University of Massachusetts Medical School

eScholarship@UMMS

2005-01-14

Regulation and Function of Stress-Activated Protein Kinase Signal Transduction Pathways: A Dissertation

Deborah Marie Brancho
University of Massachusetts Medical School

Let us know how access to this document benefits you.

REGULATION AND FUNCTION OF STRESS-

ACTIVATED PROTEIN KINASE SIGNAL

TRANSDUCTION PATHWAYS

A DISSERTATION PRESENTED

BY

DEBORAH MARIE BRANCHO

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences

Worcester, Massachusetts

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

January 14, 2005

Interdisciplinary Graduate Program

COPYRIGHT

Part of this dissertation has been published in:

Weston, C.R.[*], **Brancho, D.**[*], Xu, P., Doran, B., Goad, M.E., Flavell, R.A., Davis, R.J. The mixed-lineage protein kinase DLK is essential for early embryonic development. *In preparation.*

Brancho, D., Ventura, J.J., Jaeschke, A., Doran, B., Flavell, R.A., Davis, R.J. Role of MLK3 in the regulation of MAP kinase signaling cascades. *Molecular and Cellular Biology.* In press.

Brancho, D.[*], Tanaka, N.[*], Jaeschke, A.[*], Ventura, J.J.[*], Kelkar, N.[*], Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R.A., Davis, R.J. Mechanism of p38 MAP kinase activation *in vivo*. *Genes & Development.* 17 (16)1969-1978 (2003).

Schonhoff, C.M., Bulseco, D.A., **Brancho, D.M.**, Parada, L.F., Ross, A.H. The Ras-ERK pathway is required for the induction of neuronal nitric oxide synthase in differentiating PC12 cells. *J. Neurochem.* 78 (3)631-639 (2001).

Enslen, H., **Brancho, D.M.**, and Davis, R.J. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. *EMBO J.* 19 1301-1311 (2000).

[*] These authors contributed equally to this work.

REGULATION AND FUNCTION OF STRESS-ACTIVATED PROTEIN KINASE SIGNAL TRANSDUCTION PATHWAYS

A DISSERTATION PRESENTED

BY

DEBORAH MARIE BRANCHO

Approved as to style and content by:

_____
Tony Ip, Ph.D., Chair of Committee

_____
Stephen Jones, Ph.D., Member of Committee

_____
Kendall Knight, Ph.D., Member of Committee

_____
Robert Lewis, Ph.D., Member of Committee

_____
Craig Peterson, Ph.D., Member of Committee

_____
Roger J. Davis, Ph.D., Dissertation Mentor

_____
Anthony Carruthers, Ph.D., Dean
Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
Program in Molecular Medicine
January 14, 2005

To my dad, Fr. John S. Brancho

To my mom, Marie Brancho

And

To my brother, Stephen P. Brancho

No Ta Hey.

ACKNOWLEDGEMENTS

I would like to thank members of my dissertation committee for their time and guidance. Drs. Tony Ip, Stephen Jones, Kendall Knight, and Craig Peterson have helped direct my research work over the years. Dr. Robert Lewis from the University of Nebraska Medical School has taken the extra time to serve as my outside committee member.

Finally, I would like to thank my friends and family for their encouragement throughout these difficult years. There are too many friends and family to acknowledge here. Nonetheless, I would like to especially thank Fr. John Brancho, Marie Brancho, Stephen Brancho, and Chi-Wing Chow for their love and support.

ABSTRACT

The c-Jun NH$_2$-terminal kinase (JNK) group and the p38 group of mitogen-activated protein kinases (MAPK) are stress-activated protein kinases that regulate cell proliferation, differentiation, development, and apoptosis. These protein kinases are involved in a signal transduction cascade that includes a MAP kinase (MAPK), a MAP kinase kinase (MAP2K), and a MAP kinase kinase kinase (MAP3K). MAPK are phosphorylated and activated by the MAP2K, which are phosphorylated and activated by various MAP3K.

The work presented in this dissertation focuses on understanding the regulation and function of the JNK and p38 MAPK pathways. Two different strategies were utilized. First, I used molecular and biochemical techniques to examine how MAP2K and MAP3K mediate signaling specificity and to define their role in the MAPK pathway. Second, I used gene targeted disruption studies to determine the *in vivo* role of MAP2K and MAP3K in MAPK activation. I specifically used these approaches to examine: (1) docking interactions between p38 MAPK and MAP2K [MKK3 and MKK6 (Chapter II)]; (2) the differential activation of p38 MAPK by MAP2K [MKK3, MKK4, and MKK6 (Chapter III)]; and (3) the selective involvement of the mixed lineage kinase (MLK) group of MAP3K in JNK and p38 MAPK activation (Chapter IV and Appendix). In addition, I analyzed the role of the MKK3 and MKK6 MAP2K in cell proliferation and the role of the MLK MAP3K in adipocyte differentiation (Chapter III and Chapter IV). Together, these data provide insight into the regulation and function of the stress-activated MAPK signal transduction pathways.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter V

Appendix

LIST OF ABBREVIATIONS

AP-1	Activator protein-1
ASK	Apoptosis signal-regulating kinase
ATF	Activating transcription factor
ATP	Adenosine 5'-triphosphate
BAC	Bacterial artificial chromosome
bp	Base pair
BSA	Bovine serum albumin
CD domain	Common docking domain
cDNA	Complementary DNA
C/EBP	CCAAT/enhancer binding protein
COOH	Carboxyl
CREB	cAMP response element-binding protein
CRIB	Cdc42/Rac binding motif
DAPI	4'-6'- diamino-2-phenylindole
D domain	MAPK docking domain
Dex	Dexamethasone
DLK	Dual-leucine-zipper-bearing kinase
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
E	Embryonic day
ED domain	ERK docking domain
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eIF-4E	Eukaryotic initiation factor-4E
Elk	Ets-like transcription factor
ERK	Extracellular-signal regulated kinase
ES	Embryonic stem cell
Ets	Erythroblast transformation
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde phosphate dehydrogenase
GCK	Germinal center kinase
GCKR	GCK-related
Grb2	Growth factor bound protein-2
GSH	Glutathione
GST	Glutathione S-transferase
HPK	Hematopoietic progenitor kinase
HSP	Heat shock protein
IBMX	Isomethylbutyl-1-xanthine
Ig	Immunoglobin

IκB	Inhibitor of NFκB
IL-1	Interleukin-1
Ins	Insulin
JIP	JNK-interacting protein
JNK	c-Jun NH_2-terminal kinase
kDA	Kilodalton
LPS	Lipopolysaccharide
LZK	Leucine-zipper kinase
MAPK	Mitogen-activated protein kinase
MAP2K	MAP kinase kinase
MAP3K	MAP kinase kinase kinase
MAPKAP	Mitogen-activated protein kinase activated kinase
MEF	Murine embryo fibroblast
MEF2	Myogenic enhancer factor-2
MEK	MAP/ERK kinase
MEKK	MEK kinase
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MKP	MAPK phosphatase
MLK	Mixed lineage kinase
MNK	MAP kinase signal-integrating kinase
mRNA	Messenger RNA
MSK	Mitogen and stress activated protein kinase
MUK	MAPK-upstream kinase
Neo	Neomycin
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-chain binding protein
NGF	Nerve growth factor
NH_2	Amino
NIK	NFκB inducing kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PPAR	Peroxisome proliferator-activated receptor
PRAK	p38 regulated/activated protein kinase
RIP1	Receptor interacting protein kinase-1
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
SAM	Sterile α-motif
Sap1A	Serum response factor accessory protein-1A
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulfate
siRNA	short interfering RNA
SODD	Silencer of death domain

