BIOCHEMICAL MECHANISM OF PROTEIN KINASE ACTIVATION BY THE UBIQUITINATION SYSTEM

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by

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BIOCHEMICAL MECHANISM OF PROTEIN KINASE ACTIVATION BY THE UBIQUITINATION SYSTEM

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The University of Texas Southwestern Medical Center at Dallas, 2008

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

The NF-κB signaling pathway is important for immune, inflammatory and stress responses of cells and can be activated by a variety of extracellular stimuli. In the IL1R/TLR signaling pathway, NF-κB is activated through activation of TAK1-IKK cascade by TRAF6 and Ubc13/Uev1A in a polyubiquitination-dependent manner. Mechanistically how TAK1 is activated by the TRAF6-Ubc13/Uev1A dependent polyubiquitination system is unknown. Whether TAK1 and IKK kinases can be activated by other than TRAF6-Ubc13/Uev1A is an open

question.

By inactivating ubiquitin activating enzyme E1 and ubiquitin conjugating enzyme E2 using NEM to further dissect the system, studies on how TAK1 is activated by the TRAF6-Ubc13/Uev1A system have revealed that 1) polyubiquitination step and kinase activation step can be un-coupled; 2) Polyubiquitination step generates unanchored K63-linked polyubiquitin chains as the kinase activators; 3) Coiled-coil domain of TRAF6 is required for synthesis of active polyubiquitin chains.

Through biochemical fractionation, I purified UbcH5 as another E2 that works with TRAF6 to directly activate IKK. Mechanistic analysis on how TRAF6/UbcH5 activates IKK reveals that TRAF6-UbcH5 synthesize mixed-linkage-linked unanchored polyubiquitin chains and this polyubiquitin chains function as direct activators for IKK activation.

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PRIOR PUBLICATIONS

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LIST OF ABBREVIATIONS

DUB: Deubiquitination enzyme

E1: ubiquitin-activating enzyme

E2: ubiquitin-conjugating enzyme

E3: ubiquitin ligase

IKK: IκB kinase

IL-1β: interleukin-1β

IκB: inhibitor of NF-κB

NEMO: NF-κB essential modulator

NEM: N-ethylmaleimide

NF-κB: nuclear factor kappa B

RING: really interesting new gene

RIP1: receptor interacting protein kinase 1

TAB2: TAK1 associated protein 2

TAK1: TGFβ activated kinase 1

TNFα: tumor necrosis factor alpha

TRAF: tumor necrosis factor associated factor

Chapter I Introduction

I.A NF-κB signaling pathway.

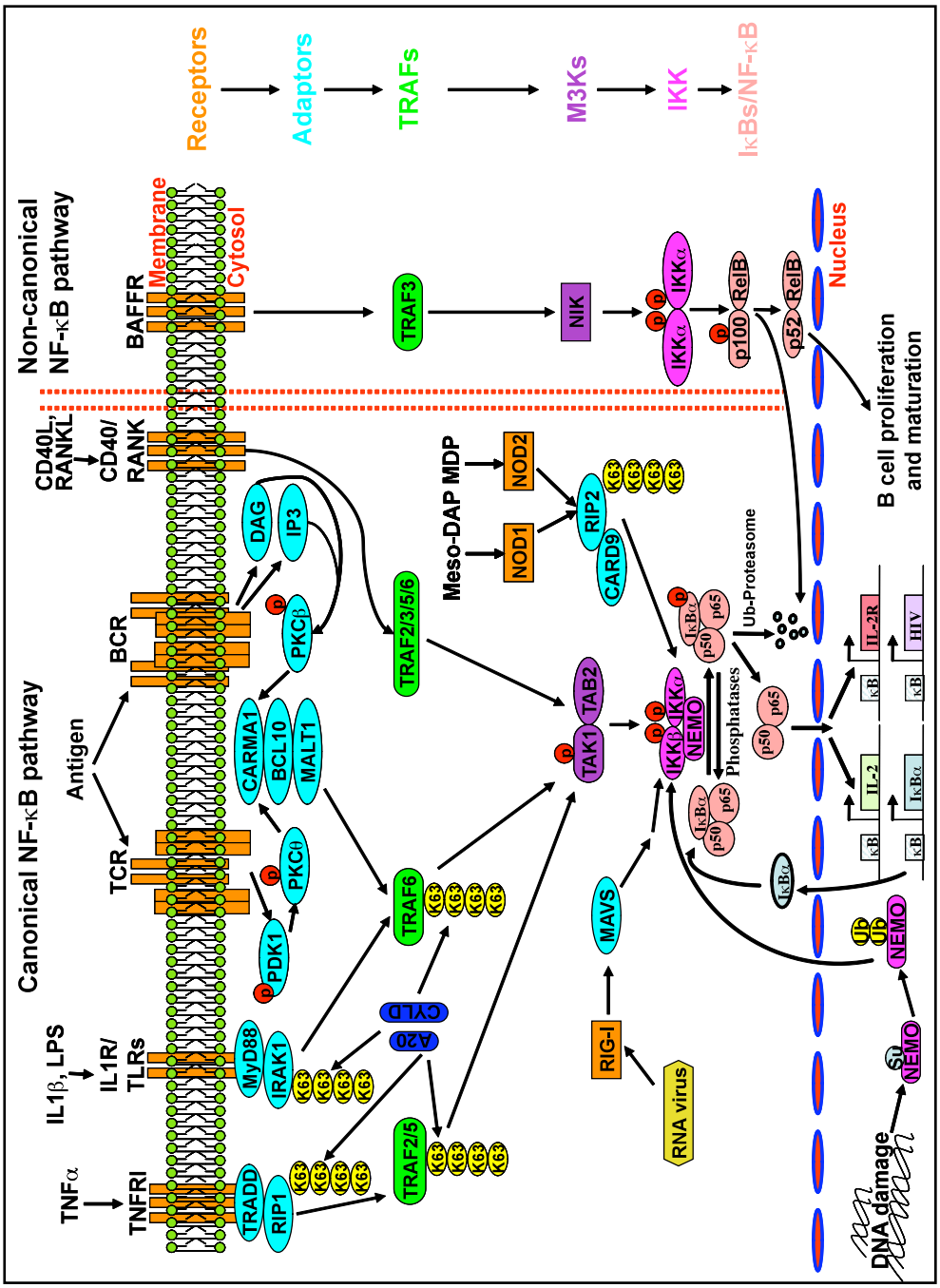


Figure 1 IKK-NF-κB signaling pathways. Two, canonical and non-canonical NF-κB signaling pathways are depicted here. In the canonical pathway, a variety of extracellular stimuli (pro-inflammatory cytokines TNFα, IL1β, bacterial products LPS, viral RNAs, UV, DNA damage etc.) activate the canonical NF-κB pathway when receptors are bound by their cognate ligands. Upon ligand binding, the receptors transduct signals to adaptors, which activate one or more TRAF family protein members and initiate polyubiquitination-dependent activation of MAPKKK (M3K) TAK1. TAK1 then phosphorylates and activates IKK. Activated IKK phosphorylates IκBα in the cytoplasm of resting cells. IκBα phosphorylation leads to its K48-linked polyubiquitination and subsequent proteasomal degradation. This allows NF-κB (shown here as a p65–p50 dimer) to translocate to the nucleus, where it induces the transcription of genes with an NF-κB consensus site in their promoter region, including IκBα. This newly synthesized IκBα can establish a negative-feedback loop by moving to the nucleus, where it removes DNA-bound NF-κB complexes from their cognate sites and exports them back to the cytoplasm. In the non-canonical pathway, cognate ligand binding leads to activation of IKKα, which then phosphorylates p100 leading to its limited degradation and then translocation to the nucleus as a dimer with p50, where p52/p50 dimer induces gene expression for B cell proliferation and maturation.

The transcription factor NF-κB (Nuclear Factor of κ sites in B cells) is a central player in diverse aspects of innate and adaptive immune responses as well as stress-responsive reactions. Since its first description as a regulator of (Ig)κ light chain gene expression in murine B lymphocytes in 1986 (Sen & Baltimore, 1986), NF-κB pathway has been one of the well-studied signaling pathways that lead to transcriptional activation and a paradigm for post-translational regulation of protein activities. NF-κB is expressed in virtually every cell type and can be activated by a variety of extracellular stimuli such as pro-inflammatory cytokines (IL1β, TNFα), bacterial products (LPS), viral infection, double-stranded RNA,

DNA damage, T- and B-cell antigens, UV radiation, and other physical and chemical stress (Figure 1). NF-κB is also activated in a number of diseases such as cancer, arthritis, neurodegenerative disease.

I.A.1 Key components in the pathway

I.A.1.a NF-κB protein family and IκB protein family

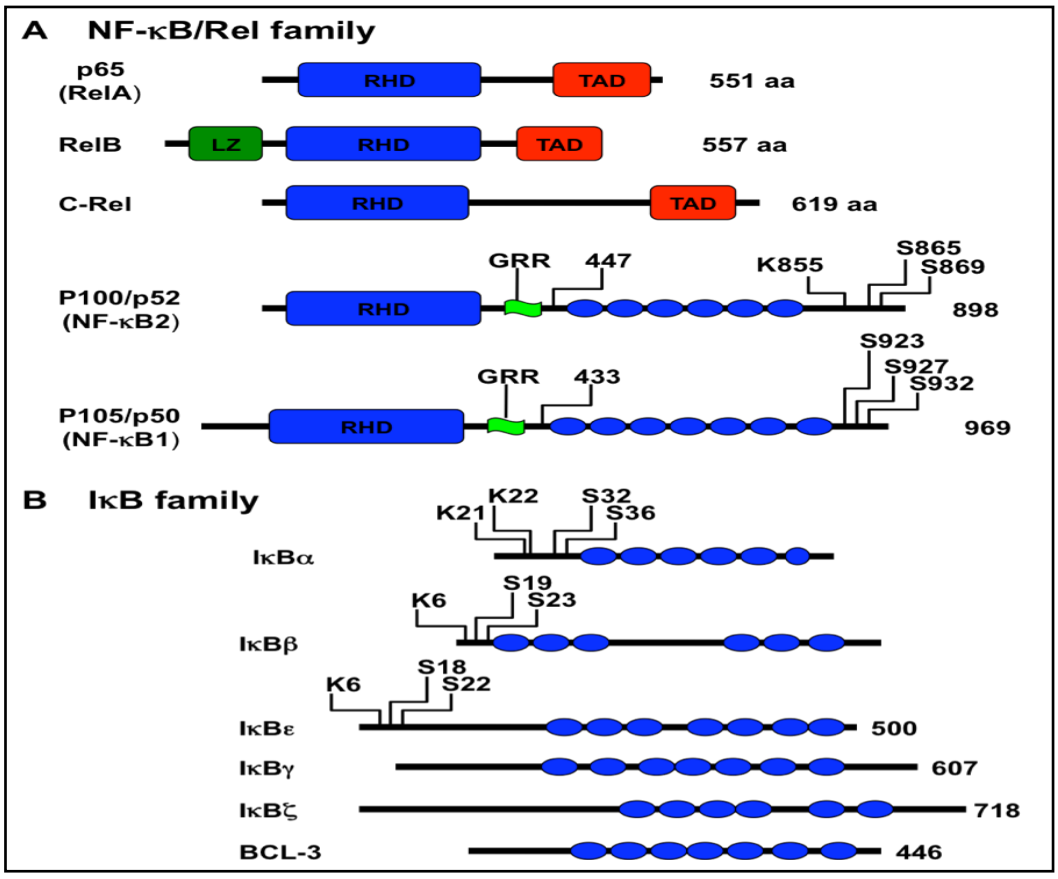


Figure 2 Proteins of NF-κB and IκB families. Members of the NF-κB and IκB protein families are shown. The number of amino acids in each protein refers to the human proteins. Presumed sites of cleavage for p100 (amino acid 447) and p105 (amino acid 433) are shown. Phosphorylation and ubiquitination sites on p100, p105, and IkB proteins are indicated. (RHD) Rel homology domain; (TAD)

transactivation domain; (LZ) leucine zipper domain on Rel-B; (GRR) glycine-rich region. Modified from Hayden & Ghosh, 2004.

NF-κB is a collection of transcription factors that are conserved from the phylum Cnidaria to humans (they do not exist in yeast and *C.elegans*, though) (Figure 2A). Five mammalian NF-κB proteins have been identified, which are RelA (p65), RelB, c-Rel (so called "Rel" proteins), and NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (Ghosh et al., 1998; Miyamoto and Verma, 1995; Siebenlist et al., 1994). Three Rel proteins have also been identified in *Drosophila melanogaster*: Dorsal, Dif, and Relish (Ghosh et al., 1998). All of these proteins share a highly conserved 300 amino acid Rel homology domain (RHD) comprised of two immunoglobulin (Ig)-like domains. The RHD domains are responsible for their dimerization, DNA binding, and interaction with their inhibitory proteins IκBs. The RHD domains also contain the nuclear localization sequence (NLS). p105 and p100 have long C-terminal domains that contain multiple copies of ankyrin repeats which inhibit activity of these proteins but undergo limited proteolysis to create active DNA-binding proteins p50 and p52, respectively. The five members form various heterodimers. Homodimers have also been reported. Among them the p65/p50 heterodimer is the major NF-κB dimer in cells and has been the most studied. NF-κB dimers bind to κB sites with the consensus sequences of GGGRNNYYCC, where R is purine nucleotide, Y is pyrimidine nucleotide, and N is any nucleotide (Gilmore,

2006). Different NF-κB complexes show distinct DNA-binding site specificities, and different affinities for the κB sites and can either activate or inhibit gene transcription depending on which dimers are bound to the κB sites. For example, p65/p50 heterodimer is a potent transcriptional activator whereas p50/p50 homodimer is inhibitory. NF-κB regulates the transcription of a large number of genes involved in inflammatory and immune responses, antiviral infection, and apoptosis.

The IκB (Inhibitor of κB binding protein) protein family includes IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3, and p105 (precursor of p50) and p100 (precursor of p52) (Gilmore, 2006) (Figure 2B). The Drosophila genome encodes one homologue called Cactus (Whiteside and Israel, 1997). All IκB proteins contain either six or seven ankyrin repeats, which form stacked helical domains and mediate binding to the RHD domains of NF-κB dimers and therefore masking their NLS sequences. Different IκBs have different affinities towards different NF-κB dimers, and show different tissue-specific expression patterns (Chen and Ghosh, 1999). The three major cytoplasmic inhibitors of NF-κB (IκBα, IκBβ and IκBε) also contain N-terminal regulatory regions (degron sequences) that are responsible for stimulus-induced degradation. These IκBs undergo different stimulus-induced degradation kinetics. For example, IκBα is degraded very fast whereas IκBβ very slow. IκBζ and Bcl-3 can form complex with p50 or p52

dimer to activate transcription in the nucleus. Except for IκBβ, the expression of all other IκBs is regulated by NF-κB.

The activity of NF-κB is tightly regulated by IκB proteins. In resting cells, NF-κB stays in the cytosol as a latent, inactive form by virtue of their interaction with inhibitor proteins IκBs, which mask the NLS sequences on NF-κB and prevent their translocation to the nucleus. When cells are stimulated, IκBs undergo site-specific phosphorylation by upstream kinase IKK (IκB kinase), which marks them as substrates for polyubiquitination and being degraded by the 26S proteasome. Degradation of IκBs then releases NF-κB, which in turn translocates to the nucleus and regulates target gene transcription. The activation of NF-κB is transient and cyclical in the presence of continued inducer. Part of the reason for this kind of behavior is due to the auto-inhibitory negative feedback loop (Hoffmann et al., 2006), such as induced rapid re-synthesis of IκBα by activated NF-κB. Re-synthesized IκBα can enter into the nucleus and bind to NF-κB and export NF-κB back to the cytoplasm by means of the export sequence (NES) present on IκBα, thus terminating NF-κB. Activation of NF-κB independent of IKK and therefore independent of serine phosphorylation has also been reported. When cells are treated with pervanadate or under hypoxia conditions IκBα undergoes phosphorylation at tyrosine-42, which then dissociates from NF-κB independent of proteasome degradation (Imbert et al., 1996; Beraud et al., 1999). UV treatment of cells induces IκBα degradation by the proteasome

without phosphorylation of either Ser32, Ser 36 or tyr-42 leading to NF-κB activation (Bender et al., 1998; Li et al., 1998).

In addition to its major regulation by IκB proteins, the activity of NF-κB is also regulated by phosphorylation, acetylation and prolyl isomerization. p65 is phosphorylated by IKK or PKA in the cytosol and by MSK1 in the nucleus, and these phosphorylation events are required for activation of gene expression. On the other hand, the un-phosphorylated p65-containing heterodimer is suppressive (Perkins, 2006).

I.A.1.b IKK kinase complex

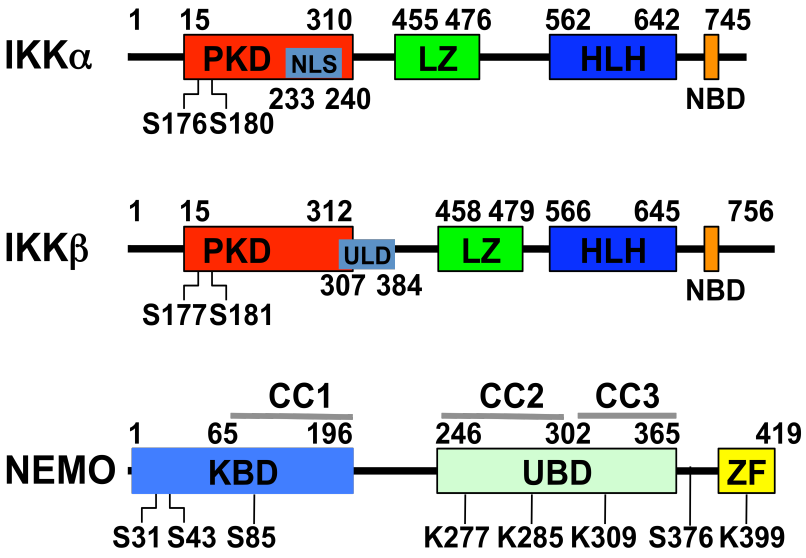


Figure 3 Functional domains in IKKα, IKKβ and NEMO/IKKγ. Numbers refer to amino acid positions in the human proteins. The activation loop serines in IKKα and β are shown. Lysine residues in NEMO which undergo SUMOylation or non-degradative ubiquitination upon stimulation, Ub^{K63} at

Lys399 by MALT1 (Zhou *et al.*, 2004) and at Lys285 by Nod2/RIP2 (Abbott *et al.*, 2004); SUMO-1 and Ub at Lys277 and Lys309 by genotoxic stress (Huang *et al.*, 2003) and serine residues in NEMO/ IKKγ that are phosphorylated upon Tax or TNFα stimulation (Ser-31, Ser-43, Ser-376) (Carter *et al.*, 2003) or by ATM (Ser-85) (Wu *et al.*, 2006) are indicated. HLH: Helix-loop-helix; PKD: Protein kinase domain; NBD: NEMO binding domain; NLS: Nuclear localization signal; ULD: Ubiquitin-like domain; LZ: Leucine zipper; CC: Coiled-coil; KBD: Kinase binding domain; UBD: Ubiquitin-binding domain; ZF: Zinc finger. Modified from Scheidereit, 2006.

The other key component in the NF-κB signaling pathway is the kinase complex IKK (IκB kinase) that is responsible for phosphorylation of IκBs. IKK was first identified through biochemical fractionation as a 700 KDa complex that was activated by ubiquitination-dependent but proteasome-independent manner in an *in vitro* system that recapitulates phosphorylation-dependent ubiquitination of IκBα (Chen et al., 1996). Subsequently through biochemical purification, two highly related kinases were identified as IKKα (IKK1) and IKKβ (IKK2) (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997, Woronicz et al., 1997; Zandi et al., 1997). IKKα is 85 KDa and IKKβ 87 KDa. IKKα and IKKβ are highly homologous to each sharing 50% sequence identity and >70% similarity. They contain N-terminal kinase domains followed by leucine zipper (LZ) and helix-loop-helix (HLH) motifs. These two kinases are the catalytic subunits of the IKK complex. The third component of IKK complex was later identified through biochemical purification and genetic complementation of a cell line unresponsive to NF-κB-activating stimuli (Rothwarf et al., 1998; Yamaoka et al., 1998). This subunit was named NEMO (NF-κB essential modulator), which

was also called IKKγ, IKK-associated protein 1 (IKKAP1), or adenovirus protein E3-14.7 KDa interaction protein 3 (FIP-3). NEMO is 47 KDa. NEMO is predominantly helical with three predicted coiled-coil domains, leucine zipper and a Zinc-finger motif at its C-terminus. NEMO doesn't have any kinase activity but is obligatory for NF-κB activation by most if not all stimuli tested so far (Ghosh & Karin 2002). The stoichiometry of the three subunits in the IKK complex is not conclusive yet. It appears that the 700-900 KDa IKK complex contains equimolar quantities of IKKα, IKKβ and NEMO subunits (Krappmann et al., 2000; Miller & Zandi, 2001). With purified NEMO, one report showed NEMO formed a trimer (Agou et al., 2002; 2004) but another report showed NEMO as a tetramer (Tegethoff et al., 2003). Without NEMO, IKKα and IKKβ can form homodimers or IKKα/β heterodimers. NEMO can form a complex with either IKKα or IKKβ. The minimal interaction domains among the three subunits have been mapped (Figure 3). Other proteins than IKKα, IKKβ and NEMO have also been reported that associate with the IKK kinase complex including IκB and Rel family proteins, MKP-1 (Heilker et al., 1999), MEKK1 (Rao et al., 1996), NIK (Regnier et al., 1997), IKAP (Cohen et al., 1998), ELKS (Ducut Sigala et al., 2004), chaperones HSP90 and Cdc37 (Chen et al., 2002). However each of these proteins' status as bode fide components of IKK complex was not consistent among different reports and might not be representative of core components (Karin & Ben-Neriah, 2000).

IKKα and IKKβ belong to the serine/threonine kinase family, with the conserved ATP-binding lysine and conserved activation loop (T-loop) within their kinase domains. The two serine residues in the T-loop (Ser176 and Ser180 in IKKα, Ser177 and Ser181 in IKKβ) need to be phosphorylated in order to activate IKKα or IKKβ (Mercurio et al., 1997; Delhase et al., 1999). Phosphorylation of these two serine residues can be achieved through either trans-autophosphorylation or by upstream kinases. Transient overexpression of either IKKα or IKKβ leads to activation of IKKα or IKKβ. Similarly, expression of either IKKα or IKKβ in sf9 cells yields active kinases. A number of kinases have been reported to be able to activate IKK when overexpressed in cells or when incubated with IKK *in vitro*. These kinases include protein kinase C isoforms (Lallena et al., 1999), and the MAPKKK family members such as NIK (Woronicz et al., 1997; Lin et al., 1998), AKT/PKB (Romashkova & Makarov, 1999), MEKK1 (Lee, et al., 1997; Nemoto et al., 1998), MEKK2 (Zhao et al., 1999), MEKK3 (Zhao et al., 1999), COT/Tpl2 (Lin et al., 1999) and TAK1 (Ninomiya et al., 1999; Sakurai et al., 1999, Wang et al., 2001). However, in genetic studies, cells deficient in MEKK1 (Xia et al., 2000; Yujiri et al., 2000) or Cot/TPL2 (Dumitru et al., 2000) didn't have defects in IKK activation. Studies with KO mice have shown that TAK1 is a relevant physiological kinase that activates IKK in response to proinflammatory stimuli (Sato et al., 2005; Shim et al., 2005; Wan et al., 2006, Liu et al., 2006) and RANK ligand stimulation (Cao et al., 2001) in B

cells in the traditional NF-κB activation pathways while NIK is the kinase that activates IKKα in the alternative NF-κB signaling pathway in response to LTβ (lymphotoxin β) (Yin et al., 2001).

Although IKKα and IKKβ share high sequence similarity, exist in the same kinase complex, and can phosphorylate IκB proteins *in vitro*, knockout studies in mice have shown that these two kinases have different physiological functions (Gerondakis et al., 2006). These studies show that IKKβ (together with NEMO) is essential for NF-κB activation in response to proinflammatory stimulation such as TNFα, IL1β, LPS, while IKKα plays an important role in B-cell development through phosphorylation of p100 in the non-canonical NF-κB activation pathway (Karin & Delhase, 2000; Senftleben et al., 2001). IKKα is also involved in epidermal differentiation through an unknown mechanism independent of its kinase activity and NF-κB activation (Hu et al., 2001). In addition, IKKα has an intact NLS sequence and accumulates in nucleus in response to TNFα stimulation where it phosphorylates histone H3 to controls induction of IκBα, IL6 and IL8 genes (Anest et al., 2003; Yamamoto et al., 2003).

Activation of IKK is highly regulated and transient. When cells are stimulated by TNFα or IL1β, IKK activation peaks around 5 to 15 minutes and then within 30 minutes drops to 15-to 20% of the peak activity (DiDonato et al., 1997; Zandi et al., 1997; Delhase et al., 1999). There are several mechanisms that may down-regulate IKK activity after it is activated: intra-molecular auto-

inhibition from auto hyper-phosphorylation of IKK at their C-termini (Delhase et al., 1999); dephosphorylation of activation loop by phosphatases such as PP2A or PP2Cb (Prajapati et al., 2004; Kray et al., 2005) and de-ubiquitination of upstream intermediates such as TRAF6 and RIP1 by the deubiquitinating enzymes CYLD and A20.

I.A.1.c TRAF proteins

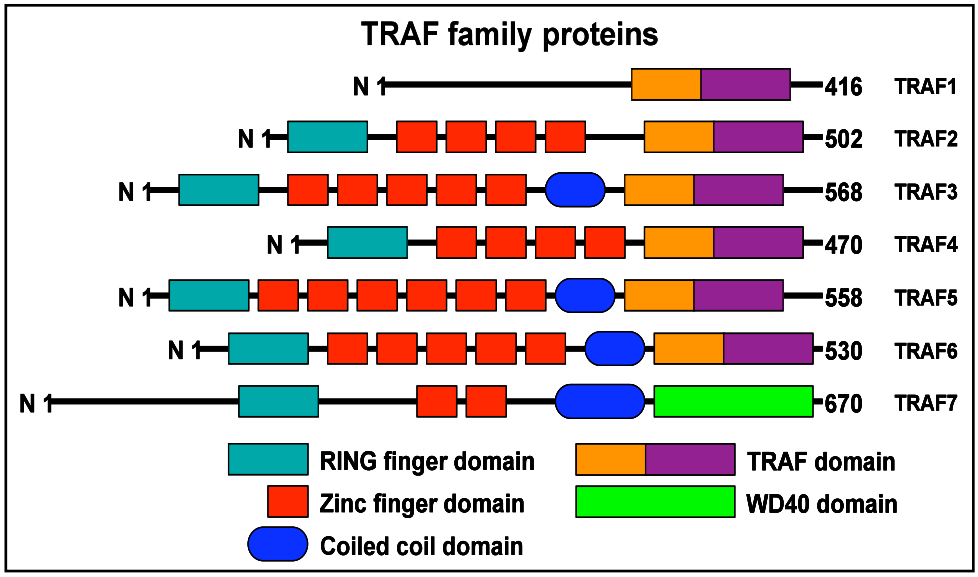


Figure 4 Domain organization of the members of the TRAF family. A total of seven members of the TRAF family (TRAF1-7) have been described in mammals. All share an N-terminal RING finger domain except TRAF1 which doesn't have it, followed by various copies of ZnF and coiled-coil motifs. TRAF1- 6 also have a C-terminal fold called the TRAF domain while in TRAF7, this domain is replaced with WD40 repeat domain.

Upstream of IKK and downstream of IL1R/TLRs and TNF receptor superfamily receptors, there is another important protein family serving as

signaling intermediates: the TRAF (TNF receptor-associated factor) proteins. There are seven known TRAF proteins in human genome named as TRAF1 to TRAF7 (Figure 4). Except TRAF1, TRAF2 to TRAF7 all have a RING (really interesting gene) domain and several zinc finger motifs important for downstream signaling (Baud et al., 1999; Zapata et al., 2002) at their N-termini. TRAF1 to TRAF6 have a "TRAF" domain at their C-termini while in TRAF7 the TRAF domain is replaced by a WD40 domain. TRAF domains are responsible for self-association and interaction with upstream signaling components such as IRAK-1, RIP1, and cytosolic domains of plasma membrane receptors CD40 and RANK (Hsu et al., 1995).

The first known TRAFs (TRAF1 and TRAF2) were identified as proteins associated with TNFRII through biochemical fractionation and yeast two-hybrid stuides (Rothe et al., 1994). Other members were later identified as CD-40 interacting proteins through yeast two-hybrid screening (Hu et al., 1994; Sato et al., 1995). TRAF7 was identified through high-throughput proteomic analysis of components in human TNF-NF-κB signaling pathway (Bouwmeester et al., 2004) TRAFs play a pivotal role in diverse processes such as inflammation, immunity and apoptosis (Bishop, 2004; Chung et al., 2002). TRAFs, especially the most studied TRAF2 and TRAF6, are critical for the regulation of NF-κB activation. TRAF2 and TRAF5 function redundantly in TNFα signaling for NF-κB activation (Wajant & Scheurich 2001). TRAF6 is essential for signaling by IL-1R, CD40,

RANK (TRANCE-R), and Toll-like receptors (Cao et al., 1996; Ishida et al., 1996; Lomaga et al., 1999; Naito et al., 1999; Schwandner et al., 2000; Naito et al., 2002). TRAF3 is required for NIK activation, and TRAF2 negatively regulates NIK abundance in cells (Xia & Chen, 2005) in the non-canonical NF-κB pathway. TRAF1 is a negative regulator of NF-κB and AP-1 activation by TNFα (Tsitsikov et al, 2001), which has been contributed to its lack of RING domain.

