UBIQUITINATION-DEPENDENT ACTIVATION OF IKK

APPROVED BY SUPERVISORY COMMITTEE

Zhijian J. Chen, Ph.D.

Eric Olson, Ph.D.

Kim Orth, Ph.D.

Jim Waddle, Ph.D.

TO MY PARENTS AND THE LOVE OF MY LIFE, HUI-TING

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by

CHEE-KWEE EA

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UBIQUITINATION-DEPENDENT ACTIVATION OF IKK

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Chee-Kwee Ea, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Supervising Professor: Zhijian J. Chen, Ph.D.

Ubiquitination plays two different roles in the nuclear factor κB (NF-κB) pathway, the traditional K48-linked polyubiquitination-mediated IκB degradation and the non-traditional K63-linked polyubiquitination-mediated IKK activation. TRAF6 is a RING domain ubiquitin ligase that mediates the activation of protein kinases such as TAK1 and IKK by promoting the formation of a unique polyubiquitin chain linked through lysine-63 of ubiquitin. Previous studies have suggested that the ubiquitin ligase and signaling activity of TRAF6 may be regulated by its oligomerization. However, it is not known whether there is an endogenous "oligomerizer" that regulates TRAF6 activity. TRAF-interacting protein with a forkhead-associated (FHA) domain (TIFA, also known as T2BP) is one of such TRAF6 "oligomerizers". Recombinant TIFA protein, but not TRAF6-binding defective mutant protein, can activate IKK in crude cytosolic extracts. Furthermore, TIFA activates IKK in an

in vitro reconstitution system consisting of purified proteins including TRAF6, the TAK1 kinase complex and the ubiquitin conjugating enzyme complex Ubc13/Uev1A. Interestingly, a fraction of recombinant TIFA protein exists as high molecular weight oligomers, and only these oligomeric forms of TIFA can activate IKK. Importantly, TIFA induces the oligomerization and polyubiquitination of TRAF6, which leads to the activation of TAK1 and IKK through a proteasome-independent mechanism.

The receptor interacting protein kinase 1 (RIP1) is essential for the activation of NF-κB in response to tumor necrosis factor α (TNFα) stimulation. RIP1 undergoes TNF-induced polyubiquitination at Lysine 377 in the intermediate domain and the polyubiquitination of RIP1 is required for proper signal transduction. Furthermore, when introducing RIPK377R mutant into $RIP^{-/-}$ Jurkat cells, it fails to restore TNF-dependent IKK activation, and these RIPK377R cells are sensitive to TNFα-induced cell death. In addition, TAK1 and IKK kinase complexes are not recruited to TNFR1 followed TNFα stimulation in the absence of RIP1 polyubiquitination. Moreover, TAB2 and NEMO bind to K63-linked polyubiquitin chains and function as receptors that bind polyubiquitinated RIP1. These results indicate a unique interaction between a polyubiquitinated protein and a polyubiquitin binding protein can trigger the activation of TAK1 and IKK.

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LIST OF ABBREVIATIONS

E1: ubiquitin-activating enzyme

E2: ubiquitin-conjugating enzyme

E3: ubiquitin ligase

FHA: forkhead-associated domain

IKK: IκB kinase

IL-1β: interleukin-1β

IκB: inhibitor of NF-κB

NEMO: NF-κB essential modulator

NF-κB: nuclear factor kappa B

PCR: polymerase chain reaction

RING: really interesting new gene

RIP1: receptor interacting protein kinase 1

TAB2: TAK1 associated protein 2

TAK1: TGFβ activated kinase 1

TIFA: TRAF-interacting protein with a FHA domain

TNF-R1: tumor necrosis factor receptor I

TNFα: tumor necrosis factor alpha

TRAF2: tumor necrosis factor associated factor 2

TRAF6: tumor necrosis factor associated factor 6

TRIKA1: TRAF6-regulated IKK activator 1

TRIKA2: TRAF6-regulated IKK activator 2

CHAPTER I: GENERAL INTRODUCTION

I.A The NF-κB pathway

I.A.1 Overview of the NF-κB pathway

Cells response to microbial or viral infections, environmental changes and stresses require rapid and accurate transmission of signals from cell-surface receptors to the nucleus. The nuclear factor kappa B (NF-κB) signaling pathway represents one of the best studied signaling cascades that elucidates how cells response to stresses by activating a transcription factor. NF-κB was first described in 1986 as a nuclear factor that regulated the transcription of immunoglobulin kappa light chain in B cells [1]. It was originally thought that NF-κB was only expressed in B cells, because the DNA-binding activity of NF-κB could not be detected in other cell lines. However, it was later found that the DNA-binding activity of NF-κB in most of the cell type was masked by the presence of an inhibitor, inhibitor of NF-κB (IκB) [2, 3]. Since the discovery of NF-κB, the NF-κB binding element (κB site, GGGRNNYYCC) is found in the promoters and enhancers of numerous genes involved in immune, inflammatory and apoptotic responses. Besides the involvement of NF-κB in these cellular responses, NF-κB also plays a role in embryonic development, and in the development and physiology of tissues including central nervous system, bone, mammary gland, and skin.

NF-κB is a transcription factor whose activity is regulated mainly by its nuclear translocation. NF-κB can be activated through canonical and non-canonical pathways (Fig.

1). In the canonical pathway (Fig. 1a), NF-κB is sequestered in the cytoplasm through its interaction with the IκB in resting cells. Stimulation of cells with a variety of ligands, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) or pathogen-associated molecular patterns (PAMPs), leads to the rapid phosphorylation of IκB by the IκB kinase complex (IKK).

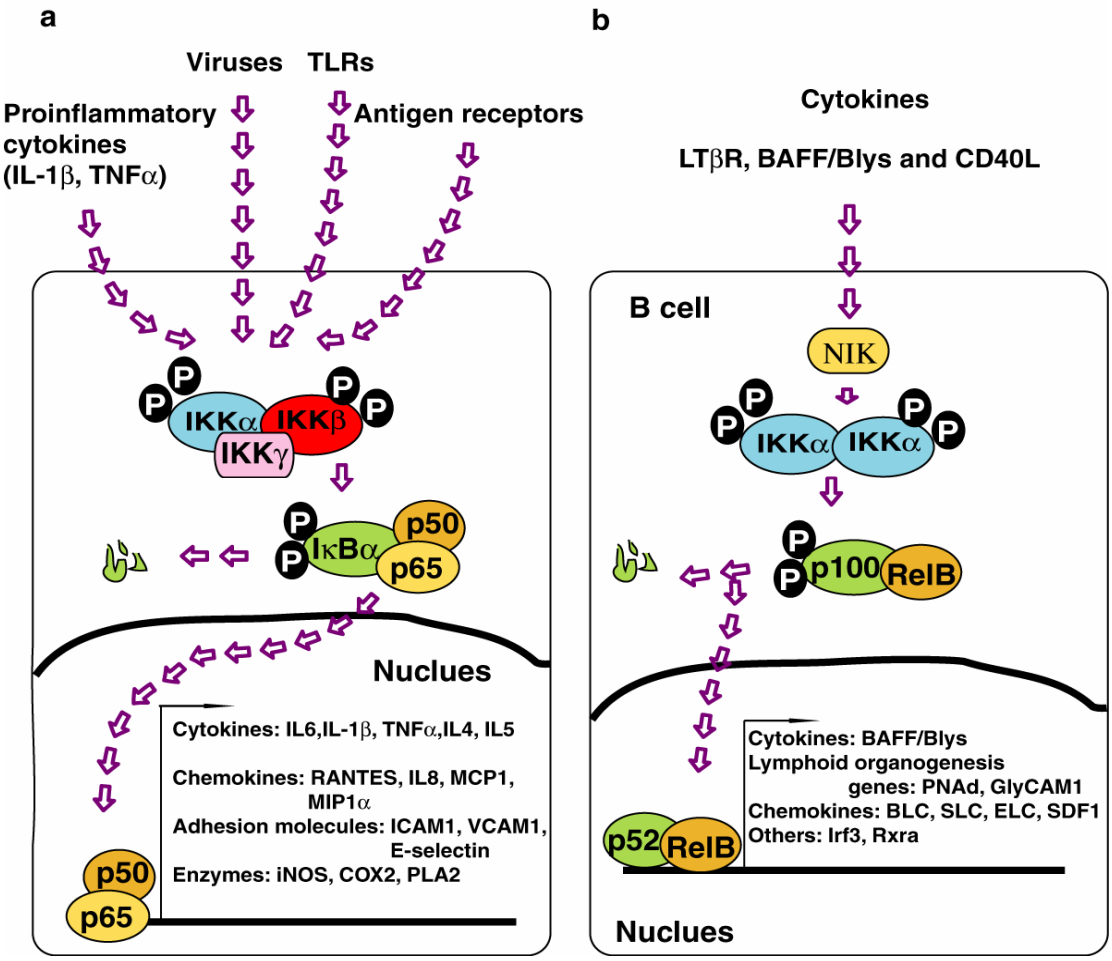

Figure 1. Canonical and non-canonical NF-κB pathways.

(a) In the canonical NF-κB pathway, NF-κB is activated in IKKβ- and IKKγ-dependent manner in most of the cell types. In resting cells, NF-κB is sequestered in the

cytoplasm through its association with IκBα. In response to stimuli, such as proinflammatory cytokines, virus and pathogen-associated molecular patterns, IκBα is phosphorylated by IKKβ and degraded by ubiquitin-proteasome. NF-κB is released and translocated into the nucleus to activate multiple inflammatory and innate immune genes. **(b)** The non-canonical NF-κB is restricted mainly in B cells. In response to a subset of TNF superfamily, including LTβ, BAFF/Blys and CD40L, NIK is activated and mediates the phosphorylation of IKKα. Activated IKKα phosphorylates p100 at the C-terminus and induces the processing of p100 into the mature p52. In conjunction with RelB, the p52/RelB heterodimer translocates into the nucleus and coordinates the expression of genes involved in development and maintenance of secondary lymphoid organs. BAFF, B-cell-activating factor belonging to the TNF family; BLC, B-lymphocyte chemoattractant; CD40L, CD40 ligand; COX-2, cyclooxygenase 2; ELC, Epstein–Barr virus-induced molecule 1 ligand CC chemokine; ICAM1, intercellular adhesion molecule 1; IKK, IκB kinase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LT, lymphotoxin; MCP-1, monocyte chemotactic protein 1; MIP1α, macrophage inflammatory protein 1a; NIK, NF-κB-inducing kinase; PLA2, phospholipase 2; SDF1, stromal cell-derived Factor 1; SLC, secondary lymphoid tissue chemokine; TLRs, Toll-like receptors; TNFα, tumor necrosis factor α; VCAM1, vascular cell adhesion molecule1.

The IKK kinase complex contains two catalytic subunits, IKKα and IKKβ, and the regulatory subunit, IKKγ. In the canonical pathway, it is mainly the IKKβ subunit of IKK that catalyzes the phosphorylation of IκB at two serine residues in the N-terminus. The phosphorylated IκB becomes ubiquitinated and subsequently degraded by 26S proteasome, thereby allowing NF-κB to enter the nucleus to turn on a large array of target genes [4, 5]. The canonical pathway is the major pathway that activates NF-κB in most cell types. On the other hand, activation of NF-κB through the non-canonical pathway is more cell type restricted (Fig. 1b). It usually occurs in B cells in response to certain subsets of TNF receptor superfamily, including LTβR, B-cell activating factor belonging to the TNF family receptor (BAFF-R) and CD40 [6]. This pathway is independent of IKKβ and IKKγ. In response to appropriate agonists, IKKα is activated by the NF-κB interacting kinase (NIK). The target of IKKα in this pathway is p100, which is phosphorylated at two C-terminal sites.

Phosphorylation of p100 induces the processing of p100 into the mature form, p52, in a ubiquitin-proteasome dependent manner. As the Rel homology domain (RHD) of p100 is most commonly associated with RelB, activation of this non-canonical pathway results in nuclear translocation of p52–RelB heterodimers.

I.A.2 NF-κB and IκB family proteins

NF-κB represents a group of evolutionarily conserved and structurally related proteins. The five members of the mammalian NF-κB , p65 (RelA), RelB, cRel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), form homo- or heterodimers that bind to IκB family proteins in unstimulated cells (Fig. 2) [4]. Homologs of mammalian NF-κB have been found in other species such as viral oncoprotein v-Rel, X-Rel1 in X*enopus laevis* and Dorsal, Dif and Relish in *Drosophila melanogaster*. NF-κB proteins are characterized by the presence of an N-terminal RHD which is responsible for DNA binding, dimerization and interacting with IκB proteins. Within the RHD of NF-κB, there is a nuclear localization sequence (NLS) which is responsible for the nuclear translocation of NF-κB after the degradation of IκB. Binding of IκB with the RHD domain of NF-κB masks the NLS, thus preventing the NF-κB-IκB complex from translocating to the nucleus.

IκB family members are characterized by the presence of six to seven ankyrin repeats that form an elongated cylinders and bind the RHD domain of NF-κB dimers (Fig. 2) [7]. Eight IκB family members have been found in mammalian. They are IκBα, IκBβ, BCL-3, IκBε, IκBγ, IκBζ, and the precursor protein p100 and p105 [4]. The structure of IκBα bound to p65-p50 dimers reveals that the ankyrin repeats of IκBα mask only the NLS of p65 [8, 9].

The presence of the accessible NLS on p50 along with the nuclear export sequence (NES) on p50 and p65 results in the constant shuttling of the ternary IκBα-NF-κB complex between the nucleus and the cytoplasm with the overall steady state localization in the cytoplasm [9, 10]. The transcriptional specificity of NF-κB is partially regulated by the ability of specific NF-κB dimers to preferentially associate with certain members of the IκB family.

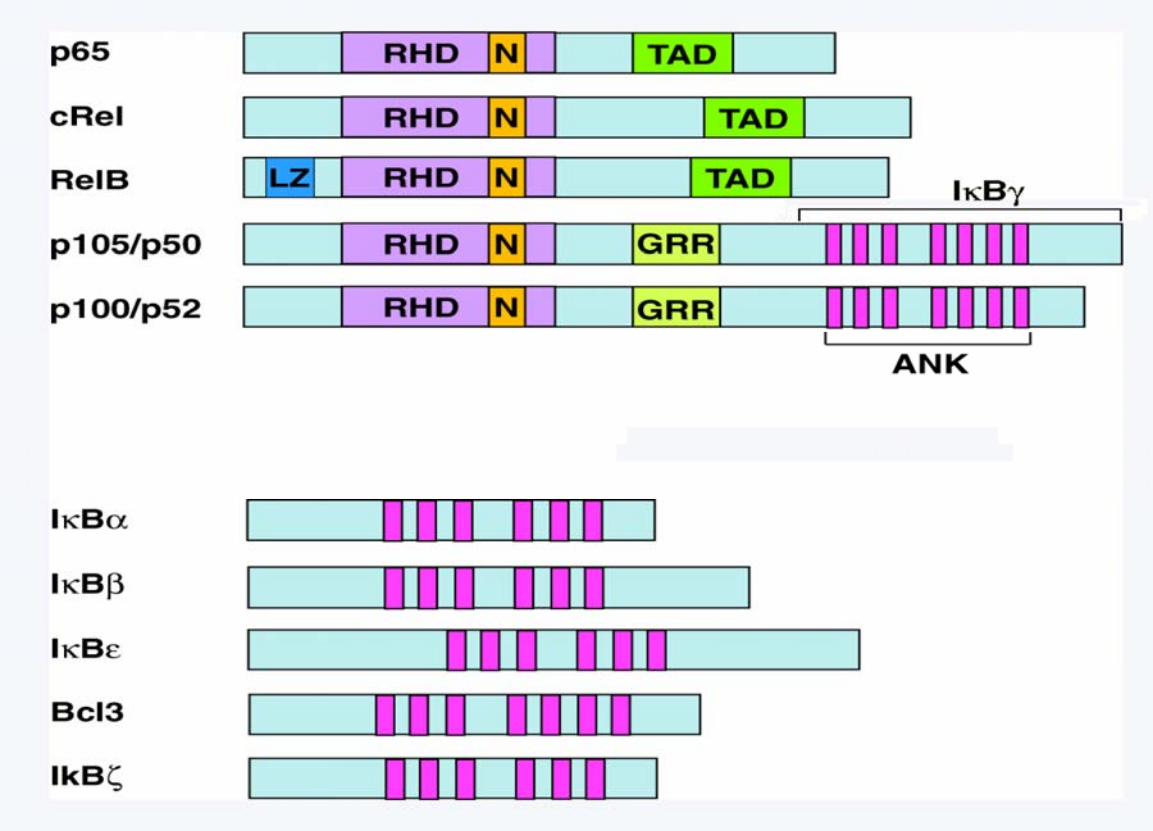

Figure 2. Structural domains of mammalian NF-κB and IκB protein families.

There are five members of the mammalian NF-κB, p65 (RelA), RelB, cRel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2); and eight members of the mammalian IκB IκBα, IκBβ, BCL-3, IκBε, IκBγ, IκBζ, and the precursor protein p100 and p105. RHD, Rel homology domain; N, nuclear localization sequence; TAD, transactivation domain; GRR, glycine rich repeat; ANK, ankyrin repeat; LZ, leucine zipper.

All of the mammalian NF-κB and IκB family members have been knocked out in mice. Genetics studies of these NF-κB deficient mice suggest that different individual NF-κB family members have distinct roles in development and in immune cells [6, 11]. The major form of NF-κB in most cell types is the p65-p50 heterodimer. Disruption of the relA locus leads to embryonic lethality at 14.5-16 days of gestation, caused by a massive degeneration of the liver by programmed cell death or apoptosis [12]. The embryonic lethality of $p65^{-/-}$ mice can be rescued by crossing with either $TNF\alpha^{-/-}$ mice [13] or $TNF\text{-}R1^{-/-}$ mice [14], suggesting that the pathology of apoptotic liver degeneration can be attributed to TNF signaling. The p65/TNF-R1 double knockout mice are born healthy but are highly susceptible to bacteria infections and they die within a few weeks after birth. The phenotypes of these mice indicate that p65 plays a role in innate immune responses and it is essential for cells of nonhemopoietic origin during the initiation of an innate immune response [14]. Further genetics study reveals its importance in class switching in B cells and in lymphocyte proliferation following various stimuli [15]. In contrast, mice lacking the p50 subunit of NF-κB show no developmental abnormality, but are more prone to infection than wild type mice [16]. Although lymphocyte populations appear normal, these mice show decreased immunoglobulin production and defective humoral immune responses. B cells from these mice are hyporesponsive to LPS [16], implicating that RelA/p50 heterodimer is involved in Toll/IL-1 signaling pathways while the antiapoptotic role of p50 is redundant.

Unlike p50 and p65, which are expressed in virtually all cell types, RelB expression is restricted to lymphoid tissue. RelB does not form homodimer and is only able to heterodimerize with p50, p52 and p100 [17, 18]. RelB does not interact with any IκB family

members other than p100 [19]. As a result, RelB-p52 heterodimer is constitutively localized in the nucleus which results in the constitutive expression of kappa B-regulated genes in lymphoid tissues. Mice with an inactivated rel-B gene have decreased basal NF-κB activity in the spleen and thymus. Furthermore, RelB plays a decisive role in the hematopoietic system as RelB-deficient mice display increased inflammatory cell infiltration in several organs, myeloid hyperplasia, splenomegaly due to extramedullary hematopoiesis, and a reduced population of thymic dendritic cells [20]. Dendritic cells and thymic UEA-1+ medullary epithelial cells are absent in mice lacking RelB, and these abnormalities accounted for the loss of cellular immune responses in these mice [21].

The p52 subunit of NF-κB has an important role in the maintenance of the peripheral B cell population, humoral responses, and normal spleen architecture. p52 null mice fail to develop B cell follicles, follicular dendritic cell networks and germinal centers, and show severe disruption of the splenic marginal zone [22, 23]. In addition, these mice have normal B-cell maturation and immunoglobulin switching, but generate inadequate humoral responses to various T-cell-dependent antigens. On the contrary, c-Rel proto-oncogene is expressed predominantly in hemopoietic cells and is crucial for T cell and B cell division and immune function. The c-Rel-null mice have normal development of cells from all hemopoietic lineages, but are impaired of humoral immunity [24]. The c-Rel-null mice exhibit defects in T- and B-lymphocyte proliferation and synthesis of IgG1 and IgG2. Mature B and T cells from these mice are unresponsive to most mitogenic stimuli.

The IκBα is the primary regulator of the classical NF-κB heterodimer (p65-p50). However, IκBα-deficient mice have increased but not constitutive active p65-p50

heterodimers suggesting that other isoforms of IκBs can compensate for the loss of IκBα [25, 26]. In the absence of IκBα, cells display a prolong NF-κB activation in response to TNFα stimulation indicating that IκBα participates in a negative feedback loop where newly synthesized IκBα terminates the activity of NF-κB [26]. IκBα-deficient mice have normal embryonic development but exhibit severe runting, skin defects, and extensive granulopoiesis postnatally, and typically die within 8 days [25].

Biochemical studies indicate that IκBβ can form complexes with p65-p50 and c-Rel-p50 heterodimers [27, 28]. However, the biological function of IκBβ remains to be seen as the phenotype of IκBβ-knockout mice has not been published. BCL-3-deficient mice are developmentally normal, but are unable to generate an appropriate humoral immune response and protection against several pathogens [29, 30]. These mice are also impaired in germinal center reactions and show abnormality in the microarchitecture of secondary lymphoid organs. The p52-null mice show partially overlapped phenotypes with Bcl-3-knockout mice implying that BCL-3 may form transcriptionally active complexes with p52 that regulate events involved in antigen-specific priming of T and B cells [22, 23, 29-31].

IκBε was first identified in two different yeast two-hybrid screens using p50 and p52 as baits respectively, but was later shown to selectively regulate p65 homodimers and c-Rel-p65 heterodimers [28, 32]. IκBε displays slower degradation kinetics in response to many NF-κB agonists compared to IκBα. The expression of IκBε is induced by NF-κB activation indicating that it may function in regulating the activity of c-Rel-p65 complex at later phases [28, 32]. IκBγ, the C-terminal region of p105 that is synthesized as an alternative mRNA

through a separate promoter, is predominantly found in lymphoid cells [33, 34]. Although it has been shown that IκBγ prevents sequence-specific DNA binding of p50-p65 heterodimer, p50 homodimer, and c-Rel i*n vitro*, it is still unclear if IκBγ has a defined biological role. The latest newly identified potential member of IκB family, IκBζ (also named as MAIL/INAP), is found predominantly in the nucleus. Its expression can only be detected in cells stimulated with IL-1 and TLR ligands but not with TNF [35-39]. IκBζ has six ankyrin-repeat motifs and shares weak amino acid sequence homology with Bcl-3 and other IκB family members. Although it has been shown *in vitro* that IκB-ζ inhibits the DNA binding of p65-p50 heterodimer as well as the p50 homodimer through its association with the p50 subunit [37], genetic study has indicated a positive role of IκB-ζ in regulating gene expression in response to pathogens [40]. The IκBζ-knockout mice grew normally but some of them tend to develop atopic dermatitis-like skin lesions with acanthosis and lichenoid changes at the age of 4 to 5 weeks [40]. Macrophages derived from these mice are impaired of production of a subset of genes, including IL-6, endothelin 1 (Edn1) and IL-12β in response to several TLR ligands and IL-1β.

I.A.3 The IKK complex

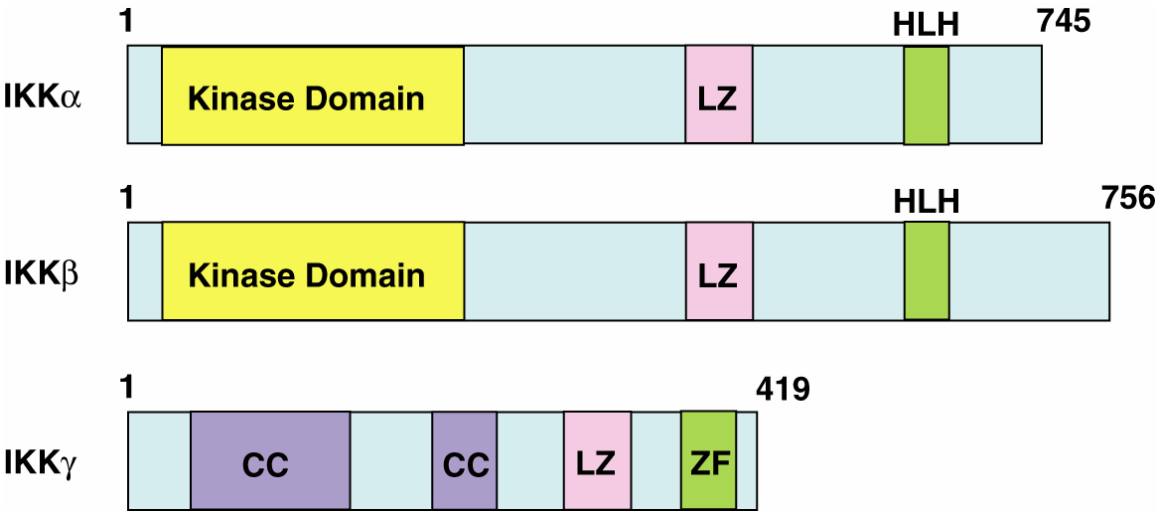

Figure 3. Structural domains of IKK subunits.

The IKK complex contains two catalytic subunits IKKα and IKKβ, and the regulatory subunit IKKγ. LZ, leucine zipper, HLH, helix-loop-helix, CC, coiled-coil domain, ZF, zinc finger

Virtually all signaling pathways that lead to the activation of NF-κB converge at the activation of IKK. Therefore, this is the most important regulatory step and represents a topic of intense investigation by numerous research groups. The IKK kinase complex is 700-900-kDa complex containing the catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ (also known as NEMO, IKKAP1, and Fip-3) (Fig. 3) [4, 6]. Since the identification of IKK a decade ago by multiple groups [41-44], it has been shown that IKK can activate NF-κB through two distinct mechanisms [6]. As discussed in I.A.1, the activated IKK complexes in the canonical pathway catalyze the phosphorylation of IκBs in IKKβ-and IKKγ-dependent manner; while in the non-canonical NF-κB signaling pathway, it is strictly acting through IKKα and is independent of IKKβ and IKKγ.

