Functional characterization of protein kinase NLK (nemo-like kinase)

Chan, Aye Thu

2011

Chan, A. T. (2011). Functional characterization of protein kinase NLK (nemo-like kinase). Doctoral thesis, Nanyang Technological University, Singapore.

https://hdl.handle.net/10356/47448

https://doi.org/10.32657/10356/47448

FUNCTIONAL CHARACTERIZATION OF PROTEIN KINASE NLK (NEMO-LIKE KINASE)

CHAN AYE THU

School of Biological Science

2011

A thesis submitted to the Nanyang Technological University in fulfillment of the requirement for the degree of Doctor of Philosophy

Acknowledgement

I thank Dr. Peter Cheung for preparation of plasmids and his helpful instructions and guidance for my project. I also would like to take this chance to show my appreciation to my supervisor especially for encouragement and motivational talk during the toughest time of my Ph.D study. I also want to thank my senior Liu Xinyu for teaching me many techniques that are essiential to perform my project, Seah Cheah Chen for purification of the proteins, Dai Liang and Xi Jia Jia for their technical support. Most importantly I really appreciate the friendship and teamwork that was built between lab members and it has created a very enjoyable and productive working environment. Last but not least, I would like to thank my family and friends who always try their best to understand me and support me throughout my long journey of Ph.D study.

Table of contents **Page**

Acknowledgement 1

List of Figures 5

List of table 6

Summary 7

Abbreviations 9

1. Introduction

1.1 Discovery of nemo-like kinase (NLK) 13

1.2 Physiological function of NLK 17

1.3 Wnt signaling and NLK 21

1.4 NLK is activated by upstream kinase TAK1 23

1.5 TAK1 signaling mediated by IL-1β and TNFα 27

1.6 Objective 32

2. Materials and Methods

2.1 Materials 34

2.2 Antibodies 34

2.3 DNA plasmids 35

2.4 Cell culture and stimulation 36

2.5 Expression of proteins in mammalian cells 36

2.6 Establishment of stable NLK knockdown cell lines 36

2.7 GST pulldown and immunoprecipitation assay 36

2.8 *in vitro* kinase assay 37

2.9 *in vitro* dephosphoryaltion assay 37

2.10 *in vitro* ubiquitin binding assay 38

2.11 Dual luciferase assay 38

2.12 Western blotting 38

2.13 Human RayBio inflammatory antibody array 39

2.14 Real time PCR reaction 39

3. Results

Part 1: NLK downregulates TAK1 activity via TAB2/3

3.1	TAB2 and TAB3 are novel binding partners of NLK	41
3.2	The catalytic domain and C-terminal extension of NLK are required to interact with C terminal region of TAB2 or TAB3	46
3.3	Phosphorylation of TAB2 and TAB3 by NLK	51
3.4	C terminal region of TAB2 is phosphorylated by NLK	54
3.5	Binding of TAB2/3 to NLK doesn't augment its kinase activity	58
3.6	Binding of TAB2/3 to NLK does not facilitate the direct activation of NLK by its upstream kinases	61
3.7	NLK does not disrupt the interaction between TAK1 and its binding proteins	64
3.8	NLK suppresses the activation of TAK1 via TAB2 or TAB3	66
3.9	NLK knockdown increases TAK1 activation	69
3.10	NLK knockdown increases the activation of p38, JNK and IKK signaling pathways	75

Part 1: NLK downregulates TAK1 activity via TAB2/3

3.11	TAB2 and TAB3 are novel binding partners of NLK	85
3.12	The catalytic domain and C-terminal extension of NLK are required to interact with C terminal region of TAB2 or TAB3	90
3.13	Phosphorylation of TAB2 and TAB3 by NLK	97
3.14	C terminal region of TAB2 is phosphorylated by NLK	100
3.15	Binding of TAB2/3 to NLK doesn't augment its kinase activity	104
3.16	Binding of TAB2/3 to NLK does not facilitate the direct activation of NLK by its upstream kinases	110

3.17 NLK phosphorylates c-jun *in vitro* 114
3.18 NLK negatively regulates c-jun activity in multiple signaling 116
3.19 NLK negatively regulate gene expression of inflammatory cytokines mediated by NFκB and AP-1 in TNFα signaling 121

4. Discussion 128
5. Perspectives and future directions 138
6. Summary 139
7. References 140

List of Figures and Tables

Introduction

Figure 1-1	Sequence alignment of NLK with ERK2 and CDK2	14
Figure 1-2	Schematic representation of NLK kinase structure	15
Figure 1-3	Schematic diagram of regulation of Wnt signaling by NLK and its homologs	20
Figure 1-4	Wnt signaling pathway	22
Figure 1-5	Schematic diagram of TAK1	23
Figure 1-6	Schematic diagrams of TAB2/3 and the models of K48 or K63 linked poly ubiquitin chains	26
Figure 1-7	TNFα and IL-1 β signaling pathways	29

Result part I

Figure 3-1	NLK binds to TAB2 and TAB3	43
Figure 3-2	Domain mapping of interaction between NLK and TAB2 or TAB3	48
Figure 3-3	TAB2 and TAB3 are phosphorylated by NLK	52
Figure 3-4	Phosphorylation of TAB2 truncates by NLK	55
Figure 3-5	TAB2 and TAB3 does not enhance the kinase activity of NLK	60
Figure 3-6	TAK1 and HIPK2 are not direct upstream kinases of NLK	62
Figure 3-7	NLK does not disrupt the interaction between TAK1 and its binding proteins	65
Figure 3-8	Inhibition of TAK1 activity by NLK requires TAB2 or TAB3	67
Figure 3-9	NLK inhibits TAK1 activitation in cells	71
Figure 3-10	Activation of p38, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα and IL-1 β	77

Result part II

Figure 3-11	NLK inhibits activation of NFκB pathway	87
Figure 3-12	Polyubiqutin chain binding by TAB2 and TAB3 are reduced by NLK	92
Figure 3-13	NLK does not abrogate the physical interaction between TRAFs and TAB2 or TAB3	98
Figure 3-14	Polyubiquitin chain binding by TAB2 and TAB3 are reduced by phosphorylation	101
Figure 3-15	NLK inhibits activation of NFκB in TNFα pathway	106
Figure 3-16	NLK interacts with c-jun and inhibit activation of AP-1 pathway	112
Figure 3-17	NLK phosphorylates c-jun *in vitro*	115
Figure 3-18	NLK negatively regulates c-jun activity	118
Figure 3-19	Effect of NLK depletion on the profile of cytokine protein secretion by HEK293 cells	123

Discussion

Figure 4-1	Schematic representation of two possible models of NLK regulation on TAK1 signaling	130
Figure 4-2	Model of NLK regulation on IL-1β and TNFα signaling	137

Tables

Table 1	Function of NLK and its characterized physiological substrates	19

Summary

Nemo-like kinase (NLK) is a species conserved serine/threonine kinase that belongs to the mitogen activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs) families. TAK1 (Transforming Growth Factor-beta activated protein kinase) acts as the upstream kinase of NLK and regulates the function of NLK in multiple signaling pathways such as Wnt-1, IL6 and TGFβ. The preliminary findings of my research work highlight the versatile function of NLK because of its substrates that range from adaptor proteins like TAB2/3 (TAK1 binding protein 2/3) to transcriptional factors like c-jun (Activator protein 1 or AP-1) in the same signaling pathway to regulate TAK1 mediated signaling.

Our study has identified NLK as an interaction partner of TAB2/3 and phosphorylation of TAB2/3 by NLK modulates the function of TAB2/3. NLK inhibits TAB2/3 from binding of polyubiquitin chain by either causing conformational changes or masking the ubiquitin binding domain of TAB2/3. Furthermore, phosphorylation of TAB2/3 by NLK possibly alters the conformation of TAK1 which is in the complex with TAB2/3, and inactivates TAK1. We have shown that over-expression of kinase active NLK, but not the kinase dead mutant NLK [D270A], with TAK1 complex suppresses the activity of TAK1 by reducing the auto-phosphorylation at the activation loop of TAK1. NLK knockdown cells also show increased activity of TAK1 and its downstream target p38 and JNK compared with control cells. Since TAK1 is a master regulator of p38, NFκB and JNK signaling pathways which control inflammation, the inhibition of TAK1 activity following the stimuli-dependent rapid activation is important.

Besides regulating TAK1 activity through phosphorylation of TAB2/3, NLK also regulates the gene expression of cytokines through two major transcriptional factors NFκB and AP-1. Over-expression of NLK downregulates TAB2 or TAB3 mediated NFκB activation in a dose dependent manner. NLK phosphorylation also modulates the binding of lysine 63 linked polyubiquitin chain by TAB2/3,

which is an essential component of TAK1-IKK-NFκB signaling activation. NLK "knockdown" cells show increased polyubiqutin binding of endogenous TAB2/3 and enhanced activation of NFκB reporter gene in response to TNFα. NLK also directly regulates AP-1 activity by phosphorylating c-jun as well as indirectly through the negative feedback regulation of TAK1 and JNK activtiy. Furthermore, NLK "knockdown" cells also show increase gene expression and secretion of several inflammatory factors such as MCP-1, TNFα, TIMP-2, IP-10, and IL-8 compared to control cells.

Taken together, our findings suggest that NLK is involved in negative regulation of inflammatory signaling triggered by TNF and IL-1 through phosphorylation of TAB2 and TAB3, or transcriptional factor AP-1.

Abbreviation

A-20	Deubiquitinating enzyme
A-myc	Proto-oncogene of vertebrate myc family member
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
B-myc	Proto-oncogene of vertebrate myc family member
Cdks	Cyclin dependent kinases
CHIP	carboxyl-terminus of HSC70 interacting protein
c-jun	Oncogene jun, nuclear protein (a major component of AP-1)
c-myc	Proto-oncogene, cellular progenitor of the v-myb oncogenes
CBP	cAMP response element-binding or CREB Binding Protein
CtBP	Carboxy terminal binding protein
CUE	Coupling of ubiquitin conjugation to ER degradation
cyclin D1	Regulatory subunit to advance the G1 phase of the cell-cycle
DLD-1	Human colorectal carcinoma cell lines
DUBs	Deubiquitinating enzymes
ERK	Extracellular signal-regulated kinase
FLAG	Flag epitope tag
GCSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GST	Glutathione S-transferase
GSK3	Glycogen synthase kinase 3
h	hour
HA	Hemaglutinin
HDAC3	Histone deacetyltransferase 3
HIPK2	Homeodomain-interacting protein kinase 2
His	6 histidine residues containing tag
I-309	Chemokine (15/16 kDa glycoprotein)

IFN-ρ	Interferon gamma
IκB	Inhibitor of κB
IKK	Ikappa-B kinase
IKK	IκB kinase
ICAM-1	Inter-Cellular Adhesion Molecule 1
IL	Interleukin
IL-12 p40	Interleukin 12 protein 40 kDa
IL-12p70	Interleukin 12 protein 70 kDa
IL-1R AcP	Interleukin 1 receptor accessory protein
IL-6sR	Interleukin 6 soluble receptor
IP	Immunoprecipitation
IP-10	Interferon inducible protein 10
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRAK	IL-1 receptor associated protein kinase
IRFs	IFN regulatory factors
IVK assay	*in vitro* kinase assay
JNK	Jun N-terminal kinase
K or lys	Lysine
kDa	kilo Dalton (molecular weight of the protein)
LEF-1	Lymphoid enhancer factor
LIT-1	*C.elegans* homolog of NLK
MAPK	Mitogen activated protein kinase
MAPKAP-K2	MAPK-activated protein kinase
MBP	Maltose binding protein
MCP-1	Monocyte chemoattractant protein 1
MCP-2	Monocyte chemoattractant protein 2
MEFs	Mouse embryonic fibroblasts
MEKK1	MAPK kinase kinase 1
MIG	Monokine induced by gamma-interferon
MIP-1 α	Macrophage Inflammatory Proteins 1 alpha
MIP-1 β	Macrophage Inflammatory Proteins 1 beta

MIP-1 δ	Macrophage Inflammatory Proteins 1 delta
min	minutes
MKK6	Mitogen-activated protein kinase kinase 6
Myc	myc epitope tag
MyD88	Myeloid differentiation primary response protein 88
NARF	NLK associated ring finger protein
NFκB	Nuclear factor kappa B
NLK	Nemo-like kinase
Nmo	Nemo
NZF	Nuclear protein localization 4 zinc finger
PDGF	Platelet-derived growth factor
POP1	*C.elegans* homolog of TCF
PPARδ	Peroxisome proliferators activated receptor δ
RANTES	Regulated upon Activation, Normal T cell expressed and Secreted
RING	Really interesting new gene
RIP	Receptor interacting protein kinase 1
Runx2	Runt-related transcription factor 2
S or Ser	Serine
SAPK2a/p38α	Stress-activated protein kinase 2α
SETDB1	SET domain bifurcated 1
Smad	Small mothers against decapentaplegic
STAT-3	Signal transducer and activator of transcription 3
T or Thr	Threonine
TAB1	TAK-1 binding protein 1
TAB2	TAK-1 binding protein 2
TAB3	TAK-1 binding protein 3
TAK1	TGF-β activated kinase 1
TCF	T cell factor
TGF-β	Transforming growth factor- beta
TIMP-2	Tissue inhibitor of metalloproteases 2

TIP	Type 2A phosphatase interacting protein
TIRAP	TIR domain containing adaptor protein
TLR	Toll like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
sTNF R I	Soluble TNF receptor I
sTNF R II	Soluble TNF receptor II
TRADD	TNF receptor associated death domain protein
TRAF	Tumor necrosis factor receptor associated factor
Ub	Ubiquitin
Ubc	Ubiquitin carrier protein
Uev	Ubiquitin conjugating enzyme variant
WRM-1	a member of the Worm aRMadillo gene class, C.elegans β-catenin
Y or Tyr	Tyrosine

1. Introduction

1.1 Discovery of nemo like kinase (NLK)

Drosophila nemo was first identified in 1994 as a gene required for proper photoreceptor cells rotation during ommatidia morphorgenesis in the eye. [1-2] The murine ortholog of Nemo (nemo like kinase) and *C.elegans* ortholog of Nemo (LIT-1) were subsequently identified in 1999 as gene products that interact genetically with the Wnt signaling pathway. [3-5]

NLK was classified as atypical mitogen activated proteins (MAP) kinases family members of serine/theronine kinases based on its ability to be phosphorylated and activated by MAP kinase kinase members MAPKK/MEK family. NLK is comprised of 515 amino acids and the central kinase domain is bordered by N and C terminal extensions of about 100 residues. Kinase domain and C terminal domain are highly conserved among other homologs such as *Homo Sapien, C.elegan, Drosophila* and *Xenopus laevis.* More than 70% similarity and 45% identity of amino acid was observed in the kinase domain and C-terminal region among the invertebrate and mammals. The high level of sequence identity found in kinase domain and its C terminal domain among the orthologs suggests it may play an important function and strict specificity for substrate in many organisms. Its kinase domain is composed of 289 amino acids (residues 127-415) and shows a high degree of sequence similarity to that of kinase domains of ERK (MAPK7) and cyclin-dependent kinases (CDKs). NLK is ~55% similar and 42% identical to Erk-2, and 50% similar and 38% identical to Cdc2. [6] The kinase domain of NLK displays more sequence similarity to the MAPK than to CDKs. Like other members of atypical MAP kinase ERK3 and ERK4, NLK also lacks the tyrosine phosphorylation site in its activation loop. The "TQE" motif found in NLK resembles the "THE" sequence of CDK2.

The amino acid sequence alignment of human MAP kinase ERK2, cdk and NLK kinase proteins is shown Figure 1-1.

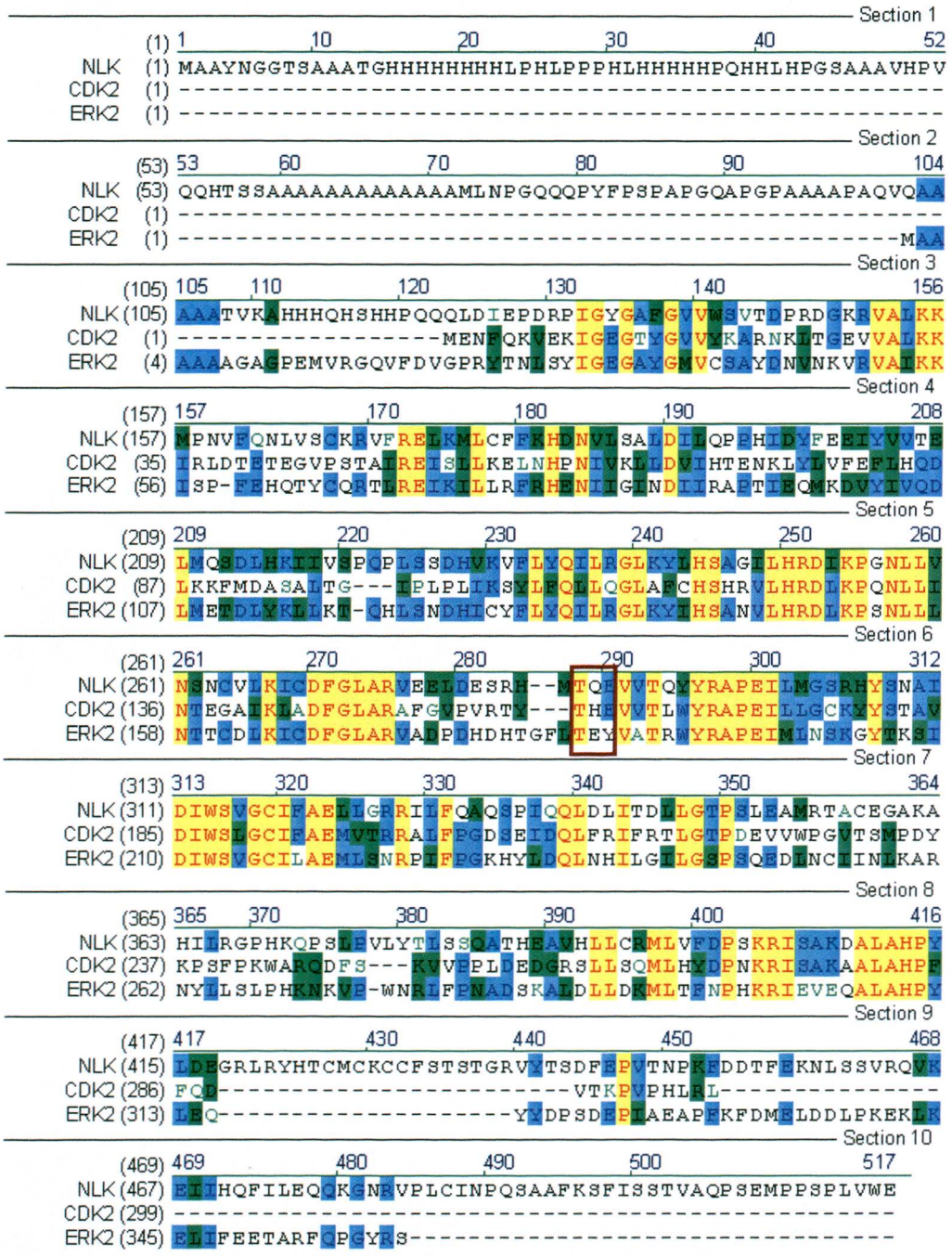


Figure 1.1 Sequence alignment of NLK with ERK2 and CDK2

Identical amino acid residues in all three proteins are highlighted in yellow, identical residues among 2 proteins are shown in blue and the amino acids with similar functional groups are shown in blue. "TEY', "THE" and "TQE" in the red box represents the phosphorylation motif in the activation loop of the kinases.

The N terminal region of the kinase is poorly conserved among NLK orthologs and its function remains unclear. It has a unique sequence that is highly enriched in alanine, glutamine, histidine and proline residues and shows high similarity to the Zn finger type transcriptional factor. Based on the available sequence information and function of known proteins, that amino acid repeat in N terminus of NLK was predicted to be important for interacting with nuclear proteins. Furthermore, it also highlights the possible functional role of NLK at the level of gene expression by regulating the function of transcriptional factors in the nucleus. [7] Mammalian NLK gene has an extended sequence in the N terminal region compared with shorter N terminal domains found in invertebrates like Xenopus or Zebrafish. Perhaps, this may indicate an additional function of NLK in mammals to cope with more sophisticated gene regulation system. [8]

Similar to other MAPK members, the activity of NLK is thought to be regulated by phosphorylation on its T loop and C terminus by upstream kinases proteins. It was predicted that phosphorylation at Thr 286 in the T-loop of NLK is important for its activation. [6, 9] Phosphorylation of Ser 510 by p38α in the C-terminus of NLK controls its binding interaction with its substrate. [10]

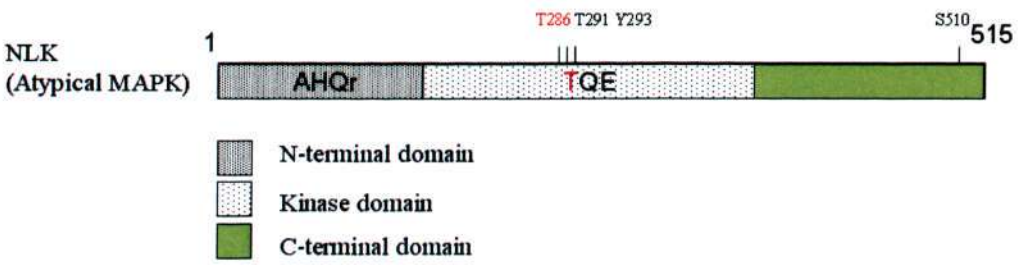


Figure 1.2 Schematic representation of NLK kinase structure

NLK comprises of 515 amino acids and its kinase domain is flanked with N and C terminus extension domain. "AHQr" in N terminal domain stands for Alanine, Histine and Glutamine repeat and phosphorylation of T286 in the kinase domain is predicted to be required for its activity. p38 phosphorylates NLK at [S510] residue and it regulates it activity. [6, 10-11]

A number of reports have suggested NLK as a nuclear protein; however, the subcellular localization of endogenous NLK is still unclear. As sensitive and specific antibodies against NLK are not available at present, its nuclear localization was shown by immunostaining and fractioning of overexpressed NLK in Cos 7 cells and HEK293 cells. [6] However, a recent study has reported that NLK is localized in the cytoplasm under basal condition at least in PC12 cells. [12] In their study, NLK was shown to translocate from cytoplasm to the nucleus and cell leading edge in PC12 neuronal cells upon stimulation with NGF. Since nuclear localization signal was not identified in its sequence, NLK is thought to be carried into the nucleus by interacting with other proteins in a similar manner to its family members such as Erk/MAPKs and Cdks proteins. [13]

Only one single functional NLK gene has been found in invertebrate (*Caenorhabditis elegans, Drosophila melanogaster*) and mammal (*Mus musculus and Homo sapiens*). With the availability of genomic sequence, one functional single copy of NLK gene and a processed pseudogene were mapped in mouse chromosome 11 and 2. Although genes encoding upstream (Wnts and Frizzleds) and downstream components such as (TCF/LEF) are diversified, the proteins controlling the surveillance of pathways such as GSK-beta and NLK are maintained in single copies. So far only one isoform of NLK gene exists in human and mammal, however, 2 isoforms [NLK1 and NLK2] were found in *Drosophila*, Xenopus and Zebrafish [8, 14]

The functional importance of the NLK gene and its physiological relevance were studied using NLK knockout mice. The importance of NLK gene in the development of embryo was shown by the fact that NLK null mice die during the third trimester of pregnancy. The NLK-/- mice were shown to have significant hematopoietic abnormalities compared with control mice. Compared with wild type mice, NLK -/- mice have reduced hematopoiesis which is indicated by reductions in total cellularity of bone marrow by 50% and reduction of lymphoid cells and myeloid cells by 66% and 75% of the population. In addition,

disruption of the NLK gene in mice gives a very complex phenotype highlighting the crucial functional role of NLK in multiple signaling pathways. Furthermore, NLK null mice are growth retarded and suffer from various neurological abnormalities. The concentrated expression pattern of NLK in mouse brain and neural tissues strengthen the possible function of NLK in nervous system and brain development. [12, 14]

1.2 Physiological Function of NLK

Our understanding of the role of NLK has improved markedly in the last decade. A number of research findings have shown the physiological role of NLK in a variety of cellular events like tumorigenesis, programmed cell death, neurogenesis and osteogenesis. [12, 15-21] NLK phosphorylates and control the fate of its substrates by several mechanisms – it regulates the activity, stability or subcellular distribution of its substrates or regulates their interaction with other binding of partners.

A wide range of NLK novel substrates were identified from research studies based on animal models and cell culture system. TCF4/LEF-1 is one of the first characterized substrate of NLK. TCF/LEF1 activity is controlled by members of the Wnt family of signaling molecules which play important roles in development and diseases. NLK can negatively regulate Wnt target gene expression through transcriptional factor TCF/LEF1, providing the first piece of information on an important role of NLK. [28, 29]

Later, it was reported that NLK also regulates the activity of transcription factors including c-Myb, A-Myb, Notch1, stat-3, SMAD4, FOXO1, NFκB, AP-1, and p53. [22-27] these transcriptional factors are important cell-cycle gate controllers in proliferation and are also involved in different stages of tumor development. The regulation of a wide range of transcription factors by NLK suggest that human NLK homolog could function as tumor suppressor and its loss

of function would lead to persistent activation of Wnt signaling and consequent tumor development.

Table 1 summarizes and provides a list of NLK substrates and their role in several signaling pathways. The phosphorylation sites in the substrates are shown together with the sequence surrounding the phosphorylation motif. Based on the current knowledge of identified NLK phosphorylation sites in substrates, we can predict that NLK prefer to phosphoryalte serine/threonine residues which are mostly followed by proline and sometimes glycine.

A major contribution to understanding of NLK function comes from genetic and biochemical analyses of its orthologs in model organisms such as *Drosophila, C.elegans and Xenopus*.

A mutant allele of *Nmo*, a *Drosophila* ortholog of NLK causes defects of epithelial planar polarity in the fly's eyes.[1-2] Another group also identified a more severe *Nmo* mutant allele which restored the wing phenotype of fruit flies overexpressing β-catenin [Armadillo], indicating that *Nmo* acts as a suppressor of Wnt signaling in wing development. [32] Nmo was also suggested to play a crucial role in regulation of apoptosis in *Drosophila* eye development. Ectopic expression of nemo causes defects in patterning and head development of flies leading to lethality. [15]

The *Caenorhabditis elegans* NLK homolog, LIT-1, has been identified as a critical regulator of embryonic cell fate determination accompanying anterior–posterior cell division [33]. In *C. elegans* development, Wnt signaling is known to determine cell polarity and differentiation [2, 33-34]. Several lines of genetic and biochemical analyses have shown that LIT-1 phosphorylates POP-1 (*C.elegans* TCF homolog) to regulate its sub cellular distribution and activity in the nucleus. [4, 33] Similarly, in *Xenopus*, NLK suppresses the transcriptional activity of the β-catenin/T-cell factor (xTCF) complex through phosphorylation of xTCF to

regulate axis formation [5]. Therefore, NLK/Nmo/LIT-1 functions as a downstream regulator of Wnt/Wingless signaling in a variety of organisms. [35] Figure 1-3 represents a schematic diagram of Wnt signaling pathways which is counteracted by NLK. The crucial role of NLK in Wnt signaling indicates NLK as an evolutionarily conserved functional protein in both vertebrates and invertebrates.

Table1. Function of NLK and its characterized physiological substrates

Substrate	Phosphorylation site	Sequence	Method	Significance or pathway
LEF1	T155 S166	SHAVHPLtPLTYSD TYSDEHFsPGSHPSH	*In vivo* and *in vitro*	Inhibit its function and/or promote its degradation in Wnt pathway [28-29]
TCF7L2	T201 T212	PHHVHPLtPLITYSN TYSNEHFtPGNPPPH	*In vivo* and *in vitro*	Inhibit its function and/or promote its degradation in Wnt pathway [28-29]
Smad4	T9 S138	DNMSITNtPTSNDAC YHYERVVsPGIDLSG	*In vitro*	Its role is still unclear[25]
FOXO1A	S329	STISGRLsPIMTEQD	*In vivo*	Negatively regulate FOXO1A function [24]
STAT-3 (mouse)	S727	NTIDLPMsPRTLDSL	*In vivo* and *in vitro*	Recruit STAT3 as scaffold protein in IL-6 pathway [22]
c-Myc A-Myb	Multiple sites	C-terminal region	*in vitro*	Inhibit transcriptional function and/or promote its degradation in Wnt1 pathway [9, 30]
Notch1ICD	Unidentified	Ser/Thr rich region and Trans-activation domain	*In vivo* and *in vitro*	Suppresses transcriptional function in Notch pathway [23]
SETDB1 (Histone methyltransferase)	T976	PSSEEtPKNKVAS	*in vitro*	Suppresses transactivation of PPARr and induce Runx2 expression in Wnt5a pathway [31]
Paxillin MAP-1B	S126 Unidentified	NKQKSAEPsPT	*in vitro* *In vitro*	Control the dynamics of the cytoskeleton organization in NGF pathway [12]
Mad (mothers against dpp) (*Drosophila*)	S25	GSLFSFTsPAVKK	*In vivo and in vitro*	Control distribution, accumulation of pMad in motor neurons and its function. In wing development, NLK antagonize BMP pathway. [16-17]

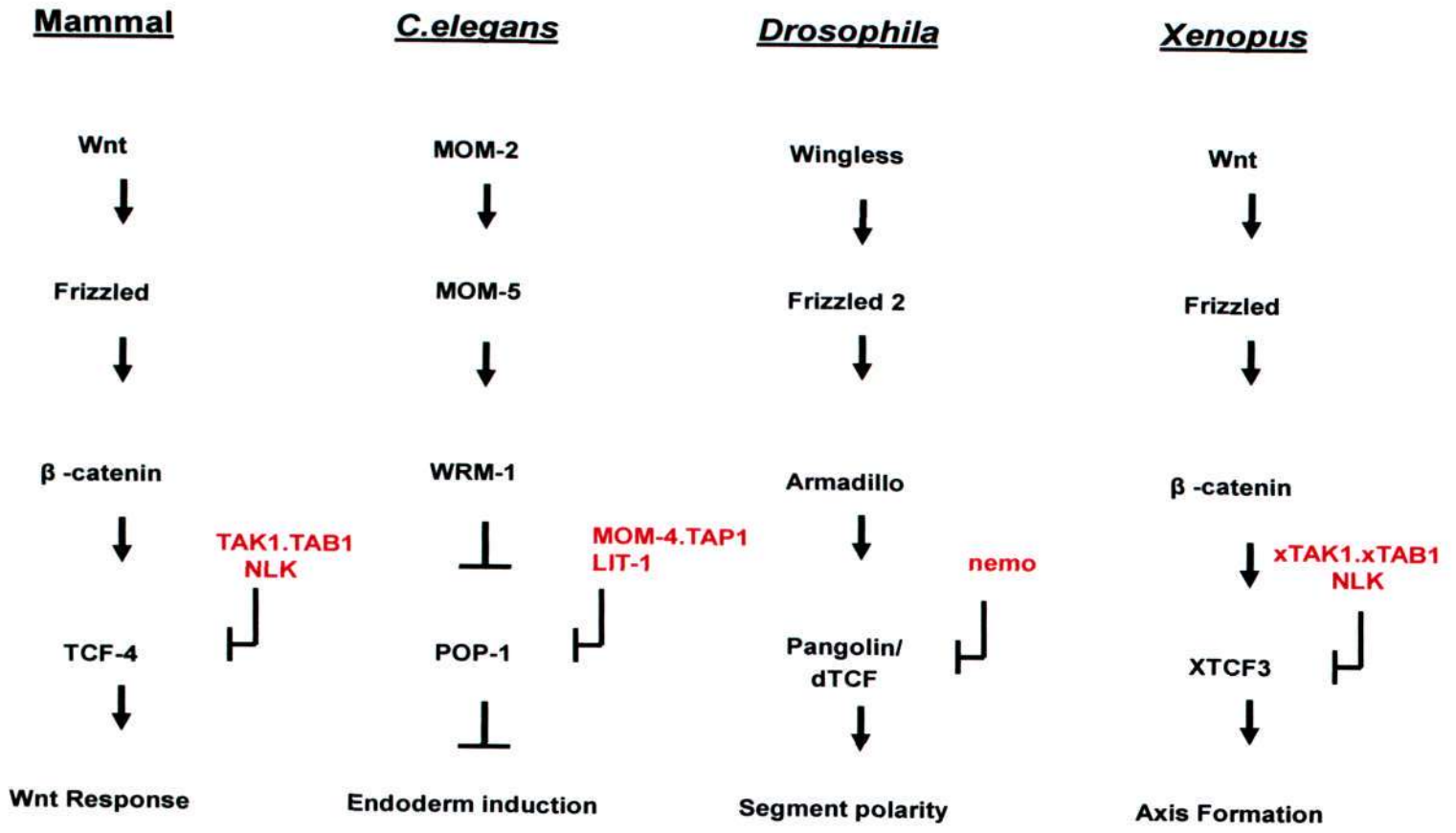


Figure 1-3. Schematic representation of Wnt signaling regulated by NLK and its homologs

Mammalian NLK and it orthologs regulates Wnt signaling in different organisms by either inhibiting TCF function through phosphorylation to determine the final outcome of signaling cellular responses. Also shown is the TAK1-TAB1 complex which functions above NLK. [2-3, 5, 15, 36-40]

1.3 Wnt signaling and NLK

Wnt signaling is evolutionarily conserved and is involved in diverse biological processes such as embryonic development and adult cellular homeostasis including determination, proliferation, migration differentiation, tumorigenesis and inflammation. [41-42] Constitutive activation of Wnt dependent gene expression in mammals is associated with hereditary and sporadic forms of colon cancer. [43-44]

Wnts are secreted cysteine rich proteins which bind to the Frizzled family of receptors. In the absence of Wnt, cells regulate β-catenin levels by a multiprotein complex consisting of the adenomatous polyposis coli (APC) tumour suppressor protein, axin and the glycogen synthase kinase GSK3-β, which phosphorylate β-catenin, marking it for subsequent ubiquitination and degradation. Due to the depletion of β-catenin, TCF is bound to the transcriptional repressor Groucho (*Drosophila* ortholog) or transducin-like enhancer TLE (mammalian ortholog) and histone deacetylases (HDACs) which silence Wnt target gene expression. [45-46] Upon binding of the Wnt ligand to its Frizzled receptor, a canonical cascade of Wnt signaling events leads to disruxption of the axin scaffold protein complex. As a result of the destruction of the protein complex, cytoplasmic β-catenin accumulates and enters the nucleus where it activates specific gene expression. β-catenin displaces the transcriptional repressors (Groucho/HDACs) from TCF/LEF1 and recruits histone acetylase CBP/p300 to activate gene expression. [39, 47]

Wnt signaling is regulated by multiple layers of safe-guard mechanisms such as cross-regulation and negative feedback inhibition. In this study, we focus on MAP kinase pathway involving the TAK1 and NLK which counteracts Wnt signaling. As shown in the diagram [Figure 1-3] Wnt signaling leads to the parallel activation of TAK1 and NLK which then phosphorylates TCFs/LEF1 complexed to β-catenin. [48] NLK regulates TCFs/LEF1 function through modification of its ability to interact with DNA and/or chromatin remodelling complex or stability in *vivo*.[28,29][Fig. 1-4] Several target genes of TCF-β catenin complexes such as c-MYC and MMP-7 have important roles

in tumorigenesis and therefore have been intensively studied. [47, 49-50] The functional involvement of NLK in the negative regulation of these proto-oncogenes suggests a possible function as a tumor suppressor *in vivo*.

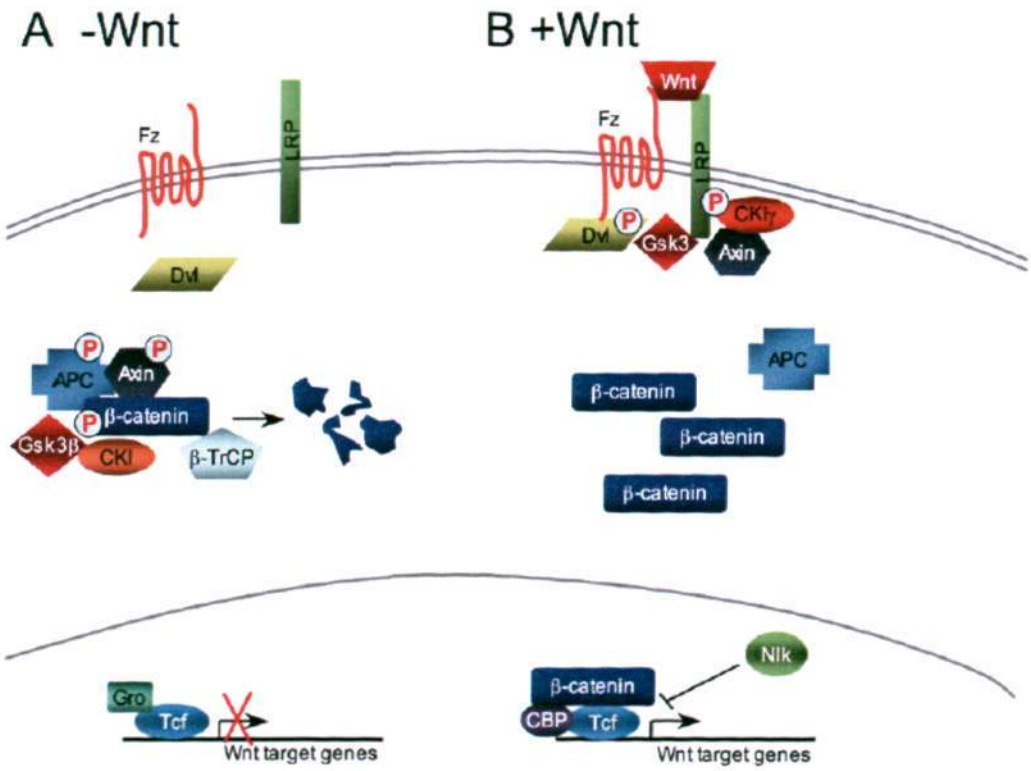


Figure 1.4 Wnt signaling pathway

In the absence of Wnt ligand, β- catenin is degraded by a proteasome mediated mechansim in the cytoplasm. When Wnt is bound to the Frizzled receptor, Dishevelled a key component of a membrane-associated Wnt receptor complex is activated and leads to the inhibition of the Axin/GSK-3/APC complex, which normally promotes the proteolytic degradation of the β-catenin in the cytoplasm. Inbition of axin/GSK3/APC protein complex by Wnt leads to stablization of cytoplasmic β-catenin. The excess β-catenin is then able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression.

1.4 NLK is activated by upstream kinase TAK1

Several lines of evidence suggest NLK as a crosstalk linkage between the Wnt and MAPK signaling pathways. Based on genetic and biochemical studies, NLK activity is shown to be modulated by its upstream kinase TGF-beta-activated kinase 1 (TAK1). TAK1 was originally identified in mouse as a kinase activated by TGF β family ligands [51] and later classified as a member of the MAPKKK family. TAK1 is also a highly conserved protein in different organisms like *Drosophila, Xenopus*, mouse and human. The human TAK1 gene is located in chromosome 6qe16.1 -6q16.3 with 17 exons [52].

Studies from TAK1 knockout mouse have shown that TAK1 is a major player in the activation of IKK and JNK in the innate and adaptive immunity pathways. Deletion of TAK1 in thymocytes affects the population and maturation of single positive CD4 or CD8 thymocytes in peripheral tissues. In addition, T-cell receptor ligation failed to activate NFκB and JNK signaling pathway in TAK1-deficient thymocytes but activated pathways which led to apotosis. [53] A similar observation is also found in B cells with depleted TAK1 where B-cell receptor ligation failed to induce the activation of IKK and JNK signaling. [54] Experiment carried out in TAK1 -/- MEFs (mouse embryonic fibroblast cells) indicates that TAK1 deletion impairs NFκB and JNK activation in the TGF-β signaling pathway. All these studies suggest that TAK1 is an upstream activating kinase for IKKβ and JNK as well as a key modulator of transcription factors NFκB and AP-1. [55]

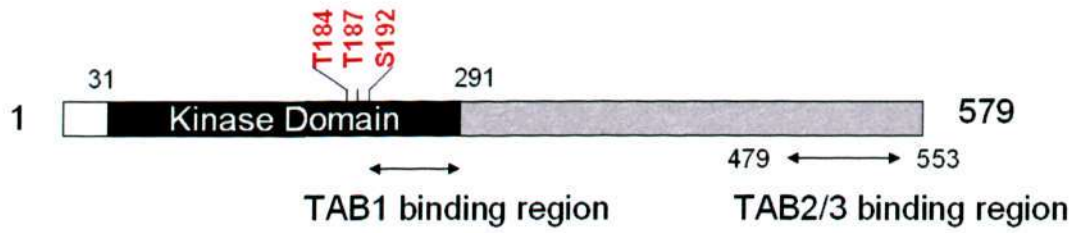


Figure 1.5 Schematic diagram of TAK1

TAK1 consists of 579 amino acids and has a N-terminal kinase domain spanning residues 31 to 291. Three well characterized autophosphorylation sites [Thr 184, Thr 187 and Ser 192] in the activation loop are important for its activity. TAB1 binding site is predicted to be in the kinase domain of TAK1 whereas TAB2/3 binds to C terminal region [497-553]. [59-61]

Three binding partners of TAK1 were identified by yeast two hybrid assay and cells-based studies and they are termed as TAK1 binding proteins (TAB1, TAB2 and TAB3). [56-58] Even though *in vitro* biochemical studies have revealed the existence of TAK1 complex (TAK1-TAB1-TAB2/3) *in vivo*, it still remains as a debatable topic of which members of this complex are essential for signaling. Using embryonic fibroblasts lacking TAK1, TAB1, or TAB2, a research group has found that TNFR1, IL-1R, TLR3, and TLR4-mediated NFκB and AP-1 activation are severely impaired in TAK1 deficient cells, but not in TAB1 or TAB2 deficient cells. Their finding highlights the functional significance of TAK1 but not TAB1 or TAB2 in inflammation pathways. [55]

Contradictory to that report, other research groups have reported that TAB1 is indispensible for TAK1 function. The stable and strong association of TAB1 induces conformation changes in TAK1 to augment its autophosphoryaltion activity *in vivo*. [58] TAB1 was experimentally shown to promote TAK1 auto-phosphorylation and acts as the activator in the TAK1 signaling pathway. [58] Three auto phosphorylation sites (Thr 184, Thr 187 and Ser 192) in its activation loop of TAK1 were reported to be required for TAK1 activity. [59-61]

Biochemical assays performed with TAB1 knockout mouse embryonic fibroblasts cells have shown that TAB1 depletion abolishes TAK1 autophosphoryaltion and activity in IL-1β and TNFα signaling. [62-63] TAK1 activity is negatively regulated by p38α by phosphorylating on the residues [Thr 431 and Ser 438] of TAB1. [64]

A second subunit TAB2 (TAK-1 binding protein 2) and third subunit TAB3 (TAK1 binding protein 3) were identified from mammalian cells and function as adaptor proteins. TAB2 and TAB3 share 48% sequence identity and they appear to play redundant roles in TAK1 activated signaling pathways *in vivo*. [65] Studies from TAB2 knockout mice and RNAi mediated knockdown indicates that TAB2/3 are necessary for TAK1 activation and the subsequent activation of IKK signaling cascades by IL-1, TNF, and RANKL [40-42, 44-45].

