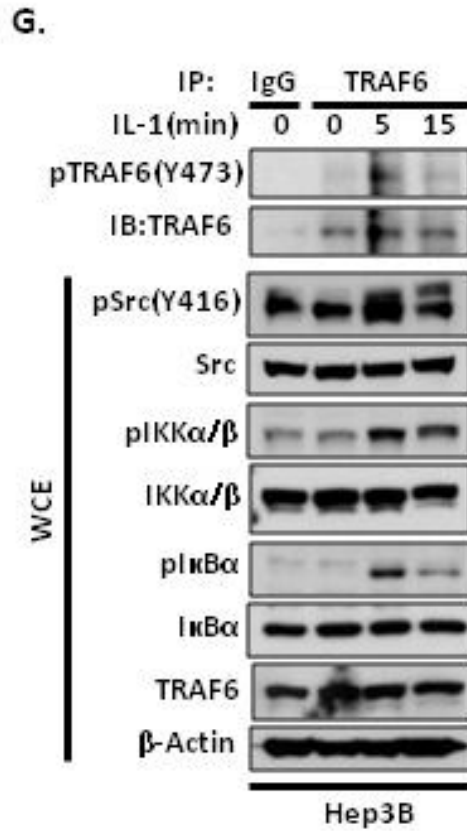
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

 (G) Hep3B cells were treated with IL-1 for indicated time points, harvested for IP, and followed by IB analysis.

H.
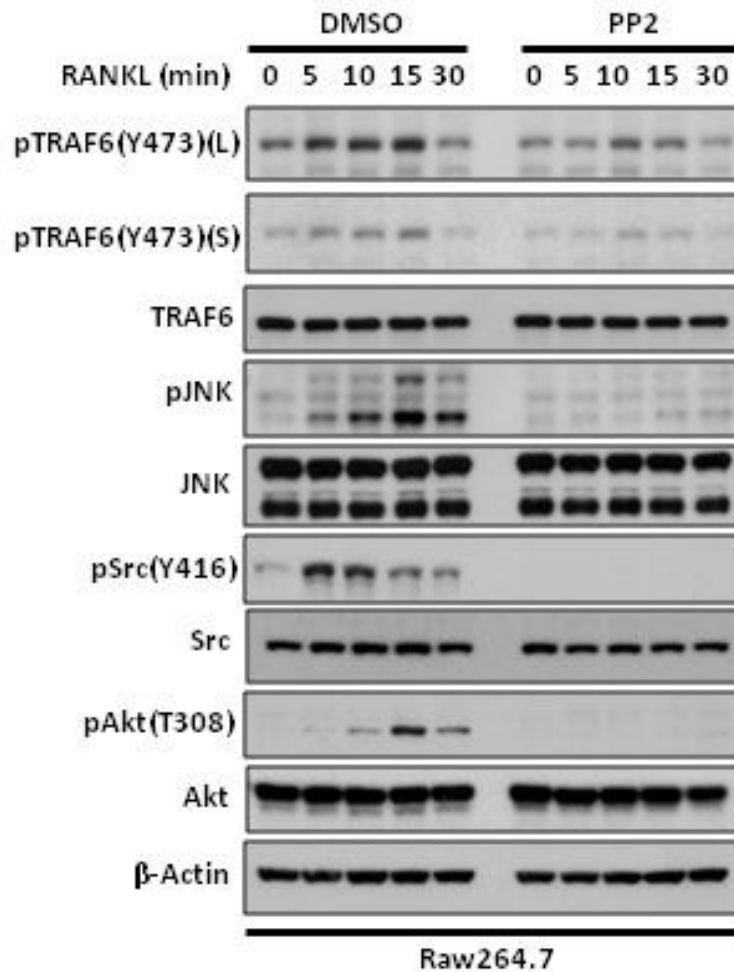
Figure 3-4. Diverse stimuli commonly induce TRAF6 Y473 phosphorylation in a Src dependent manner

(H) Src family tyrosine kinase inhibitor, PP2 inhibits induction of TRAF6 Y473 phosphorylation upon RANKL stimulation. Raw264.7 cells were pretreated with or without PP2 (10 μM) for 3 hr, stimulated with RANKL (100ng/ml) and harvested for IB analysis. L stands for long exposure and S stands for short exposure.

3-5. TRAF6 Y473 phosphorylation is critical for Akt, JNK, and IKK activation

TRAF6 E3 ligase activity is required for Akt, JNK, and IKK activation in response to various growth factors and cytokines [23,98]. Because TRAF6 Y473 phosphorylation is induced by various stimuli, I next determined whether TRAF6 Y473 phosphorylation is essential for Akt, JNK, and IKK activation upon diverse stimuli by restoring Mock, TRAF6 WT, TRAF6 Y473D, or TRAF6 Y473F into *Traf6*^{-/-} MEFs. Remarkably, viral reconstitution of TRAF6 WT or phospho-mimetic Y473D mutant into *Traf6*^{-/-} MEFs could rescue Akt phosphorylation and activation defects, as determined by Foxo3a phosphorylation, upon IGF-1 stimulation, whereas restoration of the TRAF6 Y473F mutant could not (Figure 3-5A). Similarly, restoration of TRAF6 WT and phosphomimetic Y473E, but not Y473F, in HEK293 cells with TRAF6 knockdown could also rescue Akt phosphorylation upon IGF-1 stimulation (Figure 3-5B). Moreover, reconstitution of TRAF6 WT or Y473D, but not Y473F, in *Traf6*^{-/-} MEFs also rescued the defect in IKK and JNK activation upon stimulation with LPS, IL-1, or RANKL (Figures 3-5C-E). These results highlight the critical role of TRAF6 Y473 phosphorylation in TRAF6-mediated Akt, JNK and IKK activation by distinct physiological stimuli.

I have shown that Src induces TRAF6 Y473 phosphorylation and is required for Akt, IKK, and JNK activation in response to multiple stimuli including IGF-1, IL-1, LPS, and RANKL. Given that TRAF6 Y473 phosphorylation is required for TRAF6-mediated Akt, JNK, and IKK activation, I speculated that Src may mediate activation of JNK and IKK by inducing TRAF6 Y473 phosphorylation. I indeed supported this notion by showing that ectopic expression of phospho-mimetic mutant TRAF6 Y473E, but not

phosphorylation-dead mutant TRAF6 Y473F, rescued the defect in IKK and JNK activation upon stimulation with LPS or IL-1 (Figures 3-5F and G). My data therefore offers the first insight into Src regulation of IKK, JNK activation in response to diverse stimuli.
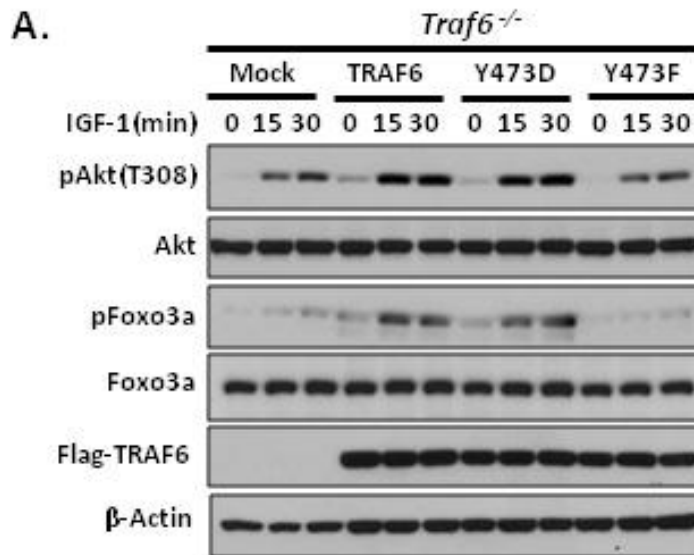
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(A) TRAF6 Y473 is required for TRAF6-mediated Akt activation. *Traf6*^{-/-} MEFs retrovirally reconstituted with empty vector (Mock) or the indicated Flag-TRAF6 constructs were serum starved overnight, treated with IGF-1 (100ng/ml) for indicated time points, and harvested for IB analysis.

B.
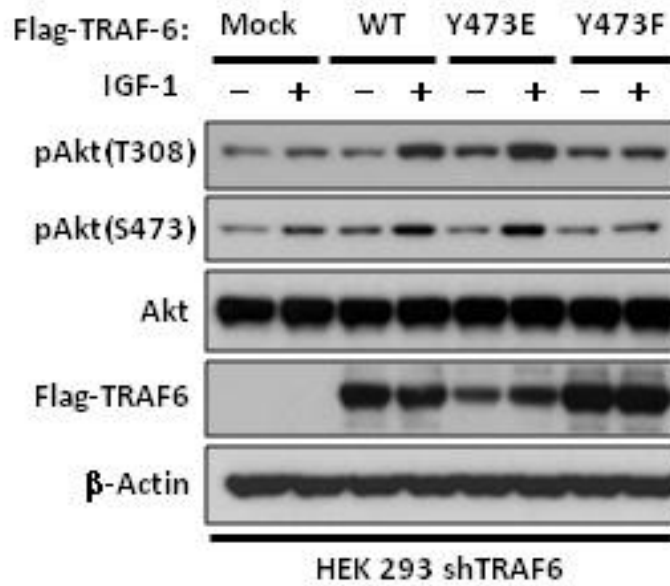
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(B) TRAF6 knockdown HEK 293 cells were transfected with indicated plasmids, serum-starved for 1day, stimulated with IGF-1, and harvested for IB analysis.
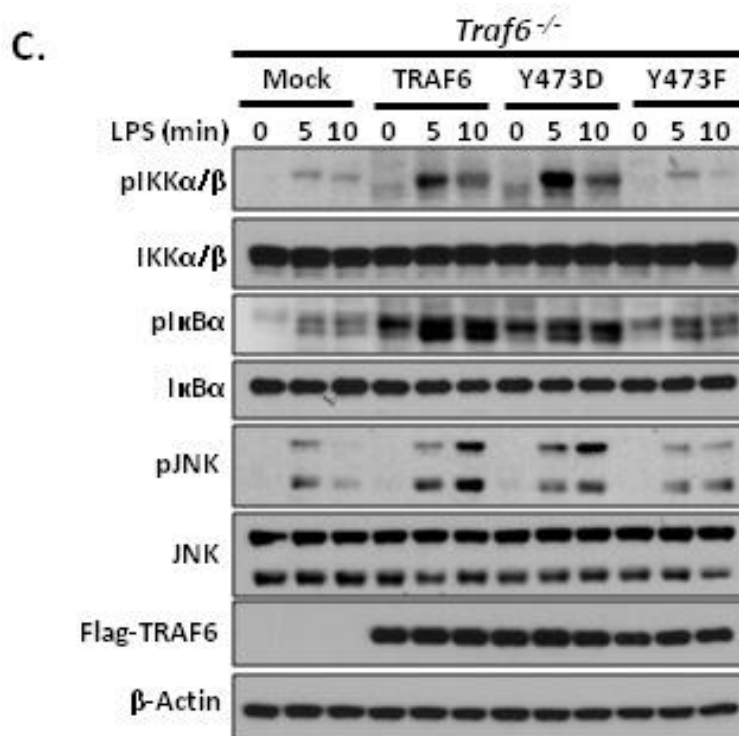
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(C) TRAF6 Y473 phosphorylation is required for TRAF6-mediated IKK and JNK activation upon LPS stimulation. *Traf6*^{-/-} MEFs retrovirally reconstituted with empty vector (Mock) or the indicated Flag-TRAF6 constructs were treated with LPS (5μg/ml) and harvested for IB analysis.
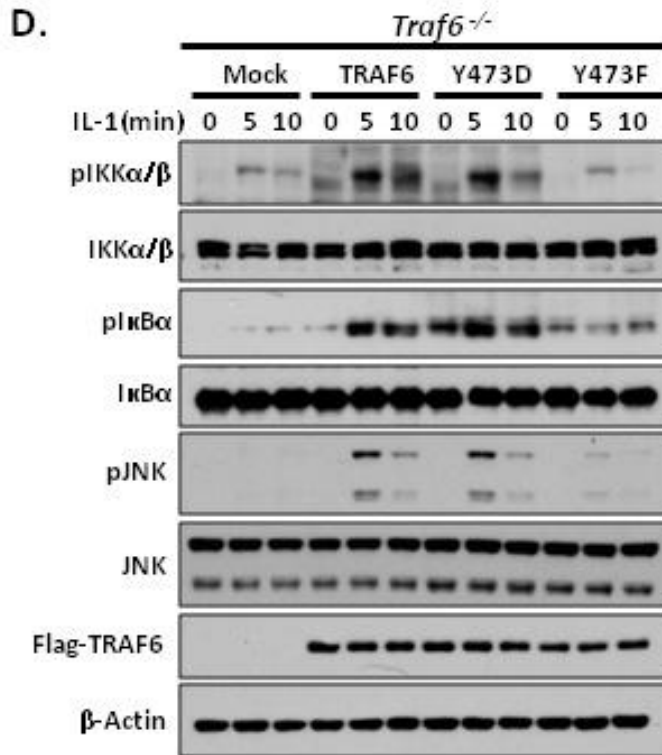
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(D) TRAF6 Y473 phosphorylation is required for TRAF6-mediated IKK and JNK activation upon IL-1 stimulation. *Traf6*^{−/−} MEFs retrovirally reconstituted with empty vector (Mock) or the indicated Flag-TRAF6 constructs were stimulated with murin IL-1 (20 ng/ml) and followed by IB analysis.
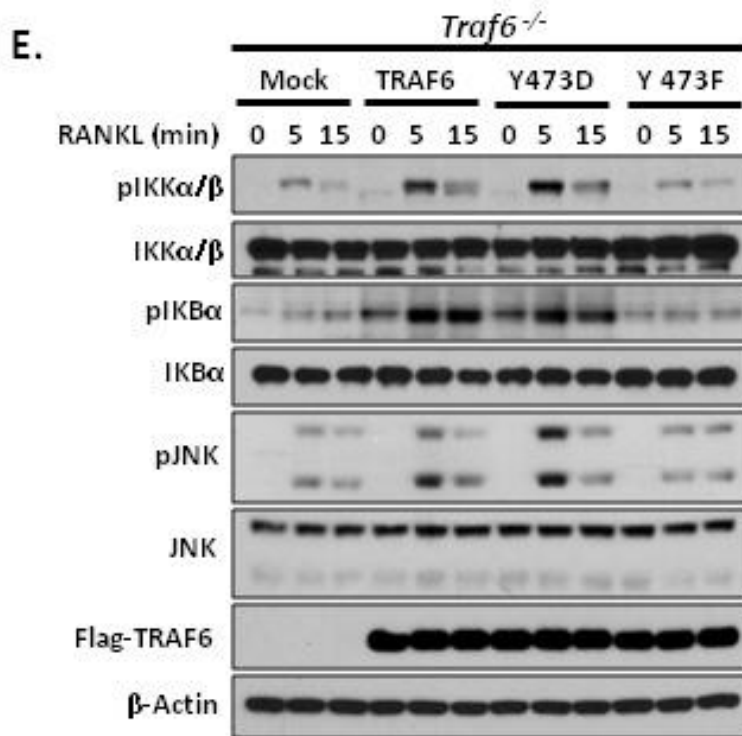
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(E) TRAF6 Y473 phosphorylation is required for TRAF6-mediated IKK and JNK activation upon RANKL stimulation. *Traf6*^{-/-} MEFs retrovirally reconstituted with empty vector or the indicated Flag-TRAF6 constructs were treated with RANKL (100 ng/ml) for indicated times.

F.
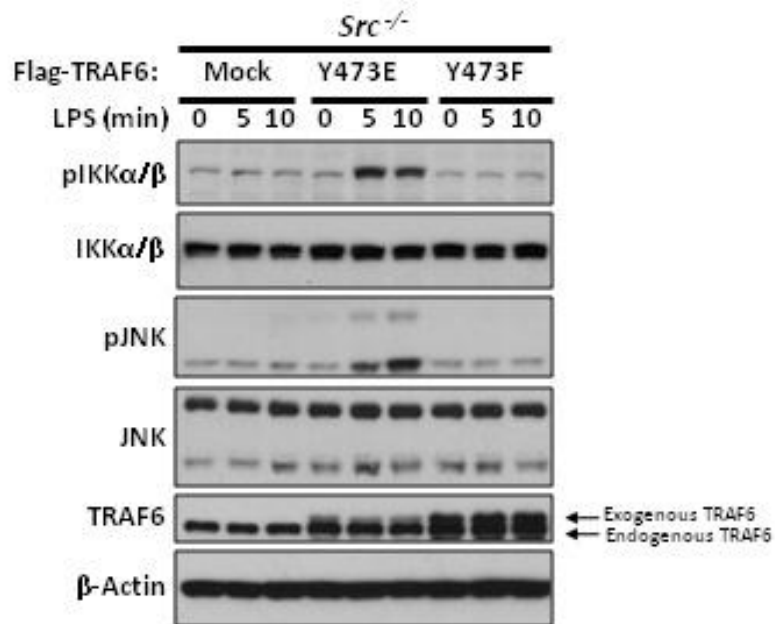
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(F) Src mediates activation of JNK and IKK by inducing TRAF6 Y473 phosphorylation upon LPS stimulation. *Src*^{-/-} MEF cells were transfected with indicated mock, Flag-TRAF6 Y473E and Flag-TRAF6 Y473F plasmids, stimulated with 5 μg/ml LPS for indicated time points and collected for IB analysis.
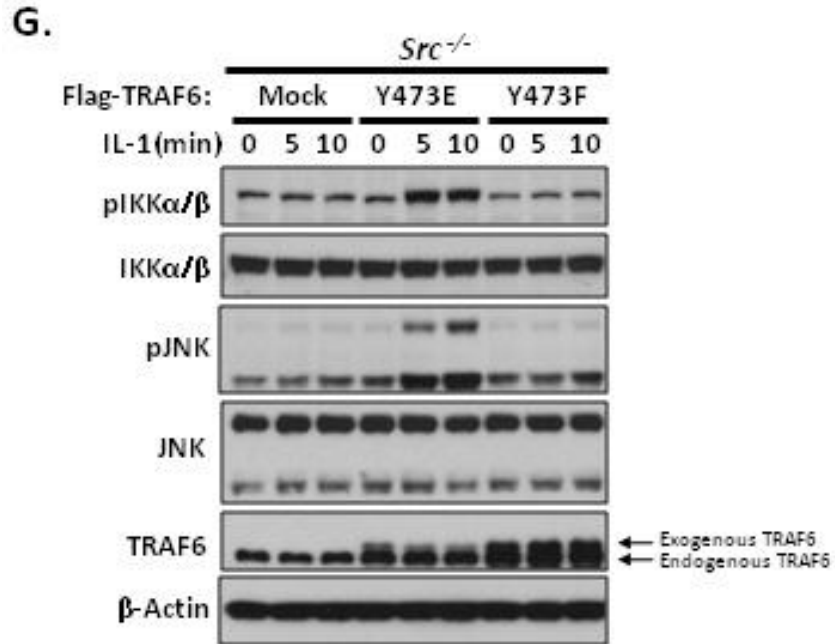
Figure 3-5. TRAF6 Y473 phosphorylation is required for Akt, IKK and JNK activation.

