8-2014

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

Yun Seong Jeong

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THE ROLE OF TRAF6 PHOSPHORYLATION IN Src/TRAFl6-MEDIATED IKK, JNK, Akt ACTIVATION AND TUMORIGENESIS

by

Yun Seong Jeong, M.S.

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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

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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 throughout 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.
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<td>AP-1</td>
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<tr>
<td>ASK-1</td>
<td>Apoptosis singal activating kinase-1</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AWP1</td>
<td>Associated with PRK1</td>
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<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
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<tr>
<td>BMH</td>
<td>Bismaleimidohexane</td>
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<td>CHIP</td>
<td>Carboxyl terminus of Hsc-70-interacting protein</td>
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<td>c-IAP1/2</td>
<td>Cellular inhibitor of apoptosis protein ½</td>
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<td>CSK</td>
<td>C-terminal Src Kinase</td>
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<td>Cylindromatosis</td>
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<td>Deubiquitinating enzyme A</td>
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<td>IFN</td>
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<td>IGF-1R</td>
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<td>IkB</td>
<td>Inhibitor of NF-κB</td>
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<td>JNK</td>
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<td>LPS</td>
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<td>Lymphotoxin-β</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<td>MEKK1</td>
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<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
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<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>OUT</td>
<td>Ovarian tumor-like protease</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDGF-R</td>
<td>Platelet derived growth factor receptor</td>
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<td>RANKL</td>
<td>Receptor activator of NF-κB ligand</td>
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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.

RING: RING domain, Zn: Zinc-finger, C-C: Coiled-coil, W: WD40 domain
TRAF-C: TRAF-C 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

<table>
<thead>
<tr>
<th>E3 ligases</th>
<th>Targets</th>
<th>Receptor signaling</th>
<th>Ubiquitin-linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act1</td>
<td>TRAF6 (Lys124)</td>
<td>IL-17R</td>
<td>K63</td>
</tr>
<tr>
<td></td>
<td>TRAF5</td>
<td>IL-17R</td>
<td>K63</td>
</tr>
<tr>
<td>cIAP1/2</td>
<td>TRAF3 and TRAF6</td>
<td>RIG-1</td>
<td>K63</td>
</tr>
<tr>
<td></td>
<td>TRAF2</td>
<td>TNF-R2</td>
<td>K48</td>
</tr>
<tr>
<td></td>
<td>TRAF2</td>
<td>M-CSFR</td>
<td>K48</td>
</tr>
<tr>
<td></td>
<td>TRAF2 and TRAF3</td>
<td>CD40 and TLR4</td>
<td>K48</td>
</tr>
<tr>
<td></td>
<td>TRAF3 and TRAF6</td>
<td>RIG-I</td>
<td>K48</td>
</tr>
<tr>
<td>Pellino3</td>
<td>TRAF6 (Lys124)</td>
<td>TLR3</td>
<td>K63</td>
</tr>
<tr>
<td>Triad3A</td>
<td>TRAF3</td>
<td>RIG-I</td>
<td>K48</td>
</tr>
<tr>
<td>AWP1</td>
<td>TRAF2</td>
<td>TNF-R1/2</td>
<td>K48</td>
</tr>
<tr>
<td>SOCS2</td>
<td>TRAF6</td>
<td>AhR</td>
<td>K48</td>
</tr>
<tr>
<td>Siva-1</td>
<td>TRAF2</td>
<td>TCR</td>
<td>K48</td>
</tr>
<tr>
<td>Numbl</td>
<td>TRAF6 and TRAF5</td>
<td>IL-1R</td>
<td>K48</td>
</tr>
<tr>
<td>CHIP</td>
<td>TRAF2</td>
<td>Cancer cell invasion</td>
<td>K48</td>
</tr>
</tbody>
</table>
Table 1-2. Deubiquiting enzymes of TRAFs

