UBIQUITIN MEDIATED REGULATION OF NF-κB SIGNALING

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DEDICATION

To my dearest Christina for your endless love and support.

UBIQUITIN MEDIATED REGULATION OF NF-κB SIGNALING

by

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DISSERTATION

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UBIQUITIN MEDIATED REGULATION OF NF-κB SIGNALING

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The University of Texas Southwestern Medical Center at Dallas, 2007

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

NF-κB signaling is involved in many vital cellular functions such as immunity, cell proliferation, inflammation, and apoptosis. The activation of NF-κB signaling requires the process of ubiquitination. K63-and K48-linked ubiquitin chains have been shown to have distinct roles and biological function in NF-κB signaling. K63-linked ubiquitin chains are required for the activation of TAK1, which leads to the activation of IKK. Activation of IKK leads to K48-linked ubiquitination, and the subsequent proteasomal degradation of IκBα. Two important areas of research focusing on ubiquitin regulation of NF-κB signaling are addressed in this dissertation. The areas addressed

include understanding how ubiquitinated substrates are targeted for proteasomal degradation and how CYLD negatively regulates NF-κB signaling.

In these studies, I investigated the molecular mechanisms involved in the regulation of IκBα degradation. Using a siRNA approach, NPL4 was shown to be required for IκBα degradation. *In vitro* proteasomal degradation assays demonstrated that the NPL4 complex is required for IκBα degradation. Evidence from both *in vitro* and *in vivo* studies suggest NPL4 is required for IκBα degradation, but not for IKK activation. These results suggest NPL4 is working at a step after ubiquitination of IκBα, but before proteasomal degradation. I propose that ubiquitinated IκBα is targeted to the proteasome by an interaction between the NPL4 complex that is mediated through the zinc finger domain of NPL4.

The cylindromatosis tumor suppressor gene (CYLD) encodes a 110 kDa deubiquitination enzyme that negatively regulates NF-κB signaling. Loss-of-function mutations in CYLD lead to the disease Familial Cylindromatosis, which is characterized by the formation of benign skin tumors that originate from the head and neck of individuals afflicted with the disease. Here I present *in vitro* evidence that CYLD inhibits both TAK1 and IKK activation by TRAF6 in a cell free system. I also demonstrate, using a highly purified *in vitro* system, that CYLD specifically cleaves K63 linked ubiquitin chains and harbors endoproteolytic activity. Furthermore, the third CAPGLY domain of CYLD was shown to be a novel ubiquitin binding domain. My results provide biochemical evidence that CYLD functions as a K63 deubiquitinase to attenuate NF-κB signaling.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

1. **Pineda, G**., and Chen, Z.J., (2007) "The Tumor Suppressor CYLD is a K63- specific Deubiquitination Enzyme that Inhibits TAK1 and IKK." (In Preparation)

2. **Pineda, G**., and Chen, Z.J., (2007) "NPL4 is Required for Proteasomal Degradation of IκBα by TNFα activation of NF-κB Signaling." (In Preparation)

3. **Gabriel Pineda**, Chee-Kwee Ea, Zhijian J. Chen, (2007) "Ubiquitination and TRAF Signaling" Adv. Exp. Med. Biol. 597: 80-92. Book Chapter

4. Ea, C-K., Deng, L., Xia, Z-P., **Pineda, G**., and Chen, Z.J., (2006) "Activation of IKK by TNFα Requires Site-Specific Ubiquitination of RIP1 and Polyubiquitin Binding by NEMO." Molecular Cell 22: 245-257.

5. Li, X-D., Sun, L., Seth, R.B., **Pineda, G**., and Chen, Z.J., (2005) "Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade the innate immunity." PNAS 102: 17717-17722.

6. Wooldridge, J. B., **Pineda, G**., Banuelas-Ornelas, J. J., Dagda, K. R., Gasanov, E. S., Rael, D. E., Lieb, S. C. (2001). Mojave rattlesnakes (Crotalus scutulatus scutulatus) lacking the acidic subunit DNA sequence lack Mojave toxin in their venom. Comp. Biochem. Physiology Part B. 130: 169 – 179.

LIST OF FIGURES

LIST OF DEFINITIONS

293T HEK - human embryonic kidney cell line expressing the large T antigen

AAA - ATPase – ATPase associated with a variety of cellular activities

ATP - adenosine triphosphate

βTrCP - beta-transducing repeat containing protein

BSA - bovine serum albumin

Cdc34 - cell division cycle mutant number 34

E1 - ubiquitin-activating enzyme

E2 - ubiquitin-conjugating enzyme

E3 - ubiquitin ligase

EDTA - ethylenediaminetetraacetic acid

DTT - dithiothreitol

IκBα - inhibitor of nuclear factor kappa B, subtype alpha

IKK - IκB kinase complex

IP - immunoprecipitation

IPTG - isopropyl β-D-thiogalactopyranoside

IL-1β - interleukin-1β

MG-132 - proteasome inhibitor compound

NEMO - NF-κB essential modulator

NF-κB - nuclear factor kappa B

Npl4 - nuclear protein localization factor 4

p65 - NF-κB family member (RelA)

PAGE - polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

RIP1 - receptor interacting protein kinase 1

PBDM - protein break down mix

SDS - sodium dodecyl sulfate

S100 - cytoplasmic extract free of all intracellular organelles

SCF - Skp1, Cul1, F-box containing ubiquitin ligase enzyme (E3)

siRNA - small interfering RNA

TRAF - tumor necrosis factor (TNF) (TNFR) associated factors

TAK1 - transforming growth factor β-activated kinase

TAB1 - TAK1 associated protein 1

TAB2 - TAK1 associated protein 2

Tris - tris(hydroxymethyl)methylglycine

TNF-R1 - tumor necrosis factor receptor I

TNFα - tumor necrosis factor alpha

TRAF2 - tumor necrosis factor-associated factor 2

TRAF6 - tumor necrosis factor-associated factor 6

TRIKA1 - TRAF6-regulated IKK activator 1

TRIKA2 - TRAF6-regulated IKK activator 2

Ub - ubiquitin

Uba - Ubiquitin activating enzyme (E1)

Ubc - Ubiquitin conjugating enzyme (E2)

Ubch - Ubc from human

Uev1a - Ubiquitin enzyme variant 1a

Ufd1 - Ubiquitin fusion degradation protein 1

VCP - Valosin Containing Protein

CHAPTER ONE
Introduction

I.A. NF-KB PATHWAY

I.A.1 Overview of NF-κB

The NF-κB pathway is a major evolutionarily conserved signaling pathway in that is activated in response to viruses, bacteria, cytokines, or UV (Courtois and Smahi, 2006). NF-κB signaling is involved in many vital cellular functions such as immunity, cell proliferation, inflammation, and apoptosis (Hayden and Ghosh, 2004; Sun and Chen, 2004). NF-κB/Rel is a family of structurally related transcription factors that is composed of p65 RelA, RelB, c-Rel, p52, and p50 (Figure 1). These transcription factors can form homo- or heterodimers, which are held in an inactive state in the cytoplasm of the cell by a member of the inhibitor κB (IκB) family. All NF-κB family members contain a conserved N-terminal Rel homology domain that is responsible for DNA binding, dimerization, nuclear localization, and binding with IκB. The activation of NF-κB pathway ultimately leads to its nuclear translocation where it can bind to a variety of host response genes that contain κB binding elements in their promoter regions and activate gene transcription (Ghosh et al., 1998; Silverman and Maniatis, 2001). IκB comprises a family of proteins containing six to seven ankyrin-repeats such as IκBα, IκBβ, IκBε, and Bcl3 depicted in Figure 1 (Ghosh et al., 1998; Hayden and Ghosh, 2004). With the exception of Bcl3, all IκB proteins normally sequester NF-κB in the cytoplasm of a cell. Bcl3, along with a mature subunit of NF-κB p52, act together in the nucleus to activate gene expression (Watanabe et al., 1997).

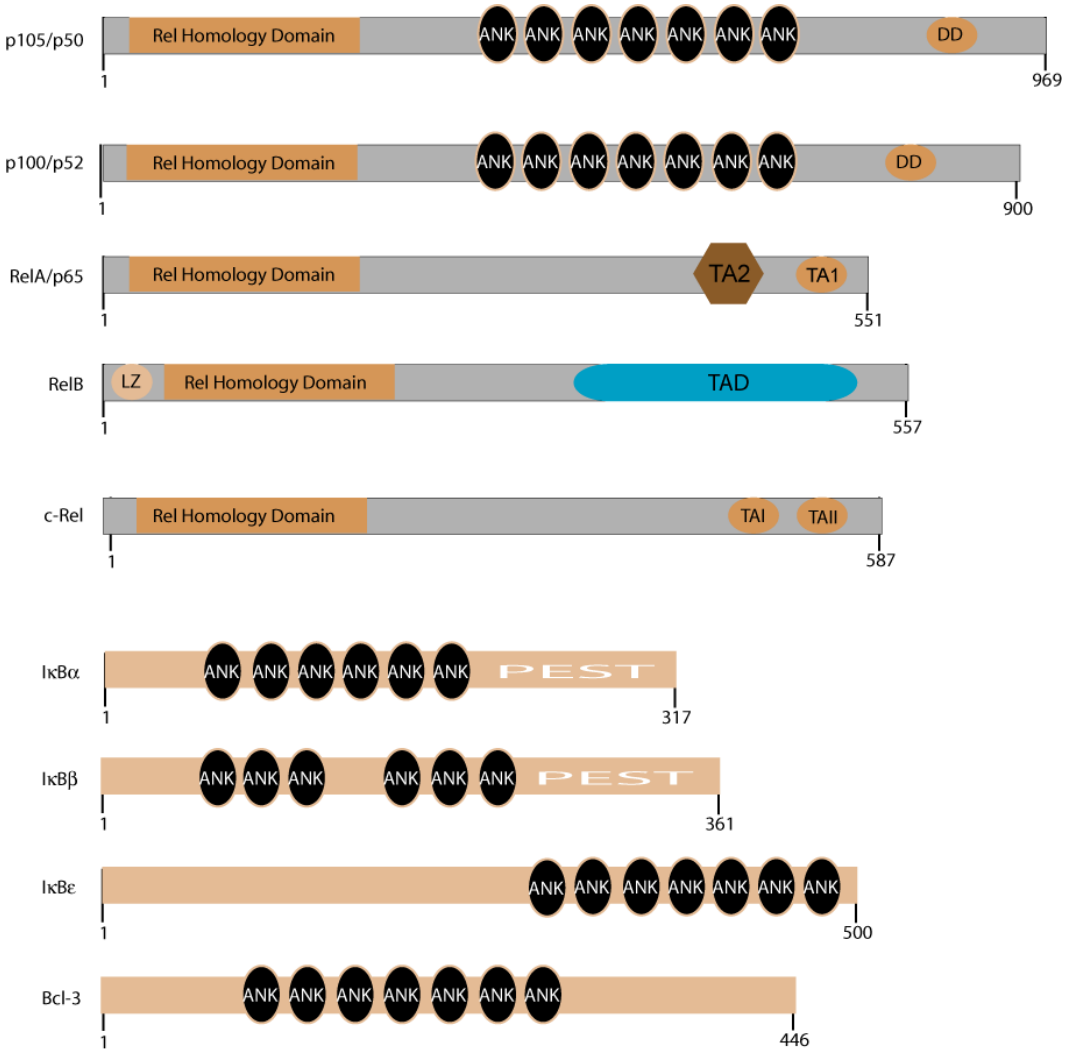


Figure 1. Structure of IκB subunits and NF-κB subunits

Shown is a diagram of structural domains of NF-κB subunits and IκB family members. Names given to proteins are indicated on the left. Abbreviations for NF-κB: DD region with homology to Death Domain, TA1 and TA2 subdomains of RelA transactivation domain (TAD), LZ, Leucine Zipper, ANK, Ankyrin repeats. Abbreviations for IκB: ANK, Ankyrin repeats, PEST, domain rich in proline.

In order for NF-κB to translocate to the nucleus, IκB must be degraded, allowing exposure of the NF-κB nuclear localization signal which is masked by IκB seen in Figure

2 (Jacobs and Harrison, 1998). Proteasomal degradation of IκB is the main regulatory step involved in the activation of NF-κB. It has been previously shown that proteasomal inhibitors block the signal dependent activation of NF-κB (Chen et al., 1995; Palombella et al., 1994).

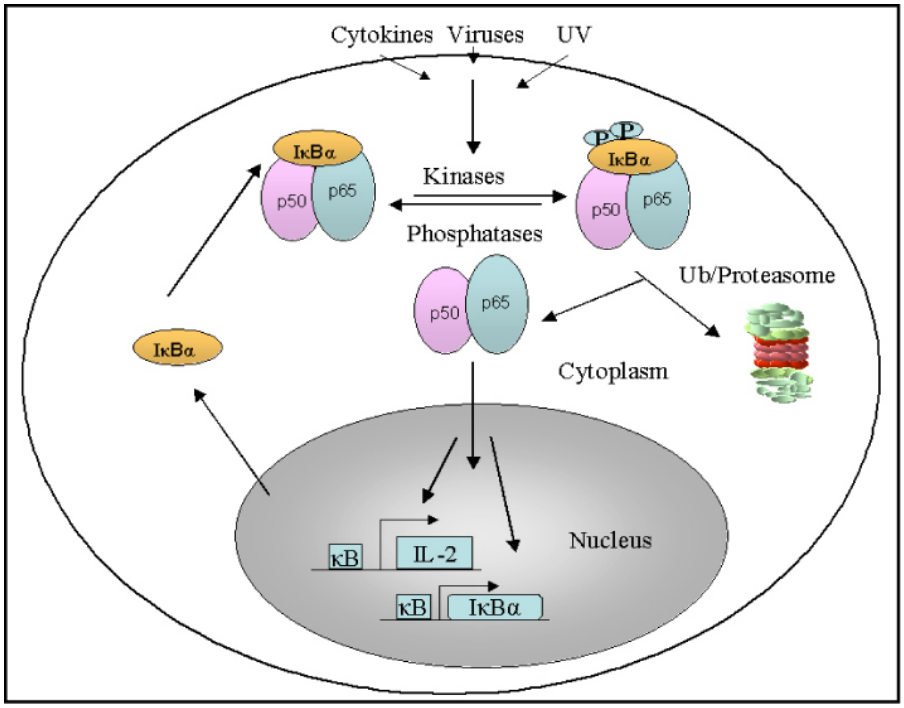


Figure 2. The NF-κB signaling pathway

The transcription factor NF-κB is a heterodimer composed of p50 and p65. This heterodimer is sequestered in the cytoplasm of unstimulated cells with IκBα. When cells are stimulated IκBα is rapidly phosphorylated and degraded by the 26S proteasome. This allows p50/p65 to be translocated into the nucleus where it turns on a variety of genes such as IL-2 receptor, and IκBα.

I.A.2 Proteasome and Ubiquitin

In eukaryotic cells the proteasome is a large multi-protein complex that is composed of over 30 subunits. It can be divided into two major subunits the 19S and 20S subunits Figure 3 (Baumeister et al., 1998; Coux et al., 1996). The 19S subunit

comprises the lid and base of the proteasome. The 20S is considered the protealytic core composed of four rings of seven subunits each. The alpha rings contact the lid while the beta rings contain the proteolytic core. The catalytic core harbors three distinct proteolytic activities characterized as typsin-like, chymotrypsin-like, and caspase-like (Kisselev et al., 2006). The proteasome is the principal ATP-dependent protease in cells that is responsible for the majority of non-lysosomal mediated protein degradation (Liu et al., 2006a). This highly specific protein degradation is fundamental to the regulation of a variety of biological processes. Proteolysis by the 26S proteasome begins by binding the ubiquitinated substrate protein to the 19S regulatory particle. This binding is followed by the unfolding and translocation of the substrate into the lumen of the 20S core.

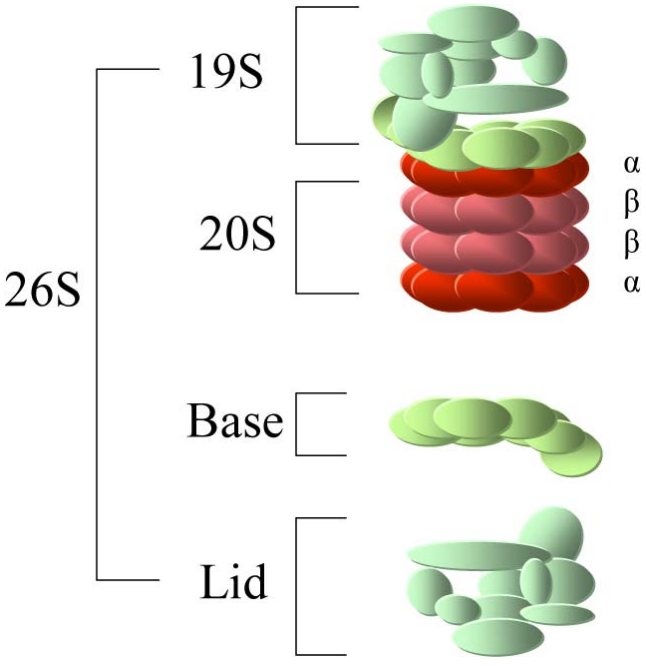


Figure 3. The 26S proteasome The 26S proteasome is a large barrel-like complex composed of two 19S caps and a 20S core complex. The core complex is composed of two α rings, which contact the 19S cap forming a gate complex and two β rings composing the catalytic core.