TAB	TGFβ-activated protein kinase 1-binding protein
TAK	TGFβ-activated kinase
TAO	Thousand and one kinase
TGFβ	Tumor growth factor-β
Th	T helper cell
TLB	Triton lysis buffer
T-loop	Activation loop
TNFα	Tumor necrosis factor alpha
TNF-R	TNF receptor type
Tpl-2	Tumor progression locus-2
TRADD	TNFR-associated death domain containing protein
TRAF	TNFR-associated factor
Trog	Troglitizone
UTR	Untranslated region
UV	Ultraviolet radiation
WT	Wild-type
ZAK	Zipper sterile-α-motif kinase
ZPK	Zipper (leucine) kinase protein

CHAPTER I

INTRODUCTION

As living organisms, cells adapt to changes in the environment. These changes include differences in temperature, oxidation, and nutrient concentration - basic necessities for maintaining cellular homeostasis. Physical and chemical fluctuations act as signals to stimulate cellular growth, differentiation, and death. A mechanism by which cells convert a signal into a biological function is a process called signal transduction. Signal transduction is critical for normal life. Specific stimuli differentially affect cellular homeostasis and any abnormal signaling may lead to a diseased state. Therefore, understanding signal transduction pathways is essential for understanding and treating human diseases. Maintaining homeostasis requires the cooperation of many signaling pathways. Previous studies have established a role for mitogen-activated protein kinase (MAPK) pathways in regulating cellular responses. Here I summarize recent advances in understanding the MAPK signal transduction pathway and the role of MAPK in diverse cellular processes.

The MAPK signal transduction pathway

Mitogen-activated protein kinases (MAPK) are serine-threonine kinases that are activated by the exposure of cells to extracellular stimuli, such as growth factors, cytokines, and cellular stress (Figure I.1). Recent reviews focus on the components and the regulation of the MAPK pathways (Davis 2000; Ono and Han 2000; Kyriakis and Avruch 2001; Weston and Davis 2002; Nebreda and Porras 2000; Manning and Davis 2003). These kinases are stimulated through a signaling cascade and regulate cell proliferation, differentiation, development, inflammation, and apoptosis. MAP kinases control these cellular processes, in part, through the activation of various transcription factors. MAP kinase signaling occurs though a "three-tiered" phosphorylation cascade (Davis 2000; Kyriakis and Avruch 2001). This protein kinase cascade involves a MAP kinase (MAPK), a MAP kinase kinase (MAP2K), and a MAP kinase kinase kinase (MAP3K). MAPK are phosphorylated and activated by MAP2K, which are phosphorylated and activated by MAP3K.

The canonical MAPK pathway is evolutionarily conserved (Davis 2000; Kyriakis and Avruch 2001). In mammals, there are three major groups of MAP kinases: (1) extracellular-signal regulated kinases (ERK), (2) c-Jun NH_2-terminal kinases (JNK), and (3) p38 MAP kinases. As serine-threonine kinases, these MAPK phosphorylate protein substrates on conserved Ser-Pro or Thr-Pro motifs. The target sequence of these substrate phosphorylation motifs is distinct for individual MAP kinases. In addition, MAP kinases are also differentiated by the dual phosphorylation motif that mediates their activation: (1) Thr-Glu-Tyr (ERK), (2) Thr-Pro-Tyr (JNK), and (3) Thr-Gly-Tyr (p38 MAPK).

MAP kinases are activated by different extracellular stimuli. The ERK pathway primarily responds to mitogens and is regulated by a Ras-dependent pathway (Cobb 1999;

Pearson et al. 2001; Chen et al. 2001; Schonhoff et al. 2001). Some examples of these mitogenic

signals are epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor

(NGF), and platelet-derived growth factor (PDGF). In contrast, the JNK and p38 MAPK

pathways are stress-activated MAP kinases, since they are activated by both inflammatory

cytokines and environmental stress (Davis 2000; Ono and Han 2000). Examples of

inflammatory cytokines that activate JNK and p38 MAPK are tumor necrosis factor alpha

(TNFα) and interleukin-1 (IL-1). Examples of environmental stress include UV radiation,

osmotic shock, heat shock, lipopolysaccharide (LPS), and protein synthesis inhibitors. The

following sections summarize the JNK and the p38 MAPK pathways and discuss their role in

cellular responses.

Figure I.1. The mitogen-activated protein kinase (MAPK) signal transduction pathway.

MAPK are activated by extracellular stimuli to regulate cell proliferation, differentiation, development, and apoptosis. MAPK control these cellular processes, in part, through the activation of various transcription factors. MAPK signaling occurs though a phosphorylation cascade that involves a MAP kinase (MAPK), a MAP kinase kinase (MAP2K), and a MAP kinase kinase kinase (MAP3K). MAPK are phosphorylated and activated by MAP2K, which are phosphorylated and activated by MAP3K. There are three major groups of MAPK in mammals: ERK, JNK, and p38 MAPK. The ERK pathway primarily responds to mitogens, while the JNK and p38 MAPK pathways are stress-activated MAPK pathways and are strongly stimulated by inflammatory cytokines and environmental stress.