The biochemical function of TRAF proteins in the canonical NF-κB signaling pathway, especially TRAF2 and TRAF6, has been investigated. The RING and Zinc-finger domains of TRAF2 and TRAF6 are required and sufficient for IKK-NF-κB and JNK activation in cell transfection studies (Baud et al., 1999). *In vitro* studies have shown that TRAF2 and TRAF6 function as ubiquitin ligases through their N-terminal RING domains that, together with E2 Ubc13/Uev1A, catalyze Lys63-linked polyubiquitin chain synthesis and lead to activation of IKK (Deng et al., 2000. See more discussion in later section).

I.A.1.d TAK1 complex

As shown in Figure 1, the kinase upstream of IKK in the canonical NF-κB pathway is the TAK1 complex. Mammalian TAK1 was originally discovered through complementation screening of a murine cDNA library in a Ste11p/MAPKKK deficient strain of yeast and as a TGFβ-activated kinase (Yamaguchi et al, 1995). In cells, TAK1 exists as a kinase complex with either

TAB1 and TAB2 or TAB1 and TAB3 (Shibuya et al, 1996; Kanayama, 2004). TAK1 is the kinase subunit of the complex while TAB1 and TAB2 or TAB3 are the regulatory subunits of the complex. TAK1 kinase belongs to MAPKKK (M3K) and so is also called MAKKK7 (M3K7). Phosphorylation of Thr.187 in the kinase domain (numbering in human TAK1) is required for TAK1 activation. TAK1 can activate MAP kinases and NF-κB. TAK1 has an important role in animal development (Lu et al., 2007). It has been shown that TAK1 is essential for mesoderm induction and patterning in early Xenopus development, for the control of cell shape and regulation of apoptosis in Drosophila, and for vascular development in mice. Knockout of TAK1 in mice is embryonic lethal.

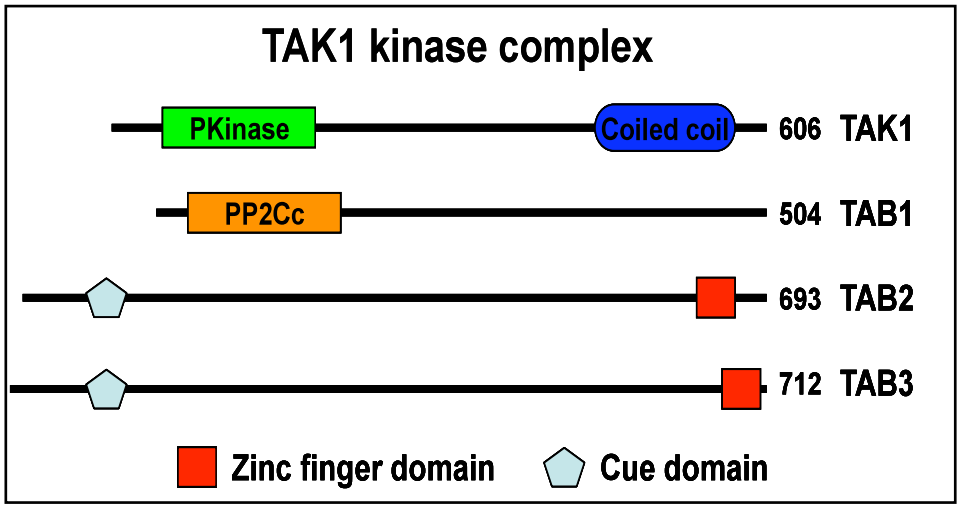


Figure 5 Structural domains of subunits of TAK1 kinase complex. TAK1 kinase exists in cells as a complex with TAB1 and TAB2, or with TAB1 and TAB3. TAK1 is the kinase subunit with a kinase domain at its N-terminus and a coiled-coil domain at its C-terminus. TAB1 has a pseudo-phosphatase domain PP2Cc at its N-terminus. TAB2 and TAB3 are two homologues sharing similar domain organization with a Cue domain at their N-termini and a ZnF domain at their C-termini. Both Cue and ZnF domains can bind polyubiquitin

chains. The polyubiquitin chain binding by the ZnF motif in TAB2 and TAB3 is required for TAK1 activation by pro-inflammatory stimuli.

TAB1 was identified through yeast-two-hybrid analysis using TAK1 as the bait (Shibuya et al., 1996). It has a pseudo phosphatase PP2Cc domain but this domain doesn't have any phosphatase activity (Lu et al., 2007). TAB1 is important for activation of TAK1 by TGFβ (transformation growth factor β) and BMP (bone morphogenic protein) (Birkey Reffey et al., 2001; Yamaguchi et al., 1999).

TAB2 was identified in the same study that isolated TAB1 (Shibuya et al., 1996). TAB3 was identified through its similarity to TAB2 (Kanayama et al., 2004). TAB2 and TAB3 are homologous and share a very similar domain organization. Both have a Cue motif and a specialized ZnF motif. TAB2 and TAB3 are important for TAK1 activation by pro-inflammatory stimuli. Biochemical studies have shown the ZnF motif of TAB2 and TAB3 have polyubiquitin chain binding activity and this binding activity is required for TAK1 activation by TRAF6 (Kanayama et al., 2004).

A number of studies using *in vitro* assays and transfected cells show TAK1 kinase plays an important role in IKK-NF-κB activation by TNFα, IL1β, or LPS. In a cell-free system for activation of IKK by incubating Hela S100 with recombinant TRAF6, the TAK1 complex was the only kinase complex responsible for IKK activation after chromatographic purification. TAK1

phosphorylates the activation loop of IKKβ directly (Wang et al., 2001). TAK1 was recruited to TRAF6 in IL1β signaling (Ninomiya et al., 1999). TAK1 complex was recruited, together with the IKK complex, to the TNFRI when cells were stimulated with TNFα (Ea et al., 2006). RNAi-based knockdown of TAK1 affected TNFα and IL1β signaling (Takaesu et al., 2003). Double knockdown of Tab2 and Tab3 by RNAi impaired TNFα and IL1β signaling to IKK-NF-κB as well as JNK/p38 (Ishitani et al., 2003; Kanayama et al., 2004). Conditional knockout studies showed that in MEF and B cells TAK1 is required for TNFα, IL1β, Toll-like receptor and CD40 signaling to IKK-NF-κB and JNK activation (Sato et al., 2005; Shim et al., 2005). Somatic genetics-based TAK1 knockout in chicken DT40 B cell line abolished IKK, JNK activation in BCR signaling (Shinohara, et al., 2005). TAK1 knockout in mouse B cells, however, showed BCR signaling to IKK-NF-κB is normal although JNK activation is impaired (Sato et al, 2005).

I.A.2 NF-κB signaling pathways

There are two, or possibly three, signaling pathways that lead to NF-κB activation (Figure 1). One is the so-called canonical (or the classical) pathway (see discussion below). The other is the non-canonical (or alternative) pathway (Figure 1). This pathway is largely for activation of p100/RelB complexes during B-and T-cell organ development and is limited to a few receptor signaling events

such as those elicited by lymphotoxin B receptor, B-cell activating factor (BAFF) receptor and CD40 signaling. When a ligand binds to a receptor, such as BAFF binding to BAFFR or CD40 ligand (CD154) binding to CD40, it leads to activation of the NF-κB-inducing kinase NIK, which then phosphorylates and activates the IKKα-only-containing kinase dimer (instead of the whole IKK complex). Activated IKKα in turn phosphorylates two serine residues adjacent to the ankyrin repeat of p100, leading to its partial proteolysis by the proteasome and liberation of the p52/RelB complex, which then translocates to the nucleus and induces target gene expression important for the proliferation and maturation of B cells. TRAF2 and TRAF3 have been implicated in the regulation of NIK activation and thus the non-canonical NF-κB pathway. Other distinct NF-κB pathways may also exist. For example, p50 and p52 homodimers enter the nucleus and become transcriptional activators after forming complex with the IκB-like co-activator Bcl-3 or IκBζ, although the upstream signaling pathways are unknown (Perkins, 2006).

The most well-studied NF-κB pathway is the canonical NF-κB signaling pathway. Several related signaling pathways that lead to IKK-NF-κB activation have been extensively studied and are shown in Figure 1 and discussed briefly here. The main modules consist of a ligand binding to its cognate receptor, either on the plasma membrane, intracellular membrane or in the cytosol, to initiate the signaling. This is then transmitted to intracellular adaptors and one or a few

TRAF proteins. TRAF proteins then catalyze polyubiquitination to activate the TAK1 complex, which in turn activates IKK. Activated IKK then phosphorylates IκB proteins which leads to activation of NF-κB (Chen, 2005).

In the TNFα pathway, TNFα binds to its receptor TNFRI or TNFRII, which triggers trimerization of the receptor and recruits TRADD, RIP1 and TRAF2. RIP1 and TRAF2 are ubiquitinated by K63-linked polyubiquitin chains, which recruits TAK1 kinase complex and IKK kinase complex leading to activation of TAK1 and IKK.

In the IL1R and TLR signaling pathway, IL1β binding to IL1R or bacterial-derived products such as LPS binding to TLR4, leads to recruitment of MyD88, IRAK4, IRAK1 and TRAF6. IRAK1 and TRAF6 are ubiquitinated by K63-linked polyubiquitin chains, which recruits TAK1 kinase complex and IKK kinase complex leading to activation of TAK1 and IKK.

In the TCR and BCR signaling, antigen binding recruits the Src family tyrosine kinases ZAP70 and Syk, leading to production of second messengers IP3 and DAG. IP3 and DAG activate PKCθ (TCR) or PKCβ (BCR). PKC then phosphorylates CAMRA1 resulting in activation and oligomerization of CARMA1-BCL10-MALT1 (CBM) complex. Oligomerized CBM recruits TRAF6 (and also probably TRAF2), and activates its ubiquitin ligase activity leading to TRAF6 autoubiquitination and NEMO ubiquitination. Ubiquitinated TRAF6, and also probably ubiquitinated NEMO, help to recruit both the TAK1 complex and

the IKK complex leading to activation of TAK1 and IKK (Sun et al., 2004; Thome, 2004).

In the CD40 and RANK pathways, ligand binding leads to recruitment of TRAF proteins through their cytosolic TRAF binding motifs and activation of TAK1 and IKK kinase complex.

Intracellular bacteria also activate IKK-NF-κB pathway through NOD pathways. NOD1, a cytosolic sensor for bacterial products meso-DAP, and NOD2 for MDP, initiates the signaling to CARD9 and RIP2, which undergoes K63-linked polyubiquitination that somehow leads to activation of IKK (Kanneganti et al., 2007).

In the DNA damage-induced NF-κB activation pathway, DNA damage triggers sequential sumoylation and ubiquitination of NEMO, which leads to translocation of NEMO from cytosol to nucleus and then back to cytosol where ubiquitinated NEMO somehow leads to activation of IKK (Huang et al., 2003).

When cells are infected by viruses, they activate both NF-κB and IRF3 pathways. In the case of RNA viruses, the cytosolic receptor for double stranded RNA, RIG-I, is activated and undergoes K63-linked polyubiquitination (Gack et al., 2007), which in turn transmits the signal to a mitochondrion-localized intermediate protein MAVS (which is also called IPS-1, VISA and CARDIFF) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). MAVS, by an unknown mechanism, activates IKK and its related kinase TBK1,

leading to activation of NF-κB and IRF3, respectively.

I.B Ubiquitination system

I.B.1 Ubiquitination machinery

In cells, many proteins undergo post-translational modifications under certain conditions. As many as 300 post-translational modifications of proteins are known to occur physiologically in cells (Witze et al., 2007): phosphorylation, acetylation, methylation, ubiquitination and so on.

Ubiquitin is a small protein of only 76 amino acids that is ubiquitously expressed in all cells and is highly conserved from yeast to human. Modification of proteins by ubiquitin is achieved through the process called ubiquitination in which the carboxyl group of the last glycine residue at the C-terminus of ubiquitin is covalently attached to the ε-amino group of a lysine residue on the target protein resulting in the formation of an isopeptide bond. The ubiquitination process is a coordinated three-step enzymatic reaction (Figure 6) (Pickart 2001). In the first step, the C-terminus of a ubiquitin is activated by formation of a Ub-adenylate intermediate catalyzed by ubiquitin-activating enzyme 1 (E1) followed by the reaction of this intermediate with the active site cysteine residue of E1 to form a high energy E1~Ub thioester. In the next step, the ubiquitin on the E1~Ub is transferred to a ubiquitin-conjugating enzyme (E2) via formation of an E2~Ub

thioester. Finally, in the presence of a ubiquitin ligase (E3), ubiquitin is ligated to a lysine residue of the target protein through an isopeptide bond.

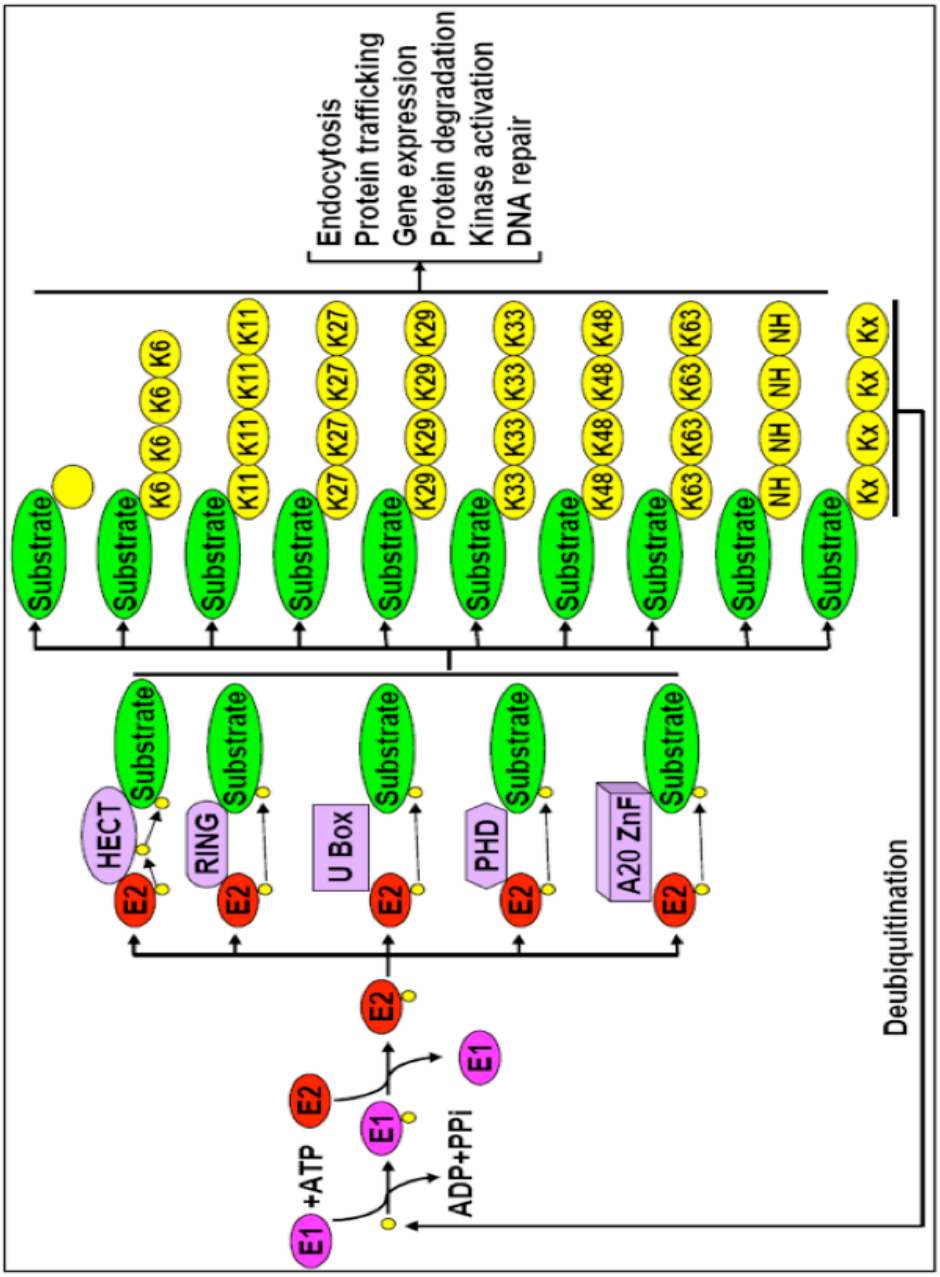


Figure 6 The protein ubiquitination system. The ubiquitination cascade involves three enzymatic steps. Ubiquitin is first activated by the ubiquitin

activation enzyme E1 with the hydrolysis of ATP. Ubiquitin is then transferred to a ubiquitin conjugating enzyme E2. Ubiquitin loaded on E2 is then transferred to a substrate with the help of ubiquitin ligase E3. Five subfamily of E3s have been described (HECT-, RING-, U box-, PHD-, and A20 ZnF-domain containing E3s). In the case of HECT-domain E3s, ubiquitin is first transferred to E3s from E2 and then passed to a substrate. Protein substrate can be mono- or poly-ubiquitinated. Polyubiquitin chains can be K6, K11, k27, k29, k33, K48, or K63 linked. Linear polyubiquitin chains (shown as 'NH' linkage) and mixed-linkage chains (shown as 'Kx' linkage) have also been reported. Mono- or poly-ubiquitination is rich in information and involved in protein degradation, endocytosis and intracellular protein trafficking, kinase activation and DNA repair.

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 a thioester, the other as an adenylate (Hershko & Ciechanover, 1998). Until recently it was believed that a single ubiquitin E1 activates ubiquitin for the entire array of downstream conjugating enzymes (E2). But in 2007 three different groups have shown in human and mouse genomes there is another E1 that can activate and transfer ubiquitin to a subset of E2s (Jin et al., 2007; Chiu et al., 2007; Pelzer et al., 2007).

In the ubiquitination cascade, the second step is catalyzed by ubiquitin-conjugating enzymes called E2. All E2s share a conserved, about 150 amino acid long core domain called Ubc domain. For some E2s, the core domain often comprises the entire protein. Some other E2s have additional N- or C-terminal or N- and C- terminal extensions which may facilitate specific interactions with cognate E3s, substrates, or regulate E2 activity (Pickart 2001). The human

genome encodes more than 40 E2s. Free E2s have relatively low affinity for free ubiquitin or free E1, but a high affinity for the ubiquitin-loaded E1 or for their cognate E3s (Haas et al., 1988; Hershko et al., 1983; Miura et al., 1999). The 3D structures of several E2s have been solved. These structures show overall similarity among the E2s and do not show dramatic conformational changes when they are in various protein complexes (VanDemark & Hill, 2002).

In the final step of the ubiquitination, a ubiquitin ligase E3 facilitates ligation of activated ubiquitin to a cognate substrate. E3, as an enzyme, binds E2, E2~Ub, and specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, to the protein substrates or existing polyubiquitin chains from a thioester intermediate. All characterized E3 can be roughly divided into five subfamilies depending on the feature of their ligase domains: HECT domain E3, RING finger E3, PHD domain E3, U-box E3 and A20-ZnF E3 (Figure 6).

HECT (Homologues to E6-AP C-terminus) domain E3s are a large family and their members harbor an approx. 350 amino acid C-terminal region homologous to that of E6-AP, the founding member of this family. The N-termini of these proteins are highly variable and may be involved in cognate protein substrate recognition and binding. HECT domains have a catalytic cysteine which forms a thioester intermediate with ubiquitin transferred from E2~Ub intermediates during the ubiquitination reaction cascade (Scheffner et al., 1995). This feature is unique among all known E3s. The prototype of this subfamily is

E6 associated protein (E6-AP). The N-terminus of E6-AP mediates substrate recruitment while its HECT domain binds E2~Ub and transfers ubiquitin to a conserved cysteine residue at ~35 residues upstream of the C-terminus (Pickart & Eddins, 2004).

RING finger E3s display a series of histidine and cysteine residues with a characteristic spacing ($C\text{-}X_2\text{-}C\text{-}X_{9\text{-}39}\text{-}C\text{-}X_{1\text{-}3}\text{-}H\text{-}X_{2\text{-}3}\text{-}C/H\text{-}X_2\text{-}C\text{-}X_{4\text{-}48}\text{-}C\text{-}X_2\text{-}C$) (Saurin et al., 1996) that allows for the coordination of two zinc ions in a cross-brace structure. The zinc ions and their ligands are catalytically inert and it is the spacing of the zinc ligands rather than any primary sequence that is conserved in the RING finger family. These features suggest that RING fingers function not as chemical catalysts but as molecular scaffolds which bring together other proteins (Borden 2000). RING domain, together with surrounding sequences, can bind E2s in a specific and catalytically productive manner. RING E3s come in two forms. One is single-subunit RING finger E3, typified by c-Cbl, the other is a multi-protein complex in which RING finger protein is just one subunit, typified by the Skp1, cullin, F-box (SCF) E3, the von Hippel-Lindau (VHL) E3 and the anaphase promoting complex (APC) (Jackson et al., 2000). 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 (Zheng et al., 2002). 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 similar 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 (Kaelin et al, 1998). Similar to the SCF E3 and VHL E3, the APC E3 contains a cullin homologue, Apc2, and a RING finger protein, Apc11 (Zachariae et al., 1999). The composition of APC is more complex than the SCF E3 and the VHL E3.

U-box E3s (UFD2 homology (U-box) E3 (Pickart & Eddins, 2004)) define a small family of proteins that share a sequence motif called the U-box. The U-box adopts a conformation similar to that of RING finger, with stabilization being achieved by electrostatic interactions rather than by metal ion coordination (Aravind and Koonin, 2000). Ufd2 was originally identified as an "E4" as it promotes the polyubiquitination of another E3's substrate (Koegl et al., 1999). Later it was shown that the U-box resembles the structure of RING domain (Aravind et al., 2000; Ohi et al., 2003). Furthermore, several U-box proteins interact directly with E2s and promote autoubiquitination (Hatakeyama et al., 2001; Pringa et al., 2001; Murata et al., 2001; Jiang et al., 2001). These findings suggest that U-box proteins are indeed conventional E3s.

PHD domain containing E3s are found in MEKK1, a MAPKKK protein kinase family member, and several viral-encoded proteins such as KSHV MIR-1

and MIR-2 (Coscoy & Ganem, 2003). MEKK1 has an N-terminal PHD (plant homeodomain) domain that displays strong sequence similarity to a RING finger domain, which displays E3 ligase activity toward ERK1 and ERK2. Mutation of cysteine 441 to alanine abolishes *in vitro* ubiquitination of ERK (Lu et al, 2002). MEKK1 also targets itself for ubiquitination and this is also dependent on the PHD domain of MEKK1 (Witowsky et al., 2003).

A20-type ZnF may represent a novel class of ubiquitin ligases. A20 has a deubiquitination domain named OTU at its N-terminus and multiple ZnF motifs at its C-terminus. *In vitro* assays have shown the ZnF region of A20 has E3 activity. This activity has been attributed to its ZnF4 motif through mutagenesis studies (Wertz et al., 2004). A20 ZnF4 is characterized by an acidic residue in the third position and a large residue in the eighth position distal to the fourth conserved cysteine. This acidic residue and the polar helix on which it is located could form a critical interaction surface required for ubiquitin transfer. A20-type ZnFs are found in all eukaryotes and form fusions with a variety of domains involved in ubiquitin signaling pathways (Wertz et al., 2004).

E3s don't share sequence similarities except in their ligase domains within the same subfamilies, making it difficult to predict new E3 members by bioinformatic approaches. Although about 500 different E3s have been predicted in the human genome based on information from known E3s, it is conceivable that new families of E3s will be uncovered with more studeis.

I.B.2 Polyubiquitination

One major feature about ubiquitination is that one ubiquitin can be linked to another ubiquitin forming inter-ubiquitin isopeptide bonds. Through multiple rounds of ubiquitination, polyubiquitin chains are formed. There are seven lysine residues in ubiquitin. All of these seven lysine residues can be used for chain synthesis resulting in K6, K11, K27, K29, K33, K48, or K63 chains. All of these linkages have been detected *in vivo* using mass spectrometry (Peng et al., 2003). Although all the seven lysine residues can be used for chain synthesis, their abundance in cells are different in which K48 linkage is the most abundant followed by K63 and K11 chains and the less frequent K33, K27, K6, and K29 chains (Peng et al., 2003). Mixed linkage chains and branched polyubiquitin chains have also been reported (Ben-Saadon et al., 2006; Kirkpatrick et al., 2006). A recent report has shown linear polyubiquitin chains in which C-terminus of one ubiquitin is linked to the N-terminal methionine of another ubiquitin through a bona fide peptide bond (Kirisako et al., 2006).

In general, ubiquitination and polyubiquitin chain synthesis requires E3s. However free ubiquitin chains can be synthesized by E2 alone without the presence of any E3s. The most studied and earliest example is the mammalian E2-25K which is capable of assembling K48-ubiquitin chains (Chen et al., 1991). The heterodimeric E2, Ubc13/Uev1a, can catalyze K63-ubiquitin chains without any

E3s (Petroski et al., 2007). In these two examples, free polyubiquitin chains as opposed to protein conjuagted ub chains are created. In another report, some ubiquitin-binding motif-containing proteins undergo monoubiquitination without any involvement of E3 (Hoeller et al., 2007). Normally, ubiquitin is conjugated to another protein on lysine residues through isopeptide bonds; an exception has been reported where ubiquitin is linked to a substrate through Cysteine (Cadwell & Coscoy, 2005)

Not much has been known about how the chain linkage specificity is determined. In some cases it is the E2 that determines the linkage specificity. The crystal structure of Ubc13/Uev1a heterodimeric E2 clearly explains why it specifically catalyzes K63 linked chains. In this system, Uev1 binds a ubiquitin molecule and only positions its K63 near the active site cysteine of Ubc13, thus limiting chain extension to only K63 (VanDemark et al., 2001).

There are many other open questions in the field of polyubiquitination, such as whether and how the length of a chain is determined, mechanistically how polyubiquitin chains are assembled, and whether polyubiquitin chains are just linear random, irregular coils or they have defined higher-order structures. If different chains catalyzed by different E3s adopt different higher-order structures, how are they achieved?

In terms of the polyubiquitin chain assembly, currently four models have been postulated (Hochstrasser 2006). The following questions are pertinent: 1), Is

ubiquitin transferred to a target protein one by one or as a pre-assembled polyubiquitin chains? 2), If the transferred entity is pre-assembled polyubiquitin chains, where does it happen: on E2s, on E3s, or as un-anchored free chains? 3) Which end is the growing site for chain extension, the proximal end or the distal end?

