THE ROLE OF NF-κB INDUCING KINASE (NIK) IN MODULATING MELANOMA TUMORIGENESIS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

December, 2011

Nashville, Tennessee

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DEDICATION

To my parents, who have always been devoted to our education

To my husband, who has always been there for me

In memory of (Saya) Win Aung

ACKNOWLEDGMENTS

First, I would like to acknowledge my advisor, Ann Richmond, who is essential in finishing this work. She has provided me an environment in which I can become an independent scientist. She has also helped me with my career development. I also would like to thank my committee members, Josiane Eid, Dell Yarbrough, Tom Thomas, Fiona Yull and Wasif Khan. They have given me valuable suggestions and guidance during my training. I am also grateful to past and present Richmond lab members. They are wonderful people to work with and help me get through difficult days. Specifically, I would like to thank Yingjun Su who has taken the time to train me during my early days in the lab. He is a great colleague, friend and teacher. I would like to thank Tammy Sobolik, who has been a great friend and colleague. I appreciate the help and support from Cancer Biology students and staff, especially Tracy Tveit, who have made my time in the department easier.

My acknowledgment would not be complete without mentioning my wonderful friends outside of the lab. These include Wan-Hsin Lin, my classmate and close friend; Seint Lwin, a wonderful friend who has always listened to my failure and success.

I am also grateful for my wonderful family who has supported me in every way that they can. Lastly, I would like to acknowledge my husband who has been incredibly supportive in every choice that I make. He has always shared my frustration and happiness during my graduate career.

This work was supported by NIH grant CA 098807-01 and the grant from Department of Veteran Affairs to AR.

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β-ME	β-mercaptoethanol
β-TrCP	β-transducin repeat containing protein
17-AAG	17-allylamino-17-demethoxygeldanamycin
A	alanine
Aly	alymphoplasia
Amp	Ampicillin
APC	adenomatous polyposis coli
BAFF	B cell activating factor belonging to the TNF family
Bcl10	B-cell CLL/lymphoma 10
Bcl2	B-cell CLL/lymphoma 2
Bcl3	B-cell CLL/lymphoma 3
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
BSA	bovine serum albumin
CCND2	cyclin D2
CD28RE	CD28-responsive enhancer
CD40L	CD40 ligand
CDK	cyclin dependent kinase
cDNA	complementary DNA
cFLIP	cellular FLICE like inhibitory protein
ChIP	chromatin immunoprecipitation
cIAP	cellular inhibitors of apoptosis
CK	Casein kinase

CO_2	Carbon dioxide
Cot1	cancer Osaka thyroid-1
COX-2	cyclooxygenase-2
DCs	dendritic cells
DDX5	DEAD box polypeptide 5
DED	death effector domain
dH_2O	deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunoadsorbent assay
EMSA	electrophoretic mobility shift assay
Erb	estrogen receptor
ERK	extracellular signal-regulated kinases
FADD	Fas-associating protein with death domain
FBS	fetal bovine serum
FDA	Federal Drug Administration
Foxp3	forkhead box p3
G	glycine
GITR	glucocorticoid-induced TNFR family-related gene
Grb	growth factor receptor bound
GSK3β	glycogen synthase kinase 3β
GST	glutathione S transferase
H_2O_2	hydrogen peroxide

HBSS	Hank's balanced salt solution
HHV8	human herpesvirus 8
Hsp90	heat shock protein 90
HVEM	herpes virus entry mediator
IκB	inhibitor IκB
ICAM-1	intercellular adhesion molecule-1
ICOSL	inducible costimulator ligand
Ig	immunoglobulin
IKAP	IKK-complex-associated-protein
IKK	inhibitor of -κB kinase
IL	interleukin
IP10	interferon-inducible protein-10
IPTG	isopropyl-beta D-thiogalactopyranoside
K	lysine
LB	lysogeny broth
LEF1	lymphoid enhancer-binding factor 1
LiCl	lithium chloride
LIGHT	homologous to Lymphotoxins, exhibits Inducible expression, and competes with HSV Glycoprotein D for HVEM, a receptor expressed by T-lymphocytes
LMB	leptomycin B
LPS	lipopolysaccharide
LTα1β2	lymphotoxin α1β2
LTβR	lymphotoxin beta receptor
M	methionine

MALT	mucosa associated lymphoid tissue lymphoma translocation gene 1
MAP3K	mitogen activated kinase kinase kinase
MAPK	mitogen activated protein kinase
MBP	maltose binding protein
MCP-1	monocyte chemotactic protein-1
MEF	mouse embryonic fibroblast
MEK	MAP/ERK kinase
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
MIP	macrophage inhibitory protein
MITF	microphthalmia-associated transcription factor
MMP	matrix metalloprotease
mTEC	medullary thymic epithelial cells
NBIP	NIK and IKKβ binding protein
NCC	neural crest cells
NF-kB	nuclear factor-κB
NHEM	normal human epidermal melanocytes
NIK	NF-κB inducing kinase
NKT	natural killer T-cells
NLR	nucleotide binding domain-leucine rich repeat
NLS	nuclear localization signal
OPN	osteopontin
Pax5	paired box 5
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PI	propidium iodide
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor γ
PTEN	phosphatase and tensin homolog
PTX	pentraxin 3
qRT-PCR	quantitative real-time PCR
R	Arginine
RAG	recombination activating gene
RANKL	receptor activator of NF-κB ligand
RANTES	regulated upon activation, normally T-cell expressed, and presumably secreted
Rb	retinoblastoma
RING	really interesting new gene
RIP	receptor interacting protein
RKIP	Raf kinase inhibitor protein
RNA	ribonucleic acid
N-RAS	neuroblastoma RAS viral (v-ras) oncogene homolog
RPS3	ribosomal protein S3
RT	room temperature
SCF	SKP1-cullin-F-box
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	serine
shRNA	short hairpin RNA
siRNA	small interfering RNA

SLC	secondary lymphoid organ chemokine
SMAC	secondary mitochondria-derived activator of caspase
Stat3	signal transducer and activator of transcription 3
TAK1	transforming growth factor beta activated kinase-1
TAMs	tumor associated macrophages
TAP	tandem affinity purification
TBK1	TANK-1 binding protein
TBS	Tris buffered saline
Tcf	transcription factor
TCR	T-cell receptor
Thr	Threonine
TLR	Toll-like receptor
TMA	tissue microarray
TNAP	TRAFs and NIK-associating protein
TNFα	tumor necrosis factor α
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
T_{reg}	regulatory T-cells
TTC4	tetratricopeptide repeat containing protein
TWEAK	TNF-related weak inducer of apoptosis
UTR	untranslated region
UV	ultra violet
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
WT	wild type
XAF1	XIAP-associated factor 1

CHAPTER I

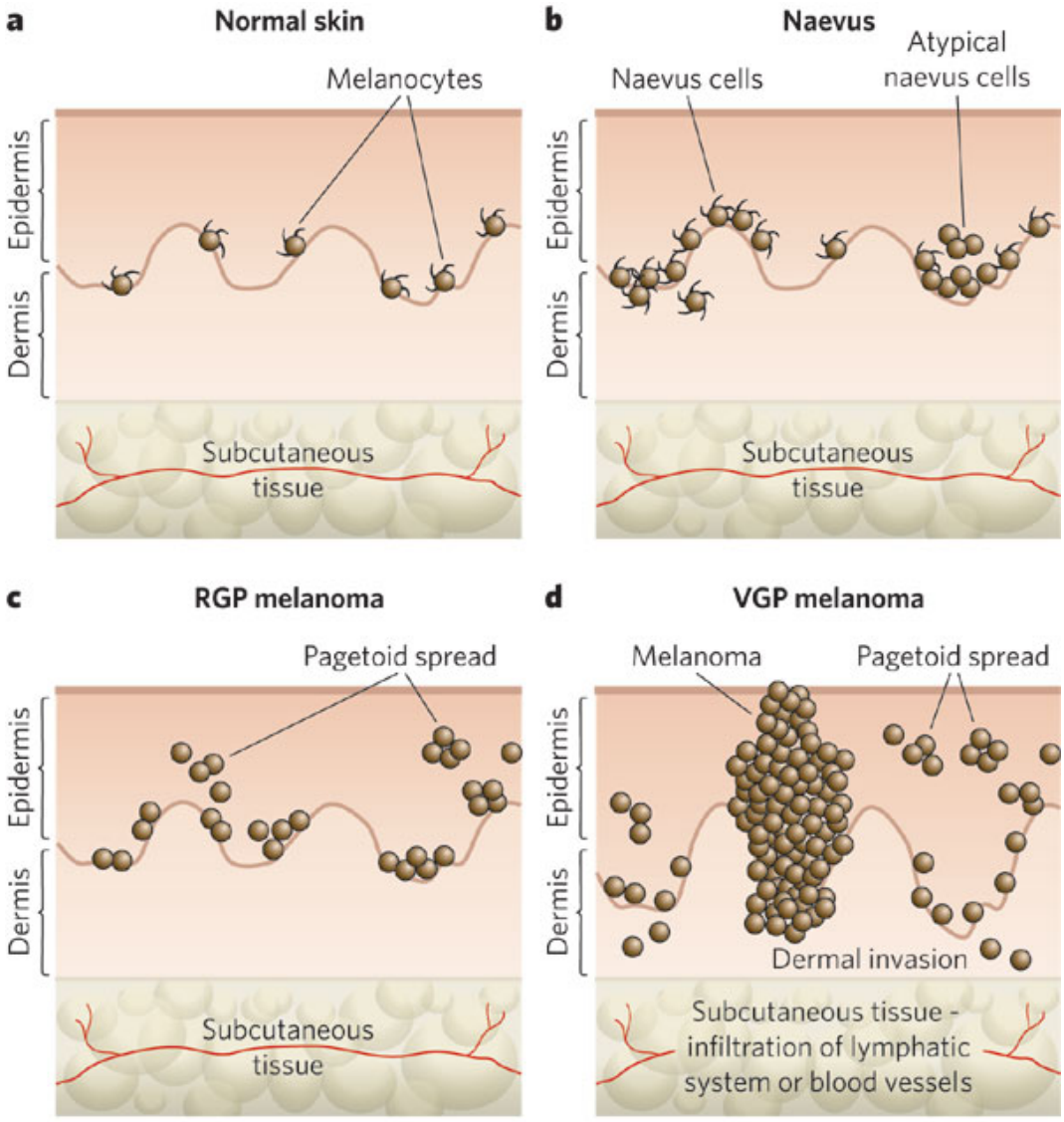
INTRODUCTION

Melanoma Tumorigenesis

Melanoma is one of the most aggressive solid tumors and median survival of a patient with advanced stages of melanoma is 6 to 8 months (Jemal et al., 2010). The incidence of melanoma is rising and it is among the ten leading cancer types in both males and females (Jemal et al., 2010). Melanoma arises from melanocytes, a specialized cell type that makes melanin pigment in an organism. Melanocytes arise from neural crest cells (NCC), which originate in the ectodermal layer. Upon signaling by ligands such as Wnt and bone morphongenic protein (BMP), NCCs reorganize cytoskeleton and undergo changes in cell adhesion properties, thus becoming more motile (Ernfors, 2010). Neural crest cells become melanoblasts and migrate along the dorso-lateral pathway during development.

Compared to other cell types within the skin, melanocytes have limited life-span and basal keratinocytes control the proliferation of melanocytes. Melanocytes are located in the epidermal-dermal junction with 5-10 basal keratinocytes surrounding every melanocyte (Hsu et al., 2002). An adhesion molecule such as E-cadherin mediates the interaction between keratinocytes and melanocytes to control the homeostasis of melanocyte proliferation (Perlis & Herlyn, 2004). Melanocytes synthesize melanin and distribute this UV-protective pigment to surrounding keratinocytes in the skin through their dendrites. Melanocytes may become malignant and progress to melanoma because of inherited mutations and/or environmental insults such as UV radiation (Figure 1).

Melanoma is commonly associated with many genetic changes such as the loss of p16 (INK4a) through mutation, deletion or epigenetic silencing (Bennett, 2008; Dahl & Guldberg, 2007; de Snoo & Hayward, 2005), mutation in BRAF that leads to constitutive activation of the MEK/ERK pathway, or constitutive activation in the MAP kinase pathway. Other common mutations of melanoma include NRAS (Q61L) in 15%-30%, phosphatase and tensin homolog (PTEN) loss in 5%-20%, p16 (INK4a) loss in 30%-70% and p53 loss in about 10% (Gray-Schopfer et al., 2007). Mutations of β-catenin that lead to stabilization of the protein (either at S37 or S45) have also been reported in 20%-30% of melanoma cell lines (Rubinfeld et al., 1997). Among these many mutations, constitutive BRAF mutant is the most common in melanoma. An activating mutation in kinase domain of BRAF has been identified in about 50-60% of malignant melanoma (Davies et al., 2002). Although many other BRAF mutations have been identified in melanoma, 95% of these BRAF mutations is the valine at residue 600 (Wellbrock et al., 2004). This dominant active BRAF ($BRAF^{V600E}$) leads to constitutive activation of the MAP kinase pathway, contributing to proliferation, survival and transformation. As a result, inhibition of BRAF activity with a kinase inhibitor or RNA interference reduces melanoma malignancy (Hingorani et al., 2003), suggesting that this mutation is crucial for melanoma growth.



Gray-Schopfer *et al., Nature Reviews*. 2007.
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Figure 1. Development of melanoma from melanocytes. Melanocytes reside along the basal layer of the epidermis. Melanocytes may form a benign nevus or a dysplastic nevus when they acquire genetic mutations. Additional mutations allow melanocytes to progress from nevi to malignant melanoma.

Much effort has been made to target constitutive BRAF in malignant melanoma to improve the disease outcome. Currently, some drugs targeting BRAF have been in clinical trials (Flemming, 2010). Although targeting BRAF may have been ineffective in the past, more specific and efficacious drugs have been developed. Recently, Zelboraf (from Roche, also known as Vemurafinib or PLX4032), a potent inhibitor of mutant BRAF, has been approved by the Federal Drug Administration (FDA) for treatment of melanoma patients with proven BRAF mutation. In phase III BRIM3 clinical trials, Zelboraf treatment resulted in progression free survival of 5.3 months and in phase II BRIM2 the progression free survival was 6.1 months with about 80% response in patients with BRAF-mutant metastatic melanoma (Bollag et al., 2010; Chapman et al., 2011). Thus, targeting BRAF is one of the promising therapeutic options for advanced metastatic melanoma.

However, similar to other oncogenes, $BRAF^{V600E}$ mutation is not sufficient to induce transformation in melanocytes. In fact, $BRAF^{V600E}$ mutation induces senescence in melanocytes (Dhomen et al., 2009; Michaloglou et al., 2005). $BRAF^{V600E}$ mutation has been identified in more than 80% of melanocytic nevi (Kumar et al., 2004; Pollock et al., 2003), suggesting that not all melanocytes with this mutation become malignant. It is believed that up-regulation of p16 prevents melanocytes with $BRAF^{V600E}$ from becoming malignant melanoma (Wellbrock & Hurlstone, 2010). Consistent with this idea, another common mutation in melanoma is the loss of p16/INK4A or mutation in p16/p19, resulting in loss of function of these proteins (McKenzie et al., 2010; Meyle & Guldberg, 2009). p16 inhibits CDK4 and CDK6 activity, thereby inhibiting the cell cycle and cellular growth (Sherr & Roberts, 1995). The function of p16 has been lost in 25% to 60% of malignant melanoma (Kamb et al., 1994; Nobori et al., 1994).

Possibly due to the survival signal mediated by the MAP kinase pathway, molecules in this pathway have been often mutated in melanoma. For example,

mutations that activate NRAS have been identified in melanoma. Although not as common as BRAF mutation, NRAS mutation has been found in 15-30% of melanoma (Davies et al., 2002). Both RAF and RAS are part of the classical RAS/RAF/MEK/ERK pathway which relays mitogenic signals from tyrosine kinase receptors to the nucleus to activate transcription factors such as Ets and Myc (Gollob et al., 2006), resulting in increased proliferation and resistance to apoptotic signals.

In addition to the above mentioned mutations, the β-catenin pathway is affected in melanoma. β-catenin regulates developmental processes as well as genes important for transformation including c-Myc, CyclinD1 and microphthalmia-associated transcription factor (MITF). β-catenin is generally an unstable protein which undergoes constant proteasomal degradation mediated by adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and β-transducin repeat containing protein (β-TrCP). During development, β-catenin regulates both proliferation and differentiation of melanocytes (Larue et al., 2003). In addition to the role in normal development, a role for β-catenin has been suggested in melanoma. Some melanoma lesions exhibit genetic alterations that activate the β-catenin pathway, such as mutations to stabilize β-catenin or mutations resulting in the absence of APC (Rubinfeld et al., 1997).

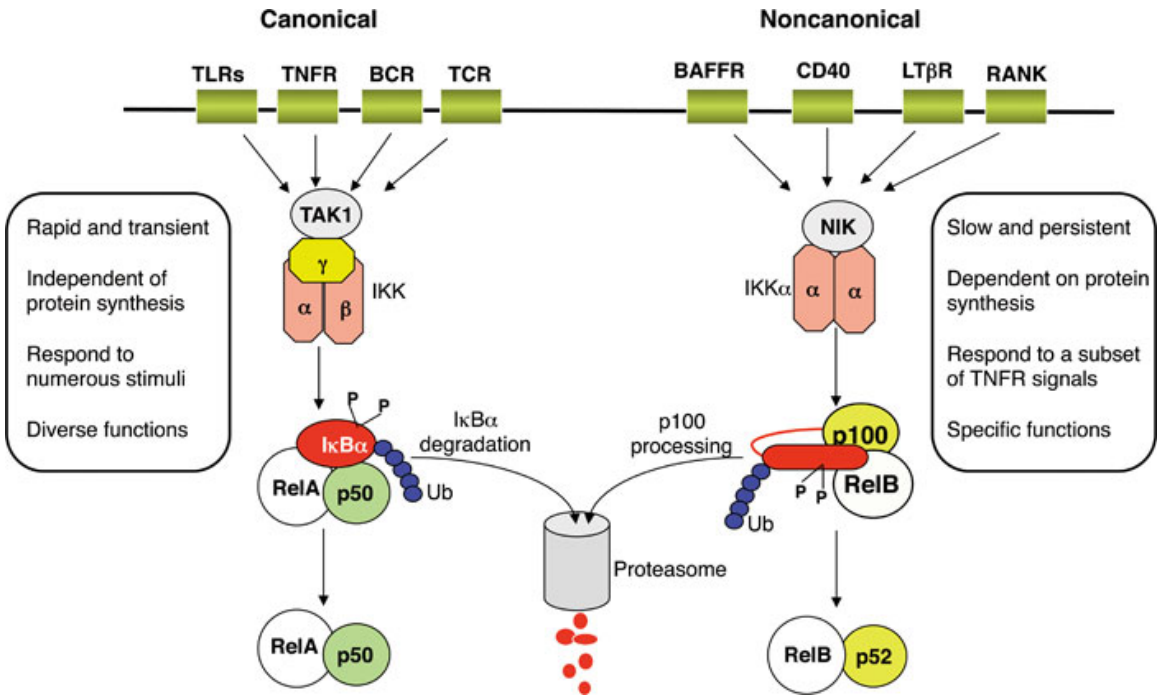
In addition to β-catenin, another developmental gene MITF contributes to melanoma growth. MITF is a lineage specific transcription factor for melanocytes, and promotes melanocyte differentiation and cell cycle arrest. During melanoma progression, MITF regulates senescence and proliferation of melanoma (Giuliano et al., 2010).

In addition to the above mentioned pathways and genes, other genes documented to contribute to melanoma include c-Kit, PTEN, pRB and AKT. Mis-regulation in these pathways supports growth and proliferation of melanoma. Similarly, due to its pleiotropic roles in cellular processes, deregulation of nuclear factor-κB (NF-

κB) has been reported in melanoma growth (Wood & Richmond, 1995; Yang & Richmond, 2001; Yang et al., 2010).

Regulation of Nuclear Factor-κB (NF-κB)

Nuclear factor κB (NF-κB) is a family of transcription factors that regulate essential biological processes such as proliferation, development/differentiation, apoptosis and inflammation. It was first described as a B-cell specific transcription factor that binds to the promoter region of heavy and kappa-light chain (Singh et al., 1986). Later studies show that NF-κB is not restricted to B cells but is ubiquitously expressed in many cell types (Sen & Baltimore, 1986; Staudt et al., 1986). The mammalian NF-κB family includes five members namely, RelA (p65), RelB, p50, p52 and c-Rel. All of these members contain the Rel homology domain which contains DNA-binding sequences, dimerization sequences and a nuclear localization signal (NLS). Likewise, the transactivation domain is present in all members except p50 and p52 and thus these NF-κB members by themselves are poor transcription factors. NF-κB proteins may form homodimers or heterodimers when acting as transcription factors. A non-conventional member of the NF-κB family, Bcl3, may also function as part of the transactivation complex. It can bind to NF-κB complexes and act as a transcriptional cofactor for DNA-bound p50 homodimers or p52 homodimers (Bours et al., 1993; Dechend et al., 1999; Franzoso et al., 1992; Nolan et al., 1993). Recently, ribosomal protein S3 (RPS3) was identified as an additional member of NF-κB complex. RPS3 is required to bind to the promoter regions of some NF-κB regulated genes for optimal transactivation (Wan et al., 2007).



Sun. *Cell Research.* 2011.
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Figure 2. Overview of the canonical and alternative pathways of NF-κB. IKK complex IKKα, IKKβ and IKKγ is a main component of the canonical pathway whereas, IKKα and NIK are essential for the activation of the non-canonical pathway.

Activation of NF-κB pathway is tightly regulated by cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1); bacterial products, such as lipopolysaccharide (LPS); and viral components, such as double stranded RNA. Generally, two major NF- κB pathways can be distinguished: the canonical pathway and the non-canonical or alternative pathway (Figure 2). The transcriptional responses mediated through the canonical pathway are known to be rapid and transient, whereas those of the alternative pathway are slow and long-lasting. For instance, NF-κB activation in response to one of the canonical pathway activators, TNFα, peaks within a few minutes after stimulation and declines within two hours (Werner et al., 2005). Under a steady state of the canonical pathway, NF-κB is sequestered in the cytoplasm by its inhibitor IκB (IκBα, IκBβ or IκBε). IκB binds to NF-κB through its ankyrin repeats, masking NLS of the transcription factor. Upon stimulation with a ligand, IκB is phosphorylated by an IκB kinase (IKK), subsequently ubiquitinated and degraded by the proteasome (Figure 2).

IKKs act as a complex which is composed of two kinases IKKα and IKKβ, and a regulatory subunit IKKγ (Nemoto et al., 1998). Although all IKKs act as a complex, only IKKβ is indispensable for activation of the canonical pathway (Li et al., 1999b). It phosphorylates IκB members (IκBα, IκBβ, IκBε and p105) and liberates NF-κBs p65/p50 heterodimer to translocate into the nucleus. It appears that IKKα is not essential in this signaling event, as observed in IKKα knock-out mice which still have an intact canonical NF-κB activity (Hu et al., 1999). Interestingly, some evidence suggests that IKKα may act as a negative regulator of NF-κB. Macrophages that lack IKKα, and have mutated IKKα exhibit higher NF-κB activity and produce pro-inflammatory cytokines and chemokines (Lawrence et al., 2005; Li et al., 2005). Unlike IKKα, studies on IKK$\gamma^{-/-}$ mice

suggest that IKKγ is required for the activation of NF-κB through the canonical pathway (Schmidt-Supprian et al., 2000). IKKγ does not have a kinase activity but serves as a scaffolding protein in activating the canonical pathway. IKKγ binds to lysine-63 linked ubiquitin on another intermediate, such as receptor-interacting protein (RIP), and brings the IKK complex in proximity of the receptor (Wu et al., 2006). When recruited to the receptor such as TNFR, IKKα and IKKβ are phosphorylated and activated by upstream kinases, such as transforming growth factor beta activated kinase-1 (TAK1) in T-loop regions (Adhikari et al., 2007) (Figure 2).

A less well-understood pathway, the alternative pathway of NF-κB, is activated through IKKα (Senftleben et al., 2001) yet does not require IKKβ and IKKγ. Expressing an IKKα mutant, which cannot be activated, in IKKα$^{-/-}$ mice shows that chemokines regulated by the alternative pathway are down-regulated (Bonizzi et al., 2004). In these mice, nuclear localization of p52 and RelB is defective, suggesting the necessity of IKKα activation in this event. Unlike the canonical pathway which can be activated by multiple ligands, only a limited number of stimuli, such as CD40 ligand (CD40L), B cell activating factor belonging to the TNF family (BAFF), receptor activator of NF-κB ligand (RANKL) and the lymphotoxin beta receptor (LTβR) ligands and TNF-related weak inducer of apoptosis (TWEAK), stimulate the alternative pathway. An additional kinase, NF-κB inducing kinase (NIK), acts as an upstream kinase of IKKα (Senftleben et al., 2001). Both NIK and IKKα phosphorylate p100, an IκB of the alternative pathway, at many serine residues. Unlike the IκB of the canonical pathway, p100 exerts an intramolecular inhibition. Once phosphorylated, the C-terminus of p100 is ubiquitinated by SCF$^{\beta\text{-TrCP}}$ and degraded by the proteasome (Figure 2). As a result, the N-terminus is freed to translocate to the nucleus as a transcription factor p52 heterodimerized with RelB.

Interestingly, overexpression of NIK in 293 cells is sufficient to induce p100 processing to p52 (Xiao et al., 2001).

Previous research has shown that alterations in the NIK gene, NIK activity or NIK protein expression occur in hematological cancers and that targeting NIK reduces tumor cell survival (Annunziata et al., 2007; Conze et al., 2010; Keats et al., 2007; Saitoh et al., 2008). While the importance of NIK in tumorigenesis of solid tumors has been reported (Neely et al., 2010; Saitoh et al., 2010), its role in solid tumors is less well-understood and detailed mechanisms of how NIK modulates tumor growth have not been fully explored. Because of the role of NIK in tumorigenesis and elevated NF-κB activity in melanoma, we speculated that understanding how NIK contributes to melanoma tumor progression might be useful for therapeutic interventions. In the following sections, we review the literature to date on the function and regulation of NIK in relation to its role in the NF-κB pathway, as well as some functions of NIK that are independent of the NF-κB pathway. We also review the functional significance of NIK in the context of physiological processes such as immune regulation, and in disease models such as autoimmunity and cancer.

NF-κB inducing kinase (NIK)

NIK as a kinase of the canonical and non-canonical NF-κB pathways

NIK was first identified as a kinase that mediates the proximal signaling downstream of TNF and IL-1 receptors and the kinase activity of NIK is required for this process (Malinin et al., 1997). NIK also mediates stimulation through other receptors such as CD27, CD30, CD40, LTβR and BAFFR (Akiba et al., 1998). When overexpressed, NIK leads to activation of NF-κB, therefore protecting cells from undergoing TNF-induced apoptosis (Malinin et al., 1997). In reverse, the kinase dead

NIK mutant (either KK429/430AA or T559A) has a dominant negative effect and over-expression of this mutant inhibits activation of NF-κB by TNFα (Lin et al., 1998; Malinin et al., 1997). Surprisingly, dominant negative NIK has a differential effect on NF-κB activation in transformed cell lines versus primary cells. Adenoviral expression of dominant negative NIK in primary macrophages did not have an inhibitory effect on NF-κB activation in response to TNFα and LPS, as suggested by normal NF-κB dependent cytokine production, IκBα degradation and NF-κB DNA (Smith et al., 2001). Although it is not clear why dominant negative NIK exerts different effects on different cell types, NIK was thought to be an essential kinase for the canonical NF-κB activity.

NIK has been demonstrated to interact with and activate IKKα and IKKβ. However, NIK phosphorylates IKKα to a greater extent than IKKβ (Woronicz et al., 1997). Thus, NIK serves as an upstream IKK complex kinase responsible for the proximal signaling of various cytokine receptors. Since the identification of NIK, many other kinases have been identified as IKK kinases acting in concert with NIK. Some of them include MAP/ERK kinase kinase 1 (MEKK1) (Nakano et al., 1998; Nemoto et al., 1998) and TAK1 (Sakurai et al., 1998). TAK1 was identified as an upstream kinase of NIK that can activate NIK/IKK/NF-κB signaling downstream of IL-1R (Ninomiya-Tsuji et al., 1999). TAK1 phosphorylates NIK at a site other than Ser-549, Thr-552 and Thr-559 since mutations of these amino acids to alanine still gave rise to phosphorylated NIK (Ninomiya-Tsuji et al., 1999). Although one study demonstrated that TAK1 did not directly phosphorylate IKKα (Ninomiya-Tsuji et al., 1999), another study suggested that TAK1 may directly activate NF-κB, independent of NIK (Sakurai et al., 1998). NIK is also downstream of TNF receptor associated factor (TRAF) 2, 5 and 6 and TANK-binding kinase-1 (TBK-1) in NF-κB activation upon certain ligand stimulation (Akiba et al., 1998; Pomerantz & Baltimore, 1999).

IKKα is a preferential substrate for NIK. NIK directly phosphorylates IKKα on Ser-176 and mutation of this residue to alanine blocks NIK activation of IKKα, thus disrupting signals from IL-1 and TNFα stimuli (Ling et al., 1998). Interestingly, NIK still phosphorylates the catalytically inactive IKKα (K44M), but does not induce phosphorylation of catalytically inactive IKKβ (K44A), suggesting that auto-phosphorylation is necessary for IKKβ response to NIK (Delhase et al., 1999). T-loop mutants of IKKα and IKKβ (SS176/180AA and SS177/181AA, respectively) show similar results. Mutations in the T-loop of IKKβ disrupt NIK-induced phosphorylation, and the kinase activation status (Delhase et al., 1999). Nevertheless, T-loop mutations in IKKα do not affect NIK's ability to phosphorylate IKKα (Delhase et al., 1999). This suggests that NIK is a better kinase for IKKα than for IKKβ (Delhase et al., 1999). Alternatively, the catalytic activity of IKKβ may be required to "prime" NIK phosphorylation of IKKβ. However, IKKβ is considered a more potent activator of NF-κB since co-expression of NIK and IKKα does not activate NF-κB to the same degree over-expression of IKKβ alone (Woronicz et al., 1997).

In contrast to the report by Delhase *et al.* (Delhase et al., 1999), O'Mahony *et al.* demonstrated that NIK can stimulate phosphorylation of IKKβ (K44A) in an IKKα dependent manner (O'Mahony et al., 2000). In fact, IKKα appears to be upstream of IKKβ in response to NIK. The IKKα (S176A) mutant is unable to phosphorylate IKKβ in response to NIK activation (O'Mahony et al., 2000). Thus, this study suggested a directional activation of IKKα towards IKKβ, stimulated by upstream kinases such as NIK (O'Mahony et al., 2000). Remarkably, NIK recruitment by IKKα to the IKK complex was thought to be required for the basal activation of IKKβ (O'Mahony et al., 2000), despite the non-essential role of NIK in response to certain stimuli.

From the preferential interaction with IKKα, it can be deduced that NIK is a more important kinase for IKKα than IKKβ. Later studies suggest that NIK is only essential for the non-canonical pathway, although it plays a role in both the canonical and non-canonical pathways of NF-κB activation. Data from studies using mouse embryonic fibroblasts (MEFs) with NIK knock-out or with a naturally occurring NIK defective mutant, alymphoplasia (*aly/aly*), support this notion. MEFs from these mice exhibit an intact response to TNFα, yet a partial defect in response to LTβR ligands (Shinkura et al., 1999; Yin et al., 2001). In addition to these mouse models, expression of dominant negative NIK in human skin fibroblasts only inhibits lymphotoxin α1β2 (LTα1β2)-induced NF-κB signaling, but does not affect TNFα-induction (Smith et al., 2001). More careful characterization suggests that in $NIK^{-/-}$ MEFs, NF-κB still binds to DNA yet mRNA of some NF-κB responsive genes such as IκBα and monocyte chemotactic protein-1 (MCP-1)/CCL2 are not induced in response to LTβR agonist antibody (Yin et al., 2001). Thus, it was speculated that NIK does not affect IκB degradation and nuclear translocation but NIK can activate the transcription of NF-κB regulated genes. These data are consistent with the findings that RelA and p50 binding to the DNA is normal in *aly/aly* mice (Muller & Siebenlist, 2003). In *aly/aly* mice, the situation may be more complex. In response to LTβR ligation, the vascular cell adhesion molecule-1 (Vcam-1) protein expression level remain unchanged in *aly/aly* cells, assessed by flow cytometry analysis (Dejardin et al., 2002; Matsumoto et al., 1999). However, LTβR ligation still increases mRNA expression of Vcam-1 by inducing IKK kinase activity in *aly/aly* MEFs (Dejardin et al., 2002). These data suggest that *aly* mutation does not affect the canonical pathway induction via LTβR, but may affect the post-transcriptional regulation of Vcam-1 (Dejardin et al., 2002).

Notably, NIK deficiency affects activation of both the canonical and non-canonical pathways in response to certain ligands in specific cell types. For instance, NIK deficient lymphocytes are defective in activation of the canonical and non-canonical pathways of NF-κB upon treatment with CD40L, BAFF and CD70 (a ligand for CD27) (Ramakrishnan et al., 2004). Deficiency of NIK results in defective IκB degradation, RelA nuclear translocation, and p100 to p52 processing in response to these ligands (Ramakrishnan et al., 2004). Intriguingly, NIK deficiency in B-cells results in slight diminution of some NF-κB members such as p100, RelB and c-Rel, all of which are NF-κB-regulated genes (Ramakrishnan et al., 2004). This observation implies that continuous and weak basal NF-κB activation exists in a NIK-dependent manner (Ramakrishnan et al., 2004). B-cells from *aly/aly* mice also exhibit diminished IκB phosphorylation in response to CD40 engagement, whereas dendritic cells do not have a similar defect (Garceau et al., 2000). Thus, the notion that NIK is non-essential for the canonical pathway is somewhat misleading. Signals via CD40 and BAFFR, in fact, rely on NIK to induce RelA activation. In other words, NIK is not required for the proximal signaling from the receptors inducing only the canonical pathway, yet it is required for the signaling from some receptors that activate both the canonical and non-canonical arms of NF-κB. Ramakrishnan *et al*. proposed NIK-dependent and NIK-independent mechanisms of receptor activation. Based on their data, they suggested that NIK is recruited together with the IKK complex only to the receptors that are NIK-dependent (i.e. CD27), whereas it does not associate with the receptors that are NIK-independent (i.e. TNFR) (Ramakrishnan et al., 2004). The necessity for NIK in the canonical NF-κB pathway may be cell type and ligand specific and how this specificity occurs is yet to be determined.

The requirement of NIK for the non-canonical pathway is more evident. NIK is responsible for the processing of p100 to p52, confirming its role in the non-canonical pathway (Xiao et al., 2001). The kinase activity of NIK or IKKα is not required for the recruitment of p100 to both kinases however it is required for the ligand-induced proteolysis to occur (Xiao et al., 2004). Surprisingly, NIK is not required for the constitutive processing of p100 to p52 (Qing et al., 2005). The mechanism by which NIK controls p100 to p52 processing is through its stability. Upon stimulation with ligands such as CD40L or BAFF, basally translated NIK is stabilized to induce p100 to p52 processing (Qing et al., 2005). The C-terminus of NIK is crucial for the interaction with p100 as well as for inducing the proteolysis of p100 (Xiao et al., 2001). In addition to regulating p100 proteolysis, NIK plays a role in histone modification to control gene expression together with IKKα. In response to the bacteria endotoxin LPS, NIK induces phosphorylation of IKKα, which in turn phosphorylates histone H3 (Park et al., 2006). This is one mechanism by which NIK controls LPS-induced NF-κB activation and gene expression.

From the above evidence, it is apparent that NIK is necessary for the non-canonical pathway. Paradoxically, NIK was first identified as a kinase which activates the canonical pathway downstream of TNF and IL-1 receptors. The physiological relevance of NIK in the canonical NF-κB activation pathway was re-investigated by Zarnegar *et al*. who demonstrated the role of NIK in amplification of signals from the canonical pathway (Zarnegar et al., 2008). They used $TRAF3^{-/-}$ mice to show the elevation of the canonical pathway activity as a result of TRAF3 deficiency and NIK stabilization (Zarnegar et al., 2008). In concert with findings from NIK over-expression studies, NIK activates the canonical pathway when present at a high amount due to stabilization. Interestingly, the response of MEFs to TNFα was markedly enhanced

when pre-treated with LTβR agonist antibody (Zarnegar et al., 2008). This enhancement was not observed in $NIK^{-/-}$ MEFs, suggesting that NIK stabilization in response to pre-treatment with LTβR agonist antibody amplified signals upon TNFα treatment (Zarnegar et al., 2008). Interestingly, recent study shows that NIK is stabilized upon TNF-treatment in $RIP^{-/-}$ MEFs, suggesting that RIP negatively regulates the non-canonical pathway through NIK stability (Kim et al., 2011). As the canonical and non-canonical pathways are traditionally thought to be inflammatory and lymphoid organogenic responses, respectively, it is possible that NIK may have a role in integrating signals from those processes.

In summary, NIK is a kinase that functions in the canonical pathway, but is required only for NF-κB activation in response to certain ligands. The physiological significance of this selective requirement remains elusive. Possibly, NIK lies at a cross road of the canonical and the non-canonical pathway of NF-κB, maintaining the positive and negative regulatory balance between these two pathways. For example, NIK activates the canonical pathway and thus possibly induces the production of p100, a fourth IκB of the NF-κB family (Basak et al., 2007)(negative feedback mechanism). At the same time, NIK is the kinase responsible for the processing of this IκB so that it will liberate NF-κB heterodimers such as RelA/p50 to activate gene transcription (positive feedback mechanism). Another significance of NIK's role in both pathways is the amplification of signals as described above (Zarnegar et al., 2008). It becomes exceedingly clear that NIK plays a dynamic role in NF-κB activation, coordinating the intricate balance between the canonical and non-canonical regulations of NF-κB. Maintaining this balance is crucial for the proper physiological responses. Moreover, tipping this balance probably leads to disease processes such as cancer.

Chemokines, cytokines and genes under the influence of NIK

NIK influences the production of inflammatory cytokines and chemokines, possibly by regulating the non-canonical pathway, or in some contexts, by regulating both the canonical and non-canonical pathways. Inflammatory factors such as interferon-inducible protein-10 (IP10)/CXCL10, MCP-1, KC and regulated upon activation, normally T-cell expressed, and presumably secreted (RANTES)/CCL5 are up-regulated in $TRAF2^{-/-}$ mice and more so in $TRAF3^{-/-}$ mice, which have a dramatic stabilization of NIK protein expression (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Similarly, the IL-12 level is elevated in TRAF3 deficient tissues (Zarnegar et al., 2008). However, the contribution of the canonical or the non-canonical pathway in up-regulating these secreted factors is not clear, since NIK significantly activates the canonical NF-κB pathway when it accumulates as a result of the loss of TRAF3 ($TRAF3^{-/-}$ mice) (Zarnegar et al., 2008). T-lymphocytes from *aly/aly* mice fail to secrete IL-2, suggesting that NIK regulates IL-2 production (Yamada et al., 2000). Additionally, Sanchez-Valdepenas *et al*. have shown that NIK regulates the phosphorylation of c-Rel, the binding of c-Rel to a regulatory region within IL-2 promoter [CD28-responsive enhancer (CD28RE)], and controls IL-2 transcription in T-lymphocytes (Sanchez-Valdepenas et al., 2006). Thus, NIK may influence cytokine expression in an IKK/IκB/NF-κB pathway independent manner. Transcription of other cytokines and chemokines such as IL-6 and IL-8/CXCL-8 are also likely to be NIK-dependent as kinase-dead NIK inhibits their production upon LTβR ligation (Smith et al., 2001).

Other genes regulated by NIK include TRAF2 and TRAF3, both of which are negative regulators of NIK (Sasaki et al., 2008; Wang et al., 1998). Moreover, NIK appears to positively regulate its own transcription (Bista et al., 2010). Microarray data from a colon carcinoma cell line with TRAF3 depletion by siRNA show that the NIK

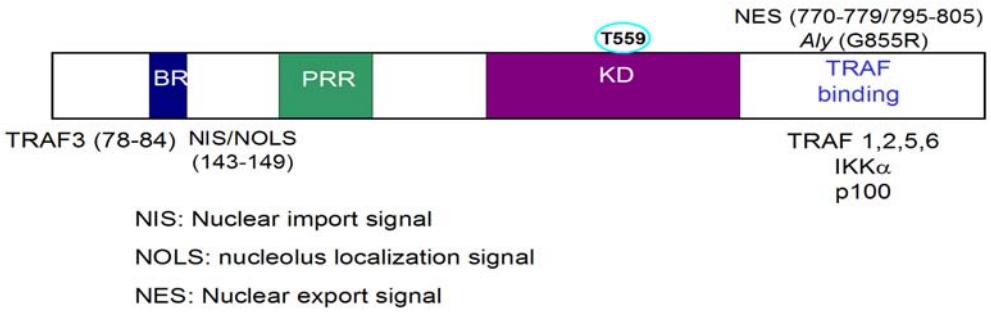
mRNA level is elevated in these cells (Bista et al., 2010). These data suggest that stabilization of NIK protein through loss of TRAF3 leads to a positive feedback loop, resulting in enhanced transcription of NIK. Although the universality of this effect remains to be elucidated, regulation of NIK appears to have built-in positive and negative feedback systems, implying that intricate control is necessary for the appropriate physiological responses.