The first component of the IKK complex, CHUK, was first identified by an RT-PCR-based strategy in an attempt to isolate Myc-like genes and was first proposed to play a role in transcriptional regulation [45, 46]. CHUK was later renamed as IKKα. Further study of IKKα reveals that it associates with IκBα in mammalian cells and specifically phosphorylates IκBα on both serine 32 and serine 36 [47, 48]. IKKβ was discovered shortly thereafter through biochemical purification and sequence homology search [41, 43, 48]. The regulatory subunit of the IKK complex, IKKγ, was initially isolated through genetic complementation cloning using two NF-κB-unresponsive cell lines, 1.3E2 and HTLV-1 Tax-transformed rat fibroblast 5R [49]. It was subsequently described as a protein interacting with IKKα and IKKβ [50, 51], and as an adenovirus protein, Ad E3-14.7K, binding protein [52]. Other kinases, namely IKKε [53] and T2K [54](also known as TBK1 [55] or NAK [56]) have been shown to be able to phosphorylate IκB at ser-36. However genetic studies have ruled out their role as an IκB kinase in the NF-κB signaling [54, 57, 58].

IKKα and IKKβ share 65% sequence identity within the kinase domain and have 52% overall sequence identity. They are serine/threonine kinases that are characterized by the presence of the kinase domain at the N-terminus, followed by a leucine zipper domain and a helix-loop-helix (HLH) domain at the C-terminus. IKKγ is a 48kDa protein with a C-terminal zinc finger-like domain, a leucine zipper, and N-terminal and C-terminal coiled-coil domains. The activation of IKK depends on the phosphorylation of two conserved serines of either IKKα (Ser176 and Ser180) or IKKβ (Ser177 and Ser181) in their activation loops [48, 59, 60]. IKKα and IKKβ are capable of phosphorylating multiple sites of members of IκB

family but with different efficiency. For example, IKKα and IKKβ are less efficient to phosphorylate IκBβ than IκBα. This difference provides an explanation for the slower degradation kinetics observed for IκBβ [61]. Furthermore, IKKα and IKKβ show preference of phosphorylating IκBα that is bound to NF-κB to free IκBα, which provide a mechanism for cells to accumulate newly synthesized IκBα in the presence of active IKK and thus allowing it to terminate the activation of NF-κB [62]. IKKα and IKKβ can form homodimer or heterodimer through the leucine zipper domain [41, 42, 48]. IKKα and IKKβ bind to IKKγ through the NEMO binding domain (NBD) at the C-terminus which encompasses a hexapeptide sequence (Leu-Asp-Trp-Ser-Trp-Leu) [63, 64]. A cell-permeable NBD peptide blocks association of IKKγ with the IKK complex and inhibits cytokine-induced NF-κB activation and NF-κB-dependent gene expression [63, 64]. The N-terminal coiled-coil motif of IKKγ interacts with IKKα and IKKβ. Competition experiments using the NBD peptide suggest that IKKβ binds to IKKγ with higher affinity than IKKα does [63]. IKKγ has been shown to form oligomers through its C-terminal coiled-coil domain, and several oligomeric states of IKKγ can be detected in vivo [65, 66]. It is proposed that the activation of IKK complex occurs through the oligomerization of IKKγ [65, 66]. Consistent with this hypothesis, it has been shown that enforced oligomerization of the N-terminal half of IKKγ fused to an artificial dimerization domain is sufficient to induce NF-κB activation [67]. Furthermore, two human pathologies, Incontinentia Pigmenti and Anhidrotic Endodermal Dysplasia with Immunodeficiency (EDA-ID), are shown to have mutations in the C-terminus of IKKγ [68, 69].

Based on the molecular weight of IKK subunits, the predicted size of IKK complex is about 200-350 kDa. However the molecular weight observed in the gel filtration chromatography is 700-900 kDa, which is much larger than the expected size. The difference could be due to the presence of undefined components or the formation of higher-order stoichoimetry complex. The stoichoimetry of IKKα, IKKβ, and IKKγ in the IKK complex continues to be controversial. Recent study has suggested that IKKγ may have a large Stoke's radius as IKKγ trimers migrate as a 550 kDa complex in the gel filtration chromatography [70]. This result further complicates the determination of the stoichoimetry of IKK components. On the other hand, IKKα, IKKβ, and IKKγ are the only definitive components of the IKK complex identified thus far. Although HSP-90/Cdc37 and ELKS have been shown to be IKK-interacting proteins [71, 72], further experiments are required to demonstrate that these proteins are *bona fida* components of IKK complex given the fact that IKK complex reconstituted solely from recombinant IKKα, IKKβ, and IKKγ has apparent molecular weight comparable to that of the endogenous IKK complex [73].

Physiological roles of each of the IKK subunits have been characterized genetically. Mice devoid of the IKKβ gene die at E14.5 from extensive liver damage which resemble the knockout phenotype of p65 knockout mice [74-76], and this phenotype can be rescued by removing the TNF-R1 locus [77]. These results suggest that IKKβ is essential for mouse development and cannot be substituted with IKKα. The IKKα-deficient mice are perinatally lethal and exhibit a wide range of developmental defects including limb and skeletal patterning abnormality, and proliferation and differentiation defect of epidermal keratinocytes [78-80]. Subsequent knock-in studies have indicated that these defects are NF-

κB-independent as these defects can be rescued by knocking in a kinase-dead IKKα [81] or an activation loop mutant of IKKα [82, 83]. Unlike IKKβ-deficient mice, IKKα-deficient mice do not develop liver apoptosis. Furthermore, biochemical analysis of $IKK\beta^{-/-}$ MEF cells reveals a dramatic defect in NF-κB activation in response to proinflammatory stimuli [74-76] while $IKK\alpha^{-/-}$ MEF cells have only mild reduction of NF-κB activation in response to TNFα [78-80]. These results indicate that IKKβ has a major role in NF-κB signaling while IKKα has some kinase-independent roles in skin development. Similar to IKKβ-deficient mice, an early embryonic lethality due to liver degeneration is observed in embryos that lack the regulatory subunit IKKγ, and this embryonic lethality can be prevented by removing the TNF-R1 gene. The IKKγ and IKKβ knockout mice have similar phenotypes indicating that in response to various proinflammatory stimuli, phosphorylation of IκBα is dependent on IKKβ and IKKγ. IKKγ has one interesting feature in which it is an X-linked gene. Female $IKK\gamma^{+/-}$ mice develop a condition analogous to Incontinentia pigmenti in human with a similar pattern of inheritance [84, 85].

I.B The role of ubiquitination in the NF-κB pathway

I.B.1 The ubiquitination machinery

As its name implies, ubiquitin is a 76-amino acid polypeptide that is ubiquitously expressed in all eukaryotes. It is highly conserved from yeast to human, and there is only three amino acids difference between yeast and human ubiquitin. It is not surprising that this small protein is involved in virtually all aspects of cell biology from the generation of new

cells through cell division to cell death via apoptosis [86, 87]. Ubiquitin achieves its functions through covalent modification of specific target proteins, a process that is usually referred to as ubiquitination. This conjugation results in the formation of an isopeptide bond between the carboxyl group of the glycine residue at the C-terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. Ubiquitination is accomplished by a three-step enzymatic cascade [88]. In the first step, the C-terminus of ubiquitin is activated by formation of a Ub-adenylate intermediate followed by the reaction of this intermediate with a ubiquitin-activating enzyme (E1) cysteine residue to form a high energy E1-Ub thioester. In the next step, the ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2) via formation of a E2-Ub thioester. Finally, in the presence of a ubiquitin ligase (E3), ubiquitin is conjugated to a lysine residue of the target protein through an isopeptide linkage. A specific lysine residue in the conjugated ubiquitin is then attached to a second ubiquitin and reiteration of this process results in the assembly of a polyubiquitin chain.

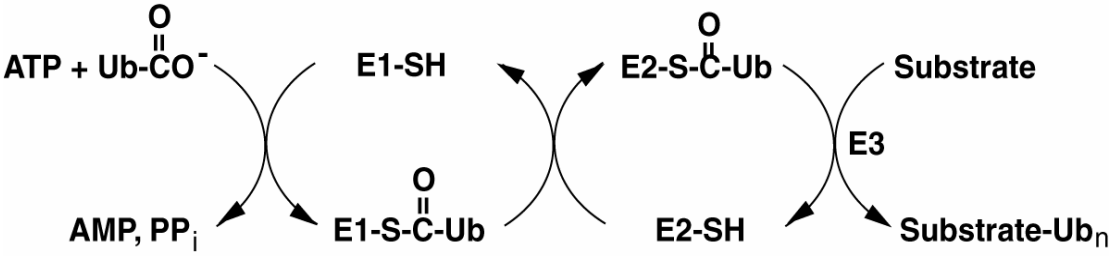

Figure 4. Ubiquitination mechanisms and machinery.

The ubiquitin cascade involves three enzymatic steps. First, ubiquitin is activated by the E1 enzyme with the hydrolysis of ATP. Then, ubiquitin is transferred to the E2 enzyme. Some E2s can directly add ubiquitin to a substrate, but generally a third step involving an E3 enzyme is required to facilitate the transfer of ubiquitin from E2 to the substrate. Ub, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; PP_i, pyrophosphate.

In most organisms, a single ubiquitin E1 catalyzes all ubiquitination reactions, and inactivation of this gene is lethal. E1 uses ATP hydrolysis to provide the chemical potential needed for the subsequent transfer steps. Each fully loaded E1 harbors two molecules of activated ubiquitin, one as thioester, the other as an adenylate [88]. All E2s have a ~150 amino acids core domain that often comprises the entire protein, but certain family members feature additional N- or C-terminal extensions which may be involved in E2 activity regulation, E3 association or substrate binding [88]. Free E2s show low affinity to free ubiquitin and free E1, but show high affinity to their cognate E3s and loaded E1 [89-91]. Several E2 structures have been solved. These structures show overall similarity and do not show dramatic conformational change in various protein complexes [92].

E3s can be categorized into three types, homology to the E6-AP carboxyl terminus (HECT) E3, really interesting new gene (RING) E3 and UFD2 homology (U-box) E3 [93]. All the members of the HECT-type E3 harbors a 350 amino acid HECT domain. HECT E3s form a thioester intermediate with ubiquitin during the ubiquitination reaction [94]. Thus, it is the E3 cysteine that functions as the last stop for activated ubiquitin in HECT E3 mediated ubiquitination. This feature is unique among all E3s. E6 associated protein (E6-AP) is the first identified HECT E3. The N-terminus of E6-AP mediates substrate recruitment while its HECT domain binds the E2~Ub and transfers ubiquitin to a conserved cysteine residue at ~35 residues upstream of the C-terminus [93]. U-box E3s contain the U-box domain whose tertiary structure resembles the structure of RING finger domain except that the structure of the U-box domain is not stabilized through Zinc-coordination but is stabilized by dynamic hydrogen bonds and salt bridges [95, 96]. The yeast Ufd2 is the founder of the U-box E3s

[97]. Although Ufd2 is considered as an 'E4' as it promotes the polyubiquitination of another E3's substrate [97], it is later shown that the U-box of Prp19 resembles the structure of RING domain [96]. Furthermore, several U-box proteins interact directly with E2s and promote autoubiquitination [98-101]. These findings suggest that U-box proteins are indeed conventional E3s.

The RING finger domain is the hallmark of RING E3s. The RING E3 is the largest group among the three different types of E3s. The RING finger domain can be defined as a short motif rich in conserved cysteine and histidine residues ($C\text{-}X_2\text{-}C\text{-}X_{9\text{-}39}\text{-}C\text{-}X_{1\text{-}3}\text{-}H\text{-}X_{2\text{-}3}\text{-}C/H\text{-}X_2\text{-}C\text{-}X_{4\text{-}48}\text{-}C\text{-}X_2\text{-}C$) [102]. Apart from the conserved residues and the spacing amid them, the conservation of the primary sequence among RING domains is limited. These conserved cysteine and histidine residues form a unique cross-brace arrangement to chelate two zinc ions [103]. The association of RING finger domain with ubiquitination was first suggested based on the observation that a plant N-end rule E3 Prt1 shared the RING finger domain with yeast proteins Ubr1p, Hrd1p/Der3p, Rad18p and Apc11p all of which have been implicated in ubiquitination [104]. It was later revealed that the RING finger domain binds E2s [105-107]. Most of the RING E3 carry out their function as a single entity, in other words, a single RING finger containing protein is sufficient to bring together the substrate and the E2~Ub intermediate. A subset of RING E3 acquires its ubiquitin-ligase activity by forming a multi-subunit complex. This subtype of RING E3 includes the Skp1, cullin, F-box (SCF) E3, the von Hippel-Lindau (VHL) E3 and the anaphase promoting complex (APC) [108]. SCF E3s are composed of at least four proteins: Skp1, Cullin, Roc1/Rbx1/Hrt1 and an F-box protein. The substrate is recruited to the SCF complex via the F-box protein. The F-

box motif of the F-box protein interacts with Skp1 which bridges the F-box protein to cullin. Cullin in turn functions as a scaffold to bind the RING finger protein Roc1/Rbx1/Hrt1 [107]. The VHL E3 is structurally similar to the SCF E3. It contains Roc1/Rbx1/Hrt1, cullin Cul2, elongin C, elongin B and the VHL. Elongin C interacts with the BC-box of VHL. This interaction is homologous to the F-box-Skp1 interaction. VHL is a tumor suppressor which is mutated in von Hippel-Lindau disease and in over 80% of sporadic renal cell carcinomas [109]. Similar to the SCF E3 and VHL E3, the APC contains a cullin homologue, Apc2, and a RING finger protein, Apc11 [110]. The constitution of APC is more complex than the SCF E3 and the VHL E3. At least 11 components of APC have been identified, however the function of most of these proteins is still unclear [111, 112].

I.B.2 Functions of ubiquitination

Protein ubiquitination shares some common features with protein phosphorylation, one of the best-studied regulatory processes in biology. First, both protein modifications are reversible. As protein phosphorylation can be removed by protein phosphatase, protein ubiquitination can be removed by members of a large family of enzymes known as isopeptidases or more specifically deubiquitination enzymes (Dubs) [113]. Second, both protein modifications can occur within seconds or minutes in response to stimuli. Third, both protein modifications are very specific and are tightly regulated by a very diverse set of enzymes. With the completion of the human genome projects, it reveals the presence of more than 500 kinases and 120 protein phosphatases. On the other hand, it is predicted that there are more than 40 E2, 500 different E3 and 80 different Dubs in the human genome. Fourth, both protein modifications can be recognized by specific protein domains. For

example, in case of protein phosphorylation, a phosphotyrosine embedded in a specific sequence can be detected by a SH2 and a PTB domain [114], or a specific sequence containing phosphoserine or phosphothreonine can be recognized by a FHA and a WD repeats domain [115]. In the case of protein ubiquitination, several domains have been identified to be capable of specifically binding to ubiquitin or polyubiquitin chains. These domains include the Ubiquitin-interacting motifs (UIM), ubiquitin-association domain (UBA), Cue1-homologous domain (CUE), polyubiquitin-associated Zinc finger (PAZ), novel Zinc finger (NZF), ubiquitin E2 variant domain (UEV), GRAM-like ubiquitin binding in Eap45 (GLUE), Gga and TOM1 (GAT), and Vps27/HRS/STAM (VHS) [116, 117]. Therefore, ubiquitin has evolved as a 'large phosphate' group that can be covalently attached to other cellular proteins, thereby changing the stability, localization, or activity of these target proteins.

Ubiquitin has seven lysine residues, all of which can be used for polymerization catalyzed by the ubiquitin-modifying system [88, 118]. Recent advances in mass spectrometry have shed the light for accurate and direct determination of ubiquitinated lysines in vivo. All seven theoretical ubiquitin-ubiquitin linkages (K6, K11, K27, K29, K33, K48 and K63) are present in the budding yeast proteome [118]. It is believed that different ubiquitin-ubiquitin linkages generate different fates to their target proteins, as different ubiquitin-linkages have been shown to be involved in diverse cellular processes including protein degradation, membrane trafficking, histone function, transcription regulation, DNA repair, DNA replication and kinase activation [116, 119]. Although proteins are usually polyubiquitinated, some are modified by a single ubiquitin moiety on one or multiple lysines.

This monoubiquitination can serve as a signal for lysosomal degradation of plasma membranes proteins or modulate protein function in the absence of promoting proteolysis [120].

Polyubiquitin chains are discovered through the studies of ubiquitin function in proteasomal proteolysis. It is well-established that the K48-linked polyubiquitin chain functions as a 'kiss of death' to target proteins for degradation by the proteasome, the best known function of ubiquitin. The first implication of ubiquitin functions in protein turnover come from the study of a temperature-sensitive E1 cell line ts85. Under the restrictive temperature, the turnover of short-lived normal proteins in ts85 cells are inhibited by more than 90%, while at the permissive temperature the turnover of these proteins is accompanied by their transient appearance in the ubiquitin conjugate pool [121, 122]. These observations reveal that the ubiquitin-proteasome pathway is the principle mechanism for turnover of normal short-lived proteins. Later on, a pioneering biochemical study indicates that a single K48-linked polyubiquitin chain conjugation is sufficient to target a model substrate to 26S proteasomes [123], suggesting that the K48-linked polyubiquitin chain is the principle proteasome delivery signal [124]. Recent studies have uncovered several new functions of ubiquitin that are independent of proteasomal degradation. Ubiquitination has been shown to be involved in several steps in endocytosis [125]. It is required for the entry of cargo into primary endocytic vesicles, budding from the plasma membrane as well as vesicle sorting. Several ion channels and signaling receptors that undergo signal-dependent internalization are ubiquitinated, and it has been shown that cargo ubiquitination is required for proper vesicles sorting [126, 127]. These mechanisms are conserved from yeast to human.

Ubiquitination also plays an unexpected role in transcriptional regulation. Studies have suggested that monoubiquitination of histone H2B is associated with transcriptional initiation and elongation [128], whereas polyubiquitination of a subset of transcriptional activator is required for transcriptional activation [129, 130]. Moreover, ubiquitination is required for DNA repair. Several proteins involved in DNA repair become ubiquitinated in response to DNA damage. The best example of these proteins is the proliferating cell nuclear antigen (PCNA), a protein that assembles into a trimeric ring that encircles DNA to recruit DNA polymerase during DNA replication and post-replicative DNA repair. It has been shown that the monoubiquitination of PCNA is required for error-prone DNA repair while the polyubiquitination of PCNA is required for error-free DNA repair [131]. Finally, ubiquitination can lead to IKK activation in the NF-κB pathway (see I.B.4) [119].

I.B.3 Proteolysis function of ubiquitination in the NF-κB pathway

I.B.3.a Ubiquitination and degradation of IκB

Ubiquitination plays two different roles in NF-κB signaling pathway. The first role is the traditional K48-linked polyubiquitination-mediated IκB degradation, and the second role is the non-traditional K63-linked polyubiquitination-mediated IKK activation. NF-κB is usually sequestered in the cytoplasm through its association with IκB. To activate NF-κB, IκB must be either removed or eliminated. Indeed, stimulation of cells with many NF-κB agonists leads to the ubiquitin-proteasome-dependent degradation of IκB [5]. The first clue showing that IκBα undergoes proteasomal degradation comes from the study using

proteasome inhibitors to prevent NF-κB activation [132]. In this study, the proteasome inhibitors were able to block signal-induced degradation of IκBα, but not the phosphorylation of IκBα. The phosphorylated IκBα remained bound to NF-κB, thereby preventing the signal-dependent activation of NF-κB. It is later demonstrated that ubiquitination of IκBα is phosphorylation-dependent, and ubiquitinated IκBα is then recognized and selectively degraded by the 26S proteasome while NF-κB itself is spared [133]. The E2 that carries out ubiquitination of IκBα belongs to Ubc4/Ubc5 family [44] and the E3 is identified as the $SCF^{Slimb/\beta TrCP}$ (Fig. 5) [134, 135].

Slimb/β-TrCP belongs to the F-box protein family. It has a F-box domain at the N-terminus and a WD40 repeats domain at the C-terminus. The F-box domain interacts with Skp1 while the WD40 repeats bind the target protein. Slimb is first described as a negative regulator in both Hh and Wnt/Wg pathways in fly [136]. In the absence of Slimb, high levels

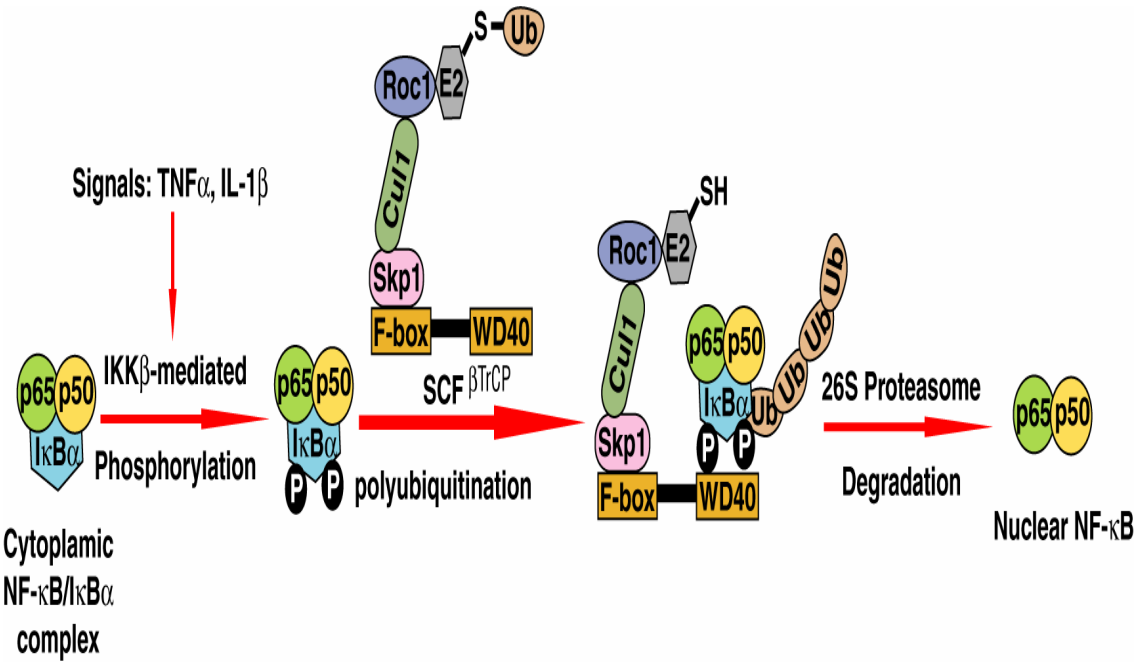

Figure 5. The biochemical pathway of IκBα ubiquitination and degradation.

In response to NF-κB stimuli, IκBα is phosphorylated by IKK at two specific N-terminal serine residues. The phosphorylated IκBα is then recruited to the $SCF^{\beta TrCP}$ ubiquitin ligase complex, which composed of Skp1, Cul1, Roc1, and the F-box protein βTrCP. βTrCP contains seven WD40 repeats that bind specifically to the phosphorylated form of IκBα. The RING domain protein Roc1 recruits the E2 Ubc5, and facilitates the transfer of ubiquitin from the E2 to two N-terminal lysine residues of IκBα. The polyubiquitinated IκBα remains bound to NF-κB and is selectively degraded by 26S proteasome. Free NF-κB then enters the nucleus to regulate the expression of target genes that mediate inflammation, immunity and cell survival.

of Ci and Arm accumulate in a cell-autonomous manner, and result in the ectopic expression of both Hh- and Wg- responsive genes suggesting that Slimb targets Ci and Arm for processing or degradation by the ubiquitin-proteasome pathway. β-TrCP, the mammalian homologue of Slimb, is essential for the ubiquitination of IκBα, IκBβ, p105, p100, and β-catenin [137-144]. The WD40 repeats of β-TrCP is involved in substrate recognition by binding to phosphorylated substrate [107]. Typically each substrate of βTrCP contains a degron motif or consensus destruction sequence DpSGXXpS, where pS represents phosphorylated serine [145]. In the case of IκBα, phosphorylation of IκBα at serine 32 and 36 creates a degron motif to recruit $SCF^{Slimb/\beta TrCP}$ complex which promotes the polyubiquitination and subsequent degradation of IκBα [139]. Two forms of βTrCP, βTrCP1 and βTrCP2, have been found in mammalian cells. Genetic studies have suggested that βTrCP1 and βTrCP2 may have redundant functions as IκBα degradation is modestly affected in βTrCP1-deficient mice [146].

I.B.3.b Ubiquitination and processing of p100 and p105

The p50 and p52 subunit of NF-κB are synthesized as p105 and p100 respectively. The processing of p100 and p105 requires the ubiquitin-proteasome pathway. The p105 processing can be accomplished either co-translationally or post-translationally [132, 147]. The p50 protein is constitutively present in resting cells. This reservoir of p50 is derived from the constitutively co-translational processing of p105 which does not require phosphorylation or ubiquitination. Under certain circumstances, including phorbol ester (PMA) and lipopolysaccharide (LPS) stimulation, post-translational processing of p105 can be induced through phosphorylation of p105 at the C-terminus by IKK [148]. In contrast to p105, the processing of p100 is tightly regulated by the non-canonical NF-κB pathway which involves mainly the activation of IKKα by stimulation of several receptors of TNFR family in B cells [22, 23, 149]. Genetic studies of NIK mutant (aly) mice have implicated the essential role of NIK in inducing p100 processing [150]. NIK is required for activating IKKα which in turn is responsible for the phosphorylation of p100 at serines 866 and 870. The phosphorylation of these two serine residues on p100 results in the recruitment of $SCF^{Slimb/\beta TrCP}$ which catalyzes the polyubiquitination of p100. The C-terminal IκB-like domain of polyubiquitinated p100 is then selectively degraded by the proteasome.