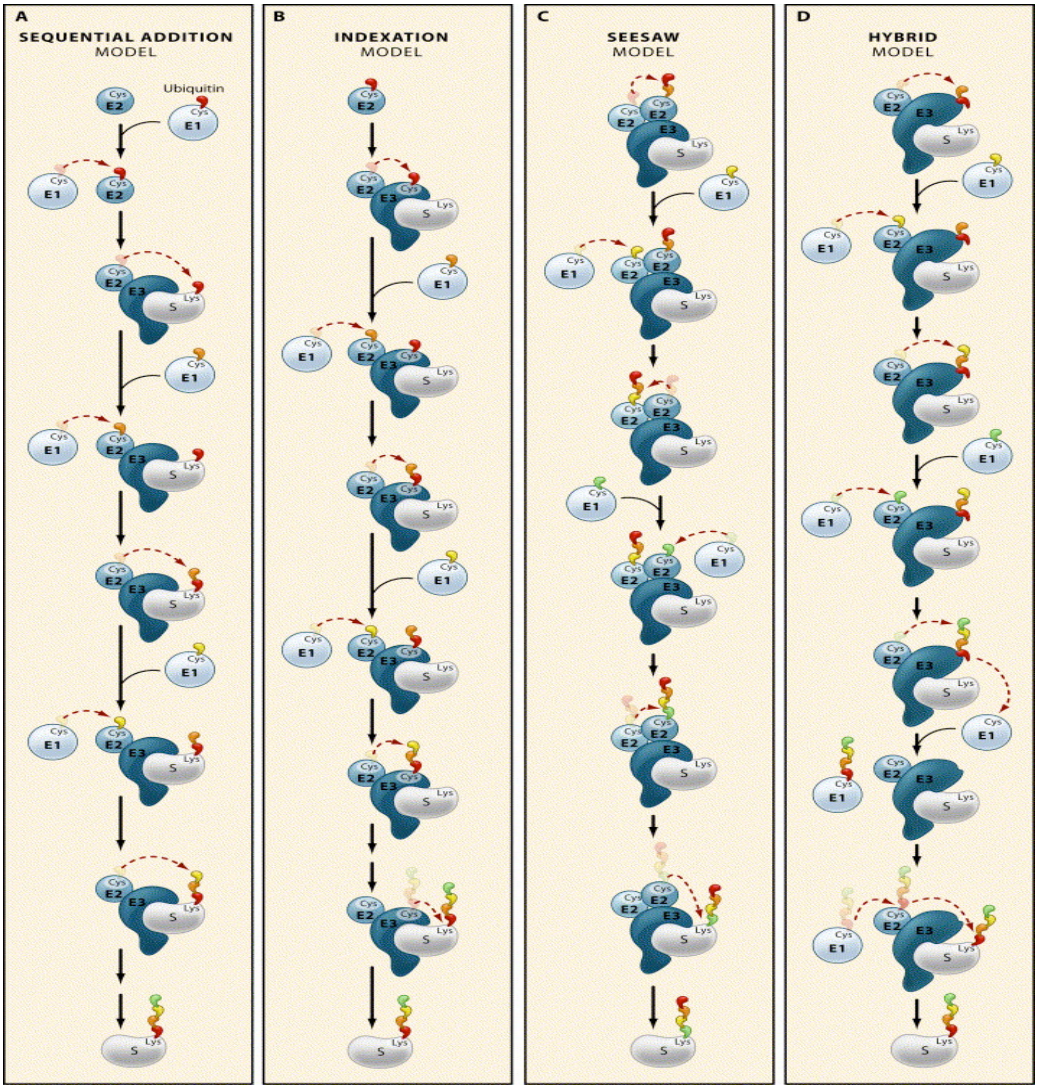


Figure 7. Models for Polyubiquitin-Chain Synthesis (A) Sequential

addition model, in which ubiquitin molecules are added one at a time, first to a lysine on the substrate protein (S) and then to a specific lysine in the ubiquitin at the distal end of the growing ubiquitin chain. A RING E3 is depicted and is assumed to remain associated with the substrate through multiple rounds of ubiquitin addition. **(B)** Indexation model (Verdecia et al., 2003). The ubiquitin chain is first built on the active-site cysteine in the HECT domain of the E3 ligase before ultimately being transferred to the substrate. A flexible hinge between two lobes of the HECT domain (not depicted) allows repositioning of the ubiquitin chain such that a lysine in the distal end of the chain is oriented for attack on the E2~ubiquitin thioester. The chain is "indexed" to a limited length because of the physical constraints imposed by the hinge in E3. **(C)** Seesaw model. Ubiquitin chains are built by a pair of E2s (either a homo- or heterodimer), which pass the growing chain back and forth between the two E2 active sites, before being transferred to the substrate. During chain assembly, the attacking lysine is always on a monomeric ubiquitin that is in thioester linkage to an E2. An E3 HECT domain could replace one of the E2s in this model. The order of ubiquitin addition is opposite to that in the other three models. **(D)** Hybrid model. The ubiquitin chain is assembled prior to transfer to the substrate, but a noncovalent interaction between (poly)ubiquitin and a site in the E2 or E3 (the latter is depicted) positions it for nucleophilic attack on the E2~ubiquitin thioester, much as in the sequential addition model. At some point, the free end of the ubiquitin chain must be activated by E1 and transferred to the E2 cysteine before the final transfer of the chain to the substrate. (Adopted from Hochstrasser, 2006).

The first model is the "Sequential addition model" which posits that ubiquitins are added one at a time, first to the substrate protein and then to the distal end of the growing ubiquitin chain. Historically this is a logical extension of a monoubiquitination reaction in which a to-be-ubiquitinated lysine of a substrate attacks ubiquitin-E2 thioester bond finishing the transfer of the first ubiquitin from E2 to the substrate. In each subsequent cycle, the ubiquitin most distal from the substrate provides lysine to attack ubiquitin-E2 thioester bond. This model can explain how a relatively short chain is assembled. However polyubiquitin chains

are normally very long (over a dozen ubiquitin moieties) (Varshavsky, 2005), and the distal ubiquitin becomes further away from the E3-substrate and upcoming ubiqutin-E2. Although this model can be accommodated by proposing that growing chains can be looped out to shorten the distance between the distal ubiquitin and the incoming ubiquitin-E2 complex, there are no strong data to support this model.

The second model is called the "indexation model" (Verdacia et al., 2003). This model propses that the polyubiquitin chain is first built on the active site cysteine in the HECT domain of E3s and then the whole ubiquitin chain is transferred to the substrate. The model is suggested by the observation that in HECT domain-containing E3s, the HECT domain has two lobes connected by a flexible linker and this flexibility is essential for E3 activity. So the sequential transfer of ubiquitin from an incoming ubiqutin-E2 to the distal ubiquitin of a ubiquitin chain that has already tethered to the E3 cysteine would be accommodated by progressive extension of the hinge between the two HECT domain lobes. However the HECT domain can only hold tetra-ubiquitin, implying the ubiquitin chain is limited to four ubiquitins, and currently there is no evidence showing tetra-ubiquitin on a substrate is the chain length limit.

The third model is called "Seesaw model". This model, like the 'indexation model", also proposes that a polyubiquitin chain is assembled first and then transferred to the substrate. But the way the polyubiquitin chain is assembled is

different from that of “indexation model”. In this model, polyubiquitin chain is assembled by a pair of E2s or an E2-HECT domain pair (there are two active cysteines in proximity) to pass a growing ubiquitin chain back-and-forth between the two active site cysteines. During chain assembly, the attacking lysine always comes from a monomeric ubiquitin that is in a thioester bond with an active site cysteine. In this model, the latest ubiquitin added is always at the proximal end of the chain unlike in other models where the latest ubiquitin added is at the distal end. The observation that E2s can form dimers both *in vitro* and *in vivo*, particularly when E2 are charged with ubiquitin favors this model. Homodimers of yeast Cdc34 E2 are induced by ubiquitin thioester formation and this homodimers are required for its function (Varelas et al., 2003). Heterodimer formation between Ubc6 and Ubc7 E2s has been reported (Chen et al, 1993).

The final model is “hybrid model”. In this model polyubiquitin chain is also synthesized first before being transferred to the substrate. Here a growing polyubiquitin chain noncovalently interacts with E2 or E3, which positions it to attack the incoming ubiquitin in the ubiquitin-E2 complex. The growing ubiquitin chain has a free un-anchored C-terminus. At some point the free un-anchored polyubiquitin chain is re-activated by E1 and then transferred to E2, E3 and finally to a substrate. Consistent with this model, some E2s such as E2-25K and Ubc1 (Merkley et al., 2005) and some E3s have ubiquitin binding domains although their functional significance in polyubiquitin chain synthesis has not

been investigated extensively yet.

Overall there are few published results addressing the mechanistic problem concerning polyubiquitin chain assembly. In one report (Petroski and Deshaies, 2006), assembly of polyubiquitin chain by Cdc34 E2-SCF^{Cdc4} E3 pair on pre-monoubiquitinated substrate was much faster than that on the same un-modified substrate. These results are consistent with the "sequential addition model". In another report, it was shown that when the E3 E6-AP was loaded with a ubiquitin on its active site cysteine, lys48 of this ubiquitin attacked the ubiquitin thioester bond on the cognate ubiquitin-E2, which resulted in ubiquitin dimer formation. This result is consistent with both the "indexation model" and "seesaw model" (Wang & Pickart, 2005). In the same report, another HECT E3, KIAA10 showed a different behavior in which K48 and K29 linked chains were synthesized as free entities (Wang & Pickart, 2005), which is consistent with the "sequential model" or the "hybrid model". In a more recent report (Rape et al., 2006), substrate ubiquitination by the RING finger-containing multisubunit APC (anaphase promoting complex) required multiple APC binding events for long chain synthesis. Pre-incubation of E1, E2, E3 and ubiquitin prior to substrate addition didn't enhance subsequent polyubiquitination of the substrate. Therefore it is possible that different E2-E3 pairs might use different mechanisms to assemble their polyubiquitin chains.

I.B.3 Ubiquitin-binding motifs

There are over a dozen classes of specific ubiquitin-binding motifs that recognize and bind ubiquitin chains and mediate the effects of protein ubiquitination. Since the first ubiquitin binding motif UBA (ubiquitin associated) was described, the number and kinds have now been expanded to at least 16: UBA (Ubiquitin associated), UIM (ubiquitin interacting motif), MIU (motif interacting with ubiquitin), DUIM (double-sided UIM), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation), GAT, NZF, A20 ZnF, UBP ZnF, UBZ, Ubc, UEV, UBM, GLUE, Jab1/MPN, PFU (Hurley et al., 2006) and NUB (Ea et al, 2006). They recognize various surfaces on ubiquitin and show a wide range of affinities for mono-ubiquitin. They also catalyze auto-ubiquitination of the proteins in which they reside.

The largest class of ubiquitin-binding domains are alpha-helical based: UBA, UIM, DUIM, MIU, CUE, and GAT and TOM (target of Myb)). All these motifs interact with ubiquitin at a single region surrounding the Ile44 hydrophobic patch of ubiquitin. Structural studies have shown that the UIM consists of a single-alpha helix, centered on a conserved alanine residue, which packs against the Ile44 of ubiquitin during the binding (Fisher et al., 2003). UIMs have low binding affinity for mono-ubiquitin which is in the 100uM to 2mM range (Fisher at al., 2003). These motifs have been found in trafficking proteins that recognize ubiquitinated cargoes, and the S5a subunit of proteasome. Two variants of UIM,

MIU and DUIM, have been described. MIU, as found in Rabex-5, is a single helix centered on the alanine (Penengo at al., 2006). In the DUIM, as reported for yeast protein Vps27, two UIM sequences are interlaid on a single helix such that both faces are capable of binding to ubiquitin (Hirano at al., 2006). UBA (Mueller at al, 2003), Cue (Kang et al., 2003) and GAT (Mattera et al., 2004) domains are all compact three-helical bundles (α1, α2, α3), in which helices α1 and α3 form a hydrophobic surface that binds to the hydrophobic patch surrounding Ile44 of ubiquitin.

ZnF domains are the second largest class of ubiquitin-binding domains and have been recognized in Npl4, A20 ZnF, UBP ZnF and TAB2 and TAB3 (TAK1 Binding protein 2 or 3) (Kanayama et al., 2004) ZnF. The ZnF ubiquitin-binding domains offer much more diversity in recognition and binding affinity than the helical ubiquitin-binding domains. ZnF domains recognize three different regions on the surface of ubiquitin and bind with affinities in the range of about 1uM to nearly millimolar, which is mirrored by their wide range of biological roles (Hurley et al., 2006). NZF domains, as found in TAB2 and TAB3, are approximately 30-residues long built around a single zinc-binding site. A Thr-Phe pair in the first 'zinc-knuckle' and a hydrophobic residue in the second knuckle (the "TFΦ fingerprint"), which make hydrophobic contacts with the ubiquitin Ile44 patch (Alam et al., 2004), seem to distinguish ubiquitin-binding ZnF motif from those NZF domains, which don't bind ubiquitin. The A20 ZnF domains use

a pair of aromatic residues and several polar residues to bind a predominantly polar patch on ubiquitin centered on Asp58, which doesn't overlap with the Ile44 patch. A20 ZnF domains contribute to the ubiquitin ligase activity of A20. The ZnF UBP domains found in some deubiquitination enzymes and in the deacetylase HDAC6 are about 130 residues in length built around a single zinc-binding site in its N-terminal half which is fused to a α/β fold (Reyes-Turcu et al., 2006). The free C-terminal glycine residue of ubiquitin is required for ZNF UBP binding.

Recently our lab also characterized a novel ubiquitin-binding motif in NEMO, NUB, (Ea et al., 2006). This motif is about 70 amino acids long and its binding to polyubiquitin chains is required for activation of IKK by the polyubiquitination system. The 3D structure of NUB, however, is not available yet.

The Ubc domains of the ubiquitin conjugating enzyme E2s can also bind ubiquitin non-covalently. UbcH5c binds non-covalently to the Ile44 patch of ubiquitin via its β-sheet (Brzovic et al., 2006) with an affinity about 30 μM. The Ubc domain variant, Uev domains, which adopt the same α/β fold as in Ubc domains except the lack of a catalytic cysteine, also bind ubiquitin. Uev1, the obligate binding partner of Ubc13 for its E2 catalytic activity, binds ubiquitin with an affinity of 100 μM (McKenna et al., 2003).

As in the case for ubiquitin E3 ligases where they don't share much sequence conservation among the subfamilies, the ubiquitin binding motifs described above also show a wide range of sequence diversities among different classes. One would expect, with high confidence, more and more ubiquitin binding motifs will be discovered through a variety of techniques including bioinformatics and biochemistry.

Most of the studies concerning ubiquitin binding motifs, their structures and affinities with ubiquitin were performed with mono-ubiquitin, outside of the environment of targeted proteins and polyubiquitin chains. How much of that information can be translated to interaction between ubiquitin binding motifs and polyubiquitin chains and/or in the presence of targets is not clear yet. There is currently not much information regarding the mechanisms for discrimination among mono-, poly-, multimono- ubiquitination targets, or among linkage-specific polyubiquitin chains by these varieties of ubiquitin binding motif-containing proteins.

I.B.4 Deubiquitination enzymes

Polyubiquitination is a reversible process. There are dozens of deubiquitination enzymes (DUBs) that can reverse the processes. About 80 different Dubs in the human genome have been predicted. These enzymes are an integral part of the ubiquitination system and function in processing inactive

ubiquitin precursors, proofreading of ubiquitin-protein conjugates, removing and dissembling of polyubiquitin chains and maintaining the pool of free monoubiquitins.

DUBs specifically cleave ubiquitin-linked molecules right after the C-terminal carbonyl of the last glycine residue of ubiquitin, such as peptide bonds in ubiquitin precursors, isopeptide bonds in polyubiquitin chains, and ester bonds in some chemically-made ubiquitin derivatives such as ubiquitin-AMC.

Based on sequence similarities and likely mechanism of cleavage, DUBs can be grouped into roughly five distinct subfamilies (Amerik & Hochstrasser, 2004). The first four subfamilies are specialized types of cysteine proteases while the fifth one is zinc-dependent metalloprotease.

1) UBP (ubiquitin-specific processing protease) subfamily. This is the largest and most diverse group of DUBs. These family members contain two short well-conserved motifs, Cys and His boxes with all the catalytic triad residues in them. The deubiquitination enzyme IsoT is in this subfamily. It acts preferentially on free, unanchored polyubiquitin chains by a sequential exo-protease mechanism starting from the end of the chains that contains a free C-terminus and very sensitive to any modifications on this free C-terminal glycine (Wilkinson et al., 1995). CYLD, the protein mutated in human familial cylindromatosis and discussed more in I.C.7, is in this family. Faf, a UBP subfamily DUB encoded in Drosophila, deubiquitinates and therefore stabilizes its substrate lqf to control fly

eye development. Mutations in either faf or lqf lead to a defect in eye development (Huang et al., 1995). Another UBP dub HAUSP, can deubiquitinate p53 that is ubiquitinated by Mdm2 to control the cellular level of p53 (Li et al., 2002). Herpes simplex virus protein ICP0 can bind HAUSP and may control HAUSP activity toward particular proteins *in vivo* (Everett et al., 1997). Ubp8 has been shown to deubiquitinate ubiquitin-H2B and therefore regulate gene transcription (Henry et al., 2003).

2) UCH (ubiquitin carboxy-terminal hydrolases) subfamily. UCHs were originally identified by their ability to hydrolyze amides and esters at the C-terminus of ubiquitin. This subfamily generally consists of small proteins. Yuh1 in yeast and UCH-L1 and UCH-L3 are in this subfamily and are responsible for generation of mature mono-ubiquitin from ubiquitin precursors, which are either expressed as linear ubiquitin with multiple copies in one polypeptide or fusion proteins with ribosome subunit (Finley and Chau, 1991). A neuronal-specific UCH, Ap-Uch in *Aplysia*, is required for long-term potentiation (Hegde et al., 1997).

3) OTU (Ovarian tumor)-related subfamily. This domain was predicted by bioinformatics based on their structural similarity in a presumed catalytic core domain containing conserved Cys, His and Asp residues thought to comprise the proteolytic triad of protease (Makarova et al., 2000). Deubiquitination enzyme A20, as discussed earlier, is in this subfamily and can cleave both K48- and K-63

linked polyubiquitin chains.

4) Ataxin-3 subfamily. Ataxin-3 is the only demonstrated member to date and is characterized by a domain called the Josephin domain. The Josephin domain has been found in over 30 predicted proteins with unknown function. This domain shows weak similarities to the His and Cys boxes of UBPs and UCHs. Ataxin-3 has typical properties of Dubs and has deubiquitination activity *in vitro* (Burnett et al., 2003).

5) JAMM/MPN+ protease subfamily. This subfamily is represented by the subunit of the proteasome, Rpn11 (yeast)/POH1 (human). Rpn11 has features of a metalloprotease specific for protein-linked ubiquitin. The JAMM motif has two conserved His residues and an Asp residue that together coordinate a zinc ion important for the function of the Rpn11 subunit when incorporated into the proteasome (Verma et al., 2002, Mayatl-Kivity et al., 2002). Rpn11/POH1 is an ATP-dependent de-ubiquitination protease and cannot be inhibit by ubiquitin aldehyde (Ubal), a potent inhibitor for most other cysteine deubiquitination enzymes.

Currently there is little information available regarding the specificity of DUBs towards different lysine-linked polyubiquitin chains as well as whether and how their deubiquitination activity is regulated in cells.

I.B.5 Functions of ubiquitination

The function of ubiquitination can be grouped into traditional and non-traditional functions. The now-well-known traditional function of ubiquitination is for polyubiquitination-dependent protein degradation by the proteasome. This emerged from efforts of Hershko and his colleagues to understand ATP-dependent intracellular proteolysis (Simpson, 1953). Through biochemical fractionation, they uncovered the enzymes and enzymology in this process in early 1980's (Hershko et al., 1983). Proteins that are destined for degradation by the proteasome are first polyubiquitinated by lys48-linked chains, which can then be recognized by and degraded inside the proteasome. Short-lived proteins, which normally have basic or bulky hydrophobic residues at their N-termini (N-end rule amino acids) (Varshavsky, 1995) are recognized by the N-end rule E3, a 200 KDa E3α, and are degraded through ubiquitination-proteasome pathway. There are many other proteins in cells, such as IκB proteins (discussed earlier) and inhibitors of the cell cycle, which are degraded by the ubiquitin-proteasome system in a highly regulated, stimulus-dependent manner.

In addition to its function in protein degradation, protein ubiquitination also regulates a wide variety of other cell processes without mediating protein degradation, which include endocytosis and vesicular trafficking (Hicke, 2001; Raiborg et al., 2003), viral budding, stress response, DNA repair (Huang and D'Andrea 2006), signaling (Di Fiore et al., 2003), transcription and gene silencing (Flick et al., 2004).

The non-proteolytic function of ubiquitin has long been recognized before reports by Hicke and Riezman (1996) and Chen et al. (Chen et al., 1996) show strong positive evidence that protein ubiquitination can have consequences other than direct targetng to the proteasome (Hochstrasser, 1996). Kicke and Riezamn showed that binding of alpha factor to Ste2p, a G protein-coupled plasma membrane receptor, induces ubiquitination of the Ste2p cytoplasmic tail and stimulates endocytosis of the receptor-ligand complex. Recent studies have shown that ubiquitination system is involved in many aspects of protein trafficking in cells including ligand-induced receptor internalization, budding of transmembrane proteins into multiple vesicular bodies (MVB), budding of enveloped viruses from the plasma membrane, transmembrane protein sorting at the trans-Golgi-network (TGN). The components of the protein trafficking machinery themselves are also regulated by the ubiquitination (Hicke & Dunn, 2003).

In the report by Chen et al. (1996), partially purified IKK kinase was activated by Ubc4/Ubc5 in a ubiquitination-dependent, proteasome-independent manner. More recent reports have shown another kinse, TAK1, was also activated by TRAF6 in a K63-linked polyubiquitination but proteasome-independent fashion (Wang et al., 2001) (more discussion in Section I.C.3).

Ubiquitination has been shown to be required for DNA repair. Proliferating cell nuclear antigen (PCNA) is a replicative processivity factor that that forms a trimeric ring which encircles DNA to recruit DNA polymerase during DNA

replication and post-replicative DNA repair. PCNA undergoes two types of ubiquitination upon DNA damage. Monoubiquitination of PCNA shifts recruitment from replicative polymerase to translesion polymerase for error-prone DNA repair (Bienko et al., 2005) while K63-linked polyubiquitination of PCNA is required for error-free DNA repair (Hoege et al., 2002).

Ubiquitination is also involved in signaling. One good example is the ubiquitination of receptor tyrosine kinase TrKA in the NGF signaling pathway. When NGF binds to the neurotrophin receptor p75, TrkA is modified by K63-linked polyubiquitin chains, which leads to TrkA internalization into the endosome. From the endosomes, TrkA initiates signaling for NGF-dependent differentiation of neuronal cells (Zhang et al., 2000, Geetha, et al., 2005).

Ubiquitination also plays an unexpected role in transcriptional regulation. Studies have suggested that monoubiquitination of histone H2B is associated with transcriptional initiation and elongation (Xiao et al., 2005), whereas polyubiquitination of a subset of transcriptional activators is required for transcriptional activation (Muratani et al., 2003; Lipford et al., 2003).

I.B.6 Ubiquitin-like proteins

Following the discovery of protein modification by the small, highly conserved ubiquitin polypeptide, more than 10 distinct ubiquitin-like proteins (Ubls) have been found to function as protein modifiers as well, such as SUMO,

ISG15, Nedd8, and Atg8. As in the case of the ubiquitination cascade, these diverse Ubls are also covalently attached via their C-termini to their targets by parallel, but specific, cascades of three classes of enzymes E1, E2, and E3. These Ubls modify a vast number of proteins, and can affect the target's half-life, subcellular localization, enzymatic activity, or ability of the target proteins to interact with other protein or DNA partners. In these ways ubls function as critical regulators of many cellular processes, including transcription, DNA repair, signal transduction, autophagy, and cell cycle control (Kerscher et al., 2006).

I.C Regulation of IKK-NF-κB activation by the ubiquitination system

The activation of IKK-NF-κB is highly regulated. In the past twelve years, its regulation by the ubiquitination system has been under intensive study and has become a paradigm for crosstalk between the ubiquitination system and a traditional signaling pathway. In this paradigm, several components and steps in the NF-κB pathway are under tight regulation, either positively or negatively, by the ubiquitination system, which involves both traditional and non-traditional functions of the ubiquitination system. The key components and steps in the NF-κB signaling, which are regulated by ubiquitin, are discussed briefly here.

I.C.1 Phosphorylation-induced ubiquitination/proteasome-dependent degradation of IκBs.

As discussed earlier, NF-κB is retained in the cytosol because of the binding to IκB proteins in resting cells. Upon stimulation, IκB proteins undergo signal-induced phosphorylation, polyubiquitination and proteasome-dependent degradation, which releases NF-κB from inhibition by IκBs. Among the IκB proteins, the best studied is IκBα.

Studies have shown that signal-induced phosphorylation sites on IκBα are specific and required for proteolysis (Beg et al., 1993; Brockman et al., 1995, Brown et al., 1995). The proteolysis of IκB can be blocked by proteasome inhibitors, which, however, don't block phosphorylation of IκBα, which still remains bound to NF-κB (Palombella et al., 1994). Subsequently, direct evidence was provided that IκBα is ubiquitinated in a phosphorylation-dependent manner and that ubiquitinated IκBα remains bound to NF-κB before being selectively degraded by the 26S proteasome (Chen et al., 1995). Site-specific phosphorylation of IκB proteins is carried out by IKK kinase complex, which becomes activated when cells are stimulated, for example, by proinflammatory cytokines such as TNFα, IL1β or LPS. The sites phosphorylated by IKK on IκBs are two serine residues residing in the degron sequences (in the case of IκBα, they are Ser32 and Ser36). Degron sequence is $DS(PO_3)GXXS(PO_3)$, where 'X' represent any amino acid residues, and it is conserved in all IκB proteins (Aberle

et al., 1997). Phosphorylation of IκBs by IKK marks them for polyubiquitination, which then is transported into 26S proteasome for degradation. Mutations of any of these serine residues to alanine residues render IκBs resistant to IKK phosphorylation and degradation both *in vivo* and *in vitro*. Polyubiquitination of IκBs is carried out by ubiquitin conjugating enzyme E2 UbcH5 (Chen et al., 1996) and ubiquitin ligase E3 $SCF^{\beta-TrCP}$ (SKP1-Cul1-Roc1/Rbx1-F-box ligase-containing the F-box protein β-TrCP) (Suzuki, et al., 2000). In the case of IκBα, two specific lysines, lys21 and 22, are ubiquitinated, which is sufficient for IκBα degradation (Deshaies, 1999; Maniatis 1999). Replacement of lys21 and 22 with arginine changes IκBs to be resistant to signal-dependent degradation. $SCF^{\beta-TrCP}$ is a RING-finger containing multisubunit E3 complex that specifically recognizes degron sequences when the two serines are phosphorylated by upstream kinases. β-TrCP has an F-box domain at its N-terminus and 7 WD40 repeats at its C-terminus. The WD40-repeats forms a β-propeller structure (Wall et al., 1995) that is responsible for binding to phosphorylated but not non-phosphorylated IκBs. The F-box domain Skp1, binds to Cul1. Cul1 in turn binds to the RING domain protein Roc1 (Rbx1). Roc1 recruits the E2 UbcH5 and promotes polyubiquitination of substrate bound at the WD40 repeat domain of the F-box proteins (Zheng et al., 2002).

I.C.2 Processing of p105 and p100 by the ubiquitin-proteasome system

NF-κB1/p50 and NF-κB2/p52 are expressed as large protein precursors p105 and p100, respectively. Processing of p105 to p50 and p100 to p52 requires the ubiquitin-proteasome system (Heusch et al., 1999). Although processing of p105 can be co-translational and doesn't require phosphorylation and ubiquitination (Lin et al., 1998), most studies support the model that the ubiquitination-proteasome system plays a major role in the processing of p105. In a cell-free system, processing of p105 requires ATP and ubiquitin and is blocked by a ubiquitin mutant that prohibits the polymerization of the ubiquitin chains (Fan and Maniatis, 1991). In cells, proteasome inhibitors block p105 processing (Palombella et al., 1994).

Processing of p100 to p52 is tightly regulated and depends on both phosphorylation and ubiquitination, which mostly happens in and is essential for the development of B cells, as mentioned in the previous section (Caamano et al., 1998; Franzoso et al., 1998; Poljak et al., 1999). When B cells are stimulated by BAFF, NIK, a MAPKKK kinase, is activated which in turn activates IKKα. Activated IKKα phosphorylates p100 at two C-terminal serine residues (Ser866 and Ser 870) located at the death domain of p100, which inhibits p100 processing (Ciechanover et al., 2001; Xiao et al., 2001). Phosphorylation of the two serine residues relieves the inhibition, and results in recruitment of F-box protein $SCF^{\beta\text{-}TrCP}$ E3 ligase to polyubiquitinate p100 at a specific lysine, which results in

selective degradation of the C-terminal IκB-like domain and generation of mature p52 (Xiao et al., 2001; Senftleben et al., 2001; Fong and Sun, 2002).

I.C.3 Activation of TAK1 and IKK complex by the polyubiquitination system

The first report that links proteasome-independent function of polyubiquitination with kinase activation was made twelve years ago (Chen et al., 1996) in which a signal-dependent site-specific phosphorylation and ubiquitination of IκBα was recapitulated in a cell-free system using Hela cell extracts. Through biochemical fractionation, an 700 KDa kinase (IKK) complex responsible for site-specific phosphorylation was partially purified. Surprisingly, activation of this IKK kinase complex in this *in vitro* system required ubiquitination components E1, E2 (Ubc4 or Ubc5) and ubiquitin in a polyubiquitination-dependent but proteasome-independent manner. This report was exciting as well as puzzling since it has not been able to fit it to any known signaling pathway (Hochstrasser 1996).

The significance of kinase activation by ubiquitin is best illustrated by the TRAF6-dependent IKK activation. In the process of elucidating the mechanism of activation of IKK by TRAF6 in the IL1R and TLR signaling pathway, a cell-free system was established in which IKK was activated by incubation of Hela S100 with recombinant TRAF6 (Deng et al., 2000). After biochemical fractionation two factors were identified: TRIKA1 (TRAF6-regulated IKK activator 1) and

TRIKA2. TRIKA1 is a heterodimeric ubiquitin conjugating enzyme E2 comprised of Ubc13 and Ubc-like protein Uev1A (Deng et al., 2000). TRIKA2 is a MAPKKK member TAK1 kinase complex containing TAK1/TAB1/TAB2 (Wang et al., 2001). Through a series of experiments to address how TAK1 and IKK were activated in this system, the connection between polyubiquitination and kinase activation was revealed again. In this *in vitro* system, TRAF6, as a RING-finger containing ubiquitin ligase E3, together with the Ubc13/Uev1a as a ubiquitin conjugating enzyme E2, catalyzes lys63-linked polyubiquitin chain synthesis which somehow leads to activation of TAK1 complex. Activated TAK1 complex in turn leads to activation of IKK through phosphorylation of serine residues at the activation loop. Here polyubiquitination leads to activation of a kinase complex instead of protein degradation (Deng et al., 2000; Wang e al., 2001).

I.C.4 Ubiquitin binding by TAB2, TAB3 and NEMO

Activation of TAK1 in a polyubiquitination-dependent manner requires the TAB2 or TAB3 subunit. TAB2 and TAB3 has a ZnF motif at its C-terminus and this ZnF motif is a polyubiquitin chain-binding motif (Kanayama et al., 2004). This motif has a stronger affinity towards lys63-linked chains than to lys48-linked chains. Through mutagenesis, deletion mutants and motif-swapping experiments *in vitro* and in cell culture, it was shown the binding to polyubiquitin

chains by this ZnF motif was required for activation of TAK1 by TRAF6 (Kanayama et al., 2004).