Intriguingly, NIK affects the expression of members of the NF-κB pathway. RelB, p50, and c-Rel expression levels are reduced in aly/aly mice (Yamada et al., 2000) as well as in NIK knock-out cells (Ramakrishnan et al., 2004). Although the list of genes regulated by NIK described here is not exhaustive, we can conclude that NIK modulates chemokines, cytokines and signaling molecules in NF-κB pathways, implying that NIK can have a profound effect on NF-κB activation.

Understanding the molecular components of NIK

NIK was first identified as a mitogen activated kinase kinase kinase (MAP3K) that binds to TRAF2 in a two-hybrid screen of a human B-cell cDNA library (Malinin et al., 1997) and was later shown to phosphorylate IKKα and IKKβ. In the two-hybrid screen, NIK did not bind to TNF receptor associated death domain protein (TRADD), Fas-associating protein with death domain (FADD) or RIP-1 (Malinin et al., 1997). NIK shares some homology to the MAP3K. From this homology and from mutagenesis studies, it was determined that Thr-559 in the activation loop of NIK is required for its kinase activity (Lin et al., 1998). Unlike RIP-1 whose activity is not required for NF-κB activation, the kinase activity of NIK is necessary for both basal and TNFα-mediated NF-κB activations (Lin et al., 1998; Malinin et al., 1997) as well as for the processing of p100 to

p52 (Xiao et al., 2001). Curiously, NIK can spontaneously homodimerize or oligomerize, but TNFα does not further induce oligomerization of NIK (Lin et al., 1998). Thus, oligomerization or dimerization of NIK may be crucial only for the basal, but not for the ligand-induced kinase activity of NIK. Another layer of regulation of NIK may come from the intramolecular interaction of NIK. The amino-terminal (N-terminal) region of NIK contains a negative regulatory domain, which interacts with the NIK carboxyl-terminal (C-terminal) end. This interaction prevents NIK from binding to its substrates, such as IKKα (Xiao & Sun, 2000).



Thu and Richmond, *Cytokine and Growth Factor Reviews.* 2010
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Figure 3. Different domains of NIK, which interact with specific substrates and regulators. The C-terminus of NIK mainly interacts with substrates such as IKKα and p100, and NIK regulators, such as TRAF1, 2, 5 and 6. The N-terminus of NIK regulates the stability of the protein by interacting with TRAF3. So far, only one phosphorylation site (Thr-559) on NIK has been identified in its kinase domain. *Aly* mutation in the C-terminus impedes the function of NIK. NIK also has sequences for nuclear and nucleolar localization signals, which enable the protein to shuttle between different cellular compartments.

Mutant forms of NIK have been generated to provide insight into the functional domains of NIK, and to determine how regulation of NIK is achieved. Based on its sequence and functions, at least four distinct domains were identified in NIK: the basic region, the proline rich region, the kinase domain and the C-terminal TRAF binding domain (Xiao & Sun, 2000) (Figure 3). Among these, the C-terminus is responsible for many important interactions. The C-terminus binds to IKKα, the downstream kinase of NIK, and also mediates homotypic dimerization or oligomerization (Lin et al., 1998). A careful characterization of NIK demonstrated that the C-terminal fragment of NIK (aa 735-945) is necessary and sufficient for binding to IKKα (Lin et al., 1998). Not surprisingly, expression of the C-terminus of NIK disrupts the activation of NF-κB by TNFα as well as by the wild type NIK over-expression (Lin et al., 1998; Malinin et al., 1997). In parallel, the kinase dead NIK mutant acts as a dominant negative mutant when over-expressed (Lin et al., 1998; Malinin et al., 1997), possibly due to its ability to bind to IKKs, while lacking the kinase activity. The kinase dead NIK (KK429/430AA) also cannot process p100 to p52 (Xiao et al., 2001), suggesting that NIK kinase activity is required for the non-canonical NF-κB pathway.

The TRAF binding domain of NIK, as the name suggests, mediates the binding of NIK to TRAFs such as TRAF1, 2 and 6 (Malinin et al., 1997). Unlike other TRAFs, TRAF3 binds NIK in the N-terminal region of NIK (aa78-84). Deletion of this domain results in the dramatic stabilization of NIK since TRAF3 mediates its degradation (Liao et al., 2004; Sasaki et al., 2008). In addition to domains that regulate NIK stability, the protein also has nuclear import and export signals at the N-terminus (aa143-149) and the C-terminus (aa770-779/aa795-805), respectively (Birbach et al., 2002). These signals enable the nucleo-cytoplasmic shuttling of NIK. A truncation mutant of NIK which lacks the nuclear export signal, thereby confining NIK to in the nucleus, still retains some

ability to activate NF-κB (Birbach et al., 2002). These data suggest that NIK may be able to activate the nuclear IKK complex, or it may have a direct effect on the transcription mediated by NF-κB. NIK also appears to localize to the nucleolus, as determined by the signal RKKRKKK (aa143-149) from the protein's N-terminus (Birbach et al., 2004). This short stretch of amino acid sequence is both necessary and sufficient for the nucleolar localization of NIK (Birbach et al., 2004). Deleting the nucleolar localization motif attenuates NIK's ability to activate NF-κB (Birbach et al., 2004), suggesting that NIK may have additional roles in different cellular compartments, aside from acting as an IKK kinase.

In addition to genetically engineered mutants, there exists a naturally occurring mutation of NIK in mice named alymphoplasia or *aly* mutation, first described by Miyawaki *et al*. in C57BL/6J mice (Miyawaki et al., 1994). Shinkura *et al*. later mapped the gene responsible for this mutation and identified a missense point mutation (G855R) in the carboxyl terminus of NIK, which is necessary for TRAF binding (Shinkura et al., 1999) (Figure 3). Despite the speculation that *aly* NIK may not bind to one of the TRAFs, co-immunoprecipitation experiments suggest otherwise. At least the C-terminus of NIK (aa624-947) (TRAF-interacting domain) carrying the *aly* mutation binds to TRAF 1, 2, 3 and 6 as equally as the wild type C-terminal fragment binds (Luftig et al., 2001). Yet, the C-terminus of *aly* NIK does not bind IKKα and binds IKKβ with less efficiency compared to the wild type C-terminus (Luftig et al., 2001), implying that phenotypes of *aly* stem from the defective NIK/IKKα interaction.

Interestingly, when over-expressed in 293T cells, *aly* NIK can still activate NF-κB, as manifested by an increased DNA binding of NF-κB in electrophoretic mobility shift assay (EMSA) (Shinkura et al., 1999). Since *aly* NIK fails to bind IKKα, perhaps with its over-expression there is sufficient NIK interaction with IKKβ to activate NF-κB. The

ability of *aly* NIK to activate NF-κB is in contrast to the kinase dead NIK, which fails to activate NF-κB. Perplexing views come from the fact that *aly* NIK inhibits CD40-mediated NF-κB activation in B-cells (Luftig et al., 2001). Likewise, this mutant form of NIK inhibits NF-κB activation, caused by TNFR1 and 2 over-expression but not LTβR-mediated NF-κB activation in 293T cells (Shinkura et al., 1999). The studies on the ability of the *aly* NIK to activate or disrupt NF-κB did not distinguish clearly the contribution of NIK to the canonical or non-canonical pathway, as the conclusions were based on EMSA or luciferase activity. Moreover, how the *aly* mutation results in a dominant negative function of NIK in response to certain ligands remains elusive. Yet, all these data suggest that the *aly* mutation may disrupt NF-κB activation in a ligand-specific manner.

Follow-up studies addressed the role of the *aly* mutation in the non-canonical NF-κB pathway. The *aly* mutation disables NIK's ability to bind either IKKα or p100, resulting in a deficient p100 to p52 processing (Luftig et al., 2001; Xiao et al., 2001). MEFs from *aly/aly* mice are defective in inducing mRNA level of VCAM-1 expression upon ligation with LTβR agonist antibody (Shinkura et al., 1999). These data are consistent with data from NIK knock-out mice, confirming the notion that NIK is indispensable for the non-canonical pathway of NF-κB. *Aly* mutation highlights the importance of the physical interaction between NIK and its substrate. Despite the presence of the kinase domain, NIK function may still be defective when the proper binding does not occur. It is very likely that the physical interaction between its substrates as well as the kinase activity is required for the ability of NIK to stimulate p100 to p52 processing. In other words, both of those criteria are required for the non-canonical pathway, whereas the interaction between NIK and its binding partner at the C-terminus may not be absolutely necessary for NIK activation of the canonical pathway of NF-κB.

Regulation of NIK by stability

Although NIK functions as a kinase and phosphorylation of NIK is required for its ability to activate IKKs and NF-κB, regulation of the NIK protein stability is the major mechanism by which NIK function is regulated. Interestingly, mRNA expression of NIK is increased in the absence of its 3' untranslated region, suggesting that NIK expression may be mediated at the post-transcriptional level (Malinin et al., 1997). The basal level of NIK protein is very low under normal physiological conditions. However, NIK becomes stabilized after stimulation with CD40L and BAFF in B-cells (Qing et al., 2005). The basal stability of NIK depends on the action of four E3 ubiquitin ligases namely; TRAF2, TRAF3, cellular inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2). Initially, TRAF3 was suggested to degrade NIK, since the direct interaction between NIK and TRAF3 suppressed NIK activation of NF-κB (Liao et al., 2004). Deletion of the TRAF3 binding domain (aa78-84) of NIK prevents NIK from being ubiquitinated and degraded (Liao et al., 2004). Consistently, in a knock-in mouse with NIK mutant lacking the TRAF3 binding domain, NIK was substantially stabilized (Sasaki et al., 2008). Additional studies suggest that TRAF3 negatively regulates NIK. TRAF3 is down-regulated in response to CD40L and BAFF (Li et al., 2005; Moore & Bishop, 2005). Interestingly, CD40L and BAFF treatment protects basally translated NIK from undergoing proteasomal degradation in B-cells (Qing et al., 2005). Since both CD40L and BAFF activate the non-canonical pathway of NF-κB, and NIK is an essential kinase for this pathway, it can be deduced that down-regulation of TRAF3 upon receptor ligation leads to the positive regulation of NIK, relaying downstream signals. Consistent with the previous data, $TRAF3^{-/-}$ mice have constitutive p100 processing due to the accumulation of NIK (He et al., 2006).

In addition to TRAF3, TRAF2 is known to play a negative role in p100 to p52 processing, as $TRAF2^{-/-}$ B-cells have the constitutive processing of p100 (Grech et al.,

2004). Similarly, cIAP1 and 2 are implicated in the negative regulation of NIK by studies using IAP inhibitors. Pharmacological inhibitors of IAPs, which lead to auto-degradation of cIAP1 and 2, markedly stabilize NIK and stimulate the activation of NF-κB through the non-canonical pathway (Varfolomeev et al., 2007). These data strongly suggest that TRAFs and cIAPs are degradative agents of NIK.

Two recent studies provide biochemical evidence of how TRAF2, TRAF3, cIAP1 and cIAP2 cooperate to affect the stability of NIK. According to data from Vallabhapurapu *et al*. and Zarnegar *et al*., TRAF2 preferentially binds to cIAP1/2 and TRAF3 preferentially binds to NIK. TRAF2 is required to recruit the TRAF3/NIK complex to the TRAF2/cIAP1/2 complex and TRAF3 is required to recruit the TRAF2/cIAP1/2 complex to TRAF3/NIK. Based on their data with knock-out MEFs, TRAF2 and TRAF3 cannot compensate for each other's loss in degrading NIK (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). On the contrary, cIAP1 and cIAP2 can compensate for the loss of one another in degrading NIK and downstream processing of p100 (Zarnegar et al., 2008). To add to the complexity of the system, NIK also regulates TRAF2 and TRAF3 expression at a transcriptional level (Sasaki et al., 2008; Wang et al., 1998). When NIK is stabilized in B-cells, it induces mRNA expression of TRAF2 and TRAF3, establishing a negative feedback loop for NIK (Sasaki et al., 2008). This negative feedback regulation is likely to be the mechanism to ensure the termination of NF-κB signaling after an appropriate duration.

In slight contrast to the requirement of TRAF3 to mediate NIK degradation by recruiting the TRAF2/cIAPs complex, Demchenko *et al*. have shown in the multiple myeloma model that TRAF2 is still capable of binding to NIK and inducing degradation even when TRAF3 interaction with NIK is absent (Demchenko et al., 2010). The investigators of this study argued that TRAF3 enhances the degradation of NIK, yet it is

not absolutely required for this process to occur (Demchenko et al., 2010). The different results regarding the requirement for TRAF3 may be due to the transformation status of the B-cell-derived multiple myeloma cells. Perhaps not all of the four E3 ubiquitin ligases are functionally equal in all cell types, some being more important for ablation of NIK than others.

The reason for the necessity of four ubiquitin ligases for the constitutive degradation of NIK is not known. Although the field has made a significant progress in elucidating the regulation of NIK, many questions remain unanswered. Among four ubiquitin ligases that are responsible for NIK degradation, which is/are the actual ubiquitin ligase(s) that conjugate ubiquitin to NIK? Liao *et al*. suggested that using purified TRAF3 in an *in vitro* ubiquitination assay did not result in significant ubiquitination of NIK (Liao et al., 2004). Consistent with this finding, Vallabhapurapu *et al*. argued that TRAF3 was simply an adaptor, as they did not observe a necessity for the TRAF3 RING domain to mediate NIK degradation (Vallabhapurapu et al., 2008). Instead, they proposed that cIAP1/2 may be responsible for K-48 ubiquitination of NIK (Vallabhapurapu et al., 2008). In support of this idea, it is demonstrated that RING mutant constructs of TRAF2 or cIAP1 do not degrade NIK (Vallabhapurapu et al., 2008; Varfolomeev et al., 2007). Hence, only some of these E3 ubiquitin ligases may serve as the critical ubiquitin ligase for NIK degradation, whereas others merely serve as scaffold proteins. It is quite challenging to reconstitute an *in vitro* ubiquitination of NIK, possibly due to the requirement of all these factors and the ubiquitination activity that they have on one another. In addition, we still do not know the identity of the lysine residue(s) on NIK required for its ubiquitination that leads to degradation.

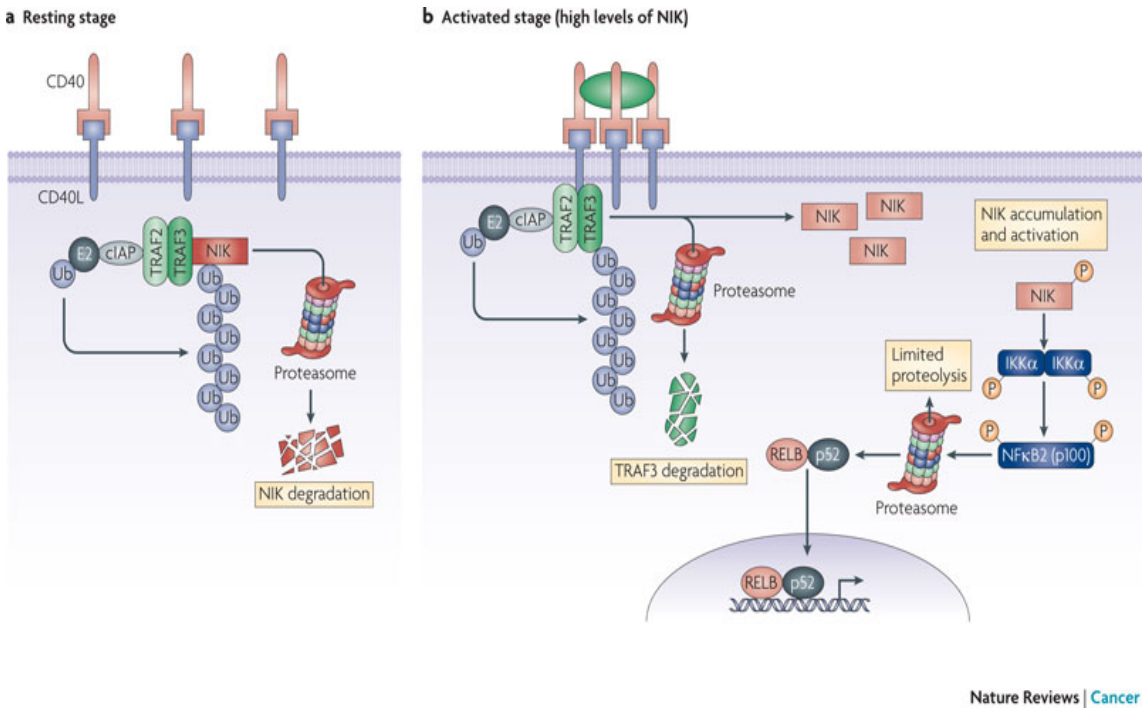

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Figure 4. Mechanisms of NIK degradation mediated by E3 ubiquitin ligases. At a resting stage, NIK undergoes ubiquitination and degradation mediated by cIAP1/2, TRAF2/3. Upon ligand stimulation, NIK is liberated from the degradative complex, thereby stabilized to mediate the downstream signaling such as p100 to p52 processing.

In addition to the basal regulation of NIK, some mechanisms by which NIK is stabilized upon ligation of upstream receptors have been demonstrated (Figure 4B). It is noted that upon ligand stimulation by CD40L or BAFF, TRAF2 and TRAF3 are degraded in a cIAP-dependent manner, resulting in the stabilization of NIK (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). TRAF3 degradation precedes TRAF2 degradation, suggesting that TRAF3 degradation first disassembles the complex bound to NIK to relieve the suppression (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). The mechanism of how cIAPs become the degradative agents for TRAFs upon ligand engagement remains elusive. Another ligand, TWEAK, mediates NIK stabilization in a manner slightly different from CD40L and BAFF, at least in the melanoma cell line A2058. Upon TWEAK ligation, cIAP1 is degraded, then stabilized NIK potentiates p100 to p52 processing (Varfolomeev et al., 2007). However, cIAP-dependent negative regulation of NIK may not be the complete story. A recent report suggests that cIAP-independent NIK stabilization may exist, as TWEAK can still stabilize NIK in cIAP1/2 double knock-out mice (Feltham et al., 2010).

Another receptor that can activate NIK is the LTβR. It was recently proposed that both LTβR and NIK bind to the same site on TRAF2 and 3. Thus, upon stimulation through LTβR, the receptor competes for the binding sites on TRAF3, thus replacing NIK from the negative regulatory complex and relieving the degradation of NIK (Sanjo et al., 2010). Similarly, the receptor replaces cIAP1/2 from TRAF2, possibly to ensure the disassembly of the degradative complex for NIK (Sanjo et al., 2010). Aside from serving as an allosteric competitor, activation through LTβR leads to ubiquitination and proteasomal degradation of TRAF3 in a TRAF2-dependent manner (Sanjo et al., 2010). Although TRAF2 is also ubiquitinated and degraded upon ligand stimulation via LTβR, it is not clear whether TRAF2 undergoes auto-ubiquitination or whether some other E3

ligases are responsible for this process. Interestingly, cIAPs were not associated with the activated LTβR and TRAF complex (Sanjo et al., 2010). Thus, the mechanism by which NIK is stabilized upon ligand induction appears to be unique for individual receptor signaling.

Another interesting question regarding NIK regulation is whether it has a dosage dependent effect on NF-κB activation. The first indication that the level of NIK may be tightly regulated comes from NIK over-expression and gene knock-out studies. NIK potently activates basal as well as ligand stimulated NF-κB upon over-expression (Malinin et al., 1997). However, data from NIK knock-out mice suggest that this kinase is not required for the activation of the canonical pathway. Thus, the level of NIK likely determines whether NIK is selectively activating one or both pathways of NF-κB. For example, in TRAF3$^{-/-}$ mice where NIK is stabilized, nuclear accumulation of RelA, p50, RelB and p52 is significantly elevated, suggesting that there is a constitutive activity of both canonical and non-canonical pathways of NF-κB (Zarnegar et al., 2008). Remarkably, the lethality resulting from TRAF3 deletion was significantly rescued by deleting just one allele of NIK (Zarnegar et al., 2008). This observation suggests that the level of NIK is tightly controlled to regulate physiological processes.

In accordance with the dosage dependent regulation of NIK, Demchenko *et al*. proposed a model by which the level of NIK protein may be tightly controlled (Demchenko et al., 2010). In this model, TRAF2, TRAF3, cIAP1 and cIAP2 cooperatively modulate the stability of NIK. Only in the functional presence of all four of them, can maximal degradation of NIK be achieved. When one of the E3 ligases is absent or non-functional, NIK stability increases, resulting in NF-κB activation. These investigators also postulated that initial stabilization of NIK robustly activates NF-κB, whereas additional stabilization of NIK has minimal additive effect of NF-κB activation (Demchenko et al.,

2010). The differential effect of NIK on NF-κB activation depending on the dosage may help explain why the expression level of NIK is maintained minimal under normal physiological conditions.

In summary, there are three ways to post-translationally regulate the function of NIK based on the literature to date; phosphorylation, interaction between NIK and its binding partners, and the stabilization of NIK. In addition the 3'UTR of NIK mRNA plays a role in regulating the stability of the NIK mRNA. The resulting NIK activity is the net effect of the interplay among these regulations.

NIK in the regulation of B-cells

Based upon data accumulated from studies of the NIK null mice and the *aly/aly* mice, subsequent studies have often focused on NIK's role in the modulation of the immune system. *Aly/aly* mice were characterized as mice without lymph nodes and Peyer's patches (Shinkura et al., 1996). These mice do not exhibit somatic hypermutation during antigen-specific immune response and germinal center formation (Shinkura et al., 1996). Presumably because of these defects, *aly/aly* mice exhibited weak antibody response and defective isotype switching (Shinkura et al., 1996). These mice also lack allogenic skin rejection, exhibit abnormal spleen and thymic development and have defective B-cell proliferation (Miyawaki et al., 1994; Shinkura et al., 1996; Yamada et al., 2000). Since NIK is a downstream activator of LTβR and LTβR is exclusively expressed by non-lymphoid cells, it was speculated that the phenotypes in *aly/aly* mice are caused by a defective signaling in non-bone marrow derived cells (Matsumoto et al., 1999). However, transferring bone marrow from *aly/aly* mice into heterozygous *aly* mice clearly shows that proper NIK activity is required intrinsically in bone marrow-derived cells as well as in stroma. Chimeric mice with bone marrow

derived cells from *aly/aly* mice have poorly developed B-cell follicles in the spleen, with lack of follicular dendritic cell clusters and germinal center formation (Yamada et al., 2000). B-cells from *aly/aly* mice also have a defect in proliferation and IgM/IgG production in response to CD40L, LPS and anti-Ig activation (Garceau et al., 2000). Interestingly, up-regulation of surface molecules such as MHC II and intercellular adhesion molecule-1 (ICAM-1) is not affected by *aly* mutation in B-cells (Garceau et al., 2000).

In addition to the necessity of NIK activity in bone-marrow derived cells, it is also required in stromal cells in the peritoneal cavity so that B1 cells can emigrate. *Aly/aly* mice have defective B-cell emigration from the peritoneal cavity, resulting in accumulation of B-cells in the cavity (Kunisawa et al., 2008). These phenotypic defects are due to the inability of NIK deficient stromal cells to express VCAM-1 and ICAM-1, therefore weakening the interaction between the stromal cells and B-cells (Kunisawa et al., 2008).

Most of the phenotypes in NIK deficient B-cells may be attributable to NIK's function as an activator of IKKα. However, intriguingly, NIK has other roles in addition to its role as an IKKα kinase in B-cells. To distinguish IKKα-dependent and IKKα-independent contribution of NIK to the development of B-cells, mice with IKKα phospho deficient mutant (S176A and S180A) were generated (Mills et al., 2007). As these serine residues are phosphorylated by NIK, these mutations should mimic the functional absence of NIK. Results showed that *aly*/*aly* mice have impaired antibody production, yet IKKα (S176A/S180A) knock-in mice do not (Mills et al., 2007). B-cells from these IKKα knock-in mice proliferate normally in response to natural ligands such as CD40L, BAFF or the bacterial product LPS, in contrast to the phenotypes in *aly/aly* mice (Garceau et al., 2000; Yamada et al., 2000). Altogether, these data suggest that NIK

may have IKKα and non-canonical NF-κB pathway independent functions, that can profoundly affect the cellular physiology, at least in B-cells (Mills et al., 2007).

In addition to its role in B-cell function, NIK provides survival signals in B-cells. Under normal physiological conditions, signal transduction events activated through CD40 and BAFFR are necessary for the induction of p100 proteolysis and for the promotion of B-cell survival (Claudio et al., 2002; Siebenlist et al., 2005). Consistent with this notion, ablation of NIK negative regulators, TRAF2 and TRAF3, specifically in B-cells markedly enhances the survival of B-cells (Gardam et al., 2008; Grech et al., 2004; Xie et al., 2007). Substantial NIK accumulation enables TRAF3$^{-/-}$ B-cells to survive in a ligand-independent manner (He et al., 2006), as NIK stabilization bypasses the necessity of CD40L and BAFF engagement.

In support of the genetic studies, treatment of primary B-lymphocytes with secondary mitochondria-derived activator of caspase (SMAC) mimetic compounds increase the proliferation and survival of these cells (Zarnegar et al., 2008). SMAC mimetics degrade cIAPs, negative regulators of NIK, and therefore stabilize NIK expression. Although accumulation of NIK enhances the survival of B-cells, it impedes the transition from the pro-B to the pre-B cell stage, thus disrupting the normal development of B-cells (Vallabhapurapu et al., 2008). NIK also has a suppressive role on the expression of the transcription factor Pax5, which regulates the pro-B to pre-B cell transition. The mRNA transcript level of Pax5 was reduced in Traf2$^{-/-}$ and Traf3$^{-/-}$ B-cells and was restored upon deletion of NIK in these cells (Vallabhapurapu et al., 2008). Conversely, transgenic mice with TRAF3 specifically over-expressed in B-cells exhibited an expanded plasma cell population despite the low expression of NIK (Zapata et al., 2009). Yet, their proliferative response to stimuli such as CD40L and BAFF *in vitro* did not differ appreciatively from wild type cells (Zapata et al., 2009). Although it remains ambiguous how the level of NIK specifically affects the B-cell population, it is apparent

that the NIK level has to be tightly regulated to give rise to a proper differentiation and expansion of B-cells.

NIK in the regulation of T-cells

During the development of T-cells, auto-reactive T-cells have to be eliminated to establish self-tolerance. One way to achieve this is through the negative selection of T-cells as a result of their interaction with the thymic stroma where self-antigens are expressed. Generation of thymic chimeras using *aly/+* or *aly/aly* embryonic thymus suggests that NIK is required in stromal cells to prevent autoimmune diseases in some organs (Kajiura et al., 2004). In addition, *aly/aly* mice have fewer $CD4^+CD25^+$ regulatory T-cells (T_{reg}) (Kajiura et al., 2004). Reduction of this T-cell subset correlated with downregulation of forkhead box P3 (Foxp3) in $CD4^+$ T-cells and down-regulation of autoimmune regulator in thymus, the former being a major transcription factor for T_{reg} and the latter being responsible for antigen production to establish self-tolerance (Kajiura et al., 2004).

Additional studies using NIK knock-out mice support the notion that NIK has a role in the T_{reg} population. T_{reg} cells have a regulatory role to suppress auto-reactive effector T-cells. NIK knock-out mice have a significantly lower ratio of T_{reg} to total $CD4^+$ T-cells, although their suppressive function is comparable to the wild-type mice (Lu et al., 2005). Similar phenotypes were observed using *aly/aly* mice (Kajiura et al., 2004). In the absence of NIK, the T_{reg} population proliferates more upon stimulation through glucocorticoid-induced TNFR family-related gene (GITR), a TNFR family member (Lu et al., 2005). Moreover, NIK knock-out mice have a lower subset of specific T_{reg} population, defined as $CD62L^{high}$ $CD4^+CD25^+$ T cells, resulting in a higher proportion of $CD62L^{low}$

$CD4^+CD25^+$ T cells. The physiological implication of this phenomenon is the impairment of the normal regulatory T-cell response, leading to autoimmunity (Lu et al., 2005).

In addition to its intrinsic role in T_{reg} maintenance, NIK also mediates self-tolerance by modulating the signals through CD40 and RANK in medullary thymic epithelial cells (mTEC) (Akiyama et al., 2008). During the development of T-cells, those reacting with the self-antigens are eliminated. mTECs are proposed to mediate this elimination of self-reactive T-cells by expressing self-antigens. Precursor mTECs culture from *aly/aly* mice did not exhibit RelB expression or nuclear localization when treated with CD40L or RANKL, thus these precursor cells did not fully develop into mTEC (Akiyama et al., 2008). As NIK is downstream of CD40 or RANK signaling, NIK function is also necessary for the development of these cells (Akiyama et al., 2008).

Another way NIK is linked to autoimmunity is through its regulation in the Th17 cell population. Using NIK knock-out mice, it was demonstrated that NIK is necessary for Th17 differentiation (Jin et al., 2009). NIK knock-out mice are resistant to a T-cell mediated autoimmune disease named experimental autoimmune encephalomyelitis (EAE) due to the intrinsic defect in naïve $CD4^+$ T-cell population to differentiate into Th17 cells (Jin et al., 2009). Intriguingly, along with T-cell receptor (TCR) stimulation, Th17 differentiation can be co-stimulated by a TNFR family member, herpes virus entry mediator (HVEM), a receptor for homologous to Lymphotoxins, exhibits Inducible expression, and competes with HSV Glycoprotein D for HVEM, a receptor expressed by Tlymphocytes (LIGHT). This signaling is impaired in NIK knock-out T-cells, implying that NIK controls Th17 cells via LIGHT ligation (Jin et al., 2009). NIK deficiency does not affect T-cell proliferation, yet it attenuates Th17-regulatory genes, such as IL-17, IL-21, IL-22, IL-23R and RORγt (Jin et al., 2009). Th17 differentiation process may not be through the p100 to p52 proteolysis although this cannot be excluded. Instead, NIK deficiency results in diminished Stat3 phosphorylation upon activation through TCR as

well as IL-6R (Jin et al., 2009). Thus, NIK is necessary for synergistic Stat3 activation by these two receptors. It has already been reported that Stat3 is crucial for Th17 differentiation (Mathur et al., 2007), and thus NIK is likely to have an impact on Th17 differentiation via this transcription factor. In addition to its effect on T_{reg} and Th17 populations, a subset of immature natural killer T-cells (NKT cells), referred to as Vα14i NKT, requires NIK-mediated RelB activation for development (Elewaut et al., 2003). Thus, NIK plays a pivotal role in various populations of T-cells, modulating their development or differentiation.

NIK not only plays a critical role in T-cell differentiation and development, but it also has an effect on T-cell activation. TCR/CD3-stimulated T-cells cannot produce IL-2 in *aly/aly* mice (Yamada et al., 2000). In normal T-cells, NIK phosphorylates c-Rel in the transactivation domain and activates its transcriptional activity at CD28RE to regulate IL-2 production upon CD28 ligation (Sanchez-Valdepenas et al., 2006). The signaling through TCR and CD28 are thought to cooperate for optimal activation of T cells and cytokine production (Acuto & Michel, 2003). NIK has a role in regulating those processes through both receptors. Moreover, NIK and protein kinase C (PKC) cooperate in T-cell activation, as suggested by the defective proliferation in NIK defective mature T-cells. The NF-κB response cannot be sustained in thymocytes from mice with *aly* mutation in response to CD3 conjugation (Matsumoto et al., 2002). In parallel, thymocytes from *aly/aly* mice have a defect in the proliferative response upon stimulation with anti-CD3 antibody (Matsumoto et al., 2002). Therefore, it is speculated that NIK regulates the function of T-cells by modulating NF-κB activation when T-cells respond to antigens and in mediating T-cell proliferation (Matsumoto et al., 2002).

Other immune regulation of NIK

Interestingly, in T and B cells isolated from the peritoneal cavity, NIK is downstream of secondary lymphoid organ chemokine (SLC)/CCL21 receptor in activating NF-κB (Fagarasan et al., 2000). Although it is well established that NIK is downstream of some cytokine receptors such as BAFFR, CD40 and LTβR, it was not previously connected to a seven-transmembrane G protein-coupled chemokine receptor. These peritoneal cavity cells (T- and B-cells) from *aly/aly* mice exhibit a defective homing to the gut-associated lymphatic tissue *in vivo* (Fagarasan et al., 2000). This homing defect partly contributes to the impaired immunoglobulin production of B-lymphocytes as they fail to migrate to the proper niches to become plasma cells (Fagarasan et al., 2000).

The contribution of NIK in dendritic cells (DCs) has been elucidated by a few studies using *aly/aly* mice. DCs with *aly* mutation still have intact DC activation in response to engagement through CD40 (Garceau et al., 2000). DCs from *aly/aly* mice do not exhibit any difference in cell surface molecules such as MHC I, MHC II, CD80/86, CD40, CD70, 41BB-L, and CD150 or upregulation of chemokine receptors such as CCR2 and CCR7 (Lind et al., 2008). Nevertheless, they are intrinsically defective in cross-priming with $CD8^+$ T cells (Lind et al., 2008). This defect is a result of *aly* mutant DCs inability to process the whole antigen (Lind et al., 2008). DCs with *aly* mutation also fail to induce many genes, some of which are implicated in the survival of DCs [cellular FLICE like inhibitory protein (cFLIP) and serpinb9] and antigen processing (cathepsin E and Lamp1) (Lind et al., 2008).

In addition to studying the functionally defective NIK, introducing wild type NIK into DCs provides insight into the function of NIK in DCs. It has been proposed that induction of NF-κB through over-expression of NIK in DCs can be an effective vaccine

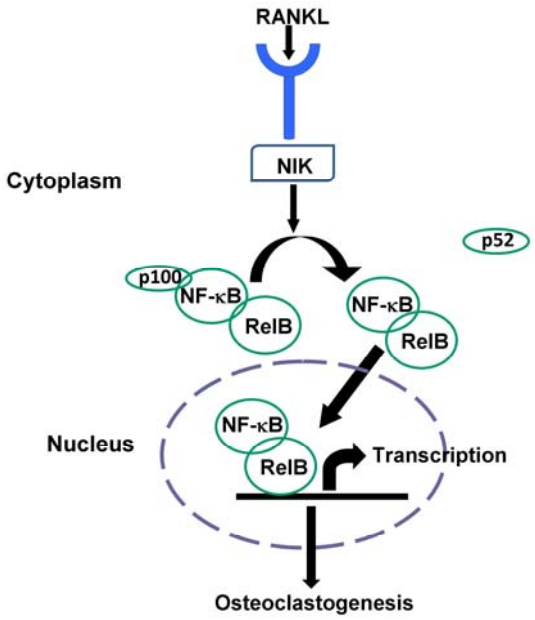
adjuvant (Andreakos et al., 2006). When expressed in DCs, NIK induces cytokine and chemokine production (such as TNFα, IL-6, IL-12, IL-15 and IL-18, IL-8, macrophage inhibitory protein-1 (MIP-1α/β)/CCL3, MCP-1 and MCP-3) (Andreakos et al., 2006). More importantly, when over-expressed in DCs, NIK enhances the antigen presentation, Th-1 type immune response, and antigen specific cytotoxic T-cell response *in vivo* (Andreakos et al., 2006).

NIK not only participates in the regulation of the adaptive immunity, it also has a role in innate immune regulation. NIK stimulates the transcription of cyclooxygenase-2 (COX-2) by phosphorylating a transcription factor PU.1, which binds to the promoter region of COX2 (Azim et al., 2007). When NIK expression was silenced by siRNA, less PU.1 was recruited to the COX-2 promoter. In parallel, COX-2 production was impaired in NIK siRNA treated macrophages in response to LPS (Azim et al., 2007). NIK also has a role in the inflammasome by interacting with a nucleotide binding domain-leucine rich repeat (NLR) protein, Monarch-1 (Arthur et al., 2007; Ye et al., 2008). Upon CD40 ligation, Monarch-1 and NIK association increases, resulting in proteasomal degradation of NIK and suppression of p100 to p52 processing in monocytes (Arthur et al., 2007).

The role of NIK in the bone microenvironment

Osteoclasts and osteoblasts reside in the bone marrow, maintaining the balance between bone resorption and bone formation, respectively. This balance is important since it affects the general bone structure as well as the bone maintenance associated with pathological conditions such as cancer metastasis and arthritis. Research relating NIK in osteoclastogenesis has been of interest, as RANKL differentiates monocyte and macrophage lineage progenitors to become osteoclasts. RANKL is one of the few known activators of the non-canonical pathway that plays a crucial role in the bone

microenvironment. NIK deficiency deters the nuclear translocation of RelA and p52 in response to RANKL stimulation, due to the role of p100 as an IκB in osteoclast precursors (Figure 5). In other words, the lack of p100 proteolysis as a result of NIK loss prevents osteoclast formation (Novack et al., 2003). In this scenario, the loss of NIK affects both pathways of NF-κB and the phenotypes observed cannot be clearly attributed to either the classical or the non-canonical pathway of NF-κB.



Thu and Richmond, *Cytokine and Growth Factor Reviews*, 2010.
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Figure 5. The requirement of the function of NIK in inducing the proteolysis of p100 to p52 for osteoclastogenesis. The IκB function of p100 deters NF-κB family members such as RelA and RelB to translocate to the nucleus. Re-expression of RelB rescues the defective osteoclastogenesis in osteoclast precursors but RelA does not, suggesting that only RelB is required for the osteoclastogenesis.

To distinguish the relative contribution of the two pathways, Vaira *et al*. re-expressed RelA or RelB in $NIK^{-/-}$ osteoclast precursors (Vaira et al., 2008). They observed that only the re-expression of RelB rescues the differentiation defect of the osteoclast precursors, suggesting that RelB is necessary for the expression of factors required for osteoclast differentiation (Vaira et al., 2008). NIK itself is not required for the differentiation of these cells. Crossing $NIK^{-/-}$ and $p100^{-/-}$ mice gave rise to mice with normal osteoclast differentiation (Vaira et al., 2008). This observation suggests that NIK stimulation of the proteolytic processing of p100, a negative regulator of osteoclast differentiation, is necessary for the osteoclastogenesis (Figure 5). Amazingly, TNFα induces TRAF3 stabilization in osteoclasts (Yao et al., 2009), possibly destabilizing NIK, diminishing p100 processing and promoting osteoclastogenesis (Tanaka & Nakano, 2009). Unlike osteoclastogenesis, tumor-induced osteolysis in a B16 melanoma model requires both NIK and RelB, evidenced by the inhibition of tumor-induced bone loss in $RelB^{-/-}$ and $NIK^{-/-}$ mice injected with B16 melanoma cells (Vaira et al., 2008). Therefore, the non-canonical pathway of NF-κB may have a therapeutic value in bone metastatic cancers.

As NIK function is needed for osteoclast differentiation, its role has been studied in an inflammatory arthritis model. Bone degradation in arthritis is performed by osteoclasts. Inhibiting the inducer of osteoclastogenesis, RANKL, ameliorates bone erosion in certain arthritis models (Kong et al., 1999; Pettit et al., 2001). In serum transfer arthritis model, NIK is not required for inflammation, but for bone erosion (Aya et al., 2005). By using B-and T-cell transfer into recombination activating $gene^{-/-}$ ($RAG2^{-/-}$) mice, Aya *et al*. elegantly demonstrated that NIK is necessary in T-cells, but not in-B cells, for antigen-induced arthritis (Aya et al., 2005).

Interestingly, NIK determines the fate of bone marrow stem cells through a peroxisome proliferator-activated receptor γ (PPARγ)-dependent mechanism. Gene targeting of PPARγ in mice revealed that PPARγ induces adipogenesis (Kubota et al., 1999). Cytokines such as TNFα and IL-1 can prevent mesenchymal stem cells from differentiating into adipocytes. The adipogenesis suppression mediated by these cytokines is through activation of TANK-1 binding protein (TBK1)/TAK1/NIK signaling (Suzawa et al., 2003). Over-expression of NIK in ST2 cells, a mesenchymal cell line, diminishes PPARγ transactivation activity by increasing NF-κB association with PPARγ and reducing its DNA binding (Suzawa et al., 2003). When the activity of PPARγ is suppressed, mesenchymal stem cells become osteoblasts instead of becoming adipocytes (Takada et al., 2007). Therefore, NIK may indirectly contribute to osteoblast genesis.