The processing of p100 and p105 is an unusual case of proteasomal protein degradation in which the protein is partially degraded. Both p100 and p105 contain a glycine rich region (GRR) at the C-terminal domain. The GRR serves as a processing signal and it is believed that the GRR forms a hairpin-like loop that inserts into the proteolytic chamber of the proteasome to initiate the degradation of polypeptides in both N- and C-terminal direction

[151]. The N-terminal RHD domain of p100 and p105 forms a tightly folded structure that may be difficult to unfold, and thus may function as a stop signal that spare the degradation of p52 and p50. On the other hand, the C-terminal degradation proceeds to the end as neither p100 nor p105 contains any tightly packed structure at the C-terminus.

I.B.4 Non-proteolytic function of ubiquitination in the NF-κB pathway

Virtually all the signals that lead to the activation of NF-κB converge at the level of IKK activation. The activation of IKK is tightly regulated and ubiquitination has been shown to be involved in this process in a proteasome-independent manner. The first indication of ubiquitination-mediated activation of IKK was observed in an early study that led to the identification of the IKK complex [44]. In this study, it was shown that the IKK can be activated by UBC4 or UBC5, which are ubiquitin conjugating enzyme (E2), through an unknown mechanism that did not involve the proteolytic function of ubiquitination. However the non-proteolytic function of ubiquitination in the NF-κB pathway remains controversial until several years later with the discovery that TNFR-associated factor (TRAF) proteins are ubiquitin RING E3 ligase [152]. TRAF proteins function as signal transducers that link the cytosolic portions of the tumor necrosis factor receptor (TNFR) and interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamilies to downstream effectors to activate NF-κB. Seven members of the TRAF family have recently been identified in mammals [153-156]. Among them, TRAF2-7 contains the RING domain at the N-termini and TRAF2, TRAF5, TRAF6 and TRAF7 have been shown to be involved in the NF-κB pathway.

In an effort to elucidate the mechanism of IKK activation by TRAF6, two TRAF6-regulated IKK activators (TRIKAs) were identified. TRIKA1 is a Ubc13 and Uev1A heterodimer and functions as ubiquitin E2. Ubc13-Uev1A heterodimer in conjunction with TRAF6 promotes K63-linked polyubiquitination of TRAF6 itself and NEMO [152, 157]. Ubc13-Uev1A is also required for polyubiquitination of TRAF2 in the TNFα pathway [158]. Moreover, *Drosophila* homologs of Ubc13 and Uev1A (Blendless-dUev1A) are required for the activation of dTAK1 and DmIKK in the Imd pathway [159]. The crystal structure of Ubc13 and Mms2 (a paralog of Uev1A) heterodimer has been solved [160, 161]. The crystal structure reveals that there are two channels on the Ubc13-Mms2 heterodimer. These channels hold two ubiquitin molecules in an orientation that facilitate the formation of K63-linkage but not the others. The acceptor ubiquitin binding channel is formed by residues from both Ubc13 and Mms2, and places the ε-nitrogen of side-chain Lys-63 of the acceptor ubiquitin within 3Å from the sulfur atom of the active-site cysteine of Ubc13. On the other hand, the donor ubiquitin binding channel is composed entirely of Ubc13 residues. It places the Gly76 carbonyl group of the donor ubiquitin within 3 Å of the active site Cys87 of Ubc13. The Lys-48 of the acceptor ubiquitin is buried within the protein-protein interface, thereby preventing the formation of canonical K48-linked polyubiquitin chain.

TRIKA2 is a kinase complex composed of TAK1, TAB1 and TAB2 [162]. TAK1 was initially identified as a TGFβ activated kinase [163], it was subsequently discovered to be involved in innate and adapt immunity [157, 162, 164]. Several lines of biochemical and genetic evidence strongly support the idea that TAK1 functions as a major kinase that regulates the activation of IKK and JNK pathways. First, biochemical characterization of

TAK1 has indicated that TAK1 activates IKK and JNK/p38 pathways through direct phosphorylation of IKKβ and MKK6 at the activation loops [162]. Second, the TRAFs→TAK1→IKK/JNK signaling cascade is conserved in fly. Mutation in the *Drosophila* dTAK1 results in severe defects in the IKK-dependent activation of the NF-κB homolog Relish [165]. In addition, RNAi of dTAK1 in *Drosophila* Schneider (S2) cells inhibited the activation of NF-κB and JNK signaling in response to peptidoglycan (PGN) stimulation [166, 167]. Third, silencing of TAK1 expression by RNAi abolishes TNFα– and IL-1β-induced IKK and JNK activation [168], as well as IKK activation and IL-2 production in response to TCR stimulation of T cells [157]. Fourth, pre-treating the cells with a specific TAK1 inhibitor, 5Z-7-oxozeaenol, blocks JNK, p38 and IKK activation in response to TNFα and IL-1β stimulation [169]. Finally, in the TAK1-deficient MEF cells, the TNFα- and IL-1β-induced activation of NF-κB is impaired [170].

TAB2 is first isolated in a yeast two-hybrid screening for TAK1 interacting partners [171, 172]. TAB3 is identified based on sequence homology to TAB2 [173, 174]. Although mice devoid of the TAB2 gene die at E12.5 from extensive liver damage that resembles the knockout phenotype of p65 and IKKβ knockout mice, TAB2 deficient MEF cells are still capable of activating IKK and JNK in response to TNFα or IL-1β [175]. However, RNAi of both TAB2 and TAB3 block TNFα- and IL-1β-induced activation of TAK1 and NF-κB, suggesting that TAB2 and TAB3 are functionally redundant in TNFα- and IL-1β-mediated signaling pathways and TAB2 has additional roles in embryonic development that are not compensated by TAB3 [173, 174]. The novel zinc finger (NZF) domains at the C-terminal of

TAB2 and TAB3 bind preferentially to K63-linked polyubiquitin chain and are required for TAK1 and IKK activation [176]. The NZF domain can be replaced by a heterogeneous ubiquitin binding domain without losing its ability to activate NF-κB. Thus, TAB2 and TAB3 function as the K63-linked polyubiquitin chain receptor to link TAK1 to TRAF6 or TRAF2/RIP1. On the other hand, the role of TAB1 in these pathways is not clear. Although TAB1 has been shown to be an activator of TAK1 that augments the kinase activity of TAK1 when it is coexpressed with TAK1 [171, 177, 178], *in vitro* reconstitution experiments revealed that TAB1 is dispensable for TRAF6-induced IKK activation [162]. It is very likely that TAB1 regulates the TAK1 activity in the signaling pathway other than NF-κB, such as TGFβ, BMP and Wnt pathway [179-182]. In fact, TAB1 knockout mice show cardiac phenotypes that resemble those of TGF-β2 knockout mice [183].

The non-proteolytic function of ubiquitination in NF-κB activation has been shown to play an important role in innate and adapt immunity (Fig. 6). In innate immunity, TRAF2 and TRAF5 promote the polyubiquitination of RIP1 in the TNF pathway, while TRAF6 undergoes self-ubiquitination in response to IL-1β stimulation. Moreover, the non-proteolytic function of ubiquitination is essential for NF-κB activation during T cell activation. The CARD domain proteins, CRAMA1 and BCL10, and the paracaspase MALT1 form a complex in response to TCR signaling. The formation of this complex leads to the activation of TRAF6 which in turn induces the polyubiquitination of IKKγ and TRAF6 itself. The polyubiquitination catalyzed by TRAF6, TRAF2 or TRAF5 in these pathways requires Ubc13-Uev1A ubiquitin E2 which favor the synthesis of K63-linked polyubiquitin chains

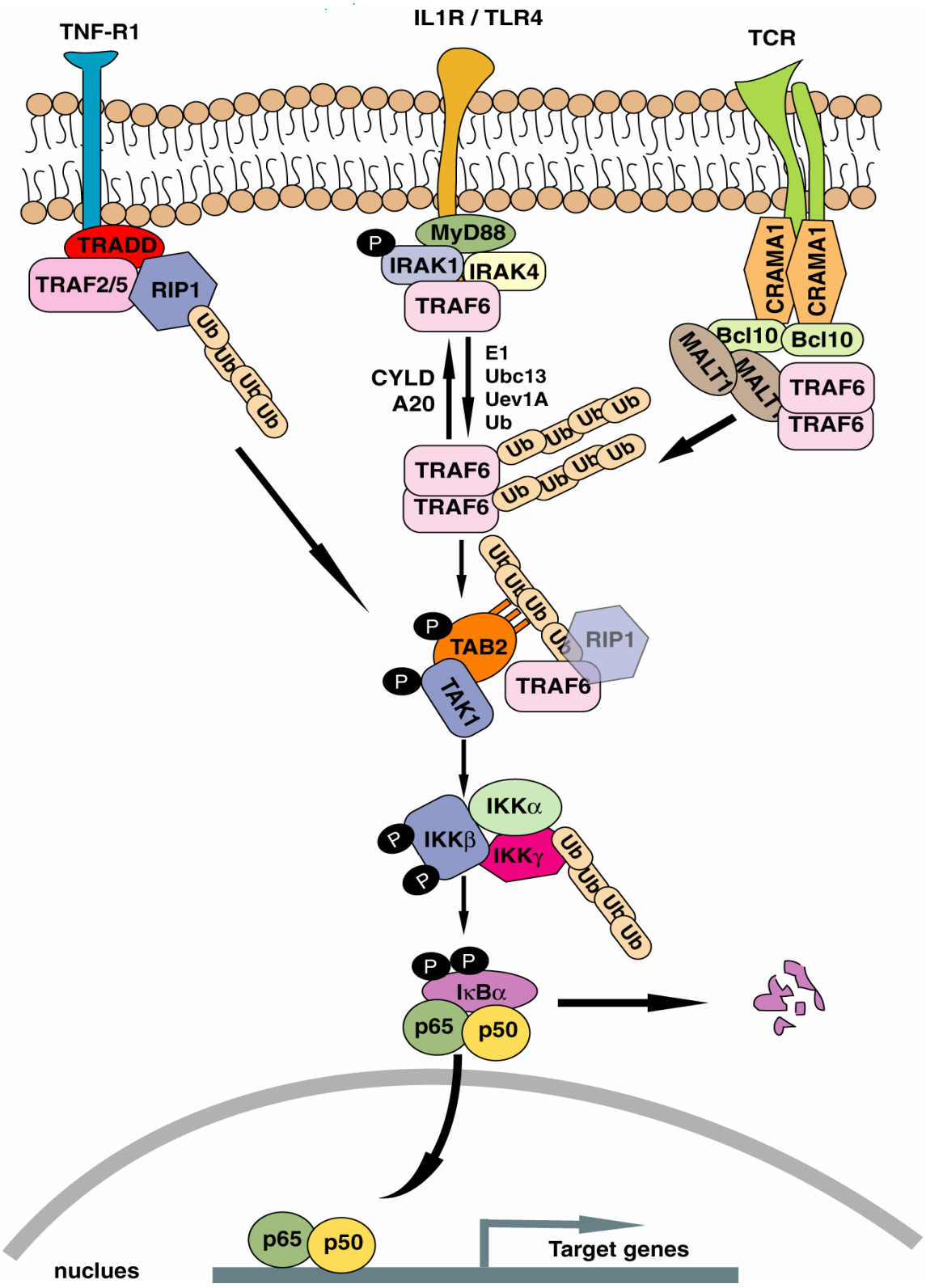
TNF-R1
IL1R / TLR4
TCR
TRADD
TRAF2/5
RIP1
Ub
MyD88
P
IRAK1
IRAK4
TRAF6
CYLD
A20
E1
Ubc13
Uev1A
Ub
CRAMA1
CRAMA1
Bcl10
Bcl10
MALT1
MALT1
TRAF6
TRAF6
TRAF6
TRAF6
TAB2
TAK1
RIP1
TRAF6
IKKα
IKKβ
IKKγ
IκBα
p65
p50
nuclues
p65
p50
Target genes

Figure 6. Ubiquitin-mediated activation of TAK1 and IKK.

In response to proinflammatory cytokines or pathogens, TNF receptors (TNFR), IL-1 receptor (IL-1R), or Toll-like receptors (TLR) bind to their cognate ligands and activate a signaling cascade leading to the activation of TRAF ubiquitin ligases, including TRAF2 and TRAF6. Similarly, in the adaptive immunity pathway, stimulation of the T cell receptors (TCR) with antigenic peptides leads to the membrane recruitment of a protein complex consisting of CARMA1, BCL10 and MALT1. These proteins regulate TRAF2 and TRAF6 by promoting their oligomerization, resulting in the activation of TRAF ubiquitin ligase activity. Activated TRAF proteins in conjunction with E1, Ubc13-Uev1A (E2), and ubiquitin catalyze the K63-linked polyubiquitination of target proteins including RIP1, IKKγ and TRAF6 itself. This process can be reversed with deubiquitination enzymes CYLD or A20. The K63-linked polyubiquitin chains serve as docking sites to recruit the TAK1-TAB2 complex through interacting with the novel zinc finger (NZF) domain of TAB2. The recruitment of TAK1-TAB2 leads to the activation of TAK1, which in turn activates IKK through direct phosphorylation of IKKβ within the activation loop. Activation of IKKβ results in the phosphorylation of IκBα and subsequent degradation of IκBα in a ubiquitin-proteasome dependent manner.

[152, 157, 162, 184]. These K63-linked polyubiquitin chains on TRAF6 facilitate the recruitment of the TAK1-TAB2 complex through interacting with the NZF domain of TAB2. The recruitment of TAK1-TAB2 to polyubiquitinated RIP1 or TRAF6 results in the activation of TAK1. Activated TAK1 subsequently activates IKKβ through direct phosphorylation of IKKβ at the activation loop and results in the activation of NF-κB.

The importance of non-proteolytic function of polyubiquitination in the NF-κB pathway is further underscored by the discovery of two K63-linked polyubiquitin chain specific deubiquitinating enzymes (DUBs), Cylindroma tumor suppressor gene (CYLD) and A20. Like phosphorylation, the effects of ubiquitination can be reversed by the removal of ubiquitin chains from a target substrate in the process of deubiquitination. CYLD and A20 have been shown to suppress NF-κB activation at a step upstream of IKK. CYLD is the first DUB identified to negatively regulate NF-κB through the deubiquitination of several proteins

including NEMO, TRAF2, and TRAF6 [185-187]. Mutations in the deubiquitination domain of CYLD, which result in enhanced NF-κB activity, have been attributed to the pathogenesis of cylindromas in patients [188]. A20 is a protein that is induced by NF-κB activation, and inhibits NF-κB in a negative-feedback loop [189, 190]. Overexpression of A20 inhibits NF-κB activation by several NF-κB agonists while removing the gene locus of A20 in mice results in enhanced and prolonged activation NF-κB by proinflammatory stimuli, such as TNFα and LPS [191]. The molecular mechanism of A20 as a negative regulator is not clear until the discovery that A20 contains a novel ovarian tumor (OTU)-type DUB domain. It has been shown that A20 inhibits IKK activation by dissembling K63-linked polyubiquitin chains from RIP1 and TRAF6 in the TNFα and LPS pathways respectively [192, 193]. Another unique feature of A20 is that it also contains ubiquitin ligase activity through its zinc finger domains which can promote the K48-linked polyubiquitination of RIP1 [193]. Therefore, A20 down regulates TNFα pathway at two steps through switching K63-linked polyubiquitin chain to K48-linked polyubiquitin chain on RIP1 that lead to the termination of IKK activation.

CHAPTER II: UBIQUITINATION-DEPENDENT ACTIVATION OF IKK BY TIFA

II.A Introduction

Tumor necrosis factor receptor-associated factors (TRAFs) are a family of adaptor proteins that participate in signal transduction of the tumor necrosis factor receptor (TNFR) and interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamilies [153, 156, 194-196]. TRAF proteins function as signal transducers that link the cytosolic portions of these receptors to downstream effectors. A wide range of biological functions, such as inflammatory response, innate and adaptive immunity and bone metabolism, rely on the proper functions of TRAF proteins to activate transcription factors including NF-κB, c-JUN and ATF2. Seven members of the TRAF family have recently been identified

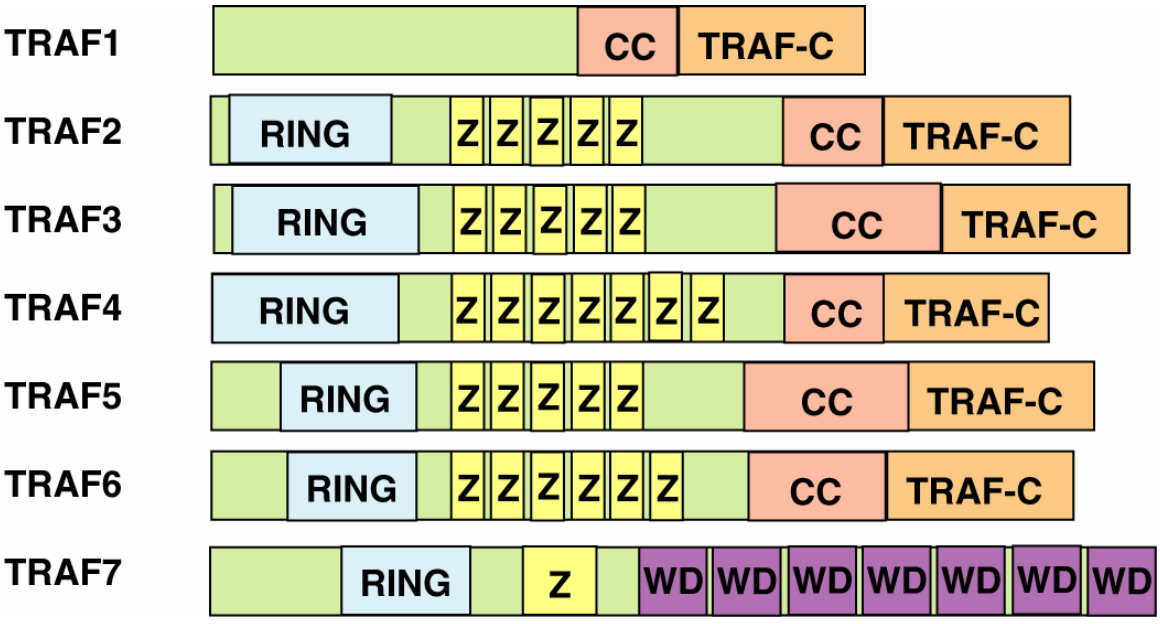

Figure 7. Structural domains of mammalian TRAF proteins.

There are seven TRAF proteins have been identified in mammalian genome. Z, Zinc finger domain; CC, coiled-coil domain; WD, WD40 repeat.

in mammals (fig. 7) [153-156]. All TRAF proteins except TRAF7 contain a highly conserved TRAF/MATH domain at the C-terminus, which mediates homotypic and heterotypic TRAF-TRAF interactions, as well as their association with members of the TNFR and IL-1/TLR receptor superfamilies. Two additional functional domains, the zinc finger domain and the RING domain, are located at the N-terminus of TRAF2-7. The RING domain, which uses a unique cross-brace arrangement to chelate zinc, is important for downstream signaling events [162, 194, 197].

TRAF-interacting protein with a FHA domain (TIFA) was identified as a TRAF6-interacting protein in a yeast two-hybrid screen [198]. The same molecule was also isolated from an independent mammalian two-hybrid screen as a TRAF2 binding protein [199]. Besides the FHA domain, which is known to bind phosphothreonine and phosphoserine [200], TIFA also contains a consensus TRAF6 binding motif [Pro-X-Glu-X-X-Ar/Ac, where X represents any amino acid, and Ar/Ac represents an aromatic or acidic amino acid. [201]]. A homologue of TIFA, termed TIFAB, has recently been identified [202]. TIFAB contains an FHA domain but lacks the consensus TRAF6 binding site. While overexpression of TIFA activates NF-κB and c-JUN N-terminal kinase (JNK), TIFAB appears to inhibit these pathways. *Xenopus laevis* homologue of TIFA (XTIFA) has been identified recently [203]. Overexpression of XTIFA leads to activation of NF-κB and the TRAF6 binding site at the C-terminal of XTIFA is required. It has been suggested that a possible function of TIFA in NF-κB activation is to link TRAF6 to IRAK-1 in an IL-1 stimulation-dependent manner [198], but the biochemical mechanism underlying TIFA-induced activation of NF-κB has remained unknown. In this study, I demonstrate that TIFA activates IKK in an in vitro reconstitution

system consisting of purified proteins including TAK1, TRAF6 and other ubiquitination enzymes. Furthermore, I show that TIFA induces the oligomerization and polyubiquitination of TRAF6, which in turn activates TAK1 and IKK.

II.B Materials and Methods

II.B.1 Plasmids and Proteins

Human TIFA, Ubc13 (by Dr. Li Deng) and Uev1A (by Dr. Li Deng) cDNAs were cloned by Polymerase Chain Reaction (PCR), and then subcloned into pET14b (Novagen) for expression in *Escherichia coli.* cDNA of TRAF6 was a gift from Dr. Jun Ichiro-Inoue (University of Tokyo). cDNAs encoding TAB1, TAB2, TAK1, and NEMO were cloned by Dr. Chen Wang and Dr. Li Deng by PCR from human placenta cDNA library (Clontech)[162]. These cDNAs were subcloned into pFAST-Bac (Invitrogen) for Sf9 expression as His_6-tagged proteins. Baculovirus harboring His_6-tagged E1 was kindly provided by Dr. Cecile Pickart (Johns Hopkins University). His_6-tagged TAK1, TAB1, TAB2, TRAF6, and E1 were expressed in Sf9 cells using the baculovirus expression system. His_6-tagged TIFAs were expressed in *E. coli*. His_6-tagged proteins were purified using nickel columns (Qiagen) according to Manufacturer's instruction. Purified proteins were dialyze with buffer containing 50mM Tris-HCl [pH7.5], 150mM NaCl, 10% glycerol, 0.5mM DTT and 0.5mM PMSF and stored at -80°C. TIFA mutants were generated by using the QuikChange Kit (Stratagene). The open reading frames of wild type and mutant TIFA were subcloned into the mammalian expression vector pEF-FLAG-IRES-Puro. All the constructs were verified by automatic DNA sequencing. Ubiquitin protein was acquired from Sigma. Endogenous IKK complex was purified form HeLa cells [162]. In vitro translation of IκBα was carried out using wheat germ lysates supplemented with ^{35}S-labeled methionine. His_6-Ubc13 and Uev1A, and GST-IκBα NT (the N-terminal 36 amino acids) were expressed in *E.*

coli and purified as described before [152]. The purity of recombinant proteins were verified by SDS-PAGE followed by coomassie blue staining or silver staining (Owl separation systems).

II.B.2 Antibodies and Cell Culture

Antibodies against IκBα (C21), TRAF6 (H274, D10), TRAF2 (H249), NEMO (FL419) and Ub (P4D1) were purchased from Santa Cruz Biotech. Antibodies against penta-His (Qiagen), FLAG (M2, Sigma), HA (Covance), and Ubc13 (4E11; Zymed Laboratories) were purchased from the respective commercial sources.

HeLa S3 cells were purchased from National Cell Culture Center (Minneapolis). Human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin G (100μg/ml), and streptomycin (100μg/ml). Human Jurkat (SVT35) and NEMO-deficient SVT 35 Jurkat cells (kindly provided by Dr. Shao-Cong Sun, Penn State University; [204]) were cultured in RPMI medium supplemented with 10% FCS, 2mM L-glutamine and antibiotics.

II.B.3 RT-PCR

HEK293 cells were transfected with vector, wild type or mutant TIFA by calcium phosphate precipitation. For the positive control, HEK293 cells were treated with 10ng/ml TNFα. After 48hr, TRIZOL LS reagent (Invitrogen) was used to isolate total RNAs. cDNAs were synthesized by using the TaqMan reverse transcription reagent (Applied Biosystems). The sequences of the primers were as follows: hICAM-1: forward

AACGACTCCTTCTCGGCCAA; reverse TCTGCAGTCTCTCCTGGCTCT. hMCP-1: forward GCCCCAGTCACCTGCTGTTAT; reverse TTGGCCACAATGGTCTTGAA. hGAPDH: forward AAAATCAAGTGGGGCGATGCT; reverse GGGCAGAGATGATGACCCTTT. RT-PCR products were separated in 2% agarose gel.

II.B.4 RNA Interference and NF-κB Reporter Assay

Double-stranded RNA oligonucleotides were synthesized at the Center for Biomedical Invention at UT Southwestern. The sequences of the sense strand of RNA oligos were as follows: GFP (471-490): GCAGAAGAACGGCAUCAAGdTdT; TRAF6 (1335-1354): CCACGAAGAGAUAAUGGAUdTdT; TRAF2 (1167-1186): GAUGUGUCUGCGUAUCUACdTdT; Ubc13 (313-332): CUGCUAUCGAUCCAGGCCUdTdT. The sense and antisense RNA oligos were annealed and then transfected into HEK293 cells by calcium phosphate precipitation at a final concentration of 50 nM. This procedure was repeated the second day to increase the efficiency of gene silencing. To measure NF-κB activation, cells were transfected with a luciferase reporter driven by three tandem repeats of NF-κB enhancer found in immunoglobulin kappa light chain gene, pCMV-LacZ and TIFA or NIK expression plasmid on the next day after the second siRNA transfection. After 24hr, the cells were lysed in 1X passive lysis buffer (Promega) and luciferase activity was measured with Anthos Lucy2 Luminescence Reader using luciferin (promega) as substrate. β-galactosidase activity was measured with a Thermo Labsystem Microplate Reader at the wavelength of 450nm using o-

nitrophenyl-β-D-galactopiranoside (ONPG prepared in 100mM Na_2PO_4, 1mM $MgCl_2$, 50mM β-mercaptoethanol, 0.66mg/ml ONPG) as substrate.

II.B.5 Ubc13-Sepharose Affinity Chromatography

Ubc13 protein was covalently conjugated to NHS-Sepharose at the density of 10 mg/ml according to Manufacturer's Instruction (Pharmacia). To purify proteins that bind to Ubc13-Sepharose, HeLa cytosolic extracts (S100) were incubated with the beads at 4^{o}C for 1hr. After a brief centrifugation, the supernatant was collected as "Ubc13-depleted extracts" and used for further assays (see below). The beads were washed with Buffer A (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF) and then eluted with Buffer A plus 0.5 M NaCl. After buffer exchange to remove NaCl by repeated cycle of concentration and dilution in Buffer A, the eluate (designated as Ubc13 eluate) was used for IKK activation assays (see below).