Both TAB2 and TAB3 contain a CUE ubiquitin-binding domain, a coiled-coil (CC) region, a TAK1-binding domain and a C-terminal Npl4 zinc finger (NZF) ubiquitin-binding domain. [66] [Figure 1-6A] The NZF domain of TAB2/3 act as ubiquitin receptor for K63 linked polyubiquitin chains and is essential for TAK1 and IKK activation *in vivo*. Several independent biochemical studies have reported that TAB2/3 exhibits strong interaction with K63 linked polyubiquitin chains, but not with K48 linked polyubiquitin chains. [66]

Upon receiving the specific extracellular stimuli, TAB2 and TAB3 interact with upstream components of TAK1 signaling pathway through K63 linked polyubiquitin chains and relay the signal to downstream components such as IKK, JNK and p38.[56-57, 67] K63 linked polyubiquitin chain is different from classical K48 linked polyubiquitin chains which is a marker for proteasome mediated degradation system *in vivo*. The different conformations of K48 and K63 linked tetra polyubiquitin chains are also shown in Figure 1-6B.

The involvement of K63 linked polyubiquitin chains in cell signaling can be explained by using an analogy to phosphorylation system in which polyubiquitin chains function like a phosphate group to determines the [ON/OFF] switch of signaling. K63 linked polyubiquitin chains can also act as scaffolds to assemble signaling components and mediate their activation through proteasome independent mechanism. Several mechanisms of ubiquitin mediated activation of kinases involve conformational changes, sub-cellular localization and association or dissociation with other binding partners.

Activation of signaling cascades by K63 linked polyubiquitin chains are counterbalanced by deubiquitinases. A-20 and CYLD (cylindromatosis) are well known examples of NFκB inducible deubiquitinases which remove the K63 linked polyubiquitin chains from molecules such as TRAF6, NEMO and RIP. [68-70]

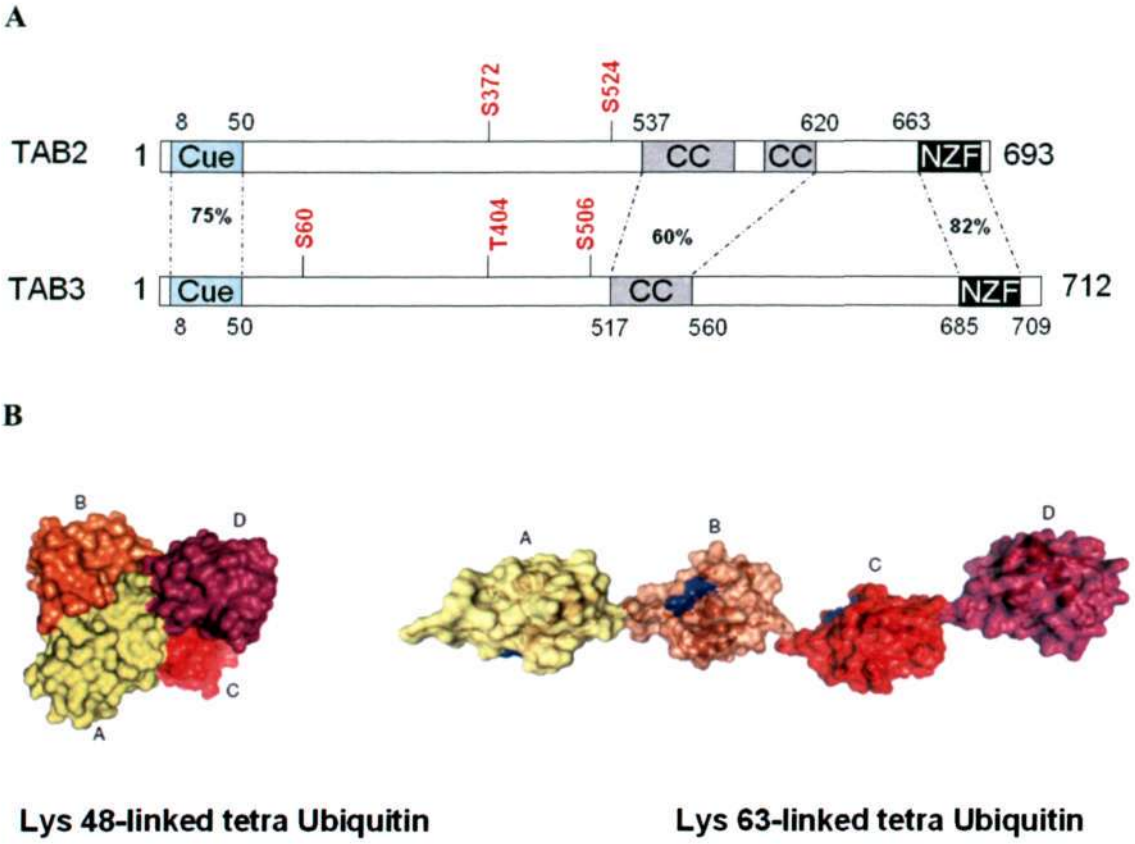


Figure 1.6 Schematic diagrams of TAB2/3 and the models of K48 or lys-63 linked polyubiquitin chains

(A) CUE domains near the N terminus and coiled coil domain or NZF domain in C terminus share high level of sequence identity as indicated. CUE and NZF domain are known as ubiquitin receptor domains which are essential for ubiquitin binding by TAB2/3. The specific interaction between K63 linked ubiquitin chains and TAB2/3 via its NZF domain has been solved by structural studies recently. [66] Two phosphorylation sites in TAB2 [S372 and S524] and three phosphorylation sites in TAB3 [S60, T404 or S506] in IL-1 activated cells are identified and predicted to be involved in regulation of its function *in vivo*. [62, 71]

(B) The different morphology of K63-linked and K48-linked tetra ubiquitin chains are adopted from reference [72]. Ubiquitin is a highly conserved protein containing 76 amino acid and 7 lysine residues. [73] The two most intensively studied polyubiquitin chains are K48 or K63 linked where a peptide bond is formed between 2 adjacent mono ubiquitin proteins through K48 or K63 residues. K48-linked ubiquitin tetramers represents a compact conformation, [72] while K63-linked ubiquitin chain adopt a different conformation, which is extended in nature and possesses a very high degree of flexibility. This flexible conformation is important for activation of signal transduction events by providing a highly ordered scaffold structure for signaling molecules.

1.5 TAK1 signaling mediated by IL-1-β and TNFα:

TAK1 is a member of the MAPKKK family and is activated by a growing number of various stimuli such as Wnt-1, Wnt5a, IL-1β, IL-6, TNFα, LPS, GCSF and TGF-β and osmotic stress. [5, 9, 51, 61, 64, 74-75] Here, we focus on TAK1 signaling mediated by TNF or IL-1. [Figure 1.7]

Upon binding of TNF to the TNFR1, SODD (the silencer of death domain) is released from cytoplasmic tail of the receptor to allow the recruitment of death domain adaptor TRADD (TNF receptor associated death-domain protein) through homophilic interaction between the death domains. TNFR1-bound TRADD then serves as a platform for binding of TRAF2 or TRAF5 and the DD-containing serine-threonine kinase RIP1 (receptor interacting protein kinase 1). TRAF2/5 are RING domain ubiquitin ligases and can ubiquitinate RIP1 at lysine 377 with K63-linked polyubiquitin chains which acts as a scaffold for the recruitment of the downstream TAK1-TAB1-TAB2/3 complex by binding the NZF domains of TAB2/3. Upon docking of IL-1β to IL-1RI, a receptor complex is formed with recruitment of IL-1R AcP (IL-1R accessory protein) which subsequently associates with the adaptor MyD88 through a homophilic TIR-TIR interaction. MyD88 recruits IRAK1 & 4 (IL-1 receptor associated kinase 1 and 4) followed by phosphorylation of IRAK1 by IRAK4. Phosphorylated IRAK1 binds to TRAF6 leading to the synthesis of K63 polyubiquitin chains and interaction with the TAK1 complex through a similar mechanism as described for TNFα signaling.

TAK1complex can transmit the signaling cascades through 3 major signaling pathways.

- **JNK and p38α pathways**

TAK1 activate **JNK** (c-Jun N-terminal kinase) and **p38α** (also called stress activated protein kinase 2a) via phosphorylation of their upstream kinases MKK4/7 and MKK3/6 (Mitogen activated protein kinase kinase) respectively, which subsequently result in the activation of transcription factors such as AP-1(Activator protein), CREB (c-AMP responsive element binding protein) and induction of inflammatory cytokines like IL-2, IL-6, IL-12 and TNF[76]. (Refer to pathway I as shown in Figure 1-7)

-IKK pathway

Activated TAK1 can also recruit NEMO (NFκB essential modulator, or **IKKγ**) which then acts as a bridge between TAK1 and the IKK (IκB kinase) complex. IKKβ (IκB Kinase β) in complex with NEMO is then phopshorylated by TAK1, leading to the phosphorylaton and degradation of IκB (inhibitor of κB) and in turn the liberation and translocation of NFκB into nucleus where it turns on a number of genes related to inflammation, for example cytokines, chemokines like IL-6 and MCP-1[77], immunoreceptor CCR5, CXCR2 [78], growth factor (VEGF, NGF) [79]. [Also refer to pathway II as shown in Figure 1-7]

TNFα and IL-1β are involved in local and systemic inflammation and also associated with molecular pathology of several chronic inflammatory disorders. For the past few years, several pharmaceutical companies have initiated huge efforts to develop small molecule inhibitors against protein kinases in IL1 or TNF signaling pathway, potentially as treatments for inflammatory diseases. Unfortunately, many small molecules kinase inhibitors that have undergone clinical trials have failed and only a few have been proven safe and effective to be used for treatment. For example, several inhibitors against p38α have failed to pass through the clinical trials because its dual activator and suppressor function in IL1 and TNF signaling was previously unknown. Cheung et al., 2003 reported that p38α plays a role in negative feedback regulation of TAK1 in IL-1β, TNFα and LPS signaling. [64] Due to its dual functional role in IL1 and TNF signaling, p38α kinase inhibitor such as VX 745 and VX-702 showed enhanced activation of NFκB *in vivo* with severe liver toxicity and undesirable effects on brain and were discontinued at phase II clinical trial [80]

Therefore, one has to be careful with designing the inhibitors or drug against protein kinases in inflammatory signaling. It is important to understand the complicated signaling network at the molecular level of protein kinases in the TNF and IL-1 pathway. Notably, our finding of NLK as a negative regulator of TAK1 in inflammatory signaling will provide knowledge which is potentially useful for desgining drug target for controlling inflammation.

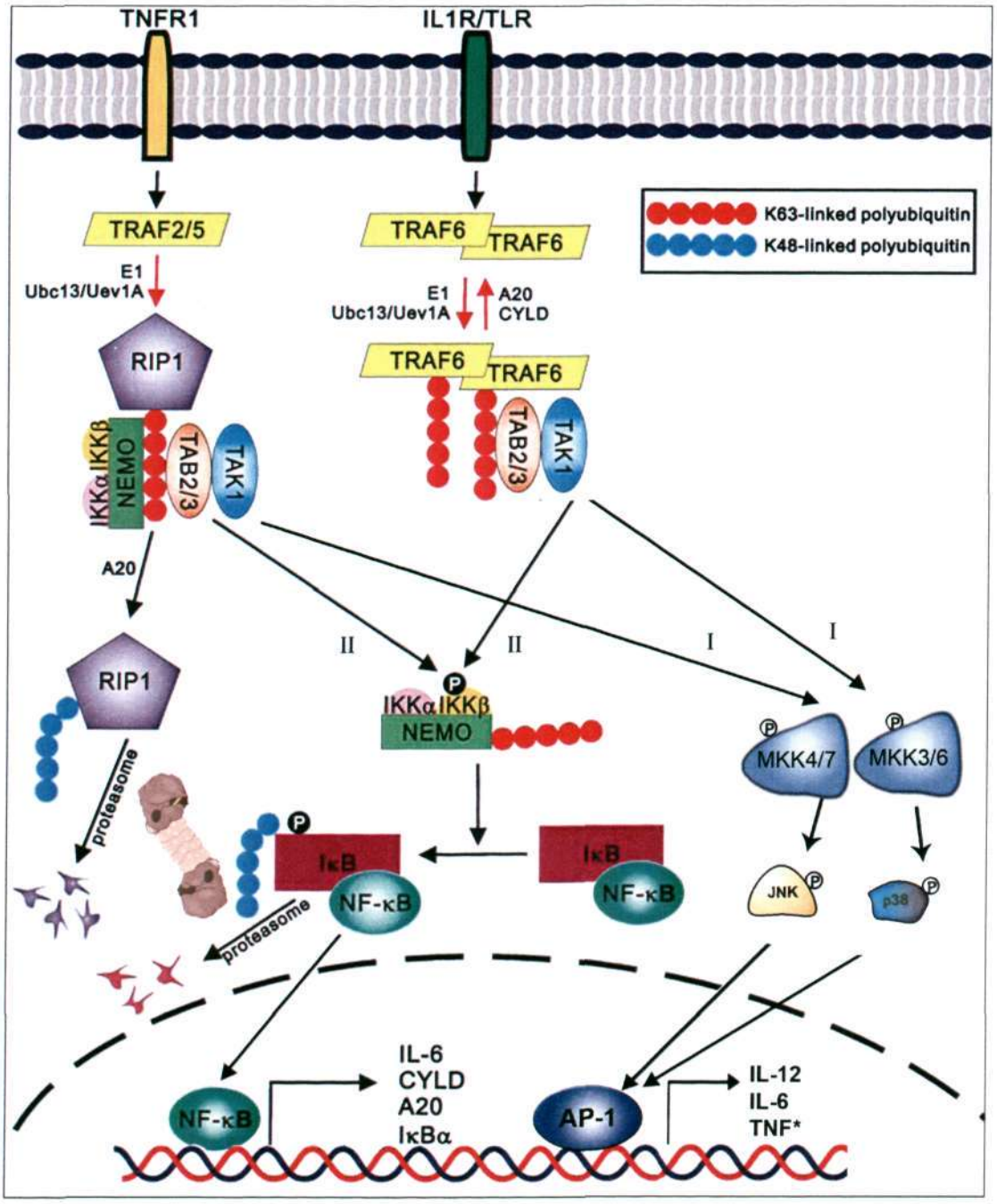


Figure 1.7 TNFα and IL-1β signaling pathways

Figure is adopted and modified from reference. [63] Binding of TNF to TNF receptor-1 leads to the recruitment of TNF receptor-1 associated death domain protein (TRADD), which in turn recruits TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP). After its ubiquitin ligase activity is activated, TRAF2 catalyses K63 polyubiquitination of itself. This causes the release of TRAF2 into the cytosol and TRAF2-mediated K63 polyubiqutination of RIP. K63 polyubiquitinated RIP is

recognized by the TAK1-binding proteins TAB2 and/or TAB3, as well as by IκB kinase γ (IKKγ). This brings TAK1 in close proximity to the IKK complex, allowing it to phosphorylate the catalytic IKKβ subunit.

Binding of IL-1β to its receptor leads to activation of ubiquitin-ligase activity of TRAF6. K63-polyubiquitinated TRAF6 recruits the TAK1 complex, which subsequently phosphorylates and activates IKKβ. **(Denoted as pathway I)** The IKKβ catalytic subunit then phosphorylates the inhibitor of κB (IκB)α protein. Phosphorylation of IκBα results in tagging with K48 linked polyubiquitin chains and subsequent proteasomal degradation. This allows NFκB to translocate to the nucleus, where it induces the transcription of genes such as IL-6, CYLD, A20 and IκBα. Some of the genes are involved in positive feedback loop and some function in negative regulatory roles to balance the cells to the equilibrium state or basal condition.

Activated TAK1 can also lead to activation of JNK and p38α via MKK4/7 and MKK3/6 pathways **(denoted as pathway II)** which activates the transcription factor AP-1 leading to the expression of inflammatory cytokines such as IL-12, IL6 and TNF.

Importance role of polyubiquitin chains in TNFα and IL-1β mediated signaling

Two different types of ubiquitin chains are shown (in 2 different colour codes) and different linkage of ubiquitin chain types determine the fates of the substrates *in vivo*. [81]

Ubiquitin is a 76-amino acid polypeptide, which is covalently attached to proteins in a three-step enzymatic cascade[82].

- First step of the ubiquitin chain reaction is initiated by the ubiquitin-activating enzyme (E1), which forms a thiol ester bond between the C-terminal glycine of ubiquitin and the active-site cysteine of E1 in an ATP-dependent reaction.
- Second step involves the transfer of the activated ubiquitin to a cysteine residue of a ubiquitin-conjugating enzyme which is termed as E2 or UBC and form an E2-ubiquitin thioester complex.
- Final step occurs with the help of ubiquitin-protein ligase (E3), ubiquitin is transferred to a target protein by forming an isopeptide bond between the C terminal glycine of ubiquitin and the ε-amino group of a lysine residue on the target protein.

Ubiquitin contains seven lysine residues, each of which can be covalently attached to another ubiquitin, resulting in the formation of a polyubiquitin chain. Tthe role of K48 (in blue) and K63 (in red) linked polyubiquitin chains in IL-1β and TNFα pathways is shown. K48 linked polyubiquitin chain is recognized by proteasomal degradation system where K63 linked polyubiquitin chain is recruited by ubiquitin receptor containing adaptor proteins such as TAB2 and TAB3 to amplify the activated signaling at the level of TRAFs and TAK1.

The diagram also highlights the role of two NFκB inducible deubiquitinases such as CYLD and A20 which play a negative feedback role in NFκB pathway via removal of K63 linked polyubiquitin chains from RIP, TRAF2, TRAF6 and NEMO. [68-70]

1.6 Objective

In the beginning of this study, the components of the signaling pathway between TAK1 and NLK and the exact mechanism of how TAK1 activates NLK have not yet been clearly defined. Scaffold proteins and intermediate kinase important for the activation of NLK have been identified. For example, in IL-6 signaling pathway, STAT3 acts as a scaffold protein for TAK1 and NLK *in vivo*. These results indicate that STAT3 enhances the efficiency of its own Ser-727 phosphorylation by acting as a scaffold for TAK1-NLK. [22] In Wnt-1 signaling, HIPK2 acts as an intermediate kinase between TAK1 and NLK to regulate the activity of NLK upon degradation of c-Myb. [9, 30] A recent research finding suggests TAB2/3 as scaffold protein to link TAK1 with NLK [83]. In *Xenopus laevis* embryos, NLK was genetically mapped as downstream kinase of p38β in the anterior formation of embryo development in NGF signaling. It also suggests the possibility of p38 as an intermediate kinase between TAK1 and NLK in mammalian cells. [10]

Therefore, the objective of our study is to look for the missing link of information in NLK activated signaling pathway. We want to look for the specific upstream activators or signaling components lie between TAK1 and NLK, which brings the signal activation to NLK in response to different external stimuli.

To meet the objective of our study, we have sought to identify proteins that interact and regulate NLK. Surprisingly, we have discovered that NLK showed the possible interaction with TAB2 and TAB3 but not with TAB1 in our initial studies. This led us examine the rational behind the physical interaction between TAB2/3 and NLK. TAB2 and TAB3 are adaptor proteins that bind to K-63 linked polyubiquitin chains of signaling proteins and play an important role in TAK1 mediated activation of NFκB gene *in vivo*. Therefore, we want to study the functional role of NLK on TAB2 or TAB3 which could contribute to the regulation of TAK1 and/or TAB2/3 *in vivo*. In the course of examining the functional role of NLK, we also look for other possible downstream components or novel substrates of NLK in TNFα and IL-1β signaling.

To achieve the goal of our project, we have performed a series of "cell based" studies using mammalian cell culture and also *in vitro* biochemical assays to identify the potential interaction partners and physiological substrates of NLK in the context of multiple signaling pathways.

2. Materials & Methods

2.1 Materials

[γ^{32}P]-ATP (150mCi/ml), ECL reagent for western blot and Glutathione sepharose 4B were from Amersham (Little Chalfont, UK); Protein G agarose, Braford assay reagent were from Pierce, Ni-NTA-agarose and plasmid Mini/Mega Prep kits were from Qiagen, DMEM media was from Gibco, fetal bovine serum was from Hyclone, trypsin was from Sigma; Nitrocellulose membrane was from Schleicher & Schuell (Dassel, Germany); all other common chemicals and reagents were purchased from Sigma or Merck.

Buffers - Buffer A [50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 1% (w/w) Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol plus 1 tablet/50 ml of EDTA free complete protease inhibitor cocktail (Roche, Germany)]. Buffer B [50 mM Tris/HCl pH 7.5, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol].

2.2 Antibodies

Ubiquitin antibody (P4D1), GST antibody (Z5), TAB2 antibody (K-20), HA antibody (Y-11), Myc antibody (9E10) and His antibody (H-3) were from Santa Cruz (Santa Cruz, CA, USA); Flag antibody (M2) was from Sigma. TAK1 antibody, TAB1 antibody, GST-TAB3 antibodies have been described previously (Cheung, *et al.*, 2003). Sheep-, rabbit-, or mouse-specific secondary antibodies conjugated to horseradish peroxidase were from Pierce (Rockford, IL, USA). TAK1 p-Thr 184 and TAK1 p-Thr 187 antibodies were purchased from cell signaling. P-JNK antibody was purchased from Bio-source. Rabbit anti-NLK antibody recognizing the C-terminus [AQPSEMPPSPLVWE] and sheep anti-TAK1 antibody recognizing the C-terminus [CKKQLEVIRSQQQKRQGTS] were custom made (Biogenes, Berlin, Germany). TAB1 and TAB3 antibodies have been described previously [56] and were generously provided by Prof. Sir Philip Cohen, University of Dundee, UK. TAB2 (K-20), ubiquitin, actin, HA, myc and GST antibodies were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Antibodies recognising total p38α, JNK1 & 2, IκB, and the phosphorylated forms of p38α [pThr180/pTyr182] and IκB [pSer32], were purchased from Cell Signaling (Beverly, MA, U.S.A.). Antibody recognising the phosphorylated forms of JNK 1 & 2 [pThr183/pTyr185] was from

BioSource (Carlsbad, California, U.S.A.). Anti-FLAG antibody was from Sigma (St Louis, MO, U.S.A.).

2.3 DNA Plasmids

The following plasmids were used in the study of Result Part 1 and 2: pEBG2T-TAB1, pEBG2T-TAK1, pEF6-TAB2-HA, pEF6-TAB3-HA, pCMV5-TAK1-myc, pCMV5-TAB1-FLAG, pCMV5-NLK-myc, pCMV5-NLK-myc [D270A], pCMV5-NLK-FLAG, pEBG2T-TAB2, pEBG2T-TAB3, pEBG2T-p38α, pEBG2T-NLK, pMAL-MKK6, pNFκB-Luc, pTK-RL. pEF6-NLK [1-454]-myc, pEF6-NLK [43-515]-myc, pEF6-NLK [43-417]-myc, pEF6-NLK [43-497]-myc, pEF6-NLK [124-515]-myc, pEF6-NLK [124-497]-myc, pEF6-NLK [124-480]-myc, pEBG2T-c-jun, pEBG2T-NLK, pCMV5-NLK-myc, pCMV5-NLK-myc [D270A], pCMV5-c-jun-myc, pAP-1-Luc. The truncated TAB2 mutant plasmids used were pEBG2T-TAB2 [1-203], pEBG2T-TAB2[1-318], pEBG2T-[1-400], pEBG2T-[283-481], pEBG2T-[283-527], pEBG2T-[283-588], pEBG2T-[283-693], pEBG2T-[341-693]. pGEX6P1-NLK [124-480], pGEX6P1-cjun, pGEX6P1-TAB2 [481-693] and pMKK6-MBP were used for purification of proteins in *E.coli*. All mammalian expression plasmids used in this study contain N-terminal FLAG, GST, HA or myc epitope tags. TAB2, NLK and MKK6 plasmids were constructed using standard molecular biology techniques and the DNA templates encoding NLK (clone ID 6527673) and MKK6 (clone ID 4499772) were obtained from the IMAGE consortium (Open Biosystems, Huntsville, AL, USA). Plasmids encoding shRNA targeting NLK (Catalogue number SHCLNG-NM_016231) were purchased from Sigma. Plasmid NLK shRNA3 and shRNA5 targets the sequences [tt tgc agg atg ttg gtc tt] and [gc tca gat cat gtc aaa gtt t] in the NLK coding region respectively. Scramble shRNA in pLKO vector was generously provided by Dr David M Sabatini (Whitehead Institute, Cambridge, U.S.A.). The shRNA resistant form of pEF6-NLK-myc had the sequence [gc tca gat cat gtc aaa gtt t] mutated to [gc tc**g** ga**c** cat gt**a** aa**g** g t**g** t]. All mammalian expression plasmids used in this study contain N-terminal GST, His or myc epitope tags.

Methods

2.4 Cell culture and stimulation

HEK293 and HeLa cells were cultured in DMEM supplemented with 10% FCS (Hyclone) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The cells were incubated at 37°C with a humidified atmosphere of 5% CO_2. 16 h prior to stimulation with TNFα (50ng/ml) and IL-1β (50ng/ml), HeLa cells were incubated in DMEM plus antibiotics but without FCS. After 16 h, the cells were stimulated with 50 ng/ml of either TNFα or IL-1β. Cells were then harvested at different timepoints with buffer A and used immediately or stored at -20°C until required. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

2.5 Expression of proteins in HEK 293 cells by transfection

HEK 293 cells were transfected by a modified calcium phosphate method (Chen et al., 1988) 20 µg/ml DNA/dish. Cells were harvested by lysis in buffer A [50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/1% (v/v) Triton X-100/1 mM Na_3VO_4/50 mM NaF/5 mM sodium pyrophosphate/0.27 M sucrose /0.1% (v/v) 2-mercaptoethanol/0.1 mM EDTA free Roche tablet of protease inhibitor cocktail]. The expression of proteins was determined by resolving on SDS-PAGE and western blot analysis.

2.6 Generation and establishment of stable NLK knockdown cell lines

HEK293 cells were transfected with shRNA by modified calcium phosphate precipitation [84]. Hela cells were transfected with fugene as described by manufacturer (Roche). Cells expressing shRNA targeting NLK or shRNA control were selected with media containing 2.5 µg/ml puromycin (Sigma) for 72 hours. Surviving cells were pooled as stable transfectants and subcultured in selective media containing puromycin for further experiments.

2.7 GST pull down assay and immunoprecipitation of lysates

HEK 293 cells were harvested by lysis in buffer A. An aliquot of 0.5 mg or 1 mg of supernatant cell lysates was added to 10 µl glutathione-Sepharose beads for GST pull down assay or 2 μg of antibody coupled with 10 μl of Protein G–Sepharose for

immunoprecipitation assay. Following the incubation for 1 h at 4◦C, the suspension was centrifuged, the supernatant was discarded and the beads were washed twice with 1 ml of buffer A containing 0.5 M NaCl. Followed by two washes with 1 ml of 50 mM Tris/HCl, pH 7.5, containing 0.27 M sucrose and 0.1 %(v/v) 2-mercaptoethanol, the proteins were boiled at 72 °C for 10 min in sample buffer and loaded on SDS-PAGE and identified by Western blot analysis.

2.8 *In vitro* kinase assay

Endogenous protein or transfected proteins were subjected to perform immunoprecipitation or GST pulldown and the bound proteins were incubated at 30°C with 40 ml of 50 mM Tris-HCl pH 7.5, 0.1% (v/v), 2-mercaptoethanol, 0.1 mM sodium orthovanadate, 0.1 mM EGTA, 0.1 mg/ml BSA, 50 mg/ml of unactivated MBP-MKK6, 10 mM magnesium acetate and 0.1 mM 0.1 mM [γ^{32}P]ATP (10^6 c.p.m./nmol). incubated with 4 µg of inactivated MBP-MKK6 in 50 mM Tris-HCl (pH7.5), 0.1%(v/v)2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P] ATP ($3x10^6$c.p.m./nmol). The total volume of 30 µl reaction was terminated for the time indicated by the addition of 10µl sample buffer and resolved on SDS-PAGE. The phosphorylated substrates were visualized via autoradiography. MBP-MKK6 was expressed in bacteria and purified by affinity chromatography using Amylose resin (NEB, Beverly, U.S.A).

2.9 *In vitro* dephosphorylation assay

Following the immunoprecipitation with anti-HA antibody, TAB2-HA and TAB3-HA were treated with 400 units* of λ-phosphatases (λ-PPase) which can be used to release phosphate groups from serine, threonine or tyrosine residues in proteins. The reaction was carried out with 1 µl of λ-PPase (400,000 units/ml) in 1xλ-PPase Reaction Buffer Pack (50 mM Tris-HCl, 100 mM NaCl, 2 mM dithiothreitol, 0.1 mM EGTA, 0.01 % Brij 35, pH 7.5), 2 mM $MnCl_2$ for 30 min at 30◦C. Then, the proteins were resolved on SDS-PAGE and visualized by Western Blotting.

2.10 *In vitro* ubiquitin binding assay

Ubiquitin binding assay- GST-TAB2 or GST-TAB3 were co-expressed in HEK293 cells and purified by "GST-pulldown". The glutathione-Sepharose bound proteins were incubated with or without λ phosphatase (NEB) for 30 mins at 30°C. The λ phosphatase was removed by extensive washing and incubated for 1 h at 4°C with protein lysates from HEK293 cells overexpressing myc-tagged ubiquitin or K63 linked polyubiquitin synthesized *in vitro* using E1, Ubc13/Uev1a and CHIP as described [85].

2.11 Dual luciferase assay

NLK "knockdown" and control HEK293 cells were transfected with NFκB or AP-1 reporter construct (pNFκB-Luc), (pAP-1-Luc), internal control plasmid encoding Renilla-luciferase (pTK-RL) and indicated plasmids in 6-well plates. The total amount of transfected DNA was kept constant by adding empty vector pCMV5. A 10:1 ratio of NFκB or AP-1 reporter construct (1 μg, pNFκB-Luc or pAP-1-Luc) and internal control (0.1 μg, pTK-RL plasmid) was used in transfection. Total DNA used was 5 μg per well. Cells stimulated with or without TNFα for 8 hours were extracted in passive lysis buffer (Promega, Madison, U.S.A.). The NFκB or AP-1 reporter gene activity was measured according to Dual-Luciferase Reporter Assay System (Promega). Three independent transfections were carried out and the results expressed as the average ± SEM.

2.12 Western blotting

The lysate was subjected to SDS-PAGE and gels are transferred to nitrocellulose membrane (Schleicher & Schuell (Dassel, Germany) in transfer buffer (500mM Glycine, 50mM TrisHCl, 0.01% SDS, 20% methanol) buffer at 70V for 3h by the use of an electroblotting apparatus. Membranes are washed in TBS-T (10mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4) and then blocked with 5% non-fat milk extract in TBS-T for 30min to 1h. Membranes are exposed to primary antibody in 5% non-fat milk prepared in TBS-T for 3h at room temperature to over night at 4°C. Membranes are washed with TBS-T three times for ten minutes. Then the membranes are incubated with appropriate secondary antibodies coupled to horse radish peroxidase

(HRP) for 1h at room temperature. After washing the membranes three times for ten minutes, signals were visualized using Western Pico Super ECL reagent (Pierce).

2.13 Cytokine array

Supernatant media were collected from the cells which were transfected with control shRNA or NLK shRNA at time 0h, 3h, 6h and (16h) overnight stimulation of TNFα. Control or NLK shRNA transfected cells were kept in the conditional media for 2-3 days before proceeding with this experiment. RayBio Human inflammatory Antibody Array was used to detect relative the level of (40 different cytokines or chemokines) secreted from both sets of cells into culture media. The array analysis and western blot were carried out according to the manufacturer's instruction and the cytokines detected by the antibody array was analyzed by using Image J software. The relative intensity ratio of each spot was calculated as the ratio of [the integrated intensity of cytokines/chemokine dot detected divided by the average integrated intensity of 4 positive respective dots highlighted in grey] from each blot. [Table shown in Figure 3-19 B]

2.14 Real time PCR

Total RNA was extracted from HEK 293 cells using Qiagen RNeasy kit according to the manufacturer's instructions. 2 μg of total RNA was reversed transcribed into cDNA for 1 h at 50°C using using oligo dT primer and reverse transciptase in the presence of RNAse inhibitor. Transcribed cDNA template (50 ng) was incubated with 200 nM primers in a total volume of 20 μl using KAPA SYBR FAST qPCR kit. GAPDH was used as internal control to minimize the error of different input amount.

Result Part 1

NLK downregulates TAK1 activity via TAB2/3

3.1 TAB2 and TAB3 are novel binding partners of NLK

NLK activity is regulated by TAK1 and shown to function in many cellular events such as neurogenesis, tumorigenesis, apoptosis and growth.[10, 12, 18-19, 23] However, the exact mechanism of how TAK1 regulates NLK is not fully understood. In addition, the roles of TAB1, TAB2 and TAB3 in the activation of NLK have never been examined before. So in this study, we have co-expressed NLK with TAK1 and its associated proteins TAB1 (TAK1 binding protein 1), TAB2 (TAK1 binding protein 2) and TAB3 (TAK1 binding protein 3) and investigated the role of TAB1/2/3 in TAK1-NLK signaling pathway.

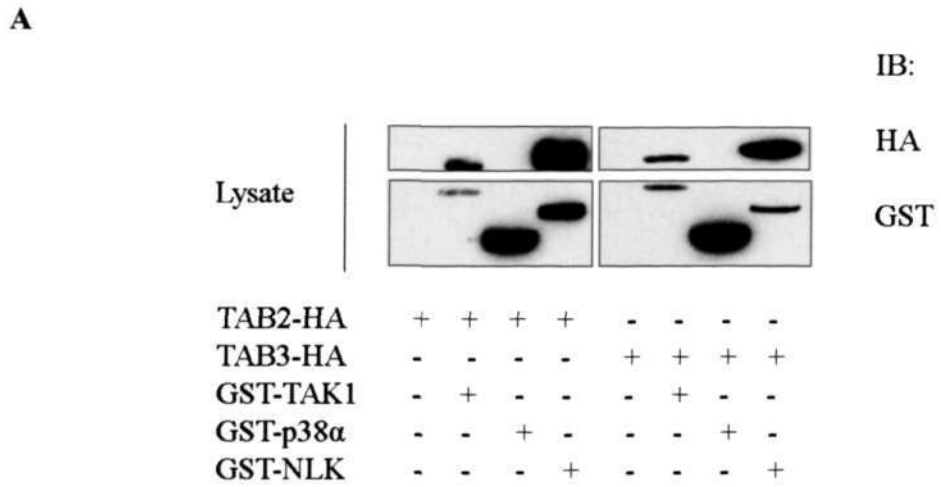
Previous studies in our lab have shown that TAB2/3 tagged with GST or HA could only be expressed at very low levels in HEK293 cells. Interestingly, NLK was shown to stabilize and enhance the expression of TAB2/3. In comparison with TAK1, NLK was able to increase the expression of TAB2/3 to a higher level as shown in Figure 3-1A. Since NLK was identified as a member of the MAPK family [6], we also investigated whether other members of MAPK can also stabilize and enhance the expression of TAB2/3. Thus, GST-p38α was co-expressed with HA-TAB2/3 in HEK293 cells. Unlike NLK, ectopic expression of p38 does not enhance the expression level of TAB2/3, suggesting the novel action of NLK on TAB2 or TAB3. We also found that NLK kinase activity is required to enhance the expression of TAB2/3. [Figure 3-1B]

To investigate their stable interaction, cellular protein lysates of HA-TAB2/3 coexpressed with GST-NLK were subjected to a GST pull-down and stained with Coomassie Blue or analyzed by western blot. Coomassie Blue staining shows the co-purified bands of GST-NLK and HA-TAB2/3 suggesting that TAB2 and TAB3 are novel interacting partners of NLK. The amount of TAB2/3 that co-purified with GST-NLK protein suggests that TAB2/3 interacts with NLK in a 0.5:1 stoichiometric ratio. [Figure 3-1C]

This finding led us to check whether NLK can also directly interact with TAK1 and its binding partner TAB1/2/3, GST-TAK1 or GST-TAB1/2/3 were co-expressed with

myc-NLK in HEK293 cells and the whole cell lysates subjected to a GST pulldown assay. As shown in Figure 3-1D, myc-NLK was pulled down by GST-TAK1, GST-TAB2/3 but not by GST-TAB1. This indicates that only TAK1 and TAB2/3 could be the direct binding partners of NLK.

To confirm their interaction, HEK293 lysates were incubated with purified GST protein, GST-NLK or GST-TAK1 and the presence of endogenous binding proteins were analyzed by western blotting using anti-NLK, TAK1, TAB1, TAB2 and TAB3 antibodies respectively. Endogenous TAK1 complex consisting of TAK1, TAB1, TAB2 and TAB3 were pulled down by GST-NLK, not by GST alone. [Figure 3-1E] Similarly, endogenous NLK was pulled down by GST-TAK1, but not by GST alone as shown in Figure 3-1 F. Altogether, our result suggests that TAB2 and TAB3 are novel binding partners of NLK.



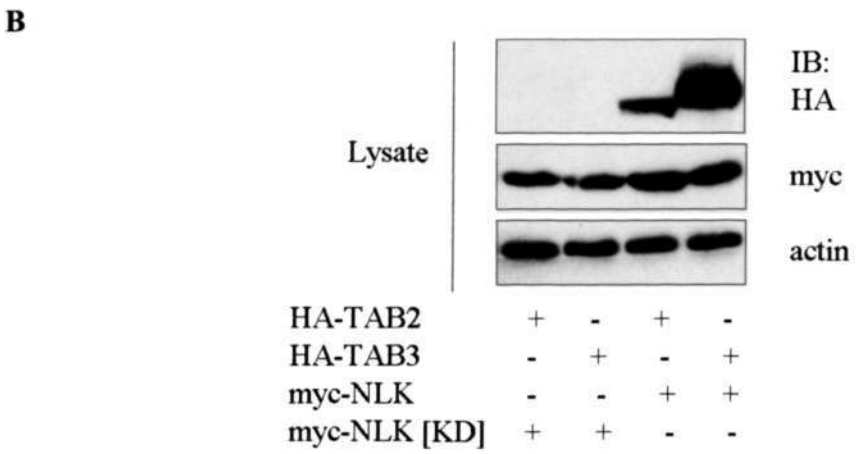


Figure 3.1 NLK binds to TAB2 and TAB3

(A) TAK1 and NLK stabilize the expression of TAB2 and TAB3 in cells –

HEK293 cells were transiently transfected with an expression vector for pEBG2T-NLK in combination with expression vectors for pEF-6 TAB2-HA or TAB3-HA. pEBG2T-TAK1/p38α with PEF6-TAB2-HA or TAB3-HA were expressed as positive and negative controls. HA-TAB2/3 can only be stably expressed together with GST-NLK or GST-TAK1 but not with GST-p38α.

(B) Kinase activity of NLK is required for stabilizing TAB2/3 expression in cells –

HEK293 cells were transiently transfected with HA-TAB2/3 and kinse active or kinase dead myc-NLK. The expression of the protein was analyzed by western blotting by using antibodies against myc and HA.

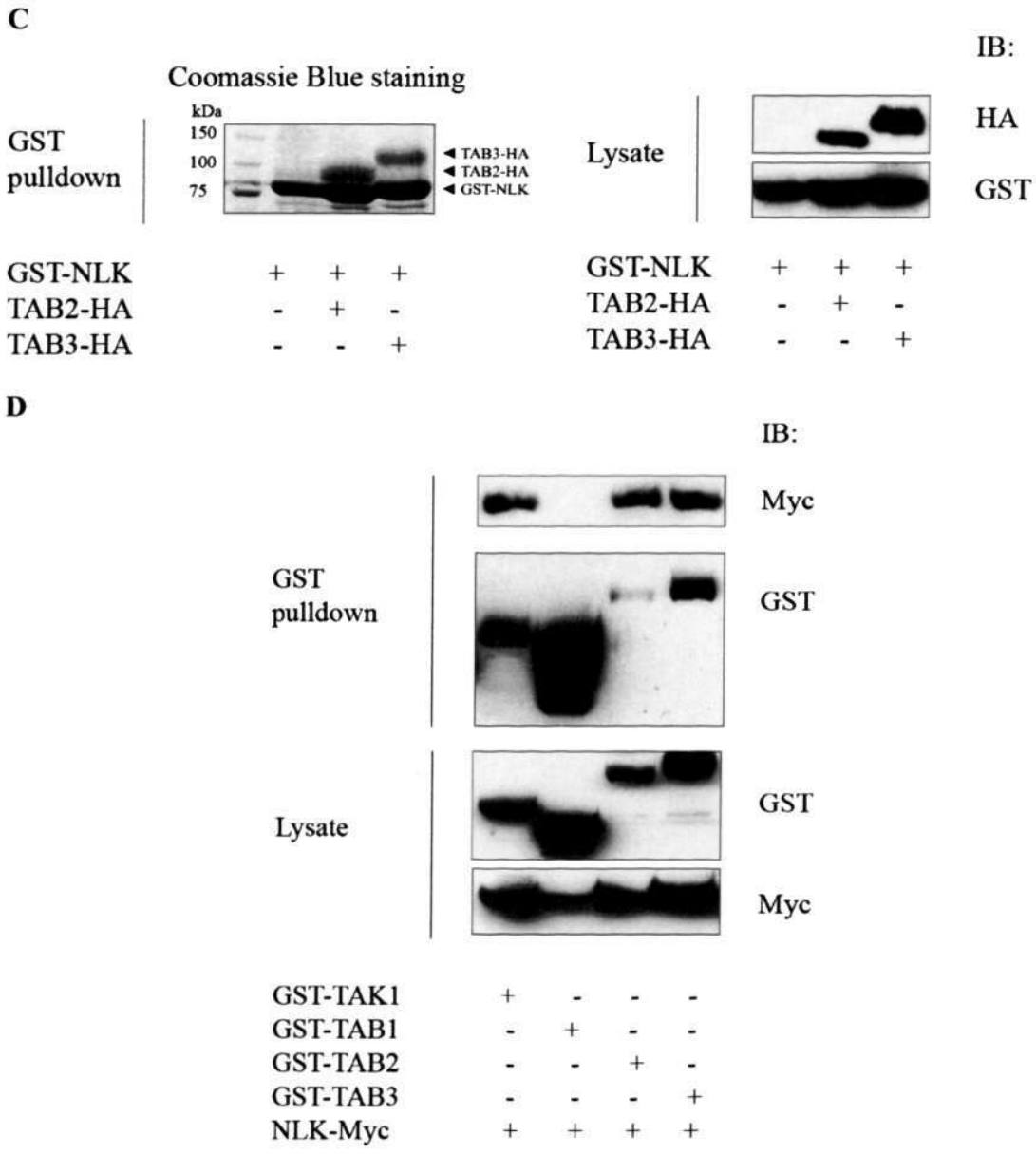


Figure 3.1 NLK binds to TAB2 and TAB3

(C) NLK interacts with TAB2/3 –

1mg of whole cell extract of GST-NLK and HA-TAB2/3 from (A) were pulled down with glutathione sepharose beads and coprecipitated proteins were stained with Coomassie Blue or analyzed by western blotting with indicated antibodies.