The importance of NIK in cancer: death versus survival (cIAPs and NIK)

Although traditionally thought of as pro-survival factors, cIAPs have anti-proliferative and anti-survival function in B-lymphocytes due to their role in degrading NIK (Zarnegar et al., 2008). It is clearly demonstrated that NIK provides survival signals, especially in B-cells. For example, B-cells with TRAF2 and TRAF3 deficiency (Gardam et al., 2008; Grech et al., 2004; Xie et al., 2007), as well as B-cells expressing a NIK mutant that lacks the TRAF3 domain (Sasaki et al., 2008), have prolonged survival due to the accumulation of NIK. B-cells from these mice become independent of CD40 or BAFFR signaling.

On the contrary, SMAC mimetic compounds, which inhibit cIAPs and stabilize NIK, are used to induce cell death in many cancer cells. The detailed mechanism of how SMAC mimetic compounds mediate cell death has been previously described

(Varfolomeev et al., 2007). These compounds potentiate auto-ubiquitination and degradation of cIAPs. As a result, both the canonical and the non-canonical pathways are activated, increasing the production of TNFα along with other chemokines such as IL-8 and MCP-1. The activation of two NF-κB pathways cannot be inhibited by the use of a TNF-blocking agent such as TNFR1-Fc. Nevertheless, cell death induced by SMAC mimetic drugs can be inhibited by blocking TNFR signaling, implying that this signaling is necessary for cell death (Varfolomeev et al., 2007). In support of these data, another study also observed the activation of NF-κB (both canonical and non-canonical) and cell death in the presence of cIAPs antagonists (Vince et al., 2007). The investigators of this study speculated that the ability of cIAP antagonists to induce the canonical NF-κB pathway is likely due to the prolonged association of RIP-1 with TNFR when cIAPs are absent (Vince et al., 2007). When cIAPs are degraded, NF-κB activation may no longer be cytoprotective to counteract TNFα-induced cell death.

Another independent study regarding SMAC mimetics examined their effect on tumor growth *in vivo*. Similarly, this study also demonstrated that TNFR signaling can lead to apoptosis, induced by SMAC mimetics (Petersen et al., 2007) and this cell death is also RIP-1 dependent, in agreement with the study by Vince *et al*. (Vince et al., 2007). SMAC mimetic-induced TNFα production triggers the signaling of TNFR to form a RIP-dependent death-inducing complex in addition to its signaling through pro-survival NF-κB activation (Petersen et al., 2007). The death signal possibly prevails over the survival function of NF-κB, leading to cell death in cancer cells (Petersen et al., 2007).

In these studies using SMAC mimetics, NIK is stabilized upon treatment with the pharmacological cIAP inhibitors, yet cell death is still induced. On one hand, NIK is thought to be a pro-survival mediator as it is a potent activator of NF-κB. On the other hand, the role of some of NIK negative regulators, such as TRAF2, cIAP1 and cIAP2

during the anti-apoptotic mediation of NF-κB has been documented (Wang et al., 1998). Although cIAPs are traditionally viewed as pro-survival factors, they antagonize the pro-survival function of NIK. Interestingly, B-cells expressing a NIK mutant with the deletion of the TRAF3 binding domain are much more susceptible to FasL-induced cell death as they have elevated Fas expression (Sasaki et al., 2008). Therefore, it is possible that stabilization of either NIK or cIAPs is necessary for the survival of different cell types. NIK undergoes continuous proteasomal degradation that is executed in part by cIAPs in non-transformed cells, thus NIK may not mediate survival in this context. However, in the event of transformation, malignant cells may become dependent on the stabilization of NIK. Therefore, for the safe use of SMAC mimetics in cancers and to target NIK as a cancer therapeutic, the question of how the coordination between NIK and cIAPs favors death versus survival needs further exploration.

NIK in hematological malignancies

Mis-regulation of NIK in multiple myeloma has been well documented by several studies. Genetic screening of multiple myeloma tumor samples and cell lines suggest that many genetic mutations occur in these tumors, leading to activation of the canonical and non-canonical NF-κB pathways. More importantly, genetic or epigenetic alterations that lead to NIK stabilization have been demonstrated in multiple myeloma (Annunziata et al., 2007; Keats et al., 2007). Some negative regulators of NIK, such as TRAF2, TRAF3, cIAP1 and cIAP2, have inactivating mutations in multiple myeloma (Annunziata et al., 2007; Keats et al., 2007). In addition, activating mutations have also been identified in positive regulators of NIK, such as CD40 and LTβR (Annunziata et al., 2007; Keats et al., 2007).

Another type of mutation is an activating mutation in NIK itself. Three of five multiple myeloma patient samples have chromosomal translocations between the NIK locus and either the IGH or IGL locus (Annunziata et al., 2007). Some multiple myeloma cell lines also have chromosomal translocations or gene amplification, leading to enhanced NIK expression (Annunziata et al., 2007). Furthermore, allelic deletions of TRAF3 and mutations resulting in a truncated form of TRAF3 that cannot bind to NIK were also identified (Annunziata et al., 2007; Keats et al., 2007). As a result, multiple myeloma tumors from patients as well as cell lines exhibit a high NF-κB index (Annunziata et al., 2007; Keats et al., 2007). Although plasma cells, from which multiple myeloma is derived, normally display high NF-κB activation, this activation is ligand-dependent. Therefore, it is thought that the mutations mentioned above render multiple myeloma cells with the ability to undergo ligand-independent proliferation and survival (Annunziata et al., 2007; Keats et al., 2007).

In support of this notion, manipulations of NF-κB regulators alter the pro-survival phenotype. For instance, TRAF3 was re-introduced in cell lines with either bi-allelic deletions or inactivating mutations. Re-expression of TRAF3 ablates the constitutive processing of p100 to p52 as well as nuclear translocation of RelA, p50, p52 and RelB, resulting in the growth arrest of these tumor cells (Annunziata et al., 2007; Keats et al., 2007). It was reported that the TRAF3 level was altered in many leukemia and lymphoma cell lines, suggesting the possibility of NIK involvement in these malignancies (Zapata et al., 2000). NIK silencing provides direct evidence for the contribution of NIK to multiple myeloma. Knocking down NIK in multiple myeloma cell lines with high NIK expression induced cell death, whereas cells with low NIK expression were unaffected (Annunziata et al., 2007). These data suggest that multiple myeloma cells with elevated

NIK expression are indeed dependent on NIK expression for their survival (Annunziata et al., 2007).

In support of the pro-survival role of NIK, expressing NIK mutant without the TRAF3 binding domain in B-cells lead to hyperplasia and independence of BAFFR signaling (Sasaki et al., 2008). Since signaling from the BAFFR is crucial for B-cell survival, BAFFR-independence as a result of NIK stabilization is one of the events important for B-cell malignancy.

Despite the increasing evidence that malignancy of myeloma can be attributed to NIK, the mechanism by which NIK is mediating tumorigenesis is still elusive. The literature to date suggests that both the canonical and non-canonical pathways may be crucial for the ability of NIK to elicit transformation. shRNA-mediated depletion of NIK in multiple myeloma cell lines with TRAF3 or cIAPs mutation abrogates both canonical and non-canonical NF-κB pathway activation (Annunziata et al., 2007; Demchenko et al., 2010). Interestingly, silencing of IKKα using shRNA was not toxic to NIK over-expressing multiple myeloma cells, suggesting that NIK does not mediate tumorigenesis via the non-canonical pathway (Annunziata et al., 2007). When NIK was over-expressed in multiple myeloma cell lines with low NF-κB activity, the gene signature induced by NIK was almost identical to the cell lines with IKKβ over-expression, implying that NIK over-expression mediated NF-κB gene transcription to induce tumorigenesis can be through the canonical pathway (Demchenko et al., 2010). However, in another cell line, the NIK over-expression-induced gene signature does not correlate well with IKKβ over-expression-induced signature (Demchenko et al., 2010). Hence, it remains elusive how NIK over-expression may lead to malignant cell phenotypes.

Another hematological malignancy in which NIK plays a role is T-cell leukemia and Hodgkin Reed-Sternberg cells. A genetically engineered mouse model suggests a

role for NIK and NF-κB in development of T lymphocyte malignancy. In thymocytes, NIK plays a role in Notch3 mediated NF-κB activation in a ligand-independent manner, resulting in high proliferative and tumorigenic potential of these cells (Bellavia et al., 2000; Vacca et al., 2006) . However, in the absence of pre-TCR/pTα, NIK does not participate in the IKK complex and p100 to p52 processing is only dependent upon IKKα (Vacca et al., 2006).

In concert with the data from the mouse model, the transcript level of NIK is highly upregulated in leukemia cell lines as well as in patients (Saitoh et al., 2008). Protein expression of NIK in these cells was observed only with proteasomal inhibitor, MG132, treatment suggesting that the turn-over rate of NIK is rapid in this type of tumor (Saitoh et al., 2008). Although NIK knock-down did not affect cell growth *in vitro,* depletion of NIK significantly decreased tumor size in a mouse model (Saitoh et al., 2008). Perhaps, NIK expression allows the tumor cells to grow in an *in vivo* tumor environment by affecting other cells in the tumor micro-environment that promote tumor growth, and this difference may not be apparent in culture where growth is more conducive or where stromal cells are absent. Although this study highlights the importance of NIK in T-cell leukemia, it remains unclear what aspect of tumor development NIK affects.

In parallel, forced expression of NIK in rat fibroblasts transformed them, as assessed by anchorage independent growth. However, the investigators of this study noted that retroviral overexpression of NIK was toxic to the cells. Thus, they used fibroblasts expressing a gene under the transcriptional control of NF-κB, which confers resistance to an antibiotic blasticidin only when NF-κB is constitutively active. Only under this selection pressure did NIK over-expression lead to transformation. The ability of NIK to transform these fibroblasts depends on NF-κB activation since over-expressing the

super-suppressor IκB hindered colony formation of NIK over-expressed cells in a soft agar assay (Saitoh et al., 2008).

NIK in solid tumors

In addition to hematological tumors, some reports suggest the role of NIK in solid tumors, albeit less extensively. For instance, constitutive activation of the non-canonical pathway of NF-κB as well as NIK accumulation upon MG132 treatment was demonstrated in pancreatic cancer cells (Nishina et al., 2009). When NIK expression was reduced by siRNA, cell growth and p100/p52 processing were also diminished, suggesting that those processes are attributable to NIK over-expression in these pancreatic cancer cells (Nishina et al., 2009). However, NIK does not regulate the canonical pathway in these cells (Nishina et al., 2009). Similar to pancreatic cancer cells, non-small cell lung cancers have elevated NIK expression. Depletion of NIK diminishes constitutive NF-κB activation of both pathways, as well as some survival or metastatic genes such as survivin and matrix metalloprotease-9 (MMP-9) (Saitoh et al., 2010). Therefore, depending on cancer types, NIK may regulate one or both NF-κB pathways. Moreover, over-expression of NIK is responsible for the constitutive activation of NF-κB in basal-like subtype breast cancer cell lines (Yamaguchi, et al., 2009).

Additionally, one study suggests that NIK may be required for invasion and migration of a murine melanoma cell line (Rangaswami et al., 2004). In this model, osteopontin (OPN) leads to enhanced production of MMP-9, possibly mediated by NIK activation (Rangaswami et al., 2004). In support of the murine melanoma model, in human melanoma cells with constitutive NF-κB activity, there is increased association of NIK with the IKK complex in melanoma as compared to normal melanocytes based upon co-immunoprecipitation assays (Dhawan & Richmond, 2002). Interestingly, melanoma

cells show higher expression of LTβR, an upstream activator of NIK. Silencing the expression of the LTβR reduces the growth and invasion of melanoma cells (Dhawan et al., 2008).

Although some advances have been made in understanding NIK's tumorigenic potential, additional studies are necessary to understand the possible contribution of NIK in solid tumors. Additional *in vivo* models are gravely needed to elucidate the function of NIK in carcinogenesis. Moreover, the mechanism by which NIK mediates tumorigenesis is poorly understood. NIK is likely to be affecting both NF-κB pathways during malignancy, possibly as a result of shifting the balance between the positive and negative feedback mechanisms. It cannot be excluded that NIK may have NF-κB independent functions in contributing to tumorigenesis. As NIK is not an essential kinase for the canonical NF-κB pathway under physiological conditions, a thorough understanding of its function may provide avenues for therapeutic interventions in cancer treatment.

Concluding remarks

NIK functions as a kinase crucial for the non-canonical pathway of NF-κB and contributes to various cellular processes, ranging from the regulation of adaptive and innate immunity to cancer growth and survival. Although significant advances have been made during the past 13 years since NIK was first cloned, many questions remain unanswered. Most importantly, regulation of NIK function is not completely clear. Is stabilization of NIK required for the phosphorylation and activation of NIK? Which ligase(s) is/are responsible for ubiquitination of NIK? Although the data are suggestive that endogenous NIK is ubiquitinated under basal conditions, this has not been formally demonstrated. Answering these questions will be valuable to understand the importance

of NIK in disease models such as cancer and autoimmunity, to eventually develop therapeutic interventions. NIK is an attractive therapeutic target since its function is not essential for activation of the canonical pathway of NF-κB, though NIK can affect this pathway upon stabilization. It appears that cells under physiological conditions do not require abundant NIK expression as evidenced by the low level of NIK in most cell types and the survival of systemic NIK knock-out mice. However, in pathological contexts, such as cancer, NIK becomes stabilized. Thus, attenuating the function of NIK is a potentially promising strategy for cancer therapy. Yet, targeting NIK to alleviate cancer should proceed with caution due to its role in physiological functions of the patient such as antibody production, T-cell regulation and osteoclastogenesis.

Summary and Dissertation Goals

Since the discovery of NIK, we have become to understand its regulation and functions in different physiological processes. However, many areas, including the role of NIK in tumorigenesis, remained to be explored. In addition, our lab has previously shown that higher amount of NIK immunoprecipitates with the IKK complex in melanoma compared to normal melanocytes (Dhawan & Richmond, 2002). We have also shown that an upstream receptor of NIK, LTβR, contributes to melanoma tumor growth (Dhawan et al., 2008). Based on the current literature and previous studies from our group, we hypothesize that NIK promotes melanoma malignancy. The goal of this thesis is to better elucidate the significance of NIK over-expression in melanoma and molecular mechanisms by which NIK mediates melanoma tumorigenesis. In addition, we also give brief insights into how systemic immune system and tumor immunology may be altered when NF-κB activation is targeted for melanoma therapy.

CHAPTER II

NIK MODULATES MELANOMA TUMOR GROWTH BY DOWN-REGULATING EXPRESSION OF PRO-SURVIVAL FACTORS THROUGH THE β-CATENIN PATHWAY

Rationale

Previous studies from our laboratory have shown that NF-κB activity is crucial for melanoma growth. Inhibiting IKKβ by pharmacological agents or genetic ablation to reduce NF-κB activity leads to significant reduction of melanoma tumor growth (Yang et al., 2006a; Yang et al., 2007a; Yang et al., 2010). Although IKKβ is a good potential target for melanoma therapy, its crucial role in the immune system may raise concerns for systemic therapy in patients. Therefore, we explored the possibility of targeting another kinase, NIK that is not essential for many physiological processes or for the activation of NF-κB. The expression of NIK is up-regulated in melanoma compared to normal melanocytes. NIK knock-out mice are viable (Yin et al., 2001) and they do not exhibit any lethal phenotypes despite some defects in lymphoid development and osteoclastogenesis. Moreover, it is consistently reported that NIK contributes to tumorigenesis of cancers such as multiple myeloma and lymphomas as mentioned in Chapter I. Based on the above knowledge, we hypothesized that NIK contributes to melanoma tumorigenesis and that NIK may be a potential therapeutic target for melanoma treatment.

(Part of this Chapter is published in Thu *et al.*, *Oncogene*, 2011)

Materials and Methods

Cell culture

Melanoma cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS) at 37°C with 95% air/5% CO_2 in a water jacketed incubator. Normal human epidermal melanocytes were obtained from Vanderbilt Skin Research Core or were purchased from Clonetics and cultured in Media 254 containing supplements for melanocytes (Cascade Biologics S-002-5) under the same conditions. Cell lines were obtained from ATCC and were tested for mycoplasma monthly. *Nik*$^{+/+}$ and *Nik*$^{-/-}$ MEFs were cultured in DMEM containing 10% FBS, 2% L-glutamine, at 37°C with 95% air/5% CO_2 in a water jacketed incubator. HEK293 cells were cultured in DMEM containing 10% FBS at 37°C with 95% air/5% CO_2 in a water jacketed incubator.

Generating NIK knock down cell lines

Melanoma cells (Hs294T or WM115) were infected with lentivirus carrying NIK shRNA, (Sigma Mission shRNA pLKO.1_733 or_731) or IKKα shRNA (Sigma Mission shRNA pLKO.1_508). Forty-eight hours after infection, cells were cultured in media containing puromycin (Sigma P9620) at a concentration of 2ug/ml to select for cells expressing the NIK shRNA, the IKKα shRNA or the control non-targeting shRNA.

IKK kinase assay

Non-silencing or knock-down cells were starved overnight and treated with vehicle or TNFα (20ng/ml) (PreproTech 300-01A) for 30 min. Using 200 µg of cytoplasmic extract prepared from untreated or treated cells, IKK kinase assay was performed as previously described (Yang et al., 2007b).

Cytokine array

Non-silencing or knock-down cells were cultured for 24 h in the same amount of serum free DMEM/F12 to obtain conditioned media. Cytokine array (RayBioTech: AAH-CYT-G2000-8) was performed according to the user manual using 50µl of collected conditioned media. Serum free media was used as a negative control.

Bromodeoxyuridine (BrdU) incorporation

BrdU incorporation was performed using the BrdU staining kit (BD Biosciences 559619). Briefly, cells were cultured until they reached 70-80% confluency, and then incubated with BrdU for 4 h in serum containing media. Following BrdU incorporation, cells were fixed, permeabilized and stained with anti-BrdU antibody conjugated with FITC, according to the manufacturer's protocol. After staining, samples were analyzed by flow cytometry (Beckman Coulter).

Cell cycle analysis

Non-silencing and NIK knock-down cells were synchronized by thymidine block (2mM for 16h). After indicated amount of time of release from thymidine block, cells were collected, fixed with 70% ethanol and stained with propidium iodide (PI) staining buffer [0.1% Triton X-100, 0.2mg/ml of RNAse, 20µg/ml of PI, 1mM of ethylenediaminetetraacetic acid (EDTA)] for 30min at room temperature (RT), followed by flow cytometry analysis. Experiments were performed at least three times and statistical significance was determined by using t-test. Significance was assumed if the p-value ≤ 0.05.

Annexin V staining

Non-silencing and knock-down cells were plated at equal density and treated with TNFα (1000ng/ml) (PeproTech 300-01A) where indicated. After the 8h of TNFα treatment, the assay was performed as described in the user manual (TACS Annexin V 4830-01-K R&D Systems). Samples were subjected to flow cytometry analysis (Beckman Coulter), following the Annexin V staining.

Western blot and immunoprecipitation

Cells were lysed with the buffer containing 10% glycerol, 50mM Tris pH 7.7, 150mM NaCl, 0.5% NP40 and a cocktail of protease (Sigma P8340) and phosphatase inhibitors (Sigma P2850 and P5726). Proteins were separated by electrophoresis and immunoblotted with appropriate antibodies (antibody information described later). Dilutions of antibodies used are as recommended by the manufacturers and the rest of the Western blot procedure was as previously described (Yang et al., 2007b). Immunoblots were repeated at least three times, scanned for band density, and mean density values were quantified using ImageJ (NIH).

Primary antibodies used were NIK (Novus NB120-7204), p100/p52 (Upstate 05-361), RelA (Santa Cruz sc-109) phospho-p65 (Ser536) (Cell Signaling 3033S), IκBα (BD Transduction Laboratories 610690), β-catenin (Abcam ab2365), Bcl2 (Santa Cruz sc-7382), Survivin (Cell Signaling 2808), cIAP1 (Alexis: ALX-803-335), Bcl-xl (Santa Cruz sc-634), FLIP (Cell Signaling 3210), actin (Santa Cruz sc-1616 or 1616R) or tubulin (Sigma T2220). Secondary antibodies are (Molecular Probes Alexa Fluor 680 or 800 A21058/ A21084; Rockland 611-732-127 or Jackson Laboratories 115-035-146 (goat anti-mouse), 705-035-003 (donkey anti-goat), 111-035-144 (goat anti-rabbit).

Transfection of HEK293 cells with Flag-NIK (kindly shared by David Goeddel) or β-catenin (purchased from Open Biosystems: MHS1010-9205712) (1.5μg each DNA) was performed using Fugene (Roche 11814443001) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were lysed with IP lysis buffer: 10% glycerol, 50mM Tris pH 7.7, 150mM NaCl, 0.5% NP40 and a cocktail of protease (Sigma P8340) and phosphatase inhibitors (Sigma P2850 and P5726). Flag-NIK was immunoprecipitated using an antibody against Flag (Sigma F1804, 1μg/1mg of total protein) whereas, β-catenin was immunoprecipitated using an antibody against β-catenin (Abcam: ab2365, 1μg/1mg of total protein). Immunoprecipitation was performed as detailed in Chapter IV.

Cytoplasmic/ nuclear extraction

Cytoplasmic and nuclear extraction was performed using NE-PER Nuclear Extraction Reagents (Pierce: 78833), following the manufacturer's protocol except the changes mentioned here. Briefly, cells were harvested, washed with 1xPBS and lysed using CERI buffer for varying amounts of time depending on the cell line (WM115 ns shRNA and shNIK: 2h, *Nik*$^{+/+}$ and *Nik*$^{-/-}$ MEFs: 30min). At the end of cytoplasmic lysis, CERII buffer was added to the lysate, supernatant was collected and nuclei were washed with 1xPBS three times. Nuclei were then lysed with NER buffer as described in the manufacturer's protocol. Lysates were subjected to sodium dodecyl sulfate (SDS)-gel electrophoresis under reducing conditions followed by Western blot for immunodetection of specific proteins. LaminA/C (Cell Signaling 4777) or Oct-1 was used as a nuclear marker. GAPDH (Cell Signaling 2118) was used as a cytoplasmic marker.

Quantitative Real-time PCR

RNA was isolated from non-silencing and knock-down cells using the RNeasy kit (Qiagen 74104) according to the manufacturer's protocol. Subsequently, cDNA was synthesized from one μg of the isolated RNA using the iScript cDNA Syn RT-PCR kit (BioRad 1708890) according to the user manual. The resulting cDNA was then amplified by quantitative real-time PCR using IQ Real Time Sybr Green PCR supermix (BioRad 1708880) and BioRad IcyclerIQ Multicolor Real-Time PCR Detection System. cDNA levels were normalized to actin that was amplified with the following primers: 5'-caccacaccttctacaatgag-3', and 5'-atagcacagcctggatagc-3'. NIK primers were: 5'tgcggaaagtgggagatcctgaat-3', and 5'-tgtactgtttggacccagcgatga-3'. For Bcl2, cIAP1, survivin, and genes from microarray analysis, primers were purchased from SABiosciences. qRT-PCR arrays for NF-κB pathway is from SABiosciences: PAHS-025. Data are shown as fold changes in NIK knock-down cells compared to the non-silencing control, and each gene was normalized to the loading control (actin or tubulin).

Orthotopic tumor implant studies in mice

Non-silencing vector control or NIK knock down WM115 cells ($3x10^6$ per mouse) were subcutaneously injected into athymic-Foxn1$^{nu/nu}$ nude mice (Harlan Laboratories) and allowed to grow until palpable tumors developed (about three weeks). Once palpable tumors formed the size was measured every two days with a microcaliper. Three weeks from the day of initial palpable tumor detection (or when tumors reached 1.5 cm in one dimension), mice were sacrificed and final tumor volumes were measured by Hank's balanced salt solution (HBSS) volume displacement.

Immunofluorescence

Cells on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.2-0.5% of Triton-X 100 in 1xPBS for 5min. Fixed and permeablized cells were blocked with 10% normal donkey serum in 1xPBS for 30 minutes and incubated with β-catenin antibody (abcam ab2365, 1:100) for 2h at RT, followed by three washes with 0.1% Tween-20 in 1x phosphate buffered saline (PBS) (1xPBST) and incubation with secondary antibody conjugated with Cy2 (Jackson ImmunoResearch 715-225-150, 1:100) for 2 h at RT. Then, coverslips were washed three times and stained with Hoechst nuclear stain during the last wash. Coverslips were then air dried and mounted onto glass slides. Images were taken using a 63x oil lens Plan-Apochromat on Zeiss Axioplan 2 microscope and Hamamatsu Orca ER fluorescence camera. Images were processed using Metamorph Software (Molecular Devices).

Immunofluorescence staining of Tissue Microarray (TMA)

Human patient samples for TMA were collected according to the protocol approved by Institutional Review Board of Vanderbilt University. Two or three cores were cut from each paraffin embedded, formaldehyde fixed tissue block and mounted into the TMA mold in a random fashion. Benign nevi (n=30), dysplastic nevi (n=22), primary melanoma lesions (n=15), and metastatic melanoma (n=13) lesions contributed to the tissue microarray block using protocols developed by Kononen et al. (Kononen et al., 1998), with the assistance of the Human Tissue Pathology Core at Vanderbilt University. The specificity and sensitivity of the NIK antibody to be used for immunofluorescent staining of TMAs was first validated in human melanoma xenograft tumor sections from WM115 tumors with or without NIK knock-down. TMA paraffin embedded block was cut into sections of 4μm thickness and immunofluorescent staining was performed as follow.

After antibody validation, paraffin embedded nevus and melanoma tissues were deparaffinized by washing two times in Xylene for 10 min each wash, followed by washing in a series of ethanol dilutions (100%, 90%, 80%, 70%, 50% ethanol). Then tissues were permeabilized in 1x Tris buffered saline with Tween-20 TBST (0.05% Tween-20) for 15min, washed with 1xTBS and rinsed with dH_2O. Antigen retrieval was performed by boiling tissue slides in 10mM sodium citrate pH 6.0 for 12 min. After the slides were cooled down, tissues were blocked with the blocking buffer containing 10mM Tris-HCl pH 7.4, 0.1M $MgCl_2$, 0.5% Tween-20, 1% bovine serum albumin (BSA) and 5% goat serum. Slides were then incubated with NIK antibody (Novus 120-7204, 1:100) in the blocking buffer overnight at 4°C. Slides were then washed with 1xTBST for 3x10min each and incubated with Alexa Flour 488 goat anti-rabbit secondary antibody (Invitrogen A-11008, 1:1000) for 2 h at RT. Slides were washed again with 1xTBST for 3x10 min each, counterstained with Hoechst and mounted with Prolong Gold antifade reagent (Invitrogen P36930). Images were scanned and quantitated using Ariol SL-50 imaging system (Genetix). Quantitative data are presented as (area of NIK staining/area of Hoechst staining)x100. Damaged tissue cores were excluded from quantitation. Statistical significance was determined by using ANOVA. Significance was assumed if the p-value ≤ 0.05.

Immunohistochemistry

Five micron sections of tissue were placed on charged slides, paraffin was removed by xylene and ethanol washes, and tissues were rehydrated. For caspase-3 staining, endogenous peroxidase was neutralized with 0.3% H_2O_2 for 20 min followed by Ultra V block for 5 min (Labvision) for blocking of nonspecific staining. For Ki-67 staining, sections were rehydrated and placed in heated Target Retrieval Solution

(Labvision). Endogenous peroxidase was neutralized with 0.03% H_2O_2 followed by a casein-based protein block (DakoCytomation) to minimize nonspecific staining. Then, sections were incubated with rabbit anti-human cleaved caspase-3 (Promega) or Ki-67 (Vector Laboratories VP-K451) for 60 min. The Dako Envision+ HRP/DAB+ System (DakoCytomation) was used to provide a substrate to develop peroxidase linked antibody and produce localized, visible staining. The slides were lightly counterstained with Mayer's hematoxylin, dehydrated and coverslipped using Vectashield Mounting Solution (H-1000).

Gene Expression Microarray Analysis

RNA was isolated from cells using the RNeasy kit (Qiagen 74104) according to the manufacturer's protocol. Affymetrix WT Sense reactions, using the Affymetrix WT Sense reaction kit (#900652), were assembled and run following Affymetrix protocol. Briefly, a sample mix of 200ng total RNA from NIK knock-down WM115 cells and from NIK shRNA control WM115 cells, and poly-A spike in controls (included in kit), were brought to a 5 μl final volume with nuclease free H_2O. First Strand Synthesis Master mix was added to the sample mix and incubated at 42º C for 1 h, enzymes were heat inactivated 10 min at 70ºC, followed by a cooling to 4º C for 2 min. Subsequently, Second Strand Synthesis Master mix was added to the sample mix, and incubated at 16ºC for 2 h. The reactions were heat-inactivated by 10 min incubation at 75ºC. The resulting cDNA was then processed in an IVT reaction to generate cRNA. The cRNA was used in first strand synthesis reactions to generate target of the correct sense for hybridization to the Affymetrix Gene and Exon arrays. The reactions were set up with 10μg cRNA, random hexamer, and first strand synthesis reagents to make cDNA. The targets were then treated with RNase to remove template RNA and cleaned up over kit

supplied columns, or Agencourt SPRI beads, following manufacturer's protocol (RNAClean, #000494). A total of 5.5μg of the clean cDNA target was then enzymatically fragmented and end-labeled using the Affymetrix kit reagents. The cRNA, cDNA, and fragmented and end-labeled targets were assessed by Agilent bioanalysis to insure that the amplified targets met the recommended smear range, and fragmentation and end-labeling were complete. For both Exon and Gene arrays, the requisite amount of target was then added to hybridization cocktail to give a final target concentration of ~25ng/μl in the hybridization cocktail. The targets in hybridization cocktail were heat denatured at 99ºC for 5 min, cooled to 45ºC for 5 min, centrifuged and then loaded on the Affymetrix Hu Gene 1.0STcartridge arrays for a hybridization period of 16 h at 45ºC, with rotation of 60RPM. The cartridge arrays were washed, stained and scanned per standard Affymetrix Protocols using Affymetrix Hybridization Wash and Stain kit reagents (#900720).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed following the protocol using Simple Enzymatic ChIP kit (Cell Signaling #9002). Briefly, cells were cross-linked using 1% paraformaldehyde, permeablized and chromatin was digested with micrococcal nuclease at 37ºC for 20min. Chromatin was immunoprecipitated using IgG, histone, or β-catenin (ab2365) antibodies for 2 h at 4ºC. Immunoprecipitated chromatin was purified and PCR was performed using the following primers amplifying the survivin promoter: 3'-GGGGCGCTAGGTGTGGG-5', 5'-TTCAAATCTGGCGGTTAATGGC-3' (β-catenin_human); 5'-CTTCTGCTTCCCTTCCAACC-3', 5'-GCCTTGCGCAAGGGTGTGATT-3' (β-catenin_mouse). PCR was performed with 30-35 cycles.

Statistical analysis

Non-parametric statistical analyses (Mann-Whitney or ANOVA) were performed using the Graphpad Prism software (La Jolla, CA). Statistically significant differences were assumed if the p-value ≤ 0.05.

Results

Evaluation of NIK expression in non-malignant nevi and melanoma lesions

To determine whether NIK levels are elevated in melanoma compared to non-malignant nevi, we examined NIK expression in tissue microarray of nevi (n=30), dysplastic nevi (n=22), primary melanoma (n=15) and metastatic melanoma (n=13) lesions by immunofluorescence staining. Intriguingly, NIK expression was higher in dysplastic nevi ($p<0.05$) and in primary and metastatic melanoma tissues compared to benign nevi ($p<0.05$) (Figures 6a and b). Likewise, Western blot analysis on lysates of 10 human melanoma patient tumors and 4 melanoma cell lines (Hs294T, SKMel5, SKMel28, WM115) showed that NIK protein expression was elevated in some melanoma tissues (M2, M5, M6, M7) and all melanoma cell lines compared to normal human epidermal melanocytes (NHEM) (Figures 6c and d). Nevertheless, NIK mRNA expression in melanoma cells was not significantly up-regulated in melanoma (Figure 7a), suggesting that NIK over-expression may be due to protein stability.

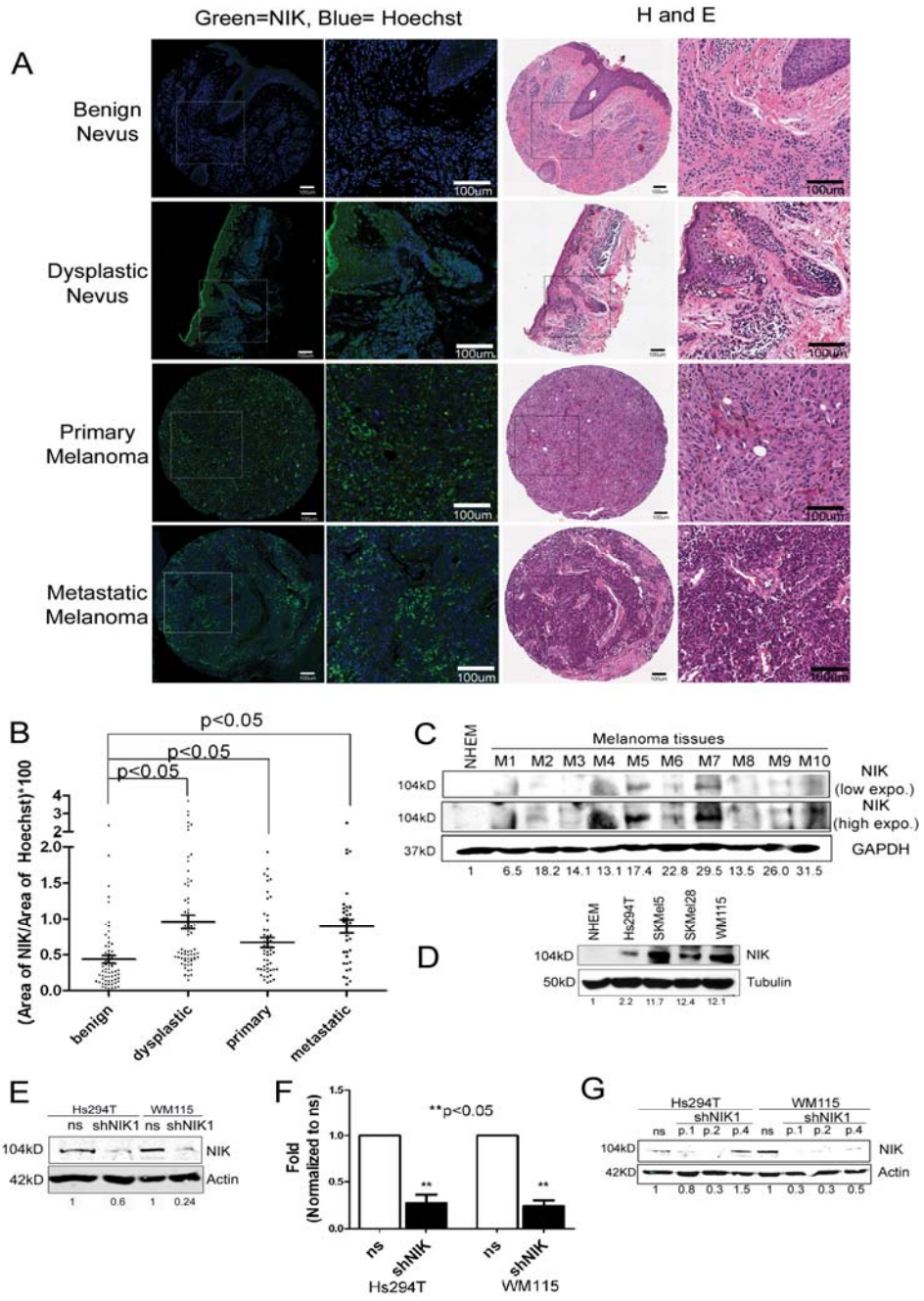


Figure 6. NIK expression is up-regulated in melanoma tissues and cells. **A.** Left to right: first column: Immunofluorescent analysis of NIK expression in benign (n=30), and dysplastic (n=22) nevi, primary (n=15) and metastatic (n=13) melanoma tissue samples from TMA. NIK positive cells are green, based upon Alexa 488 anti-rabbit antibody recognition of NIK antibody. Nuclei are blue based upon Hoechst stain. Second column: Enlargement of area denoted by box in the adjacent left panel showing fine cellular details of the histology of the lesions. Third column: H&E stain of the tissues shown in the first column. Fourth column: Enlargement of area denoted by box in the adjacent left panel showing fine cellular details of the histology of the lesions. [Scale bar =100μm]

B. Quantitation of NIK staining from TMA, based upon scans of images from duplicate or triplicate core samples of each lesion using the Ariol SL-50 imaging system (Genetix) calculated as described in Materials and Methods: (area of NIK staining/area of Hoechst) x100. Statistical significance was determined by ANOVA, where $p<0.05$ indicates significance. **C and D.** Western blot analysis of NIK expression in melanoma tissues (**C**) and cell lines (Hs294T, SKMel5, SKMel28, WM115) (**D**) compared to normal human epidermal melanocytes (NHEM). Densitometric scans from triplicate assays were quantitated, normalized to the loading control and calculated as fold difference from NHEM cells. **E.** Western blot and **F.** qRT-PCR analysis of NIK expression in NIK knock-down cells compared to the non-silencing (ns) control in Hs294T and WM115. Ns shRNA is a mock shRNA with the same vector backbone as the NIK shRNA. **G.** Western blot analysis of NIK in different passages of Hs294T_shNIK1 and WM115_shNIK1 cells (p=passage). Densitometric scans from triplicate assays were quantitated, normalized to the loading control and calculated as fold difference from ns.

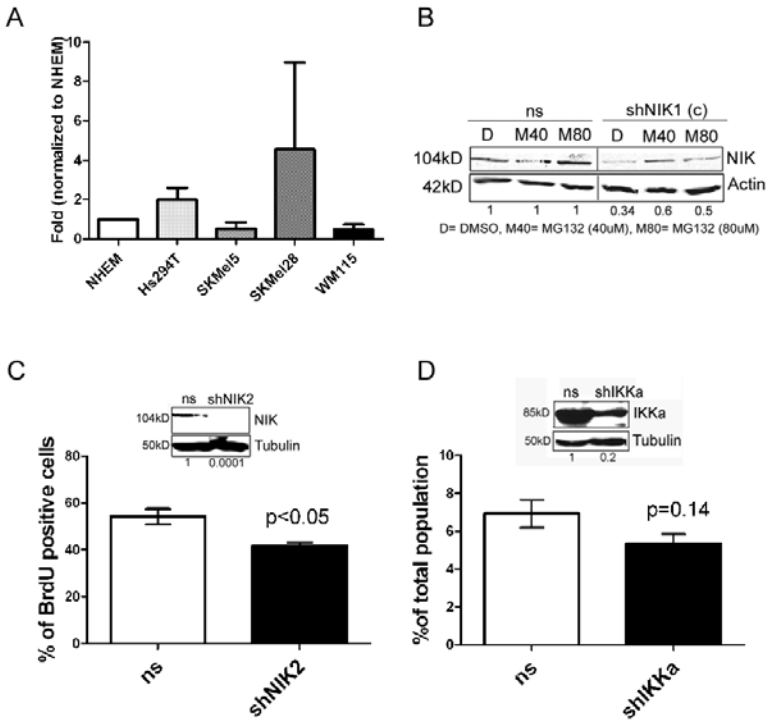


Figure 7. NIK mRNA expression in melanoma, NIK protein expression and BrdU incorporation in NIK knock-down WM115 cells and apoptosis of IKKα knock-down WM115 cells. **A**.qRT-PCR analysis of NIK mRNA in NHEM and melanoma. Fold changes were calculated by normalizing to the loading control and comparing it to NHEM. **B**. Western blot analysis of NIK knock-down WM115 clones treated with MG132 (40μM or 80μM) or DMSO. Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from non-silencing control cells. **C.** BrdU incorporation analysis of WM115 cells with another NIK-targeting shRNA (shRNA2) compared to the non-silencing control. Error bars are ± S.E.M. (n=6) **D.** Apoptosis analysis of IKKα knock-down WM115 compared to the control. Error bars are ± S.E.M. (n=12) In Figures C and D, insets are Western blot showing the efficiency of NIK knock-down. Statistical significance was determined by Mann-Whitney test, where $p<0.05$ indicates significance.

Development of melanoma cell lines with NIK knock-down

To understand the importance of NIK over-expression in melanoma, NIK was knocked down in two melanoma cell lines, Hs294T (derived from a metastatic melanoma) and WM115 (derived from a primary melanoma) with NIK targeting shRNA. Quantitative RT-PCR (qRT-PCR) and Western blot analyses showed that NIK expression was efficiently reduced at the mRNA and protein levels in Hs294T and WM115 (Figures 6E and F). Surprisingly, NIK knock-down was gradually lost within the population of Hs294T and WM115 cells selected to stably express the shRNA (Figure 6G), suggesting that NIK depletion slowed growth or enhanced death of melanoma cells and resulted in overgrowth of the cells without NIK knock-down.