Ubiquitin is a highly conserved compact globular protein consisting of 76 amino acids (Vijay-Kumar et al., 1987). It was initially isolated from calf thymus and is found in all eukaryotic cells (Goldstein et al., 1975). Ubiquitin is one of the most conserved proteins known. In fact, comparisons

between yeast and human ubiquitin have revealed only 3 amino acid differences. Ubiquitin is synthesized as precursor protein that must be postranslationally processed by ubiquitin specific hydrolases, resulting in a functional ubiquitin molecule that possesses a C-terminal glycine which is required for ubiquitination of target proteins (Wiborg et al., 1985).

The enzymatic process of ubiquitination requires a series of ATP-dependent reactions (Hershko and Ciechanover, 1998). This cascade of reactions in Figure 4 begins when a high energy thiol ester bond is formed between the C-terminus of ubiquitin and a cysteine residue located in the active site of ubiquitin activating enzyme (E1). Once the E1 is loaded with ubiquitin, the next reaction occurs with the transfer of ubiquitin from E1 to the ubiquitin conjugating enzyme (E2). As with the first reaction, a thiol ester bond is formed with the C-terminus of ubiquitin and a cysteine residue located in the active site of the E2. Finally, the ubiquitin ligase (E3) catalyzes the transfer of ubiquitin from the E2 to the ε-amino group on the lysine residue of the substrate. In mammalian cells, there are two E1 enzymes, several dozen E2s, and many E3s (Chiu et al., 2007; Jin et al., 2007; Pickart, 2001).

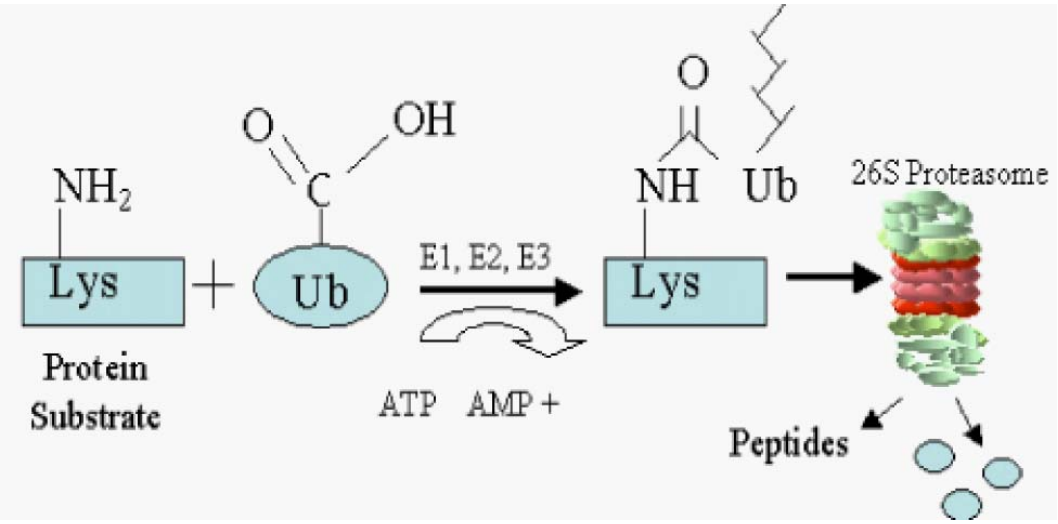


Figure 4. The ubiquitin-proteasome pathway

A protein substrate (e.g., IκBα) is conjugated by multiple molecules of ubiquitin via an enzymatic cascade composed of E1, E2, and E3. The multiubiquitinated protein is then recognized and degraded by the 26S proteasome. Ubiquitin that form the multi-ubiquitin chains is recycled via the action of isopeptidases (Figure Adapted from Pickart, 2000).

Substrates that are recognized by the 26S proteasome are tagged by K48-linked polyubiquitin chains (Hershko and Ciechanover, 1992). The preferred ubiquitin linkage for proteasomal degradation is K48 (Chau et al., 1989). Ubiquitin contains seven lysines all of which can be used to assemble various linkage specific polyubiquitin chains, but only K48 and K63 chain functions have been determined in the cell (Figure 5) (Pickart, 2000). Unlike K48-linked polyubiquitin chains, K63-linked polyubiquitin chains have been shown to be involved in DNA repair, stress response, and the activation of protein kinases such as IκB kinase (IKK) (Arnason and Ellison, 1994; Deng et al., 2000; Hofmann and Pickart, 1999; Spence et al., 1995).

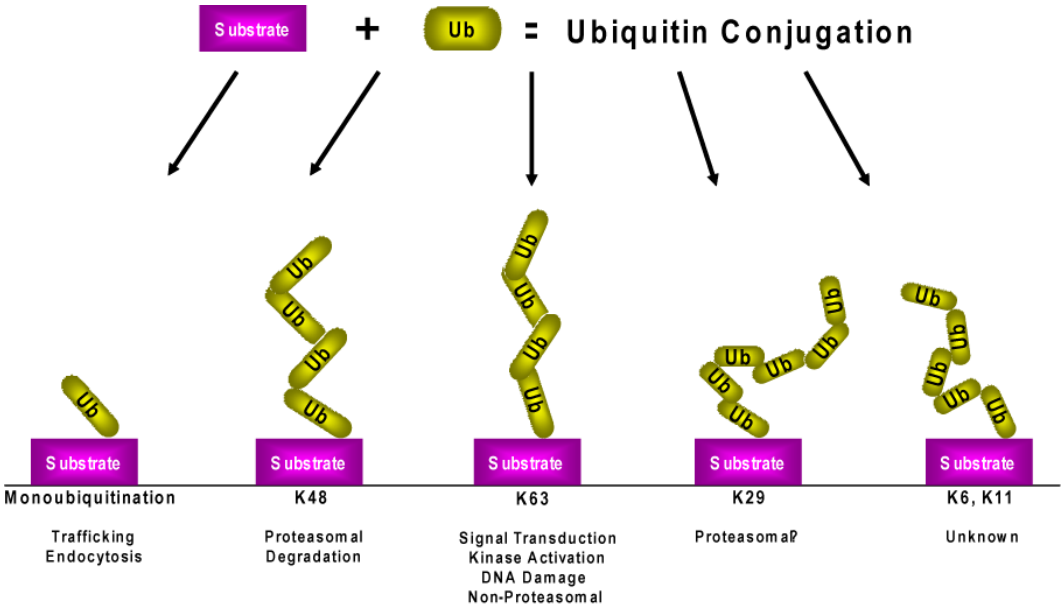


Figure 5. Different Forms of Ubiquitin Chains

Ubiquitin has seven lysine residues that can be used to generate a variety of ubiquitin chains of various linkage. Each ubiquitin chain has a specific function as described above.

Ubiquitination of IκBα requires an E2 that belongs to the Ubc4/Ubc5 ubiquitin conjugating enzyme family (Chen et al., 1996). The E3 ubiquitin ligase responsible for IκBα ubiquitination is a protein complex called $SCF^{\beta TrCP}$ (Deshaies, 1999; Maniatis, 1999). This multi-protein complex is composed of the following proteins Skp1, Cul1, Roc1/Rbx1, and an F-box protein Slimb/βTrCP. βTrCP is the mammalian homologue of slimb and was found to mediate the ubiquitination of IκBα, IκBβ, p105, p100, and β-catenin (Fong and Sun, 2002; Latres et al., 1999; Orian et al., 2000; Shirane et al., 1999; Spencer et al., 1999; Winston et al., 1999; Wu and Ghosh, 1999; Yaron et al., 1998). Phosphorylation of IκBα is required for its subsequent ubiquitination and degradation by

the 26S proteasome. Serine residues 32 and 36 are two key amino acids phosphorylated in response to stimuli (Figure 6) (Chen et al., 1995).

From the crystal structure of SCF^{Skp2} it was determined that Cul1 acts as a rigid scaffold protein which generates the backbone of the SCF complex (Zheng et al., 2002). Skp1 acts as a hinge to bring together the F-box protein Skp2 and Cul1. A RING containing protein Roc1/Rbx1 sits at the C-terminal region of Cul1 interacting with Ubch5 conferring ubiquitin ligase activity to the SCF complex (Petroski and Deshaies, 2005). From modeling $SCF^{\beta TrCP}$ from the SCF^{Skp2} structure, it was determined that both F-box proteins WD40 repeat motifs form a β-propeller like structure that binds phosphorylated substrate (Zheng et al., 2002). Typically, each substrate of βTrCP contains a degron motif or consensus destruction sequence DpSGXXpS, where pS represents phosphorylated serine (Huang et al., 2003). Ubiquitin ligase substrate specificity is determined by the recognition of both phosphorylated serine residues leading to phosphorylation dependent ubiquitination of the substrate.

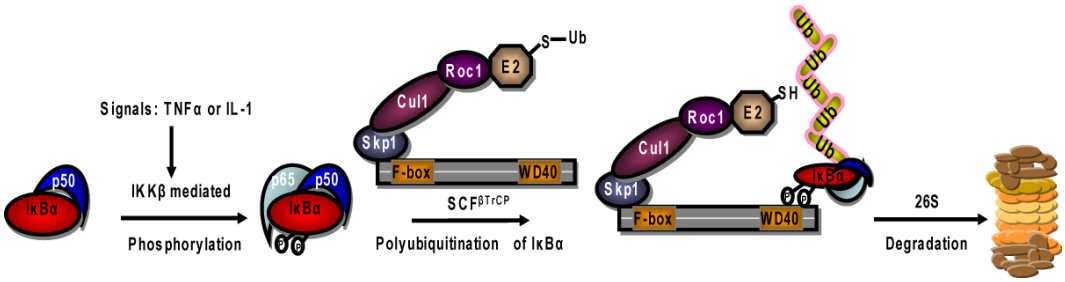


Figure 6. The ubiquitin/proteasome-dependent degradation of IκBα

In response to stimuli, IκBα is phoshorylated by IKK at two serine residues located in the degron motif. The phosphorylated IκBα recruits the $SCF^{\beta TrCP}$ through interaction between the degron motif of IκBα and the WD40 repeat motifs of βTrCP. $SCF^{\beta TrCP}$ functions as ubiquitin E3 ligase to promote K48-linked polyubiquitination of

IκBα. $SCF^{\beta TrCP}$ is a protein complex composed of Skp1/Cul1/Roc and the F-box protein βTrCP. Ubiquitinated IκBα is then degraded by the 26S proteasome.

I.A.3 Proteasomal Degradation in ERAD/RUP

Ubiquitin mediated proteasomal degradation has also been shown to regulate other biologically important processes such as ER-associated degradation (ERAD) and regulated ubiquitin mediated processing (RUP). A series of experiments have demonstrated that all proteins composing the NPL4/UFD1/VCP complex are essential in both ERAD and RUP (Bays and Hampton, 2002; Jarosch et al., 2003). NPL4 was first identified in a genetic screen for nuclear transport mutants (Nelson et al., 1993). Observations from original NPL4 mutants displayed general defects in nuclear import and membrane integrity (DeHoratius and Silver, 1996). NPL4 is conserved from C. elegans to humans and in yeast NPL4 is 35% similar and 56% identical to human NPL4. Among adult human tissues, NPL4 is expressed as several isoforms in brain, heart, skeletal muscle, and kidney (Meyer et al., 2000). Also, it has been observed that the majority of the NPL4 protein resides in the cytoplasm of the cell. NPL4 has also been implicated in unsaturated fatty acid biosynthesis (Hitchcock et al., 2001). Specifically, it has been shown that the NPL4 complex in yeast is required for proteasome mediated processing of ER membrane bound transcription factors Mga2 and Spt23 (Rape et al., 2001). Ufd1 was originally discovered in screens of Saccharomyces cerevisiae mutants with defects in the ubiquitin fusion degradation pathway (Johnson et al., 1995). Using deletion mutant analysis of UFD1 it has been shown that the N-terminal portion of UFD1 contains a ubiquitin binding site and the C-terminal portion is important for binding to VCP and NPL4 (Park et al., 2005; Ye et al., 2003). VCP, or valosin-containing protein is

the mammalian homolog of the cell division cycle protein Cdc48 in yeast and p97 in Xenopus. Cdc48 was first identified in a screen for cold sensitive cell division cycle mutants (Moir et al., 1982). The heterocomplex between NPL4/UFD1 is considered a co-factor for VCP, which belongs to a family of proteins known as the ATPases-Associated with diverse cellular Activities (AAA). The interaction between NPL4/UFD1 and VCP is considered critical in directing AAA activity toward many different cellular functions. For example, VCP functions in multiple cellular processes including cell division cycle, membrane fusion, and the regulation of IκBα degradation (Dai et al., 1998). Also, recombinant Cdc48 was shown to be capable of distinguishing between native and non-native conformations of model proteins, suggesting that Cdc48 functions as a co-chaperone protein (Dai and Li 2001).

I.A.4 Ubiquitination and processing of NF-κB precursors.

The ubiquitin proteasome pathway is required for both co-translational and post-translational processing of NF-κB1/p105 to p50 (Ciechanover et al., 2001). The co-translational processing of p105 is constitutive and does not require either phosphorylation or ubiquitination. Both p105 and the mature p50 can be detected in unstimulated cells. The source of p50 in unstimulated cells is generated from constitutive processing and it has been shown that a 23 amino acid glycine rich region (GRR) in the C-terminal domain of p105 functions as a processing signal for the generation of p50 (Lin and Ghosh, 1996). The ubiquitination of p105 is thought to recruit the proteasome, generating the processing of the C-terminal portion of p105 (Lin et al., 1998). An endoproteolytic cleavage downstream of the GRR is required for this processing and is thought to occur in an unstructured region allowing access to the proteasome for

processing (Lee et al., 2001; Rape and Jentsch, 2002). p105 processing can also be activated post-translationally through the activation of IKK by the following cell stimuli such as phorbol ester (PMA) and lipopolysaccharides (LPS) (Beinke et al., 2002; Belich et al., 1999; MacKichan et al., 1996; Mercurio et al., 1993; Salmeron et al., 2001).

The processing of NF-κB2/p100 to p52 also requires the proteasome and is a highly regulated process that is essential for B cell development (Caamano et al., 1998; Franzoso et al., 1998; Heusch et al., 1999; Poljak et al., 1999). This processing leads to the activation of what is considered the non-canonical pathway of NF-κB signaling. The C-terminal domain of p100 contains a death domain which negatively regulates its processing (Xiao et al., 2001). Phosphorylation of p100 on Ser866 and Ser870 by IKKα relieves this negative inhibition and allows for βTrCP ubiquitin ligase to ubiquitinate p100 (Fong and Sun, 2002). NIK activation leads to the phosphorylation of IKKα, which directly phosphorylates p100 (Senftleben et al., 2001). It has been shown that NF-κB inducing kinase (NIK) is also involved in the activation of p100 processing. The alymphoplasia (aly) mouse mutant has been characterized as having a mutation in the C-terminus of NIK, which display a phenotype of developmental defects and are defective in p100 processing (Shinkura et al., 1999). Overexpression of NIK leads to p100 processing. However, a NIK mutant that is catalytically inactive is incapable of activating p100 processing (Xiao et al., 2001).

I.A.5 Ubiquitin in IKK activation

All signals that lead to the activation of NF-κB signaling converge on IκB kinase (IKK) depicted in Figure 7. IKK activation leads to the direct phosphorylation of IκBα, which is a key regulatory step in NF-κB activation. Mutating either Serine residue that

gets phosphorylated by IKK blocks NF-κB activation. IKK is a large complex composed of two catalytic subunits IKKα and IKKβ (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). The third subunit IKKγ otherwise called NEMO (Rothwarf et al., 1998; Yamaoka et al., 1998), is considered to be the regulatory subunit of the complex. Both NEMO and IKKβ are required for NF-κB activation (Ben-Neriah, 2002; Silverman and Maniatis, 2001). IKKα activation plays less of a role in cytokine activation of NF-κB, but plays a significant role in p100 processing. Activation of IKK can be independently achieved through direct phosphorylation by MEKK1, a kinase in the JNK pathway (Lee et al., 1997), or by a novel mechanism involving ubiquitination independent of proteasomal degradation.