II.B.6 Purification and Identification of TRAF6

The purification and identification of TRAF6 was carried out together with Dr. Lijun Sun. All procedures were carried out at 4°C. HeLa cells from 50 liters were resuspended in 70ml of hypotonic buffer A and were lysed in a Dounce homogenizer. Crude S100 were generated by ultracentrifugation at 100,000g for 1hr. S100 was loaded onto a Ubc13-sepharose column (5ml bed volume; 5mg Ubc13 per ml) equilibrated with buffer A. The column was wash extensively with buffer A, and the bound proteins were eluted with buffer A plus 0.5mM NaCl. It was then precipitated with 40% saturation of ammonium sulfate. The

pellets were resuspended in buffer B (20mM Tris-HCl [pH 7.5], 0.15M NaCl, 10% glycerol, 0.5mM DTT, and 0.1mM PMSF), and applied to a Superdex-200 column (24 ml bed volume; Pharmacia) preequilibrated with buffer B. Fractions containing IKK stimulatory activity were pooled and loaded onto a heparin-sepharose column (1ml) preequilibrated with buffer C (10mM Tris-HCl [pH 7.5], 10% glycerol, 0.02% CHAPS, 0.5mM DTT, and 0.1mM PMSF). Active fractions were eluted with 0.2M NaCl in buffer C and were further fractionated on a MonoQ column (0.1ml) suing the SMART system (Pharmacia). Bound proteins were eluted with a gradient of NaCl (0.2-0.5M in 2.5ml) in buffer C. the fraction containing the peak IKK stimulatory activity was subjected to SDS-PAGE followed by colloidal blue staining (Invitrogen). The 60 kDa band that copurified with the IKK stimulatory activity was excised for identification by Nano-HPLC/electrospray mass spectrometry on a ThermoFinnigan LCQ Deca XP MS instrument (UT Southwestern Protein ID core facility).

II.B.7 Gel Filtration Chromatography and Glycerol Gradient Ultracentrifugation

Wild type or mutant TIFA (1 μg) was added to 20 μl Ubc13 eluate (6.4 mg/ml; containing endogenous TRAF6). After 1hr incubation at 4^{o}C, the mixture was applied to Superdex-200 (2.4 ml bed volume) equilibrated with buffer B using the SMART system (Pharmacia), and fractions (100μl) were collected for analysis by immunoblotting with anti-TRAF6 antibody (D10).

Wild type or mutant TIFA (5μg) was incubated with 50 μl Ubc13 eluate as described above and then the mixtures were applied to a 2ml 10-50% glycerol gradient in 50 mM Tris-

HCl, pH7.5, 150 mM NaCl. After centrifugation at 250,000 x g for 3hr in a Beckman TLS-55 rotor at 4°C, fractions (200μl) were analyzed by immunoblotting with an antibody against penta-His (detecting His_6-TIFA) or TRAF6.

II.B.8 Immunodepletion of TRAF6

HeLa S100 (100 μl) was mixed with 10 μg of TRAF6 antibody (H274) and 10 μl of protein A/G-Sepharose (Pierce). After mixing end-to-end at 4°C for 1hr, the beads were removed by centrifugation. This process was repeated twice and the depletion of TRAF6 from the extracts was confirmed by immunoblotting of the supernatants with the TRAF6 antibody.

II.B.9 *In vitro* Assay for IKK Activation

To measure the activation of IKK by TIFA *in vitro*, Jurkat or HeLa cytosolic extracts (10 mg/ml in 20 mM Tris-HCl [pH7.5], 150 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1% NP40) were incubated with recombinant TIFA protein (50 ng) and ^{35}S-labeled IκBα (0.2 μl) in a 10 μl reaction mixture containing an ATP regenerating system (50mM Tris-HCl [pH7.5], 5mM $MgCl_2$, 2mM ATP, 10mM phosph-creatine, 0.6U/ml pyrophosphatase, 3.5U/ml creatine phosphakinase). The reaction mixture was incubated at 30°C for 1hr, followed by SDS-PAGE and PhosphoImaging. To identify the factor required for the activation of IKK by TIFA, the same IKK kinase assay was used except that the cell extracts were replaced by Ubc13-depleted HeLa S100, Ubc13/Uev1A (1μM) and column fractions.

II.B.10 Ubiquitination Assay

To detect the ubiquitin-ligase activity of TRAF6, reaction mixtures containing E1 (0.1 μM), Ubc13/Uev1A (0.4 μM), TRAF6 (approximately 0.1 μM), Ub (60 μM) and ATP (2 mM) were incubated in the presence or absence of TIFA at 30^{o}C for 1hr. The reaction products were then resolved by SDS-PAGE and detected by an antibody against TRAF6 (D10) or Ub (P4D1).

II.C Results

II.C.1 TIFA Activates IKK in a Cell Free System

TIFA is a 20 kDa protein with a forkhead-associated (FHA) domain and a TRAF6 binding site (Fig. 8a). Overexpression of TIFA leads to activation of IKK and NF-κB in a manner that depends on the FHA domain and TRAF6 binding site [198]. For example, a point mutation in TIFA, E178A, abolishes its binding to TRAF6 as well as its ability to activate the expression of an NF-κB reporter (11). To determine whether overexpression of TIFA can induce the expression of endogenous NF-κB target genes, I transfected expression vectors encoding wild-type or mutant TIFA into human embryonic kidney (HEK) 293 cells, and then used RT-PCR to examine the expression of two NF-κB target genes, hICAM-1 (human intercellular adhesion molecule 1) and hMCP-1 (human monocyte chemoattractant protein 1). As shown in Fig. 8b, overexpression of wild-type TIFA induced the expression of hICAM-1 and hMCP-1 without affecting the constitutive expression of GAPDH. In contrast, the FHA-domain mutant (G50E/S66A) and TRAF6-binding-defective mutant (E178A) of TIFA failed to activate any of the NF-κB target genes. These results indicate that overexpression of TIFA is sufficient to activate the expression of endogenous NF-κB target genes.

To identify the factors that link TIFA to IKK activation, I developed an in vitro kinase assay that measures IKK activation following the addition of TIFA protein to crude cell extracts. Recombinant proteins of His_6-tagged wild type, FHA domain mutant (G50E/S66A), and TRAF6 binding defective mutant (E178A) of TIFA were expressed in

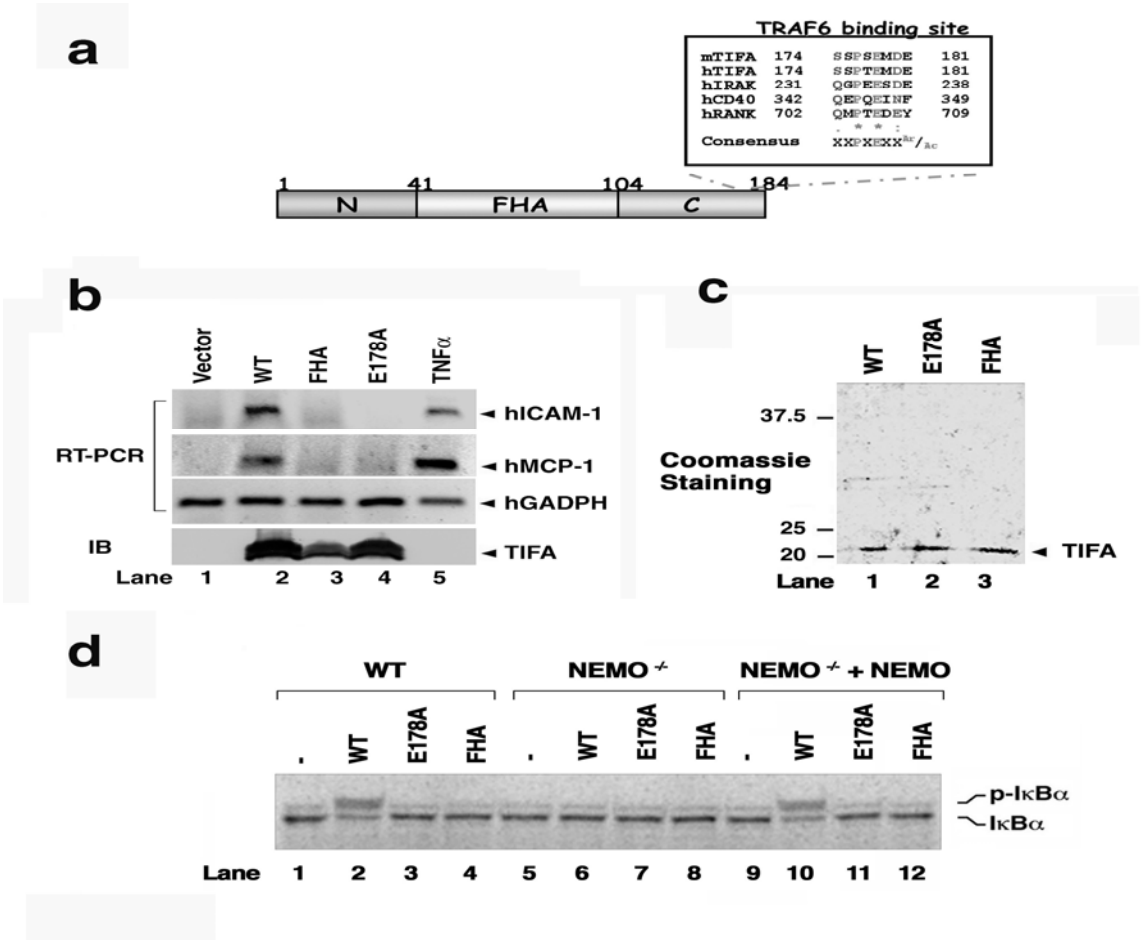

Figure 8. TIFA activates IKK in a cell-free system.

(a) Diagram of the primary structure of TIFA and alignment of the TRAF6-binding site. Ar/Ac represents an aromatic or acidic amino acid. **(b)** Overexpression of TIFA is sufficient to induce the expression of endogenous NF-κB target genes. Plasmids encoding Flag-tagged wild-type or mutant TIFA were transfected into HEK 293 cells. At 48 h after transfection, the expression of hICAM-1, hMCP-1, and human GAPDH (hGAPDH) RNA was analyzed by RT-PCR, and the expression of TIFA proteins was examined by immunoblotting with the Flag antibody. As a positive control, HEK 293 cells were stimulated with TNFα (10 ng/ml). WT, wild type; E178A, TRAF6-binding-defective mutant; FHA, FHA-domain mutant G50E/S66A. **(c)** Expression and purification of recombinant TIFA. His-tag TIFA and mutants were expressed in *E. coli* and purified with nickel nitrilotriacetic acid (Ni-NTA) resin. **(d)** Phosphorylation of IκBα in crude cell extracts. *In vitro* translated, ^{35}S-labeled IκBα was used as the substrate in the reactions that contain protein extracts from wild-type Jurkat cells (lanes 1–4) or NEMO-deficient Jurkat cells (lanes 5–12), TIFA or its mutants, and ATP. In lanes 9–12, recombinant NEMO was added to the NEMO-deficient extracts to restore IKK activation. *p*-IκBα, phosphorylated IκBα.

E. coli, and then purified using nickel columns (Fig. 8c). Addition of wild type TIFA, but not any of the mutants, to Jurkat T cell extracts led to the phosphorylation of ^{35}S-labeled IκBα as indicated by the electrophoretic mobility shift (Fig. 8d). Furthermore, TIFA-dependent activation of IKK in the cell free system depends on an intact IKK complex as the extracts prepared from NEMO-deficient Jurkat cells [204] failed to phosphorylate IκBα in response to TIFA. Addition of recombinant NEMO protein to the NEMO-deficient extracts restored TIFA-dependent phosphorylation of IκBα. Therefore, the in vitro TIFA-inducible system shown here recapitulates the functional domain requirement of TIFA and the requirement of NEMO for IKK activation.

II.C.2 Ubc13-Uev1A and TRAF6 Link TIFA to IKK Activation

It has been shown previously that TIFA interacts with TRAF2 [199] and TRAF6 [198], and that activation of IKK by TRAF2 and TRAF6 requires the ubiquitin-conjugating enzyme complex Ubc13-Uev1A [152]. To determine whether TIFA-induced activation of IKK requires Ubc13-Uev1A, I carried out in vitro IKK activation assay using HeLa cytosolic extracts (S100) which are depleted of Uev1A by Sepharose beads coated with Ubc13 (15; see Materials and Methods). As shown in Figure 9a, TIFA was unable to activate IKK in Uev1A-depleted extracts (lane 2). However, even when recombinant Ubc13 and Uev1A proteins were added back to the reaction, TIFA still failed to activate IKK (lane 5), whereas recombinant TRAF6 was able to restore IKK activation (lane 6). To test if the Ubc13 column retained another factor that is required for IKK activation by TIFA, I eluted proteins bound to the column with 0.5 M NaCl and used the eluate (designated as Ubc13 eluate) in the IKK

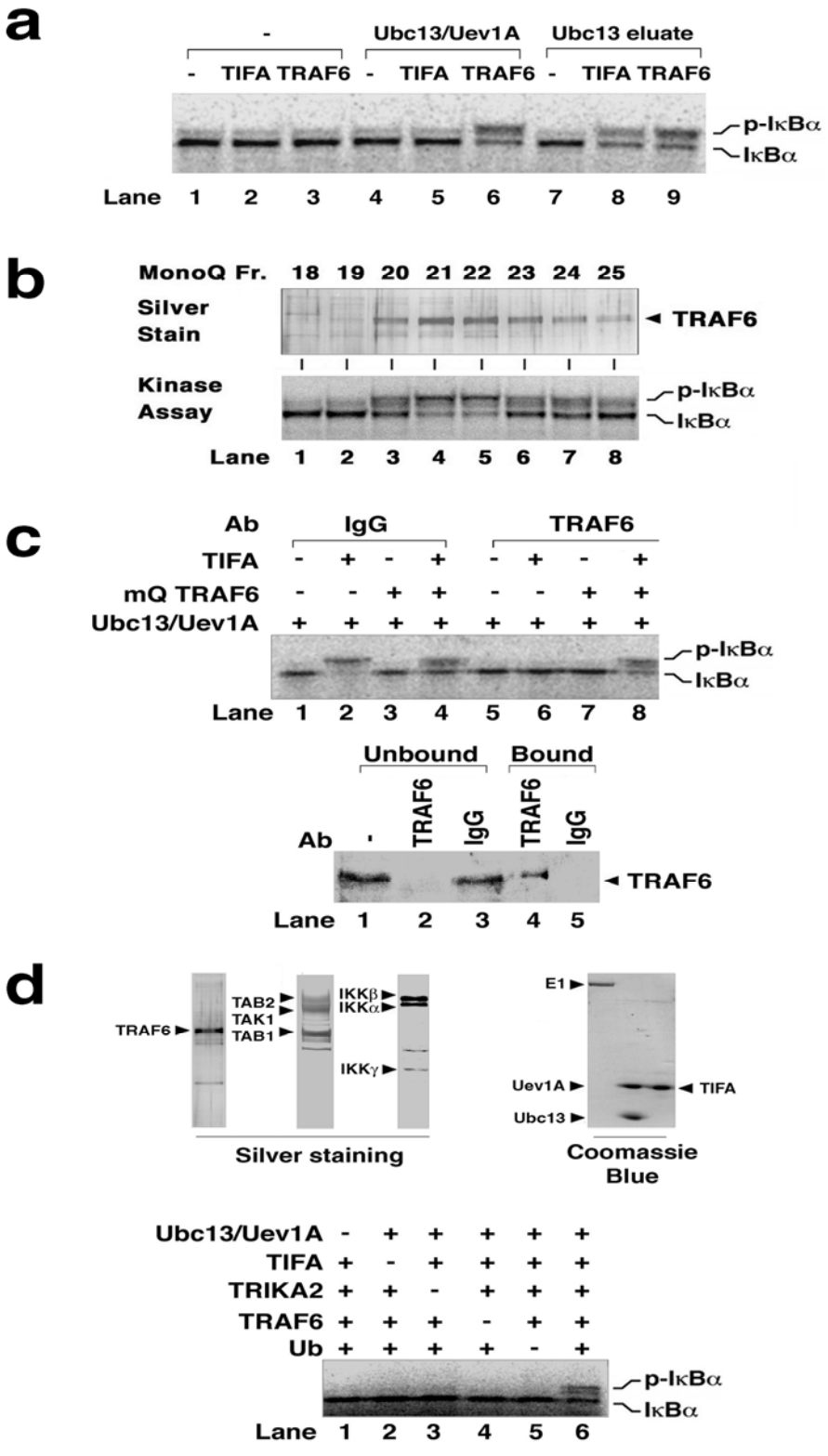
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Figure 9. TIFA activates IKK through Ubc13–Uev1A and TRAF6.

(a) Activation of IKK by TIFA requires Ubc13–Uev1A and another Ubc13-associated factor. HeLa S100 was incubated with Ubc13–Sepharose to remove endogenous Uev1A and other proteins that bound to the resin. The supernatant was used as ‘‘Ubc13-depleted extract’’ for IKK assays in the presence of recombinant TRAF6 (lanes 3, 6, and 9) or TIFA (lanes 2, 5, and 8). In lanes 4–6, recombinant Ubc13–Uev1A protein was added to the reaction, whereas in lanes 7–9, protein that bound to the Ubc13 column was eluted (Ubc13 eluate) and added to the assays. **(b)** Identification of TRAF6 as a Ubc13-interacting protein that is required for IKK activation by TIFA. Endogenous TRAF6 protein was purified as described in ref. 16, and fractions from the last MonoQ step were analyzed by silver staining (*Upper*) or IKK kinase assays (*Lower*). **(c)** TRAF6 is required for IKK activation by TIFA *in vitro*. HeLa S100 was subjected to immunoprecipitation with an antibody against TRAF6 (lanes 5–8) or a control IgG (lanes 1–4). The supernatants were then analyzed by immunoblotting with a TRAF6 antibody (*Lower*) or by IKK assays (*Upper*) in the presence of Ubc13–Uev1A, TIFA, and purified endogenous TRAF6, as indicated. **(d)** (*Upper*) Silver or Coomassie blue staining of purified proteins. TRAF6 (native, purified from HeLa S100; lane 1), IKK (native, purified from HeLa S100; lane 2), TRIKA2 (recombinant, purified from Sf9; lane 3), E1 (recombinant, purified from Sf9; lane 4), Ubc13–Uev1A (recombinant, purified from *E. coli*; lane 5), and TIFA (recombinant, purified from *E. coli*; lane 6). (*Lower*) Reconstitution of TIFA induced IKK pathway *in vitro*. Purified native IKK complex and TRAF6 were incubated with recombinant proteins of TIFA, Ubc13–Uev1A, Ub, and TAK1–TAB1–TAB2 complex (TRIKA2) as indicated. The reconstitution reaction mixture also contained ATP, E1, and ^{35}S-labeled IκBα.

assay. Indeed, the Ubc13 eluate restored IKK activation by TIFA (lane 8), indicating that another Ubc13-associated factor distinct from Ubc13-Uev1A is required for TIFA activation of IKK. In a separate series of experiments, Dr. Lijun Sun and I have further purified the IKK stimulatory factor from the Ubc13 eluate and identified this factor as TRAF6, which mediates IKK activation by BCL10 [157]. To determine if TRAF6 also mediates TIFA-dependent IKK activation, I tested MonoQ fractions containing purified TRAF6 for their ability to activate IKK in the presence of TIFA. As shown in Figure 9b, the TIFA-dependent IKK stimulatory activity (bottom panel) co-purified very well with TRAF6 protein [top panel; the identity of the TRAF6 protein was confirmed by mass spectrometry and immunoblotting; [157]. To confirm that TRAF6 was indeed required for IKK activation by TIFA in the extracts, I immunodepleted TRAF6 from the extracts and examined the resulting effect on IKK activation (Figure 9c). Depletion of TRAF6 from HeLa S100 abolished its ability to support TIFA-dependent IKK activation (Fig. 9c; lane 6), whereas the control extracts retained full IKK activation potential (lane 2). Addition of purified TRAF6 (Mono-Q fraction 21) to the TRAF6-depleted extracts restored IKK activation in response to TIFA (lane 8). These results indicate that endogenous TRAF6 is required for IKK activation by TIFA in vitro, whereas recombinant TRAF6 overexpressed and purified from insect cells is able to activate IKK independently of TIFA or other upstream regulators. It has previously shown that a fraction of recombinant TRAF6 protein forms oligomers which activate IKK constitutively, thus explaining the difference in the regulation of endogenous versus recombinant TRAF6 [157].

II.C.3 Reconstitution of IKK Activation by TIFA in vitro

I next attempted to reconstitute TIFA-dependent IKK activation in vitro using purified proteins. The reconstitution reactions contain E1, Ubc13-Uev1A (E2), TRAF6 (E3), Ub, TRIKA2 (TAK1-TAB1-TAB2 complex expressed and purified from baculovirus-infected insect cells), purified endogenous IKK complex, TIFA, ^{35}S-IκBα (purified from in vitro translation system) and ATP (see Materials and Methods). As shown in Figure 9d, the presence of all of these components in the reaction was required to reconstitute IKK activation by TIFA (compare lane 6 with other lanes in the lower panel). These results demonstrate that TIFA activates IKK through TRAF6 and TAK1 complex in a ubiquitination-dependent manner.

II.C.4 NF-κB Activation by TIFA Requires Ubc13, TRAF2 and TRAF6

To determine if Ubc13, TRAF2 and TRAF6 are required for TIFA-mediated NF-κB activation in living cells, I used RNAi to silence the expression of these genes in HEK293 cells transfected with TIFA expression vector and a luciferase reporter gene under the control of three tandem repeats of NF-κB binding sites (Fig 10a). Small interfering RNA (siRNA) oligos targeting Ubc13, TRAF6 or TRAF2 decreased the expression of the corresponding proteins (Fig. 10b). Overexpression of TIFA activated the NF-κB-luciferase reporter in cells transfected with GFP siRNA (as a control, Fig. 10a). In contrast, siRNA targeting Ubc13, TRAF6 or TRAF2 strongly inhibited TIFA-mediated NF-κB activation (Fig. 10a). As a control, none of the siRNA oligos had significant effect on NF-κB activation by NIK (Fig. 10a), which is likely to activate NF-κB through direct phosphorylation of IKKα

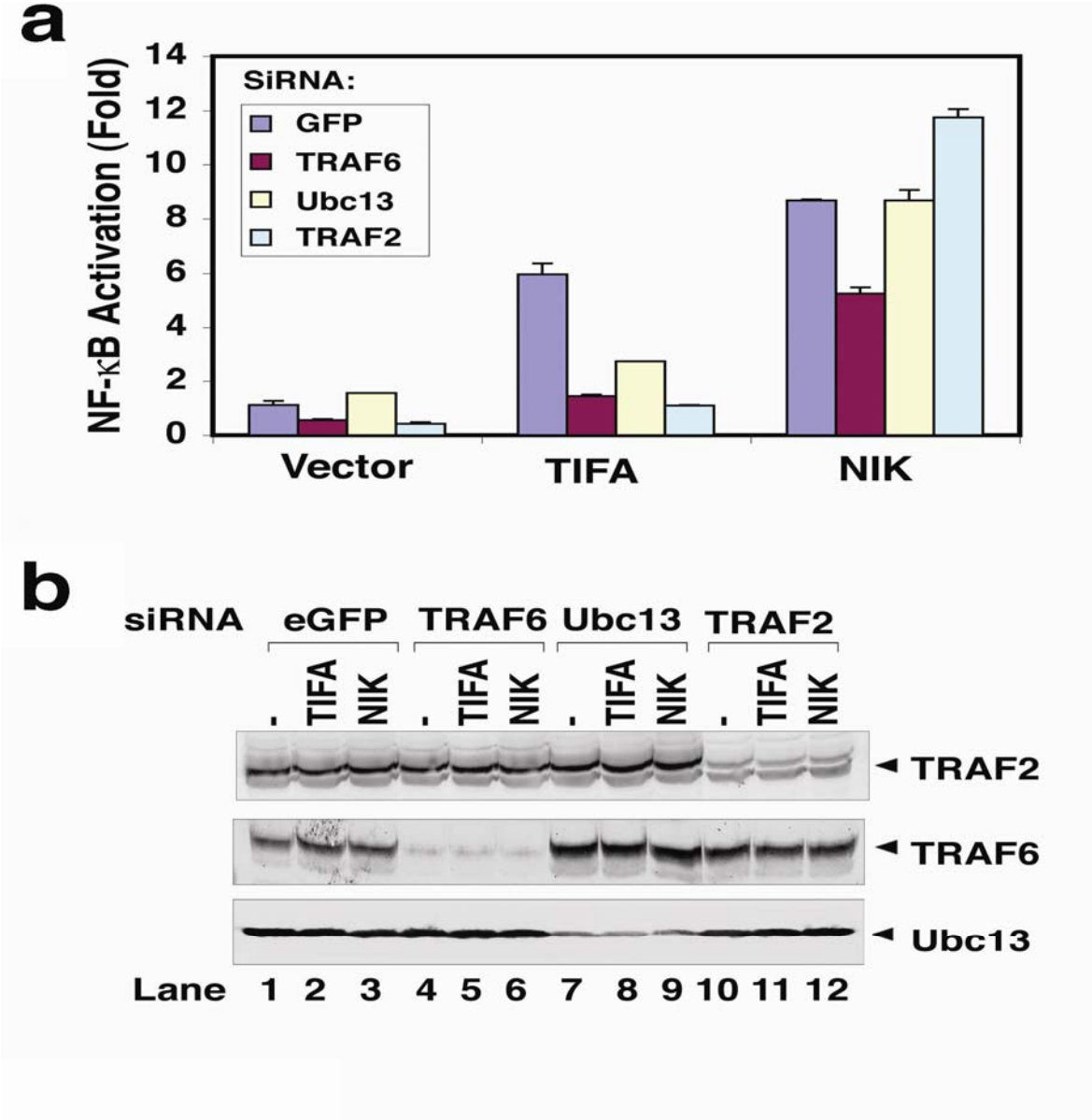


Figure 10. Ubc13, TRAF6, and TRAF2 are required for NF-κB activation by TIFA.