Generation of clonal and polyclonal NIK knock-down cells

To prevent the selection against NIK depleted cells, a clonal population of NIK deficient WM115 cells was selected by in-cell-Western, followed by confirmation using Western blot. As NIK expression level is regulated by the proteasome, non-silencing and clonal knock-down [shNIK1(c)] cells were treated with a proteasome inhibitor (MG132) to stabilize NIK expression. MG132 treatment did not significantly stabilize the level of NIK protein in WM115 with NIK shRNA (Figure 7B), confirming the efficiency of NIK silencing. This NIK depleted WM115 clone was used to examine the functional significance of NIK in melanoma cells. In some experiments, polyclonal NIK knock-down WM115 cells [shNIK1(p)] were included to ensure that the observed phenomena were not simply due to clonal variation. Likewise, Hs294T cells with NIK knock-down by shRNA were also included in some experiments to evaluate the effect of NIK depletion in another melanoma cell line.

NIK deficiency in melanoma cells decreased proliferation and increased apoptosis

The loss of NIK-depleted cells from polyclonal lines over-time suggests that NIK may regulate proliferation and/or apoptosis. Consistently, NIK deficient cells exhibited a significant lag in doubling time compared to non-silencing control cells (Figure 8A). Both Ki67 and BrdU staining suggest that the percentage of cells undergoing proliferation was lower in knock-down populations than in the non-silencing control (Figures 8B and C). This phenotype was confirmed in WM115 with another shRNA targeting NIK (shNIK2) (Figure 7C). Depletion of NIK in another melanoma cell line Hs294T also resulted in decreased proliferation, determined by Ki67 staining (Figure 8B).

To determine whether NIK deficiency affected apoptosis, Annexin V and PI staining were performed. For these experiments, only polyclonal NIK knock-down cells in early passages were used, as we reasoned that the clonal knock-down cell line had already developed a mechanism to maintain equilibrium between survival and death. At a basal level, the proportion of apoptotic cells was two-fold higher in the knock-down population than in the control (Figure 8D, white bars). This suggests that over time the cumulative number of cells undergoing apoptosis would be much greater, providing an explanation for the growth disadvantage of NIK deficient cells. Interestingly, knocking down IKKα in WM115 did not induce apoptosis (Figure 7D), suggesting that NIK-mediated apoptosis is not likely to result from decreased IKKα activity.

NIK knock-down cells were sensitive to TNFα-induced cell death

It has been documented that NF-κB activation and NIK activity protect cells from TNFα-induced cell death (Malinin et al., 1997; Van Antwerp et al., 1998; Wang et al., 1996). To determine whether NIK deficient WM115 cells were sensitive to TNFα-induced

apoptosis, WM115 non-silencing or NIK knock-down cells were treated with TNFα (1000ng/ml) for 8h and apoptosis was determined by Annexin V and PI staining. TNFα treatment markedly increased the percentage of apoptotic cells in NIK deficient population (Figure 8D, dark bars).

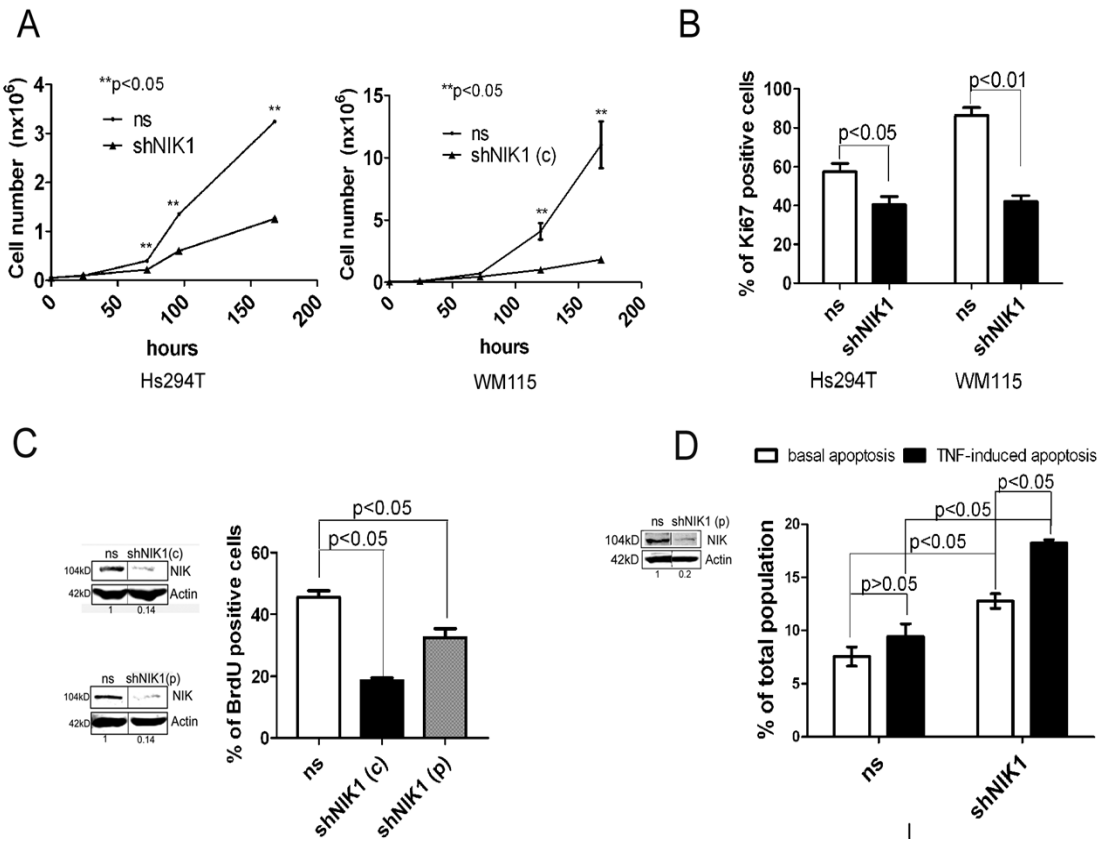


Figure 8. Depletion of NIK decreases growth and survival of melanoma cells. **A.** Growth curves of NIK knock-down and non-silencing Hs294T and WM115 cells. Error bars are ± S.E.M. In Hs294T graph, S.E.M.<0.02. (n=9) **B.** Quantification of Ki67 staining in NIK deficient Hs294T or WM115 cells compared to the non-silencing control. Error bars are ± S.E.M. (n=10) **C.** BrdU incorporation analysis of NIK knock-down WM115 cells compared to WM115 with non-silencing control. Error bars are ± S.E.M. (n=7) **D.** Apoptosis analysis of basal and TNFα-treated NIK knock-down WM115 compared to the control. Percentage of apoptosis is defined by % of cells that are Annexin V^+ and Annexin V^+ PI^+. Error bars are ± S.E.M. (n=9) In Figures C and D, insets are Western blot showing the efficiency of NIK knock-down.

NIK deficient WM115 cells exhibit a defect in cell cycle progression

In addition to a defect in proliferation and apoptosis, NIK knock down WM115 cells exhibited a delay in cell cycle progression compared to the control, most prominent at 12h after the release from thymidine block (Figure 9). It appears that NIK depleted cells have a delay in progression from G1 to S (Figure 9). By 12 h after release, cells with the control shRNA have completed the cell cycle, whereas a significant percentage of NIK knock-down cells are still in G1 and S phase (Figure 9).

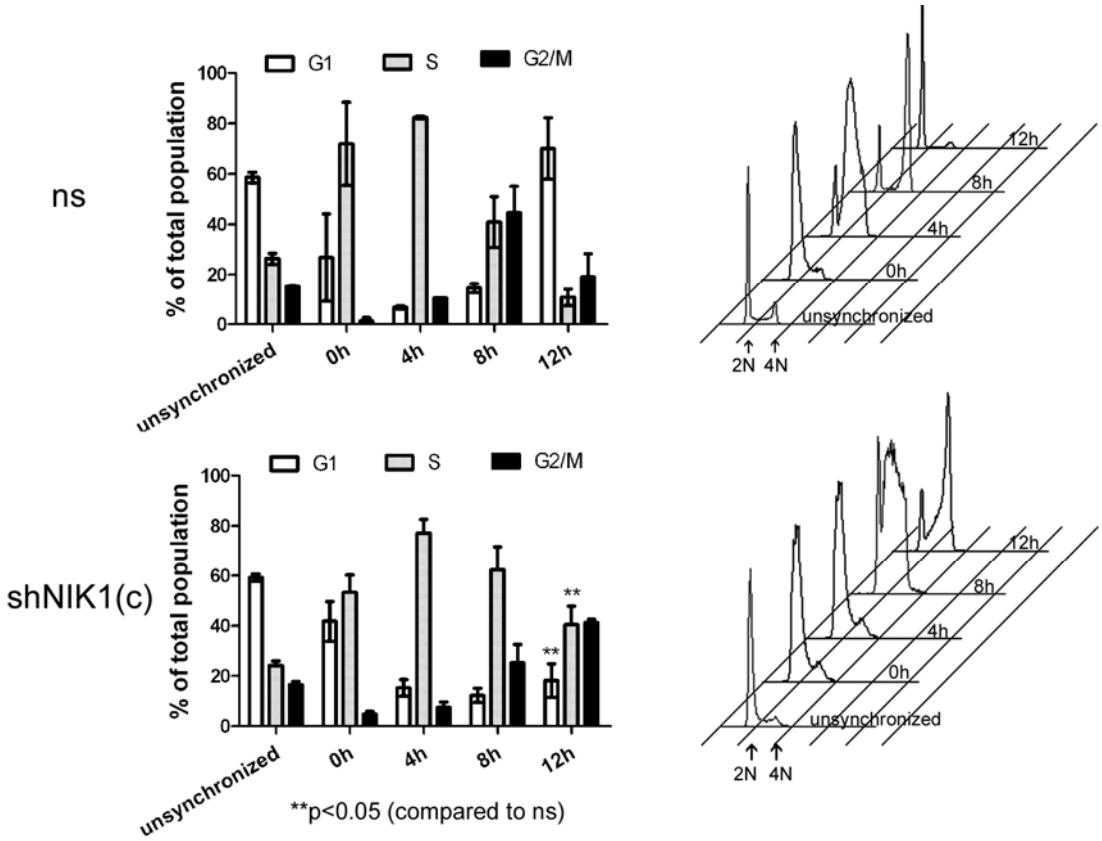


Figure 9. NIK depletion delays cell cycle progression in WM115 cells. Left panel: Percentage of unsynchronized and synchronized (thymidine block, 16h) cells in each phase of cell cycle. Error bars are ± S.E.M. (n=3) Right panel: Time course graphs showing the progression of cell cycle in ns and NIK depleted WM115 populations. Times indicated (0h, 4h, 8h, 12h) were hours after the release from the thymidine block of 16h. Scales for number of non-silencing control WM115 cells: unsynchronized: 0-4500, 0h: 0-1600, 4h: 0-1000, 8h: 0-2500, 12h: 0-5000. Scales for number of clonal shNIK1(c) WM115 cells: unsynchronized: 0-4000, 0h: 0-2200, 4h: 0-1200, 8h: 0-600, 12h: 0-1500.

NIK depletion in WM115 cells reduced xenograft tumor growth in nude mice

In agreement with *in vitro* results, depletion of NIK significantly decreased melanoma tumor growth and burden in nude mice (Figure 10A and B). NIK deficiency in tumor samples was confirmed by both Western blot and qRT-PCR analyses (Figures 10C and D). Immunostaining of Ki67 or cleaved-caspase 3 was lower in NIK knock-down tumor sections than non-silencing control tumor sections (Figures 10E and F). These data suggest that tumors from NIK knock-down WM115 grew and turned over slower than control tumors. In summary, NIK deficiency substantially attenuated melanoma tumor growth of WM115 cells both *in vitro* and *in vivo*. To determine molecular mechanisms by which NIK depletion reduced tumor growth, gene expression changes and signaling pathways were next examined.

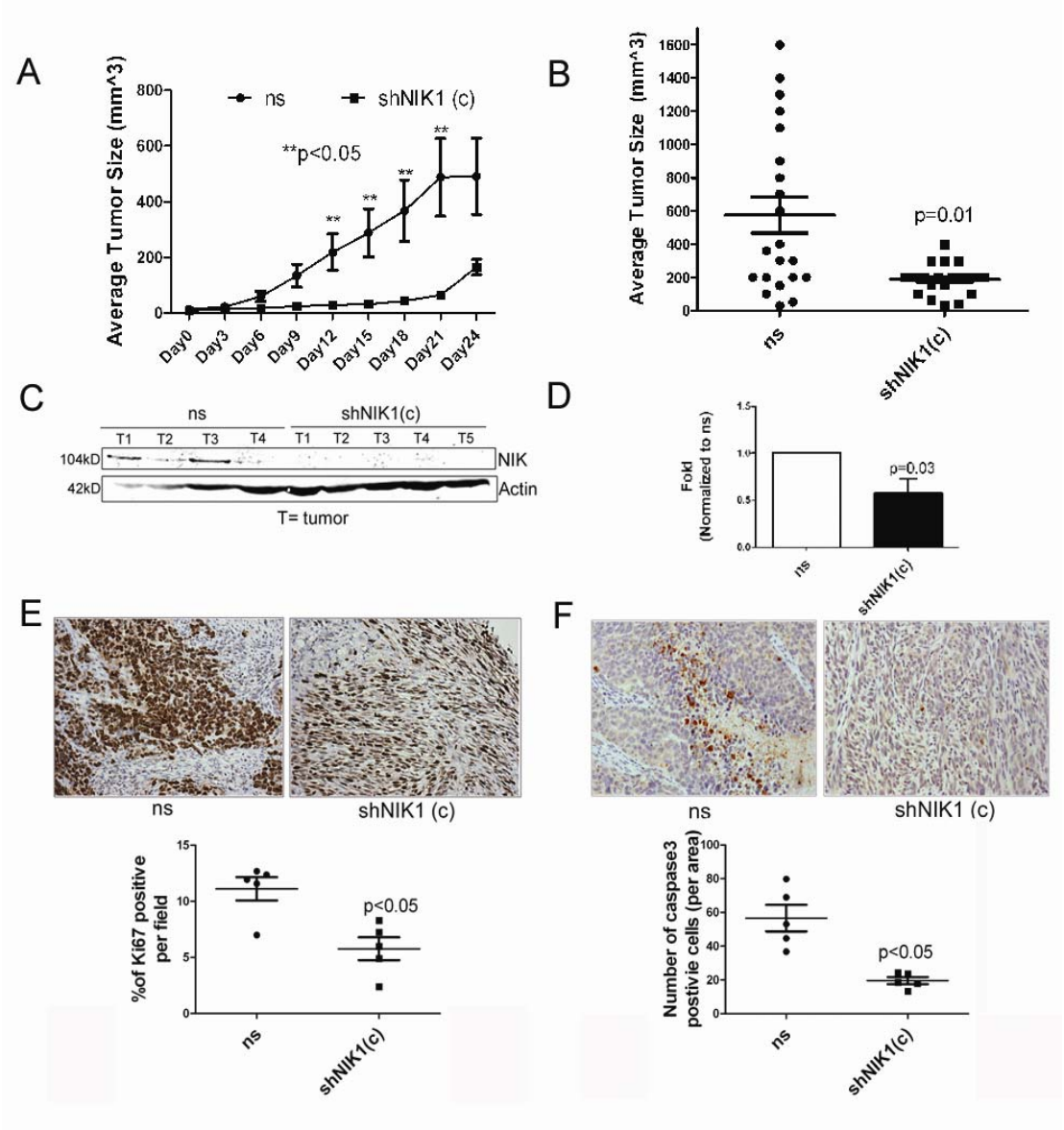


Figure 10. NIK depletion significantly reduces tumor burden in a melanoma xenograft model of NIK knock-down WM115 [shNIK1 (c)] and non-silencing WM115. **A**. Tumor growth over 24 days. **B**. size of tumors at the 24 day end point. Error bars are ± S.E.M. (ns n=21 and shNIK1(c) n=22). **C.** Western blot and **D.** qRT-PCR analyses of NIK in tumor samples. Fold changes in Figure D were calculated by normalizing to the loading control and comparing it to ns. **E**. Ki67 (proliferation marker) and **F**. cleaved caspase-3 (apoptosis marker) staining of non-silencing control WM115 tumors compared to WM115 with NIK knock-down. Error bars are ± S.E.M. (n=5). Statistical significance was determined by Mann-Whitney test, where $p<0.05$ indicates significance.

NIK depletion altered expression of genes important for melanoma tumor growth

To elucidate potential mechanisms by which attenuation of NIK expression contributed to reduced tumorigenicity of melanoma cells, the global gene expression profile of polyclonal and clonal NIK knock-down WM115 cells as compared to the non-silencing control was examined by using gene expression microarray analyses. While results of these experiments suggested clear fold differences in the expression of many genes, the differences were not statistically significant when stringent statistical analyses ($p<0.05$) were applied, possibly due to the variations between polyclonal and clonal knock-down populations. However, using less stringent criteria for analysis ($p<0.15$), several genes that play a crucial role in tumor growth or progression (*CXCR4, c-MYC, c-MET, CCND2, LIN28B*) (Pastorino et al., 2003; Puri et al., 2007; Scala et al., 2006; Scala et al., 2005; Viswanathan et al., 2009) were markedly reduced in NIK knock-down cells (Table 1). NIK knock-down cells also exhibited up-regulated expression of genes such as XIAP-associated factor 1 (*XAF1*), which plays a negative role in cancer cell survival (Plenchette et al., 2007). Similar trends of gene expression were observed with control and NIK depleted melanoma tumor samples (Table 1). qRT-PCR analysis of selected genes revealed statistically significant changes in expression for several genes identified by microarray analyses (fold changes relative to the non-silencing control): *c-MYC* (-3.39), *c-MET* (-4.30), *CXCR4* (-620.35), *CCND2* (-58.08), *XAF1* (5.78) and *LIN28B* (-3.2×10^7) with p-values <0.05 for all genes) (Table 1). Taken together, microarray and qRT-PCR data suggest that NIK depletion altered the expression of genes crucial for tumor growth.

Table 1. Gene expression microarray data from NIK depleted cells and tumors confirmed by qRT-PCR (normalized to non-silencing control) to examine gene expression changes which are indicative of less tumorigenecity compared to the control.

	Microarray (cells)	p-value	Microarray (tumors)	p-value	qRT-PCR	p-value
c-Myc	-3.90	0.14	-2.20	0.06	-3.39	p<0.05
c-Met	-3.72	0.12	-6.80	0.002	-4.30	p<0.05
CXCR4	-14.50	0.08	-5.80	0.01	-620.35	p<0.05
CCND2	-9.60	0.13	-1.04	0.92	-58.08	p<0.05
Xaf1	3.78	0.17	3.65	0.01	5.78	p<0.05
CD36	7.80	0.07	1.70	0.003	5.11	p<0.05
TSC22D3	-10.40	0.04	-7.30	0.02	-33.05	p<0.05
TRIM22	5.30	0.15	1.03	0.73	5.26	p<0.05
ZNF711	-11.20	0.06	-7.10	0.003	-28.57	p<0.05
LIN28B	-18.02	0.09	-5.20	0.02	-32436417	p<0.05

NIK depletion in WM115 did not reduce the canonical NF-κB activation

To further explore the mechanism by which NIK knock-down affects NF-κB pathways, the canonical NF-κB activation in NIK knock-down melanoma cells as compared to the control was determined by IKK kinase assay and by examining nuclear p-p65 (Ser536) (Figures 11A and B). TNFα treatment was used as a positive control. Both assays suggest that the basal and TNFα-induced canonical NF-κB activity was not diminished by NIK knock-down (Figures 11A and B).

Similarly, the levels of some NF-κB-regulated chemokines and cytokines (IL-6, IL-8/CXCL8, CCL2 and CXCL1) in the conditioned media from either WM115 clonal or polyclonal knock-down cells were not consistently reduced between these two cell lines as compared to the control (Figures 11C and D). The above data suggest that NIK depletion did not diminish the canonical NF-κB activation in WM115 melanoma cells.

NIK depletion decreased the non-canonical NF-κB activation

In contrast to the canonical NF-κB activation, depletion of NIK in WM115 cells reduced the basal processing of p100 to p52 and nuclear localization of p52 (Figures 11E and F). NIK deficient cells still responded to the ligation through LTβR although to the less extent than the control (Figure 11F). Knock-down of NIK in another melanoma cell line, Hs294T, resulted in a similar phenotype (Figures 11E and F).

To determine whether NIK deficiency in melanoma cells exhibited expected changes in the gene expression profile associated with NIK activity, we compared the gene expression from NIK knock-down WM115 cells to the gene expression pattern from lymphoma, where cIAP2-MALT1 fusion protein induces NIK activation (Rosebeck et al., 2011). In agreement with their observations, genes (such as *TLR4*, *IL1β*, *PTX3*) up-

regulated in cIAP2-MALT1 negative tumors (NIK low) were increased in NIK deficient melanoma, whereas genes (such as *HMOX1*, *PIM2*, *CXCR4*) up-regulated in cIAP2-MALT1 positive tumors (NIK high) were decreased in NIK deficient cells compared to the control ($p<0.05$ except *PTX3*) (Figure 11G). Based on above data, we conclude that knocking-down NIK reduced the non-canonical NF-κB activation in melanoma cells.

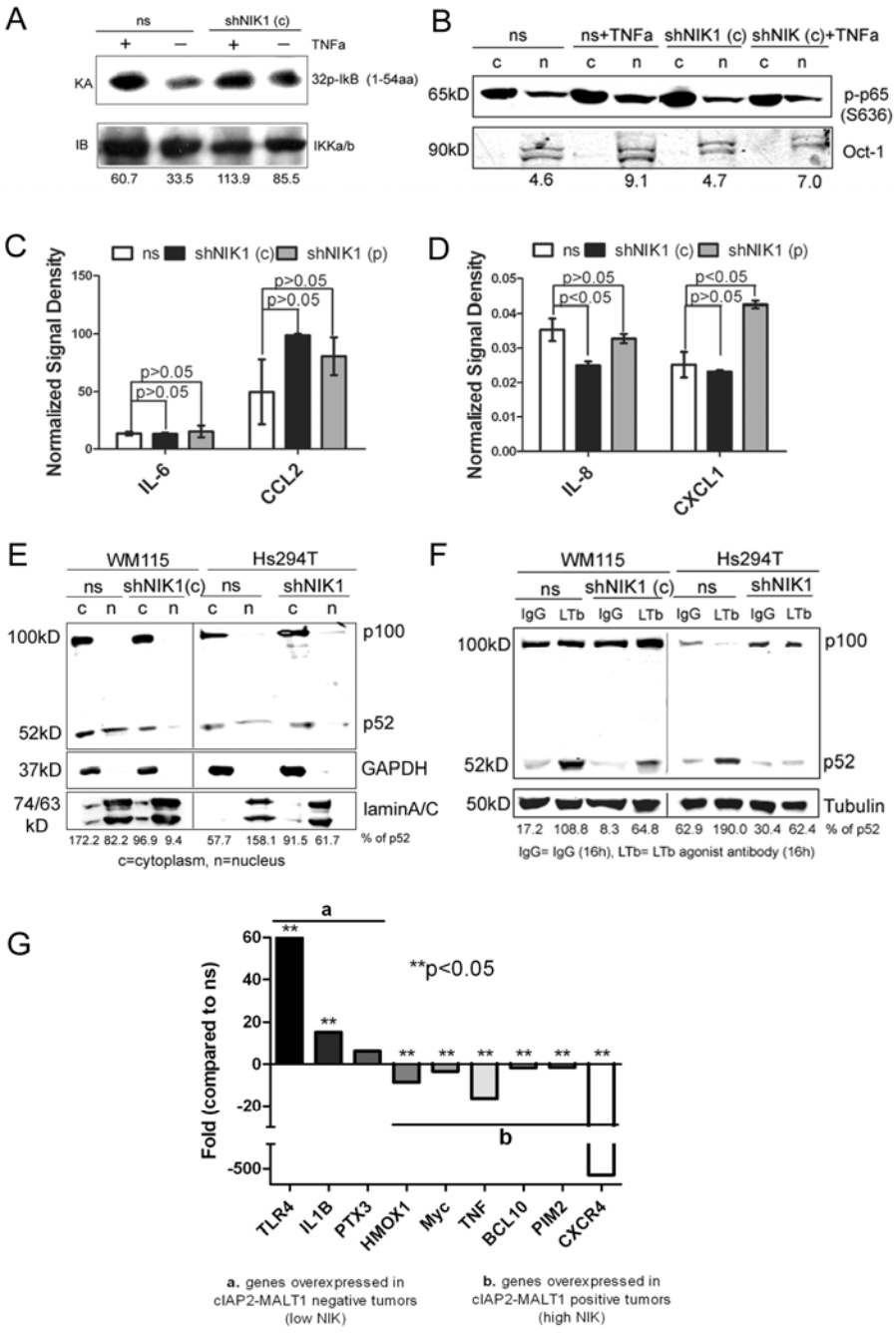


Figure 11. Knocking-down NIK in WM115 melanoma reduces the non-canonical but not the canonical NF-κB activation. **A.** Basal IKK activity in NIK knock-down WM115 compared to WM115 non-silencing control in an *in vitro* kinase assay. Densitometric scans from duplicate assays were quantitated and normalized to the total immunoprecipitated IKKs. **B.** Nuclear p-p65 in NIK knock-down WM115 compared to the non-silencing control (c=cytoplasm, n=nucleus). Densitometric scans from four assays were quantitated and normalized to the loading control. TNFα-treatment was used as a positive control in both A and B.

C and **D.** Cytokine array data from conditioned media of NIK knock-down cells (clonal and polyclonal) compared to the non-silencing control. Signal intensity for each cytokine was normalized to the internal positive control. Error bars are ± S.E.M. (n=4) Statistical significance was determined by Mann-Whitney test, where $p<0.05$ indicates significance. **E.** Nuclear and cytoplasmic localization of p52 in non-silencing and NIK knock-down WM115 and Hs294T (c=cytoplasm, n=nucleus). Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from ns. **F.** The proteolytic processing of p100 to p52 in non-silencing and NIK knock-down WM115 and Hs294T. Cells were treated with LTβR agonist antibody or IgG (2μg/ml) for 16h. Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from ns. **G.** qRT-PCR analysis of gene expression in NIK deficient WM115 cells normalized to the control, compared to study by Rosebeck et al., 2011.

Some NF-κB regulated genes were transcriptionally up-regulated in NIK knock-down WM115

Since NF-κB transcriptional activity was still active in knock-down cells, the mRNA expression of several key NF-κB regulated chemokines and cytokines was examined using qRT-PCR analysis in both clonal and polyclonal NIK knock-down or control cells. The mRNA expression of chemokines and cytokines examined [IL-1α, IL-1β, IL-6, IL-8, IL-10, CCL2, CXCL1 and vascular endothelial growth factor (VEGF)] was up-regulated in NIK knock-down cells (Table 2). In contrast to other studies (Viemann et al., 2004), mRNA expression of pro-survival factors (cIAP1, cFLIP, A20) and NF-κB negative regulators (IκBα, p100 and A20) did not change significantly in NIK knock-down cells despite the activity of NF-κB (Table 2). These data suggest that NIK may be involved in differential regulation of some NF-κB-regulated genes.

Table 2. qRT-PCR anslysis of gene expression in NIK knock-down WM115 cells compared to the non-silencing control.

Chemokines and cytokines

mRNA	Fold	p-value
IL1A	38.8	p<0.05
IL1B	15.2	p<0.05
IL6	45.1	p<0.05
IL8	56.8	p<0.05
IL10	-2.2	p>0.05
CCL2 (MCP-1)	57.7	p<0.05
CXCL1	57.4	p>0.05
VEGF	2.9	p>0.05

NF-κB inhibitors

mRNA	Fold	p-value
A20	1.5	p>0.05
IκBα	-2.2	p<0.05
p100	-1.1	p>0.05

Survival factors

mRNA	Fold	p-value
cIAP1	-1.1	p>0.05
cFLIP	7.1	p>0.05
A20	1.5	p>0.05

NIK knock-down attenuated the expression of pro-survival factors

One of the mechanisms by which tumor cells escape apoptosis is by up-regulating the expression of survival factors. To understand the apoptotic phenotype of NIK depleted cells, protein expression of a panel of pro-survival factors was examined. Some anti-apoptotic proteins such as BCL-XL and cFLIP expression remained unchanged (Figure 12A). However, pro-survival proteins such as cIAP1, survivin, BCL2 were markedly decreased in NIK knock-down WM115 cells (Figure 12A). Depletion of NIK in another melanoma cell line, Hs294T, also diminished the protein expression of survivin (Figure 12A). qRT-PCR demonstrates that there is no difference in cIAP1 mRNA in NIK-depleted melanoma cells, though both BCL2 and survivin mRNA levels were significantly diminished (Figure 12B). While NIK depletion may affect cIAP1 at a post-transcriptional level, the above data suggest that NIK regulates pro-survival proteins BCL2 and survivin at the transcriptional level. A similar decrease in pro-survival proteins and mRNA was observed in *Nik*$^{-/-}$ MEFs, suggesting that NIK regulates the expression of these proteins and that the regulation is not melanoma specific (Figures 12C and D).

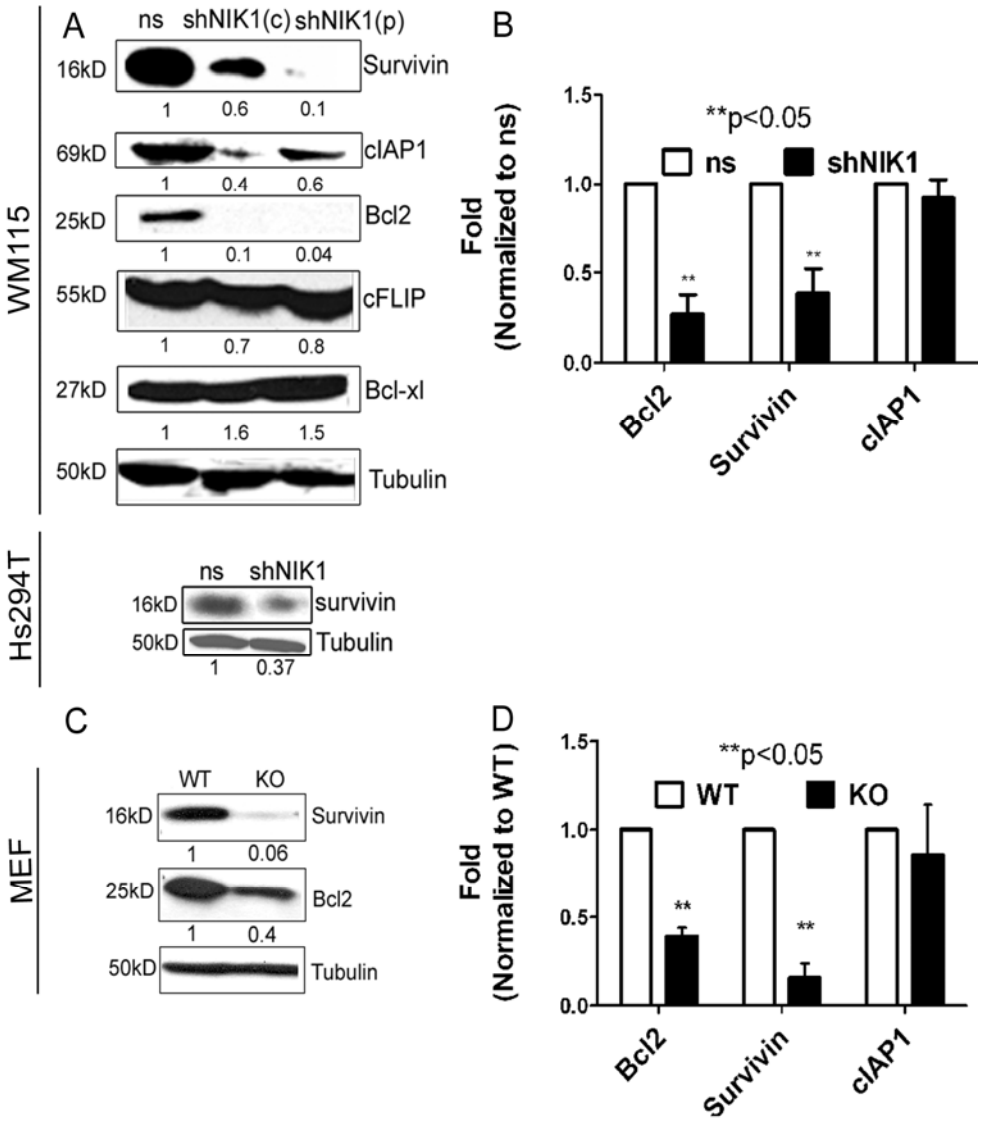


Figure 12. NIK modulates the expression of pro-survival factors. **A**. Protein expression of survival factors such as BCL2, cIAP1 and survivin in NIK knock-down WM115 (both clonal and polyclonal) compared to the non-silencing control. Similar to WM115, Hs294T cells with NIK knock-down exhibited decreased protein expression of survivin. Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from ns. **B.** mRNA levels (qRT-PCR) of BCL2, survivin and cIAP1 in NIK depleted and non-silencing WM115 cells. Fold changes were calculated by normalizing to the loading control and comparing it to ns. **C.** Western blot and **D.** qRT-PCR of BCL2 and survivin in *Nik*$^{+/+}$ and *Nik*$^{-/-}$ MEFs. In Figure C, densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from WT. In Figure D, fold changes were calculated by normalizing to the loading control and comparing it to WT.

NIK modulated melanoma tumorigenesis by regulating β-catenin activity

Although NIK knock-down WM115 cells exhibited decreased non-canonical NF-κB activity, this pathway has not been directly linked to the expression of pro-survival proteins such as survivin. Therefore, we postulated that NIK modulates the expression of survivin through an alternative pathway. Firestein *et. al.* have identified NIK (MAP3K14) as one of the kinases that regulates β-catenin activation in colorectal cancer cells (Firestein et al., 2008). To explore the possibility of β-catenin regulation by NIK, we examined the expression of genes which are typically targeted by β-catenin in NIK knock-down melanoma cells and *Nik*$^{-/-}$ MEFs by qRT-PCR. Data suggest that deficiency of NIK attenuated expression of β-catenin regulated genes such as *AXIN2* and *TCF7* (Figures 13A and B). Interestingly, down-regulation of β-catenin targets, such as c-*MYC*, *c-MET* and *CCND2* in NIK knock-down WM115 cells (Table 1), further supports the idea that NIK regulates β-catenin activity (Cohen et al., 1998; Matsumoto et al., 2002; Scala et al., 2006).

In addition, the expression of some pro-survival factors such as survivin can be transcriptionally regulated by β-catenin (Zhang et al., 2001). To determine whether NIK modulates the expression of survivin through affecting β-catenin transcriptional activity, ChIP analysis was performed using β-catenin antibody to monitor β-catenin binding to the survivin promoter. Both NIK knock-down melanoma cells and *Nik*$^{-/-}$ MEFs exhibited a decreased β-catenin binding to the promoter of the survivin gene (Figure 13C and D), supporting the notion that NIK regulates survivin expression, and possibly other β-catenin targets such as c-*MYC*, *c-MET* and *CCND2*, through β-catenin activity.

To understand how NIK may affect β-catenin activity, nuclear and cytoplasmic extraction and immunofluorescence staining of β-catenin was performed in NIK knock-

down melanoma cells and *Nik*$^{-/-}$ MEFs. The ratio of nuclear to cytoplasmic pool of β-catenin is less in NIK deficient cells as shown by both analyses (Figure 13E and F), implying that NIK regulates sub-cellular localization of β-catenin.

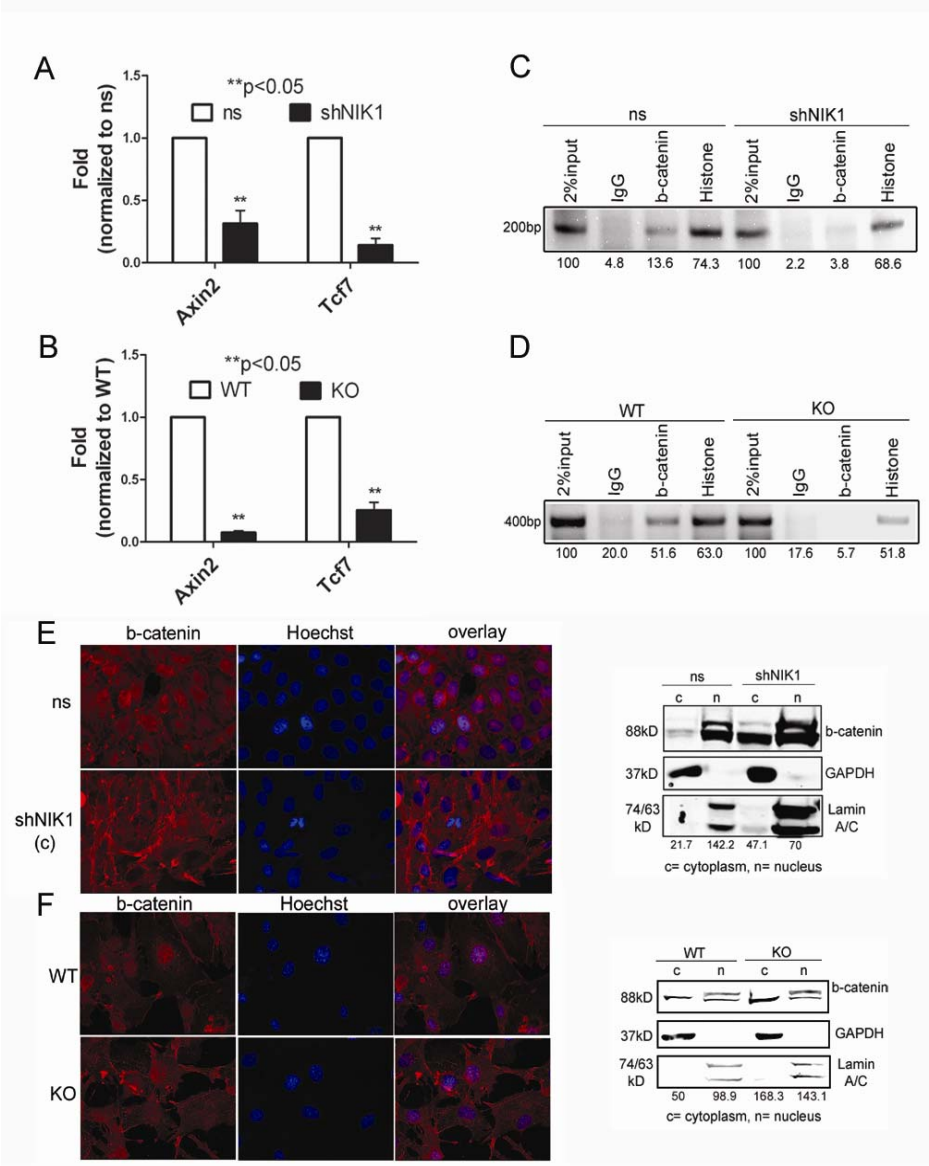


Figure 13. NIK regulates survivin expression through β-catenin activity. **A and B.** Expression of traditional β-catenin transcriptional targets such as *AXIN2* and *TCF7* in NIK knock-down WM115 (**A**) and *Nik*$^{-/-}$ MEFs (**B**). Fold changes were calculated by normalizing to the loading control and comparing it to ns or WT. **C and D.** ChIP analysis using β-catenin antibody and primers flanking the survivin promoter exhibited decreased β-catenin occupancy at the survivin promoter in NIK knock-down WM115 (**C**) and *Nik*$^{-/-}$ MEFs (**D**). Amount of DNA bound to IgG, β-catenin or Histone was calculated as percentage of the 2% input. **E and F.** Immunofluorescent staining and Western blot analyses showing sub-cellular localization of β-catenin in NIK knock-down WM115 (**E**) and *Nik*$^{-/-}$ MEFs (**F**). Images for immunofluorescence staining were taken using a 63x oil lens and pseudo-colored. Densitometric scans of Western blot from triplicate assays were quantitated and normalized to the loading control.

Effect of NIK on stability and post-translational modification of β-catenin

Since NIK influences the activity of β-catenin, we determine the mechanisms by which NIK may modulate β-catenin. Previous literature suggest that β-catenin activity can be regulated by stability, sub-cellular localization and post-translational modification such as phosphorylation. In normal physiological conditions, the cytoplasmic pool of β-catenin undergoes proteasomal degradation mediated by a destruction complex composed of GSK3β, APC, Axin2 and casein kinase I (CKI). In the presence of positive signals such as Wnt signaling, cytoplasmic β-catenin becomes stabilized and translocates into the nucleus to mediate transcription of its target genes.

Based on the changes within the β-catenin pathway in NIK deficient cells, we postulated that one or more of the above mentioned mechanisms may be utilized by NIK to modulate β-catenin activity. Hence, we examined each possibility of β-catenin regulation in cells with altered NIK expression.