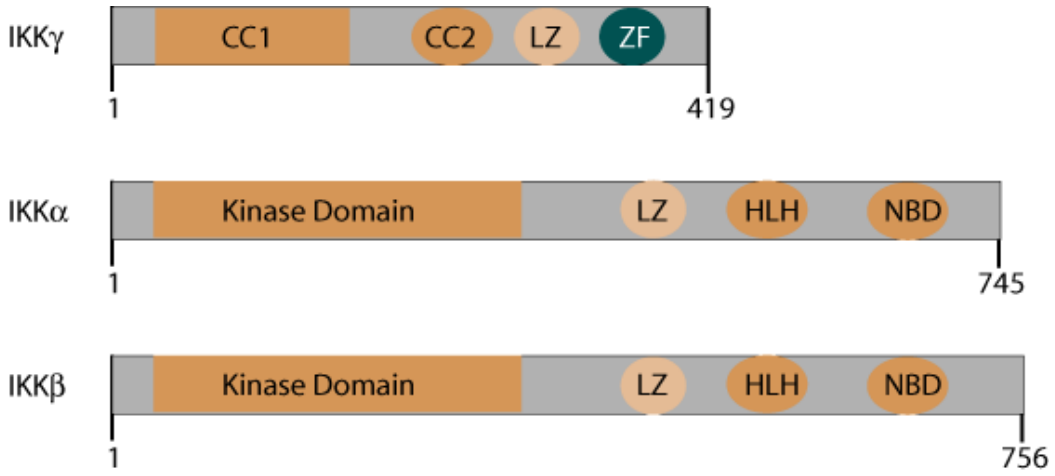


Figure 7. Structural domains of IKK subunits

Shown is a diagram of structural domains and subunits of IKK consisting of IKKγ (NEMO), IKKα, and IKKβ. Names given to proteins are indicated on the left. The IKK complex contains two catalytic subunits IKKα and IKKβ, and the regulatory subunit NEMO. Abbreviations: LZ, leucine zipper domain, HLH, helix-loop-helix domain, CC, coiled-coil domain, ZF, zinc finger domain.

I.A.6 TRAF Family

A family of important proteins that act as intracellular adaptors that mediate signaling upstream of IKK in response to TNFα or IL-1β are tumor necrosis factor receptor associated factors (TRAF). TRAF1 and TRAF2 were first identified in this family of proteins that associated with the TNF-R2 complex (Rothe et al., 1994). TNF-R2 is a member of the TNF receptor superfamily (TNFR). Using yeast two-hybrid screens four TRAF proteins were identified (Cao et al., 1996b; Cheng et al., 1995; Hu et al., 1994; Ishida et al., 1996a; Ishida et al., 1996b; Mosialos et al., 1995; Nakano et al., 1996; Regnier et al., 1995). The most recent TRAF protein, TRAF7, was identified by affinity purification and bioinformatics approaches (Bouwmeester et al., 2004; Xu et al., 2004). One of the most distinguishing characteristics of TRAF proteins is that they all contain a C-terminal TRAF domain. This domain is composed of two portions an N-terminal coiled-coiled region and a C-terminal TRAF region. The C-terminal region of the TRAF domain is required for association to membrane complexes and for signal transduction to downstream molecules. All TRAF proteins except TRAF1 contained a conserved N-terminal RING domain followed by several zinc fingers. TRAF7 is the only TRAF protein that contains seven WD forty repeats in the C-terminus of the protein.

I.A.7 Ubiquitin-mediated activation of IKK by TRAF2 and TRAF6

TRAF2 and TRAF6 play key roles in transducing signals from cell surface receptors, which lead to the activation of IKK. Ligand binding to cognate receptor initiates cellular signaling which can be seen in the TNF receptor superfamily (TNFR), IL-1 receptors (IL-1R), and Toll-like receptors (TLRs). In response to TNFα stimulation TRAF2 is recruited to the TNF receptor complex by an adaptor protein called TRADD.

TRADD contains a death domain which mediates direct interaction with the death domain of TNFR (Hsu et al., 1995). Both TRAF2 and TRADD interact with a kinase called receptor interacting protein kinase-1 (RIP1) (Stanger et al., 1995). It has been shown genetically that RIP1 is essential and signals through a kinase independent mechanism in the TNF pathway. It was also genetically demonstrated that TRAF2 and TRAF5 act redundantly in the TNF pathway, but TRAF2 is essential for JNK activation (Tada et al., 2001; Yeh et al., 1997). TRAF6 is also essential for activation of JNK and IKK in both IL-1 and TLR signaling (Cao et al., 1996b; Lomaga et al., 1999; Naito et al., 1999; Regnier et al., 1997). Once IL-1β binds to the IL-1 receptor a complex of proteins are recruited to the membrane through an adaptor protein MyD88 (Wesche et al., 1997). MyD88 interacts with a kinase IRAK4, which phosphorylates and activates IRAK1 (Cao et al., 1996a; Li et al., 2002b). Ultimately, activation of IRAK1 leads to the recruitment of TRAF6, which is required for activation of IKK.

I.A.8 TRAF proteins are Ub ligases that activate IKK.

Once it was established that TRAF6 was required for IKK activation, an effort was taken to understand the link between TRAF6 and the activation of IKK. A biochemical assay was developed using cell free components to identify which proteins are required for IKK activation in a TRAF6 dependent manner *in vitro* (Deng et al., 2000). Subsequent biochemical fractionation of HeLa S100 using this cell free system lead to the identification of two factors termed (TRAF6 regulator of IKK activation) TRIKA1 and TRIKA2 (Deng et al., 2000; Wang et al., 2001). TRIKA1 is composed of a dimeric protein complex that contains Ubc13 and Uev1a. Uev1a is considered an ubiquitin conjugating enzyme variant due to its active site lacking catalytic cysteine

residue as compared to a typical E2 enzyme such as Ubc13. The yeast homologue of Uev1a is Mms2 and has been shown to function in DNA repair signaling (Hofmann and Pickart, 1999). The RING domain of TRAF6 is required for E3 ubiquitin ligase activity and together with Ubc13/Uev1a, specifically generates K63-linked polyubiqutin chains. This RING domain is essential for K63-linked ubiquitin chain synthesis and is required for activation of IKK.

I.A.9 Ub targets: RIP1, NEMO, TRAF6/2

Ubiquitination of several signaling proteins leads to IKK activation by K63 linked polyubiquitin chains in both the TNF and IL-1 pathways. TNFα induces RIP1 ubiquitination and this form of RIP1 has been shown to associate with TNFR (Ea et al., 2006; Lee et al., 2004; Zhang et al., 2000). Recently, the ubiquitination site of RIP1 was mapped to Lys377 and mutation of this residue to K377R abolished RIP1 ubiquitination, as well as its ability to activate IKK (Ea et al., 2006; Li et al., 2006). The recruitment of TAK1/TAB2 complex may occur based on the evidence that TAB2 associates with ubiquitinated RIP1 (Kanayama et al., 2004). TRAF2 ubiquitination by Ubc13/Uev1a is also observed to be essential for JNK, germinal center kinase related (GCKR), and p38, but not NF-κB activation by TNFα (Habelhah et al., 2004; Shi and Kehrl, 2003). In TRAF2 deficient cells, NF-κB activation is not affected, but JNK and p38 activation is blocked. TRAF5 may also be a target of ubiquitination since TRAF2/TRAF5 double knockout cells are defective in NF-κB activation.

NEMO is also polyubiquitinated in response to TNFα (Tang et al., 2003) and has also been shown to be a target for ubiquitination by RIP2 and NOD2 (Abbott et al., 2004). NOD2 serves as receptor to detect intracellular bacteria. RIP2 is similar to the

RIP1 kinase. Both molecules are required for induction of NF-κB activation by intracellular bacteria. NEMO polyubiquitination also mediates IKK activation during T cell receptor signaling in adaptive immunity (Sun et al., 2004; Zhou et al., 2004). It was shown that Bcl10 activation leads to the recruitment and oligomerization of TRAF6 to MALT1 (a paracaspase) which is essential for IKK activation in T cell signaling. Again from this evidence, TRAF6 acts as the E3 ubiquitin ligase responsible for NEMO ubiquitination. Activation of TRAF6 in several signaling pathways such as TCR, IL-1, and toll-like receptor signaling leads to K63 polyubiquitination of targets proteins including TRAF6 itself (Ea et al., 2004; Sun and Chen, 2004; Wang et al., 2001).

I.A.10 DUBS: CYLD and A20

The effects of ubiquitination can be reversed by the removal of ubiquitin chains from a target substrate in the process of deubiquitination by a family of proteins called deubiquitinating enzymes (DUBs). Two DUBs have been identified which target K63-linked polyubiquitin chains. Cylindroma tumor suppressor gene (CYLD) was the first DUB identified to negatively regulate NF-κB signaling (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). CYLD has been shown to interact with TRAF2 and NEMO (Saito et al., 2004). Mutations in the deubiquitination domain of CYLD have been identified in patients with cylindromas (Leonard et al., 2001). These mutations block the deubiquitination activity of CYLD leading to the over activation of NF-κB.

A20 is a protein that is induced by NF-κB activation (Dixit et al., 1990; Opipari et al., 1992) and identified as a second DUB that targets K63-linked polyubiquitin chains (Boone et al., 2004; Evans et al., 2004; Wertz et al., 2004). A unique feature of A20 is

that it also contains ubiquitin ligase activity (Wertz et al., 2004). One target of A20 is RIP1, which is deubiquitinated and then ubiquitinated for degradation. Mice deficient in A20 suffer from severe inflammation from the overstimulation of LPS and TNFα.(Lee et al., 2000) This is a result of the prolonged and unregulated activation of NF-κB. A20 also negatively regulates IRF3 activation by interacting with tank binding kinase (TBK), which leads to the inhibition of IRF3 phosphorylation (Saitoh et al., 2005).

I.A.11 Signaling pathways downstream of TRAFs - TAK1

TAK1 was initially identified as a TGFβ activated kinase, but was subsequently discovered to be involved in both innate and adaptive immunity (Ninomiya-Tsuji et al., 1999; Sun and Chen, 2004; Wang et al., 2001; Yamaguchi et al., 1995). TAK1 is a major kinase downstream of TRAF proteins and has been shown to interact with TRAF2 and TRAF6 in a signal-dependent manner (Ninomiya-Tsuji et al., 1999). Recently, TRAF6 was knocked out in T cells, which resulted in multiorgan inflammatory disease (King et al., 2006). Although T cells from these mice were still able to activate NF-κB and MAPK, studies using siRNA in Jurkat cell targeting both TRAF2 and TRAF6 led to a suppression of NF-κB (Sun et al., 2004). It is possible that both TRAF2 and TRAF6 compensate for each other functionally. Three independent genetic studies using conditional knockout strategies have provided evidence that TAK1 functions as a major kinase that regulates the activation of IKK and JNK pathways. All three studies observed defects in thymocyte development and activation in T cells lacking TAK1 (Liu et al., 2006b; Sato et al., 2006; Wan et al., 2006). A higher incidence of colitis was observed in mice lacking TAK1 stemming from developmental defects in regulatory T cells (Sato et al., 2006). Effectory T cells do not require TAK1 for the activation of both NF-κB and

JNK unlike mature thymocytes (Wan et al., 2006). B cells were also shown to have similar defects in JNK activation, but not NF-kB when stimulated with antigen (Sato et al., 2006). Biochemically, TAK1 has been shown to activate IKK and JNK/p38 pathways through direct phosphorylation of IKKβ and MKK6 at the activation loop (Wang et al., 2001). The TAK1 signaling cascade is conserved in *Drosophila* and mutations in dTAK1 resulted in a severe defect in the IKK-dependent activation of the NF-κB homolog Relish (Vidal et al., 2001). Additionally, RNAi of dTAK1 in *Drosophila* Schneider (S2) cells inhibited the activation of NF-κB and JNK signaling in response to PGN stimulation (Chen et al., 2002; Silverman et al., 2003). The use of either an inhibitor 5Z-7-oxozeaenol or siRNA targeting TAK1 have been shown to inhibit both TNFα and and IL-1β-induced IKK and JNK activation (Sun and Chen, 2004). Taken together, all these independent results demonstrate TAK1 plays a major role in the activation of IKK and JNK in the innate and adaptive immune response pathways.

CHAPTER TWO
NPL4 Regulation of IκBα Degradation

II. A INTRODUCTION

In this report, I investigated the molecular mechanisms involved in the regulation of IκBα degradation. Using siRNA approaches, NPL4 was identified to be required for IκBα degradation, and *in vitro* proteasomal degradation assays demonstrate that the NPL4 complex is required for IκBα degradation. Evidence from both *in vitro* and *in vivo* studies suggest NPL4 is required for IκBα degradation, but not for IKK activation. These results suggest NPL4 is working at a step after ubiquitination of IκBα, but before proteasomal degradation. Previously, only VCP was implicated in IκBα degradation. I propose that ubiquitinated IκBα is targeted to the proteasome by an interaction between the NPL4 complex that is mediated through the zinc finger domain of NPL4.

II.B Materials and Methods

II.B.1 Materials and Cell Culture

Antibodies against IκBα (C21) were purchased from Santa Cruz Biotechnology and antibodies against FLAG antigen (M2) were purchased from Sigma. NPL4 polyclonal antibodies were raised in rabbits against purified recombinant full length NPL4 expressed in *E.coli*. Polyclonal antibodies were purified using an affinity column generated from recombinant NPL4 coupled to NHS Sepharose. Antibodies against VCP and UFD1 were generously provided by Dr. George Demartino. Okadaic acid and MG-132 were purchased from Alexis Biochemicals. Human embryonic kidney 293T cells were cultured and maintained in DMEM supplemented with 10% Cosmic Calf Serum (Hyclone) and 100μg/ml penicillin and 100μg/ml streptomycin.

II.B.2 Plasmids and Proteins

Full length human NPL4 was subcloned from pET28a into pcDNA3.0. The first round of PCR primers used were forward primer: HindIII/Flag/NPL4 (5'-CCC AAG CTT ATG GAC TAC AAG GAC GAC GAT GAC AAG GAA TTC CTT AAG ATG GCC GAG AGC ATC ATA ATT CGT-3') and reverse primer: NotI/NPL4 (5'-ATAAGA ATG CGG CCG CCT AGG TCC TGG GGA GGC T-3'). The subsequent PCR product was gel purified and digested with HindIII/NotI and ligated into pcDNA3.0 The resulting construct pcDNA3.0FlagNPL4 was used to establish stable 293T cells lines expressing Flag-NPL4* and Flag-ΔZincNPL4*. NPL4* RNAi resistant mutant were generated by standard based PCR mutagenesis using the following PCR primers forward: (5'-CTT GAG GAT CCA AAA GCA GAG GTC GTC GAC GAG ATA GCT GCC AAA CTT GGC-3') and reverse primer (5'-GCC AAG TTT GGC AGC TAT CTC GTC GAC GAC CTC TGC TTT TGG ATC CTC AAG-3'). Resulting constructs were verified on ABI capillary DNA sequencing instruments. Both VCP and UFD1 were expressed using *E.coli* from pET28a.

II.B.3 Immunodepletion of NPL4 from S100

NPL4 Sepharose was prepared by using NPL4 polyclonal antibody (2µg eluate pH3.2) coupled with 10µl of Protein A/G-Sepharose (Pierce) in 200µl TBST with 1-2% Milk using a 1.5ml tube. The antibody and Sepharose mixture was rotated at room temperature for 1 hour. The beads were subsequently washed 3X TBST and with 1X cell lysis buffer. Immunodepletion of HeLa S100 (15µl) began by mixing the NPL4-Sepharose beads with HeLa S100 then rotating at $4^{\circ C}$ for 1 hour. Beads were collected by centrifugation at 3000 RPM for 5 minutes. NPL4 immunodepleted supernate was

collected and confirmed by immunoblotting the supernate using NPL4 polyclonal pH 5.0 antibodies.

II.B.4 Preparation of Cell Extracts

HEK293 cell cytoplasmic extracts were prepared by lysing the cells in hypotonic buffer (10mM HEPES pH7.5, 1mM EDTA, 10mM KCl, 1mM DTT, and 1X complete protease inhibitor cocktail (Roche)). Following incubation on ice for 10 minutes, 0.2% NP-40 was added to the lysate and the mixture was pipetted up and down 10 times and incubated on ice for another 5 minutes. After incubation on ice cell extracts were centrifuged at 20,000g for 15 minutes at $4^{\circ C}$. The supernate (cytoplasmic extract) was stored at $-80^{\circ C}$.

II.B.5 RNA Interference and NF-κB Reporter Assay

Double-stranded RNA oligonucleotides were purchased from Dharmacon. The sense strand of RNAi oligos were as follows: NPL4 (877- 898): GCU GAA GUG GUC GAU GAA A; GFP (471-489): GCA GAA GAA CGG CAU CAA G and RNA oligos were annealed at then transfected into 293T cells at a final concentration of 20 nM. Cells were seeded in a 12 well plate at a cell density of 1.0-1.5 X 10^5 cells per well and transfected using the calcium phosphate method. On the second day, cells were transfected again with 40ng of each 3X-NFκB-Luc and pCMV-LacZ reporter constructs. 36 hours after transfection cellular extracts were collected for luciferase and β-Galactosidase assays. Luciferase assays for NF-κB-luc were performed in duplicate and results were normalized to co-transfected β-Galactosidase. Luciferase activity was measured with a FLUOstar

OPTIMA luminometer (BMG LABTECH) using Promega's luciferase assay system. β-galactosidase activity was measured using a Thermo Labsystems microplate reader using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate.