Double-stranded siRNA oligomers corresponding to the sequence of GFP (control), Ubc13, TRAF2, or TRAF6 were transfected into HEK 293 cells. After 2 consecutive days of transfection of siRNA, cells were transfected with a NF-κB luciferase reporter with the expression constructs encoding Flag-tagged TIFA, NF-κB-interacting kinase (NIK), or vector alone. **(a)** The luciferase activity was measured and normalized for transfection efficiency, as determined by cotransfection of a constitutively expressed β-galactosidase reporter. **(b)** The efficiency of RNA interference was verified by immunoblotting.

[60]. Taken together, these results suggest that Ubc13, TRAF6 and TRAF2 are essential for TIFA-mediated activation of NF-κB.

II.C.5 TIFA Forms Oligomers to Activate IKK

Previous studies have suggested that TRAF proteins may be regulated by oligomerization, which may in turn be controlled by some upstream signaling molecules [157, 162]. To determine if TIFA forms oligomers, I analyzed the molecular sizes of purified TIFA protein by glycerol gradient ultracentrifugation. While the majority of TIFA protein was present in fractions containing low concentration of glycerol (top of the gradient), a very small amount of TIFA forms oligomers or aggregates that migrated towards the bottom of the gradient where the percentage of glycerol was higher (Fig. 11a). Interestingly, only these oligomeric forms of TIFA, whose apparent molecular size was similar to that of the 26S proteasome, were able to activate IKK. The TIFA mutant defective in TRAF6 binding, E178A, had similar distribution pattern to that of wild type TIFA in the glycerol gradient, but was unable to activate IKK. Mutations in the FHA domain caused a shift in the distribution of TIFA towards the middle of the gradient, but none of the fractions containing TIFA-FHA mutant was able to activate IKK.

II.C.6 TIFA Promotes the Oligomerization of TRAF6

TIFA was identified as a TRAF6-binding protein. Thus, one possible mechanism by which TIFA oligomers activate IKK is to induce the oligomerization of TRAF6. To test this possibility, I incubated a partially purified TRAF6 fraction with wild type or mutant TIFA

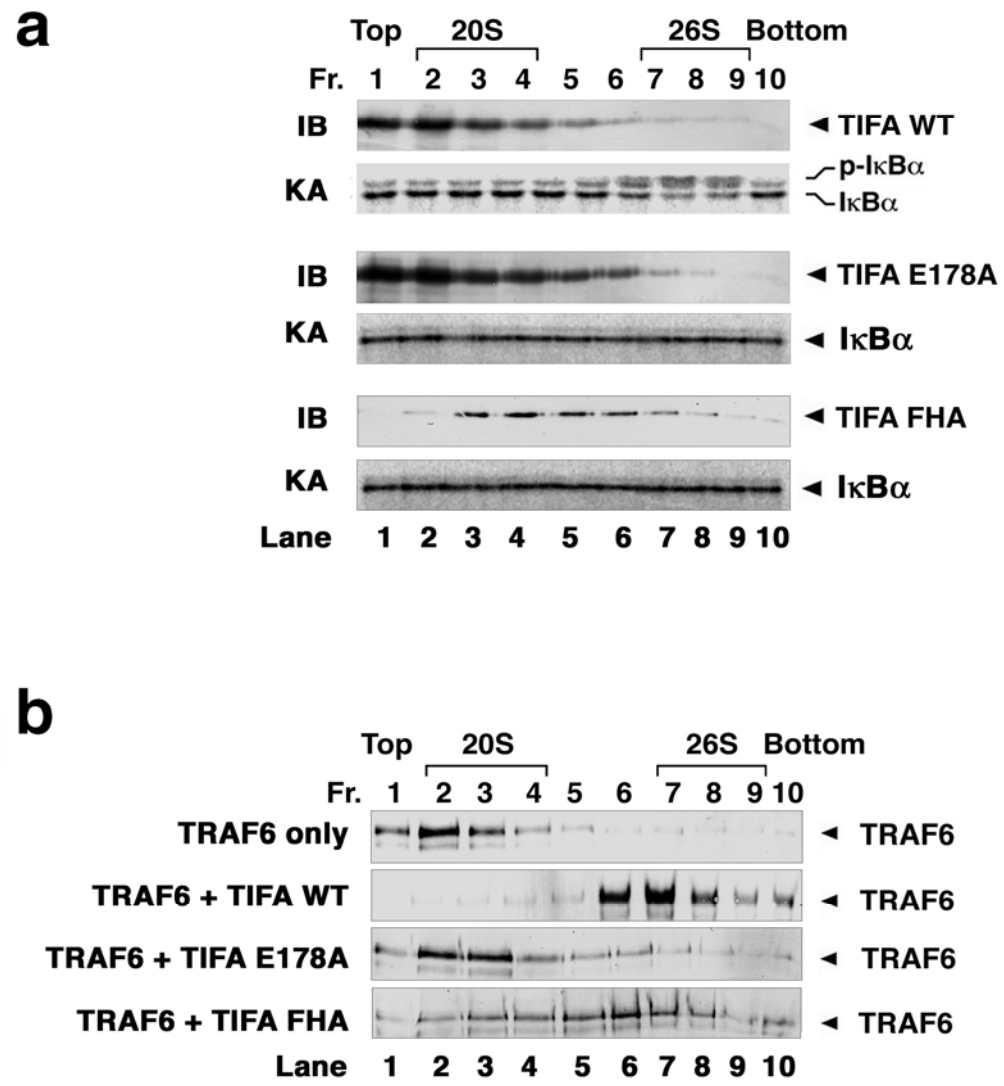


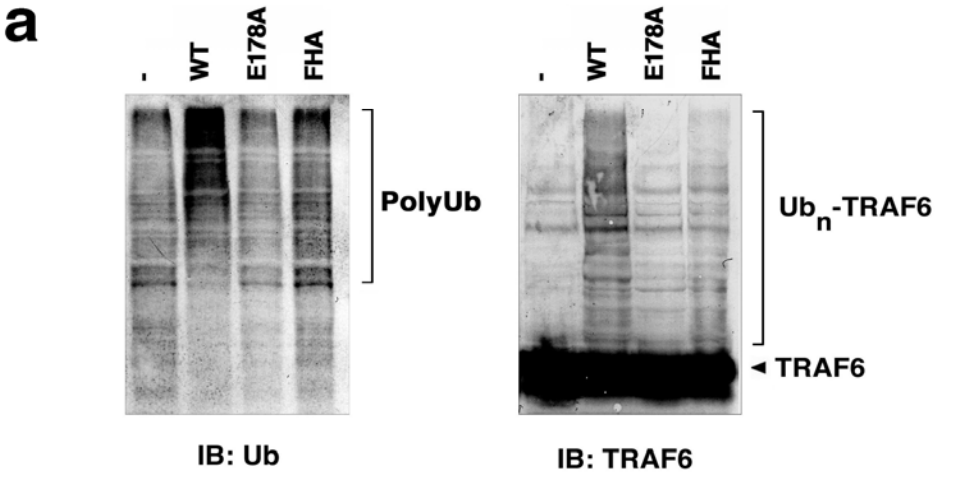
Figure 11. TIFA induces TRAF6 oligomerization and IKK activation.

(a) Oligomerized TIFA activates IKK *in vitro*. Recombinant wild-type or mutant (E178A or FHA) His_6-TIFA proteins were fractionated by glycerol-gradient ultracentrifugation (10–50%). Fractions were collected (percentage of glycerol increases from top to bottom) and analyzed by immunoblotting with a penta-His-specific antibody (IB) or by IKK kinase assays (KA). **(b)** TIFA induces the oligomerization of TRAF6. Ubc13 eluate enriched in native TRAF6 (labeled as TRAF6) was incubated alone or with TIFA proteins as indicated at 4°C for 1 h. The mixtures were fractionated by glycerol-gradient ultracentrifugation as described above. Fractions were collected and analyzed by immunoblotting with a TRAF6 antibody.

protein and then determine the molecular distribution of TRAF6 by glycerol gradient ultracentrifugation. In the absence of TIFA, the majority of TRAF6 was present in low molecular weight fractions (Fig. 11b). Significantly, when wild type TIFA was added, TRAF6 migrated to the high molecular weight fractions. The TIFA mutant that does not bind to TRAF6 was also unable to induce TRAF6 oligomerization, whereas the FHA mutant of TIFA, which still binds to TRAF6, caused TRAF6 to sediment in the glycerol gradient in a pattern that is similar to that of TIFA-FHA mutant itself (Fig. 11a). These results suggest that TIFA binds to TRAF6 and induces TRAF6 oligomerization. It is interesting to note that although the majority of TIFA was present as low molecular weight species (Fig. 11a, top), only the high molecular weight forms of TIFA co-sedimented with TRAF6 (Fig. 11b), suggesting that oligomerization of TIFA greatly enhances its ability to bind to TRAF6.

II.C.7 TIFA Enhances the Ubiquitin-Ligase Activity of TRAF6

TRAF6 is a RING domain ubiquitin ligase (E3), which in conjunction with Ubc13/Uev1A catalyzes K63-linked polyubiquitination that is required for IKK activation [152]. It has previously shown that the ubiquitin ligase activity of TRAF6 can be enhanced by forced dimerization of an artificial dimerization domain [162] or by oligomerization induced by BCL10 and MALT1, two signaling proteins in the T cell receptor pathway [157]. To determine if TIFA enhanced the ubiquitin-ligase activity of endogenous TRAF6, I performed in vitro ubiquitination assay in the presence of E1, Ubc13-Uev1A, purified endogenous TRAF6, Ub and ATP (Fig. 12a). Addition of wild type, but not mutant TIFA into the reaction significantly enhanced the synthesis of polyubiquitin chains as well as the polyubiquitination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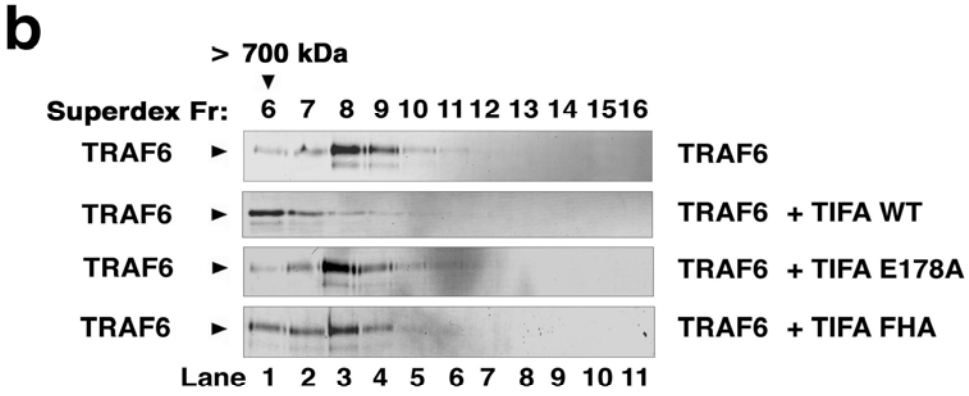
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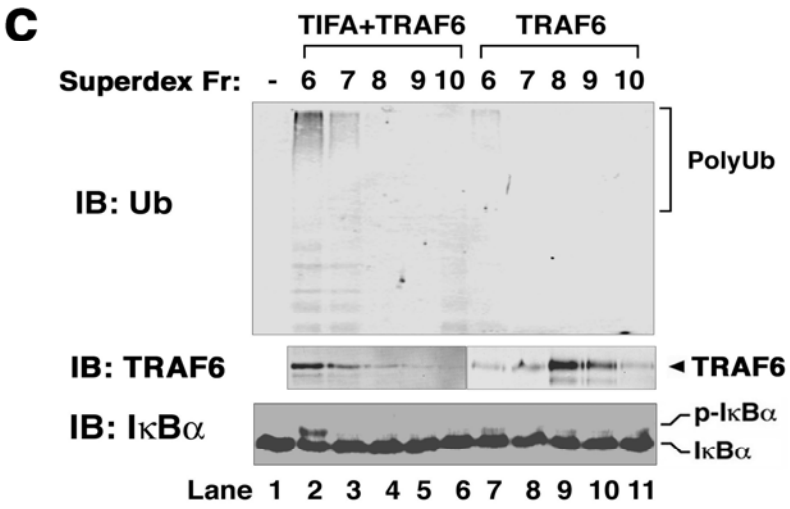
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TRAF6
IB: IκBα
p-IκBα
IκBα
Lane 1 2 3 4 5 6 7 8 9 10 11

Figure 12. Oligomerization of TRAF6 by TIFA activates its Ub ligase activity.

(a) TIFA promotes polyubiquitination of TRAF6. Recombinant wild-type or mutant TIFA proteins were incubated with TRAF6 in a reaction mixture containing E1, Ubc13–Uev1A (E2), Ub, and ATP. After incubation at 30°C for 1 h, reaction products were analyzed by immunoblotting with an antibody against Ub (*Left*) or TRAF6 (*Right*). **(b)** TIFA induces oligomerization of TRAF6. TIFA was incubated with TRAF6 as described in the legend of Fig. 4*B*, and the mixture was fractionated by gel-filtration chromatography on Superdex 200. Fractions from the column were analyzed by immunoblotting with a TRAF6 antibody. **(c)** The Ub ligase and IKK-stimulatory activities of TRAF6 are induced by TIFA-mediated oligomerization. (*Top*) The Superdex 200 fractions shown in **b** were depleted of His_6-TIFA by using nickel-affinity resin and then incubated in a reaction mixture containing E1, Ubc13–Uev1A, Ub, and ATP. Polyubiquitin chain synthesis was analyzed by immunoblotting with a Ub antibody. (*Bottom*) Aliquots containing TRAF6 of different molecular sizes from the Superdex 200 column were also incubated with HeLa S100 to determine the IKK stimulatory activity of TRAF6 fractions. After incubation at 30°C for 1 h in the presence of ATP, phosphorylation of endogenous IκBα was analyzed by immunoblotting with an IκBα-specific antibody.

of TRAF6. To determine if the enhanced ubiquitin ligase activity of TRAF6 was due to TIFA-induced oligomerization, I incubated TRAF6 with wild type or mutant TIFA proteins and then separated the mixtures by gel filtration chromatography (Superdex-200). As shown in Figure 12b, addition of wild type, but not mutant, TIFA protein led to oligomerization of TRAF6, which eluted from the gel filtration column in the void volume (> 700 kDa). Importantly, these high molecular weight forms of TRAF6 displayed greatly increased ubiquitin ligase activity as compared to TRAF6 of lower molecular weight species, despite similar amount of TRAF6 protein in these fractions (Fig. 12c, top two panels). Moreover, only the fraction with significantly higher ubiquitin ligase activity of (Fraction 6, lane 2) was able to activate IKK. This activity was not due to the presence of TIFA since I had removed His_6-TIFA from the fraction using Nickel affinity column (data not shown). Taken together, these results indicate that TIFA activates IKK by inducing the oligomerization and ubiquitination of TRAF6, which then activates the IKK kinase, TAK1 (Fig. 13).

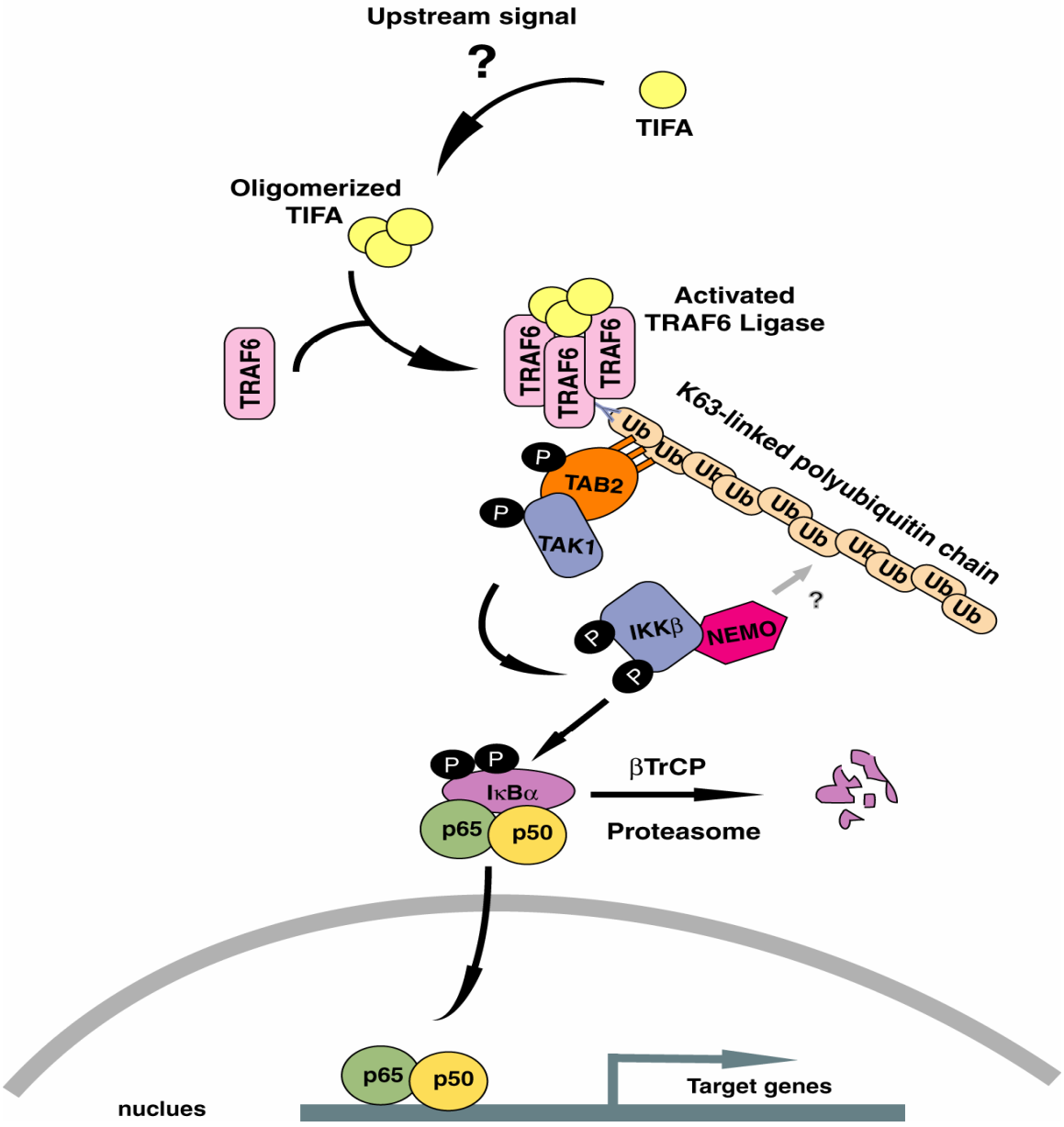


Figure 13. A biochemical model of IKK activation by TIFA.

TIFA forms oligomers upon stimulation of cells with some NF-κB agonists. The oligomerized forms of TIFA bind to TRAF6 and promote TRAF6 oligomerization. As a result, the TRAF6 Ub ligase is activated to catalyze K63-linked polyubiquitination in conjunction with the Ubc13–Uev1A E2 complex (TRIKA1). Ubiquitinated TRAF6 is recruited to the TRIKA2 complex, which contains the Ub receptor TAB2 and the protein kinase TAK1. TAK1 is then activated to phosphorylate IKKβ at key serine residues within the activation loop, thereby activating IKK.

II.D Discussion

In this study, I have investigated the biochemical mechanism of IKK activation by TIFA. I show that TIFA activates IKK in crude cell extracts in a manner that depends on an intact FHA domain and a TRAF6 binding motif. Using this in vitro activation system, I have identified Ubc13-Uev1A and TRAF6 as the E2 and E3, respectively that mediate IKK activation by TIFA through a ubiquitin-dependent mechanism. Importantly, I have succeeded in the reconstitution of IKK activation by TIFA in vitro using purified proteins including the TAK1 kinase complex, TRAF6 and other ubiquitination enzymes. Furthermore, I have provided evidence that TIFA forms oligomers that activate IKK by inducing the oligomerization and polyubiquitination of TRAF6.

My data showed that the FHA domain and TRAF6 binding site of TIFA are required for IKK activation in vitro. A single point mutation in the TRAF6 binding site of TIFA abolished its ability to induce TRAF6 oligomerization and ubiquitination, indicating that the direct binding between TIFA and TRAF6 is essential for its function. On the other hand, the biochemical function of FHA domain in TIFA is less clear. FHA domain is known to bind directly to phosphoserine/phosphothreonine residues [200]. However, none of the proteins known to be involved in the TRAF6 pathways have been shown to interact with the FHA domain in a phosphorylation-dependent manner, although the binding of IRAK1 to TIFA appears to be IL-1β dependent [198]. Interestingly, it has been shown that mutations of TIFA in the FHA domain cause this mutant protein to form pentamer or hexamer, whereas the wild type TIFA appears to be a trimer [198]. In this study, I found that a small fraction of TIFA formed high molecular weight oligomers with sedimentation coefficient of approximately

26S. Remarkably, only these large oligomers of TIFA were capable of inducing TRAF6 oligomerization and activating IKK. Although the FHA domain mutation causes TIFA to form intermediate-sized multimers that sediment in the middle of the glycerol gradient after ultracentrifugation, these multimers fail to activate IKK or induce the formation of high molecular weight species of TRAF6. Thus, it appears that the activity of TRAF6 as a RING domain ubiquitin ligase and an IKK activator depends on its highly oligomerized forms.

Over 70 members of RING domain proteins have been observed in discrete subcellular structures that can be visualized by immunostaining and confocal microscopy [102, 205]. Studies on the smallest known RING protein, the arenaviral protein Z, showed that this protein self-assembled into spherical structures that resemble functional bodies formed by the same protein in infected cell [206]. The zinc coordinating residues are required for the generation of these supramolecular assemblies. Similar phenomena have been described in the RING domain of other proteins such as promyelocytic leukemia protein (PML), KAP-1/TIF1β, Mel18, BRCA1 and BARD1 [207]. In the case of BRCA1, these proteins form discrete subnuclear foci termed “BRCA1 nuclear dots” in the S phase [208]. In addition, purified RING domain of BRCA1 self-assembles in vitro into supramolecular assemblies, which have higher ubiquitin-ligase activity than do the tetrameric or dimeric forms of BRCA1 [207]. A cancer predisposing mutation (C64G) in BRCA1 abolishes the self-assembly and ubiquitin-ligase activity of the protein in vivo and in vitro [208]. The ubiquitin-ligase activity of this BRCA1 mutant can be partially recovered by forced oligomerization [207]. These studies, together with our finding that the higher molecular weight forms of TRAF6 have much increased ubiquitin ligase activity (Figure 12), suggest

that RING domain proteins may facilitate polyubiquitination by coupling multiple ubiquitin thioester-bound E2 to each other when the RING domains are assembled into supramolecular structures. In support of this model, multiple gold-stained UbcH5C molecules and nanogold-stained ubiquitin chains have been found to co-localize with the BRCA1-BARD1 supramolecular structures by electron microscopy [207].

In addition to binding to TRAF6, TIFA has also been found to interact with TRAF2 [199], although the exact TRAF2 binding site has not been mapped. In keeping with the role of TRAF2 in TIFA signaling, I have found that RNAi of TRAF2 blocked NF-κB activation by TIFA. However, the *in vitro* studies did not reveal a requirement of TRAF2 in IKK activation by TIFA. Unlike TRAF6, TRAF2 does not bind to the Ubc13 column (data not shown). In addition, TRAF2 is not required to reconstitute IKK activation by TIFA in vitro, although it remains to be determined whether TRAF2 could enhance IKK activation in the reconstitution system. Although TRAF2 and TRAF6 appear to be involved separately in the TNFα and IL-1 pathways, respectively, both are required for CD40 signaling [209-212]. Thus, TRAF2 and TRAF6 cooperate in certain signaling pathways, some of which may involve TIFA. However, the physiological functions of TIFA remain to be determined by further genetic studies.

My studies on TIFA have uncovered a mechanism of TRAF6 ubiquitin ligase activation that involves inducible oligomerization. TRAF6 then functions together with Ubc13-Uev1A to catalyze the synthesis of K63-linked polyubiquitin chains that are conjugated to target proteins including NEMO and TRAF6 itself [152, 157, 162]. Polyubiquitination of TRAF6 then leads to the activation of IKK through the TAK1-TAB1-

TAB2 kinase complex [162]. It was recently found that TAB2 and its homologue TAB3 bind preferentially to K63-linked polyubiquitin chains through a conserved novel zinc finger (NZF) domain, which is essential for TAK1 and IKK activation [176]. Thus, polyubiquitination of TRAF6 likely facilitates its interaction with the TAB2-TAK1 complex, thereby leading to TAK1 activation. TAK1 then phosphorylates IKKβ at two conserved serine residues in the activation loop, resulting in IKK activation. Such an oligomerization→ ubiquitination→ phosphorylation cascade has also been shown to be operating in T lymphocytes, in which antigenic stimulation of T cell receptors leads to the recruitment and clustering of essential signaling proteins including CARMA1, BCL10 and MALT1 [157, 213]. The oligomerization of BCL10 and MALT1 induces TRAF6 oligomerization and ubiquitination, leading to the activation of TAK1 and IKK in T cells. Together, these studies suggest that different signaling pathways may utilize distinct adaptor proteins, such as MALT1 and TIFA, as the endogenous “oligomerizers” to activate ubiquitin ligases such as TRAF6, which then initiate protein kinase cascades that are important for stress responses, inflammation and immunity.

CHAPTER III: UBIQUITINATION-DEPENDENT ACTIVATION OF IKK BY RIP1

III.A Introduction

TNFα regulates many cellular functions including apoptosis, cell growth, inflammation, immune response, and cell differentiation [214]. TNFα-induced cellular responses are mediated by two receptors of the TNF receptor superfamily: TNF-R1 and TNF-R2 [215]. Stimulating cells with TNFα activates NF-κB and MAP kinases, including ERK, p38 and JNK. In the TNF-R1 signaling, engagement of TNFα with TNF-R1 leads to the recruitment of the TNF-R1-associated death domain (TRADD) protein [216]. TRADD subsequently serves as a platform for the recruitment of the FAS-associated death domain (FADD) protein, the TNF receptor-associated factor 2 (TRAF2) protein, and the death domain kinase RIP1 [217, 218]. While association of FADD with TRADD triggers the apoptosis program, binding of TRAF2 and RIP to TRADD activates NF-κB and JNK [217, 219, 220]. On the other hand, stimulation of TNF-R2 results in the recruitment of TRAF2 and TRAF1 as well as cellular inhibitor of apoptosis proteins: c-IAP1 and c-IAP2 [194, 221].