(D) NLK interacts with TAK1, TAB2/3 –

HEK293 cells were transiently transfected with GST-TAK1 or its binding partners GST-TAB1/2/3 with NLK-Myc. 0.5mg of cell extracts were affinity purified by using glutathione-sepharose beads and analyzed the bound proteins by western blotting with indicated antibodies.

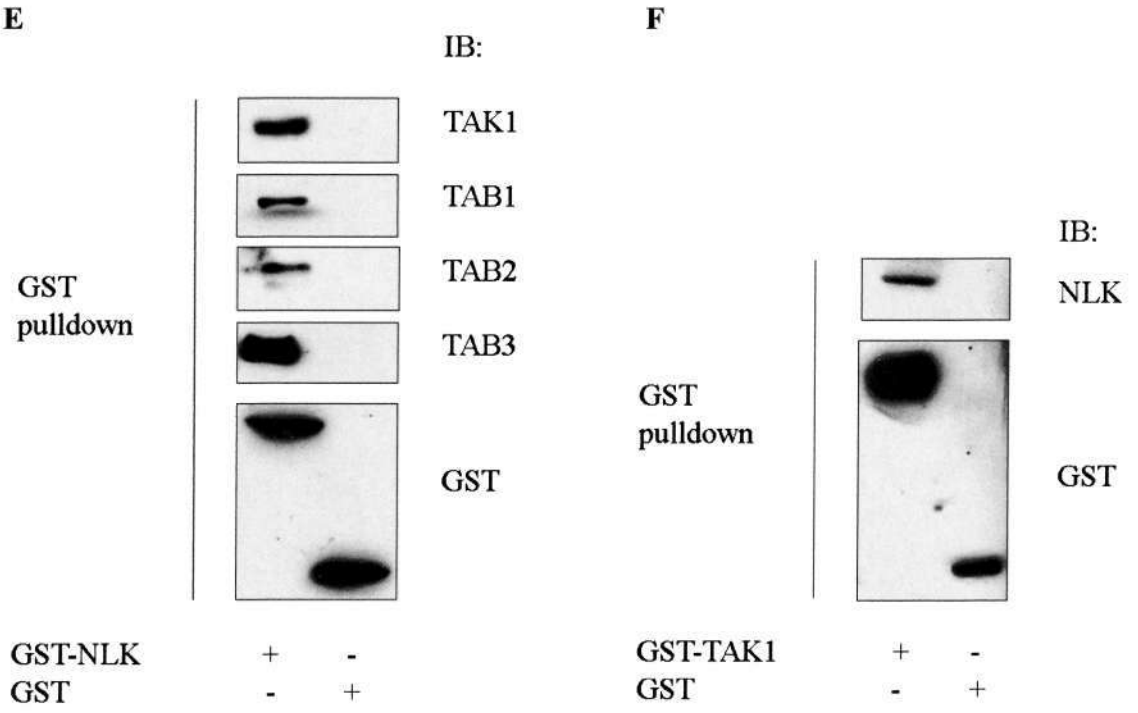


Figure 3.1 NLK binds to TAB2 and TAB3

(E) NLK forms a complex with endogenous TAK1 complex –

HEK293 cells were transiently transfected with GST, GST-TAK1 and 1 mg of cell extracts were pulled down with glutathione beads and analyzed the western blotting by using antibodies against TAK1, TAB1, TAB2 and TAB3.

(F) TAK1 forms a complex with endogenous NLK –

HEK293 cells were transiently transfected with GST, GST-NLK and 1 mg of cell extracts were pulled down with glutathione beads and analyzed the western blotting by using antibodies against NLK.

3.2 The catalytic domain and C-terminal extension of NLK are required to interact with C terminal region of TAB2 or TAB3

After showing that TAB2/3 stably interacts with NLK, we attempted to map their interaction sites. It was reported that TAK1 interacts with the C-terminal region of TAB2 and TAB3 as the binding region is located between residues 574-693 of TAB2. [71]

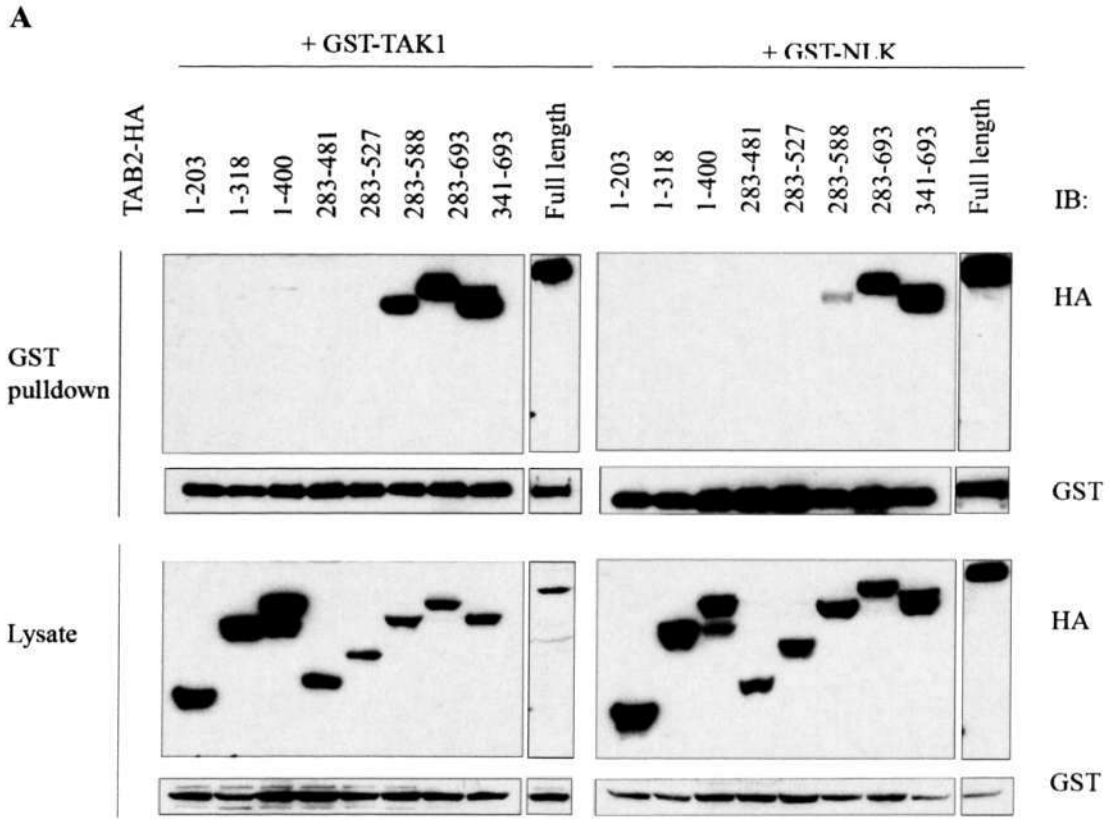
We tried to map their interaction using truncates of HA-TAB2 and GST-NLK or GST-TAK1. Deletion mutants of HA-TAB2 [1-203], [1-318], [1-400], [283-481], [283-527], [283-588], [283-693], [341-693] and full length HA-TAB2 co-expressed with GST-NLK or GST-TAK1 in HEK293 cells and the lysates were subject to GST pulldown assay. As shown in Figure 3-2A, TAK1 and NLK interacts with C-terminal of TAB2 [283-588], [283-693] and [341-693] fragments. Therefore, the minimum binding region of NLK in TAB2/3 is located between residues 341-588. This data demonstrates that both TAK1 and NLK binding region are located in the C terminal region of TAB2. Since the TAK1 binding region in TAB2 was previously mapped to residues [574-693] [71], we hypothesize that NLK binding site in TAB2 could be located in between residues 341 to 574. [Figure 3-2A, B]

In order to prove our hypothesis, we also attempted to map their interaction by using the shorter fragments of TAB2 mutants. Unfortunately the result obtained from the shorter deletion mutants was not very consistent and we conclude that further deletions of TAB2 protein might disrupt the structure of the protein or affect their proper folding.

We then mapped the TAB2/3 binding site in NLK. Full length GST-TAB2 and GST-TAB3 were co-expressed with the truncated mutants NLK [1-454], NLK [43-515], NLK [43-417], NLK [43-497], NLK [124-515] and NLK [124-497] and NLK [124-480]. As shown in the Figure 3-2C & D, apart from C terminal deletion mutant 1-454, 43-417 and 124-480, the rest of truncates were all able to enhance the expression of GST-TAB2/3.

The lysates of GST-TAB2/3 and full length myc-NLK or smallest deletion mutant myc-NLK [124-497] were subjected to immunoprecipitation (IP) by using anti-HA antibodies and the presence of co-IP, myc-NLK was detected by western blotting. This result also shows that the kinase domain and C-terminus of NLK might be critical for interaction with TAB2/3. [Figure 3-2E]

We conclude that NLK interacts with C terminal region of TAB2 or TAB3. The kinase domain and minimal fragment of C terminal region of NLK [124-497] is essential for its interaction with TAB2/3.



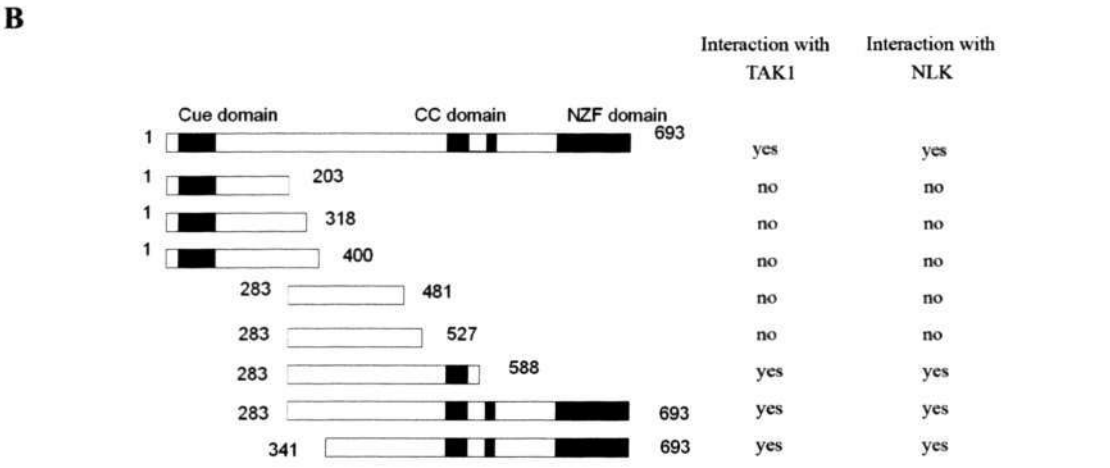


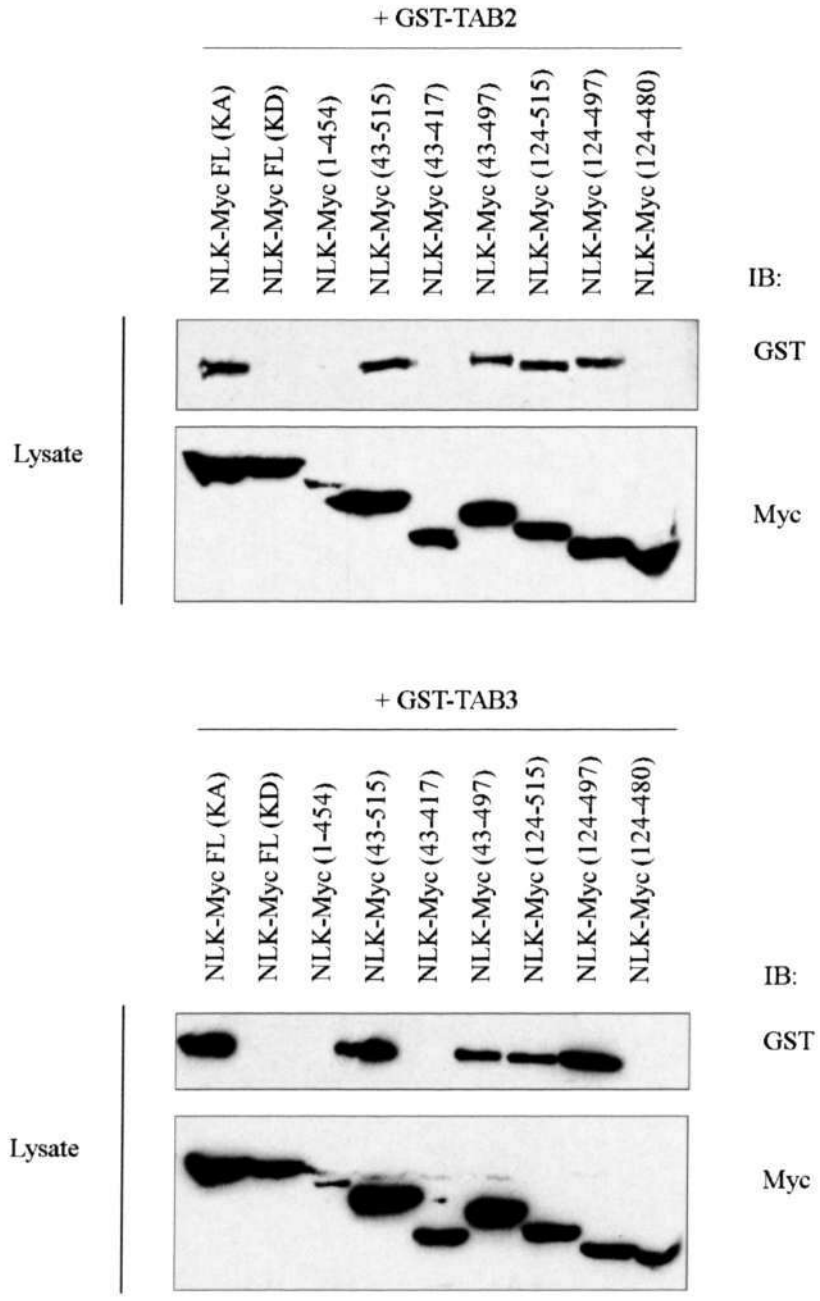
Figure 3.2 Domain mapping of interaction between NLK and TAB2 or TAB3

(A) C terminus of TAB2 interacts with TAK1 and NLK –

HEK293 cells were transiently transfected with full length or deletion mutants HA-TAB2 [1-203], [1-318], [1-400], [283-481], [283-527], [283-588], [283-693], [341-693] and GST-NLK or GST-TAK1. 0.5 mg of cell extracts were pulled down with glutathione beads and co-purifying proteins were stained with Coomassie Blue or analyzed by western blot.

(B) Schematic digrams of TAK1 and NLK interacting domains in TAB2 were shown.

C

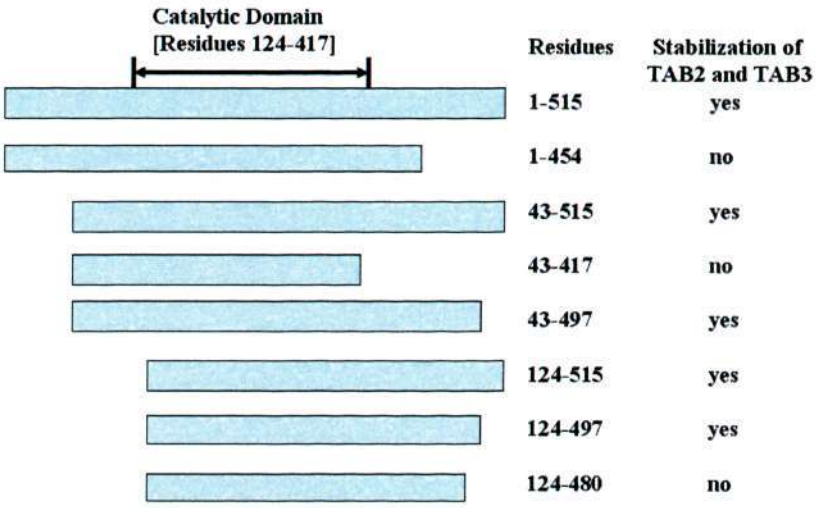


3.2 Domain mapping of interaction between NLK and TAB2 or TAB3

(C) Mapping the binding region of NLK and TAB2/3 by accessing the stabilization of TAB2/3 expression by NLK –

HEK293 cells were transiently transfected with myc-NLK Kinase Active, myc-NLK Kinase Dead, myc-NLK [1-454], myc-NLK [1-515], myc-NLK [43-417], myc-NLK [43-497], myc-NLK [124-515], myc-NLK [124-497], myc-NLK [124-480] and full length GST-TAB2/3. The expression of the proteins was confirmed by western blotting with antibodies against myc and GST.

D



E

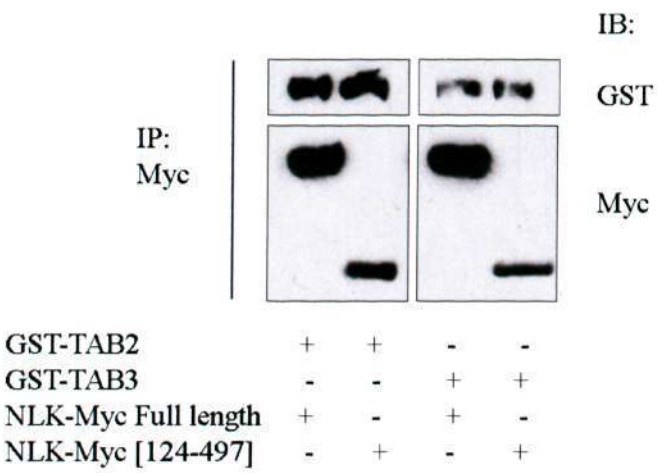


Figure 3.2 Domain mapping of interaction between NLK and TAB2 or TAB3

(D) Schematic representation of NLK mutants which are used to studied and summarizes the result obtained from Figure 3-2C.

(E) Kinase domain and C terminal extension of NLK is essential for its interaction with TAB2/3- 0.5mg of lysates from (C) were used to perform immunoprecipitation with anti-myc antibody to detect the interaction between smallest fragment or full length NLK and GST-TAB2/3.

3.3 Phosphorylation of TAB2 and TAB3 by NLK

After showing that NLK interacts with TAB2/3, we examined whether NLK can directly phosphorylate TAB2/3. Interestingly, as shown in Figure 3-1A, we found that HA-TAB2/3 co-expressed with GST-NLK migrated at a slower mobility rate compared to proteins co-expressed with GST-TAK1.

To determine whether the slower migration of HA-TAB2/3 on SDS-PAGE is due to the hyper-phosphorylation of TAB2/3 by NLK, HA-TAB2/3 was immunoprecipitated using anti-HA antibodies followed by treatment with λ-phosphatase. As shown in Figure 3-3A, the slow migration of HA-TAB2/3 on SDS-PAGE was reversed by treatment with λ-phosphatase suggesting that the band shift was caused by the presence of extra phosphate groups possibly catalyzed by GST-NLK on HA-TAB2/3. To further demonstrate that phosphorylation of TAB2/3 is catalyzed by NLK, we purified GST-TAB2/3 separately and performed *in vitro* kinase assay by incubating with kinase active or kinase dead myc-NLK [D270A]. Figure 3-3B shows that phosphorylation of TAB2/3 by NLK can only be achieved by full length kinase active NLK but not by the kinase dead mutant of NLK.

We next investigate whether exogenous NLK is sufficient to phosphorylate endogenous TAB2/3 proteins in cells. Therefore, kinase active myc-NLK, kinase dead myc-NLK and pCMV-5 empty vector were expressed in HEK293 cells. The lysates were immunoprecipitated with anti-TAB1 antibody to purify TAK1 complex and then employed to perform the "mobility shift" assay. As shown in the Figure 3-3C lane 2, ectopic expression of kinase active myc-NLK caused the slower migration of TAB2 and TAB3 on SDS-PAGE. This result further proves that NLK phosphorylates endogenous TAB2 and TAB3 proteins.

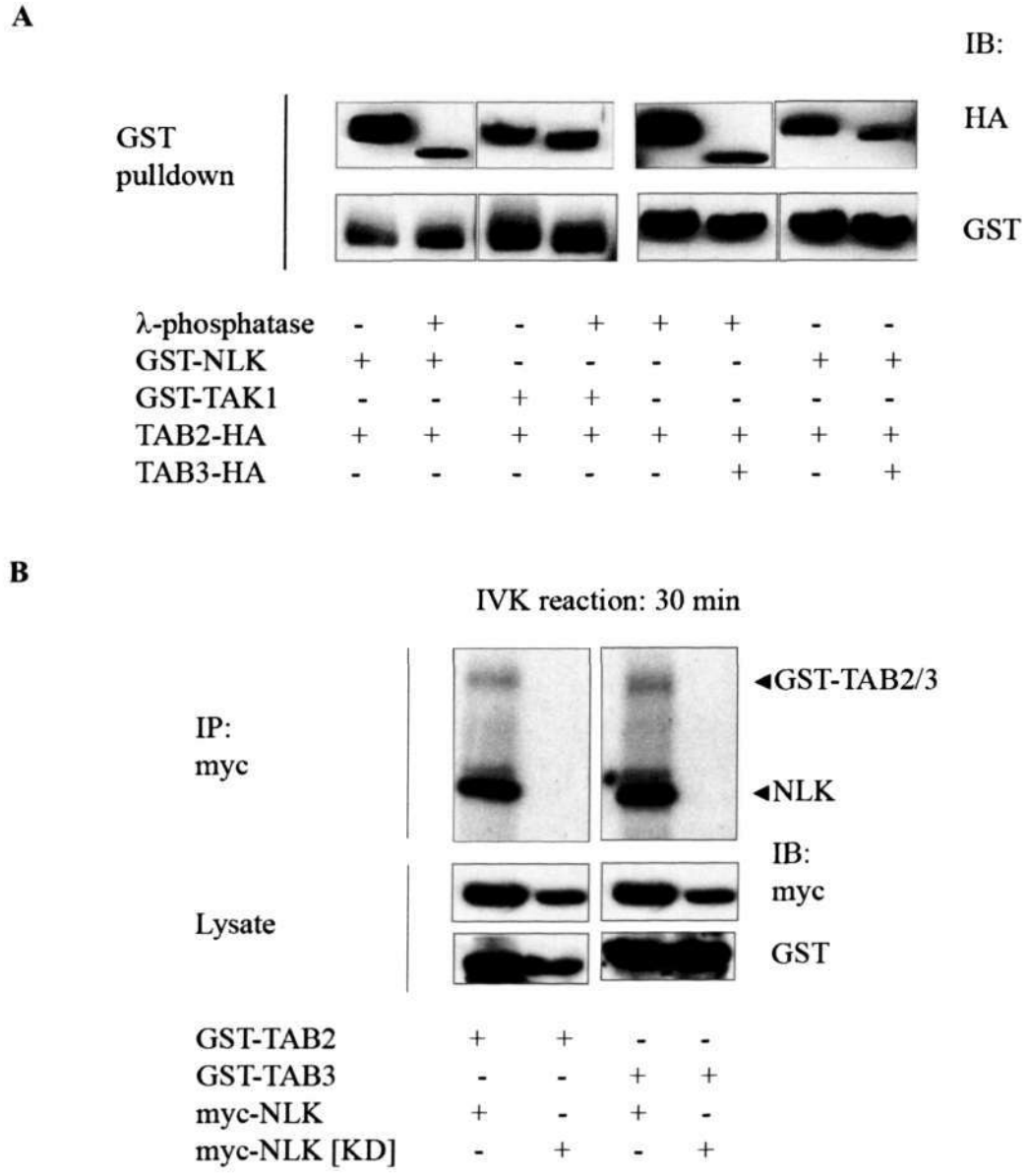


Figure 3.3 TAB2 and TAB3 are phosphorylated by NLK

(A) Dephosphorylation of TAB2/3 which is coexpressed with TAK1 or NLK–

HEK293 cells were transfected with expression vectors coding for NLK-Myc and TAB2/3-HA. 0.5mg of cell lysates were immunoprecipitated with anti-HA antibody and the immunoprecipitates treated with λ-phosphatases or wash buffer followed by immunoblotting with anti-HA antibody.

(B) Kinase active NLK phosphorylates TAB2/3 *in vitro* –

HEK293 cells were used to overexpress GST-TAB2/3. 0.5mg of cell lysates were subjected to GST-pulldown and the purified proteins were incubated with NLK kinase active or kinase dead in the presence of [γ^{32}P] labeled ATP for the indicated times. The loading input of GST tagged proteins and Myc tagged NLK.KA or NLK.KD proteins are indicated in the immunoblots.

C

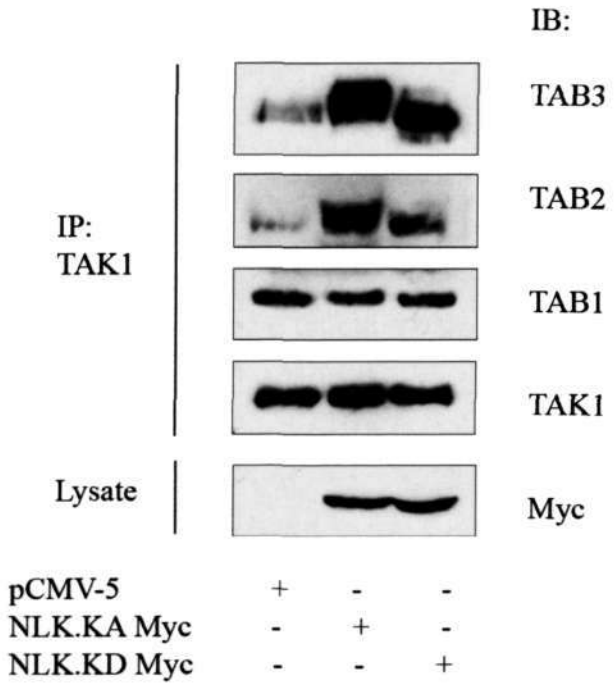


Figure 3.3 TAB2 and TAB3 are phosphorylated by NLK

(C) Ectopic expression of kinase active NLK causes a slow mobility bandshift in endogenous proteins TAB2 and TAB3 –

HEK293 cells were transiently transfected with expression vectors coding for NLK-Myc, kinase dead NLK [D270A]. 2 mg of lysates were used to perform immunoprecipitation with anti-TAB1 antibody and were checked for the presence of TAK1, TAB1 and TAB2/3 with their respective antibodies.

3.4 C terminal region of TAB2 is phosphorylated by NLK

We also attempted to map NLK phosphorylation sites on TAB2. GST-TAB2 truncates co-expressed with or without myc-NLK in HEK293 cells were subjected to GST pulldown and performed an *in vitro* kinase assay. As indicated in Figure 3-4A lane 4&5, GST-TAB2 [283-693] and [516-693] were heavily phosphorylated by NLK suggesting the NLK phosphorylation sites in the residues between 283 and 693 of TAB2. The *in vitro* kinase assays results are in line with the data obtained from 'mobility shift' assay when GST-TAB2 truncates were co-expressed with myc-NLK. As shown in the Figure 3-4 B, C terminal fragment of TAB2 [401-693] migrated slower on the SDS-PAGE in lane 6 and 7 in the presence of kinase active NLK.

The results obtained from in vitro kinase assay data and "mobility shift" assay indicates that C terminal region of TAB2 contains putative phosphoryaltion site of NLK. To further map the putative phosphorylation sites, bacteria purified C terminal fragment of TAB2 [481-693] was incubated with [γ^{32}P] labeled ATP (hot ATP) or unlabeled ATP (cold ATP) in the presence of active GST-NLK purified from mammalian cells. [Figure 3-4C] To further map the NLK phosphorylation residue/residues on TAB2 fragment, [γ^{32}P] labeled GST-TAB2 protein that had been phosphorylated by NLK was digested with trypsin and chromatographed on C_{18} column, which resolve one major tryptic phosphopeptide at the fraction number F78. (Figure 3-4D) Unlabled GST-TAB2 proetin that have been phosphorylated by NLK was also digested with trypsin and chromatographed accordingly. The unlabeled trypic phosphopeptide collected from the same fraction number F76 was analyzed by Mass Spectormetry. The peptide was identified as residuces 580-596 of NLK phosphorylated at Ser 582 (Figure 3-4D lower panel).

A

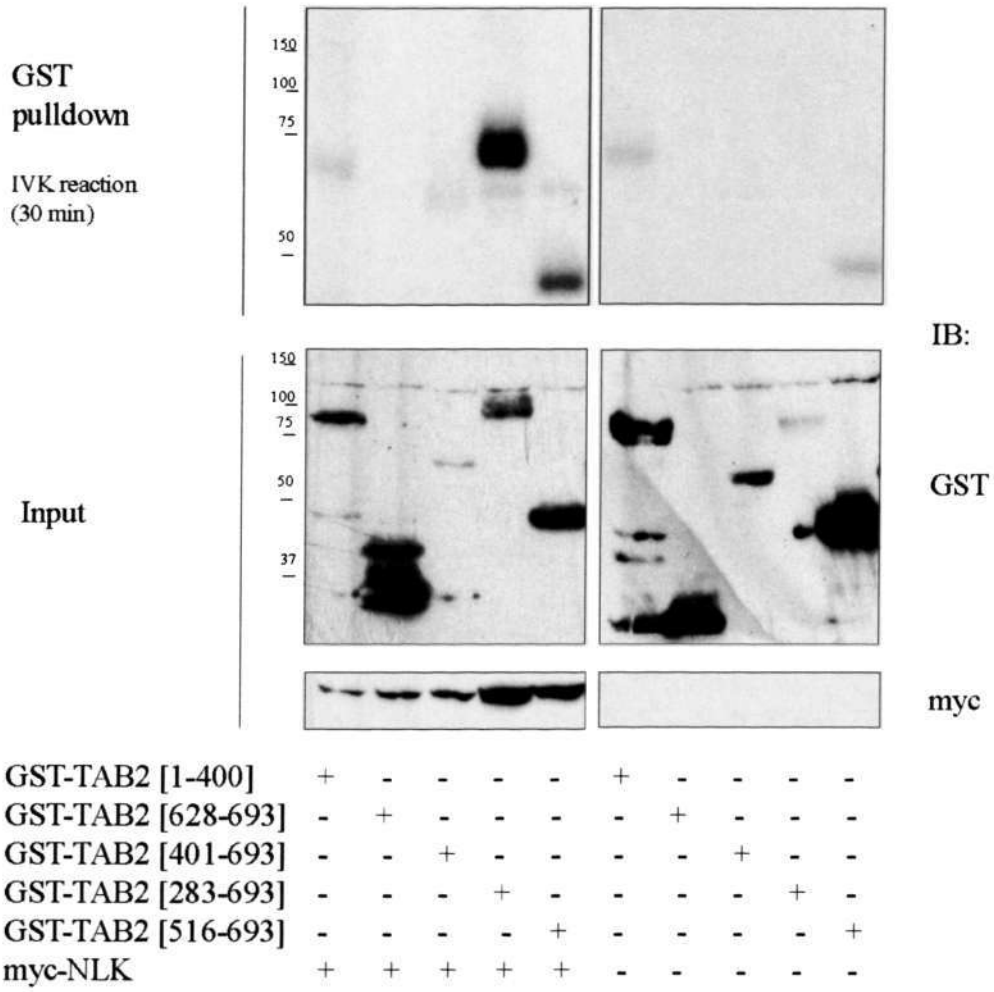


Figure 3.4 Phosphorylation of TAB2 truncates by NLK

(A) *In vitro* phosphorylation of TAB2 truncates by NLK –

HEK293 cells were transfected with the plasmids indicated and subjected to GST pulldown. The bound proteins were subjected to *in vitro* kinase assay at at 37°C for 30min in the presence of radioactive labeled [γ^{32}P] ATP or unlabelled ATP. Gels were stained with Coomassie Blue and autoradiographed at -80°C for overnight. The input of the GST-TAB2 truncates proteins and myc-NLK was shown in the anti-GST and anti-myc immunoblots respectively.

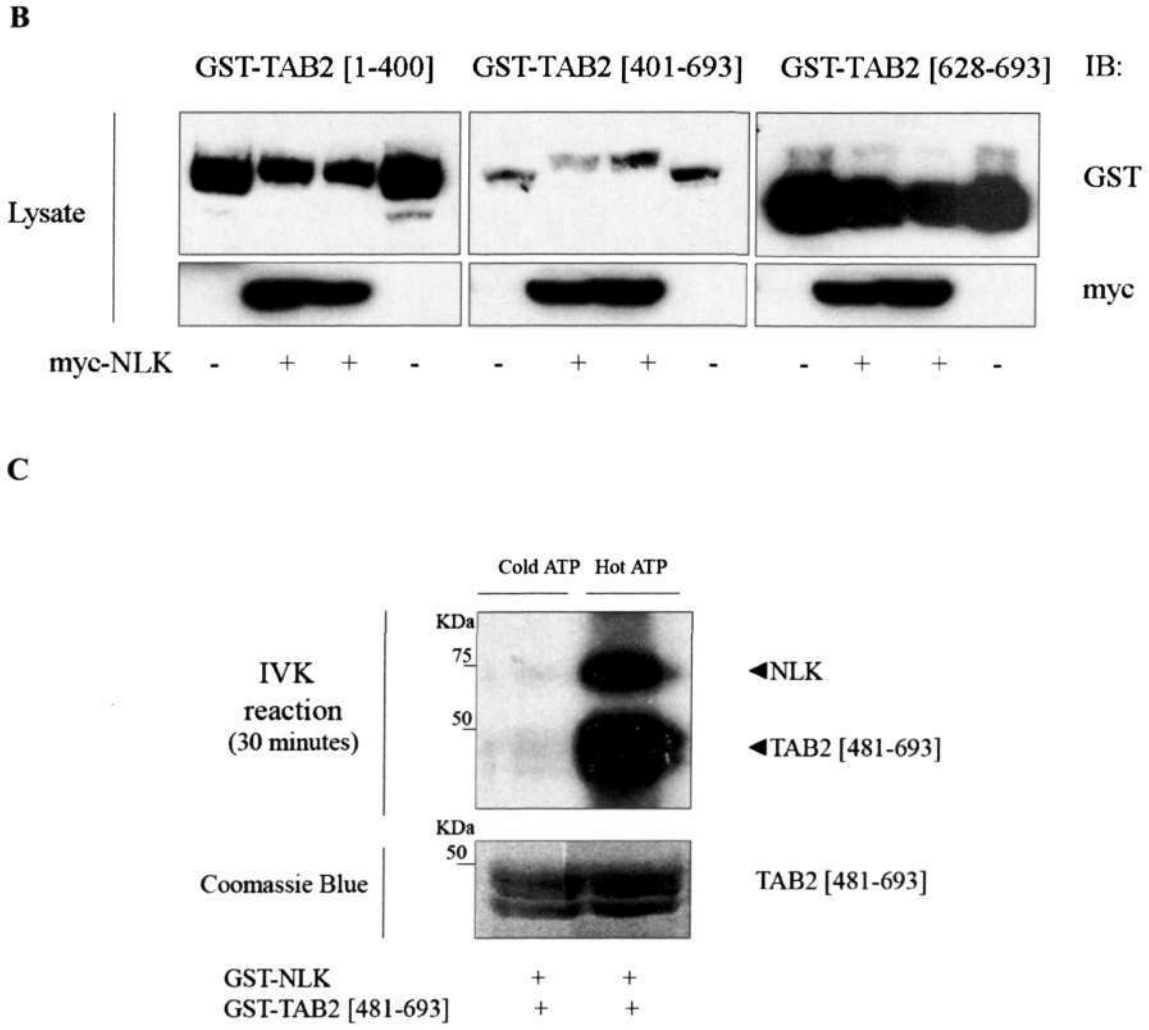


Figure 3.4 Phosphorylation of TAB2 truncates by NLK

(B) NLK phosphorylates C terminal region of TAB2 in cells upon overexpression– HEK293 cells were transiently transfected with deletion mutants of GST-TAB2 [1-400], [481-693], [628-693] and myc-NLK in HEK293 cells. Cell extracts were analyzed by western blot.

(C) NLK phosphorylates C terminal region of TAB2 *in vitro*– 2.0 μg of GST-NLK purified from HEK293 cells was incubated with E.coli purified GST-TAB2 [481-693] at 37°C for 30min in the presence of radioactive labeled [γ^{32}P] ATP or unlabelled ATP. Gels were stained with Coomassie Blue and exposed for overnight. The input of purified GST-TAB2 [481-693] was shown after Coomassie Blue staining.

D

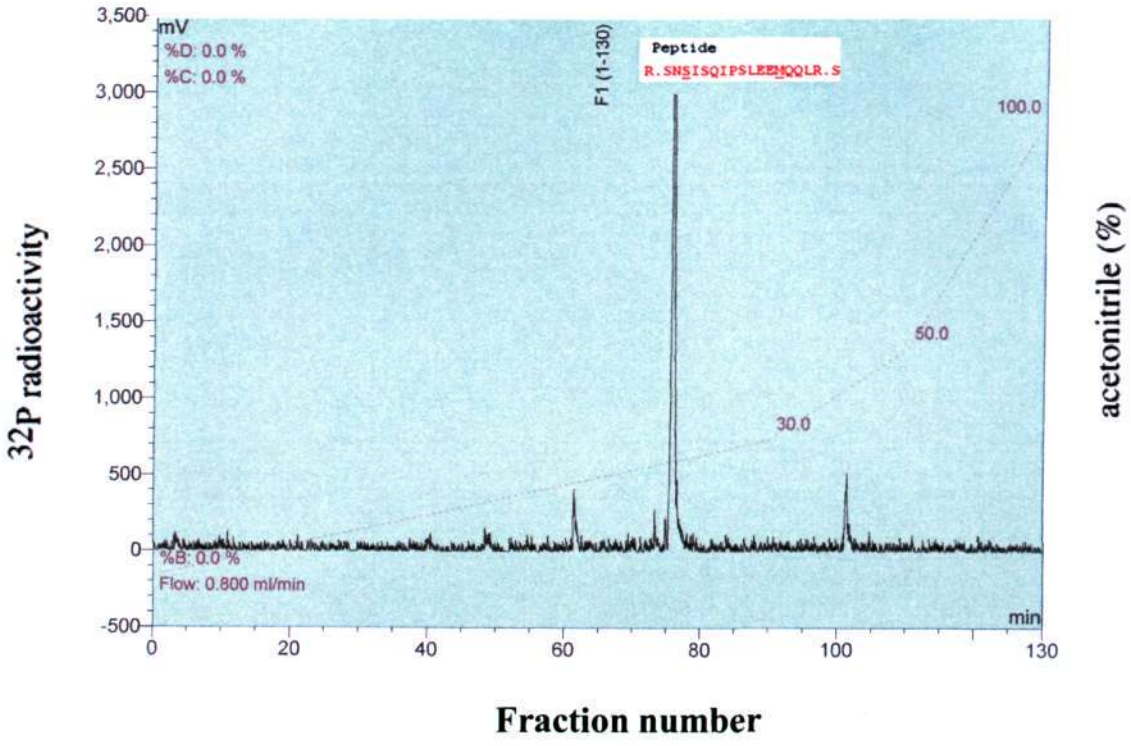


Figure 3.4 Phosphorylation of TAB2 truncates by NLK

(D) Identification of the residues on TAB2 phosphorylated by NLK –

GST-TAB2 [481-693] was phosphorylated with active GST-NLK from (C), denatured in SDS and subjected to SDS-PAGE. The band corresponding to [γ^{32}P] labelled GST-TAB2 was visualized by staining with Coomassie blue [Figure 3-4C], excised and subjected to digestion with trypsin. The tryptic phosphopeptides were separated by HPLC on a Vydac C18 column equilibrated in 0.1% (v/v) trifluoroacetic acid. The Column was developed with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (borken line).

The mass of the peptide from peak fraction determined by mass spectrometry corresponded to residues 580-596 [SNSISQIPSLEEMQQLR] of TAB2 plus one phosphate group. Ser582 was identified as the phosphorylation site by mass spectrometry. Mass Spectrometry analysis was kindly performed by Dr. Newman Sze laboratory, SBS, NTU.