II.B.6 Isolation of *in vitro* translated ^{35}S-labeled IκBα

In vitro TNT synthesized IκBα was purified from wheat germ extract using His-p65 for affinity purification. 200µl of TNT WGE ^{35}S-IκBα product was mixed with 20µg His_6-p65 and incubated on ice for 60 minutes, allowing p65/ IκBα complex to form. 1.0ml IP Buffer (20mM Tris pH 8.0, 150mM NaCl, 1mM DTT, 20mM Immidazole, 0.5% NP-40) was added with 55µl Ni-Agarose Suspension (50% Washed Slurry) and rotated at 4^{oC} for 1 hour. Agarose was washed 3X with IP buffer and beads were eluted with (20mM Tris pH 8.0, 50mM NaCl, 1mM DTT, 150mM Immidazole, Glycerol 10%). Elution was concentrated down to 100µl and buffer exchanged with (20mM Tris pH 8.0, 20mM NaCl, 5% Glycerol) to get Immidazole concentration to about 25mM.

II.B.7 *In Vitro* Signal Dependent Ubiquitination of IκBα

In vitro ubiquitination of IκBα was carried out for 1 hour at 30^{oC} and analyzed by SDS-PAGE and autoradiography. 10ul reaction mixtures consisted of 1ul of purified TNT His-p65/^{35}S- IκBα complex, ubiquitin 0.5µM, 0.5µM IKKβ, 0.1µM E1, 0.5µM Ubc3, 0.2µM $SCF^{\beta TrCP}$ in the presence of 1X PBDM. PBDM is an ATP regenerating system containing 50mM Tris pH 7.6, 5mM $MgCl_2$, 2mM ATP (Sigma A-2383), 10mM creatine phosphate (Sigma P-7936), 3.5 U/ml creatine kinase(Sigma C-3755), and 0.6 U/ml inorganic pyrophosphatase(Sigma I-1643).

II.B.8 Conjugate Degradation Assay

Purified *in vitro*-translated His-p65/^{35}S- IκBα was incubated with HeLa extract in the presence of ATP regenerating system. The reactions were incubated at 30°^{C} for the indicated time. At the desired time points, the reaction was quenched by addition of 125µl of 4% BSA and 575µl of 12% TCA. After removal of the TCA precipitates by centrifugation, 600 µl of the supernates were counted in a scintillation counter. The results are expressed as percentage of the conjugates that are degraded to TCA-soluble counts.

II.C. Results

II.C.1 Activation of NF-κB by TNFα requires NPL4 in HEK293 cells

To test if NPL4 is required for NF-κB activation, a siRNA based luciferase assay was used. It is well established that cytokine induced activation of NF-κB signaling can be monitored using a NF-κB -Luc reporter construct. This reporter construct has expression of luciferase under the control of a 3X-κB binding element. Prior to co-transfecting HEK293 cells with both the NF-κB -Luc reporter construct and pCMV-LACZ construct to normalize the co-tranfection, they were transfected twice with siRNA oligos. NPL4 oligos were designed to silence the expression of endogenous NPL4 and GFP oligos were used as a negative control. NPL4 siRNA inhibited NF-κB activation by TNFα whereas GFP siRNA had no effect on NF-κB activation (Figure 8A).

One other method used to assay for NF-κB activation *in vivo* was to immunoblot cells extracts for IκBα that were generated during a time course stimulation of TNFα. Activation of NF-κB leads to the phosphorylation of IκBα, as represented by a slower migrating IκBα species. Again, to examine if NPL4 is required for NF-κB activation, a

siRNA based approach was used to silence NPL4. Controls included siRNA against TAK1, p62 and GFP (Figure 8B). TAK1 was included as a positive control since TAK1 is a key kinase required for IKK activation during cytokine induced activation of NF-κB. Both GFP and p62 were targeted as negative controls. p62 functions as a scaffold protein that is required for NF-κB activation by nerve growth factor. As expected, TNFα stimulated IκBα degradation was inhibited in extracts generated from TAK1 RNAi transfected cells. Extacts generated from cells transfected with RNAi oligos targeting NPL4 also displayed a distinct inhibition of IκBα degradation. IκBα degradation was not affected in extracts from both GFP and p62 siRNA transfected cells. Taken together, these results suggest that NPL4 is required for NF-κB activation by TNFα.

Figure 8A

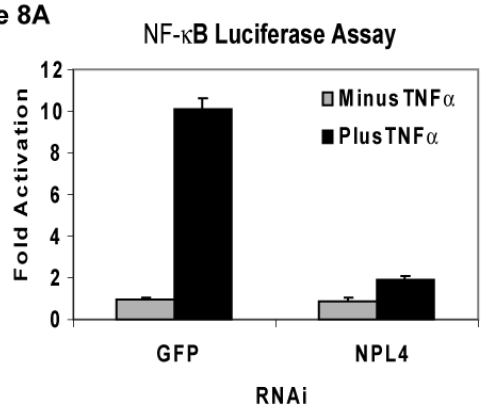


Figure 8B

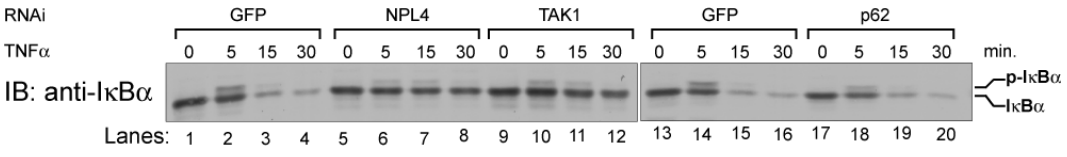


Figure 8. NPL4 is Required for Degradation of IκBα and NF-κB Activation

(A) siRNA oligos targeting NPL4 and GFP as negative control were transfected into HEK293 cells together with NF-κB-dependent luciferase reporter (3X-kB-Luc) and

β-galactosidase reporter (pCMV-LacZ). Luciferase assays were performed as described in Materials and Method and normalized against β-galactosidase activity. Results shown represent an average of duplicate experiments. **(B)** HEK293 cells were transfected with siRNA oligos corresponding to GFP, NPL4, TAK1, and p62. siRNA of TAK1 and NPL4 blocked TNFα-induced degradation of IκBα. HEK293 cells were transfected with siRNA oligos as indicated and then stimulated with TNFα (20ng/ml) for the indicated times before harvest. IκBα degradation was measured by immunoblotting of cell extracts with an IκBα specific antibody.

II.C.2 Roles of NPL4/VCP/UFD1 in IKK activation in HEK293 cells

In order to determine if the NPL4 complex is involved in IKK activation by TNFα (Figure 9A), siRNA oligos targeting NPL4, VCP, and UFD1 were transfected in HEK293 cells. These cells were subsequently stimulated with TNFα and cytosolic extracts from the siRNA cells were used for immunoprecipitation (IP) kinase assays to assess endogenous IKK activity. As shown in (Figure 9B), IKK activation by TNFα was not inhibited by siRNA directed against NPL4 nor the GFP control. Similar results were observed in cell extracts that were generated from cells that had been transfected with either VCP or UFD1 siRNA oligos.

In order to determine whether NPL4 is required for IKK activation an *in vitro* assay was utilized. In this assay the addition of recombinant TRAF6 to cell extracts leads to the activation of IKK. Cytosolic extracts were then prepared from cells that had been transfected with oligos targeting NPL4 and GFP as a control. As shown, siRNA of NPL4 or GFP did not inhibit IKK activation (Figure9C). Both the *in vivo* and *in vitro* data suggest that the NPL4 complex in not involved in IKK activation.

Figure 9A

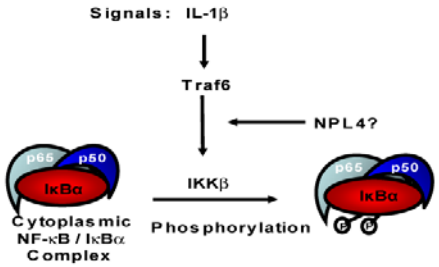


Figure 9C

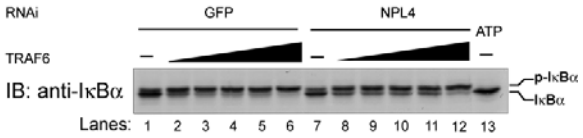


Figure 9B

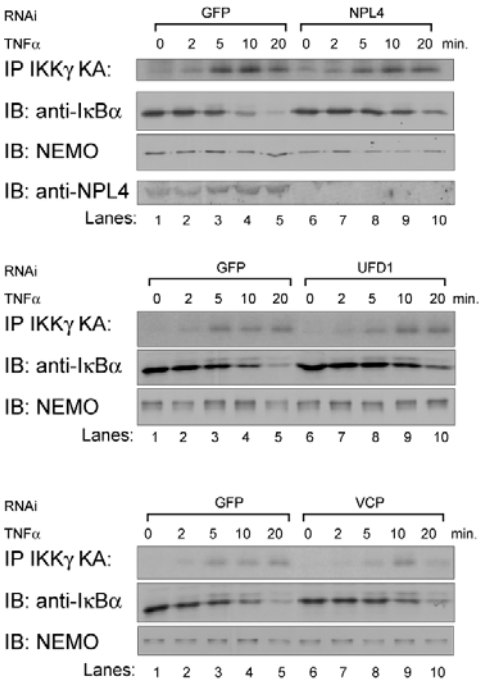
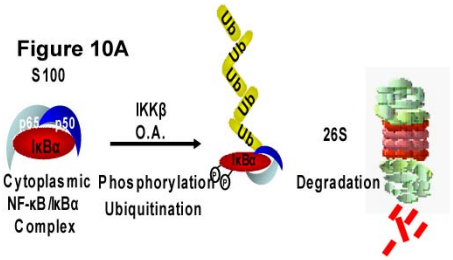


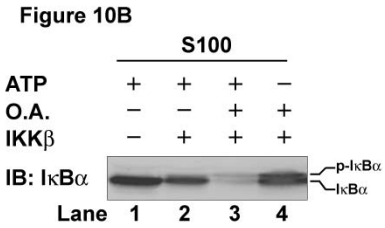
Figure 9. NPL4 is Not Required for IKK Activation

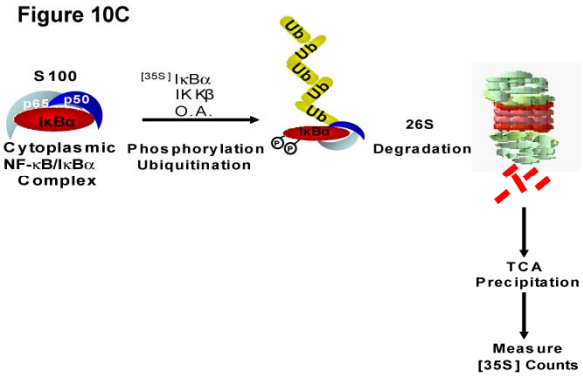
(A) Cartoon depiction of NF-κB activation by IL-1β. **(B)** IKK is activated by TNFα (20 ng/ml) stimulation for indicated times in HEK293 cells that were transfected with siRNA oligos targeting GFP, NPL4, UFD1, and VCP. Antibodies against NEMO were added to the cell extracts to immunoprecipitate IKK complex. The kinase assays were carried out in the presence of γ-^{32}P-ATP and GST-IκBα-NT. **(C)** Cell extracts generated from HEK293 cells that were transfected with siRNA targeting GFP and NPL4 were used for TRAF6 activation of IKK *in vitro*. IKK activation was measured by immunoblotting of cell extracts with an IκBα specific antibody.

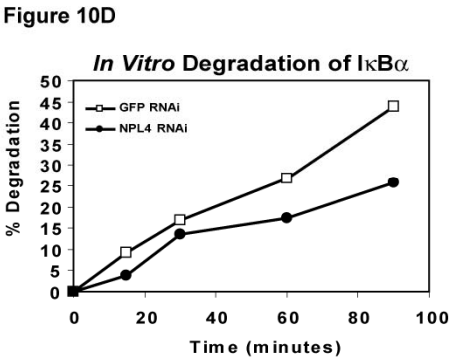
II.C.3 NPL4 is required for Degradation of Ubiquitinated IκBα

To determine whether NPL4 is required for the degradation of IκBα, an *in vitro* degradation assay was established. A cartoon depiction of this assay is shown (Figure10A). This *in vitro* degradation assay faithfully recapitulates signal dependent degradation of IκBα, which can be seen in lane 3 (Figure 10B). This degradation assay requires a HEK293 cytoplasmic fraction (S100) that is activated by the addition of both okadaic acid (phosphatase inhibitor) and recombinant IKKβ expressed and purified in insect cells. In order to directly measure the degradation of IκBα, a modification of this assay was added to include the addition of *in vitro* translated radiolabeled substrate ^{35S}IκBα (Figure 10C). The degradation products were separated from undegraded IκBα by trichloroacetic acid (TCA) precipitation, and the TCA-soluble radioactivity was determined. To establish whether NPL4 is required for IκBα degradation *in vitro*, siRNA oligos targeting NPL4 and or GFP as a negative control were transfected into the in HEK293 cells. Cytosolic extracts were then prepared from the siRNA transfected cells and used to measure IκBα degradation over time. As shown in (Figure 10D), siRNA of NPL4 substantially inhibited IκBα degradation, as expected in the GFP negative control IκBα degradation was not inhibited. To determine if the NPL4 complex is required for IκBα degradation, NPL4 was depleted from HeLa cell extracts (S100) using protein A/G beads coated with polyclonal antibodies toward NPL4. As shown in (Figure 10E) depletion of NPL4 from HeLa S100 significantly inhibited IκBα degradation over time by 25% compared to wild type. As expected, the addition of the proteasome inhibitor MG-132 blocked the degradation of IκBα. Both *in vitro* data suggest that NPL4 is required for IκBα degradation.









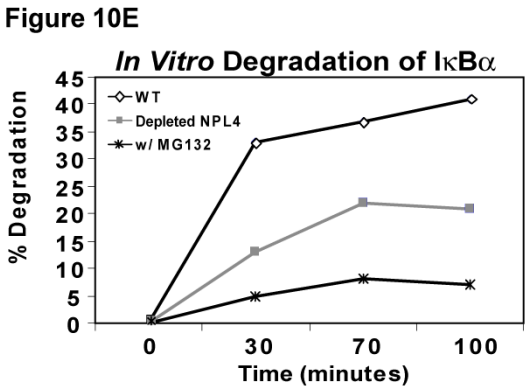


Figure 10. NPL4 is Required for IκBα Degradation

(A) Cartoon describing *in vitro* degradation of IκBα by 26S proteasome. **(B)** HEK293 cytoplasmic extracts were used for an *in vitro* IκBα degradation assay. In (lane 3) IκBα degradation was observed in extracts programmed with both okadaic acid and IKKβ. IκBα degradation was analyzed by immunoblotting of cell extracts with an IκBα specific antibody. **(C)** Cartoon describing *in vitro* degradation of p65/^{35}S-IκBα. ^{35}S-

IκBα was incubated with immunodepleted NPL4 extracts, wild type extracts, or wild type extracts with MG-132. At indicated time points, an aliquot of the reaction was precipitated by 10% TCA. The TCA soluble radioactivity was then determined by liquid scintillation counting. **(E)** Similar *in vitro* assay to **(D)** but extracts were generated from cells that were transfected with either NPL4 or GFP siRNA oligos.

II.C.4 Reconstitution of Signal Dependent Degradation of IκBα *in vitro*

In order to directly analyze NPL4 requirement in IκBα degradation, a series of reagents were generated for reconstitution experiments. In (Figure 11A) highly purified recombinant proteins E1, Ubc3 (E2), $SCF^{\beta TrCP}$ (E3), IKKβ, and Ub, were used to reconstitute signal dependent ubiquitination of IκBα. A rigorous purification scheme was used to reconstitute the NPL4 complex with proteins of high purity (Figure 11B). When combined, the proteins formed tertiary complexes (Figure 11C).