(G) Src mediates activation of JNK and IKK by inducing TRAF6 Y473 phosphorylation upon IL-1 stimulation. $Src^{-/-}$ MEF cells were transfected with indicated mock, Flag-TRAF6 Y473E and Flag-TRAF6 Y473F plasmids, stimulated with 20ng/ml of murin IL-1 for indicated time points and collected for IB analysis.

3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

The finding that TRAF6 Y473 phosphorylation is critical for Akt, IKK, and JNK activation upon diverse stimuli suggests that TRAF6 Y473 phosphorylation is required for TRAF6 E3 ligase activation. To test this notion, I examined whether mutation of TRAF6 Y473 affects its E3 ligase activity by examining TRAF6 autoubiquitination, a readout for TRAF6 activation, and ubiquitination of Akt, a substrate of TRAF6 [23]. While TRAF6 WT and TRAF6 Y473E displayed substantial autoubiquitination, the TRAF6 Y473F mutant profoundly lost this autoubiquitination ability (Figure 3-6A). Interestingly, I found that TRAF6 Y473E mutant displayed even higher autoubiquitination compared to TRAF6 WT (Figure 3-6A). Similarly, TRAF6 WT and TRAF6 Y473E, but not TRAF6 Y473F, promoted ubiquitination of Akt (Figure 3-6B). These results suggest that TRAF6 Y473 phosphorylation is critical for TRAF6 E3 ligase activation.

TRAF6 exists as a dimer and/or an oligomer in cells, which is a prerequisite for TRAF6 E3 ligase activity [108,109]. Because TRAF6 Y473 residue resides in TRAF-C domain, which mediates TRAF6 oligomerization [110], and interaction with upstream activators and downstream effectors [107], we envisioned that Y473 phosphorylation may be required for the formation of TRAF6 dimer and/or oligomerization, thereby regulating TRAF6 E3 ligase activity. To test this notion, I determined whether mutation of Y473 residue affects TRAF6 dimerization and/or oligomerization by using a well-established BMH cross-linking method [111,112,113,114]. I found that oligomerization of TRAF6 or TRAF6 Y473D, but not of TRAF6 Y473F, was induced upon IGF-1

stimulation (Figure 3-6C). Remarkably, upon LPS or IL-1 treatment, while most of TRAF6 Y473F was maintained in the monomer condition, the formation of TRAF6 dimer, trimer, and oligomer was increased in TRAF6 wild type (Figures 3-6D and 3-6E). These results suggest that Y473 phosphorylation is required for TRAF6 dimerization and/or oligomerization in response to diverse stimuli. I confirmed these results by co-immunoprecipitation assay using two different HA- and Flag-tagged TRAF6 constructs (Figures 3-6F and 3-6G). Similarly upon IGF-1 stimulation, induction of TRAF6 dimerization decreased in TRAF6 Y473F mutant in comparison to TRAF6 WT and TRAF6 Y473D.

Since TRAF6 autoubiquitination and activation are also dependent on its association with ubiquitin-conjugating enzyme (E2) UBC13 [115], I examined whether TRAF6 Y473 phosphorylation regulates the interaction between TRAF6 and UBC13. Co-immunoprecipitation assay showed that while TRAF6 WT or TRAF6 Y473E interacts with UBC13, TRAF6 Y473F does not (Figure 3-6H). Notably, LPS or IL-1 treatment induced the interaction of UBC13 with TRAF6 wild type, but not with TRAF6 Y473F (Figures 3-6I and 3-6J). Thus, TRAF6 Y473 phosphorylation mediates the interaction between TRAF6 and UBC13 in response to stimuli.

Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(A) In vivo TRAF6 ubiquitination assay in HEK 239T cells co-transfected with His-ubiquitin (His-Ub) along with indicated Flag-TRAF6 plasmids, and harvested for nickel bead precipitation, followed by IB analysis. Ni-nitrilotriacetic acid (NTA) indicates nickel bead precipitate. C70A indicates TRAF6 C70A mutant, which loses E3 ligase activity
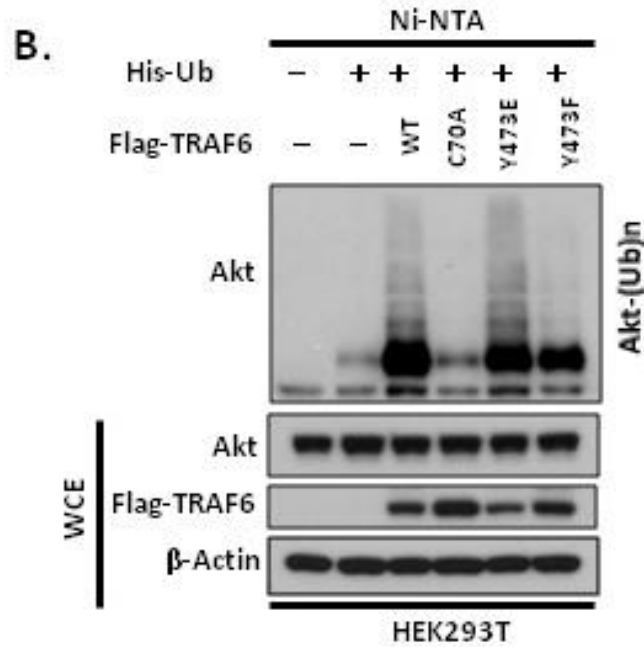
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

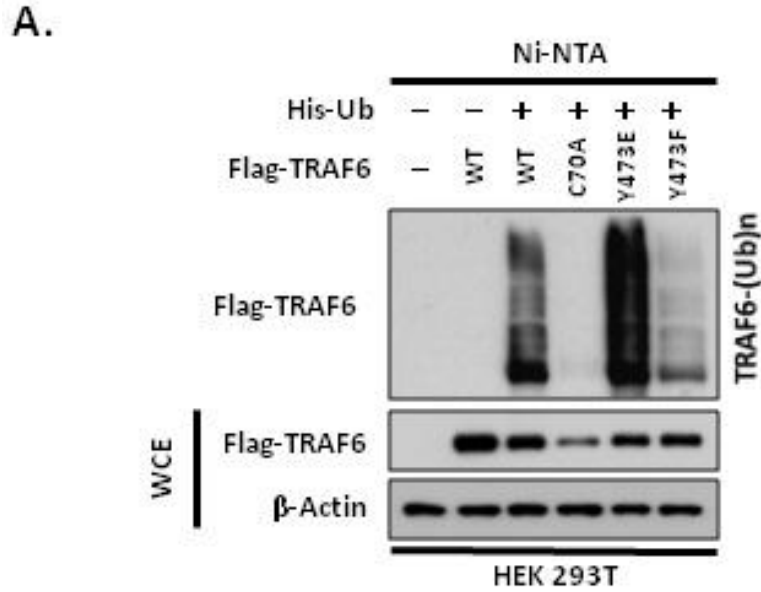 (B) In vivo Akt ubiquitination assay in 293T cells co-transfected with His-Ub along with indicated Flag-TRAF6 plasmids, and harvested for nickel bead precipitation, followed by IB analysis. C70A indicates TRAF6 C70A mutant, which loses E3 ligase activity

Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(C) TRAF6 Y473 phosphorylation affects TRAF6 oligomerization upon IGF-1 stimulation. *Traf6^-/-* MEFs retrovirally-reconstituted with empty vector or the indicated Flag-TRAF6 constructs were serum starved and treated with IGF-1 (100 ng/ml) for 15 min. Cells were then lysed, incubated with 0.2 mM BMH cross-linker at room temperature for 1hr and followed by immunoblotting analysis.

Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(D) TRAF6 Y473 phosphorylation affects TRAF6 oligomerization upon LPS stimulation. *Traf6*^{-/-} MEFs retrovirally-reconstituted with empty vector or the indicated Flag-TRAF6 constructs were treated with LPS (5 μg/ml) for 5 min. Cells were then lysed, incubated with BMH cross-linker for 1hr and followed by immunoblotting analysis.
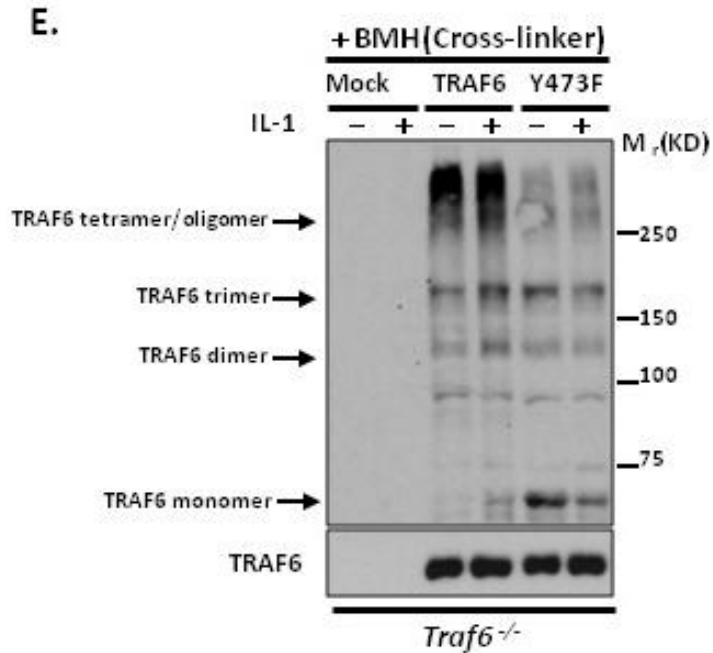
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(E) TRAF6 Y473 phosphorylation affects TRAF6 oligomerization upon IL-1 stimulation. *Traf6*^{-/-} MEFs retrovirally-reconstituted with empty vector or the indicated Flag-TRAF6 constructs were treated with IL-1 (20 ng/ml) for 5 min. Cells were then lysed, incubated with BMH cross-linker for 1hr and followed by immunoblotting analysis.
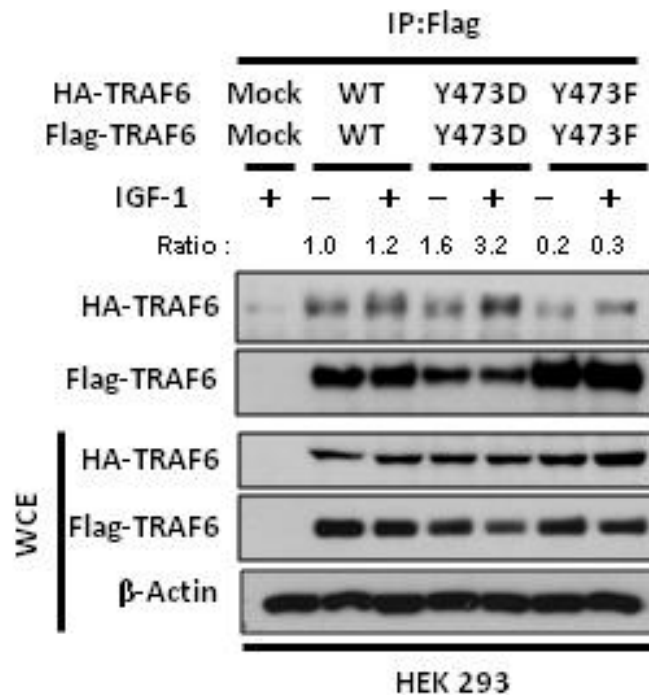
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(F) TRAF6 Y473 phosphorylation affects TRAF6 dimerization upon IGF-1 stimulation. HEK 293 cells were transfected with empty vector or indicated Flag-TRAF6 and HA-TRAF6 plasmids, serum starved for 1 day, stimulated with IGF-1 and harvested for co-IP, followed by IB analysis.
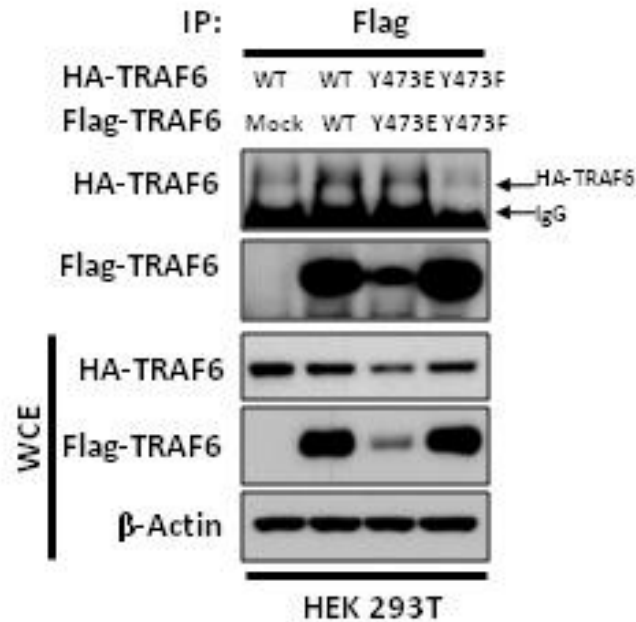
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(G) TRAF6 Y473 phosphorylation affects TRAF6 dimerization. HEK 293T cells were transfected with empty vector or indicated Flag-TRAF6 and HA-TRAF6 plasmids, and harvested for co-IP, followed by IB analysis.
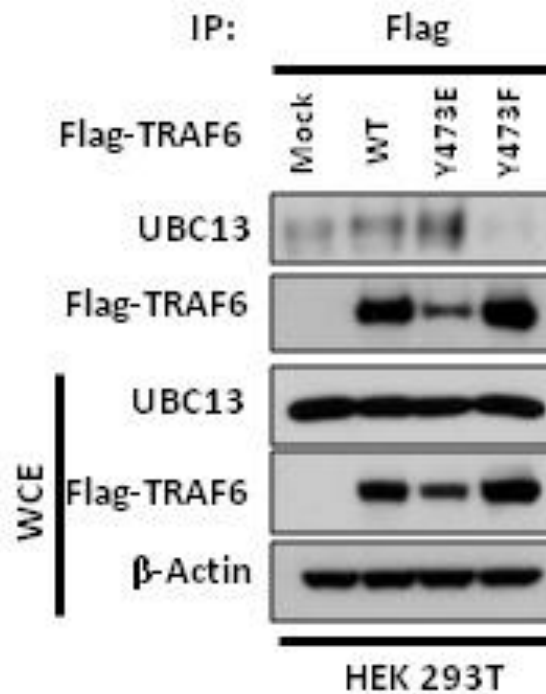
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

(H) TRAF6 Y473 phosphorylation regulates the interaction between TRAF6 and UBC13. HEK 293T cells were transfected with empty vector or indicated Flag-TRAF6 plasmids, and harvested for co-IP, followed by IB analysis.
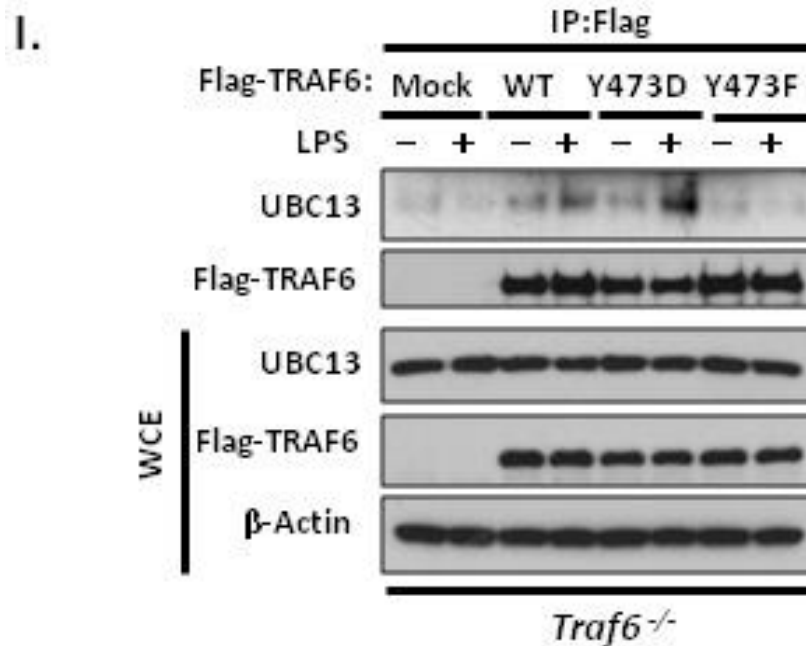
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding

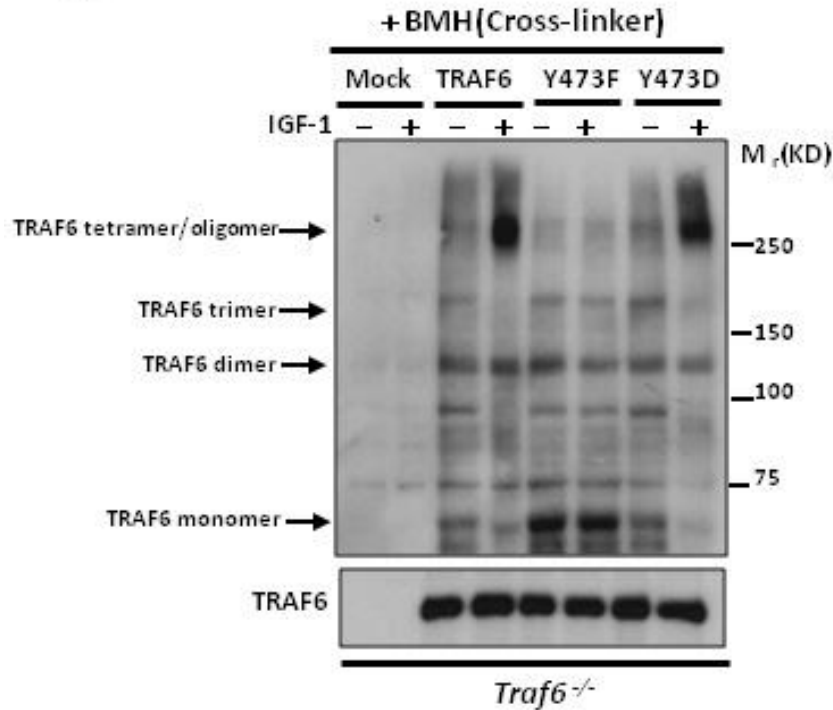(I) TRAF6 Y473 phosphorylation regulates the interaction between TRAF6 and UBC13 upon LPS stimulation. *Traf6$^{-/-}$* MEFs retrovirally-reconstituted with empty vector or indicated Flag-TRAF6 constructs were treated without or with LPS (5 μg/ml) for 5 min and harvested for IP and IB analysis.