First, to determine whether β-catenin and NIK interact, we performed co-immunoprecipitation between Flag-NIK and β-catenin over-expressed in HEK293. Figure 14A shows that Flag-NIK and β-catenin indeed interact (Figure 14A). To examine the basal level of β-catenin in cells with altered NIK expression, cells were treated for 4h with 10μM of MG132, a proteasomal inhibitor, and the β-catenin expression level was determined by Western blot analysis. In HEK293 with Flag-NIK and β-catenin over-expression, cells with Flag-NIK exhibited stabilized exogenous β-catenin compared to cells with β-catenin over-expression only (Figure 14B). MG132 treatment stabilizes β-catenin in cells without Flag-NIK overexpression whereas it does not further stabilize β-catenin in cells with Flag-NIK overexpression, implying that NIK inhibits β-catenin proteasomal degradation (Figure 14B). Consistent with these findings, NIK knock-down WM115 cells do not exhibit stabilized β-catenin in response to MG132 treatment

whereas, this treatment increased β-catenin expression in non-silencing cells (Figure 14C). However, no apparent difference in β-catenin expression was observed in *Nik*$^{-/-}$ MEFs (Figure 14D). These data suggest that NIK may not be required for stability of β-catenin although it can increase the stability of the protein.

As β-catenin can be ubiquitinated and degraded by the proteasome, we asked whether ubiquitination status of β-catenin is different in cells with altered NIK expression. We examined the level of β-catenin ubiquitination in NIK over-expressed (Flag-NIK over-expressed) and NIK deficient cells (NIK knock-down WM115 cells and *Nik*$^{-/-}$ MEFs). To normalize the effect of degradation after ubiquitination, cells were first treated with MG132 (where indicated) before β-catenin was immunoprecipitated and immunoblotted for ubiquitin. Interestingly, β-catenin is ubiquitinated less in NIK over-expressed cells (Figure 14E) whereas, the reverse is true in NIK deficient cells (Figure 14F and G). These data suggest that NIK can alter the ubiquitination status of β-catenin.

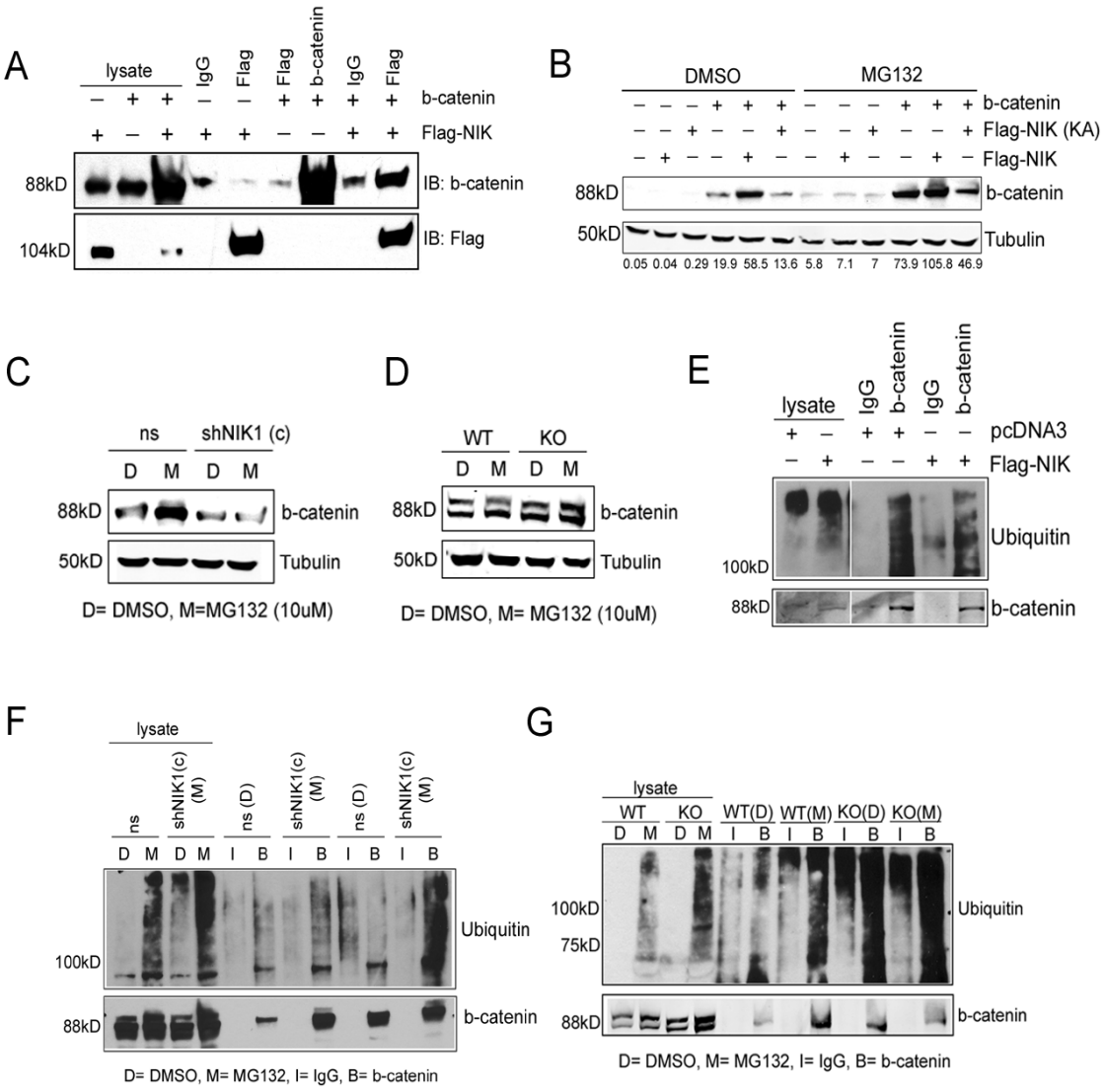


Figure 14. NIK alters the stability and ubiquitination status of β-catenin. **A.** Flag-NIK interacts with β-catenin in HEK293 over-expressing both proteins. **B.** Flag-NIK overexpression stabilizes β-catenin expression in HEK293. HEK293 cells overexpressing FlagNIK, β-catenin or both were treated with dimethyl sulfoxide (DMSO) and MG132 and β-catenin expression was analyzed by Western blot. **C.** and **D.** NIK knock-down WM115 cells (**C**) or *Nik*$^{+/+}$ and *Nik*$^{-/-}$ MEFs (**D**) were treated with DMSO or MG132 (10μM) for 4h and β-catenin expression was analyzed by Western blot. **E.** β-catenin was immunoprecipitated from HEK293 cells overexpressing Flag-NIK or empty plasmid were treated with MG132 and immunoblotted for ubiquitin. **F** and **G.** NIK knock-down cells (**F**) or *Nik*$^{-/-}$ MEFs (**G**) were treated with DMSO or MG132 (10μM) for 4h. From these lysates, β-catenin was immunoprecipitated and immunoblotted for ubiquitin.

NIK favors nuclear localization of β-catenin

As nuclear β-catenin is associated with its transcriptional activity and sub-cellular distribution of β-catenin is altered in NIK deficient cells, we further examined localization of β-catenin in NIK deficient cells. *Nik*$^{-/-}$ MEFs were treated with leptomycin B (LMB), an inhibitor of exportin 1/CRM1-dependent nuclear export, and cellular β-catenin distribution was deteremined by immunofluorescence staining. NIK deficient cells are defective in nuclear accumulation of β-catenin in response to LMB treatment compared to cells with NIK expression (Figure 15). This data suggest that NIK deficient has defect in nuclear import of β-catenin.

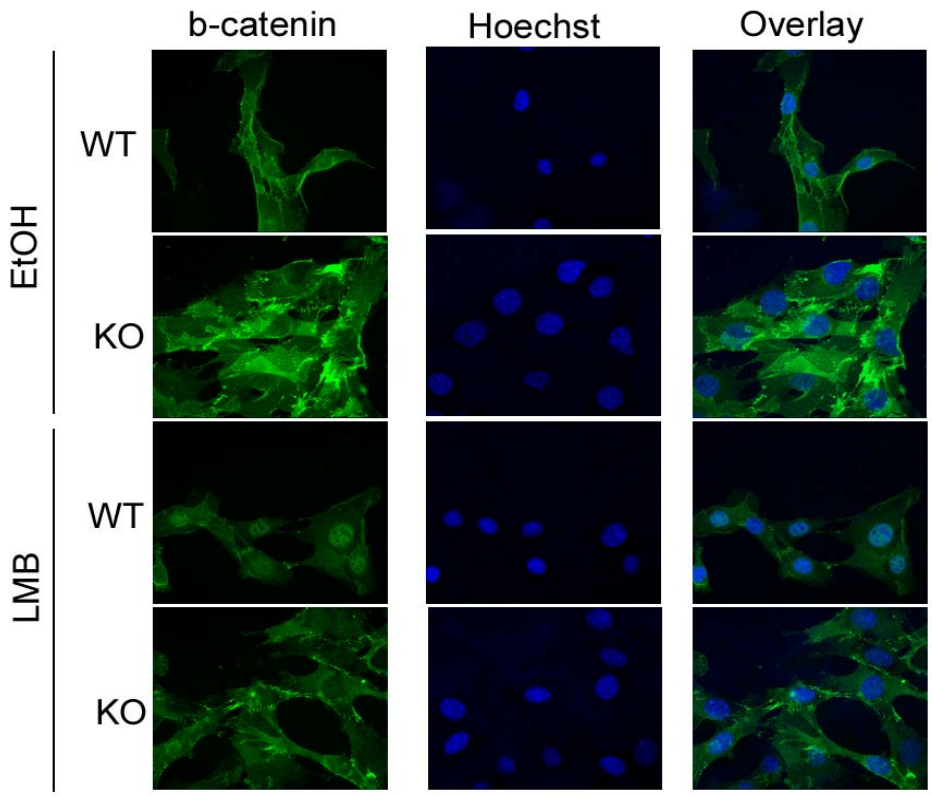


Figure 15. NIK deficient cells exhibit defect in nuclear localization of β-catenin compared to cells with wild-type NIK. Immunofluorescent staining showing sub-cellular localization of β-catenin in *Nik*$^{+/+}$ and *Nik*$^{-/-}$ MEFs. Cells were treated with either EtOH or LMB (100nM/ml) for 5h and β-catenin localization was determined by immunofluorescent staining with antibody against β-catenin. Hoechst stain was used to depict nuclei. Images were taken using a 63x oil lens and pseudo-colored.

Discussion

In this study, we demonstrate for the first time that NIK regulates melanoma tumor cell growth and survival. We also demonstrate that the mechanism for this effect on tumor growth involves NIK regulation of pro-survival gene expression, in part through modulation of nuclear β-catenin transcriptional activity. NIK depletion decreases proliferation and increases apoptosis in melanoma cells *in vitro* and sensitizes melanoma cells to TNFα-induced apoptosis. NIK knock-down also affects the progression of cell cycle and this effect may be likely at G1 to S phase transition. Interestingly, CCND2, a cyclin which is responsible for G1 to S transition, is down-regulated in NIK knock-down melanoma.

Although the canonical NF-κB pathway is not decreased in NIK knock-down cells, pro-survival proteins such as BCL2, cIAP1 and survivin are diminished, suggesting that the canonical NF-κB pathway lacks the cyto-protective function in response to TNFα when pro-survival effectors are absent. Other studies have also reported that cell death can be induced by TNFα in the presence of NF-κB activation when other survival signals such as those of cIAPs are disrupted in cancer cells (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007).

Consistent with *in vitro* data, a melanoma tumor xenograft model shows that NIK silencing markedly decreases melanoma tumor growth. Ki67 staining of tumor tissues suggests that decreased proliferation accounts for the decreased growth of NIK deficient tumors. Despite the significant decrease in tumor volume, NIK deficient tumors showed diminished cleaved caspase 3 staining. It is possible that NIK depleted cancer cells undergoing apoptosis were already eliminated by the time tumors were harvested, or the turn-over rate of melanoma tumors with the control shRNA was faster due to the limited nutrients in the microenvironment, resulting in less cleaved caspase 3 staining in tumors

with NIK knock-down. Overall, NIK depletion markedly decreases the net melanoma tumor growth.

Similarly, studies from other groups suggest that NIK mediates proliferation and survival of multiple myeloma, T-cell leukemia and lymphoma (Annunziata et al., 2007; Conze et al., 2010; Demchenko et al., 2010; Keats et al., 2007; Saitoh et al., 2008). As a result, depleting NIK in these cancers leads to decreased tumor growth (Annunziata et al., 2007; Keats et al., 2007; Saitoh et al., 2008). Consistent with the previous literature, our data clearly demonstrate that NIK contributes to melanoma tumor growth. However, prior studies have not demonstrated which downstream effectors of NIK mediate tumorigenesis. Here, we demonstrate that NIK mediates expression of genes that are pro-tumorigenic and pro-survival in melanoma and thus, depletion of NIK leads to melanoma tumor cell apoptosis.

NIK regulates specific patterns of gene expression

Gene expression data from NIK deficient melanoma cells compared to NIK expressing melanoma cells suggest that NIK modulates the expression of genes such as *CXCR4, c-MET, c-MYC, LIN28B* and *XAF1*, all of which are important for melanoma and/or tumor malignancy. For instance, high CXCR4 expression predicts poor prognosis in malignant melanoma (Scala et al., 2005). The c-MET tyrosine kinase receptor and the c-MYC transcription factor stimulate survival and proliferation (Lai et al., 2009; Ruggero, 2009). It was reported that both of these genes were amplified in metastatic melanoma from patients (Moore et al., 2008). Specifically, both c-MET and c-MYC are proposed to be targets for melanoma therapy (Pastorino et al., 2003; Puri et al., 2007). Depletion of NIK significantly diminishes the transcriptional expression of *CXCR4, c-MET* and *c-MYC*.

In addition, XAF1, which plays a negative role for cellular survival by counteracting the function of the survival factor XIAP (Liston et al., 2001), is increased at the mRNA level in NIK depleted WM115 cells. Consistently, it is reported that XAF1 expression is significantly decreased in melanoma (Ng et al., 2004). Remarkably, a potent tumor promoter, LIN28B, is down-regulated in NIK knock-down WM115 cells. LIN28B de-represses oncogenes such as K-RAS and c-MYC through a miRNA, let-7 (Viswanathan et al., 2009).

Since changes in *CXCR4, c-MET, c-MYC, LIN28B* and *XAF1* observed in cultured NIK knock-down melanoma cells were retained in NIK knock-down tumors, our data indicate that these genes contribute to tumorigenicity. Based on its ability to regulate the expression of genes important for melanoma progression, we propose that NIK is a promising target for melanoma therapy.

NIK modulates melanoma growth by altering the expression of survivin through β-catenin activity

Up-regulation of survival factors is one of the mechanisms by which cancer cells resist apoptosis (Baldwin, 2001). Previous studies suggest that pro-survival proteins such as BCL2, cIAP1 and survivin contribute to tumorigenesis of melanoma. As a result, functional disruption of these proteins impairs melanoma tumor cell growth or survival (Grossman et al., 2001; Hilmi et al., 2008; Lecis et al.). Consistent with these findings, BCL2, cIAP1 and survivin levels were markedly decreased in NIK deficient melanoma cells. Yet, the canonical NF-κB transcriptional activity, which typically regulates the expression of survival proteins (Baldwin, 2001), is not diminished in NIK deficient melanoma cells, in agreement with the genetic data (Yin et al., 2001). The activity of the non-canonical pathway is down-regulated in NIK deficient melanoma cells and it possibly

contributes to downstream effects of NIK on melanoma growth. However, it has not been directly linked to the expression of genes such as survivin. Interestingly, *Nik*$^{-/-}$ MEFs which do not exhibit a defect in the canonical NF-κB activation (Yin et al., 2001), also have decreased survivin expression. Therefore, we hypothesize that the decrease in pro-survival gene expression is attributable to a non-NF-κB function of NIK.

Here our data suggest that NIK regulates β-catenin activity, a novel mechanism by which NIK may mediate cancer progression. This non-NF-κB-function of NIK is similar to findings of other NF-κB kinases such as IKKα and β which have NF-κB-independent functions (Chariot, 2009). Specifically, NIK depletion reduces β-catenin binding to the promoter region of survivin, diminishing the expression of survivin. Down-regulation of β-catenin transcriptional targets in NIK deficient cells based on microarray data (*c-MYC, CCND2, c-MET*) and down-regulation of traditional β-catenin targets (*AXIN2* and *TCF7*) in NIK deficient cells further support the notion that NIK mediates β-catenin activity. In addition, several studies have reported that β-catenin activity contributes to melanoma tumor growth (Dhomen et al., 2009; Rubinfeld et al., 1997; Sinnberg et al., 2010). Although our data suggest that NIK regulates the subcellular localization of β-catenin, detailed mechanisms by which NIK activates β-catenin are yet to be determined. As a kinase, NIK may affect the phosphorylation of β-catenin regulators such as GSK3β. Alternatively, NIK may directly or indirectly regulate the nuclear transport of β-catenin.

NIK regulation of β-catenin

Data from NIK over-expressed and deficient cells suggest that NIK can modulate stability and ubiquitination status of β-catenin. It is important to note that β-catenin expression is not significantly altered in NIK deficient cells although it is stabilized upon

Flag-NIK over-expression. These data suggest that β-catenin stability can be modulated by NIK, but NIK is not necessary for stabilization of β-catenin. Kinases such as Akt can mediate β-catenin activity by phosphorylating and degrading its negative regulator GSK3β. Residues on the N-terminus of β-catenin such as Ser33, Ser37, Thr41 and Thr47, phosphorylated by CKI and GSK3β, are involved in regulating the stability of β-catenin. It is already reported that NIK can influence the activation status of Akt through PTEN (Kim et al., 2004). Therefore, it is possible that NIK may modulate β-catenin stability indirectly by affecting PTEN and Akt.

In addition to NIK modulation of β-catenin activity through effects on the stability of this protein, β-catenin activity may also be directly regulated through its nuclear localization. It has been reported that APC and Axin can regulate nuclear localization of β-catenin, thereby affecting the activity of β-catenin (Cong & Varmus, 2004; Henderson, 2000). In addition to APC and Axin, other novel proteins can regulate nuclear translocation of β-catenin. These proteins include Chibby (Li et al., 2010) and menin (Cao et al., 2009), both of which export β-catenin out of the nucleus. We propose here that NIK may also be important for the appropriate nuclear localization of β-catenin.

Although stabilized β-catenin accumulates in the nucleus, whether the stabilization of β-catenin is required for nuclear accumulation of β-catenin still remains unclear. A recent study suggests that positive regulators of the β-catenin pathway such as lithium chloride (LiCl), an inhibitor of GSK3β, decrease nuclear export of β-catenin (Jamieson et al., 2011). Interestingly, the S45A mutant of β-catenin, which cannot be phosphorylated by GSK3β, still enters the nucleus, suggesting that GSK3β regulates nuclear β-catenin accumulation independent of N-terminal phosphorylation (Jamieson et al., 2011). This study also shows that lymphoid enhancer-binding factor 1 (LEF-1) retains nuclear β-catenin, therefore favoring the nuclear activity of β-catenin (Jamieson

et al., 2011). Therefore, one potential mechanism by which NIK may affect β-catenin activity could be through altering the dynamics of nuclear transport of β-catenin. Intriguingly, a NIK-interacting protein, DEAD box polypeptide 5 (DDX5) reported in Chapter III, favors nuclear translocation of β-catenin through GSK3β (Yang et al., 2006b).

NIK as a therapeutic target in cancer

Results from our experiments have therapeutic implications in melanoma as well as in other cancers, since both NIK and β-catenin are also involved in multiple myeloma and colon cancer (Annunziata et al., 2007; Keats et al., 2007; Malbon, 2005). Studying the mechanism by which these two proteins interact may further elucidate how to interfere with the progression of those cancers for which NIK and β-catenin are mis-regulated.

Recently, significant progress has been made in melanoma therapy for those patients that exhibit BRAF mutation (Dhomen et al., 2009). However, it is imperative to also increase our understanding of other pathways that are involved in melanoma growth so that therapies for patients that cannot be effectively targeted with $BRAF^{V600E}$ inhibitors can be effectively treated. Due to its non-essential role in the canonical NF-κB activation and its ability to regulate survival factors, NIK is an attractive target to induce melanoma cell death without substantially reducing NF-κB activity, which is important for normal cellular processes. However, effects of systemic inhibition of NIK on the immune cells must be taken into consideration when devising any clinical trials with NIK inhibitors.

CHAPTER III

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST NIK

Introduction

Commercially available antibodies against NIK do not exhibit high immunoreactivity and do not immunoprecipitate endogenous NIK. In addition, there is no commercial monoclonal antibody against NIK. Therefore, we decided to generate monoclonal antibody against NIK, in collaboration with the Vanderbilt Monoclonal Antibody Core. We initially screen sera from mice immunized with purified NIK protein. Using the positive sera, we generated hybridoma clones producing NIK antibodies by fusing B cells with myeloma cells.

Materials and Methods

Molecular cloning and bacteria expression of recombinant MBP-NIK

Full length NIK was cloned into pAT-108 vector using SalI and NotI sites to produce maltose binding protein (MBP)-tagged NIK. The construct was subsequently transformed into competent BL21 bacteria. As a control, the construct with MBP tag only was used. Production of MBP or MBP-NIK protein was induced with 0.4mM of isopropyl-beta D-thiogalactopyranoside (IPTG) at 37°C for 2-4 h. The protein expressed from this induction was purified by using amylose resin. Briefly, bacteria carrying MBP or MBP-NIK were lysed and soluble lysate was incubated with amlysose resin for 1 hour at 4°C. Bound MBP or MBP-NIK was eluted from amylose resin by incubating with maltose buffer. Purified MBP and MBP-NIK (full length) were used to immunize mice.

Immunization and Fusion

Four A/J or BALB/c mice were immunized with the antigen described above. For primary injections, 50 μg of purified antigen were emulsified in 50% PBS, 50% Freund's complete adjuvant and injected subcutaneously into the nape of the neck (50%) and intramuscularly to the gluteal muscles (50%). In subsequent booster injections, Freund's incomplete adjuvant was substituted for Freund's complete adjuvant. Fourteen days after each booster injection, serum was collected from each mouse and assayed for reactivity with the antigen by enzyme-linked immunoadsorbent assay (ELISA), and Western blot analysis as described below.

The mouse with the highest level of reactivity was chosen for final boosting by intraperitoneal injection of the antigen diluted in 1xPBS. Four days after the final immunization, spleen cells were harvested and electro-fused/fused by standard methods with SP2/0 myeloma cells. The products of the fusion were plated for under selection for fourteen days in semi-solid media (Stemcell Technologies). Resulting colonies were picked and distributed individually into 96-well plates. Hybridomas producing antigen-specific antibodies were initially identified by ELISA, and subsequently verified by Western blot analysis as described below. Clones producing antibodies with the desired properties were subcloned to ensure monoclonality and cryopreserved.

Western Blot

HEK293 cells were lysed with the lysis buffer containing 10% glycerol, 50mM Tris pH 7.7, 150mM NaCl, 0.5% NP40 and cocktail of protease and phosphatase inhibitors (Sigma 8340, 2850 and 5726) at 4°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and blocked with 5% non-fat dried milk in 1xTBS at RT for 1h. The

membranes were immunoblotted using slot blot apparatus with mouse sera or supernatant from hybridoma cells (undiluted or diluted 1:1 with 5% non-fat dried milk in 1xTBS) or control primary antibodies: NIK (Novus: NB120-7204) and actin (Santa Cruz: sc-1616 or 1616R) for 4 h at RT or overnight at 4°C. The membranes were washed three times with 1xTBS containing 0.05% Tween-20 followed by incubation with secondary antibodies conjugated with IR680 and IR800 (Molecular Probes A21058 or A21084) for one hour at RT. Subsequent to washing three times with 1xTBS containing 0.05% Tween-20, membranes were scanned using the Odyssey system (Licor).

Immunoprecipitation

HEK293 or HEK293 transfected with Flag-NIK cells were lysed as mentioned above and pre-cleared with 30-40 µl of protein A/G Sepharose beads for 1h at 4°C. Then, 1ml of hybridoma supernatant or 1-1.5µg of antibody per 1mg of total protein was used to immunoprecipitate NIK for 1-2 h at 4°C, followed by pull-down with 30-40µl of protein A/G Sepharose beads for another hour at 4°C. The same amount of IgG was used as a negative control. The immunoprecipitate was washed three times with the lysis buffer, eluted with 4x Laemmli loading buffer, resolved by 8% SDS-PAGE and analyzed using Western blot analysis.

Immunofluorescence

HEK293 cells plated on coverslips were fixed with 4% paraformaldehyde or methanol and permeabilized with 0.2% of Triton-X 100 in 1xPBS. The cells were then blocked with 10% normal donkey serum in 1xPBS for 30 minutes at RT and incubated with supernatant from hybridoma cultures (undiluted) for 2 h at RT. After the secondary

antibody incubation, slides were washed three times with 0.1% Tween-20 in 1xPBS and stained with Hoescht nuclear stain during the last wash. Coverslips were air dried and mounted onto glass slides. Images were taken using a 20x oil lens Plan-Apochromat on Zeiss Axioplan 2 microscope and Hamamatsu Orca ER fluorescence camera. Images were processed using Metamorph Software.

Results and Discussion

Bacterial expression of recombinant MBP (50kD) or MBP-NIK (154kD) was amplified by IPTG treatment (Figure 16A). Proteins were isolated over amylose resin and eluted with maltose buffer as described in Methods and purity was characterized by SDS-PAGE with coomassie staining (Figure 16A). We observed that bacterially purified MBP-NIK resulted in some lower molecular weight forms of NIK despite the use of protease inhibitors during lysis and the attempts to use lower temperature during IPTG induction. This data suggests that bacteria were not able to transcribe or translate the full length protein due to high molecular weight or that the full length protein was unstable (Figure 16A). Purified MBP-NIK (both full length and smaller fragments) was used to immunize mice to produce NIK antibody as described in Materials and Methods.

Sera from mice immunized with NIK antigen were examined for immunoreactivity with MBP-NIK and endogenously expressed NIK by Western blot analysis. Results show that sera from all immunized mice react with purified and recombinant NIK (Figure 16B and C). However, only sera from four mice (all BALB/c mice) exhibit antigenicity towards endogenous NIK (Figure 16D). Among these, splenic B cells from a BALBL/c mouse, which exhibited the strongest antigenicity, were subsequently isolated and hybridoma cells were generated from these B cells. Supernatant from these hybridoma cells was screened for recognizing endogenous and recombinant NIK expression by Western blot.

Among many clones tested, two of them (8H3A2 and 10A2E12) recognize both endogenous (asterisk) and exogenous (arrow) NIK in Western blot analysis (Figure 17A and B). The signal intensity from the endogenous band of NIK protein (104kD) recognized by 8H3A2 clone was weak compared to 10A2E12, while both clones reacted strongly with recombinant NIK protein (Figure 17A and B). The commercial rabbit polyclonal NIK antibody (Novus Biological) shows a similar pattern of NIK expression at the same molecular weight (compare Figure 17A and B, top and bottom panels).

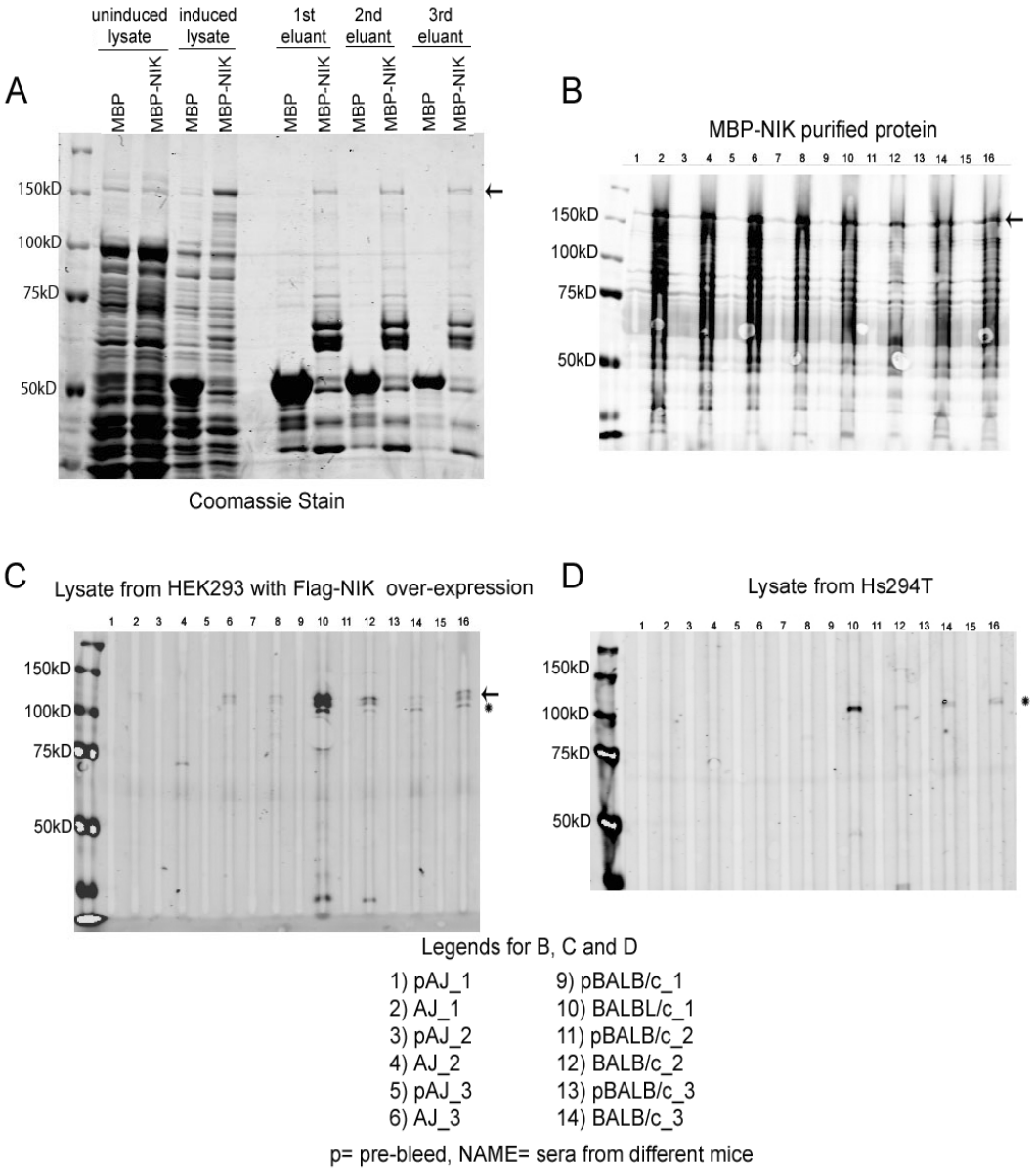


Figure 16. Purification of NIK antigen and characterization of sera from mice immunized with NIK antigen. **A.** Coomassie stain of purified MBP or MBP-NIK. Protein expression was induced by using IPTG and proteins were eluted with amylose. **B-D.** Immunoblot of purified MBP-NIK (**B**), Flag-NIK over-expressed HEK293 lysate (**C**) and Hs294T lysate (**D**) using sera from mice immunized with antigens.

Immunoprecipitation experiments reveal that both 8H3A2 and 10A2E12 monoclonal antibodies can precipitate exogenously expressed Flag-NIK (Figure 17C and D, arrows). Although endogenous NIK also appears to be precipitated by these antibodies, the intensity of the band is weak (Figure 17C and D, asterisks), suggesting that these antibodies may not be optimal in immunoprecipitation. Immunofluorescent staining using these antibodies shows a strong positive staining specific for Flag-NIK expressed in these cells (Figure 17E). However, 8H3A2 antibody does not show immunoreactivity towards endogenous NIK expressed by HEK293 cells in immunofluorescence (Figure 17E). 10A2E12 appears to recognize endogenous NIK in immunofluorescence although the specificity of this staining should be further characterized using NIK knock-down cells (Figure 17E).

Conclusions

We characterized two monoclonal antibodies against NIK, 8H3A2 and 10A2E12. These two antibodies recognize the endogenous NIK by Western blot although the specificity of the recognition should be further verified by using NIK deficient cells. To date, we were not successful in detecting endogenous NIK expression in melanoma cells with wild type NIK or NIK silencing, described in Chapter II. Our monoclonal NIK antibodies may not be superior to commercially available polyclonal antibody from Novus biological because polyclonal NIK antibody recognizes multiple epitopes of the protein. However, for assays that specifically require monoclonal antibodies, our monoclonal NIK antibody will be useful since, to our knowledge, there is no commercially available monoclonal NIK antibody.

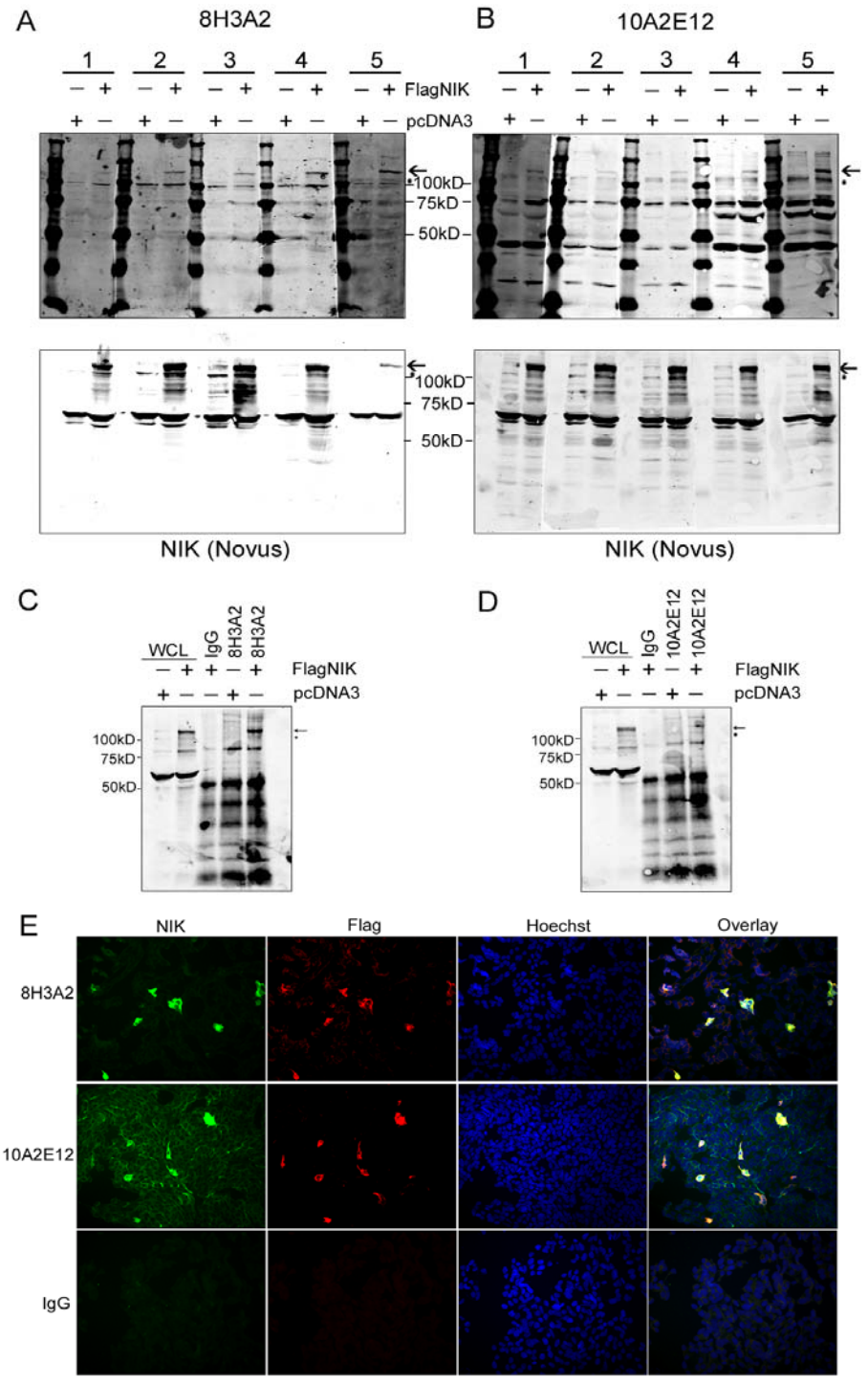

A
8H3A2
B
10A2E12
1 2 3 4 5
FlagNIK
pcDNA3
100kD
75kD
50kD
NIK (Novus)
C
WCL
IgG
8H3A2
8H3A2
D
10A2E12
10A2E12
E
NIK
Flag
Hoechst
Overlay
8H3A2
10A2E12
IgG

Figure 17. Characterization of NIK monoclonal antibodies, 8H3A2 and 10A2E12. **A and B.** Western blot analysis of HEK293 over-expressing an empty plasmid or Flag-NIK using 8H3A2 (**A**) or 10A2E12 (**B**) NIK monoclonal antibodies. Lower panels are immunoblots using polyclonal NIK antibody. (Blocking conditions: 1 and 2) 5% milk in 1xTBS 3) 5% milk in 1xPBS 4) 5% milk in 1xTBST; Antibody incubation: 1) 5% milk in 1xTBS 2) 1% milk in 1xTBS 3) 1% milk in 1xPBS 4) 1% milk in 1x TBST. **C and D.** Immunoprecipitation of NIK using 8H3A2 (**A**) or 10A2E12 (**B**) NIK monoclonal antibodies and immunoblotted with NIK polyclonal antibody. **E.** Immunofluorescence staining of HEK293 over-expressing Flag-NIK using 8H3A2 (**A**) or 10A2E12 (**B**) NIK monoclonal antibodies. Flag antibody was used to confirm the specificity NIK monoclonal antibodies. Hoechst staining was used to depict nuclei. Images were taken using a 20x oil lens and pseudo-colored.

CHAPTER IV

CHARACTERIZATION OF NIK ASSOCIATING PROTEINS

Introduction

Although the role of NIK in activating the NF-κB pathway has been extensively studied, its functions in relation to other signaling pathways have been largely unexplored. Therefore, identifying NIK associating partners using an unbiased approach will give insights on novel functions of NIK. As NIK has been implicated in cancer such as multiple myeloma and melanoma as described in Chapter II, identification of NIK associating proteins will be useful to better understand the tumorigenic functions of NIK.

Yeast-two-hybrid and mass spectrometry analyses have identified expected as well as novel NIK interacting proteins. Other NIK regulating proteins, which are not detected by mass spectrometry or yeast-two-hybrid analyses, have been also identified. Many of these NIK interacting proteins were identified based on their ability to modulate NF-κB activity. Some NIK interacting proteins, namely IKKα/β and p100, are expected based upon our knowledge that NIK functions to regulate NF-κB signaling. However, other NIK protein/protein interactions, such as interaction with heat shock protein 90 (Hsp90), are somewhat unexpected. Although some of these proteins have been known to interact with the NF-κB pathway, their functions in relation to NIK have not been fully explored. Elucidating the nature of these interactions may reveal novel functions for NIK and may uncover key information on the modulation of NIK protein.

NIK interacts with IKKα and IKKβ (details discussed in Chapter I) in addition to its negative regulators, cIAP1, cIAP2, TRAF2 and TRAF3, all of which are E3 ubiquitin

ligases (details discussed in Chapter I). In addition to TRAF2 and 3, NIK associates with other TRAF family members, TRAF1, 5 and 6, and those interactions have been implicated in TNFα and IL-1 induced NF-κB activation (Song et al., 1997). It was also demonstrated that IKKγ can bind to NIK (Bouwmeester et al., 2004; Li et al., 1999a). These interactions modulate NF-κB activation in response to different stimuli.

A number of the NIK interacting proteins modulate the stability of NIK protein. In addition to the E3 ubiquitin ligases, NIK binds to a negative regulator named Monarch-1, which induces the proteasomal degradation of NIK (Arthur et al., 2007). Another NIK interacting protein, Hsp90, also affects NIK stability by protecting NIK from autophagic degradation (Qing et al., 2007). From the literature, we can deduce that many layers of degradative mechanisms may be in place to maintain a physiologically appropriate expression of NIK.