Cytokines/Cellular Stress

Rac1/Cdc42

ATF2

MKK4/MKK7

JNK

c-Jun

Growth Factors

Ras

Raf MEKK

MEK1/MEK2

ERK1/ERK2

Elk1

GTPase

MAP3K

MAP2K

MAP Kinase

Scaffolding Proteins
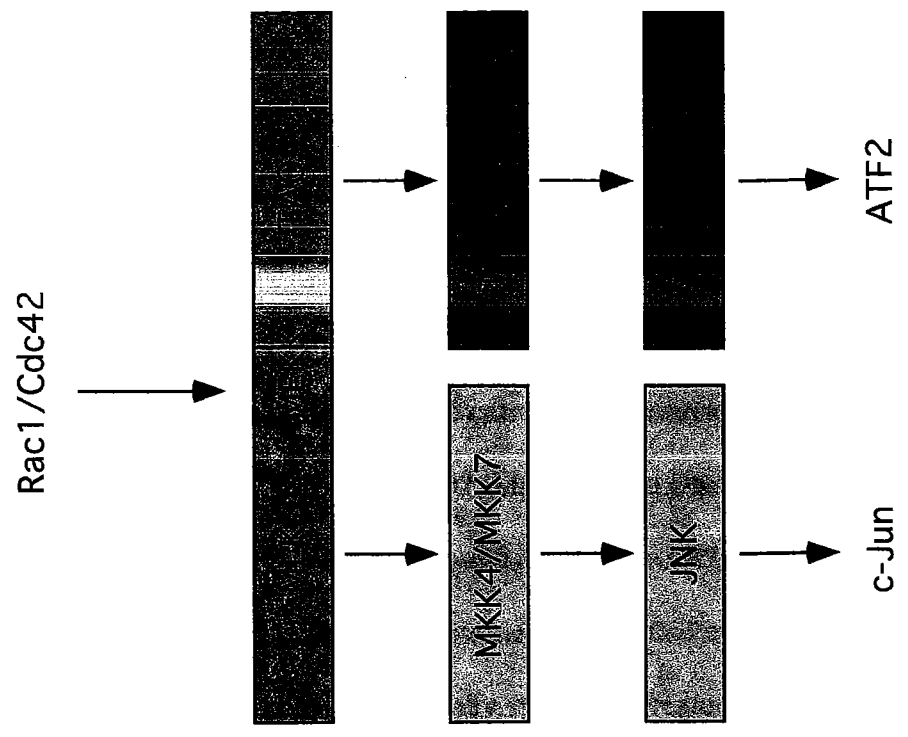
Transcription Factors
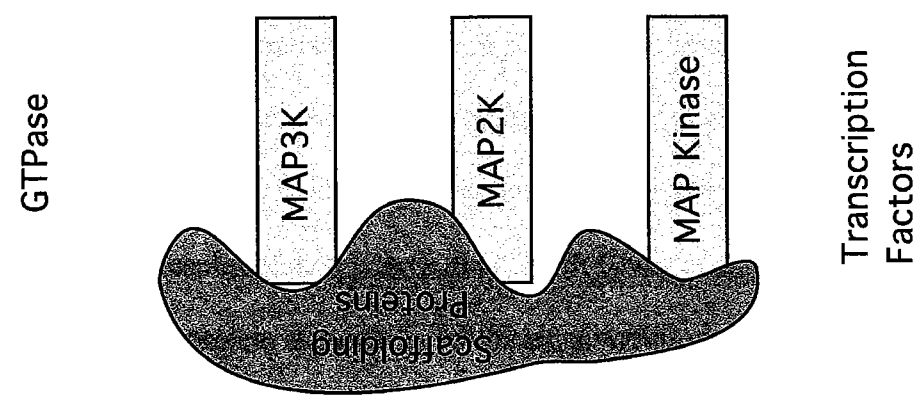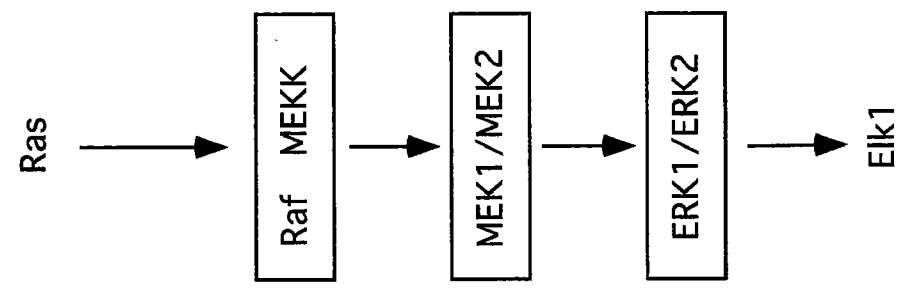
The c-Jun NH₂-terminal kinase (JNK) group of MAP kinases

The c-Jun NH$_2$-terminal kinases (JNK) were first identified as p54 protein kinases that were activated in response to cycloheximide (Kyriakis and Avruch 1990). These protein kinases bind to and phosphorylate the c-Jun transcription factor at two sites, Ser-63 and Ser-73. (Adler et al. 1999; Pulverer et al. 1991; Hibi et al. 1993). Cloning of the p54 kinase revealed it as a member of the MAP kinase family (Derijard et al. 1994; Kyriakis et al. 1994). The p54 kinase, named c-Jun NH$_2$-terminal kinase (JNK), has been extensively characterized (Davis 2000; Kyriakis and Avruch 2001; Weston and Davis 2002; Manning and Davis 2003). Three genes encode the JNK protein kinase family, *Jnk1*, *Jnk2*, and *Jnk3*. The *Jnk1* and *Jnk2* genes are ubiquitiously expressed, while the *Jnk3* gene is restricted to a limited number of tissues (e.g. brain, heart, testis). These *Jnk* genes are alternatively spliced to create ten different mRNAs. Each gene generates a p46 kDa and a p54 kDa JNK isoform (Gupta et al. 1996).

Inflammatory cytokines and cellular stress activate the JNK pathway through the MAP kinase cascade. Activation of JNK leads to phosphorylation of protein substrates, particularly the c-Jun components of the activator protein-1 (AP-1) transcription factor complex. Phosphorylation of c-Jun by JNK directly enhances AP-1 transcriptional activity (Pulverer et al. 1991; Smeal et al. 1991). In addition to c-Jun phosphorylation, JNK also phosphorylates other AP-1 proteins (JunD and ATF2) that subsequently enhance AP-1 gene transcription (Gupta et al. 1995; Whitmarsh et al. 1995; Whitmarsh and Davis 1996). Thus, AP-1 activation involves a direct phosphorylation, as well as, an induced expression of AP-1 transcription factors. In contrast to activation of AP-1, JNK inhibits transcriptional activity of NFATc1 (NFAT2) and NFATc3 (NFAT4) upon phosphorylation (Chow et al. 1997; Chow et al. 2000). Furthermore, activated JNK also regulates protein stability by inhibiting ubiquitin-mediated degradation

(Musti et al. 1997; Fuchs et al. 1998).

The p38 group of MAP kinases

The p38 MAP kinase was originally characterized as a 38 kDa protein that was phosphorylated in response to LPS treatment (Han et al. 1993). Independent studies identified this protein as a target of anti-inflammatory drugs that inhibit cytokine production (Lee et al. 1994), and as an activator of MAPKAP kinase 2 (Rouse et al. 1994; Freshney et al. 1994). Cloning of this 38 kDA protein identified it as a member of the MAP kinase family (Han et al. 1994). This kinase is named p38 MAP kinase and has been extensively characterized (Ono and Han 2000) (Nebreda and Porras 2000; Kyriakis and Avruch 2001). Four genes encode p38 MAP kinases, *p38α*, *p38β*, *p38γ*, and *p38δ*. The p38α and p38β MAPK consist of one sub-group, while the p38γ and p38δ MAPK represent a second subgroup. The p38α and p38β MAPK are sensitive to the anti-inflammatory drug SB203580, while the p38γ and p38δ MAPK are resistant to its inhibition. Furthermore, the p38α and p38β MAPK are ubiquitiously expressed, while the p38γ and p38δ MAPK display a more limited pattern of expression. For example, the p38γ MAPK is expressed in skeletal and heart muscle and the p38δ MAPK is expressed in endocrine and exocrine tissues.