J.
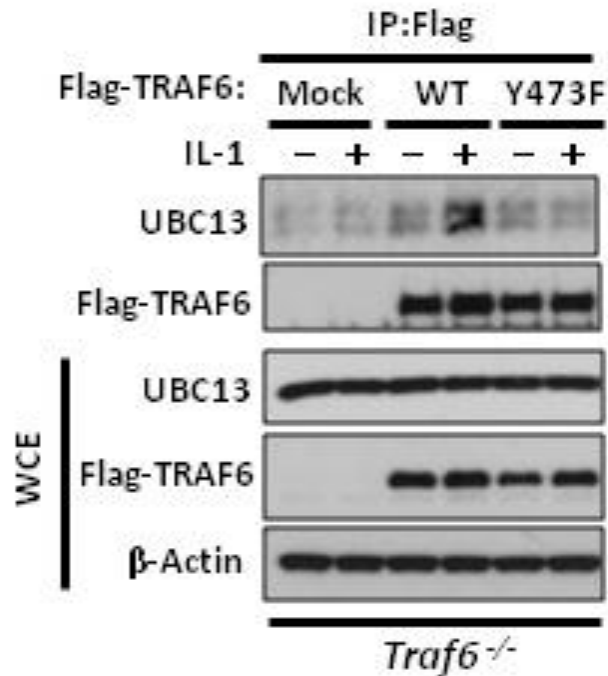
Figure 3-6. TRAF6 Y473 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its oligomerization and UBC13 binding
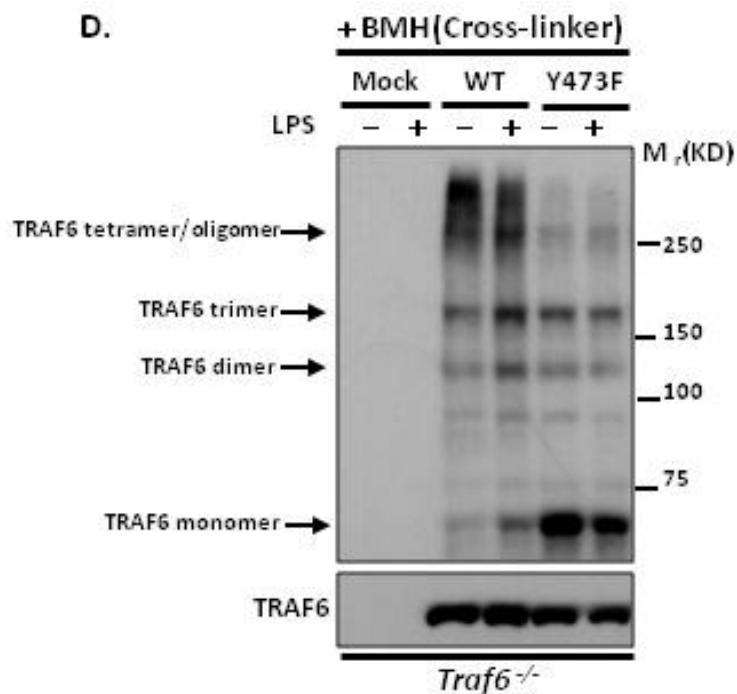
(J) TRAF6 Y473 phosphorylation regulates the interaction between TRAF6 and UBC13 upon IL-1 stimulation. *Traf6*^{-/-} MEFs retrovirally-reconstituted with empty vector or the indicated Flag-TRAF6 constructs were treated without or with IL-1 (20 ng/ml) for 5 min. Cells were then harvested for IP, followed by IB analysis.

3-7. TRAF6 Y473 phosphorylation is required for TRAF6-mediated cell proliferation, migration and tumorigenesis

We have previously shown that TRAF6 depletion reduces tumorigenic potential in PC-3 prostate cancer cell model [101]. In order to determine whether TRAF6 phosphorylation regulates TRAF6-mediated oncogenic potential in various cancer cell models, I generated PC-3, Hep3B, and MDA-MB-231 cancer cells with stable expression of Mock, TRAF6, TRAF6 Y473D, or TRAF6 Y473F. I found that overexpression of TRAF6 wild type or Y473D, but not TRAF6 Y473F, promoted cell proliferation, migration, and invasion in PC-3 prostate cancer cell model (Figures3-7A-7C). Similarly, I also found that overexpression of TRAF6 wild type or Y473D, but not TRAF6 Y473F, promoted cell proliferation and migration in Hep3B liver cancer cells and MDA-MB-231 breast cancer cells (Figures3-7D-7G). Notably, overexpression of TRAF6 WT or TRAF6 Y473D markedly promoted cancer development in the xenograft mouse model, but TRAF6 Y473F overexpression failed to do so (Figure 3-7H). These results suggest that TRAF6 phosphorylation is imperative for TRAF6-mediated cancer cell proliferation, migration, invasion, and tumorigenesis.
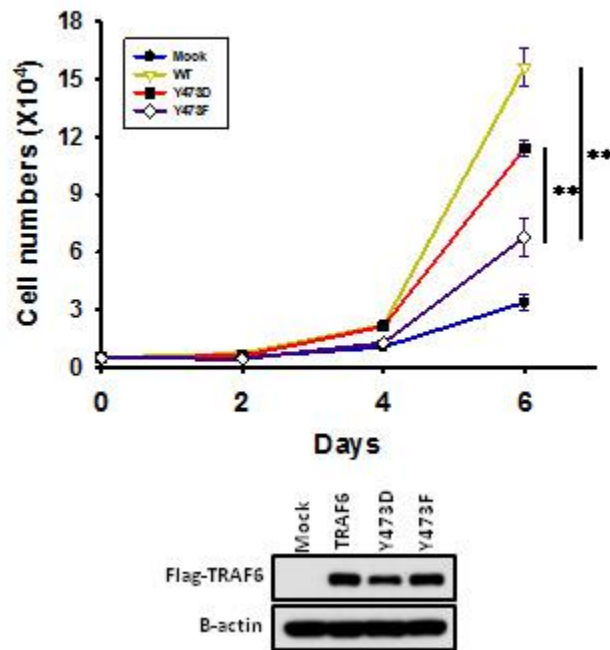
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(A) Cell proliferation of PC-3 prostate cancer cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retrovirus vectors. The quantified results are shown as means $\pm$ SD (n=3), **P<0.01.
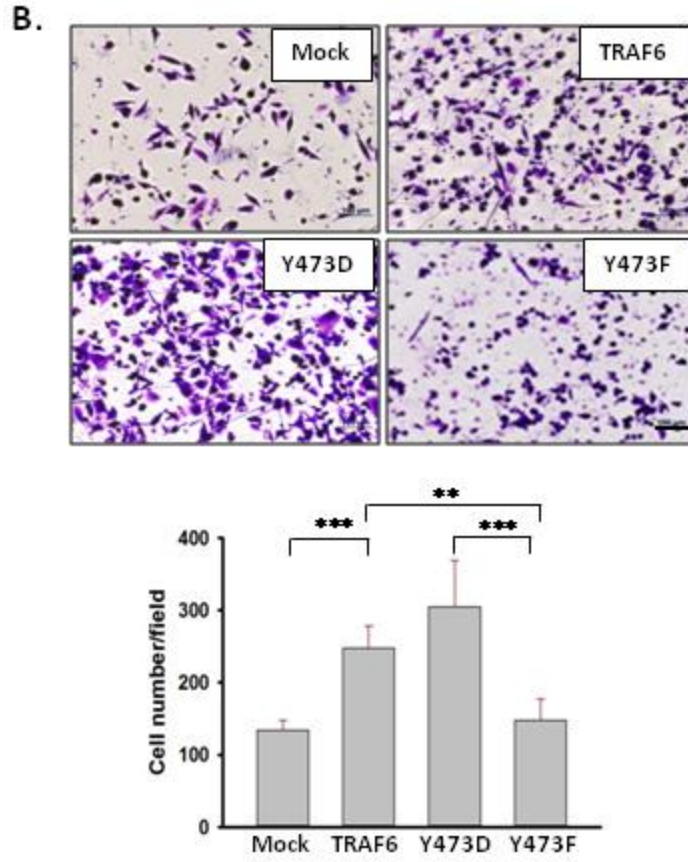
Figure 3-7.   TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(B) Transwell migration assay in PC-3 cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retroviral vectors. The quantified results are shown as means ± SD (n=3), **P<0.01; ***P<0.001. Scale bar represents 100μm.
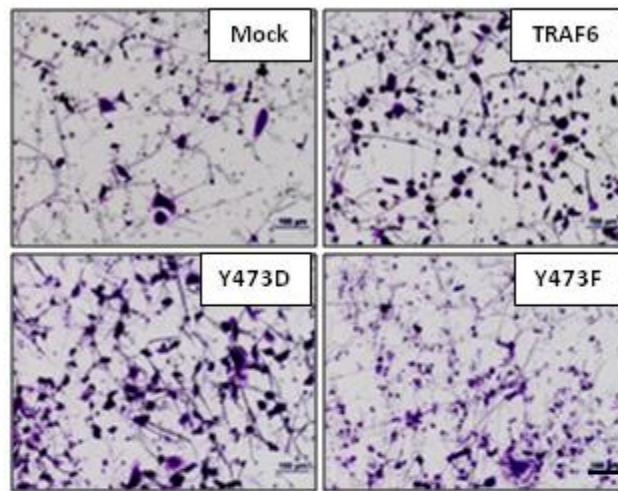
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(C) Transwell invasion assay in PC-3 cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retroviral vectors. The quantified results are shown as means ± SD (n=3), **P<0.01; ***P<0.001. Scale bar represents 100μm.
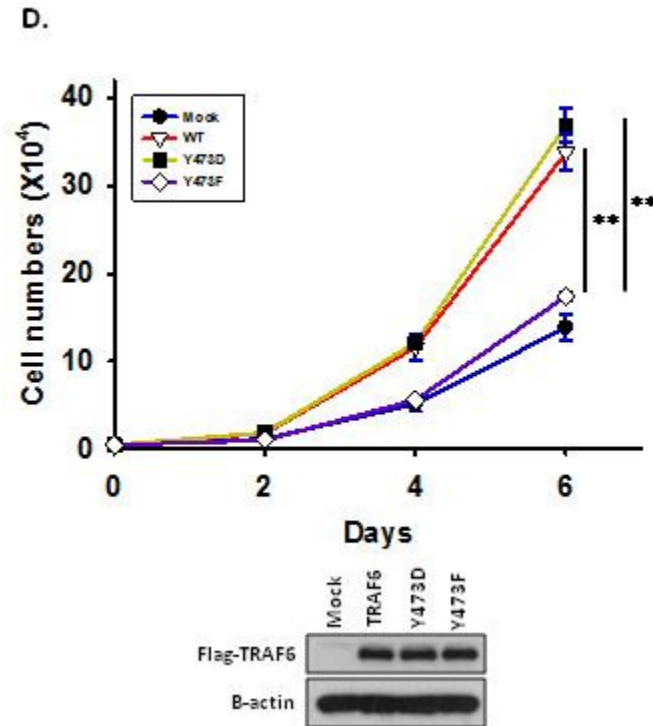
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(D) Cell proliferations of Hep3B liver cancer cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retrovirus vectors. The quantified results are shown as means ± SD (n=3), **P<0.01.
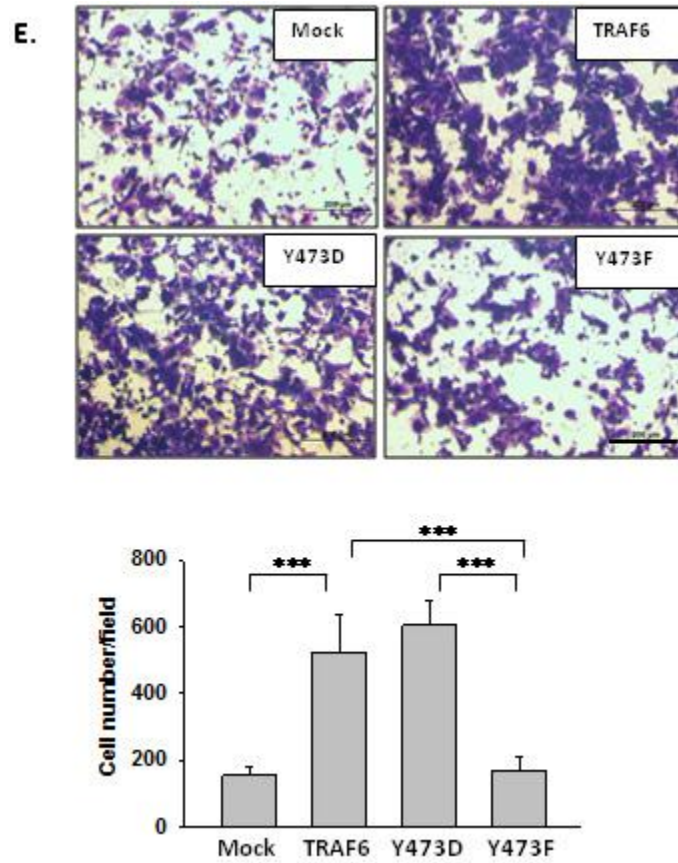
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(E) Transwell migration assay in Hep3B cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retrovirus vectors. The quantified results are shown as means ± SD (n=3), ***P<0.001. Scale bar represents 200μm.
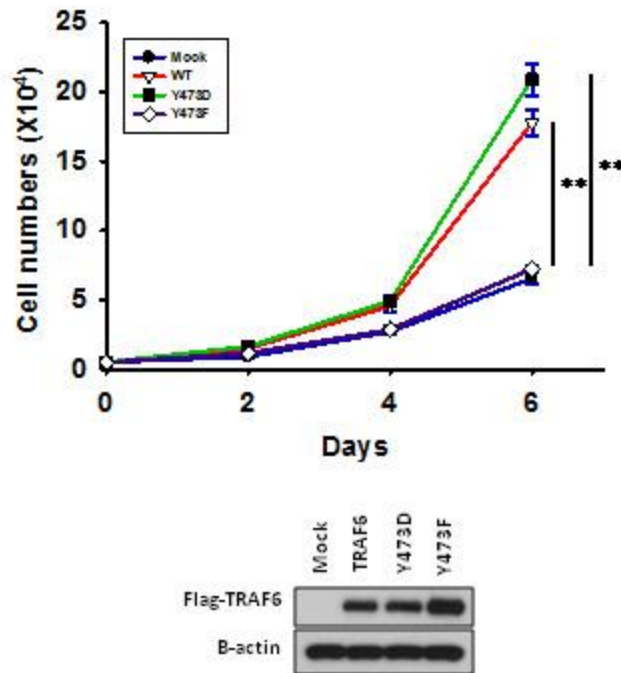
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(F) Cell proliferations of MDA-MB-231breast cancer cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retrovirus vectors. The quantified results are shown as means ± SD (n=3), **P<0.01.
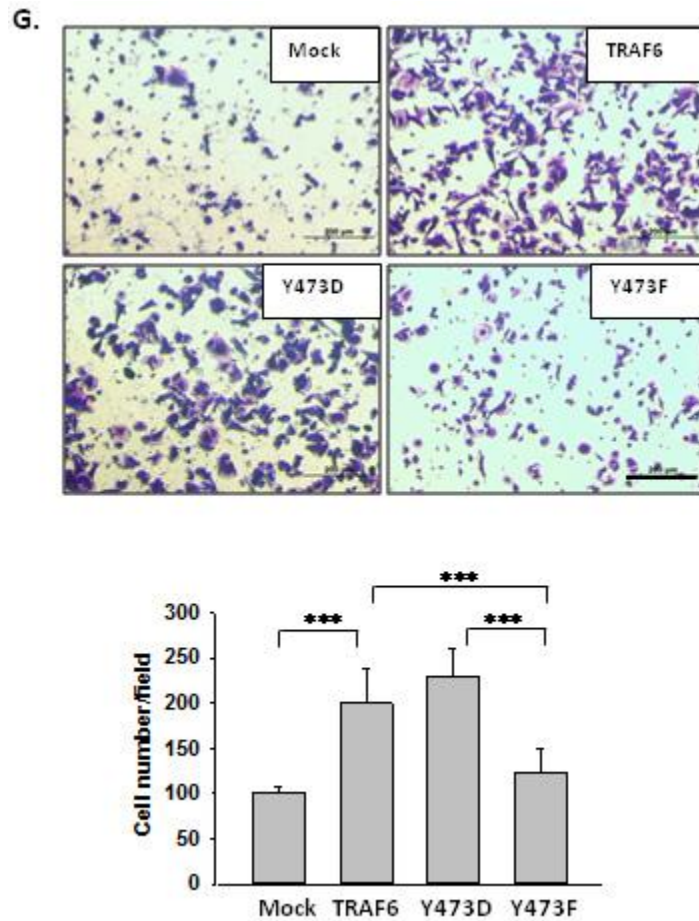
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