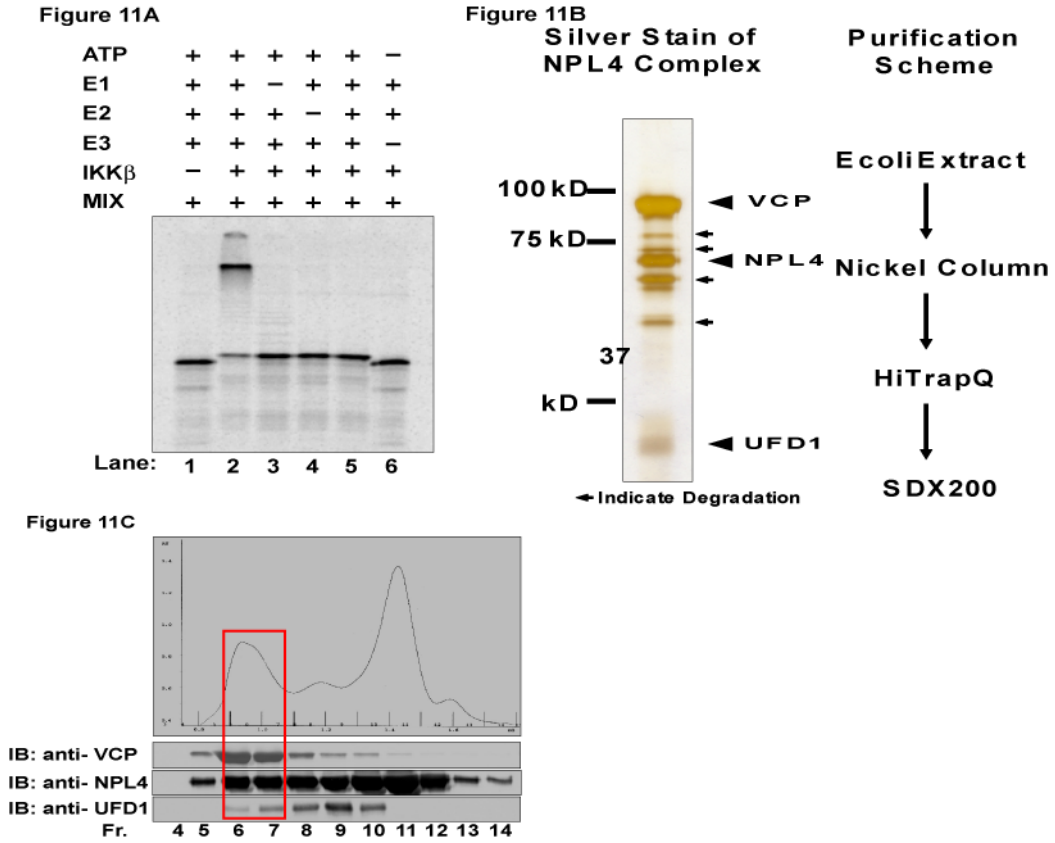


Figure 11. Signal Dependent Ubiquitination of IκBα

(A) *In vitro* signal dependent ubiquitination of IκBα. Recombinant proteins were mixed together His-E1 (0.1uM), GST-Ubc3 (0.5uM), $SCF^{\beta TrCP}$ (0.2uM), IKKβ and incubated at $30^{\circ C}$ for one hour with wild type ubiquitin. Ubiquitination reactions were resolved with 12% SDS-Page and analyzed using phosphoimager. **(B)** Purification scheme of NPL4 protein complex and silver stain of reconstituted NPL4/UFD1/VCP complex. **(C)** Reconstitution profile of NPL4/UFD1/VCP complex from Superdex 200. Complex assembly was analyzed by immunoblotting superdex fractions with specific antibodies against NPL4/UFD1/VCP.

II.C.5 NPL4 Zinc Finger is Required for Degradation of IκBα

To determine if the zinc finger (ZnF) domain of NPL4 is important for IκBα degradation, mutants of NPL4 were generated that lacked the ZnF domain (Figure 12A). To determine if the zinc finger domain of NPL4 is required for IκBα degradation, *in vivo* stable cell lines expressing RNAi resistant forms of either wild type NPL4 or ΔZincNPL4

were generated in HEK293 cells. siRNA oligos targeting NPL4 and GFP as a control were transfected into the stable cell lines WT NPL4* and ΔZincNPL4* to block endogenous expression of NPL4. As expected, exogenous expressed WT NPL4* rescued IκBα degradation during TNFα stimulation (lanes 4-6, Figure 12B). Strikingly, exogenous expressed ΔZincNPL4* blocked IκBα degradation during TNFα stimulation (lanes 10-12, Figure 12B). Taken together these results suggest that NPL4 binds ubiquitinated IκBα through its zinc finger domain and is required for IκBα degradation (Figure 12C).

Figure 12A

Figure 12B

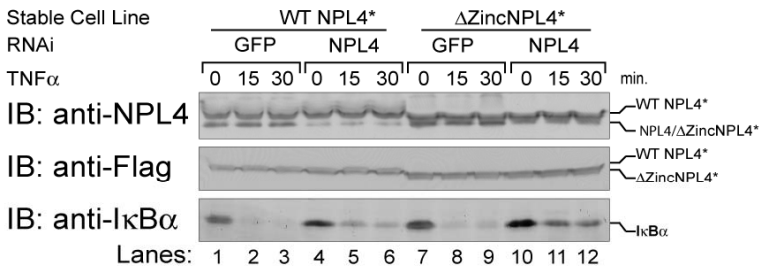


Figure 12C

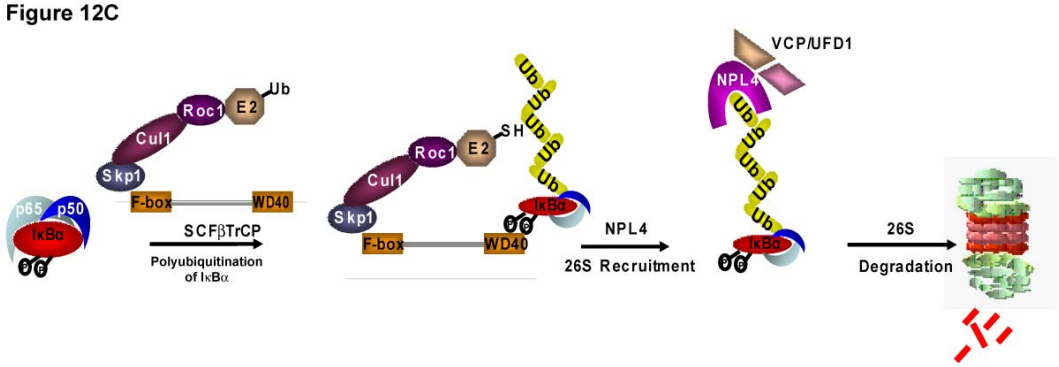


Figure 12. NPL4 Zinc Finger Domain is Required for IκBα Degradation

(A) Diagram of structural domains of wild type NPL4 as compared to zinc finger deletion mutant of NPL4. **(B)** Stable HEK293 cell line expressing NPL4* and mutant ΔZincNPL4 (*denotes siRNA resistant) were transfected with NPL4 and GFP siRNA to block endogenous expression of NPL4 and stimulated with TNFα. IκBα degradation was analyzed by immunoblotting of cell extracts with an IκBα specific antibody. Ectopic expression of NPL4 was analyzed by immunoblotting of cell extracts with a Flag specific antibody. **(C)** Model for role of NPL4 complex in IκBα degradation. Activation of IKK leads to phosphorylation and ubiquitination of IκBα. The NPL4 complex binds to ubiquitinated form of IκBα and chaperones the substrate to the 26S proteasome for degradation.

II.D. DISCUSSION

In this study, I investigated the regulation of proteasomal mediated degradation of IκBα by TNFα activation of NF-κB signaling. I show, using siRNA, that NPL4 is required for both NF-κB activation and the degradation of IκBα by TNFα. Importantly, the analysis of IKK activity using cell extracts generated from HEK293 cells transfected with siRNA oligos targeting individual components of the NPL4 complex suggest they are not required for activation of IKK. Using an *in vitro* degradation assay, I have provided biochemical evidence, through both immunodepletion of the NPL4 complex and RNAi of NPL4 in HEK293 extracts, that NPL4 is required for degradation of IκBα. In addition I have also shown using siRNA and rescue experiments in HEK293 cells that NPL4 is required for IκBα degradation by TNFα dependent activation of NF-κB signaling.

In this report, I reconstituted signal dependent ubiquitination of IκBα *in vitro*, which when combined with the reconstituted NPL4 complex, should contribute as the key reagents to help conclude what are the minimum factors required for ubiquitination and proteasomal degradation of IκBα.

My data has shown that the zinc finger domain of NPL4 is required for IκBα degradation. Inhibition of IκBα degradation was observed when the deletion zinc finger NPL4 mutant was unable to rescue IκBα degradation in response to TNFα stimulation. It has been previously been shown that the NPL4 zinc finger domain binds ubiquitin and is considered an ubiquitin binding motif (Wang et al., 2003). Also, it has been shown that VCP/p97 a member of the heterotrimeric NPL4 protein complex, associates with ubiquitinated IκBα in immunoprecipitation experiments (Dai et al., 1998). And in yeast

it has been shown that an escort pathway controls activation and degradation of the NF-κB related yeast transcription factor Spt23 and that it is involved in ER-associated degradation (Richly et al., 2005). Taken together, these studies suggest that degradation of IκBα occurs through a similar escort pathway described in (Figure 12C).

Although considerable effort was taken to reconstitute signal dependent degradation of IκBα *in vitro,* the molecular mechanisms responsible for the recognition of ubiquitinated proteins and processes leading to proteasome mediated degradation are still unclear. A more extensive effort will be required to nail down specifically, which proteins recognize ubiquitinated IκBα and escort the substrate to the proteasome. From the studies presented I have identified NPL4 as a factor required for IκBα degradation.

CHAPTER THREE
CYLD is a K63 Specific Deubiquitination Enzyme that Inhibits TAK1 and IKK

III.A. INTRODUCTION

Cylindroma tumor suppressor gene (CYLD) was the first DUB identified to negatively regulate NF-κB through the deubiquitination of several proteins including NEMO, TRAF2, and TRAF6 (Brummelkamp et al., 2003; Kovalenko et al., 2003; Regamey et al., 2003). CYLD has been shown to interact with TRAF2 and NEMO (Brummelkamp et al., 2003; Kovlaenko et al., 2003, Regamey et al., 2003). The gene for familial cylindromytosis was first mapped by linkage analysis to chromosome 16q12-q13 (Biggs et al., 1995). Familial Cylindromatosis, also know as Brooke Speigler Syndrome, is an autosomal dominant disorder characterized by individuals that have tumors which form skin appendages that originate from hair follicles on the head and neck region. The CYLD gene was first identified to be a tumor suppressor by positional cloning and loss of heterozygosity analysis in families with the disease (Bignell et al., 2000). Mutations in the deubiquitination domain of CYLD have been identified in patients with cylindromas (Courtois and Gilmore, 2006). These mutations inhibit the deubiquitination activity of CYLD, resulting in the constitutive activation of NF-κB, which leads to cell proliferation. Recently, it has been shown that CYLD regulates JNK activation (Reiley et al., 2004; Xue et al., 2007) and *in vivo* studies of CYLD have included studies on ketratinocytes, B cells, and bacterial induced lethality in mouse (Hovelmeyer et al., 2007; Massoumi et al., 2006; Reiley et al., 2007; Reiley et al., 2006).

Activation of NF-κB signaling by TNFα or IL1-β requires either TRAF2 or TRAF6 proteins to transduce signals from cell surface receptors to the IKK complex.

Both TRAF proteins contain an N-terminal RING domain, which is necessary for ubiquitin ligase activity. This specific ligase activity together with the E2 protein complex Ubc13/Uev1a is responsible for generating K63 specific ubiquitin chains (Deng et al., 2000). Several key NF-κB signaling components are ubiquitinated by these unique K63 ubiquitin chains including NEMO, TRAF2, TRAF6, and RIP1 (Adhikari et al., 2007). It has been shown that these K63 specific ubiquitin chains are required for the activation of TAK1 (Wang et al., 2001). Activated TAK1 directly phosphorylates and activates IKK in a non-proteasomal ubiquitin dependent manner. The IκB kinase (IKK) is responsible for directly phosphorylating the inhibitor of NF-κB (IκBα), which is the priming event required for recognition by the $SCF^{\beta TrCP}$ ubiquitin ligase. Once IκBα is ubiquitinated and degraded, NF-κB can translocate into the nucleus of the cell and bind a variety of host response genes that are involved in many vital cellular functions such as cell survival, inflammation, and immunity.

In the TNFα pathway, K63 ubiquitination of RIP1 is required for NEMO binding and activation of IKK (Ea et al., 2006; Wu et al., 2006). NEMO polyubiquitination also mediates IKK activation during T cell receptor signaling in adaptive immunity (Zhou et al., 2004). It was shown that Bcl10 activation of IKK leads to the recruitment and oligomerization of TRAF6 to MALT1, a paracaspase that is essential for signaling at T cell immunocomplexes (Sun et al., 2004).

In this report, I investigated the molecular mechanisms of how CYLD negatively regulates NF-κB signaling using a biochemical approach. No direct evidence has been shown to explain the underlying mechanism that leads to inactivation of NF-κB signaling. In this chapter I demonstrate that CYLD inhibits activation of TAK1 and IKK

by TRAF6 *in vitro* in a cell free system. Furthermore, I provide direct evidence that CYLD harbors K63 specific endoprotease activity and preferentially binds K63 polyubiquitin chains.

III.B. Materials and Methods

III.B1. Materials and Cell Culture

Antibodies against IκBα (C21) and ubiquitin (P4D1) were purchased from Santa Cruz Biotechnology. Antibodies against FLAG antigen (M2) were purchased from Sigma. Antibodies against phosphorylated Thr187 on TAK1 were purchased from Cell Signaling Technology. Okadaic acid was purchased from Alexis Biochemicals. Human embryonic kidney (Shekhtman and Cowburn) 293T cells were cultured and maintained in DMEM supplemented with 10% Cosmic Calf Serum (Hyclone) and 100µg/ml penicillin and 100µg/ml streptomycin.

III.B2. Plasmids and Proteins

Full length human CYLD were subcloned into Gateway® entry vector pDONR™221 by two rounds of PCR using attB modified custom primers from pcDNA3FLAGCYLD (Trompouki et al., 2003). The first round of PCR primers used was forward primer: B1-TEV-CYLD (5'-TAT TTT CAG GGC ATG AGT TCA GGC TTA TGG AGC CAA-3') and reverse primer: B2-CYLDFLAG (5'-AGA AAG CTG GGT TTA CTT GTC ATC GTC GTC CTT GTA GTC TTT GTA CAA ACT CAT TGT TGG ACT-3'). The second round of PCR primers used was forward primer: attB1-TEV: (5'-ACA AGT TTG TAC AAA AAA GCA GGC TCC GAG AAT CTT TAT TTT CAG GGC-3') and reverse primer: attB2 (5'-ACC ACT TTG TAC AAG AAA GCT GGG T-3'). pDONR™221-CYLDFLAG was recombined with pDEST™20 baculoviral

expression vector. The fusion protein GST-CYLD was expressed in Sf9 insect cells from pDEST™20-CYLDFLAG and purified using glutathione Sepharose (GE Healthcare, Piscataway, NJ) according to manufacturer's instructions. CYLD point mutants were generated by standard based PCR mutagenesis using oligos designed by the program The Primer Generator (Turchin and Lawler, 1999) and verified on ABI capillary DNA sequencing instruments. Deletion mutants of CYLD were generated by cloning PCR generated fragments of CYLD into XhoI digested pcDNA3 and ligated in frame with the FLAG epitope to generate: pcDNA3FLAGΔN303CYLD, pcDNA3FLAGΔN540, pcDNA3FLAGΔC583, pcDNA3FLAGΔC783.

III.B3. *In Vitro* Assay for TAK1 and IKK Activation

TAK1 and IKK activation were measured *in vitro* by mixing Hela cytosolic extracts 10mg/ml generated in (10mM Tris pH7.5, 1.5mM $MgCl_2$, 10 mM KCl, 0.5mM DTT, 0.5mM PMSF, 1μg/ml leupeptin) with 1X ATP Buffer (50mM Tris pH 7.5, 5mM $MgCl_2$, 2mM ATP, 0.5 mM DTT, 0.01μM okadaic acid), and 0.1μM recombinant TRAF6 in a 10μl reaction and incubated at $30^{\circ C}$ for one hour. The reaction mixture was analyzed by SDS Page and immunoblotted with anti-IκBα and anti-Phospho-TAK1. To observe inhibition of TAK1 and IKK activation, recombinant CYLD (0.1 μM) was added to the same assay.

III.B4. Polyubiquitin Chain Synthesis and Conjugation to NHS Sepharose resin.

In vitro ubiquitination reactions were carried out for 2 hours at $30^{\circ C}$ to generate K63 and K48 specific ubiquitin chains essentially as (Kanayama et al., 2004). K63 linked ubiquitin chains were generated by mixing His-E1 (0.1 μM), His-Ubc13/Uev1a (0.2μM), His-TRAF6 (0.1μM), and ubiquitin (0.5μM) in the presence of 1X ATP buffer.

K48 linked ubiquitin chains were generated as above, but with GST-Ubc3 (0.5μM) and $SCF^{\beta TrCP}$ were used as E2 and E3 respectively. Before ubiquitin chains were conjugated to activated NHS Sepharose (GE Healthcare, Piscataway, NJ), as directed by manufacture instructions, ubiquitination enzymes were depleted using GST and Nickel Sephrose. Ubiquitin chains were then concentrated and underwent buffer exchange with 20mM Tris pH7.5, 150mM NaCl using Millipore concentrators.