RIP1 was initially identified in a yeast two-hybrid screen for proteins interact with the death domain of Fas [222]. It is later shown to be involved in the TNF pathway, DNA damage-induced responses, Trif signaling, and anti-viral response [223]. RIP1 has an N-terminal kinase domain, an α-helical intermediate domain, and a C-terminal death domain (DD). Overexpression of RIP1 activates NF-κB, c-Jun N-terminal kinase (JNK), and apoptosis [218]. The kinase domain of RIP1 is dispensable for NF-κB activation while the intermediate domain is sufficient to activate NF-κB [218, 219]. The physiological significant

of RIP1 in the TNF pathway is elegantly demonstrated with the analysis of the RIP1-deficient Jurkat cell line and the generation of RIP1 knockout mice [219, 224]. The TNF-induced NF-κB activation is defective in the cell lacking RIP1 while the activation of JNK is normal.

TGFβ-activated kinase (TAK1) and MEKK3 have been implicated to be involved in the TNF-mediated IKK activation. In TAK1-deficeint MEF cells, TNF-induced activation of IKK, JNK and p38 are impaired [170]. In MEKK3-deficeint MEF cells, the TNF-induced activation of IKK is reduced [225]. Biochemical study suggests that TAK1 activates IKKβ by direct phosphorylation at the activation loop [162]. TAK1 forms a complex with TAB1, TAB2 and TAB3 and its kinase activity is regulated by the TAB proteins. TAB2 and TAB3 contain of two ubiquitin binding domain: an N-terminal CUE domain and a C-terminal novel zinc finger (NZF) domain. The NZF domain is required for TAK1 activation, and it binds preferentially to K63-linked polyubiquitin chains [176]. The IKK complex contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (IKKγ). NEMO has two coil-coiled domain, CC1 (residues 93-231) and CC2 (residues 234-286), a leucine zipper (residues 303-337), and zinc finger motifs (residues 390-412). NEMO does not containing any catalytic domain, and has been found to interact with a variety of signaling molecules, thus it has been proposed that NEMO regulates the IKK complex by bridging upstream activators to the IKK complex.

In response to TNFα stimulation, RIP1 is recruited to the TNF-R1 and has a ladder-like appearance, a characteristic of polyubiquitinated proteins [71, 176, 226-228]. In addition, TNFα stimulation promotes the interaction between TAB2 and polyubiquitinated RIP1.

However the physiological significance of RIP1 polyubiquitination is not clear. In this chapter, I show that RIP1 undergoes K63-linked polyubiquitination in response to TNFα stimulation. I map the polyubiquitination site of RIP1 to lysine 377 in the intermediate domain and show that polyubiquitination of RIP1 is needed for TNF-induced IKK activation. Furthermore, RIPK377R cells are sensitive to TNFα-induced cell death. In addition, TAK1 and IKK kinase complexes are not recruited to the TNF-R1 in response to TNFα stimulation in the absence of RIP polyubiquitination. Moreover, TAB2 and NEMO bind to polyubiquitin chains and function as receptors that bind polyubiquitinated RIP1. These results indicate a unique interaction between a polyubiquitinated protein and a polyubiquitin binding protein triggers the activation of TAK1 and IKK.

III.B Materials and Methods

III.B.1 Biological Reagents and Cell Culture

TRAF2 (H249), NEMO (FL419), RIP1 (H207), p-IκBα (B9), IκBα (C21), TAK1 (M579) and Ub (P4D1) antibodies were bought from Santa Cruz Biotech. RIP1, TRADD, IKKβ antibodies were purchased from BD Bioscience. GST (4C10) was purchased from Covance. TAB2 antibody was generated by Dr. Chen Wang and Dr. Li Deng from rabbits immunization using a His_6-TAB2 N-terminal fragment (residues 1-450) as an antigen. The serums were purified using a TAB2-conjugated sepharose column [162]. Anti-Flag (M2) antibody was acquired from Sigma. Anti-HA antibody was purchased from Cell Signaling Technology. Anti-Ubc13 (4E11) antibody was obtained from Zymed Laboratories Ins. HEK293 cells and Phoenix cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin G (100μg/ml), and streptomycin (100μg/ml). Human SVT35 Jurkat, RIP1-deficient SVT35 Jurkat cells (kindly provided by Dr. Adrian Ting, Mount Sanai Medical School) and NEMO-deficient SVT35 Jurkat cells (kindly provided by Dr. Shao-Cong Sun, Penn State University; [204]) were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2mM L-glutamine and antibiotics.

III.B.2 Plasmids and Retro-Viruses

RIP1 mutants were generated by using the QuikChange Kit (Stratagene) in the pCDNA3 backbone. The entire open reading frame of wild type and mutant RIP1 were subcloned into the retro-virus expression vector pT7Retro-FLAG-IRES-Puro. FLAG-tagged

TRAF2 was made by Dr. Li Deng as described previously [152]. Full-length TRADD cDNA was purchased from ATCC and was subcloned into pCDNA3 with a FLAG-tagged at the N-terminus. cDNA corresponded to hTNFα (77-233) was cloned by Polymerase Chain Reaction (PCR) using cDNAs derived from TNFα stimulated HeLa cells and then subcloned into pGEX4T1 (GE) vector for expression in *Escherichia coli*. All constructs were verified by automatic DNA sequencing.

Retro-viruses were generated by transfecting the RIP1 wild type or mutant retro-virus expression plasmids into phoenix cells using calcium phosphate precipitation transfection method. Medium was replaced with fresh medium 24hr after transfection and viruses containing culture medium was collected 48 hr later.

III.B.3 Generation of RIP1 Wild Type or Mutant Stable Cell Lines

$RIP^{-/-}$ SVT35 Jurkat cells were infected with retro-viruses harboring wild type or mutant RIP1 in the medium containing 2μg/ml polybrene. After 48 hr incubation, cells were spun down and viruses containing medium was removed. Cells were resuspended in fresh medium containing 0.5μg/ml puromycin and the medium was changed every two days. Cells were selected under this condition for two weeks. Single cell clones were picked and the expression levels were verified by immunoblotting using an antibody against RIP1.

III.B.4 TNF-R1 Recruiting Assay

Ten million Cells treated with 1μg/ml GST-TNFα were harvested at different time point (for t = 0 GST-TNFα was added after cells were lysed). Cells were lysed in kinase

assay lysis buffer (20mM Tris HCl pH7.5, 150mM NaCl, 25mM β-glycerophosphate, 1mM sodium orthovanadate, 10% glycerol and 0.5mM DTT supplement with 10ug/ml leupeptin and 1mM PMSF) containing 1% Triton X100. The cell debris was removed by centrifugation at 14000rpm for 5 minutes at 4℃. Five hundred microgram of lysates were incubated with 10μl Glutathione beads (GE). After mixing end-to-end at 4℃ for 1hr, the beads were washed with PBS plus 1% Triton X100 once and twice with PBS plus 0.5% Triton X100. The bound proteins were analyzed by immunoblotting with various antibodies as indicated.

III.B.5 λ Phosphatase Treatment

TNF-R1 bound TAB2 was purified as described in III.B.4 and washed beads were incubated with 40U λ phosphatase (NEB) in a 50μl reaction at 30℃ for 1hr. The beads were then washed with PBS to remove λ phosphatase and analyzed by immunoblotting with an antibody against TAB2.

III.B.6 TNFα-induced polyubiquitination of RIP1

TNF-R1 bound RIP1 was purified as in III.B.4. Complexes on the beads were disrupted by the addition of 1% SDS in 50mM Tris-HCl [pH7.5] and 1mM DTT. After removal of beads by centrifugation, the supernatant was diluted 10-fold in PBS before the addition of a RIP1-specific antibody. After mixing end-to-end at 4°C for 1hr, 10μl of protein A/G beads were added. The mixtures were further mixing end-to-end at 4°C for 1hr followed

by washing the beads three times with PBS plus 0.2% NP40. The immunoprecipitated proteins were detected by immunoblotting with a ubiquitin-specific antibody.

III.B.7 IKK kinase assay

Cells were treated with 10ng/ml TNFα for the different time as indicated and were lysed in kinase assay lysis buffer containing 0.2% NP40. Two hundred microgram of lysate was incubated with 1μl anti-NEMO (FL419) antibody at 4°C for 30min. Ten microliter of protein A/G beads (Pierce) were added. After mixing end-to-end at 4°C for 30min, the beads were washed twice with kinase assay lysis buffer containing 0.2% NP40 and once with kinase assay buffer (20mM HEPES pH7.6, 50mM NaCl, 20mMb-glycerophosphate, 1mM sodium orthovanadate, 10 mM MgCl2, 1mM DTT). The beads was then incubated with 1ug GST-IκBαN, 100uM ATP, 5uCi γ-^{32}P ATP in 10μl kinase assay buffer at 30°C for 40min. Reaction products were analyzed by SDS-PAGE and PhosphoImaging.

III.B.8 Immunoprecipitation

For immunoprecipitation of RIP1, HEK293 cells were transfected with HA-tagged wild type or mutant ubiquitins by calcium phosphate transfection method. Twenty four hours later, cells were treated with TNFα (10ng/ml) and lysed in kinase assay lysis buffer containing 0.5% Triton X100. One milligram of lysates were incubated with 2μg RIP1 antibody at 4 °C for 1hr. Five microliter of protein A/G beads (Pierce) were added. After mixing end-to-end overnight at 4°C, the beads were washed three times with kinase assay

lysis buffer containing 0.5% Triton X100. Bound proteins were examined by immunoblotting with RIP1 and HA antibodies.

For immunoprecipitation of TAB2, a TAB2-specific antibody was used to immunoprecipitate endogenous TAB2 from TNFα (10ng/ml) treated or untreated RIPWT or RIPK377R cells. After mixing end-to-end at 4°C for 1hr, 10μl of protein A/G beads were added. The mixtures were further mixing end-to-end at 4°C for 1hr followed by washing the beads three times with PBS plus 0.2% NP40. Associated proteins were analyzed by immunoblotting with different antibodies as indicated. Immunoprecipitation of NEMO was carried out similar to immunoprecipitation of TAB2 except that an antibody against NEMO (FL419) was used.

In case of TRAF2 and TRADD immunoprecipitation, 1μg of FLAG-tagged TRAF2 or FLAG-TRADD was transfected into HEK293 cells in the presence of 1μg FLAG-tagged RIPWT or FLAG-tagged RIPK377R using calcium phosphate transfection method. After 24hr, cells were lysed in kinase assay lysis buffer with 0.2% NP40. The lysates were incubated with 1μl of TRAF2, TRADD or control IgG respectively at 4℃ for 30min. Ten microliter of protein A/G beads (Pierce) were added. After mixing end-to-end at 4℃ for 30min, the beads were washed three times with kinase assay lysis buffer containing 0.2% NP40 and examined by immunoblotting using a FLAG-specific antibody.

III.B.9 TNFα Cytotoxicity Assay

Cells were treated with or without 10ng/ml of TNFα in the presence or absence of 100ng/ml cycloheximide in a 96 well format. After incubation for 24 hr, 50μl of 2mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in RPMI medium was added and cells were further incubated for 3hr at 37℃ in a CO_2 incubator. Cells were spun down at 1500rpm for 5 minutes and the medium was carefully removed. Two hundred microliter of DMSO was added to each well. After pipetting up and down several times, the absorbance was measured with a Thermo Labsystem Microplate Reader at the wavelength of 540nm.

III.B.10 RT-PCR

Cells were treated with TNFα (10ng/ml) for 24hr. Total RNAs were isolated using Total RNA mini kit (BIO-RAD). cDNAs were synthesized using iScript cDNA synthesis kit (BIO-RAD). The primers for hGAPDH, hMCP-1 and hICAM-1 were as described in II.B.3. RT-PCR products were separated in 2% agarose gel.

III.B.11 RNA Interference

Double stranded RNA oligonucleotides were synthesized at the Center for Biomedical Invention at UT Southwestern. Each RNA oligo has 19 nucleotides followed by dTdT. The sequences of the sense strand of RNA oligos are as follows: TAB2 (2037-2055): AGCCUUAAUUCGCUGUG-AA; TAB3 (817-835): CAGAACUAUCAGCCUUCUC; GFP

(471-489): GCAGAAGAACGGCAUCAAG. The sense and antisense strands of each pair of RNA oligos were annealed and then transfected into 293 cells by calcium phosphate precipitation at a final concentration of 15 nM. Calcium phosphate precipitation was repeated the second day after the first transfection, and cells were harvested on the third day for further analyses.

III.C Results

III.C.1 RIP1 is Ubiquitinated at Lys 377 in Response to TNFα Stimulation

Stimulating cells with TNFα leads to the recruitment of RIP1 to the TNF-R1. The receptor bound RIP1 undergoes covalent modification and exhibits a ladder-like appearance. To verify that the covalent modification of RIP1 is indeed polyubiquitination, Jurkat cells were treated with GST-TNFα and TNF-bound TNF-R1 was isolated by affinity purification using glutathione-conjugated beads. Treating Jurkat cells with GST-TNFα activated IKK (Fig. 14a, lower panel) and RIP1 was recruited to the TNF-R1 (upper panel) indicating that the GST-tagged on TNFα did not interfere with its biological function. As shown in figure 14a, some high molecular weight species of RIP1 were observed. When I dissociated TNF-R1 bound proteins with 1% SDS and carried out RIP1 immonuprecipitation using a RIP1-specific antibody after the dilution of SDS, the high molecular weight species of RIP1 cross reacted with a ubiquitin-specific antibody. These results confirm that RIP1 undergoes polyubiquitination in response to TNFα stimulation. Next I determined the linkage specificity of RIP1 polyubiquitination. HEK293 cells were transfected with HA-tagged wild type or mutant ubiquitins. Twenty four hours later, cells were treated with TNFα. The endogenous RIP1 was enriched by RIP1 immunoprecipitation, and the polyubiquitination of RIP1 was detected by immunoblotting with a HA antibody. TNFα stimulation induced polyubiquitination of RIP1 in wild type ubiquitin overexpressed cells (Fig. 14b, left panel, lane 1 and 2). The polyubiquitination of RIP1 was also detected in cells overexpressed K63Ub (ubiquitin mutant containing only the lysine 63, lane 4) and R48Ub (ubiquitin mutant whose lysine 48 is mutated into arginine, lane5) ubiquitin mutants, but not in cells

overexpressed R63Ub (ubiquitin mutant whose lysine 63 is mutated into arginine, lane 3) and K48Ub (ubiquitin mutant containing only lysine 48, lane 6) ubiquitin mutants. As indicated in figure 14b (right panel), wild type and mutant ubiquitins have similar expression level. These results reveal that RIP1 undergoes K63-linked polyubiquitination in response to TNFα stimulation.

Next I sought to map the polyubiquitination site of RIP1. I focused on the intermediate domain of RIP1 given the fact that this domain itself is sufficient to activate NF-κB [218, 219]. As indicated in figure 14c, there are five conserved lysine residues in the intermediate domain of RIP1 among human, mouse and rat. I mutated each of these lysine residues into arginine and then introduced them into $RIP^{-/-}$ Jurkat cells using the retro-virus system. Stable RIP1 mutant cell lines were established and the RIP1 expression was verified by immunoblotting using a RIP1-specific antibody (Fig. 14d). I selected stable cell lines expressing wild type or mutant RIP1 with RIP1 expression comparable to the endogenousRIP1 and examined their ability to undergo TNF-induced polyubiquitination (see above). Among five conserved lysine residues, mutation of lysine 377 abolished TNF-mediated polyubiquitination suggested that lysine 377 is the target site for RIP1 polyubiquitination (Fig. 14e).

III.C.2 Polyubiquitination of RIP1 is Essential for TNF-Mediated IKK and NF-κB Activation

Once the polyubiquitination site of RIP1 had been identified, it provided a powerful tool to address the physiological significant of the RIP1 polyubiquitination *in vivo*.

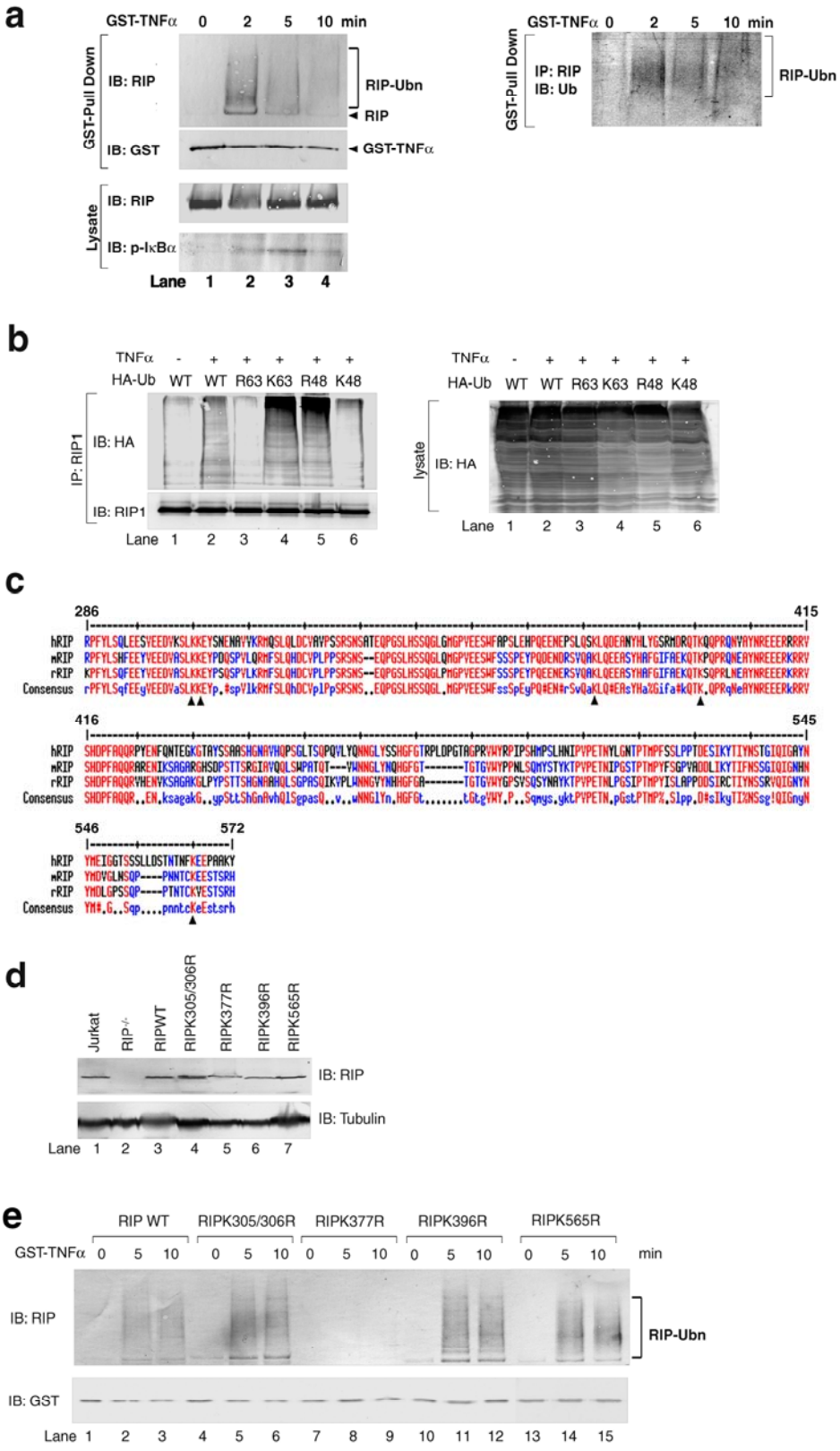

a
GST-TNFα 0 2 5 10 min
GST-Pull Down
IB: RIP
RIP-Ubn
RIP
IB: GST
GST-TNFα
Lysate
IB: RIP
IB: p-IκBα
Lane 1 2 3 4
GST-TNFα 0 2 5 10 min
GST-Pull Down
IP: RIP
IB: Ub
RIP-Ubn
b
TNFα - + + + + +
HA-Ub WT WT R63 K63 R48 K48
IP: RIP1
IB: HA
IB: RIP1
Lane 1 2 3 4 5 6
TNFα - + + + + +
HA-Ub WT WT R63 K63 R48 K48
lysate
IB: HA
Lane 1 2 3 4 5 6
c
286 415
hRIP RPFYLSQLEESYEEDVKSLKKEYSNENAYVKRMQSLQLDCVAVPSSRSNSATEQPGSLHSSQGLGMGPVEESWFAPSLEHPQEENEPSLQSKLQDEANYHLYGSRMDRQTKQQPRQNVRAYNREEERKRRV
mRIP RPFYLSHFEEYVEEDVASLKKEYPDQSPVLQRMFSLQHDCVPLPPSRSNS--EQPGSLHSSQGLQMGPVEESWFSSSPEYPQDENDRSYQAKLQEERSYHAFGIFREKQTKPQPRQNEAYNREEERKRRV
rRIP KPFYLSQFEEYVEEDVASLKKEYPSQSPVLKRMFSLQHDCVPLPPSRSNS--EQPGSLHSSQGLPMGPVEESWFSSSPEYPQEENERSYQAKLQEERSYHAFGIFREKQTKSQPRLNEAYNREEERKRRV
Consensus rPFYLSqfEEyVEEDVaSLKKEYp.#spVlkRMfSLQhDCVplPpSRSNS..EQPGSLHSSQGL.MGPVEESWFssSpEyPQ#EN#rSvQaKLQ#EAsYHa%Gifa#kQTK.QPRqNeAYNREEERkRRV
416 545
hRIP SHDPFAQQRPYENFQNTEGKGTAYSSRASHGNAYHQPSGLTSQPQVLYQNNGLYSSHGFGTRPLDPGTAGPRVWYRPIPSWMPSLHNIPVPETNYLGNTPTMPFSSLPPTDESIKYTIYNSTGIQIGAYN
mRIP SHDPFAQQRARENIKSAGARGHSDPSTTSRGIAYQQLSWPATQT---VWNNGLYNQHGFGT-------TGTGVWYPPNLSQMYSTYKTPVPETNIPGSTPTMPYFSGPYAQDLIKYTIFNSSGIQIGNHN
rRIP SHDPFAQQRVHENVKSAGAKGLPYPSTTSHGNARHQLSGPASQIKYPLWNNGYYNHHGFGA-------TGTGVWYGPSYSQSYNRYKTPVPETNLPGSIPTMPYISLAPPDDSIRCTIYNSSRVQIGNYN
Consensus SHDPFAQQR..EN.ksagakG..ypStt.ShGnAvhQlSgpasQ..v..wNNGlYn.HGFGt.....tGtgVWY.P..Sqmys.ykt.PVPETN.pGst.PTMP%.Slpp.D#sIkyTI%NSsg!QIGnyN
546 572
hRIP YMEIGGTSSSLLDSTNTNFKEEPRAKY
mRIP YMDVGLNSQP----PNNTCKEESTSRH
rRIP YMDLGPSSQP----PTNTCKVESTSRH
Consensus YM#.G..Sqp....pnntcKeEstsrh
d
Jurkat
RIP-/-
RIPWT
RIPK305/306R
RIPK377R
RIPK396R
RIPK565R
IB: RIP
IB: Tubulin
Lane 1 2 3 4 5 6 7
e
RIP WT
RIPK305/306R
RIPK377R
RIPK396R
RIPK565R
GST-TNFα 0 5 10 0 5 10 0 5 10 0 5 10 0 5 10 min
IB: RIP
RIP-Ubn
IB: GST
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 14. RIP1 is ubiquitinated at Lys 377 in response to TNFα stimulation.

(a) TNFα induces ubiquitination of RIP1. Jurkat cells were treated with GST-TNFα (1μg/ml) as indicated. Cell lysates were analyzed by immunoblotting with RIP1 and p-IκBα antibodies (lower left panel). TNF-R1 bound proteins were purified by glutathione affinity purification and examined by immunoblotting with RIP1 and GST antibodies (upper left panel). TNF-R1 bound proteins were dissociated in the presence of 1% SDS. After 10 fold dilution, RIP1 immunoprecipitation was carried out. Immunoprecipitated proteins were analyzed by immunoblotting with a ubiquitin antibody (right panel). RIP-Ubn, polyubiquitinated RIP1; p-IκBα, phosphorylated IκBα. **(b)** RIP1 undergoes TNF-induced K63-linked polyubiqutination. HEK293 cells were transfected with HA-tagged wild type or mutant ubiquitin as indicated. Twenty four hours later, cells were treated with TNFα (10ng/ml) for 2 minutes. Lysates were subjected to RIP1 immunoprecipitation and immunoprecipitated proteins were analyzed by immunoblotting with HA and RIP1 antibodies (left panel). The expression of HA-tagged ubiquitin was verified by immunoblotting with a HA antibody (right panel). **(c)** Diagram of the alignment of the intermediate domain of RIP1 among human, mouse and rat. hRIP, human RIP1; mRIP, mouse RIP1; rRIP, rat RIP1. **(d)** Expression level of wild type or mutant RIP1 in $RIP^{-/-}$ Jurkat stable cell lines. Wild type or mutant RIP1 stable cell lines were generated using retro-virus system. Expression level of RIP1 in each stable cell lines was verified by immunoblotting with RIP1 (upper panel) and tubulin (lower panel) antibodies. **(e)** TNFα induces polyubiquitination of RIP1 at Lys 377. Wild type (lane 1-3) or mutant RIP1 (lane 4-15) stable cell lines were stimulated with GST-TNFα for the indicated time. TNF-R1 bound proteins were purified as in **a** and were examined by immunoblotting with RIP1 (upper panel) and GST (lower panel) antibodies.