(G) Transwell migration assay in MDA-MB-231 cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retrovirus vectors. The quantified results are shown as means ± SD (n=3), ***P<0.001. Scale bar represents 200μm.
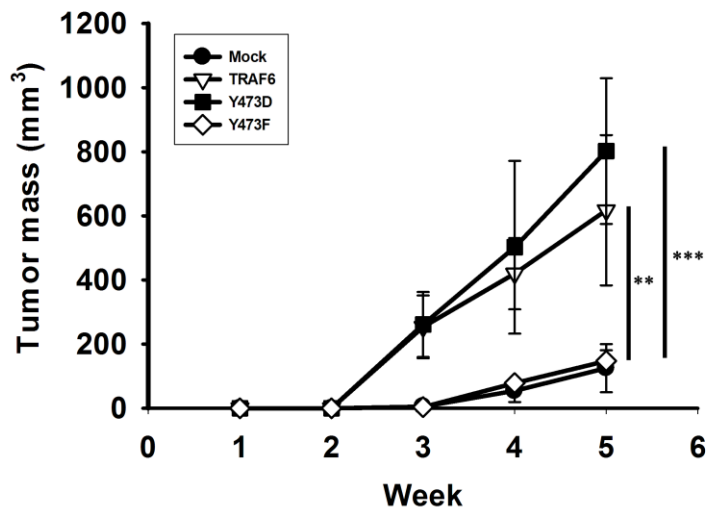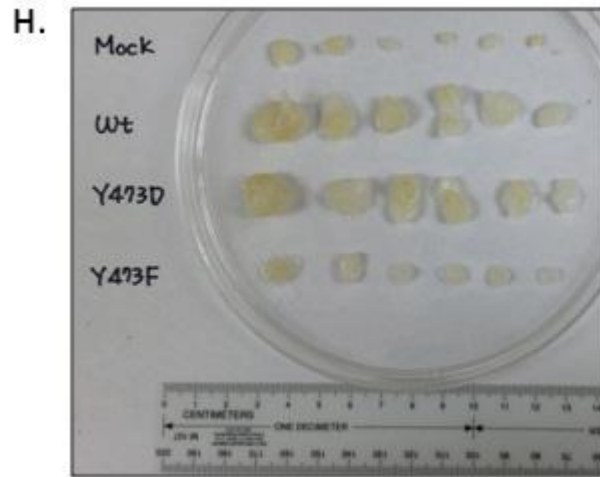
Figure 3-7. TRAF6 Y473 phosphorylation is critical for TRAF6-mediated cell proliferation, migration and tumorigenesis

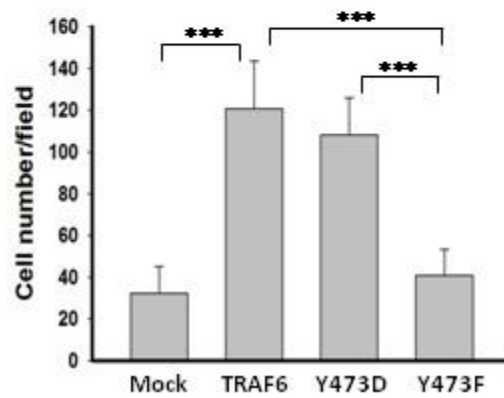(H) PC-3 cells infected with mock, TRAF6, TRAF6 Y473D, and TRAF6 Y473F retrovirus vectors were injected into nude mice and monitored for tumorigenesis. The quantified results are shown as means $\pm$ SD (n=6), **P<0.01; ***P<0.001.

3-8. TRAF6 Y473 phosphorylation is highly correlated with activation of Src, Akt and IKK and predicts poor survival outcome and metastasis-free survival in breast carcinoma patients.

Since TRAF6 Y473 phosphorylation regulated TRAF6-mediated oncogenic potential in various cell models and xenograft mouse model, we next investigated whether TRAF6 Y473 phosphorylation correlates with activation of Src, Akt, and IKK in the human cancer samples. To achieve this aim we carried out immunohistochemistry analysis to determine whether the expression level of TRAF6 Y473 phosphorylation and correlation with Src, Akt, and IKK activation in the cohort of breast cancer samples using phospho-TRAF6 (Y473), phospho-Src (Y416), phospho-Akt (S473), and phospho-IKKα/β (S176/180) antibodies. In the 212 breast carcinoma samples, elevated expression both phospho-Src (Y416) and phospho-TRAF6 (Y473) was significantly associated with increments of primary tumor status (pT, p=0.024 and 0.013, respectively), nodal metastasis (pN, p=0.024 and 0.044, respectively), and stage (p=0.031 and 0.004, respectively). High phospho-Akt (S473) expression was also associated with the presence of nodal metastasis (p=0.034). Interestingly, the phospho-IKKα/β (S176/180) high expression also positively correlated with increments in nodal status (p-0.0010) and tumor stage (p=0.012) (Table 3-1). As summarized in Table 3-2, Figures 3-8A-D, phospho-Src (Y416) expression was positively associated with phospho-TRAF6 (Y473) (r=0.515, p<0.001), phospho-Akt (S473) (r=0.454, p<0.001), and phospho-IKKα/β (S176/180) expression (r=0.528, p<0.001). Phospho-TRAF6 (Y473) expression was also significantly associated with phospho-Akt (S473) (r=0.272, p<0.001) and phospho-IKKα/β (S176/180) (r=0.367, p<0.001) expression (Figures 3-8 E and F). Importantly,

expression of all phospho-Src (Y416), phopho-TRAF6 (Y416), phopho-Akt (S473), and phospho-IKKα/β (S176/180) significantly predicted disease-specific (DSS) and metastasis-free survival (MeFS), as summarized in Table 3-3, Figures 3-8 G-J together with pT status, pN status, and stage. In the multivariate analysis, high expression of phospho-Src (Y416) (p=0.001) and phopho-TRAF6 (Y473) (p=0.018), together with higher stage (p<0.001) remained to be prognostically significant for MeFS (Table 3-4). Only tumor stage independently predicted DSS. These results underscore the importance of TRAF6 Y473 phosphorylation and Src activation in breast cancer progression and disease outcome.

Table 3-1. Associations between pSrc, pTRAF6, pAkt, and pIkkα/β expressions with important clinicopathologic variables.

Parameters	Category	Case No.	pSrc LI		pTRAF6 LI		pAkt LI		pIkkα/β LI	
			H-Score	p-value	H-Score	p-value	H-Score	p-value	H-Score	p-value
Primary tumor (T)[#]	T1	90	195.3±66.4	**0.024***	208.9±63.9	**0.013***	216.6±56.6	0.116	191.1±57.8	0.323
	T2	97	212.9±87.4		228.5±63.6		222.6±61.8		202.7±64.1	
	T3-T4	25	246.8±74.7		250.8±61.1		240.6±64.2		205.4±55.4	
Nodal status (N)[&]	N0	123	197.4±71.7	**0.024***	215.2±65.1	**0.044***	214.8±55.6	**0.034***	1898.9±59.6	**0.010***
	N1-N2	89	226.2±85.5		233.3±62.9		232.4±64.7		209.4±60.3	
Stage[&]	I	69	189.5±64.4	**0.031***	204.5±67.0	**0.004***	213.2±53.832	0.234	182.5±54.1	**0.012***
	II-III	143	226.5±83.5		231.6±61.8		226.5±62.628		205.6±62.2	

Table 3-2. Associations between pSrc, pTRAF6, pAkt, and pIkkα/β expressions in breast cancer

Parameters	pSrc LI		pTRAF6 LI		pAkt LI	
	Correlation coefficient	p-value	Correlation coefficient	p-value	Correlation coefficient	p-value
pTRAF6 LI	r=0.515	**<0.001***	-	-	-	-
pAkt LI	r=0.454	**<0.001***	r=0.272	**<0.001***	-	-
pIkkα/β LI	r=0.528	**<0.001***	r=0.367	**<0.001***	r=0.271	**<0.001***

Pearson's correlation coefficient test; *, Statistically significant
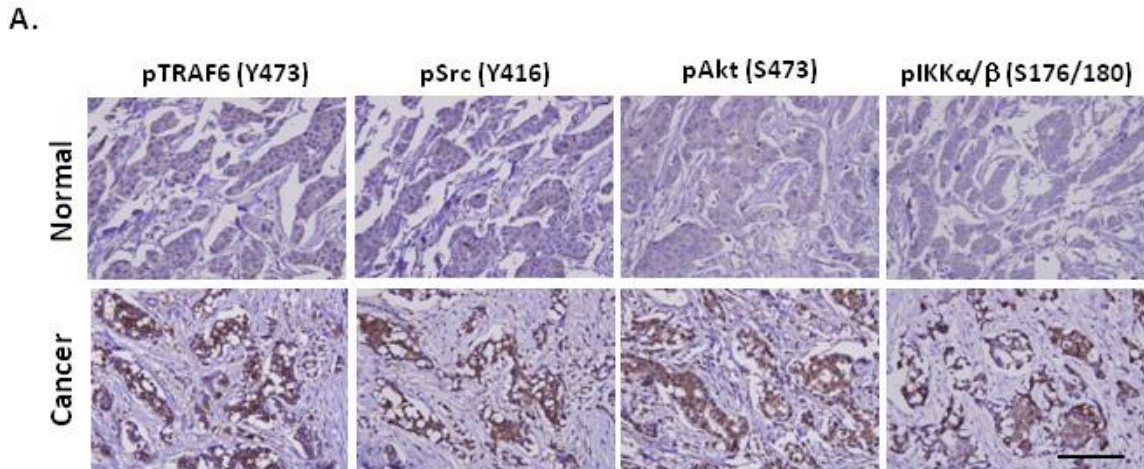
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(A) Immunohistochemistry analysis of phospho-TRAF6 (Y473), phospho-Src (Y416), phospho-Akt (S473), and phospho-IKKα/β (S176/180) in breast carcinoma samples. Scale bar represents 200μm.
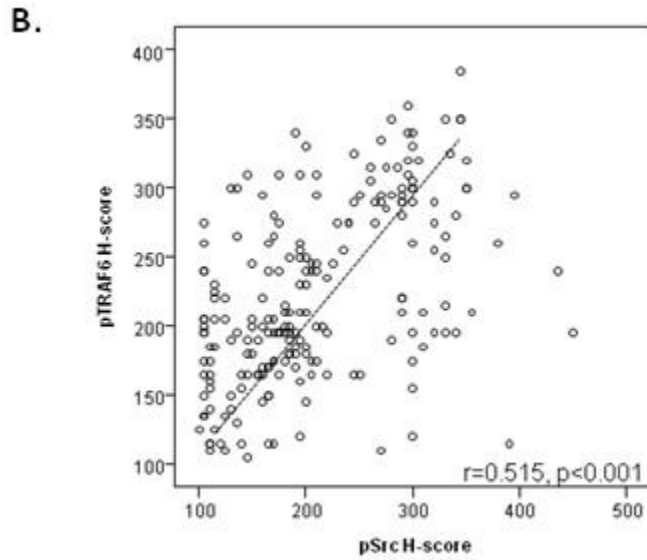
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(B) Positive correlation between phopho-TRAF6 (Y473) and phospho-Src (Y416) in histological score.
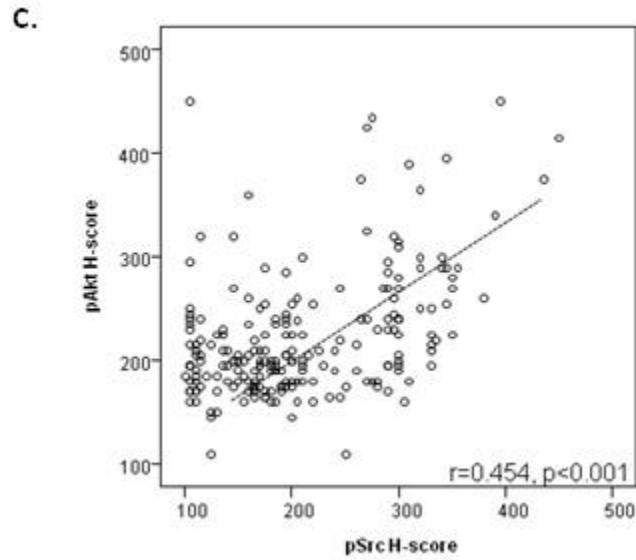
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(C) Positive correlation between phospho-Src (Y416) and phospho-Akt (S473).
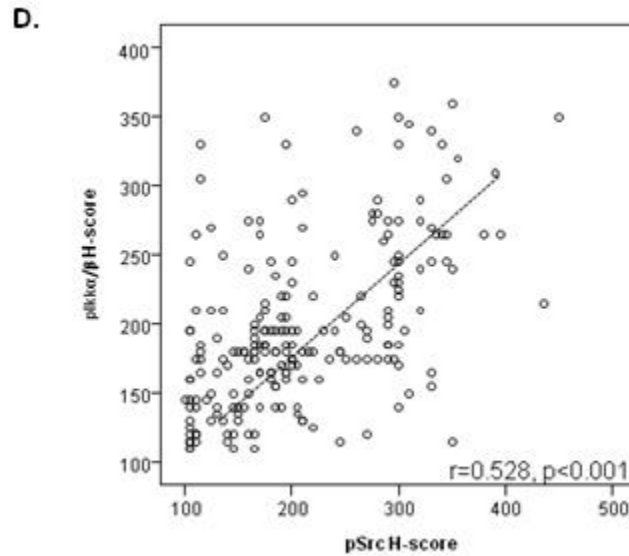
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and

IKK activation and predicts poor survival outcome in breast cancer patients

(D) Positive correlation between phospho-Src (Y416) and phospho-IKKα/β (S176/180).
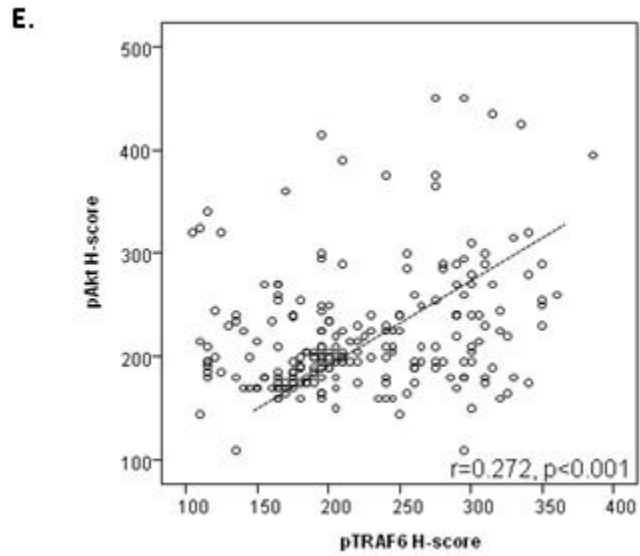
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(E) Positive correlation between phospho-TRAF6 (Y473) and phospho-Akt (S473).
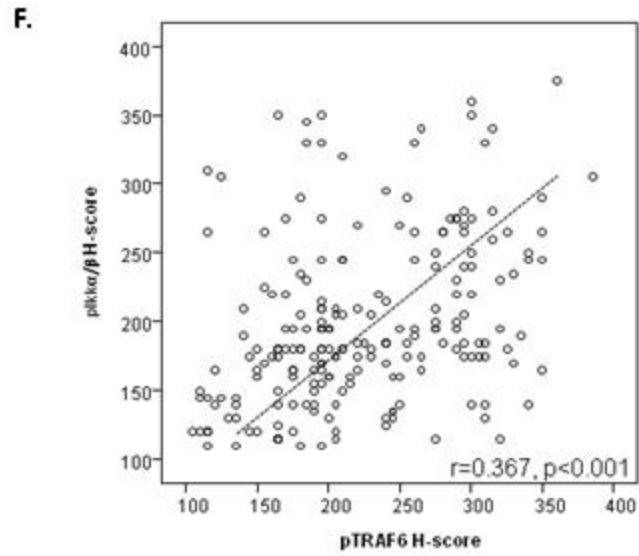
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(F) Positive correlation between phospho-TRAF6 (Y473) and phospho-IKKα/β (S176/180).