In addition to destabilizing interactions, NIK can associate with a negative regulator that does not affect its stability. A yeast-two-hybrid screen of a human brain cDNA library using the N-terminus of NIK identified a novel NIK interacting protein named TRAFs and NIK-associating protein (TNAP). A glutathione S transferase (GST)-pull down assay reveals that TNAP directly interacts with NIK (Hu et al., 2004). TNAP can markedly decrease basal, TNFα-induced and NIK over-expression-mediated NF-κB activation (Hu et al., 2004). TNAP attenuates the kinase activity of NIK, as evidenced by reduced phosphorylation of the NIK substrate, IKKα (Hu et al., 2004), yet it does not appear to affect NIK stability. Furthermore, TNAP negatively regulates p100/p52 processing as well as NIK-mediated RelA phosphorylation, suggesting that it inhibits both canonical and non-canonical NIK activities (Hu et al., 2004). Another protein identified from this yeast-two-hybrid screen is NIK and IKKβ binding protein (NBIP). NIK directly binds to NBIP along with IKKβ, and NBIP potentiates NF-κB activation induced

by these two kinases upon cytokine treatment (Hu et al., 2005). Similar to TNAP, Raf kinase inhibitor protein (RKIP) antagonizes NIK in its ability to activate NF-κB by interacting with the kinase (Yeung et al., 2001). In addition to NIK, RKIP inhibits the ability of TAK1, another IKK kinase, thus hampering NF-κB activation (Yeung et al., 2001). Yet, RKIP may not directly associate with these kinases, based upon negative data from the *in vitro* pull down assay using recombinant proteins (Yeung et al., 2001).

Intriguingly, NIK interacts with death effector domain (DED)-containing proteins such as caspases 8, 10 and cFLIP, all of which activate NF-κB, possibly through their interaction with NIK and IKKs (Chaudhary et al., 2000). Over-expression of dominant negative NIK disrupts the activation of NF-κB, mediated by these proteins (Chaudhary et al., 2000). Similarly, NIK associates with the viral DEDs-containing protein vFLIP from human herpesvirus 8 (HHV8), which has an ability to activate NF-κB (Chaudhary et al., 1999). These results suggest that NIK has a potential role in the signaling from death receptors, determining survival or cell death through NF-κB activation.

Interestingly, epidermal growth factor receptor (EGFR) binds to NIK and RIP-1, forming a signaling complex to induce NF-κB activation (Habib et al., 2001). Over-expression of a dominant negative NIK disrupts EGFR-induced NF-κB (Habib et al., 2001). Thus, NIK may mediate cellular processes through tyrosine kinase receptor signaling. Consistently, screening NIK associating proteins by using antibody arrays identified new binding partners of NIK that are also part of the EGFR complex (Chen et al., 2003). Flag-NIK expressing HEK293 was incubated with the antibody array and novel NIK binding partners were identified in the screen. In an over-expression system, NIK binds to growth factor receptor bound (Grb) family members such as Grb7, Grb10 and Grb14 (Chen et al., 2003). Endogenous NIK also interacts with Grb7 and estrogen receptor 2 (Erb2), suggesting that NIK is part of the EGFR complex (Chen et al., 2003).

Experiments using NIK knock-out MEFs strongly suggest that NIK is required for EGFR activation of NF-κB (Chen et al., 2003). As EGFR is over-expressed in many breast cancer types, NIK may have a regulatory role in breast cancer progression.

Moreover, another activator of NIK, cancer Osaka thyroid-1 (Cot-1/Tpl-2) physically associates with NIK and IKKα (Lin et al., 1999). Cot-1 induces phosphorylation of NIK, resulting in the activation of IKKα (Lin et al., 1999). Kinase deficient NIK disrupts Cot-1-induced activation of IKKα and subsequent NF-κB activation in CD3/CD28 stimulation pathway (Lin et al., 1999).

Mass-spectrometry analysis of NIK associating proteins has been performed using the tandem affinity purification (TAP) tag system (Bouwmeester et al., 2004). Some interesting proteins that were identified in this analysis include ribosomal proteins, DDX and Cdc37 [for complete list, see (Bouwmeester et al., 2004)]. The functional significance of these interactions has not been fully explored. Biochemical and functional studies are necessary to identify new roles of NIK in the context of its interacting partners.

Here, we identified NIK associating proteins by mass spectrometry analysis using Flag-tagged NIK over-expressed in HEK293 cells. Due to its effect on tumorigenesis and non-essential role in the canonical NF-κB activation, we speculated that NIK plays a role in other signaling pathways in addition to NF-κB activation. We reasoned that some of these interacting proteins may give insights into the contribution of NIK to tumorigenesis of melanoma or other tumors such as multiple myeloma.

Materials and Methods

Immunoprecipitation and mass spectrometry

Flag-NIK was overexpressed in HEK293 cells using Fugene 6 (Roche 11814443001) according to the manufacturer's protocol. 48h after transfection, cells were lysed with IP lysis buffer: 10% glycerol, 50mM Tris pH 7.7, 150mM NaCl, 0.5% NP40 and a cocktail of protease (Sigma P8340) and phosphatase inhibitors (Sigma P2850 and P5726). Flag-NIK was immunoprecipitated from this lysate using an antibody against Flag (Sigma F1804). Prior to immunoprecipitation, Flag antibodies were conjugated to NHS-activated Sepharose beads (GE health care 17-0906-01) to minimize the amount of IgG contamination in samples eluted from Sepharose beads. Immunoprecipitated samples were eluted from Sepharose beads using 4x Laemmli loading buffer and subjected to gel electrophoresis. Lysates run on the gel were either transferred to the nitrocellulose membrane to be immunoblotted with NIK antibody (sc-6363), TRAF3 (sc-949 or sc-1828), IKKα/β (sc-7607) or subject to staining with Colloidal Coomassie (Invitrogen LC-6025). Once the efficiency of immunoprecipitation was confirmed, each lane was cut and subjected to tandem mass spectrometry analysis in collaboration with Vanderbilt Mass Spectrometry Research Center. An in gel tryptic digest was performed and the resulting peptides were subjected to tandem mass spectrometry by linear ion trap. The results were analyzed using Sequest and peptide identity was searched against the IPI human protein database. These assays were performed three times on Flag-NIK immunoprecipitates from HEK293 cells. Immunoprecipitates using IgG in Flag-NIK over-expressed HEK293 and immunoprecipitates using Flag antibody in empty vector expressed HEK293 were included as negative controls.

Molecular Cloning of NIK truncated mutants and full length Hsp90

Truncated mutants of NIK were generated by using PCR amplification of truncated portions from full-length NIK (kindly shared by David Goeddel, Tularik). The primers used were forward primer: 5'-aaggggggatccatggcagtg-3'; reverse primers: NIK_I: 5'-acgctcgagttagccctctgtag-3'; NIK_II: 5'-gctctcgagttaccgtttcttccg-3'; NIK_III: 5'-gggctcgagttaggggcct-3'; NIK_IV: 5'-gctctcgagttagtgtgggccagg-3'; NIK_V: 5'-gcgctcgagttacgtggc-3'; NIK_VI: 5'-ttgctcgagttaccggttcac-3'. These NIK mutants were cloned into pGEX-6P-1 vector (GST-tag vector) using BamHI and XhoI sites. To generate His-Hsp90, full-length human Hsp90 construct (kindly shared by Len Neckers, NCI) was double digested with BamHI and SmaI sites and shuttled into pQE-80L vector (His-tag vector) using the same restriction sites.

Purification of GST-NIK truncated mutants or His-Hsp90

GST-NIK truncated mutants: An over night culture of BL-21 bacteria transformed with GST-NIK truncated mutant DNA was diluted to 1:10 with lysogeny broth (LB)+Ampicillin (Amp) (0.1mg/ml) and grown at 37^{o}C to an OD_{600} reading=0.6-0.8. The production of recombinant protein in the bacterial culture was subsequently induced by addition of IPTG (0.2mM) for 6h at 37^{o}C. After the induction, bacteria were pelleted, re-suspended in 10ml of 1xPBS with protease inhibitor (Sigma P8465) and lysed by sonication (10sec, 4 times). After lysis, 0.5% of Triton X-100 was added to the lysate, which was subsequently incubated with 50µl of glutathione beads at 4^{o}C for 1h. After separating the unbound from the bound lysate, beads were re-suspended in 1xPBS and used in GST pull down assays or stored at -80^{o}C for future use.

His-Hsp90: An over night culture of BL-21 bacteria transformed with His-Hsp90 was diluted to 1:20 with LB+Amp (0.1mg/ml)+chloramphenicol (34ug/ml) and grown at 37°C to an OD_{600} reading=0.6-0.8. The production of recombinant protein in the bacterial culture was induced by addition of IPTG (0.1mM) for 2h at 37°C. After the induction, bacteria were pelleted, re-suspended in 10ml of 1xPBS with EDTA-free protease inhibitor (Roche 04693159001)+20U DNAse+2mM β-mercaptoethanol (β-ME) and lysed by sonication (10sec, 4 times). Lysate with 10mM of imidazole (pH 8.0) was incubated with TALON beads (equilibrated with binding buffer containing 50mM NaH_2PO_4, 500mM NaCl, 20mM imidazole, pH 8.0, 2mM β-ME) for 1h at 4°C. After the unbound lysate was separated from the bound lysate, beads were washed with binding buffer overnight at 4°C. His-Hsp90 was eluted from the beads with the elution buffer containing 50mM NaH_2PO_4, 500mM NaCl, 250mM imidazole, pH 8.0 and 2mM β-ME. Eluted protein was dialyzed overnight at 4°C and used in direct binding assays or stored at -80°C for future use.

GST pull down

500pmol of full length His-Hsp90 was incubated with 5pmol of GST or GST-NIK truncated mutants immobilized on glutathione beads and incubated for 1h at 4°C. After the beads were centrifuged and the unbound portion was removed, beads were washed 3 times with 1xPBS. His-Hsp90 directly bound to GST-NIK truncated mutants was resolved by 10% SDS-PAGE and analyzed by Western blot analysis.

Results

To identify new NIK-interacting proteins by mass-spectrometry, Flag-NIK was first immunoprecipitated with Flag antibody, then subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with NIK antibody to verify the efficiency of the immunoprecipitation (Figure 18A). Once the efficiency of the immunoprecipitation was confirmed, immunoprecipitates were separated on a 8% acrylamide gel, stained with Colloidal Coomassie (Figure 18B) and subjected to in-gel trypsin digest, followed by tandem mass spectrometry. Negative controls included Flag-NIK over-expressed HEK293 cell lysate immunoprecipitated with IgG and empty plasmid expressed HEK293 cell lysate immunoprecipitated with Flag antibody. Consistent with previous studies, IKKα/β and TRAF3 co-immunoprecipitated with Flag-NIK, verifying the reliability of our immunoprecipitation conditions and techniques (Figure 18C).

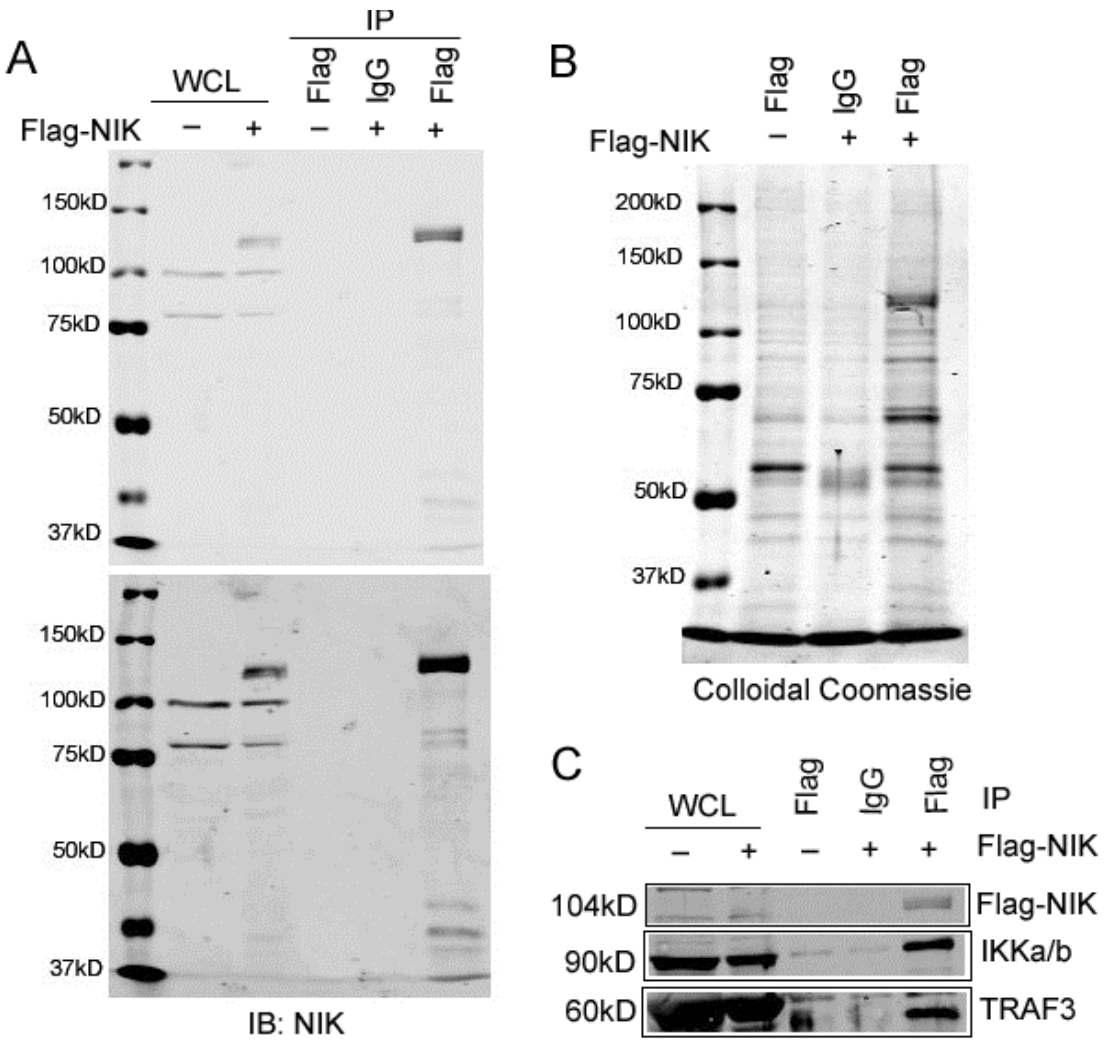


Figure 18. Verification of Flag-NIK co-immunoprecipitation with known interacting proteins. **A**. Immunoprecipitation of Flag-NIK using Flag antibody and immunoblotting with NIK antibody. **B**. Coomassie staining of Flag-NIK immunoprecipitates. **C**. Co-immunoprecipitation of Flag-NIK, IKKα/β and TRAF3 in HEK293. Flag-NIK was immunoprecipitated using antibody against Flag and immunoblotted with NIK, IKKα/β and TRAF3 antibodies. Immunoprecipitation from Flag-NIK over-expressing lysates using IgG or immunoprecipitation from lysates without Flag-NIK over-expression using Flag antibody were used as negative controls.

Proteins identified by immunoprecipitation with Flag-NIK followed by mass spectrometry in at least 2 separate experiments were as follows: HSP90, IKKα, IKKβ, IKKγ, NIK, RPS3, ADP/ATP translocase, ATP-dependent RNA helicase DDX5, isoform of polyadenylate-binding protein, NCL protein, heterogeneous nuclear ribonucleoprotein M isoform a, cDNA FLJ 45706fis, interleukin enhancer-binding factor 2, isoform 5 of interleukin enhancer-binding factor 3, insulin receptor substrate 4, insulin-like growth factor 2 mRNA binding protein (Table 3). It is reassuring that proteins reported to interact with NIK such as IKKα , IKKβ and IKKγ (Li et al., 1999a; Ling et al., 1998; Nakano et al., 1998) were identified (Table 3). We next determined whether some of these interactions can be verified in immunoprecipitation and direct binding assays.

Table 3. NIK associating proteins identified by mass spectrometry analysis. Proteins identified in two and three runs were listed.

Proteins identified in three runs	Proteins identified in two runs
Heat Shock Protein 90α (Hsp90α)	ADP/ATP translocase (1 and 2)
NF-κB inducing kinase (Zlotnik et al.)	ATP-dependent RNA helicase
Similar to 40S ribosomal protein SA	CDNA FLJ 45706 fis, clone FEBRA 2028457, highly similar to Nu
Ribosomal Protein S3 (RPS3)	Hsp90 co-chaperone Cdc37
	IKKα
	Interleukin enhancer-binding factor 2
	Isoform of Polyadenylate-binding protein 1
	Isoform 5 of Interleukin enhancer-binding factor 3
	NCL protein
	NEMO/IKKγ
	Probable ATP-dependent RNA helicase DDX5
	Heterogeneous Nuclear Ribonucleoprotein M Isoform a
	Insulin Receptor Substrate 4
	Insulin-like Growth Factor 2 mRNA Binding Protein 1

Verification of NIK-associating proteins

Our preliminary data suggest that Flag-NIK interacts with both endogenous and exogenous Hsp90, as shown by the co-immunoprecipitation (Figure 19A and B). To examine whether NIK directly interacts with Hsp90 and to characterize which domains of NIK interacts with Hsp90, we generated truncated mutants of NIK with GST tag and full-length Hsp90 with His tag. Schematic of NIK truncated mutants (NIK I-VI) are shown in Figure 19C. We purified GST-tagged truncated mutants of NIK (NIKI, NIK II and NIK IV) (Figure 19D and E) and full length His-tagged Hsp90 (Figure 19F). Our preliminary data from GST-binding experiments suggest that Hsp90 directly binds to GST-NIKII (Figure 19F).

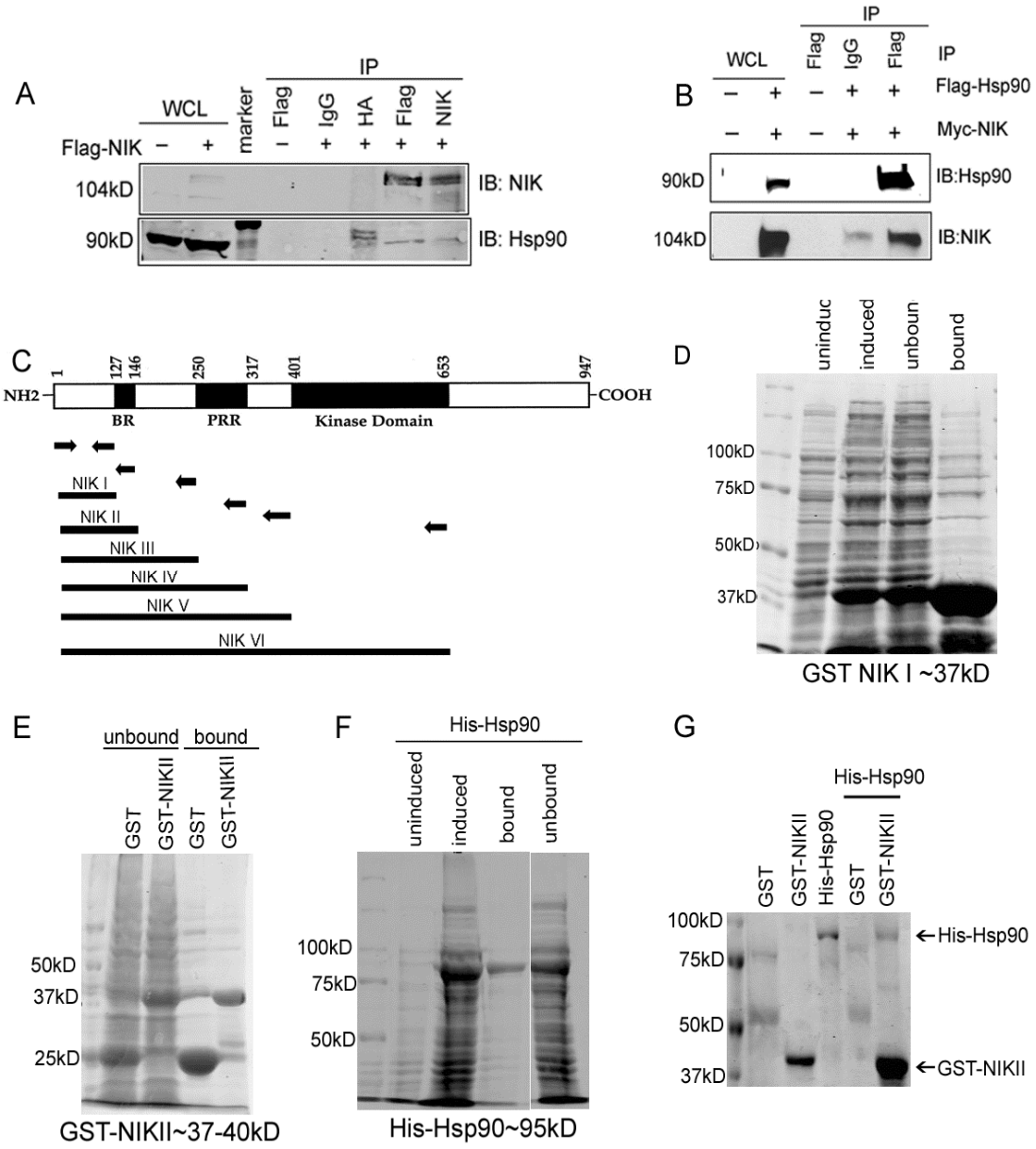


Figure 19. Verification of Flag-NIK co-immunoprecipitation with known interacting proteins. **A and B.** Co-immunoprecipitation of Flag-NIK and endogenous Hsp90 (**A**) and exogenous His-Hsp90 (**B**). **C.** Different domains of NIK: BR (basic region), PRR (proline rich region) and kinase domain (Xiao, et al. 2000). The regions of primers to generate truncated mutants of NIK are shown in dark arrows. **D, E and F.** Coomassie stain of purified GST-NIKI (**D**), GST-NIKII (**E**) and His-Hsp90 (**F**) resolved on SDS-PAGE gels. Protein expression was induced by IPTG and purified using GST or TALON beads. **G.** Coomassie stain of direct binding between GST-NIKII and His-Hsp90.

Previous studies suggest that Hsp90 regulates the stability of NIK. NIK physically interacts with Hsp90 through its C-terminal domain (Qing et al., 2007), although whether this interaction is direct or indirect is yet to be examined. Treatment of cells with the Hsp90 inhibitor geldanamycin dissociates NIK and Hsp90, and results in marked NIK degradation (Qing et al., 2007). Thus, Hsp90 appears to have a positive regulation on the stability of NIK. In contrast, another study demonstrated that Hsp90 is required for the negative regulatory role of Monarch-1 to induce NIK degradation (Arthur et al., 2007). Thus, depending on the context, Hsp90 can positively or negatively regulate the stability of NIK. By modulating NIK stability, these proteins regulate p100 to p52 processing and possibly the expression of downstream target genes. Intriguingly, Hsp90 and another protein identified in mass spectrometry, Cdc37, are part of the IKK complex and geldanamycin treatment impairs TNFα-induced NF-κB activation (Chen et al., 2002). These two proteins are essential components of NF-κB activation.

It is interesting to note that Hsp90 has been reported to be involved in melanoma malignancy. Its co-chaperone tetratricopeptide repeat containing protein (TTC4) is over-expressed in melanoma and other cell lines derived from cancerous tissues, such as breast, colon and ovaries (Crevel et al., 2008). In melanoma, mutated TTC4 retains the interaction with Hsp90 (Crevel et al., 2008), suggesting that Hsp90 may be playing an important role in melanoma. Consistent with this notion, immunohistochemistry identifies Hsp90 expression in primary melanoma as well as in melanoma cell lines, and its inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) reduces proliferation rates, migratory and invasive abilities (Faingold et al., 2008). Similarly, monoclonal antibody against Hsp90 inhibits melanoma invasion and metastasis (Stellas et al., 2007). Hsp90 is also required for stabilizing mutated BRAF (Grbovic et al., 2006), which is found in about 50-70% of melanoma lesions (Davies et al., 2002). Hence, studying the interaction

between Hsp90 and NIK may give more insight into deregulated molecular mechanisms of NIK in melanoma.

Another protein identified in all three runs, aside from Hsp90, is RPS3. To date, we cannot verify the interaction between NIK and RPS3 by immunoprecipitation. Recently, RPS3 was identified as part of NF-κB complex (Wan et al., 2007). When RPS3 is knocked down, expression of some NF-κB regulated genes are defective, suggesting that RPS3 is necessary for the transcription of a subset of NF-κB targeted genes (Wan et al., 2007). Recently, it was reported that IKKβ phosphorylates RPS3 and regulates RPS3 nuclear translocation (Wan et al., 2011). As NIK is an upstream kinase of IKKβ and NIK interacts with RPS3, studying the interaction between NIK and RPS3 may elucidate novel functions of NIK in NF-κB as well as non-NF-κB pathways.

In addition to Hsp90 and RPS3, another interesting protein identified in mass spectrometry is DDX5. DDX5, also known as P68 RNA helicase, belongs to the DEAD-box family of RNA helicases, which have RNA-dependent ATPase activity to unwind RNA structures or disrupt RNA-protein interactions (Rocak & Linder, 2004). Intriguingly, DDX5 disrupts β-catenin from the Axin complex, which normally mediates β-catenin destruction, and leads to nuclear accumulation of β-catenin (Yang et al., 2006b). Based upon our observations that NIK may affect the nuclear translocation of β-catenin and that NIK binds to DDX5, we postulate that NIK may regulate β-catenin nuclear transport through DDX5.

Another NIK-interacting protein, interleukin enhancer-binding factor 2, can be a part of a transcriptional complex along with p300 (Karmakar et al., 2010) as well as control mitotic regulation (Guan et al., 2008). Heterogeneous Nuclear Ribonucleoprotein M Isoform a is part of heterogeneous nuclear ribonucleoproteins, which bind to heterogeneous nuclear RNA and function in RNA processing (Dery et al., 2011).

Interestingly, another NIK interacting protein, ADP/ATP translocase (1 and 2) regulates apoptosis through recruiting IkB/NF-kB into mitochondria (Zamora et al., 2004). The diverse functions of these NIK interacting proteins suggest that NIK may be involved in cellular processes that have not been linked to NIK before.

Conclusions

Here, we identify novel NIK associating proteins such as Hsp90, RPS3 and DDX5 by using mass spectrometry approach. The candidates from our list of NIK associating proteins greatly overlapped with the list reported by Bouwmeester et al (Bouwmeester et al., 2004), suggesting that the interactions are likely to be specific. Confirming these interactions by using co-immunoprecipitation in cell lysate, GST pull down and studying the functional significance will possibly lead to identification of novel NIK functions. The interactions between these proteins and NIK may also be important for tumorigenesis mediated by NIK.

CHAPTER V

DETERMINING THE EFFECT OF NF-κB/IKKβ INHIBITION ON THE HOST IMMUNE SYSTEM

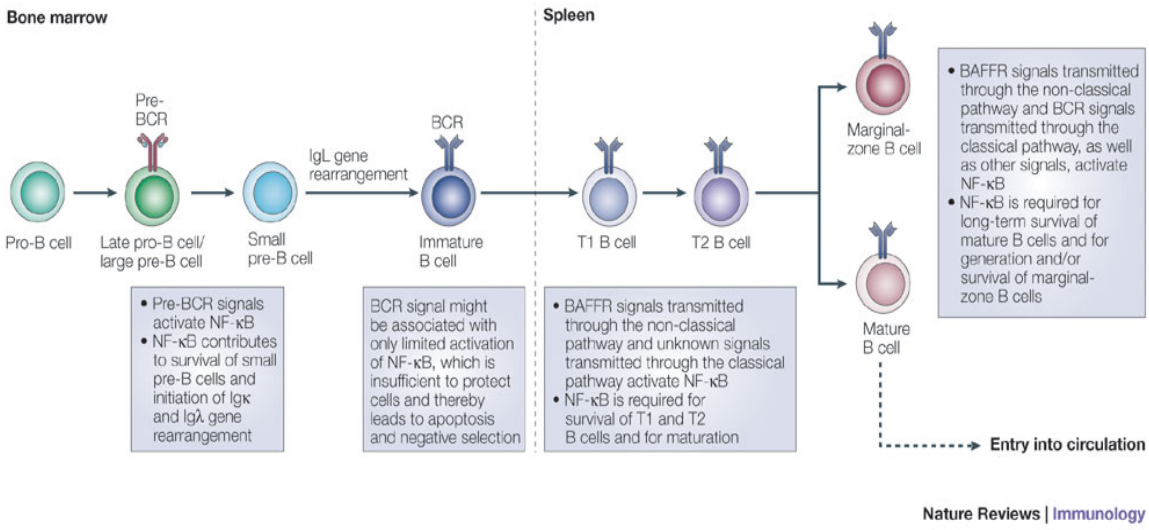
Introduction

NF-κB is crucial for tumorigenesis of many tumor types, including melanoma (Basseres & Baldwin, 2006; Yang & Richmond, 2001).Therefore, targeting the major kinase of NF-κB signaling, IKKβ, turns out to be a promising target (Yang et al., 2006a; Yang et al., 2007a; Yang et al., 2010). As NF-κB is an important mediator of inflammation, cancer and other inflammatory diseases, various drugs targeting NF-κB have been developed in an attempt to alleviate diseases where constitituve activation of NF-κB may play a causal role. Drugs targeting different levels of the NF-κB pathway include both natural products as well as synthetic compounds (Gilmore & Herscovitch, 2006). Some of these inhibitors are general inhibitors of inflammation whereas others are specific inhibitors of the NF-κB pathway (Gilmore & Herscovitch, 2006). Among these, some specific inhibitors have drawn attention for the treatment of certain cancers and inflammatory diseases. For example, MLN120B is an IKKβ inhibitor that has been reported to reduce the growth of multiple myeloma in vitro and in vivo (Hideshima et al., 2006). Bortezomib (VELCADE), a proteasonal inhibitor that blocks NF-κB activation , has been approved for treatment of myeloma (Mulligan et al., 2007) and recently was shown to be useful in mouse models of lung cancer (Xue et al., 2011). Interestingly, the Bay-117082 IKK inhibitor had similar effects to bortezomib in the lung model.

Similar to MLN120B, BMS-345541 is an allosteric small molecule inhibitor of IKKβ, preferably exerts its effect on IKKβ over IKKα and blocks NF-κB mediated transcription when administered in mice (Burke et al., 2003). Although BMS-345541 appears to be a potential drug for melanoma therapy and other cancer therapy, systemic effects when used for cancer treatment are still unknown. Inhibiting NF-κB through IKKβ may have a potent effect on immune system, notably on B cells as they depend on NF-κB activity for their development, activation and survival. It is established that NF-κB activity protects B cells from undergoing apoptosis when challenged with high amount of TNFα (Siebenlist et al., 2005). In addition, NF-κB controls B cells' proliferation in response to stimulators of NF-κB activation. For instance, CD40L- and LPS-induced proliferation as well as the regulation of proliferation related genes are greatly diminished in $cRel^{-/-}$ and $p50^{-/-}$ $cRel^{-/-}$ B cells (Zarnegar et al., 2004).

Our lab has also demonstrated that BMS-345541 induces melanoma cells to undergo mitochondria-dependent apoptosis, thus inhibiting melanoma growth in mice (Yang et al., 2006a). Yet, in the context of the tumor microenvironment, the effect that BMS-345541 may have on the expression of cyotokines and effectors that regulate the infiltration of immune cells into the tumor should be considered. For instance, BMS-345541 may inhibit immune infiltrates, which may support tumor cells proliferation and metastasis, thereby improving the cancer therapy. On the reverse side, BMS-345541 may inhibit the infiltration of leukocytes with anti-tumor activity into the tumor. If this occurs, this drug may need to be administered with other immunotherapies to eliminate undesirable effects on tumor growth. Thus, to optimize the efficacy of the drug, the effect of BMS-345541 on the immune system of the tumor bearing host needs to be determined. Many studies suggest that NF-κB and IKKβ activity play an important role in both B and T cell development. Figure 20 depicts different stages of B cell development

and the role of NF-κB in these stages. Normally, pro-B cells are developed in the bone marrow up to an immature B cell stage. Then they complete their development in splenic follicles where they can either become resident marginal zone B cells or follicular B cells. Follicular B cells can enter the circulation and the follicles of other secondary lymphoid organs (Siebenlist et al., 2005).



Siebenlist *et al*., *Nat Rev Immunol.*, 2005.
(Reprint with permission from *Nature*)

Figure 20. The role of NF-κB activation in different developmental stages of B cells. NF-κB activity determines the survival and apoptosis of B cells at early stages (pre-B cells and immature B cells). In addition, NF-κB activity (both canonical and non-canonical activation) is required to maintain mature population of B cells.

Different transgenic mouse models reveal that IKKβ is important for mature B cell homeostasis. First, conditional IKKβ knock-out mice have a reduction in peripheral B cells due to their susceptibility to apoptosis (Burke et al., 2003). These mice also have a defect in mounting antibody responses to both T-cell dependent and independent antigens (Burke et al., 2003). Second, B-cell specific disruption of IKKβ leads to disappearance of marginal zone and follicular zone B lymphocytes, suggesting IKKβ is needed autonomously in mature B cells for their survival (Pasparakis et al., 2002). Third, expressing a dominant negative form of IKKβ, specifically in B cells of mice suggests that IKKβ is crucial for B cell proliferation and antibody response (Ren et al., 2002). Yet, neither developmental defect nor basal immunoglobulin production was observed in these mice (Ren et al., 2002). Pharmacological inhibition of IKKβ in mice with another IKKβ inhibitor, MLN120, reveals significant reduction of the number of B cells of all stages of development (Nagashima et al., 2006).

In addition to the B cell population, NF-κB activity is important for some subsets of T cells. Adoptive transfer experiments suggest that IKKβ is dispensable for T cell development (Schmidt-Supprian et al., 2003). However, regulatory and memory T cells depend on IKKβ for their development and survival (Schmidt-Supprian et al., 2003; Schmidt-Supprian et al., 2004). Of note, T_{reg} cells suppress helper and cytotoxic T cells function in melanoma, thus therapeutic efforts have been made to deplete the regulatory T cell population (Lizee et al., 2006). Therefore, the drug targeting IKKβ and thus attenuating NF-κB activation may be beneficial for melanoma therapy because of its potential effect on both tumor growth and the immunosuppression through T_{reg}.

In addition to the potential effect of BMS-345541 on the host immune system, the drug may alter the immune infiltrate profile within the tumor. The importance of the immune system during tumor development has been increasingly appreciated. Tumor

tissues are often infiltrated with both innate and adaptive immune cells, and the presence of these immune constituents can be pro- or anti-tumorigenic (Mantovani et al., 1992). NF-κB can regulate various cytokines and chemokines in tumor cells, and these factors are involved in recruiting immune cells, such as macrophages, neutrophils and dendritic cells to the tumor microenvironment (Rollins, 2006). As a specific example, MCP-1/CCL2 is a chemokine regulated by NF-κB (Ueda et al., 1997) and is produced by melanoma (Nesbit et al., 2001). Its level influences tumor associated macrophage recruitment, angiogenesis and melanoma progression (Gazzaniga et al., 2007; Nesbit et al., 2001). Studies have shown that melanoma is infiltrated with other immune cells, such as cytotoxic T cells (Ladanyi et al., 2004) and dendritic cells which can participate in rejecting the tumor (Preynat-Seauve et al., 2007; Preynat-Seauve et al., 2006; Vermi et al., 2003).

Many studies have suggested that the regulation of NF-κB is crucial not only in tumors but also in the ability of immune cells to influence the growth and survival of cancer cells. Many studies using genetic mouse models elegantly demonstrated that targeted deletion of IKKβ in macrophages in both hepatocellular carcinoma and colitis-associated colon cancer reduce the tumor burden (Hagemann et al., 2009). These data suggest that IKKβ plays a tumor promoting role in macrophages and that inhibiting IKKβ in macrophages is likely to be beneficial for cancer treatment. However, it is important to note that these models are genetically engineered models in which IKKβ is systemically absent throughout tumor development.

Therefore, in addition to these genetic models, the role of IKKβ in tumor associated immune cells has also been studied in other models. For instance, defective NF-κB activity has been reported in tumor associated macrophages (TAMs) in a fibrosarcoma model (Saccani et al., 2006). This defect prevents TAMs from exerting

anti-tumor activity (Saccani et al., 2006). Therefore, this study implies that the activity of NF-κB is required for TAMs to become tumor-killing M1 macrophages. Similarly, another study using a mouse model of mammary carcinoma also suggests that reactivating NF-κB in tumor-promoting M2-like TAMs polarize these cells towards M1-like phenotypes (Guiducci et al., 2005). In contrast, in a mouse model of ovarian cancer, Hagemann et al have demonstrated that tumor promoting macrophages can be reverted to anti-tumor immune cells by targeting IKKβ (Hagemann et al., 2008).

Similarly, the activity of IKKβ is also important for T cells to modulate tumor growth. For instance, anergic T cells from tumor bearing mice have defective NF-κB activation and therefore, are unable to express cytokines to mediate tumor rejection (Ghosh et al., 1994; Sundstedt et al., 1996). From the literature, it is not clear whether NF-κB activity in myeloid or lymphoid cells is pro- or anti-tumorigenic. It is very likely that the nature of NF-κB activity on tumor growth depends on tumor types as well as the tumor stage. Systemically inhibiting NF-κB activity by IKKβ inhibitor to hinder melanoma growth may alter the tumor immunology during the development of malignancy.

Based on the literature and the studies from our lab, it is clear that NF-κB plays a critical role in tumorigenesis. Using genetic models and pharmacological inhibition, our lab has demonstrated that inhibition of NF-κB through IKKβ is a potential therapeutic agent for treatment of melanoma. However, we also predict that inhibiting NF-κB systemically may have an impact on the host immune system as well as on the tumor immunology in modulating malignancy of melanoma.

Materials and Methods

Xenograft model

To determine the effect of the IKKβ inhibition on B cells, C57BL/6 mice were injected subcutaneously with the C57BL/6-derived MelA/MIP2 mouse melanoma cells. For each mouse, $10x10^6$ cells were injected into the right flank. After about two weeks when the tumor mass reached a detectable size (~7-8mm), BMS-345541 or solvent control was administered by oral gavage at the dosage of 50mg/kg (twice per day) for 15 days (PBS treatment: n=7, BMS treatment: n=8). Experiment was performed once. During the drug treatment, tumor growth of the animal was monitored every five days by microcaliper measurements.

Xenograft experiments using Hs294T human melanoma cells were performed as below. Briefly, $3x10^6$ Hs294T cells were injected subcutaneously on the right flank of each nude mouse. After, xenograft tumors were allowed to grow for about two weeks, the tumor bearing mice were oral gavaged with solvent control or 100 mg/kg of BMS-345541 (twice per day). Eight mice were in each treatment and the treatment lasted for 30days (Control and BMS treatment: n=4). Experiment was performed once.

The weight of the animals and their general health was monitored according to standards set by IACUC to ensure that the dosage of the drug did not have a severe toxicity. All mice were housed in the Vanderbilt AAALAC accredited animal facility and studies were performed in keeping with the principals of human care of animals approved by IACUC.

Flow cytometry analysis

At the end of the treatment with vehicle control or BMS-345541, splenocytes were collected and stained with fluorescent antibodies; CD19-PerCP-Cy5, CD21-APC,

CD23-PE, IgM-FITC and AA4.1-PECy7 (BD Pharmigen) to analyze mature and immature B cell populations. Flow cytometry analysis was performed on these splenocytes to examine mature B cell subsets such as, marginal zone ($CD19^{+}IgM^{hi}AA4.1^{-}CD23^{-}CD21^{+}$), follicular zone ($CD19^{+}IgM^{low}AA4.1^{-}CD23^{+}CD21^{int}$) and; immature B cell subsets such as, T_1 ($CD19^{+}IgM^{hi}AA4.1^{hi}CD23^{-}CD21^{lo}$) and T_2 ($CD19^{+}IgM^{hi}AA4.1^{+}CD23^{+}CD21^{int}$). In addition, splenic T cell population was examined in these treated and control mice by staining with CD3-FITC (BD Pharmigen: 110-81-31). Flow cytometry analysis was performed using Beckman coulter machine.

Measurement of immunoglobin levels in BMS-345541 treated mice

To study the effect of BMS-345541 on B cell functions, the basal level of immunoglobulins from sera of BMS-345541 or vehicle treated mice was measured. Sera collected from mice were diluted 1:100 in 1xPBS and the immunoglobulin level was determined by ELISA (Southern Biotech 5300-01; 5300-05), following the manufacture's protocol.

Results

Although the effect of BMS-345541 was tested using human melanoma cells, its effect has not been determined using a mouse melanoma cell line such as MelA/MIP2. To examine the drug effect on this cell line *in vitro*, cells were treated with a range of drug concentrations (25μM to 100μM) or DMSO. Figure 21A shows that BMS-345541 kills MelA/MIP2 tumor cells *in vitro* in a dosage dependent manner.

Although the effect of BMS-345541 on tumor growth in immunodeficient mice was tested using human melanoma cells, the effect of the drug had not been determined in immunocompetent mice. To determine whether systemic IKKβ inhibition in

immunocompetent mice would inhibit tumor growth, we examined the effect of BMS345541 on the growth of the Mel/MIP2 cell in C57Bl/6 mice. Once palpable tumors were formed, we treated tumor-bearing mice with vehicle control or BMS-345541 for 15 days. There was not a statistically significant difference in tumor size between control and BMS-345541 treated mice (Figure 21B). Long-term treatment, a larger sample size and/or a higher dosage of the drug may be necessary to observe a significant response of melanoma to the drug.

