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Mechanisms of Non-canonical NF-kappaB Regulation

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Abstract

NF-kappaB is activated through two signaling cascades: the classical and non-canonical pathways, which are distinguished based on the Inhibitor of kappaB Kinase (IKK) components required to activate each pathway. Whereas classical NF-kappaB requires NF-kappaB Essential Modulator (NEMO) and IKKbeta, non-canonical NF-kappaB requires IKKalpha and upstream stabilization of NF-kappaB Inducing Kinase (NIK), but not NEMO. However, we have previously shown that IKKalpha contains a functional NEMO binding domain and associates with NEMO and IKKbeta as part of the heterotrimeric IKK complex. The overarching goal of the work described in this thesis was to determine whether the interaction between NEMO and IKKalpha was required for non-canonical NF-kappaB signaling. We used a reconstitution approach in knockout cells to show that IKKalpha, independent of its association with NEMO, is sufficient to control the non-canonical NF-kappaB pathway. Thus we provide biochemical evidence to support the tenet that IKKalpha alone regulates non-canonical NF-kappaB signaling. Importantly, while pursuing these results we made the surprising finding that NEMO, and furthermore, IKKbeta, play a compulsory role in the constraint of basal non-canonical NF-kappaB activity. These studies reveal that the previous model depicting a NEMO-independent non-canonical NF-kappaB signaling cascade is incomplete. We show that an intact and catalytically competent classical IKK complex constrains basal NIK accumulation. This novel mechanism of non-canonical NF-kappaB regulation requires classical NF-kappaB transcription driven by the prototypic NF-kappaB family member p65. Thus classical NF-kappaB activity is essential to maintain the quiescent state of the non-canonical NF-kappaB signaling pathway. Upon activation, the duration of non-canonical signaling is controlled by a recently described negative feedback mechanism that promotes NIK turnover. We used a combination of translation and proteasome inhibitors to show that turnover of active NIK is proteasome-dependent. Furthermore, we utilized a Smac mimetic compound to show that degradation of active NIK does not require cellular inhibitor of apoptosis (cIAP)1/2, which distinguishes this feedback mechanism from the cIAP1/2-dependent mechanism of basal NIK turnover. Collectively, these studies advance our understanding of a recently described mechanism of non-canonical NF-kappaB down-regulation and reveal a novel interplay between classical and non-canonical NF-kappaB activity, which revises the current model of non-canonical NF-kappaB signaling.

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MECHANISMS OF NON-CANONICAL NF-κB REGULATION

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Carolyn Margaret Gray

DEDICATION

This thesis is dedicated to my family.

Mom and Dad, you believed in me from the beginning and continue to have faith I will succeed in

all I undertake, no matter how ambitious the goal.

Thank you for your patience and support.

Stephanie, Christina, and John, thank you for being siblings I am proud to call friends. Your

strength and humor have carried me through the most difficult parts of this process.

Levi, you always find a way to make me smile. Thank you for being part of the adventure.

ABSTRACT

MECHANISMS OF NON-CANONICAL NF-κB REGULATION

Carolyn M. Gray

Michael J. May, Ph.D.

NF-κB is activated through two signaling cascades: the classical and non-canonical pathways, which are distinguished based on the Inhibitor of κB Kinase (IKK) components required to activate each pathway. Whereas classical NF-κB requires NF-κB Essential Modulator (NEMO) and IKKβ, non-canonical NF-κB requires IKKα and upstream stabilization of NF-κB Inducing Kinase (NIK), but not NEMO. However, we have previously shown that IKKα contains a functional NEMO binding domain and associates with NEMO and IKKβ as part of the heterotrimeric IKK complex. The overarching goal of the work described in this thesis was to determine whether the interaction between NEMO and IKKα was required for non-canonical NF-κB signaling. We used a reconstitution approach in knockout cells to show that IKKα, independent of its association with NEMO, is sufficient to control the non-canonical NF-κB pathway. Thus we provide biochemical evidence to support the tenet that IKKα alone regulates non-canonical NF-κB signaling. Importantly, while pursuing these results we made the surprising finding that NEMO, and furthermore, IKKβ, play a compulsory role in the constraint of basal non-canonical NF-κB activity. These studies reveal that the previous model depicting a NEMO-independent non-canonical NF-κB signaling cascade is incomplete. We show that an intact and catalytically competent classical IKK complex constrains basal NIK accumulation. This novel mechanism of non-canonical NF-κB regulation requires classical NF-κB transcription driven by the prototypic NF-κB family member p65. Thus classical NF-κB activity is essential to maintain the quiescent state of the non-canonical NF-κB signaling pathway. Upon activation, the duration of non-canonical signaling is controlled by a recently described negative feedback mechanism that promotes NIK turnover. We used a combination of translation and proteasome inhibitors to show that turnover of active NIK is

proteasome-dependent. Furthermore, we utilized a Smac mimetic compound to show that degradation of active NIK does not require cellular inhibitor of apoptosis (cIAP)1/2, which distinguishes this feedback mechanism from the cIAP1/2-dependent mechanism of basal NIK turnover. Collectively, these studies advance our understanding of a recently described mechanism of non-canonical NF-κB down-regulation and reveal a novel interplay between classical and non-canonical NF-κB activity, which revises the current model of non-canonical NF-κB signaling.

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Table 1. List of Abbreviations

aly	alymphoplasia
ANOVA	Analysis of Variance
APPL1	Adaptor Protein Containing Pleckstrin Homology Domain, Phosphotyrosine Dinding Domain, and Leucine Zipper Motif 1
BAFF	B cell Activating Factor
BAFF-R/BR3	B cell Activating Factor Receptor
β-TrCP	Skp1-Cul1-F-box (SCF) E3 Ubiquitin Ligase Complex Containing β-TrCP
BM	Bone Marrow
CHX	Cycloheximide
cIAP	Cellular Inhibitor of Apoptosis
CSR	Class Switch Recombination
DKO	IKKα/IKKβ Double Knockout
DLBCL	Diffuse Large B cell Lymphoma
DMSO	Dimethyl Sulfoxide
DUB	Deubiquitinating Enzyme
ECL	Enhanced Chemiluminescence
EMSA	Electrophoretic Mobility Shift Assay
FF	Firefly Luciferase
HUVEC	Human Umbilical Vein Endothelial Cell
HVEM	Herpes Simplex Virus Entry Mediator
IκB	Inhibitor of κB
IKK	Inhibitor of κB Kinase
IBM	IAP Binding Motif

KO	Knockout
IL-1	Interleukin - 1
IP	Immunoprecipitation
LIGHT/TNFSF14	Homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LPS	Lipopolysaccharide
LTβR	Lymphotoxin-beta Receptor
MALT	Mucosal Associated Lymphoid Tissue
MEF	Murine Embryonic Fibroblast
NBD	NEMO Binding Domain
NBDp	NBD peptide
NEMO	NF-κB Essential Modulator, or IKKγ
NEMO-ID	NEMO Immunodeficiency
NF-κB	Nuclear Factor kappa B
NIK	NF-κB Inducing Kinase
ODC	Ornithine Decarboxylase
PBMC	Peripheral Blood Mononuclear Cell
RANK	Receptor Activator of NF-κB
RHD	Rel Homology Domain
RL	Thymidine Kinase Renilla Luciferase
RLU	Relative Luciferase Units
RQ	Relative Quantitation
SCF	Skp1-Cul1-F-box
Smac	Second Mitochondria-derived Activator of Caspase
TAD	Transactivation Domain

TK	Thymidine Kinase
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TNFR	TNF Receptor
TRAF	TNF Receptor Associated Factor
TWEAK	TNF-related Weak Inducer of Apoptosis, or Fn14
WT	Wild Type

LIST OF ILLUSTRATIONS

CHAPTER 1. INTRODUCTION

        Activation of Nuclear Factor kappa B (NF-κB) [See Table 1 for common abbreviations] is essential for the development and maintenance of lymphocyte populations and to mount effective immune responses (Claudio, Brown et al. 2006; Hayden and Ghosh 2011). NF-κB is most often activated by extracellular stimuli such as pro-inflammatory cytokines but can also be activated by intrinsic signals of cellular stress (Hayden and Ghosh 2008). NF-κB signaling leads to changes in gene expression that are required for normal physiological processes such as cellular survival and differentiation, inflammation, and innate and adaptive immunity (Fig. 1). An important phase of NF-κB signal transduction is the down regulation of active signaling, and unconstrained NF-κB activity is associated with cancer and chronic inflammatory pathogenesis (Dejardin, Droin et al. 2002; Braunersreuther and Mach 2006; Dejardin 2006; Acharyya, Villalta et al. 2007; Volger, Fledderus et al. 2007; Kim, Kang et al. 2008). Thus since its discovery nearly thirty years ago, intense effort has been made to understand the molecular mechanisms involved in NF-κB activation and regulation. Furthermore, modulation of the NF-κB signaling pathway has become an attractive target for the development of drugs designed to treat cancer and inflammatory diseases.

        There are two modes of NF-κB signaling, the classical and non-canonical pathways, which will be discussed in detail below. Each of these pathways is activated by a distinct set of receptors and drives the expression of unique gene programs. This dissertation focuses on regulatory mechanisms of the non-canonical NF-κB signaling pathway, as dysregulated non-canonical NF-κB has been implicated in a growing number of diseases including multiple myeloma, Hodgkin lymphoma, pancreatic cancer, ovarian cancer, and rheumatoid arthritis (Annunziata, Davis et al. 2007; Keats, Fonseca et al. 2007; Jin, Zhou et al. 2009; Nishina, Yamaguchi et al. 2009; Wharry, Haines et al. 2009; Ranuncolo, Pittaluga et al. 2012; Noort, van Zoest et al. 2014). The studies presented here have unveiled a critical layer of cross talk between

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Inflammatory or antigenic
insult

$\downarrow$

NF-κB
activation

$\downarrow$

Pro-survival, pro-inflammatory,
and immunoregulatory
gene expression
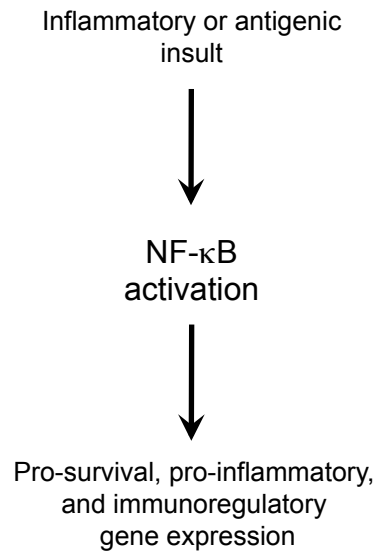
Figure 1. Inducible NF-κB signal transduction. Diverse cellular signals activate NF-κB. NF-κB signaling promotes the expression of genes involved in cellular survival, inflammation, immune regulation, and other physiological responses.

the classical and non-canonical NF-κB pathways. This previously unappreciated interplay will be important to consider in disease states affecting classical NF-κB (Orange and Geha 2003) and the development of novel therapeutics aimed at selectively targeting NF-κB signaling pathways.

1.1. NF-κB signal transduction

NF-κB is a family of transcription factors activated by a wide range of stimuli to drive the expression of numerous genes, including those involved in inflammation, cellular survival and proliferation, and lymphoid organogenesis (Claudio, Brown et al. 2006; Hayden and Ghosh 2011). NF-κB is ubiquitously expressed, but activation may be cell-type specific due to limited receptor expression. The NF-κB family contains five members: RelA (or p65), RelB, c-Rel, p50/p105 (or NF-κB1), and p52/p100 (or NF-κB2), which homo- and hetero-dimerize through their shared N-terminal Rel homology domains (RHD) to control inducible gene expression (Hayden and Ghosh 2008). p65, c-Rel, and RelB contain a C-terminal transactivation domain (TAD) through which they recruit transcriptional coactivators. Because they lack the TAD, p50 and p52 must bind to p65, c-Rel, or RelB to drive target gene transcription. Homodimers of p50 or p52 can thus only act as transcriptional repressors (Smale 2012).

Under resting conditions, NF-κB proteins are inactive due to their cytoplasmic localization. NF-κB proteins are sequestered in the cytoplasm through their association with an inhibitor of κB (IκB) protein. There are three classical IκBs: IκBα, IκBβ, IκBe, and three atypical IκBs: IκBz, Bcl3, and IκBns (Hinz, Arslan et al. 2012). Additionally, the precursor proteins p105 and p100 contain IκB-like regions that function to retain the unprocessed protein and associated NF-κB dimers in the cytoplasm (Savinova, Hoffmann et al. 2009). The proteasomal processing of p105 and p100 ceases at a glycine rich region, liberating the active transcription factors p50 and p52, respectively (Kanarek and Ben-Neriah 2012). Constitutive p105 processing to p50 is signal-independent but signal-induced activation leads to the complete degradation of p105 and

enhanced NF-κB activity (Heissmeyer, Krappmann et al. 2001; Kanarek and Ben-Neriah 2012). Conversely, receptor ligation is necessary for the induced processing of p100 to p52 to activate NF-κB (Xiao, Harhaj et al. 2001; Xiao, Fong et al. 2004).

In the simplest activation scheme, the stimulation-induced phosphorylation of the prototypical inhibitor IκBα promotes K48-linked polyubiquitylation of IκBα by the Skp1-Cul1-F-box (SCF) E3 ubiquitin ligase complex containing β-TrCP (hereafter referred to as β-TrCP) (Kanarek and Ben-Neriah 2012). Ubiquitylated IκBα is degraded via the 26S proteasome, and loss of IκBα reveals a nuclear localization sequence on p50/p65, thus permitting the nuclear translocation of the transcription factor pair. In the nucleus, NF-κB binds conserved κB sequences (5' GGGRNWYYC 3' where N, any base; R, purine; W, adenine or thymine; and Y, pyrimidine) in the promoters of genes involved in inflammation, proliferation, and cell survival (Hayden and Ghosh 2008; Hayden and Ghosh 2011). Promoter occupancy may be further regulated by accessibility and cofactor engagement, leading to context-specific gene signatures for particular transcription factor pairs (Smale 2012).

1.2. The IKK complex

A watershed discovery in NF-κB biology was the identification of the kinase that phosphorylates IκB. The Inhibitor of κB Kinase, or IKK, was isolated by multiple groups using various molecular and biochemical techniques and was shown to have *in vitro* kinase activity against IκBα after stimulation with the pro-inflammatory cytokines Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1) (DiDonato, Hayakawa et al. 1997; Mercurio, Zhu et al. 1997; Regnier, Song et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al. 1997; Rothwarf, Zandi et al. 1998). IKK is a heterotrimeric complex comprised of two catalytic subunits IKKα (or IKK1) and IKKβ (or IKK2), and a third non-catalytic regulatory subunit NF-κB Essential Modulator (NEMO, or IKKγ) (Fig. 2). IKKα and IKKβ are 85 kDa and 87 kDa, respectively, and share more than 50% sequence homology (Mercurio, Zhu et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al.

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1997). NEMO is a 45 kDa protein with numerous splice variants (Rothwarf, Zandi et al. 1998; Yamaoka, Courtois et al. 1998). IKKα and IKKβ associate with NEMO through a conserved six-amino acid sequence (LDWSWL) in their C termini (May, D'Acquisto et al. 2000; May, Marienfeld et al. 2002). The catalytic IKKs associate stoichiometrically with NEMO in a 1:1:2 ratio (Rothwarf, Zandi et al. 1998) and NEMO may oligomerize further, as the IKK complex has a final molecular weight of 600-900 kDa (DiDonato, Hayakawa et al. 1997; Woronicz, Gao et al. 1997; Rothwarf, Zandi et al. 1998).

Distinct receptor-proximal signaling events converge on the IKK complex, and activation of this complex is the precipitating event in NF-κB signal transduction (Hinz and Scheidereit 2014). Importantly, *in vivo* deletion of each IKK component has delineated two pathways to NF-κB activation, named classical (or canonical) and non-canonical (or alternative) NF-κB (Senftleben, Cao et al. 2001). These transduction cascades are activated through separate cell surface receptors and each controls the expression of a distinct panel of genes. The classical and non-canonical NF-κB pathways are discussed in detail in the next two sections.

1.3. Classical NF-κB signaling

Signaling through a multitude of receptors has been shown to activate classical NF-κB, including innate and adaptive immune receptors and cytokine receptors. Through diverse biochemical mechanisms, receptor-proximal signaling events converge to activate the IKK complex. Once active, the IKK complex phosphorylates the quintessential inhibitor IκBα on Ser-32 and Ser-36 (DiDonato, Mercurio et al. 1996); IκBβ and IκBϵ are also classical NF-κB inhibitors that are phosphorylated by activated IKK. β-TrCP catalyzes the formation of K48-linked ubiquitin chains on phosphorylated IκBα, which is thus targeted for degradation via the 26S proteasome (Kanarek and Ben-Neriah 2012). IκBα degradation releases p50/p65 complexes for nuclear translocation and target gene transcription (Fig. 3). While p50/p65 is the prototype, other NF-κB dimers are also sequestered by IκB and are thus activated by classical signaling (Smale 2012).

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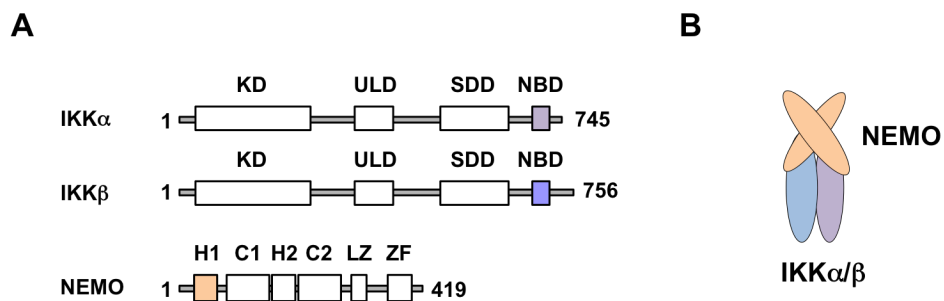
Figure 2. The IKK complex. (A) Domain structure of the IKK proteins. Highlighted are the NEMO binding domains (NBD) of IKKα and IKKβ, which are required for their association with the regulatory subunit NEMO. (KD, kinase domain; ULD, ubiquitin-like domain; SDD, scaffold/dimerization domain; H, α-helix; C, coil; LZ, leucine zipper; ZF, zinc finger). (Adapted from Hinz and Scheidereit, EMBO 2013) **(B)** Schematic representation of the heterotrimeric IKK complex. IKKα (purple oval) and IKKβ (blue oval) associate with NEMO (peach ovals) in a 1:1:2 ratio.

Because the classical signaling pathway only requires IκB degradation to release NF-κB dimers, activation is rapid, occurring within minutes of receptor ligation (Hayden and Ghosh 2008). Classical NF-κB-driven genes include those encoding pro-survival and pro-proliferative proteins (cyclin-D1, Bcl-xL, c-FLIP, IAPs), cell adhesion molecules (VCAM, ICAM, E-selectin), inflammatory cytokines (TNF, IL-1, IL-6), lymphocyte activation molecules (IL-2, B7, MHC), anti-microbial products (Defensins, COX-2), and complement proteins (Hayden and Ghosh 2011). Classical NF-κB activity also drives the transcription of important regulatory molecules to dampen the immune response. These negative regulators of NF-κB include the de-ubiquitinating enzyme (DUB) A20, and the inhibitory proteins IκBα and p100 (Dejardin, Droin et al. 2002; Ruland 2011).

Biochemical analyses of individual IKK proteins have revealed a central role for IKKβ and NEMO in classical NF-κB signal transduction. IKKβ isolated from TNF or IL-1 stimulated HeLa (Zandi, Rothwarf et al. 1997) or 293 (Woronicz, Gao et al. 1997) cells can phosphorylate IκBα and IκBβ *in vitro.* Mutations in either the activation loop (S177A, S181A) or catalytic domain (K44M) of IKKβ block TNF-induced IκBα phosphorylation, p65 nuclear localization, and transcriptional activity measured by a luciferase reporter in HeLa cells (Mercurio, Zhu et al. 1997). A similar inactivating mutation (K44A) of IKKβ was found to block TNF or IL-1 –induced luciferase reporter activity in 293 cells (Woronicz, Gao et al. 1997). Moreover, a mutant rat fibroblast cell line (5R) and murine pre-B cell line (1.3E2) that were defective in TNF, IL-1, or Lipopolysaccharide (LPS) –induced IκBα or IκBβ degradation and luciferase reporter activity could be rescued by complementation with a cDNA encoding NEMO (Yamaoka, Courtois et al. 1998). Finally, NEMO suppression by antisense oligonucleotides in 293 cells blocked TNF-induced IκBα degradation and decreased DNA binding of radiolabeled κB probes measured by Electrophoretic Mobility Shift Assay (EMSA) (Rothwarf, Zandi et al. 1998). Together these seminal studies have lead to the model that NEMO and the catalytic activity of IKKβ are

absolutely required to phosphorylate IκB downstream of classical NF-κB stimuli (Fig. 3).

Mice deficient in NEMO, IKKβ, or p65 die between embryonic day 10 and 15 due to massive hemorrhaging of the liver (Beg, Sha et al. 1995; Li, Van Antwerp et al. 1999; Li, Chu et al. 1999; Tanaka, Fuentes et al. 1999; Rudolph, Yeh et al. 2000). Lethality of each of these knockouts can be rescued by crossing them onto the TNF receptor (TNFR) null background, suggesting that TNF-induced hepatocyte apoptosis is the cause of death in animals that lack classical IKK signaling components (Gerondakis, Grumont et al. 2006). Subsequent studies have shown that IKKβ- and NEMO-mediated classical NF-κB signaling through p65 is absolutely required for pro-survival transcriptional programs downstream of the TNFR (Hayden and Ghosh 2008), providing *in vivo* evidence to support the central role for NEMO and IKKβ in classical NF-κB activation.

Mice lacking IKKα survive until birth, but die shortly thereafter due to severe defects in epidermal keratinocyte differentiation affecting limb development (Hu, Baud et al. 1999; Li, Lu et al. 1999; Takeda, Takeuchi et al. 1999). Interestingly, this epidermal role for IKKα does not require IKK activity or NF-κB activation (Hu, Baud et al. 2001). Though not exclusively required for IκBα degradation induced by most stimuli (Solt, Madge et al. 2007), IKKα is still part of the heterotrimeric IKK complex that regulates classical pathway activation (Fig. 2) (Hu, Baud et al. 1999; Li, Van Antwerp et al. 1999; Takeda, Takeuchi et al. 1999; Rudolph, Yeh et al. 2000; Muller and Siebenlist 2003; Solt, Madge et al. 2009). Furthermore, IKKα plays a nuclear role in Histone-H3 phosphorylation, which is critical for cytokine-induced gene expression (Anest, Hanson et al. 2003; Yamamoto, Verma et al. 2003). The unique phenotype of IKKα-deficient animals in conjunction with developmental defects in other mouse models revealed a second NF-κB signal transduction pathway, termed non-canonical NF-κB.
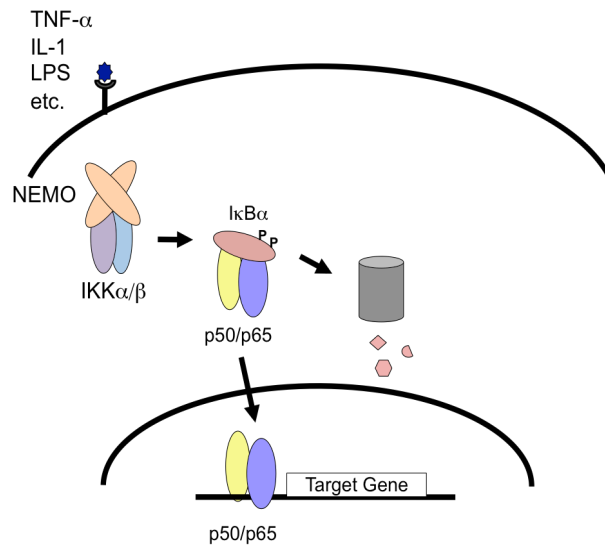
Figure 3. Classical NF-κB signaling. Ligation of pro-inflammatory cytokine receptors leads to IKK activation. IKKβ (blue oval) phosphorylates (P) IκBα (pink oval), which is degraded via the proteasome (gray cylinder). IκBα degradation permits the nuclear translocation of the prototypic p50/p65 NF-κB heterodimer (yellow and blue ovals).

1.4. Non-canonical NF-κB signaling

A collection of genetic evidence led to the discovery of the non-canonical NF-κB pathway. The naturally occurring *alymphoplasia* (*aly*) mutation in the gene encoding NF-κB Inducing Kinase (NIK, *Map3k14*) interferes with lymph node and Peyer's Patch development in mice (Shinkura, Kitada et al. 1999; Yamada, Mitani et al. 2000). This phenotype is similar to animals lacking p100, Lymphotoxin (LT)β, or the LTβ receptor (LTβR) (Koni, Sacca et al. 1997; Caamano, Rizzo et al. 1998; Franzoso, Carlson et al. 1998; Futterer, Mink et al. 1998; Rennert, James et al. 1998). The immunodeficiency in these animals is largely due to defects in B cell development (Franzoso, Carlson et al. 1998; Yamada, Mitani et al. 2000; Senftleben, Cao et al. 2001). Moreover, splenocytes isolated from *aly/aly* mice cannot be induced to process p100 *ex vivo* (Xiao, Harhaj et al. 2001; Claudio, Brown et al. 2002). NIK was discovered as a serine/threonine kinase that was activated downstream of the LTβR and was required for the phosphorylation and activation of IKKα (Malinin, Boldin et al. 1997; Matsushima, Kaisho et al. 2001). It was later shown to induce the regulated processing of p100 via IKKα (Xiao, Fong et al. 2004). Finally, bone marrow (BM) chimeras using BM from animals with an activation loop mutant version of IKKα (S176A, S180A) recapitulated the immunological defects in *aly/aly* mice: B cells developed but did not mature, and over-expression of NIK did not induce p100 processing in donor-derived lymphocytes that lacked functional IKKα (Senftleben, Cao et al. 2001). Thus non-canonical NF-κB signaling requires NIK and the subsequent activation of IKKα to phosphorylate p100, which is then ubiquitylated and processed to p52. Together with RelB, p52 translocates to the nucleus. p52/RelB drives the transcription of a panel of genes whose products are involved in lymphoid organization and lymphocyte development, including the homeostatic chemokines CXCL12, CXCL13, CCL19, CCL21, and B cell activating factor (BAFF) (Fig. 4) (Dejardin, Droin et al. 2002; Bonizzi, Bebien et al. 2004).