Table 3-3. Univariate survival analyses

Parameters	Category	DSS		MeFS	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Primary tumor (T)	T1 vs. T2 vs. T3-T4	2.159 (1.067-4.348)	**0.032***	3.934 (2.680-5.775)	**<0.001***
Nodal status (N)	N0 vs. N1-N2	3.946 (1.550-10.043)	**0.004***	4.873 (2.796-8.492)	**<0.001***
Stage	Stage I vs. Stage II vs. Stage III	4.886 (2.278-10.483)	**<0.001***	4.708 (3.079-7.2004)	**<0.001***
pSrc LI	Continuous	1.011 (1.005-1.016)	**<0.001***	1.010 (1.007-1.013)	**<0.001***
pTRAF6 LI	Continuous	1.013 (1.006-1.021)	**<0.001***	1.012 (1.008-1.016)	**<0.001***
pAkt LI	Continuous	1.009 (1.003-1.014)	**0.001***	1.007 (1.004-1.010)	**<0.001***
pIkkα/β LI	Continuous	1.011 (1.005-1.018)	**0.001***	1.006 (1.003-1.010)	**0.001**
pSrc LI	Low (<median) vs. High (>=median)	-	**0.0052***	-	**<0.0001***
pTRAF6 LI	Low (<median) vs. High (>=median)	-	**0.0114***	-	**<0.0001***
pAkt LI	Low (<median) vs. High (>=median)	-	**<0.0001**	-	**<0.0001***
pIkkα/β LI	Low (<median) vs. High (>=median)	-	**0.0029***	-	**0.0398***

DSS, disease-specific survival; MeFS, metastasis-free survival; *, statistically significant
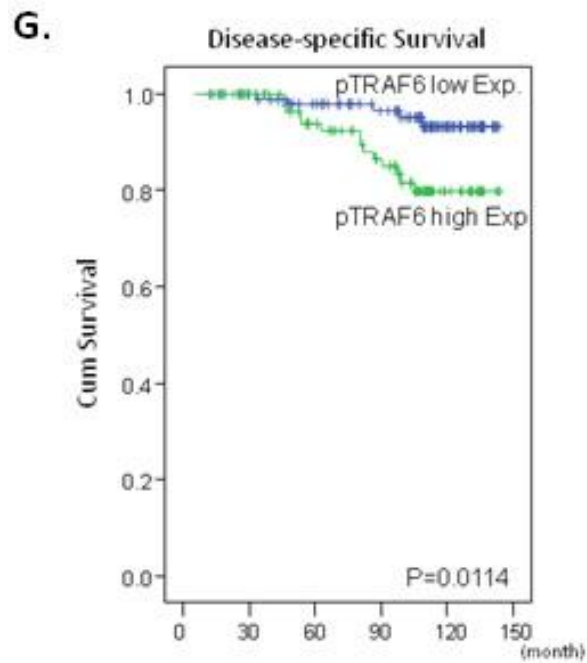
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(G) Kaplan-Meier plot analysis of disease-specific survival of breast carcinoma patient samples with low or high expression of phospho-TRAF6 (Y473)
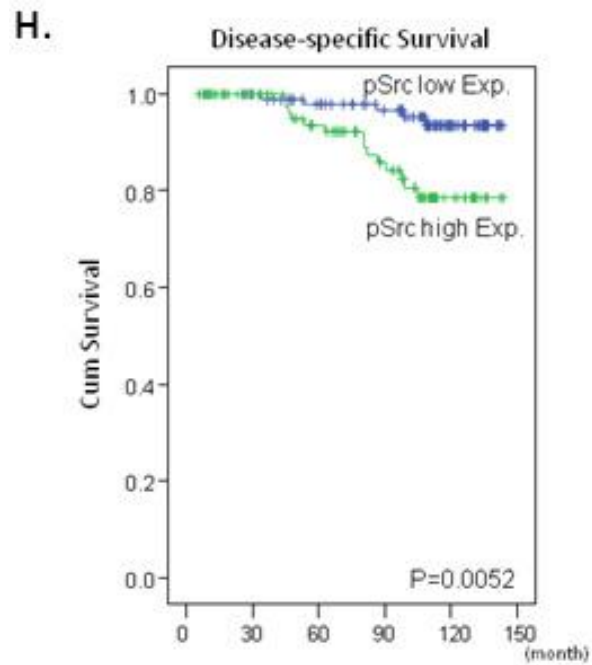
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(H) Kaplan-Meier plot analysis of disease-specific survival of breast carcinoma patient samples with low or high expression of phospho-Src (Y416).
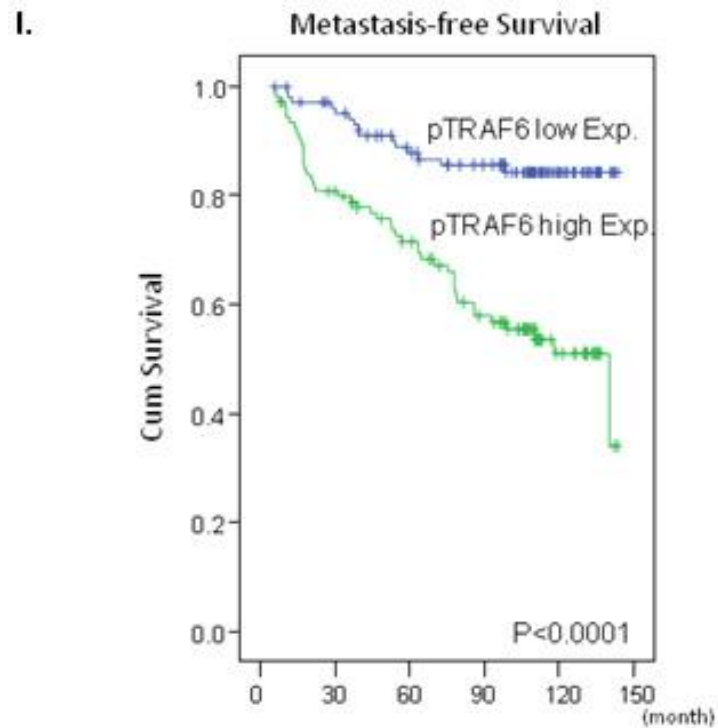
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(I) Kaplan-Meier plot analysis of metastasis-free survival of breast carcinoma patient samples with low or high expression of phospho-TRAF6 (Y473).

J.

Metastasis-free Survival

pSrc low Exp.

pSrc high Exp.

P<0.0001
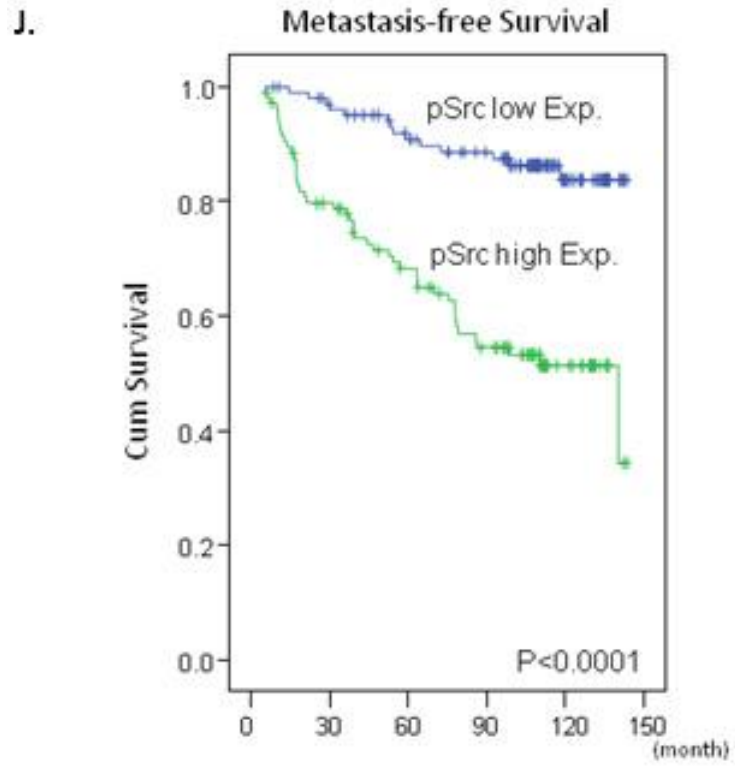
Figure 3-8. TRAF6 Y473 phosphorylation is highly correlated with Src, Akt and IKK activation and predicts poor survival outcome in breast cancer patients

(J) Kaplan-Meier plot analysis of metastasis-free survival of breast carcinoma patient samples with low or high expression of phospho-Src (Y416).

Table 3-4. **Multivariate survival analyses**

Parameters	Category	DSS		MeFS	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Stage	Stage I vs. Stage II vs. Stage III	3.771 (1.491-9.537)	**0.005***	3.482 (2.174-5.576)	**<0.001***
pSrc LI	Continuous	1.005 (0.998-1.013)	0.159	1.007 (1.003-1.011)	**0.001***
pTRAF6 LI	Continuous	1.006 (0.997-1.014)	0.187	1.006 (1.001-1.011)	**0.018***
pAkt LI	Continuous	0.999 (0.992-1.006)	0.768	0.999 (0.995-1.002)	0.466
pIkkα/β LI	Continuous	1.005 (0.998-1.013)	0.164	1.000 (0.995-1.004)	0.882

DSS, disease-specific survival; MeFS, metastasis-free survival; *, Statistically significant

CHAPTER 4

DISCUSSION

4-1. Diverse receptor signalings trigger TRAF6 activation by Src-mediated TRAF6 phosphorylation

TRAFs are well known cytoplasmic adaptor family proteins that are essential for receptor-mediated immune response. Among the TRAFs, TRAF6 is the unique TRAF family member involved in TLR-4/IL-1- and TNFR-mediated NF-κB and AP-1 activation, and IGF-1R-mediated Akt activation. Therefore, the proper regulation of TRAF6 activity is imperative to maintain and regulate normal cellular response and physiology. Although TRAF6 has critical roles in maintaining and regulating normal cellular response and physiology, the mechanism by which TRAF6 activation is achieved through multiple physiological stimuli remains largely unclear.

In my study, we have revealed for the first time the novel molecular mechanism by which TRAF6 E3 ligase is activated by diverse receptor signaling stimuli. I have shown that distinct stimuli commonly trigger Src activation can also induce TRAF6 tyrosine phosphorylation at Y473. The Y473 phosphorylation of TRAF6 represents a critical event for TRAF6 dimerization/oligomerization and its binding to E2 UBC13, thereby leading to TRAF6 activation. Active TRAF6 then triggers activation of Akt, IKK, and JNK to participate in various biological processes such as cell proliferation, migration, and tumorigenesis (Figure 4-1).
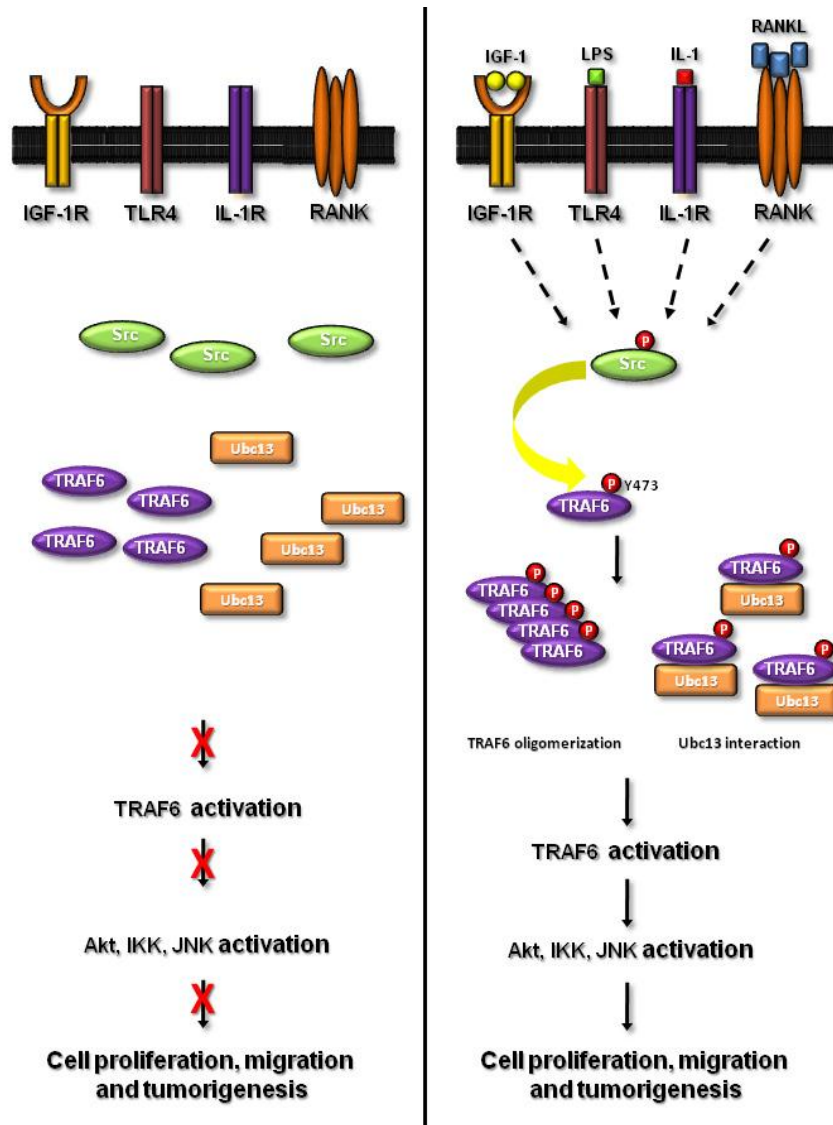
Figure 4-1. Working model.

Src activation is induced by multiple receptors including IGF-1R, IL-1R, TLR4 and RANK. Src then induces TRAF6 tyrosine phosphorylation at Y473, which serves as a critical event for TRAF6 dimerization/oligomerization and its binding to E2 UBC13, thereby leading to TRAF6 activation. Active TRAF6 then triggers activation of Akt, IKK and JNK to participate in various biological processes such as cell proliferation, migration and tumorigenesis

4-2. Src tyrosine kinase phosphorylates TRAF6 at Y473

Phosphorylation is an important posttranslation modification to regulate TRAF proteins in signaling. It is reported that PKN1-mediated TRAF1 S146 phosphorylation negatively regulates TNF-R2-dependent NF-κB and JNK signaling activation in HeLa cells and T cells [50,51]. Phosphorylation of TRAF2 by PKCζ, IKKε, PKCδ, or PKCε promotes K63-linked ubiquitination of TRAF2 and NF-κB activation in TNF-R signaling and transformed cells [52,53,54,55]. IKKα-mediated TRAF4 S426 phosphorylation upon NOD2 activation inhibits NF-κB activation, cytokine production, and antibacterial activity in macrophage [56].

In my study, I have also demonstrated that tyrosine phosphorylation is a key regulatory mechanism of TRAF6 E3 ligase function in various signaling pathways. I have shown that TRAF6 tyrosine phosphorylation is commonly induced by various signaling stimuli (Figures.3-1A-1D) and identified that Src is a direct kinase that induces TRAF6 Y473 phosphorylation (Figures.3-2C-2E). I further showed that this Src-mediated TRAF6 Y473 phosphorylation positively regulates TRAF6 E3 ligase function (Figures 3-6A and 6B). However, I also observed that Src deficiency or inhibition of Src activity could not completely abolish TRAF6 Y473 phosphorylation (Figures 3-4A, 4C, and 4D). This suggested kinase(s) other than Src may also be involved in this Y473 phosphorylation. Given that other Src family kinases including Fyn and Syk have shown to interact with TRAF6 [100,116], it is highly possible that they may be involved in TRAF6 regulation through phosphorylation. Further investigation is needed to explore this possibility.

4-3. Src tyrosine kinase is required for Akt, IKK and JNK activation

Src tyrosine kinase role in Akt activation under diverse growth factor receptors [117,118] is widely known. Previous studies have implicated Src in NF-κB signaling activation upon simulation with IL-1 and LPS [102,103,104]. However, the mechanism which Src engages in these diverse signaling pathways is not well understood. Notably, my results support and provide important new insights into how Src is engaged in Akt, JNK, and IKK/NF-κB activation. I have shown that diverse stimuli, such as IGF-1, IL-1, LPS, and RANKL, commonly induces Src activation in various cell models, triggers TRAF6 tyrosine phosphorylation at Y473 as well to induce TRAF6 activation, in turn eliciting activation of Akt, IKK, and JNK (Figures.3-4A-4H).

It is valuable to mention that I observed TRAF6 phosphomimetic mutant itself could not be constitutively active in the downstream of TRAF6 signaling events in our rescue experiments (Figures 3A-3D). IGF-1, LPS, IL-1, and RANKL stimulation are still required for Akt, IKK and JNK activation. This suggests that the receptor-dependent signaling activation is still required for TRAF6 downstream signaling activation.

Because previous study showed that TRAF6 also regulates Src kinase activity in RANK signaling pathway [100], I also tested whether Src-mediated TRAF6 phosphorylation functions as a positive feedback loop for Src activation. Indeed, I observed TRAF6 Y473F mutant showed defect in Src activation upon RANKL stimulation (unpublished observation). Future investigation is needed to determine whether Src-mediated TRAF6 phosphorylation may serve as a positive feedback loop for Src activation in other signaling pathway.