3.5 Binding of TAB2/3 to NLK doesn't augment its kinase activity

It was reported that functional interaction between TAK1 and TAB2/3 is essential for TAK1 activity in IL-1 and TNF signaling. In addition, co-expression of TAB2/3 with TAK1 also enhances the kinase activity of TAK1. [71] Based on this knowledge, we asked whether TAB2/3 can enhance the kinase activity of NLK. Two methods are commonly used to assay protein kinase activity. Firstly, phospho antibodies which detect the phosphorylation sites in the activation loop of the kinases are used to measure the kinase activity of the protein. Based on its homology to MAP kinases and cyclin dependent kinases, NLK is predicted to be regulated by phosphorylation on its activation loop at Thr 286. Although no one has yet shown a direct correlation of NLK activity and Thr 286 phosphorylation in biochemically, mutation of Thr 286 was shown to abolish NLK activity and its function *in vivo*. [6] Since no commercial antibody was available in the market at the time of our study, we attempted to raise the phospho Thr 286 antibodies against a peptide sequence (CDESRHM**pT**QEVVTQY). However, the sensitivity of the phospho antibody against endogenous NLK protein was very low and did not provide any satisfactory results in our study. [Data not shown]

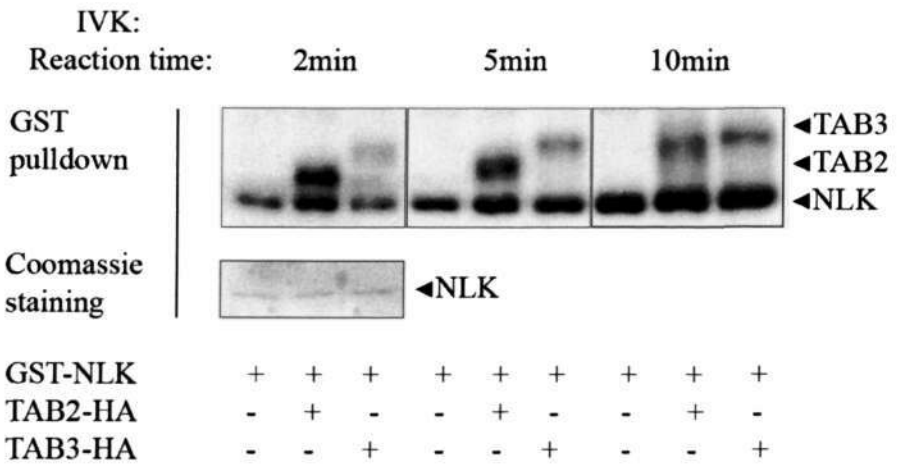
Therefore, we used a second method, conventional *in vitro* kinase assay to measure NLK activity and its autophosphorylation *in vitro*. A GST pull down was carried out by using co-transfected lysates of GST-NLK and HA-TAB2/3. The co-precipitated lysates were subjected to an *in vitro* kinase assay by incubation with [γ^{32}P] labeled ATP. We carried out the *in vitro* kinase assay for different reaction time [from 2 min to 10 min] to avoid signal saturation. As shown in the Figure 3-5, the kinase activity of GST-NLK as was not further enhanced by co-expression of HA-TAB2/3 for all the time points indicated. However, phosphorylation of TAB2 and TAB3 by NLK was detected in all the reactions.

It is a possibility that TAB2/3 did not further increase NLK activity because NLK was already fully activated upon expression in HEK293 cells. Therefore, we replaced purified GST-NLK with *in vitro* translated NLK and coincubated with HA-TAB2/3. In this case, we used myelin binding protein as the substrate to measure NLK kinase activity.

Similar to the results using proteins purified mammalian cells, NLK kinase activity was not further enhanced by the presence of TAB2 or TAB3. [Data not shown]

Collectively, observations from this study suggest that interaction with TAB2 and TAB3 does not augment NLK kinase activity.

A



B

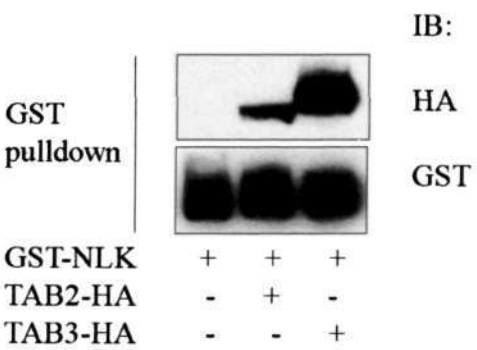


Figure 3.5 TAB2 and TAB3 do not enhance the kinase activity of NLK

A. GST-NLK was co-expressed with or without HA-TAB2/3 in HEK293 cells. 0.5 mg of cell lysates was mixed with glutathione sepharose beads and GST-pulldown carried out. Purified proteins were used to perform an *in vitro* kinase assay in the presence of radioactive labeled [γ^{32}P] ATP at 30°C for 2 minutes, 5 minutes and 15 minutes respectively. Proteins were stained with Coomassie Blue and autoradiographed at -80°C for overnight.

B. Aliquots of the proteins purified from GST pulldown assay from (A) were checked by western blotting with anti-GST and anti-HA antibodies.

3.6 Binding of TAB2/3 to NLK does not facilitate the direct activation of NLK by its upstream kinases

Our previous finding show that TAB2 and TAB3 are not required for NLK kinase activity and their presence does not enhance NLK activity. Therefore, we asked whether TAB2/3 acts as scaffold between TAK1 and NLK to facilitate the direct activation of NLK by TAK1. We performed "i*n vitro* kinase assay" to check whether TAK1 can directly phosphorylate NLK *in vitro*. TAK1 complex consisting of TAK1-TAB1-TAB2/3 was over-expressed and purified from HEK293 cells and incubated with *E.coli* purified GST-NLK [124-480] in the presence of cold ATP (unlabeled ATP) or hot ATP (γ^{32}P radioactive labeled ATP) at 30°C for 30 min. However, phosphorylation of NLK by TAK1 was not detected in Figure 3-6A, suggesting that TAB2 or TAB3 does not mediate NLK activation by TAK1 and TAK1 may not be a direct upstream kinase of NLK.

And previously, protein kinase HIPK2 has also been reported to function as an intermediate between TAK1 and NLK in Wnt signaling. [9] The MAPKK, MKK6 is a well known characterized substrate of TAK1 and there is a possibility that MKK6 may activate MAPK member NLK. [64] Therefore, it supports the idea of the existence of an intermediate kinase between TAK1 and NLK and we tested our hypothesis by including MKK6 or HIPK2 in our *in vitro* kinase assay. [86] [Figure 3-6E]

Mammalian purified TAK1 complex and *E.coli* purified GST-NLK [124-480] were co-incubated with MBP-MKK6 or HIPK2 in the presence of [γ^{32}P] ATP at 30°C for 30 min reaction. However, phosphorylation of NLK by TAK1 was not detected in the presence of either MKK6 or HIPK2 as shown in Figure 3-6C & D. MKK6 phosphorylation by TAK1 [Figure 3-6C lane 5&7] or autophosphorylation of HIPK2 [Figure 3-6D lane 3&4] was readily detectable in the autoradiographs indicating that purified TAK1 complex or HIPK2 is active. The protein input used in the *in vitro* kinase assay is shown in the SDS-PAGE stained with Coomassie Blue. Altogether our *in vitro* kinase assay results suggest that TAB2/3 does not facilitate the direct activation of NLK by its upstream kinases TAK1 or MKK6 or HIPK2.

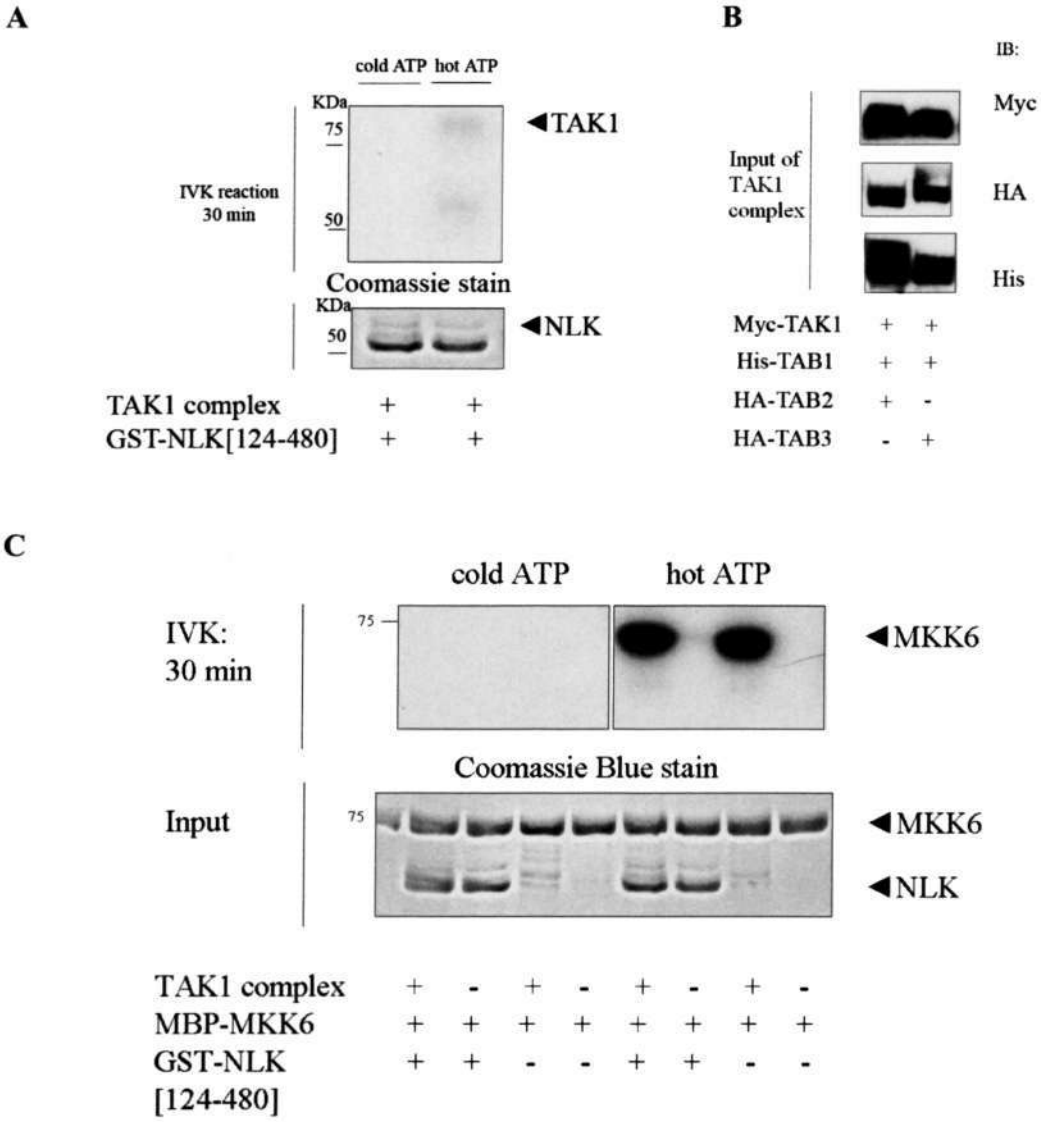


Figure 3.6 TAK1 and HIPK2 are not direct upstream kinases of NLK

(A) TAK1 is not a direct upstream kinase of NLK –

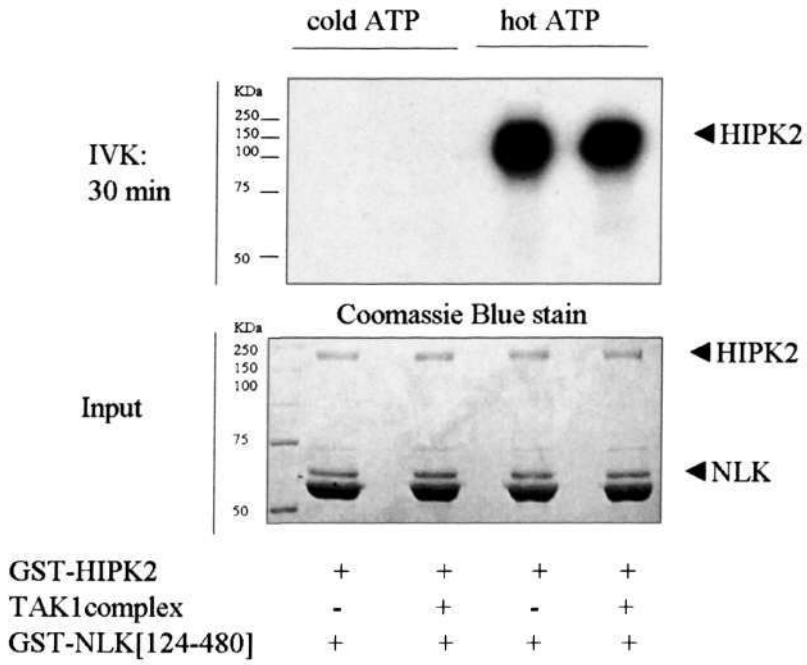
0.2 μg of TAK1 complex purified from HEK293 cells was incubated with 10 μg of GST-NLK [124-480] at 37°C for 30min in the presence of radioactive labeled [γ^{32}P] ATP or unlabelled ATP. Gels were stained with Coomassie Blue and dried before autoradiography at -80°C for overnight. The input of purified MBP-MKK6 and GST-NLK were shown after Coomassie Blue staining.

(B) The input of TAK1 complex used in the assay was shown in the western blots.

(C) TAK1 and MKK6 are not direct upstream kinases of NLK –

0.2 μg of TAK1 complex purified from HEK293 cells was incubated with 3 μg of MKK6 and 10 μg of GST-NLK [124-480] at 37°C for 30min in the presence of radioactive labeled [γ^{32}P] ATP or unlabelled ATP. Gels were processed similarly as described in (A).

D



E

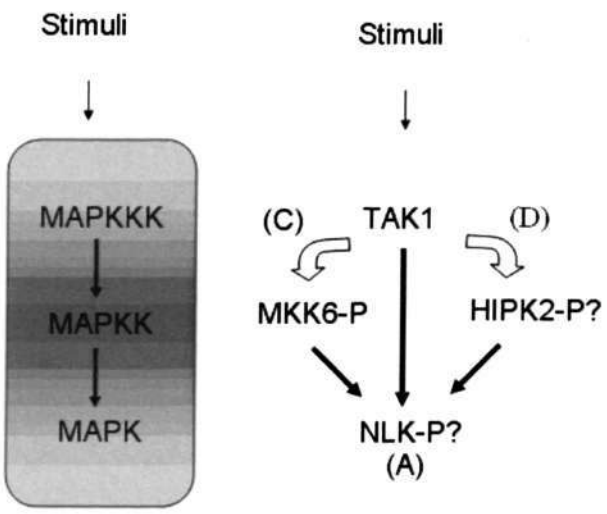


Figure 3.6 TAK1 and HIPK2 are not direct upstream kinases of NLK

(D) HIPK2 is not a direct upstream kinase of NLK –

0.2 µg of TAK1 complex purified from HEK293 cells and/or GST-HIPK2 purified from 0.5 mg of lysates were incubated with substrates 10 µg of GST-NLK [124-480] at 37°C for 30min in the presence of radioactive labeled [γ^{32}P] ATP or unlabelled ATP. Gels were stained with Coomassie Blue and dried before autoradiography at -80°C for overnight. The input of purified MBP-MKK6 and GST-NLK were shown after Coomassie Blue staining.

(E) Summary of the *in vitro* kinase assay reactions performed in Fig A, C and D. Their identities are shown as representatives of members of MAPK signaling cascade.

3.7 NLK does not disrupt the interaction between TAK1 and its binding proteins

Next we asked whether NLK binding to TAB2/3 abrogates their interaction with TAK1. HEK293 cells were transfected with GST-TAK1, Flag-TAB1, HA-TAB2/3 and myc-NLK and the cell lysates used for GST pulldown. NLK-myc co-precipitated together with TAB2/3 and GST-TAK1 in lane 2 and 3 but not with TAB1 and GST-TAK1 in lane 1. Since all three proteins TAK1, TAB2/3 and NLK are co-purified as shown in Figure 3-7A, it suggests that NLK does not compete with TAK1 for TAB2/3 and they may have independent binding sites on TAB2/3.

The independent binding of NLK to TAB2/3 was accompanied by additional phosphorylation on TAB2/3 in Figure 3-7B. HEK293 cells were transfected with indicated plasmids and the cell lysates were subjected to GST pull down assay. As shown in the Figure 3-7B, lanes 3&5 compared with lane 4&6, the slower migration of TAB2 on SDS-PAGE suggests that NLK is able to interact with and phosphorylate TAB2 when it is bound to TAK1. The similar result obtained for TAB3 as shown in the Figure 3-7B, lane 7&9 compared with lane 8&10.

The result shows that interaction between TAK1 and TAB1/2/3 is not disrupted even when NLK modifies TAB2/3 by phosphorylation. This interaction study suggests that NLK could form a supercomplex with TAK1-TAB1-TAB2 or TAK1-TAB1-TAB3 where it specifically phosphorylates TAB2/3.

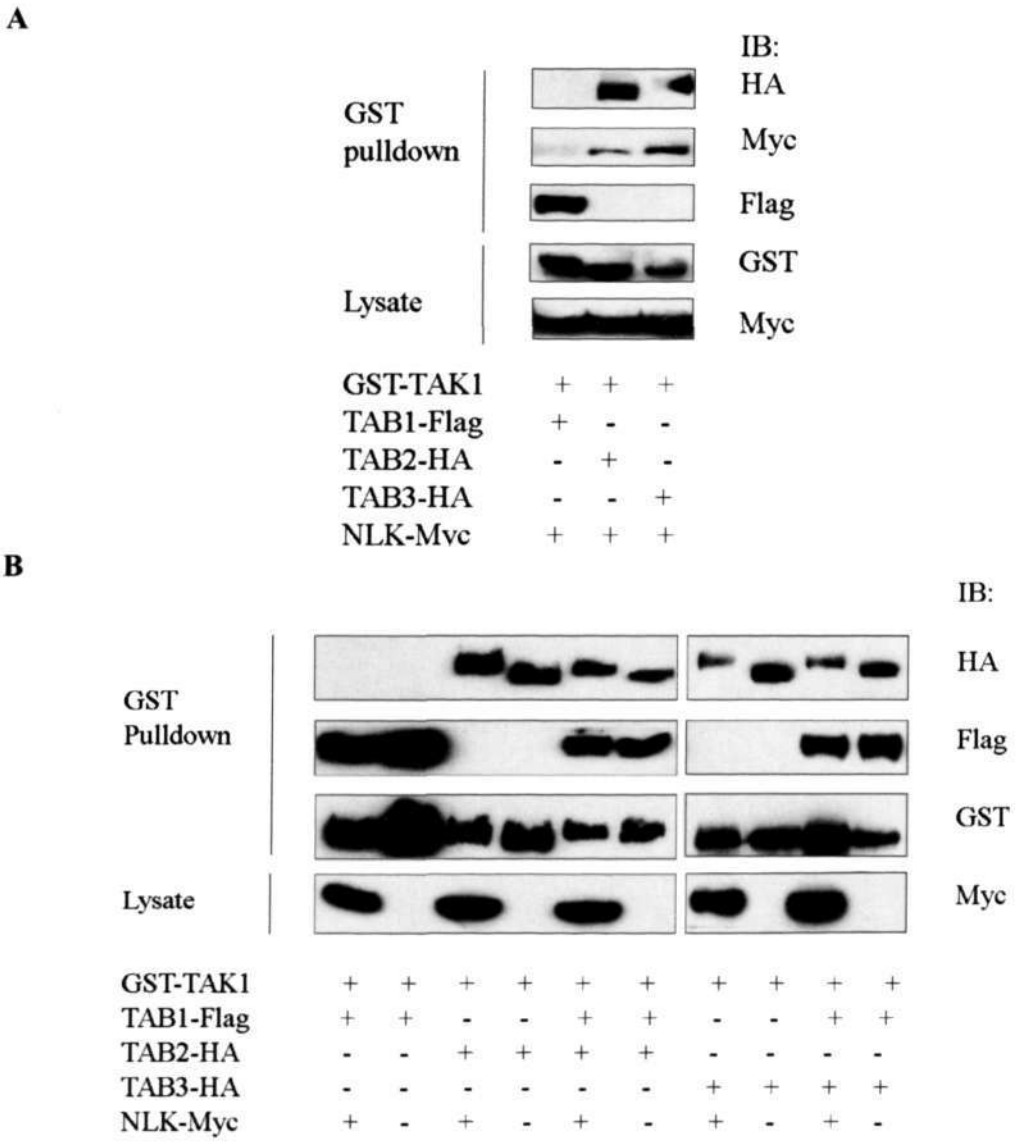


Figure 3.7 NLK does not disrupt the interaction between TAK1 and it binding proteins

(A) NLK does not compete with TAK1 for TAB2/3 –

HEK293 cells were transiently transfected with plasmids encoding myc-NLK, GST-TAK1 and HA-TAB2/3. Whole cell lysates were mixed with glutathione-sepharose beads and GST pulldown carried out. The input of the proteins was also shown by western blotting with antibodies against GST, myc and HA.

(B) NLK does not disrupt the interaction between TAK1 and TAB1/2/3 –

HEK293 cells were transiently transfected with plasmids encoding GST-TAK1-TAB1-Flag and/or TAB2/3-HA in the presence or absence of NLK-myc as indicated. Whole cell lysates subjected to GST pulldown with glutathione sepharose beads and bound proteins were determined by anti-GST, anti-HA, anti-Flag and anti-Myc antibodies respectively.

3.8 NLK suppresses the activation of TAK1 via TAB2 or TAB3

To gain insight into the functional significance of TAB2 and TAB3 phosphorylation by NLK, we systematically examined the activity of TAK1 and its downstream kinases p38α, JNK and IKK in the presence of exogenous NLK. It has been previously shown that phosphorylation of TAB2/3 by p38α is a negative feedback control mechanism of TAK1 activity in TAK1-MAPK pathway. [57, 64]

In this study, we have examined the possibility of negative feedback regulation of TAK1 by NLK. Mutational experiments defined Thr-184, Thr-187 and Ser-192 in the activation loop as residues important for TAK1 kinase activity. [59, 87] The TAK1 pThr-187 antibody is widely used by many research groups due to its sensitivity and availability; therefore, we have used anti pT187 TAK1 antibody to detect the kinase activity of TAK1 in cells.

To demonstrate that NLK regulates the kinase activity of TAK1 through phosphorylation of TAB2/3, we have expressed NLK in a dose-dependent manner with the fixed equal amount of GST-TAK1 and HA-TAB2. As shown in the Figure 3-8A lane 1 and 2, pThr 187 of TAK1 signal was drastically reduced when TAB2 is heavily phosphorylated in the presence of high dose of NLK. However, when GST-TAK1 was co-expressed with NLK in dose-dependent manner, TAK1 activity or phosphorylation was unchanged in the Figure 3-8A from lane 6 to 10.

To confirm our results obtained with pThr187 antibody, we have measured the kinase activity of TAK1 using MKK6 as substrate [64] in the presence of exogenous NLK. Whole cell lysates from HEK293 cells expressing GST-TAK1-TAB1-Flag and/or HA-TAB2, with or without myc-NLK were used to perform GST pulldown assay followed by an *in vitro* kinase assay. As shown in Figure 3-8B lane 8 and 11, MKK6 phosphorylation by TAK1 and autophosphorylation of TAK1 (measured by Thr 187 antibodies) were drastically reduced in the presence of kinase active myc-NLK, but not with kinase dead NLK. Collectively, our result demonstrates that NLK down-regulates the kinase activity of TAK1 via modification of TAB2/3.

A

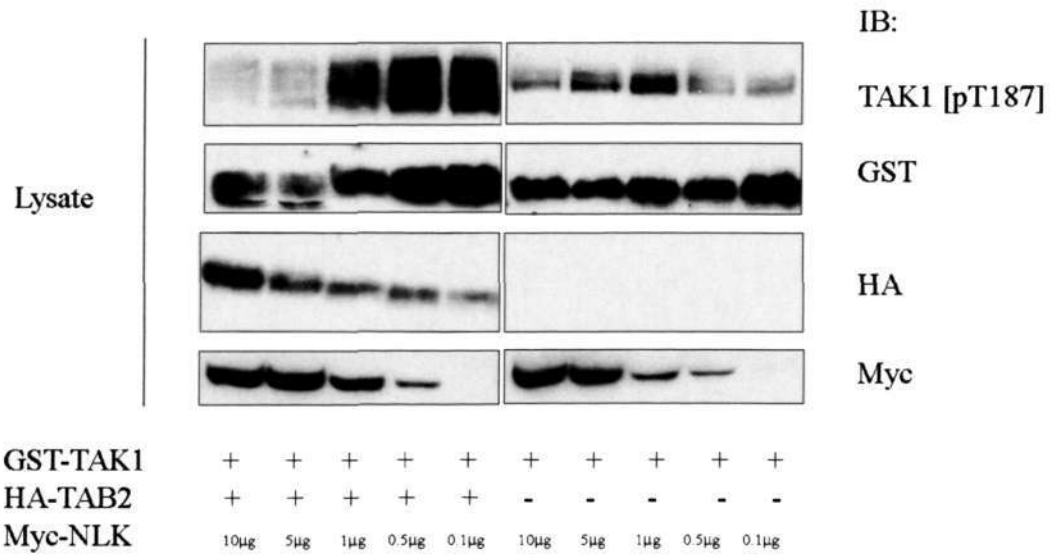


Figure 3.8 Inhibition of TAK1 activity by NLK requires TAB2 or TAB3

(A) NLK suppresses activation of TAK1 via TAB2 in dose dependent manner –

HEK293 cells were transfected with fixed amount of plasmid encoding GST-TAK1 and HA-TAB2 while myc-NLK was transfected in a dose dependent manner. Whole lysates were immunoblotted anti-phospho Thr 187 TAK1, anti-GST, HA & myc antibodies respectively.

B

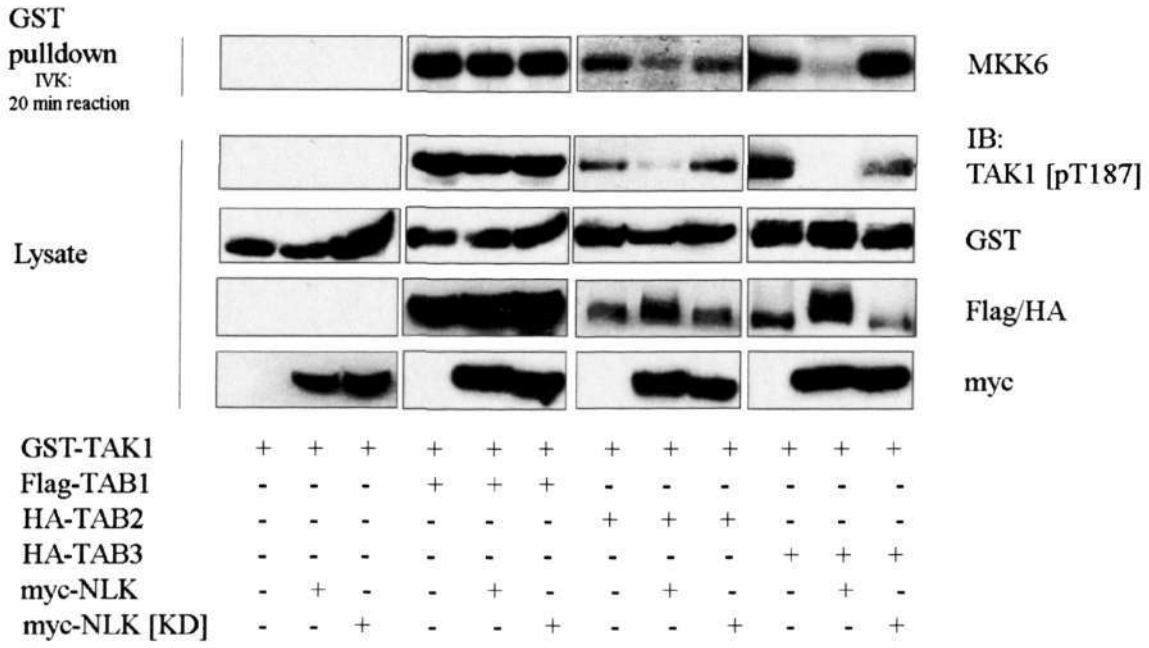


Figure 3.8 Inhibition of TAK1 activity by NLK requires TAB2 or TAB3

(B) NLK suppresses the activation of TAK1 via TAB2 or TAB3–

HEK293 cells were transfected with plasmids encoding GST-TAK1-TAB1-Flag, GST-TAK1-TAB2-HA and/or TAB1-Flag in the presence or absence of NLK-Myc. 0.5 mg of cell lysates were subjected to GST-pulldown assay and TAK1 kinase activity was measured by an *in vitro* kinase assay using MKK6 as a substrate and [γ^{32}P]ATP at 30°C for 5 minutes. The lysates were also used to measure TAK1 kinase activity by anti-phospho Thr 187 TAK1 antibody. The lysates were immunoblotted with anti-GST, anti-flag, anti-HA and anti-myc antibodies to ensure equal loading of input.

3.9 NLK knockdown increases TAK1 activation

We then examined the effect of shRNA mediated NLK knockdown on TAK1 activity in cells. To check the efficiency of the knockdown in cells, HEK293 cells were transfected with five shRNA constructs targeting different regions of the NLK mRNA and a plasmid bearing GST-NLK or myc-NLK [Fig. 3-9A]. Among the five contructs, NLK shRNA5 construct was found to be the most effective followed by NLK shRNA3 and subsequently used to silence expression of endogenous NLK in this study.

We tested the effect of NLK knockdown on TAK1 activity by using pT187 antibody or performing *in vitro* kinase assay. Endogenous TAK1 was immunoprecipitated from the cells expressing NLK shRNA or scrambled shRNA and the activity of TAK1 probed with pThr187 antibody. Cells with NLK "knocked down" showed a drastic increase in Thr187 phosphorylation both in unstimulated or stimulated with TNFα for 2 min and 5 min [Figure 3-9B]. The activity of immunoprecipitated endogenous TAK1 was also assayed directly using [γ^{32}P] ATP and MKK6 as substrate. TAK1 from cells with NLK "knocked down" displayed a higher activity (indicated by increased phosphorylation of its substrate MKK6) compared to cells expressing scrambled shRNA at zero time point or indicated time points of TNFα stimulation (Figure 3-9B). This shows that TAK1 is negatively regulated by NLK even under basal resting conditions.

The experiments performed in Figure 3-9B were repeated by using shRNA 3 which achieved more than 50% NLK knockdown efficiency as shown in Fig 3-9A. Consistent with the results obtained with shRNA5, increased TAK1 activity (indicated by increased phosphorylation of TAK1 T187) was also observed in NLK depleted cells compared with control cells. [Figure 3-9D]

Next we checked whether NLK also negatively regulate duration of TAK1 activation upon cell stimulation. Cells transfected with NLK shRNA5 or control scramble shRNA were stimulated with TNFα for different times [2, 5, 10, 20, 60 and 120 min] and TAK1 activity was measured by using pT187 antibodies. The result shown in Figure 3-

9C illustrates that phosphorylation of Thr187 on TAK1 is highest after 5-10 min of stimulation. The phosphorylation was diminished at 60 min or 120 min in both NLK knocked down or control cells suggesting that NLK does not affect the duration of TNFα mediated TAK1 activation in cells.

We also proved that an increase in Thr187 phosphorylation or activity of TAK1 is due to the depletion of NLK. NLK knockdown cells were rescued by expression of a wild type or NLK plasmid that is sensitive or resistant to shRNA knockdown respectively. After stimulation with TNFα, phosphorylation of TAK1 at T187 residue was lower in cells expressing the shRNA resistant NLK plasmid compared to the wild type NLK plasmid. [Figure 3-9E] Depletion of endogenous NLK expression in knockdown cells was shown by western blotting of cell lysates using anti-NLK antibody. [Figure 3-9B-E]

To summarize the results obtained from this section, the band intensity of immunoblot of phospho T187 TAK1 and total TAK1 were analyzed by image J software. As shown in Figure 3-9F, the bar graphs represents the two to three fold increase in TAK1 activity in NLK knockdown cells with respect to control cells [Figure 3-9 B or D] or cells which were rescued with shRNA resistant NLK [Figure 3-9 E]. In this regard, our finding reveals that NLK suppresses the activity of TAK1 in TNFα signaling.

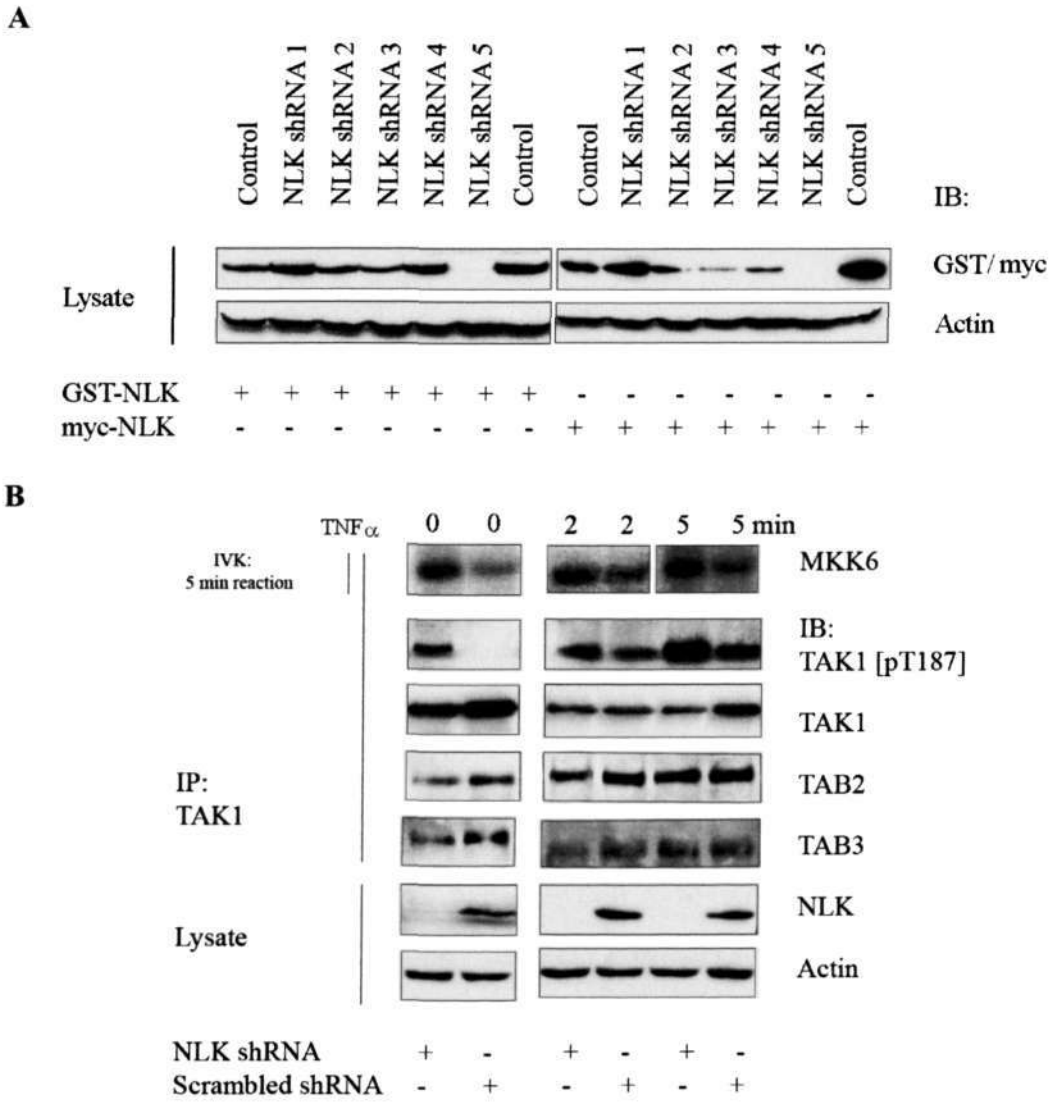


Figure 3.9 NLK inhibits TAK1 activation in cells

(A) Testing the efficiency of 5 different constructs of NLK shRNA –

HEK293 cells were co-transfected with plasmids encoding myc-tagged or GST-tagged NLK and five different shRNA plasmid constructs targeting NLK or control "scrambled" plasmid. Protein lysates were probed by western blotting with anti-myc, anti-GST and anti-actin antibodies.

(B) NLK knockdown increases TAK1 activation in cells -

HEK293 cells expressing "scrambled" shRNA or shRNA targeting NLK (NLK shRNA 5) were incubated in complete media and starved in media without FBS and stimulated with TNFα for the time indicated. Endogenous TAK1 was immunoprecipitated from 0.1 mg or 1 mg of protein lysates with anti-TAK1 antibody and subjected to an *in vitro* kinase assay using MKK6 as substrate followed by SDS-PAGE and autoradiography or probed for phosphorylation on Thr187 by western blotting with phospho-TAK1 [pThr187] antibody.

C

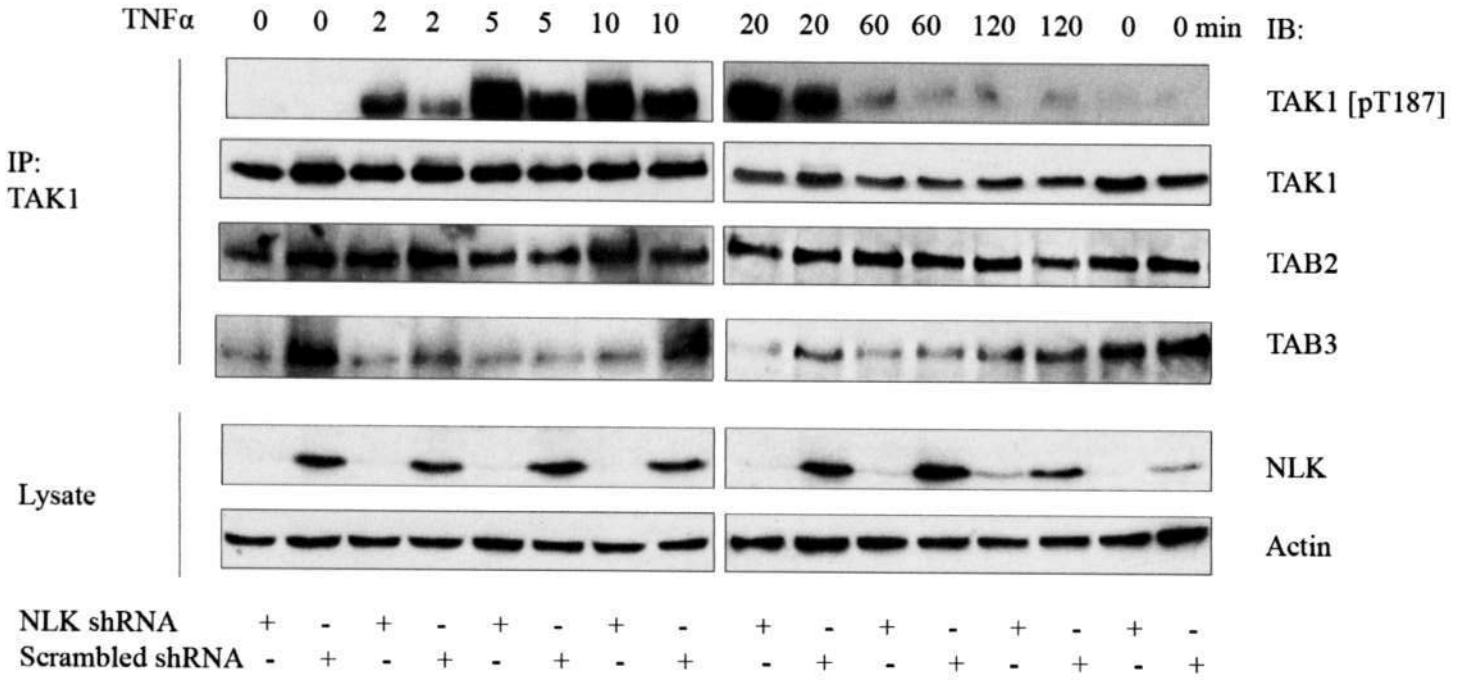
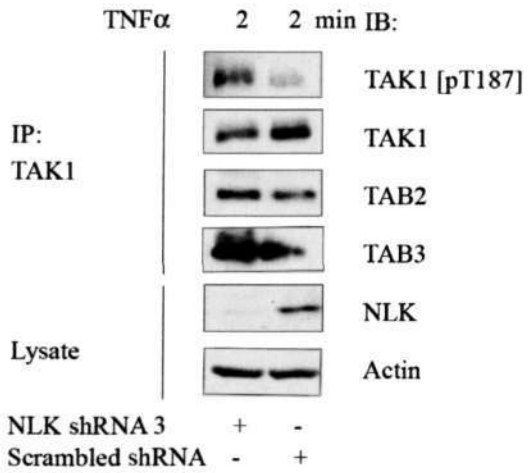


Figure 3.9 NLK inhibits TAK1 activation in cells

(C) NLK knockdown increases TAK1 activation mediated by TNFα -

HEK293 cells expressing "scrambled" shRNA or shRNA targeting NLK (NLK shRNA 5) were incubated in complete media or starved in media without FBS and stimulated with TNFα for 2 minutes to 120 minutes respectively. Endogenous TAK1 was immunoprecipitated from 1 mg protein lysates with anti-TAK1 antibody and subjected to an *in vitro* kinase assay using MKK6 as substrate followed by SDS-PAGE and autoradiography or probed for phosphorylation on Thr187 by western blotting with phospho-TAK1 [pThr187] antibody.

D



E

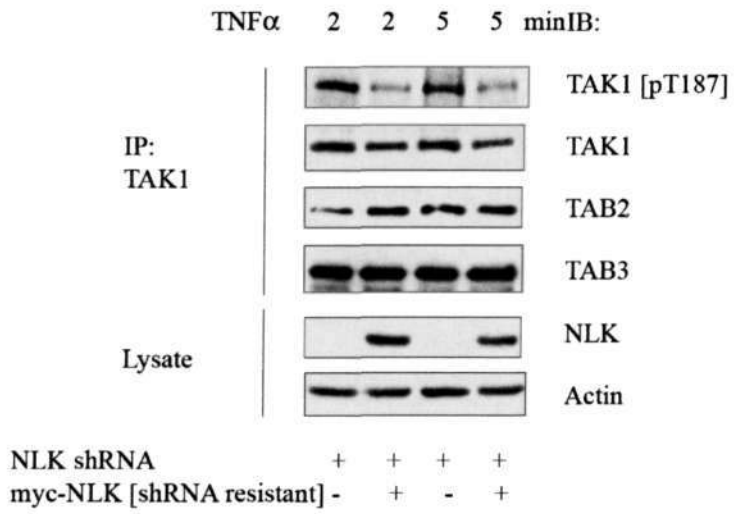


Figure 3.9 NLK inhibits TAK1 activation in cells

(D) NLK knockdown increases TAK1 activation mediated by TNFα –

HEK293 cells expressing "scrambled" shRNA or shRNA targeting NLK (NLK shRNA 3) were starved in media without FBS and stimulated with TNFα for the times indicated.