In addition to the LTβR, other members of the TNFR superfamily can activate the non-canonical NF-κB pathway including CD40, receptor activator of NF-κB (RANK), TNF-related weak inducer of apoptosis (TWEAK) receptor, or Fn14, and B cell activating factor receptor (BAFF-R) (Sun 2012). Signaling through BAFF-R is required for B cell development and maintenance (Hayden and Ghosh 2011). Early work showed that BAFF-R signaling induced p100 processing in splenic B cells, which was required for their maturation beyond the transitional stage in the spleen (Claudio, Brown et al. 2002). Unlike the rapid degradation of IκBα observed after classical NF-κB activation, which occurs within minutes of stimulation (Hayden and Ghosh 2008), p100 processing does not occur until 4-24 hours after receptor ligation (Claudio, Brown et al. 2002). Moreover, p100 processing is blocked by the translational inhibitor cycloheximide (CHX), indicating that protein synthesis is required for non-canonical NF-κB activation (Claudio, Brown et al. 2002; Muller and Siebenlist 2003; Liang, Zhang et al. 2006).

Unlike classical NF-κB, non-canonical signaling does not require NEMO and IKKβ, as p100 processing can be induced in NEMO-deficient pre-B cell lines (Claudio, Brown et al. 2002) and Murine Embryonic Fibroblasts (MEFs) lacking either NEMO or IKKβ (Senftleben, Cao et al. 2001; Dejardin, Droin et al. 2002; Saitoh, Nakano et al. 2002; Muller and Siebenlist 2003). Instead IKKα is the critical IKK component required for p100 phosphorylation and non-canonical NF-κB activity mediated by p52/RelB (Senftleben, Cao et al. 2001; Dejardin, Droin et al. 2002; Muller and Siebenlist 2003). Collectively, these genetic studies have led to a model for non-canonical NF-κB signaling whereby upon receptor ligation, NIK activates IKKα to phosphorylate p100 and promote its proteasomal processing to p52 (Fig. 4). In this scheme, IKKα is often depicted as a cytoplasmic homodimer, however this "IKKα-alone" complex has yet to be supported by definitive biochemical evidence (Hayden and Ghosh 2008; Razani and Cheng 2010; Sun 2012).
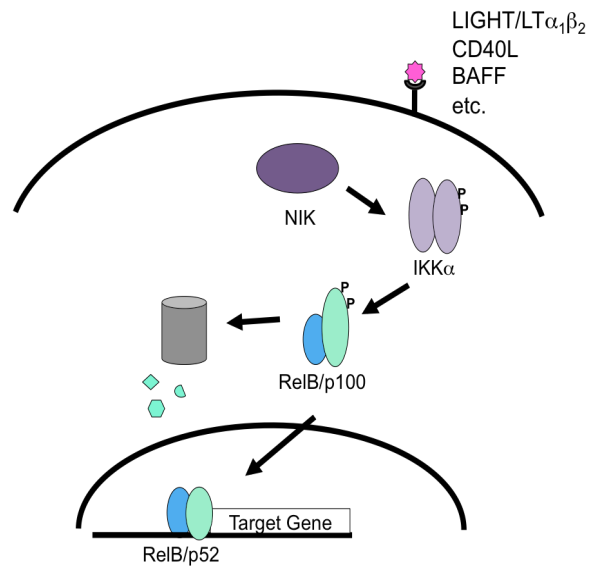
Figure 4. Non-canonical NF-κB signaling. Ligation of the LTβR, CD40, or BAFF-R promotes NIK stabilization (dark purple oval). NIK phosphorylates (P) IKKα (purple oval), which in turn phosphorylates the IκB region of the NF-κB precursor protein p100 (green oval). Phosphorylated p100 is ubiquitylated and partially processed by the proteasome to liberate p52/RelB (blue oval) NF-κB dimers.

1.5. NIK Regulation

The delayed kinetics of induced p100 processing and its dependence on *de novo* protein synthesis (Claudio, Brown et al. 2002; Liang, Zhang et al. 2006) coupled with the observation that exogenously expressed NIK is sufficient to induce p100 processing (Xiao, Harhaj et al. 2001) led to the key discovery that non-canonical NF-κB signaling required NIK stabilization upstream of IKKα activation (Liao, Zhang et al. 2004). Structural analysis of the active site has shown that NIK is a constitutively active kinase (Liu, Sudom et al. 2012); thus NIK protein levels are tightly controlled to maintain the latent state of non-canonical NF-κB.

In resting cells NIK is constitutively degraded through the coordinated activity of a complex comprised of TNFR Associated Factor (TRAF)3, TRAF2, cellular Inhibitor of Apoptosis (cIAP)1, and cIAP2 (Liao, Zhang et al. 2004; Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008). In this model, TRAF3 associates directly with NIK, and TRAF2 associates with the cIAPs. TRAF2 and TRAF3 interact, which promotes NIK ubiquitylation by the cIAPs (Fig. 5A). Ubiquitin-conjugated NIK is constitutively degraded by the 26S proteasome in a resting cell (Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008). Receptor ligation releases the negative regulation of the TRAF:cIAP complex on NIK, although whether this occurs by cIAP-mediated TRAF3 degradation or by competitive displacement of NIK by the ligated receptor is unclear and may be cell type or receptor-specific (Liao, Zhang et al. 2004; Vallabhapurapu, Matsuzawa et al. 2008; Sanjo, Zajonc et al. 2010). Nevertheless, once NIK is freed from the basal degradation complex, NIK protein amounts increase (Fig. 5B). Because NIK is a constitutively active kinase (Liu, Sudom et al. 2012), protein stabilization controls NIK activity. NIK phosphorylates IKKα on Ser-178 (Ling, Cao et al. 1998), which activates the kinase that in turn phosphorylates p100, triggering its partial processing to p52 and activation of the non-canonical NF-κB pathway (Fig. 5B).
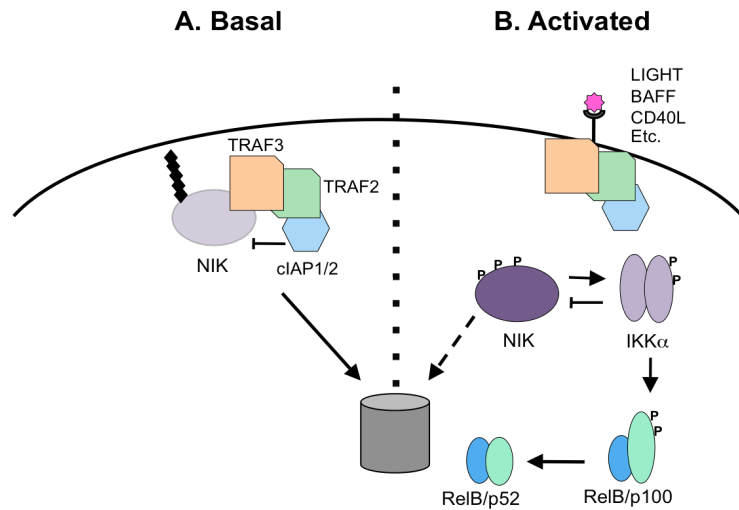
Figure 5. NIK regulation. (A) In resting cells, newly synthesized NIK (pale purple oval) associates with the TRAF3:TRAF2:cIAP complex. cIAP1/2 constitutively ubiquitylate NIK (black diamonds), leading to its proteasomal degradation. Thus NIK protein is undetectable in unstimulated cells and is depicted as a purple shadow. **(B)** Upon receptor ligation, the TRAF:cIAP complex localizes to the receptor, releasing NIK from its negative regulation. NIK protein stabilizes (dark purple oval), activating the non-canonical signaling pathway. Active IKKα phosphorylates (P) p100 and also feeds back to phosphorylate NIK, leading to the destabilization of NIK and down-regulation of non-canonical NF-κB activity. The dashed arrow indicates NIK turnover, but it has not been determined whether this occurs via the proteasome.

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More recently, a negative feedback loop has been described in the non-canonical NF-κB pathway (Razani, Zarnegar et al. 2010). In this model active IKKα phosphorylates p100 to induce non-canonical signaling but also feeds back to phosphorylate active NIK (Fig. 5B). Though the molecular details remain unclear, this phosphorylation event leads to NIK destabilization and has been suggested to promote the down-regulation of non-canonical NF-κB activity (Razani, Zarnegar et al. 2010). Furthermore, defects in NIK turnover have been shown to promote kidney nephropathy (Jin, Xiao et al. 2012) and gut associated lymphoid hyperplasia (Conze, Zhao et al. 2010; Hu, Brittain et al. 2013) in mice. Collectively, this evidence underlies the crucial role NIK regulation plays in the proper balance of non-canonical NF-κB activity.

1.6. Non-canonical NF-κB signaling in human disease

Due to its well-documented role in cell survival and proliferation, perturbations in classical NF-κB signaling have been associated with inflammatory pathogenesis and cancer (Staudt 2010; DiDonato, Mercurio et al. 2012; Lim, Yang et al. 2012). However, strong evidence has accumulated to implicate the non-canonical NF-κB pathway in certain lymphoid malignancies and chronic inflammatory diseases (Razani, Reichardt et al. 2011).

Genetic abnormalities have been shown to promote NIK stabilization and non-canonical NF-κB activation in human multiple myeloma, diffuse large B cell lymphoma (DLBCL), and mucosal associated lymphoid tissue (MALT) lymphoma (Annunziata, Davis et al. 2007; Keats, Fonseca et al. 2007; Compagno, Lim et al. 2009; Demchenko, Glebov et al. 2010; Pham, Fu et al. 2011). Diverse mutations associated with disease have been found both in proteins that promote basal NIK degradation and in NIK itself. Furthermore, unconstrained signaling through BAFF-R has been shown to activate the non-canonical signaling pathway to drive B cell growth and survival in DLBCL (Pham, Fu et al. 2011). Patient samples from Hodgkin lymphoma exhibit constitutive NIK stabilization and RelB transcriptional activity (Ranuncolo, Pittaluga et al. 2012). In

addition to lymphoid malignancies, aberrant NIK activity has been identified in Pancreatic (Nishina, Yamaguchi et al. 2009; Wharry, Haines et al. 2009) and Ovarian cancers (Uno, Saitoh et al. 2014).

Aberrant non-canonical NF-κB signaling has also been described in chronic inflammatory and autoimmune diseases. Our group has shown non-canonical NF-κB activation in human endothelium in response to LTβR ligation (Madge, Kluger et al. 2008). Non-canonical NF-κB inducing stimuli, including LTαβ, LIGHT (TNFSF14), and CD40L are expressed in atherosclerotic lesions (Volger, Fledderus et al. 2007; Kim, Kang et al. 2008; Grabner, Lotzer et al. 2009). Human plaque endothelial cells exhibit nuclear p100/p52 (Rossi, Marziliano et al. 2004; Volger, Fledderus et al. 2007), and non-canonical regulated genes, including those expressed in endothelial cells, are expressed in chronic inflammatory lesions (Reape, Rayner et al. 1999; Braunersreuther, Mach et al. 2007; Damas, Smith et al. 2007). The non-canonical NF-κB gene target CXCL13 is expressed in lymphoid aggregates of patients with Sjogren's syndrome (Salomonsson, Larsson et al. 2002). Finally, NIK is highly expressed in synovial tissue from patients with rheumatoid arthritis and is thought to drive tertiary lymphoid structure formation in this chronic inflammatory disease (Noort, van Zoest et al. 2014). These diverse pathological roles for NIK in human disease underpin the importance of elucidating the biochemical mechanisms underlying non-canonical NF-κB activation and down-regulation, as they may ultimately reveal novel therapeutic targets.

1.7. Goals and Hypotheses

Mounting evidence implicates aberrant non-canonical NF-κB signaling in disease progression. Understanding the biochemical and molecular requirements for precise non-canonical NF-κB regulation will reveal critical insight for the design of novel therapeutics selectively targeting this pathway. Therefore the overarching goal of this thesis is to dissect the

biochemical and molecular mechanisms governing non-canonical NF-κB activation and down-regulation.

Our lab has a longstanding interest in understanding the composition and functional regulation of the IKK complex (May, D'Acquisto et al. 2000; May, Marienfeld et al. 2002; Solt, Madge et al. 2007; Solt, Madge et al. 2009). We have identified the residues through which IKKβ and IKKα associate with NEMO to form the heterotrimeric IKK complex (May, D'Acquisto et al. 2000; May, Marienfeld et al. 2002), and we described a cell permeable NEMO Binding Domain (NBD) peptide spanning this region that potently blocks signal-induced classical NF-κB activity (May, D'Acquisto et al. 2000). Moreover, this peptide can disrupt pre-formed IKK complexes, suggesting that it competes with both IKKα and IKKβ to bind NEMO (May, D'Acquisto et al. 2000). Genetic studies in cells lacking individual IKK components indicate that IKKβ is the exclusive kinase regulated by NEMO for classical NF-κB signaling and suggest that IKKα is redundant in this pathway (Hayden and Ghosh 2008). However, we have shown that IKKα contains a functional NEMO-binding domain (NBD) (Fig. 2), through which the association between IKKα and NEMO is critical for IL-1 mediated classical NF-κB activity (Solt, Madge et al. 2007; Solt, Madge et al. 2009). Therefore the paradigm based on the original genetic studies delineating two pathways to NF-κB activation may be incomplete.

The model for non-canonical NF-κB signaling indicates that IKKα involved in non-canonical signaling as a cytoplasmic homodimer (Fig. 4). However, we currently lack any biochemical evidence to support this "IKKα-alone" scheme. An additional void in our understanding of non-canonical NF-κB activation is how NIK specifically targets IKKα for phosphorylation. As we have previously shown that IKKα contains a functional NBD that directs its assembly into the heterotrimeric complex and promotes classical NF-κB activation by IL-1 (Solt, Madge et al. 2007; Solt, Madge et al. 2009), we surmise that the IKKα involved in non-

canonical NF-κB activation is also associated with NEMO. Specifically, we hypothesized that the association between NEMO and IKKα is necessary to regulate non-canonical NF-κB signaling.

To ask whether the NEMO:IKKα association was important for the induction of non-canonical NF-κB, we created a cell line in which IKKα lacked the NBD and thus cannot associate with NEMO and IKKβ as part of the heterotrimeric IKK complex. Non-canonical NF-κB activation occurs with normal kinetics in these cells, and we definitively show that the NEMO:IKKα association is dispensable for IKKα-mediated turnover of active NIK (Chapter 3). These studies therefore support the current model that NEMO is not required for inducible non-canonical NF-κB signaling. However in parallel experiments using NEMO-deficient cells, we identified a role for NEMO in the negative regulation of non-canonical NF-κB signaling via NIK protein amounts (Chapter 3). We show that in addition to NEMO, IKKβ is required for the basal constraint of NIK (Chapter 4). This unexpected regulatory mechanism requires a signal-competent IKK complex and transcriptional activity mediated by the prototypical NF-κB family member p65 to maintain the quiescent state of the non-canonical NF-κB pathway (Chapter 4). Importantly, our studies provide compelling evidence that this novel interplay between the classical and non-canonical NF-κB pathways occurs in patients with hypomorphic mutations in the NEMO protein (Chapter 4) (Gray, Remouchamps et al. 2014).

Our overarching goal is to understand molecular mechanisms of non-canonical NF-κB regulation. A negative feedback loop to control the duration of non-canonical signaling was recently described that implicates IKKα in the negative turnover of active NIK (Fig. 5B) (Razani, Zarnegar et al. 2010), which we show does not require the association of IKKα with NEMO (Chapter 3). However, this newly proposed model is poorly defined and it remains to be determined how phosphorylated NIK is degraded and which other cellular factors facilitate this process. We hypothesized that like basal NIK, active NIK is degraded via cIAP1/2-dependent proteasomal degradation. In chapter 5, we show that active NIK turnover is indeed proteasome-

dependent but does not require cIAP1/2. These findings therefore delineate the molecular requirements for basal NIK degradation from those required for the turnover of stimulation-induced NIK.

This thesis describes novel regulatory mechanisms of basal and active non-canonical NF-κB signaling. The studies presented here expand our understanding of a new mechanism that controls the duration of non-canonical NF-κB activity as well as revise our former understanding of basal NIK regulation and the interplay between the classical and non-canonical NF-κB signaling pathways. Ultimately, cross talk between classical and non-canonical NF-κB signaling will be critical to consider when manipulating these pathways to treat NF-κB-associated pathologies.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell Lines and Culture

3T3-NIH Mouse Embryonic Fibroblasts and Hela cells were purchased from ATCC (Manassas, VA). The 3T8 Jurkat T cell line has been described elsewhere (He and Ting 2002) and had been previously mutagenized to create the NEMO^{KO} cell line 8321 (He and Ting 2002). These 8321 cells were reconstituted with full-length NEMO to create 8321^{WT} cells in earlier studies (Hanson, Monaco-Shawver et al. 2008). TRAF3^{KO}, p65^{KO}, and p65^{WT} MEFs were kindly provided by Emmanuel Dejardin (University of Liège, Belgium).

All fibroblasts and Plat-E cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 2mM L-glutamine, penicillin (50 units/mL) and streptomycin (50 units/mL). Jurkat T cells were cultured in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, penicillin (50 units/mL) and streptomycin (50 units/mL). Cells were passaged with 0.25% Trypsin (Invitrogen). Unless otherwise noted, cells were stimulated with TNF (10ng/mL), LIGHT (100ng/mL), anti-LTβR (300ng/mL), or Smac mimetic (1μg/mL) when they reached 80% confluence.

2.2. Human Umbilical Vein Endothelial Cell (HUVEC) Isolation

HUVECs were isolated from discarded tissue in accordance with a protocol approved by the University of Pennsylvania Institutional Review Board. Following collagenase digestion (1 mg/ml in PBS) from the canulated umbilical vein, endothelial cells were serially cultured on gelatin-coated tissue culture plastic in Vasculife VEGF-MV complete media supplemented with penicillin (50 units/mL) and streptomycin (50 units/mL). All experiments were performed on cells between passages 2-3.

2.3. Patient Samples

Patient peripheral blood mononuclear cells (PBMCs) were obtained with parental consent and with approval by the Children's Hospital Committee on Clinical Investigation, Children's Hospital Boston, as described elsewhere (Orange, Jain et al. 2004). Clinical presentations and immunological findings from these patients were previously reported (Orange, Jain et al. 2004; Hanson, Monaco-Shawver et al. 2008).

2.4. Antibodies and Reagents

Antibodies against NIK (4994S), Histone-H3 (9715S), phospho-IKKα/β (2697S) and mouse-p100/p52 (4882S) were purchased from Cell Signaling Technology (Beverly, MA). Anti-human p100/p52 (05-361) was from Millipore (Billerica, MA). Anti-NEMO antibody (K0159-3) was purchased from MBL International (Woburn, MA). Anti-IKKα (IMG-136A) and anti-IKKβ (IMG-129A) antibodies were purchased from Imgenex (San Diego, CA). Monoclonal anti-cIAP1 antibody (mAb 1E1-10) was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-NEMO antibody was purchased from MBL International (Woburn, MA). Antibodies against NEMO (sc-8330), IκBα (sc-371), IKKα (sc-7218), TRAF3 (sc-947), TRAF2 (sc-876), cIAP-2 (D19), and TBK1 (sc-52957) were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-cIAP2 (AF8171) and pan-cIAP1/2 (Mab 3400) antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-α-Tubulin (T5168) was from Sigma-Aldrich (St. Louis, MO). Horseradish Peroxidase-conjugated secondary antibodies and normal rabbit serum were from Jackson Immuno Research (West Grove, PA). Fluorescently labeled secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Agonistic antibody against the LTβR (clone 5G11) was purchased from Abcam (Cambridge, MA).

Cycloheximide was purchased from Calbiochem (239764). Protein λ-phosphatase (NEB P0753S) was purchased from New England Biosciences (Ipswich, MA). Recombinant human LIGHT (TNFSF14) and TNF-α were purchased from R&D Systems (Minneapolis, MN). Protein-G

agarose beads, and MG132 (10μM) were from Sigma-Aldrich (St. Louis, MO). Fugene-6 (E-269A) and the dual luciferase reporter assay kit (E1980) were purchased from Promega (Madison, WI). Fast-digest restriction enzymes were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Bortezomib was a generous gift from Katherine A. High, M.D. (The Perelman School of Medicine at The University of Pennsylvania). The active Smac mimetic GT13072 and the inactive Smac mimetic GT13199 were generously provided by TetraLogic Pharmaceuticals (Malvern, PA).

2.5. Generation of Stable Cell Lines

All cloning procedures were performed using the *Pfu* Turbo PCR procedure as previously described (Solt, Madge et al. 2009). Briefly, cDNAs encoding full length (WT) and ΔNBD (1-734 lacking the NEMO-binding domain) IKKα were subcloned into the EcoR1 and Xho1 restriction sites of the Super MigR1 vector (kindly provided by Warren Pear, The University of Pennsylvania). Platinum-E cells were transfected with the appropriate vector using Fugene-6 with a 3:1 ratio of Fugene:DNA, according to the manufacturer's instructions. Twenty-four and 48 hours later, retroviral supernatants were collected and filtered through sterile cell strainers (40μm). If not used immediately, retrovirus was stored at -80°C. IKKα^{KO} MEFs were transduced with retroviral supernatants containing 8μg/mL Polybrene. After two rounds of transduction, IKKα^{WT} and IKKα^{ΔNBD} cells were FACS sorted at the Flow Cytometry and Cell Sorting Resource Laboratory at The University of Pennsylvania on a BD FACSVantage with DiVa option sorter based on GFP expression. IKKα protein expression was confirmed by immunoblot.

Complimentary DNAs encoding full length (NEMO^{WT}), NEMO 86-419 (NEMO^{ΔNBD}), and WT IKKβ were subcloned into the EcoR1 and Xba restriction sites of the Super MigR1 vector, and Platinum-E cells were transfected to create retroviral supernatants as described above. NEMO^{KO} MEFs were retrovirally transduced and FACS sorted to create stable NEMO^{WT}, NEMO$^{86-419}$, or NEMO$^{KO-IKKβ}$ cell lines as described above.

Complimentary DNAs containing full-length or Kinase dead (K44M) IKKβ were ligated

into the HindIII and Not1 restriction sites of LZRS-pBMN-lacZ retroviral vector (kindly provided by

Dr. Garry Nolan, Stanford University, La Jolla, CA). Pheonix cells were transfected as described

above. Vector-containing cells were selected using Puromycin (8 μg/mL) 24 hours after

transfection. Conditioned medium from Puromycin-resistant cells was used to transduce IKKβ^{KO}

MEFs and create stable IKKβ^{WT} or IKKβ^{K44M} cell lines.

2.6. Immunoblotting and Immunoprecipitation

Cells were harvested on ice in a lysis buffer consisting of 150mM NaCl, 50mM TrisCl pH

7.5, 1% Triton X-100, and complete protease inhibitors (Roche). Proteins were separated by

SDS-PAGE in Tris-Glycine running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) and

transferred onto PVDF membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20%

(v/v) methanol). Membranes were blocked in 5% non-fat milk and incubated with primary

antibodies overnight at 4°C with gentle rocking. Membranes were washed three times in TBS-T

(25 mM Tris–HCl, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20) and incubated with

secondary antibody for one hour at room temprature. Membranes were washed three times with

TBS-T and developed using enhanced chemiluminescence (ECL). ECL was made by mixing

equal volumes of solution 1 and solution 2. (Solution 1: 200 μL 250 mM luminol (in DMSO), 88 μL

90 mM p-coumaric acid (in DMSO), 2 mL 1M Tris-HCl pH 8.5, 17.7 mL dH$_2$O. Solution 2: 12 μL

30% H$_2$O$_2$ (v/v), 2 mL Tris-HCl pH 8.5, 18 mL dH$_2$O). Unless otherwise noted, the immunoblots

presented are one of three representative experiments.

For immunoprecipitations, 500 μg total protein was cleared using normal rabbit serum.

Lysates were then incubated with 1ug of indicated antibody and Protein-G agarose beads for 12

hours at 4°C under rotary agitation. Beads were washed four times in 0.1% TNT (150 mM NaCl,

50 mM TrisCl pH 7.5, 0.1% Triton X-100), boiled in reducing sample buffer (5x; 0.3 M TrisCl pH

6.8, 5% SDS, 50% Glycerol, 100 mM DTT), and samples were divided onto two SDS-PAGE gels for immunoblot as described above.

2.7. Luciferase Reporter Assay

Cells cultured in 12-well plates were transiently transfected using Fugene 6 according to the manufacturer's protocol. Cells were transfected with a total of 500 ng DNA per well comprised of the NF-κB-dependent pBIIX-Firefly luciferase reporter construct (FF, 450 ng/well) and the Thymidine Kinase (TK)-dependent Renilla Luciferase reporter (RL, 50ng/well). Twenty-four hours after transfection, cells were treated with TNF (10 ng/mL) for five hours and lysed in 1X Passive Lysis Buffer (Promega) for 15 minutes at room temperature. Samples were run in quadruplicate using a Luminoscan 96-well automated luminometer (Thermo LabSystems, Franklin, MA), and FF:RL ratios were calculated using Ascent software (Thermo LabSystems). Means are from at least three independent experiments and P-values were calculated using a student's two-tailed t-test or one-way ANOVA in Prism software.

2.8. Preparation of Nuclear and Cytosolic Extracts

Cells were scraped into PBS at 4°C and pelleted (425 x g for 10 min). Pellets were resuspended and swollen for 10 min on ice in 100μl Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 2 mM NaF, 2 mM β-glycerophosphate, and complete mini protease inhibitors) plus 0.1% Nonidet P-40 and centrifuged (3800 x g) for 1 min. Supernatants (cytoplasmic fraction) were snap frozen and retained. Pelleted nuclei were washed four times with Buffer A plus 0.1% Nonidet P-40 before being resuspended in Buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 2 mM NaF, 2 mM β-glycerophosphate, and complete mini protease inhibitors) and vortexed on high at 4°C for 60 minutes. Supernatants were centrifuged (14000 RPM) for twenty minutes at 4°C. Nuclear lysates were either used immediately or snap frozen and stored at -80°C. Lysates were immunoblotted as described above.