III.B5. Polyubiquitin Binding by CYLD

Binding studies were carried out as in (Ea et al., 2006) with Flag-CYLD, Flag-NEMO, and deletion mutants of CYLD. These fusion proteins were expressed and purified from 293T cells and incubated with polyubiquitin Sepharose beads (2μl at 1μg/μl) for 20 minutes at room temp. Beads were washed with buffer B (20mM Tris pH 7.5, 150mM NaCl, and 0.5mM DTT). CYLD and NEMO bound to the beads were analyzed by SDS-Page and then immunoblotted with anti-Flag (M2 antibodies). The CAP GLY (a.a. 472-540) domain of CYLD was expressed in E. coli as a GST fusion protein and purified with glutathione Sepharose. Similar binding conditions were used as previously mentioned with GST-CAPGLY and GST also analyzed via SDS PAGE and immunoblotted with anti-GST.

III.B6. Deubiquitination Assay

Linkage specific ubiquitin chains were incubated for 1 hour at $30^{\circ C}$ with full length baculoviral expressed GST-CYLD and point mutants to analyze deubiquitination activity of CYLD. Deubiquitination reaction mixture was analyzed by SDS-Page and immunoblotted with anti-Ubiquitin.

III.C. Results

III.C1. CYLD Inhibits TAK1 and IKK Activation by TRAF6.

CYLD is an 110kDa protein that contains several distinct structural domains (Figure. 13A). The N-terminus of CYLD encodes three cytoskeletal-associated-protein-glycine-rich (CAP-GLY) domains, which have been shown to bind microtubules (Li et al., 2002a; Scheel et al., 1999). The C terminus of the protein encodes an ubiquitin specific protease domain designated UBP. As shown, WT CYLD inhibits NF-κB activation by TRAF6 and the UBP catalytic activity is required for this inhibition as observed using the catalytic inactive mutant, which has a cysteine to serine (C601S) mutation that abolishes catalytic activity (Figure 13B). In order to determine which functional domain of CYLD is required for its inhibitory activity a series of deletion mutants were generated using standard PCR methods (Figure. 13C). To analyze the activity of each CYLD deletion mutant, expression constructs of each mutant were transfected into and subsequently purified from human embryonic kidney 293T cells using agarose beads coated with flag antibodies (M2 Agarose). Purified deletion mutants of CYLD were added to an *in vitro* system that has been shown to faithfully recapitulate TRAF6 activation of IKK as *in vivo* (Deng et al., 2000). The addition of TRAF6 to Hela cell extracts leads to the activation of TAK1 and IKK, which is indicated by the autophosphorylation of TAK1 on Threonine 187 and phosphorylation of IκBα as indicated by the electrophoretic mobility shift (Figure 13C). Addition of wild type CYLD inhibits the activation of TAK1 and IKK (lane 3). Both the third CAPGLY domain and catalytic UBP domain are the minimum domains required for CYLD inhibition of TAK1 and IKK (lane 4). Deletion of either the third CAPGLY domain or

partial deletion of the catalytic UBP domain leads to the inactivation of CYLD inhibitory activity on TAK1 and IKK (lanes 5-8).

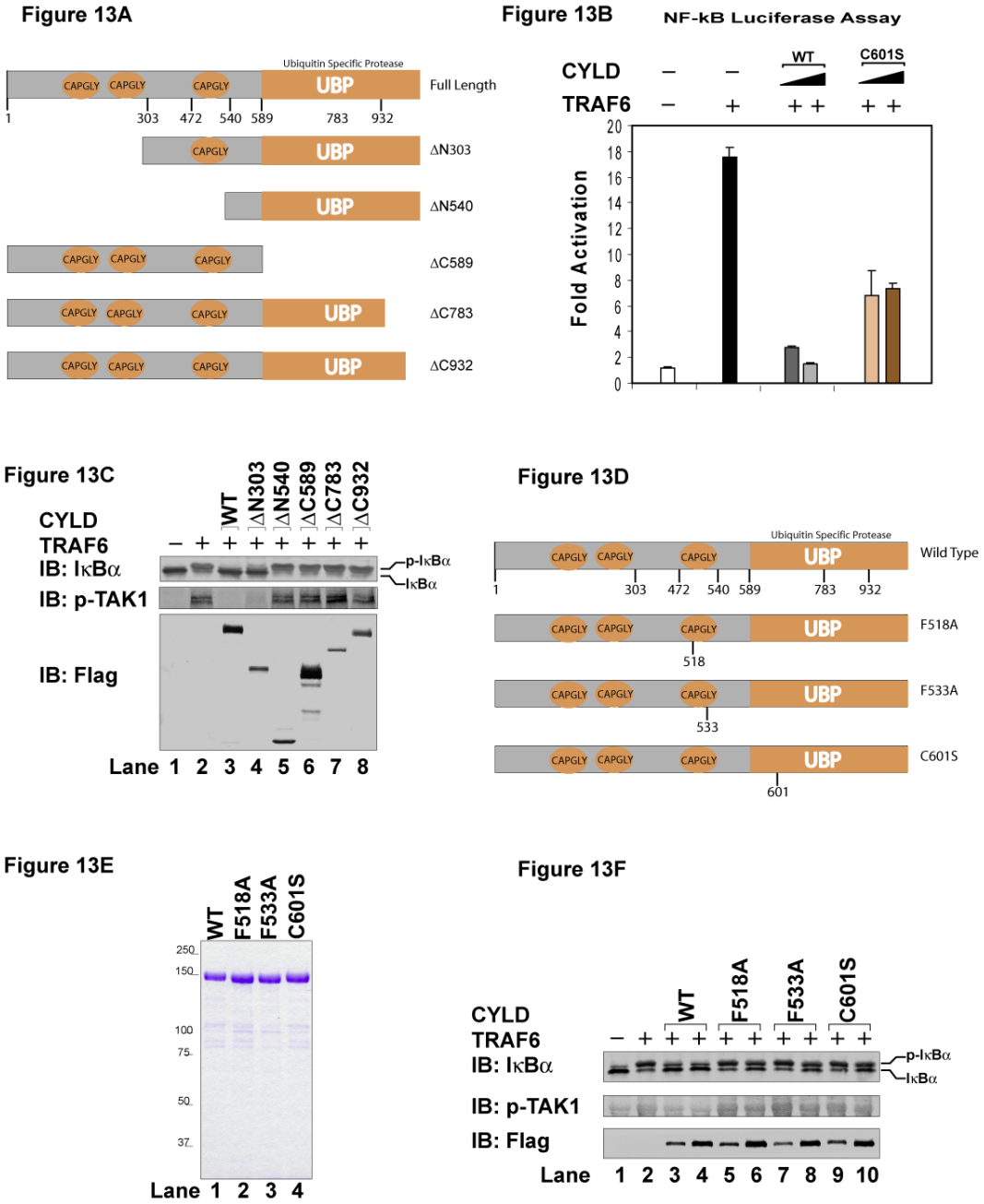


Figure 13. CYLD inhibits TAK1 and IKK activation by TRAF6

(A) Diagram of structural domains of wild type CYLD as compared to deletion

mutants. Names given to proteins are indicated on the right. **(B)** NF-κB activation by TRAF6 measured using NF-κB reporter luciferase assay. Full length CYLD WT inhibits NF-κB activation and is not inhibited by catalytic inactive CYLD mutant C601S **(C)** The third CAPGLY domain and UBP catalytic domain of CYLD are required for inhibition of TAK1 and IKK. Flag tagged WT, wild type CYLD, and deletion mutants of CYLD; ΔN303 ΔN540, ΔC589 ΔC783, ΔC932, expressed and purified from 293T cells using M2 agarose were added to an *in vitro* activation assay of TAK1 and IKK by recombinant TRAF6. (lane 1) control (lane 2) TRAF6 activation of TAK1 and IKK, (lane 3) WT CYLD inhibits activation of TAK1 and IKK, (lanes 4-8) deletion mutants of CYLD. Samples were resolved by SDS-Page and immunoblotted with anti-Flag, anti-IκBα, and anti-PhosphoTAK1. Activation of IKK is noted by phosphorylation of IκBα represented on the gel as a distinct mobility shift of IκBα and activation of TAK1 is represented by immunoreactivity with anti-PhosphoTAK1 which specifically reacts when Thr187 is phosphorylated on TAK1. **(D)** Diagram of WT, wild type CYLD compared to point mutants, F518A and F533A CAPGLY domain mutants, C601S, inactive catalytic mutant. Names indicated on the right. **(E)** Full length wild type GST-CYLD with point mutants expressed and purified from SF9 cells using glutathione Sepharose resolved on SDS-Page and stained with Coomassie Blue. Names of proteins are indicated on top and molecular weight marker is indicated on the left. **(F)** CAPGLY and catalytic domain point mutants of CYLD block its inhibitory activity. CYLD and point mutants from D were titrated in an *in vitro* activation assay of TAK1 and IKK as used in B. (lanes 1-2) control activation of TAK1 and IKK, (lanes 3-4) WT CYLD inhibits activation of TAK1 and IKK,(lanes 5-8) CAPGLY domain mutants block CYLD inhibitory activity (lanes 9-10) catalytic domain mutant blocks CYLD inhibitory activity. Sample reactions were terminated and analyzed as in B.

To reassess if indeed the third CAPGLY domain and UBP domain of CYLD were required for its inhibitory activity, point mutants were generated (Figure 13D). Recombinant full length wild type GST fusion CYLD proteins and points mutants (F518A, F533A, C601S,) were expressed and purified from Sf9 cells using glutathione resin (Figure 13E). A titration of an increasing amount of purified wild type CYLD and point mutants were added to an *in vitro* IKK activation assay (Figure 13F). TRAF6 addition to Hela cell extracts leads to the activation of both TAK1 and IKK (lane 2). The addition of wild type CYLD inhibits the activation of both TAK1 and IKK (lanes 3-4). The CAPGLY and catalytic point mutants lose their inhibitory activity against activation

of TAK1 and IKK (lanes 5-10). Therefore the inhibitory activity observed by full length CYLD mutants recapitulate the activities demonstrated by the functional domain requirements of the CYLD deletion mutants.

III.C2. CYLD has K63-specific endoprotease activity.

In order to directly examine the specific deubiquitination activity of CYLD and test which linkage specificity CYLD has toward ubiquitin chains, I decided to enzymatically generate specific ubiquitin chains *in vitro* using highly purified recombinant ubiquitination enzymes (Figure 14A). It has been shown that ubiquitination enzymes can produce K63 or K48 linkage specific ubiquitin chains.

In order to confirm the ubiquitin linkage to K63 or K48, *in vitro* ubiquitination were done using ubiquitin that contained either K48R or K63R point mutations. K63 specific ubiquitin chains were generated using His-E1, His-Ubc13/Uev1a, and His-TRAF6. K48 specific ubiquitin chains were generated using His-E1, GST-Ubc3, and reconstituted E3-$SCF^{\beta TrCP}$ (Figure 14B). In order to test directly the deubiquitination activity of CYLD, I titrated increasing amounts of either wild type CYLD or point mutants (F518A, F533A, C601S) expressed and purified from SF9 cells with either K63 or K48 specific ubiquitin chains (Figure 14C). Wild type CYLD cleaved the K63 ubiquitin chains (lanes 2-4). Point mutations in the third CAPGLY domain display a reduced efficiency to cleave K63 ubiquitin chains (lanes 5-10). The CYLD catalytic mutant (C601S) was unable to cleave K63 ubiquitin chains (lanes 11-13). All CYLD proteins were unable to cleave K48 ubiquitin chains (lanes 15-26). An *in vitro* deubiquitination assay with both K48 and K63 substrates were used to determine if CYLD would specifically cleave K63 linked ubiquitin chains when challenged with both

K48 and K63 linkage. As depicted in (Figure 14D) tetra ubiquitin that either contained K63 linkage labeled K63-Ub4 or a

Figure 14A

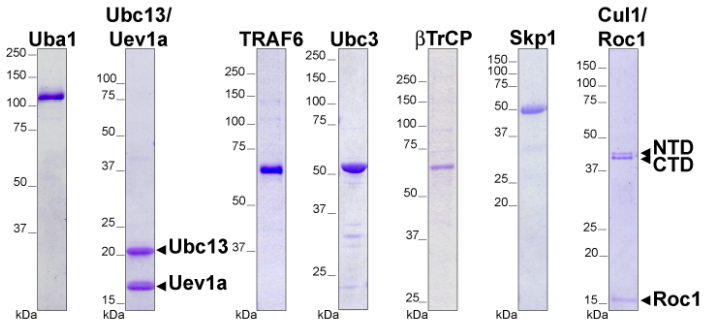


Figure 14B

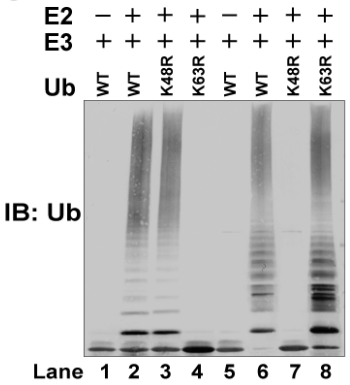


Figure 14C

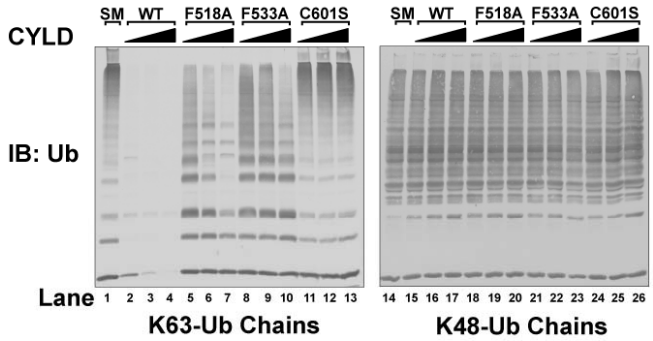


Figure 14D

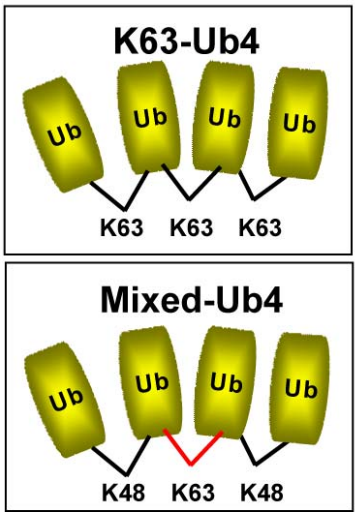


Figure 14E

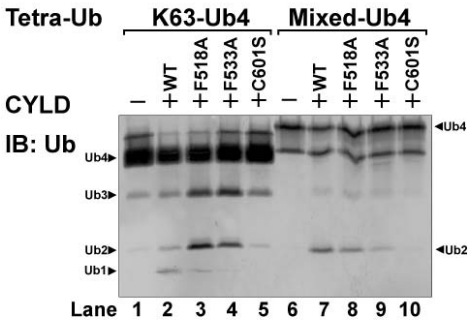


Figure 14. CYLD has K63-specific endoprotease activity

(A) Coomassie Blue staining of purified recombinant ubiquitination enzymes used to generate linkage specific K63 and K48 ubiquitin chains *in vitro*. Names of proteins are indicated on bottom and molecular weight marker is indicated on the left.

His-tagged proteins were purified with nickel-nitrilotriacetic acid (Ni-NTA) resin and GST-tagged proteins were purified with glutathione Sepharose. His-Uba1, His-TRAF6, His-βTrCP, and GST-Skp1 were expressed and purified from Sf9 cells. His-Ubc13/Uev1a and Cul1(NTD, CTD)/Roc1 were expressed and copurified from E. coli. GST-Ubc3 was also expressed and purified from E. coli. **(B)** Generation of linkage specific K63 and K48 ubiquitin chains *in vitro*. Recombinant ubiquitination enzymes were mixed and incubated at $30^{\circ C}$ for one hour with wild type ubiquitin, K48R or K63R ubiquitin. K63 linked ubiquitin chains were generated with His-E1 (0.1uM), His-Ubc13/Uev1A (0.2uM), and His-TRAF6 (0.1uM). K48 linked ubiquitin chains were generated with His-E1 (0.1uM), GST-Ubc3 (0.5uM) and reconstituted $SCF^{\beta TrCP}$ (0.2uM). Ubiquitination reactions were resolved with SDS-Page and immunoblotted using ubiquitin specific antibody P4D1 to visualize ubiquitination reaction. **(C)** CYLD catalyzes the cleavage of K63 linked ubiquitin chains *in vitro*. Full length recombinant CYLD and point mutants were titrated into either K63 or K48 linked ubiquitin chains and subjected to SDS-Page. Cleavage reactions were immunoblotted with anti-ubiquitin. **(D)** Tetra ubiquitin that is only K63 linked labeled (K63 Ub4) or mixed ubiquitin linked labeled (Ub4 Mix K63) were mixed together *in vitro* with recombinant wild type CYLD or point mutants. **(E)** Diagram of enzymatically generated tetra ubiquitin purchased from Boston Biochem UC-310 and UCM-310 as used in D. "K63 Ub4" labeled ubiquitin is four monomers of ubiquitin linked through K63. "Ub4 Mix K63" labeled ubiquitin is made from two monomers of ubiquitin linked by K48 forming dimers that are linked together by K63.

mixed linkage ubiquitin label mixed-Ub4. The mixed-Ub4 tetra ubiquitin has only one K63 linkage that joins to dimers of ubiquitin that are K48 linked. These tetra ubiquitin substrates were mixed together with wild type CYLD or point mutants of CYLD (Figure 14E). Wild type CYLD was able to cleave K63 tetra ubiquitin (lane 2). Both CAPGLY point mutants (F518A, F533A) were capable of cleaving K63 tetra ubiquitin, but less efficiently (lanes 3-4). The catalytic point mutant of CYLD (C601S) was unable to cleave K63 tetra ubiquitin (lane 5). These results using K63 tetra ubiquitin also recapitulate the cleavage results using K63 polyubiquitin chains. Results from mixed Ub4 demonstrate that CYLD has endoproteolytic activity. Wild type CYLD cleaves mixed Ub4 (lane7) and the point mutants cleave less efficiently (lanes 8-9). Once again the catalytic mutant is unable to cleave mixed Ub4 (lane 10).