Cytokines and cellular stress activate p38 MAPK through a protein kinase cascade. Activation of p38 MAPK is important for the production of inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNFα (Kumar et al. 2003). Induction of these cytokines may be mediated, in part, through the stabilization and translation of the short-lived cytokine mRNAs (Clark et al. 2003; Kumar et al. 2003). The p38 MAPK also phosphorylates many transcription factors, including several AP-1 proteins (e.g. ATF2). In addition to phosphorylating transcription factor

substrates, p38 MAPK phosphorylates other protein kinases (e.g. MAPKAP2, PRAK, MSK1, and MNK1/2) to amplify its signal. MAPKAP2 and PRAK phosphorylate the small heat shock proteins HSP27 (Rouse et al. 1994; Freshney et al. 1994; New et al. 1998) and MNK1/2 phosphorylate the translation initiation factor eIF-4E (Waskiewicz et al. 1997; Waskiewicz et al. 1999). Similarly, MAPKAP2 and MSK1 activate the CREB transcription factors ATF1 and CREB (Tan et al. 1996; Deak et al. 1998).

The MAP2K: MAP kinase kinases

MAP kinases are activated by dual phosphorylation at conserved Thr-Xaa-Tyr motifs in the activation loop (Davis 2000; Kyriakis and Avruch 2001). This dual phosphorylation is mediated by a MAP kinase kinase (MAP2K). At least six protein kinases function as MAP2K: MEK1 and MEK2 (for ERK1/2 MAPK signaling pathway); MKK3 and MKK6 (for p38 MAPK signaling pathway); MKK4 and MKK7 (for JNK MAPK signaling pathway).

The JNK protein kinases are activated by MKK4 and MKK7. Nine alternatively spliced isoforms with distinct NH_2-terminal regions (MKK4 and MKK7) and COOH-terminal regions (MKK7) have been identified by degenerate PCR analysis (Lin et al. 1995; Sanchez et al. 1994; Derijard et al. 1995; Tournier et al. 1999; Tournier et al. 1997). MKK4 and MKK7 phosphorylate all three JNK isoforms, although they are differentially activated by specific stimuli. For example, MKK4 is strongly activated by environmental stress, while MKK7 is activated by both inflammatory cytokines and environmental stress (Tournier et al. 2001). Recent studies indicate that although MKK4 and MKK7 phosphorylate JNK on both Thr and Tyr residues, MKK4 preferentially phosphorylates JNK on Tyr and MKK7 preferentially targets JNK on Thr (Lawler et al. 1998; Tournier et al. 2001). Since dual phosphorylation is necessary for

maximal JNK activation, MKK4 and MKK7 may cooperate to synergistically activate JNK. Thus, in response to cytokine stimulation, the basal level of MKK4 activity may be important for maximal JNK activation.

The p38 MAPK kinases are activated by MKK3 and MKK6. Four alternatively spliced isoforms with distinct NH_2-terminal regions have been identified by degenerate PCR analysis. (Moriguchi et al. 1996b; Moriguchi et al. 1996; Han et al. 1997; Han et al. 1996; Derijard et al. 1995; Raingeaud et al. 1996). MKK3 and MKK6 are strongly activated by both inflammatory cytokines and environmental stress, although they differentially activate different p38 MAPK isoforms. MKK3 phosphorylates p38α, p38γ, and p38δ, whereas MKK6 phosphorylates all four p38 MAPK isoforms (Enslen et al. 1998; Jiang et al. 1997). One aim of this thesis is to determine how MKK3 and MKK6 mediate selective activation of p38 MAP kinases (Chapter II).

MKK3 and MKK6 are specific activators of the p38 MAP kinase, and neither kinase activates the JNK pathway. Interestingly, MKK4 also phosphorylates p38 MAPK *in vitro* (Derijard et al. 1995). The mechanism of p38 MAPK activation by MAP2K is different than the mechanism of JNK activation by MAP2K. There appears to be no preferential phosphorylation of Thr or Tyr residues by the MAP2K in p38 MAPK activation (Enslen et al. 2000; Tournier et al. 2001). MKK3, MKK4, and MKK6 phosphorylate p38 MAPK equally on Tyr and Thr residues. The absence of preferential p38 MAPK phosphorylation may lead to redundancy among the MAP2K. For example, p38 MAPK activation is not significantly affected in MKK4-deficient cells (Yang et al. 1997a; Tournier et al. 2001). A second aim of this thesis is to determine whether MKK4 is a relevant activator of p38 MAPK (Chapter III).

The MAP3K: MAP kinase kinase kinases

MAP2K are activated by dual phosphorylation on conserved Ser-Xaa_3-Thr motifs in the activation loop (Davis 2000; Kyriakis and Avruch 2001). This dual phosphorylation is mediated by a MAP kinase kinase kinase (MAP3K). Many protein kinases may function as MAP3K in the JNK and p38 MAPK pathways. These MAP3K are divided into several broad groups: (1) the MEK kinase family (MEKK); (2) the *Tumor progression locus-2* (Tpl-2); (3) the thousand and one kinase family (TAO); (4) the TGFβ-activated kinase (TAK); (5) the apoptosis signal-regulating kinase (ASK); and (6) the mixed lineage kinase family (MLK).

Many MAP3K were initially identified as MAPK activators in transfection assays (Fanger et al. 1997). The biochemical properties of these MAP3K have been recently reviewed (Kyriakis and Avruch 2001; Gallo and Johnson 2002). The MEKK, MEKK1 - MEKK4, are a diverse group of MAP3K. MEKK share a common kinase domain and activate all different MAPK pathways. MEKK contain different regulatory regions such as SH3-domains and Pro-rich regions that mediate interactions with other proteins. Tpl-2 is a protein kinase that is encoded by a protooncogene and activates all different MAPK pathways. In contrast, TAO1 specifically activates the p38 MAPK pathway. Common features of TAO kinases include an NH_2-terminal kinase domain, followed by a very large (700 amino acid) COOH-terminal region. TAK1 contains a short NH_2-terminal regulatory motif that is essential for interacting with the TAB adaptor proteins, coupling TAK1 to upstream signals. ASK1 binds to several adaptor proteins as well, coupling ASK1 to downstream apoptotic signaling. TAK1 and ASK1 activate the JNK and p38 MAPK pathways. Lastly, seven MLK have been identified; however, only a few of these protein kinases have been characterized.

The large number of MAP3K suggests an inherent redundancy in MAPK regulation. This idea of redundancy is supported by the presence of common substrates for these protein kinases. However, much remains unclear regarding the role of MAP3K in MAPK signaling. A third aim of this thesis is to elucidate the individual role of the MLK MAP3K, particularly the MLK3 MAP3K, in JNK and p38 MAPK activation (Chapter IV, Appendix I). Characteristics of the MLK MAP3K family will be discussed in detail below.