2.9. Electrophoretic Mobility Shift Assay (EMSA)

Single stranded complimentary oligonucleotides encompassing a consensus NF-κB site (upper strand, 5'-AGTTGAGGGGACTTTCCCAGGC-3') were annealed and labeled with [γ−^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled probe was then purified using the mini-Quick Spin columns (Roche Applied Science) according to the manufacturer's protocol. Nuclear extracts were prepared as described in section 2.8. 5 µg of lysate supplemented with 1 µg of poly (dI:dC) (Roche Applied Science) was incubated with an equal volume of 2X binding buffer (40 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM MgCl$_2$, 2 mM EDTA, 10% glycerol, 0.2% NP-40, 2 mM DTT, 100 µg/ml BSA) on ice for 10 minutes. After the incubation, 1 µl of labeled probe was added and then incubated at room temperature for 20 minutes. The resulting DNA:protein complexes were separated on 5% polyacrylamide non-denaturing gels by electrophoresis. The gels were then dried and visualized by autoradiography.

2.10. NBD peptide

NBD peptides (NBDp) were obtained from the Howard Hughes Medical Institute Biopolymer-Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). Immediately prior to use, the peptides were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 200 mM. The sequences of the WT and mutant NBD peptides (NBDp and mutNBDp, respectively) have been described previously (May, D'Acquisto et al. 2000). Peptides were used at a final concentration of 100 µM.

2.11. mRNA isolation and Quantitative Real Time PCR analysis

RNA was isolated using the RNase Easy Spin Kit (Qiagen) according to the manufacturer's instructions. Samples were subject to on-column DNase digestion. Semi-quantitative amplification of target genes was performed using Power SYBR Green (Applied Biosystems) according to the manufacturer's instructions. PCR products were generated in quadruplicate and normalized to β-actin (*actb*) using the ABI 7500 Real-Time PCR system.

Relative quantitation (RQ) was derived from the difference in cycle threshold (Ct) between the gene of interest and β-actin using the equation RQ = 2-$\Delta\Delta$Ct and analyzed using SDS v1.3 software. PCR product specificity was confirmed by performing a dissociation curve with each experiment. The *map3k14* QuantiTect Primer Assay was purchased from Qiagen; the *actb* primer pair (5'-ACC CAC ACT GTG CCC ATC TA-3', 3'-ATC GGA ACC GCT CGT TGC-5'), *p100* primer pair (5'-CAG AAA CTT CAG AGG CAG CGT-3' and 5'-GCA AAT AAA CTT CGT CTC CAC CG-3') and *birc3* primer pair (5'-CTG GCC AAA GCA GGC TTC TAC TAC-3', 3'-CAC GCT ACC CTT TGA CTC GTT GAC-5') were from Integrated DNA Technologies.

2.12. Protein quantification and statistical analyses

Western blots were quantified with background subtraction using the LI-COR Odyssey imaging system or ImageJ software (National Institutes of Health). For each method the intensity of the band of interest was normalized to its corresponding control band (α-Tubulin, β-Actin, or Histone-H3). Each value was normalized back to the unstimulated (WT) control and relative arbitrary units are shown. Statistical analyses for at least three independent experiments were performed using Prism software. A student's two-tailed t-test or one-way ANOVA with Dunnett's post-test to the WT control were performed for all data sets with a significance cutoff of $P < 0.05$.

CHAPTER 3. IKK REQUIREMENTS FOR NON-CANONICAL NF-κB REGULATION

Portions of this chapter have previously been published as: Gray CM, Remouchamps C, McCorkell KA, Solt LA, Dejardin E, Orange JS, and May MJ. Non-canonical NF-κB signaling is limited by classical IKK activity. *Sci Signal*. **7**, ra13(2014).

3.1. Introduction

Diverse upstream signals converge on the IKK complex to activate NF-κB pathways. Analyses of NF-κB signaling in MEFs lacking individual components of the IKK complex have established that inducible p100 processing can occur in the absence of either NEMO or IKKβ (Senftleben, Cao et al. 2001; Dejardin, Droin et al. 2002; Derudder, Dejardin et al. 2003). Thus it has been concluded that IKKα alone is required for regulation of the non-canonical pathway and that the major IKK complex responsible for NIK-induced activation is an IKKα homodimer (Fig. 4). Despite this model, very little biochemical evidence exists to support a definitive role for an "IKKα-alone" complex in the non-canonical NF-κB pathway. Furthermore, our previous work established that IKKα contains a functional NBD (Fig. 2), suggesting that NEMO may associate with IKKα homodimers (Solt, Madge et al. 2007; Solt, Madge et al. 2009). We therefore sought to determine whether the association of NEMO with IKKα played any role in regulating the non-canonical NF-κB pathway. We hypothesized that the IKKα:NEMO association was necessary for non-canonical NF-κB activation and down-regulation mediated by IKKα.

In this chapter, we utilized cell lines lacking IKKα or NEMO to determine whether the association between these key signaling mediators was important for p100 processing and NIK turnover. We show here that a mutant version of IKKα that cannot associate with NEMO is sufficient for non-canonical NF-κB activation and NIK turnover. Thus we provide biochemical evidence to support the current model that IKKα alone is required for non-canonical NF-κB signaling. However, parallel studies led us to the surprising finding that the classical IKK complex

is critical for the negative regulation of non-canonical NF-κB signaling through the basal stability of NIK.

3.2. Results

3.2.1. Association of IKKα with NEMO is dispensable for non-canonical NF-κB activation

Upon LTβR cross-linking, IKKα is required to phosphorylate p100, leading to its proteasomal processing to p52 and, ultimately, non-canonical NF-κB-dependent gene expression (Dejardin, Droin et al. 2002). Despite its conserved NBD (May, D'Acquisto et al. 2000; Solt, Madge et al. 2009), genetic evidence has implicated IKKα homodimers in non-canonical NF-κB activity (Sun 2012). To determine whether the association of NEMO with IKKα specifically plays a role in the induction of non-canonical NF-κB signaling, we stably reconstituted IKKα^{KO} MEFs with either MigR1 vector alone, wild-type IKKα (IKKα^{WT}), or a version of IKKα that lacks the C-terminal NBD (IKKα$^{\Delta NBD}$) (Fig. 6A) (Solt, Madge et al. 2009). IKKα$^{\Delta NBD}$ retains the ability to interact with IKKβ but is unable to interact with NEMO specifically (Fig. 6B). This cell line allows us to specifically test, at the molecular level, whether the association of NEMO and IKKα is required for non-canonical NF-κB signaling.

We assessed the ability of IKKα-reconstituted cells to induce p100 processing after stimulation with LIGHT. Stimulation of WT MEFs with LIGHT led to transient stabilization of NIK and p100 processing to p52 (Fig. 7, lanes 1-7), indicating non-canonical NF-κB activity. Basal NIK was detected in un-stimulated MigR1-transduced IKKα^{KO} cells and was further stabilized in response to LIGHT (Fig. 7, lanes 8-14). This observation is consistent with a role for IKKα in the destabilization of active NIK and has been described previously (Razani, Zarnegar et al. 2010). Notably, reconstitution of IKKα^{KO} cells with either IKKα^{WT} or IKKα$^{\Delta NBD}$ restored undetectable basal levels of NIK. In addition, stimulation of both IKKα^{WT} and IKKα$^{\Delta NBD}$ MEFs with LIGHT induced NIK stabilization and p100 processing with kinetics similar to untransduced WT cells (Fig.
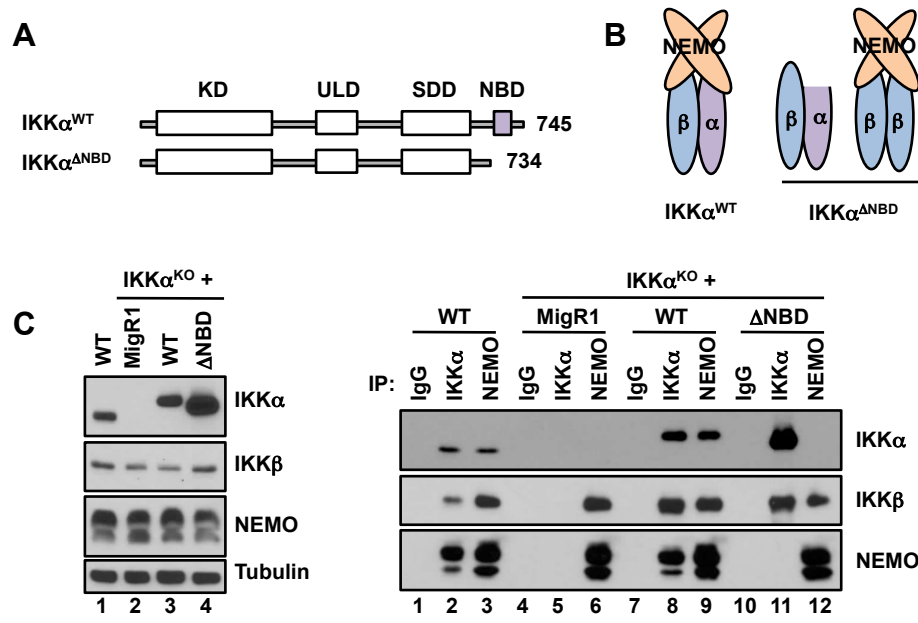
Figure 6. Generation of IKKα^WT and IKKα^ΔNBD MEF cell lines. (**A**) Schematic representation of the domain structure of IKKα. The NEMO-binding domain (NBD) is absent in IKKα^ΔNBD. (**B**) Depiction of IKK complexes in IKKα^KO MEFs reconstituted with IKKα^WT or IKKα^ΔNBD. In addition to these classical complexes, we previously established that IKKα^WT and IKKα^ΔNBD homodimers are also present in these respective reconstituted MEF lines (Solt *et al.* 2009). (**C**) Left: WT and IKKα^KO MEFs retrovirally transduced with MigR1 or with MigR1 encoding either IKKα^WT or IKKα^ΔNBD were lysed, and whole-cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. Right: Immunoprecipitations (IP) were performed with anti-IKKα and anti-NEMO antibodies, as indicated, to show IKK complex formation. Blots are representative of four independent experiments.
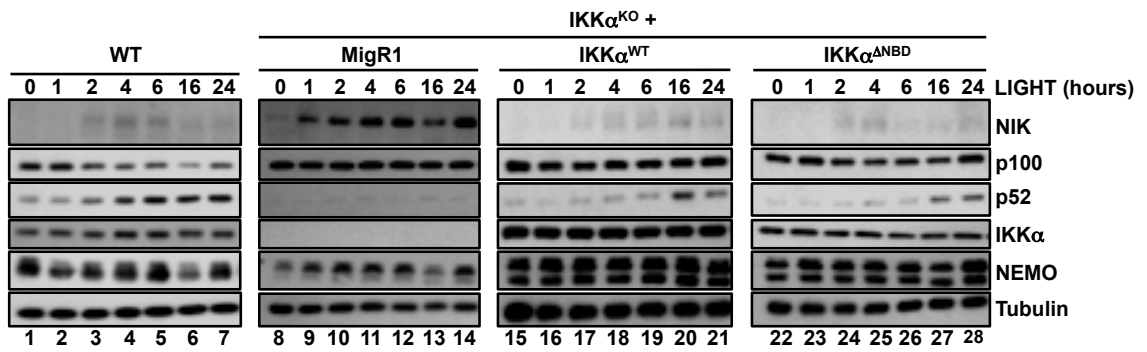
Figure 7. The association between NEMO and IKKα is dispensable for inducible p100 processing. WT and IKKα-deficient (IKKα^{KO}) MEFs retrovirally transduced with either MigR1 alone or MigR1 expressing IKKα^{WT} or IKKα^{ΔNBD} were incubated with LIGHT for the indicated times. Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins.

7, lanes 15-28). Thus we conclude that binding to NEMO is not required for IKKα to regulate

p100 processing in response to LIGHT. This dissection of the NEMO:IKKα association provides

biochemical evidence to support the current model of NEMO-independent activation of the non-

canonical NF-κB pathway by IKKα.

3.2.2. IKK$\alpha^{\Delta NBD}$ is sufficient to promote NIK turnover

We next questioned whether the association of IKKα with NEMO was required for the

IKKα-dependent turnover of active NIK. This negative feedback loop was described based on the

observation that LTβR stimulation permits NIK protein accumulation in WT MEFs, but protein

amounts decrease after sustained activation, whereas NIK protein levels continually increase in

stimulated IKKα-deficient cells (Razani, Zarnegar et al. 2010). Mutational analyses established

that IKKα phosphorylates NIK on three serine residues to promote NIK destabilization, but the

investigators never actually visualized the rate of endogenous NIK turnover to confirm this model

(Razani, Zarnegar et al. 2010). Assessing active NIK turnover is complicated because NIK is

continually synthesized in a resting cell; protein levels are constrained due to the E3 ubiquitin

ligase activity of the basal degradation complex and thus NIK is undetectable until an activating

stimulus is encountered. When a cell is activated through the LTβR, for example, the basal

regulatory machinery is disrupted and NIK protein accumulates. However, this pool of active NIK

continues to be filled by newly synthesized protein (Fig. 8). Thus we established a CHX chase

strategy to inhibit new protein synthesis, permitting us to detect active NIK turnover and

determine whether the interaction between IKKα and NEMO was required for this process.

To visualize active NIK turnover we incubated MEFs with LIGHT for four hours to induce

NIK accumulation, then incubated cells with CHX for up to one hour to prevent new protein

synthesis. Supporting the model of IKKα-dependent feedback regulation (Razani, Zarnegar et al.

2010), LIGHT-induced NIK was rapidly lost in WT MEFs whereas NIK remained detectable after
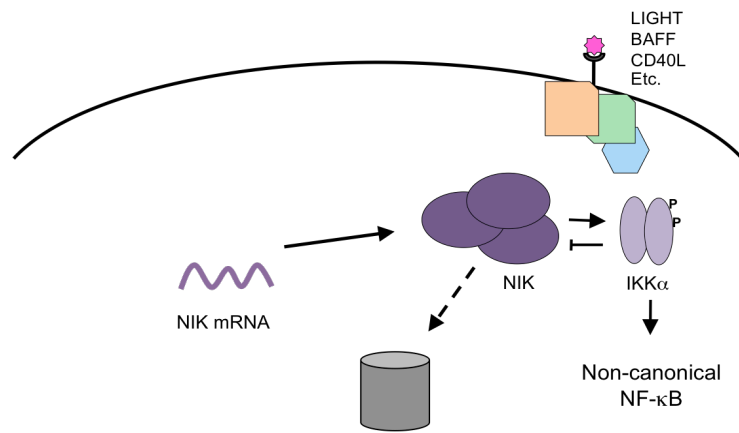
Figure 8. The pool of active NIK is continually filled with newly translated protein. Upon receptor ligation, the TRAF:cIAP complex localizes to the receptor, releasing NIK from its negative regulation. NIK protein stabilizes (dark purple oval), activating the non-canonical signaling pathway. Newly synthesized NIK continues to accumulate. Active IKKα phosphorylates (P) p100 and also feeds back to phosphorylate NIK, leading to the destabilization of NIK. The dashed arrow indicates NIK turnover, but it has not been determined whether this occurs via the proteasome.

| | | | | | | | | | IKKα^{KO} + | | | | | | | | | | | | | | | | | | | | | | |

The figure shows Western blots with the following layout:

WT — 4h LIGHT; **MigR1** — 4h LIGHT; **IKKα^{WT}** — 4h LIGHT; **IKKα^{ΔNBD}** — 4h LIGHT

CHX (min): 0 0 5 15 30 45 60 V (repeated for each group)

Rows: NIK, IKKα, Tubulin

Lane numbers: 1 2 3 4 5 6 7 8 | 9 10 11 12 13 14 15 16 | 17 18 19 20 21 22 23 24 | 25 26 27 28 29 30 31 32
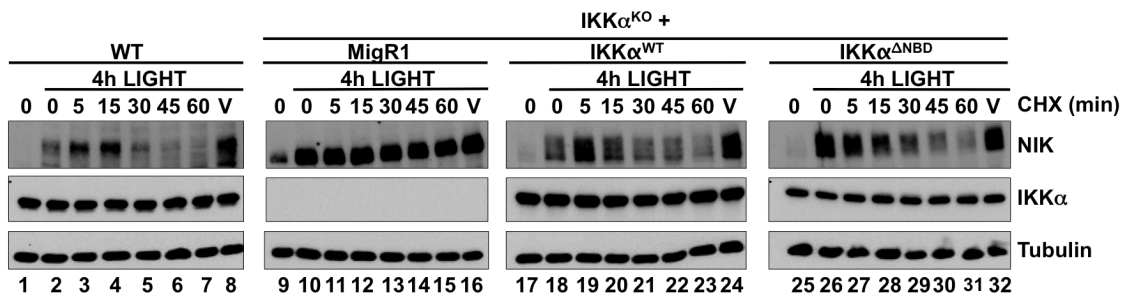
Figure 9. An association between NEMO and IKKα is dispensable for active NIK turnover. WT, IKKα^{KO}, and IKKα^{KO} MEFs transduced with the indicated constructs were incubated for 4 hours with LIGHT and then were treated either with cycloheximide (CHX) for the indicated times or with ethanol for 60 min as a vehicle control (V). Lysates were then analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of four independent experiments.

sixty minutes of CHX incubation IKKα-deficient cells (Fig. 9). Furthermore, both IKKα^{WT} and IKKα$^{\Delta NBD}$ restored NIK turnover in IKKα^{KO} cells, demonstrating that the ability of active IKKα to feedback and destabilize LIGHT-induced NIK (Razani, Zarnegar et al. 2010) is independent of its association with NEMO (Fig. 9). Collectively, these results establish that the association with NEMO is dispensable for both the activation and down-regulation of non-canonical NF-κB by IKKα.

3.2.3. Elevated p100 processing in the absence of NEMO

We performed parallel experiments in NEMO-deficient (NEMO^{KO}) MEFs to discern whether the NEMO:IKKα association might have a role in non-canonical NF-κB activity. Unexpectedly, as we pursued these studies, we observed elevated basal p52 amounts in unstimulated NEMO^{KO} MEFs compared with resting WT cells (Fig. 10, A and B). LIGHT or an agonistic anti-LTβR antibody (α-LTβR) could further enhance p100 processing in NEMO^{KO} MEFs (Fig. 10A, lane 6). However, in some experiments basal p52 levels were already maximal in NEMO-deficient cells and could not be increased by activating ligands (Fig. 11, A and B). In light of the prevailing model that NEMO plays no role in non-canonical NF-κB activity (Fig. 4), basal p100 processing in the absence of NEMO is surprising, as this result suggests that NEMO is actually required to suppress non-canonical NF-κB activity.

To further examine the activation status of non-canonical NF-κB signaling in the absence of NEMO, we isolated cytoplasmic and nuclear fractions from resting and anti-LTβR stimulated MEFs. Fractions were immunoblotted with antibodies against the non-canonical NF-κB proteins p52 and RelB to assess whether elevated p52 in NEMO-deficient cells had localized to the nucleus, where it might be transcriptionally active. As shown in Figure 12A, p52 was absent in nuclear samples from unstimulated WT or IKKα^{KO} MEFs, and as expected, anti-LTβR induced the nuclear translocation of p52 in WT but not IKKα^{KO} cells. Consistent with elevated basal p100
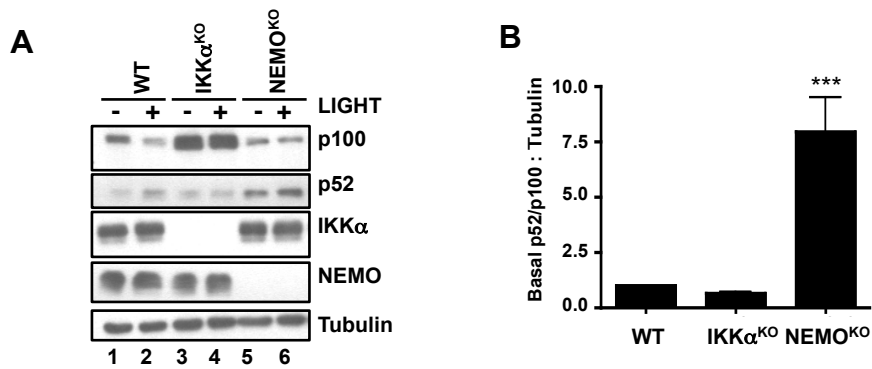
Figure 10. NEMO constrains basal p100 processing in MEFs. (A) WT, IKKα[KO], and NEMO[KO] MEFs were either untreated (-) or were stimulated with LIGHT for 12 hours (+). Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins. **(B)** Basal extents of p100 processing in unstimulated WT, IKKα[KO], and NEMO[KO] MEFs graphed to present the mean ratios of the abundances of p52 and p100 proteins normalized to the abundance of tubulin. Data are means ± SEM from five independent experiments. ***$P < 0.001$ by one-way ANOVA and Dunnett's post-test.

Figure 11. Inducible p100 processing in NEMO-deficient MEFs. (A) WT, IKKα[KO], and NEMO[KO] MEFs were either left untreated (-) or incubated with anti-LTβR antibody for 12 hours (+). Whole-cell lysates were then prepared and analyzed by Western blotting with antibodies against the indicated proteins. **(B)** Quantification of the extent of p100 processing in the indicated cells before (-) or after (+) treatment with anti-LTβR antibody. Data are graphed to present the mean ratios of the abundances of p52 and p100 proteins normalized to the abundance of tubulin protein. Data are means ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.001$ by one-way ANOVA and Dunnett's post-test.
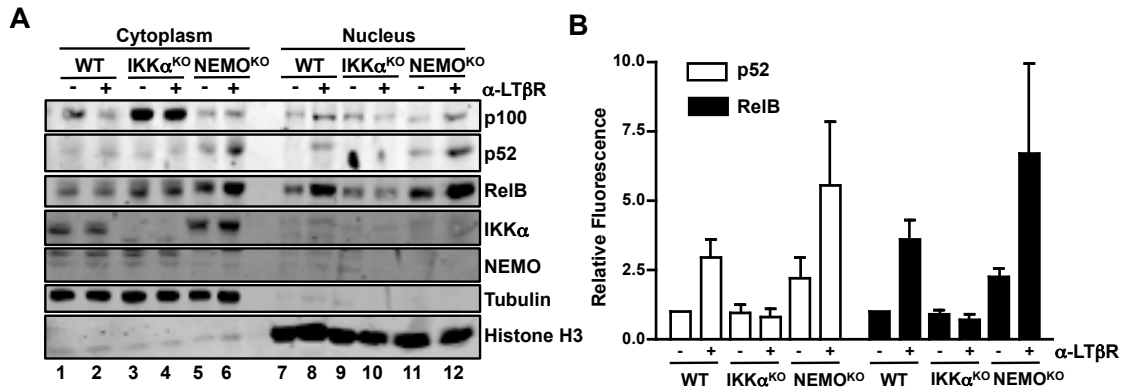
Figure 12. Elevated nuclear p52/RelB amounts in NEMO-deficient MEFs. (A) WT, IKKα[KO], and NEMO[KO] MEFs were either left untreated (-) or were incubated with anti-LTβR antibody (α-LTβR) for 12 hours (+). Cytoplasmic and nuclear extracts were then prepared and analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of three independent experiments. **(B)** Quantification of the relative fluorescence of nuclear fractions similar to those shown in (A). Data are mean fluorescence values ± SEM of p52 (white bars) and RelB (black bars) relative to that of Histone H3 from three independent experiments. $P > 0.05$ by one-way ANOVA.

processing in cells lacking NEMO (Fig. 10), nuclear p52 was present in unstimulated NEMO[KO] MEFs and increased following LTβR ligation (Fig. 12A, lanes 11 and 12). Furthermore, nuclear RelB was present in resting NEMO[KO] MEFs compared with unstimulated WT and IKKα[KO] cells, and RelB nuclear translocation was markedly enhanced by anti-LTβR (Fig. 12A, compare lane 1 with lanes 11 and 12). These data reveal that the basal amounts of nuclear p52 and RelB in NEMO[KO] MEFs are similar to the LTβR-induced levels in WT cells (Fig. 12B). Consistent with elevated nuclear p52 and RelB, transcription of the non-canonical NF-κB gene target *Cxcl12* (Dejardin, Droin et al. 2002; Madge, Kluger et al. 2008; Madge and May 2010) was significantly increased in unstimulated NEMO[KO] MEFs compared to resting WT cells (Fig. 13). Overall these data indicate that the non-canonical NF-κB signaling pathway is basally active in NEMO-deficient MEFs, which challenges the prevailing model for non-canonical NF-κB regulation.

To confirm these findings are not unique to MEFs, we assessed p100 processing in the NEMO-deficient Jurkat T cell line 8321. 8321 cells were identified in a mutagenesis screen of the parental 3T8 cell line for mutations that prevented the induction of a NF-κB-dependent reporter (He and Ting 2002). Immunoblotting confirmed that these cells lacked full-length NEMO (He and Ting 2002). As in NEMO[KO] MEFs (Fig. 10), p52 was elevated in unstimulated 8321 cells compared with the parental 3T8 line containing WT NEMO (Fig. 14). Reconstitution of 8321 cells with wild-type NEMO (8321[WT]) (He and Ting 2002) restored basal p100 processing (Fig. 14). Collectively, these findings establish that intact NEMO maintains the inactive state of non-canonical NF-κB signaling in resting cells.