It has been well known that without NEMO, activation of IKK-NF-B cannot be achieved by a variety of stimulations. As in the case of TAB2 and TAB3, it is found that NEMO, the structural subunit of IKK kinase complex, also has polyubiquitin chain binding activity. Through serial deletion and site-directed mutagenesis, a minimal polyubiquitin binding motif has been mapped. This motif is different from any known ubiquitin binding motif and so is named NUB (NEMO Ubiquitin Binding) (Ea et al., 2006). This NUB motif and its binding to polyubiquitin chains are required for IKK activation by IL1β and TNFα *in vivo* and TRAF6 *in vitro* (Ea et al., 2006; Wu et al., 2006).

I.C.5 Ubiquitination of NEMO

In addition to its polyubiquitin chain binding function in IKK-NF-κB activation, NEMO is also ubiquitinated with K63 chains at various sites in different signaling pathways. In the TCR and BCR signaling, polyubiquitination of NEMO at Lys399 is essential for TCR signaling leading to NF-κB activation (Zhou et al., 2004; Sun et al., 2004). In Zhou et al. report (Zhou et al., 2004), Malt1 functions as the E3, together with Ubc13/Uev1a, to ubiquitinate NEMO. In Sun et al., report (Sun et al., 2004), TRAF6 is the E3 that is responsible for NEMO ubiquitination, where Malt1 induces E3 activity of TRAF6 through

oligomerization. In the intracellular NOD2-dependent IKK-NF-κB activation pathway, NEMO is ubiquitinated at Lys285 in a RIP2-dependent manner (Abbott et al., 2004). Site-specific NEMO ubiquitination is also required for DNA damage-induced IKK-NF-κB activation (Huang et al., 2003).

I.C.6 Ubiquitination of RIP1, RIP2 and IRAK-1

In the TNFα pathway, RIP1 is ubiquitinated upon stimulation of cells with TNFα. A single lysine ubiquitination site, Lys377, was mapped and its ubiquitination was required for IKK-NF-κB activation by TNFα (Ea et al., 2006; Li et al., 2006). Ubiquitination of Lys377 recruits both the TAK1 complex and the IKK complex to the TNFR. Mutation of this site to arginine abolished recruitment of TAK1 and IKK and activation of NF-κB. Similarly, in the NOD2-dependent IKK/NF-κB activation pathway, the intermediate RIP2 is also ubiquitinated at a single specific lysine residue and its ubiquitination was shown to be important for activation of IKK (Hasegawa et al., 2007).

In parallel, IRAK-1, as an intermediate between MyD88 and TRAF6 in IL1R and TLR signaling, also undergoes K63-linked polyubiquitination. Here polyubiquitinated IRAK-1 recruits IKK through polyubiquitin binding by NEMO, and polyubiquitinated TRAF6 recruits TAK1 through polyubiquitin binding by TAB2, thus facilitating TAK1-IKK-NF-κB activation (Windheim et al., 2008).

I.C.7 Deubiquitination enzymes CYLD and A20 in the down-regulation of IKK-NF-κB

As an integral part of the ubiquitination system, deubiquitination enzymes (DUBs) also play an important role as negative regulators in the IKK-NF-κB activation pathway. CYLD was originally identified as a tumor suppressor mutated in familial cylindromatosis (Courtois &Gilmore, 2006) where elevated NF-κB activation drove proliferation of hair epithelial cells through induction of sonic hedgehog (Shh) and cyclin D1 expression (Schmidt-Ullrich et al., 2006). CYLD was also identified through its interaction with NEMO in a yeast-two-hybrid screening and by searching for DUBs which interfered with NF-κB signaling (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). In these studies CYLD was shown to interact with NEMO and TRAFs and remove Lys63 polyubiquitin chains from NEMO, TRAF2, TRAF6 and TRAF7 thus repress activation of IKK-NF-κB by a variety of stimuli (Figure 1).

The OTU domain-containing DUB A20 is another negative feedback regulator of NF-κB activation. A20 can deubiquitinate RIP1 and TRAF6 to negatively regulate TNF-R and IL1R/TLR signaling (Boone et al., 2004, Wertz et al., 2004) (Figure 1). A20 knockout mice have prolonged activation of IKK and NF-κB (Lee et al., 2000). Interestingly A20 has ubiquitin E3 ligase activity towards RIP1. In TNFα signaling, A20 removes Lys63-linked polyubiquitin chains from RIP1 through its N-terminal DUB domain and puts Lys48 ubiquitin

chains back onto RIP1 through its C-terminal ubiquitin ligase domain targeting RIP1 for degradation (Wertz et al., 2004).

Expression of both CYLD and A20 are induced by activation of NF-κB and this may form an auto-regulatory circuit providing a prolonged negative feedback regulation of IKK-NF-κB activation (Jono et al., 2004).

Chapter II Materials and Methods

II.A. Materials

II.A.1 Buffers

Lysis Buffer A	50mM Tris-Cl, pH7.5, 150mM NaCl, 10% glycerol, 0.5% NP-40. Fresh DTT, PMSF and protein inhibitor cocktail were added to final concentration of 1mM, 1mM and 1X, respectively.
His6 protein purification protein A	50mM Na2HPo4/NaH2PO4, pH8.2, 500 mM NaCl. Fresh DTT, PMSF and protein inhibitor cocktail were added to final concentration of 0.5mM, 1mM and 1X, respectively.
His6 protein elution buffer	His6 protein purification protein A plus 250mM imidazole.
Tev cleavage buffer	50mM Tris-Cl, pH7.5, 10% glycerol, 0.5mM EDTA. Fresh DTT was added to a final concentration of 1mM before use.
M2 agarose beads elution buffer	50mM Tris-Cl, pH7.5, 200mM NaCl, 10% glycerol, 0.02% NP-40. Fresh DTT and PMSF were added to a final concentration of 1mM, 0.5mM, respectively before use.
Hypotonic buffer	20mM HEPES/KOH, pH7.4, 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA. Fresh DTT, PMSF and protein inhibitor cocktail were added to final concentration of 1mM, 1mM and 1X, respectively.
10X kinase assay buffer	50mM Tris-Cl, pH7.5, 20mM ATP, 50mM MgCl2, 5mM DTT, 1uM Okadeic acid. 0.5 ml aliquots were stored at 20°C or -80°C.
Buffer A & B for Column Q, Heparin	20mM HEPES, pH7.4, 10% glycerol. DTT and PMSF were added to a final concentration of 1mM, 0.5mM, respectively before use. B is A plus 1M NaCl
Buffer A & B for Column SP, momoS	20mM HEPES, pH6.5, 10% glycerol. DTT and PMSF were added to a final concentration of 1mM, 0.5mM, respectively before use. B is A plus 1M NaCl
Buffer for superdex 75 & 200	20mM HEPES, pH7.4, 100 mM NaCl, 10% glycerol. DTT and PMSF were added to a final concentration of 1mM, 0.5mM, respectively before use.

II.A.2 Antibodies

Antibody name	Source	Dilution factor	Supplier
TAK1 sc-579	Rabbit	1:1000	Santa Cruz
p-Thr187 TAK1	Rabbit	1:1000	Cell signaling
TAB1	Rabbit	1:1000	Santa Cruz
TAB2	Rabbit	1:1000	Home-made
IKKα	Rabbit	1:1000	Santa Cruz
IKKβ	Rabbit	1:1000	Santa Cruz
p-IKKα & β	Rabbit	1:1000	Cell signaling
NEMO FL419	Rabbit	1:1000	Santa Cruz
IκBα (C-21)	Rabbit	1:2000	Santa Cruz
p-IκBα	Mouse	1:1000	Cell signaling
TRAF6 H274	Rabbit	1:1000	Santa Cruz
Ubc13	Mouse	1:1000	Zymed laboratories
Uev1A	Rabbit	1:1000	Home-made
UbcH5	Rabbit	1:1000	Home-made
Ub (P4D1)	Mouse	1:2000	Santa Cruz
p-MKK6	Rabbit	1:500	Santa Cruz
Flag-M2	Mouse	1:1000	Sigma
Penta-His	Mouse	1:1000	Qiagen
HSP70	Mouse	1:1000	Santa Cruz

II.A.3 Chemicals and other reagents

NEM (N-ethylmaleimide) was purchased from Sigma, catalogue# E3876, and dissolved in H2O at a concentration of 100 mM before use.

Ubiquitin mutants (K48, R48, K63, R63, KO) were gifts from Wenwen Zeng. CYLD and its mutant form CYLD(C601S) were gifts from Gabriel Pineda. Deubiquitination enzyme IsoT was purchased from Boston Biochem.

II.B. Methods

II.B.1 Purification of TAK1/TAB1/TAB2 complex

TAK1/TAB1/TAB2 complex was purified from 293T/TAP-TAK1-Flag stable cell line, in which the N-terminus of mouse TAK1 was fused to protein A-calmodulin tandem tag and the C-terminus of TAK1 was fused to a Flag tag to facilitate protein purification, in which a Tev protease cleavage site was inserted between protein A and calmodulin. Cells were grown in DMEM media supplemented with 10% FBS, penicillin and streptomycin. After cells reached confluency (100 10-cm plates), they were harvested and lysed in lysis buffer A at a ratio of cell pellet:lysis buffer=1:5. Lysate was centrifuged at 14000RPM to remove insoluble cell debris and supernatant was saved to a new clean tube. The supernatant was then mixed with 200 μl of protein A sepharose beads (pre-washed with lysis buffer A) and incubated for 4 hours at 4°C. At the end of incubation, beads were transferred to an empty column and washed with 30 ml of lysis buffer A, followed by 5ml of Tev cleavage buffer. Beads were then transferred to a 1.5 ml Eppendorf tube and resuspended in 1ml of Tev cleavage buffer. Tev was added and incubated at 4°C overnight. The second day, beads were pelleted down and supernatant, which contained Calmodulin-TAK1-Flag, was saved and diluted to 5 ml with lysis buffer A. The product was then mixed with 100 μl of anti-Flag M2 agarose beads and incubated for 2 hours at 4°C. After incubation, M2 beads were washed with 30 ml lysis buffer A followed by 5ml M2 beads elution buffer. M2 beads were then eluted with 6 volume of M2 beads

elution buffer containing 0.2 mg/ml Flag peptide. Eluate was collected and concentrated to about 50 μl and further purified using superdex200 column.

II.B.2 Purification of IKK

IKK complex was purified from a stable cell line expressing Flag-tagged NEMO generated in NEMO-deficient 1.3E2 cells. 10 liters of cells grown to $2x10^6$/ml in suspension in RPMI/10% FBS/penicillin/streptomycin were harvested and lysed in lysis buffer A in a pellet:buffer=1:5 ratio. After centrifugation for 30 minutes at 20000g, supernatant was filtered through 0.22 μM membrane and incubated with M2 beads (1.0 ml pre-washed with lysis buffer A) for 6 hours at 4°C. Beads were transferred to a clean empty column and washed with lysis buffer extensively (50 times of bead volume). Beads were then washed again with M2 bead elution buffer (5 times of bead volume). Bound proteins were eluted with M2 bead elution buffer containing 0.2 mg/ml of Flag peptide (6 times of bead volume). The eluate containing Flag-NEMO/IKK was concentrated to 500 μl and loaded onto superdex200. Superdex fractions containing IKK complex were pooled and loaded onto monoQ column for further purification. Fractions containing IKK complex were used for assay or stored at -80°C.

II.B.3 Purification of Ubc13, Uev1A, MKK6(K82A)

Ubc13, Uev1A and MKK6(K82A) were purified from *E.coli* BL21/pLys strain. Ubc13, Uev1A and MKK6(K82A) were tagged with 6xHis tag to facilitate purification. 1 L of cells harboring expression vector for His6-Ubc13, His6-Uev1A or His6-MKK6(K82A) were induced overnight with 0.2 mM IPTG at room temperature, harvested, and lyzed in His6-protein purification lysis buffer at pellet:lysis buffer=1:5 ratio. Lysate was cleared by centrifuge for 30 minutes at 12000g. Cleared supernatant was mixed with Nickel-agarose beads (Qiagen) (0.5 to 1ml beads for 1 L cell culture depending on protein induction level) and incubated at 4°C. After 2 hours of incubation, beads were transferred to an empty column and washed with lysis buffer until no protein reading based on protein concentration measurement. Bound proteins were eluted with His6 elution buffer and collected as 1 ml fraction each. Purity and yield were checked by Coomassie blue staining after fractions were resolved on SDS-PAGE gels. Purified proteins were dialyzed with buffer containing 50mM Tris-HCl, pH7.5, 10% glycerol, 0.5mM DTT and 0.5mM PMSF and stored at -80°C.

To make Ubc13/Uev1A complex, roughly equal molars of His6-Ubc13 and His6-Uev1A were mixed and farther purified on HiTRAP Q HP anion exchange column. Fractions containing roughly equal molar of Ubc13 and Uev1A were saved and stored in Q/buffer A at -80°C.

For His6-MKK6, it was further purified using HiTRAP Q HP anion exchange column and stored in Q/buffer A at -80°C.

II.B.4 Purification of ubiquitin

Ubiquitin without any tags was expressed in *E.coli.* For purification of ubiquitin, cells were lysed in hypotonic buffer and proteins were released by sonication. After initial clearup of insoluble pellets by centrifugation at 15000g for 30 minutes, supernatant was further centrifuged at 100,000g for 1 hour to make S100. Ubiquitin was further purified by sequential HiTRAP Q HP anion exchange column, HiTRAP SP HP cation exchange column and superdex 75 sizing column. Fractions from superdex 75 containing ubiquitin were pooled, buffer-exchanged to H_2O and stored at -20°C.

II.B.5 Purification of UbcH5b and UbcH5c

UbcH5b and UbcH5c were expressed as non-tagged proteins in *E.coli*. After induction of *E.coli* harboring expression vector for UbcH5b or UbcH5c with 0.1 mM overnight at 25°C, cells were lysed in hypotonic buffer and proteins were released by sonication. After initial clearup of insoluble pellets by centrifugation at 15000g for 30 minutes, supernatant was further centrifuged at 100,000g for 1 hour to make S100. UbcH5b and UbcH5c were further purified by sequential HiTRAP SP HP cation exchange column and superdex 75 sizing column. Fractions from superdex 75 containing UbcH5b and UbcH5c were pooled, buffer-exchanged to Q/buffer A and stored at -80°C.

II.B.6 Purification of TRAF6 and E1

6xHis-tagged TRAF6 and E1 were expressed in sf9 cells using baculoviral protein expression system. Sf9 cells were grown to $2x10^6$/ml and infected with freshly amplified viruses and harvested 48 hours post infection. Then the same protocol for 6xHis Ubc13 protein purification was followed to purify TRAF6 and E1.

II.B.7 Purification of Flag-tagged protein

Flag-tagged proteins used in the studeis described here include IκBα, TRAF6 WT, TRAF6(1-365) and TRAF6(1-291). 293T cells were transfected with pcDNA3-based plasmids encoding IκBα, TRAF6 WT, TRAF6(1-365) or TRAF6(1-291) tagged with Flag at their N-termini and harvested after 48 hours post transfection. Cells were harvested and lyzed in Lysis Buffer A. After centrifugation at 14000 RPM to remove insoluble cell debris, cell lysate was incubated with anti-Flag antibody M2-immobilized agarose beads according to a ratio of 5 μl of bead per plate. After rotation for 2 hours at 4°C, beads were washed with 50 times volume of lysis buffer A followed with 5 volumes of Flag-elution buffer. Flag-tagged proteins were then eluted with 0.2 mg/ml Flag peptide dissolved in Flag elution buffer, buffer-exchanged to Q/Buffer A and stored at -80°C

II.B.8 Ubiquitination Assay

For TRAF6-Ubc13/Uev1A system, for a typical ubiquitination assay, purified E1 (0.1 μM), Ubc13/Uev1A (0.5 μM), TRAF6 (0.2 μM), Ub (12.5 μM) were mixed with 1x ATP buffer in a final volume of 10 μl and incubated for 30 minutes at 30°C. Ubiquitination was detected by immunoblotting using mouse anti-ubiquitin antibody after reaction products were resolved on 4-20% SDS-PAGE.

For TRAF6-UbcH5 system, it is the same as TRAF6-Ubc13/Uev1A system except Ubc13/Uev1A was replaced with UbcH5c (5 nM).

II.B.9 TAK1 kinase activation assay

TAK1 activation assay was similar to ubiquitination assay for TRAF6-Ubc13/Uev1A system except TAK1/TAB1/TAB2 complex was added. After incubation for 1 hour at 30°C, reaction was stopped by adding 2.5 μl of 5x SDS sample buffer. After boiling for 4 minutes, products were separated by 10% SDS-PAGE gel for immunoblotting. Activation of TAK1 was detected by anti-phosphor-Thr187 antibody. Total TAK1 was also detected using rabbit anti-TAK1 antibody. For some assays, TAK1 substrate, 0.5 μg of kinase-dead MKK6 (MKK6(K82A)) was added together with TAK1 complex. Phosphorylation of MKK6 at Ser207 was monitored by immunoblotting using anti-phosphoMKK3/6(Ser189) antibody.

II.B.10 IKK activation assay

IKK activation assay was similar to ubiquitination assay for TRAF6-UbcH5 system except IKK complex was included. After incubation for 1 hour at 30°C, IKK was separated on 10% SDS-PAGE for immunoblotting of IKKα/β, p-IKKα/β, and NEMO to detect activation of IKK. In some assays, IKK substrate IκBα, purified from transient overexpression in 293 cells was included. Immunoblotting of IκBα and p-IκBα was carried out to detect IKK activation.

II.B.11 Polyubiquitin chain synthesis and NEM treatment

The reaction was the same as "Ubiquitination Assay" except the reaction volumn was scaled up to 100 μl. The reaction was stopped by addition of EDTA to a final concentration of 10mM from a stock solution of 0.5M. DTT was then added to a final concentration of 5mM from a freshly made 1M stock and waited for 15 minutes at room temperature followed by addition of freshly-made NEM to final 10mM. After further incubation for 15 minutes at room temperature, DTT was added again to final 10mM. Following 15 more minutes incubation at room temperature, the final product was gone through extensive buffer exchange (>10000x) and concentrated to final volume of 25 μl, which was used for TAK1 kinase activation assay or stored at -80°C.

II.B.12 Purification of polyubiquitin chains

Polyubiquitin chain was synthesized under the same condition for "Polyubiquitin chain synthesis and NEM treatment" except the reaction volume was scaled up to 2 ml and concentration of each component (E1, Ubc13/Uev1A, TRAF6) was doubled. The final product was mixed with 1ml of Nickel beads and incubated for 2 hours at 4°C to remove His6-tagged E1, Ubc13/Uev1A and TRAF6. The supernatant was separated from the beads by centrifugation and concentrated to about 500 μl before applied to superdex200. Fractions was collected and tested for TAK1 activation assay. Active fractions were pooled and buffer-exchanged to SP buffer A before applied to monoS column. Fractions from monoS were buffer-exchanged to Q/buffer A and used for TAK1 activation assay or stored at -80°C.

II.B.13 Depletion of Ubc13 and Uev1A from S100

Purified Ubc13 or Uev1A protein was covalently immobilized to NHS-Sepharose at the density of 10 mg/ml according to Manufacturer's Instruction (Pharmacia). To deplete Uev1A from Hela S100 or Ubc13 from U937 S100, 50 μl of S100 were mixed with 5 μl of beads and incubated for 2 hours at 4°C. After centrifugation at 1000g for 2 minutes, supernatant was saved and used for assay or stored at -80°C.

Chapter III TAK1 activation by polyubiquitin synthesized by Ubc13/Uev1A-TRAF6

III.A Introduction

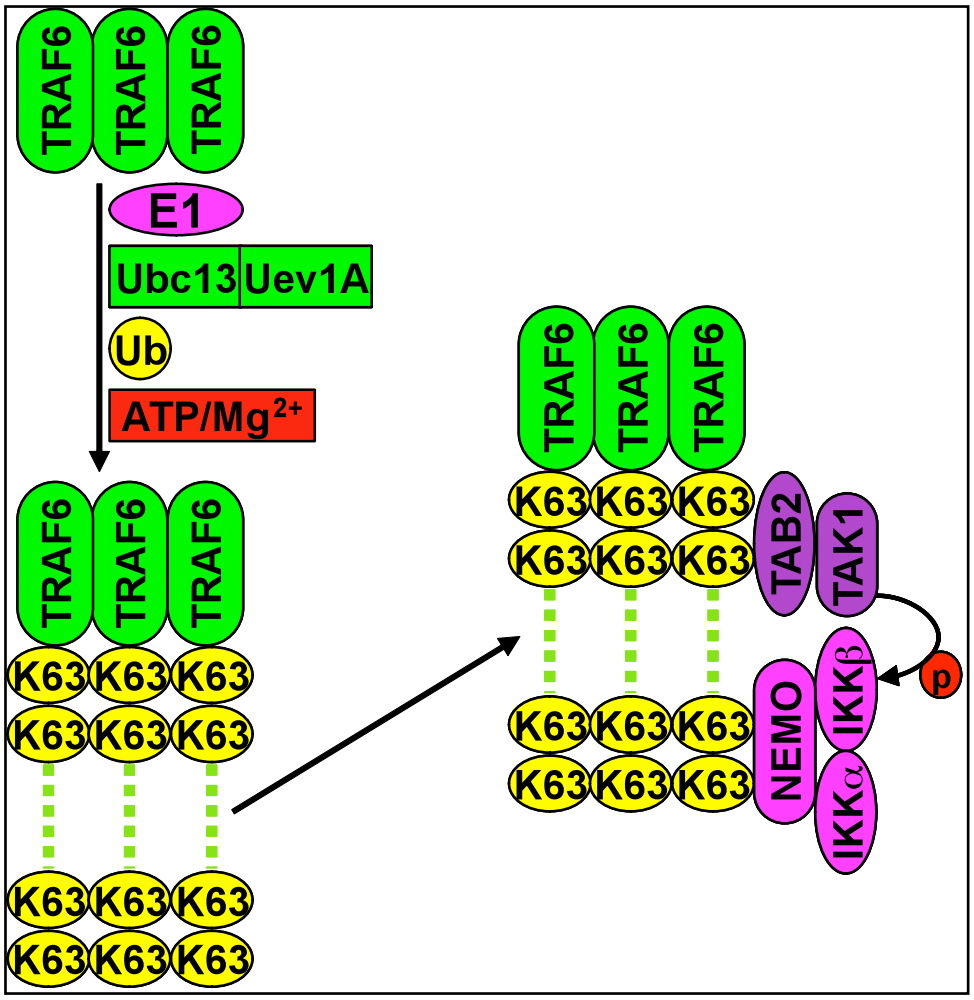


Figure 8 A current model explaining TAK1-IKK activation by TRAF6. TRAF6, as a RING finger ubiquitin ligase E3, probably as a trimer, together with Ubc13/Uev1A, E1, Ubiquitin and ATP, catalyze K63-linked polyubiquitination and results in auto-ubiquitination of TRAF6. Polyubiquitinated TRAF6 recruits both TAK1 kinase complex through TAB2, which has a polyubiquitin chain-

binding motif (ZnF) at its C-terminus, and IKK through NEMO, which also has a polyubiquitin-binding motif (NUB). In a yet-unknown mechanism, TAK1 is activated and then phosphorylates IKK leading to IKK activation.

Previously our lab has established a cell-free system to recapitulate IKK activation by incubating recombinant TRAF6 with Hela S100 (Deng et al., 2000). After chromatographic fractionation, the minimal components required for IKK activation *in vitro* have been identified (Deng et al., 2000; Wang et al., 2001). These components are TRAF6, Ubc13/Uev1A, ubiquitin activating enzyme E1, ubiquitin, and a kinase complex TAK1/TAB1/TAB2. The current model on how this system works to activate IKK is shown in Figure 8. In brief, ubiquitin activating enzyme E1, Ubc13/Uev1A as a heterodimeric E2 complex, and TRAF6 as a RING-finger containing E3, together with ubiquitin catalyze K63-linked polyubiquitin chain synthesis. One of the ubiquitination targets is TRAF6 itself. Polyubiquitinated TRAF6 then recruits both TAK1 through TAB2 subunit of the TAK1 kinase complex, which has a ubiquitin binding motif, ZnF, at its C-terminus, and IKK kinase complex through the NEMO subunit, which also has a polyubiquitin chain binding motif NUB. TAK1 is then activated through an unknown mechanism. Activated TAK1 in turn phosphorylates and activates IKKβ. However, the mechanism by which polyubiquitination leads to TAK1 activation is still a mystery. My goal in this chapter is to describe my efforts to address this question. Through further dissection of the IKK activation system

into polyubiquitination step and kinase activation step, I found, after chromatographic purification, the actual activation species (activator) for TAK1 activation generated during the ubiquitination step is free, un-anchored polyubiquitin chains.

III.B Results

III.B.1 Purification of proteins

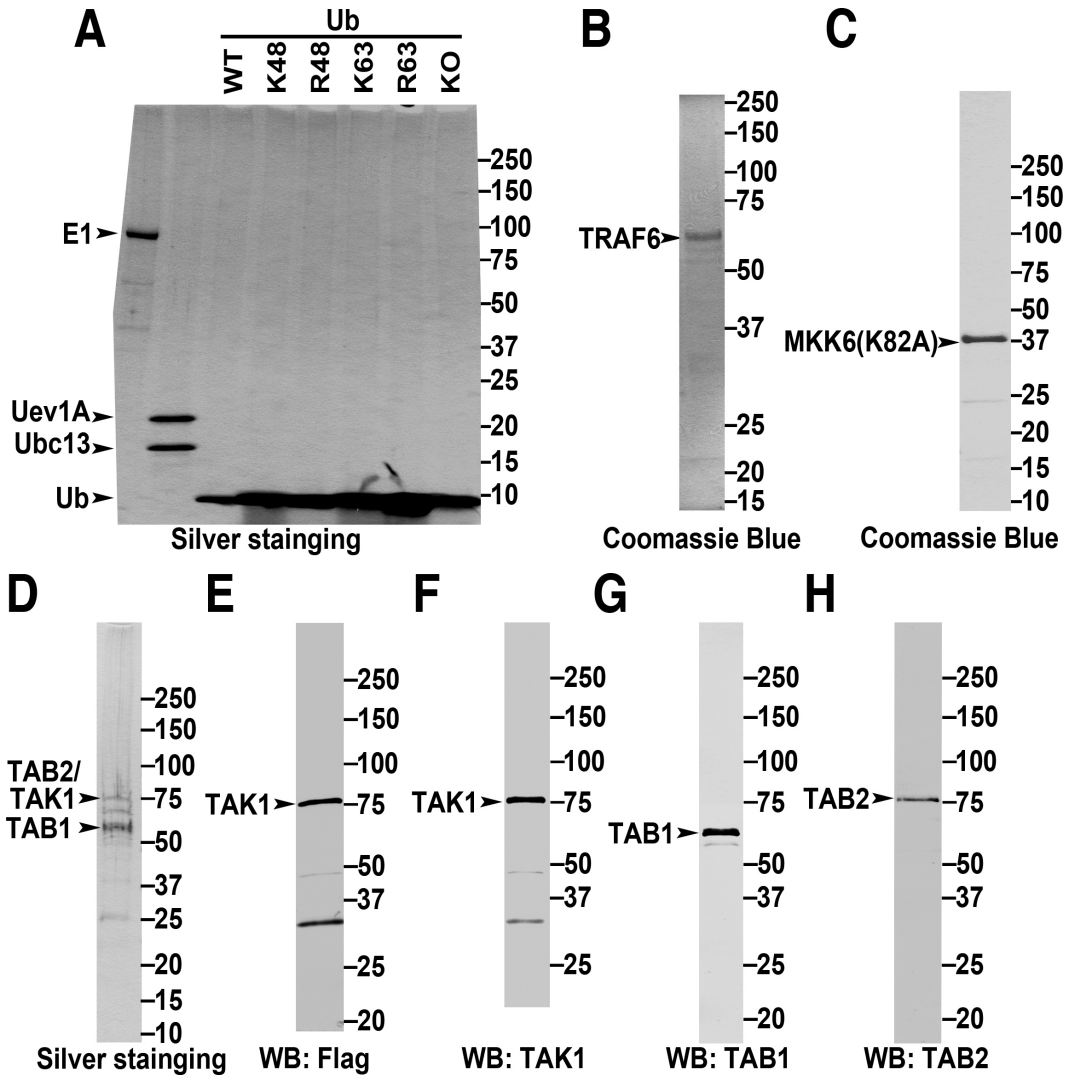


Figure 9: Purified proteins used in the study. A): Silver staining of E1, Ubc13/Uev1A complex, Ubiquitin wild type (WT) and its mutants. B): Coomassie blue staining of purified TRAF6. C): Coomassie blue staining of purified MKK6(K82A). D): Silver staining of TAK1 kinase complex. TAK1 and TAB2 are close in size and cannot be separated. The band labeled by '*' is HSP70 based on immunoblotting using anti-HSP70 antibody. E, F, G, H): Immunoblotting of purified TAK1 complex to show TAK1, TAB1 and TAB2.