The mixed lineage kinase (MLK) group of MAP3K

Mixed lineage kinases (MLK) are serine/threonine kinases that regulate the JNK pathway. MLK share two structural features: a kinase catalytic domain and a leucine zipper region (Gallo and Johnson 2002). The kinase catalytic domain shares similarities to both Ser/Thr and Tyr kinases. Protein kinase subdomains 1-7 resemble the MEKK serine/threonine kinases, while protein kinase subdomains 8-11 resembles the Src tyrosine kinases. Thus, when MLK were first cloned, their dual phosphorylation specificity was unclear and MLK were named "mixed lineage kinases" (Dorow et al. 1993). Autophosphorylation on Ser and Thr residues of MLK, however, demonstrate its Ser/Thr specificity (Gallo et al. 1994). MLK also share a leucine zipper region that is required for dimerization and subsequent autophosphorylation and activation of the JNK pathway (Nihalani et al. 2000; Nihalani et al. 2001; Ikeda et al. 2001; Leung and Lassam 1998; Vacratsis and Gallo 2000).

Within the MLK family of protein kinases, seven different MLK have been identified (Gallo and Johnson 2002). They are further divided into three subgroups based on their domain characteristics: MLK, DLK, and ZAK (Figure I.2). All of the MLK share a common kinase catalytic domain and leucine zipper region. The MLK (MLK1 - MLK4) have additional

domains that mediate protein-protein interactions. The DLK (DLK and LZK) contain two leucine zipper motifs, while ZAK may feature a sterile-α motif (SAM) in addition to the leucine zipper region that may mediate dimerization.

The MLK subgroup, MLK1 - MLK4, contains a SH3-domain, a Pro-rich region, and a Cdc42/Rac (CRIB) binding motif. The SH3 domain of MLK3 has been shown to bind intramolecularly to the Pro-rich region and autoinhibit its kinase activity (Zhang and Gallo 2001). In addition, MLK3 also binds activated Cdc42, a member of the Rho family of GTPases (Teramoto et al. 1996; Bock et al. 2000). Overexpression of Cdc42 has been shown to increase MLK3 activity; however, the mechanism of activation is not completely understood. The interaction of MLK3 with activated Cdc42 may disrupt the SH3-mediated autoinhibition, since the CRIB motif is located adjacent to the Pro-rich region (Gallo and Johnson 2002).

As MAP3K, MLK phosphorylate MKK, such as MKK7, a preferred substrate of many MLK (Merritt et al. 1999). Thus, all known MLK strongly activate JNK. However, some MLK, such as MLK3, also activate ERK and p38 MAPK in transfection assays. Consistent with multiple MAPK activation, MLK3 has been shown to phosphorylate *in vitro* several MAP2K (MEK1, MKK3, MKK4, MKK6, and MKK7), but the physiological relevance of the phosphorylation remains unclear (Shen et al. 2003; Tibbles et al. 1996; Merritt et al. 1999). MLK-mediated JNK activation also appears to have a negative regulatory role on MLK function. The COOH-terminal of MLK2 and MLK3 has been proposed to be phosphorylated by JNK in a negative feedback loop (Vacratsis et al. 2002; Phelan et al. 2001).

Figure I.2. The mixed lineage kinase (MLK) family of MAP3K.

Mixed lineage kinases (MLK) are MAP3K that regulate the ERK, JNK, and p38 MAPK pathways. Within the MLK family of MAP3K, seven different MLK have been identified. They are further divided into three subgroups based on their domain characteristics: MLK, DLK, and ZAK. All of the MLK share a common kinase catalytic domain and leucine zipper region. The MLK (MLK1 - MLK4) have additional domains 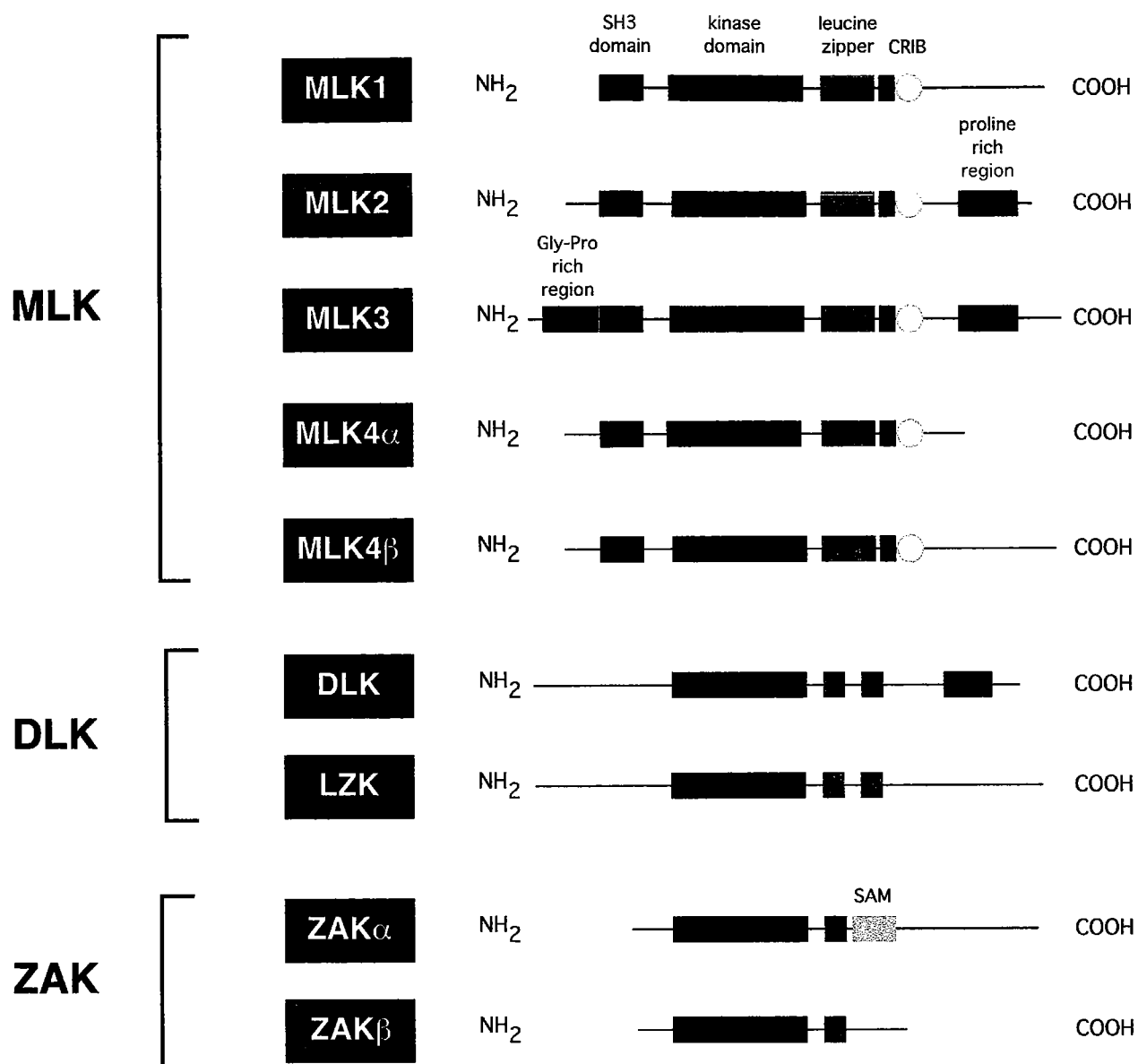such as SH3-domains, Pro-rich regions, and Cdc42/Rac (CRIB) binding motifs that mediate protein-protein interactions. The DLK (DLK and LZK) contain two leucine zipper motifs, while ZAK may feature a sterile-α motif (SAM).