3.2.4. NIK is present in cells lacking NEMO

Non-canonical NF-κB activation requires ligand-induced NIK stabilization (Senftleben, Cao et al. 2001; Xiao, Harhaj et al. 2001; Dejardin, Droin et al. 2002). Since genetic loss of NEMO results in elevated p100 processing and *cxcl12* expression in the absence of stimulating
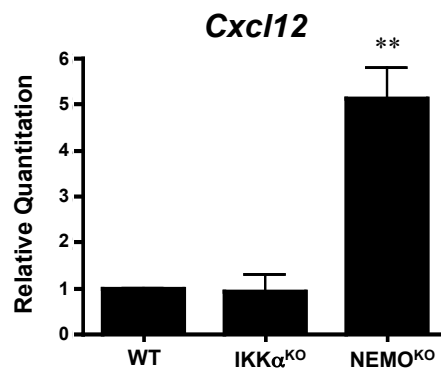
Figure 13. Basal *Cxcl12* transcript amounts in unstimulated MEFs. Relative amounts of *Cxcl12* mRNA in unstimulated WT, IKKα[KO], and NEMO[KO] MEFs relative to that of *Actb* mRNA. Data are means ± SEM from six independent experiments. **P < 0.01 by one-way ANOVA and Dunnett's post-test.
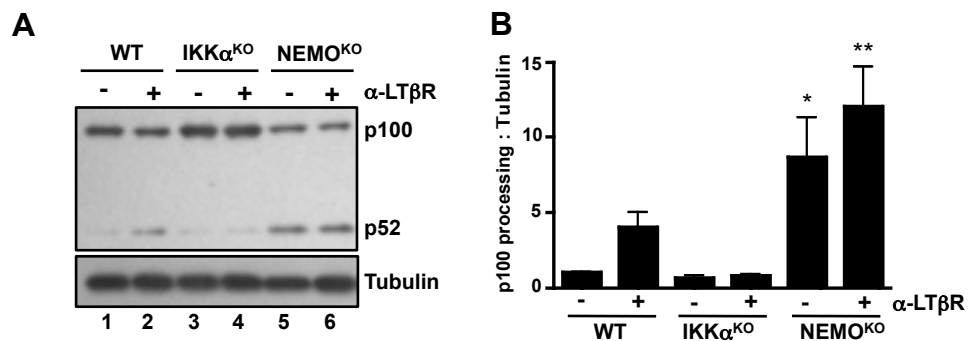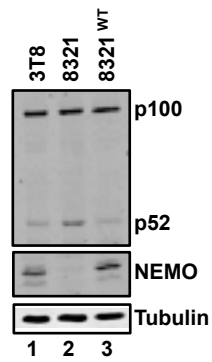
Figure 14. Basal p100 processing in NEMO-deficient T cells can be rescued by WT NEMO.
Whole-cell lysates from the 3T8 and NEMO-deficient 8321 Jurkat T cell lines and from 8321 cells
reconstituted with WT NEMO (8321[WT]) were analyzed by Western blotting with antibodies against
the indicated proteins.

ligands (Figs. 10-14), we asked whether NIK protein amounts were also dysregulated in the absence of NEMO. As expected, NIK was undetected in resting WT MEFs but was stabilized by treatment with LIGHT (Fig. 15A, lanes 1 and 2). Consistent with the recently reported role for IKKα in regulating NIK turnover (Razani, Zarnegar et al. 2010), NIK was present in unstimulated IKKα-deficient cells, and further enhanced following LIGHT stimulation (Fig. 15A, lanes 3 and 4). Strikingly, NIK was present in resting NEMO^{KO} MEFs (Fig. 15A, lane 5), and these amounts were either unchanged (Fig. 15A, lane 6) or minimally enhanced by LIGHT stimulation. Despite significantly elevated NIK protein in NEMO^{KO} MEFs compared to WT MEFs (Fig. 15B), quantitative RT-PCR showed similar abundance of *Map3k14* in WT, IKKα^{KO}, and NEMO^{KO} cells (Fig. 16), indicating that constitutively stabilized NIK in NEMO-deficient MEFs is not due to increased transcription of the gene that encodes NIK.

We next analyzed NIK in the NEMO-mutant 8321 cells that exhibited elevated p100 processing (Fig. 14). Basal NIK was markedly higher in 8321 cells lacking NEMO than in unstimulated parental 3T8 cells (Fig. 17A). Similarly, NIK was elevated in resting NEMO-deficient Rat5R fibroblasts compared with parental Rat1 cells (Fig. 17B). These data from separate cell types and different species strongly suggest that NEMO negatively regulates NIK protein levels.

To confirm this novel function of NEMO, we reconstituted NEMO^{KO} MEFs with WT NEMO (NEMO^{WT}), and as shown in Figure 18 (A and B), basal p100 processing was limited in unstimulated NEMO^{WT} MEFs. Aberrantly stabilized NIK was also ablated in unstimulated NEMO^{WT} MEFs (Fig. 19). Moreover, LIGHT stimulation led to NIK stabilization in NEMO^{WT} MEFs analogous to the activation of WT MEFs (Fig. 19, compare lanes 7 and 8 with lanes 1 and 2). Hence exogenous NEMO rescues basal NIK regulation in NEMO-deficient MEFs.

We next questioned whether the role of NEMO in NIK protein regulation was related to its ability to interact with the catalytic IKKs. To address this we reconstituted NEMO^{KO} MEFs with a

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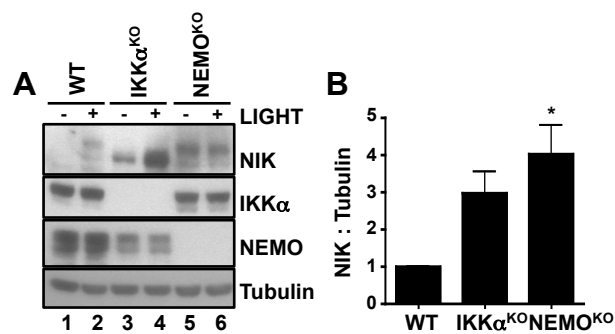
Figure 15. NEMO constrains the accumulation of NIK protein. (A) WT, IKKα^{KO}, and NEMO^{KO} MEFs were either left untreated (-) or incubated with LIGHT for 12 hours (+). Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins. **(B)** Quantification of the abundance of NIK protein relative to that of tubulin in the experiments represented by the Western blot in (A). Data are means ± SEM from three independent experiments. *P < 0.05 by one-way ANOVA and Dunnett's post-test.
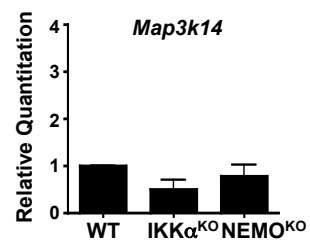
Figure 16. NEMO deficiency does not affect *Map3k14* transcription. Analysis of the abundance of *Map3k14* mRNA (which encodes NIK) relative to *Actb* mRNA in WT, IKKα[KO], and NEMO[KO] MEFs. Data are means ± SEM from four independent experiments, $P > 0.05$ by one-way ANOVA.
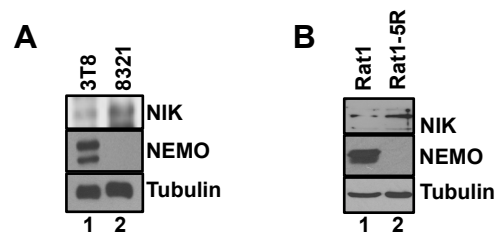
Figure 17. Elevated basal NIK in NEMO-deficient cells. (A and **B)** Lysates from WT and NEMO-deficient Jurkat cells (A) and Rat1R fibroblasts (B) were analyzed by Western blotting with antibodies against the indicated proteins.
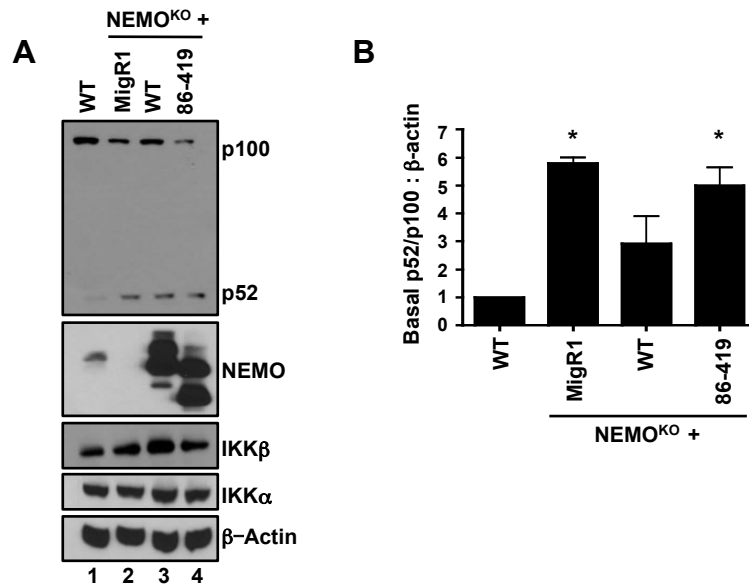
Figure 18. The association between NEMO and IKKα/β is required to constrain basal p100 processing. (A) Lysates from WT or NEMO[KO] MEFs retrovirally transduced with MigR1 (as a control) or with MigR1 encoding WT NEMO or NEMO 86-419 were analyzed by Western blotting with antibodies against the indicated proteins. **(B)** Quantification of the extent of p100 processing to generate p52 in cell lysates from three independent experiments represented by the Western blot in (A). Data are graphed to present the mean ratios of the abundances of p52 and p100 proteins normalized to the amount of β-actin protein. Data are means ± SEM from three independent experiments. *$P < 0.01$ by one-way ANOVA and Dunnett's post-test.
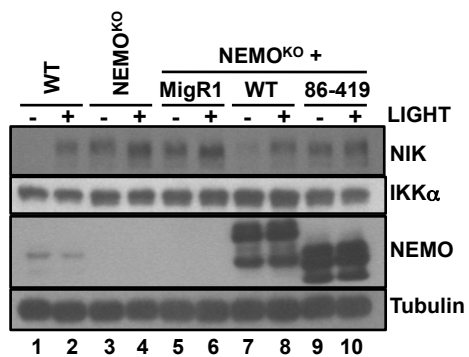
Figure 19. The association between NEMO and IKKα/β is required to constrain NIK amounts. WT, NEMO[KO], and NEMO[KO] MEFs retrovirally transduced with either control MigR1 vector or with MigR1 encoding WT NEMO or a truncated mutant NEMO (86-419) were either untreated (-) or incubated with LIGHT for 12 hours (+) before cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of four independent experiments.

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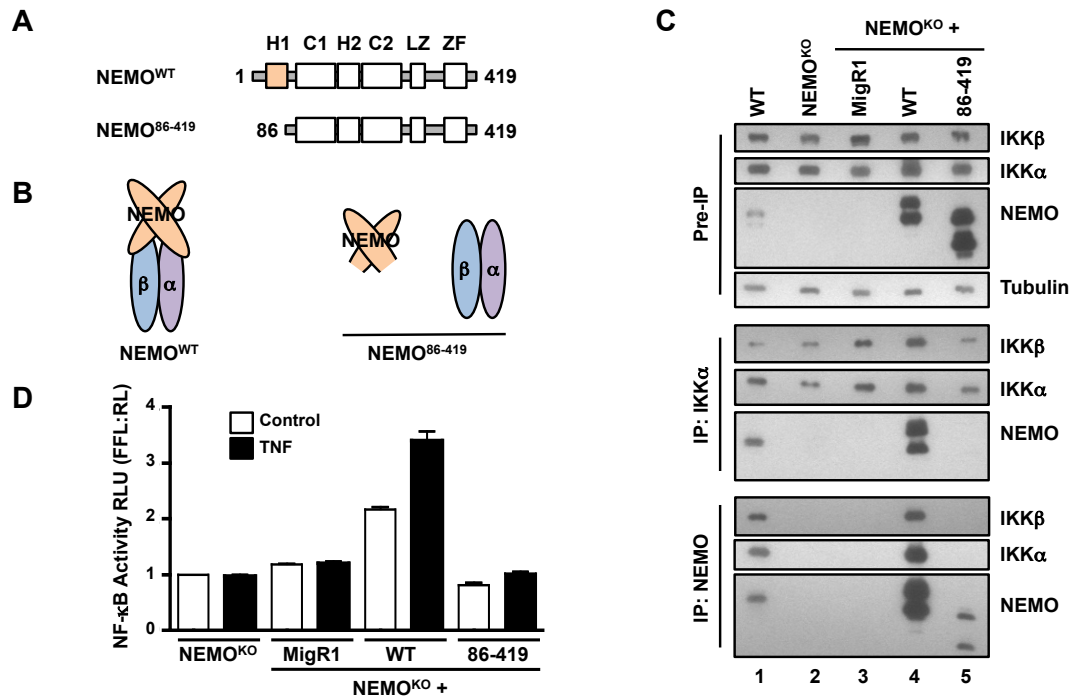
Figure 20. Classical NF-κB signaling in NEMO-reconstituted MEFs. (**A**) Schematic representation of the domain structure of NEMO showing the α-helical domain absent in NEMO[86-419] (H1). (C, coil; LZ, leucine zipper; ZF, zinc finger (**B**) Depiction of the classical IKK complexes in NEMO[KO] MEFs reconstituted with NEMO[WT] or NEMO[86-419]. (**C**) WT, NEMO[KO], and NEMO[KO] MEFs retrovirally transduced with MigR or with MigR encoding either NEMO[WT] or NEMO[86-419] were lysed, and immunoprecipitations (IP) were performed with anti-IKKα or anti-NEMO antibodies, as indicated. Samples before immunoprecipitation (Pre-IP) and immunoprecipitated samples were then analyzed by Western blotting with antibodies against the indicated proteins. Blots are representative of three independent experiments. (**D**) The indicated cells were transfected with the pBIIx-κB firefly luciferase (FFL) and the TK renilla luciferase (RL) vectors. Twenty-four hours later, cells were either left untreated (Control; white bars) or were stimulated with TNF-α for five hours (black bars). NF-κB transcriptional activity was determined as the fluorescence ratio of firefly:renilla luciferase (RLU, relative luciferase units). Data are means ± SD of four replicates from one of three independent experiments.

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NEMO truncation mutant unable to bind either IKKα or IKKβ (NEMO[86-419]) and thereby unable to rescue classical NF-κB activation (Fig. 20). Notably, NIK remained detectable in unstimulated NEMO[86-419] MEFs, similar to NEMO-deficient cells (Fig. 19, lane 9). Further, basal p100 processing remained elevated in NEMO[86-419] MEFs (Fig. 18, A and B). These data indicate that NEMO must associate with either IKKα or IKKβ to properly constrain basal NIK.

3.2.5. The classical IKK complex is required for basal NIK regulation

As dissociation of NEMO from IKKα alone does not affect basal p100 processing or NIK stability (Figs. 7 and 9), the findings in Figures 18 and 19 led us to ask whether IKKβ might be involved in the NEMO-dependent negative regulation of NIK. To address this we examined non-canonical NF-κB signaling in resting IKKβ-deficient (IKKβ[KO]) MEFs, and similar to NEMO[KO] cells, basal p52 was elevated (Fig. 21). Consistent with previous reports (Dejardin, Droin et al. 2002), p100 processing was further enhanced by LIGHT stimulation in IKKβ[KO] MEFs, confirming that inducible non-canonical NF-κB signaling was intact in the absence of IKKβ (Fig. 21).

To determine whether basal p100 processing in IKKβ-deficient cells was caused by a similar mechanism observed in NEMO-deficient cells, we investigated NIK protein amounts in IKKβ[KO] MEFs. Consistent with our observations in NEMO[KO] cells, NIK was significantly elevated in resting IKKβ[KO] MEFs, and treatment with LIGHT or anti-LTβR led to additional NIK stabilization (Fig. 22A, lanes 10-12 and 22B). Examination of a second independently generated IKKβ[KO] MEF line confirmed these findings (Fig. 22C). Constitutive IKKα phosphorylation was detected in IKKβ[KO] cells prior to stimulation (Fig. 22A, lane 10), further indicating that the non-canonical NF-κB pathway is basally active in these cells.

We hypothesized that reconstitution of IKKβ[KO] MEFs with WT IKKβ could restore NIK regulation. As expected, stable transduction of IKKβ[KO] MEFs with WT IKKβ (IKKβ[WT]) but not a
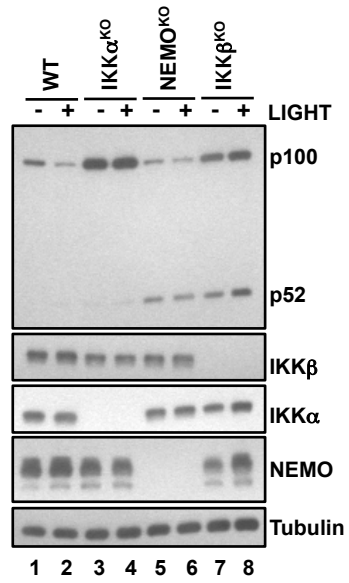
Figure 21. Non-canonical NF-κB activity in IKKβ^{KO} MEFs. WT, IKKα^{KO}, NEMO^{KO}, and IKKβ^{KO} MEFs were either untreated (-) or incubated with LIGHT for 12 hours (+) before cell lysates were analyzed by Western blotting with antibodies against the indicated proteins.
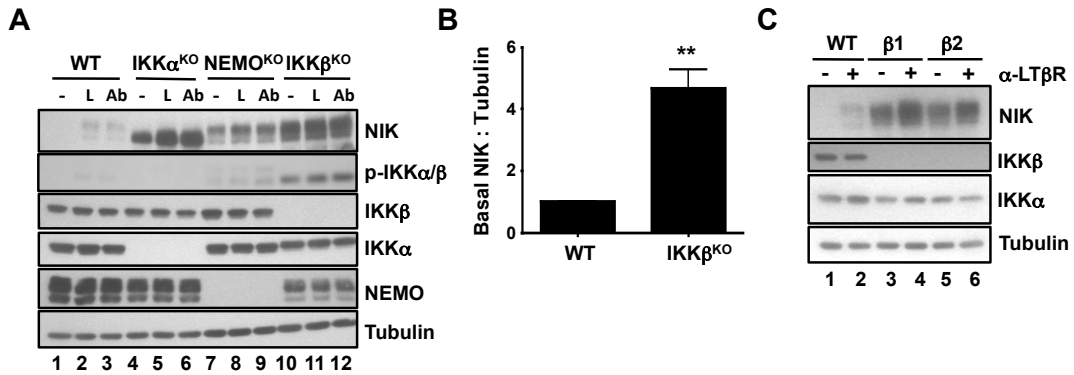
Figure 22. Elevated NIK amounts in IKKβ^{KO} MEFs. (A) The indicated MEF cell lines were either untreated (-) or were stimulated with LIGHT (L) or anti-LTβR antibody (Ab) for 12 hours before cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. Active IKK was detected with an antibody specific for phosphorylated IKKα/β (p-IKKα/β). **(B)** Quantification of NIK protein abundance in WT and IKKβ^{KO} MEFs. Data are means ± SEM from three independent experiments. **$P < 0.01$ by a student's two-tailed t-test. **(C)** WT MEFs and two independently derived IKKβ^{KO} MEF cell lines (β1 and β2) were either left untreated (-) or were treated with anti-LTβR antibody for 8 hours (+). Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins. Blots are representative of three independent experiments.
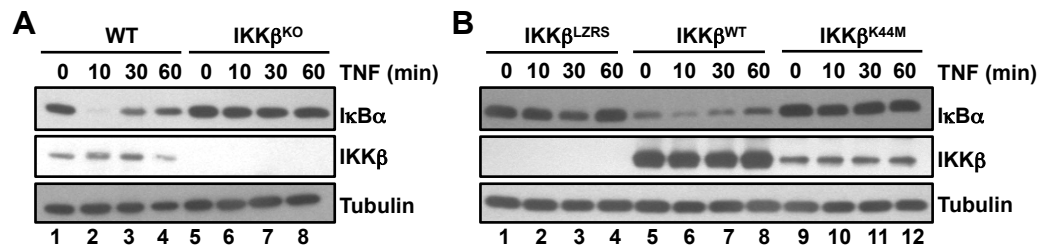
Figure 23. IκBα degradation in WT and IKKβ-reconstituted MEFs. (A) WT and IKKβ^{KO} MEFs and **(B)** IKKβ^{KO} MEFs reconstituted with IKKβ^{WT} or IKKβ^{K44M} were incubated with TNF-α for the indicated times, and IκBα degradation was detected by Western blotting analysis with antibodies against the indicated proteins. Blots are representative of data from three experiments.
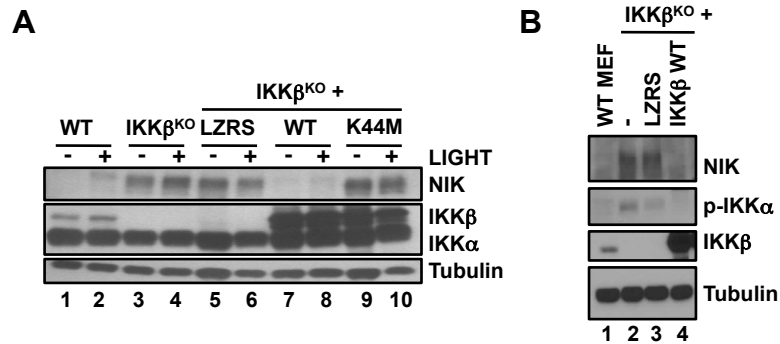
Figure 24. The catalytic activity of the IKK complex is required to constrain basal non-canonical NF-κB activity. (A) WT, IKKβ^{KO}, and IKKβ^{KO} MEFs retrovirally transduced with LZRS or LZRS expressing either IKKβ^{WT} or a catalytically inactive mutant IKKβ (IKKβ^{K44M}) were either untreated (-) or incubated with LIGHT for 12 hours (+) before cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. **(B)** Lysates from WT, IKKβ^{KO} MEFs, and IKKβ^{KO} MEFs reconstituted with LZRS or WT IKKβ were analyzed by Western blotting with antibodies against the indicated proteins. Blots are representative of three independent experiments.

catalytically inactive version of the kinase (IKKβ^{K44M}) rescued classical NF-κB signaling (Fig. 23). Importantly IKKβ^{WT}, but not IKKβ^{K44M}, reduced basal NIK (Fig. 24A), demonstrating that the catalytic activity of IIKKβ is required to properly regulate NIK. Moreover, reconstitution of IKKβ-deficient cells with WT IKKβ reduced basal IKKα phosphorylation (Fig. 24B), indicating that elevated NIK is required for the robust IKKα activation seen in these cells. Collectively these findings establish that basal regulation of the non-canonical NF-κB pathway requires the intact and catalytically active classical IKK complex (Fig. 25).

3.3. Discussion

In this chapter we sought to determine whether the conserved NBD in IKKα played a functional role in non-canonical NF-κB signaling. We took two complementary approaches to understand whether IKKα could promote p100 phosphorylation independent of the heterotrimeric IKK complex. First, we complemented IKKα-deficient MEFs with a mutant version of IKKα that is unable to associate with NEMO. Signal-induced p100 processing was intact in IKKα-deficient cells reconstituted with the mutant IKKα. Our findings therefore provide definitive biochemical support for the tenet that IKKα alone, independent of its association with the classical IKK complex, is sufficient to induce p100 processing. These data do not determine whether "IKKα-alone" complexes exist *in vivo*, but rather they support the genetic model in which IKKα is capable of fully activating the non-canonical NF-κB pathway.

Since the NEMO:IKKα interaction is not required for the activation of the non-canonical NF-κB pathway, we questioned a potential role for this association in the newly described negative feedback loop to terminate active signaling. Feedback inhibition of NIK requires its IKKα-mediated phosphorylation (Razani, Zarnegar et al. 2010). Here we describe an experimental strategy using LIGHT-induced NIK accumulation coupled with the translational inhibitor cycloheximide (CHX) to directly visualize active NIK turnover. We show that the IKKα-

mediated turnover of endogenous NIK occurs in the absence of IKKα binding to NEMO. Thus we conclude that IKKα does not need to associate with NEMO to negatively regulate non-canonical NF-κB activity.

In addition to complementing IKKα-deficient cells, we assessed non-canonical NF-κB activation in NEMO-deficient MEFs. In light of the prevailing model in which the classical IKK complex plays no role in the non-canonical pathway (Sun 2011), we were surprised to detect elevated basal p100 processing in cells lacking NEMO. In most cases p100 processing could be further enhanced by stimulation with LIGHT or anti-LTβR; however, in some instances p52 in NEMO- or IKKβ-deficient cells appeared saturated. Moreover, the ratio of p52 to p100 in untreated NEMO^{KO} MEFs was similar to that in maximally stimulated WT MEFs, suggesting that non-canonical signaling is fully active in the absence of NEMO. Careful examination of the literature indicates that these findings are consistent with earlier studies in which IKKα was catalytically active and p100 was basally processed to p52 in unstimulated NEMO-deficient cells (Senftleben, Cao et al. 2001; Claudio, Brown et al. 2002; Dejardin, Droin et al. 2002; Derudder, Dejardin et al. 2003). However, these observations were never highlighted or addressed in the original papers and the notion that non-canonical NF-κB signaling is intact in the absence of NEMO has been the prevailing conclusion. Thus, because activating stimuli could further induce p100 processing in NEMO-deficient cells over elevated basal amounts, the widely accepted model states that non-canonical NF-κB signaling is NEMO-independent. Our data establish that this model is incomplete and should include a role for NEMO in basal suppression of the non-canonical pathway (Fig. 25).

In seeking to determine the mechanism for enhanced basal p100 processing, we found NIK to be significantly elevated in resting cells lacking either NEMO or IKKβ. Consistent with active p100 processing, NIK detected in the absence of NEMO or IKKβ resembled NIK induced by stimulation of WT MEFs with LIGHT or anti-LTβR; this further corroborates that non-canonical

NF-κB signaling is basally active in these cells. We also found NEMO and IKKβ must interact, and the catalytic activity of IKKβ is required for basal NIK regulation. Hence we conclude that in resting cells, the intact and catalytically competent classical IKK complex maintains basal NIK regulation and prevents constitutive p100 processing to p52 (Fig. 25).
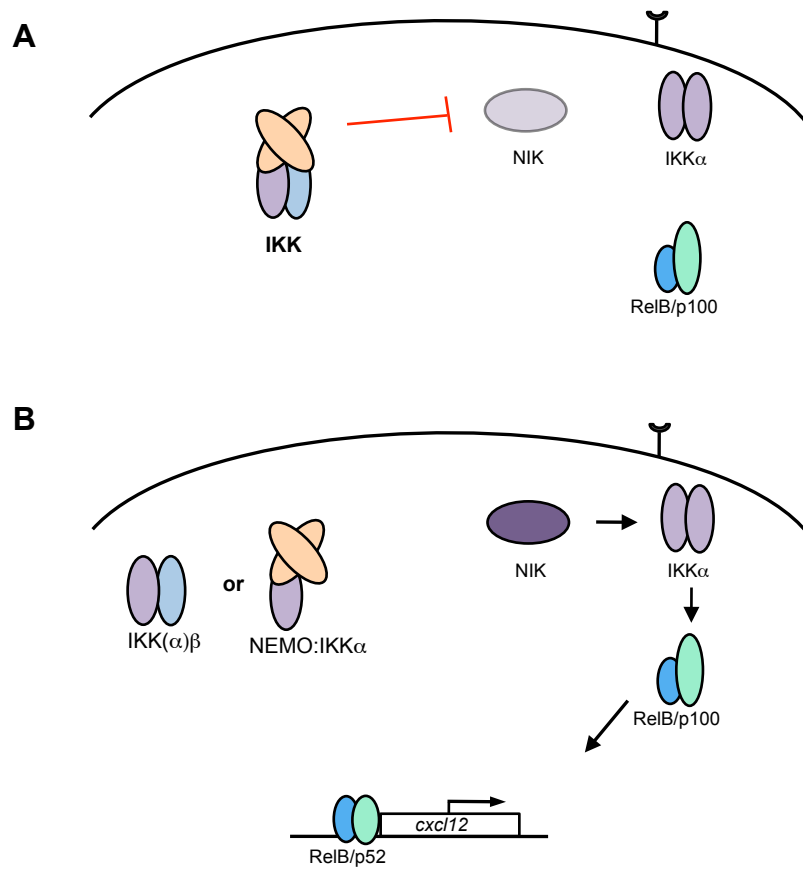
Figure 25. The non-canonical NF-κB signaling pathway is basally active in the absence of NEMO or IKKβ. (A) The classical IKK complex constrains NIK levels in unstimulated cells. **(B)** In the absence of NEMO or IKKβ, NIK protein levels stabilize, leading to p100 processing and activation of the non-canonical NF-κB pathway.