Effect of systemic IKKβ inhibition on the host immune system

As IKKβ is crucial for the development and survival of B and T cells, how the IKKβ inhibitor may affect the host adaptive immune system was determined by examining the composition of splenic B-cells and T-cells. Splenocytes from PBS and BMS-345541 treated mice were stained with B220, IgM, AA4.1, CD21 and CD23 to examine B cell population or with CD45, CD3 and 7AAD to examine T cell population. Figure 21C suggests that marginal zone and immature T1 cell populations are slightly diminished in BMS-345541 treated mice compared to the PBS treatment group. These data agree with the studies reporting that interfering IKKβ activity by genetic disruption or pharmacological inhibition using MLN120B (300mg/kg) leads to apoptosis in B cell population (Li et al., 2003; Nagashima et al., 2006; Pasparakis et al., 2002; Ren et al., 2002). Likewise, total T cell population was slightly decreased in drug treated mice (Figure 21E). These data suggest that IKKβ inhibition may have an effect on the host immune system.

As IKKβ is involved in antibody production (Ren et al., 2002), basal immunoglobulin response was measured in mice treated with PBS or BMS-345541. No apparent difference was observed in basal immunoglobulin production between these

two groups, suggesting that BMS-345541 does not have a significant effect on antibody production (Figure 21D).

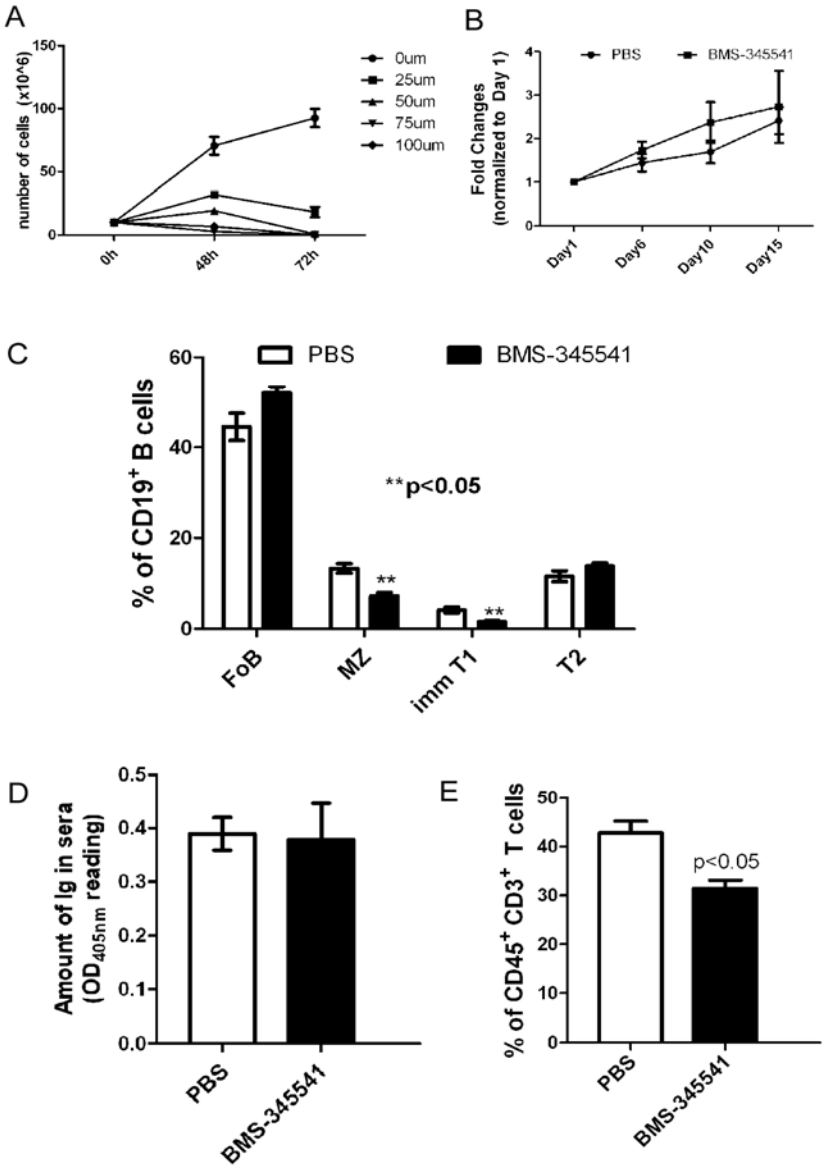


Figure 21. Effects of BMS-345541 on melanoma tumor growth and the host immune system. **A.** BMS-345541 causes MelA/MIP2 tumor cell death *in vitro*. $0.1x10^6$ cells were plated in a 12-well plate and treated with different concentrations of BMS-345541. After 48 and 72 h, cells were trypsinized and counted using a hemacytometer. DMSO was used a control. **B.** Tumor size of control and BMS-345541 treated mice over time. MelA/MIP2 cells were subcutaneously injected into C57/BL6 mice. Once the tumors reached a palpable size, mice were treated with BMS-345541 or 1xPBS for 15 days (50mg/kg, twice a day). **C.** Compared to the control, immature T1 B cell ($CD19^+IgM^+CD21^{lo}$) and marginal zone B cell number ($CD19^+CD23^-CD21^{hi}$) decrease in mice treated with BMS-345541. There is no statistically significant difference in number of follicular ($CD19^+CD23^+CD21^{int}$) and T2 ($CD19^+IgM^+CD21^{int}$) cells between treated and untreated groups.

D. Immunoglobulin response by B cells was not affected by treatment with BMS-345541. Serum was collected from mice treated with either PBS or BMS-345541 after 15days of treatment. Immunoglobulin levels were determined by ELISA (SouthernBiotech). **E.** The total number of T-cells ($CD45^+CD3^+7\text{-}AAD^-$) in BMS-345541 treated mice is reduced compared to the control mice treated with PBS. (PBS: n=7, BMS-345541: n=8). Tumor bearing C57/BL6 mice were treated with either PBS or BMS-345541 (75mg/kg twice per day) for 15days. On the 16th day, mice were treated with either PBS or BMS-345541 three hours before they were sacrificed. Subsequently, splenocytes were isolated from these mice and stained with A) CD45, CD3 and 7-AAD or B) IgM, CD19, CD21, CD23 and AA4.1, followed by FACS analysis. The data was analyzed by Mann-Witney test (n=7 in PBS treated; n=8 in BMS-345541 treated).

Effect of IKKβ inhibition on immune infiltrates in melanoma

As IKKβ and NF-κB are important in tumor associated immune cells, we hypothesized that the composition of immune infiltrates would be different in BMS-345541 treated tumors. This hypothesis was tested using nude mice carrying Hs294T human melanoma xenograft tumors. Hs294T cells were subcutaneously injected into nude mice and they were treated with BMS-345541 for 30 days once the tumors reached palpable sizes. Vehicle treatment was used as a control. Tumors were harvested at the end of the treatment, sectioned and immuno-stained with antibodies against neutrophil antigen or F4/80 (macrophages). A marked increase in the number of immune infiltrates (both neutrophils and macrophages) was observed in BMS-345541 treated tumors (Figure 22A and B). These data suggest that inhibiting IKKβ may influence the immune effectors during melanoma growth, thereby altering the tumor response to the drug.

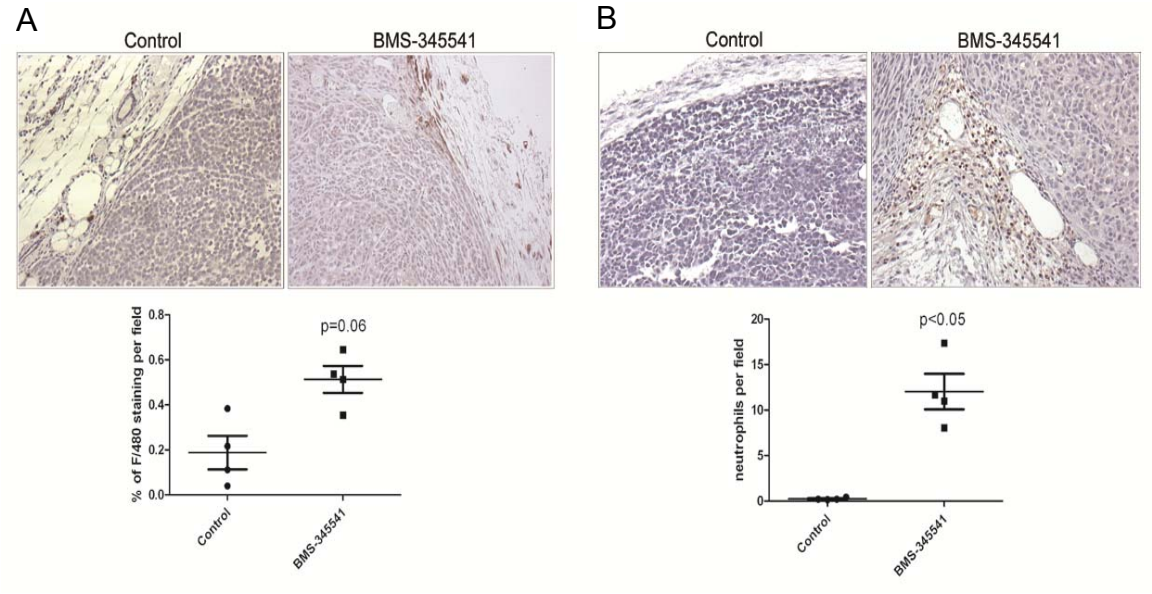


Figure 22. Effects of BMS-345541 on the immune infiltrates of melanoma tumors. **A** and **B**. The amount of the macrophage (A) and neutrophil (B) infiltrates is higher in tumors treated with BMS-345541 compared to the vehicle control. Hs294T melanoma cells were injected into nude mice (1×10^6 cells per mice). After about two weeks, treatment of tumor bearing mice with vehicle or BMS-345541 (100mg/kg, twice per day) was initiated. After 30 days of treatment, mice were sacrificed and tumors were embedded in paraffin. Tumor slides were stained with antibodies to the neutrophil marker (Abcam: ab2557-50) or the F4/80 macrophage marker (Serotec: MCAP497). Images were taken at 20x and 20 fields per slide were counted for the positive staining. A total of four tumor sections were counted for each stain analysis. Mann-Witney test was performed to assess the statistical significance.

Discussion and Conclusions

The activity of NF-κB potently promotes tumorigenesis in various cancer types due to its role as a master regulator of many growth factors, cytokines and chemokines and pro-survival proteins (Basseres & Baldwin, 2006). In parallel, its functions are vital to many normal biological functions including immune regulation. Therefore, disruption of NF-κB through IKKβ inhibitor may not only inhibit tumor growth but also alter the tumor microenvironment through regulation of the immune system. The role of NF-κB in tumor immunology has been increasingly apparent. Yet, the controversy of whether NF-κB activity in immune constituents is pro-tumorigenic or anti-tumorigenic remains unsettled. For example, whether NF-κB activation in TAMs is growth promoting or cytotoxic for tumor cells is a topic of dispute (Lawrence, 2011). Cancer is a complex disease, constantly evolving along with its microenvironment. Hence, altering its microenvironment can immensely change the tumor biology. As we have now begun to understand that chronic inflammation can lead to cancer progression and that NF-κB is a molecular link to inflammation (Balkwill & Coussens, 2004), better elucidation of the role of NF-κB in tumor immunology will advance the field of cancer therapy.

Here, we demonstrated that inhibition of IKKβ using BMS-345541 diminishes the number of some B-cell subsets in the host and increases immune infiltrates into melanoma tumors. Although the results are preliminary, our data suggest that the use of BMS-345541 in cancer therapy should proceed with caution as it may influence the tumor immunology and host defense mechanism. Further analysis of the effects of IKKβ inhibition on tumor immunology is warranted to provide insight into appropriate ways to use combination therapy to achieve optimal efficacy.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

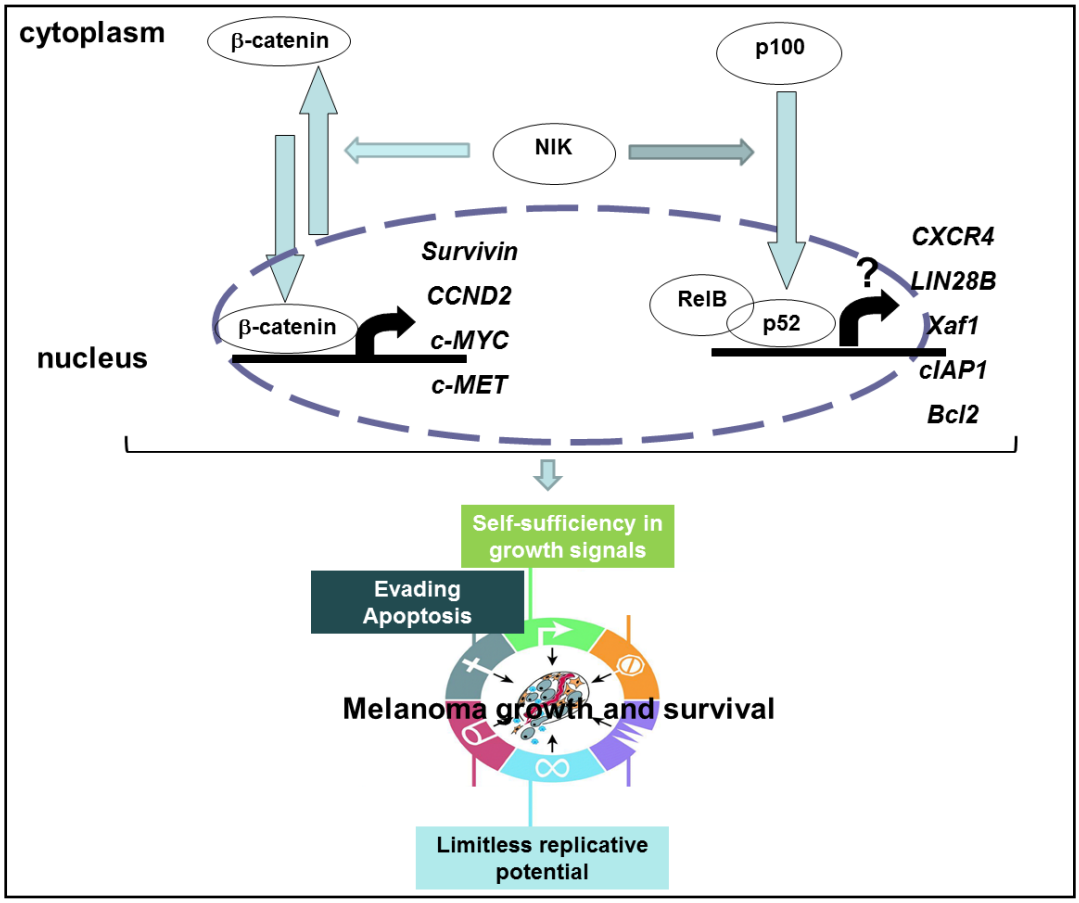
In our efforts to find an improved therapy for melanoma, we have identified the transcription factor NF-κB and its major regulator IKKβ kinase as potential therapeutic targets. NIK is one of the upstream kinases of NF-κB which offers a therapeutic opportunity. We hypothesized that NIK is important for melanoma tumorigenesis and that disruption of NIK attenuates melanoma growth. In this study, we have demonstrated that NIK expression is up-regulated in dysplastic nevi, primary melanoma and metastatic melanoma compared to benign nevi. NIK depletion in melanoma decreases proliferation, increases apoptosis, and results in a cell cycle progression defect. Gene expression analysis of NIK knock-down melanoma cells and xenograft tumors suggests that NIK modulates expression of pro-tumorigenic genes such as *CXCR4*, *c-MYC*, *c-MET* and *LIN28B*. In addition to these genes, NIK regulates the expression of some pro-survival proteins such as cIAP1, survivin and BCL2. By modulating the expression of above mentioned genes, NIK contributes to multiple aspects of cancer progression, as detailed in the "Hallmarks of Cancer" by Hanahan and Weinberg (Hanahan & Weinberg, 2000) such as evasion from apoptosis, self-sufficiency in growth signals and limitless replicative potential (Figure 23).

We also show for the first time that NIK modulates the occupancy of β-catenin on the survivin promoter, thereby regulating the expression of this pro-survival/pro-tumorigenic protein. We also show that NIK may modulate the stability of β-catenin through ubiquitination. Intriguingly, nuclear accumulation of β-catenin is lower in NIK

deficient cells. These data suggest that NIK regulates the sub-cellular localization of β-catenin, either directly or indirectly**.**

Here, we identify novel NIK-associating proteins by mass spectrometry analysis. In addition to known NIK interacting proteins including IKKα, IKKβ and IKKγ, novel proteins such as Hsp90, RPS3 and DDX5, which play important roles in NF-κB or β-catenin activation, are identified. Further analysis of these interactions should reveal novel functions of NIK and may give additional insight into the biological functions and potential pro-tumorigenic roles of NIK. Increased understanding of these protein interactions will be useful when considering NIK as a target for cancer therapy.

Due to an interest in using IKKβ inhibitor in treating melanoma or cancer with aberrant NF-κB activation, determining the effects of the inhibitor on the host is of interest. Here, we demonstrate that an IKKβ inhibitor, BMS-345541, alters the host immunity as well as immune responses to tumors. Specifically, BMS-345541 treatment decreases subsets of B cell and total T cell population. It also alters the composition of immune infiltrates to the tumors. Although detailed mechanisms need to be explored to determine whether IKKβ inhibition creates a pro- or anti-tumor microenvironment, these results generate new hypotheses regarding the role of NF-κB and IKKβ in tumor immunology of melanoma.



Modified from Hanahan and Weinberg, *Cell.* 2000.

Figure 23. Working model of NIK roles in melanoma tumorigenesis. We demonstrate that NIK mediates melanoma tumor growth and expression of genes crucial for tumorigenesis. We also demonstrate, for the first time, that NIK can regulate expression of some pro-survival genes through the β-catenin pathway. By regulating pro-tumorigenic/pro-survival gene expression, NIK impacts on some important processes of cancer progression (self-sufficiency in growth signals, evasion from apoptosis and limitless replicative potential). As NIK regulates the expression of pro-survival factors, yet is not an essential kinase for the canonical NF-κB pathway, our data suggest that NIK offers a promising therapeutic target for malignant melanoma.

Future Directions

Contribution of NIK to melanoma tumorigenesis

Although we have demonstrated that NIK plays an important role in melanoma growth, many questions remain unanswered. Most importantly, mouse models to verify the importance of NIK in melanoma are necessary before NIK can be pursued as a valid therapeutic target. Using the existing mouse models, NIK knock-out mice (Yin et al., 2001) can be crossed with immunocompetent mice that spontaneously develop melanoma, $HRas^{V12}$ in *Ink4a/Arf*$^{-/-}$ background (Chin et al., 1997). To specifically identify NIK oncogenic functions in melanocytes, mice with *Nik*$^{f/f}$ expressing *Tyr-rtTA* and *TetO-Cre* genes, which will have a deletion of *Nik* only in melanocytes, are needed. Then, these mice can be interbred with mice carrying $HRas^{V12}$ in *Ink4a/Arf* background. These *in vivo* models will confirm the necessity of NIK functions in Ras-mediated melanoma growth and progression. Similar studies might also be informative using the BRAF/PTEN mouse model where mutant BRAF is expressed in an inducible manner in melanocytes where PTEN is inducibly lost (Dankort et al., 2009). Since this mouse model leads to metastatic phenotype, it will also address the question of whether or not NIK over-expression is vital for metastatic potential of melanoma. It is interesting to note that NIK expression is elevated starting from the dysplastic nevus stage and its expression remains elevated in metastatic melanoma. Therefore, it is likely that the functions of NIK are necessary throughout the progression of melanoma.

In vitro studies reported in this dissertation raise many biologically important questions. For example, we have not determined how NIK affects cell cycle progression of melanoma. It is likely that reduction of cyclinD2 and survivin in NIK deficient cells impairs some aspects of cell cycle. CyclinD2 is a cyclin that mediates progression from

G1 to S phase and survivin regulates mitosis as being part of the chromosomal passenger complex. To determine whether cell cycle progression in NIK deficient cells was impaired through the functions of cyclinD2 and survivin, these proteins can be re-expressed in cells with NIK depletion followed by cell cycle analyses.

Another interesting question is whether the non-canonical pathway of NF-κB contributes to the melanoma growth. It has been reported that p100 or p52 is pro-tumorigenic in some tissue types such as breast (Connelly et al., 2007; Demicco et al., 2005). Some of the downstream targets of the non-canonical NF-κB pathway [CCL21/SLC, CCL19/ELC, CXCL13/BLC, BAFF (Bonizzi et al., 2004; Dejardin et al., 2002), RANKL (Senftleben et al., 2001)] may play a more prominent role in *in vivo*, due to their interaction with appropriate receptors on cells in the microenvironment such as immune cells and tumor cells (Zlotnik et al., 2011). However, some genes that are downstream of p100/p52 [PIM2 (Chen et al., 2008; Lesley et al., 2004), CXCR4 (Luftig et al., 2004), CXCL12/SDF1 (Bonizzi et al., 2004; Dejardin et al., 2002), BCL2 (Viatour et al., 2003)] are more likely to have cell autonomous effects. Therefore, targeting p100 in melanoma cells will clarify whether the non-canonical pathway contributes to melanoma tumor growth.

In this study, we have not addressed how NIK activation occurs in melanoma. Literature to date suggests that NIK can be phosphorylated at Thr559 to be activated or NIK stabilization leads to downstream functions such as p100 to p52 processing. Lack of reliable antibody to detect Thr559 on NIK makes it difficult to determine whether NIK phosphorylation is required for NIK pro-tumorigenic activity in melanoma or whether stabilization of NIK is sufficient to execute its subsequent functions. Mass-spectrometry analysis in combination with NIK immunoprecipitation may be employed to determine whether phosphorylation status of NIK is different in melanocytes compared to

melanoma cells. However, antibodies currently available to immunoprecipitate endogenous NIK are not highly reliable. Therefore, production of reliable phospho-NIK antibody will help greatly in answering many questions regarding NIK functions during cancer development.

Mass spectrometry analysis to identify NIK associating proteins also generates interesting questions. While our study utilized HEK293 cells to identify NIK interacting proteins, if it were possible to do these experiments in melanoma tumor cells and normal melanocytes, additional insights might be gained. Due to the technical challenges of immunoprecipitating endogenous NIK, it will be useful to express a tagged NIK protein in melanoma cells to achieve this, possibly under the control of an inducible promoter to overcome any negative effects of long-term over-expression of NIK.

Fundamental questions of NIK regulation

Although NIK has been reported to be stabilized in different tumor types, two fundamental questions regarding NIK regulation remain unanswered. What is/are the lysine residue(s) on NIK that are responsible for NIK ubiquitination and degradation? Which ubiquitin ligases are absolutely necessary for NIK ubiquitination and degradation? As we have discussed extensively in Chapter I, four E3 ubiqiutin ligases cIAP1/2 and TRAF2/3, to date, have been reported to be responsible for NIK degradation. However, we have yet to identify which of these ligases conjugate a ubiquitin chain to NIK in an *in vitro* ubiquitination assay. In addition, mutagenesis study of different lysine residues on NIK in combination with mass spectrometry analysis will identify definite residue(s) leading to ubiquitination and degradation of the protein. In fact, it has not been demonstrated that endogenous NIK is ubiquitinated and we have inferred from ectopically expressed NIK ubiquitination (Liao et al., 2004) that endogenous NIK is poly-

ubiquitinated. Answering the above mentioned questions will shed light on how to screen for mutations in NIK and/or other proteins which lead to NIK accumulation in many types of cancers, and subsequently how to rationally design targeted therapy to inhibit NIK activity.

NIK regulation of β-catenin activity

Since we have demonstrated that NIK influences β-catenin occupancy on the survivin promoter, more detailed mechanisms of how NIK regulates β-catenin will be intriguing to pursue. Our initial experiments show that NIK and β-catenin interact, and that loss of NIK results in diminished nuclear localization of β-catenin. Yet, detailed mechanisms still remain elusive. Does NIK directly interact with β-catenin and modulate its nuclear accumulation? Alternatively, is this modulation through another protein which is in complex with both β-catenin and NIK? Interestingly, NIK-interacting protein DDX5 (reported in Chapter III) can determine the level of nuclear β-catenin (Yang et al., 2006b). Therefore, verifying interaction between NIK and DDX5 and studying how this interaction may influence β-catenin will be worth pursuing.

In addition to nuclear localization, NIK may regulate the stability and post-translation modification of β-catenin. Increased β-catenin ubiquitination observed in NIK deficient cells is consistent with the model that NIK depletion destabilizes β-catenin through ubiquitination. To further support of this model, we have observed stabilization of β-catenin in NIK over-expressing cells. However, we have not observed consistently to date that β-catenin expression is decreased in NIK deficient cells. It is likely that β-catenin expression is dynamic. The difference in expression may not be observed unless β-catenin protein expression levels are determined after treatment with MG132. Our preliminary data suggest that MG132 treatment fails to stabilize β-catenin expression in

NIK knock-down cells. Biochemical assays to monitor β-catenin half-life and to determine β-catenin post-translational modifications are necessary to determine whether or not NIK promotes β-catenin activity by regulating its stability.

NIK as a therapeutic target

NIK is proposed to be a therapeutic target for diseases such as cancer due to its non-essential role in the canonical NF-κB activation crucial for diverse biological processes, while it has an essential role in the modulation of expression of pro-tumorigenic genes. This proposal is further supported by findings in multiple myeloma and lymphoma which suggest that frequent genetic mutations in these tumors (such as TRAF3, cIAPs and MALT1-cIAP2 fusion protein) stabilize the expression of NIK, resulting in downstream oncogenic functions mediated through stabilized NIK. Here, we also demonstrated that NIK mediates melanoma growth and survival.

While further analyses are in place to validate NIK as a target in cancer, effects of NIK systemic inhibition should also be examined when NIK inhibitors are used to treat diseases. Characterization of NIK knock-out mice or *aly/aly* mice, which have defective NIK, suggest that NIK plays a central role in many immunological processes such as antibody production (Garceau et al., 2000), regulation of T_{reg} (Lu et al., 2005) and functions of Th17 (Jin et al., 2009). A recent report on NIK suggests that the activity of NIK is critical in DCs to stimulate effector functions of $CD4^+$ T cells (Hofmann et al., 2011). In parallel, another recent report demonstrates that NIK supports the generation of follicular helper T cells by regulating inducible costimulator ligand (ICOSL) in B cells (Hu et al., 2011). Both of these reports suggest that systemic use of NIK inhibitors may impair T cell functions and subsequently weaken tumor rejection. In light of these recent reports and previous literature, NIK inhibitors should not be used long-term, when

utilized as a cancer therapy, since NIK orchestrates the proper functioning of the immune system. However, NIK knock-out mice are not embryonic lethal, suggesting that the deleterious effects from the use of NIK inhibitors can be alleviated. Thus, studies addressing the side effects of systemic inhibition of NIK will provide directions for the safe and efficacious use of NIK inhibitors.

Although NIK is a non-essential kinase of the canonical NF-κB activation based on the genetic study (Yin et al., 2001), depletion of NIK in some cancers such as multiple myeloma and T cell leukemia decreases canonical NF-κB activity (Annunziata et al., 2007; Keats et al., 2007; Saitoh et al., 2008). Yet, we did not observe a reduction of the canonical NF-κB activation in melanoma cells. As the canonical NF-kB acitivity plays more diverse roles in various cellular processes, it is important to closely examine the effects of NIK on the canonical NF-κB functions when NIK is targeted for cancer therapy. Understanding this aspect of NIK will be useful in assessing the safety of NIK inhibition.

Recently, a new oncogenic mutant of NIK has been reported in lymphoma with MALT1-cIAP2 fusion protein (Rosebeck et al., 2011). This mutant form of NIK lacks TRAF3-binding domain and therefore it cannot be degraded. Yet, it still functions as a kinase, resulting in mis-regulated activity of NIK (Rosebeck et al., 2011). Discovery of this mutant expands our view on oncogenic potential of NIK. Not only stabilization of NIK but also dominant active NIK can be pro-tumorigenic. Similar dominant active forms of NIK may yet to be discovered in other types of tumors.

In this dissertation, we have demonstrated that NIK plays a central role in melanoma growth and survival by modulating expression of genes crucial for different aspects of tumor progression. We also link NIK to another transcriptional regulator, β-catenin and identify novel NIK-interacting proteins. This study has provided insights and

opportunities to advance the field in understanding NIK functions and its oncogenic potential.

APPENDIX

Table A1. Proteins identified in mass spectrometry analysis of HEK293 over-expressing Flag-NIK. Proteins identified in IgG control or Flag immunoprecipitation using HEK293 without Flag-NIK over-expression are substracted. Peptide hits that were used to identify the protein were shown.

First Run		
Protein Name	**Accession Number**	**Peptides**
12 kDa protein	IPI:IPI00373857.1	K.SDGIYIINLK.R
		R.KSDGIYIINLK.R
14-3-3 protein epsilon	IPI:IPI00000816.1	K.LIC*C*DILDVLDK.H
		R.NLLSVAYK.N
25 kDa protein	IPI:IPI00026138.4	K.FKLITEDVQGK.N
		K.LIPDSIGKDIEK.A
		K.TSYAQHQQVR.Q
		R.EVQTNDLK.E
40S ribosomal protein S10	IPI:IPI00008438.1	K.AEAGAGSATEFQFR.G
		K.KAEAGAGSATEFQFR.G
		R.DYLHLPPEIVPATLR.R
		R.IAIYELLFK.E
40S ribosomal protein S11	IPI:IPI00025091.2	K.C*PFTGNVSIR.G
		K.EAIEGTYIDKK.C
		R.AYQKQPTIFQNK.K
		R.DYLHYIR.K
40S ribosomal protein S12	IPI:IPI00013917.1	K.ESQAKDVIEEYFK.C
		K.LGEWVGLC*K.I
		K.TALIHDGLAR.G
40S ribosomal protein S13	IPI:IPI00221089.4	K.GLAPDLPEDLYHLIK.K
		K.GLSQSALPYR.R

		K.GLTPSQIGVILR.D
		K.KGLTPSQIGVILR.D
		K.LTSDDVKEQIYK.L
		R.DSHGVAQVR.F
40S ribosomal protein S14	IPI:IPI00026271.4	K.ADRDESSPYAAMLAAQDVAQR.C
		K.TPGPGAQSALR.A
		R.C*KELGITALHIK.L
		R.TKTPGPGAQSALR.A
40S ribosomal protein S15a	IPI:IPI00221091.8	K.WQNNLLPSR.Q
		R.FDVQLKDLEK.W
		R.M#NVLADALK.S
40S ribosomal protein S16	IPI:IPI00221092.7	K.ALVAYYQK.Y
		K.EIKDILIQYDR.T
		K.GPLQSVQVFGR.K
		K.LLEPVLLLGK.E
40S ribosomal protein S18	IPI:IPI00013296.2	K.IAFAITAIK.G
		K.IPDWFLNR.Q
		K.RAGELTEDEVER.V
		K.YSQVLANGLDNK.L
		R.AGELTEDEVER.V
		R.VITIMQNPR.Q
		R.VLNTNIDGR.R
40S ribosomal protein S19	IPI:IPI00215780.4	K.DVNQQEFVR.A
		K.HKELAPYDENWFYTR.A
		K.LKVPEWVDTVK.L
		R.IAGQVAAANK.K
		R.RVLQALEGLK.M
		R.VLQALEGLK.M
40S ribosomal protein S2	IPI:IPI00013485.3	K.AEDKEWMPVTK.L

		K.ATFDAISK.T
		K.SLEEIYLFSLPIK.E
		K.SPYQEFTDHLVK.T
		K.TYSYLTPDLWK.E
		R.GC*TATLGNFAK.A
		R.GTGIVSAPVPK.K
40S ribosomal protein S20	IPI:IPI00012493.1	K.DTGKTPVEPEVAIHR.I
		K.TPVEPEVAIHR.I
		K.VC*ADLIR.G
		R.LIDLHSPSEIVK.Q
40S ribosomal protein S23	IPI:IPI00218606.6	K.ANPFGGASHAK.G
		K.VANVSLLALYK.G
		R.KGHAVGDIPGVR.F
40S ribosomal protein S25	IPI:IPI00012750.3	K.LITPAVVSER.L
		R.AALQELLSK.G
		R.GSLARAALQELLSK.G
		R.NTKGGDAPAAGEDA.-
40S ribosomal protein S3	IPI:IPI00011253.3	K.AELNEFLTR.E
		K.DEILPTTPISEQK.G
		K.GC*EVVVSGK.L
		K.IMLPWDPTGK.I
		K.LLGGLAVR.R
		R.AC*YGVLR.F
		R.ELAEDGYSGVEVR.V
		R.ELTAVVQKR.F
		R.FGFPEGSVELYAEK.V
		R.GLC*AIAQAESLR.Y
		R.TEIIILATR.T
40S ribosomal protein S3a	IPI:IPI00419880.5	K.AC*QSIYPLHDVFVR.K

		K.APAM#FNIR.N
		K.APAMFNIR.N
		K.ATGDETGAKVER.A
		K.DWYDVK.A
		K.TTDGYLLR.L
		R.LFC*VGFTK.K
		R.VFEVSLADLQNDEVAFR.K
40S ribosomal protein S4, X isoform	IPI:IPI00217030.9	K.DANGNSFATR.L
		K.FDTGNLC*MVTGGANLGR.I
		K.GIPHLVTHDAR.T
		K.LTGVFAPR.P
		R.EC*LPLIIFLR.N
		R.HPGSFDVVHVK.D
		R.LKYALTGDEVKK.I
		R.LSNIFVIGK.G
		R.TIRYPDPLIK.V
40S ribosomal protein S5	IPI:IPI00008433.3	K.AQC*PIVER.L
		K.TIAEC*LADELINAAK.G
		R.VNQAIWLLC*TGAR.E
40S ribosomal protein S6	IPI:IPI00021840.1	K.DIPGLTDTTVPR.R
		K.LFNLSKEDDVR.Q
		K.LIEVDDER.K
		K.NKEEAAEYAK.L
		R.M#ATEVAADALGEEWK.G
40S ribosomal protein S7	IPI:IPI00013415.1	K.DVNFEFPEFQL.-
		K.LTGKDVNFEFPEFQL.-
40S ribosomal protein S8	IPI:IPI00216587.8	K.ISSLLEEQFQQGK.L
		K.LTPEEEEILNK.K

		K.NC*IVLIDSTPYR.Q
		R.ADGYVLEGK.E
		R.IIDVVYNASNNELVR.T
40S ribosomal protein S9	IPI:IPI00221088.4	K.LIGEYGLR.N
		K.QVVNIPSFIVR.L
		R.IGVLDEGK.M
		R.LFEGNALLR.R
		R.RLFEGNALLR.R
		R.RLQTQVFK.L
60S acidic ribosomal protein P0	IPI:IPI00008530.1	K.AFLADPSAFVAAAPV AAATTAAPAAAAAPAK. V
		K.C*FIVGADNVGSK.Q
		K.IIQLLDDYPK.C
		K.TSFFQALGITTK.I
		R.GHLENNPALEK.L
		R.GNVGFVFTK.E
		R.GTIEILSDVQLIK.T
60S ribosomal protein L10a	IPI:IPI00412579.5	K.KYDAFLASESLIK.Q
		K.VSRDTLYEAVR.E
		K.YDAFLASESLIK.Q
		R.DTLYEAVR.E
60S ribosomal protein L12	IPI:IPI00024933.3	K.EILGTAQSVGC*NVDG R.H
		K.IGPLGLSPK.K
		R.C*TGGEVGATSALAP K.I
		R.QAQIEVVPSASALIIK. A
60S ribosomal protein L13	IPI:IPI00465361.3	K.LATQLTGPVM#PVR.N
		R.GFSLEELR.V
60S ribosomal protein L13a	IPI:IPI00304612.8	K.VFDGIPPPYDKK.K
		K.YQAVTATLEEK.R

		R.LAAIVAK.Q
60S ribosomal protein L18	IPI:IPI00215719.5	K.GC*GTVLLSGPR.K
		K.ILTFDQLALDSPK.G
		K.TAVVVGTITDDVR.V
		K.TAVVVGTITDDVRVQEVPK.L
		R.TNRPPLSLSR.M
		R.TNSTFNQVVLK.R
		R.TNSTFNQVVLKR.L
		R.VQEVPK.L
60S ribosomal protein L18a	IPI:IPI00026202.1	R.DLTTAGAVTQC*YR.D
		R.IFAPNHVVAK.S
60S ribosomal protein L19	IPI:IPI00025329.1	K.LLADQAEAR.R
		K.VWLDPNETNEIANANSR.Q
60S ribosomal protein L21	IPI:IPI00247583.4	R.TNGKEPELLEPIPYEFM#A.-
		R.TNGKEPELLEPIPYEFMA.-
		R.VYNVTQHAVGIVVNK.Q
60S ribosomal protein L23	IPI:IPI00010153.5	K.GSAITGPVAK.E
		K.NLYIISVK.G
		R.ISLGLPVGAVINC*ADNTGAK.N
		R.LNRLPAAGVGDM#VM#ATVK.K
		R.LPAAGVGDMVMATVK.K
60S ribosomal protein L23a	IPI:IPI00021266.1	K.EAPAPPKAEAK.A
		K.KLYDIDVAK.V
		K.VNTLIRPDGEKK.A
		R.LAPDYDALDVANK.I
		R.LAPDYDALDVANKIGII.-
60S ribosomal protein L24	IPI:IPI00306332.4	K.VFQFLNAK.C
		R.AITGASLADIM#AK.R

		R.AITGASLADIMAK.R
		R.TDGKVFQFLNAK.C
60S ribosomal protein L27	IPI:IPI00219155.4	K.VVLVLAGR.Y
		R.YSVDIPLDK.T
60S ribosomal protein L3	IPI:IPI00550021.3	K.IGQGYLIK.D
		K.NNASTDYDLSDK.S
		R.ERLEQQVPVNQVFGQDEM#IDVIGVTK.G
		R.HGSLGFLPR.K
		R.LEQQVPVNQVFGQDEM#IDVIGVTK.G
		R.LEQQVPVNQVFGQDEMIDVIGVTK.G
60S ribosomal protein L30	IPI:IPI00219156.6	K.LVILANNC*PALR.K
		K.SLESINSR.L
		R.KSEIEYYAM#LAK.T
		R.KSEIEYYAMLAK.T
		R.VC*TLAIIDPGDSDIIR.S
60S ribosomal protein L36	IPI:IPI00216237.4	R.EELSNVLAAMR.K
		R.EVC*GFAPYER.R
60S ribosomal protein L37a	IPI:IPI00414860.5	K.KIEISQHAK.Y
		K.TVAGGAWTYNTTSAVTVK.S
60S ribosomal protein L4	IPI:IPI00003918.5	K.AAAAAAALQAK.S
		K.M#INTDLSR.I
		R.IEEVPELPLVVEDK.V
		R.KLDELYGTWR.K
		R.NIPGITLLNVSK.L
		R.PLISVYSEK.G
		R.PLISVYSEKGESSGK.N
		R.QPYAVSELAGHQTSAESWGTGR.A
		R.YAIC*SALAASALPALVM#SK.G

60S ribosomal protein L5	IPI:IPI00000494.5	R.DIIC*QIAYAR.I
		R.VTNRDIIC*QIAYAR.I
		R.YLM#EEDEDAYKK.Q
60S ribosomal protein L6	IPI:IPI00329389.7	K.AVDSQILPK.I
		K.FVIATSTK.I
		K.HLTDAYFK.K
		R.HQEGEIFDTEK.E
		R.HQEGEIFDTEKEKYEITEQR.K
		R.YYPTEDVPR.K
60S ribosomal protein L7	IPI:IPI00030179.3	K.AGNFYVPAEPK.L
		K.ASINMLR.I
		K.EANNFLWPFK.L
		K.SVNELIYK.R
		K.SVNELIYKR.G
		R.IALTDNALIAR.S
		R.IVEPYIAWGYPNLK.S
		R.KAGNFYVPAEPK.L
60S ribosomal protein L7a	IPI:IPI00299573.11	K.VPPAINQFTQALDR.Q
		R.AGVNTVTTLVENK.K
		R.AGVNTVTTLVENKK.A
		R.LKVPPAINQFTQALDR.Q
60S ribosomal protein L8	IPI:IPI00012772.7	R.AVVGVVAGGGR.I
		R.KGAGSVFR.A
60S ribosomal protein L9	IPI:IPI00031691.1	K.FLDGIYVSEK.G
		K.TILSNQTVDIPENVDITLK.G
		R.TIC*SHVQNM#IK.G
9 kDa protein	IPI:IPI00176662.1	R.LVQSPNSYFM#DVK.C
		R.LVQSPNSYFMDVK.C
ADP/ATP translocase 2	IPI:IPI00007188.4	K.DFLAGGVAAAISK.T