MLK

MLK1
MLK2
MLK3
MLK4α
MLK4β

DLK

DLK
LZK

ZAK

ZAKα
ZAKβ

SH3 domain   kinase domain   leucine zipper   CRIB

proline rich region

Gly-Pro rich region

SAM

NH₂ ... COOH

Regulation of MAPK pathways by upstream signaling components

MAP3K directly bind to upstream regulatory proteins to mediate MAPK activation. For example, Cdc42 and Rac, members of the Rho family of GTPases, are upstream activators of the JNK and p38 MAPK pathways (Coso et al. 1995; Minden et al. 1995). Activated Cdc42 and Rac directly bind to the MLK or the MEKK (Porter et al. 1999). In addition, MAP3K bind to adaptor proteins to indirectly mediate MAPK activation. The TNF-receptor adaptor proteins TRAF2 and RIP1 interact with MEKK1/ASK1 and MEKK1/MEKK3, respectively (Baud et al. 1999; Nishitoh et al. 1998; Lee et al. 2003).

In addition to upstream GTPases, MAPK3K are also regulated by upstream kinases (Fanger et al. 1997; Kyriakis 1999; Kyriakis and Avruch 2001). For example, the germinal center kinases (GCK) HPK1, GCK, and NIK have been shown to activate MEKK1 and MLK3 (Kiefer et al. 1996; Tibbles et al. 1996; Su et al. 1997; Yuasa et al. 1998). GCK also bind to adaptor proteins, coupling MAPK pathways to additional upstream components. The GCK and GCKR protein kinases bind the TNF-receptor adaptor protein TRAF2, while HPK1 binds the SH2/SH3 adaptor proteins, Grb2 and Crk (Ling et al. 1999; Shi et al. 1999). The mechanism of MAP3K activation by GCK is not completely clear; however, the GCK protein kinase, which binds both TRAF2 and MEKK1, may directly phosphorylate MEKK1 (Chadee and Kyriakis 2004). TRAF2 may also mediate MAP3K activation by increasing GCK protein stability (Zhong and Kyriakis 2004).

MAP3K activation represents the entry point into the canonical MAPK pathway and thus integrates signals from multiple stimuli (Davis 2000; Kyriakis and Avruch 2001). For example, Rho GTPases and the SH2/SH3 adaptor proteins could be regulated by tyrosine kinases and may therefore relay signals from receptor tyrosine kinases to the MAPK pathways. In contrast, the

TNF-receptor adaptor proteins, TRAF2 and RIP1, mediate signals from the cytokine receptors to the MAPK pathways. Thus, due to the large number of protein kinases involved, mechanisms that account for MAPK signaling specificity are not completely understood.

Regulation of MAPK pathways by scaffold proteins

Signaling specificity is mediated, in part, by protein-protein interactions. Scaffold proteins interact with different components of the MAPK pathways to assemble functional signaling complexes (Whitmarsh and Davis 1998; Morrison and Davis 2003). For example, the JNK-interacting protein (JIP) is a family of scaffold proteins that regulate the JNK pathway. The JIP family consists of four proteins (JIP1 - JIP4), which differentially bind to components at all three levels of the JNK pathway: MAPK-MAP2K-MAP3K (Dickens et al. 1997; Whitmarsh et al. 1998; Yasuda et al. 1999; Kelkar et al. 2003; Ito et al. 1999; Lee et al. 2002). Each JIP binds to JNK, MKK7, and the MLK MAP3K. Several MLK, including MLK2, MLK3, and DLK, also interact with both JIP1 and JIP2, consistent with their ability to strongly activate JNK (Whitmarsh et al. 1998; Yasuda et al. 1999). In contrast, the structurally distinct JIP3 and JIP4 proteins have been shown to interact with JNK-MKK7-MLK3 and JNK-MKK4-MEKK1/MEKK3 (Kelkar et al. 2003; Ito et al. 1999; Lee et al. 2002). In addition, some studies suggest that JIP2 also binds to p38 MAPK-MKK3-MLK3 to potentiate p38 MAPK activation (Schoorlemmer and Goldfarb 2001; Schoorlemmer and Goldfarb 2002; Buchsbaum et al. 2002).

Transfection studies indicate that JIP potentiate MAPK activation, perhaps by increasing the "effective" local kinase concentration. Interestingly, JIP1 also inhibits MAPK activation by preventing DLK dimerization, suggesting that scaffold proteins may act as molecular switches in response to specific stimuli (Nihalani et al. 2003; Nihalani et al. 2001). In addition, scaffold

proteins may mediate the subcellular localization of MAPK activation. For example, JIP

proteins bind to kinesin light chain, a component of kinesin motor complex, suggesting that JIP

may act as cargo proteins for kinesin-mediated transport (Whitmarsh and Davis 2001; Verhey et

al. 2001; Bowman et al. 2000; Lee et al. 2002).

Regulation of MAPK pathways by docking interactions

Functional signaling complexes are also created through binary interactions between

individual members in the MAPK cascade. These binary interactions are mediated by specific

MAPK docking motifs that are present in MAPK, MAPK activators, MAPK substrates, MAPK

phosphatases, and MAPK scaffolds (Enslen and Davis 2001). Although MAPK docking sites

are found throughout the MAPK family, there are specific motifs for individual MAPK

subgroups to regulate signaling specificity (Enslen and Davis 2001; Sharrocks et al. 2000;

Tanoue and Nishida 2003). These docking sites are physically distinct from kinase active sites

and are necessary for efficient substrate recognition and subsequent phosphorylation.

MAPK docking motifs were initially identified in MAP kinase substrates. The NH_2-

terminal δ domain in c-Jun was the first domain identified for JNK interaction (Hibi et al. 1993;

Dai et al. 1995). This domain contains a conserved MAPK docking site, the D domain, and is

found on many MAPK interacting proteins. The D domain consists of a hydrophobic motif

separated from a cluster of basic residues (L/I-X-L/I-X_{2-6}-R/K_{2+}) (Enslen and Davis 2001;

Tanoue and Nishida 2003). Similar D domains have been identified in other MAPK

transcription factor substrates. Examples of these D domains include the D domain on

MEF2A/C that binds p38 MAPK (Yang et al. 1999), the D domain on Sap1 that binds p38

MAPK (Galanis et al. 2001; Barsyte-Lovejoy et al. 2002), and the D domain on Elk1 that binds

JNK and ERK (Yang et al. 1998b; Yang et al. 1998c). The NH_2-terminal regions of MAPK activators (MEK1, MEK2, MKK4, and MKK7) and MAPK phosphatases (MKP1, MKP3, and MKP5) have also been proposed to contain D domains (Tanoue et al. 2000). Interestingly, a second docking motif, the F-X-F-P motif has been recently identified in Elk1-ERK interactions and SAP1-p38 MAPK interactions (Jacobs et al. 1999; Galanis et al. 2001). This suggests that multiple docking domains may act synergistically to mediate MAPK interaction and activation.