CHAPTER 4. CLASSICAL NF-κB SIGNALING NEGATIVELY REGULATES NON-CANONICAL NF-κB ACTIVITY

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4.1. Introduction

NIK is a constitutively active kinase required for non-canonical NF-κB signal transduction. Therefore, NIK protein levels are under stringent post-translational regulation (Fig. 4). In resting cells, newly synthesized NIK is rapidly degraded by a complex consisting of TRAF2, TRAF3, cIAP1, and cIAP2 (Liao, Zhang et al. 2004; Varfolomeev, Blankenship et al. 2007; Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008), and upon receptor ligation (e.g. LTβR) NIK is freed from this complex and accumulates in the cytoplasm (Liao, Zhang et al. 2004; Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008). Stabilized NIK phosphorylates IKKα, which in turn leads to p100 processing and non-canonical NF-κB activation. Recently, IKKα has also been shown to phosphorylate active NIK, triggering its degradation (Razani, Zarnegar et al. 2010). The precise phosphorylation-dependent mechanism of NIK turnover is unclear, but this negative feedback loop is crucial to eliminate NIK and is thought to down-regulate non-canonical NF-κB signaling (Fig. 5B).

Our results in chapter 3 reveal an unprecedented role for the classical IKK complex in the basal constraint of non-canonical NF-κB signaling. These findings prompt reassessment of the mechanisms that control NIK protein stability. In this chapter, we investigate the role of the classical IKK complex in basal NIK stabilization and active NIK turnover. We hypothesized that the classical IKK complex directly phosphorylated active NIK, facilitating its turnover by IKKα. However, our results reveal that classical NF-κB transcriptional activity mediated by p65 is required for the negative regulation of basal NIK, which is a surprising interplay between the

classical and non-canonical NF-κB pathways. Moreover, we identify a population of patients with

mutations in NEMO for whom this novel regulatory function may be important clinically.

4.2. Results

4.2.1. NEMO and NIK interact via IKKα

To begin to identify how the classical IKK complex may regulate NIK protein levels, we

asked whether NEMO could associate with endogenous NIK. Because NIK is constitutively

degraded in resting cells, we treated cells with the proteasome inhibitor MG132 for four hours to

stabilize NIK. Immunoprecipitation (IP) using an antibody against NEMO accessed the

heterotrimeric IKK complex consisting of IKKα and IKKβ as well as NIK (Fig. 26A). However,

reciprocal pull-downs with an antibody raised against the C terminus of the NIK protein

immunoprecipitated NIK itself but none of the IKKs (Fig. 26A). This may be due to antibody

interference of NIK interactions, as IP of endogenous NIK with this antibody has also never co-

immunoprecipitated any members of the basal regulatory complex (TRAF3, TRAF2, or cIAP1, not

shown). NIK and IKKα have been shown to interact when exogenously expressed (Lin, Mu et al.

1998; Ling, Cao et al. 1998; Xiao, Harhaj et al. 2001) to induce p100 processing (Xiao, Harhaj et

al. 2001). We thus performed control pull-downs with an anti-IKKα antibody to IP NIK.

Immunoprecipitation of IKKα pulled down NIK, NEMO, and a small amount of IKKβ (Fig. 26A lane

5).

Because the association with NEMO is not required for IKKα to function in the non-

canonical NF-κB pathway (Figs. 7 and 9), we questioned whether NEMO pulled down NIK via an

association with IKKα or whether NEMO associated with NIK directly. To test this, we performed

the same biochemical analysis on lysates from IKKα^{KO} MEFs. The NEMO:NIK association was

lost in the absence of IKKα (Fig. 26A, lane 8), suggesting that IKKα may serve as a molecular

bridge between NIK and NEMO.
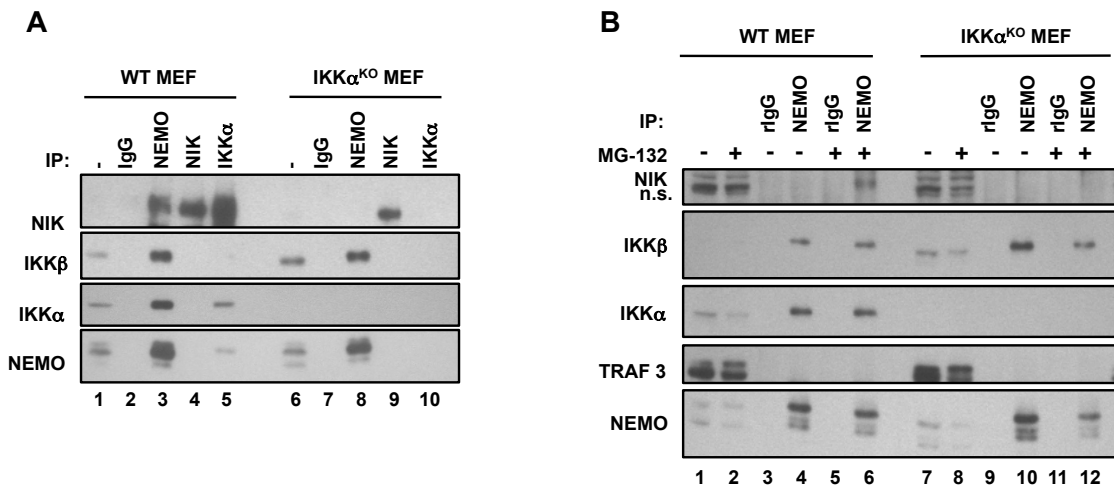
Figure 26. The classical IKK complex may associate with endogenous NIK but does not interact with the basal degradation complex. (A) WT and IKKα[KO] MEFs were treated with MG132 (25 μM) for four hours to stabilize NIK. Cells were lysed, and immunoprecipitations (IP) were performed with anti-NEMO, anti-NIK, or anti-IKKα antibodies, as indicated. Samples before immunoprecipitation (-) and immunoprecipitated samples were then analyzed by Western blotting with antibodies against the indicated proteins. **(B)** WT and IKKα[KO] MEFs were either untreated (-) or treated with MG132 (25 μM, +) for four hours to stabilize NIK. Cells were lysed and analyzed as in (A).

To investigate whether the association with NEMO might be important for basal NIK turnover, we asked whether NEMO associated with TRAF3, which is required for the constitutive degradation of NIK in resting cells (Liao, Zhang et al. 2004; Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008). We therefore immunoprecipitated NEMO and immunblotted for endogenous NIK and TRAF3. Despite binding NIK, we have been unable to detect an association between NEMO and endogenous TRAF3, even in the absence of MG132 (Fig. 26B). Because TRAF3 is a critical component of the basal NIK regulatory complex and NEMO does not associate with TRAF3, we thought this result indicated that the classical IKK complex might not be important for basal NIK degradation. Furthermore, because IKKα is required for the association of NEMO and NIK (Fig. 26A), and IKKα promotes the destabilization of active NIK, we hypothesized that the classical IKK complex may facilitate IKKα-mediated turnover of NIK.

4.2.2. Classical IKK is not required for NIK phosphorylation by IKKα

The recently described negative feedback loop leading to degradation of NIK requires phosphorylation by IKKα (Razani, Zarnegar et al. 2010). This prevents prolonged non-canonical NF-κB signaling and can be visualized by a single unphosphorylated NIK band detected in IKKα^{KO} MEFs (Fig. 15A, lanes 3 and 4). Our results from Figure 23 suggest that NEMO, which associates with NIK via IKKα, may pay a role in this process. As shown in Figure 27A, λ-phosphatase treatment of LIGHT-stimulated MEFs caused NIK in WT, NEMO^{KO}, and IKKβ^{KO} cells to migrate with the same electrophoretic mobility as unphosphorylated NIK in IKKα^{KO} MEFs. Moreover, λ-phosphatase treatment reduced the molecular weight of stabilized NIK in TRAF3-deficient MEFs, in which basal NIK degradation is defective (Fig. 27B) (He, Zarnegar et al. 2006). Contrary to our original hypothesis that the classical IKK complex facilitates IKKα-mediated NIK turnover, these data indicate that active NIK is properly phosphorylated by IKKα in MEFs lacking NEMO, IKKβ, or TRAF3, similar to WT cells.
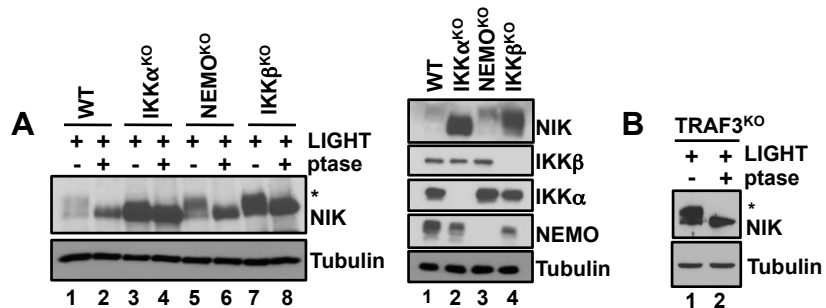
Figure 27. NEMO and IKKβ are dispensable for the IKKα-mediated phosphorylation of active NIK. (A) Left: The indicated cells were incubated with LIGHT for 4 hours, and then cell lysates were prepared and either left untreated (-) or incubated with λ-phosphatase (ptase; +) before being analyzed by Western blotting with antibodies against the indicated proteins. The asterisk indicates the band corresponding to phosphorylated NIK. Right: Loading controls to show the abundances of NIK, IKKα, IKKβ, and NEMO in lysates of the indicated LIGHT-stimulated cells before treatment with phosphatase. **(B)** TRAF3[KO] MEFs were processed and analyzed as described in (A).

As our results suggest both IKKα and IKKβ are required for the overall regulation of NIK

protein, we assessed NIK stability in IKKα/β double-knockout (DKO) MEFs. Consistent with these

findings, NIK was detectable in unstimulated DKO MEFs (Fig. 28A). Similar to IKKα^{KO} MEFs,

there was no electrophoretic shift indicating IKKα-mediated NIK phosphorylation in DKO MEFs

when compared to NIK in stimulated WT cells (Fig. 28B). λ-phosphatase treatment enhanced the

electrophoretic mobility of NIK from unstimulated NEMO^{KO} and IKKβ^{KO} MEFs but not from DKO

MEFs (Fig. 28B). These data demonstrate that in cells lacking either NEMO or IKKβ, NIK is

basally phosphorylated in an IKKα-dependent manner.

4.2.3. Active NIK turnover is independent of the classical IKK complex

Our accumulated findings establish that NIK is present and basally phosphorylated by

IKKα in NEMO- or IKKβ-deficient MEFs. As NIK associates with NEMO in the presence of IKKα

(Fig. 26), we asked whether the intact classical IKK complex is required for the IKKα-dependent

degradation of active NIK (Razani, Zarnegar et al. 2010). Consistent with inducible activation of

the non-canonical pathway in NEMO^{KO} and IKKβ^{KO} cells, NIK was enhanced in these cells

following LIGHT treatment; this active NIK was degraded in the presence of CHX with similar

kinetics to NIK in WT MEFs (Fig. 29). As expected, NIK remained stable with CHX treatment in

DKO MEFs confirming that negative feedback in the absence of the intact classical IKK complex

requires IKKα. Furthermore, activation-induced turnover is independent of the basal NIK

regulatory complex, as active NIK in TRAF3^{KO} cells was degraded similar to NIK in WT, NEMO^{KO}

and IKKβ^{KO} MEFs (Fig. 29). These data definitively show that the classical IKK complex is not

required for IKKα-mediated turnover of activated NIK.

We next questioned whether the basally elevated and IKKα-phosphorylated NIK present

in resting NEMO^{KO} and IKKβ^{KO} MEFs resulted from a general defect in protein turnover. As

shown in Figure 30, CHX treatment of unstimulated NEMO^{KO} and IKKβ^{KO} MEFs led to loss of
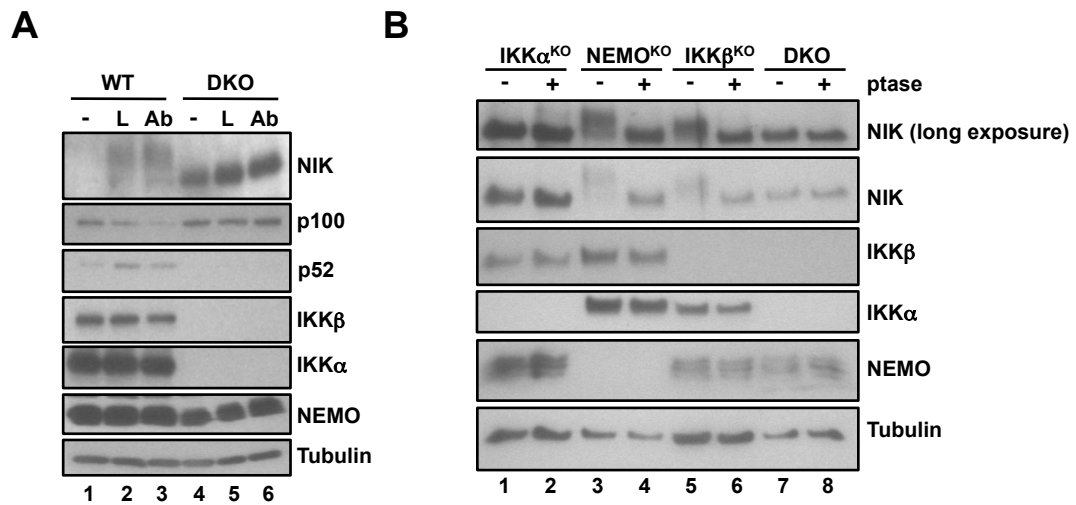
Figure 28. Increased NIK abundance in IKKα/β DKO MEFs. (A) WT and IKKα/β DKO MEFs were either untreated (-) or were stimulated with LIGHT (L) or anti-LTβR antibody (Ab) for 4 hours. Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins. **(B)** Lysates from resting IKKα[KO], NEMO[KO], IKKβ[KO], and DKO MEFs were treated with λ-phosphatase before being analyzed by Western blotting with antibodies against the indicated proteins. Blots are representative of three independent experiments.
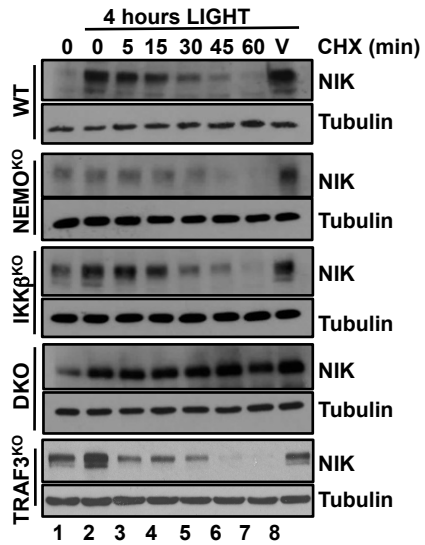
Figure 29. The classical IKK complex does not facilitate the turnover of active NIK. WT, NEMO[KO], IKKβ[KO], DKO, and TRAF3[KO] MEFs were incubated for 4 hours with LIGHT and then were either treated with cycloheximide (CHX) for the indicated times or were treated for 60 min with ethanol as a vehicle control (V). Cell lysates were analyzed by Western blotting with antibodies against the indicated proteins.

basal NIK. In contrast unphosphorylated NIK in both resting IKKα^{KO} and DKO cells was highly stable and remained detectable after two hours of CHX treatment (Fig. 30). Thus we conclude that the classical IKK complex does not regulate IKKα-mediated NIK turnover but instead plays a separate, obligate role in limiting the basal pool of newly synthesized NIK.

4.2.4. Classical NF-κB activity is required for basal NIK regulation

Our accumulated results suggest that classical IKK activity is required to constrain basal NIK and thus non-canonical NF-κB signaling. We reasoned that this could occur via one of two mechanisms. First, the IKK complex may phoshorylate NIK directly via IKKβ, facilitating its basal turnover via the TRAF3:TRAF2:cIAP1/2 complex. While NEMO associates with endogenous NIK, we have never identified an interaction between NEMO and TRAF3 (Fig. 26). Additionally, elevated NIK is basally phosphorylated in NEMO^{KO} and IKKβ^{KO} MEFs (Fig. 27A), suggesting that IKKβ kinase activity does not target NIK directly. An alternative model for basal NIK constraint by the classical IKK complex is that a gene product of classical NF-κB signaling is required to regulate NIK amounts.

To address this second possibility, we assessed non-canonical NF-κB activity in MEFs lacking the prototypic classical NF-κB subunit p65 (p65^{KO}). Similar to unstimulated NEMO^{KO} and IKKβ^{KO} MEFs, NIK was elevated in p65^{KO} MEFs prior to stimulation with anti-LTβR (Fig. 31A). Quantitative RT-PCR in p65^{KO} MEFs showed classical NF-κB signaling did not control *Map3k14* transcription directly (Fig. 31B). As in NEMO^{KO} and IKKβ^{KO} MEFs (Fig. 27A), λ-phosphatase treatment revealed that active NIK was phosphorylated in p65^{KO} MEFs (Fig. 31C). Furthermore, low resting NIK was partially rescued by stable reconstitution of p65-deficient MEFs with a WT version of p65 (Fig. 31D). Collectively, these results indicate that p65-driven gene transcription is required for basal NIK regulation.
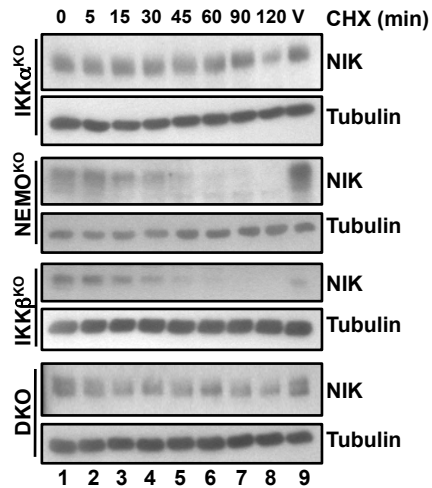
Figure 30. Basal NIK stability in the absence of NEMO or IKKβ. Unstimulated IKKα[KO], NEMO[KO], IKKβ[KO], and DKO MEFs were incubated with CHX for the indicated times or with ethanol (V) for 120 min before cell lysates were prepared and analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of four independent experiments.
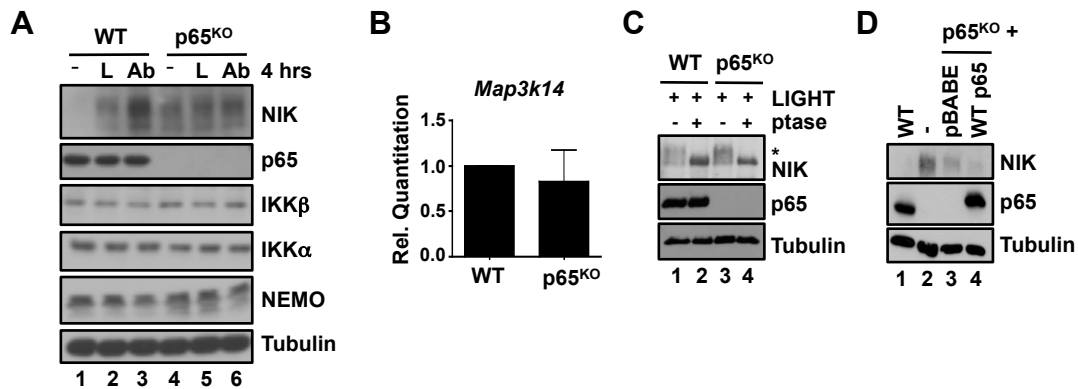
Figure 31. Classical NF-κB–dependent gene expression is required to regulate basal NIK abundance. (A) WT or p65[KO] MEFs were left unstimulated or were treated with either LIGHT (L) or the anti-LTβR antibody (Ab) for 4 hours. Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins. **(B)** RNA from WT or p65[KO] MEFs was isolated and analyzed by quantitative RT-PCR to determine the abundance of *Map3k14* mRNA in comparison to that of *actb* mRNA. Data are means ± SEM from three independent experiments. *P* > 0.05 by student's two-tailed t-test. **(C)** WT and p65[KO] MEFs were stimulated with LIGHT for 4 hours. Whole-cell extracts were then left untreated or were treated with λ-phosphatase (ptase) and analyzed by Western blotting with antibodies against the indicated proteins. The asterisk indicates the band corresponding to phosphorylated NIK. **(D)** p65[KO] MEFs were transduced with empty pBABE vector or the pBABE vector encoding WT p65. Cell lysates were then analyzed by Western blotting with antibodies against the indicated proteins.

Since classical NF-κB activity does not affect *Map3k14* transcription directly (Figs. 16 and 31B), we sought to determine whether any of the known modulators of basal NIK expression were deregulated in p65^{KO} MEFs. The molecular components regulating basal NIK form the TRAF2:TRAF3:cIAP1:cIAP2 E3 ubiquitin ligase complex (Sun 2012). We found TRAF2, TRAF3, cIAP1, and cIAP2 amounts to be similar or elevated in p65^{KO} MEFs compared to WT (Fig. 32A), consistent with a potential role for non-canonical NF-κB signaling in TRAF3 expression (Sasaki, Calado et al. 2008). As cIAP2 (*Birc3*) is a classical NF-κB gene target (Chu, McKinsey et al. 1997), we assessed *Birc3* transcripts in p65^{KO} MEFs and found similar amounts in p65^{KO} and WT MEFs (Fig. 32B). In addition, *Birc3* transcription was intact in NEMO^{KO} MEFs, suggesting that disruption of the classical IKK complex does not affect basal *Birc3* transcription. We therefore conclude that cIAP2 is not the molecular target of classical NF-κB signaling that controls basal NIK regulation. cIAP1, TRAF2 and TRAF3 protein amounts were similar if not elevated among the cell lines we studied (Fig. 33), and TRAF3 stability was unaffected by loss of classical NF-κB activity (Fig. 34). Notably, over-expressed NIK was able to associate with endogenous TRAF3, TRAF2, and cIAP1 in the absence of NEMO or p65 (Fig. 35). Together these data suggest that aberrant NIK detected in the absence of NEMO, IKKβ, or p65 is not due to alteration of the currently known NIK regulatory machinery.

4.2.5. Classical NF-κB transcriptional activity rescues basal NIK amounts

Loss of p65 in MEFs permits basal NIK stabilization and p100 processing, similar to loss of the upstream signaling components NEMO or IKKβ, indicating that classical NF-κB transcriptional activity is required to actively suppress basal non-canonical NF-κB signaling. We therefore hypothesized that enforced classical NF-κB activity could rescue aberrant non-canonical NF-κB signaling in NEMO^{KO} MEFs. To test this we transduced NEMO^{KO} MEFs with
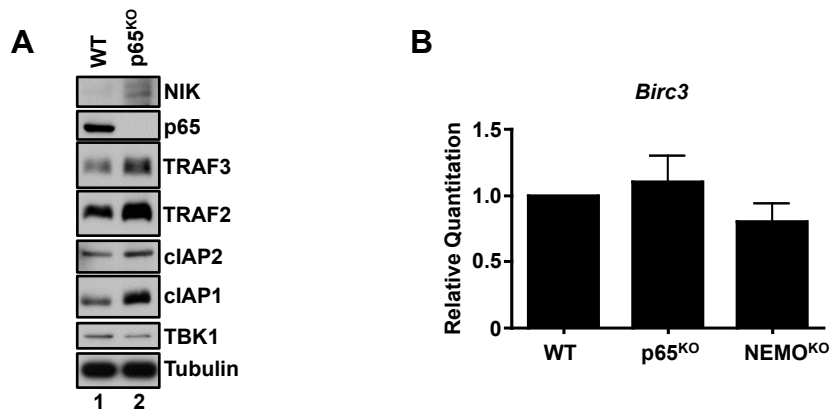
Figure 32. p65 does not regulate the expression of the basal NIK regulatory complex. (A) Whole-cell extracts from WT or p65^{KO} MEFs were analyzed by Western blotting with antibodies against the indicated proteins. **(B)** RNA was isolated from unstimulated WT, p65^{KO}, and NEMO^{KO} MEFs for quantitative RT-PCR analysis of *Birc3* expression. Data are means ± SEM of *Birc3* mRNA abundance relative to that of *actb* mRNA from three independent experiments. *P* > 0.05 by one-way ANOVA.
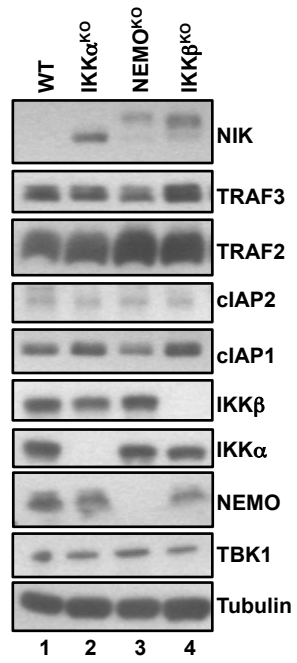
Figure 33. Basal NIK regulatory complex expression in IKK-deficient MEFs. Lysates from WT, IKKα[KO], NEMO[KO], and IKKβ[KO] MEFs were analyzed by Western blotting with antibodies against the indicated proteins.
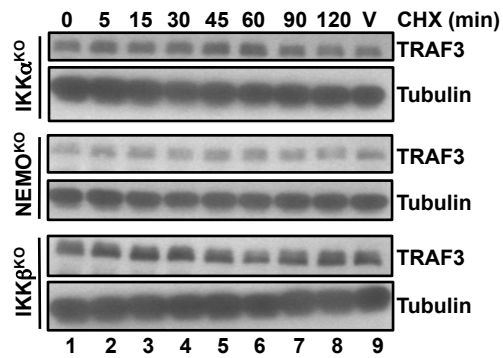
Figure 34. TRAF3 stability in IKK-deficient MEFs. Unstimulated IKKα^{KO}, NEMO^{KO}, and IKKβ^{KO} MEFs were incubated with cycloheximide (CHX) for the indicated times or with ethanol as a vehicle control (V) for 120 min. Cell lysates were then analyzed by Western blotting with anti-TRAF3 and anti-tubulin antibodies. Blots are representative of four independent experiments.
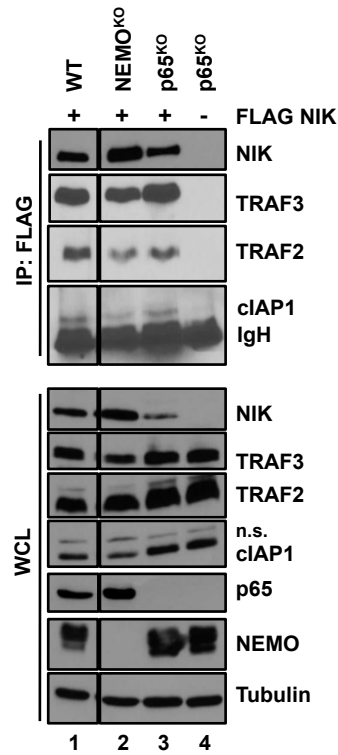
Figure 35. FLAG-NIK associates with the endogenous basal NIK regulatory complex in NEMO[KO] and p65[KO] MEFs. WT, NEMO[KO], and p65[KO] MEFs were transfected with pFLAG-CMV2-NIK. Top: FLAG-tagged NIK was immunoprecipitated (IP) with FLAG (M2) beads, and co-immunoprecipitation of NIK with endogenous TRAF3, TRAF2, and cIAP1 was determined by Western blotting analysis. Bottom: Western blotting analysis of the abundances of the indicated proteins in whole-cell lysates (WCL) of samples before they were subjected to immunoprecipitation. Blots are representative of three experiments.