In my study, I did not explore the possibility that other physiological stimuli also can activate Src/TRAF6 activation. It is known that Src tyrosine kinase is involved in multiple receptor tyrosine kinase (RTK) signaling pathways. Src has shown to be downstream of IGF-1R, PDGF receptor (PDGF-R), fibroblast growth factor receptor (FGF-R), epidermal growth factor receptor (EGF-R), hepatocyte growth factor receptor (HGF-R), stem cell factor receptor (SCF-R),colony-stimulatingfactor-1 receptor (CSF-1R), muscle specific kinase (MuSK)[119,120,121,122,123,124,125,126,127]. Given that TNF-α, DNA damage and hypoxia can trigger Src and TRAF6 activation [128,129,130], it will be interesting to investigate whether these physiological stimuli can trigger Src-mediated TRAF6 activation and play critical roles in those pathways.

4-4. Src-mediated TRAF6 phosphorylation regulates TRAF6 E3 ligase activity by enhancing its dimerization/oligomerization and UBC13 binding

TRAF6 dimerization/oligomerization is critical for TRAF6 E3 ligase activity [108,109]. Since Src mediated TRAF6 phosphorylation site is located in TRAF-C domain, which is known to mediate TRAF6 oligomerization and interaction with upstream activators and downstream effectors, I first tested whether mutating the Src-mediated TRAF6 phosphorylation site would affect their dimerization/oligomerization ability. I showed that Src-mediated TRAF6 phosphorylation is critical for its dimerization and/or oligomerization in response to diverse stimuli (Figures 3-6C-6H). I further showed that Src-mediated TRAF6 phosphorylation also affected TRAF6 interaction with E2 enzyme, UBC13 (Figures 3-6I and 6J). These findings are conflicted to previous independent reports showing that UBC13 interacts with TRAF6 through the N-terminal RING and zinc finger as well as the coiled-coil domain [131,132]. How does Src-mediated TRAF6 phosphorylation regulate UBC13 interaction? Previous report suggests that TRAF6 is autoinhibited by intramolecular interaction between RING-Zinc (RZ) region and carboxyl-terminal MATH domain. This inactive closed form can be disrupted and converted to an active form, upon binding of proteins containing TRAF interacting motif (TIM). In this open active structure, TRAF6 can interact with UBC13 and downstream kinases [133]. Because TRAF6 phosphorylation site reside in TRAF6 substrate binding motif, it is highly possible that Src-mediated TRAF6 phosphorylation promotes association with upstream activator proteins leading to open active conformation. Indeed, I observed that association of TRAF6 with TRAF6 binding

proteins including IRAK-1, and TAB2 is decreased in TRAF6 Y473F mutant in comparison to TRAF6 WT and TRAF6 Y473D (unpublished observation).

4-5. Src-mediated TRAF6 Y473 phosphorylation is required for oncogenic role of TRAF6

Although deregulation of NF-κB signaling is involved in human cancer development [134,135], it was not clear whether TRAF6 is also related to tumorigenesis until recently, our lab showed that TRAF6 depletion decreased Akt activation and reduced tumorigenic potential in PC-3 prostate cancer cell model [23].

In my study, my data support our previous study and extend our current knowledge on the oncogenic role of TRAF6. I have shown that overexpression of TRAF6 wild type or Y473D, but not TRAF6 Y473F, promoted cell proliferation, migration, and invasion in various cancer cell models (Figures 3-7A-7G). These data suggest Src-mediated TRAF6 phosphorylation is imperative to regulate TRAF6-mediated cancer cell proliferation, migration, invasion, and tumorigenesis. Moreover, I also found that TRAF6 overexpression promotes cell proliferation and migration not only in PC-3 cells but also MDA-MB-231 and Hep3B cells. My findings are further supported by recent reports. It has been reported that TRAF6 is involved in many human cancer including gastric cancer, lung cancer, prostate cancer, breast cancer, osteosarcoma and acute myeloid leukemia [136,137,138,139,140,141].

4-6. Src-Mediated TRAF6 Y473 phosphorylation is a marker for poor survival outcome and a potential therapeutic target for breast cancer

It has been well documented that Src activity and Src protein levels are elevated in several human cancers including colon and breast cancer [142,143,144,145,146,147]. It is also reported that overexpression and gene amplification of TRAF6 are observed in lung cancer and osteosarcoma cells [138,140,141]. In my study, my data provide new insights into how Src and TRAF6 activations are correlated in human cancer. We showed Src-mediated TRAF6 tyrosine Y473 phosphorylation is a critical factor for TRAF6-mediated oncogenic activity in various cell models and mouse xenograft model. We also showed that the clinical relevance of TRAF6 Y473 phosphorylation. We found that Src Y416 and TRAF6 Y473 phosphorylation are upregulated in high stage of human breast cancer samples and correlate with IKK and Akt activation, supporting the notion that Src-TRAF6-Akt and Src-TRAF6-IKK pathways are highly activated in advanced human breast cancer. Importantly, upregulation of Src Y416 phosphorylation or TRAF6 Y473 tyrosine phosphorylation predicts poor survival outcome and metastasis-free survival. Taken together, our data suggest that Src-mediated TRAF6 Y473 phosphorylation can be regarded as a useful biomarker for predicting disease progression and survival outcome of breast cancer patients.

In summary, my study identifies Src-mediated TRAF6 Y473 phosphorylation as a common mode for oncogenic TRAF6 activation and Src-mediated Akt, JNK and IKK activation under diverse physiological stimuli. Therefore, pharmacological targeting of Src-mediated TRAF6 Y473 phosphorylation may represent an effective strategy for cancer therapy.

CHAPTER 5

Future direction

TRAF6 is a well-known cytoplasmic mediator protein that is involved in TLR-4/IL-1R and TNFR-mediated NF-κB and AP-1 activation, and IGF-1R-mediated Akt activation. Therefore, a number of studies have revealed the importance of TRAF6 in maintaining and regulating normal cellular response and physiology. However, the mechanism TRAF6 activation is achieved through multiple physiological stimuli remains largely unclear.

In this study, I demonstrated that TRAF6 Y473 phosphorylation can be commonly induced by IGF-1, IL-1, LPS and RANKL, and Src, which is activated by these stimuli, acts as a direct kinase for this TRAF6 phosphorylation. Importantly, TRAF6 or Src deficiency impairs the activation of Akt, IKK and JNK, and such defects can be rescued by phosphomimetic TRAF6, but not phospho-dead mutant TRAF6, suggesting that Src induces the activation of Akt, IKK and JNK through promoting TRAF6 Y473 phosphorylation. Notably, I found that TRAF6 Y473 phosphorylation is required for TRAF6 dimerization/oligomerization and its binding to UBC13, a ubiquitin conjugating enzyme (E2), in order to activate TRAF6 E3 ligase. Finally, I showed that TRAF6 Y473 is a critical event for TRAF6-mediated tumorigenesis in mouse models and serves as a poor prognosis marker for breast cancer progression and disease outcome. My study, therefore, provides an answer to a long-standing puzzle of how TRAF6 activation is achieved and of how Src/TRAF6 mediates Akt, IKK and JNK activation in response to multiple physiological ligands.

However, some important questions still remain to be addressed for future investigation. Here, I discuss some of these questions for the future direction.

5-1. Can IGF-1R signaling stimulus also trigger IKK/NF-κB activation through Src-mediated TRAF6 Y473 phosphorylation?

In this study, I have shown that Src activation is induced by multiple receptors including IGF-1R, IL-1R, TLR4 and RANK. Src then induces TRAF6 tyrosine phosphorylation at Y473, which serves as a critical event for TRAF6 activation. Active TRAF6 then triggers activation of Akt, IKK and JNK to participate in various biological processes such as cell proliferation, migration and tumorigenesis. However, it is still unclear whether IGF-1R signaling stimulus can also trigger the IKK/NF-κB activation through Src-mediated TRAF6 Y473 phosphorylation.

It has been reported that IGF-1R pathway can activate PI-3K/Akt, which in turn stimulates NF-κB signaling through phosphorylating and activating the IKK complex [148,149]. This upregulation of NF-κB signaling is achieved through several mechanisms including stimulating p65 transactivation, as well as phosphorylation and activation of IKKβ. Moreover, many reports have shown that Akt phosphorylates and activates IKKα, which enhances the processing of p100 to p52 [150,151,152]. Interestingly, it is also demonstrated that PDK1 can directly phosphorylate IKKβ and subsequently induce the nuclear translocation of NF-κB complexes to turn on the expression of NF-κB target genes [153]. My data along with these observations suggest that IGF-1R-driven IKK/NF-κB activation is achieved through either or both Src/TRAF6/IKK pathway or/and PDK1/IKK pathway. Additional investigations are required to determine which pathway governs the IGF-1R-driven IKK/NF-κB activation.

5-2. Are there other kinases or phosphatases also involved in regulation of TRAF6 activation?

Initial TRAF6 sequence analysis revealed that several putative kinase motifs are within the TRAF6 sequence including EGFR, JAK2, and Src kinase. Among these candidate kinases, I identified, in my study, the Src tyrosine kinase is a direct TRAF6 kinase that regulates TRAF6 E3 ligase activity through TRAF6 Y473 phosphorylation. However, we did not explore the possibility that other kinases or phosphatases are also involved in regulation of TRAF6 activity. A recent study showed that the gamma catalytic subunit of protein phosphatase 1(PP1-γ) physically associates with TRAF6 and enhances the TRAF6 E3 ligase activity [154]. This result suggests that there might be unknown inhibitory phosphorylation sites of TRAF6, E2 enzyme UBC13, or one of its substrates, requiring for E3 ligase function. Moreover, given the fact that PP1-γ is a serine/threonine phosphatase, it is also possible that serine/threonine phosphorylation event by unknown serine/threonine kinases is also involved in regulation of TRAF6 activation. Further investigation will be needed to explore this possibility.

5-3. Does the Src-mediated TRAF6 Y473 phosphorylation regulate TRAF6 localization and activation?

Activation of Src kinase is known to associate with its membrane localization, shorter disease survival, and increasing tumor size [155,156]. It is also known that upon stimulation with IL-1 or a TLR ligand triggers TRAF6 recruitment to the receptor through interacting with myddosome complex which consists of adaptor protein MyD88 and the protein kinases IRAK1 and IRAK4. IRAKs have been reported to recruit TRAF6 with assistance by a TRAF6-interacting protein with a forkhead-associated domain (TIFA) [157,158]. Moreover, another report showed that upon RANKL ligand stimulation, TRAF6 directly interacts with RANK, leading to downstream activation of signaling pathway including NF-κB [159]. However, it is still unclear whether the TRAF6 membrane localization is associated with its activation. My study showed that various receptor stimulations activate Src tyrosine kinase and Src then phosphorylates and activates TRAF6. Since IRAKs and TIFA promote TRAF6 oligomerization and activation [157,158], it is highly possible that Src-mediated TRAF6 phosphorylation may also regulate TRAF6 membrane recruitment and activation. Our recent findings also support this hypothesis that IGF-1 stimulation increases TRAF6 membrane localization [23], and I have also observed that IL-1 stimulation increases membrane localization of TRAF6 wild-type or TRAF6 phospho-mimetic mutant, but not TRAF6 Y473F mutant (unpublished observation). Further investigation is needed to explore this possibility.

5-4. What is the in vivo impact of Src-mediated TRAF6 Y473 phosphorylation?

During my thesis study, I revealed the novel molecular mechanism of how TRAF6 is activated under diverse physiological stimuli including IGF-1, IL-1, LPS, and RANKL. In this study, I showed that Src-mediated TRAF6 Y473 phosphorylation is critical to regulate the TRAF6 E3 ligase activity and its oncogenic potential. I further showed the clinical relevance of Src activation and TRAF6 Y473 phosphorylation. These findings highlight the importance of TRAF6 Y473 phosphorylation in regulation of TRAF6 activation and TRAF6-mediated tumorigenesis.

To further elucidate the in vivo relevance of Src-mediated TRAF6 Y473 phosphorylation in tumorigenesis, future studies include generating and examining TRAF6, TRAF6 Y473D, and TRAF6 Y473F transgenic mice models. In addition, given the relevance of TRAF6 in host immunity, bone metabolism, autoimmune disease, and neurodegenerative diseases, generating and examining those TRAF6 transgenic mouse models will give us valuable information to advance our current knowledge of TRAF6 in human diseases.

BIBLIOGRAPHY

[1] V.G. Bhoj, Z.J. Chen, Ubiquitylation in innate and adaptive immunity, Nature 458 (2009) 430-437.

[2] B.I. Giasson, V.M. Lee, Are ubiquitination pathways central to Parkinson's disease?, Cell 114 (2003) 1-8.

[3] D. Hoeller, I. Dikic, Targeting the ubiquitin system in cancer therapy, Nature 458 (2009) 438-444.

[4] V. Grunwald, L. DeGraffenried, D. Russel, W.E. Friedrichs, R.B. Ray, M. Hidalgo, Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells, Cancer Res 62 (2002) 6141-6145.

[5] I. Vivanco, C.L. Sawyers, The phosphatidylinositol 3-Kinase AKT pathway in human cancer, Nat Rev Cancer 2 (2002) 489-501.

[6] J.P. Van Brussel, G. Jan Van Steenbrugge, C. Van Krimpen, J.F. Bogdanowicz, T.H. Van Der Kwast, F.H. Schroder, G.H. Mickisch, Expression of multidrug resistance related proteins and proliferative activity is increased in advanced clinical prostate cancer, The Journal of urology 165 (2001) 130-135.

[7] C.M. Pickart, Mechanisms underlying ubiquitination, Annual review of biochemistry 70 (2001) 503-533.

[8] A. Adhikari, Z.J. Chen, Diversity of polyubiquitin chains, Developmental cell 16 (2009) 485-486.

[9] W.L. Yang, X. Zhang, H.K. Lin, Emerging role of Lys-63 ubiquitination in protein

kinase and phosphatase activation and cancer development, Oncogene 29 (2010) 4493-4503.

[10] N. Allende-Vega, M.K. Saville, Targeting the ubiquitin-proteasome system to activate wild-type p53 for cancer therapy, Semin Cancer Biol 20 (2010) 29-39.

[11] C. Raiborg, H. Stenmark, The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins, Nature 458 (2009) 445-452.

[12] H.V. Heemers, D.J. Tindall, Unraveling the complexities of androgen receptor signaling in prostate cancer cells, Cancer Cell 15 (2009) 245-247.

[13] K. Xu, H. Shimelis, D.E. Linn, R. Jiang, X. Yang, F. Sun, Z. Guo, H. Chen, W. Li, H. Chen, X. Kong, J. Melamed, S. Fang, Z. Xiao, T.D. Veenstra, Y. Qiu, Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination, Cancer Cell 15 (2009) 270-282.

[14] S. Zucchelli, M. Codrich, F. Marcuzzi, M. Pinto, S. Vilotti, M. Biagioli, I. Ferrer, S. Gustincich, TRAF6 promotes atypical ubiquitination of mutant DJ-1 and alpha-synuclein and is localized to Lewy bodies in sporadic Parkinson's disease brains, Human molecular genetics 19 (2010) 3759-3770.

[15] S. Zucchelli, F. Marcuzzi, M. Codrich, E. Agostoni, S. Vilotti, M. Biagioli, M. Pinto, A. Carnemolla, C. Santoro, S. Gustincich, F. Persichetti, Tumor necrosis factor receptor-associated factor 6 (TRAF6) associates with huntingtin protein and promotes its atypical ubiquitination to enhance aggregate formation, J Biol Chem 286 (2011) 25108-25117.

[16] S. Adhikary, F. Marinoni, A. Hock, E. Hulleman, N. Popov, R. Beier, S. Bernard, M. Quarto, M. Capra, S. Goettig, U. Kogel, M. Scheffner, K. Helin, M. Eilers, The

ubiquitin ligase HectH9 regulates transcriptional activation by myc and is essential for tumor cell proliferation, Cell 123 (2005) 409-421.

[17] M.S. Huen, R. Grant, I. Manke, K. Minn, X. Yu, M.B. Yaffe, J. Chen, RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly, Cell 131 (2007) 901-914.

[18] N. Mailand, S. Bekker-Jensen, H. Faustrup, F. Melander, J. Bartek, C. Lukas, J. Lukas, RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins, Cell 131 (2007) 887-900.

[19] M.J.M. Bertrand, S. Milutinovic, K.M. Dickson, W.C. Ho, A. Boudreault, J. Durkin, J.W. Gillard, J.B. Jaquith, S.J. Morris, P.A. Barker, cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination, Molecular cell 30 (2008) 689-700.

[20] J.i. Inoue, T. Ishida, N. Tsukamoto, N. Kobayashi, A. Naito, S. Azuma, T. Yamamoto, Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling, Experimental cell research 254 (2000) 14-24.

[21] H. Wajant, F. Henkler, P. Scheurich, The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators, Cellular signalling 13 (2001) 389-400.

[22] H. Ha, D. Han, Y. Choi, TRAF-mediated TNFR-family signaling, Current protocols in immunology / edited by John E Coligan [et al] Chapter 11 (2009) Unit11.19D.

[23] W.-L. Yang, J. Wang, C.-H. Chan, S.-W. Lee, A.D. Campos, B. Lamothe, L. Hur, B.C. Grabiner, X. Lin, B.G. Darnay, H.-K. Lin, The E3 ligase TRAF6 regulates Akt ubiquitination and activation, Science (New York, N Y) 325 (2009) 1134-

1138.

[24] J.R. Arron, M.C. Walsh, Y. Choi, TRAF-mediated TNFR-family signaling, Current protocols in immunology / edited by John E Coligan [et al] Chapter 11 (2002) Unit 11.19D.