<table>
<thead>
<tr>
<th>DUBs</th>
<th>TRAFs</th>
<th>Signaling</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20</td>
<td>TRAF6</td>
<td>TLR2, TLR4</td>
<td>[25,46,49]</td>
</tr>
<tr>
<td>CYLD</td>
<td>TRAF2,TRAF6</td>
<td>CD40,XEDAR,EDAR,RANK IL-1R, TNF-α</td>
<td>[44,45]</td>
</tr>
<tr>
<td>DUBA (OUTD5)</td>
<td>TRAF3</td>
<td>IL-1R,TLR9,TLR3,TLR4, TLR7, RIG-I, MDA-5</td>
<td>[46,47,48]</td>
</tr>
<tr>
<td>MCPIP1</td>
<td>TRAF2,TRAF3,TRAF6</td>
<td>IL-1R, TLR4</td>
<td>[58]</td>
</tr>
<tr>
<td>OTUB1</td>
<td>TRAF3,TRAF6</td>
<td>RIG-I</td>
<td>[59]</td>
</tr>
<tr>
<td>OTUB2</td>
<td>TRAF3,TRAF6</td>
<td>RIG-I</td>
<td>[59]</td>
</tr>
<tr>
<td>USP2a</td>
<td>TRAF2</td>
<td>TNFR1</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>TRAF6</td>
<td>IL-1R and RIG-I</td>
<td>[61]</td>
</tr>
<tr>
<td>USP4</td>
<td>TRAF2,TRAF6</td>
<td>TNF-α</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>TRAF6</td>
<td>IL-1R,TLR4</td>
<td></td>
</tr>
<tr>
<td>USP20</td>
<td>TRAF6</td>
<td>IL-1R</td>
<td>[63]</td>
</tr>
<tr>
<td>USP25</td>
<td>TRAF5,TRAF6</td>
<td>IL-17R</td>
<td>[64]</td>
</tr>
</tbody>
</table>
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 phosphorylates 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).
**Table 1-3. Known functions and expression pattern of Src family kinases**

<table>
<thead>
<tr>
<th>SFKs</th>
<th>Expression</th>
<th>Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Src</td>
<td>Ubiquitous</td>
<td>Cell proliferation, adhesion, migration, chemotaxis, phagocytosis, and survival</td>
<td>[80]</td>
</tr>
<tr>
<td>Fyn</td>
<td>Ubiquitous</td>
<td>T-cell inhibition and Rac and stress kinase activation</td>
<td>[81]</td>
</tr>
<tr>
<td>Lyn</td>
<td>Platelet, granulocyte; Monocyte, macrophage, B cell</td>
<td>Neutrophil recruitment, Inhibition of platelet aggregation</td>
<td>[82]</td>
</tr>
<tr>
<td>Lck</td>
<td>T cell, B cell</td>
<td>Regulation of signaling and B-1 cell fate</td>
<td>[83]</td>
</tr>
<tr>
<td>Hck</td>
<td>Granulocyte, monocyte, macrophage</td>
<td>Inhibition of Hck and Fgr kinase activity</td>
<td>[84]</td>
</tr>
<tr>
<td>Fgr</td>
<td>Granulocyte, monocyte, macrophage</td>
<td>Inhibition of Hck and Fgr kinase activity, Inhibition of β2 integrin receptor and Syk kinase signaling</td>
<td>[84,85]</td>
</tr>
<tr>
<td>Blk</td>
<td>Granulocyte, monocyte, macrophage, B cell</td>
<td>Control of B cell proliferation</td>
<td>[86]</td>
</tr>
<tr>
<td>Frk</td>
<td>Ubiquitous</td>
<td>Unclear</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>Ubiquitous</td>
<td>LTB4-mediated degranulation</td>
<td>[87]</td>
</tr>
</tbody>
</table>
Figure 1-4. The structure of Src kinase.

The Src is consists of an N-terminal myristoylation 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 motifs. 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<sup>−/−</sup> MEFs were prepared as described previously [23]. Src<sup>−/−</sup> 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


2-4. Immunoprecipitation (IP) and Immunoblotting (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 Na3VO4, 10mM MgCl2, 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\textsuperscript{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.