(E) NLK knockdown increases TAK1 activation mediated by TNFα –

NLK knockdown NLK HEK293 cells, expressing NLK targeting shRNA5 with wild type pCMV-5 myc-NLK or shRNA resistant pCMV5-myc-NLK were starved and stimulated with TNFα for the times indicated. Endogenous TAK1 was immunoprecipitated from 1 mg of protein lysates with anti-TAK1 antibody and probed for phosphorylation on Thr187 by western blotting with phospho-TAK1 [pThr187] antibody. The amount of immunoprecipitated TAK1 with associated TAB2 and TAB3 were checked by western blotting with their respective antibodies. Total NLK and actin were checked in the protein lysates by immunoblotting with anti-NLK and anti-actin antibodies.

F

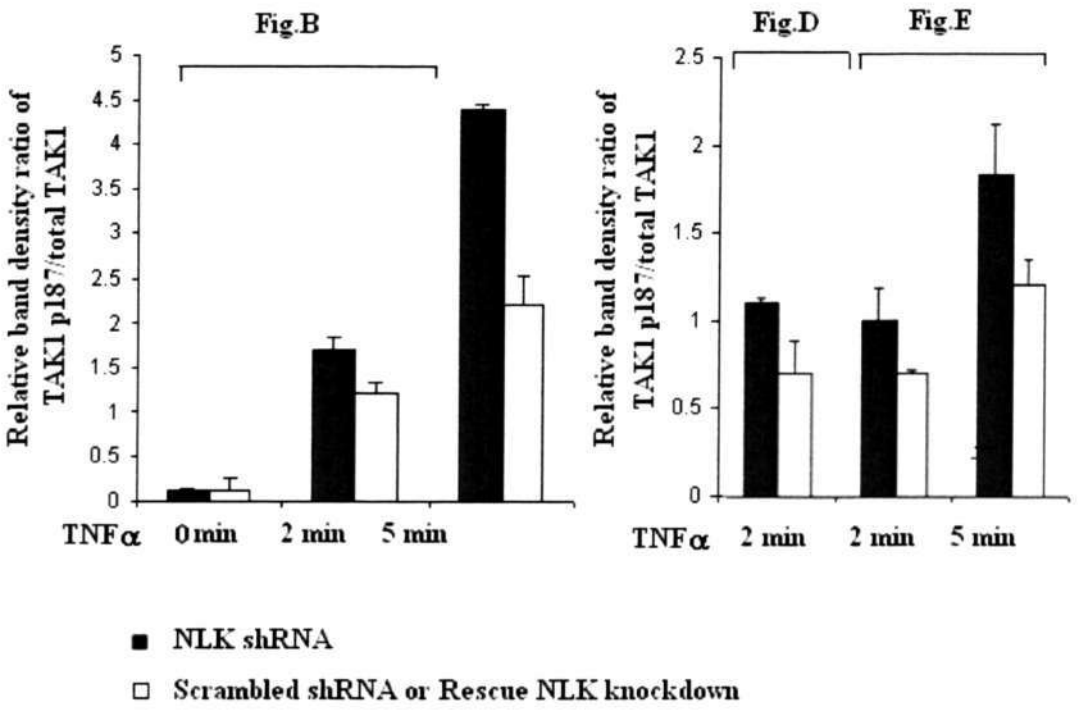


Figure 3.9 NLK inhibits TAK1 activation in cells

(F) Densitometric analysis of pThr187 and total TAK1 immunoblots in NLK knockdown cells and control cells from Figs [B, C, and D] is analyzed by using image J software. The Y axis represents the relative band intensity of pThr187 signal divided by total TAK1 signal. The results are representative of two indpendent experiments.

3.10 NLK knockdown increases the activation of p38, JNK and IKK signaling pathways

Since p38, JNK and NFκB are known downstream effector molecules of TAK1; we examined the activity of those proteins in NLK knockdown cells. By using phospho-antibodies against p38, IκB and JNK, we have assayed their activation levels upon stimulating the cells with cytokine TNFα or IL-1β. The protein lysates were probed with phospho-antibodies against Thr180/Tyr182 of p38α, Thr183/Tyr185 of JNK and Ser32 of IκB respectively. [Refer to the simple signaling digram shown in Figure 3-10E]

Phosphorylation of p38 and JNKs was increased throughout the time course of TNFα stimulation in cells which are transfected with shRNA 5 or shRNA 3 compared with control cells. [Figure 3-10 A&B] But we failed to detect drastic changes in the phosphorylation of IκB in NLK knockdown cells compared with control. It could be due to the fact that the half life of IκB is very short upon phosphorylation. Phosphorylated IκB is quickly recognized and degraded by proteasome dependent mechanism.[63] Phosphorylation on Ser32 of IκB was moderately higher at 2 mins and 5 mins after TNFα stimulation in NLK "knockdown" cells compared to control. [Figure 3-10 A and B] Densitometric analysis and western blots from Figure 3-10A suggest that NLK knockdown cells show two to three fold increase in phosphorylation of p38α, JNKs and IκB after activation by TNFα compared to the control cells.

As control, the cells were rescued by expression of NLK plasmid that is sensitive or resistant to shRNA knockdown respectively. After stimulation with TNFα, phosphorylation of p38α, JNK1 and JNK2 were lower in cells expressing the shRNA resistant NLK plasmid compared to the wild type NLK plasmid. The disappearance of total IκB was slightly faster in cells expressing the wild type NLK plasmid. [Figure 3-10 C] These results show that in "NLK knockdown" cells which are rescued with the shRNA resistant NLK plasmid, TAK1 activity is suppressed and subsequently leads to the inhibition of downstream signaling.

To show that the results obtained from this study is not cell line specific and reflects the true physiological function of NLK, we have performed the same experiments using HeLa cells. And we tested the activation level of p38α, JNKs and phosphorylation of IκB mediated by IL-1β. Upon stimulation with IL-1β at 3 min, 10 min and 20 min, NLK knocked down cells showed enhanced phosphorylation of p38α, JNK2 or IκB compared with control cells. [Figure 3-10D] Densitometric analysis indicates that IL-1β induced phosphorylation of p38α, JNK2 and IκB is enhanced by approximately two-fold in NLK depleted cells. Figure 3-10F illustrates the TNF and IL-1 mediated phosphorylation of kinases used in this section and summarizes the experimental findings.

Overall, NLK depletion in HEK293 or HeLa cells leads to enhanced TNF and IL-1 mediated activation TAK1 and its downstream kinases approximately up to two to three fold. The result also suggests that the enhanced activation of the p38α, JNKs and the NFκB pathways in the absence of NLK is mediated by upregulation of TAK1 activity.

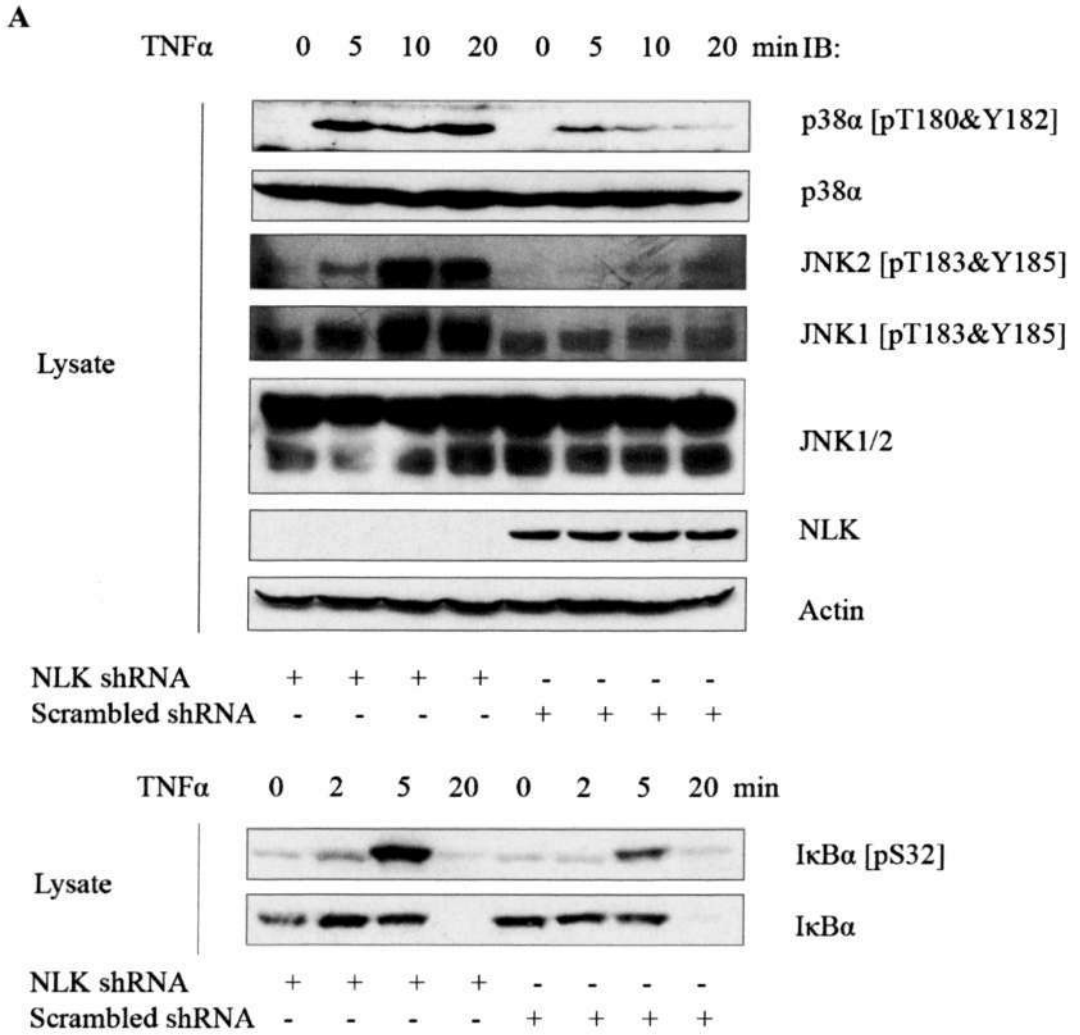


Figure 3.10 Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα and IL-1β

(A) Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα –

HEK293 cells, expressing "scrambled" or NLK targeting shRNA5 were starved and stimulated with TNFα for the times indicated. Protein lysates were examined for activation of p38α, JNK1 & 2 or NFκB by western blotting with phospho-p38α [pThr180/pTyr182], phospho-JNK [pThr183/pTyr185] and phospho-IκB [Ser32] antibodies respectively. Protein lysates were also probed for total p38α, JNK1 & 2 and IκB. NLK knockdown efficiency and loading control was checked by immunoblotting with anti-NLK and anti-actin antibodies.

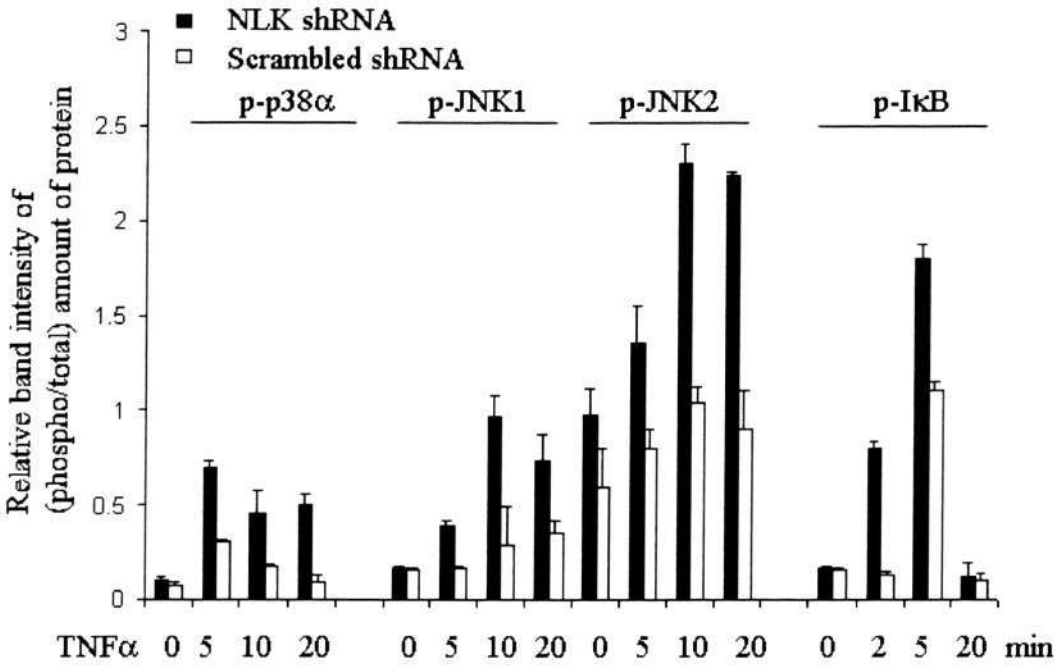


Figure 3.10 (A) Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα –

Relative band density of the phospho-proteins divided by total integrated density of the total proteins [p38α, JNK and IκB] from Figure 3.10 (A) were analyzed by Image J software and represented in the bar graph. Western blots with the same exposure time from both "knockdown" and control cells were used in densitometric analysis. Results are representative of two independent experiments.

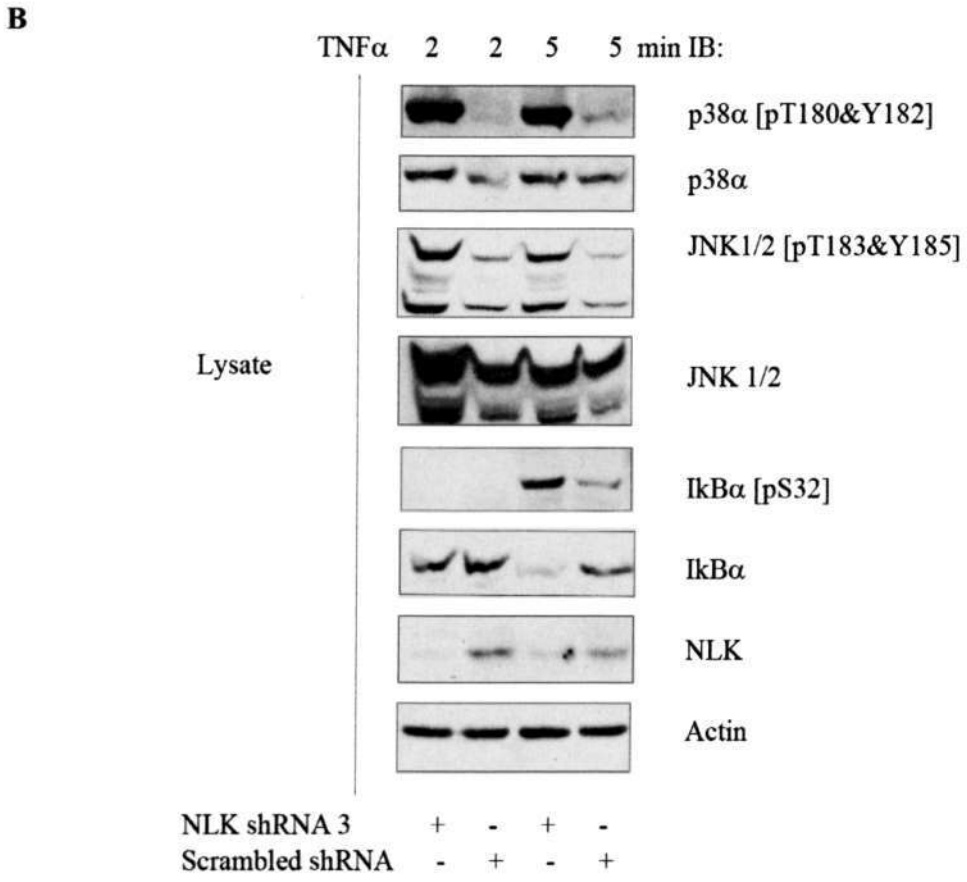


Figure 3.10 Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα and IL-1β

(B) Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα –

HEK293 cells, expressing "scrambled" or NLK targeting shRNA3 were starved and stimulated with TNFα for the times indicated. Protein lysates were examined for activation of p38α, JNK1 & 2 or NFκB by western blotting with phospho-p38α [pThr180/pTyr182], phospho-JNK [pThr183/pTyr185] and phospho-IκB [Ser32] antibodies respectively. Protein lysates were also probed for total p38α, JNK1 & 2 and IκB. NLK knockdown efficiency and loading control was checked by immunoblotting with anti-NLK and anti-actin antibodies.

C

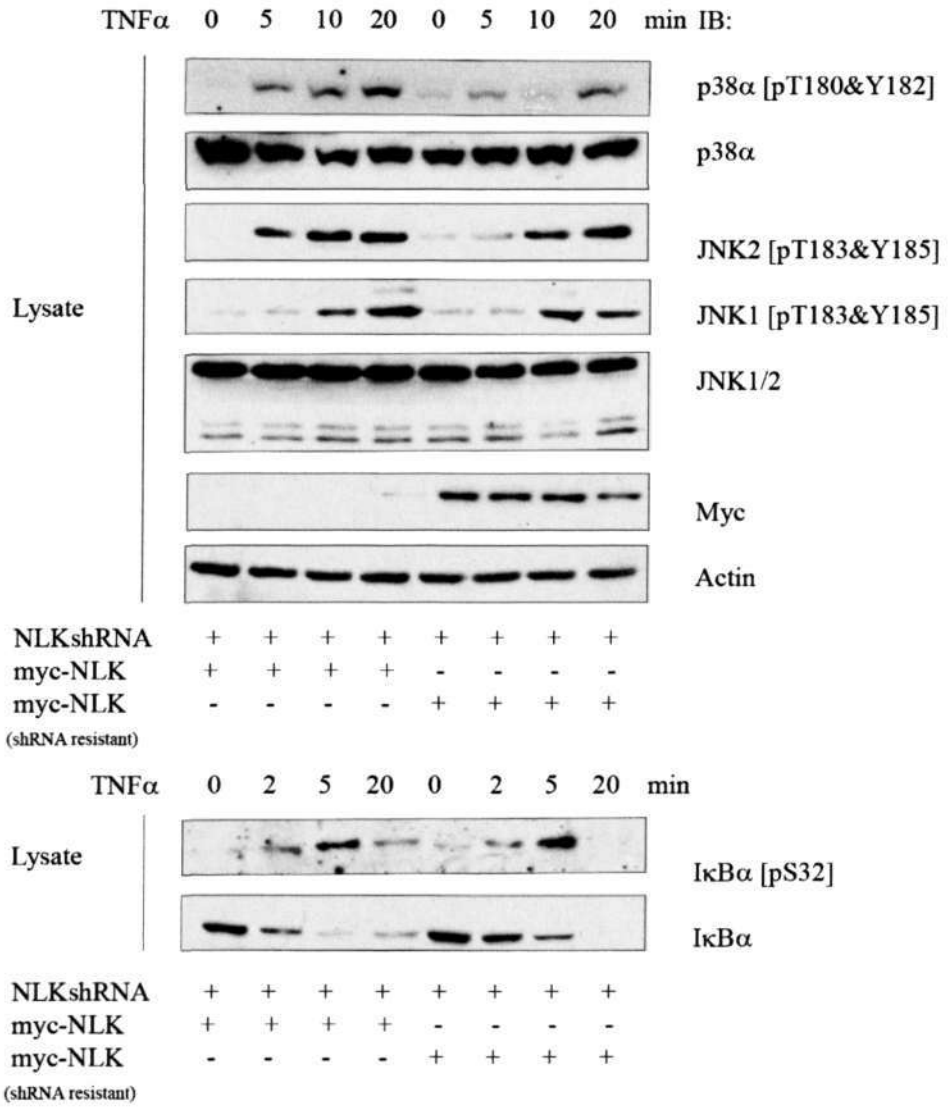


Figure 3.10 Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα and IL-1β

(C) Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα –

HEK293 cells, expressing NLK targeting shRNA5 with wild type pCMV-5 myc-NLK or shRNA resistant pCMV5-myc-NLK were starved and stimulated with TNFα for the times indicated. Protein lysates were examined for activation of p38α, JNK1 & 2 or NFκB by western blotting with phospho-p38α [pThr180/pTyr182], phospho-JNK [pThr183/pTyr185] and phospho-IκB [Ser32] antibodies respectively. Protein lysates were also probed for total p38α, JNK1 & 2 and IκB. NLK knockdown efficiency and loading control was checked by immunoblotting with anti-NLK and anti-actin antibodies.

D

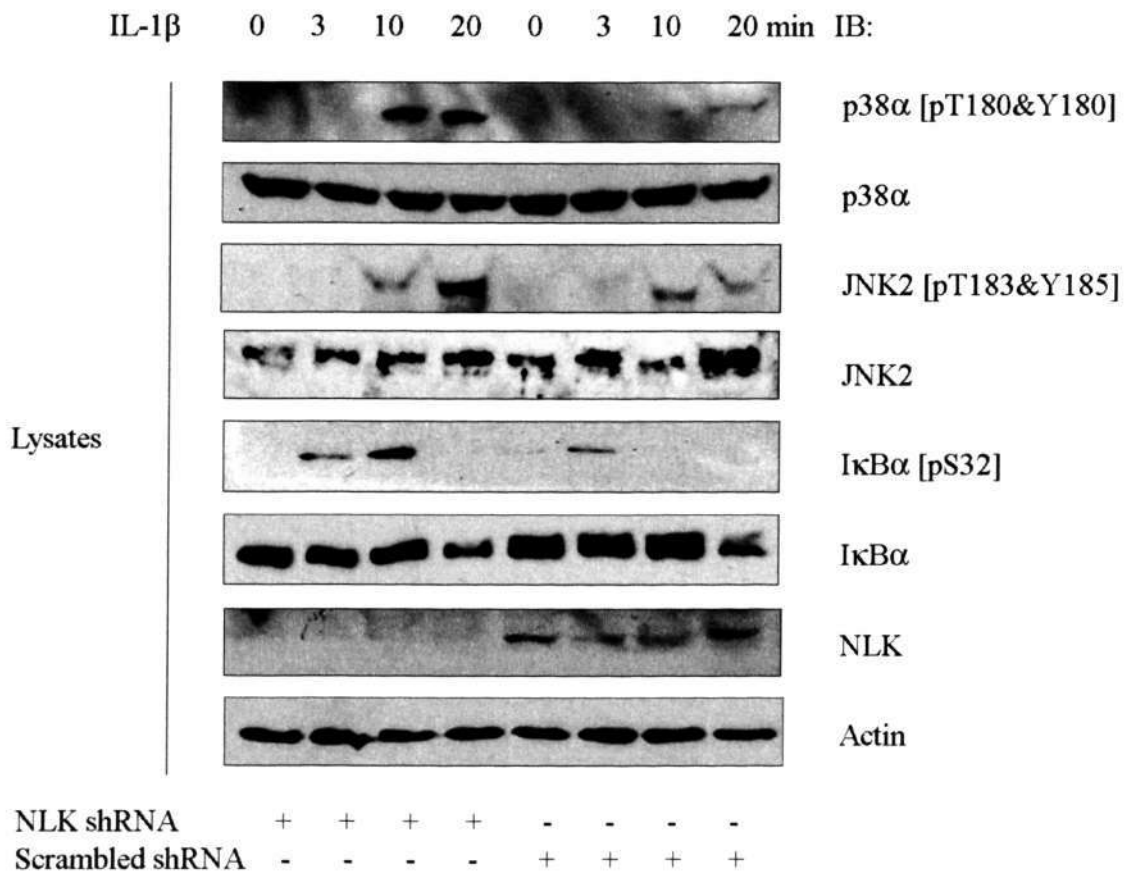


Figure 3.10 Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNFα and IL-1β

(D) Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with IL-1β –

HeLa cells expressing "scrambled" or NLK targeting shRNA5 were starved and stimulated with TNFα for the times indicated. Protein lysates were examined for activation of p38α, JNK 2 or NFκB by western blotting with phospho-p38α [pThr180/pTyr182], phospho-JNK [pThr183/pTyr185] and phospho-IκB [Ser32] antibodies respectively. Protein lysates were also probed for total p38α, JNK 2 and IκB. NLK knockdown efficiency and loading control was checked by immunoblotting with anti-NLK and anti-actin antibodies.

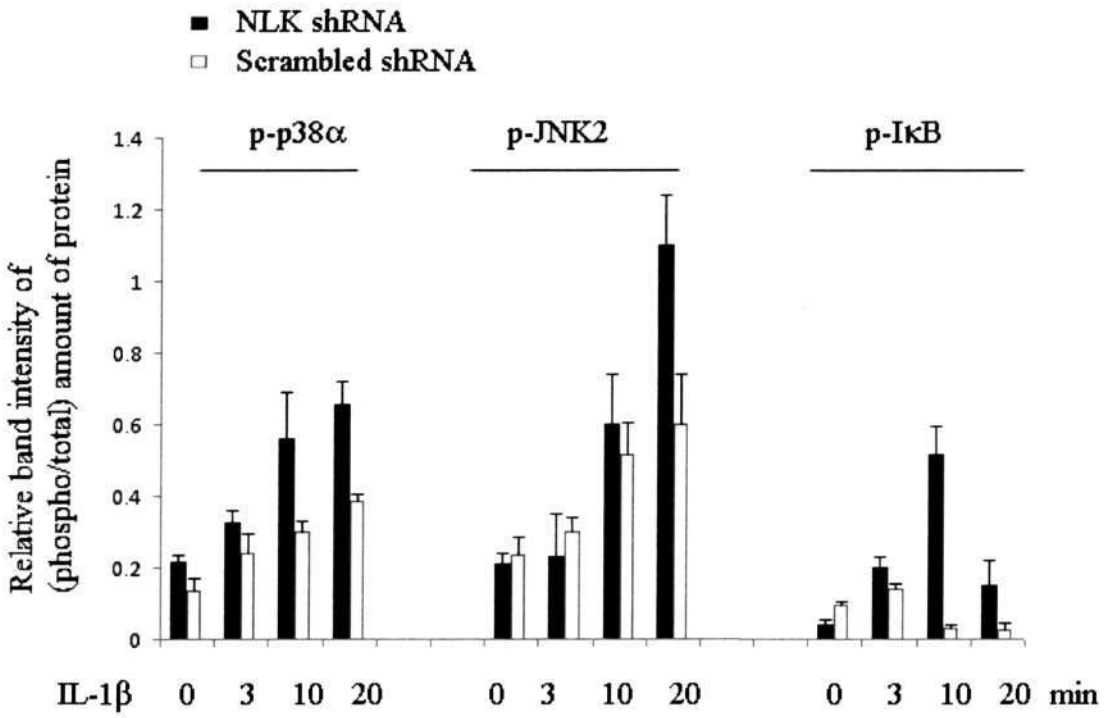
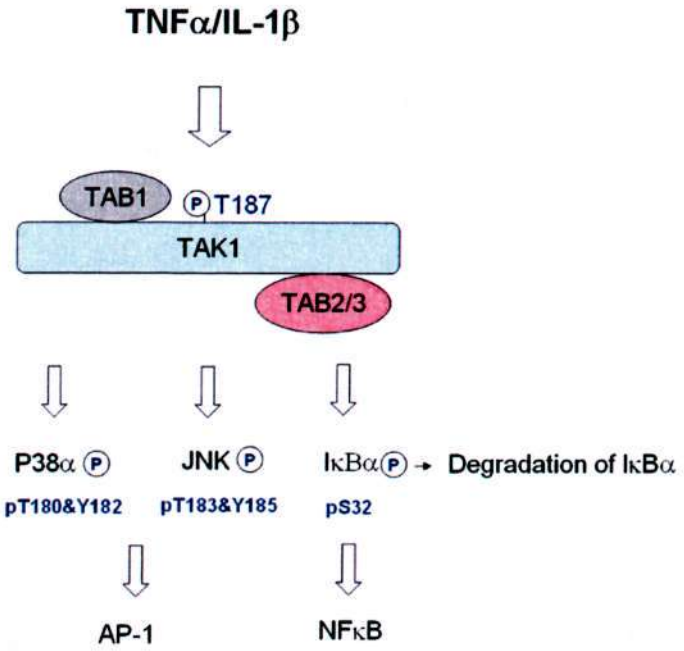


Figure 3.10 (D) Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with IL-1β

The relative band density of the phospho-protein divided by total integrated density of the total proteins [p38α, JNK2 and IκB] from Figure 3.10 (D) were analyzed by Image J software and represented in the bar graph. Results are representatives of two independent experiments.

E



F

TNFα or IL-1β mediated phosphorylation of protein kinases	NLK "knockdown" cells	Control cells
TAK1 [pT187]	+++	+
p38α [pT180&Y182]	+++	+
JNK1&2 [pT183&Y185]	+++	+
IκBα [pS32]	++	+

Figure 3.10 Activation of p38α, JNKs and IKK are increased in NLK knockdown cells stimulated with TNF α and IL-1β

(E) Schematic diagram of IL-1 and TNF induced activation and/or phosphorylation of TAK1 and its downstream signaling protein p38, JNK and IκB. The phosphorylation sites that are used to measure their activity in this study are also indicated in the diagram.

(F) The summary of results obtained from Figure 3.10 A to D. The different intensity of the protein phosphorylation detected in our study is indicated in (+) sign. Higher level of phosphorylation is denoted as (+++, ++) while lower level of phosphorylation signal detected is represented as (+).

Result Part 2

NLK negatively regulates NFκB and AP-1 signaling

3.11 NLK inhibits TAB2/3 mediated activation of NFκB

In cells, TAK1 is activated by TRAF6 (an E3 ubiquitin ligase) which synthesizes K63-linked polyubiquitin chains to which TAB2/3 binds preferentially through the NZF domain. Mutation of the NZF domain abolishes the ability of TAB2 and TAB3 to activate TAK1 and IKK, whereas the replacement of the NZF domain with a heterologous ubiquitin-binding domain restores the function of TAB2 and TAB3. This suggests that ubiquitin-binding by TAB2/3 is necessary for activating TAK1 and NFκB. [56-57, 65, 88] Many reports have shown that TAB2 and TAB3 are indispensable for activation of IKK and subsequent activation of NFκB genes. [65, 67, 89] Based on this knowledge, we tested whether NLK directly inhibits polyubiquitin binding by TAB2/3 and the inhibition of the NFκB pathway.

Using a NFκB luciferase linked reporter gene, activation was observed when TAB2 or TAB3 were expressed alone. When TAB2 or TAB3 were co-expressed with NLK, there was a significant reduction in reporter gene activation. Similarly, when TAK1, TAB1 and/or TAB2/3 were co-expressed with NLK, a decrease in activation of NFκB luciferase reporter gene was observed as shown in [Figure 3-11A]. GST protein with or without myc-NLK showed no activation of the NFκB reporter and is included as a negative control in this experiment. The expression level of proteins used in the assay are shown in Figure 3-11B. The hyper-phosphorylation of TAB2/3 by NLK was observed in lanes 2, 4, 6, 8, 10 and 12 of Figure 3-11B and correlates with the inhibition of NFκB reporter gene activation seen in Figure 3-11A. Therefore, this finding led us to the hypothesis that NLK might impair the function of TAB2/3 through phosphorylation.

To find out the possible molecular mechanisms of NLK inhibition on TAB2/3, GST pull down assay was carried out with GST-TAK1 after cells were transfected with TAB1 and/or TAB2 either in the presence or absence of NLK. The precipitated proteins were probed with anti-ubiquitin antibodies. As shown in Figure 3-11C, the characteristic ubiquitin smear was only observed in lane 3 to 6 when TAB2 was co-expressed with TAK1 or TAK1-TAB1. The experimental data suggests that polyubiquitin chain is attached to TAB2, but not on TAK1 or TAB1.

To our surprise, we failed to detect the presence of ubiquitin smear where NLK was co-expressed with TAK1-TAB2 or TAK1-TAB1-TAB2 in Figure 3-11C lane 3 and 5. Collectively, our results suggest that NLK may affect the polyubiquitin binding function of TAB2 and TAB3 in cells and inhibit TAB2/3 mediated activation of NFκB.

A

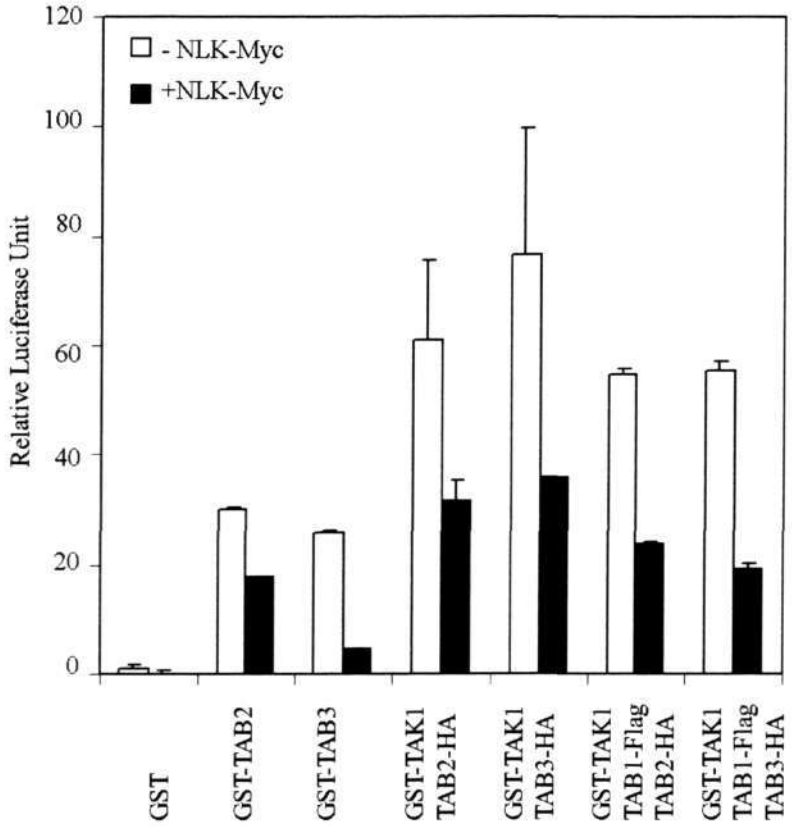


Figure 3-11 NLK inhibits activation of NFκB pathway

(A) NLK suppresses TAK1 mediated activation of NFκB gene through TAB2 and TAB3- HEK293 cells were co-transfected with plasmids encoding the indicated combinations of proteins, NFκB reporter activated firefly luciferase and control renilla luciferase. The cells were harvested and the protein lysates assayed for expression of firefly luciferase. Assays were done in triplicate and the results shown are the mean ± SEM from three independent experiments.

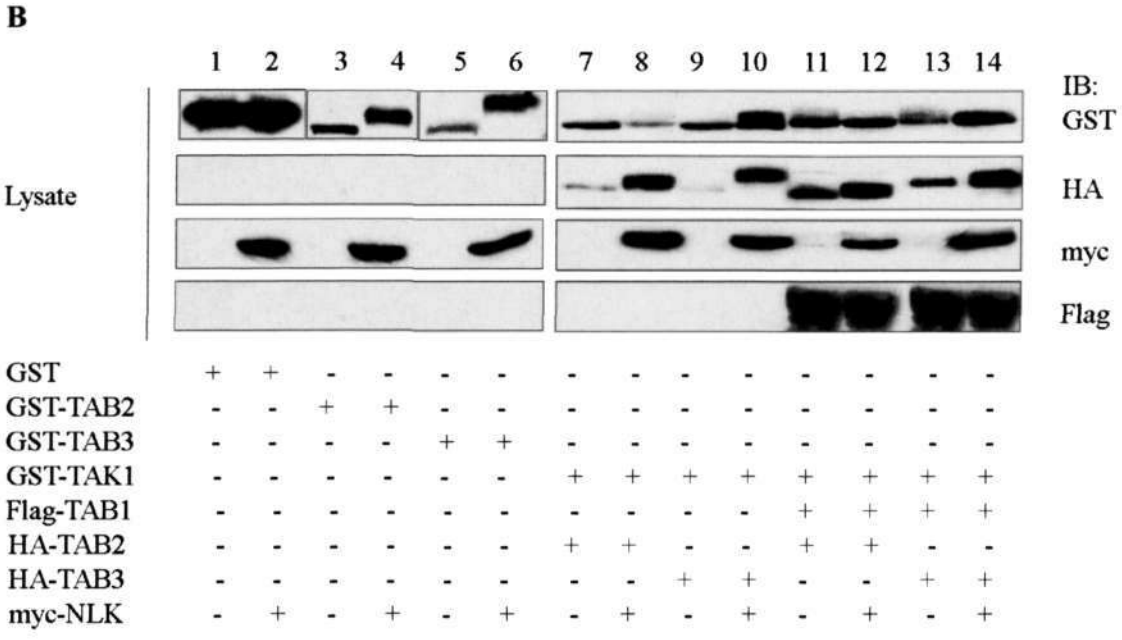
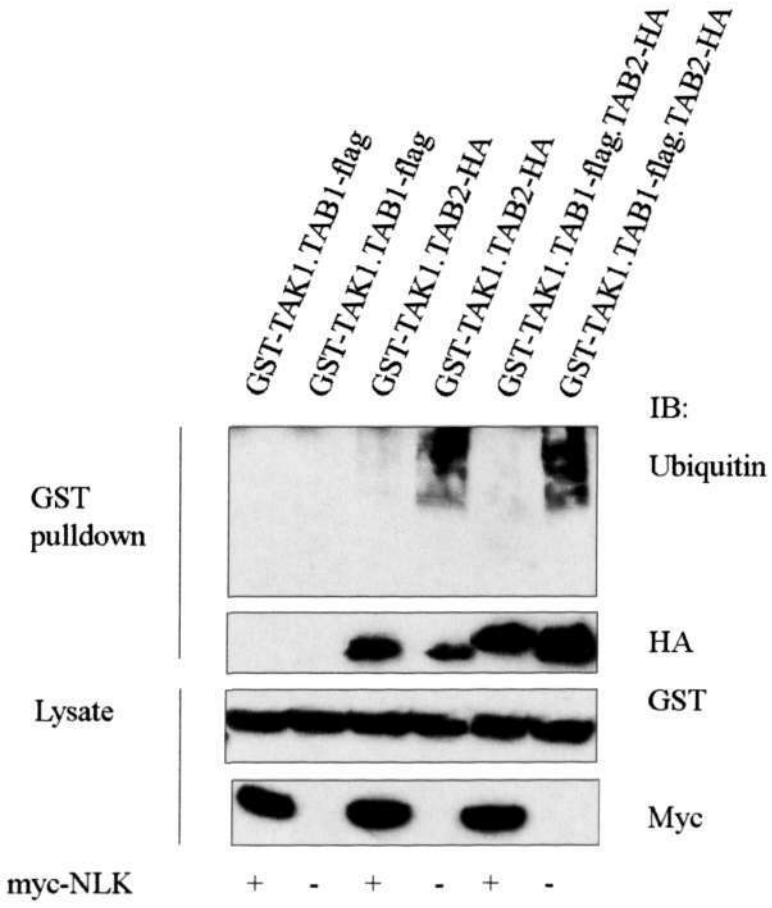


Figure 3-11 NLK inhibits activation of NFκB pathway

(B) Protein lysates from (A) were probed for protein expression with GST, FLAG, HA and Myc specific antibodies.

C



Proteins used in GST pulldown assay	Non-covalent Polyubiquitin binding
TAK1 and/ or TAB1	No
TAK1 and TAB2	Yes
TAK1-TAB1-TAB2	Yes

Figure 3-11 NLK inhibits activation of NFκB pathway

(C) NLK reduces polyubiqutin binding of TAB2 in cells –

HEK293 cells were transfected with plasmids encoding GST-TAK1, FLAG-TAB1 and/or HA-TAB2. 0.5 mg of cell extracts were subjected to GST pull down and immunoblotted with anti-ubiqutin, HA, myc and GST antibodies.

3.12 NLK reduces the polyubiquitin binding ability of TAB2 and TAB3

To further confirm the experimental findings from result section 3.11, we investigated whether polyubiqutin binds to TAB2/3 by non-covalent interaction and is required for NFκB activation. NZF and CUE domains of TAB2 and TAB3 are essential for non covalent interaction with polyubiquitin chains and their optimum function in IL 1 and TNF signaling. Therefore, wild type or NZF and CUE domain deletion mutants of TAB3 were transfected with NFκB luciferase reporter gene in HEK293 cells and measure the activiation of NFκB. As shown in the Figure 3-12A, wild type TAB3 can achieve high level of activation of NFκB reporter gene. However, NZF and/or CUE domain deletion mutants of TAB3 show lower activation of the NFκB reporter compared to wild type TAB3.

Next we examined whether NLK directly modulates the ubiquitin binding ability of TAB2 and TAB3. GST-TAK1-TAB2-HA or GST-TAK1-TAB3-HA were co-expressed with wild type or ubiquitin binding domain deletion mutants of TAB2 and TAB3 in the presence or absence of exogenous myc-NLK (Fig. 3-12B and C). TAK1 complexes were then subjected to GST pulldown and immunoblotted for the presence of ubiquitin chains. Polyubiquitin chains bound to TAB2 (Fig. 3-12B, lane 2) or TAB3 (Fig.3-12C, lane 2) complexed with GST-TAK1 were readily detectable but reduced in the presence of NLK (Fig. 3-12B, lane 1 and Fig. 3-12C, lane 1). NZF and CUE-NZF mutants of TAB2 and TAB3 show no binding of ubiquitin chain (Fig. 3-12B, lanes 3-6 and Fig. 3-12C, lanes 3-6) This result is consistent with low level activation of NFκB gene by NZF and CUE-NZF mutants of TAB3 [Figure 3-12A] indicating the relationship between polyubiquitin binding by TAB2/3 and NFκB gene activation. In agreement with other reports [65, 67]; we also showed here that non-covalent poly ubiqutin binding is essential for NFκB gene activation.