To reconstitute TAK1 activation *in vitro*, all the proteins required for the assay were purified. Ubiquitin activating enzyme E1 and ubiquitin ligase E3 TRAF6 were purified from Sf9 cells using baculovirus protein expression system (both were tagged with 6xHis). Ubiquitin conjugating enzyme Ubc13/UevA tagged with 6xHis and un-tagged ubiquitin were purified from *E.coli*. The TAK1 complex were purified from a stable 293 cell line expressing TAK1, in which mouse TAK1 was tagged with a TAP tag at its N-terminus and a Flag tag at its C-terminus to facilitate purification process. TAK1 kinase substrate, kinase-dead MKK6 (MKK6(K82A)) tagged with 6xHis, was purified from *E.coli*. As shown in Figure 9, all the proteins have been purified to apparent homogeneity.

III.B.2 Dissection of polyubiquitination and TAK1 activation

In order to understand the molecular details of TAK1 kinase activation by the Ubc13/Uev1A-TRAF6-dependent ubiquitination system, a TAK1 activation assay was set up according to the published protocol from our lab using recombinant proteins (Deng et al., 2000; Wang et al., 2001). Briefly, purified TAK1 complex was mixed with E1, Ubc13/Uev1A, TRAF6, ubiquitin and ATP/Mg^{2+} and incubated at 30°C for one hour. To check for TAK1 activation, reaction products were separated on 10% SDS-PAGE and probed with anti-TAK1 phosphor-Thr187 specific antibody. TAK1 Thr187 is located in the kinase activation loop and its phosphorylation is required for TAK1 activation. After its

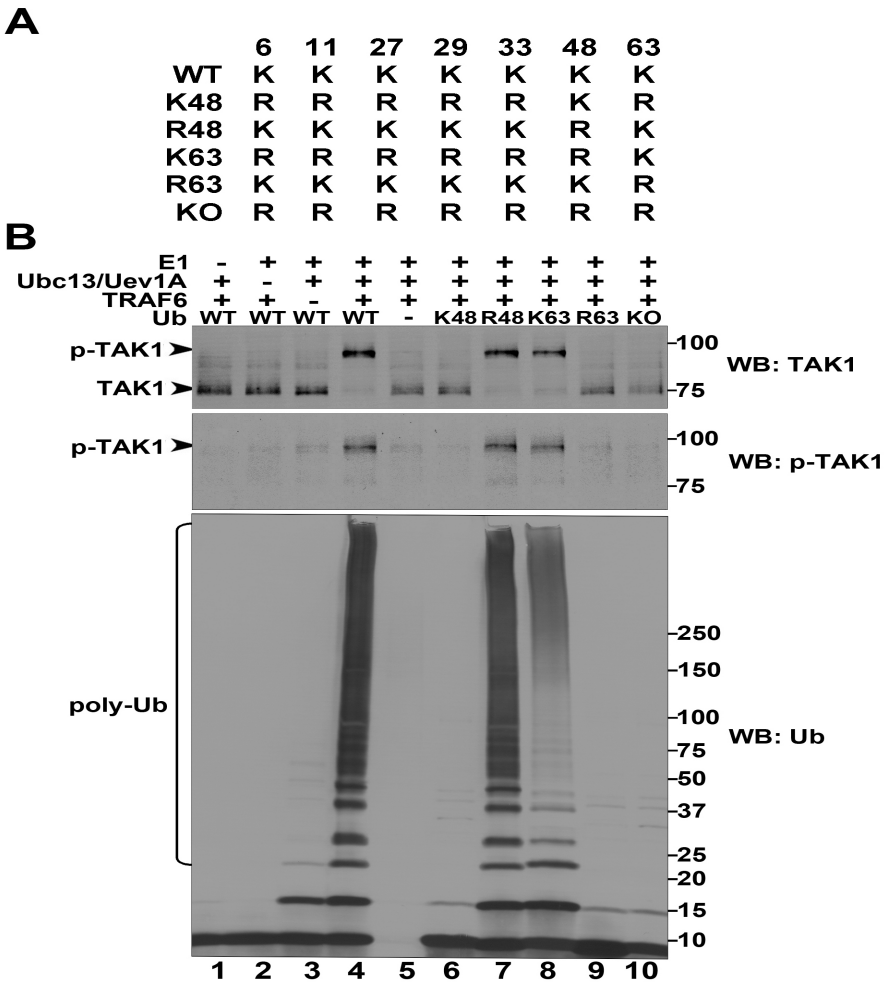
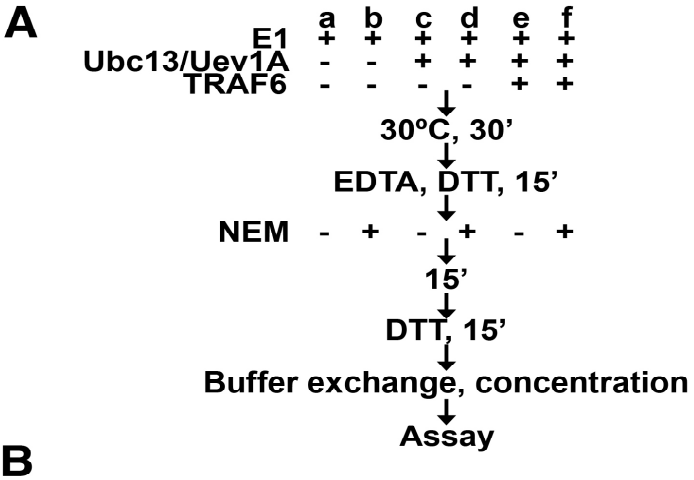


Figure 10: Activation of TAK1 by TRAF6-Ubc13/Uev1A catalyzed K63-linked polyubiquitin chain. A): Diagram showing ubiquitin WT and mutants with lysine(K) to Arginine(R) mutation. B): TAK1 activation by TRAF6 and Ubc13/Uev1A. Purified TAK1 kinase complex was incubated with E1, Ubc13/Uev1A, TRAF6, Ubiquitin or its mutants, ATP/Mg^{2+} for 1 hour at 30°C. Aliquots of reaction products were resolved on 10% SDS-PAGE for TAK1, p-TAK1 immunoblotting (upper and middle panels) and 4-20% SDS-PAGE for ubiquitin immunoblotting (bottom panel). Activation of TAK1 was indicated by its slower mobility shift on TAK1 immunoblotting (upper panel,) or TAK1 phospho-Thr187 (p-TAK1) immunoblotting (middle panel, lanes 4, 7 and 8).

phosphorylation, TAK1 also runs more slowly than its un-phosphorylated

counterpart on SDS-PAGE, which is also an indicator of TAK1 activation. As shown in Figure 10B lane 4, TAK1 was activated as detected by TAK1 anti-phosphor-Thr187 specific antibody (Figure 10B, middle panel) and also by its slower mobility shift on TAK1 immunoblotting (Figure 10B, upper panel) when all the ubiquitination components (E1, Ubc13/Uev1A, TRAF6, ubiquitin) were present. Omission of any of the ubiquitination reaction components did not lead to TAK1 activation (Lanes 1, 2, 3 & 5). The activation seemed to be K63 polyubiquitin chain-dependent since TAK1 was activated when ubiquitin wild-type (WT) was replaced with either K63 or R48 ubiquitin mutants but not with K48, R63 or KO mutants (lane 6-10). In correlation with TAK1 activation, polyubiquitin chain synthesis only happened with ubiquitin WT and R48 and K63 mutants but not with other mutants (Figure 10B, bottom panel). This is consistent with prior observation (Deng et al., 2000; Wang et al., 2001), that is, TAK1 kinase complex is activated during the K63-linked polyubiquitination process catalyzed by Ubc13/Uev1A-TRAF6 E2/E3 pair.

In this *in vitro* TAK1 activation system, two events must happen: polyubiquitination and kinase activation. The first question I asked was to see if these two events could further be separated into two steps. In other words, do these two events have to be coupled? To test that a two-step protocol for TAK1 activation assay has been carried out. In the first step, ubiquitination components are incubated together to synthesize polyubiquitin chains (polyubiquitination



B

STEP I	p35	-	-	+	+	+	+	-	-	+	+
	TRAF6	-	-	-	-	+	+	-	-	-	-
NEM treatment		-	+	-	+	-	+	-	+	-	+
STEP II (TAK1)	p35	-	-	-	-	-	-	+	+	-	-
	TRAF6	-	-	-	-	-	-	+	+	+	+
		a	b	c	d	e	f	a	b	c	d

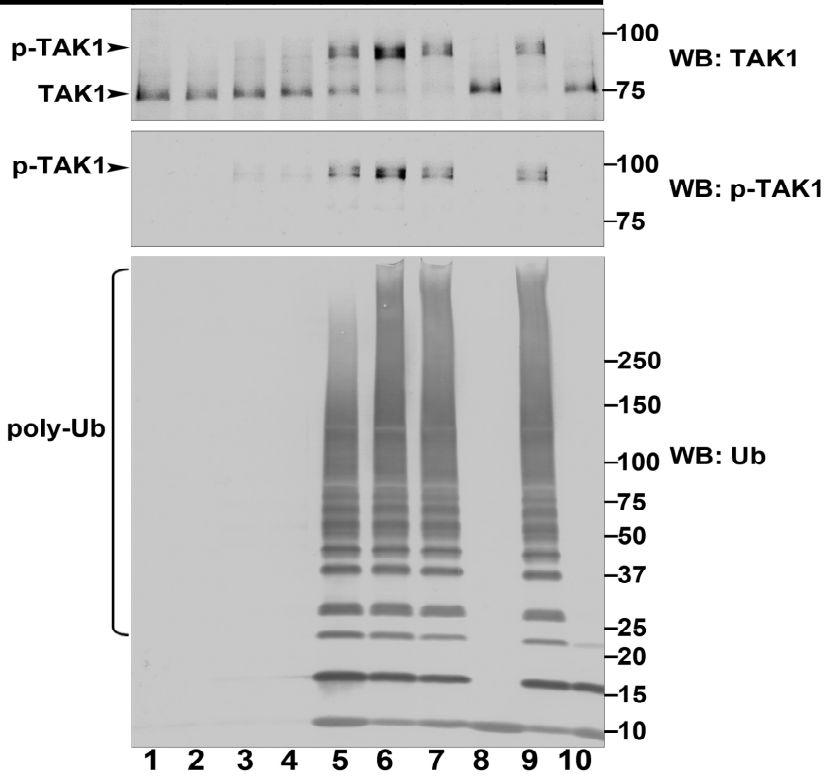


Figure 11: Activation of TAK1 by polyubiquitin chains synthesized by TRAF6-Ubc13/Uev1A. A): Protocols used for synthesis of pre-assembled polyubiquitin chains. B): TAK1 activation by polyubiquitin chains. TAK1 complex was incubated with products from step A and incubated for 1 hour at 30°C. Additional TRAF6 was added to reactions 7-10 and additional Ubc13/Uev1A was added to reactions 7 and 8 during the second step. Aliquots of reaction products were resolved on 10% SDS-PAGE for TAK1, p-TAK1 immunoblotting (upper and middle panels) and 4-20% SDS-PAGE for ubiquitin immunoblotting. Activation of TAK1 was indicated by its slower mobility shift on TAK1 immunoblot (upper panel, lanes 5, 6, 7, and 9) or TAK1 phospho-Thr187 (p-TAK1) immunoblot (middle panel, lanes 5, 6, 7, and 9).

reaction). After the polyubiquitination reaction, products are treated with NEM to inactivate the enzymes E1 and Ubc13 so they would not catalyze any new ubiquitination reaction if they were incubated again. NEM irreversibly reacts with cysteine and inactivates any enzymes depending on active cysteine residues. In the second step, polyubiquitination products from the first step were then incubated with the TAK1 complex in the presence of ATP/Mg^{2+} to determine whether TAK1 can be activated or not. The prediction is TAK1 will be activated if the two steps can be separated or say are not coupled.

As shown in Figure 11A, for the first polyubiquitination reaction step in this particular experiment, six reactions (a to d) were set up, in which a and b only had E1 and ubiquitin, c and d had E1, E2 Ubc13/Uev1A and ubiquitin, while e and f had E1, E2, E3 TRAF6 and ubiquitin. The reactions were incubated at 30°C for 30 minutes and stopped by addition of EDTA, which were then treated with DTT for 15 minutes to release any possible active site cysteine residues that were

conjugated by ubiquitin through iso-peptide bonds. After that, reactions b, d and f were treated with NEM for 15 minutes to inactivate E1 and Ubc13. Excess NEM was then treated with excess DTT for 15 minutes. The post-NEM treatment products underwent extensive buffer exchange to remove residual NEM. The products were then incubated with TAK1 complex during the second step of the TAK1 activation assay. As shown in Figure 11B, 10 reactions were set up in which reactions 1 to 6 were incubation of TAK1 with reaction products of a to f, respectively, from the first step (Figure 11A). Reactions 7 and 8 were incubation of TAK1 with products a and b, respectively, and fresh Ubc13/Uev1A and TRAF6. Reactions 9 and 10 were incubation of TAK1 with products e and f, respectively, and fresh TRAF6. The prediction was that TAK1 would be activated by product from reaction f if the polyubiquitination step and kinase activation step could be separated and were not coupled. As shown in Figure 11B lane 6, which was incubation of TAK1 and product f, TAK1 was activated based on both p-TAK1 and TAK1 immunoblotting (Figure 11B, lane 6, middle and upper panels). Without Ubc13/Uev1A and TRAF6 (Fig11.A a, b) or TRAF6 (Figure 11A c, d) in the first ubiquitination reaction step, TAK1 was not activated in the second step (Figure 11B lanes 1-4). Addition of fresh Ubc13/Uev1A and TRAF6 back to reactions 7 to 10 in the second step, TAK1 was activated by products of a and c (Figure 11B lanes 7 & 9), but not b and d (Figure 11B 8 & 10). These controls showed NEM was working and had inactivated E1 and Ubc13/Uev1A, suggesting

that TAK1 activation in Figure 11B 6 was not due to a new ubiquitination event during the second step catalyzed by residual amount of active E1 and Ubc13 carryover due to incomplete inactivation by NEM in the first step. Anti-ubiquitin immunoblot on aliquots from the second step showed a correlation between polyubiquitin chain synthesis and TAK1 activation (Figure 11B, bottom panel). This result strongly suggests that the two events in the TAK1 activation by the Ubc13/Uev1A-TRAF6-dependent polyubiquitination can be uncoupled and further dissected into two steps: a polyubiquitination step and a kinase activation step. It also suggests that an activator for TAK1 kinase activation has been generated during the polyubiquitination reaction step. Further experiments have shown that the activation species can undergo several cycles of freezing and thawing without obvious loss of activity for TAK1 kinase activation and that the activator is sensitive to heat treatment (data not shown).

III.B.3 Purification of activator

In order to characterize the identity of the TAK1 activator generated during polyubiquitination process, chromatographic purification was carried out. For this purpose, polyubiquitination reaction was scaled up to 2ml and TAK1 activator was purified according to the scheme shown in Figure 12A. After the final monoS chromatography step, fractions were tested for their activity for TAK1 activation, used for silver staining and immunoblotting of ubiquitin. As

shown in Figure 12D upper (TAK1 immunoblot) and middle (p-TAK1 immunoblot) panels, TAK1 was activated by fractions 14, 15, 16 and 17 with peak activity in fraction 15. Silver staining of these fractions, as shown in Figure 12B, shows staining of high molucular weight region on the top of the 4-20% gradient gel (Figure 12B, bottom panel), suggesting the possibility that ubiquitin chains could be part of the activation species. Immunoblotting of ubiquitin on these fractions shows a similar pattern as to that of silver staining (Figure 12 C).

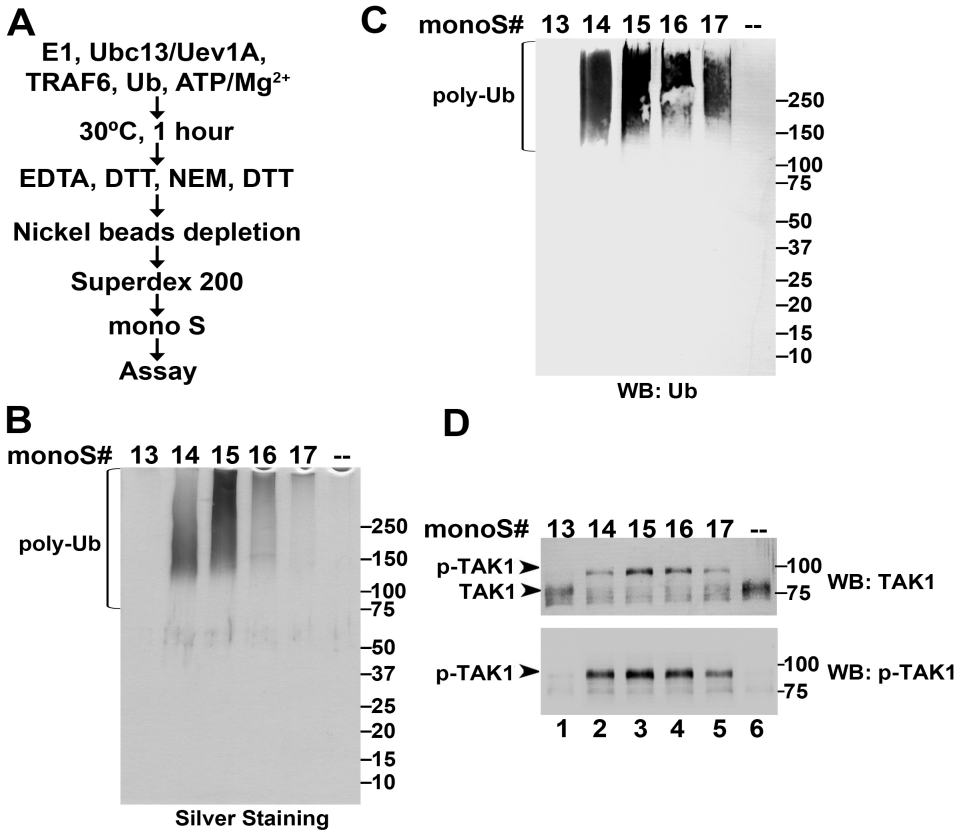


Figure 12: Purification of polyubiquitin chains. A): Scheme for purification of polyubiquitin chains synthesized by TRAF6-Ubc13/Uev1A. B): TAK1 activation assay after final monoS chromatography step. Aliquots of monoS fractions (2 µl) were incubated with TAK1 complex for 1 hour at 30°C.

Aliquots of reaction products were resolved on 10% SDS-PAGE for TAK1, p-TAK1 immunoblotting (upper and middle panels) and ubiquitin immunoblotting. Activation of TAK1 was indicated by its slower mobility shift on TAK1 immunoblotting blot (upper panel, lanes 2-5) or TAK1 phospho-Thr187 (p-TAK1) immunoblotting blot (middle panel, lanes 2-5).

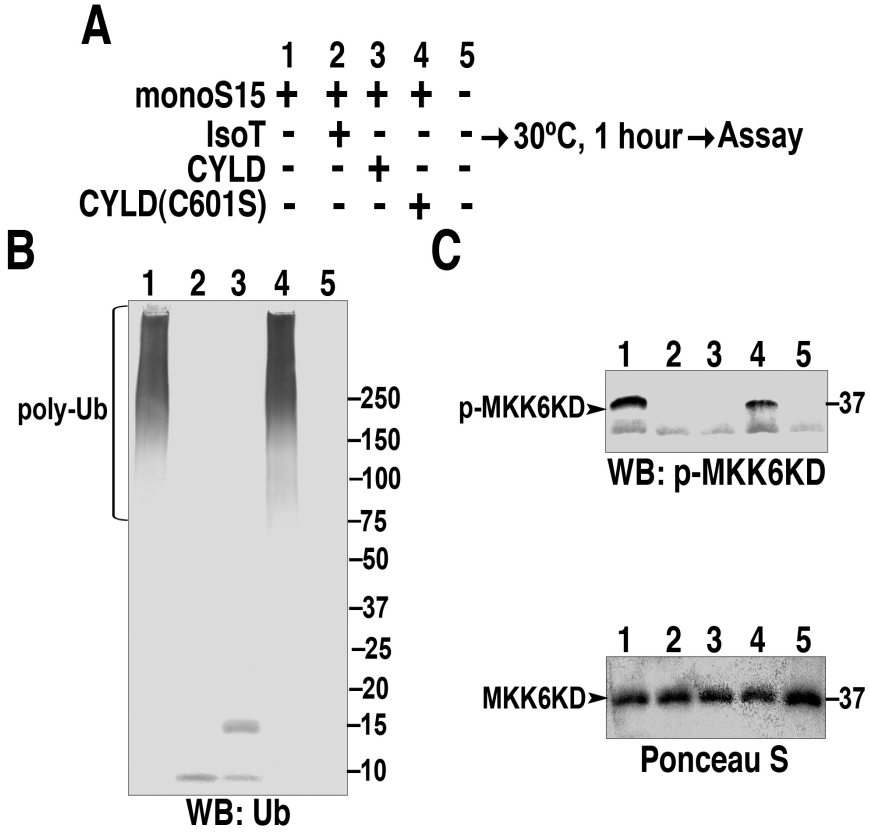
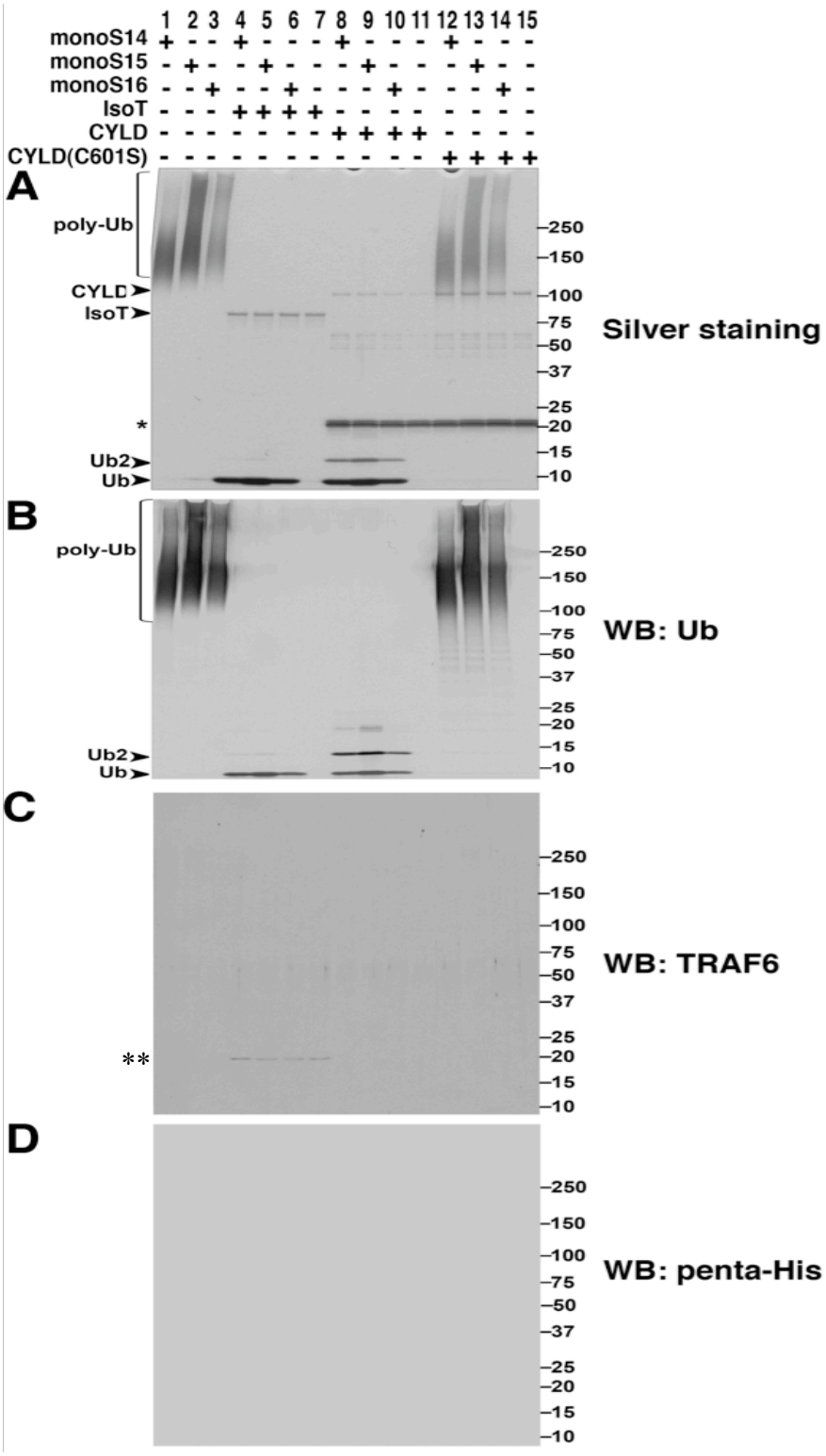


Figure 13: Polyubiquitin chains are required for TAK1 activation. A): Cleavage of polyubiquitin chains by de-ubiquitin enzymes. Aliquots of purified polyubiquitin chains (monoS#15) were incubated alone, with IsoT, CYLD or CYLD(C601S) for 1 hour at 37°C. A small fraction of the treated samples were resolved on 4-20% SDS-PAGE and immunoblotted for ubiquitin, as shown in B). C): Products from step A were incubated with TAK1 complex and its substrate MKK6(K82A) (kinase-dead mutant of MKK6) for 1 hour at 30°C. After incubation, samples were separated on 10% SDS-PAGE and activation of TAK1 was determined by immunoblotting on phosphorylation of MKK6 using MKK6 phospho-Ser207 specific antibody (upper panel). Before immunoblotting, the membrane was stained with Ponceau S to show equal loading (bottom panel).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
monoS14 + - - + - - - + - - - + - - -
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monoS16 - - + - - + - - - + - - - + -
IsoT - - - + + + + - - - - - - - -
CYLD - - - - - - - + + + + - - - -
CYLD(C601S) - - - - - - - - - - - + + + +
A
poly-Ub
CYLD
IsoT
*
Ub2
Ub
250
150
100
75
50
37
25
20
15
10
Silver staining
B
poly-Ub
Ub2
Ub
WB: Ub
C
**
WB: TRAF6
D
WB: penta-His

Figure 14: Characterization of polyubiquitin chains after purification. Aliquots (6 μl. For monoS 15, it is about 1 μg/μl of total proteins) of purified polyubiquitin chains from monoS fractions 14, 15, and 16 were treated with IsoT, CYLD, or CYLD(C601S) for 2 hours at 37°C. After that, 50% were resolved on 4-20% SDS-PAGE for silver staining (A). 15% were separated on 4-20% SDS-PAGE and immunoblotted for ubiquitin (B). 25% were resolved on 10% SDS-PAGE and immunoblotted for TRAF6 (C). The same membrane for C was also immunoblotted using anti-penta-His antibody (D). '*' in A indicates a co-purified contaminating proteins in CYLD and CYLD(C601S). '**' in C indicates a crossing reaction protein band from IsoT preparation.

III.B.4 Polyubiquitin chains are required for TAK1 activation

The data so thus far has shown a direct correlation between polyubiquitin chain synthesis/polyubiquitin chains and TAK1 activation. However there is no direct evidence to show if polyubiquitin chains are required for TAK1 activation. To address directly if the polyubiquitin chains are required for TAK1 activation, one of the active monoS fractions, fraction 15, was treated with two different deubiquitin (DUB) enzymes, IsoT and CYLD, respectively. As a control, the CYLD active site mutant CYLD(C601S) was included (Figure 13A). Following treatment, the products were then used in the TAK1 activation assay. IsoT is a deubiquitination enzyme that prefers free, unanchored polyubiquitin chains as its substrates and cleaves ubiquitin off from the proxy end. IsoT activity requires unmodified Gly-Gly of the ubiquitin C-terminus. CYLD is a deubiquitination enzyme that specifically cleaves isopeptide bonds linked through K63 and it can be endo-isopeptidase. If polyubiquitin chains are required for TAK1 activation, treatment of polyubiquitin chains with a deubiquitinating enzyme should abolish

their activity. As shown in Figure 13B for ubiquitin immunoblotting, IsoT and CYLD treatment of monoS fraction15 resulted in dramatic reduction of high molecular weight polyubiquitin signals and accumulation of small molecular weight polyubiquitin signals (lanes 2 and 3) while CYLD(C601S) didn't cause obvious reduction of high molecular weight polyubiquitin chains. In the TAK1 activation assay, TAK1 was activated by products without treatment or treated with CYLD(C601S) detected by phosphorylation of TAK1 substrate MKK6(K82A) (Figure 13C, lane 1 & 4). However TAK1 activation was abolished by products treated with IsoT and CYLD (Fig13C, lane 2 & 3). This result suggests that polyubiquitin chains are required for TAK1 activation. The observation that IsoT treatment resulted in reduction of activity for TAK1 activation suggests that the activators might be free, unanchored polyubiquitin chains instead of polyubiquitin chains conjugated to some target proteins such as TRAF6, as what is in the current model regarding the mechanism of kinase activation by ubiquitination system (see Figure 8).