Therefore, I examined the TNF-induced activation of IKK among the RIP1 mutant stable cell lines. I immunoprecipitated endogenous IKK complex from various stable cell lines treated with or without TNFα using a NEMO-specific antibody, the IKK kinase activity was measured using GST-IκBα as a substrate (see materials and methods). As demonstrated in figure 15a, IKK activity was rapidly induced by TNFα and RIP1 was required for TNF-induced IKK activation as $RIP1^{-/-}$ Jurkat cells failed to activate IKK. Introducing a wild type RIP1 into $RIP1^{-/-}$ Jurkat could rescue the defect. Interestingly, introducing polyubiquitination defective RIPK377R did not restore the IKK activation while introducing other conserved lysine mutants (RIPK305/306R, RIPK396R and RIPK565R) could rescue the defect. The differences of IKK activity among the different cell lines were not due to the different efficiency of IKK immunoprecipitation as immunoblotting of IKKβ indicated that similar amount of endogenous IKK had been pulled-down. To further confirm the defect of RIPK377R in the TNF signaling, I performed RT-PCR to analyze the induction of two endogenous NF-κB regulated genes, human intercellular adhesion molecule 1 (hICAM-1) and human monocyte chemoattractant protein 1 (hMCP-1). Stimulation of Jurkat cells with TNFα for 24 hours led to the induction of hMCP1 and hICAM-1 (Fig. 15b, lane 1 and 2) and $RIP1^{-/-}$ Jurkat cells failed to induce the expression of these two genes (Fig. 15b, lane 3 and 4). Introducing wild type but not the RIPK377R mutant could restore the TNFα-dependent expression of hMCP-1 and hICAM-1 (Fig 15b, lane 5 to 6). In contract to hMCP-1 and hICAM-1 expression, the expression of the house keeping gene GAPDH was normal in the $RIP1^{-/-}$ Jurkat and RIPK377R cells. A very good correlation between RIP1 polyubiquitination and NF-κB activation in response to TNFα stimulation were observed indicated that

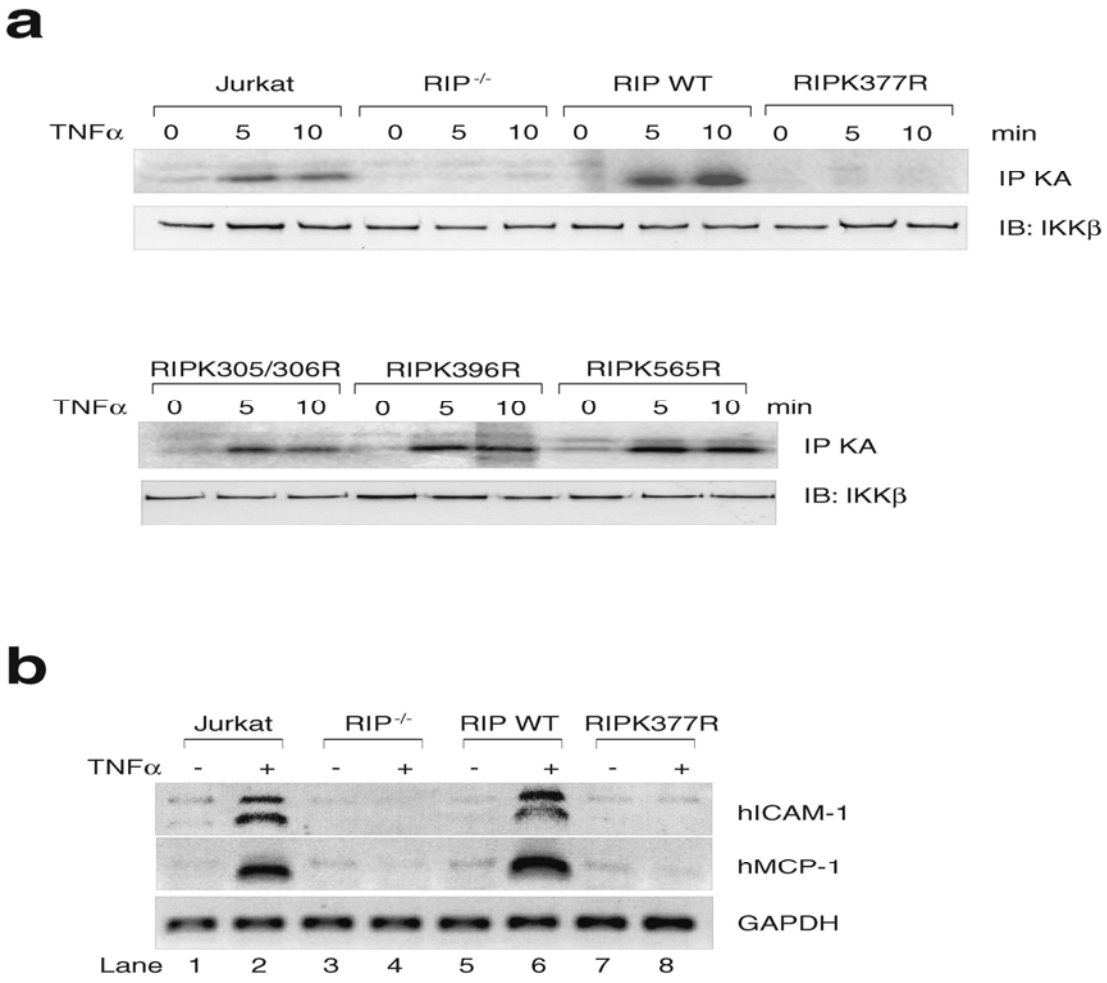


Figure 15. Polyubiquitination of RIP1 is required for IKK and NF-κB activation in response to TNFα stimulation.

(a) Polyubiquitination of RIP1 is essential for TNF-induced IKK activation. Cells were treated with TNFα (10ng/ml) as indicated. Two hundred micrograms of the cell lysates were immunoprecipitated with a NEMO antibody to isolate the endogenous IKK complex, whose activity was measured by using GST-IκBα and γ-^{32}P-ATP as substrates. An aliquot of the IKK immunoprecipitate was immunoblotted with an IKKβ antibody. **(b)** Polyubiquitination of RIP1 is required for TNF-induced NF-κB activation. Cells were treated with TNFα (10ng/ml) for 24 hours. The expression of hICAM-1, hMCP-1 and hGAPDH RNA was analyzed by RT-PCR.

polyubiquitination of RIP1 is important for the activation of IKK and NF-κB in response to TNFα stimulation.

III.C.3 RIPK377R Cells are Sensitive to TNFα-Induced Cell Death

It is well known that the activation of NF-κB prevents TNFα-induced cell death and RIP1-deficient cells are susceptible to TNFα treatment [224, 229-232]. To test the susceptibility of RIPK377R cells to TNFα treatment, I performed the TNFα cytotoxicity assay. In the absence of cycloheximide, Jurkat and RIPWT cells were resistant to TNFα treatment (Fig. 16a). In contrast, treating RIP1$^{-/-}$ and RIPK377R cells with TNFα for 24hr resulted in a 40% decrease in cell viability. The cytotoxicity of TNFα could be further enhanced in the presence of CHX. In addition, both RIP1$^{-/-}$ and RIPK377R cells were slightly more sensitive to CHX treatment alone compared to Jurkat and RIPWT cells. TNFα can induce cell death through apoptosis and necrosis. To further show that apoptosis is involved in TNF-induced cell death, I monitored the cleavage of the nuclear caspase substrate poly-ADP-ribose polymerase (PARP), a biochemical marker for apoptosis. In consistent with the TNFα cytotoxicity assay, Jurkat cells and RIPWT cells did not undergo apoptosis in response to TNFα treatment as only the full length PARP was detected (Fig 16b, lane 1, 2, 5, and 6). On the other hand, prolong TNFα stimulation induced apoptosis in RIP1$^{-/-}$ cells and RIPK377R cells as indicated by the generation of a shorter form of PARP (p85) (Fig. 16b, lane 3, 4, 7, and 8). Thus, the absence of RIP1 polyubiquitination sensitizes cells to TNFα-induced apoptosis resulted from the failure of these cells to activate NF-κB.

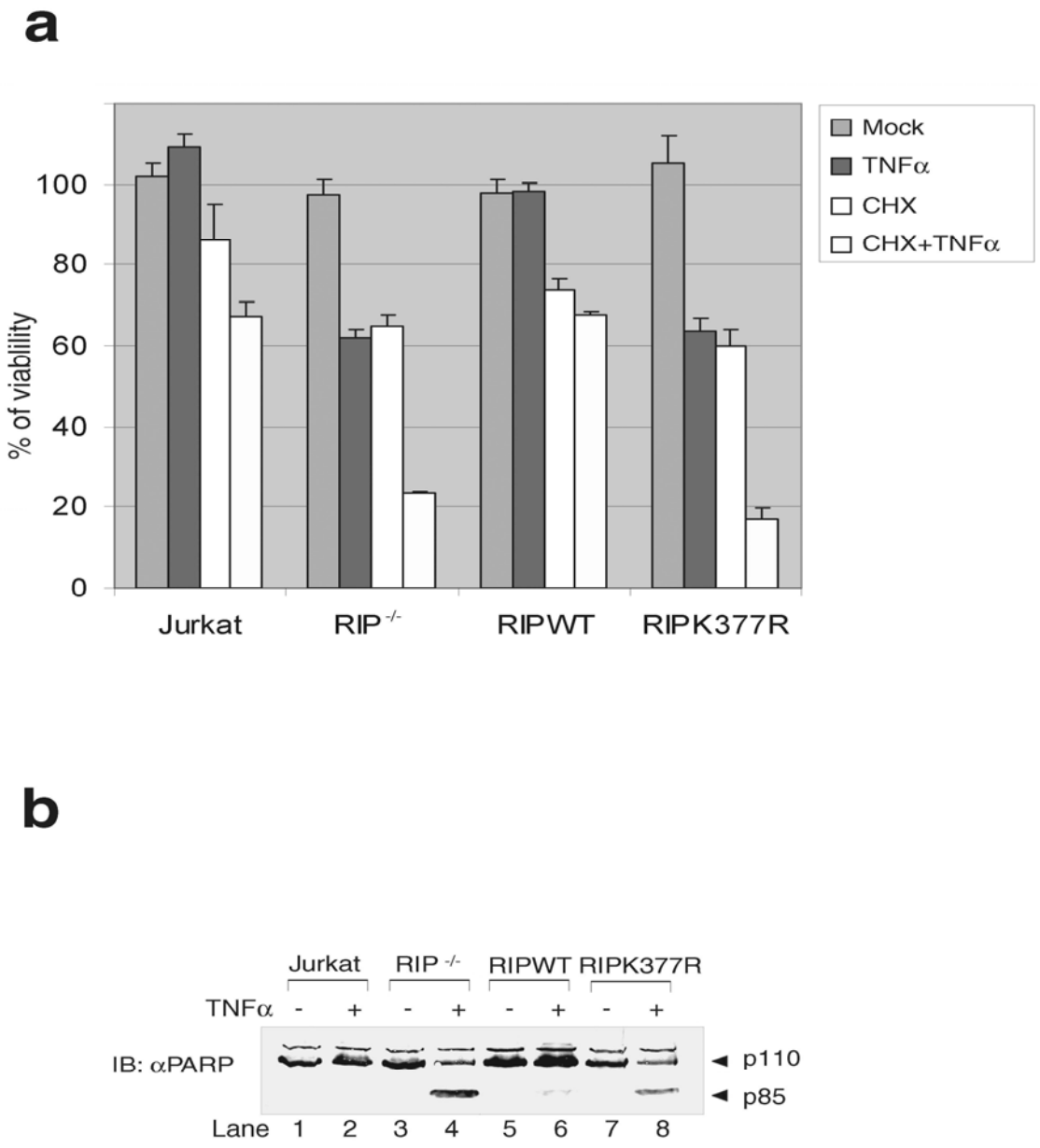


Figure 16. Polyubiquitination of RIP1 is required for protecting cells against TNF-induced cell death.

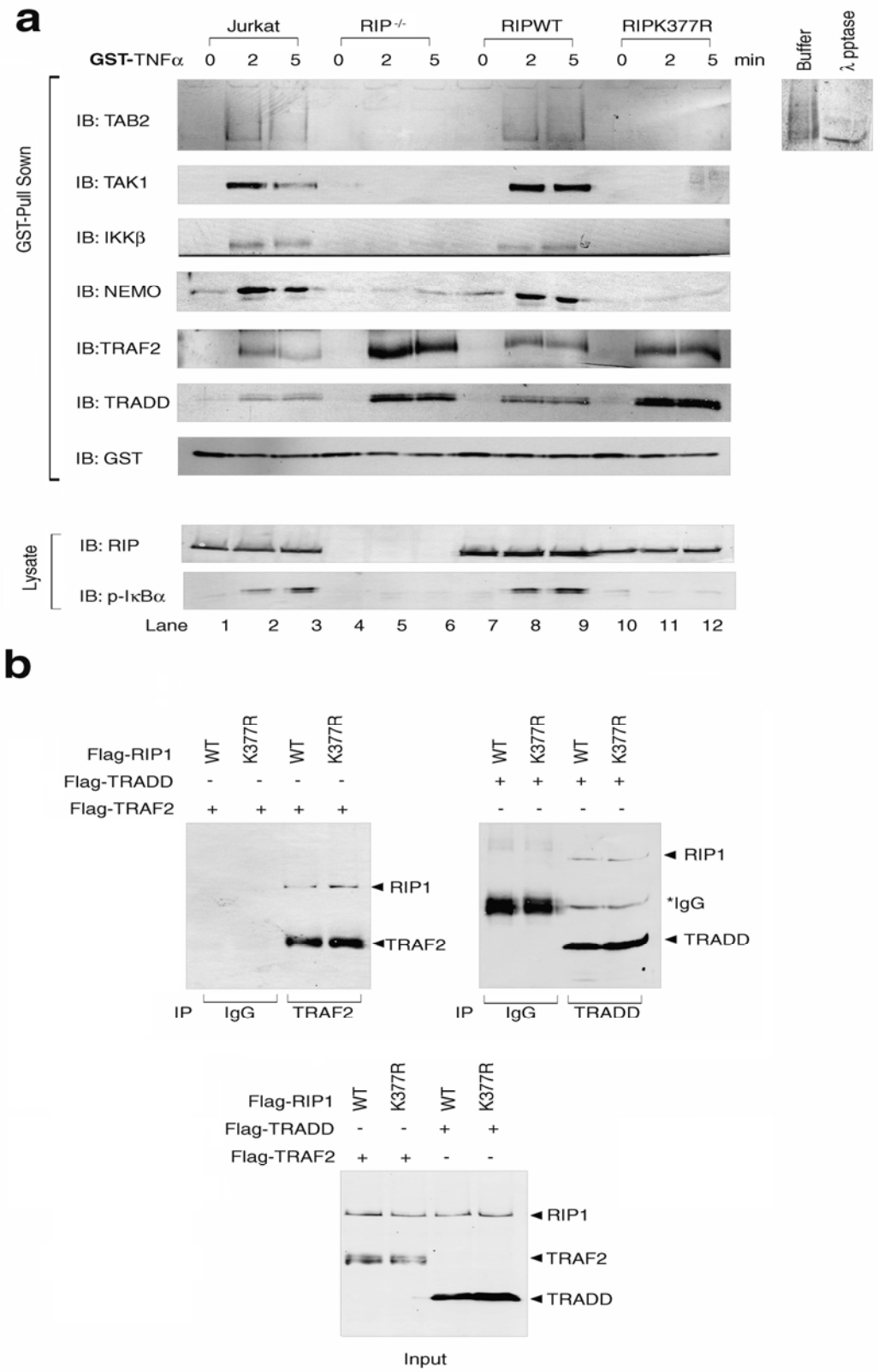
(a) RIPK377R cells were sensitive to TNF-induced cell death. Cells were treated with TNFα (10ng/ml) in the presence or absence of CHX for 24 hours. The viability of cell was measured by incubating cells with MTT at 37℃ in a CO_2 incubator. After 3hr cells were lysed with DMSO and the absorbance was measured at the wavelength of 540nm. CHX, cycloheximide. **(b)** RIPK377R cells were sensitive to TNF-induced apoptosis. Cells were stimulated with TNFα (10ng/ml) for 24hr. Cell lysates were examined by immunoblotting with a PARP antibody.

III.C.4 Polyubiquitination of RIP1 is Required for Recruitment of TAK1 and IKK Kinase Complexes to TNF-R1

In response to TNFα binding to TNF-R1, a signaling complex containing TRADD, RIP, TRAF2, IKK and TAK1 kinase complexes is rapidly formed [220, 233]. Moreover, TAB2 binds to polyubiquitinated RIP1 in a signal dependent manner and my results indicated that polyubiquitination of RIP1 is essential for IKK activation. Thus, I purposed that the polyubiquitin chain on RIP1 might serve as a docking site to recruit TAK1 kinase complex. To test if the polyubiquitination of RIP1 is required for recruiting downstream signaling molecules, I did the TNF-R1 recruitment assay (see materials and methods). Cells were treated with GST-TNFα, TNF-R1 bound proteins were purified using glutathione beads. As demonstrated in figure 17a, TNFα treatment led to the recruitment of TAK1, TAB2, IKKβ, NEMO, TRAF2 as well as TRADD to the TNF-R1 in Jurkat cells. TAB2 became phosphorylated in response to TNFα stimulation as slow migrate smearing of TAB2 was observed and these TAB2 species were sensitive to λ phosphatase treatment. Conversely, TAK1 kinase complex was not recruited to TNF-R1 in RIP1-deficient cells while recruitment of TRADD and TRAF2 were normal (Fig. 17, lane 4-6). This result is consistent with current model that TRADD mediates TRAF2 and RIP1 recruitment and it confirmed that recruitment of TAK1 kinase complex required RIP1. Surprisingly, the recruitment of IKKβ and NEMO were also impaired in $RIP^{-/-}$ Jurkat cells suggesting that RIP1 is required for recruitment of IKK kinase complex to TNF-R1 in response to TNFα stimulation. Furthermore, recruitment of TAK1 and IKK kinase complexes were abolished in RIPK377R cells while the RIPWT could rescue the defect. The defect in recruitment of

TAK1 and IKK kinase complexes to TNF-R1 co-related with the absence of IKK activation as indicated by immunoblotting with a phosphorylated-IκBα antibody (Fig. 17a, lower panel). These results are consistent with our model that the polyubiquitined RIP1 serves as a docking site to recruit TAK1 complex, and it is required for the recruitment of IKK kinase complex as well.

To rule out the possibility that the failures of RIPK377R undergoing TNF-dependent polyubiquitination and subsequent recruitment of TAK1 and IKK kinase complexes were resulted from the misfolding of RIPK377R, I tested the binding ability of RIPK377R to TRAF2 and TRADD. RIP1 has been shown to interact with the TRAF domain of TRAF2 and the death domain of TRADD [218]. Human embryonic kidney 293 cells were transfected with FLAG-tagged wild type RIP1 or RIPK377R in the presence of either FLAG-tagged TRAF2 or FLAG-tagged TRADD. TRAF2 or TRADD was immunoprecipitated using a TRAF2-specific antibody or a TRADD-specific antibody respectively, and mouse or rabbit IgGs were used as a negative control. Immunoprecipitated proteins and the expression of transfected proteins were detected by immunoblotting using a FLAG-specific antibody. As shown in figure 17b, wild type RIP1 and RIPK377 had similar expression level, and the RIPK377R behaved similarly to the wild type RIP1. It interacted specifically with TRAF2 and TRADD as none of the control IgGs could pull down any RIP1. In summary, these results suggest that the Lysine 377 to Arginine point mutation does not result in the global misfolding of the RIP1.


a
Jurkat
RIP-/-
RIPWT
RIPK377R
GST-TNFα 0 2 5 0 2 5 0 2 5 0 2 5 min
Buffer
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GST-Pull Sown
IB: TAB2
IB: TAK1
IB: IKKβ
IB: NEMO
IB:TRAF2
IB: TRADD
IB: GST
Lysate
IB: RIP
IB: p-IκBα
Lane 1 2 3 4 5 6 7 8 9 10 11 12
b
Flag-RIP1 WT K377R WT K377R
Flag-TRADD - - - -
Flag-TRAF2 + + + +
RIP1
TRAF2
IP IgG TRAF2
Flag-RIP1 WT K377R WT K377R
Flag-TRADD + + + +
Flag-TRAF2 - - - -
RIP1
*IgG
TRADD
IP IgG TRADD
Flag-RIP1 WT K377R WT K377R
Flag-TRADD - - + +
Flag-TRAF2 + + - -
RIP1
TRAF2
TRADD
Input

Figure 17. Polyubiquitination of RIP1 mediates recruitment of TAK1 and IKK kinase complexes to TNF-R1.

(a) Cells were treated with TNFα for the indicated time. Cell lysates were analyzed by immunoblotting with RIP1 and p-IκBα antibodies (lower panel). TNF-R1 bound proteins were purified by glutathione affinity purification and examined by immunoblotting with TAB2, TAK1, IKKβ, NEMO, TRAF2, TRADD and GST antibodies (upper panel). For the λ phosphatase treatment, glutathione beads incubated with cell lysates prepared from Jurkat cells treated with TNFα for 2 min were further incubated in the presence or absence of 40U λ phosphatase at 30℃ for 1hr. Reaction products were analyzed with a TAB2 antibody (right panel). **(b)** RIPK377R interacts with TRAF2 and TRADD. Wild type FLAG-RIP1 or FLAG-RIPK377R were transfected into HEK 293 along with either FLAG-TRAF2 or FLAG-TRADD. Twenty-four hours later, cells were harvested and subjected to TRAF2 (upper left panel) or TRADD (upper right panel) immunoprecipitation respectively. Mouse or rabbit IgG was used as a negative control. Immunoprecipitated proteins were examined by immunoblotting with a FLAG antibody. Cell lysates were immunoblotted with a FLAG antibody to detect the expression of the transfected cDNA.

III.C.5 TAB2 and NEMO Bind to Polyubiquitinated RIP1 in Response to TNFα Treatment

The failure of the recruitment of TAK1 kinase complex to the TNF-R1 in RIPK377R cells can be explained by the impairment of the interaction between TAB2 and polyubiquitinated RIP1. In case of the IKK kinase complex recruitment, there are two possible explanations that can account for the failure of the recruitment of IKK kinase complex to TNF-R1 in RIPK377R cells. First, the recruitment of IKK kinase complex is mediated by TAK1 kinase complex, therefore IKK kinase complex is not recruited to TNF-R1 in the absence of TAK1 kinase complex at the receptor complex in RIPK377R cells. Alternatively, the recruitment of IKK kinase complex to TNF-R1 is mediated by direct interaction of the IKK kinase complex with the polyubiquitinated RIP1. To test the first possibility, I inhibited the TAK1 kinase recruitment by knocking down the expression of TAB2 and TAB3 with RNA interference, and examined the recruitment of IKK kinase complex to TNF-R1. Small interfering RNA (siRNA) oligos targeting TAB2 and TAB3 decreased the expression of the corresponding proteins (Fig 18a, lower panel). Silencing the expression of TAB2 and TAB3 significantly reduced TNF-induced IKK activation as measured by immunoprecipitation IKK kinase assay (Fig. 18 a, upper panel). However, the recruitment of IKK kinase complex to TNF-R1 was unaffected in cells transfected with TAB2 and TAB3 siRNA (Fig. 18a, middle panel). These results suggest that the recruitment of IKK kinase complex is independent of the TAK1 kinase complex. To test the second possibility, lysates from TNFα treated or untreated RIPWT cells or RIPK377R cells were subjected to NEMO and TAB2 immunoprecipitation. In the control TAB2

immunoprecipitation, similar amount of TAK1 could be immunoprecipitated by the TAB2-specific antibody from RIPWT and RIPK377R cells (Fig. 18b). On the contrary, signal-dependent interaction between RIP1 and TAB2 could only be detected in RIPWT cells and not in RIPK377R cells. Furthermore, only the polyubiquitinated form of RIP1 associated with TAB2. These results imply that the interaction occurrs between TAB2 and the polyubiquitin chain of the polyubiquitinated RIP1. As shown in figure 18c, similar results were observed with NEMO immunoprecipitation. NEMO immunoprecipitation pulled down similar amount of IKKβ and only polyubiquitinated RIP1 interacted with NEMO in a TNF-dependent manner. These results imply that NEMO may be a ubiquitin receptor. In fact, Dr. Li Deng and Zongping Xia have shown that NEMO can bind to polyubiquitin chain *in vitro* and the binding to polyubiquitin chain is essential for the activation of IKK by TRAF6 *in vitro* (Dr. Li Deng and Zongping Xia personal communication). To test if the recruitment of IKKβ to TNF-R1 is mediated by NEMO, lysates prepared from Jurkat or NEMO-deficient Jurkat cells treated with GST-TNFα were subjected to the TNF-R1 recruitment assay. As revealed in figure 18d, the recruitment of IKKβ to TNF-R1 was impaired in NEMO-deficient Jurkat cells while the recruitment of RIP1 and TAB2 were unaffected. In summary, these results indicate that polyubiquitinated RIP1 recruits TAK1 and IKK kinase complexes through the interaction between polyubiquitin chain and TAB2 or NEMO respectively (Fig. 19).