To demonstrate that NLK regulates the ubiquitin binding ability of TAB2/3, a dose dependent expression of NLK plasmid was performed with fixed amount of TAK1 complex in HEK293 cells. GST-TAK1 complex from cell lysates were affinity purified with GST beads and checked for ubiquitin binding by TAB2 and TAB3. The amount of

detectable ubiquitin smears on the western blot was proportionally reduced with the increasing level of phosphorylation as indicated by slower migrating bands of TAB2/3 in Figure 3-12 D and E.

In conclusion, these results indicate that the ubiquitin binding ability of TAB2/3 was modulated by NLK.

B

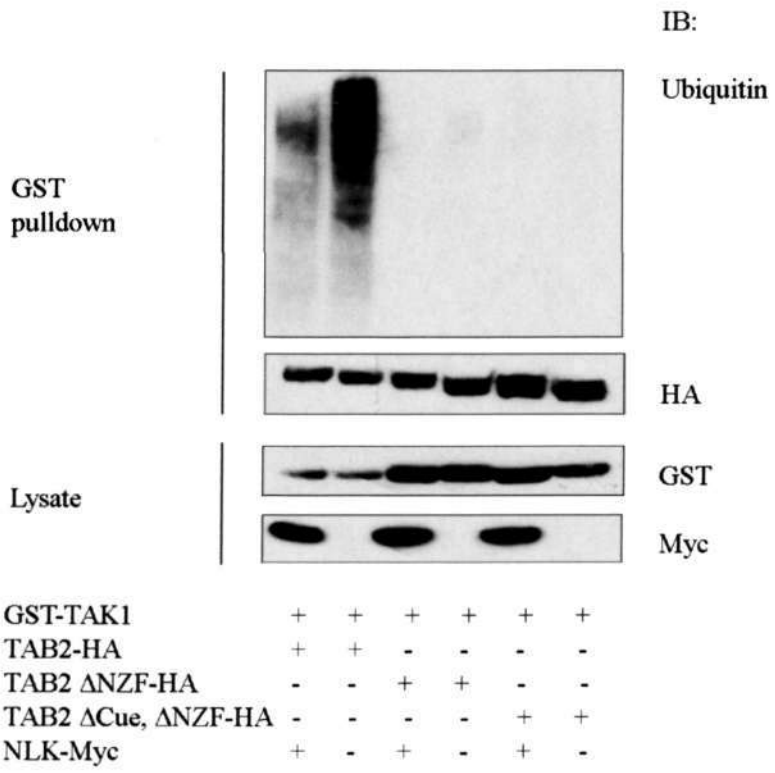


Figure 3.12 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by NLK

(B) NLK decreases polyubiquitin binding of TAB2 –

HEK293 cells were transfected with plasmids encoding the indicated proteins. GST-tagged TAK1 and TAB2 were purified from 0.5 mg of cell lysates by "GST pulldown" with glutathione-Sepharose beads and denatured in SDS sample buffer. The purified proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for ubiquitin and HA. Protein lysates were immunoblotted for expression of epitope tagged proteins with anti-GST, anti-myc and anti-HA antibodies.

C

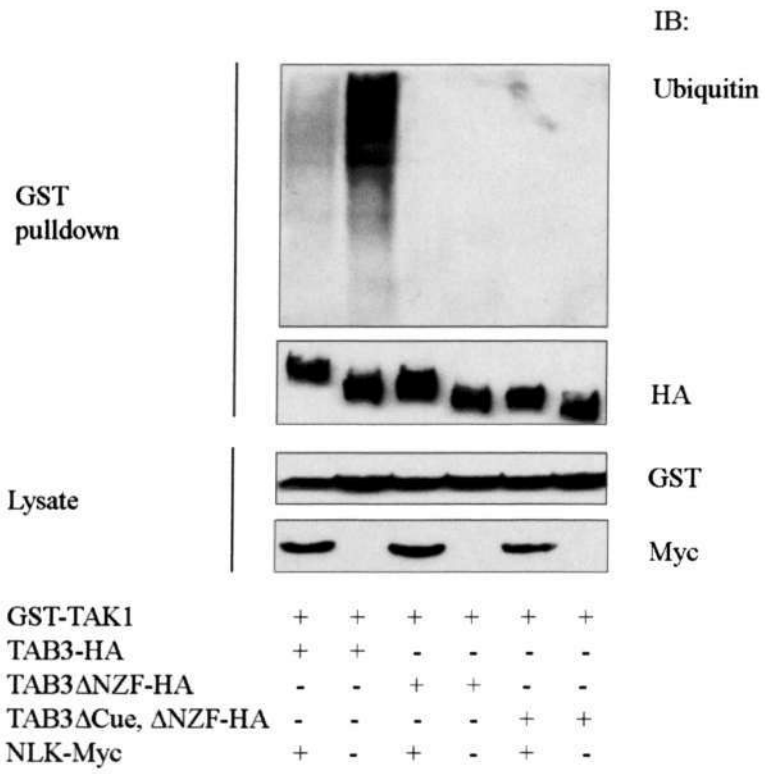


Figure 3.12 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by NLK

(C) NLK decreases polyubiquitin binding of TAB3 –

HEK293 cells were transfected with plasmids encoding the indicated proteins. GST-tagged TAK1 and TAB3 were purified from 0.5 mg of cell lysates by "GST pulldown" with glutathione-Sepharose beads and denatured in SDS sample buffer. The purified proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for ubiquitin and HA. Protein lysates were immunoblotted for expression of epitope tagged proteins with anti-GST, anti-myc and anti-HA antibodies.

D

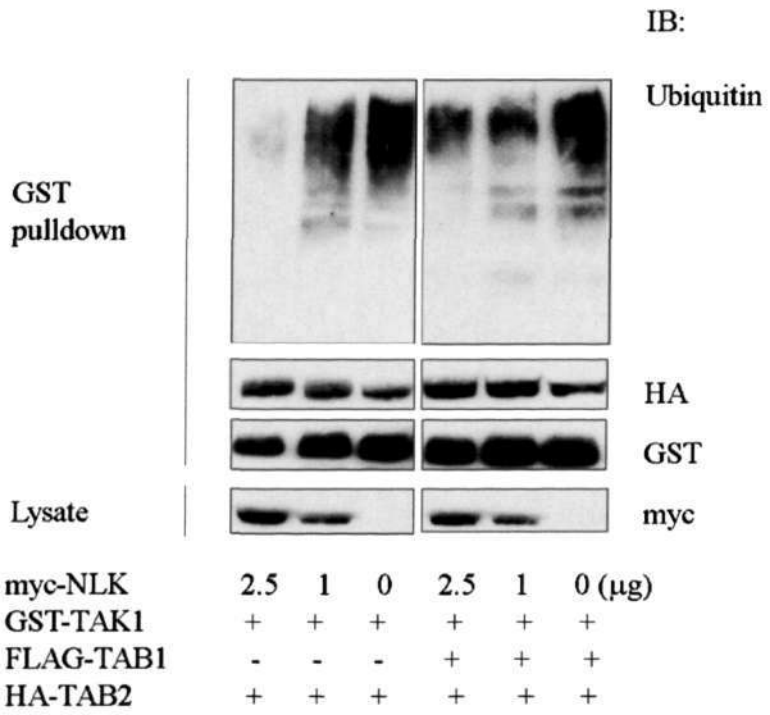


Figure 3.12 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by NLK

(D) NLK reduces polyubiqutin binding of TAB2 in dose dependent manner –

HEK293 cells were transfected with plasmids encoding the indicated proteins with kinase active myc-NLK in a dose dependent manner. Empty plasmid pCMV-5 was used to normalize with the different amount of myc-NLK for transfection. GST-tagged TAK1 and TAB2 complex were purified from 0.5mg of cell lysates by "GST pulldown" with glutathione-Sepharose beads and denatured in SDS sample buffer. The purified proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for ubiquitin and HA. Protein lysates were immunoblotted for expression of epitope tagged proteins with anti-GST, anti-myc and anti-HA antibodies.

E

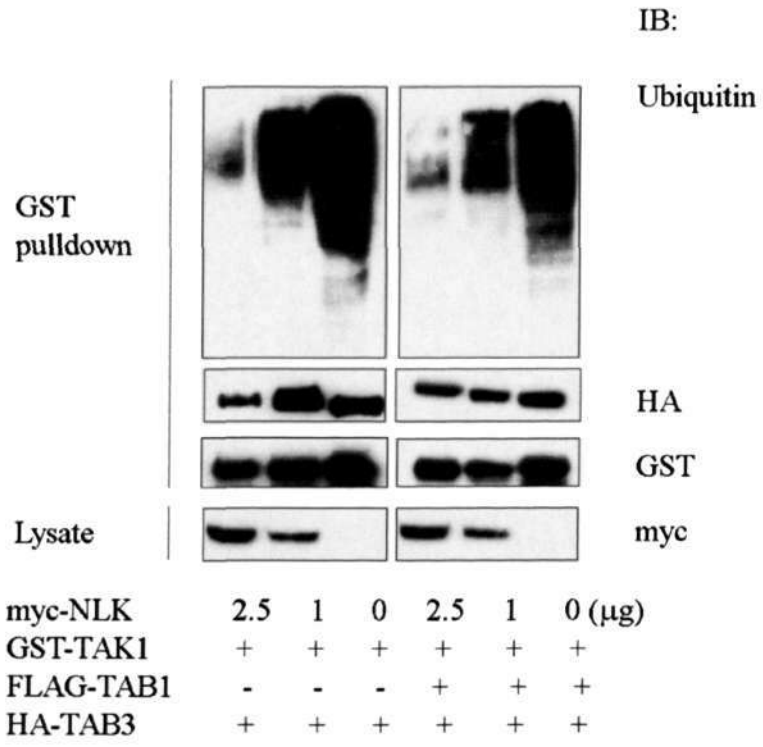


Figure 3.12 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by NLK

(E) NLK reduces polyubiquitin binding of TAB3 in dose dependent manner –

HEK293 cells were transfected with plasmids encoding the indicated proteins with kinase active myc-NLK in a dose dependent manner. Empty plasmid pCMV-5 was used to normalize with the different amount of myc-NLK for transfection. GST-tagged TAK1 and TAB3 complex were purified from 0.5mg of cell lysates by "GST pulldown" with glutathione-Sepharose beads and denatured in SDS sample buffer. The purified proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for ubiquitin and HA. Protein lysates were immunoblotted for expression of epitope tagged proteins with anti-GST, anti-myc and anti-HA antibodies.

3.13 NLK does not abrogate the physical interaction between TAB2 and TRAF2/6

Previous finding led us to test two possible hypotheses of NLK action on TAB2/3. Firstly we asked whether NLK alters the conformation of TAB2/3 by phosphorylation and subsequently abrogates the ubiquitin binding function of TAB2/*3*. Alternatively, phosphorylation on TAB2/3 disrupts the interaction between TAB2/3 and its interacting partners TRAFs which catalyze K63-linked polyubiquitin chain.

As we described earlier, polyubiquitin binding of TAB2 and TAB3 and activation of TAK1 and IKK in IL-1 or TNFα pathway is dependent on the physical interaction with ubiquitin ligases TRAFs which synthesize K63 linked polyubiquitin chains upon signal activation. [65, 67] Therefore, NLK might abrogate the physical interaction between TRAFs and TAB2/3 and lead to subsequent reduction of polyubiquitin binding by TAB2/3. Previously, SMAD7 was shown to down-regulate NFκB activity by blocking the interaction between TAB2 or TAB3 and upstream signaling components TRAF2 in TNF pathway. [91]

To address our second hypothesis, GST-TAK1, HA-TAB2 and Flag-TRAF2/6 were co-expressed in the presence of kinase active or kinase dead myc-NLK. The cell lysates were used to perform GST pulldown and immunoprecipitation followed by immunoblotting with specific antibodies. As shown in Figure 3-13B and C, TAB2 was efficiently pulled down by Flag-TRAF2/6 or GST-TAK1 regardless of whether exogenous NLK is present. The physical interaction between (TAK1-TAB2) and TRAF2/6 is not affected by ectopic expression of NLK. Altogether our finding indicates that NLK does not abrogate the physical interaction between TRAFs and TAB2/3.

A

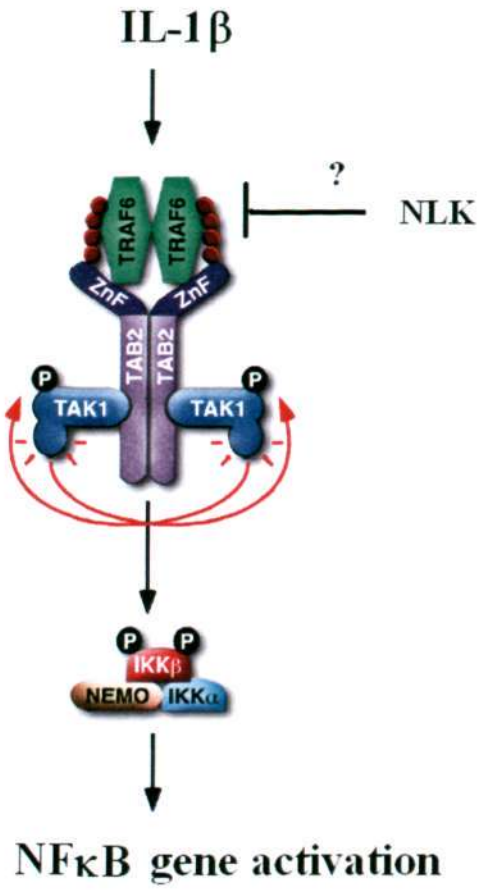


Figure 3.13 NLK does not abrogate the physical interaction between TRAFs and TAB2/3

(A) The schematic diagram illustrates the signal activation of IKK by TAK1 activation complex (TRAF6, TAB2/3 and TAK1-TAB1). TAB2 recruits K63 linked poly ubiquitinated TRAF6 through direct interaction or interaction with polyubiquitin chains via NZF domain and facilitates the autophosphorylation of TAK1. Assembly of dimers or oligomers of TAK1 and TAB2/3 on TRAF6 is important for activation of IL-1 mediated TAK1 and NFκB gene activation *in vivo*. The figure is adopted and modified from reference [88].

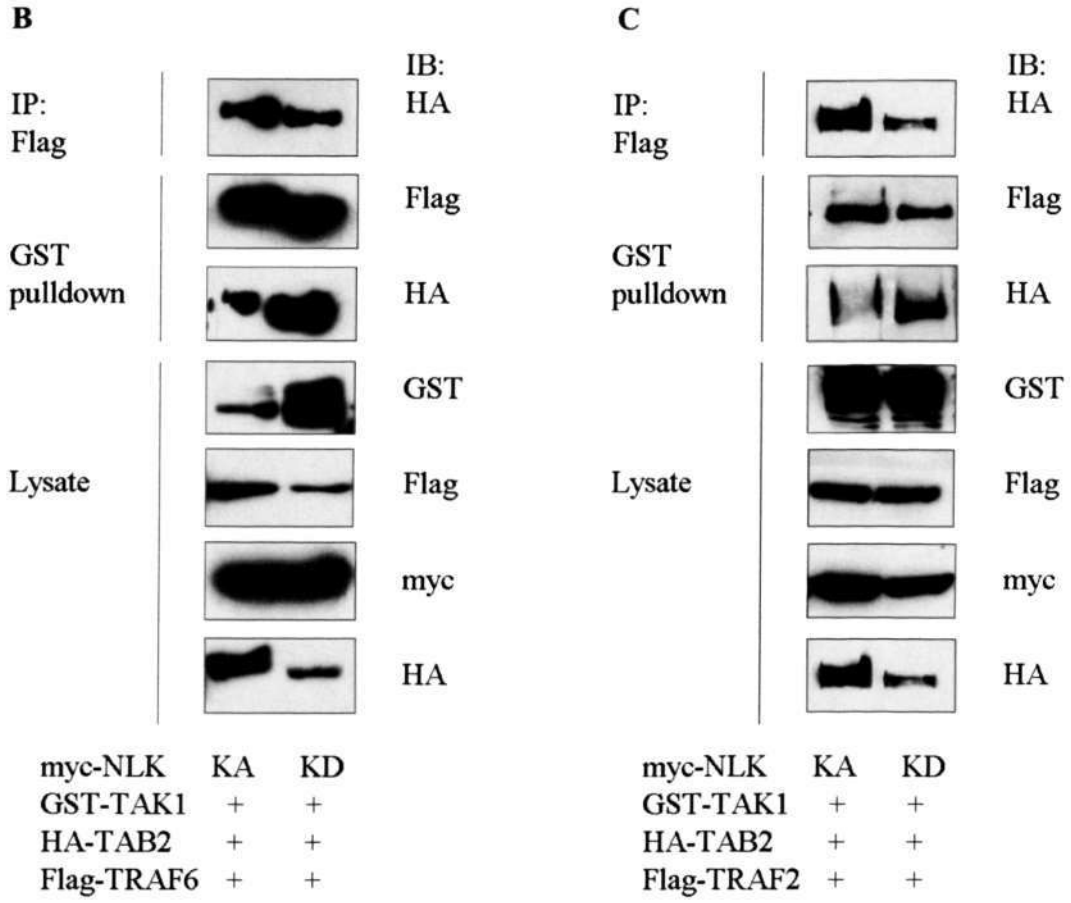


Figure 3.13 NLK does not abrogate the physical interaction between TRAFs and TAB2/3

(B) NLK does not abrogate the physical interaction between TRAF6 and TAK1 or TAB2 – HEK293 cells were transiently transfected with GST-TAK1, HA-TAB2, Flag-TRAF6 and myc-NLK KA or NLK KD [D270A]. 0.5mg of cell lysates were used to perform GST pull down and immunoprecipitation. The purified proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for Flag and HA. Protein lysates were immunoblotted for expression of epitope tagged proteins with anti-GST, anti-Flag, anti-myc and anti-HA antibodies.

(C) NLK does not abrogate the physical interaction between TRAF2 and TAK1 or TAB2 - HEK293 cells were transiently transfected with GST-TAK1, HA-TAB2, FLAG-TRAF2 and myc-NLK KA (kinase active) or NLK KD [D270A]. 0.5mg of cell lysates were used to perform GST pull down and immunoprecipitation. The purified proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for FLAG and HA. Protein lysates were immunoblotted for expression of epitope tagged proteins with anti-GST, anti-Flag, anti-myc and anti-HA antibodies.

3.14 Phosphorylation directly modulates ubiquitin binding ability of TAB2/3 *in vitro*

We next examined the first hypothesis of whether TAB2 and TAB3 phopshorylation by NLK directly modulates their ability to bind the polyubiquitin chains. Since TAB2 and TAB3 contain the NZF and CUE ubiquitin binding domains [56], NLK phosphorylation could cause conformational changes in ubiquitin binding domain of TAB2/3and inhibits its binding to poly ubiquitin chains.

GST-TAB2 or GST-TAB3 was co-expressed with NLK in HEK293 cells followed by GST pulldown and dephosphorylated with λ phosphatase. Both phosphatase treated and untreated GST-TAB2 and GST-TAB3 were then washed extensively to remove λ phosphatase followed by incubation with K48 linked and K63 linked polyubiquitin chains synthesized *in vitro* using bacterial expressed Ubc13/Uev1a and CHIP [85]. In line with the findings from other groups, we also demonstrated that GST-TAB2 and GST-TAB3 show increased binding to K63 linked polyubiquitin chain, not to K48 linked poly ubiqutin chains in an *in vitro* assay. [Figure 3-14A lane 3&4 compared to lane 5&6 both upper and lower panels] In addition, dephosphorylated TAB2 and TAB3 shows the increased binding of K63 linked polyubiquitin chains in Figure 3-14A lane 4.

A similar experiment was performed with protein lysates containing over-expressed myc-tagged ubiquitin. Dephosphorylated GST-TAB2 and GST-TAB3 treated with λ phosphatase displays faster electrophoretic mobility (Figure 3-14C, lanes 2, 4, 6, 8) compared with untreated phosphorylated proteins (Figure 3-14C, lanes 1, 3, 5, 7). Polyubiquitin chains bound much more readily to dephosphorylated GST-TAB2 (Figure 3-14C, lane 2) and GST-TAB3 (Figure 3-14C, lane 6) compared to the phosphorylated GST-TAB2 (Figure 3-14C, lane 1) and GST-TAB3 (Figure 3-14C, lane 5). The result obtained from Figure 3-14A and C are summarized in table 3-14D.

This finding from *in vitro* ubiquitin binding assay suggests for the first time that phosphorylation on TAB2/3 has a negative effect on their ubiquitin binding ability.

Therefore, the evidence led us to conclude that NLK regulates NFκB signaling through phosphorylating TAB2 and TAB3.

A

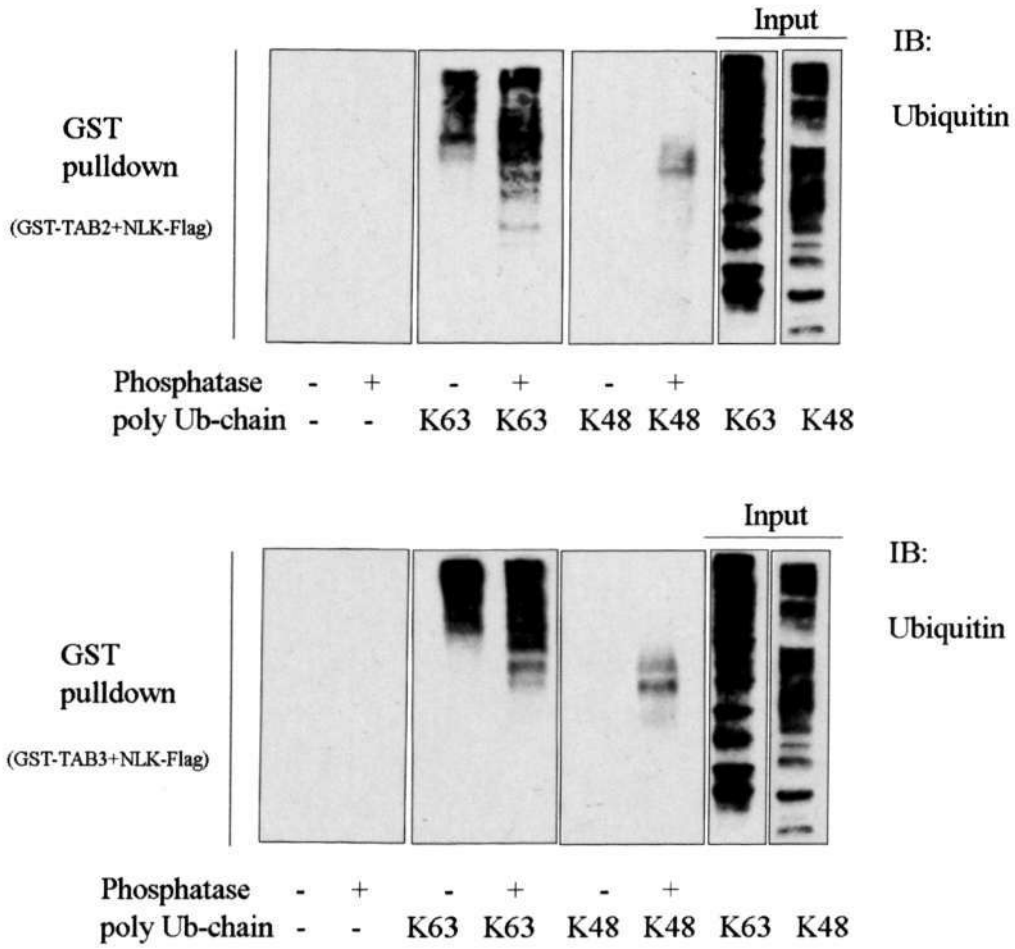


Figure 3.14 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by phosphorylation

(A) Dephosphorylated TAB2 and TAB3 bind K-63 linked polyubiquitin chain more efficiently – HEK293 cells were co-transfected with plasmids encoding Flag-NLK and GST-TAB2 or GST-TAB3. The GST-tagged proteins were affinity purified from 1 mg of lysates by glutathione-Sepharose beads and treated with or without λ phosphatase and incubated with K48 linked or K63 linked polyubiquitin chains synthesized by Ubc13/Uev1a and CHIP. In K48 linked reaction, purified K48 only ubiquitin mutant proteins were used while wild type ubiquitin proteins were used for K63 linked polyubiquitin chain synthesis. 1μl of K48 and K63 linked polyubiquitin chains from 20 μl reaction was used in the experiment. The protein bound glutathione-Sepharose beads were washed extensively and subjected to SDS-PAGE followed by immunoblotting with anti-ubiquitin, GST and FLAG antibodies.

B

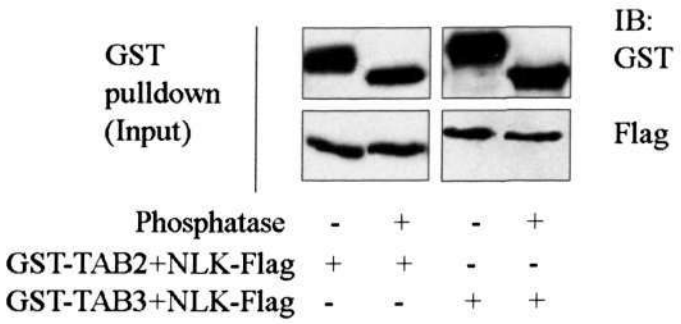
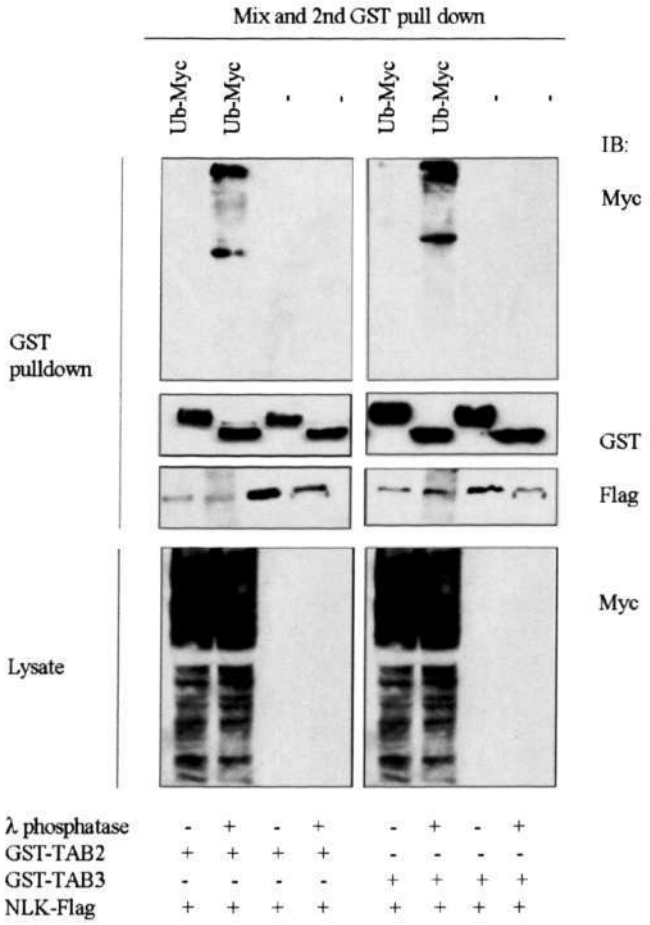


Figure 3.14 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by phosphorylation

(B) The inputs of the proteins used in Fig A were detected by western blotting with anti-GST and anti-FLAG antibodies.

C



D

Type of polyubiquitin chains	Binding to p-TAB2/3	Binding to de-p TAB2/3
K-63 chains Synthesized *in vitro*	+	+++
K-48 chains Synthesized *in vitro*	-	+
Ub-myc purified form HEK293 cells	+	+++

Figure 3.14 Polyubiquitin chain binding by TAB2 and TAB3 are reduced by phosphorylation

(C) Dephosphorylated TAB2 and TAB3 bind polyubiqutin chains more efficiently - The protein lysates were prepared and the experiment performed as in (A) except polyubiquitin chains were prepared from 0.1 mg of HEK293 cellular lysates containing over-expressed myc-tagged ubiquitin. The protein bound glutathione-Sepharose beads were washed extensively, subjected to SDS-PAGE and immunoblotted with anti-myc, GST and FLAG antibodies.

(D) Table summarizes the results obtained from Figure A and C.

3.15 NLK inhibits NFκB signaling by modulating TAB2/3 function

To validate the hypothesis that NLK suppresses NFκB signaling by inhibiting the ubiquitin binding funciton of TAB2 and TAB3, NLK shRNA or scrambled shRNA were expressed in HEK293 cells and the cells were stimulated with TNFα for 0 to 10 min. TAK1 was immunoprecipitated from whole cell extracts and probed for the presence of polyubiquitin chains in respone to TNFα signaling. Ubiquitin binding was undetectable in the control cells expressing scrambled shRNA. [Figure 3-15A lane 5 and 6] However, in NLK "knockdown" cells ubiquitin bound to the TAK1 complex was readily observed and increased with the duration of TNFα stimulation. [Figure 3-15A lane 2 and 3] Phosphorylation of TAK1 on Thr187 was also enhanced in NLK "knockdown" cells showing that the amount of polyubiquitin chain binding has a direct effect on TAK1 activity. [Figure 3-15A]

We also tested the activation of the NFκB reporter gene in NLK knockdown and control cells after stimulation with TNFα for 8 hours. In control cells, TNFα stimulation caused a robust increase in NFκB activity. However, in NLK "knockdown" cells, NFκB activation was increased a further two fold compared to control cells. [Figure 3-15B]

We also demonstrate that the increase in NFκB activity in NLK knockdown cells is due to the depletion of NLK, not because of secondary effects from off-target protein depletion in our protein knockdown study. We rescued the cells depleted of NLK by co-expression of RNAi resistant modified NLK plasmids and repeated the NFκB reporter gene assay. As expected, NFκB activation is reduced by two fold in the NLK depletion rescue experiment confirming the fact that high level NFκB activition in Fig.3-15B is due to inhibition of NFκB activity by endogenous NLK. [Fig. 3-15C]

The protein level of NLK is indicated in western blot and actin was used as a loading control. The similar expression levels of endogenous TAK1, TAB2 and TAB3 in both sets of experiment were checked and indicates that NLK depletion has no effect on the stability of the TAK1 complex [Fig. 3-15B and C]

Figure 3-15D illustrates the model of ubiquitin mediated TAK1 activation. Binding of free unchored polyubiquitin chain [92] or polyubiquitin chains binding to TAB2/3 [76] provides the scaffold for dimerization or oligomerization of the TAK1 kinase complex, and subsequent autophosphorylation of TAK1 at Thr 187, resulting in TAK1 and NFκB gene activation. We suggest here that NLK regulates TAK1-IKK-NFκB signaling cascade by inhibiting ubiquitin receptor function of TAB2/3 through phosphorylation.

A

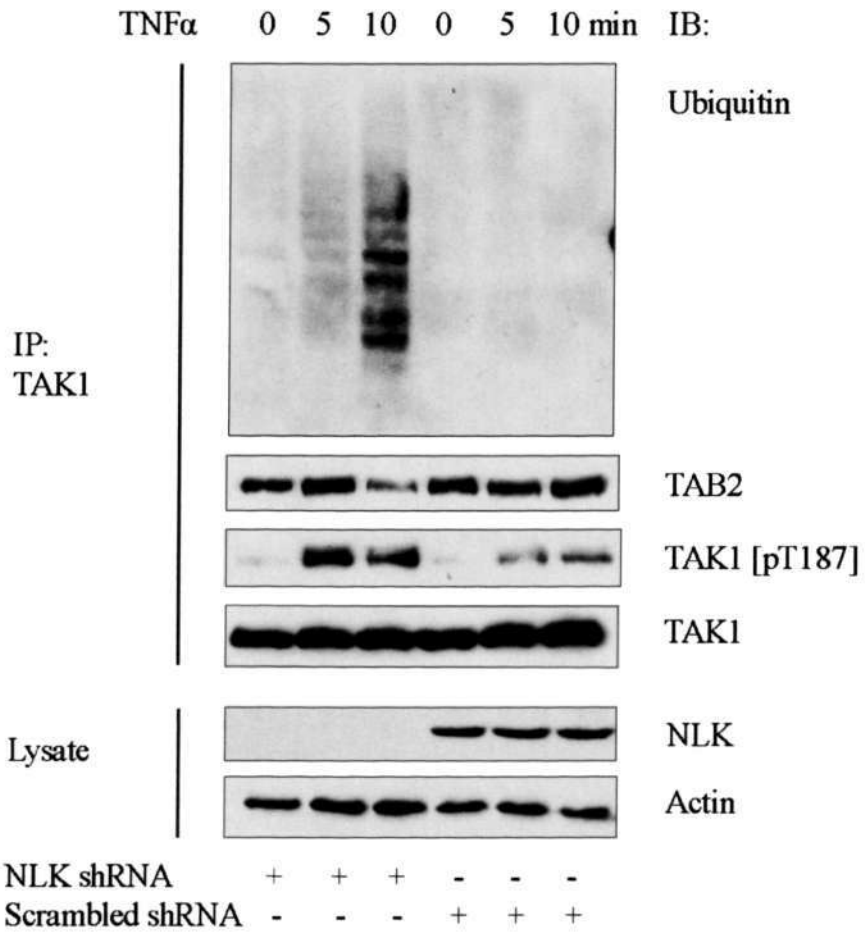


Figure 3.15 NLK inhibits activation of NFκB in TNFα pathway

(A) NLK knockdown enhances the polyubiqutin binding of TAB2 upon TNFα stimulation – HEK293 cells expressing "scrambled" or NLK targeting shRNA 5 were stimulated with TNFα for the times indicated. Endogenous TAK1 complex was immunoprecipitated from 2 mg of protein lysates with anti-TAK1 antibody and immunoblotted with specific antibodies against ubiquitin, TAK1, TAB2 and TAK1 [pThr187]. Protein lysates were immunoblotted with anti-NLK and actin antibodies to check for NLK knockdown and as loading control respectively.

B

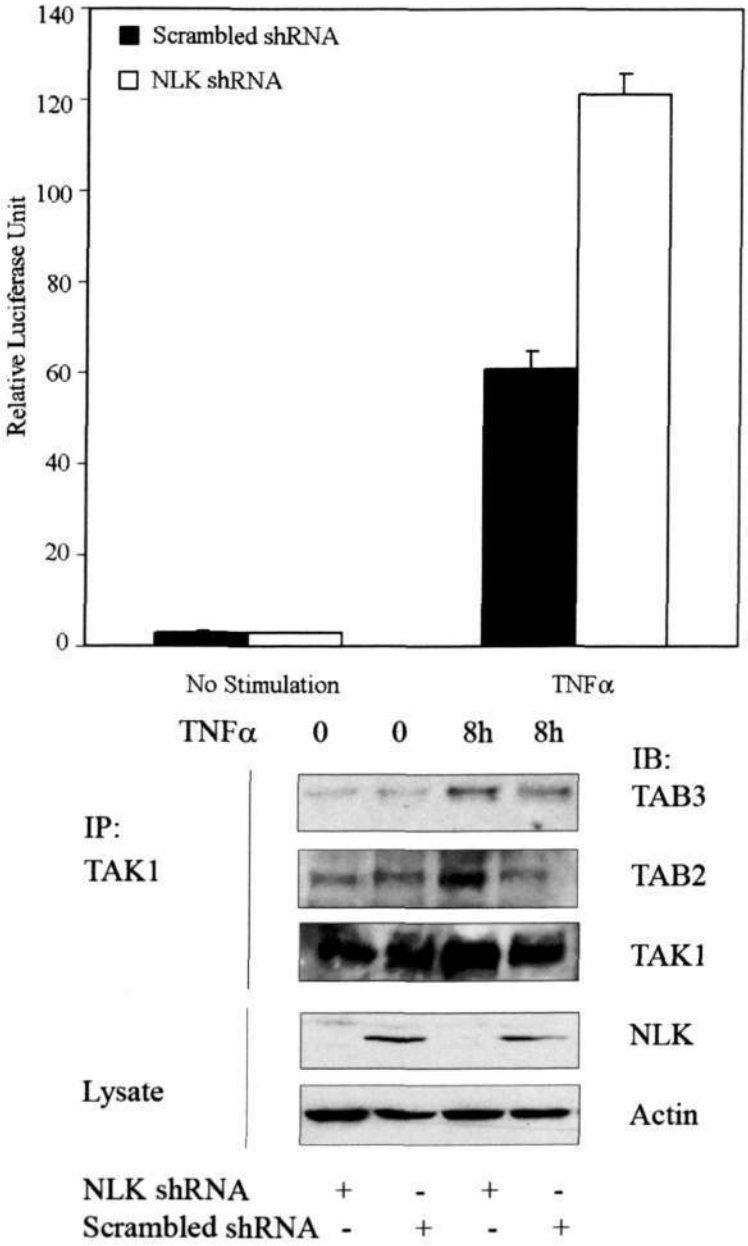


Figure 3.15 NLK inhibits activation of NFκB in TNFα pathway

(B) NLK knockdown increases activation of NFkB gene mediated by TNFα –

HEK293 cells expressing "scrambled" or NLK targeting shRNA 5 were stimulated with TNFα for the times indicated. Endogenous TAK1 complex was immunoprecipitated from 2 mg of protein lysates with anti-TAK1 antibody and immunoblotted with specific antibodies against ubiquitin, TAK1, TAB2 and TAK1 [pThr187]. Protein lysates were immunoblotted with anti-NLK and actin antibodies to check for NLK knockdown and as loading control respectively.

C

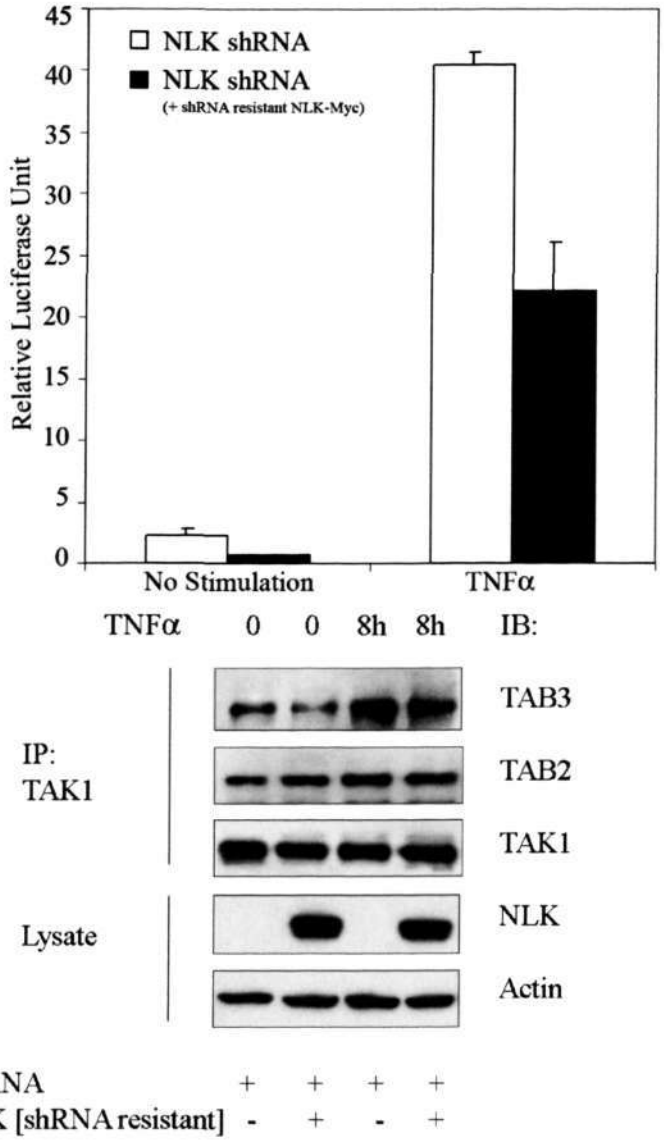


Figure 3.15 NLK inhibits activation of NFκB in TNFα pathway

(C) NLK knockdown increases activation of NFkB gene mediated by TNFα –

HEK293 cells expressing "scrambled" shRNA or shRNA 5 targeting NLK were transfected with NFκB reporter and control plasmids with or without plasmid encoding shRNA resistant form of NLK. The cells were stimulated with TNFα for 0 h and 8 h and the protein lysates processed as in (A). Assays were done in triplicate and the results shown are the mean ± SEM from three independent experiments. The protein lysates were immunoprecipitated with TAK1 antibody and western blotted for TAK1, TAB2 and TAB3. Total protein lysates were western blotted for NLK and actin. (bottom)

D

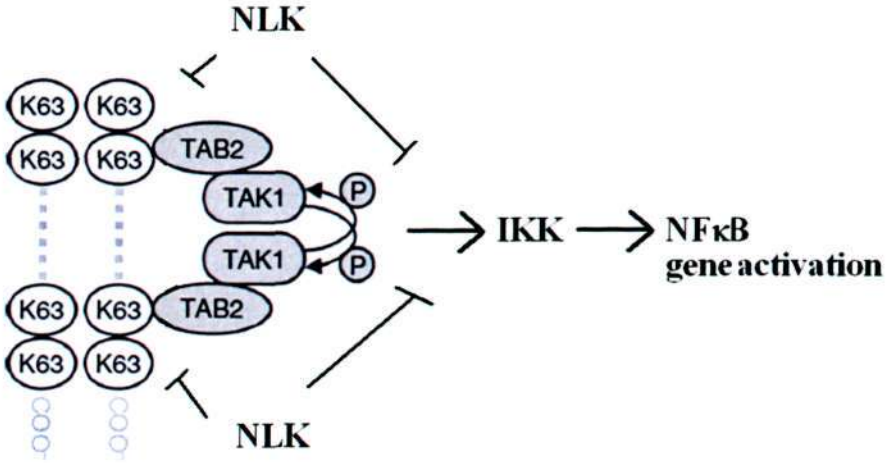


Figure 3.15 NLK inhibits activation of NFκB in TNFα pathway

(D) Diagram illustrates the possible regulatory mechanism of TAK1 and NFκB signaling pathway by NLK. TAK1 autophosphorylation mediated by cross phosphorylation is facilitated by the scaffold structure formed between TAB2/3 and K 63 linked polyubiquitin chains. Our model supports the hypothesis that NLK inhibits TAK1 and its downstream kinase IKK activity via phosphorylation of TAB2/3.