III.B.5 Unanchored polyubiquitin chains are the TAK1 activators

The result obtained with IsoT treatment suggests that the polyubiquitin chains, as the TAK1 activators, are un-anchored. To further confirm this, a direct search for potential ubiquitination targets was carried out. The final purified monoS fractions were treated with IsoT or CYLD extensively to deubiquitinate

any targets and then used for silver staining and immunoblotting for ubiquitin, TRAF6 and 6xHis (Figure 14). In the ubiquitination reaction, the only known proteins added were ubiquitin and 6xHis-tagged TRAF6, E1, Ubc13 and Uev1A. The prediction was if any of these proteins were in the fractions, they should be detected on anti-6xHis immunoblotting. If any of them were ubiquitinated, CYLD treatment should deubiquitinate them and be detected by silver staining and immunoblotting as distinctive bands. If it were TRAF6, it should be detected by anti-TRAF6 immunoblotting. If there were other unknown proteins present, either un-ubiquitinated or ubiquitinated, they should be detected by silver staining, especially after deubiquitination by CYLD. Total proteins of fraction15 for this experiment were about 6μg. After treatment 50% were used for silver staining (figure 14A), 15% for ubiquitin immunoblotting (figure 14B), and 25% for TRAF6 and 6xHis western blotting (figure 14C & D). As shown in Figure 14 B, anti-ubiquitin immunoblotting shows the cleavage of polyubiquitin chains by IsoT is almost complete and more than 95% of the chains have been reduced to monoubiquitins (lanes 4, 5 & 6). CYLD treatment also has reduced 95% of the polyubiquitin chains to mono- or di-ubiquitin (lanes 8, 9 & 10). Immunoblotting on TRAF6 (Figure 14 C) and 6xHis (Figure 14 D) didn't show any signals even after long time development. On silver staining gel only ubiquitin, IsoT and CYLD themselves were detected (Figure 14 A). These results suggest that there are not any TRAF6 or other 6xHis tagged proteins or other unknown proteins

except ubiquitin in the final monoS fractions (or say above detectable levels) and are consistent with the idea that the activators for TAK1 kinase complex activation are unanchored polyubiquitin chains themselves.

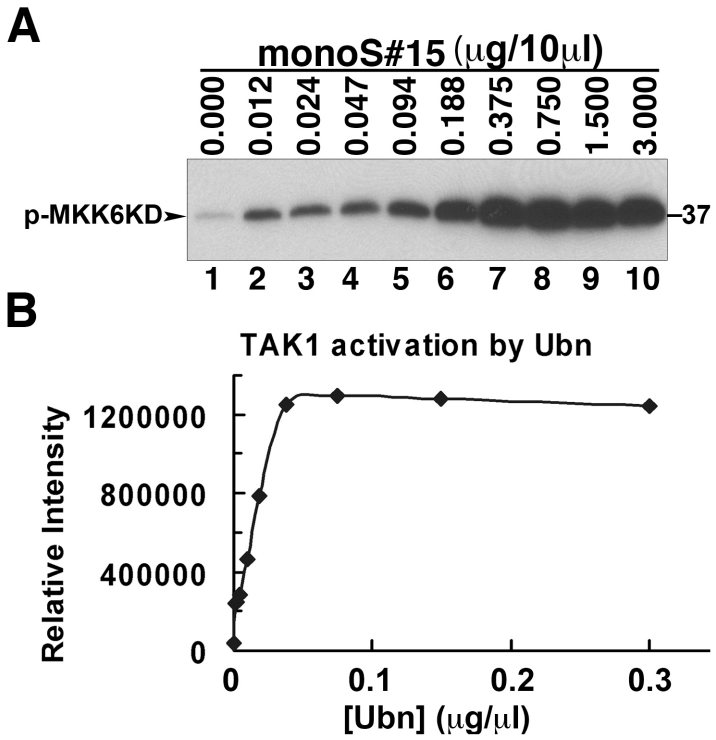

Figure 15: Titration of polyubiquitin chains on TAK1 activation. TAK1 complex and its substrate MKK6(K82A) were incubated alone or with increasing amount (2-fold increment) of purified polyubiquitin chains (monoS fraction 15) for 1 hour at 30°C. Products were resolved on 10% SDS-PAGE and immunoblotted for phospho-MKK6(K82A) using ECL (A). Intensity of each phosphorylated MKK band was quantified using ImagQuant software and plotted against the concentration of polyubiquitin chains used in each reaction (B).

III.B.6 Titration of polyubiquitin chains on TAK1 activation

In order to know how potent the polyubiquitin chains are as TAK1 kinase activators, purified TAK1 complex and its substrate MKK6(K82A) were

incubated with increasing concentrations of purified polyubiquitin chains (monoS fraction 15) and phosphorylation of MKK6(K82A) was detected using anti-phospho-MKK6 antibody after separation on 10% SDS-PAGE. As shown in Figure 15A, phosphorylation of MKK6(K82A) increases first as polyubiquitin chain concentration increases and then reaches a plateau at about 0.04 μg/μl of polyubiquitin chains. These bands were quantified and plotted and are shown in Figure 15B. The plot suggests that activation of TAK1 by polyubiquitin chains is saturable. Estimation of the concentration of polyubiquitin chains for half-maximal TAK1 activation is about 0.016 μg/μl or 2 μM of mono-ubiquitin or 40 nM of polyubiquitin chains assuming the average length of polyubiquitin chains is 50 ubiquitins.

III.B.7 Coiled-coil domain of TRAF6 is required for synthesis of TAK1 activators

To characterize which domain or domains in TRAF6 are important for catalyzing polyubiquitin chain synthesis that activates TAK1, TRAF6 WT and two C-terminal deletion mutants, one with TRAF domain deletion (TRAF6(1-365) and one with further coiled-coil domain deletion (TRAF6(1-291) (Figure 16A) were tested. Flag-tagged proteins were over-expressed in 293T cells and purified using anti-Flag antibody-conjugated M2 agarose beads. Purified proteins were tested first for their ability to activate IKK by incubating them with Hela S100. As shown

in Figure 16B top panel, IKK was activated by TRAF6 WT and TRAF6(1-365) (lanes 2 & 3) but not by TRAF6(1-291) (lane 4) determined by immunoblotting for IκBα using anti-IκBα antibody shown as slower mobility shift bands because

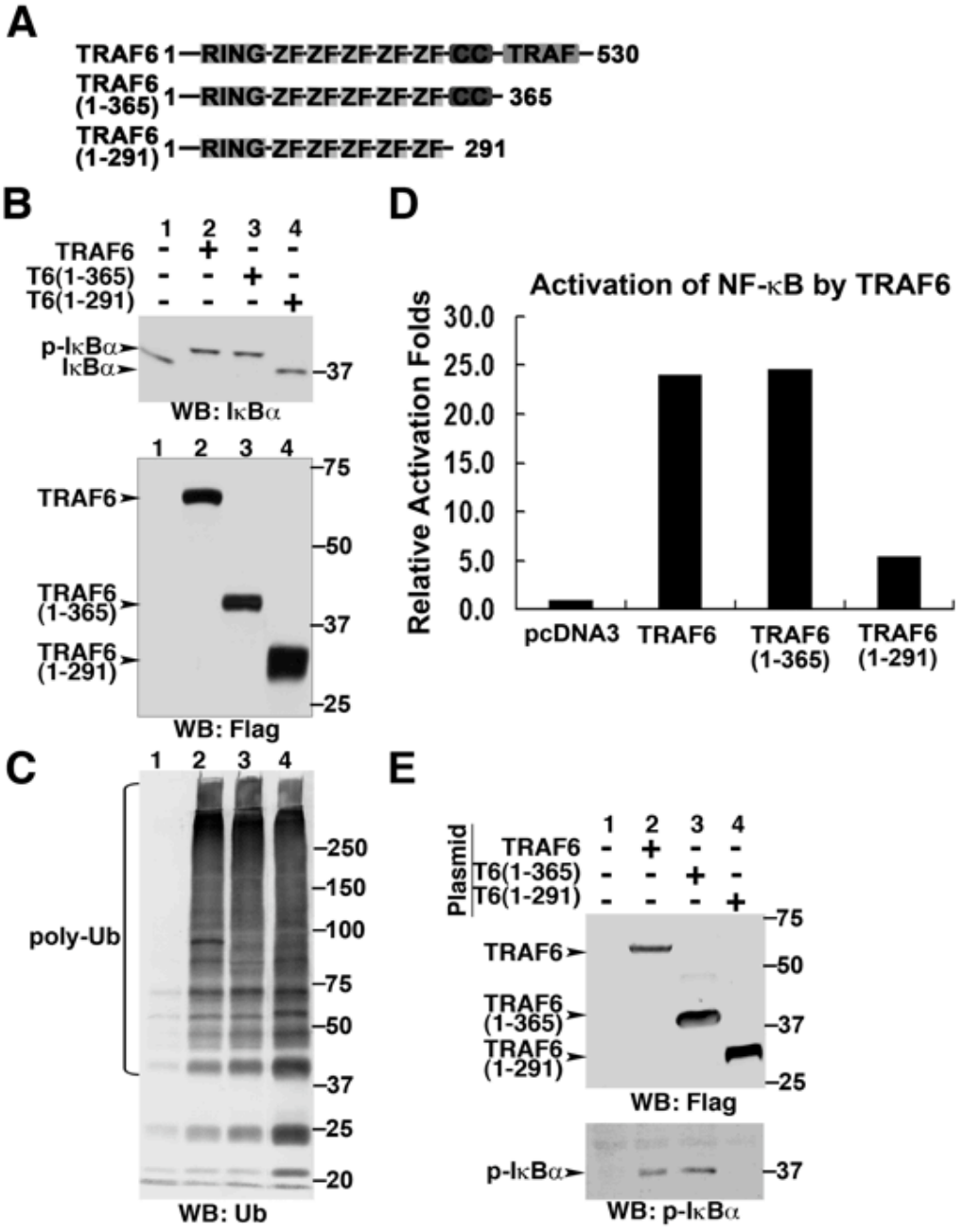

Figure 16: Coiled-coil domain of TRAF6 is required for NF-κB activation. A): Diagram showing the domain structure of TRAF6 and its deletion mutants used in this study. RING, RING-finger domain. ZF: Zinc-finger domain. CC, Coiled-coil domain. TRAF, TRAF domain. B): Flag-tagged TRAF6 and its mutants were transiently overexpressed in 293T cells and purified using anti-flag antibody-conjugated M2-agarose beads. Aliquots of purified TRAF6s were incubated with Hela S100 for 1 hour at 30°C. Products were separated on 10% SDS-PAGE and immunoblotted for IκBα (upper panel) and Flag (middle panel). Activation of IKK was indicated by slower mobility shift of IκBα after phosphorylation by activated IKK. C): Equal amount of TRAF6 as used in B was incubated with E1, Ubc13/Uev1A, Ubiquitin and ATP/Mg^{2+} for 30 minutes at 30°C. Products were separated on 10% SDS-PAGE and immunoblotted for ubiquitin to show polyubiquitin chain synthesis by the TRAF6 proteins. D): NF-κB reporter assay. 293 cells were transfected with pcDNA3, TRAF6, or TRAF6 deletion mutants together with pCMV-β-gal and p3xκB-Luciferase reporter plasmid. After 24 hours, cells were harvested and activity of luciferase was measured and normalized against β-gal expression. Relative activation of NF-κB by TRAF6s was normalized against that by pcDNA3. E): Cell lysate from D was separated on 10% SDS-PAGE and immunoblotted for expression of TRAF6s using anti-flag antibody (upper panel) and for phosphorylation of IκBα (bottom panel) using anti-phospho-IκBα antibody.

of phosphorylation by activated IKK. All three proteins catalyzed similar amounts and size distributions of polyubiquitin chain synthesis (Figure 16 C). Consistent with the *in vitro* assay, a luciferase-based reporter assay for NF-κB activation shows strong activation of NF-κB by TRAF6 WT and TRAF6(1-365) but weak activation by TRAF6(1-291) (Figure 16 D). This is not due to difference in protein expression levels since all three proteins have similar levels of protein expression (Figure 16E, top panel).

To further characterize the three proteins for their ability to catalyze synthesis of polyubiquitin chains that can activate TAK1, polyubiquitin chain synthesis by

these three proteins was scaled up and purified according to the protocol shown in Figure 17 A. Silver staining of aliquots from purified products (monoS fractions)

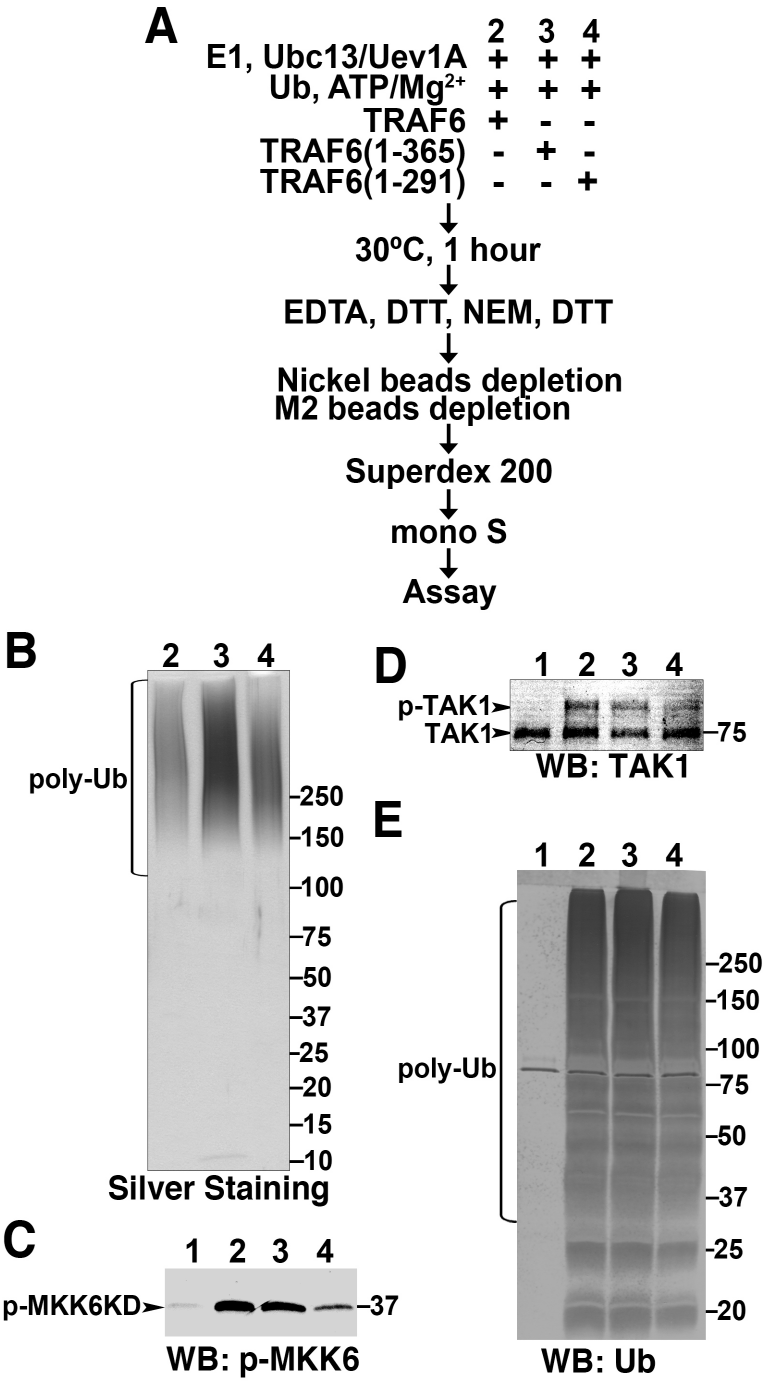

Figure 17: Characterization of polyubiquitin chains synthesized by TRAF6 and its deletion mutants. A): Diagram showing the protocol used for purification of polyubiquitin chains synthesized by TRAF6 and its deletion mutants. B): Purified polyubiquitin chains from step A were separated on 4-20% SDS-PAGE and detected by silver staining. C, D, E): Aliquots of purified polyubiquitin chains from step A were incubated with TAK1 complex and MKK6(K82A) for 1 hour at 30°C. Products were separated on 10% SDS-PAGE and activation of TAK1 was determined by immunoblotting of phospho-MKK6(K82A) (C) and of TAK1 (D). Aliquots were also separated on 10% SDS-PAGE and immunoblotted for ubiquitin (E).

shows all three proteins catalyzed synthesis of similar level and size distribution of polyubiquitin chains (Figure 17 B). In the TAK1 activation assay, as shown in Figure 17C and D, TAK1 was strongly activated by polyubiquitin chains synthesized by TRAF6 WT and 1-365 (lanes 2 & 3) and weakly by polyubiquitin chains synthesized by TRAF6(1-291) (lane 4) as determined by immunoblotting of phospho-MKK6(K82A) (figure 17C) and of TAK1 (figure 17D). Immunoblotting for ubiquitin in aliquots from the same set of reactions shows comparable polyubiquitin chains synthesized by the three proteins were added in the assay (Figure 17E).

These results suggest that the coiled-coil domain of TRAF6 is required for synthesis of polyubiquitin chains that can activate TAK1. The difference cannot simply contribute to their ability to catalyze polyubiquitin chain synthesis since all three proteins used can synthesize similar levels of polyubiquitin chains (although more TRAF6(1-291) is needed *in vitro* in order to synthesis polyubiquitin chains by the three proteins to similar levels). The observation

raises an interesting and intriguing question as to what difference there is between active polyubiquitin chains and inactive polyubiquitin chains.

III.C Discussion

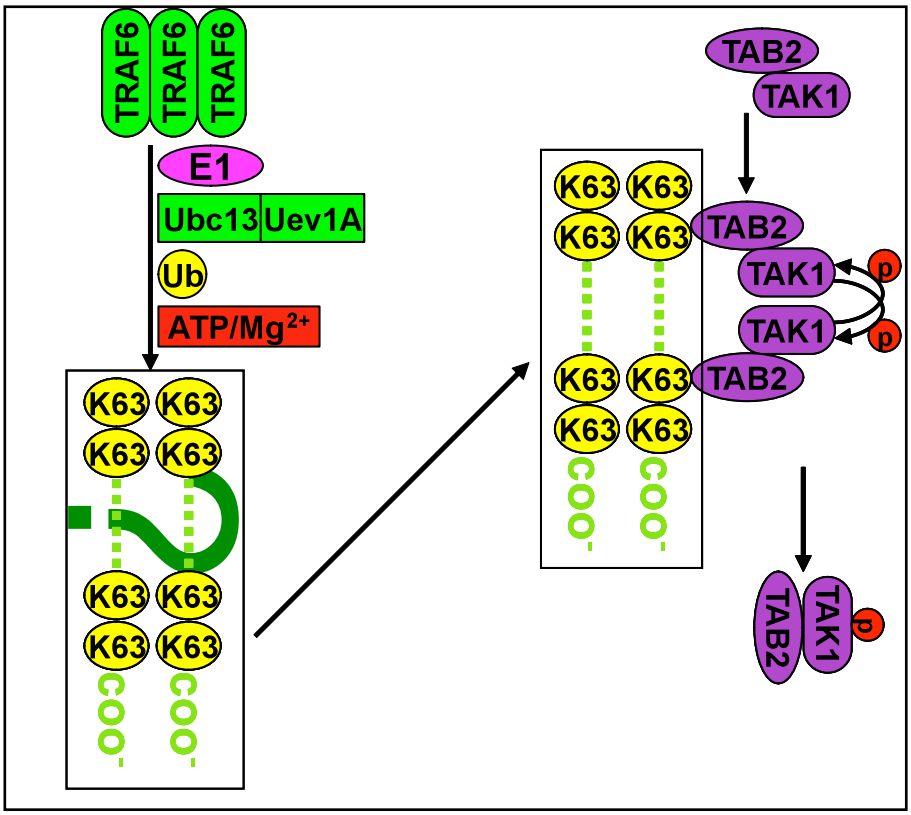

Figure 18: A new model for TAK1 activation by TRAF6. In this modified model, TRAF6, together with E1, Ubc13/Uev1A, catalyzes K63-linked un-anchored polyubiquitin chain synthesis. These un-anchored polyubiquitin chains may adopt a particular higher-order conformation, labeled by a question mark (?), that is unknown yet. The un-anchored polyubiquitin chains then recruit TAK1 complex through TAB2. Binding of TAK1 complex to polyubiquitin chains may cause their dimerization or oligomerization, which can then do trans-autophosphorylation resulting in their activation.

In this chapter, I reported the successful use of NEM in helping dissect the molecular mechanism of TAK1 kinase activation by TRAF6-Ubc13/Uev1A-dependent polyubiquitination. This has led to the following observations: 1)

polyubiquitination and kinase activation can be un-coupled; 2) the actual activation species (activators) generated from the polyubiquitination step are un-anchored K63-linked polyubiquitin chains; 3) the coiled-coil domain of TRAF6 is required for synthesis of active polyubiquitin chains for TAK1 activation. Based on this new finding, I propose a modified model, as shown in Figure 18, for TAK1 activation by the TRAF6-Ubc13/Uev1A-dependent ubiquitination system. In this modified model, TRAF6, together with E1, Ubc13/Uev1A, catalyzes K63-linked un-anchored polyubiquitin chain synthesis. These un-anchored polyubiquitin chains may adopt a particular higher-order conformation that is at present unknown. The un-anchored polyubiquitin chains then recruit TAK1 complex through binding to TAB2. Binding of TAK1 complex to polyubiquitin chains may cause their dimerization or oligomerization, which can then undergo trans-autophosphorylation resulting in their activation.

It is known that TRAF6 activates TAK1 kinase complex through its ubiquitin ligase activity. However before this study, all the minimal components required for TAK1 activation are incubated together directly and it is not known what polyubiquitination actually does during the activation process. A requirement for polyubiquitin has never been demonstrated directly. All prior evidence was indirect and correlative. We also don't know what the actual activation species for TAK1 activation that was generated during the polyubiquitination process. By using NEM to inactivate E1 and Ubc13, I

demonstrate directly for the first time that polyubiquitination is required for TAK1 activation. The polyubiquitination step generates polyubiquitin chains and it is the polyubiquitin chains themselves that are required for TAK1 activation.

The current model for how TRAF6 activates TAK1 is that TRAF6 auto-ubiquitinates itself and it is the ubiquitinated TRAF6 that then activates TAK1. However, I found, after purification, the active species are un-anchored polyubiquitin chains. They don't conjugate to any target. I could not detect any ubiquitinated or even un-ubiquitinated TRAF6 in the final purified active fractions. In fact I could not detect any proteins other than ubiquitin in the final purified active fractions. Even before purification, I could not detect any obvious ubiquitination of TRAF6. A recent study addressing the mechanism of polyubiquitination by Ubc13/Uev1A and TRAF6 also reports that TRAF6 auto-ubiquitination in this system is rare and the reason is because the Uev1A subunit inhibits TRAF6 auto-ubiquitination but facilitates processive free polyubiquitin chain assembly (Pertrosky et al., 2007).

In another recent report, site-specific polyubiquitination of TRAF6 has been reported to be required for mediating IL-1β signaling (Lamothe et al., 2007). This is in contrast to what I am reporting here. It is also in contrast to what we have observed in our laboratory. Dr. Li (unpublished data) in the laboratory has systematically investigated whether any site-specific auto-ubiquitination of TRAF6 is required for IKK activation through site-directed mutagenesis and has

not found any mutant(s) that loses activity. We don't have a good explanation to interpret the differences among these observations and more studies are needed to address them. One possibility is that site-specific ubiquitination of TRAF6 is not required for ubiquitin ligase activity *in vitro* but is required for communication with and recruitment to upstream signaling intermediates such as IRAK-1 in the IL1R and TLR signaling pathways *in vivo*.

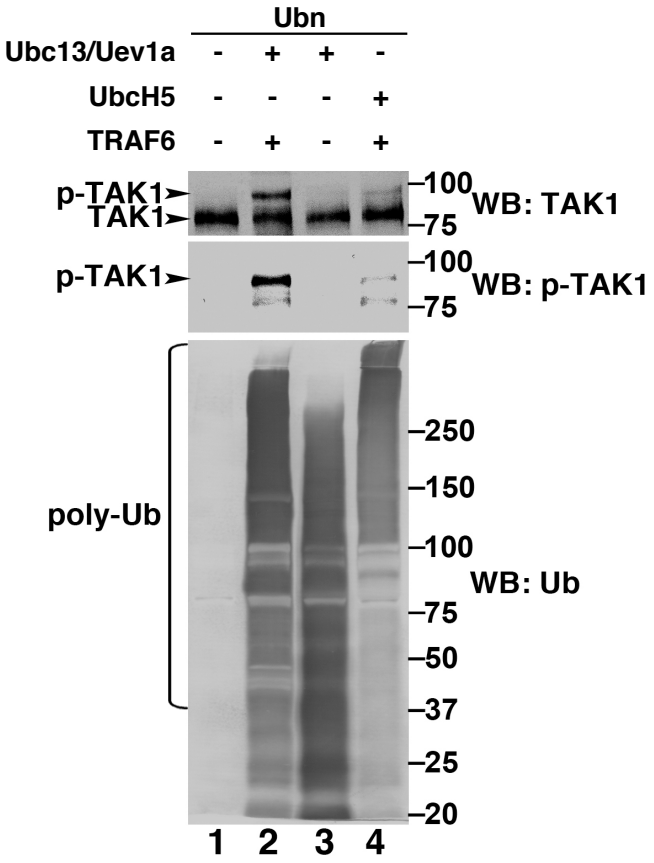

Figure 19: TAK1 activation by polyubiquitin chains. Polyubiquitin chains were synthesized by TRAF6-Ubc13/Uev1A (bottom panel, lane 2), or Ubc13/Uev1A alone (lane 3), or TRAF6-UbcH5 (lane 4). These pre-synthesized polyubiquitin chains were then incubated with TAK1 complex to check for their

TAK1 activation activity. Activation of TAK1 was determined using anti-TAK1 (upper panel) immunoblotting and anti-phospho-TAK1 immunoblotting (middle panel).

The finding that un-anchored polyubiquitin chains are the activators raises a very interesting question as to what is special about this kind of chains or whether other polyubiquitin chains can activate TAK1 kinase or not. Two observations suggest not any kind of polyubiquitin chains can activate TAK1 kinase.

1) Polyubiquitin chains synthesized by TRAF6-UbcH5 cannot (or very weakly) activate TAK1 kinase either (see Figure 19, lane 4). One explanation for that is TAB2 and TAB3 prefer K63-linked to K48-linked polyubiquitin chains. However K63 linkage itself is not sufficient to explain the specificity. Although Ubc13/Uev1A alone can only catalyze some polyubiquitin chains under normal conditions, they can catalyze very efficient and reasonably long polyubiquitin chains when used at high concentration and incubated for a longer time. However, chains generated under this condition are not able to activate TAK1 (Figure 19 lane 3). One might argue that the size distribution of polyubiquitin chains synthesized in the absence and presence of TRAF6 are different (Figure 19, bottom panel, compare lane 2 to lane 3). However, TRAF6 mutant with coiled-coil deletion can synthesize polyubiquitin chains comparable to TRAF6 WT in terms of size distribution, but the chains are barely active towards TAK1

activation (see Figure 17). This suggests to us that TRAF6 does not simply function as an E3 to facilitate chain extension; it is doing more than that and may facilitate assembly of higher order polyubiquitin chains with a particular conformation. Structural studies of these chains are needed to reveal the difference between active and inactive polyubiquitin chains. Polyubiquitin chains as the sole signal for downstream effects can also be found in ubiquitinated proteins as substrates for proteasome degradation where the sole signal recognized by proteasome is polyubiquitin chains but not the to-be degraded substrates.

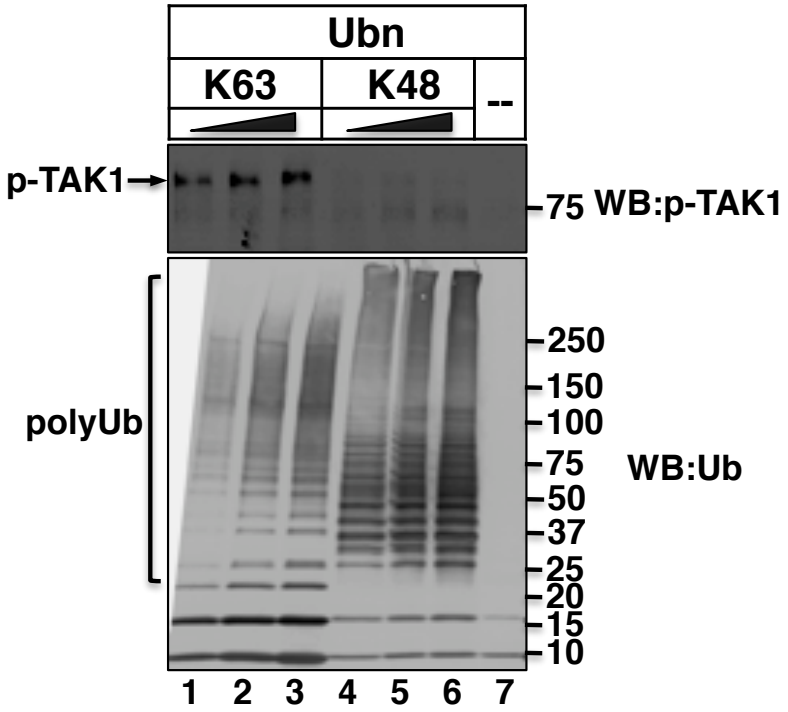

Figure 20: TAK1 activation by K63- but not K48-linked polyubiquitin chains. TAK1 kinase complex was incubated with increasing concentration of

either K63-linked polyubiquitin chains (lanes 1-3) or K48-linked polyubiquitin chains (lanes 4-6) or alone (lane 7). Activation of TAK1 was determined by immunoblotting of phosphrylation of TAK1 using anti-TAK1 phospho-Thr183 antibody (upper panel). Immunoglotting of ubiquitin (bottom panel) shows comparable concentration of K63- or K48-linked chains were used.