III.C3. CYLD preferentially bind K63 polyubiquitin chains.

It has been shown that SH3 domains can bind ubiquitin, secondary structure comparisons between CAPGLY domains and SH3 domains suggest that they adopt similar conformations. Sequence alignment between SH3 domains that have been shown to bind ubiquitin and all three of CYLD CAPGLY domains are aligned in Figure 15A. The ability of CYLD to cleave ubiquitin chains suggests that CYLD may contain ubiquitin binding domains. To test this possibility, free polyubiqutin chains were synthesized that were either K48 or K63 linkage specific. To generate K48-linked ubiquitin chains, a reaction containing E1, (Ubc3) E2, and $SCF^{\beta TrCP}$ with ubiquitin was used. A similar reaction was used to generate K63 ubiquitin chains, using Ubc13/Uev1a and TRAF6 as E2 and E3. In Figure15B linkage specific ubiquitin chains were coupled to NHS Sepharose and used to pull down either wild type CYLD or a catalytic mutant of CYLD. NEMO was used as a positive control as it binds K63 specific ubiquitin chains. As shown in Figure 15B, a very small percent of wild type CYLD can be pulled down by ubiquitin Sepharose. This is due to the fact that WT CYLD is active and cleaves ubiquitin. The catalytic mutant of CYLD that is unable to cleave ubiquitin chains pulled-down significantly more K63-linked ubiquitin chains than with K48-linked ubiquitin chains. These results from this experiment indicate that CYLD preferentially binds K63 ubiquitin chains (lanes 4-9). In order to map the ubiquitin binding domain of CYLD, a series of CYLD deletion

mutants were used in the ubiquitin Sepharose pull down experiments. CYLD deletion mutants were expressed and purified from HEK 293T cells as flag tagged fusion proteins. The N-terminal deletion of the first two CAPGLY domains are not required to preferentially bind K63 ubiquitin Sepharose (lane 10-12, Figure 15C). Interestingly, once all three CAPGLY domains are deleted, the ΔN540 CYLD deletion mutant loses ubiquitin specificity as observed by the pull down of equal amounts of K63 and K48 ubiquitin Sepharose as compared to ΔN303 mutant (lanes 13-15). Complete C terminal deletion of the UBP domain in ΔC589 results in binding of only K63 ubiquitin Sepharose (lanes 16-18). Sequential deletions of the C terminus as seen in ΔC783 and ΔC932 also still preferentially bind K63 ubiquitin (lanes 19-24). In order to determine if the third CAPGLY domain itself is a ubiquitin binding domain, we expressed both the third wild type CAPGLY domain and point mutant (F518A, F533A) domain as GST-fusion proteins in *E.coli* and purified using glutathione Sepharose (Figure 15D). These fusion proteins were tested in ubiquitin Sepharose pull down experiments. Wild type CAPGLY domain bound preferentially to K63 Sepharose as both point mutants F518A and F533 lost specificity toward K63 ubiquitin Sepharose (Figure 15E). GST protein was used as a negative control in these binding experiments. In order to determine if mutations in the CAPGLY domain affect CYLD inhibitory activity *in vivo,* a NF-κB luciferase assay was used.

TRAF6 activation of NF-κB luciferase was inhibited by full-length CYLD WT, but not CYLD mutants F518A, F533A, and C601S (Figure 15E).

Figure 15A

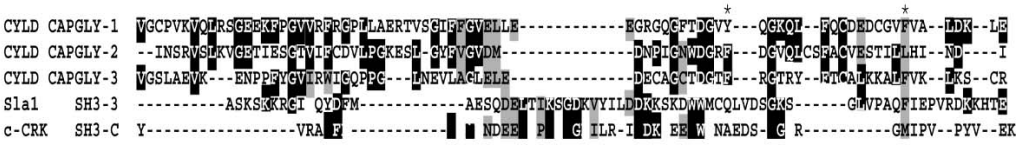


Figure 15B

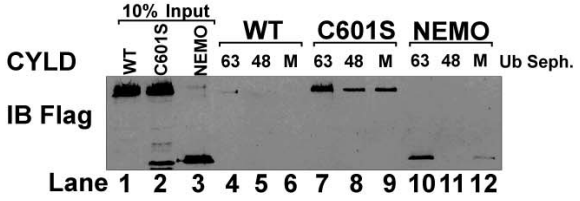


Figure 15C

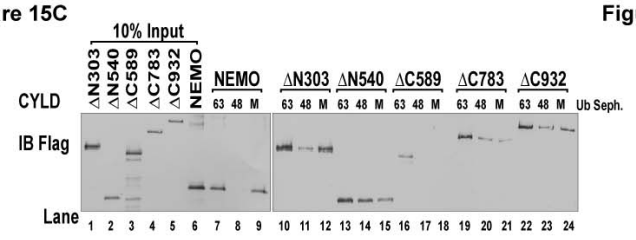


Figure 15D

Figure 15E

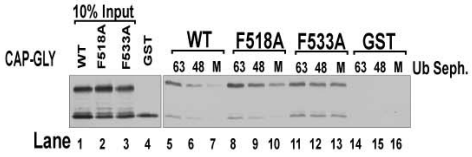


Figure 15F

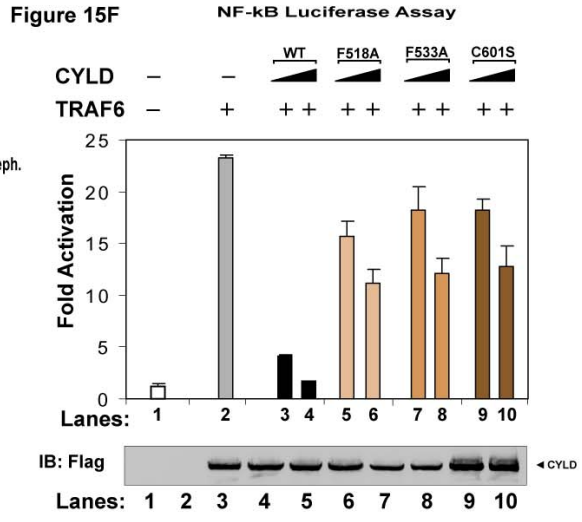


Figure 15. CYLD preferentially binds K63 linked polyubiquitin chains

(A) Sequence alignment between three CAPGLY domains from CYLD and 3rd SH3 domain from Sla1 and SH3 domain from c-CRK. **(B)** To analyze binding between CYLD and either K63 or K48 linkage, specific ubiquitin chains were mixed and incubated at room temp. Recombinant CYLD and catalytically inactive CYLD expressed and purified from 293T cells were used in binding experiments. As a positive control for ubiquitin binding NEMO was used as a positive control. **(C)** In order to identify ubiquitin binding domains of CYLD deletion mutants of CYLD were mixed and incubated with ubiquitin Sepharose as in A. **(D)** GST-CAPGLY domain of CYLD was expressed and purified from *E.coli* along with point mutants and coomassie stained. **(E)** As with previous ubiquitin binding experiments GST-CAPGLY fusion proteins were mixed with ubiquitin Sepharose and analyzed as in (A). **(F)** TRAF6 activation of NF-κB luciferase assay using full length CYLD WT, F518A, F533A, and C601S.

III.D. Discussion

In this report, the molecular mechanism of how CYLD negatively regulates NF-κB signaling was investigated using a biochemical approach. Previously, no direct evidence had been shown to explain the underlying mechanism that leads to inactivation of NF-κB signaling. I demonstrate that CYLD inhibits activation of TAK1 and IKK by TRAF6 using a cell free system *in vitro*. Furthermore, I provide direct evidence that CYLD harbors K63 specific endoprotease activity and preferentially binds K63 polyubiquitin chains. Ubiquitin binding studies also revealed the CAPGLY domain as a new, previously uncharacterized ubiquitin binding domain.

From these studies, several new conclusions can be made. In other studies, using overexpression of ubiquitin mutants had concluded that CYLD targeted K63 ubiquitin chains, but not until the data presented in this report has direct evidence been presented to support the conclusion that CYLD has K63 specific endoproteolytic activity (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). Using secondary structure predictions data it had been suggested the 3rd CAPGLY domain is

similar to the SH3 domain, which has been shown to bind ubiquitin, but no direct binding had been demonstrated until the data presented in this report (Saito et al., 2004).

My studies on CYLD have identified TAK1 as the point in the NF-κB signaling pathway that is negatively regulated. Presumably, TAK1 ubiquitination is modulated by CYLD, leading to its inactivation. During the course of these studies, it has been demonstrated in T-cells that CYLD inhibits the ubiquitination and autoactivation of TAK1 (Reiley et al., 2007).

CHAPTER FOUR
CYLD Negatively Regulates IRF3 Signaling

IV.A. INTRODUCTION

IFN α/β are cytokines that play a key role in establishing innate immunity, which help protect the cell from viral or bacterial infection (Taniguchi et al., 2001). IRF-3 is a critical transcription factor involved in the primary induction of type I IFN (Sen, 2001). IRF-3 is expressed ubiquitously in human tissues. When the cell is in a resting state, IRF-3 is held in the cytoplasm in an inactive state. Once the cell is challenged with viral pathogen, a signaling cascade is initiated, which involves the phosphorylation of IRF-3 leading to its dimerization and transport into the nucleus of the cell where it binds and enhances the transcription of a variety of target genes that contain IFN stimulation response element (ISRE). IRF-3 has been shown to be phosphorylated by two IKK-like kinases TBK1 and IKK-i/IKKε (Fitzgerald et al., 2003; Sharma et al., 2003). IRF-7 is another member of the IRF transcription factor family that is very similar to IRF-3 in sequence homology. It has been shown that IRF-7 homodimers bind to IFNα promoter and activate IFNα production in the cell. IRF-7 can also form heterodimers with IRF-3 to bind to IFNβ promoter and activate the transcription of IFNβ. Similar to IRF-3 activation, IRF-7 activation requires phosphorylation by TBK1 and IKK-i/IKKε.

In this report, I investigated the regulation of interferon signaling by CYLD. Currently, there are only two known negative regulators of NF-κB signaling, which are CYLD and A20. A20 is known to inhibit both IRF3 and NF-κB activation. Evidence from both knockdown and overexpression studies suggest CYLD is a negative regulator of interferon signaling. These results suggest CYLD is working at a step upstream of both

IKK and TBK. Previously, only A20 had been implicated in regulation of IRF-3 signaling.

IV.B. Materials and Methods

IV.B1. Materials and Plasmids

Antibodies against CYLD were a kind gift from Dr. Sun (UT Houston). Sendai virus (Cantell strain) was purchased from Charles River laboratories. Human embryonic kidney 293T cells were cultured and maintained in DMEM supplemented with 10% Cosmic Calf Serum (Hyclone) and 100μg/ml penicillin and 100μg/ml streptomycin. IFNβ-Luc reporter construct was generated by Dr. Giridhar Akkaraju and plasmids for Gal4-Luc, Gal4-IRF3, Flag-TBK1, and Flag-IKKi were kindly provided by Dr. Kate Fitzgerald (University of Massachusetts at Worcester) and Dr. Tom Maniatis (Harvard University).

IV.B2. RNA Interference and Luciferase Reporter Assays

Double-stranded RNA oligonucleotides were purchased from Dharmacon. The sense strand of RNAi oligos were as follows: CYLD (877- 898): GCU GAA GUG GUC GAU GAA A; GFP (471-489): GCA GAA GAA CGG CAU CAA G. RNA oligos were annealed and transfected at a final concentration of 20 nM. Cells were seeded in a 12 well plate at a cell density of 1.0-1.5 X 10^5 cells per well and transfected with siRNA oligos using the calcium phosphate method. On the second day, cells were transfected again with 40ng of each Luc-Reporter and pCMV-LacZ reporter constructs. 36 hours after transfection cellular extracts were collected for luciferase and β-Galactosidase assays. Luciferase assays for Gal4IRF3-luc, IRF3-luc, NF-κB-luc were performed in duplicate and results were normalized to co-transfected β-Galactosidase. GAL4-IRF3 is a chimeric

fusion construct consisting of IRF-3 fused to the DNA binding domain of Gal4, which drives the expression of the UAS-luciferase reporter gene. GAL4-IRF-3 activation mimics native IRF3 activation. Luciferase activity was measured with a FLUOstar OPTIMA luminometer (BMG LABTECH) using Promega's luciferase assay system. β-galactosidase activity was measured using a Thermo Labsystems microplate reader using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate.

IV.B3. Overexpression of TBK/IKKi and CYLD

Overexpression experiments with TBK/IKKi and CYLD were performed in 293T cells. 293T cells were cotransfected using the calcium phosphate method with 5 μg of either TBK/IKKi or CYLD. Cells were harvested 24hrs later by adding 500 μl of lysis buffer (20mM Tris pH7.5, NaCl 150mM, 10% glycerol, 0.5% NP-40) to a 10cm dish of confluent cells. 500 μg of lysates were incubated with 10μl M2 Sepharose beads and rotated at 4°C for 1hr. The beads were washed three times with lysis buffer. Proteins were eluted with 0.1 mg/ml flag peptide. CYLD phosphorylation was analyzed by immunoblotting extracts with anti-flag.

IV.B4. In vitro kinase assay with TBK/IKKi and CYLD

In vitro kinase assays were performed by mixing 10 ng of purified kinase (TBK/IKKi) with 1μg CYLD substrate in 10 μl kinase assay buffer (20mM HEPES pH7.6, 50mM NaCl, 20mM β-glycerophosphate, 1mM sodium orthovanadate, 10 mM MgCl2, 1mM DTT)., 100uM ATP, 5uCi γ-^{32}P ATP at 30°C for one hour. Reaction products were analyzed by SDS-PAGE and PhosphoImaging

IV.C. RESULTS

IV.C1. Over-expression of CYLD blocks activation of IRF3 and NF-κB

Upregulation of CYLD can be observed when HEK293 cells are either infected with Sendai, virus a positive strand RNA virus of the paramyxoviradae family, or transfected with RIGI (Figure 16A/B). To determine if CYLD could inhibit NF-κB and IRF3, a CYLD expression plasmid was transfected into HEK293 cells together with an IFN-β. reporter in which the expression of luciferase is under the control of the IFN-β promoter. The IFN-β. promoter contains enhancer elements that bind to several transcription factors including NF-κB, IRF3 and ATF2. All three of these transcription factors are required for the formation of an enhanceosome that activates IFN- β. Over-expression of CYLD in HEK293 cells inhibits IFN-β. luciferase (Figure 16C). As a control, the same cells were infected with Sendai virus, which activates IFN- β. Similarly, overexpression of the N-terminal CARD domains of RIG-I induced IFN-β. Surprisingly, overexpression of TBK a serine/threonine kinase that is responsible for phosphorylation of IRF3, was not inhibited by overexpression of CYLD, this suggests that CYLD is acting upstream of TBK or at the level of TBK. TBK could potentially regulate the activity of CYLD. CYLD also inhibited NF-κB (Figure 16D) luciferase. CYLD was also capable of inhibiting the activation of IRF3 by RIGI, but not TBK1, as determined with a GAL4-IRF-3 reporter construct (Figure 16E). Similarly, CYLD inhibited IFNα luciferase by overexpression of the N-terminal CARD domains of RIG-I, but once again not TBK (Figure 16F). Collectively, these results show that CYLD is an inhibitor of IRF3 and NF-κB.

IV.C2. CYLD is a negative regulator of antiviral signaling pathways and NF-κB

To determine if endogenous CYLD negatively regulates IFN-β induction by viruses, siRNA was used to silence the expression of endogenous CYLD. CYLD siRNA oligos as expected enhanced IFN-β induction by Sendai virus (Figure 16G). As controls, siRNA of GFP did not enhance viral induction of IFN-β. To determine if endogenous CYLD negatively regulates NF-κB induction by viruses, siRNA was used to silence the expression of endogenous CYLD. CYLD siRNA oligos, as expected, enhanced NF-κB induction by Sendai virus (Figure 16H). As controls, siRNA of GFP did not enhance viral induction of NF-κB.