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MigR1 empty vector or MigR1 containing WT IKKβ to create stable cell lines over-expressing

IKKβ. Over-expression of WT IKKβ alone was sufficient to drive classical NF-κB transcriptional

activity, even in the absence of NEMO (Fig. 36A). Furthermore we observed reduced NIK

amounts and lower basal p100 processing in NEMO^{KO} MEFs that over-expressed WT IKKβ

compared to controls (Fig. 36B), which further confirms that classical NF-κB transcriptional

activity is required to actively suppress basal NIK and thus non-canonical NF-κB signaling.

4.2.5. Hyperactive non-canonical NF-κB activity in NEMO-ID

Non-canonical NF-κB has recently been found to control immunoglobulin class-switch

recombination to the IgA isotype in activated B cells (Jin, Xiao et al. 2012). Prolonged NIK

stability enhances non-canonical NF-κB signaling, and mice with elevated NIK display high serum

titers of IgA (Jin, Xiao et al. 2012). Notably, a subset of patients with inherited mutations in NEMO

exhibits hyper-IgA (Orange, Jain et al. 2004). These patients have a complex immunodeficiency

termed NEMO-immunodeficiency (NEMO-ID) due to defects in classical NF-κB signaling;

however, the mechanisms underlying the unusual clinical presentation of hyper-IgA have not

been explained by the lack of classical NF-κB activity (Orange, Jain et al. 2004). Our studies in

MEFs reveal a novel layer of cross talk between classical NF-κB signaling and basal NIK

regulation; we therefore hypothesized that the defects in class-switch observed in NEMO-ID

patients was due to basal activity of non-canonical NF-κB.

To assess the status of the non-canonical NF-κB pathway in samples from NEMO-ID

patients previously shown to have impaired classical NF-κB activation and elevated serum IgA

(Orange, Jain et al. 2004; Hanson, Monaco-Shawver et al. 2008), we compared p100 processing

in unstimulated peripheral blood mononuclear cells (PBMCs) from two patients to those from a

healthy donor. Strikingly, the amounts of p52 compared with p100 were greater in both NEMO-

mutant PBMC samples (Q403X and C417R) compared with control donor cells or LIGHT-

activated human umbilical vein endothelial cells (HUVEC) (Madge, Kluger et al. 2008) (Fig. 37).
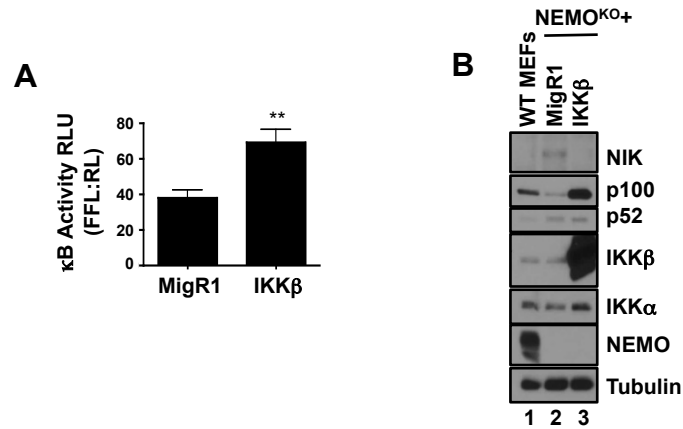
Figure 36. Constitutive classical NF-κB transcription rescues low basal NIK. (A) NEMO[KO] MEFs stably transduced with either MigR1 or MigR1 encoding WT IKKβ were transfected with the pBIIx-κB firefly luciferase (FFL) and the TK renilla luciferase (RL) vectors. Twenty-four hours later, classical NF-κB transcriptional activity was determined as the fluorescence ratio of FFL:RL. (RLU, Relative Luciferase Units). Data are means ± SEM of six replicates from two independent experiments. **$P < 0.01$ by student's two-tailed t-test. **(B)** WT or NEMO[KO] MEFs stably transduced with either MigR1 or MigR1 encoding WT IKKβ were analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of three independent experiments.
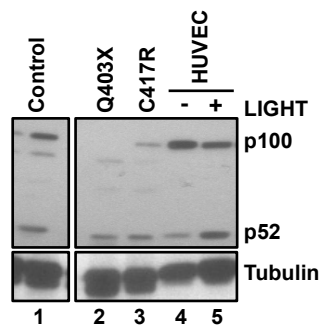
Figure 37. The extent of p100 processing is enhanced in NEMO-ID cells. Lysates of PBMCs from patients with distinct NEMO mutations (Q403X and C417R), PBMCs from a healthy donor (Control), and HUVECs that were either untreated (-) or were stimulated with LIGHT for 8 hours (+) were analyzed by Western blotting with antibodies against the indicated proteins.

Notably, p100 was not detected in NEMO^{Q403X} PBMCs, indicating that cellular p100 was completely processed to p52 in these cells (Fig. 37, lane 2). These data indicate that basal non-canonical NF-κB signaling is hyperactive in NEMO-ID.

Due to the scarcity of patient-derived material, we could not definitively determine the status of basal NIK in NEMO-mutant PBMCs. For this reason, we reconstituted NEMO^{KO} MEFs with the hyper-IgA-associated Q403X and C417R mutants to further evaluate the effects of NEMO-ID mutations on non-canonical NF-κB signaling. Unlike the NEMO$^{86-419}$ truncation mutant that cannot bind to IKKα or IKKβ, NEMO^{Q403X} and NEMO^{C417R} each associated with IKKα and IKKβ to form a heterotrimeric IKK complex in reconstituted cells (Fig. 38A). Despite being able to form IKK complexes, neither NEMO^{Q403X} nor NEMO^{C417R} rescued TNF-induced NF-κB activation in NEMO^{KO} MEFs, confirming that these mutations ablate stimulated classical IKK activity (Fig. 38B).

While proper NIK regulation was rescued in NEMO^{WT} MEFs, elevated basal NIK was maintained in NEMO^{KO} MEFs reconstituted with either NEMO^{Q403X} or NEMO^{C417R} (Fig. 39, lanes 3, 7, and 9). We generated an additional NEMO-ID mutant MEF line harboring a mutation (NEMO^{L153R}) in a distinct functional domain of the protein. This mutation did not permit classical NF-κB signaling in previous reports (Orange, Brodeur et al. 2002; Hanson, Monaco-Shawver et al. 2008), and importantly, NEMO^{L153R} also failed to restore control of basal NIK levels (Fig. 39, lane 11). These findings establish that the ability of NEMO to form an IKK complex is insufficient to properly regulate the non-canonical NF-κB pathway; a signal-competent IKK complex and intact classical NF-κB activity are essential to maintain the quiescent state of the non-canonical NF-κB pathway. Furthermore, they suggest that aberrant non-canonical NF-κB activity may underlie the hyper IgA phenotype observed in patients with NEMO-ID.
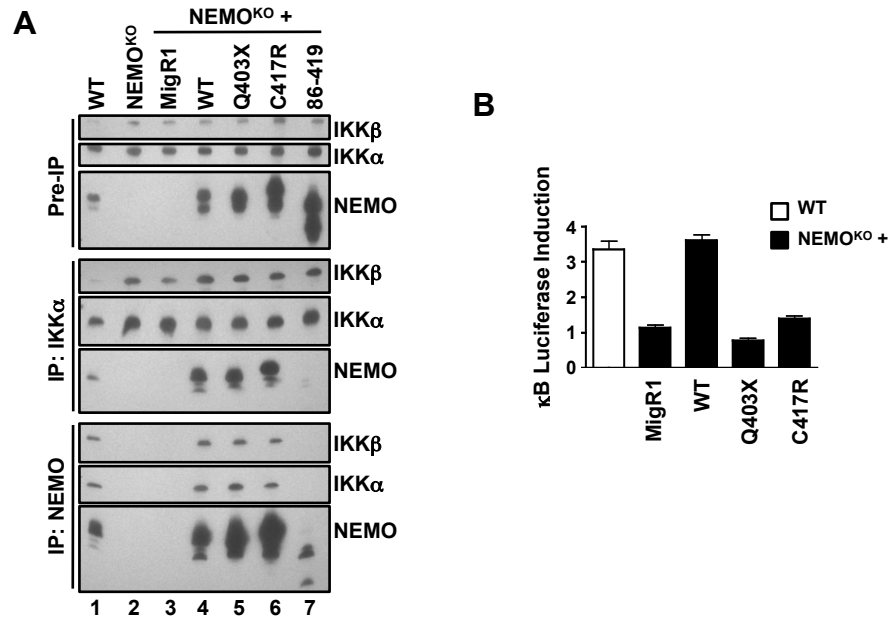
Figure 38. Mutant NEMO can form IKK complexes but not support classical NF-κB activity.
(**A**) WT, NEMO[KO], and NEMO[KO] MEFs retrovirally transduced with MigR1 or MigR1 encoding WT NEMO or the indicated NEMO mutants were lysed and then subjected to immunoprecipitation (IP) with anti-IKKα and anti-NEMO antibodies. Cell lysates and the immunoprecipitated samples were analyzed by Western blotting with antibodies against the indicated proteins. (**B**) WT and NEMO[KO] cells were transfected with the pBIIx-κB firefly luciferase (FFL) and the TK Renilla luciferase (RL) vectors. Twenty-four hours later, cells were either left untreated or were treated with TNF-α for 5 hours. NF-κB transcriptional activity was determined by measuring the fluorescence ratio of FFL:RL, and the extent of κB luciferase induction was defined as the fold-difference between the TNF-α–stimulated samples and the controls. Data are means ± SD of four replicates in one of three independent experiments.
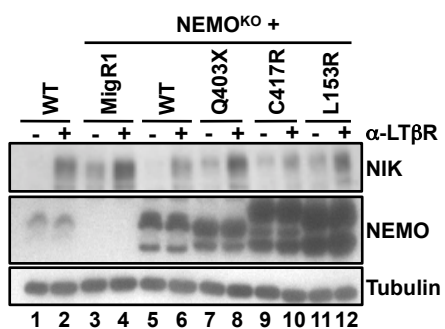
Figure 39. Elevated NIK in cells containing hypomorphic NEMO mutations. WT and NEMO[KO] MEFs retrovirally transduced with MigR1 or MigR1 encoding WT NEMO or the indicated NEMO mutants were either untreated (-) or incubated with anti-LTβR antibody for 8 hours (+) before being lysed and analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of four independent experiments.

4.3. Discussion

In this chapter, we sought to understand the mechanism underlying aberrant NIK expression in cells that lack components of the classical IKK complex. Through co-immunoprecipitation we have found that endogenous NEMO and NIK interact when NIK is stabilized by MG132 treatment. NEMO and NIK have previously been shown to interact when both proteins are over-expressed in 293 cells (Li, Kang et al. 1999). They have also been shown to interact directly *in vitro* (Li, Kang et al. 1999), however our results indicate that IKKα is required for the endogenous association of NEMO and NIK. Since we have been unable to detect association of NEMO with TRAF3 in the basal NIK regulatory complex, we hypothesized that the classical IKK complex might facilitate the IKKα-mediated turnover of active NIK.

Unlike stable NIK present in IKKα^{KO} MEFs, the NIK we detected in IKKβ-or NEMO-deficient cells was phosphorylated, as it is in stimulated WT cells. Our experiments using CHX revealed that although NIK turnover in both resting and stimulated IKKα^{KO} MEFs was defective, turnover occurred normally in cells lacking IKKβ or NEMO. We thus conclude that the classical IKK complex does not regulate IKKα-mediated NIK turnover but instead plays an obligate role in limiting the basal pool of newly synthesized NIK. Supporting this interpretation we found that the stable NIK in TRAF3^{KO} MEFs (Liao, Zhang et al. 2004) is phosphorylated and turned over normally. Hence, basal NIK in cells lacking the intact classical IKK complex is identical to newly synthesized NIK that has escaped the TRAF2:TRAF3:cIAP1/2 basal regulatory machinery. These findings also confirm that TRAF3 plays no role in the IKKα-driven degradation of active NIK and imply that a novel regulatory mechanism is responsible for this phase of non-canonical pathway de-activation.

IKKα (Razani, Zarnegar et al. 2010) and more recently, the IKK-like kinase TBK1 (Jin, Xiao et al. 2012) have been shown to phosphorylate NIK, leading to its controlled turnover. While

our results do not support a role for classical NF-κB signaling in negative feedback of the non-canonical NF-κB pathway, NIK is clearly subject to multiple layers of regulation by IKK or IKK-like kinases. We have not directly ruled out a role for novel catalytic function by the heterotrimeric IKK complex, however our studies in p65^{KO} MEFs reveal that ultimately, classical NF-κB activity is required to maintain undetectable NIK in resting cells. To accomplish this, classical NF-κB signaling may regulate a specific gene or gene panel that functions in basal NIK regulation. Likely candidate genes include TRAF2, TRAF3, the cIAPs or OTUD7B, a de-ubiquitinase that has been shown recently to regulate TRAF3 stability (Hu, Brittain et al. 2013). However we found that TRAF2, TRAF3, cIAP1, and cIAP2 protein amounts are similar or somewhat greater than WT MEFs among all of the cell lines we tested. Importantly, over-expressed NIK associates with endogenous TRAF3, TRAF2, and cIAP1 in WT MEFs or cells lacking NEMO or p65, suggesting that defective classical NF-κB signaling does not prevent the basal regulatory complex from forming in these cells.

Mounting evidence has shown that cIAP1 and cIAP2 are functionally redundant in their role as E3 ubiquitin ligases regulating basal NIK; individual knockout cells must be complemented with siRNA or Smac mimetics to degrade the other cIAP and stabilize NIK (Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008; Gardam, Turner et al. 2011). We observe similar levels of *Birc3* transcription among p65^{KO} and NEMO^{KO} MEFs compared to WT MEFs, confirming the presence of this E3 ubiquitin ligase required for basal NIK regulation. Our data further suggest that the deubiquitinase OTUD7B (Hu, Brittain et al. 2013) is not involved in basal NIK regulation by the classical IKK complex, since TRAF3 stability is unaffected by the loss of NEMO or IKKβ. We therefore surmise that classical NF-κB signaling drives the expression of a unique gene or set of genes involved in non-canonical NF-κB regulation. Though the exact gene target remains to be determined, our results establish an obligate role for classical NF-κB activity to facilitate the quiescent state of non-canonical NF-κB in unstimulated cells (Fig. 40).
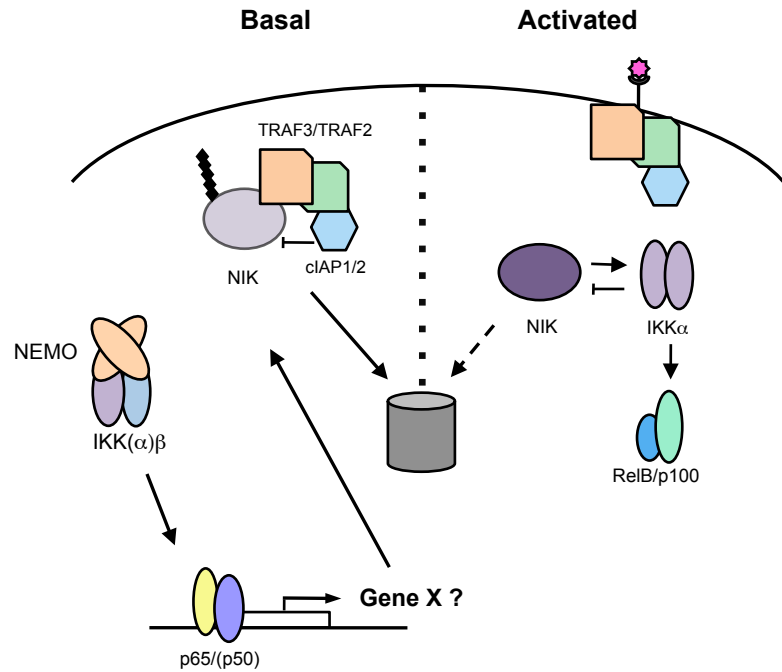
Figure 40. Classical NF-κB activity limits NIK protein amounts and constrains basal non-canonical NF-κB activity. Classical NF-κB signaling mediated by p65 induces the expression of a novel gene target (X) required for the constraint of basal NIK amounts. This negative regulatory mechanism maintains the quiescent state of the non-canonical NF-κB activity.

Our findings reveal an essential role for the NEMO-dependent classical NF-κB pathway in negatively regulating basal non-canonical NF-κB activity. We show that naturally occurring NEMO-ID mutations prevent negative regulation of the non-canonical NF-κB pathway by the classical IKK complex in human disease. Patients with hypomorphic NEMO mutations exhibit a complex immunologic phenotype resulting in widespread immunodeficiency (Orange and Geha 2003). The clinical presence of hyper-IgA in NEMO-ID is intriguing, as non-canonical NF-κB signaling has recently been shown to directly control class switch to the IgA isotype (Jin, Xiao et al. 2012). B cell-specific deletion of TBK1 in mice leads to NIK stabilization and nephritis due to prolonged non-canonical signaling and elevated IgA levels (Jin, Xiao et al. 2012). We found that unstimulated PBMCs from patients with NEMO mutations associated with hyper-IgA exhibit extremely high p100 processing, supporting a defect in basal regulation of non-canonical NF-κB. Thus we conclude that in a subset of NEMO-ID patients, defective classical NF-κB activation leads to elevated basal non-canonical NF-κB activity. This finding provides a mechanism for aberrant class switch in NEMO-ID and represents an *in vivo* human demonstration of the dysregulated non-canonical NF-κB signaling pathway.

As limited sample availability precluded definitive determination of the status of NIK in NEMO-ID patient PBMCs, we established stable MEF lines expressing pathological NEMO mutations. In each case the NEMO-ID mutants assembled with endogenous IKKα and IKKβ to form a heterotrimeric IKK complex; however, consistent with previous studies these complexes were unable to activate classical NF-κB (Makris, Roberts et al. 2002; Cordier, Vinolo et al. 2008). Furthermore, high basal NIK was detected in the NEMO-mutant cells. Hence our data establish that functional mutations of NEMO that do not prevent IKK complex assembly alter the basal regulation of the non-canonical NF-κB pathway. We have only been able to assess this defect retrospectively in clinical samples, but our studies support future assessment of non-canonical

NF-κB signaling in NEMO-ID patients. Moreover, these accumulated findings prompt a re-evaluation of the current view that the classical IKK complex plays no role in non-canonical NF-κB regulation.

CHAPTER 5. THE TURNOVER OF ACTIVE NIK IS PROTEASOME-DEPENDENT BUT DOES NOT REQUIRE CIAP1/2

Data from this chapter have been submitted for publication with the following authors: Gray CM, McCorkell KA, Chunduru SK, McKinlay MA, and May MJ.

5.1. Introduction

Our studies exploring the functional relevance of the IKKα:NEMO association in non-canonical NF-κB activity led us to discover a novel interplay between the classical and non-canonical NF-κB signaling pathways required to constrain basal NIK amounts (Chapters 3 and 4). In addition to these mechanisms required to maintain the latent phase of non-canonical NF-κB signaling in resting cells, the duration of non-canonical activity is controlled by a crucial negative regulatory mechanism in which activated IKKα feeds back to phosphorylate NIK at specific C-terminal serine residues (Razani, Zarnegar et al. 2010). These modifications promote NIK turnover to terminate non-canonical signaling; however, the precise mechanism of IKKα-induced NIK degradation remains unknown. In this chapter, we investigate the mechanism of active NIK turnover. As basal NIK is constitutively degraded via the proteasome, we hypothesize that active NIK turnover is also proteasome-dependent.

Over-expression and inhibitor studies suggest that the TRAF2:TRAF3:cIAP1/2 complex that controls the basal turnover of newly synthesized NIK does not require prior NIK phosphorylation (Razani, Zarnegar et al. 2010). Importantly however, these experiments did not directly address a potentially distinct role for the cIAP1/2 in specifically controlling the turnover of activated endogenous NIK. As the cIAPs are to date, the only known regulators of basal NIK stability, we further hypothesized that cIAP1 and cIAP2 were required for the negative turnover of signal-induced NIK.

5.2. Results

5.2.1. Active NIK turnover occurs via the proteasome

We employed the proteasome inhibitor MG132 to determine whether the proteasome

plays a role in the IKKα-dependent turnover of endogenous signal-induced active NIK. Consistent

with previous reports (Matsushima, Kaisho et al. 2001; Liao, Zhang et al. 2004; Qing, Qu et al.

2005; Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008; Sanjo, Zajonc et al.

2010), pharmacological blockade of the proteasome using MG132 led to the accumulation of NIK

in resting cells (Fig. 41A, lane 2), which confirms that basal NIK is constitutively degraded through

the proteasome. However, analysis proteasome inhibition on the fate of signal-induced NIK is

complicated by concomitant effects on basal NIK levels (Fig. 41A). We therefore used a

combination of proteasome and translation inhibition to isolate signal-induced NIK. Importantly,

NIK was undetectable in resting cells co-treated with MG132 and CHX, confirming that in the

absence of proteasome function, protein synthesis inhibition prevented NIK accumulation (Fig.

41A). As this inhibitor combination allowed us to specifically ablate newly synthesized NIK, we

employed this strategy to determine the effects of proteasome inhibition on LIGHT-induced active

NIK turnover. As shown in Figure 41B, NIK accumulation was induced by incubation of MEFs for

four hours with LIGHT, and further incubation for one hour in CHX led to the loss of detectable

NIK (lane 3). Moreover, inhibition of the proteasome with concomitant CHX treatment completely

inhibited LIGHT-induced NIK turnover (compare lanes 3 and 4), indicating that active NIK is

degraded in a proteasome-dependent manner. Notably, inhibition of the proteasome in the

absence of CHX led to increased accumulation of NIK in LIGHT-stimulated cells (Fig. 41B, lane

6), further indicating that CHX treatment isolated active NIK from the constitutively translated

basal protein.

Although MG132 is the most widely used proteasome inhibitor, it has been reported to

have off-target effects on cathepsins and calpains *in vitro* (Kisselev, van der Linden et al. 2012).
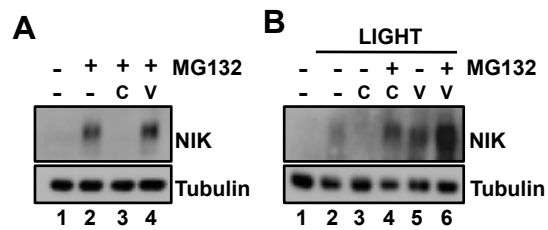
Figure 41. Turnover of endogenous NIK is proteasome-dependent. (A) WT MEFs were treated with 10µM MG132 (+), MG132 plus 2.5 mg/ml cycloheximide (C), or MG132 plus vehicle control (V) for one hour. Whole cell lysates were immunoblotted with antibodies against the indicated proteins. **(B)** WT MEFs were treated with LIGHT for four hours followed by one hour of cycloheximide (C) or vehicle (V) one hour of cycloheximide plus MG132 (+) to inhibit the proteasome. Lysates were immunoblotted as in (A).

Therefore we also utilized the more selective inhibitor bortezomib (Velcade, PS-341) (Adams, Palombella et al. 1999; Goldberg 2012) to confirm the role of the proteasome in active NIK turnover. Similar to MG132, bortezomib inhibits the chymotrypsin-like enzymatic site of the 20S core particle of the 26S proteasome (Goldberg 2012) and, as expected, both inhibitors blocked TNF-induced proteasome-dependent degradation of IκBα in the classical NF-κB signaling pathway (Hayden and Ghosh 2008) (Fig. 42A). As above (Fig. 41B), LIGHT treatment promoted NIK stabilization and subsequent incubation with CHX led to the loss of LIGHT-induced activated NIK (Fig. 42B). Importantly, NIK turnover during CHX incubation was blocked by pretreatment with either MG132 or bortezomib (Fig. 42B). These data establish, with two different pharmacological inhibitors, that the rapid IKKα-dependent turnover of endogenous NIK occurs in a proteasome-dependent manner.