2) K48-linked polyubiquitin chains are not capable of TAK1 activation. As shown in Figure 20 upper panel, TAK1 incubation with increasing amount of K63-linked polyubiquitin chains leads to increased activation of TAK1 (lanes 1-3) while TAK1 incubation with K48-linked polyubiquitin chains does not (lanes 4-6).

I want to emphasize that all of these observations have been obtained *in vitro,* and more studies are needed to address their existence *in vivo* when cells are stimulated by IL1β or other TLR ligands.

Chapter IV: IKK activation by polyubiquitin synthesized by UbcH5-TRAF6

IV.A Introduction

In the original report by Deng et al. (Deng et al., 2000), IKK activation by TRAF6 was established in a cell-free system using Hela S100 fractionation. Chromatographic fractionation resulted in purification of the Ubc13/Uev1A heterodimer and TAK1 kinase complex. The biochemical function of Ubc13/Uev1A in this TRAF6-dependent IKK activation system is ubiquitin conjugating enzyme E2 activity, in which TRAF6 and Ubc13/Uev1A catalyze K63-linked polyubiquitination. One of the ubiquitination targets is TRAF6 itself. Polyubiquitinated TRAF6 then activates TAK1 kinase complex, which in turn activates IKK and also p38 and JNK MAPK kinases.

Overexpression of a dominant negative mutant of Ubc13 in 293T cells resulted in reduction of NF-κB activation by IL1β and TNFα stimulation and by TRAF2 and TRAF6 overexpression (Deng et al., 2000). Overexpression of a dominant negative mutant of Ubc13 in Jurkat T cells also resulted in reduction of NF-κB activation by TCR signaling (Sun et al., 2004). RNAi silencing of Ubc13 resulted in defective NF-κB activation in 293 cells (Zhou et al., 2004). RNAi silencing of either Ubc13 or Uev1A in mouse microglia cells resulted in defective NF-κB activation by LPS (Anderson et al., 2005). RNAi of Ubc13 and Ubc13

knockout in MEF cells abolished NF-κB activation by the HTLV-encoded Tax protein (Shembade et al., 2007). These reports support the importance of Ubc13/Uev1A in NF-κB activation *in vivo* in a variety of cells and by a variety of stimuli.

Conditional knockout studies of Ubc13 gene, however, suggest that the function of Ubc13 in NF-κB activation is more complex. In one report (Yamamoto et al., 2006a), TLR-, BCR- and CD40-induced NF-κB activation were mostly preserved or modestly affected in Ubc13 knockout macrophages and B cells. TRAF6 ubiquitination by IL1β stimulation was normal in Ubc13 knockout MEF cells. *In vitro* assay with extracts from Ubc13 knockout MEF cells showed normal TAK1 and IKK activation. In conditional Ubc13 knockout T cells, NF-κB activation was modestly affected and TAK1 activation was largely abolished in TCR signaling (Yamamoto et al., 2006b). Defective JNK and p38 MAPK activation and ubiquitination of NEMO were consistently observed by all the stimuli tested (Yamamoto et al, 2006a and b). However, in another heterozygous $Ubc13^{+/-}$-based studies (Fukushima et al., 2007), TRAF6 ubiquitination *in vivo* was reduced in response to LPS challenge. Macrophages and splenocytes from the $Ubc13^{+/-}$ mice had defects in NF-κB as well as JNK and p38 MAPK activation in the TRAF signaling pathways.

The explanation for these conflicting observations in different studies is complex. It may be due to differences in cell types used in their studies. It may also be due to compensation by another E2 or E2s.

In this chapter, I describe my efforts to the identification of a Ubc13-independent IKK activation system by TRAF6. Through biochemical fractionation, I purified another E2, UbcH5, that works with TRAF6 for the activation of IKK in a ubiquitination dependent manner. Like in the system of TAK1 activation by TRAF6-Ubc13/Uev1A, characterization of the TRAF6-UbcH5-dependent IKK activation system also shows the system can be dissected into two steps: a ubiquitination step and an IKK kinase activation step. The activation species generated from the ubiquitination step are also un-anchored polyubiquitin chains. The linkage in these chains is neither uniformly K48- nor K63- linked but rather is probably mixed-linkage-linked.

IV.B Results:

IV.B.1 Ubc13-independent activation of IKK by TRAF6

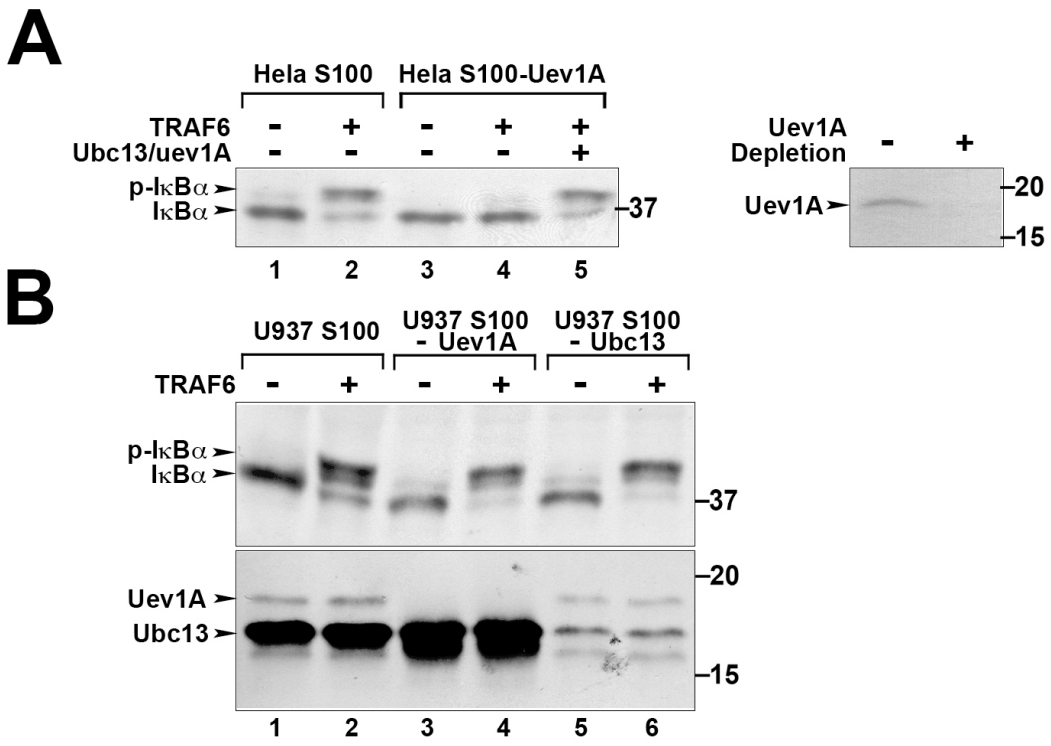

Figure 21. Ubc13-Independent IKK Activation by TRAF6. A) Activation of IKK by TRAF6 in Hela S100 requires Ubc13/Uev1a. Cytosolic extracts were prepared from Hela cells (S100) and then incubated with recombinant TRAF6 in the presence of ATP (Lane 1&2). In lane 3, 4, and 5, Hela S100 was depleted of Uev1a using immobilized Ubc13 sepharose beads and then incubated with TRAF6. In lane5, recombinant Ubc13/Uev1a were added back to the cell extracts. Phosphorylation of IκBα by IKK was determined by immunoblotting with an IκBα antibody. Right panel shows the Uev1a depletion efficiency. B). Ubc13-independent IKK activation by TRAF6 in U937 cell extracts. Cytosolic extracts were prepared from U937 cells (S100) and then undergone Ubc13 or Uev1a depletion using immobilized Uev1a or Ubc13-sepharose beads. Cell extracts were then incubated with TRAF6 in the presence of ATP (upper panel). Activation of IKK was determined by immunoblotting of IκBα as in A. Depletion efficiency was determined by immunoblotting with a Ubc13 or Uev1a antibody (bottom panel).

In the process of another project, I found that IKK activation by TRAF6 happened in the absence of Ubc13/Uev1A. As shown in Figure 21A, in Hela S100, IKK is activated by incubation with TRAF6 (lane 2) determined by immunoblotting using anti-IκBα antibody. IKK activation was abolished when Uev1A was depleted from Hela S100 using immobilized Ubc13 sepharose beads even in the presence of TRAF6 (lane 4), which can be restored when recombinant Ubc13/Uev1a is added back to the reaction (lane5). Uev1A immunoblot on the right side of panel A shows the depletion is more than 95%. This is consistent with the idea that Ubc13/Uev1A is required for IKK activation by TRAF6.

However IKK activation by TRAF6 is not affected in S100 generated from U937, a monocyte cell line, in the absence of Ubc13/Uev1A. As shown in Figure 21 B, in U937 S100, IKK is activated by incubation with TRAF6 (lane 2). However, IKK is still activated by TRAF6 when either Uev1A is depleted using Ubc13-sepharose beads (lane 4) or Ubc13 is depleted using immobilized Uev1A-sepharose beads (lane 6) from U937 S100. These results suggest that there is a Ubc13-independent IKK activation system by TRAF6 in U937 cells, consistent with the idea that different cell lines or cell types might have redundant pathways to activate IKK by TRAF6.

IV.B.2 Identification of UbcH5 as a second E2 for IKK activation by TRAF6

In order to discover what other components might be required for IKK activation by TRAF6 independent of Ubc13, U937 S100 fraction were initially fractionated into three fractions (Q/I, Q/II and Q/III) on HiTRAP Q column (Figure 22A). Q column is an anion exchange column in which negatively charged molecules bind and can then be eluted by increased concentration of salt such as NaCl. Q/I contains materials that don't bind the Q column. Q/II are materials that were eluted by 0.1M NaCl. Q/III are materials that are eluted by 0.1-0.5M NaCl. Previous experience has shown Ubc13/Uev1A complex should be in Q/II. Indeed, when Q/II and Q/III were incubated together with TRAF6, IKK was activated (Figure 22B lane 2). However, depletion of Ubc13 from Q/II using Uev1A-sepharose beads abolished IKK activation by TRAF6 (lane 6), which could be restored when Q/I was added back (lane8). Q/I itself was not sufficient to activate IKK by TRAF6 when incubated with Q/III (lane 4). These results suggest that multiple factors are required for Ubc13-independent IKK activation by TRAF6.

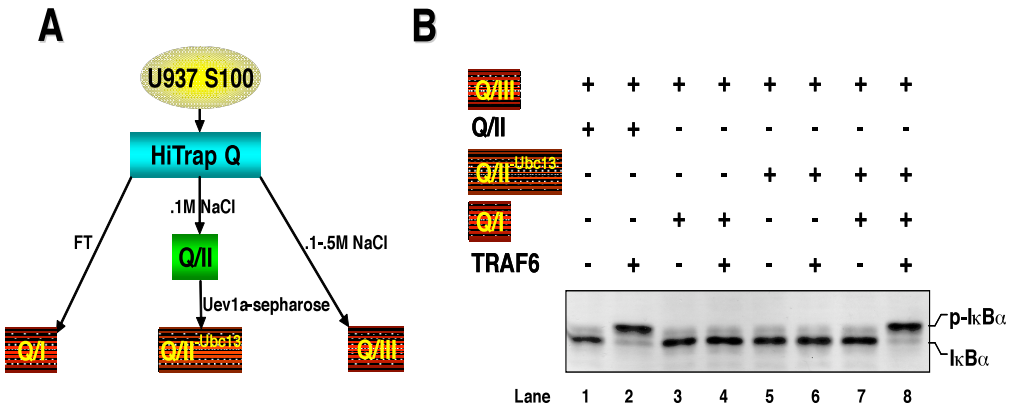

Figure 22. Ubc13-Independent IKK Activation by TRAF6 requires multiple factors. A) Scheme of initial fractionation of U937 S100 on HiTRAP Q column. U937 S100 was loaded onto Q column. Unbound materials (FT) were collected as Q/I. Materials eluted by 0.1M NaCl were collected as Q/II. Those eluted at 0.1-0.5M NaCl were collected as Q/III. Q/II^{-Ubc13} were materials that didn't bind immobilized Uev1a-sepharose beads. B). Q/III, Q/II^{-Ubc13} and Q/I are required for IKK activation by TRAF6. Fractions were incubated with recombinant TRAF6 individually or in combination to determine the requirement for restoration of IKK activation. Activation of IKK was determined by immunoblotting of IκBα using anti- IκBα antibody.

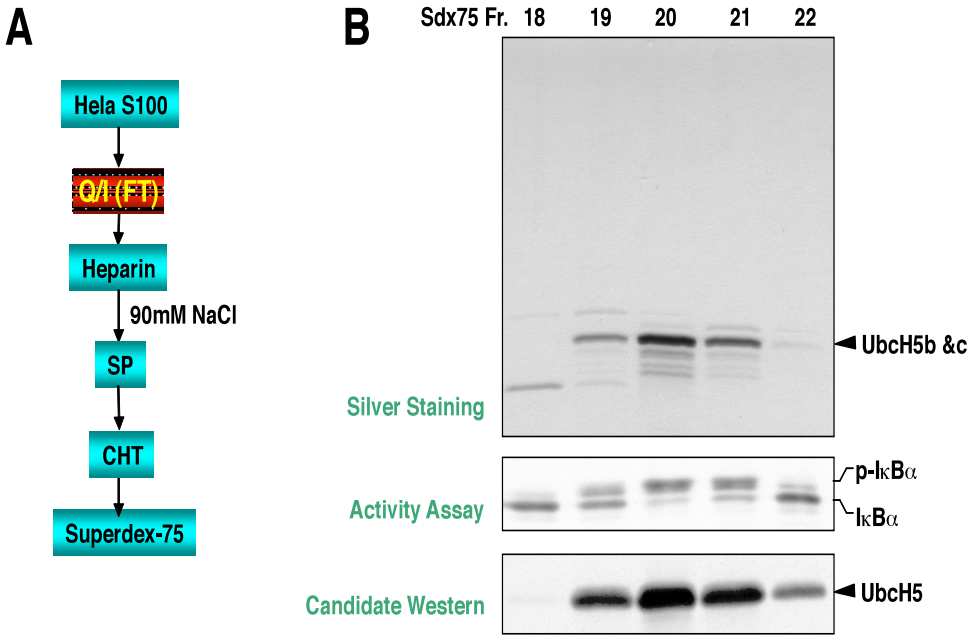

Figure 23. Identification of UbcH5b and UbcH5c as the factors in Q/I responsible for IKK activation. A) Purification scheme of Q/I. Swapping experiments (not shown) between Hela S100 fractions and U937 S100 fractions suggested Hela Q/I could replace U937 Q/I to restore IKK activation. So Hela S100 instead of U937 S100 was used for Q/I fractionation. B). Identification of UbcH5b and UbcH5c. The Fractions from the superdex-75 column were subjected to SDS-PAGE, followed by silver staining (top panel). The same fractions were also assayed for IKK activity in the presence of TRAF6, Q/II^{-Ubc13} and Q/III (middle panel). The arrowhead indicates the protein band that was correlated with IKK activity and identified as UbcH5 by mass spectrometry and by immunoblotting of UbcH5 using anti-UbcH5 antibody (bottom panel).

Swapping experiments using Q/I fractions between Hela cells and U937 cells have indicated that Q/I from Hela cells is able to replace U937 Q/I for restoration of IKK activation by TRAF6 (data not shown). Since Q/I was readily available from Hela cells, fractionation of Hela Q/I was carried out. After several trials, a fractionation scheme for purification of factor(s) in Q/I was worked out and is shown in Figure 23A. After the final superdex-75 step, IKK activation assay was carried out. As shown Figure 23B, middle panel, IKK is activated by superdex-75 fractions 19, 20 and 21 with peak activity in fraction 20. Silver staining on these fractions, as shown on Figure 23B upper panel, shows one major band (arrow head) around 15KDa that correlates with the activity of IKK activation by TRAF6. I used mass spectrometry to identify this band as UbcH5b and UbcH5c. Immunoblotting using anti-UbcH5 antibody on these fractions also shows the presence of UbcH5 in fractions having activity and their intensity correlates very well with the activity strength of each fraction (Figure 23B bottom panel).

In order to determine whether UbcH5 is the sole factor in Q/I that is required for IKK activation, recombinant UbcH5b and UbcH5c were expressed, purified from *E.coli,* and then used in the IKK activation assay. As shown in Figure 24A, IKK can be activated when either recombinant UbcH5b or UbcH5c is incubated with Q/II^{-Ubc13} and Q/III in the presence of TRAF6 (lanes 2 and 4).

SiRNA-based knockdown of UbcH5b and UbcH5c also supported UbcH5 is the factor required for IKK activation by TRAF6. 293 cells were transfected with RNAi oligo against UbcH5b and c or UbcH5a or GFP as controls and used for generation of S100. Q/I fractions were then generated from the S100 and used in the IKK assay. As shown in Figure 24B, Q/I generated from cells transfected with UbcH5bc siRNA oligoes lost the capacity to activate IKK by TRAF6 (lane 2), while those from UbcH5a or GFP siRNA transfection showed TRAF6-dependent IKK activation (lane 4 & 6). The bottom panel in Figure 24B shows the knockdown efficiency of UbcH5b & c.

All the data presented above strongly support that UbcH5b and UbcH5c are the factors in Q/I fraction that support IKK activation by TRAF6.

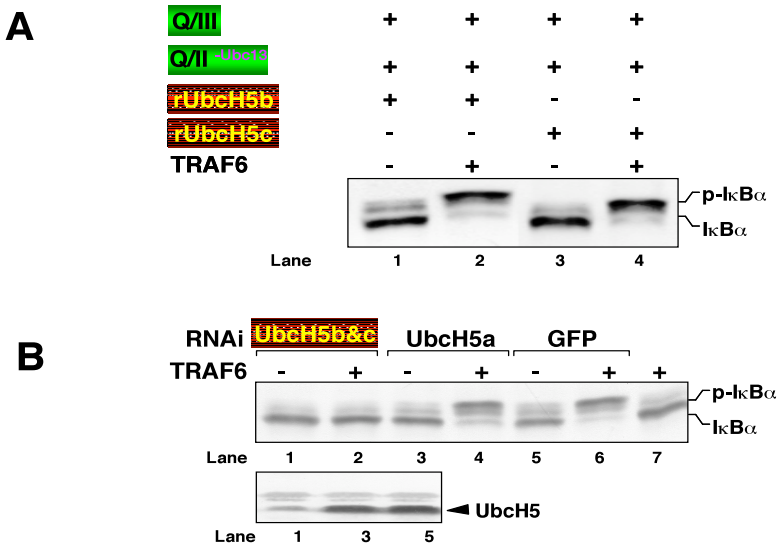

Figure 24. UbcH5b and UbcH5c are the factors in Q/I for IKK activation. A). Either recombinant UbcH5b or recombinant UbcH5c can replace

Q/I to activate IKK. Recombinant UbcH5b (1 & 2) or UbcH5c (3 & 4) was incubated with Q/III, Q/II^{-ubc13} in the absence or presence of TRAF6 to assay IKK activation. Activation of IKK was determined by immunoblotting of IκBα using anti-IκBα antibody. B). Knockdown of UbcH5b and UbcH5c abolishes Q/I activity in IKK activation. Cell extracts (S100) were prepared from 293 cells transiently transfected with GFP siRNA, UbcH5a siRNA or UbcH5b &c siRNA and used to generate Q/I, which were then incubated with U937 Q/III, Q/II^{-Ubc13} in the absence or presence of TRAf6 to determine IKK activity (top panel). Knockdown efficiency of UbcH5 was determined by immunoblotting using anti-UbcH5 antibody (bottom panel).

IV.B.3 TRAF6 and UbcH5 are sufficient for IKK activation in a ubiquitination dependent manner

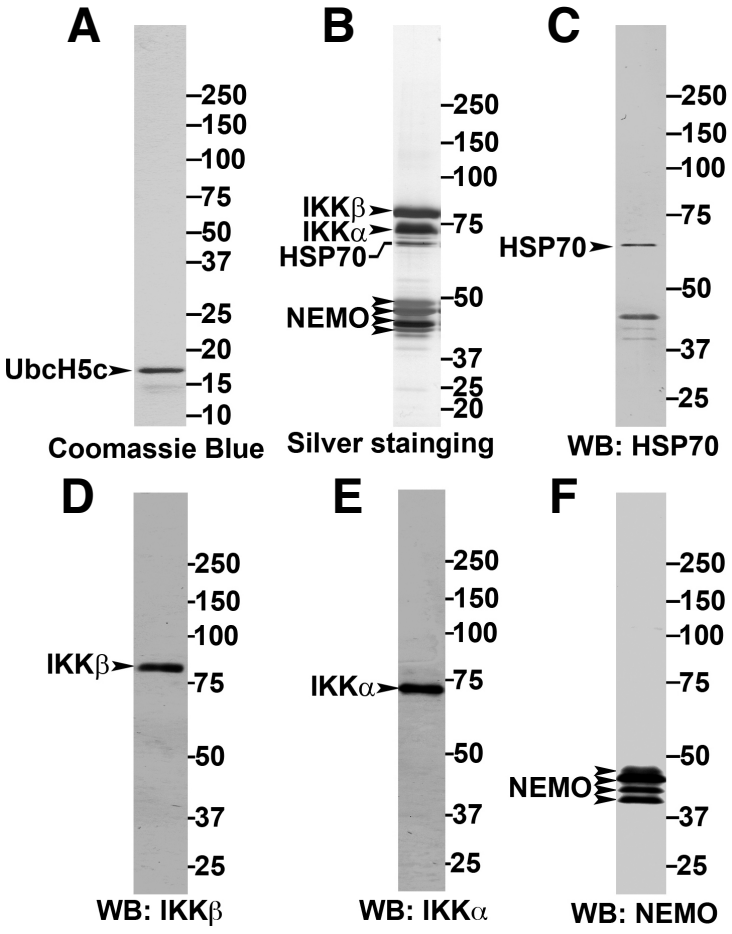

Figure 25: Purified UbcH5c and IKK used in the studies. A): Coomassie blue staining of UbcH5c purified from *E.coli*. B): Silver staining of IKK complex purified from a stable cell line expressing Flag-NEMO. C, D, E, and F): Immunoblotting of IKK complex showing the three subunits of IKKα, IKKβ and NEMO and the co-purified HSP70.

Preliminary results have indicated that the IKK complex might be the only component that is needed from Q/III for activation by TRAF6-UbcH5 system (data not shown). To test this possibility directly, the purified IKK complex was used in the IKK activation assay. IKK was purified through chromatography from a stable cell line expressing Flag-NEMO established in 1.3E2, a pre-B lymphoma cell line deficient in NEMO expression (For convenience, IKK purified this way will be called Flag-IKK). The purity of Flag-IKK was determined by silver staining and is shown in figure 25B. This highly pure IKK was incubated with UbcH5, TRAF6, E1, Ubiquitin and IKK substrate IκBα. Flag-IKK activation was determined by immunoblotting of IκBα using anti-IκBα antibody. As shown in Figure 26A lane2, IKK is activated when all components are present in the reaction. Controls in lanes 1, 3 and 4 show the activation is dependent on UbcH5 and TRAF6. Immunoblotting of NEMO shows that NEMO has undergone modification (Figure 25C, lane 2), probably due to phosphorylation and/or ubiquitination. This modification correlates with IKK activation since it is not seen in other reactions where IKK is not activated (Figure 26C lanes 1, 3 & 4). This result supports the idea that the minimal components required for activation

of IKK are UbcH5 and TRAF6 in a ubiquitination-dependent fashion. The probable ubiquitination of NEMO that correlates with the activation of IKK raises the question of whether ubiquitination of NEMO is required for IKK activation by the TRAF6-UbcH5 system.

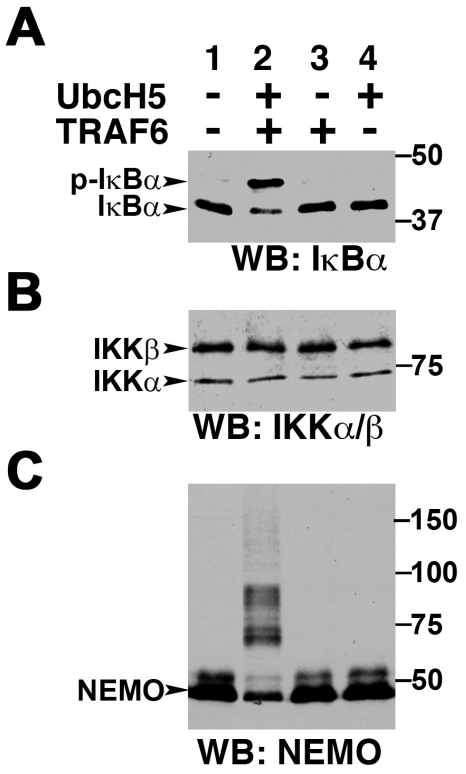

Figure 26: IKK activation by TRAF6-UbcH5. Highly-purified IKK complex and its substrate IκBα were incubated with TRAF6, UbcH5, E1, ubiquitin and ATP/Mg^{2+} for 1 hour at 30°C. Products were separated on 10% SDS-PAGE and immunoblotted for IκBα (A), IKKα and β (B) and NEMO (C). IKK activation was indicated by the slower mobility shift of IκBα in A (lane 2).

IV.B.4 Dissection of polyubiquitination and IKK activation by TRAF6 and UbcH5

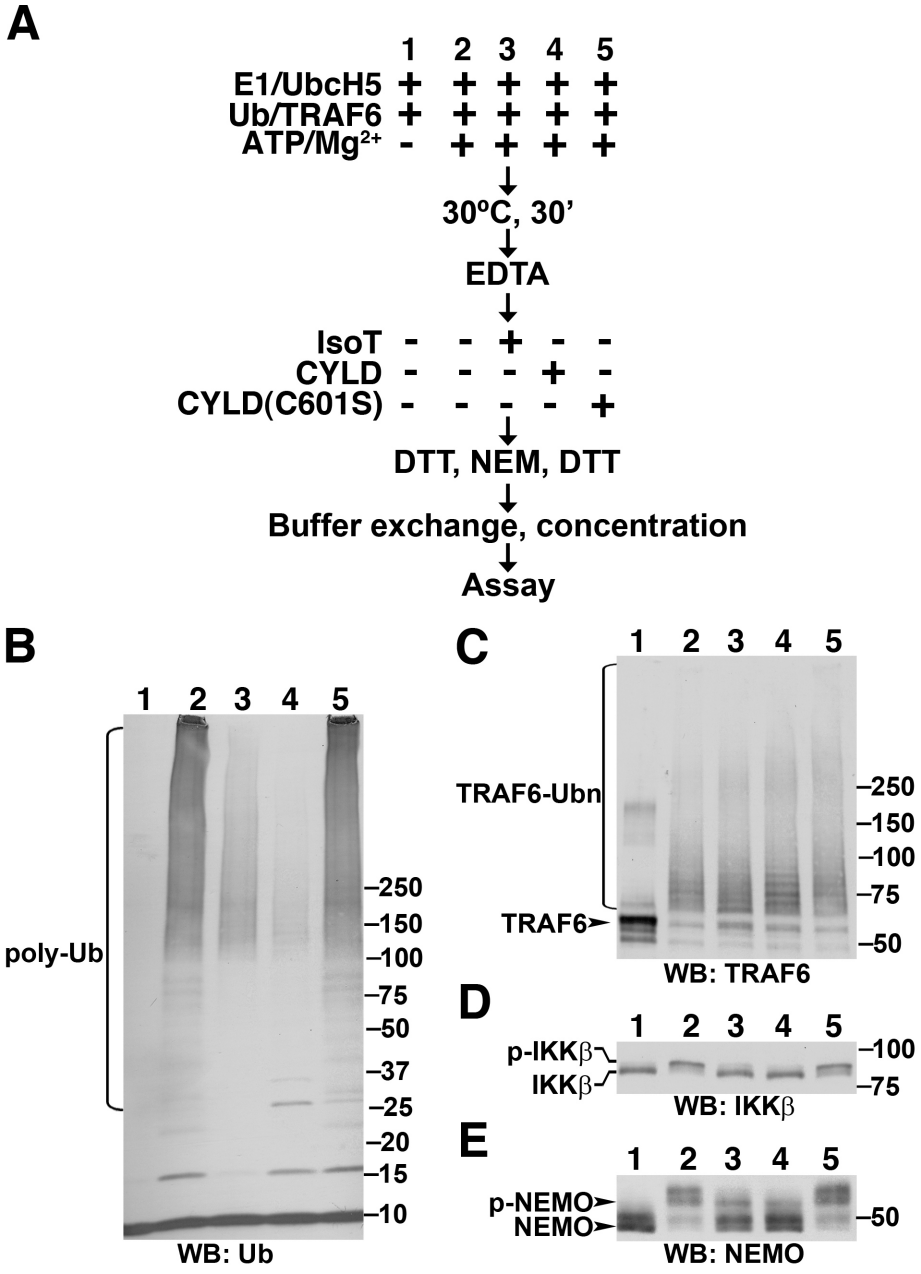

Figure 27: Activation of IKK by polyubiquitin chains synthesized by TRAF6 and UbcH5. A): Diagram showing the protocol used for polyubiquitin chain synthesis by TRAF6 and UbcH5 and treatment of polyubiquitin chains by de-ubiquitin enzymes. B, C): Aliquots from step A were resolved on 4-20% SDS-PAGE and immunoblotted for ubiquitin (B) or 10% SDS-PAGE for TRAF6 (C). D, E): Aliquots from step A were incubated with IKK for 1 hour at 30°C.