		K.EQGVLSFWR.G
		K.LLLQVQHASK.Q
		K.QIFLGGVDKR.T
		R.AAYFGIYDTAK.G
		R.GLGDC*LVK.I
		R.LAADVGK.A
		R.YFPTQALNFAFK.D
ALB protein	IPI:IPI00022434.2	K.KVPQVSTPTLVEVSR.N
		K.YLYEIAR.R
ATP-dependent RNA helicase A	IPI:IPI00215638.6	K.LAAQSC*ALSLVR.Q
		R.DFVNYLVR.I
		R.ELDALDANDELTPLGR.I
		R.LGGIGQFLAK.A
ATP-dependent RNA helicase DDX3X	IPI:IPI00215637.4	K.QYPISLVLAPTR.E
		K.SPILVATAVAAR.G
		R.VGSTSENITQK.V
CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nu	IPI:IPI00444262.1	K.ALELTGLK.V
		K.EVFEDAAEIR.L
		K.FGYVDFESAEDLEK.A
		K.GIAYIEFK.T
		K.NDLAVVDVR.I
		K.TGISDVFAK.N
		K.VEGTEPTTAFNLFVGNLNFNK.S
		K.VTQDELK.E
		K.VTQDELKEVFEDAAEIR.L
		R.KFGYVDFESAEDLEK.A
Chaperonin containing TCP1, subunit 8 (Theta) variant	IPI:IPI00302925.3	K.FAEAFEAIPR.A
		K.LFVTNDAATILR.E

		R.DIDEVSSLLR.T
DNA replication licensing factor MCM3	IPI:IPI00013214.1	K.VALLDVFR.E
		R.SKDIFDQLAK.S
DnaJ homolog subfamily A member 1	IPI:IPI00012535.1	K.QISQAYEVLSDAK.K
		R.TIVITSHPGQIVK.H
		R.YEEIVKEVSTYIK.K
Elongation factor 1-gamma	IPI:IPI00000875.5	K.ALIAAQYSGAQVR.V
		R.ILGLLDAYLK.T
Endoplasmin precursor	IPI:IPI00027230.3	R.ELISNASDALDK.I
		R.ELISNASDALDKIR.L
F-actin capping protein alpha-1 subunit	IPI:IPI00005969.2	K.FITHAPPGEFNEVFNDVR.L
		R.LLLNNDNLLR.E
Far upstream element-binding protein 2	IPI:IPI00298363.2	K.DAFADAVQR.A
		R.IGGGIDVPVPR.H
GPI-anchored membrane protein 1	IPI:IPI00030910.1	K.YQEVTNNLEFAK.E
		R.SFM#ALSQDIQK.T
HECT domain and RCC1-like domain-containing protein 5	IPI:IPI00008821.1	R.DQLM#PLPVK.V
		R.TTEM#M#PVYLDLNK.A
Heat shock 70 kDa protein 7 (Fragment)	IPI:IPI00011134.1	K.ATAGDTHLGGEDFDNR.L
		R.VEILANDQGNR.T
Heat shock protein 86 (Fragment)	IPI:IPI00031523.3	K.ADLINNLGTIAK.S
		K.YIDQEELNK.T
		R.ELISNSSDALDK.I
Heterogeneous nuclear ribonucleoprotein R	IPI:IPI00012074.3	K.AGPIWDLR.L
		R.DLYEDELVPLFEK.A
Histone H2B type 2-E	IPI:IPI00003935.5	K.AM#GIM#NSFVNDIFER.I
		R.LLLPGELAK.H
Hsp90 co-chaperone Cdc37	IPI:IPI00013122.1	K.ASEAKEGEEAGPGDPLLEAVPK.T

		K.DVQM#LQDAISK.M
		K.EGEEAGPGDPLLEAVPK.T
		K.TEEDSEEVREQK.H
		R.LGPGGLDPVEVYESLPEELQK.C
		R.SWEQKLEEM#R.K
Hypothetical protein	IPI:IPI00003362.2	K.DAGTIAGLNVM#R.I
		K.DAGTIAGLNVMR.I
		K.ELEEIVQPIISK.L
		K.FEELNM#DLFR.S
		K.NQLTSNPENTVFDAK.R
		K.SQIFSTASDNQPTVTIK.V
		K.VTHAVVTVPAYFNDAQR.Q
		R.IINEPTAAAIAYGLDK.R
		R.LIGDAAK.N
		R.NELESYAYSLK.N
		R.TWNDPSVQQDIK.F
Importin beta-1 subunit	IPI:IPI00001639.2	R.VAALQNLVK.I
		R.VLANPGNSQVAR.V
Inhibitor of nuclear factor kappa B kinase subunit beta	IPI:IPI00024709.1	K.ALDDILNLK.L
		R.DLKPENIVLQQGEQR.L
		R.LGTGGFGNVIR.W
		R.M#M#ALQTDIVDLQR.S
Inhibitor of nuclear factor kappa-B kinase alpha subunit	IPI:IPI00005104.1	K.IIDLGYAK.D
		K.IIDLLPK.V
		K.IIVHTVQSQDR.V
		K.NTLISASQQLK.A
		K.TVYEGPFASR.S
		K.VEVALSNIK.E

		R.AAMLSLLR.Y
		R.DLKPENIVLQDVGGK.I
		R.GGPVDLTLKQPR.C
		R.YNANLTK.M
Interferon-induced 17 kDa protein precursor	IPI:IPI00375631.5	K.IGVHAFQQR.L
		R.LAVHPSGVALQDR.V
		R.LTQTVAHLK.Q
Interleukin enhancer-binding factor 2	IPI:IPI00005198.2	K.VLQSALAAIR.H
		R.NQDLAPNSAEQASILSLVTK.I
Isoform 1 of 40S ribosomal protein S24	IPI:IPI00029750.1	K.TTGFGM#IYDSLDYAK.K
		K.TTGFGMIYDSLDYAK.K
Isoform 1 of Heterogeneous nuclear ribonucleoprotein Q	IPI:IPI00018140.3	R.DLFEDELVPLFEK.A
		R.NLANTVTEEILEK.A
Isoform 1 of Plasminogen activator inhibitor 1 RNA-binding	IPI:IPI00410693.3	K.SAAQAAAQTNSNAAGK.Q
		R.FDQLFDDESDPFEVLK.A
		R.TDKSSASAPDVDDPEAFPALA.-
Isoform 1 of Polyadenylate-binding protein 1	IPI:IPI00008524.1	K.EFSPFGTITSAK.V
		K.FSPAGPILSIR.V
		K.GFGFVC*FSSPEEATK.A
		K.GFGFVSFER.H
		K.GYGFVHFETQEAAER.A
		K.SGVGNIFIK.N
		R.AKEFTNVYIK.N
		R.ALDTM#NFDVIK.G
		R.ALDTMNFDVIK.G
		R.SKVDEAVAVLQAHQAK.E
		R.SLGYAYVNFQQPADAER.A
		R.YQGVNLYVK.N

Isoform 2 of Protein disulfide-isomerase A6 precursor	IPI:IPI00299571.5	K.GSFSEQGINEFLR.E
		K.LAAVDATVNQVLASR.Y
		R.TGEAIVDAALSALR.Q
Isoform 5 of Interleukin enhancer-binding factor 3	IPI:IPI00219330.2	K.AYAALAALEK.L
		K.VLQDM#GLPTGAEGR.D
		R.LNQLKPGLQYK.L
Isoform Long of Double-stranded RNA-binding protein Staufen	IPI:IPI00000001.2	K.NAAIAVLEELKK.L
		R.ELLYGGTSPTAETILK.N
Isoform Long of Trifunctional purine biosynthetic protein a	IPI:IPI00025273.1	K.AAVAGLDKAER.A
		R.AIAFLQQPR.S
Keratin 24	IPI:IPI00004550.5	R.LAADDFR.L
		R.QSVEADINGLR.K
Keratin, type II cytoskeletal 2 oral	IPI:IPI00008359.1	K.KYEDEINKR.T
		K.QLDSLLGERGNLEGELK.S
Lung cancer oncogene 7	IPI:IPI00641950.3	K.DVLSVAFSSDNR.Q
		K.IIVDELKQEVISTSSK.A
		K.LTRDETNYGIPQR.A
		R.DETNYGIPQR.A
		R.LWDLTTGTTTR.R
		R.VWQVTIGTR.-
		R.YWLC*AATGPSIK.I
Mitochondrial aspartate-glutamate carrier protein	IPI:IPI00007084.2	K.ASGDSARPVLLQVAESAYR.F
		R.AGQTTYSGVIDC*FR.K
		R.IAPLEEGTLPFNLAEAQR.Q
		R.LQVAGEITTGPR.V
Mitogen-activated protein kinase kinase kinase 14	IPI:IPI00016099.2	K.EESGM#WEPLPLSSLEPAPAR.N
		K.EESGMWEPLPLSSLEPAPAR.N
		K.EGSEAGPAAISIIAQAEC*ENSQEFSPTFSER.I

		K.EQGC*LPEDR.A
		K.HGQLENRP.-
		K.IASEPPPVR.E
		K.KQSSVYKLEAVEK.S
		K.LAC*VDSQKPLPDPHLSK.L
		K.LEAVEK.S
		K.LEAVEKSPVFC*GK.W
		K.QYSQSESLDQIPNNVAHATEGK.M
		K.SLAHAGVALAK.P
		K.SLAHAGVALAKPLPR.T
		K.SLLTGDYIPGTETHMAPEVVLGR.S
		K.SPPLTLSK.E
		K.SPVFC*GK.W
		K.SPWRGEYKEPR.H
		K.TEDNEGVLLTEK.L
		K.VGDIATGISSQIPAAAFSLVTK.D
		K.WEILNDVITK.G
		R.AEELM#AC*AGLTSPR.I
		R.ALQQVGGLK.S
		R.ALQQVGGLKSPWR.G
		R.APGPRPAEETTGR.A
		R.APGPRPAEETTGRAPK.L
		R.DTLSSGVHSWSSQAEAR.S
		R.EEVHWATHQLR.L
		R.EIPPSC*APLTAQAIQEGLR.K
		R.GEYKEPR.H
		R.GRPTDTPSYFNGVK.V

		R.GSFGEVHR.M
		R.IFIAGSK.Q
		R.ILHGDVK.A
		R.IVPLYGAVR.E
		R.M#EDKQTGFQC*AVK.K
		R.PAEETTGRAPK.L
		R.SREPSPKTEDNEGVLLTEK.L
		R.SSSWNM#VLAR.G
		R.SSSWNMVLAR.G
		R.TPEQESC*TIPVQEDESPLGAPYVR.N
		R.VKHGQLENRP.-
		R.VSAAELGGK.V
		R.VSAAELGGKVNR.A
NCL protein	IPI:IPI00183526.5	K.ATFIKVPQNQNGK.S
		K.GFGFVDFNSEEDAK.A
		K.GLSEDTTEETLK.E
		K.GLSEDTTEETLKESFDGSVR.A
		K.NSTWSGESK.T
		K.TEADAEKTFEEK.Q
		K.TFEEKQGTEIDGR.S
		R.IVTDRETGSSK.G
		R.LELQGPR.G
		R.SISLYYTGEK.G
NF-kappa-B essential modulator	IPI:IPI00002411.3	K.AQVTSLLGELQESQSR.L
		K.ELLQEQLEQLQR.E
		R.HVEVSQAPLPPAPAYLSSPLALPSQR.R
Nucleosome assembly protein 1-like 4	IPI:IPI00017763.4	K.FYEEVHDLER.K
		R.LDNVPHTPSSYIETLPK.A

Phenylalanyl-tRNA synthetase alpha chain	IPI:IPI00031820.2	K.LGITQLR.F
		K.SLQALGEVIEAELR.S
		R.LDAEPRPPPTQEAA.-
Probable ATP-dependent RNA helicase DDX5	IPI:IPI00017617.1	K.APILIATDVASR.G
		K.QVSDLISVLR.E
		R.GDGPIC*LVLAPTR.E
Ribosomal protein homolog PD-1	IPI:IPI00394699.1	K.NAESNAELK.G
		K.YLKDVTLQK.Q
Stress-70 protein, mitochondrial precursor	IPI:IPI00007765.5	K.DAGQISGLNVLR.V
		K.VLENAEGAR.T
		K.VQQTVQDLFGR.A
		K.VQQTVQDLFGRAPSK.A
		R.TTPSVVAFTADGER.L
T-complex protein 1 subunit beta	IPI:IPI00297779.6	R.GATQQILDEAER.S
		R.VQDDEVGDGTTSVTVLAAELLR.E
TSR1, 20S rRNA accumulation, homolog	IPI:IPI00292894.4	K.QIDAPGDPFPLNPR.G
		R.LEEM#FPDEVDTPR.D
chaperonin containing TCP1, subunit 3 isoform b	IPI:IPI00290770.3	R.IVLLDSSLEYK.K
		R.NLQDAM#QVC*R.N
		R.TLIQNC*GASTIR.L
heterogeneous nuclear ribonucleoprotein H1	IPI:IPI00013881.6	R.EGRPSGEAFVELESEDEVK.L
		R.STGEAFVQFASQEIAEK.A
heterogeneous nuclear ribonucleoprotein M isoform a	IPI:IPI00171903.2	K.QGGGGGGGSVPGIER.M
		K.VGEVTYVELLMDAEGK.S
		R.AFITNIPFDVK.W
		R.INEILSNALK.R
		R.M#GAGM#GFGLER.M
		R.M#GANSLER.M

heterogeneous nuclear ribonucleoprotein U isoform b	IPI:IPI00479217.1	K.NGQDLGVAFK.I
		K.SSGPTSLFAVTVAPPGAR.Q
		K.VSELKEELK.K
		K.YNILGTNTIM#DK.M
		R.NFILDQTNVSAAAQR.R
insulin receptor substrate 4	IPI:IPI00020729.1	R.AAVSAFPTDSLER.D
		R.GLDKEVSYNWDPK.D
insulin-like growth factor 2 mRNA binding protein 1	IPI:IPI00008557.4	K.ILAHNNFVGR.L
		K.LYIGNLNESVTPADLEK.V
		K.QQQVDIPLR.L
		K.TVNELQNLTAAEVVVPR.D
		R.DQTPDENDQVIVK.I
		R.LLVPTQYVGAIIGK.E
		R.M#VIITGPPEAQFK.A
poly(rC)-binding protein 2 isoform b	IPI:IPI00012066.2	R.AITIAGIPQSIIEC*VK.Q
		R.IITLAGPTNAIFK.A
		R.INISEGNC*PER.I
pyruvate kinase 3 isoform 2	IPI:IPI00220644.8	K.GADFLVTEVENGGSLGSK.K
		K.IYVDDGLISLQVK.Q
		R.APIIAVTR.N
		R.TATESFASDPILYRPVAVALDTK.G
ribosomal protein L11	IPI:IPI00376798.3	K.AEEILEK.G
		K.VLEQLTGQTPVFSK.A
		K.YDGIILPGK.-
similar to 40S ribosomal protein S7	IPI:IPI00008293.4	K.EIEVGGGR.K
		R.ELNITAAK.E
similar to 40S ribosomal protein SA	IPI:IPI00398958.3	R.ADHQPLTEASYVNLPTIALC*NTDSPLR.Y
		R.FTPGTFTNQIQAAFR.E

		R.LLVVTDPR.A
similar to 60S ribosomal protein L7a	IPI:IPI00075558.8	K.KVVNPLFEK.R
		K.NFGIGQDIQPK.R
similar to ribosomal protein L13 isoform 1	IPI:IPI00397611.2	K.EAAEQDVEK.K
		K.KGDSSAEELK.L
		K.STESLQANVQR.L
		R.AKEAAEQDVEK.K
		R.TIGISVDPR.R
		R.VATWFNQPAR.K
		R.VITEEEKNFK.A
similar to ribosomal protein S15a	IPI:IPI00156232.1	K.ILGFFF.-
		K.IVVNLTGR.L
ubiquitin and ribosomal protein S27a precursor	IPI:IPI00179330.6	K.IQDKEGIPPDQQR.L
		K.TITLEVEPSDTIENVK.A
		R.TLSDYNIQK.E
ubiquitin specific protease 9, X-linked isoform 4	IPI:IPI00003964.3	R.LAQQISDEASR.Y
		R.VVIQSNDDIASR.A

Second Run		
Protein Name	**Accession Number**	**Peptides**
40S ribosomal protein S19	IPI:IPI00215780.4	R.ALAAFLKK.S
		R.IAGQVAAANK.K
		R.RVLQALEGLK.M
		R.VLQALEGLK.M
40S ribosomal protein S3	IPI:IPI00011253.3	K.GGKPEPPAM#PQPVPTA.-
		K.GGKPEPPAMPQPVPTA.-
		R.ELAEDGYSGVEVR.V
60S ribosomal protein L23a	IPI:IPI00021266.1	R.LAPDYDALDVANK.I

		R.LAPDYDALDVANKIGII.-
60S ribosomal protein L24	IPI:IPI00306332.4	R.AITGASLADIM#AK.R
		R.AITGASLADIMAKR.N
60S ribosomal protein L4	IPI:IPI00003918.5	K.AAAAAAALQAK.S
		K.M#INTDLSR.I
		R.VDKAAAAAAALQAK.S
60S ribosomal protein L8	IPI:IPI00012772.7	R.KVGLIAAR.R
		R.LRAVDFAER.H
ADP/ATP translocase 2	IPI:IPI00007188.4	K.DFLAGGVAAAISK.T
		R.LAADVGK.A
Actin, aortic smooth muscle	IPI:IPI00008603.1	K.AGFAGDDAPR.A
		R.AVFPSIVGRPR.H
CDNA FLJ45706 fis, clone EBRA2028457, highly similar to Nu	IPI:IPI00444262.1	K.NDLAVVDVR.I
		K.NLPYKVTQDELKEVFEDAAEIR.L
		K.TGISDVFAKNDLAVVDVR.I
EEF1A1 protein	IPI:IPI00025447.6	R.VETGVLKPGM#VVTFAPVNVTTEVK.S
		R.VETGVLKPGMVVTFAPVNVTTEVK.S
Elongation factor 1-alpha 2	IPI:IPI00014424.1	K.IGGIGTVPVGR.V
		K.STTTGHLIYK.C
Heat shock 70 kDa protein 1	IPI:IPI00304925.4	K.FGDPVVQSDMK.H
		K.LDKAQIHDLVLVGGSTR.I
		K.LLQDFFNGR.D
		K.NQVALNPQNTVFDAK.R
		K.NQVALNPQNTVFDAKR.L
		R.ARFEELC*SDLFR.S
		R.FEELC*SDLFR.S
		R.IINEPTAAAIAYGLDR.T
		R.LIGDAAKNQVALNPQNTVFDAK.R

		R.LVNHFVEEFK.R
		R.LVNHFVEEFKR.K
		R.MVQEAEKYKAEDEVQR.E
Heat shock 70 kDa protein 1L	IPI:IPI00301277.1	K.AFYPEEISSM#VLTK.L
		K.AFYPEEISSMVLTK.L
		K.DAGVIAGLNVLR.I
		K.NALESYAFNM#K.S
		K.NALESYAFNMK.S
		R.AMTKDNNLLGR.F
		R.LSKEEIER.M
		R.NSTIPTKQTQIFTTYSDNQPGVLIQVYEGER.A
		R.QATKDAGVIAGLNVLR.I
Heat shock 70 kDa protein 7 (Fragment)	IPI:IPI00011134.1	K.ATAGDTHLGGEDFDNR.L
		R.VEILANDQGNR.T
Heat shock protein 86 (Fragment)	IPI:IPI00031523.3	K.ADLINNLGTIAK.S
		K.YIDQEELNK.T
Histone H1.2	IPI:IPI00217465.4	K.ALAAAGYDVEKNNSR.I
		K.ASGPPVSELITK.A
		K.SLVSKGTLVQTK.G
		R.KASGPPVSELITK.A
		R.SGVSLAALKK.A
Isoform 1 of Heat shock cognate 71 kDa otein	IPI:IPI00003865.1	K.DAGTIAGLNVLR.I
		K.ITITNDKGR.L
		K.MKEIAEAYLGK.T
		K.NQVAMNPTNTVFDAK.R
		K.SFYPEEVSSMVLTK.M
		K.STAGDTHLGGEDFDNR.M
		K.TVTNAVVTVPAYFNDSQR.Q

		K.VQVEYKGETK.S
		R.ARFEELNADLFR.G
		R.IINEPTAAAIAYGLDKK.V
		R.LSKEDIER.M
		R.QATKDAGTIAGLNVLR.I
		R.TTPSYVAFTDTER.L
		R.TTPSYVAFTDTERLIGDAAK.N
Keratin, type I cytoskeletal 10	IPI:IPI00009865.1	K.VTM#QNLNDR.L
		R.SQYEQLAEQNRK.D
Mitogen-activated protein kinase kinase nase 14	IPI:IPI00016099.2	K.ADNVLLSSDGSHAALCDFGHAVCLQPDGLGK.S
		K.GTAKEGSEAGPAAISIIAQAEC*ENSQEFSPTFSER.I
		K.HGQLENRP.-
		K.IASEPPPVR.E
		K.LAC*VDSPKPLPGPHLEPSC*LSR.G
		K.LAC*VDSQKPLPDPHLSK.L
		K.QYSQSESLDQIPNNVAHATEGK.M
		K.SLAHAGVALAKPLPR.T
		K.SLLTGDYIPGTETHMAPEVVLGR.S
		K.TEDNEGVLLTEK.L
		K.VGDIATGISSQIPAAAFSLVTK.D
		K.VQIQSLNGEHLHIR.E
		K.WEILNDVITK.G
		R.ALQQVGGLK.S
		R.ALQQVGGLKSPWR.G
		R.ALYYLGQALEGLEYLHSR.R
		R.APGPRPAEETTGR.A
		R.APGPRPAEETTGRAPK.L

		R.DTLSSGVHSWSSQAEAR.S
		R.GRPTDTPSYFNGVK.V
		R.IFIAGSK.Q
		R.NTPQFTKPLKEPGLGQLC*FK.Q
		R.SREPSPKTEDNEGVLLTEK.L
		R.SSSWNM#VLAR.G
		R.SSSWNMVLAR.G
		R.VKHGQLENRP.-
		R.VKVGDIATGISSQIPAAAFSLVTK.D
		R.VSAAELGGK.V
		R.VSAAELGGKVNR.A
NCL protein	IPI:IPI00183526.5	K.ALVATPGKK.G
		K.GFGFVDFNSEEDAK.A
		K.TLVLSNLSYSATEETLQEVFEK.A
		R.AIRLELQGPR.G
		R.IVTDRETGSSK.G
		R.LELQGPR.G
		R.SISLYYTGEK.G
Probable ATP-dependent RNA helicase DX5	IPI:IPI00017617.1	K.LLQLVEDR.G
		R.MLDMGFEPQIR.K
TUBA6 protein	IPI:IPI00166768.2	K.DVNAAIATIK.T
		R.AVFVDLEPTVIDEVR.T
Tubulin beta chain	IPI:IPI00011654.2	R.AILVDLEPGTM#DSVR.S
		R.AILVDLEPGTMDSVR.S
		R.ALTVPELTQQVFDAK.N
similar to 40S ribosomal protein SA	IPI:IPI00398958.3	K.FAAATGATPIAGR.F
		R.LLVVTDPR.A

Third Run		
Protein Name	**Accession Number**	**Peptides**
40S ribosomal protein S2	IPI:IPI00013485.3	K.LLM#M#AGIDDC*YTSAR.G
		K.SPYQEFTDHLVK.T
40S ribosomal protein S3	IPI:IPI00011253.3	K.GGKPEPPAM#PQPVPTA.-
		K.GGKPEPPAMPQPVPTA.-
		K.KPLPDHVSIVEPKDEILPTTPISEQK.G
		R.FGFPEGSVELYAEK.V
40S ribosomal protein S3a	IPI:IPI00419880.5	K.VERADGYEPPVQESV.-
		R.VFEVSLADLQNDEVAFR.K
40S ribosomal protein S5	IPI:IPI00008433.3	K.TIAEC*LADELINAAK.G
		K.WSTDDVQINDISLQDYIAVK.E
40S ribosomal protein S7	IPI:IPI00013415.1	K.IVKPNGEKPDEFESGISQALLELEM#NSDLK.A
		K.LTGKDVNFEFPEFQL.-
40S ribosomal protein S8	IPI:IPI00216587.8	K.ISSLLEEQFQQGK.L
		K.LTPEEEEILNKKR.S
		R.ADGYVLEGKELEFYLR.K
60S acidic ribosomal protein P0	IPI:IPI00008530.1	K.AFLADPSAFVAAAPVAAATTAAPAAAAAPAK.V
		K.AFLADPSAFVAAAPVAAATTAAPAAAAAPAKVEAK.E
		R.GHLENNPALEK.L
		R.GTIEILSDVQLIK.T
60S ribosomal protein L3	IPI:IPI00550021.3	R.ERLEQQVPVNQVFGQDEM#IDVIGVTK.G
		R.ERLEQQVPVNQVFGQDEMIDVIGVTK.G
60S ribosomal protein L5	IPI:IPI00000494.5	K.GAVDGGLSIPHSTK.R
		R.YLM#EEDEDAYKK.Q
60S ribosomal protein L7	IPI:IPI00030179.3	K.TTHFVEGGDAGNREDQINR.L

		R.IALTDNALIAR.S
60S ribosomal protein L8	IPI:IPI00012772.7	R.ASGNYATVISHNPETK.K
		R.AVVGVVAGGGR.I
85 kDa protein	IPI:IPI00334775.6	K.AQALRDNSTMGYMMAK.K
		K.EQVANSAFVER.V
		K.HLEINPDHPIVETLR.Q
		K.IDIIPNPQER.T
		K.KHLEINPDHPIVETLR.Q
		K.SIYYITGESKEQVANSAFVER.V
		K.SLTNDWEDHLAVK.H
		K.SLVSVTK.E
		K.SLVSVTKEGLELPEDEEEKKKMEESK.A
		R.GVVDSEDLPLNISR.E
		R.NPDDITQEEYGEFYK.S
		R.RAPFDLFENK.K
		R.RLSELLR.Y
		R.TLTLVDTGIGM#TK.A
		R.YHTSQSGDEMTSLSEYVSR.M
ATP-dependent RNA helicase A	IPI:IPI00215638.6	K.LAQFEPSQR.Q
		R.ELDALDANDELTPLGR.I
		R.LGGIGQFLAK.A
		R.RISAVSVAER.V
Heat shock protein 86 (Fragment)	IPI:IPI00031523.3	K.ADLINNLGTIAK.S
		K.YIDQEELNK.T
		K.YIDQEELNKTKPIWTR.N
Heat shock protein HSP 90-alpha 2	IPI:IPI00382470.3	K.DQVANSAFVER.L
		K.ELHINLIPNKQDR.T
		K.HIYYITGETK.D

		K.TLVSVTK.E
		R.NPDDITNEEYGEFYK.S
		R.RAPFDLFENR.K
		R.YYTSASGDEMVSLKDYC*TR.M
Histone H1x	IPI:IPI00021924.1	K.ALVQNDTLLQVK.G
		R.GAPAAATAPAPTAHK.A
Hsp90 co-chaperone Cdc37	IPI:IPI00013122.1	K.ASEAKEGEEAGPGDPLLEAVPK.T
		K.ASEAKEGEEAGPGDPLLEAVPKTGDEKDVSV.-
		K.DVQMLQDAISK.M
		K.EGEEAGPGDPLLEAVPK.T
		K.SM#PWNVDTLSK.D
		K.TADRQYMEGFNDELEAFKER.V
		R.C*IDSGLWVPNSK.A
		R.LGPGGLDPVEVYESLPEELQK.C
		R.LQAEAQQLR.K
Inhibitor of nuclear factor kappa-B kinase alpha subunit	IPI:IPI00005104.1	K.AC*DVPEELNILIHDVPLLAM#EYC*SGGDLR.K
		R.DLKPENIVLQDVGGK.I
		R.ETGINTGSQELLSETGISLDPR.K
		R.GGPVDLTLKQPR.C
		R.IERETGINTGSQELLSETGISLDPR.K
		R.RQGDLMESLEQR.A
Interleukin enhancer-binding factor 2	IPI:IPI00005198.2	R.VKPAPDETSFSEALLK.R
		R.VKPAPDETSFSEALLKR.N
Isoform 1 of DNA-binding protein A	IPI:IPI00031801.4	R.SVGDGETVEFDVVEGEK.G
		R.SVGDGETVEFDVVEGEKGAEAANVTGPDGVPVEGSR.Y

Isoform 1 of Keratin, type I cytoskeletal 13	IPI:IPI00009866.6	R.ALEEANADLEVK.I
		R.LEQEIATYR.S
Isoform 1 of Polyadenylate-binding protein 1	IPI:IPI00008524.1	K.GFGFVC*FSSPEEATK.A
		R.ALDTM#NFDVIK.G
		R.KEFSPFGTITSAK.V
		R.RSLGYAYVNFQQPADAER.A
		R.SLGYAYVNFQQPADAER.A
Isoform 1 of Polyadenylate-binding protein 4	IPI:IPI00012726.4	K.EFSPFGSITSAK.V
		R.KAHLTNQYMQR.V
Isoform 1 of Probable ATP-dependent RNA helicase DDX17	IPI:IPI00023785.5	R.GGGGLPPK.K
		R.SSQSSSQQFSGIGR.S
Isoform 1 of STIP1 homology and U box-containing protein 1	IPI:IPI00025156.4	R.LNFGDDIPSALR.I
		R.VGHFDPVTR.S
Isoform 2 of Nucleophosmin	IPI:IPI00220740.1	K.GPSSVEDIKAK.M
		K.TPKGPSSVEDIKAK.M
		K.VDNDENEHQLSLR.T
		R.M#TDQEAIQDLWQWR.K
		R.MTDQEAIQDLWQWR.K
Isoform 5 of Interleukin enhancer-binding factor 3	IPI:IPI00219330.2	K.AYAALAALEK.L
		K.VLAGETLSVNDPPDVLDR.Q
Keratin, type I cytoskeletal 14	IPI:IPI00384444.4	K.DAEEWFFTKTEELNR.E
		R.APSTYGGGLSVSSSR.F
		R.GQVGGDVNVEM#DAAPGVDLSR.I

		R.ILNEMRDQYEK.M
		R.LLEGEDAHLSSSQFSSGSQSSR.D
Keratin, type I cytoskeletal 16	IPI:IPI00217963.2	K.TEELNKEVASNSELVQSSR.S
		R.APSTYGGGLSVSSR.F
		R.GQTGGDVNVEM#DAAPGVDLSR.I
		R.LLEGEDAHLSSQQASGQSYSSR.E
		R.TDLEM#QIEGLKEELAYLR.K
Keratin, type II cytoskeletal 6B	IPI:IPI00293665.6	R.ALYDAELSQM#QTHISDTSVVLSM#DNNR.N
		R.ATGGGLSSVGGGSSTIK.Y
		R.SGFSSISVSR.S
		R.SLYGLGGSKR.I
Mitogen-activated protein kinase kinase kinase 14	IPI:IPI00016099.2	K.ADNVLLSSDGSHAALC*DFGHAVC*LQPDGLGK.S
		K.ADNVLLSSDGSHAALCDFGHAVCLQPDGLGK.S
		K.ASQSSRDTLSSGVHSWSSQAEAR.S
		K.DGQPVRYDM#EVPDSGIDLQC*TLAPDGSFAWSWR.V
		K.EESGM#WEPLPLSSLEPAPAR.N
		K.EESGMWEPLPLSSLEPAPAR.N
		K.EESGMWEPLPLSSLEPAPARNPSSPER.K
		K.EGSEAGPAAISIIAQAEC*ENSQEFSPTFSER.I
		K.GTAKEGSEAGPAAISIIAQAEC*ENSQEFSPTFSER.I
		K.IASEPPPVR.E
		K.IASEPPPVREIPPSC*APLTAQAIQEGLR.K
		K.IASEPPPVREIPPSC*APLTAQAIQEGLRK.E
		K.KQSSVYKLEAVEK.S
		K.LAC*VDSPKPLPGPHLEPSC*LSR.G
		K.LAC*VDSQKPLPDPHLSK.L

		K.LHHPQDGGPLPLPTHPFPYSR.L
		K.LKPVDYEYR.E
		K.LKPVDYEYREEVHWATHQLR.L
		K.QSSVYKLEAVEK.S
		K.QSSVYKLEAVEKSPVFC*GK.W
		K.QYSQSESLDQIPNNVAHATEGK.M
		K.SLAHAGVALAKPLPR.T
		K.SLLTGDYIPGTETHMAPEVVLGR.S
		K.SPPLTLSKEESGMWEPLPLSSLEPAPAR.N
		K.SPVFC*GKWEILNDVITK.G
		K.SPWRGEYKEPR.H
		K.TEDNEGVLLTEK.L
		K.VQIQSLNGEHLHIR.E
		K.WEILNDVITK.G
		R.AEELM#AC*AGLTSPR.I
		R.AEELMAC*AGLTSPR.I
		R.ALQQVGGLK.S
		R.ALQQVGGLKSPWR.G
		R.ALQQVGGLKSPWRGEYKEPR.H
		R.APGPRPAEETTGR.A
		R.APGPRPAEETTGRAPK.L
		R.DTLSSGVHSWSSQAEAR.S
		R.EEVHWATHQLR.L
		R.EIPPSC*APLTAQAIQEGLR.K
		R.EPSPKTEDNEGVLLTEK.L
		R.GRPTDTPSYFNGVK.V
		R.GSFGEVHR.M
		R.GSFGEVHRM#EDKQTGFQC*AVK.K

		R.GSFGEVHRM#EDKQTGFQC*AVKK.V
		R.GSFGEVHRMEDKQTGFQC*AVK.K
		R.HPPPNQANYHQTLHAQPR.E
		R.IFIAGSK.Q
		R.IFIAGSKQYSQSESLDQIPNNVAHATEGK.M
		R.LEVFRAEELM#AC*AGLTSPR.I
		R.M#EDKQTGFQC*AVK.K
		R.MEDKQTGFQC*AVK.K
		R.MEDKQTGFQC*AVKK.V
		R.NTPQFTKPLKEPGLGQLC*FK.Q
		R.SREPSPKTEDNEGVLLTEK.L
		R.SSSWNM#VLAR.G
		R.SSSWNMVLAR.G
		R.TPEQESC*TIPVQEDESPLGAPYVR.N
		R.VSAAELGGK.V
		R.VSAAELGGKVNR.A
NF-kappa-B essential modulator	IPI:IPI00002411.3	K.AQVTSLLGELQESQSR.L
		R.MQGQSVEAALR.M
		R.QLESEREALQQQHSVQVDQLR.M
Stress-induced-phosphoprotein 1	IPI:IPI00013894.1	K.ALDLDSSC*KEAADGYQR.C
		R.KAAALEAMKDYTK.A
heterogeneous nuclear ribonucleoprotein M isoform a	IPI:IPI00171903.2	K.GC*GVVKFESPEVAER.A
		K.QGGGGGGGSVPGIER.M
		R.M#GAGM#GFGLER.M
		R.M#GPAM#GPALGAGIER.M
		R.MGPAMGPALGAGIER.M
		R.MGPLGLDHMASSIER.M

insulin receptor substrate 4	IPI:IPI00020729.1	R.GDNQAGGAAAAAAAPEPPPR.S
		R.WFQPVANAADAEAVR.G
insulin-like growth factor 2 mRNA binding protein 1	IPI:IPI00008557.4	K.SGYAFVDC*PDEHWAMK.A
		K.TVNELQNLTAAEVVVPR.D
		R.MVIITGPPEAQFK.A
similar to 40S ribosomal protein SA	IPI:IPI00398958.3	K.FAAATGATPIAGR.F
		R.LLVVTDPR.A
		R.YVDIAIPC*NNK.G
tripartite motif-containing 28 protein	IPI:IPI00438229.2	K.IVAERPGTNSTGPAPM#APPRAPGPLSK.Q
		K.LSPPYSSPQEFAQDVGR.M
		K.VFPGSTTEDYNLIVIER.G

Table A2. miRNA array analysis of *Nik*$^{+/+}$ and *Nik*$^{-/-}$MEFs. Log fold changes represent the changes in *Nik*$^{-/-}$MEFs normalized to *Nik*$^{+/+}$ MEFS.

Name	Log fold change (normalized to WT)	p-value
hsa-miR-140-3p/mmu-miR-140*/rno-miR-140*	2.06	0.008
hsa-miR-382/mmu-miR-382/rno-miR-382	-1.78	0.009
hsa-miR-487b/mmu-miR-487b/rno-miR-487b	-1.82	0.008
mmu-miR-1193	-1.89	0.008
mmu-miR-382*	-1.94	0.010
mmu-miR-376a*/rno-miR-376a*	-1.98	0.009
hsa-miR-196b/mmu-miR-196b/rno-miR-196b	-2.01	0.007
mmu-miR-341/rno-miR-341	-2.06	0.008
hsa-miR-409-5p/mmu-miR-409-5p	-2.12	0.006
hsa-miR-136/mmu-miR-136/rno-miR-136	-2.17	0.007
mmu-miR-540-5p	-2.20	0.008
mmu-miR-329/rno-miR-329	-2.25	0.006
mmu-miR-673-5p	-2.27	0.004
hsa-miR-380*	-2.31	0.010
mmu-miR-434-3p/rno-miR-434	-2.44	0.007
hsa-miR-376c	-2.44	0.006
mmu-miR-676	-2.44	0.004
mmu-miR-380-3p	-2.49	0.008
hsa-miR-369-5p/mmu-miR-369-5p/rno-miR-369-5p	-2.51	0.010
hsa-miR-431*/mmu-miR-431*	-2.52	0.009
hsa-miR-154*/mmu-miR-154*	-2.60	0.004
mmu-miR-136*	-2.76	0.005
rno-miR-379*	-2.79	0.010
mmu-miR-337-3p	-2.98	0.008
hsa-miR-369-3p/mmu-miR-369-3p/rno-miR-369-3p	-3.06	0.009
hsa-miR-431/mmu-miR-431/rno-miR-431	-3.12	0.004
hsa-miR-136*/rno-miR-136*	-3.36	0.003
hsa-miR-376a	-3.38	0.003
mmu-miR-299	-3.44	0.002
hsa-miR-433/mmu-miR-433/rno-miR-433	-3.45	0.003
mmu-miR-300/rno-miR-300-3p	-3.48	0.002
mmu-miR-434-5p	-3.54	0.002
mmu-miR-376c/rno-miR-376c	-3.59	0.002
mmu-miR-540-3p/rno-miR-540	-3.66	0.004
rno-miR-337	-3.78	0.008
hsa-miR-127-5p/mmu-miR-127*	-3.83	0.002
mmu-miR-337-5p	-3.85	0.002
hsa-miR-299-5p/mmu-miR-299*/rno-miR-299	-3.87	0.004
hsa-miR-377/mmu-miR-377/rno-miR-377	-3.90	0.001
hsa-miR-410/mmu-miR-410/rno-miR-410	-4.03	0.004
hsa-miR-134/mmu-miR-134/rno-miR-134	-4.05	0.001

hsa-miR-495/mmu-miR-495/rno-miR-495	-4.17	0.001
hsa-miR-127-3p/mmu-miR-127/rno-miR-127	-4.50	0.003
hsa-miR-409-3p/mmu-miR-409-3p/rno-miR-409-3p	-4.57	0.001
hsa-miR-10a/mmu-miR-10a/rno-miR-10a-5p	-4.75	0.002
hsa-miR-379*	-4.94	0.004
hsa-miR-411*/mmu-miR-411*	-5.10	0.001
mmu-miR-376b/rno-miR-376b-3p	-5.23	0.001
hsa-miR-379/mmu-miR-379/rno-miR-379	-5.25	0.001
hsa-miR-411/mmu-miR-411/rno-miR-411	-5.34	0.001
mmu-miR-376a/rno-miR-376a	-5.41	0.001
mmu-miR-541/rno-miR-541	-5.63	0.001
mmu-miR-665	-1.52	0.028
hsa-miR-132/mmu-miR-132/rno-miR-132	-1.53	0.015
hsa-miR-1206	-1.65	0.028
hsa-miR-329	-1.69	0.048
mmu-miR-384-5p/rno-miR-384-5p	-1.71	0.011
hsa-miR-381/mmu-miR-381/rno-miR-381	-1.97	0.042
hsa-miR-1197/mmu-miR-1197	-2.08	0.025
hsa-miR-539/mmu-miR-539/rno-miR-539	-2.11	0.022
mmu-miR-743b-3p	-2.19	0.011
hsa-miR-376b	-2.20	0.033
hsa-miR-641	-2.36	0.032
hsa-miR-335/mmu-miR-335-5p/rno-miR-335	-2.44	0.028
hsa-miR-378*/mmu-miR-378*/rno-miR-378*	-2.46	0.027
rno-miR-148b-5p	-3.20	0.043

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