3.16 NLK interacts with c-jun and inhibits AP-1 activity directly

Since IL1 and TNF upregulate both AP-1 and NFκB gene *in vivo* [63], we extended our interest by looking at the role of NLK in AP-1 mediated gene activation in mammalian cells.

The mammalian AP-1 proteins are homodimers and heterodimers composed of basic region-leucine zipper (bZIP) proteins and belong to the Jun (c-jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) subfamilies [93-98]. These AP-1 dimers regulate gene expression in response to different stimuli such as growth factors, cytokines, and UV irradiation or stress. The activity of AP-1 is regulated by phosphorylation of c-jun, c-Fos or ATF2 proteins by specific upstream kinases induced by specific extra cellular stimuli. [93, 98]

To investigate the relationship between NLK and c-jun, we test whether NLK regulates the transcriptional activity of c-jun by using AP-1 luciferase linked reporter gene. Single expression of c-jun upregulates AP-1 gene activation as shown in Figure 3-17 A. however, co-expression of NLK with c-jun leads to reduction of AP-1 transcriptional activity. [Fig 3-16 A]

To validate the above finding, we also examined AP-1 gene activation in NLK "knockdown" or control cells which are co-transfected with AP-1-luciferase reporter and renila control plasmid. AP-1 activity was measured upon activation of the both sets of cells with TNFα for 0h or 8h. In line with the findings from the over-expression study, AP-1 activity is increased in NLK knockdown cells compared with control cells. [Figure 3-16B] Notably, the result led us to the conclusion that NLK also negatively regulates AP-1 activity.

Next, we investigate the molecular mechanism of NLK regulation on AP-1 activity. A recent finding has suggested the novel function of c-jun in canonical Wnt signaling and showed that c-jun function as a scaffold in the β-catenin-TCFs transcriptional complex linking the Dvl (disheveled) protein to TCF/ β-catenin activated

gene expression. [99] Another group has also reported that phosphorylated c-jun interacts with the HMG-box transcription factor TCF4 and forms a ternary complex [c-jun, TCF4 and β-catenin] to activate c-jun mediated expression in intestinal tumorigenesis. [100]

Therefore, the interesting function of c-jun in Wnt signaling and its physical interaction with TCF and β-catenin (first and best characterized substrates of NLK) led us to examine the possible role of NLK in regulation of c-jun activity. First, we examine the possible interactions between NLK and c-jun. GST-NLK/GST-c-jun were co-expressed with myc-c-jun/myc-NLK in HEK293 cells and subjected to GST pulldown. As indicated in the Figure 3-16 C lane 4, myc-c-jun shows very strong interaction with NLK. This interaction was also observed when we pulled down kinase active or kinase dead myc-NLK with GST-c-jun. [Figure 3-16B lane 2 and 3]

Altogether, we identified c-jun as a novel interacting partner of NLK for the first time and also provide more insight into the regulation of TNFα and IL-1β signaling by NLK.

A

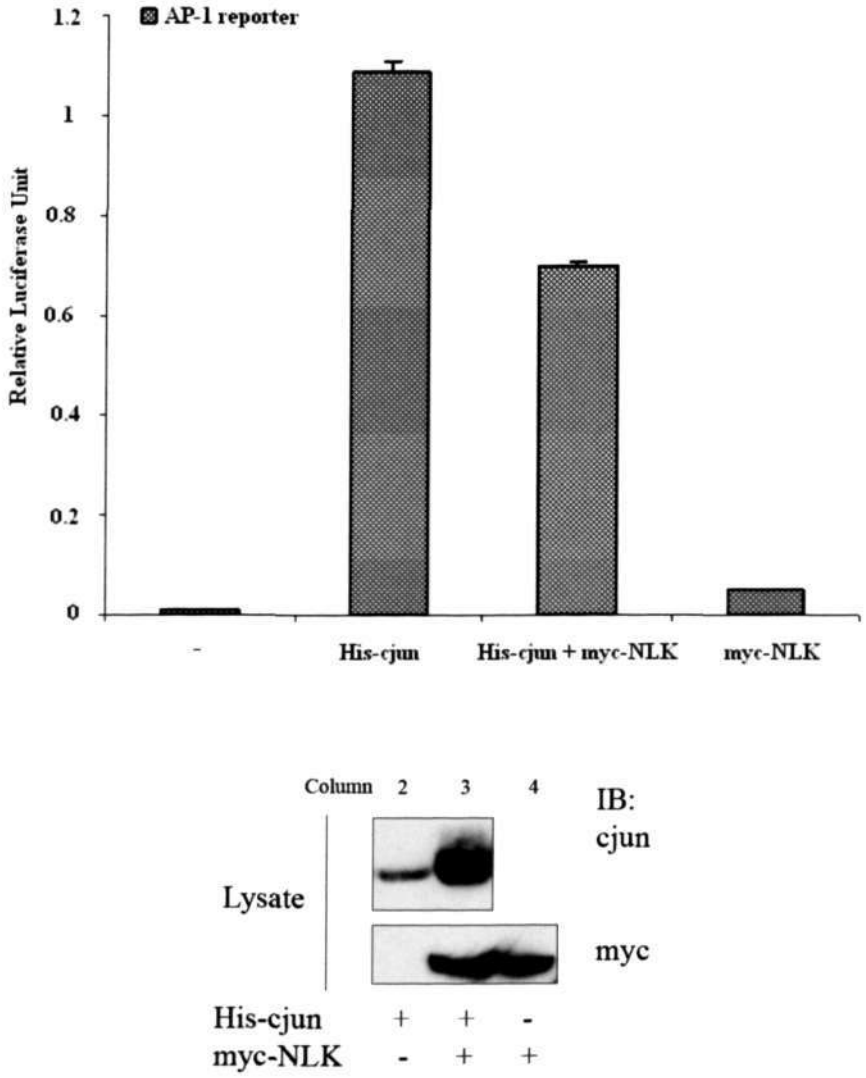


Figure 3.16 NLK interacts with c-jun and inhibits activation of AP-1 pathway

(A) NLK suppresses AP-1 reporter gene activation in cells –

HEK293 cells were transfected with the indicated combination of the plasmids in the presence of AP-1 reporter and control renilla luciferase plasmid constructs. The cells were harvested and the protein lysates assayed for expression of firefly luciferase. Assays were done in triplicate and the results shown are the mean ± SEM from three independent experiments. Protein lysates were probed for expression of proteins transfected by using anti-myc and His specific antibodies.

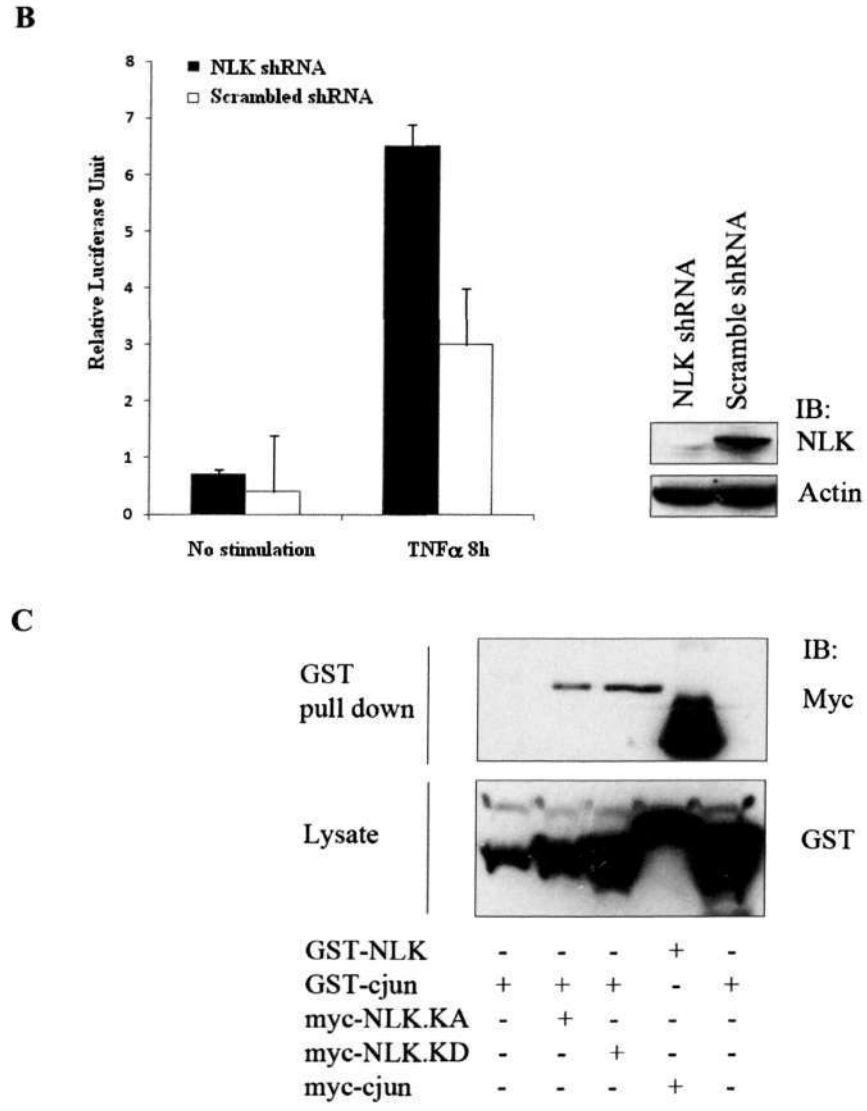


Figure 3.16 NLK interacts with c-jun and inhibits activation of AP-1 pathway

(B) NLK knockdown increases the AP-1 reporter gene activation mediated by TNFα – HEK293 cells expressing shRNA targeting NLK or scrambled shRNA were transfected with AP-1 reporter activated firefly luciferase and control renilla luciferase. The cells were stimulated with and without TNFα for 0 h or 8 h and the cells were harvested and the protein lysates assayed for expression of firefly luciferase. Assays were done in triplicate and the results shown are the mean ± SEM from three independent experiments. Total protein lysates were western blotted for c-jun and actin.

(C) HEK293 cells were transfected with different combination of plasmids as indicated, followed by GST pulldown assay using 0.5 mg of total cellular lysates. The co-precipitated proteins were analyzed by western blot with anti-myc antibodies. The expressions of GST tagged proteins were shown in GST immunoblot.

3.17 NLK phosphorylates c-jun *in vitro*

The strong interaction between NLK and c-jun in our study led us to check whether c-jun is a possible substrate of NLK. c-jun activation is instrumental in many cellular events such as cell growth and differentiation, apoptosis, cell transformation, tissue morphogenesis, and inflammatory responses. [101]

His-tagged c-jun and GST-NLK were separately expressed in HEK293 cells and the whole cell extracts were used to perform immunoprecipitation with anti-His antibody or GST pulldown assay. The precipitated proteins were subjected to an *in vitro* kinase assay. As shown in Figure 3-17A lane 2, c-jun was strongly phosphorylated by NLK *in vitro*.

To validate the result from A, we repeated the NLK kinase assay by using bacterial purified GST-c-jun as substrate. The Coomassie staining indicates the amount of GST-NLK and GST-c-jun used in the assay. All togheter, our result suggests that c-jun is a novel substrate of NLK.

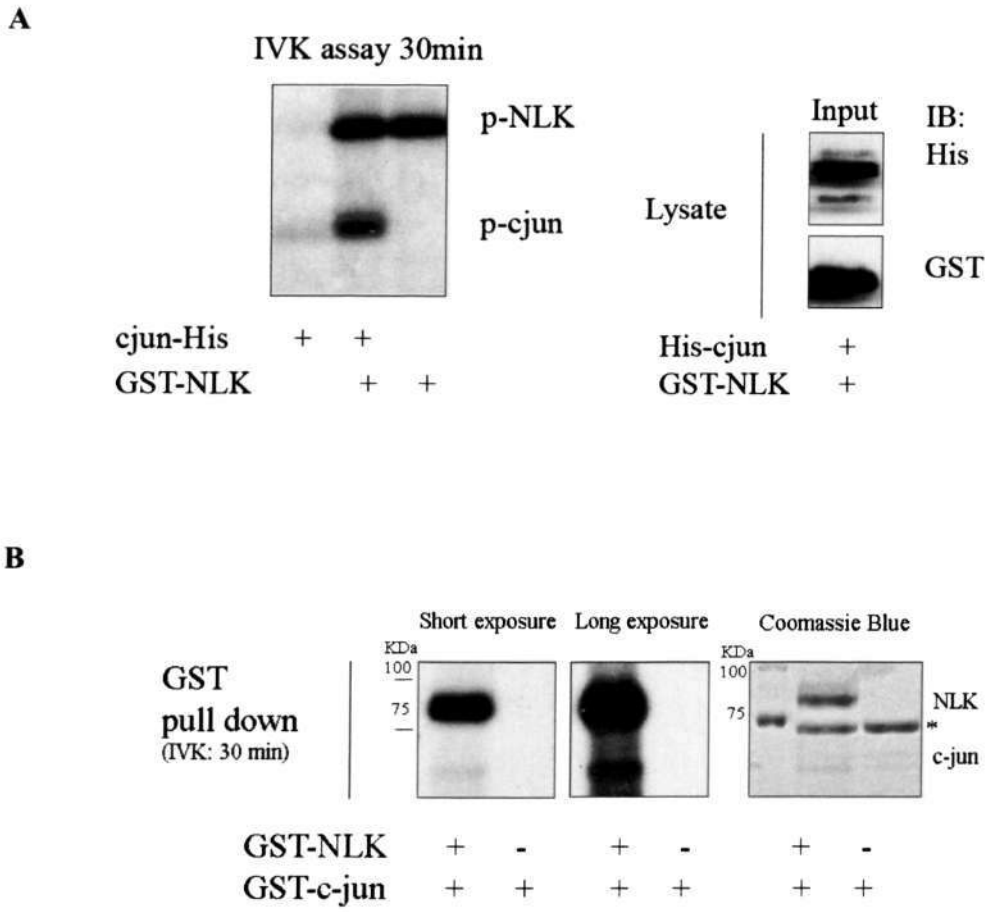


Figure 3.17 NLK phosphorylates c-jun *in vitro*

(A) HEK293 cells were transfected with the plasmids encoding the indicated proteins. Cellular lysates containing over-expressed His-c-jun (0.25 mg) and GST-NLK (0.1 mg) was subjected to immunoprecipitation and GST pulldown respectively. The purified proteins were incubated with [γ^{32}P] labeled ATP (0.1 mM) at 37°C for 30 minute. The gels were stained with Coomassie Blue and autoradiographed at -80°C for overnight. The amount of protein used in the assay is indicated in the western blot.

(B) 0.1 mg of HEK293 cells over-expressing GST-NLK were subjected to GST pulldown followed by an *in vitro* kinase assay as described in (A) and incubated with 10 µg of GST-c-jun affinity purified from *E.coli*. The protein input stained with Coomassie Blue is shown. Non specific band is indicated by asterisk.

3.18 NLK negatively regulates c-jun activity in multiple signaling pathways

To gain further insights into the molecular mechanism of AP-1 regulation by NLK, we checked the activity of c-jun in cells treated with different stimuli. It has been proved that phosphorylation of c-jun at Ser 63 and Ser 73 residues by JNKs enhance AP-1 transcriptional activity. [93] We used the commercial c-jun S-63 phospho antibody to measure c-jun activity in NLK knockdown cells compared with control cells. In this experiment, we used several external stimuli such as anisomycin, sorbitol and TNFα to stimulate c-jun phosphorylation in HEK293 cells.

Densitometric analysis and western blots shown in Figure 3-18A indicate that c-jun [pS-63] phosphorylation was drastically increased in NLK knockdown cells compared to control cells in response to all stimuli used. NLK knockdown cells undergo three to five fold increases in phosphorylation of c-jun mediated by TNF and anisomycin. However, a two fold increase in c-jun phosphorylation was observed for sorbitol stimulation. [Figure 3-18A] It has been shown that anisomycin can activate JNK and c-jun through pathways that are independent of TAK. Figure 3-18B also demonstrates that TAK1 autophosphorylation using the phospho-Thr187 antibody was not detected in response to anisomycin stimulation. Therefore, TAK1 itself is not activated by anisomycin; anisomycin stimulated increase in c-jun activity observed in NLK knockdown cells suggests the direct regulation of c-jun activity by NLK. However, in our study, we are still uncertain about the pathway that leads to NLK activation in cells stimulated with anisomycin.

And, we also cannot ignore the fact that inhibition of c-jun activity by NLK in TNFα signaling could be partly attributed to negative feedback regulation of TAK1-JNK pathway by NLK. [Figure 3-18C] Nevertheless, a drastic increase in c-jun activity or phosphorylation mediated by anisomycin signaling has strengthened the direct physiological connection between NLK and c-jun.

This finding led us to conclude that NLK may regulate c-jun activity in both TAK1 activity dependent signaling (for example, TNFα) and TAK1 activity independent signaling (for example, Anisomycin) pathways. [Figure 3-18C]

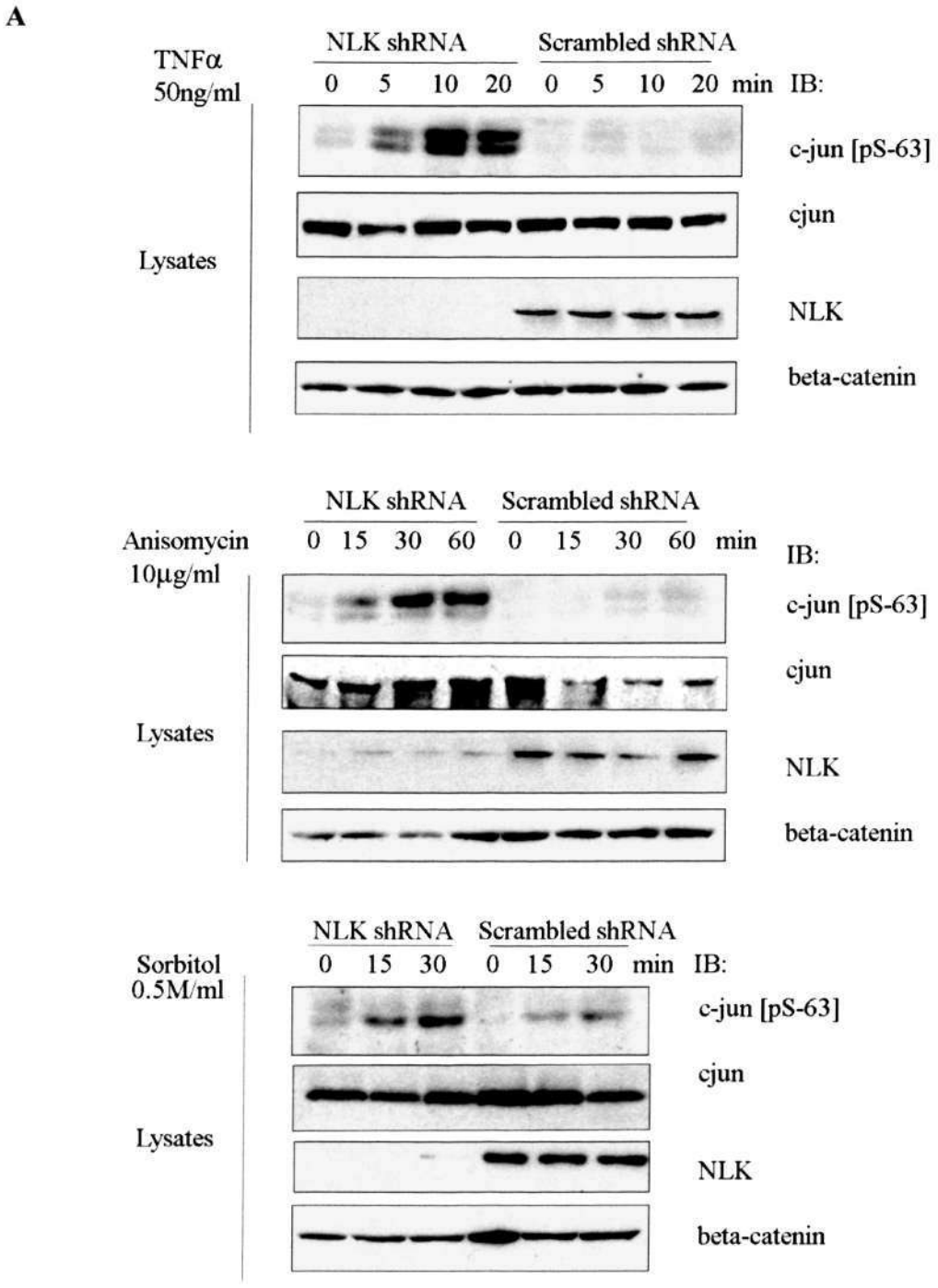


Figure 3.18 NLK negatively regulate c-jun activity

(A) NLK knockdown increaseas the phosphorylation of c-jun mediated by different stimuli – HEK293 cels were transfected with NLK or scrambled shRNA and c-jun total and phospho S63 antibodies were used to probe whole cell extracts. The knock down efficiency of NLK shRNA and loading control of protein input are indicated by NLK and beta-catenin immunoblots.

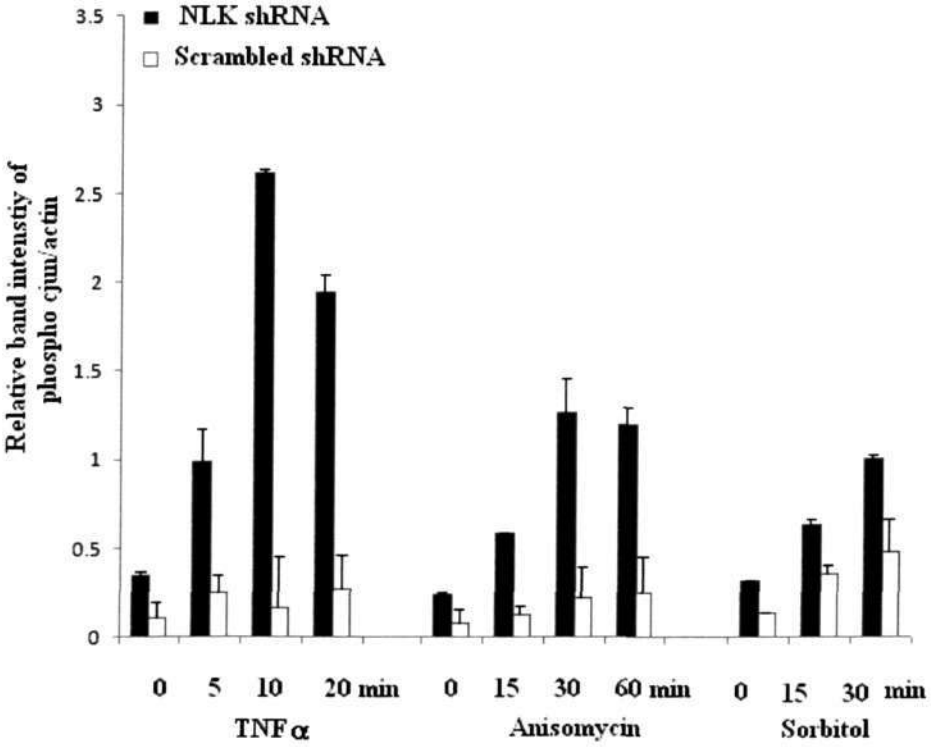
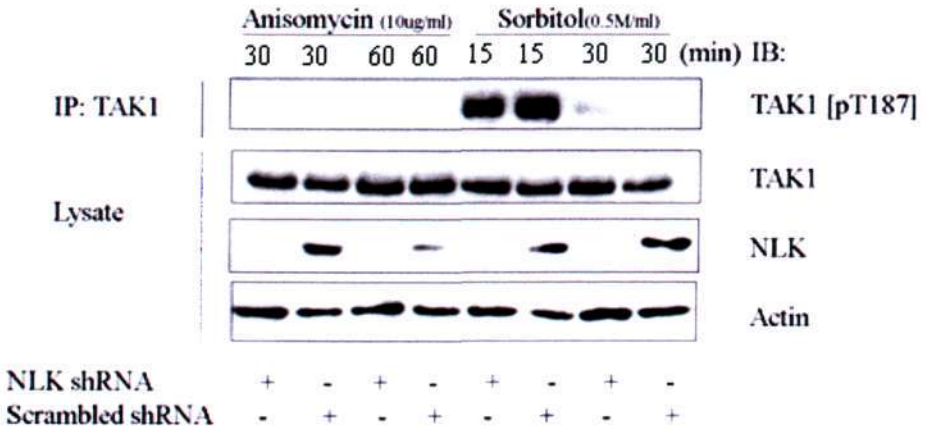


Figure 3.18 (A) NLK knockdown increaseas the phosphorylation of c-jun mediated by different stimuli –

Densitometric analysis of phospho c-jun S63 and total c-jun immunoblots in NLK knockdown cells and control cells are analyzed by using image J software. The Y axis represents the relative band intensity of pS63 signal divided by total cjun signal. The results are representative of two indpendent experiments.

B



C

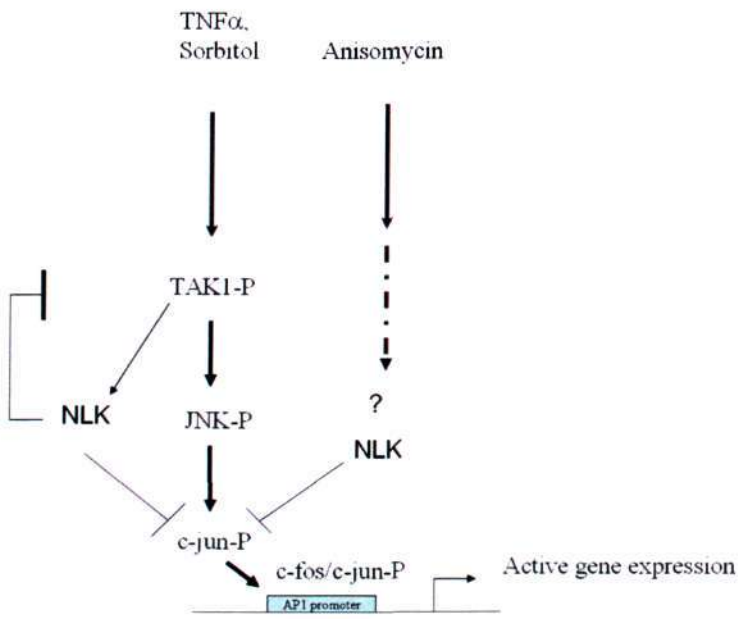


Figure 3.18 NLK negatively regulate c-jun activity

(B) TAK1 is activated by sorbitol, but not by anisomycin –

HEK293 cells were transfected with NLK or scrambled shRNA. The whole cell extracts were subjected to immunoprecipitation with anti-TAK1 antibodies and probed for TAK1 activation with TAK1 phospho T187 antibodies. The efficiency of shRNA knockdown and loading control are indicated by TAK1, NLK and actin immunoblots.

(C) Simple illustration of regulation of c-jun activity by NLK –

NLK negatively regulates c-jun activity in anisomycin activated signaling through phosphorylation. In TNFα signaling, NLK down-regulates c-jun activity directly or indirectly by suppressing the activation of TAK1-MKK4/7-JNK signaling.

3.19 NLK negatively regulate the expression and secretion of inflammatory factors mediated by NFκB and AP-1

Previous findings indicate that NLK regulate NFκB and AP-1 activity in inflammatory signaling activated by TNFα or IL-1β. To gain further insights into NLK regulation of TAK1 activity and its downstream transcriptional factors NFκB and AP-1, we examined the expression and secretion of cytokines and/or chemokines mediated by TNFα or IL1 β in stable "NLK knockdown" cells.

HEK293 cells were transfected with NLK shRNA or Scramble shRNA plasmids and stably transfected cells were selected in condition media for 72 hours. The cells were starved for 6 hours prior to stimulation with TNFα (HEK293 cells) for 3 hours, 6 hours and overnight (16 hours). The media were collected from both set of cells and secretion of inflammatory cytokines and chemokines quantitated with a human inflammation antibody array as described in Fig. 3-19A. No significantly detectable amounts of inflammatory factors was found in media which collected from either NLK "knockdown" or control plates at 3 h and 6 h time points [data not shown].

The inflammatory antibody array can quantitate a total of 40 inflammation factors shown in the table. The inflammatory proteins which are examined in our study are highlighted with grey box in the table. [Figure 3-19B] Among all the inflammatory cytokines and chemokines, TNFα, TIMP-2, IP-10, MCP-1 and IL-8 are highly secreted from both sets of cells. [Figure 3-19B&C] The relative amount of secreted cytokines and chemokines induced by TNFα in NLK knockdown and control cells were analyzed and represented in the bar chart [Fig. 3-19C].

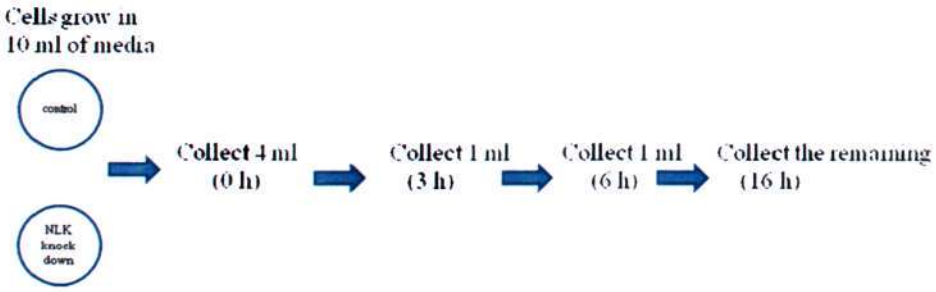
After overnight stimulation, MCP1 secretion in NLK knockdown cells is significantly increased by five fold compared with control cells. [Highlighted the duplicated dots in black box as shown in Figure 3-19 B and D: Row 5-6, Column F] It has been well characterized that MCP-1 gene expression and secretion is tightly regulated by AP-1 and NFκB in response to TNFα signaling. [102-103] In addition, a two to three

fold increase in secretion of TNFα, TIMP-2 and IP-10 and IL-8 were also observed in NLK knockdown cells compared to control cells in response to TNFα. [Figure 3-19 B&C]

To further confirm the above result, we also measured the upregulation of MCP-1 gene in NLK knock down cells by performing quantitative real time PCR reaction. Total RNA was extracted from HEK293 cells stably expressing NLK shRNA or Scramble shRNA and stimulated with TNFα. In agreement with the inflammatory antibody array analysis, we also observe the upregulation of MCP1 gene expression by two fold in NLK knockdown cells compared to the control cells in response to TNFα stimulation. [Figure 3-19D]

Collectively, the finding from cytokine array strengthens the inhibitory role of NLK on TNFα mediated activation of TAK1 and gene expression by NFκB and AP-1 by modifying the function of TAB2/3 and c-jun. [Figure 3-19E]

A



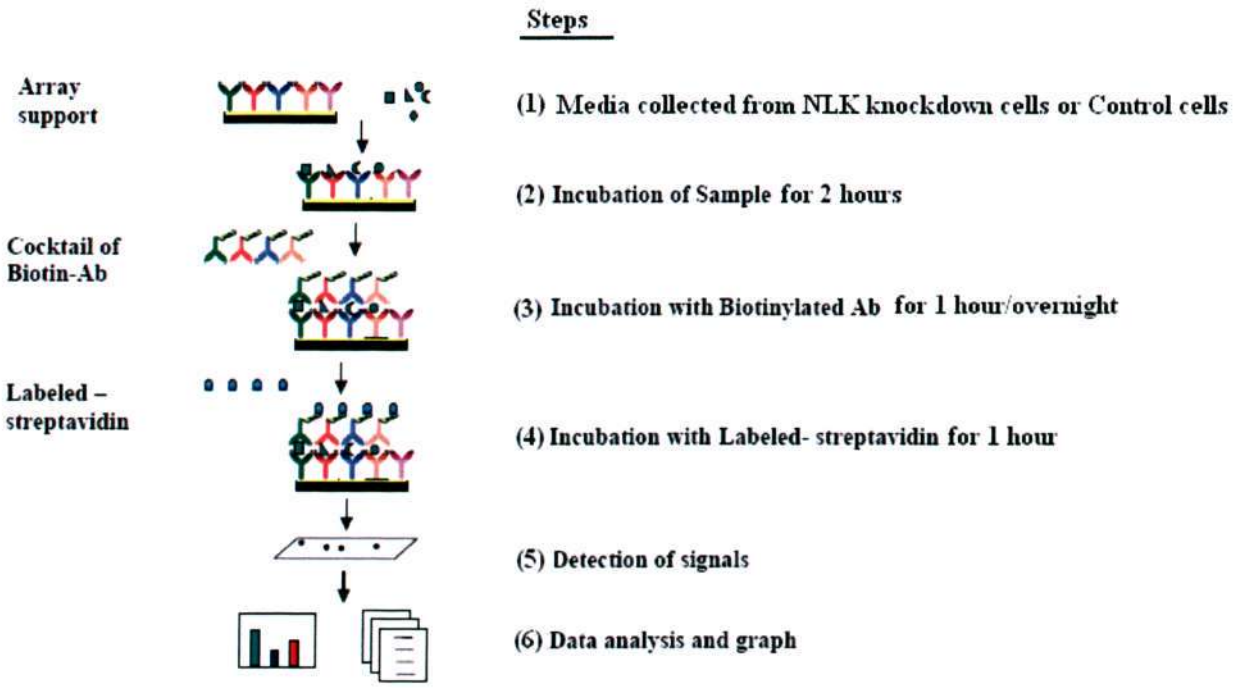


Figure 3.19 Effect of NLK depletion on the profile of cytokine protein secretion by HEK293 cells

(A) Protocol and experimental steps performed in the analysis of human inflammatory antibody array.

B

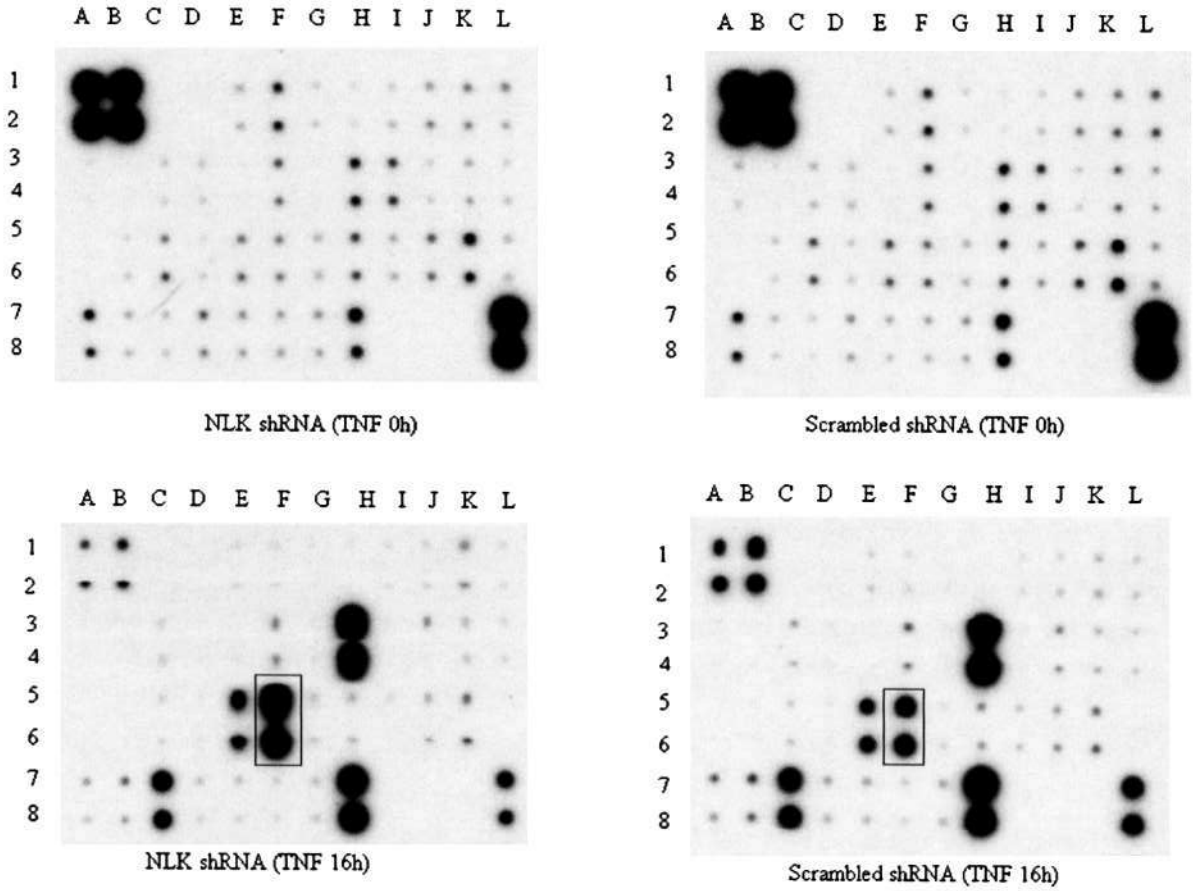


Figure 3.19 Effect of NLK depletion on the profile of cytokine protein secretion by HEK293 cells

(B) HEK293 cell cultures stably expressing NLK shRNA or control scrambled shRNA were treated with TNFα (50 ng/ml) for 0 hour and 16 hours. Cell culture supernatants were collected and analyzed using a RayBio Human Cytokine Protein Array. The blots were processed as described in the manual.

B

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	Neg	Neg	Eotaxin	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-r	I-309	IL-1α
2	POS	POS	Neg	Neg	Eotaxin	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-r	I-309	IL-1α
3	IL-1b	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1b	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1	MIP-1β	MIP-1D
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1	MIP-1β	MIP-1D
7	RANTES	TGF-β1	TNF-α	TNF-β	S TNF RI	S TNF RII	PDGF-BB	TIMP-2	BLANK	BLNAK	NEG	POS
8	RANTES	TGF-β1	TNF-α	TNF-β	S TNF RI	S TNF RII	PDGF-BB	TIMP-2	BLANK	BLNAK	NEG	POS

Identity of inflammatory factors used to study in cytokine array

(The secretion of cytokines or chemokines quantitated in this study are highlighted in grey)

C

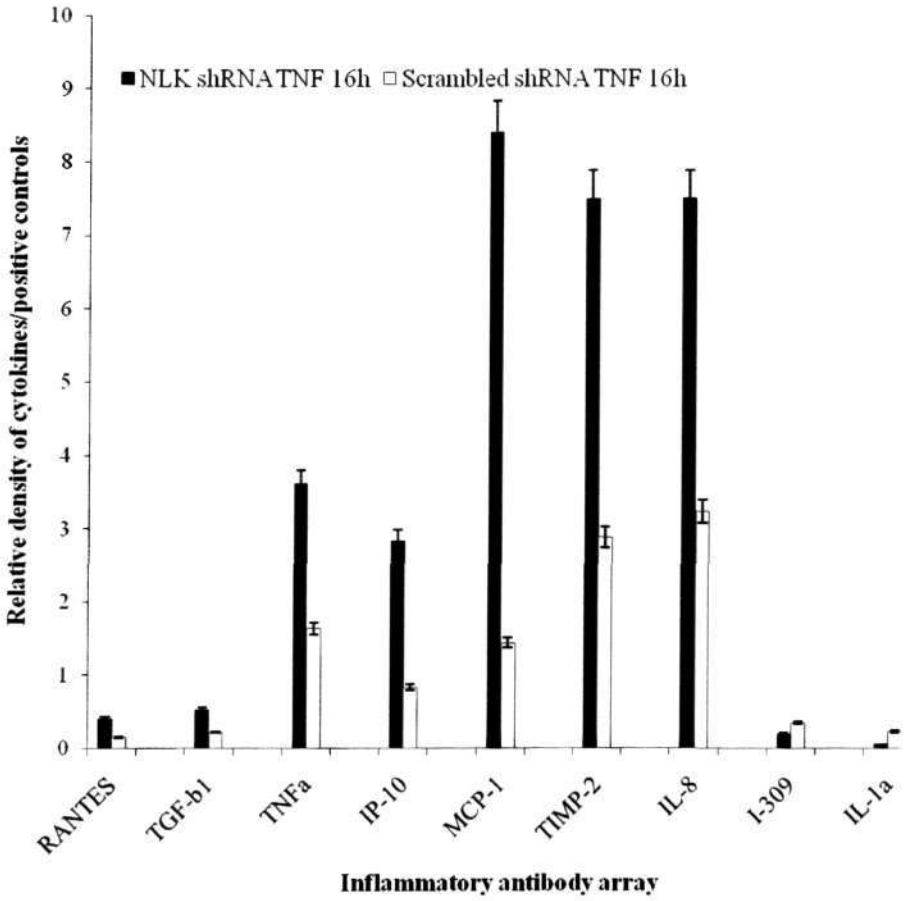
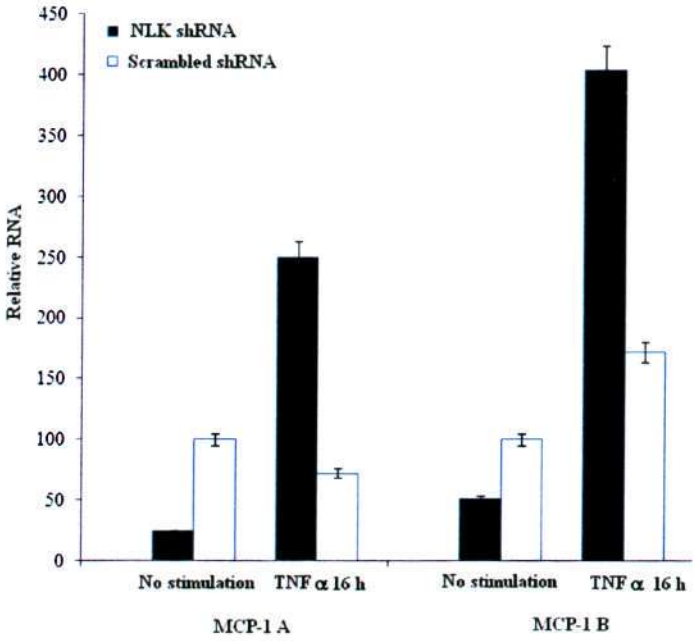


Figure 3.19 Effect of NLK depletion on the profile of cytokine protein secretion by HEK293 cells

(C) Cytokine expression was quantified by densitometric analysis. The signal intensity of images or dots from both sets of experiment was scanned as a high resolution image and the relative intensity of the respective dots (cytokine signal/ positive signal integrated density) qunatitated using Image J software. Data are expressed as mean ± SEM of two independent experiments.