Products were separated on 10% SDS-PAGE and immunoblotted for IKKβ (D) and NEMO (E). IKK activation was indicated by slower mobility shift of IKKβ (D) and NEMO (E).

As discussed in chapter III, in the TRAF6-Ubc13/Uev1A-dependent TAK1 activation system the polyubiquitination and kinase activation can be un-coupled and separated into two steps in which the ubiquitination step generates unanchored polyubiquitin chains as the TAK1 activators. In order to determine whether this is the case for TRAF6-UbcH5-dependent activation of IKK, dissection of ubiquitination and IKK activation was carried out using NEM, as was used for characterization of TRAF6-Ubc13/Uev1A-dependent TAK1 activation system. As shown in figure 26A, 5 reactions were set up for the polyubiquitination reaction by TRAF6 and UbcH5 in which reaction 1 didn't have ATP and was used as a control. After incubation at 30°C for 30 minutes, the reaction was stopped with EDTA; reactions 3, 4, and 5 were then treated with IsoT, CYLD, or CYLD(C601S), respectively. Enzymes E1, UbcH5 and also the DUBs were then inactivated by NEM treatment. After removal of excess NEM, the final products were then used in the IKK activation assay. As shown in Figure 27B, immunoblotting of ubiquitin shows TRAF6 and UbcH5 catalyze efficient synthesis of polyubiquitin chains (lane 2) in an ATP-dependent manner since there is no polyubiquitin chain synthesis in the absence of ATP (lane1). More than 80% of the polyubiquitin chains are reduced after either IsoT (lane3) or CYLD

(lane4) treatment. As expected, CYLD(C601S) doesn't cause any visible reduction of polyubiquitin chains (lane5). As shown in Figure 27D and E, IKK was activated when it was incubated with pre-synthesized polyubiquitin chains, either not treated or pre-treated with CYLD(C601S), as determined by phosphorylation of IKKβ and NEMO (lanes 2 & 5, upper and bottom panels, slower mobility shift). IKK activation was abolished when the polyubiquitin chains were pre-treated with either IsoT or CYLD (lane3 and 4). These results suggest that TRAF6-UbcH5-dependent IKK activation system can be separated into two steps: polyubiquitin chain synthesis step and kinase activation step and that polyubiquitin chains are required for IKK activation. The results also suggest that ubiquitination of NEMO per se is not required for IKK activation by the TRAF6-UbcH5 system.

In the TRAF6-UbcH5-dependent polyubiquitination system, TRAF6 itself undergoes efficient auto-ubiquitination. As shown in Figure 27C, TRAF6 is ubiquitinated (compare lanes 1 & 2). The ubiquitination of TRAF6 doesn't decrease much even after treatment with IsoT, CYLD or CYLD(C601S) (lanes 3 to 5). These results suggest that ubiquitinated TRAF6 is not responsible for the IKK activation observed. The results also suggest most of the polyubiquitin chains synthesized by TRAF6 and UbcH5 are free, unanchored chains, which are responsible for IKK activation.

Taken together, these results suggest that the IKK activation species synthesized by TRAF6-UbcH5 are unanchored, free polyubiquitin chains, as is the case for TAK1 activation species synthesized by TRAF6-Ubc13/Uev1A system.

IV.B.5 Characterization of polyubiquitin chain linkage synthesized by TRAF6-Ubch5

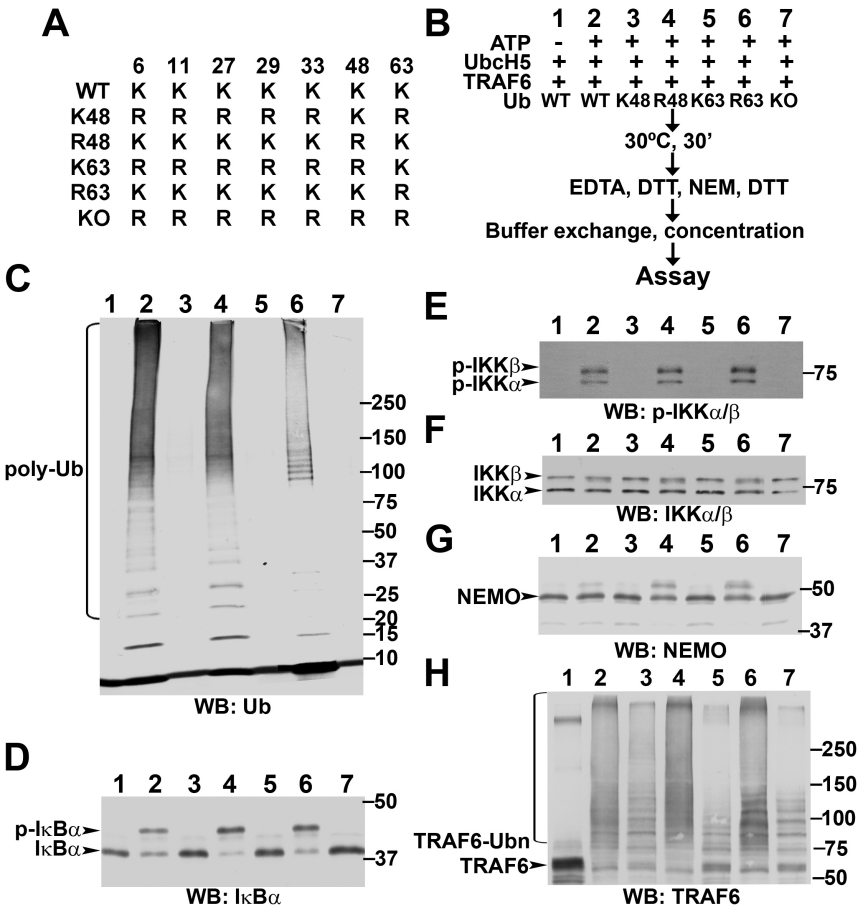

Figure 28: Characterization of polyubiquitin chain linkage catalyzed by TRAF6 and UbcH5. A): Diagram showing ubiquitin WT and mutants with

lysine (K) to Arginine (R) mutation used in the study. B): Protocol used for polyubiquitin chain synthesis by TRAF6, UbcH5, and ubiquitin WT or mutants. C, H): Aliquots from step B were resolved on 4-20% SDS-PAGE and immunoblotted for Ubiquitin (C) or 10% SDS-PAGE for TRAF6 (H). D, E, F, G): Aliquots from step B were incubated with IKK and its substrate IκBα for 1 hour at 30°C. Products were separated on 10% SDS-PAGE and immunoblotted for IκBα (D), phospho-IKKα/β (E), IKKα/β (F), and NEMO (G). IKK activation was indicated by slower mobility shift of IκBα (E) and phosphorylation of IKKα/β (G).

In the TRAF6-Ubc13/Uev1A-dependent TAK1 activation system, the activation species are K63-linked polyubiquitin chains. What chain linkage is it in the polyubiquitin chains synthesized by the TRAF6-UbcH5 system? Is it also K63-linked or another linkage? To address this question, several ubiquitin mutants in which one or more lysine residues have been mutated to arginine residues (Figure 28A) were tested in the polyubiquitination reaction and IKK activation assay. As shown in Figure 28B, TRAF6-UbcH5-dependent ubiquitination reactions were set up with either WT ubiquitin or the mutants. E1 and UbcH5 were inactivated after reaction by NEM treatment. Immunoblotting of ubiquitin shows ubiquitin WT, R48 and R63 (Figure 28C, lanes 2, 4 and 6) but not K48, K63 or KO (lanes 3, 5 and 7) support polyubiquitin chain synthesis catalyzed by TRAF6 and UbcH5. In the IKK activation assay using these pre-synthesized polyubiquitin chains, IKK was activated by polyubiquitin chains synthesized with WT, R48 or R63 ubiquitin (Figure 28 D, E, F & G, lanes 2, 4 and 6) but not with K48, K63 or KO ubiquitin (lanes 3, 5, 7). TRAF6 was

ubiquitinated by all the ubiquitins tested, even by KO ubiquitin (Figure 28H), implying TRAF6 may be mono-ubiquitinated at multiple sites. These results suggest that neither K48 chains nor K63 chains can be synthesized by the TRAF6-UbcH5 ubiquitination system. TRAF6 and UbcH5 might use other than K48 and K63 linkages for chain synthesis. In combination with information about UbcH5-dependent polyubiquitination in other systems (data not shown), the polyubiquitin chains catalyzed by TRAF6 and UbcH5 might be mixed-linkage linked.

IV.B.6 IKK activation requires NEMO ubiquitin binding

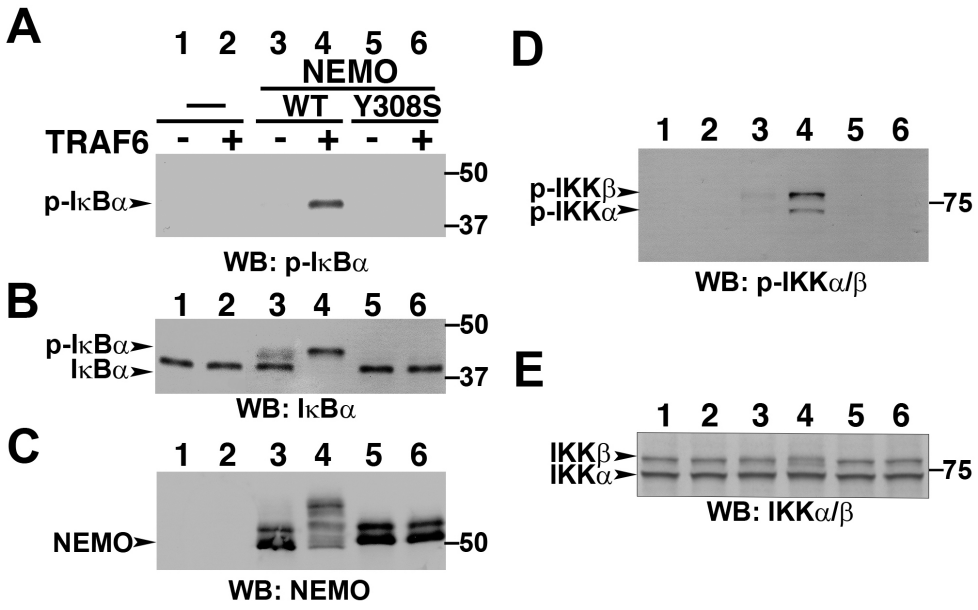

Figure 29: IKK activation by TRAF6 and UbcH5 requires polyubiquitin chain binding by NEMO. Partially purified IKKα/β complex

from 1.3E2 cells were incubated with TRAF6, UbcH5, IκBα, NEMO wild type (WT) or Y308S mutant for 1 hour at 30°C. Products were separated on 10% SDS-PAGE and immunoblotted for phosphor-IκBα (A), IκBα (B), NEMO (C), phospho-IKKα/β (D) and IKKα/β (E). IKK activation was indicated by phosphorylation of IκBα (A), slower mobility shift of IκBα (B) and phosphorylation of IKKα/β (D).

We have reported previously that NEMO has a polyubiquitin chain binding motif called NUB and that this polyubiquitin chain binding is required for IKK activation *in vivo* in the TNFα signaling pathway and *in vitro* in the TRAF6-Ubc13/Uev1A-dependent system. We found a point mutant (NEMO(Y308S)) and showed that the single site mutation dramatically reduced NEMO polyubiquitin chain binding and consequently dramatically reduced its capacity to support IKK activation by TRAF6-Ubc13/Uev1A. In order to know if NEMO polyubiquitin chain binding is required for IKK activation by TRAF6-UbcH5 system, NEMO WT and Y308S mutant were tested for their ability to rescue IKK activation in the IKK activation assay using partially purified IKKα/β complex from NEMO-deficient cell line 1.3E2. As shown in Figure 29A, IKK cannot be activated without addition of NEMO (lanes 1 and 2). Addition of NEMO WT (lanes 3 and 4) leads to activation of IKK in the presence of TRAF6 (lane 4). However addition of the polyubiquitin chain binding deficient mutant NEMO(Y308S) (lanes 5 and 6) did not rescue IKK activation even in the presence of TRAF6 (lane 6). Immunoblotting of p-IKK and NEMO on the same samples also showed only the WT but not the Y308S mutant rescued IKK activation by TRAF6-UbcH5

(Figure 29 C & D, lane 4). This result suggests that polyubiquitin chain binding by NEMO is required for IKK activation by the TRAF6-UbcH5 system.

IV.C Discussion

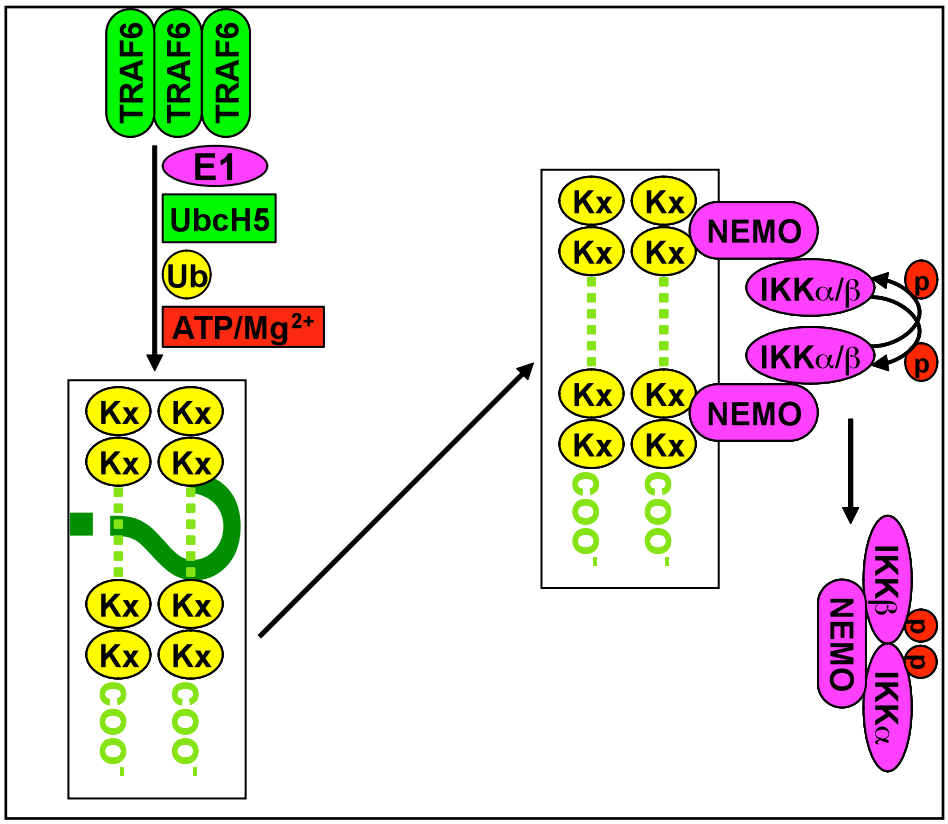

Figure 30: A model for IKK activation by TRAF6 and UbcH5. In this model TRAF6, together with E1, UbcH5, catalyzes Kx-linked un-anchored polyubiquitin chains. These un-anchored polyubiquitin chains may adopt a particular higher-order conformation labeled by a question mark (?) that is unknown yet. The polyubiquitin chains then recruit IKK complex through NEMO. Binding of IKK complex to polyubiquitin chains may cause their dimerization or oligomerization, which can then undergo trans-autophosphorylation resulting in their activation.

In this chapter, I have reported the purification of UbcH5, through biochemical fractionation, as another ubiquitin conjugating enzyme E2 that works with TRAF6 to catalyze IKK activation. In this system, IKK is directly activated

without upstream kinases such as TAK1. As in the case for TAK1 activation by TRAF6-Ubc13/Uev1A, TRAF6 and UbcH5 also catalyzed assembly of unanchored polyubiquitin chains as the IKK activators. Taken together, I propose a model, as shown in Figure 30, for IKK activation by TRAF6-UbcH5 ubiquitination system. TRAF6 and UbcH5, together with E1 and ubiquitin, synthesize non-K63-linked unanchored polyubiquitin chains. These polyubiquitin chains may adopt a particular higher-order conformation (labeled by a question mark (?) in Figure 29) that is as yet unknown. The polyubiquitin chains then recruit IKK complex through binding to NEMO. Binding of IKK complex to polyubiquitin chains may cause their dimerization or oligomerization, which can then undergo trans-autophosphorylation resulting in IKK activation.

Purification of UbcH5 as an alternative E2 for TRAF6-dependent IKK activation offers an alternative explanation for the observation with Ubc13 knockout studies, as described in the introduction section of the chapter. Different cell types may exploit multiple pathways to activate the IKK-NF-κB pathway. The observation that Ubc13 knockout affects JNK and p38 but not IKK activation fits well with my finding with the TRAF6-UbcH5-dependent IKK activation system. As shown in Figure 19, lane 4, polyubiquitin chains synthesized by TRAF6-UbcH5 cannot activate TAK1 kinase complex, which is required for JNK and p38 MAP kinase activation. However whether this is the true alternative pathway *in vivo* for Ubc13-independent IKK activation in the IL1R and TLR

signaling pathways awaits further investigation. My preliminary RNAi-based knockdown experiments *in vivo* have not given me clean and thus convincing data yet. Considering the simplicity of this TRAF6-UbcH5 system for IKK-NF-κB activation, it is also possible that other pathways may exploit it such as in tumor cells and chronic neurodegenerative diseases where constant NF-κB activation is often observed but without knowledge of how it is activated.

My purification of UbcH5 extends the finding of an earlier report (Chen et al., 1996) in which UbcH5 catalyzed ubiquitination-dependent IKK activation without TRAF6. In my initial project to purify other potential factor(s) that works with UbcH5 for IKK activation, I ended up purifying IKK itself without any obvious ubiquitin ligase E3 candidates. The requirement of TRAF6 in the new system reported here is probably due to the concentration of UbcH5 used. In the UbcH5-only-dependent IKK activation system, a high concentration of UbcH5 is required. However, in the presence of TRAF6, 100 to 1000 times lower concentration of UbcH5 (in the range of 5 nM to 0.5 nM) is sufficient to activate IKK.

In the case of TAK1 activation by TRAF6-Ubc13/Uev1A system, TRAF6 ubiquitination is not detectable. In the case of IKK activation by TRAF6-UbcH5, however, TRAF6 does undergo obvious auto-ubiquitination, making it tempting to propose that ubiquitinated TRAF6 is the actual IKK activator. But two observations exclude the possibility that ubiquitinated TRAF6 is the activator: 1)

IsoT and CYLD treatment of polyubiquitin chains don't de-ubiquitinate polyubiquitinated TRAF6 efficiently but abolish the activity of IKK activation. 2) Although TRAF6 auto-ubiquitination is readily detected, the majority of TRAF6-ub conjugates are in low molecular weight region and may represent multi-site mono-ubiquitination (see Figure 26, panel C). The ubiquitination pattern of TRAF6 by different ubiquitin K to R mutants supports the latter observation (Figure 27, panel H).

TRAF6 and Ubc13/Uev1A catalyze synthesis of K63-linked unanchored polyubiquitin chains that can activate TAK1 complex, while TRAF6 and UbcH5 catalyze synthesis of non-K63-linked unanchored polyubiquitin chains that can activate IKK complex. So are these two systems different? If so, how much are the differences? In the course of characterization of these two systems, several differences between the two kinase activation systems were observed:

1) TAK1 can be activated by polyubiquitin chains synthesized by TRAF6-Ubc13/Uev1A but not TRAF6-UbcH5 (Figure 19, compare lanes 2 & 4). Can this be explained by different preferences of polyubiquitin chain binding between TAB2 (or TAB3) and NEMO? Experiments by swapping of ubiquitin binding motifs may be able to provide the answer.

2) Endogenous IKK (IKK complex purified from Hela cells) can be activated by polyubiquitin chains synthesized by TRAF6-UbcH5 but not by TRAF6-Ubc13/Uev1A (Figure 30 panel B, compare lane 5 to lane 4). However,

IKK purified using Flag-NEMO from 1.3E2 cells (as was used in this chapter) (for convenience, it will be called Flag-IKK) can be activated by polyubiquitin chains synthesized by either TRAF6-Ubc13/Uev1A or TRAF6-UbcH5 (Figure 31 panel A, lanes 4 & 5). Also, endogenous IKK can be activated by direct incubation with TRAF6 and Ubc13/Uev1A instead of polyubiquitin chains (Figure 31 panel B, compare lanes 3 to 4; and Figure 31, compare lanes 2 & 4 to lanes 8 & 10). What are the differences between endogenous IKK and Flag-IKK that make Flag-IKK competent for activation by polyubiquitin chains? During direct incubation of IKK and TRAF6-Ubc13/Uev1A, what has happened to IKK that makes it capable for activation by polyubiquitination? More studies are needed before we can answer these questions.

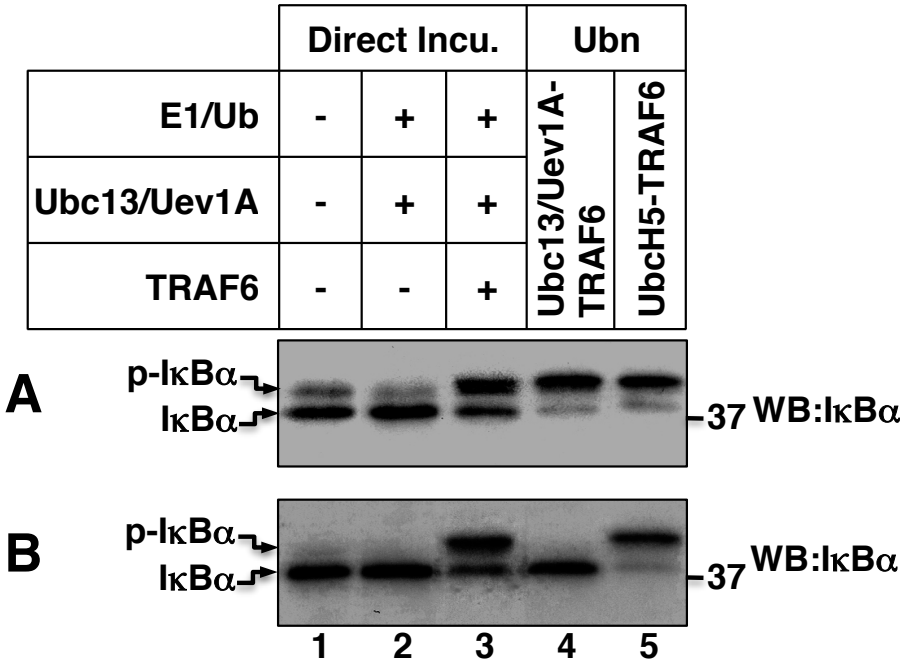

Figure 31: Comparison between Flag-IKK and endogenous IKK. Flag-IKK was purified from 1.3/E2 stably expressing Flag-NEMO, designated as Flag-IKK for convenience. Endogenous IKK was purified from Hela S100 through sequential Q, ATP sepharose, and superdex 200 steps. Flag-IKK (panel A) or endogenous IKK (panel B), together with substrate IκBα, was incubated with E1, Ub, Ubc13/Uev1A and TRAF6 (lanes 1 to 3) or with polyubiquitin chains (Ubn) by TRAF6-Ubc13/Uev1A (lane 4) or TRAF6-UbcH5 (lane 5). Activation of IKK was determined by immunoblotting of IκBα using anti-IκBα antibody.

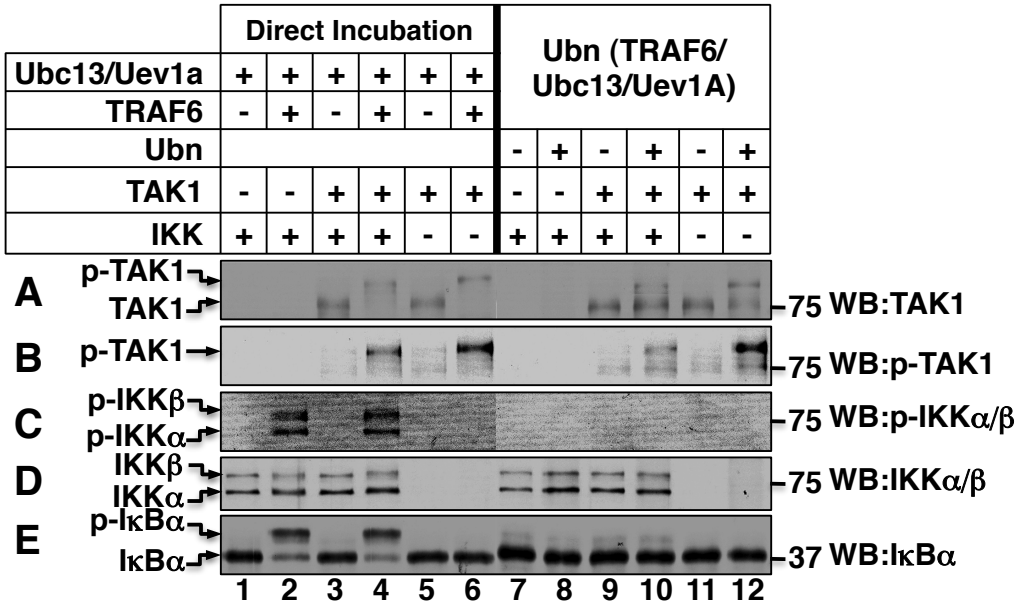

Figure 32: Comparison of activation of TAK1 and IKK by TRAF6-Ubc13/Uev1A. IKK complex alone (lanes 1, 2, 7, & 8), or IKK and TAK1 (lanes 3, 4, 9 & 10), or TAK1 alone (lanes 5, 6, 11, & 12) were incubated with Ubc13/Uev1A, ubiquitin and TRAF6 (lanes 1 to 6), or polyubiquitin chains (Ubn) (lanes 7 to 12). Activation of TAK1 was determined by immunoblotting of TAK1 (panel A) and phospho-TAK1 (B). IKK activation was determined by immunoblotting of p-IKKα/β (C), and IκBα (E).

3). TAK1 activated by polyubiquitin chains cannot activate IKK (Figure 31, lane 10). *In vivo* and *in vitro* data have shown that TAK1 is required for IKK

activation by TRAF6-Ubc13/Uev1A system. This suggests that activation of TAK1 is not sufficient for IKK activation. Since IKK can be activated directly by TRAF6-Ubc13/Uev1A when they are incubated directly under certain conditions, it raises the possibility that IKK activation by activated TAK1 requires another polyubiquitination events. It also raises the question as to what TAK1 really does to IKK activation by the TRAF6-Ubc13/Uev1A system. More studies are required to address these questions.

Chapter V. Conclusion and future directions

V.A Conclusion

In chapters III, I have provided data to show that un-anchored polyubiquitin chains synthesized by TRAF6 with Ubc13/Uev1A are the direct activators for TAK1 activation. In chapter IV, I have provided data to show that un-anchored polyubiquitin chains synthesized by TRAF6 and UbcH5 are direct activators of IKK activation. Putting the data together, I conclude that un-anchored polyubiquitin chains are kinase activators.

V.B Future directions

V.B.1 Chemistry of C-termini of un-anchored polyubiquitin chains

In both TRAF6-Ubc13/Uev1A-TAK1 and TRAF6-UbcH5-IKK systems, the activators are un-anchored polyubiquitin chains. Although we think the C-termini of the un-anchored polyubiquitin chains are Gly-Gly-COO^-, we don't have direct experimental data for that and it needs to be addressed in the future. If the C-termini are COO^-, we need to know whether this chemical feature is required or not for their activity toward kinase activation. In other words, we want to know whether polyubiquitin binding by TAB2/TAB3 in TAK1 complex or NEMO in IKK complex requires recognition and binding of free, Gly-Gly-COO^- feature of polyubiquitin chains. The ZnF ubiquitin binding motif found in IsoT

requires Gly-Gly-COO^- for efficient polyubiquitin chain binding (Reyes-Turcu et al., 2006). In TAB2 and TAB3, it is also a ZnF motif that is responsible for polyubiquitin chain recognition and binding. Structural studies should provide further information.

V.B.2 Structural studies of polyubiquitin chains

The two ubiquitination-dependent kinase activation systems reported here demonstrate that all polyubiquitin chains are not equal. There are differences between polyubiquitin chains synthesized in the presence or absence of E3 (TRAF6). There are differences between polyubiquitin chains synthesized by different E2-E3 pairs (TRAF6-Ubc13/Uev1A versus TRAF6-UbcH5). These raise the question as to whether polyubiquitin chains are random coils and differ only by different lysine linkages or whether they adopt specific higher-order conformations, which are different among different E2-E3 pairs. Structural studies with cryo-EM or other high-resolution technology will be required.

V.B.3 Mechanism of kinase activation by polyubiquitin chains

The biggest question remaining is how the unanchored polyubiquitin chains lead to kinase activation. For kinase activation, the only thing that occurs chemically, is the phosphorylation of serine or threonine residues at the activation loop of the kinase domain. Our current model is that kinase undergoes

intermolecular auto-phosphorylation in a kinase complex dimer or oligomer. Free polyubiquitin chains thus function as triggers for kinase dimerization or oligomerization. Testing of these ideas are under way.

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Vitae

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