IV.C3 Both TBK and IKKi phosphorylate CYLD

In order to take a closer look at why CYLD cannot inhibit IRF3 activation by TBK an overexpression experiment was performed. In Figure 17A CYLD was either overexpressed in 293T cells with either TBK or IKKi. As a negative control kinase dead mutants of either IKKi or TBK were used. In lanes 4 and 8 a clear mobility shift of CYLD is observed when cell extracts are immunoblotted with anti-flag. This mobility shift represents a modification of CYLD, which is presumably phosphorylation, since this mobility shift is not observed in lanes 5 and 9 which have kinase dead mutants. To determine if TBK and IKKi can directly phosphorylate CYLD an *in vitro* kinase assay was used. In figure 17B lanes 2 and 6 CYLD is phosphorylated by either TBK or IKKi. Again as a negative control kinase dead mutants of each TBK and IKKi did not phosphorylated CYLD.

Figure 16A

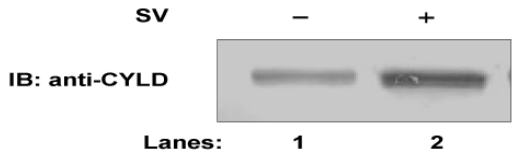


Figure 16B

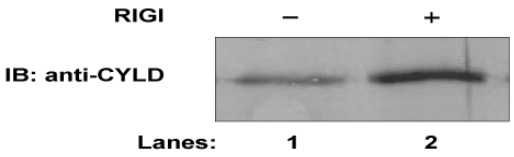


Figure 16C

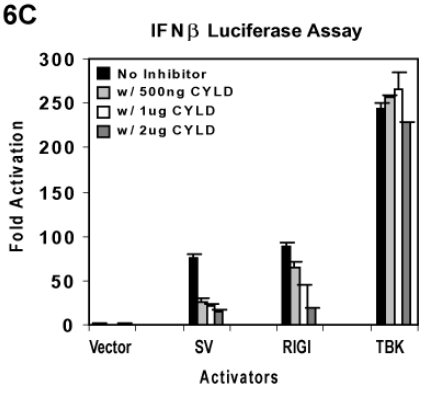


Figure 16D

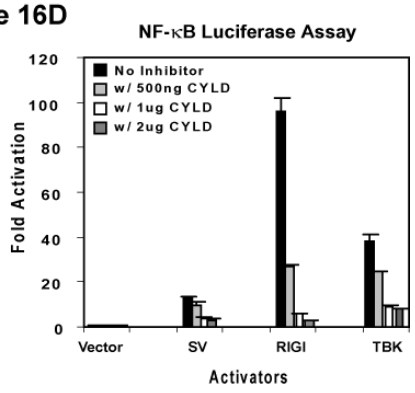


Figure 16E

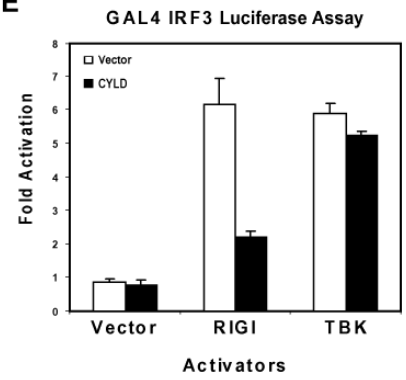


Figure 16F

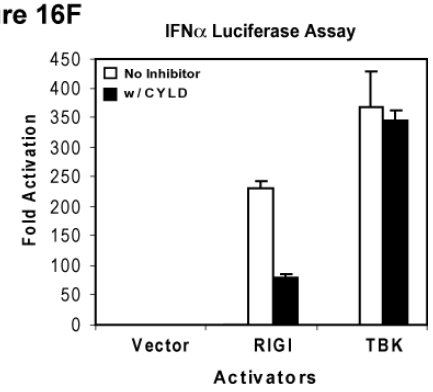


Figure 16G

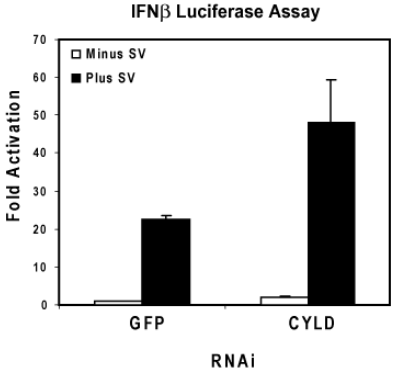


Figure 16H

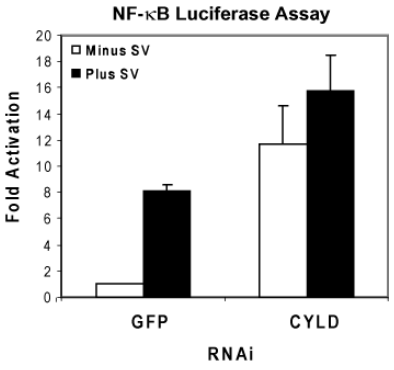


Figure 16. IRF-3 and NF-κB Luciferase Assays

Figure 17A

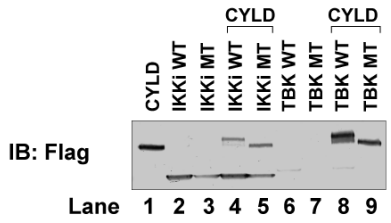


Figure 17B

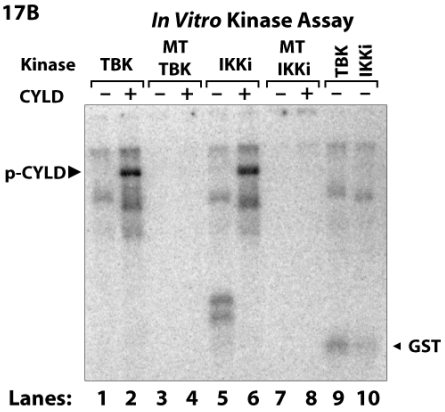


Figure 17C

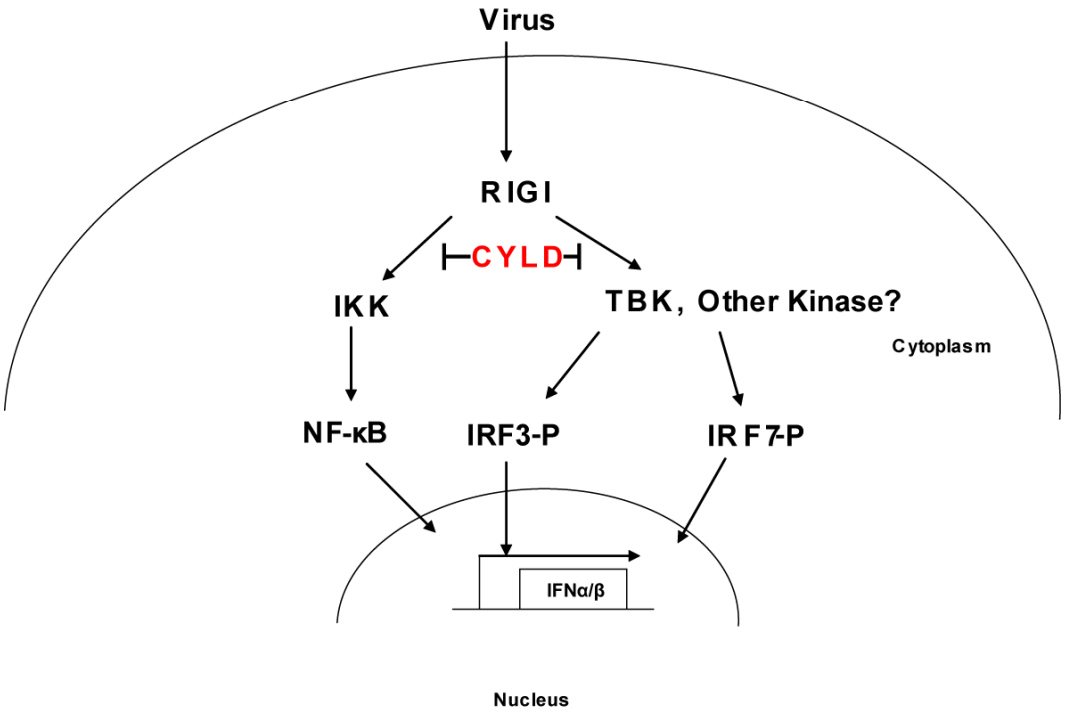


Figure 17. TBK/IKKi phosphorylates CYLD and Model of CYLD Inhibition of IFNα/β

(A) CYLD is phosphorylated in lanes 4 and 8 by both IKKi and TBK but not kinase dead mutants. **(B)** CYLD is directly phosphorylated by TBK and IKK in lanes 2 and 6 in an *in vitro* kinase assay. **(C)** The transcription factor IRF-3 is sequestered in the cytoplasm of unstimulated cells. When cells are stimulated IRF-3 is rapidly phosphorylated and forms dimers, which allow IRF-3 to be translocated into the nucleus with NF-κB where it turns on a variety of genes containing ISRE such as IFNα/β. CYLD inhibits this signaling cascade by acting upstream of both IKK and TBK.

IV.D. DISCUSSION

Previous studies have identified A20 as a negative regulator of IRF3 signaling (Saitoh et al., 2005). Mice deficient in A20 exhibit a hypersensitivity to LPS and TNFα treatment from the overactivation of NF-κB signaling. A20 inhibits the phosphorylation of IRF3 through its interaction with TBK.

In this study, CYLD was identified as an additional negative regulator of IRF3 signaling. Since CYLD is induced upon virus infection and activation of IRF3 signaling by RIGI, it may play a role in negative feedback regulation of IRF3 signaling. CYLD was shown to inhibit both IFNα/β promoter in luciferase studies using either SV or overexpression of RIGI as activators of IRF3 signaling. Surprisingly, CYLD did not inhibit IRF3 signaling that was activated by the over expression of TBK. From these results it can be concluded that CYLD is acting downstream of RIGI but upstream of TBK. Results from CYLD inhibition of TBK activation of NF-κB luciferase are still unclear. One possible explanation for the loss of CYLD inhibition of IRF3 activation by TBK could suggest TBK regulates CYLD activity. From both overexpression and in *vitro* kinase assays using TBK/IKKi and CYLD it can be concluded that CYLD is phosphorylated by TBK/IKKi. This modification of CYLD may inhibit its activity, presumably loss of deubiquitination activity. Although this possibility was not proven future experiments may reveal the physiological function behind the phosphorylation of CYLD by TBK/IKKi. One future experiment that could test this possibility would be to phosphorylate CYLD *in vitro* by TBK/IKKi and then test if CYLD can still cleave K63 ubiquitin chains or inhibit IRF3 activation.

It would be expected that deletion of a negative regulator in a particular pathway would lead to an enhancement of activity. This predicted result was observed in both studies using IFNβ or NF-κB luciferase studies when cells were stimulated with either SV or RIGI that had been transfected using siRNA targeting CYLD. One other result that supported the conclusion that CYLD is a negative regulator of IRF3 signaling came from using GAL4-IRF3 luciferase assay. This particular assay is composed of IRF3 fused to GAL4 DNA binding domain, which should mimic the activation of IRF3 signaling by monitoring the phosphorylation and dimerization of IRF3 by UAS-luciferase. Once again, activation of IRF3 signaling by the overexpression of RIGI was inhibited by CYLD, but TBK activation of IRF3 was not inhibited.

Although a particular target in the IRF3 signaling pathway was not directly identified from the studies presented in this chapter, there are several predictions that can be made from our understanding of how CYLD modulates cell signaling events. Since CYLD is known to target K63 specific ubiquitin chains, it is possible that an ubiquitination event is required for the activation of IRF3 signaling that has not been previously described. This ubiquitination event would presumably be the generation of K63 linked ubiquitin chains that are required for the activation of IRF3 signaling. Future detailed studies of how CYLD inhibits IRF3 signaling should lead to a greater understanding of how CYLD negatively regulates IRF3 signaling. Such studies could include cytokine measurements from CYLD knockout MEF cells that were stimulated with Sendai virus. Also it would be important to determine the phosphorylation status of endogenous IRF3 after stimulation.

CHAPTER FIVE
Conclusions and Future Directions

NF-κB signaling is involved in many key cellular processes such as inflammation, immunity, cell proliferation, and cell survival. The activation of NF-κB signaling by cytokines, viral pathogens, or UV lead to the transcription of many stress response genes in the cell. It has been clearly shown that ubiquitin protein has two distinct biological functions in NF-κB signaling. The first role is to target the inhibitor protein (IκBα) of NF-κB for proteasomal degradation once it has been ubiquitinated by K48 linked ubiquitin chains. The second role of ubiquitin functions in the activation of TAK1 and IKK by the formation of K63 linked ubiquitin chains.

In this dissertation, I show two examples of proteins that are involved in both ubiquitin related roles in NF-κB signaling. The first protein is NPL4, which is a ubiquitin binding protein that is involved in regulation of proteasomal degradation of IκBα. I show that the knock down of NPL4 by siRNA leads to the inhibition of IκBα degradation by TNFα activation of NF-κB signaling. In conjunction with siRNA studies, I utilized an *in vitro* degradation assay to show NPL4 was shown to be involved in IκBα degradation. NPL4 is typically found in a heterotrimeric protein complex composed of NPL4/VCP/UFD1. Also, the systematic knockdown of each component of the NPL4 complex does not affect IKK activation as shown by utilizing immunoprecipitation kinase assays. No effect on IKK activation was seen in an *in vitro* TRAF6 activation assay of IKK using cell extracts that had been generated from siRNA NPL4 transfected cells. Interestingly, this complex has already been shown to be involved in the ERAD

pathway, which is responsible for targeting proteins to the proteasome that are misfolded. VCP has been shown to associate with the proteasome and distinguish between native and non-native protein conformations. Its ATPase activity is required for its chaperone-like activity. UFD1 is a protein that was identified in ubiquitin degradation genetic screen and has been shown to bind ubiquitin through an N-terminus ubiquitin binding domain. Taken together, these data suggest the NPL4 complex act as a molecular chaperone to escort ubiquitinated IκBα to the proteasome for degradation.

The second protein is CYLD, which from my studies I conclude is a K63 specific deubiquitinating enzyme that negative regulates both NF-κB and IRF3 signaling. I demonstrate that CYLD has K63 specific endoproteolytic activity and targets NF-κB signaling at the level of TAK1. TAK1 activation by TRAF6 is inhibited by CYLD and requires both an intact UBP catalytic domain and 3rd CAPGLY domain to retain its inhibitory activity. From my studies I also demonstrate that the third CAPGLY domain from CYLD binds ubiquitin.

Deletions in CYLD which is considered a tumor suppressor leads to a disease called familial cylindromytosis. The manifestation of this disease is the formation of large benign skin tumors that form on the neck and face of the afflicted individual. Interestingly, mutations in many of the proteins involved in NF-κB signaling lead to human disease. For example, mutations in the NEMO the regulatory subunit of IKK leads to incontinentia pigmenti, anhidrotic ectodermal dysplasia (EDA), or EDA with immunodeficiency. Similar phenotypes are displayed with mutations in IRAK4 or IκBα. Typically these disease phenotypes exhibit some defect in immunity, inflammation, or cell proliferation. To date, no human pathologies have been linked to mutations in NPL4,

although members of the NPL4 complex have been linked to human disease. For example, mutations in VCP have been linked to inclusion body myopathy and patients with velocardiofacial syndrome have a chromosomal deletion which maps to the UFD1 locus. Mutations in proteins involved in ubiquitin signaling via kinase activation or proteasomal degradation could lead to human pathologies.

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VITAE

Gabriel Pineda was born in El Paso, Texas on September 27, 1975, the second son of Guadalupe and Robert Pineda. Immediately, following his graduation from J. M. Hanks High School in El Paso, Texas in 1994, he entered the University of Texas at Austin on a Student Success Scholarship. While completing coursework for both a Medical Technology and Microbiology degree he worked in the laboratory of Dr. James Holcombe in the Department of Analytical Chemistry, working on improving analyte transport between an ETV and ICP-MS. He received his Bachelor of Science degree in Microbiology in May 1998. Following graduation he moved to Beaumont, Texas to start his clinical training for Medical Technology at St. Elizabeth's Hospital to complete his requirements to sit for the national certification exam from the American Society of Clinical Pathologists (ASCP). In the summer 1999 following completion of the program he returned to El Paso, Texas to work full-time as a Medical Technologist at Providence Memorial Hospital in the laboratory sections of Hematology and Clinical Chemistry. He successfully passed the national ASCP exam in October 1999. In the spring of 2000 he entered the graduate school at the University of Texas at El Paso. He joined the lab of Dr. Eppie Rael in the Department of Biology and worked on the cloning and characterization of phospholipase A_2 genes from rattlesnakes. In the summer of 2001 he graduated with a Master of Science degree in Biology. He enrolled in the graduate program at the University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, in August 2001. During the winter break of 2001 he married Christina Uranga of El Paso, Texas. During the first summer of graduate school he was accepted to the

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