[25] R. Ostuni, I. Zanoni, F. Granucci, Deciphering the complexity of Toll-like receptor signaling, Cellular and molecular life sciences : CMLS 67 (2010) 4109-4134.

[26] H. Hacker, P.-H. Tseng, M. Karin, Expanding TRAF function: TRAF3 as a tri-faced immune regulator, Nature reviews Immunology 11 (2011) 457-468.

[27] M. Rothe, S.C. Wong, W.J. Henzel, D.V. Goeddel, A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor, Cell 78 (1994) 681-692.

[28] C.H. Regnier, C. Tomasetto, C. Moog-Lutz, M.P. Chenard, C. Wendling, P. Basset, M.C. Rio, Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma, J Biol Chem 270 (1995) 25715-25721.

[29] T.K. Ishida, T. Tojo, T. Aoki, N. Kobayashi, T. Ohishi, T. Watanabe, T. Yamamoto, J. Inoue, TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling, Proc Natl Acad Sci U S A 93 (1996) 9437-9442.

[30] H. Nakano, H. Oshima, W. Chung, L. Williams-Abbott, C.F. Ware, H. Yagita, K. Okumura, TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor, J Biol Chem 271 (1996) 14661-14664.

[31] S. Ghosh, M.J. May, E.B. Kopp, NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses, Annu Rev Immunol 16 (1998) 225-

260.

[32] N. Silverman, T. Maniatis, NF-kappaB signaling pathways in mammalian and insect innate immunity, Genes & development 15 (2001) 2321-2342.

[33] Z.J. Chen, Ubiquitin signalling in the NF-kappaB pathway, Nature cell biology 7 (2005) 758-765.

[34] S.C. Sun, S.C. Ley, New insights into NF-kappa B regulation and function, Trends in Immunology 29 (2008) 469-478.

[35] M. Karin, Z.G. Liu, E. Zandi, AP-1 function and regulation, Current Opinion in Cell Biology 9 (1997) 240-246.

[36] G. Takaesu, S. Kishida, A. Hiyama, K. Yamaguchi, H. Shibuya, K. Irie, J. Ninomiya-Tsuji, K. Matsumoto, TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway, Molecular Cell 5 (2000) 649-658.

[37] H. Nishitoh, M. Saitoh, Y. Mochida, K. Takeda, H. Nakano, M. Rothe, K. Miyazono, H. Ichijo, ASK1 is essential for JNK/SAPK activation by TRAF2, Molecular Cell 2 (1998) 389-395.

[38] K.P. Hoeflich, W.C. Yeh, Z.B. Yao, T.W. Mak, J.R. Woodgett, Mediation of TNF receptor-associated factor effector functions by apoptosis signal-regulating kinase-1 (ASK1), Oncogene 18 (1999) 5814-5820.

[39] V. Baud, Z.G. Liu, B. Bennett, N. Suzuki, Y. Xia, M. Karin, Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain, Genes & Development 13 (1999) 1297-1308.

[40] T. Yuasa, S. Ohno, J.H. Kehrl, J.M. Kyriakis, Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38, The Journal of biological chemistry 273 (1998) 22681-22692.

[41] C. Liu, W. Qian, Y. Qian, N.V. Giltiay, Y. Lu, S. Swaidani, S. Misra, L. Deng, Z.J. Chen, X. Li, Act1, a U-box E3 ubiquitin ligase for IL-17 signaling, Science signaling 2 (2009) ra63.

[42] J. Siednienko, R. Jackson, M. Mellett, N. Delagic, S. Yang, B. Wang, L.S. Tang, J.J. Callanan, B.P. Mahon, P.N. Moynagh, Pellino3 targets the IRF7 pathway and facilitates autoregulation of TLR3- and viral-induced expression of type I interferons, Nature immunology 13 (2012) 1055-1062.

[43] P. Nakhaei, T. Mesplede, M. Solis, Q. Sun, T. Zhao, L. Yang, T.-H. Chuang, C.F. Ware, R. Lin, J. Hiscott, The E3 ubiquitin ligase Triad3A negatively regulates the RIG-I/MAVS signaling pathway by targeting TRAF3 for degradation, PLoS pathogens 5 (2009) e1000650.

[44] J. Wang, Y. Hu, W.W. Deng, B. Sun, Negative regulation of Toll-like receptor signaling pathway, Microbes and infection / Institut Pasteur 11 (2009) 321-327.

[45] C. Chapard, D. Hohl, M. Huber, The role of the TRAF-interacting protein in proliferation and differentiation, Experimental dermatology 21 (2012) 321-326.

[46] J. Maelfait, R. Beyaert, Emerging role of ubiquitination in antiviral RIG-I signaling, Microbiology and molecular biology reviews : MMBR 76 (2012) 33-45.

[47] N. Kayagaki, Q. Phung, S. Chan, R. Chaudhari, C. Quan, K.M. O'Rourke, M. Eby, E. Pietras, G. Cheng, J.F. Bazan, Z. Zhang, D. Arnott, V.M. Dixit, DUBA: a deubiquitinase that regulates type I interferon production, Science (New York, N Y) 318 (2007) 1628-1632.

[48] J.M. Gonzalez-Navajas, J. Law, K.P. Nguyen, M. Bhargava, M.P. Corr, N. Varki, L. Eckmann, H.M. Hoffman, J. Lee, E. Raz, Interleukin 1 receptor signaling regulates DUBA expression and facilitates Toll-like receptor 9-driven antiinflammatory cytokine production, The Journal of experimental medicine 207 (2010) 2799-2807.

[49] N. Shembade, A. Ma, E.W. Harhaj, Inhibition of NF-kappaB signaling by A20 through disruption of ubiquitin enzyme complexes, Science (New York, N Y) 327 (2010) 1135-1139.

[50] T. Kato, Jr., Y. Gotoh, A. Hoffmann, Y. Ono, Negative regulation of constitutive NF-kappaB and JNK signaling by PKN1-mediated phosphorylation of TRAF1, Genes to cells : devoted to molecular & cellular mechanisms 13 (2008) 509-520.

[51] N.A.E. Oussa, Y. Soumounou, L. Sabbagh, TRAF1 phosphorylation on Serine 139 modulates NF-kappa B activity downstream of 4-1BB in T cells, Biochemical and Biophysical Research Communications 432 (2013) 129-134.

[52] G.S. Thomas, L.Q. Zhang, K. Blackwell, H. Habelhah, Phosphorylation of TRAF2 within Its RING Domain Inhibits Stress-Induced Cell Death by Promoting IKK and Suppressing JNK Activation, Cancer Research 69 (2009) 3665-3672.

[53] S. Li, L.Y. Wang, M.E. Dorf, PKC Phosphorylation of TRAF2 Mediates IKK alpha/beta Recruitment and K63-Linked Polyubiquitination, Molecular Cell 33

(2009) 30-42.

[54] L.Q. Zhang, K. Blackwell, A. Altaeva, Z.H. Shi, H. Habelhah, TRAF2 phosphorylation promotes NF-kappa B-dependent gene expression and inhibits oxidative stress-induced cell death, Molecular Biology of the Cell 22 (2011) 128-140.

[55] R.R. Shen, A.Y. Zhou, E. Kim, E. Lim, H. Habelhah, W.C. Hahn, I kappa B Kinase epsilon Phosphorylates TRAF2 To Promote Mammary Epithelial Cell Transformation, Molecular and Cellular Biology 32 (2012) 4756-4768.

[56] J.M. Marinis, J.E. Hutti, C.R. Homer, B.A. Cobb, L.C. Cantley, C. McDonald, D.W. Abbott, I kappa B Kinase alpha Phosphorylation of TRAF4 Downregulates Innate Immune Signaling, Molecular and Cellular Biology 32 (2012) 2479-2489.

[57] E. Chantzoura, E. Prinarakis, D. Panagopoulos, G. Mosialos, G. Spyrou, Glutaredoxin-1 regulates TRAF6 activation and the IL-1 receptor/TLR4 signalling, Biochemical and Biophysical Research Communications 403 (2010) 335-339.

[58] J.A. Liang, Y. Saad, T.H. Lei, J. Wang, D.F. Qi, Q.L. Yang, P.E. Kolattukudy, M.G. Fu, MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF-kappa B signaling, Journal of Experimental Medicine 207 (2010) 2959-2973.

[59] S. Li, H. Zheng, A.P. Mao, B. Zhong, Y. Li, Y. Liu, Y. Gao, Y. Ran, P. Tien, H.B. Shu, Regulation of virus-triggered signaling by OTUB1- and OTUB2-mediated deubiquitination of TRAF3 and TRAF6, J Biol Chem 285 (2010) 4291-4297.

[60] A.L. Mahul-Mellier, E. Pazarentzos, C. Datler, R. Iwasawa, G. AbuAli, B. Lin, S.

Grimm, De-ubiquitinating protease USP2a targets RIP1 and TRAF2 to mediate cell death by TNF, Cell Death Differ 19 (2012) 891-899.

[61] X. He, Y. Li, C. Li, L.J. Liu, X.D. Zhang, Y. Liu, H.B. Shu, USP2a negatively regulates IL-1-and virus-induced NF-B activation by deubiquitinating TRAF6, Journal of Molecular Cell Biology 5 (2013) 39-47.

[62] N. Xiao, H. Li, J. Luo, R. Wang, H. Chen, J. Chen, P. Wang, Ubiquitin-specific protease 4 (USP4) targets TRAF2 and TRAF6 for deubiquitination and inhibits TNFalpha-induced cancer cell migration, The Biochemical journal 441 (2012) 979-986.

[63] J. Yasunaga, F.C. Lin, X. Lu, K.-T. Jeang, Ubiquitin-specific peptidase 20 targets TRAF6 and human T cell leukemia virus type 1 tax to negatively regulate NF-kappaB signaling, Journal of virology 85 (2011) 6212-6219.

[64] B. Zhong, X. Liu, X. Wang, S.H. Chang, X. Liu, A. Wang, J.M. Reynolds, C. Dong, Negative regulation of IL-17-mediated signaling and inflammation by the ubiquitin-specific protease USP25, Nature immunology 13 (2012) 1110-1117.

[65] Z.J.J. Chen, Ubiquitin signalling in the NF-kappa B pathway, Nature Cell Biology 7 (2005) 758-U719.

[66] Z.J.J. Chen, L.J.J. Sun, Nonproteolytic Functions of Ubiquitin in Cell Signaling, Molecular Cell 33 (2009) 275-286.

[67] M.A. Lomaga, W.C. Yeh, I. Sarosi, G.S. Duncan, C. Furlonger, A. Ho, S. Morony, C. Capparelli, G. Van, S. Kaufman, A. van der Heiden, A. Itie, A. Wakeham, W. Khoo, T. Sasaki, Z.D. Cao, J.M. Penninger, C.J. Paige, D.L. Lacey, C.R. Dunstan, W.J. Boyle, D.V. Goeddel, T.W. Mak, TRAF6 deficiency results in osteopetrosis

and defective interleukin-1, CD40, and LPS signaling, Genes & Development 13 (1999) 1015-1024.

[68] A. Sorrentino, N. Thakur, S. Grimsby, A. Marcusson, V. von Bulow, N. Schuster, S. Zhang, C.H. Heldin, M. Landstrom, The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner, Nature Cell Biology 10 (2008) 1199-1207.

[69] M. Yamashita, K. Fatyol, C.Y. Jin, X.C. Wang, Z.G. Liu, Y.E. Zhang, TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta, Molecular Cell 31 (2008) 918-924.

[70] C.H. Heldin, M. Landstrom, A. Moustakas, Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition, Curr Opin Cell Biol 21 (2009) 166-176.

[71] R. Mao, Y. Fan, Y. Mou, H. Zhang, S. Fu, J. Yang, TAK1 lysine 158 is required for TGF-beta-induced TRAF6-mediated Smad-independent IKK/NF-kappaB and JNK/AP-1 activation, Cell Signal 23 (2011) 222-227.

[72] Y. Fan, Y. Yu, Y. Shi, W. Sun, M. Xie, N. Ge, R. Mao, A. Chang, G. Xu, M.D. Schneider, H. Zhang, S. Fu, J. Qin, J. Yang, Lysine 63-linked polyubiquitination of TAK1 at lysine 158 is required for tumor necrosis factor alpha- and interleukin-1beta-induced IKK/NF-kappaB and JNK/AP-1 activation, J Biol Chem 285 (2010) 5347-5360.

[73] Y. Mu, R. Sundar, N. Thakur, M. Ekman, S.K. Gudey, M. Yakymovych, A. Hermansson, H. Dimitriou, M.T. Bengoechea-Alonso, J. Ericsson, C.H. Heldin, M. Landstrom, TRAF6 ubiquitinates TGFbeta type I receptor to promote its

cleavage and nuclear translocation in cancer, Nat Commun 2 (2011) 330.

[74] S. Lim, E. Bae, H.S. Kim, T.A. Kim, K. Byun, B. Kim, S. Hong, J.P. Im, C. Yun, B. Lee, B. Lee, S.H. Park, J. Letterio, S.J. Kim, TRAF6 mediates IL-1beta/LPS-induced suppression of TGF-beta signaling through its interaction with the type III TGF-beta receptor, PLoS One 7 (2012) e32705.

[75] S.J. Parsons, J.T. Parsons, Src family kinases, key regulators of signal transduction, Oncogene 23 (2004) 7906-7909.

[76] S.J. Corey, S.M. Anderson, Src-related protein tyrosine kinases in hematopoiesis, Blood 93 (1999) 1-14.

[77] T.J. Boggon, M.J. Eck, Structure and regulation of Src family kinases, Oncogene 23 (2004) 7918-7927.

[78] M.D. Resh, Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, Biochim Biophys Acta 1451 (1999) 1-16.

[79] E. Giannoni, M.L. Taddei, P. Chiarugi, Src redox regulation: again in the front line, Free Radic Biol Med 49 (2010) 516-527.

[80] S.M. Thomas, J.S. Brugge, Cellular functions regulated by Src family kinases, Annu Rev Cell Dev Biol 13 (1997) 513-609.

[81] P.L. Stein, H.M. Lee, S. Rich, P. Soriano, pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells, Cell 70 (1992) 741-750.

[82] Z. Ming, Y. Hu, J. Xiang, P. Polewski, P.J. Newman, D.K. Newman, Lyn and PECAM-1 function as interdependent inhibitors of platelet aggregation, Blood 117 (2011) 3903-3906.

[83] J.M. Dal Porto, K. Burke, J.C. Cambier, Regulation of BCR signal transduction in B-1 cells requires the expression of the Src family kinase Lck, Immunity 21 (2004) 443-453.

[84] F. Chiaradonna, L. Fontana, C. Iavarone, M.V. Carriero, G. Scholz, M.V. Barone, M.P. Stoppelli, Urokinase receptor-dependent and -independent p56/59(hck) activation state is a molecular switch between myelomonocytic cell motility and adherence, EMBO J 18 (1999) 3013-3023.

[85] C.J. Fitzer-Attas, M. Lowry, M.T. Crowley, A.J. Finn, F. Meng, A.L. DeFranco, C.A. Lowell, Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn, J Exp Med 191 (2000) 669-682.

[86] S.N. Malek, D.I. Dordai, J. Reim, H. Dintzis, S. Desiderio, Malignant transformation of early lymphoid progenitors in mice expressing an activated Blk tyrosine kinase, Proc Natl Acad Sci U S A 95 (1998) 7351-7356.

[87] E. Gaudreault, C. Thompson, J. Stankova, M. Rola-Pleszczynski, Involvement of BLT1 endocytosis and Yes kinase activation in leukotriene B4-induced neutrophil degranulation, J Immunol 174 (2005) 3617-3625.

[88] P. Chiarugi, Src redox regulation: there is more than meets the eye, Mol Cells 26 (2008) 329-337.

[89] W. Xu, S.C. Harrison, M.J. Eck, Three-dimensional structure of the tyrosine kinase c-Src, Nature 385 (1997) 595-602.

[90] S.E. Byeon, Y.S. Yi, J. Oh, B.C. Yoo, S. Hong, J.Y. Cho, The role of Src kinase in macrophage-mediated inflammatory responses, Mediators Inflamm 2012 (2012) 512926.

[91] M. Okada, H. Nakagawa, A Protein Tyrosine Kinase Involved in Regulation of Pp60c-Src Function, Journal of Biological Chemistry 264 (1989) 20886-20893.

[92] S. ZrihanLicht, J. Lim, I. Keydar, M.X. Sliwkowski, J.E. Groopman, H. Avraham, Association of Csk-homologous kinase (CHK) (formerly MATK) with HER-2/ErbB-2 in breast cancer cells, Journal of Biological Chemistry 272 (1997) 1856-1863.

[93] D.R. Stover, P. Furet, N.B. Lydon, Modulation of the SH2 binding specificity and kinase activity of Src by tyrosine phosphorylation within its SH2 domain, Journal of Biological Chemistry 271 (1996) 12481-12487.

[94] M. Baker, J. Gamble, R. Tooze, D. Higgins, F.T. Yang, P.C. O'Brien, N. Coleman, S. Pingel, M. Turner, D.R. Alexander, Development of T-leukaemias in CD45 tyrosine phosphatase-deficient mutant lck mice, The EMBO journal 19 (2000) 4644-4654.

[95] J. Su, M. Muranjan, J. Sap, Receptor protein tyrosine phosphatase alpha activates Src-family kinases and controls integrin-mediated responses in fibroblasts, Current biology : CB 9 (1999) 505-511.