For the cell growth assay, 5×10\textsuperscript{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 2 days. 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 (4×10⁵) 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). Immunoreexpression 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\(^{\text{Tyr416}}\) (Cell Signaling, polyclonal, 1:50), pTRAF6 \(^{\text{Tyr473}}\) (New England Peptide, 1:50), pAkt\(^{\text{Ser473}}\) (Cell Signaling, clone D9E, 1:25), and pIk\(\kappa\alpha/\beta\) (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 = ΣPi (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 pSrcTyr416, pTRAF6Tyr473, pAktSer473, and pIkka/β expression in relation to important clinicopathologic variables. The Pearson’s correlation coefficient was used to clarify the association between pSrcTyr416, pTRAF6Tyr473, pAktSer473, and pIkka/β 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
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 15 min, 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 (5 μg/ml) for 5 min, 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.
### 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) andSrc−/− 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-κB, and JNK in response to IGF-1, LPS, IL-1, and RANKL (Figures 3-4A-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.
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.
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.
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.
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 1 day, 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.
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^−/− MEV 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.
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<sup>−/−</sup> 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 ± 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-231 breast 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 ± 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), phospho-TRAF6 (Y416), phospho-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 phospho-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.

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<tr>
<th>Parameters</th>
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<th>pAkt LI</th>
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<td>p-value</td>
<td>H-Score</td>
<td>p-value</td>
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<td>90</td>
<td>195.3±66.4</td>
<td>0.024*</td>
<td>208.9±63.9</td>
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<td>T2</td>
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<td>212.9±87.4</td>
<td></td>
<td>228.5±63.6</td>
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<tr>
<td></td>
<td>T3-T4</td>
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<td>246.8±74.7</td>
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<td>197.4±71.7</td>
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<td>215.2±65.1</td>
<td>0.044*</td>
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<td>233.3±62.9</td>
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<td>Stage</td>
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<td>204.5±67.0</td>
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Table 3-2. Associations between pSrc, pTRAF6, pAkt, and pIkκα/β expressions in breast cancer

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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>pAkt LI</td>
<td>r=0.454&lt;0.001*</td>
<td>r=0.272&lt;0.001*</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>r=0.528&lt;0.001*</td>
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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 phosho-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

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<td>p-value</td>
</tr>
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<td>T1 vs. T2 vs. T3-T4</td>
<td>2.159 (1.067-4.348)</td>
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<td>Nodal status (N)</td>
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<td>Stage</td>
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<tr>
<td>pSrc LI</td>
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</tr>
<tr>
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<td>1.013 (1.006-1.021)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>pAkt LI</td>
<td>Continuous</td>
<td>1.009 (1.003-1.014)</td>
<td>0.001*</td>
</tr>
<tr>
<td>pIkka/β LI</td>
<td>Continuous</td>
<td>1.011 (1.005-1.018)</td>
<td>0.001*</td>
</tr>
<tr>
<td>pSrc LI</td>
<td>Low (&lt;median) vs. High (&gt;=median)</td>
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<td>0.0052*</td>
</tr>
<tr>
<td>pTRAF6 LI</td>
<td>Low (&lt;median) vs. High (&gt;=median)</td>
<td>-</td>
<td>0.0114*</td>
</tr>
<tr>
<td>pAkt LI</td>
<td>Low (&lt;median) vs. High (&gt;=median)</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>pIkka/β LI</td>
<td>Low (&lt;median) vs. High (&gt;=median)</td>
<td>-</td>
<td>0.0029*</td>
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</table>

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).
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

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<tbody>
<tr>
<td></td>
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<td>HR (95% CI)</td>
<td>p-value</td>
</tr>
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<td>Stage</td>
<td>Stage I vs. Stage II vs. Stage III</td>
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<tr>
<td>pTRAF6 LI</td>
<td>Continuous</td>
<td>1.006 (0.997-1.014)</td>
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<td>plkka/β LI</td>
<td>Continuous</td>
<td>1.005 (0.998-1.013)</td>
<td>0.164</td>
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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).
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 posttranslational 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, IKKe, 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-stimulating factor-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.


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1138.


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VITA

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 university 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.

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