D



E

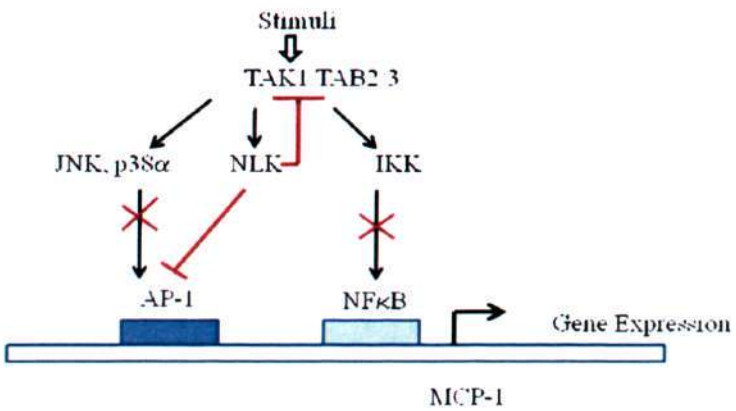


Figure 3.19 Effect of NLK depletion on the profile of cytokine protein secretion by HEK293 cells

(D) HEK293 cell cultures which are stably expressing NLK shRNA or control shRNA were stimulated with TNFα (50 ng/ml) for 16 hours. Total RNA was isolated and analyzed by qRT-PCR. Quantities of MCP-1 mRNA were normalized by the expression of actin and GAPDH mRNA. The average of three experiments is shown with ± SEM.

MCP-1_A represents the relative RNA ratio of MCP-1/Actin

MCP-1_B represent relative RNA ratio of MCP-1/GAPDH

(E) A proposed model suggesting the mechanism of AP-1 and NFκB gene regulation by NLK. NLK directly regulates AP-1 activity by phosphorylating c-jun as well as indirectly through the negative feedback regulation of TAK1 and JNK activtiy.

4. Discussion

The present study has identified a negative feedback regulation of TAK1 by NLK. In the first part of our study, we showed that NLK interacts and phosphorylates TAB2 and TAB3. In the second part, we demonstrated that NLK phosphorylation on TAB2 and TAB3 modulates their conformational changes and abrogates their interaction with K63 linked polyubiquitin chains. Therefore, we hypothesized that phosphorylation induced allosteric changes in adaptor protein TAB2/3 by NLK decreases binding of K63 linked poly ubiquitin chains. Consequently, the ability of TAB2/3 to activate TAK1 and its downstream signaling pathways p38α, JNK and NFκB is diminished.

In our study, we have shown the evidence of NLK interaction with TAB2 and TAB3. [Figure 3-1D] Due to the limitation of NLK antibodies, we can only conclude their stable and strong interaction based on proteins over-expressed in HEK293 cells. As indicated in Figure 3-1E and F, over-expressed NLK was found to be complexed with endogenous TAK1 and vice versa.

We also mapped the region mediating the interaction between TAB2/3 and NLK. The minimal fragment of NLK [124-497] consisting of the kinase domain and surrounding C terminal region is required to interact with the C terminal region of TAB2 between residues 481-588 [Fig 3-2C].

Recently, a few novel phosphorylation sites on TAB2/3 have been identified and these sites are thought to be involved in negative regulation of TAK1 in IL-1 signaling pathway. Ser60 and Thr404 of TAB3 were reported to be phosphorylated directly by p38α, whereas Ser506 of TAB3 may be phosphorylated by MAPKAP-K2/MAPKAPK3. In addition, Ser 372 and Ser 524 on TAB2 are also phosphorylated in IL1 signaling but the kinases responsible for their phosphorylation are still unknown. These reports led us to examine whether Ser 372 and Ser 524 on TAB2 sites are phosphorylated by NLK.

Therefore, we used to coexpress the single phosphorylation mutants of TAB2 [S372A] and [S524A] with NLK in cells and examined whether NLK is responsible for

phosphorylation of Ser 372 and Ser 524 on TAB2. However, we did not find any evidence of NLK phosphorylation on Ser 372 or Ser 524 residue of TAB2. [Data not shown] Interestingly, *in vitro* kinase assay indicates that C-terminal fragment of TAB2 [481-693] is phosphorylated by NLK [Figure 3-4C] and subsequently, we identified Ser 582 residue as NLK phosphoryaltion site on TAB2 fragment. [Figure 3-4D] Since Ser 582 is not followed by proline and NLK consensus phosphorylation sequence is still unknown at present, we still cannot rule out whether NLK indeed phosphoryaltes TAB2 on Ser 582 residue *in vivo*. Therefore, further studies are needed to be done by using the NLK phosphorylation mutant of TAB2 in cells and also required to validate the result by using the phospho Ser 582 TAB2 antibodies *in vivo*. Furthermore, we also want to repeat *in vitro* kinase assay and phospho peptide purification experiments for full length TAB2 protein and TAB3 protein to validate our result in future.

Though NLK was discovered as one of the regulatory signaling components in Wnt signaling in many organisms [5, 104], recent reports have shown that NLK can be activated by inflammatory cytokines such as IL-1, IL-6 and TNFα via TAK1 [22]. However, the questions still remain as to how NLK is activated by TAK1 in extracellular stimuli and whether an intermediate protein kinase such as HIPK2 is involved. Previous studies on TAK1-NLK pathway have largely ignored the role of TAB2 and TAB3. Therefore, in this study, we also attempt to carefully examine and characterize the function of TAB2 and TAB3 in NLK activation.

In our study, we also tried to address the missing link in TAK1-NLK signaling and the physiological significance of NLK phosphorylation on TAB2 and TAB3 by raising two possible hypotheses.

1. TAB2 or TAB3 acts as scaffold to facilitate the activation of NLK by TAK1
   or
2. NLK negatively regulates TAK1 activity via modification of TAB2 or TAB3.

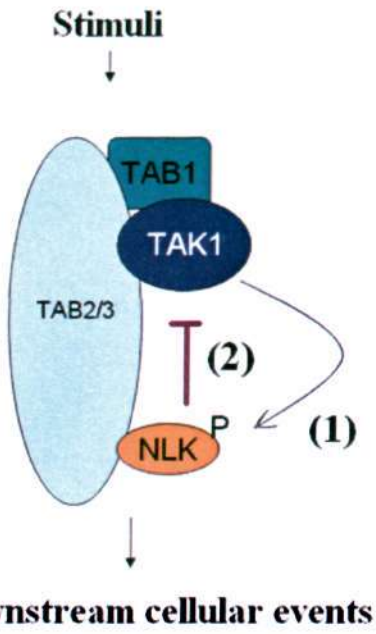


Figure 4-1. Schematic representation of two possible models of NLK regulation on TAK1 signaling

1. Upon stimulation, NLK interacts with TAB2/3 thus bringing TAK1 and NLK into close proximity and facilitates the activation of NLK by TAK1.
2. Upon stimulation of cells with specific stimuli, activated NLK phosphorylates TAB2 or TAB3 and modulates the conformational changes of TAK1 which is in the complex with TAB2/3 and negatively regulates TAK1 activity.

Scaffold proteins such as JIP1 and JIP2 which bind the ASK1/MLK, MKK4/7, JNK protein kinases have previously been shown to enhance signaling efficiency and specificity in MAPK signaling pathways. [105] Previously NLK was also reported to recruit STAT3 as scaffold molecule to facilitate its own activation by TAK1 in IL-6 signaling. [75]

Therefore, we tested our first hypothesis by performing *in vitro* kinase assay using purified NLK protein as substrate in the presence of active purified TAK1 complex from HEK293 cells [TAK1-TAB1-TAB2/3]. Unexpectedly, TAK1 complex failed to phosphorylate *E.coli* purified NLK [124-480] *in vitro* as shown in Figure 3-6A and it disagrees with our first hypothesis.

Even though TAK1complex is unable to activate NLK directly in our *in vitro* kinase system, we do not exclude the possibility that TAB2 and TAB3 may function as scaffold proteins to facilitate the activation of NLK by TAK1 for two remaining reasons. Firstly a minute amount of TAB2/3 associated with TAK1 kinase complex used in the *in vitro* kinase assay, and secondly general limitations of *in vitro* kinase assay approach widely used in biochemical studies.

However, recent studies have indicated that HIPK2 [9] may function as an intermediate kinases between TAK1 and NLK when cells are activated by Wnt 1. In contrast to their data, we did not observe any direct phosphorylation or activation of NLK by HIPK2 in an *in vitro* kinase assay. [Fig. 3-6 D] Alternatively, we introduced MKK6 as the intermediate kinase and co-incubated together with TAK1 complex in an *in vitro* kinase system to check the phosphorylation of NLK by activated MKK6. However, our data shown in Figure 3-6C demonstrated that TAK1 does not activate NLK through MKK6.

Since we could not find any evidence of TAB2/3 as scaffold protein to facilitate NLK activation by TAK1, we moved on to test the second hypothesis. In the course of testing the second hypothesis, we found several evidences to show NLK as negative regulator of pro-inflammatory signaling induced by TNFα or IL-1β. As shown in Figure 3-8A, over-expression of NLK with TAK1-TAB2/3 suppresses TAK1 activity in a dose dependent manner as indicated by a decrease in TAK1 Thr 187 phosphorylation. Similarly, as shown in Figure 3-8B we also illustrated a reduction in TAK1 Thr 187 phosphorylation and its substrate MKK6 in the presence of kinase active NLK. In this experiment, we also highlighted that NLK inhibits TAK1 activity only through phosphorylation of TAB2 or TAB3, but not TAB1.

The novel inhibitory role of NLK on TAK1 and its downstream signaling molecules p38α, JNK and NFκB was also validated in our biochemical assays using NLK knockdown cells or control cells which are stimulated with TNFα or IL-1β. When NLK knockdown cells and Scrambled shRNA transfected cells are stimulated with TNFα

for several time points, TAK1 activity or Thr 187 phosphorylation of TAK1 is increased drastically in NLK knockdown cells. [Figure 3-9 B-F] Similarly, approximately a two-fold increase in phosphorylation of p38, JNKs and IκB were detected in NLK knockdown cells induced by TNFα and IL-1β stimulation as shown in Figure 3-10A and D.

Since TAK1 is an essential intermediate kinase of IL-1 and TNFα signaling pathway, several mechanisms of TAK1 down-regulation are previously suggested in TAK1 mediated signaling.

1) Targeting TAB2/3 for degradation
2) Blockage of interaction between signaling modules at the point of TRAFs and TAK1-TAB1-TAB2/3
3) Phosphorylation or dephosphoryaltion of TAK1 and binding subunits TAB1 or TAB2/3.
4) Deubiqutination of ubiquitin ligase TRAFs or disassembly of ubiquitin chains from adaptor proteins.

The first mechanism was suggested by Yang Tian et al., 2007. RBCK1, which has been shown to act as an E3 ubiquitin ligase, negatively regulates TAK1 and TAB2/3-mediated and TNF- and IL-1-induced NFκB activation. RBCK1 physically interacts with TAB2/3 and facilitates degradation of TAB2/3 through a proteasome-dependent process. [106]

The second mechanism has been suggested by Hong et al., 2007. Smad7 is a critical mediator of TGF-β signals that block pro-inflammatory TNF signals by causing the dissociation of TAK1 complex from its upstream TRAF2 signaling complex. Smad 7 interacts with both TAB2 and TAB3, thereby blocking TNFα-induced recruitment of the TAK1 complex to TNFR1 and TRAF2 and the transmission of proinflammatory TNF signals. Over-expression of Smad7 in mouse skin suppressed inflammation and NFκB activation demonstrated the importance role of Smad7 as the negative regulator of TAK1 mediated inflammation pathway. [91]

The third mechanism highlights the regulatory role of phosphatases and kinases in TAK1 mediated cell signaling. Takaesu et al., 2001 and Taisuke Kajino et al., 2004 identified PP2C and PP6 phosphatases as negative regulators of TAK1 in IL-1 signaling pathway. PP6 dephosphorylates TAK1 at Thr 187 thus inactivating the kinase activity of TAK1. [107-108] p38α MAPK phosphorylates TAB1 at Ser423 and Thr431 and this modification serves to inhibit catalytically active (autophosphorylated) forms of TAK1, which may enhance the down-regulation of TAK1. p38α was also suggested to phosphorylate TAB2 and/or TAB3 to inactivate TAK1 in a negative feedback loop regulation of cell signaling. [56, 61, 64] So far two phosphorylation sites Ser 60 & Thr 404 of TAB3 are reported to be phosphorylated directly by p38α MAPK in IL-1 signaling. [62]

The fourth mechanism involves the removal of ubiqutin chains from signaling molecules by deubquitin ligase such as CYLD (cylindromatosis tumor suppressor protein) and A20. CYLD inhibits IKK activation by cleaving K63-linked polyubiquitin chains on several proteins including TRAF2, TRAF6 and NEMO. In patients with cylindromas, impaired function of CYLD was linked to activation of NFκB and pathogenesis of the tumors. [69-70, 109-110] Negative regulatory role of A20 was also validated in A20 deficient mice which develop severe inflammatory diseases in multiple organs with prolonged activation of NFκB. [68, 111]

Therefore, in this study, we proposed a novel negative feedback mechanism of NLK regulation on TAK1 which adopts a combined strategy from No. 3 and 4. NLK phosphorylates TAB2/3 and inhibits TAK1 activity by either inducing the conformational changes in TAK1 or causing the disassembly of K63 linked poly ubiquitin chains from TAB2/3.

As shown in Figure 3-12 D& E of dose-dependent phosphorylation of TAB2 and TAB3 by NLK which was accompanied by concomitant decreases in their binding to polyubiquitin chains as well as TAK1 Thr187 phosphorylation. The opposite effect was found in NLK knockdown cells where ubiquitin binding to TAK1 complex is enhanced

together with increased TAK1 T187 phosphorylation. [Figure 3-15A] In addition, we have also proved that reduced polyubiquitin binding by TAB2/3 is not due to disruption of the physical interaction between TRAFs and TAK1-TAB2/3 complex by NLK. [Figure 3-13 B&C]

We also pointed out in our *in vitro* ubiquitin binding assay and NFκB reporter gene assay that phosphorylation of TAB2/3 by NLK directly modulate their ability to bind polyubiquitin chains which leads to downregulation of TAK1 activity in TNF and IL-1 signaling. As shown in Figure 3-12B to E, co-expression of NLK with TAK1-TAB2/3 or TAK1 complex (TAK1-TAB1-TAB2/3) leads to decrease in poly ubiquitin binding of TAB2/3. In addion, co-expression of NLK with TAB2/3, TAK1-TAB2/3 or TAK1 complex (TAK1-TAB1-TAB2/3) also leads to suppression of NFκB reporter gene and it is shown in Figure 3-11A. In accordance with the over-expression study, increase in activation of NFκB reporter gene was observed in NLK knockdown cells compared to the control cells or NLK knockdown cells which are rescued by shRNA resistant NLK overexpression. [Fig. 3-15B and C]

Besides regulating the function of TAB2/3, NLK was found to interact and phosphorylate c-jun thus negatively regulates AP-1 activity. [Result section 3-16 to 3-18] Although we could not demonstrate the exact molecular mechanism by which NLK phosphorylation of c-jun leads to inhibition of its activity, our findings have potentially opened a novel connection between c-jun and NLK.

To conclude, we propose that NLK negatively regulates TAK1 activated signaling at multiple levels. Inhibitory role of NLK in inflammatory signaling mediated by TNF and IL1 can be divided into 2 phases of signal transduction events. [Figure 4-2]

- In early phases of signal transduction, NLK phosphorylates TAB2 and TAB3 to inhibit TAK1 activity by modulating poly ubiquitin binding of TAB2/3 or disassembly of poly ubiquitin chains from TAB2/3.
- In later phases of signal transduction, NLK directly suppresses transcriptional factor activity (c-jun) to regulate the inflammatory gene expression by AP-1. The

evidence of NLK regulating AP-1 target gene expression is demonstrated in result section 3-19 and Figure 3-19B & C. Several inflammatory factors MCP-1, TIMP2 and IL-8 are up-regulated in NLK depleted cells by two to five fold in response to TNFα signaling. [Figure 3-19B & C]

TAK1 represents a point of convergence for multiple signaling processes that are activated during inflammation and serves as a key target for regulation of inflammatory gene mediated by NFκB and AP-1. Therefore, the selective activation of NLK in negative feedback loop control of TAK1, NFκB and AP-1 regulatory pathways would serve as a potential therapeutic target for inflammatory diseases.

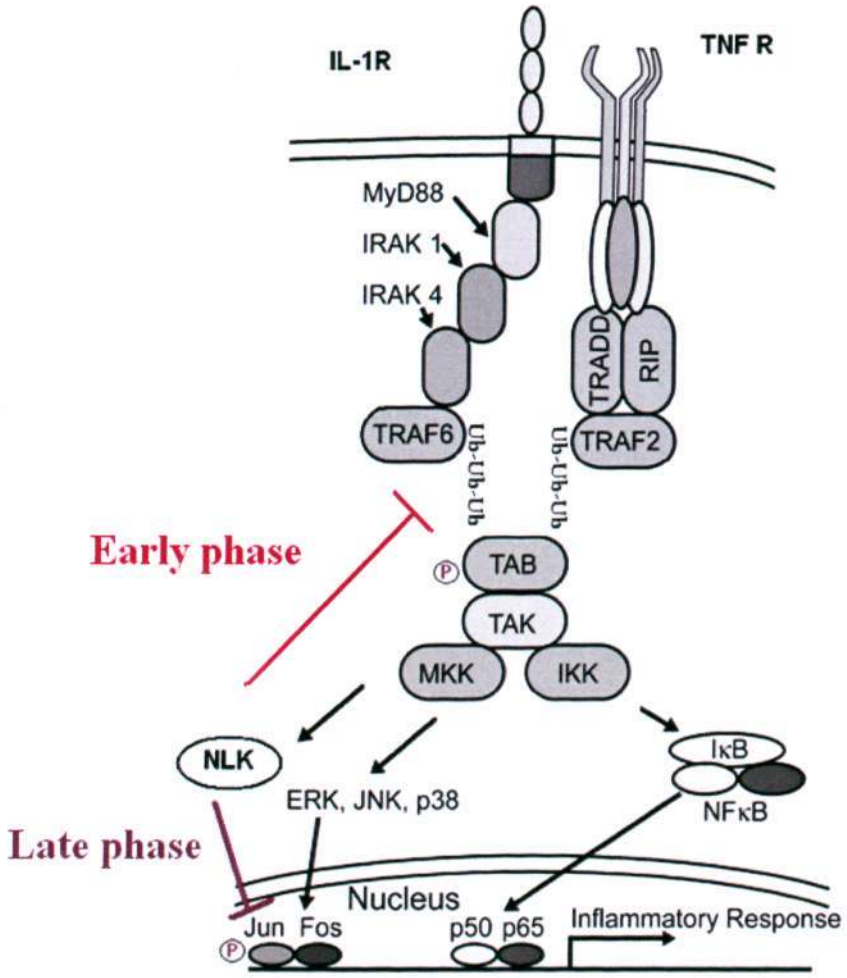


Figure 4-2. Model of NLK regulation on IL-1β and TNFα signaling

NLK regulation of TAK1 and inflammatory response by targeting TAB2/3 and directly regulating the transcriptional factor c-jun. Ubiquitin-mediated activation of transforming growth factor-β-activated kinase-1 (TAK1) and IκB kinase (IKK) in the IL-1R/TNFR pathway. Binding of interleukin-1β (IL-1β) to IL-1R causes recruitment of MyD88, IRAK, and TRAF6 to the receptor. Docking of TNF to its receptor recruits TRADD, TRAF2 and RIP. The E3 ubiquitin ligases TRAF2 and TRAF6, catalyzes the synthesis of K63-linked polyubiquitin chains, some of which are conjugated to TRAF6 or bound to TAB2. The polyubiquitin chains function as a scaffold to recruit the TAK1 and IKK complexes through binding to the regulatory subunits, TAB2/3 (shown here as TAB for simplicity). Recruitment of the kinase complexes facilitates autophosphorylation of TAK1 and subsequent phosphorylation of IKKβ by TAK1, leading to IκB degradation and subsequent activation of NFκB (represented by the p50/p65 dimer). Active TAK1 also activate p38 and JNK and subsequently leads to gene activation of AP-1. NLK regulates disassembly of K63-linked polyubiquitin chains from TAB2/3 complex by phosphorylation of TAB2/3. In addition, NLK also regulate inflammatory response at the level of gene regulation by phosphorylation of c-jun. Figure is adopted and modified from [112].

5. Perspectives and future direction

The most interesting feature of IL-1 and TNFα signaling arises from the identification of K63 linked polyubiquitination chain as a regulator of activation of TAK1 and NFκB mediated gene expression. One of the well known negative regulatory mechanisms in K63 linked ubiquitin chains in the activation of TAK1 and its downstream pathways are demonstrated by (Deubiquitin ligases) DUBs. However, degradation of poly ubiquitin chain by DUBs may be a slower process compared to phosphorylation. As we have suggested in our model [Fig.4-2], alternative strategy proposed by our study indicates that IL1 and TNF signaling can be inhibited by blocking the interaction between K63 linked polyubiquitin chains and adaptor proteins such as as TAB2/3. This event provides a more rapid and transient regulatatory approach for cells. Therefore, this study contributes to a better understanding of negative feedback regulation of pro-inflammatory signaling pathways mediated by IL1 and TNF and could provide an alternative therapeutic approach for the treatment of inflammatory diseases.

Our understanding of phosphorylation mediated regulation of K63 linked polyubiquitin chain binding by the adaptor proteins TAB2 and TAB3 needs to be improved with more future experiments. For instance, it would be crucial to identify NLK phosphorylation residues on TAB2/3 and validate most of our finding in this study by using cell based assays or *in vivo studies*. In addition to the use of NLK knockdown cells, it will be worthy to study the functional role of NLK in inflammation by using primary cell lines or NLK knockout cell lines in future. In addition, we also want to study spatial distribution of TAK1 complex and NLK in cells by performing live cell imaging studies.

6. Summary

1. NLK directly interacts and phosphorylates TAB2 and TAB3 in cells and *in vitro*.
2. NLK suppresses TAK1 and its downstream kinases activity via phosphorylation of TAB2 and TAB3.
3. NLK negatively regulates TAK1 and NFκB pathway by modulating poly ubiquitin binding of TAB2 and TAB3.
4. Collectively, NLK negatively regulates TNFα mediated inflammation responses or gene expression of inflammatory cytokines by direct modification of TAK1 activity through TAB2/3 or activity of transcriptional factor c-jun (AP-1)
5. NLK interacts and phosphorylates c-jun in cells and *in vitro*.
6. NLK negatively regulates c-jun in TAK1 dependent pathway or independent pathway. Therefore, it remains to be explored the functional significance of c-jun phosphorylation by NLK in TAK1 independent signaling pathway e.g. in anisomycin signaling and how NLK is activated in anisomycin signaling.
7. Remaining questions to be answered:

   Since TAK1 and NLK activity is regulated in multiple and diverse pathways such as Wnts [5, 104], TGFβ [113], IL-1 and IL-6 [22] signaling, it is currently unknown whether TAK1 would also be subject to the same feedback mechanism in these pathways by NLK as described in this study. TAK1 activity is shown to be upregulated in NLK knockdown cells even under resting basal conditions [Figure. 3-9B] indicating that more unidentified growth factors or cytokines might also activate TAK1-NLK signaling pathway which might also subsequently lead to suppression of TAK1 by NLK.

8. References

1. Choi, K.W. and S. Benzer, *Rotation of photoreceptor clusters in the developing Drosophila eye requires the nemo gene.* Cell, 1994. **78**(1): p. 125-36.
2. Rocheleau, C.E., et al., *WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans.* Cell, 1999. **97**(6): p. 717-26.
3. Shin, T.H., et al., *MOM-4, a MAP kinase kinase kinase-related protein, activates WRM-1/LIT-1 kinase to transduce anterior/posterior polarity signals in C. elegans.* Mol Cell, 1999. **4**(2): p. 275-80.
4. Meneghini, M.D., et al., *MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans.* Nature, 1999. **399**(6738): p. 793-797.
5. Ishitani, T., et al., *The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF.* Nature, 1999. **399**(6738): p. 798-802.
6. Brott, B.K., B.A. Pinsky, and R.L. Erikson, *Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus.* Proc Natl Acad Sci U S A, 1998. **95**(3): p. 963-8.
7. Hanna-Rose, W. and U. Hansen, *Active repression mechanisms of eukaryotic transcription repressors.* Trends Genet, 1996. **12**(6): p. 229-34.
8. Hyodo-Miura, J., et al., *Involvement of NLK and Sox11 in neural induction in Xenopus development.* Genes Cells, 2002. **7**(5): p. 487-96.
9. Kanei-Ishii, C., et al., *Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK.* Genes & Development, 2004. **18**(7): p. 816-829.
10. Ohnishi, E., et al., *Nemo-like kinase, an essential effector of anterior formation, functions downstream of p38 mitogen-activated protein kinase.* Mol Cell Biol, 2010. **30**(3): p. 675-83.
11. Oppermann, F.S., et al., *Large-scale proteomics analysis of the human kinome.* Mol Cell Proteomics, 2009. **8**(7): p. 1751-64.
12. Ishitani, T., et al., *Nemo-like kinase is involved in NGF-induced neurite outgrowth via phosphorylating MAP1B and paxillin.* J Neurochem, 2009. **111**(5): p. 1104-18.
13. Maridor, G., et al., *Nuclear localization of vertebrate cyclin A correlates with its ability to form complexes with cdk catalytic subunits.* J Cell Sci, 1993. **106 (Pt 2)**: p. 535-44.
14. Kortenjann, M., et al., *Only one nemo-like kinase gene homologue in invertebrate and mammalian genomes.* Gene, 2001. **278**(1-2): p. 161-5.
15. Mirkovic, I., et al., *Drosophila nemo is an essential gene involved in the regulation of programmed cell death.* Mechanisms of Development, 2002. **119**(1): p. 9-20.
16. Merino, C., et al., *Nemo kinase interacts with Mad to coordinate synaptic growth at the Drosophila neuromuscular junction.* J Cell Biol, 2009. **185**(4): p. 713-25.
17. Zeng, Y.A., et al., *Drosophila Nemo antagonizes BMP signaling by phosphorylation of Mad and inhibition of its nuclear accumulation.* Development, 2007. **134**(11): p. 2061-2071.

18. Nifuji, A., et al., *Nemo-like kinase (NLK) expression in osteoblastic cells and suppression of osteoblastic differentiation.* Exp Cell Res, 2010. **316**(7): p. 1127-36.
19. Emami, K.H., et al., *Nemo-like kinase induces apoptosis and inhibits androgen receptor signaling in prostate cancer cells.* Prostate, 2009. **69**(14): p. 1481-92.
20. Takada, I., A.P. Kouzmenko, and S. Kato, *Molecular switching of osteoblastogenesis versus adipogenesis: implications for targeted therapies.* Expert Opin Ther Targets, 2009. **13**(5): p. 593-603.
21. Yasuda, J., et al., *Nemo-like kinase induces apoptosis in DLD-1 human colon cancer cells.* Biochem Biophys Res Commun, 2003. **308**(2): p. 227-33.
22. Kojima, H., et al., *STAT3 regulates Nemo-like kinase by mediating its interaction with IL-6-stimulated TGFbeta-activated kinase 1 for STAT3 Ser-727 phosphorylation.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4524-9.
23. Ishitani, T., et al., *Nemo-like kinase suppresses Notch signalling by interfering with formation of the Notch active transcriptional complex.* Nat Cell Biol, 2010. **12**(3): p. 278-85.
24. Kim, S., et al., *Regulation of FOXO1 by TAK1-Nemo-like kinase pathway.* J Biol Chem, 2010. **285**(11): p. 8122-9.
25. Shi, Y., et al., *Human SMAD4 is phosphorylated at Thr9 and Ser138 by interacting with NLK.* Mol Cell Biochem, 2010. **333**(1-2): p. 293-8.
26. Kanei-Ishii, C., et al., *Fbxw7 acts as an E3 ubiquitin ligase that targets c-Myb for nemo-like kinase (NLK)-induced degradation.* J Biol Chem, 2008. **283**(45): p. 30540-8.
27. Yasuda, J., et al., *Nemo-like kinase suppresses a wide range of transcription factors, including nuclear factor-kappaB.* Cancer Sci, 2004. **95**(1): p. 52-7.
28. Ishitani, T., J. Ninomiya-Tsuji, and K. Matsumoto, *Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling.* Mol Cell Biol, 2003. **23**(4): p. 1379-89.
29. Yamada, M., et al., *NARF, an nemo-like kinase (NLK)-associated ring finger protein regulates the ubiquitylation and degradation of T cell factor/lymphoid enhancer factor (TCF/LEF).* J Biol Chem, 2006. **281**(30): p. 20749-60.
30. Kanei-Ishii, C., et al., *Differential sensitivity of v-Myb and c-Myb to Wnt-1-induced protein degradation.* Journal of Biological Chemistry, 2004. **279**(43): p. 44582-44589.
31. Takada, I., et al., *A histone lysine methyltransferase activated by non- canonical Wnt signalling suppresses PPAR-gamma transactivation.* Nature Cell Biology, 2007. **9**(11): p. 1273-U118.
32. Verheyen, E.M., et al., *The tissue polarity gene nemo carries out multiple roles in patterning during Drosophila development.* Mech Dev, 2001. **101**(1-2): p. 119-32.
33. Kaletta, T., H. Schnabel, and R. Schnabel, *Binary specification of the embryonic lineage in Caenorhabditis elegans.* Nature, 1997. **390**(6657): p. 294-8.
34. Thorpe, C.J. and R.T. Moon, *nemo-like kinase is an essential co-activator of Wnt signaling during early zebrafish development.* Development, 2004. **131**(12): p. 2899-909.

35. Kortenjann, M., et al., *Abnormal bone marrow stroma in mice deficient for nemo-like kinase, Nlk.* Eur J Immunol, 2001. **31**(12): p. 3580-7.
36. Wodarz, A. and R. Nusse, *Mechanisms of Wnt signaling in development.* Annual Review of Cell and Developmental Biology, 1998. **14**: p. 59-88.
37. Nakamura, K., et al., *Wnt signaling drives WRM-1/beta-catenin asymmetries in early C. elegans embryos.* Genes Dev, 2005. **19**(15): p. 1749-54.
38. Behrens, J., *Cross-regulation of the Wnt signalling pathway: a role of MAP kinases.* J Cell Sci, 2000. **113 (Pt 6)**: p. 911-9.
39. Daniels, D.L. and W.I. Weis, *Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation.* Nat Struct Mol Biol, 2005. **12**(4): p. 364-71.
40. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.
41. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
42. Cadigan, K.M., *Wnt-beta-catenin signaling.* Curr Biol, 2008. **18**(20): p. R943-7.
43. Nishisho, I., et al., *Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients.* Science, 1991. **253**(5020): p. 665-9.
44. Kinzler, K.W., et al., *Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers.* Science, 1991. **251**(4999): p. 1366-70.
45. Willert, K. and R. Nusse, *Beta-catenin: a key mediator of Wnt signaling.* Curr Opin Genet Dev, 1998. **8**(1): p. 95-102.
46. Cadigan, K.M. and R. Nusse, *Wnt signaling: a common theme in animal development.* Genes Dev, 1997. **11**(24): p. 3286-305.
47. Giles, R.H., J.H. van Es, and H. Clevers, *Caught up in a Wnt storm: Wnt signaling in cancer.* Biochim Biophys Acta, 2003. **1653**(1): p. 1-24.
48. Smit, L., et al., *Wnt activates the Tak1/Nemo-like kinase pathway.* J Biol Chem, 2004. **279**(17): p. 17232-40.
49. He, T.C., et al., *Identification of c-MYC as a target of the APC pathway.* Science, 1998. **281**(5382): p. 1509-12.
50. Crawford, H.C., et al., *The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors.* Oncogene, 1999. **18**(18): p. 2883-91.
51. Yamaguchi, K., et al., *Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction.* Science, 1995. **270**(5244): p. 2008-11.
52. Dempsey, C.E., et al., *Alternative splicing and gene structure of the transforming growth factor beta-activated kinase 1.* Biochim Biophys Acta, 2000. **1517**(1): p. 46-52.
53. Liu, H.H., et al., *Essential role of TAK1 in thymocyte development and activation.* Proc Natl Acad Sci U S A, 2006. **103**(31): p. 11677-82.
54. Shinohara, H., et al., *PKC beta regulates BCR-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1.* J Exp Med, 2005. **202**(10): p. 1423-31.
55. Shim, J.H., et al., *TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo.* Genes Dev, 2005. **19**(22): p. 2668-81.

56. Cheung, P.C., A.R. Nebreda, and P. Cohen, *TAB3, a new binding partner of the protein kinase TAK1.* Biochem J, 2004. **378**(Pt 1): p. 27-34.
57. Takaesu, G., et al., *TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway.* Mol Cell, 2000. **5**(4): p. 649-58.
58. Shibuya, H., et al., *TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction.* Science, 1996. **272**(5265): p. 1179-82.
59. Kishimoto, K., K. Matsumoto, and J. Ninomiya-Tsuji, *TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop.* J Biol Chem, 2000. **275**(10): p. 7359-64.
60. Yu, Y., et al., *Phosphorylation of Thr-178 and Thr-184 in the TAK1 T-loop is required for interleukin (IL)-1-mediated optimal NFkappaB and AP-1 activation as well as IL-6 gene expression.* J Biol Chem, 2008. **283**(36): p. 24497-505.
61. Singhirunnusorn, P., et al., *Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor-beta-activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2.* J Biol Chem, 2005. **280**(8): p. 7359-68.
62. Mendoza, H., et al., *Roles for TAB1 in regulating the IL-1-dependent phosphorylation of the TAB3 regulatory subunit and activity of the TAK1 complex.* Biochem J, 2008. **409**(3): p. 711-22.
63. Wang, C., et al., *TAK1 is a ubiquitin-dependent kinase of MKK and IKK.* Nature, 2001. **412**(6844): p. 346-51.
64. Cheung, P.C., et al., *Feedback control of the protein kinase TAK1 by SAPK2a/p38alpha.* EMBO J, 2003. **22**(21): p. 5793-805.
65. Kanayama, A., et al., *TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains.* Mol Cell, 2004. **15**(4): p. 535-48.
66. Kulathu, Y., et al., *Two-sided ubiquitin binding explains specificity of the TAB2 NZF domain.* Nat Struct Mol Biol, 2009. **16**(12): p. 1328-30.
67. Ishitani, T., et al., *Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling.* EMBO J, 2003. **22**(23): p. 6277-88.
68. Zetoune, F.S., et al., *A20 inhibits NF-kappa B activation downstream of multiple Map3 kinases and interacts with the I kappa B signalosome.* Cytokine, 2001. **15**(6): p. 282-98.
69. Kovalenko, A., et al., *The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination.* Nature, 2003. **424**(6950): p. 801-5.
70. Trompouki, E., et al., *CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members.* Nature, 2003. **424**(6950): p. 793-6.
71. Besse, A., et al., *TAK1-dependent signaling requires functional interaction with TAB2/TAB3.* J Biol Chem, 2007. **282**(6): p. 3918-28.
72. Komander, D., et al., *Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains.* EMBO Rep, 2009. **10**(5): p. 466-73.
73. Pickart, C.M. and M.J. Eddins, *Ubiquitin: structures, functions, mechanisms.* Biochim Biophys Acta, 2004. **1695**(1-3): p. 55-72.

74. Ishitani, T., et al., *The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling.* Mol Cell Biol, 2003. **23**(1): p. 131-9.
75. Ohkawara, B., et al., *Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction.* Genes & Development, 2004. **18**(4): p. 381-386.
76. Adhikari, A., M. Xu, and Z.J. Chen, *Ubiquitin-mediated activation of TAK1 and IKK.* Oncogene, 2007. **26**(22): p. 3214-26.
77. Alvarez-Guardia, D., et al., *The p65 subunit of NF-kappaB binds to PGC-1alpha, linking inflammation and metabolic disturbances in cardiac cells.* Cardiovasc Res, 2010. **87**(3): p. 449-58.
78. Maxwell, P.J., et al., *HIF-1 and NF-kappaB-mediated upregulation of CXCR1 and CXCR2 expression promotes cell survival in hypoxic prostate cancer cells.* Oncogene, 2007. **26**(52): p. 7333-45.
79. Heese, K., N. Inoue, and T. Sawada, *NF-kappaB regulates B-cell-derived nerve growth factor expression.* Cell Mol Immunol, 2006. **3**(1): p. 63-6.
80. Gaestel, M., A. Kotlyarov, and M. Kracht, *Targeting innate immunity protein kinase signalling in inflammation.* Nat Rev Drug Discov, 2009. **8**(6): p. 480-99.
81. Pickart, C.M., *Back to the future with ubiquitin.* Cell, 2004. **116**(2): p. 181-90.
82. Hershko, A., *Ubiquitin: roles in protein modification and breakdown.* Cell, 1983. **34**(1): p. 11-2.
83. Li, M., et al., *TAB2 scaffolds TAK1 and NLK in repressing canonical WNT signaling.* J Biol Chem, 2010.
84. Chen, C. and H. Okayama, *High-efficiency transformation of mammalian cells by plasmid DNA.* Mol Cell Biol, 1987. **7**(8): p. 2745-52.
85. Zhang, M., et al., *Chaperoned ubiquitylation--crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex.* Mol Cell, 2005. **20**(4): p. 525-38.
86. Karin, M., *Mitogen-activated protein kinase cascades as regulators of stress responses.* Ann N Y Acad Sci, 1998. **851**: p. 139-46.
87. Sakurai, H., et al., *Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1.* FEBS Lett, 2000. **474**(2-3): p. 141-5.
88. Chen, Z.J., *Ubiquitin signalling in the NF-kappaB pathway.* Nat Cell Biol, 2005. **7**(8): p. 758-65.
89. Kishida, S., et al., *TAK1-binding protein 2 facilitates ubiquitination of TRAF6 and assembly of TRAF6 with IKK in the IL-1 signaling pathway.* Genes Cells, 2005. **10**(5): p. 447-54.
90. Jin, G., et al., *Identification of a human NF-kappaB-activating protein, TAB3.* Proc Natl Acad Sci U S A, 2004. **101**(7): p. 2028-33.
91. Hong, S., et al., *Smad7 binds to the adaptors TAB2 and TAB3 to block recruitment of the kinase TAK1 to the adaptor TRAF2.* Nat Immunol, 2007. **8**(5): p. 504-13.
92. Xia, Z.P., et al., *Direct activation of protein kinases by unanchored polyubiquitin chains.* Nature, 2009. **461**(7260): p. 114-9.
93. Karin, M., *The regulation of AP-1 activity by mitogen-activated protein kinases.* J Biol Chem, 1995. **270**(28): p. 16483-6.

94. Aronheim, A., et al., *Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions.* Mol Cell Biol, 1997. **17**(6): p. 3094-102.
95. Angel, P. and M. Karin, *The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.* Biochim Biophys Acta, 1991. **1072**(2-3): p. 129-57.
96. Liebermann, D.A., B. Gregory, and B. Hoffman, *AP-1 (Fos/Jun) transcription factors in hematopoietic differentiation and apoptosis.* Int J Oncol, 1998. **12**(3): p. 685-700.
97. Wisdom, R., *AP-1: one switch for many signals.* Exp Cell Res, 1999. **253**(1): p. 180-5.
98. Karin, M. and M. Delhase, *JNK or IKK, AP-1 or NF-kappaB, which are the targets for MEK kinase 1 action?* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9067-9.
99. Gan, X.Q., et al., *Nuclear Dvl, c-Jun, beta-catenin, and TCF form a complex leading to stabilization of beta-catenin-TCF interaction.* J Cell Biol, 2008. **180**(6): p. 1087-100.
100. Nateri, A.S., B. Spencer-Dene, and A. Behrens, *Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development.* Nature, 2005. **437**(7056): p. 281-5.
101. Ip, Y.T. and R.J. Davis, *Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development.* Curr Opin Cell Biol, 1998. **10**(2): p. 205-19.
102. Yoshimura, H., et al., *Transforming growth factor-beta stimulates IL-1beta-induced monocyte chemoattractant protein-1 expression in human synovial cells via the ERK/AP-1 pathway.* Inflamm Res, 2006. **55**(12): p. 543-9.
103. Ping, D., et al., *Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene.* J Immunol, 1999. **162**(2): p. 727-34.
104. Meneghini, M.D., et al., *MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans.* Nature, 1999. **399**(6738): p. 793-7.
105. Yasuda, J., et al., *The JIP group of mitogen-activated protein kinase scaffold proteins.* Mol Cell Biol, 1999. **19**(10): p. 7245-54.
106. Tian, Y., et al., *RBCK1 negatively regulates tumor necrosis factor- and interleukin-1-triggered NF-kappaB activation by targeting TAB2/3 for degradation.* J Biol Chem, 2007. **282**(23): p. 16776-82.
107. Kajino, T., et al., *Protein phosphatase 6 down-regulates TAK1 kinase activation in the IL-1 signaling pathway.* J Biol Chem, 2006. **281**(52): p. 39891-6.
108. Kim, S.I., et al., *Protein phosphatase 2A is a negative regulator of transforming growth factor-beta1-induced TAK1 activation in mesangial cells.* J Biol Chem, 2008. **283**(16): p. 10753-63.
109. Poblete Gutierrez, P., et al., *Phenotype diversity in familial cylindromatosis: a frameshift mutation in the tumor suppressor gene CYLD underlies different tumors of skin appendages.* J Invest Dermatol, 2002. **119**(2): p. 527-31.
110. Leonard, N., et al., *Loss of heterozygosity at cylindromatosis gene locus, CYLD, in sporadic skin adnexal tumours.* J Clin Pathol, 2001. **54**(9): p. 689-92.

111. Boone, D.L., et al., *The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses.* Nat Immunol, 2004. **5**(10): p. 1052-60.
112. Wu, S., et al., *Atorvastatin reduces lipopolysaccharide-induced expression of cyclooxygenase-2 in human pulmonary epithelial cells.* Respir Res, 2005. **6**: p. 27.
113. Ohkawara, B., et al., *Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction.* Genes Dev, 2004. **18**(4): p. 381-6.