5.2.2. Turnover of active NIK is independent of cIAP1 and cIAP2

The sensitivity of NIK turnover to proteasome inhibition suggests that active NIK is ubiquitylated, thereby targeting it for degradation. As cIAP1 and cIAP2 are currently the only E3 ubiquitin ligases implicated in basal NIK degradation (Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008), we hypothesized that in addition to controlling the degradation of newly synthesized NIK, cIAP1/2 also ubiquitylated active, phosphorylated NIK. To assess the requirement of cIAP1/2 in NIK turnover, we used a Second mitochondria-derived activator of caspase (Smac or DIABLO) mimetic to degrade endogenous cIAP1/2 (Varfolomeev, Blankenship et al. 2007). Smac is a mitochondrial protein that is released upon induction of apoptosis. In the cytoplasm, Smac associates with and inhibits IAP proteins, thus permitting caspase activation and apoptosis (Gyrd-Hansen and Meier 2010). Because Smac potently induces apoptotic signaling, small molecule IAP antagonists mimicking the N-terminal tetrapeptide IAP binding motif (IBM) of Smac have been developed as anti-cancer therapeutics (Chen and Huerta 2009). These Smac mimetics bind to IAPs and promote their auto-ubiquitylation and proteasomal degradation
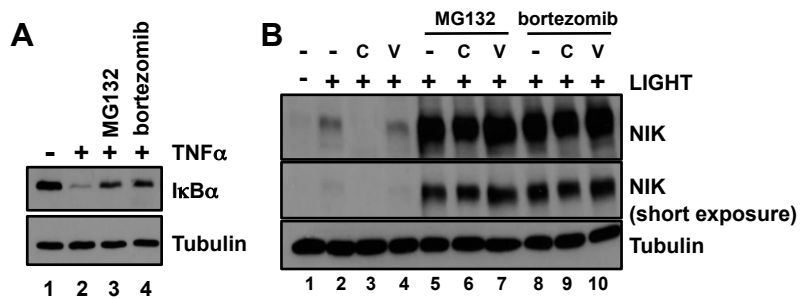
Figure 42. Two different proteasome inhibitors block active NIK degradation in MEFs. (A) WT MEFs were either left untreated or incubated with 500 nM MG132 or 500 nM bortezomib for 8 hours. Cells were then stimulated with TNF for 15 minutes. Lysates were immunoblotted with antibodies against the indicated proteins. **(B)** WT MEFs were treated with 500 nM MG132 or 500 nM bortezomib, with (+) or without (-) LIGHT for 6.5 hours. Cells were subsequently incubated with cycloheximide (C) or vehicle control (V) for 90 minutes. Whole cell lysates were immunblotted as in (A).

(Gyrd-Hansen and Meier 2010). We therefore obtained a novel Smac mimetic, GT13072, to specifically ablate cIAP1/2 and assess their requirement in active NIK turnover.

In resting MEFs, GT13072 treatment led to rapid loss of cIAP1 and induction of NIK, whereas an inactive version of the compound (GT13199) did not (Fig. 43A). Notably, treatment with GT13072 induced NIK stabilization and p100 processing more rapidly than LIGHT (Fig. 43B). Furthermore, GT13072 treatment induced a second NIK band of greater apparent molecular weight in WT MEFs that was not detected in GT13072-treated IKKα^{KO} MEFs (Fig. 43C). Together, these results suggest that release of NIK from the TRAF2/3:cIAP1/2 complex promotes its phosphorylation by IKKα in a manner similar to that induced by the natural ligand LIGHT or a cross-linking antibody against the LTβR (Fig. 43C, lane 11). We therefore used GT13072 to determine if cIAPs are further required to promote the turnover of NIK once it has been released from the TRAF2/3:cIAP1/2 complex. To specifically isolate GT13072-induced NIK from basally translated NIK, we employed the CHX chase assay established in Figure 9. Similar to LIGHT-induced NIK, NIK stabilized by pre-treatment with GT13072 was turned over within sixty minutes of CHX treatment, despite the absence of cIAP1 (Fig. 44). These experiments show that cIAP1 is dispensable for the turnover of active NIK in MEFs.

Although we were unable to detect resting cIAP2 protein in MEFs, NIK was stabilized by GT13072 treatment alone (Fig. 43A). As cIAP1 and cIAP2 have been shown to be functionally redundant in basal NIK regulation (Zarnegar, Wang et al. 2008), this suggested that GT13072 also degraded any endogenous cIAP2. However, to ensure that cIAP1 and cIAP2 did not play distinct roles in the turnover of active NIK, we assessed NIK regulation in HeLa cells, which express cIAP1 and cIAP2 (Fig. 45A). GT13072 treatment led to the rapid loss of both cIAP1 and cIAP2 in HeLa cells followed by NIK stabilization (Fig. 45A). As in MEFs, treatment with GT13072 induced p100 processing similar to the natural ligand LIGHT (Fig. 45B). Importantly, CHX chase
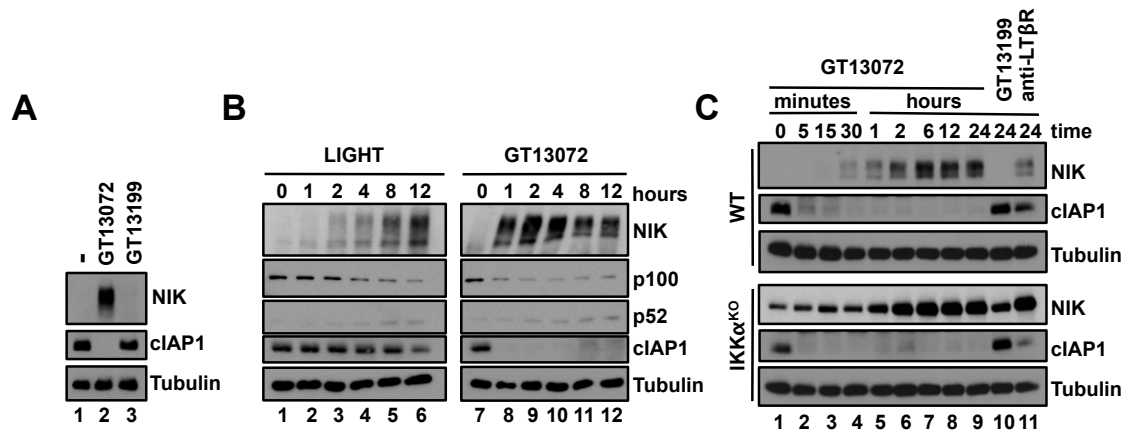
Figure 43. The Smac mimetic GT13072 stabilizes NIK. (A) WT MEFs were treated for four hours with either 1 µM smac mimetic GT13072 or the inactive compound GT13199. Cells were lysed and immunoblotted with antibodies against the indicated proteins. **(B)** WT MEFs were treated with LIGHT or GT13072 for up to twelve hours. Lysates were immunoblotted with antibodies against the indicated proteins. **(C)** WT (top) or IKKα^{KO} (bottom) MEFs were treated with GT13072, GT13199, or anti-LTβR Ab for up to 24 hours. Whole cell extracts were immunblotted for NIK, cIAP1, and tubulin.
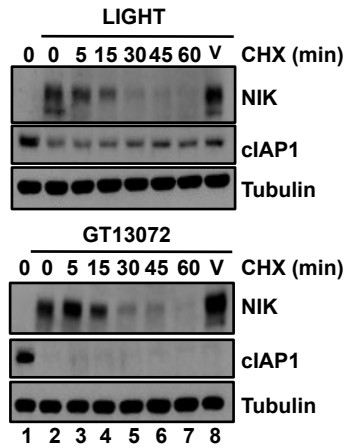
Figure 44. cIAP1 is not required for endogenous NIK turnover in MEFs. WT MEFs were stimulated with either LIGHT (top) or GT13072 (bottom) for four hours to stabilize NIK. Cells were then treated with 2.5 µg/ml cycloheximide (CHX) or vehicle control (V) for up to one hour. Cells were lysed and immunoblotted for the indicated proteins.
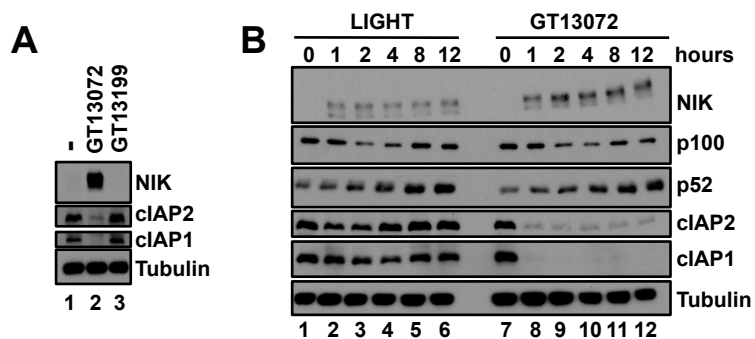
Figure 45. The Smac mimetic GT13072 stabilizes NIK in HeLa cells. (A) HeLa were treated for four hours with either 1 µM smac mimetic GT13072 or the inactive compound GT13199. Cells were lysed and immunoblotted with antibodies against the indicated proteins. **(B)** HeLa cells were treated with LIGHT or GT13072 for up to twelve hours. Lysates were immunoblotted with antibodies against the indicated proteins.

experiments that allow active NIK to be distinguished from the basally translated protein revealed

that negative regulation of NIK was intact in HeLa cells after GT13072-induced cIAP1/2

degradation (Fig. 46). These results thereby establish that endogenous cIAP1 and cIAP2 are

dispensable for the turnover of active NIK.

5.2.3. NIK turnover in the absence of cIAP1/2 occurs through the proteasome

Thus far we have established that active NIK turnover induced by LIGHT requires the

proteasome. Moreover, turnover of GT13072-stabilized NIK occurs in the absence of cIAP1 and

cIAP2 in both MEFs and HeLa cells. We therefore questioned whether GT13072-induced NIK

turnover, like that stimulated by LIGHT, required the proteasome. To specifically follow the

destabilization of active NIK induced by GT13072, we employed the co-treatment strategy

described in Figure 41. Similar to the effects in MEFs (Fig. 41A), treatment of HeLa cells with

MG132 stabilized basal NIK, a process that was dependent on new protein synthesis (Fig. 47).

Therefore, we next activated NIK using GT13072 and assessed the effect of MG132 on NIK

turnover following CHX treatment. Figure 48 shows that in both HeLa cells and MEFs, NIK

turnover is blocked by co-incubation of MG132 with CHX (compare lanes 3 and 4). Thus we

conclude that unlike basal NIK turnover, which absolutely requires cIAP1/2 (Vallabhapurapu,

Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008), proteasome-dependent turnover of active

endogenous NIK does not require the cIAPs. Together, our data support a model in which the

negative feedback control of IKKα-induced NIK turnover occurs via a novel proteasome-

dependent but cIAP-independent mechanism (Fig. 49).

5.3. Discussion

A growing body of evidence that the non-canonical NF-κB pathway plays a pathological

role in disease underscores the importance of fully elucidating mechanisms of NIK regulation.

Genetic alterations in the basal regulatory complex have been associated with multiple myeloma
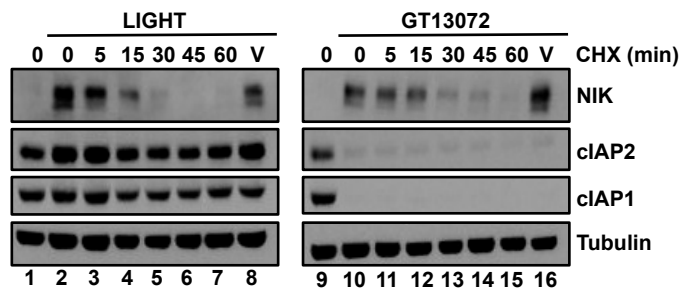
Figure 46. cIAP1/2 are not required for NIK turnover in HeLa cells. HeLa cells were stimulated with either LIGHT (left) or GT13072 (right) for four hours to stabilize NIK. Cells were then treated with 2.5 μg/ml cycloheximide (CHX) or vehicle (V) for up to one hour. Cells were lysed and immunoblotted with antibodies against the indicated proteins.
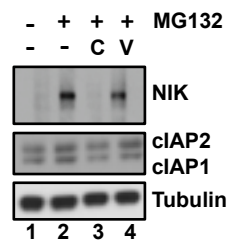
Figure 47. Concomitant translation and proteasome inhibition prevents NIK accumulation. HeLa cells were treated with 10 µM MG132 (+), MG132 plus 2.5 μg/ml cycloheximide (C), or MG132 plus vehicle control (V) for one hour. Whole cell lysates were immunoblotted with antibodies against the indicated proteins.
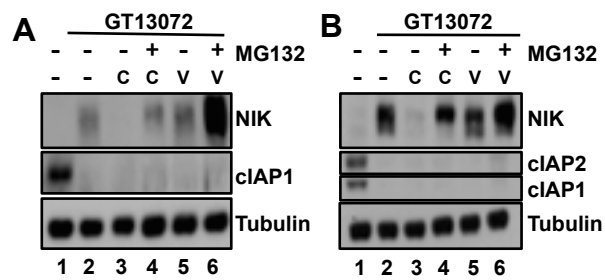
Figure 48. MG132 blocks NIK turnover in the absence of cIAP1/2. (A and B) WT MEFs (A) or Hela cells (B) were treated with GT13072 for four hours to stabilize NIK. Cells were then stimulated with 2.5 µg/ml cycloheximide (C) or cycloheximide plus 10 µM MG132 (+) for one hour. (V, vehicle control). Whole cell extracts were immunoblotted with antibodies against the indicated proteins.
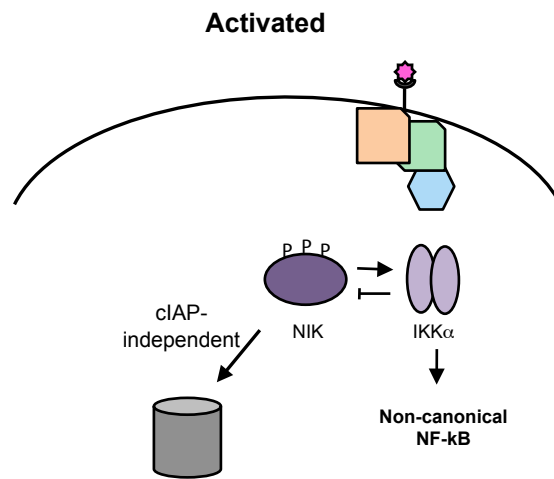
Figure 49. Model for IKKα-mediated NIK turnover. Upon receptor ligation, stabilized NIK (purple oval) activates the non-canonical NF-κB pathway. Once active, IKKα phosphorylates NIK (P), leading to its degradation via the proteasome (gray cylinder), which does not require cIAP1/2.

and melanoma in humans. (Annunziata, Davis et al. 2007; Keats, Fonseca et al. 2007; Dhawan, Su et al. 2008; Thu and Richmond 2010). More recently, blocking either the E3 ubiquitin ligase activity of cIAP2 or the negative turnover of active NIK have been shown to result in B cell hyperplasia and kidney nephropathy in mice (Conze, Zhao et al. 2010; Jin, Xiao et al. 2012). As regulatory mechanisms of non-canonical NF-κB signaling precisely control NIK stability, defining these mechanisms is crucial for the development of novel therapeutic strategies aimed at selectively manipulating this pathway.

IKKα-mediated negative feedback control of non-canonical NF-κB signaling requires the phosphorylation of NIK (Razani, Zarnegar et al. 2010). Here we show in both MEFs and HeLa cells that this post-translational modification directs the active kinase to the proteasome for degradation. The most common mechanism of signal-induced proteasomal targeting is ubiquitylation (Pickart 1997; Finley 2009), and previous overexpression studies have shown that NIK can be ubiquitylated by exogenous cIAP1 or cIAP2 (Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008; Razani, Zarnegar et al. 2010). Yet these experiments did not distinguish between ubiquitylation of basally translated NIK and NIK stabilized by activating ligands.

We have previously shown that TRAF3 is dispensable for active NIK turnover (Gray, Remouchamps et al. 2014). However, since cIAP1/2 are the only E3 ubiquitin ligases known to target NIK directly, we hypothesized that cIAP1/2 might play a TRAF3-independent role in the turnover of active NIK. Our results using the Smac mimetic GT13072 show that loss of cIAP1/2 has no effect on the turnover of active NIK. Thus we conclude that ubiquitylation of NIK by cIAP1 and cIAP2 is limited to only the newly synthesized protein controlled by the basal TRAF3:TRAF2 regulatory complex. Our data therefore suggest that phosphorylated NIK is ubiquitylated by a

currently unidentified E3 ubiquitin ligase that targets it for proteasomal degradation. One potential candidate is β-TrCP, which is required for the ubiquitylation of IκBs in the classical NF-κB pathway (Hayden and Ghosh 2008). Active NIK is phosphorylated by IKKα on three distinct serines (Ser^{809}, Ser^{812}, and Ser^{815}) (Razani, Zarnegar et al. 2010) that resemble the β-TrCP consensus sequence (DSGXXS) (Hayden and Ghosh 2008). Whether β-TrCP or another E3 ubiquitin ligase controls induced NIK turnover will be an important area of future research to selectively manipulate the duration of non-canonical NF-κB activity.

As we have ruled out the cIAPs as E3 ubiquitin ligases targeting NIK, an alternative possibility exists whereby active NIK is phosphorylated by IKKα and degraded by the 20S proteasome via a ubiquitin-independent mechanism. While such a mechanism is rare, several proteins including p53 and Ornithine Decarboxylase (ODC) can be degraded "by default" (Asher, Reuven et al. 2006; Finley 2009; Tsvetkov, Reuven et al. 2010). Our results cannot conclusively eliminate this possibility and ubiquitin-independent degradation of active NIK is an intriguing mechanism to consider.

Collectively our findings separate the molecular requirements for active NIK turnover from those involved in the basal regulation of newly translated protein, thus revealing a previously unappreciated layer of NIK regulation. Increasing reports of dysregulated non-canonical NF-κB signaling in disease (Thu and Richmond 2010; Razani, Reichardt et al. 2011; Sun 2011) and the critical role of NIK stabilization in non-canonical signaling (Sun 2010) underscore the importance of fully elucidating the mechanism of active NIK turnover to develop novel therapeutic strategies to terminate the non-canonical NF-κB signaling pathway.

CHAPTER 6. CONCLUSIONS AND IMPLICATIONS

6.1. Progress and outstanding questions

        In this thesis, we set out to investigate regulatory mechanisms of non-canonical NF-κB

signaling. We have contributed to the current understanding of non-canonical NF-κB signal

transduction with three major findings: IKKα, independent of its association with NEMO, is

sufficient to activate the non-canonical signaling pathway and feed back to promote the negative

turnover of active NIK (Chapter 3); Homeostatic classical NF-κB activity is required to suppress

basal NIK amounts and maintain the quiescent state of the non-canonical pathway in resting cells

(Chapter 4); Active NIK turnover is proteasome-dependent but does not require cIAP1/2 (Chapter

5). These findings advance our understanding of a newly described mechanism of negative

feedback regulation on non-canonical NF-κB activity and importantly, prompt reevaluation of the

current paradigm, which indicates that NEMO plays no role in non-canonical NF-κB signaling.

        While these results increase our knowledge of non-canonical NF-κB biology, they also

raise important questions that will be discussed in detail below. For example, IKKα does not need

to associate with NEMO to promote non-canonical signaling, however IKKα does have a

functional NBD. Therefore it may be activated by NIK as part of the heterotrimeric IKK complex;

alternatively, the possibility remains that IKKα exists as a cytoplasmic homodimer to promote

non-canonical NF-κB signaling. Furthermore, we show that NEMO and NIK associate in an IKKα-

dependent manner but the functional relevance of this remains unclear.

        A major finding from this thesis work is that homeostatic classical NF-κB activity is

required to negatively regulate basal NIK, however we do not know what gene(s) are driven by

classical NF-κB to suppress NIK. In that regard, it will be important to understand how this gene

or genes control basal NIK. We also correlate hyperactive non-canonical NF-κB signaling due to

loss of classical NF-κB activity with hyper-IgA in NEMO-ID, but it remains to be determined

whether dysregulated cross talk between NF-κB signaling pathways drives class switch to IgA in

NEMO-ID patients.

In seeking to understand the mechanism of active NIK turnover, we show that

phosphorylated NIK is degraded via the proteasome in a cIAP-independent manner. It will be

important to elucidate whether active NIK is directed to the proteasome by ubiquitylation; if so,

what E3 ubiquitin ligase facilitates this process.

Finally, our results suggest that an intact IKK complex is required to maintain the resting

state of non-canonical NF-κB. Therefore, we reconsider use of the NBD peptide (NBDp) as an

anti-inflammatory therapeutic in diseases where classical NF-κB signaling is targeted by this

peptide. Additionally, we consider whether small molecular inhibitors of classical NF-κB remain

an appropriate approach to cancer therapy. Ultimately, the elucidation of these answers will lead

to exciting advances in our understanding of non-canonical NF-κB signal transduction.

6.2. IKKα homodimers may exist *in vivo*, but may not be functionally relevant for non-canonical NF-κB activity

We used a biochemical approach to show that the interaction between IKKα and NEMO

is dispensable for induced p100 processing and NIK turnover. However, this complementation

approach in IKKα^{KO} MEFs does not determine whether IKKα exists independent of the

heterotrimeric IKK complex to perform these functions *in vivo*. It is possible that cells contain a

cytoplasmic pool of IKKα that, separate from NEMO and IKKβ, facilitates non-canonical NF-κB

signaling. To begin to investigate whether cells contain a cytoplasmic pool of IKKα independent

of the heterotrimeric IKK complex, we performed a series of NEMO immunodepletions in WT

MEFs. We surmised that elimination of NEMO-bound IKKα would allow us to determine whether

the proposed "IKKα-alone" complex remained in the immunodepleted samples. We

immunoprecipitated NEMO from WT MEF whole cell extracts to deplete cells of the heterotrimeric

IKK complex. Subsequent rounds of IP completely eliminated detectable levels of NEMO and IKKβ (Fig. 50A). Importantly, after three rounds of successive NEMO IP, samples still contained a detectable amount of IKKα (Fig. 50B, lane 4). This experiment indicates that WT MEFs do indeed contain a pool of IKKα that is independent of the heterotrimeric IKK complex; it must be repeated to see if this is a consistent result. While this finding in isolation does not determine whether IKKα-alone or NEMO-bound IKKα is activated by NIK, it still supports the genetic model that IKKα-alone does exist in whole cells.

Alternatively, the cytoplasmic IKKα observed in this experiment may carry out the kinase-independent function of IKKα described for keratinocyte differentiation (Hu, Baud et al. 2001) or the nuclear role of IKKα in Histone-H3 phosphorylation (Anest, Hanson et al. 2003; Yamamoto, Verma et al. 2003). Thus, while the association between NEMO and IKKα is not required for non-canonical signaling, IKKα engaged in non-canonical signaling may be part of the heterotrimeric IKK complex. In support of this notion, we have found that NIK associates with NEMO via IKKα when NIK is stabilized with MG132 (Chapter 4). It will be important to investigate whether this interaction persists when cells are stimulated through the LTβR to activate the non-canonical NF-κB pathway. If it does, this would suggest that IKKα involved in non-canonical signaling actually does bind NEMO *in vivo*, despite the association with NEMO not being absolutely necessary for non-canonical activation.

6.3. Classical IKK activity constrains basal NIK

While investigating the role of the IKKα:NEMO interaction by reconstitution of a C-terminal truncation mutant of IKKα, we performed parallel experiments in NEMO-deficient cells (Chapter 3). These studies led to the surprising result that NEMO and catalytically competent IKKβ are required to suppress basal p100 processing. Interestingly, IKKα kinase activity and elevated p52 levels were both observed in resting NEMO^{KO} MEFs in a seminal study that led to the discovery of the non-canonical NF-κB pathway (Dejardin, Droin et al. 2002). At that time the
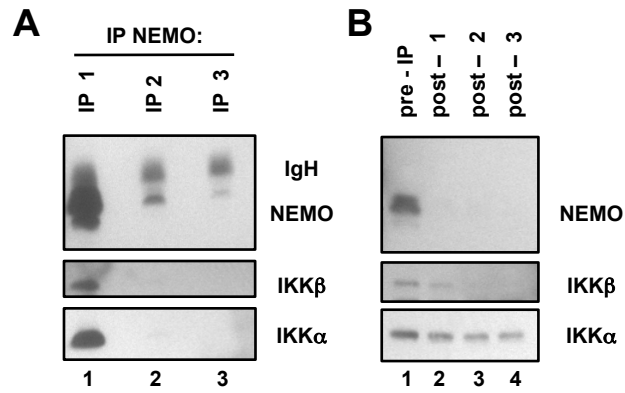
Figure 50. A cytoplasmic pool of IKKα exists independent of the heterotrimeric IKK complex. (A) WT MEFs were lysed and the heterotrimeric IKK complex was immunoprecipitated using anti-NEMO antibodies. Two subsequent rounds of immunodepletion were performed on the same samples. **(B)** Whole cell lysates prior to immunodepletion (pre-IP) and lysates following each round of IP (post-) were immunoblotted using the indicated antibodies.

authors suggested that these results were due to inherent IKKα activity that is constrained when the kinase associates with NEMO (May, D'Acquisto et al. 2000; Dejardin, Droin et al. 2002). However, we show that IKKα is basally active in IKKβ-deficient MEFs, and we have previously shown that NEMO:IKKα complexes exist in these cells (Solt, Madge et al. 2007). Therefore, loss of the NEMO:IKKα association does not fully explain the basal IKKα catalytic activity in NEMO^{KO} cells. Moreover, we show that NEMO- and IKKβ-deficient MEFs have elevated NIK amounts prior to stimulation, which could explain how IKKα becomes activated in these cells. Finally, reconstitution of IKKβ^{KO} MEFs with a WT version of IKKβ reduces basal NIK amounts and IKKα activity. Together these results lead us to conclude the classical IKK complex is required to constrain non-canonical NF-κB activity via NIK regulation, which challenges the current model of NEMO-independent non-canonical NF-κB signaling.

We have shown that NEMO and IKKβ must associate to regulate non-canonical NF-κB signaling. Furthermore, the IKK complex must be catalytically competent for this function, as basal NIK is not properly constrained in cells that express a kinase dead mutant of IKKβ or have hypomorphic NEMO mutations that preclude classical NF-κB activity. Our finding that NEMO associates with NIK suggests that IKKβ may phosphorylate NIK directly to promote basal NIK turnover. We cannot rule out this mechanism based on the studies presented here, and it will be interesting to ascertain whether IKKβ, like IKKα (Razani, Zarnegar et al. 2010), has any kinase activity towards NIK *in vitro*. However, the similar phenotype of elevated basal NIK in cells that lack the prototypic NF-κB protein p65 led us to pursue the alternative hypothesis that classical NF-κB transcriptional activity was required for basal NIK regulation.

6.4. Homeostatic NF-κB activity drives the expression of a novel factor required for basal NIK degradation

Due to its prominent role in activation-induced gene expression, NF-κB is considered a

family of inducible transcription factors (Hayden and Ghosh 2008). Despite our minimal

mechanistic understanding, the concept of basal NF-κB activity is not new (Baltimore 2011). For

example, resting cells in the latent phase of signaling exhibit basal NF-κB activity measured by

EMSA (Claudio, Brown et al. 2002; Notarangelo, Lanzi et al. 2006). Similarly, a cold competitor

probe decreases basal κB binding in WT MEFs (Dejardin, Droin et al. 2002). Moreover, a

decrease in homeostatic NF-κB activity is observed in cell lines when individual IKK subunits are

deleted (Hu, Baud et al. 1999; Li, Chu et al. 1999; Tanaka, Fuentes et al. 1999). The gene targets

of this homeostatic signaling have yet to be defined, however the important interplay we describe

here between the classical and non-canonical pathways highlights the importance of investigating

this basal NF-κB activity.