[96] C.H. Chan, C.F. Li, W.L. Yang, Y. Gao, S.W. Lee, Z. Feng, H.Y. Huang, K.K. Tsai, L.G. Flores, Y. Shao, J.D. Hazle, D. Yu, W. Wei, D. Sarbassov, M.C. Hung, K.I. Nakayama, H.K. Lin, The Skp2-SCF E3 ligase regulates Akt ubiquitination, glycolysis, herceptin sensitivity, and tumorigenesis, Cell 149 (2012) 1098-1111.

[97] J.Y. Chung, Y.C. Park, H. Ye, H. Wu, All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction, Journal of Cell Science 115 (2002) 679-688.

[98] H. Wu, J.R. Arron, TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology, Bioessays 25 (2003) 1096-1105.

[99] S. Akira, K. Takeda, Toll-like receptor signalling, Nature Reviews Immunology 4 (2004) 499-511.

[100] B.R. Wong, D. Besser, N. Kim, J.R. Arron, M. Vologodskaia, H. Hanafusa, Y. Choi, TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src, Molecular Cell 4 (1999) 1041-1049.

[101] W.L. Yang, C.Y. Wu, J. Wu, H.K. Lin, Regulation of Akt signaling activation by ubiquitination, Cell Cycle 9 (2010) 486-497.

[102] J.L. Kang, H.W. Lee, H.J. Kim, H.S. Lee, V. Castranova, C.M. Lim, Y. Koh, Inhibition of SRC tyrosine kinases suppresses activation of nuclear factor-kappa B, and serine and tyrosine phosphorylation of I kappa B-alpha in lipopolysaccharide-stimulated raw 264.7 macrophages, Journal of Toxicology and Environmental Health-Part a-Current Issues 68 (2005) 1643-1662.

[103] H.S. Lee, C. Moon, H.W. Lee, E.M. Park, M.S. Cho, J.L. Kang, Src tyrosine kinases mediate activations of NF-kappa B and integrin signal during lipopolysaccharide-induced acute lung injury, Journal of Immunology 179 (2007) 7001-7011.

[104] D.I. Jalal, B.C. Kone, Src activation of NF-kappa B augments IL-1 beta-induced nitric oxide production in mesangial cells, Journal of the American Society of Nephrology 17 (2006) 99-106.

[105] M. Funakoshi-Tago, K. Tago, K. Andoh, Y. Sonoda, S. Tominaga, T. Kasahara, Functional role of c-src in IL-1-induced NF-kappa B activation: c-Src is a

component of the IKK complex, Journal of Biochemistry 137 (2005) 189-197.

[106] K.Z.Q. Wang, N. Wara-Aswapati, J.A. Boch, Y. Yoshida, C.D. Hu, D.L. Galson, P.E. Auron, TRAF6 activation of PI 3-kinase-dependent cytoskeletal changes is cooperative with Ras and is mediated by an interaction with cytoplasmic Src, Journal of Cell Science 119 (2006) 1579-1591.

[107] H. Ye, J.R. Arron, B. Lamothe, M. Cirilli, T. Kobayashi, N.K. Shevde, D. Segal, O.K. Dzivenu, M. Vologodskaia, M. Yim, K. Du, S. Singh, J.W. Pike, B.G. Darnay, Y. Choi, H. Wu, Distinct molecular mechanism for initiating TRAF6 signalling, Nature 418 (2002) 443-447.

[108] Q. Yin, S.C. Lin, B. Lamothe, M. Lu, Y.C. Lo, G. Hura, L.X. Zheng, R.L. Rich, A.D. Campos, D.G. Myszka, M.J. Lenardo, B.G. Darnay, H. Wu, E2 interaction and dimerization in the crystal structure of TRAF6, Nature Structural & Molecular Biology 16 (2009) 658-U697.

[109] R. Ferrao, J.X. Li, E. Bergamin, H. Wu, Structural Insights into the Assembly of Large Oligomeric Signalosomes in the Toll-Like Receptor-Interleukin-1 Receptor Superfamily, Science Signaling 5 (2012).

[110] M. Takeuchi, M. Rothe, D.V. Goeddel, Anatomy of TRAF2 - Distinct domains for nuclear factor-kappa B activation and association with tumor necrosis factor signaling proteins, Journal of Biological Chemistry 271 (1996) 19935-19942.

[111] J.I.J. Leu, P. Dumont, M. Hafey, M.E. Murphy, D.L. George, Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex, Nature Cell Biology 6 (2004) 443-450.

[112] M.G. Annis, E.L. Soucie, P.J. Dlugosz, J.A. Cruz-Aguado, L.Z. Penn, B. Leber,

D.W. Andrews, Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis, EMBO J 24 (2005) 2096-2103.

[113] K. John, V. Alla, C. Meier, B.M. Putzer, GRAMD4 mimics p53 and mediates the apoptotic function of p73 at mitochondria, Cell Death Differ 18 (2011) 874-886.

[114] T. Moldoveanu, C.R. Grace, F. Llambi, A. Nourse, P. Fitzgerald, K. Gehring, R.W. Kriwacki, D.R. Green, BID-induced structural changes in BAK promote apoptosis, Nat Struct Mol Biol 20 (2013) 589-597.

[115] T. Fukushima, S. Matsuzawa, C.L. Kress, J.M. Bruey, M. Krajewska, S. Lefebvre, J.M. Zapata, Z. Ronai, J.C. Reed, Ubiquitin-conjugating enzyme Ubc13 is a critical component of TNF receptor-associated factor (TRAF)-mediated inflammatory responses, Proceedings of the National Academy of Sciences of the United States of America 104 (2007) 6371-6376.

[116] Y.C. Lin, D.Y. Huang, C.L. Chu, Y.L. Lin, W.W. Lin, The Tyrosine Kinase Syk Differentially Regulates Toll-like Receptor Signaling Downstream of the Adaptor Molecules TRAF6 and TRAF3, Science Signaling 6 (2013).

[117] E. Giannoni, T. Fiaschi, G. Ramponi, P. Chiarugi, Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals, Oncogene 28 (2009) 2074-2086.

[118] D. Johnson, M. Agochiya, K. Samejima, W. Earnshaw, M. Frame, J. Wyke, Regulation of both apoptosis and cell survival by the v-Src oncoprotein, Cell Death and Differentiation 7 (2000) 685-696.

[119] A.P. Belsches, M.D. Haskell, S.J. Parsons, Role of c-Src tyrosine kinase in EGF-induced mitogenesis, Front Biosci 2 (1997) d501-518.

[120] J.T. Parsons, S.J. Parsons, Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways, Curr Opin Cell Biol 9 (1997) 187-192.

[121] G.W. Krystal, C.S. DeBerry, D. Linnekin, J. Litz, Lck associates with and is activated by Kit in a small cell lung cancer cell line: inhibition of SCF-mediated growth by the Src family kinase inhibitor PP1, Cancer research 58 (1998) 4660-4666.

[122] J.S. Biscardi, D.A. Tice, S.J. Parsons, c-Src, receptor tyrosine kinases, and human cancer, Advances in cancer research 76 (1999) 61-119.

[123] C.L. Abram, S.A. Courtneidge, Src family tyrosine kinases and growth factor signaling, Experimental cell research 254 (2000) 1-13.

[124] A. Dey, H. She, L. Kim, A. Boruch, D.L. Guris, K. Carlberg, S.M. Sebti, D.T. Woodley, A. Imamoto, W. Li, Colony-stimulating factor-1 receptor utilizes multiple signaling pathways to induce cyclin D2 expression, Molecular biology of the cell 11 (2000) 3835-3848.

[125] L. Hong, V. Munugalavadla, R. Kapur, c-Kit-mediated overlapping and unique functional and biochemical outcomes via diverse signaling pathways, Molecular and cellular biology 24 (2004) 1401-1410.

[126] A.S. Mohamed, K.A. Rivas-Plata, J.R. Kraas, S.M. Saleh, S.L. Swope, Src-class kinases act within the agrin/MuSK pathway to regulate acetylcholine receptor phosphorylation, cytoskeletal anchoring, and clustering, The Journal of neuroscience : the official journal of the Society for Neuroscience 21 (2001) 3806-3818.

[127] Y. Maejima, H. Ueba, M. Kuroki, T. Yasu, S. Hashimoto, A. Nabata, N. Kobayashi, N. Ikeda, M. Saito, M. Kawakami, Src family kinases and nitric oxide production are required for hepatocyte growth factor-stimulated endothelial cell growth, Atherosclerosis 167 (2003) 89-95.

[128] Y. Abu-Amer, F.P. Ross, K.P. McHugh, A. Livolsi, J.F. Peyron, S.L. Teitelbaum, Tumor necrosis factor-alpha activation of nuclear transcription factor-kappaB in marrow macrophages is mediated by c-Src tyrosine phosphorylation of Ikappa Balpha, The Journal of biological chemistry 273 (1998) 29417-29423.

[129] C. Fan, Q. Li, D. Ross, J.F. Engelhardt, Tyrosine phosphorylation of I kappa B alpha activates NF kappa B through a redox-regulated and c-Src-dependent mechanism following hypoxia/reoxygenation, J Biol Chem 278 (2003) 2072-2080.

[130] J.L. Kang, H.J. Jung, K. Lee, H.R. Kim, Src tyrosine kinases mediate crystalline silica-induced NF-kappaB activation through tyrosine phosphorylation of IkappaB-alpha and p65 NF-kappaB in RAW 264.7 macrophages, Toxicol Sci 90 (2006) 470-477.

[131] K. Yang, J. Zhu, S. Sun, Y. Tang, B. Zhang, L. Diao, C. Wang, The coiled-coil domain of TRAF6 is essential for its auto-ubiquitination, Biochem Biophys Res Commun 324 (2004) 432-439.

[132] Q. Yin, S.C. Lin, B. Lamothe, M. Lu, Y.C. Lo, G. Hura, L. Zheng, R.L. Rich, A.D. Campos, D.G. Myszka, M.J. Lenardo, B.G. Darnay, H. Wu, E2 interaction and dimerization in the crystal structure of TRAF6, Nat Struct Mol Biol 16 (2009) 658-666.

[133] K.Z.Q. Wang, D.L. Galson, P.E. Auron, TRAF6 is autoinhibited by an

intramolecular interaction which is counteracted by trans-ubiquitination, Journal of cellular biochemistry 110 (2010) 763-771.

[134] V. Baud, M. Karin, Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls, Nature reviews Drug discovery 8 (2009) 33-40.

[135] M. Karin, Y.X. Cao, F.R. Greten, Z.W. Li, NF-kappa B in cancer: From innocent bystander to major culprit, Nature Reviews Cancer 2 (2002) 301-310.

[136] D.R. Hurst, M.D. Edmonds, G.K. Scott, C.C. Benz, K.S. Vaidya, D.R. Welch, Breast Cancer Metastasis Suppressor 1 Up-regulates miR-146, Which Suppresses Breast Cancer Metastasis, Cancer Research 69 (2009) 1279-1283.

[137] D.T. Starczynowski, F. Kuchenbauer, B. Argiropoulos, S. Sung, R. Morin, A. Muranyi, M. Hirst, D. Hogge, M. Marra, R.A. Wells, R. Buckstein, W. Lam, R.K. Humphries, A. Karsan, Identification of miR-145 and miR-146a as mediators of the 5q-syndrome phenotype, Nature Medicine 16 (2010) 49-U84.

[138] D.T. Starczynowski, W.W. Lockwood, S. Delehouzee, R. Chari, J. Wegrzyn, M. Fuller, M.S. Tsao, S. Lam, A.F. Gazdar, W.L. Lam, A. Karsan, TRAF6 is an amplified oncogene bridging the RAS and NF-kappa B pathways in human lung cancer, Journal of Clinical Investigation 121 (2011) 4095-4105.

[139] Y.S. Sun, Z.Y. Ye, Z.Y. Qian, X.D. Xu, J.F. Hu, Expression of TRAF6 and ubiquitin mRNA in skeletal muscle of gastric cancer patients, Journal of Experimental & Clinical Cancer Research 31 (2012).

[140] Q.B. Meng, M.Q. Zheng, H.B. Liu, C.Z. Song, W.S. Zhang, J. Yan, L. Qin, X.L. Liu, TRAF6 regulates proliferation, apoptosis, and invasion of osteosarcoma cell, Molecular and Cellular Biochemistry 371 (2012) 177-186.

[141] L. Zhong, F. Cao, Q.S. You, Effect of TRAF6 on the biological behavior of human lung adenocarcinoma cell, Tumor Biology 34 (2013) 231-239.

[142] J.S. Biscardi, D.A. Tice, S.J. Parsons, c-Src, receptor tyrosine kinases, and human cancer, Advances in Cancer Research, Vol 76 76 (1999) 61-+.

[143] J.B. Bolen, A. Veillette, A.M. Schwartz, V. Deseau, N. Rosen, Activation of Pp60c-Src Protein-Kinase Activity in Human-Colon Carcinoma, Proceedings of the National Academy of Sciences of the United States of America 84 (1987) 2251-2255.

[144] C.A. Cartwright, M.P. Kamps, A.I. Meisler, J.M. Pipas, W. Eckhart, Pp60c-Src Activation in Human-Colon Carcinoma, Journal of Clinical Investigation 83 (1989) 2025-2033.

[145] C. Egan, A. Pang, D. Durda, H.C. Cheng, J.H. Wang, D.J. Fujita, Activation of Src in human breast tumor cell lines: elevated levels of phosphotyrosine phosphatase activity that preferentially recognizes the Src carboxy terminal negative regulatory tyrosine 530, Oncogene 18 (1999) 1227-1237.

[146] B.S. Verbeek, T.M. Vroom, S.S. AdriaansenSlot, A.E. OttenhoffKalff, J.G.N. Geertzema, A. Hennipman, G. Rijksen, c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis, Journal of Pathology 180 (1996) 383-388.

[147] B. Elsberger, Translational evidence on the role of Src kinase and activated Src kinase in invasive breast cancer, Critical Reviews in Oncology Hematology 89 (2014) 343-351.

[148] L.V. Madrid, C.Y. Wang, D.C. Guttridge, A.J. Schottelius, A.S. Baldwin, Jr., M.W.

Mayo, Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB, Mol Cell Biol 20 (2000) 1626-1638.

[149] L.V. Madrid, M.W. Mayo, J.Y. Reuther, A.S. Baldwin, Jr., Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38, J Biol Chem 276 (2001) 18934-18940.

[150] O.N. Ozes, L.D. Mayo, J.A. Gustin, S.R. Pfeffer, L.M. Pfeffer, D.B. Donner, NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase, Nature 401 (1999) 82-85.

[151] C.M. Cahill, J.T. Rogers, Interleukin (IL) 1beta induction of IL-6 is mediated by a novel phosphatidylinositol 3-kinase-dependent AKT/IkappaB kinase alpha pathway targeting activator protein-1, J Biol Chem 283 (2008) 25900-25912.

[152] H.C. Dan, M.J. Cooper, P.C. Cogswell, J.A. Duncan, J.P. Ting, A.S. Baldwin, Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK, Genes Dev 22 (2008) 1490-1500.

[153] H. Tanaka, N. Fujita, T. Tsuruo, 3-Phosphoinositide-dependent protein kinase-1-mediated IkappaB kinase beta (IkkB) phosphorylation activates NF-kappaB signaling, J Biol Chem 280 (2005) 40965-40973.

[154] A.M. Opaluch, M. Schneider, C.Y. Chiang, Q.T. Nguyen, A.M. Maestre, L.C. Mulder, I. Secundino, P.D. De Jesus, R. Konig, V. Simon, V. Nizet, G. MacLeod, S. Varmuza, A. Fernandez-Sesma, S.K. Chanda, Positive regulation of TRAF6-dependent innate immune responses by protein phosphatase PP1-gamma, PLoS One 9 (2014) e89284.

[155] M.C. Frame, Src in cancer: deregulation and consequences for cell behaviour, Biochim Biophys Acta 1602 (2002) 114-130.

[156] B. Elsberger, R. Fullerton, S. Zino, F. Jordan, T.J. Mitchell, V.G. Brunton, E.A. Mallon, P.G. Shiels, J. Edwards, Breast cancer patients' clinical outcome measures are associated with Src kinase family member expression, Br J Cancer 103 (2010) 899-909.

[157] C.K. Ea, L. Sun, J. Inoue, Z.J. Chen, TIFA activates IkappaB kinase (IKK) by promoting oligomerization and ubiquitination of TRAF6, Proc Natl Acad Sci U S A 101 (2004) 15318-15323.

[158] H. Takatsuna, H. Kato, J. Gohda, T. Akiyama, A. Moriya, Y. Okamoto, Y. Yamagata, M. Otsuka, K. Umezawa, K. Semba, J.-I. Inoue, Identification of TIFA as an adapter protein that links tumor necrosis factor receptor-associated factor 6 (TRAF6) to interleukin-1 (IL-1) receptor-associated kinase-1 (IRAK-1) in IL-1 receptor signaling, The Journal of biological chemistry 278 (2003) 12144-12150.

[159] B.G. Darnay, J. Ni, P.A. Moore, B.B. Aggarwal, Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif, The Journal of biological chemistry 274 (1999) 7724-7731.

<u>VITA</u>

Yun Seong Jeong was born in Seoul city, South Korea on Aug 5, 1974, the youngest son of Pyo-Young Jeong and Keun-Nam Lee. He received degree of Bachelor of Science from Yonsei University in Seoul, South Korea in 2001 and master degree from the same universtiy in 2003. He then worked as a research assistant in the college of pharmacy at Kentucky University in the U.S. and department of chemistry at Konkuk University in South Korea. In August 2006, he enrolled in the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. He joined Dr. Hui-Kuan Lin's lab in 2010 to complete his Ph. D. dissertation.

➢ Permanent address: 436-65 Changsin 1-dong, Jongno-gu, Seoul, South Korea 110-842