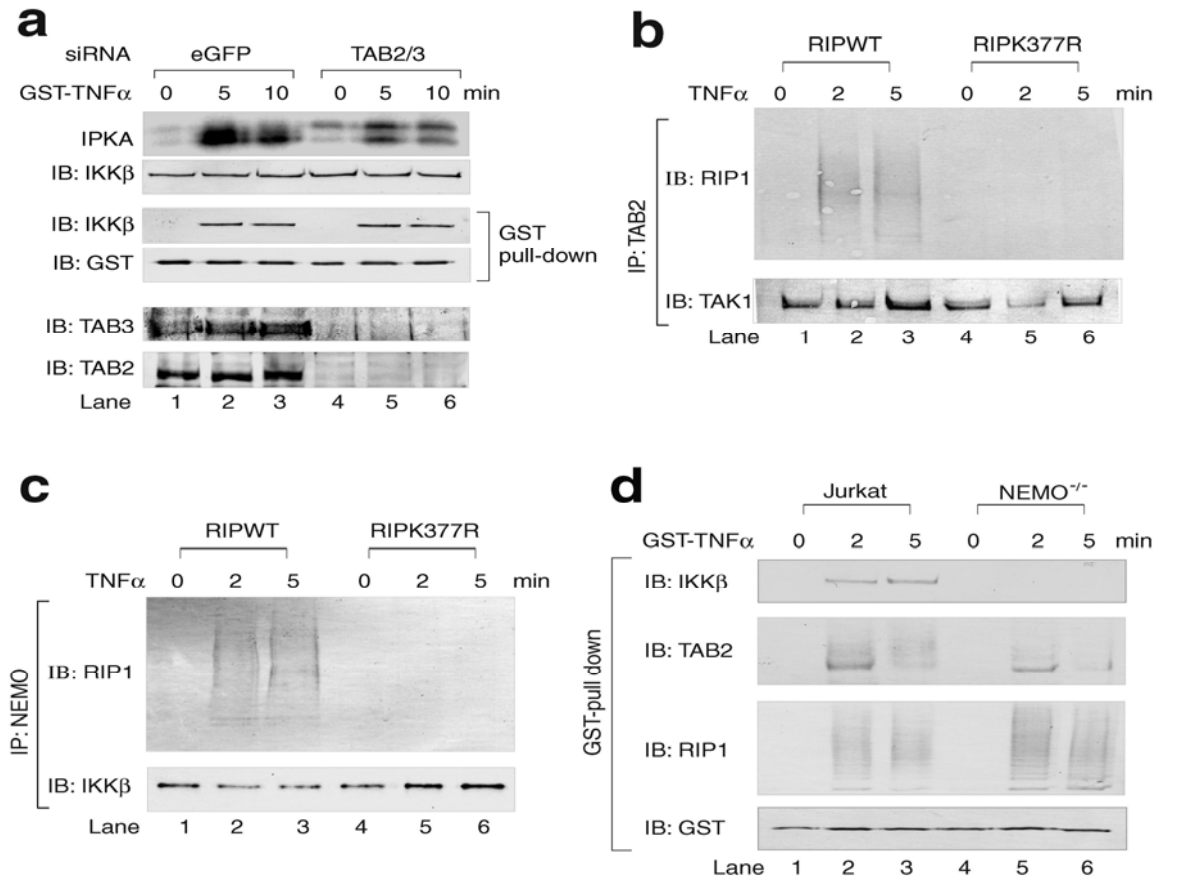

Figure 18. TAB2 and NEMO interact with polyubiquitinated RIP1.

(a) The IKK kinase complex is recruited to TNF-R1 independent of the TAK1 kinase complex. Double-stranded siRNA oligomers corresponding to the sequence of GFP (control), TAB2 and TAB3 were transfected into HEK 293 cells. After 2 consecutive days of transfection of siRNA, cells were treated with GST-TNFα (1μg/ml) for the indicated time. Two hundred microgram lysates were subjected to IKK kinase assay (upper panel) and 500μg lysates were used for TNF-R1 recruitment assay (middle panel). The efficiency of RNAi was verified by immunoblotting with TAB2 and TAB3 antibodies (lower panel). **(b)** TAB2 binds to polyubiquitinated RIP1. RIPWT or RIPK377R cells were treated with TNFα (10ng/ml) for the indicated time. Five hundred microgram lysates were used in a TAB2 immunoprecipitation. Immunoprecipitated proteins were analyzed by immunoblotting with RIP1 and TAK1 antibodies. **(c)** NEMO binds to polyubiquitinated RIP1. Five hundred microgram lysates prepared from RIPWT or RIPK377R cells treated with TNFα (10ng/ml) were used in a NEMO immunoprecipitation. Immunoprecipitated proteins were analyzed by immunoblotting with RIP1 and IKKβ antibodies. **(d)** Jurkat or NEMO-deficient Jurkat cells were treated with GST-TNFα (1μg/ml) for the indicated time. TNF-R1 bound proteins were purified with glutathione affinity purification and were examined by immunoblotting with TAB2, IKKβ, RIP1 and GST antibodies.

III.D Discussion

In this chapter, I sought to study the physiological role of TNF-induced polyubiquitination of RIP1. I mapped the ubiquitination site to the lysine 377 in the intermediate domain of RIP1. A single lysine to arginine point mutation at lysine 377 is sufficient to abolish TNF-induced RIP1 polyubiquitination. In the absence of RIP1 polyubiquitination, TNF-mediated IKK and NF-κB activation is impaired; and the cells are sensitive to bthe TNF-induced cell death. Moreover, polyubiquitinated RIP1 serves as a docking site to recruit TAK1 and IKK kinase complexes. Interestingly, TAB2 and NEMO bind to polyubiquitin chains and function as ubiquitin receptors that link TAK1 and IKK to polyubiquitinated RIP1 respectively.

The first case of ubiquitination-mediated kinase activation was discovered from the biochemical study of TRAF6 mediated IKK activation [152]. TRAF6 together with TRIKA1 and TRIKA2, activate IKK in a K63-linked polyubiquitination-dependent manner. The ubiquitination dependent kinase activation is also found in the TCR pathway and TIFA-induced IKK activation [157, 234]. In all cases, TRAF6 is the E3 ligase that promotes K63-linked polyubiquitination. In the TNF pathway, TRAF2 and TRAF5 are the E3 ligases that catalyze the polyubiquitination of RIP1 [233]. TNF-induced polyubiquitination of RIP1 is impaired in TRAF2-TRAF5-deficient MEF cells and overexpression of TRAF2 promotes polyubiquitination of RIP1. RIP1 undergoes K63-linked polyubiquitination when it is overexpressed, and this K63-linked polyubiquitination is susceptible to the deubiquitination activity of A20 [193]. In this study, I showed that, at least in the early phase of TNFα stimulation, the predominant form of polyubiquitinated RIP1 was the K63-linked

polyubiquitination. It has been proposed that A20 negatively regulates TNF-induced IKK activation by first disassembling the K63-linked polyubiquitin chains conjugated to RIP1 to shut off K63-linked polyubiquitination-mediated activation of IKK, and then by catalyzing the K48-linked polyubiquitination of RIP1 to promote degradation of RIP1 thus preventing the reactivation of RIP1 [193]. How can the K63-linked polyubiquitinated RIP1 be an activator of IKK while the K48-linked polyubiquitinated RIP1 be a substrate for proteasome? The simplest explanation is that differently linked polyubiquitin chains can be recognized by different ubiquitin receptors. Although TAB2 can bind both K48-linked and K63-linked polyubiquitin chains, it binds stronger to the K63-linked polyubiquitin chain than to the K48-linked polyubiquitin chain [176]. The same is true for endogenous NEMO (Dr. Li Deng personal communication). Thereby, TAB2 and NEMO can be considered as K63-linked polyubiquitin chain-specific ubiquitin receptors. On the other hand, the K48-linked polyubiquitinated RIP1 can be recognized by proteasome through direct binding to Rpn10, a stoichiometric component of the 26S proteasome and a K48-linked polyubiquitin chain-specific ubiquitin receptor [235]. It can also be recognized by other K48-linked polyubiquitin chain-specific ubiquitin receptors that facilitate the shuttling of RIP1 to the 26S proteasome. Thus, by recruiting different subset of ubiquitin receptors, differently linked polyubiquitin chains can confer different fates to the target protein. It remains to be tested if the two differently linked polyubiquitination occur at the same lysine residue of RIP1 or not. It has been shown that the intermediate domain of RIP1 binds TRAF2 and NEMO [52, 218, 226]. The identification of the polyubiquitination site of RIP1 is consistent with these observations and provides a possible biochemical mechanism for the interaction among the intermediate

domain of RIP1, TRAF2 and NEMO. TRAF2 binds to the intermediate domain of RIP1 and catalyzes the polyubiquitination of RIP1 at lysine 377; while the polyubiquitin chain conjugated to lysine 377 mediates the recruitment of NEMO. In fact, there are two potential TRAF2 binding sites (P/S/A/T-X-Q/E-E) located upstream of lysine 377 (353-PVEE and 366-PQEE).

RIP1 is also involved in pathways other than TNFα signaling. These pathways include TLR3, TLR4, and DNA damage response [223]. It will be interesting to know if the polyubiquitination of RIP1 is required for these pathways. RIP1 is involved in the activation of NF-κB triggered by genotoxic stress [236]. The DNA damage-induced response leads to the formation of a complex containing RIP1 and NEMO. This process is dependent on the presence of Ataxia-telangiectasia-mutated kinase (ATM) but is independent of the presence of TRAF2, TNF-R1 and TRADD. Further studies are required to test if the polyubiquitination of RIP1 mediates the recruitment of NEMO in response to DNA damage. RIP1 interacts with Trif through the homotypic interaction motif (RHIM) of both proteins [237]. Trif links RIP1 to TLR3 and TLR4, two TIR-domain-containing receptors that recognize dsRNA and LPS respectively. RIP1 is essential for poly(I:C)-induced NF-κB activation, but not the activation of JNK [237]. Similar to the TNF pathway, RIP1 undergoes polyubiquitination and is recruited to TLR3 in response to poly(I:C) stimulation [238]. TRAF6 may be responsible for the polyubiquitination of RIP1 in TLR3 pathway as it is recruited to TLR3 in response to poly(I:C) stimulation and is required for the TLR3-mediated NF-κB activation. Moreover, there are three potential TRAF6 binding sites (PXEXXAr/Ac, Ar/Ac represents an aromatic or acidic amino acid) in human RIP1, two of them are in the

intermediate domain of RIP1 (353-PVEESW and 366-PQEENE) and are overlapping with the potential TRAF2 binding sites. However, a direct interaction between TRAF6 and RIP1 has not been reported.

RIP1 is crucial for Trif-dependent TLR4-induced NF-κB as LPS fails to stimulate NF-κB activation in $rip1^{-/-}MyD88^{-/-}$ cells [238]. TRAF6 is also important for LPS-induced NF-κB activation, thus it is possible that TRAF6 may also promote the polyubiquitination of RIP1 in the TLR4 pathway. In the TLR4 pathway, LPS-induced IKK activity has a biphasic profile. The early phase of IKK activity is insensitive to cycloheximide while the second phase IKK activity is dependent on new protein synthesis. TNFα is induced by the first phase IKK activity and mediates the second phase IKK activity by a positive autocrine feedback [239]. Thereby the polyubiquitination of RIP1 may regulate both phases IKK activity. RIP1 may regulate the first phase IKK activity through Trif-dependent TLR4 pathway, and regulate the second phase IKK activity through TRAF2-dependent TNF-R1 pathway. Furthermore, genetics study of TAK1-deficient MEF cells reveals that TAK1 is required for TNF-R1, IL-1R, TLR3, and TLR4-mediated NF-κB activation [240]. It is very likely that my model of ubiquitination-dependent activation of IKK by RIP1 in the TNF pathway (Fig. 19) can be extended to TLR3 and TLR4 pathways. In response to TNFα stimulation, TRADD is recruited to the TNF-R1. TRADD is then served as a platform to recruit TRAF2/5 and RIP1. The recruitment of TRAF2/5 to the TNF-R1 activates the E3 ligase activity of TRAF2/5 presumably through oligomerization. Activated TRAF2/5 catalyzes polyubiquitination of RIP1 at lysine 377. Polyubiquitinated RIP1 in turn functions as a signal to recruit TAK1 and IKK kinase complexes through the ubiquitin binding domain of TAB2 (NZF) and NEMO

(LZ). The binding of TAK1 and IKK kinase complexes to polyubiquitinated RIP1 facilitates the sequential activation of TAK1 and IKK which ultimately leads to the activation of NF-κB.

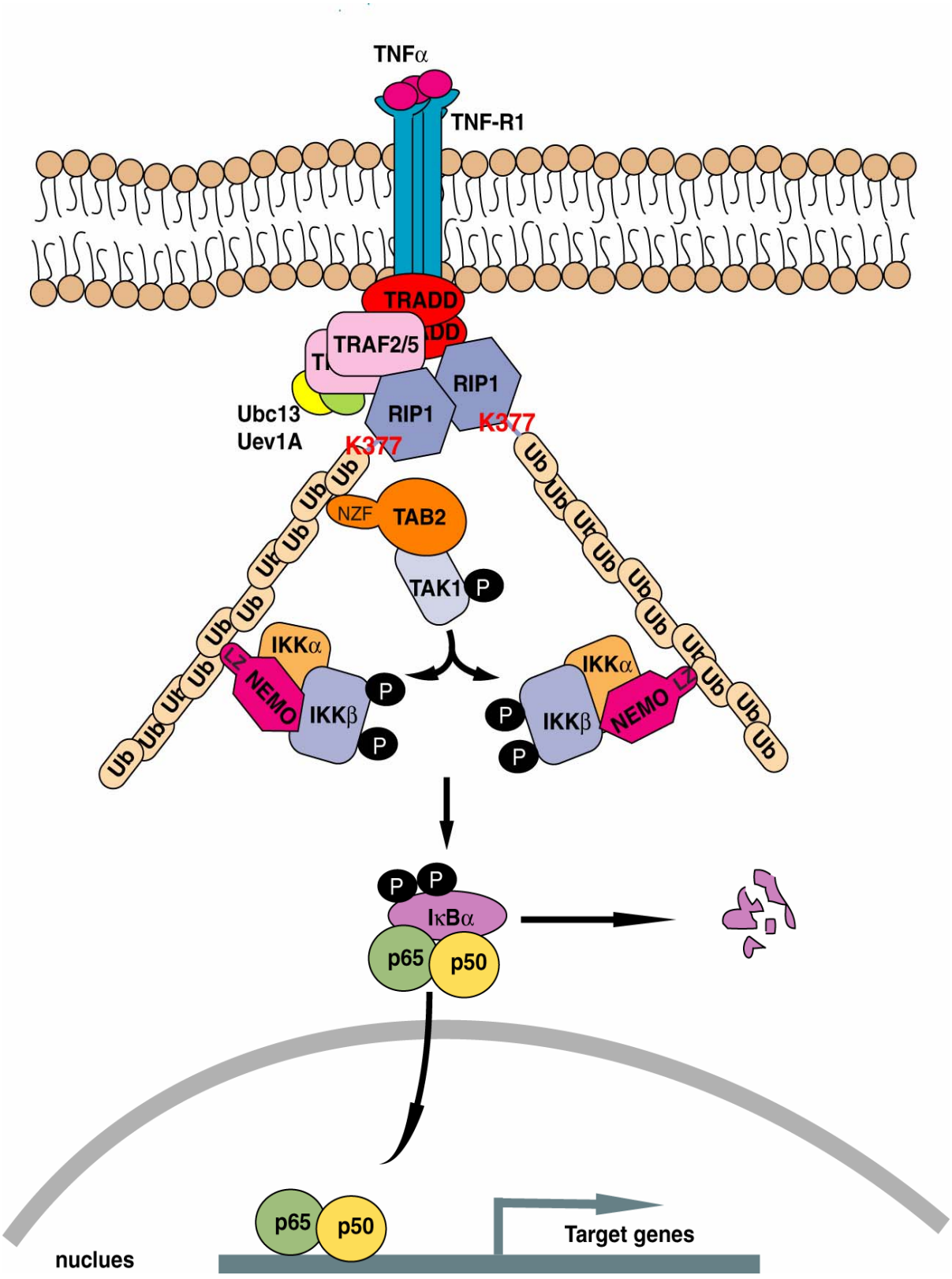
TNFα
TNF-R1
TRADD
TRAF2/5
RIP1
RIP1
K377
K377
Ubc13
Uev1A
NZF
TAB2
TAK1
P
IKKα
NEMO
LZ
IKKβ
IKKα
NEMO
LZ
IKKβ
IκBα
p65
p50
p65
p50
Target genes
nuclues

Figure 19. A biochemical model of TNFα signaling.

Engagement of TNFα to the TNF-R1 leads to the recruitment of TRADD. TRADD in turn serves as a platform to recruit TRAF2/5 and RIP1. RIP1 undergoes TNF-induced polyubiquitination at Lys377. The polyubiquitinated RIP1 is then served as a docking site to recruit TAK1 and IKK kinase complexes. The recruitment of TAK1 kinase complex is mediated by the interaction between the NZF domain of TAB2 and the polyubiquitinated RIP1 while the recruitment of IKK kinase complex is directed by the interaction between the leucine zipper of NEMO and polyubiquitinated RIP1. The recruitment of the TAK1 and IKK kinase complexes to the TNF-R1 facilitates the sequential activation of TAK1 and IKK which ultimately leads to the activation of NF-κB.

CHAPTER IV: CONCLUSION AND FUTURE DIRECTIONS

Ubiquitin is a Nobel protein. It is small but mighty as it can be 'linked' to virtually every aspect of eukaryotic cell biology. The ubiquitin field has gained a lot of attention and achieves tremendous progression in the past decade. It is appreciated by the scientific community as the 2004 Nobel Prize in Chemistry was awarded to I. Rose, A. Ciechanover and A. Hershko for their ground breaking discovery of ubiquitin-proteasome dependent proteolysis. However, the ubiquitin-proteasome pathway is not the only function of ubiquitin. Discovery of non-proteolytic functions of ubiquitin has opened a new chapter for the ubiquitin field. It is speculated that by attaching differently linked polyubiquitin chain (K6, K11, K27, K29, K33, K48, and K63) or monoubiquitin to the target protein, ubiquitination changes the molecular landscape of a protein and alters the biological function of the target protein.

In this dissertation, I show examples of how two proteins activate IKK by a novel non-protoelytic function of ubiquitination. First, I show that TIFA activates IKK through activating the ubiquitin ligase activity of TRAF6 by promoting oligomerization of TRAF6. In conjunction with Ubc13-Uev1A, E1 and ubiquitin, activated TRAF6 promotes self-ubiquitination in a K63-linked manner. The K63-linked polyubiquitinated TRAF6 serves as a docking site to recruit the TAK1 kinase complex through the interaction between the NZF of TAB2 and the K63-linked polyubiquitin chain. The recruitment of the TAK1 kinase complex leads to the activation of TAK1 presumably through either intramolecular or intermolecular autophosphorylation of TAK1. Activated TAK1 in turn activates IKK through direct phosphorylation of IKKβ at the activation loop, and ultimately leads to the activation of NF-

κB. Second, I show that RIP1 undergoes TNF-induced K63-linked polyubiquitination at lysine 377 in the intermediate domain. The polyubiquitination of RIP1 is essential for TNF-induced IKK and NF-κB activation. In the absence of RIP1 polyubiquitination, cells are sensitive to TNF-induced cell death. The polyubiquitinated RIP1 functions as a signal to recruit the TAK1 kinase complex as well as the IKK kinase complex. The TAK1 kinase complex is recruited through interaction of TAB2 with the polyubiquitin chain, while recruitment of the IKK kinase complex involves the interaction between the leucine zipper of NEMO and the polyubiquitin chain. The recruitment of TAK1 and IKK kinase complexes to the receptor by RIP1 facilitates the sequential activation of TAK1 and IKK (Fig. 19).

TIFA and RIP1 represent two examples of how ubiquitination can lead to the activation of a kinase in a non-proteolytic manner. Observations from several studies suggest that the ubiquitination-mediated kinase activation may be a more general mean to activate kinases than expected. For example, germinal center kinase-related (GCKR) can be activated by TNFα and overexpression of TRAF2 [241]. GCKR itself undergoes Ubc13-mediated ubiquitination, suggesting that ubiquitination may be involved in the activation of GCKR. Moreover, apoptosis signal-regulating kinase 1 (ASK1) interacts with TRAF1-6, and overexpression of TRAF2, TRAF5 and TRAF6 lead to the activation of ASK1 [242]. Furthermore, endogenous ASK1 undergoes TNF-dependent interaction with TRAF2. Interestingly, a RING-deleted TRAF2 mutant inhibits the TNF-dependent activation of ASK1. Recently, a reactive oxygen species (ROS)-dependent activation of the TRAF6-ASK1-p38 pathway has been described [243]. These results indicate that TRAF2 and TRAF6 may activate ASK1 in a ubiquitination-dependent manner. In addition, TRAF6 and Ubc13

are essential for the production of interferon-α induced by TLRs in response to viral RNA or bacterial DNA. The expression of interferon-α is regulated by the transcription factor IRF7. IRF7 is activated by phosphorylation-induced dimerization and nuclear translocation. Recent studies indicated that upon ligand binding to TLR7, 8, and 9, IRF7 forms a complex with MyD88, TRAF6, IRAK1, and IRAK4 [244-246]. IRF7 is then activated within this complex through phosphorylation mediated by an IRF7 kinase whose identity remained to be determined. Interestingly, Ubc13 and the E3 ligase activity of TRAF6 are required for the activation of IRF, indicating that K63-linked ubiquitination may be involved in the activation of the IRF7 kinase. All these potential ubiquitination-mediated kinase activations discussed above involve the TRAF proteins; however, a non-TRAF E3 ligase-dependent kinase activation may also exist. In fact, overexpression of the inhibitor of apoptosis (IAP) E3 ligases induces the activation of NF-κB [247, 248]. RNAi of cIAP-1 impairs TNFα-induced IKK activation and cIAP-1 promotes the ubiquitination of NEMO [248]. It has been shown that cIAP-1 and cIAP-2 catalyze the ubiquitination of RIP1 in vitro. These results indicate that IAPs may activate IKK in a ubiquitination-dependent manner. Moreover, this mechanism is evolutionarily conserved as *drosophila* homologue of cIAP, dIAP2, is essential for the activation of Relish in the IMD pathway [249, 250].

How can different polyubiquitin linkages confer different fates to the target protein? A possible explanation is that different linkage of polyubiquitin chains might have different conformation or topology that can be recognized by specific subset of ubiquitin receptors. In fact, the structural studies of the K48-linked and the K63-linked diubiquitin have clearly indicated that these alternatively linked diubiquitin chains adopt distinct conformations [251,

252]. To generate a K48-linked ubiquitin chain, an 90° turn is created between the donor and the acceptor ubiquitin molecules that result in a closed confirmation of the K48-linked polyubiquitin chain [252]. In contrast, the K63-linked polyubiquitin chain adopts an open linear conformation with no clear interface between two ubiquitin molecules [251]. Thereby these conformational differences may serve as different signals to recruit distinct ubiquitin receptors. The concept of the presence of linkage-specific ubiquitin receptors has emerged. These ubiquitin receptors function as signaling modules that interpret signals generated by differently linked polyubiquitin chains and transduce the signals to corresponding downstream effectors. A survey of 30 UBA domain containing proteins reveals diverse polyubiquitin binding properties of the UBA domains [253]. Some of these UBA domains bind specifically to either K48 or K63-linked polyubiquitin chain. Moreover, the identification of Rpn10 and Rad23 as the K48-linked polyubiquitin chain receptors while TAB2 as a K63-linked polyubiquitin chain receptor has underscored the importance of these ubiquitin receptors in transducing the cues generated by the differently linked polyubiquitin chains to different downstream effectors [176, 254]. These ubiquitin receptors fulfill their function through bipartite interaction with the ubiquitin chain and the downstream effectors. For example, Rad23 interacts with proteasome through ubiquitin-like domain (UBL) and interacts with K48-linked polyubiquitin chain through UBA domain. Similarly, TAB2 can simultaneously interact with K63-linked polyubiquitin chain and TAK1. Therefore, numerous biological consequences can be achieved by generating different combinations of polyubiquitin chains and ubiquitin receptors. There are seven lysines residues in ubiquitin. All of them can be utilized in the ubiquitination reaction. However, the physiological role of

most of these modifications is poorly understood. For example, although the abundance of K11-linked polyubiquitin chain is comparable to the abundance of K63-linked polyubiquitin in yeast, the significant of this modification is totally unclear [118]. Therefore, determining the biological function of these modifications will be an exciting challenge for the ubiquitin field. I believe that identification of linkage specific ubiquitin receptors and target proteins will shed light on understanding the function of differently linked polyubiquitin chains in vivo. In addition, generation of linkage-specific antibodies will be a powerful tool for this purpose.

Ubiquitination usually modifies the target protein through formation of an isopeptide bond between the lysine residue of the substrate and the carboxyl terminus of ubiquitin. Recently, a nonlysine mediated ubiquitination has been reported [255]. In this study, a Kaposi's sarcoma-associated herpesvirus encoded E3 ligase, MIR1, promotes the degradation of major histocompatibility complex class I (MHC I) molecule through formation of thioester linked polyubiquitin chain conjugation at a cysteine residue of the cytosolic portion of MHC I. Only a subset of E3 ligases can catalyze the cysteine mediated ubiquitination as a closely related E3 ligase from the same virus, MIR2, fails to induce degradation of MHC I by cysteine mediated ubiquitination. Since two cysteine residues can form a disulfide bond which is usually used as a means to stabilize the three dimension structure of a protein, it is likely that formation of cysteine-linked polyubiquitin chain may destabilize the folding of the target protein which may facilitate the subsequent degradation of the target protein by the 26S proteasome. Furthermore, the disulfide bond is sensitive to the redox state of the environment. The accessibility of the target cysteine may be regulated

by the redox state and thus provide a mechanism to regulate the stability of the target protein by the redox state of the cell. There is no cysteine residue in ubiquitin, so the covalent modification between two ubiquitin molecules can only occur through the formation of an isopeptide bond. It remains to be determined how common is the cysteine mediated ubiquitination and if there is a preference for a cysteine residue over a lysine residue or vice versa by a given E3 ligase.

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VITAE

Chee-Kwee Ea was born in Johor Bahru, Johor, Malaysia on December 31, 1976, the son of Tiam-Siong Ea and Ah-Leng Gan. After completing his work at Chong-Hwa High School, Kluang, Johor, Malaysia in 1994, he entered Tatung University (previously known as Tatung Institute of Technology) at Taipei, Taiwan in 1995. He received the degree of Bachelor of Science with a major in Bioengineering from Tatung University in June, 1999. During the following year he was employed as a research assistant at Cancer Research Center, National Taiwan University Hospital, Taipei, Taiwan. In September, 2000 he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. He married Hui-Ting Goh in 2003.

Permanent Address: 9, Jalan Pelandok, Taman Mohd Yassin, 86200 Simpang Renggam, Johor, Malaysia.