Docking motifs have also been identified in the MAP kinases. For example, common docking (CD) domains have been identified in the COOH-terminal regions of ERK, JNK, and p38 MAPK (Enslen and Davis 2001; Tanoue and Nishida 2003). The CD domain contains hydrophobic and acidic residues that are proposed to interact with hydrophobic and basic residues in the D domains of MAPK interacting proteins. Recently, studies using X-ray crystallography and mass spectrometry provide further structural insight into MAPK docking interactions (Weston and Davis 2002; Chang et al. 2002; Lee et al. 2004; Heo et al. 2004). These studies confer a direct interaction between hydrophobic residues in the CD domain and the L-X-L motif in the D domain of MAPK interacting proteins. These studies also indicate that ionic interactions between acidic residues in the CD domain and basic residues in D domain can directly influence MAPK binding.

Biological Functions of the JNK and p38 MAPK pathways

MAP kinases regulate many physiological processes in response to physical and chemical changes in a cell's environment(Kyriakis and Avruch 2001; Davis 2000). These environmental changes are able to stimulate cellular growth, differentiation, development, and death. The MAP kinases control these processes, in part, through the activation of various transcription factors.

As stress-activated MAP kinases, JNK and p38 MAPK play a key role in regulating gene transcription in response to cellular stress and inflammatory cytokines. For example, activated JNK and p38 MAPK increase AP-1-dependent gene transcription and cytokine expression.

The physiological roles of JNK and p38 MAPK are quite complex. The JNK pathway has been implicated in the regulation of apoptosis, cell survival, differentiation, inflammation, and embryonic morphogenesis (Weston et al. 2002; Davis 2000). The p38 MAPK pathway is also proposed to regulate similar processes (Ono and Han 2000; Nebreda and Porras 2000). Thus, the JNK and p38 MAPK pathways may have both redundant and non-redundant functions. These functions appear to be cell-type specific and context dependent.

Many studies have used overexpression and dominant-negative approaches to analyze MAPK function (Davis 2000; Kyriakis and Avruch 2001). However, mechanisms for the diverse cellular response to MAPK activation remain unclear. This may be due, in part, to the cell-type and cell environment, or may be caused by the promiscuity of the protein kinases in the MAPK cascade. In contrast, studies of MAPK gene targeted disruption have provided strong insight into the physiological roles of individual MAPK (Figure I.3) (Weston and Davis 2002; Davis 2000). Since this thesis analyzes the *in vivo* roles of several MAPK (Chapter III, Chapter IV, Appendix), characteristics of gene targeted MAPK will be discussed in detail below. In addition, since no MLK MAP3K gene targeted disruption has been reported, studies of MLK function using overexpression and dominant-negative approaches will also be discussed.

Figure I.3. Gene targeted disruption of the JNK and the p38 MAPK pathways in mice.

The physiological roles of JNK and p38 MAPK are quite complex and appear to be cell-type specific and context dependent. The JNK pathway has been implicated in the regulation of apoptosis, cell survival, differentiation, inflammation, and embryonic morphogenesis. The p38 MAPK pathway is also proposed to regulate similar processes. Recent studies of MAPK gene targeted disruption have provided strong insight into the physiological roles of individual MAPK. Illustrated are some characteristics of gene targeted MAPK in mice.

MAPK

JNK1⁻/⁻	Defects in T-cell differentiation and activation.	Dong et al. 1998 Dong et al. 2000 Sabapathy et al. 2001 Conze et al. 2002
JNK2⁻/⁻	Defects in T-cell differentiation and activation.	Yang et al. 1998 Dong et al. 2000 Sabapathy et al. 2001 Conze et al. 2002
JNK3⁻/⁻	Increased resistance to excitotoxic apoptosis in hippocampus.	Yang et al. 1997
JNK1⁻/⁻ + JNK2⁻/⁻	Embryonic lethal due to neural tube disclosure; Increased apoptosis in forebrain; decreased apoptosis in hindbrain; Increased resistance to stress-induced apoptosis in MEF; Reduced JNK activation in MEF.	Kuan et al. 1999 Sabapathy et al. 1999 Dong et al. 2000 Tournier et al. 2000 Lamb et al. 2003 Ventura et al. 2003 Ventura et al. 2004
p38α⁻/⁻	Embryonic lethal due to defects in vascularization and development; Reduced IL-1-stimulated cytokine production in ES cells.	Adams et al. 2000 Allen et al. 2000 Mudgett et al. 2000 Tamura et al. 2000

MAP2K

MKK3⁻/⁻	Increased resistance to apoptosis in peripheral T-cells; Reduced TNF-stimulated cytokine production in MEF.	Lu et al. 1999 Wysk et al. 1999
MKK4⁻/⁻	Embryonic lethal due to liver apoptosis.	Yang et al. 1997 Ganiatsas et al. 1998 Nishina et al. 1999
MKK6⁻/⁻	Increased resistance to apoptosis in thymocytes.	Tanaka et al. 2002
MKK7⁻/⁻	Embryonic lethal due to defective hepatocyte proliferation; Reduced TNF-stimulated JNK activation in MEF.	Dong et al. 2000 Sasaki et al. 2001 Wada et al. 2004
MKK4⁻/⁻ + MKK7⁻/⁻	Embryonic lethal; Reduced TNF-stimulated JNK activation in MEF.	Tournier et al. 2001
MKK3⁻/⁻ + MKK6⁻/⁻	Embryonic lethal due to defects in vascularization and development; Reduced TNF-stimulated p38 MAPK activation in fibroblasts.	Brancho et al. 2003

MAP3K

ASK1⁻/⁻	Increased resistance to TNF-induced apoptosis; Reduced TNF-stimulated sustained JNK activation in MEF.	Tobiume et al. 2001
MEKK1⁻/⁻	Defects in eyelid closure and cell migration; Reduced TGFβ/activin-stimulated JNK activation in keratinocytes.	Yujiri et al. 2000 Zhang et al. 2003
MEKK2⁻/⁻	Reduced FGF-stimulated JNK activation in MEF.	Kesavan et al. 2004
MEKK3⁻/⁻	Embryonic lethal due to defects in vascularization and development; Increased sensitivity to TNF-induced apoptosis; Reduced TNF-stimulated NF-κB activation in MEF.	Yang et al. 2000 Yang et al. 2001
MEKK4⁻/⁻	Reduced IL-18-stimulated p38 MAPK activation in T-cells.	Chi et al. 2004
Tpl2⁻/⁻	Reduced LPS-stimulated ERK activation in macrophages.	Dumitru et al. 2000
MLK3⁻/⁻	Reduced TNF-stimulated JNK activation in MEF.	Brancho et al. In press
DLK⁻/⁻	Early embryonic lethal.	Weston et al. In preparation

Gene targeted disruption of the JNK pathway

Mice with targeted disruption of the *Jnk1*, *Jnk2*, or *Jnk3* genes were viable and appeared to be developmentally normal (Yang et al. 1998a; Yang et al. 1997b; Dong et al. 1998). This suggested that there is functional redundancy among the JNK genes. However, the loss of JNK appeared to have cell-type specific defects. For example, mice deficient in *Jnk1* or *Jnk2* exhibit defects in T-cell differentiation. *Jnk1*$^{-/-}$ mice exhibited defects in the differentiation of T helper (Th) cells into Th2 effector T-cells (Dong et al. 1998), while *Jnk2*$^{-/-}$ mice exhibited defects in the differentiation of T helper cells into Th1 effector T-cells (Yang et al. 1998a). In addition, mice deficient in *Jnk3* were resistant to kainic acid-induced neuronal apoptosis in the hippocampus (Yang et al. 1997b).