In accordance with the hypothesis that NIK is constrained by classical NF-κB-driven

transcription, we found that enforced classical NF-κB activity by the over-expression of WT IKKβ

in NEMO-deficient cells restored basal NIK amounts. We thus considered that classical NF-κB

might drive the expression of genes encoding the proteins required for basal NIK degradation.

However, cIAP1/2, TRAF2, and TRAF3 protein amounts were similar in all of the cell lines we

tested. Furthermore, exogenously expressed NIK was able to associate with these proteins in

NEMO- and p65-deficient MEFs. These results suggest that classical NF-κB transcriptional

activity is not required for the interaction of NIK with the previously described basal degradation

complex. It remains possible that classical NF-κB is required for the transcription of genes whose

products are required to promote NIK ubiquitylation by this complex. We favor this possibility

because diverse genetic mutations affecting components of this complex lead to NIK stabilization

and multiple myeloma in humans (Annunziata, Davis et al. 2007; Keats, Fonseca et al. 2007).

Furthermore, the studies that first described the TRAF2:TRAF3:cIAP1/2 basal regulatory complex

used over-expression and complementation of knockout MEFs to show that the coordinated

efforts of these proteins promoted NIK ubiquitylation and degradation (Varfolomeev, Blankenship

et al. 2007; Vallabhapurapu, Matsuzawa et al. 2008; Zarnegar, Wang et al. 2008). Thus an endogenous NF-κB-dependent factor required to activate the ubiquitin ligase complex may not have been obvious in this system.

A growing body of literature suggests that basal NIK degradation facilitated by the TRAF2:TRAF3:cIAP1/2 complex may be regulated by additional factors (Lavorgna, De Filippi et al. 2009; Hupalowska, Pyrzynska et al. 2012; Choudhary, Kalita et al. 2013; Yamaguchi, Oyama et al. 2013). TRAF1 has been proposed to positively regulate non-canonical NF-κB activity in B (Lavorgna, De Filippi et al. 2009) and T (Choudhary, Kalita et al. 2013) lymphocytes. In B cells, TRAF1 may interact with BAFF-R, promoting TRAF3 degradation and NIK stabilization, though the physiologic source of TRAF1 is unknown (Lavorgna, De Filippi et al. 2009). In T cells, TRAF1 is induced by CD3 signaling. In this model, TRAF1 associates with NIK, displacing it from the TRAF2:TRAF3:cIAP1/2 complex and thus relieving the negative regulation on basal NIK protein (Choudhary, Kalita et al. 2013). Additionally, the anti-apoptotic protein A20 has been shown to positively regulate non-canonical signaling, as LTβR, Fn14, or RANK signaling do not induce NIK accumulation in A20-deficient MEFs (Yamaguchi, Oyama et al. 2013). In WT MEFs, LTβR engagement induces A20 concomitant with NIK expression. Exogenously expressed A20 associates directly with cIAP1, blocking its interaction with TRAF2 and inhibiting the function of the TRAF2:TRAF3:cIAP complex (Yamaguchi, Oyama et al. 2013). Disruption of the basal degradation complex by A20 permits NIK stabilization and has been described as a "molecular switch" between classical and non-canonical NF-κB activity downstream of the LTβR (Yamaguchi, Oyama et al. 2013). Finally, Adaptor Protein Containing Pleckstrin Homology Domain, Phosphotyrosine Binding Domain, and Leucine Zipper Motif 1 (APPL1) has been shown to promote NIK stabilization by disruption of the basal regulatory complex (Hupalowska, Pyrzynska et al. 2012). Because each of these proteins positively influences non-canonical NF-κB signaling, we do not anticipate TRAF1, A20, or APPL1 being the classical NF-κB gene target implicated in our studies. However, a negative regulator of any one of these factors may be

maintained by classical NF-κB transcription, the loss of which could prevent constitutive NIK turnover and promote non-canonical signaling in resting cells. Ultimately, these experiments illustrate that basal NIK regulation by TRAF2:TRAF3:cIAP1/2 may be more complex than originally described.

6.5. Hyperactive non-canonical NF-κB may induce hyper IgA in NEMO-ID

The presentation of hyper IgA in NEMO-ID is especially perplexing in light of the positive role for NEMO in class switch recombination (Notarangelo, Lanzi et al. 2006). Coupled with the recently reported role for NIK in class switch to the IgA isotype in the mouse model (Jin, Xiao et al. 2012), we investigated non-canonical NF-κB signaling in NEMO-ID. We found that samples from patients with two distinct mutations in the C-terminal zinc finger of NEMO (Q403X and C417R) exhibited high ratios of p52:p100, indicative of basal p100 processing and non-canonical NF-κB activity. Furthermore, NEMO^{Q403X} and NEMO^{C417R} MEFs have elevated basal NIK compared to NEMO^{WT} controls. Our findings correlate classical NF-κB signaling defects with dysregulated NIK and hyper IgA in NEMO-ID, and we think it will be important to monitor NIK expression and non-canonical NF-κB signaling in future clinical cases of this disease. However, it remains to be proven that hyper IgA is directly driven by elevated NIK due to decreased classical NF-κB activity. To test this, we would assess class switch recombination (CSR) in B cells from mice that have a *lox*P-flanked *Ikbkb* gene. *Ex vivo* deletion of IKKβ in mature splenic B cells using the Tat-Cre recombinase would permit us to test whether loss of IKKβ induces NIK accumulation and p100 processing. Importantly, we would perform CSR assays to see if these cells preferentially switch to the IgA isotype, further implicating aberrant NIK expression in this process. If IgA switching was not preferred in IKKβ-deficient cells crossed onto a NIK-deficient background, we would conclude that aberrant NIK expression, due to disrupted classical NF-κB activity, drives hyper IgA.

6.6. Hypomorphic NEMO mutations may reveal novel factors required for basal NIK regulation

While we describe dysregulated NIK and basal p100 processing associated with three NEMO-ID mutations, Hyper IgA is not a ubiquitious clinical finding in NEMO-ID. In a database of 45 patients with common hypomorphic NEMO mutations, 30% (6/20) were reported to have Hyper IgA, whereas approximately 12% of patients (2/17) reportedly had hyper IgM (Hanson, Monaco-Shawver et al. 2008). The latter finding is consistent with a role for NF-κB signaling in CSR downstream of CD40 (Notarangelo, Lanzi et al. 2006). Interestingly, classical NF-κB signaling is critical for B cell development and maintenance (Pasparakis, Schmidt-Supprian et al. 2002; Claudio, Brown et al. 2006), however B lymphocyte numbers were normal in most patients with hypomorphic NEMO mutations (Hanson, Monaco-Shawver et al. 2008). Therefore it is clear that these particular NEMO mutants can transmit some classical NF-κB signals to support B cell development.

As NEMO-ID is a heterogeneous disease with various defects leading to immunodeficiency, it is possible that certain mutations permit particular classical NF-κB signals required for basal NIK regulation that are disrupted in other hyper IgA-associated NEMO mutations like Q403X and C417R. Accordingly, it would be intriguing to assess non-canonical NF-κB signaling in a cell with a hypomorphic NEMO mutation that has not been associated with hyper IgA. We would like to analyze different mutations in the zinc finger of NEMO because we, and others (Makris, Roberts et al. 2002), have shown that mutations in this domain prevent classical NF-κB signaling but not IKK complex formation. There are competing reports as to whether nonsense mutations at E391X result in Hyper IgM (Orange, Jain et al. 2004) or Hyper IgA (Hanson, Monaco-Shawver et al. 2008). However, 6/6 patients with frameshifts at the adjacent residue E390 (E390fs) reportedly had hypogammaglobulenemia and Hyper IgM with no Hyper IgA (Hanson, Monaco-Shawver et al. 2008). Additionally, the R319Q mutation is

associated with classical NF-κB defects but normal IgA serum levels. We hypothesize that non-canonical NF-κB signaling would be properly regulated in NEMO^{KO} MEFs reconstituted with E390fs or R319Q mutant NEMO. If basal NIK amounts were low and p100 was unprocessed in resting NEMO^{E390fs} or NEMO^{R319Q} cells, we would compare RNA expression profiles of WT and NEMO-deficient cells reconstituted with E390fs or R319Q versus Q403X or C417R (Nagalakshmi, Wang et al. 2008; Wilhelm, Marguerat et al. 2008). We anticipate that this analysis may reveal a novel gene product or products important for basal NIK regulation that are expressed at similar amounts in WT, NEMO^{E390fs} and NEMO^{R319Q} cells but are under-expressed in the NEMO^{Q403X} and NEMO^{C417R} cells. We believe this will be a more targeted approach to identifying the gene(s) required for basal NIK regulation than comparing WT and NEMO^{KO} or p65^{KO} MEFs, for example, because of the broad spectrum of genes controlled by classical NF-κB signaling.

6.7. Does ubiquitin target active NIK for proteasomal degradation?

Similar to constitutive NIK degradation in a resting cell, we have shown that active NIK turnover occurs via the proteasome (Chapter 5). However, unlike the basal mechanism, cIAP1/2 are dispensable for the IKKα-mediated turnover of active NIK. Thus we conclude that active, phosphorylated NIK is ubiquitylated by a different E3 ubiquitin ligase, which targets NIK for proteasomal degradation. In chapter 5 we suggested that the most likely candidate for this ubiquitin-dependent mechanism is β-TrCP. β-TrCP is the E3 ubiquitin ligase responsible for IκB ubiquitylation during the induction of NF-κB responses (Kanarek and Ben-Neriah 2012). Furthermore, the serine residues phosphorylated by IKKα (Razani, Zarnegar et al. 2010) resemble the β-TrCP recognition sequence (DSGXXS) (Kanarek and Ben-Neriah 2012). Future studies should be performed to assess active NIK turnover in the absence of β-TrCP.

Our finding that the turnover of NIK absolutely requires the proteasome strongly suggests that active NIK is ubiquitylated. Despite intense efforts, we have been unable to detect

ubiquitylation of endogenous NIK under any conditions. However, we have preliminary evidence to suggest that exogenous NIK can be ubiquitylated under activating conditions, even in the absence of cIAP1/2 (Fig 51). While extensive work remains to establish that this experimental system definitively shows the ubiquitylation of active, exogenous NIK, we believe this may be a promising approach to answer this important remaining question. We aim to compare the ubiquitylation status of exogenous WT and mutant NIK 3SA, in which the serine residues phosphorylated by IKKα are mutated to alanines (Razani, Zarnegar et al. 2010) and therefore NIK 3SA is unable to be turned over. We surmise that the mutant NIK will only be ubiquitylated under basal conditions when the proteasome is inhibited and will not be ubiquitylated under activating conditions, whereas WT NIK will be ubiquitylated under both basal and activating conditions. Such a result would indicate that this experimental approach accurately assesses active NIK ubiquitylation downstream of IKKα-phosphorylation. Ultimately, we hypothesize that exogenous active WT NIK will be ubiquitylated in the absence of cIAP1/2, as our studies show that active NIK turnover via the proteasome is cIAP-independent (Fig. 49).

While our preliminary data suggests that active NIK may be ubiquitylated prior to degradation, ubiquitin-independent proteasomal processing has been described. This type of degradation "by default" occurs via the 20S catalytic core component of the proteasome (Asher, Reuven et al. 2006). The 26S proteasome is comprised of the 20S core particle and two 19S regulatory caps, which bind to and unfold ubiquitylated proteins for regulated degradation via the 26S proteasome (Kisselev, van der Linden et al. 2012). The most common proteasomal inhibitors MG132 and bortezomib block enzymatic activity of the 20S core particle. Thus they inhibit regulated proteasomal degradation as well as "default" protein turnover.

We have shown that inhibition of the 20S core particle using MG132 and bortezomib prevents NIK turnover (Chapter 5). A new class of proteasome inhibitors blocks the ubiquitin
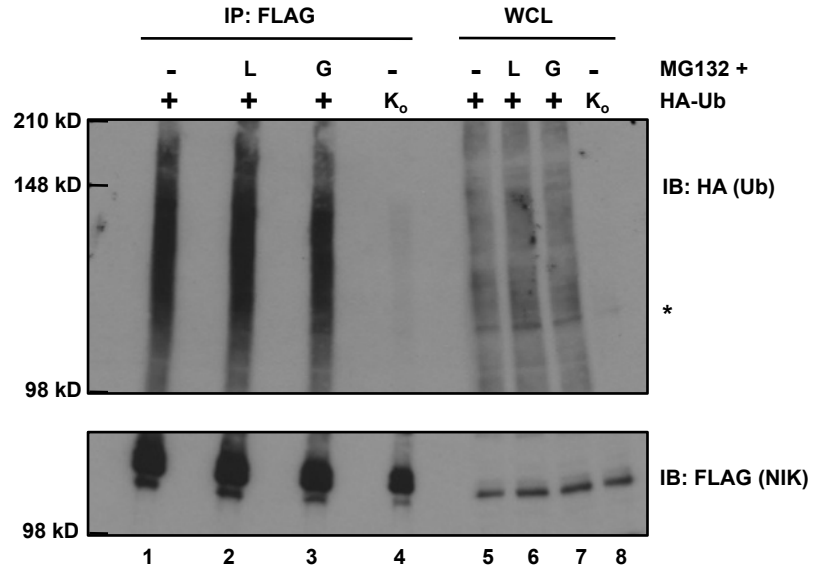
Figure 51. NIK may be ubiquitylated in the absence of cIAP1/2. HeLa cells were transfected with CMV-pFLAG WT NIK and either WT HA-Ub (+) or mutant HA-Ub (K_o). Twenty-four hours later, cells were stimulated with 10 μM MG132, with or without (-) either LIGHT (L) or GT13072 (G) for 4 hours. Cells were subsequently incubated with CHX for 90 minutes and lysed. Exogenous NIK was immunoprecipitated (IP) with anti-FLAG (M2) beads, and proteins were immunoblotted with antibodies against HA and FLAG. Ten percent of the IP input (whole cell lysate, WCL) was run in parallel to indicate transfection efficiency. An HA-Ub band corresponding to the electrophoretic mobility of FLAG NIK is indicated by the asterisk (*).

binding function of the 19S cap protein (Kisselev, van der Linden et al. 2012). We have tested

one such inhibitor to take an endogenous approach to determine whether proteasome-dependent

active NIK turnover is regulated by ubiquitin, however the results are inconclusive and require

further work. We hypothesize that a block in NIK turnover in the presence of the 19S cap inhibitor

would indicate that active NIK degradation occurs via the 26S proteasome and is therefore

facilitated by ubiquitin. Selectively targeting the 19S cap will be important to determine whether

ubiquitin facilitates endogenous NIK turnover because we are currently limited to using

exogenously expressed NIK in ubiquitylation assays. A more complete understanding of the

molecular requirements for endogenous NIK turnover is necessary to determine whether this

negative feedback mechanism is a potential therapeutic target for autoimmune diseases

associated with accumulated NIK (Salomonsson, Larsson et al. 2002; Jin, Zhou et al. 2009).

6.8. Implications for the NBDp as a classical NF-κB inhibitor

IKKα and IKKβ associate with the N-terminal α-helix of NEMO via a six amino-acid

sequence in their C-termini (LDWSWL) (May, D'Acquisto et al. 2000; May, Marienfeld et al.

2002). Our lab has developed a cell-permeable peptide encompassing this NBD sequence, which

disrupts the NEMO:IKK interaction (Fig. 52A) and prevents inducible classical NF-κB activation

(May, D'Acquisto et al. 2000). The NBDp has been employed in many cellular and *in vivo* studies

and is now well regarded as a potent and effective classical NF-κB inhibitor (Tas, Vervoordeldonk

et al. 2006; Acharyya, Villalta et al. 2007; Gaurnier-Hausser, Patel et al. 2011). However, the

effects of classical NF-κB inhibition via the NBDp on non-canonical NF-κB signaling have never

been described. We now understand that classical NF-κB activity is required for basal regulation

of the non-canonical NF-κB pathway, which prompts evaluation of non-canonical signaling after

NBDp treatment.

In preliminary studies we found that treatment with the NBDp alone led to p100

processing in WT MEFs (Fig. 52B). NBDp-induced p100 processing was similar to that induced

by cross-linking the LTβR (Fig. 52B lane 8). NBDp efficiency was measured by its ability to block TNF-induced classical NF-κB activity (Fig. 52C), and classical NF-κB inhibiton by the NBDp was not complete in this experiment. Importantly, p100 was not processed to p52 when cells were treated with a mutant version of the NBDp that could not disrupt IKK activity (Fig. 52B lane 7, and Fig. 52C). NBDp treatment also induced p100 processing to p52 in HUVEC (M.J.M., unpublished observations), leading us to believe that disruption of the resting heterotrimeric IKK complex by NBDp administration may lead to NIK accumulation.

While we observed p100 processing after NBDp treatment, we have been unable to observe NIK stabilization in NBDp-treated cells. Several possible explanations exist for this. First, the NBDp did not completely block classical NF-κB activity measured by EMSA (Fig. 52C). Thus, weakened classical activity may permit NIK stabilization such that IKKα becomes activated to phosphorylate p100, but NIK protein amounts remain below the level of detection by immunoblot. In fact, WT NBDp treatment alone was originally shown to enhance basal NF-κB activity two-fold (May, D'Acquisto et al. 2000). While this was interpreted as intrinsic kinase activity that is normally constrained by the association of IKKα and IKKβ with NEMO, it is interesting to imagine this increase in κB-driven luciferase activity was due to stabilization of NIK, as aberrant NIK expression has been shown to induce classical NF-κB activity (Malinin, Boldin et al. 1997; Ramakrishnan, Wang et al. 2004; Zarnegar, Yamazaki et al. 2008; Neely, Brose et al. 2010). This cross-talk between NIK and classical NF-κB is important to consider in potential cases of NBDp-resistance.

We can also envision a scenario whereby the NBDp blocks basal NF-κB activity such that NIK accumulates, but is rapidly degraded by IKKα-mediated negative feedback and thus NIK remains below the level of detection by immunoblot in this timecourse. Further experimentation is required to fully understand the effects of classical NF-κB inhibition by NBDp treatment on non-canonical NF-κB activity.

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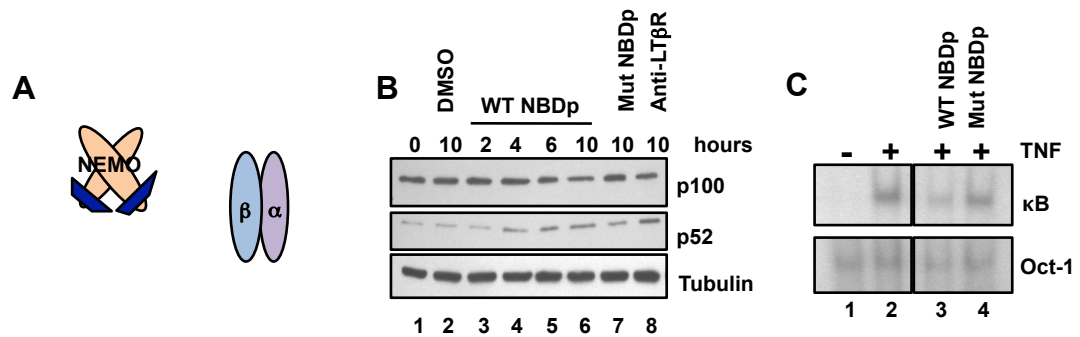
Figure 52. The NEMO Binding Domain peptide (NBDp) induces p100 processing in MEFs.
(A) Model showing how the NBDp (blue bars) disrupts the interaction of NEMO with IKKα/β. **(B)** WT MEFs were incubated with either the WT or mutant (Mut) NBDp, DMSO (vehicle) or anti-LTβR for the times indicated. Whole cell lysates were immunoblotted for p100 processing and tubulin was visualized as a loading control. **(C)** WT MEFs were either untreated (lanes 1 and 2) or pretreated with WT or mutant NBDp for two hours before stimulation with TNF for 30 minutes (+). Classical NF-κB activation was detected by EMSA using a specific κB probe and equal loading was determined using a probe bound by Oct-1. These data demonstrate that the WT NBD peptide used in panel **(B)** inhibited TNF-induced NF-κB activation.

While our preliminary results indicate the NBDp should be used with caution, this inhibitor has been proven to potently inhibit inducible classical NF-κB activity (May, D'Acquisto et al. 2000). This makes it a desirable therapeutic for inflammatory diseases driven by excessive classical NF-κB signaling. Furthermore, because off-target effects of the peptide have not been reported, we do not forsee the interplay of classical and non-canonical NF-κB signaling hindering the utility of this approach *in vivo.*

6.9. Classical NF-κB activity as a target for cancer therapy

An important advance in cancer treatment has been the development of Smac mimetics. These cIAP antagonists have been developed as anti-cancer drugs because cIAPs are often overexpressed in human malignancies and protect cancer cells from normal inducers of cell death (Gyrd-Hansen and Meier 2010). Smac mimetics lead to cIAP auto-ubiquitylation and degradation, which in turn promotes caspase activation and induction of apoptotic signaling. cIAP antagonists also block NF-κB activity downstream of the TNFR; thus these small molecules have a two-pronged approach to cancer treatment by directly promoting cell death and inhibiting pro-survival gene programs mediated by classical NF-κB (Gyrd-Hansen and Meier 2010). Accordingly, Smac mimetics have exquisite specificity for cancerous cells and transformed cell lines, with little effect on unmalignant primary cell cultures (Varfolomeev, Blankenship et al. 2007) (McCorkell *et al.* – manuscript in preparation). This is attributed to the apparent addiction of cancer cells to cIAP-dependent pro-survival signaling mediated by classical NF-κB. When cIAPs are ablated by Smac mimetics, autocrine TNF signaling promotes cell death, thus enhancing the effectiveness of this novel class of inhibitors.

We and others (Varfolomeev, Blankenship et al. 2007) show a Smac mimetic also activates the non-canonical NF-κB pathway through loss of cIAP and stabilization of NIK. While loss of cIAP function will sensitize that particular cell to TNF-induced cell death, rapid non-

canonical NF-κB activity after Smac mimetic treatment may lead to the induction of genes (ie., CXCL12, CXCL13, CCL19, and CCL21) whose products may promote lymphocyte recruitment to the tumor microenvironment. It will be important to discern whether this corollary activation of non-canonical NF-κB actually enhances treatment or causes certain tumors to be resistant to Smac mimetic therapy.

Diffuse Large B cell Lymphoma (DLBCL) relies on classical NF-κB for the transcription of pro-proliferative (ie., CyclinD1) and anti-apoptotic genes (ie., Bcl-2, cFLIP, XIAP) (Feuerhake, Kutok et al. 2005; Lam, Davis et al. 2005; Davis, Ngo et al. 2010; Gaurnier-Hausser, Patel et al. 2011). Therefore inhibitors of IKKβ have been developed to treat this lymphoid malignancy (Lam, Davis et al. 2005). The studies presented here spark concern for the efficacy of IKK inhibitors in lymphoma, as we find elevated basal NIK in cells that lack classical NF-κB activity. Moreover, constitutive BAFF-R signaling promotes NF-κB activity and B cell proliferation via NIK stabilization in DLBCL (Pham, Fu et al. 2011). Thus exacerbated non-canonical NF-κB activity due to IKKβ inhibition may promote BAFF-independent B cell survival and tumor persistence in this disease.

6.10. Conclusion

The overarching goal of these studies was to understand the molecular mechanisms regulating non-canonical NF-κB signal transduction. We demonstrate that classical NF-κB signaling limits the basal pool of NIK, identifying a novel function for the classical IKK complex and furthermore, classical NF-κB signaling in the negative regulation of the non-canonical NF-κB pathway. Thus the previous paradigm for two distinct NF-κB signaling pathways is incomplete and must be revised to include the contribution of classical NF-κB activity to basal NIK suppression in order to maintain the quiescent state of the non-canonical NF-κB pathway. Our studies also advance the current understanding of a negative feedback loop to control duration of non-canonical NF-κB signaling. We show that like basal NIK, active NIK is degraded via the

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proteasome, but the molecular events facilitating this turnover are unique. The comprehensive signaling model described in this thesis (Fig. 53) will be especially critical to consider when developing inhibitors of classical NF-κB activity, as perturbation of this pathway promotes non-canonical NF-κB signaling.
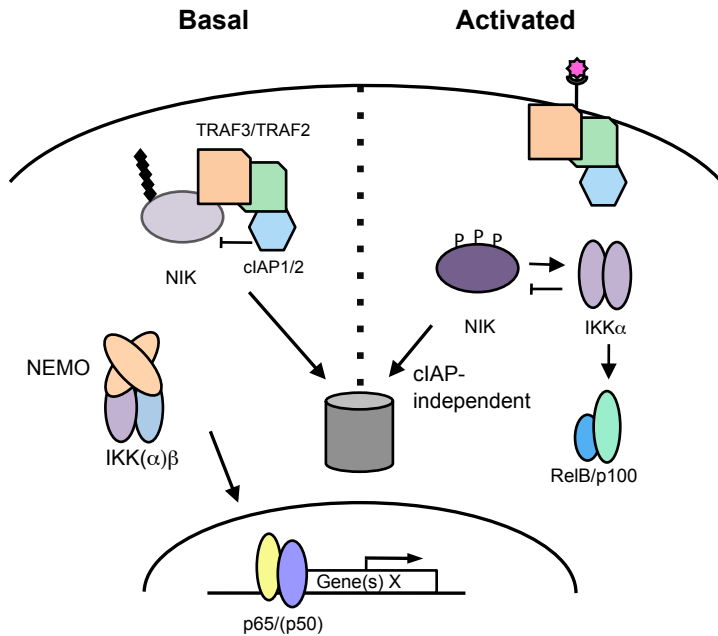
Figure 53. Comprehensive model for non-canonical NF-κB regulation. Under resting conditions (Basal), NIK (pale purple oval) is bound to TRAF2:TRAF3:cIAP1/2. This association facilitates the constitutive ubiquitylation and proteasomal degradation of NIK. However, the constraint of basal NIK protein amounts also requires p65-mediated classical NF-κB transcription. The exact gene target of classical NF-κB (Gene X) remains to be determined. Upon receptor ligation (Activated), NIK accumulates (dark purple oval) and activates IKKα, which in turn feeds back to phoshporylate (P) NIK. Phosphorylated NIK is degraded by the proteasome in a cIAP-independent manner.

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