Mice with compound mutations in the *Jnk1* and *Jnk2* genes are early embryonic lethal due to defects in neural tube closure (Sabapathy et al. 1999; Kuan et al. 1999). These mice exhibited opposite apoptotic defects in the developmental forebrain and hindbrain. *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mice have increased apoptosis in the forebrain and decreased apoptosis in the hindbrain. Together, these data suggest a context-dependent role for JNK in cell survival and cell death.

Recent studies of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mouse embryonic fibroblasts (MEF) demonstrate a lack of JNK activation and defects in AP-1-dependent gene transcription (Tournier et al. 2000; Ventura et al. 2003). The *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ MEF are resistant to stress-induced apoptosis due to a failure of mitochondria membrane depolarization and cytochrome c release (Tournier et al. 2000). In contrast, these MEF are not resistant to Fas-induced apoptosis, suggesting a distinct role for JNK signaling in response to specific apoptotic stimuli. This suggests that JNK is required for apoptosis mediated by the mitochondrial pathway, but is not required for apoptosis mediated by death receptor signaling. In addition, *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ MEFs exhibit defects in growth

arrest that include increased p53 expression and decreased proliferation (Tournier et al. 2000). These cells are sensitive to TNFα-induced apoptosis in presence of protein synthesis inhibitors, suggesting that JNK mediates cell survival under certain conditions (Lamb et al. 2003).

Mice with targeted disruption of the JNK activators, *Mkk4* and *Mkk7*, are embryonic lethal due to impaired liver development (Dong et al. 2000; Yang et al. 1997a; Ganiatsas et al. 1998; Nishina et al. 1999; Wada et al. 2001). The *Mkk4*$^{-/-}$ mice have increased hepatic apoptosis, while *Mkk7*$^{-/-}$ mice exhibited decreased hepatocyte proliferation. MEF isolated from these mice demonstrated partial defects in stress-stimulated JNK activation (Tournier et al. 2001). In addition, MKK7 has been shown to be essential for cytokine-stimulated JNK activation, while MKK4 contributes to maximal cytokine-stimulated JNK activation. Not surprisingly, mice with compound mutations of the *Mkk4* and *Mkk7* genes exhibited defects similar to that seen in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mice (Tournier et al. 2001). Fibroblasts isolated from these mice exhibited a lack of JNK activation and resistance to stress-induced apoptosis. Together, these studies suggest that the protein kinases involved in JNK signaling have both redundant and non-redundant functions.

Gene targeted disruption of the p38 MAPK pathway

The p38 MAPK kinase pathway also plays a key role in embryonic development. Mice with a targeted disruption of *p38α* MAPK are early embryonic lethal due to placental defects (Tamura et al. 2000; Allen et al. 2000; Adams et al. 2000; Mudgett et al. 2000). The *p38α*$^{-/-}$ embryos appear pale and anemic and are developmentally delayed. The *p38α*$^{-/-}$ placentas display a lack of blood vessels and increased apoptosis. The labryrinthine layer of placenta is also severely reduced. These phenotypes are consistent with a decrease in vascularization of both the embryo and the placenta. In one study, tetraploid wild-type embryos were fused to

diploid $p38\alpha^{-/-}$ embryos and rescued the embryonic lethality (Adams et al. 2000). This suggests

that the abnormal embryonic development seen in $p38\alpha^{-/-}$ embryos is a secondary affect of

defective placental function. Nevertheless, p38α MAPK is essential for vascularization and

development of the placenta.

Cultured $p38\alpha^{-/-}$ ES cells also exhibit defects in p38 MAPK activation and function

(Allen et al. 2000). These cells have reduced MAPKAP-2 activation in response to cellular

stress. In addition, the $p38\alpha^{-/-}$ ES cells display severe defects in IL-6 cytokine production in

response to IL-1 activation. This suggests that p38 MAPK may play a key role in the regulation

of IL-6 cytokine expression.

Studies of gene targeted disruption of *Mkk3* or *Mkk6* also suggest that the p38 MAPK

pathway plays a key role in the inflammatory response (Tanaka et al. 2002; Wysk et al. 1999; Lu

et al. 1999). *Mkk3*$^{-/-}$ and *Mkk6*$^{-/-}$ mice are viable with no obvious developmental defects.

However, these mice do exhibit defects in inflammatory cytokine production and T-cell

apoptosis. *Mkk3*$^{-/-}$ MEF displays reduced IL-1, IL-6, and TNFα production in response to

TNFα-stimulation (Wysk et al. 1999). In contrast, LPS-stimulated *Mkk3*$^{-/-}$ macrophages exhibit

decreased IL-12 production (Lu et al. 1999). Furthermore, peripheral T-cells from *Mkk3*$^{-/-}$ mice

are resistant to activation-induced cell death (Tanaka et al. 2002). Interestingly, thymocytes

from *Mkk6*$^{-/-}$ mice are also resistant to apoptosis (Tanaka et al. 2002), suggesting a non-

redundant role for MKK3 and MKK6 in T-cell development and cell death.

Gene targeted disruption of the MAP3K and biological functions of the MLK

Examining the biological function of individual MAP3K is particularly difficult due to

the inherent redundancy observed among the kinases at this level. Gene targeted disruption

studies of a few MAP3K, particularly the MEKK group of MAP3K, have provided some insight into their function. For example, $Mekk1^{-/-}$ mice exhibited defects in eyelid closure, a process associated with epithelial cell migration (Yujiri et al. 2000). MEF derived from the $Mekk1^{-/-}$ mice displayed defects in cell migration and reduced JNK activation in response to taxol, a drug that affects microtubule stability. In $Mekk1^{\Delta KD}$ keratinocytes, MEKK1 is also essential for TGFβ/activin-stimulated JNK activation and cell migration (Zhang et al. 2003). In contrast, $Mekk3^{-/-}$ mice are embryonic lethal due to placental and embryonic defects in vascularization and development (Yang et al. 2000). This phenotype is similar to that observed in $p38\alpha^{-/-}$ mice, suggesting that MEKK3 may contribute to p38 MAPK activation *in vivo*.

Other MAP3K null mice are viable with no obvious phenotypic effects. However, these mice do display altered MAPK activation in response to specific stimuli. MEF isolated from $Mekk2^{-/-}$ mice have reduced FGF-stimulated JNK activation (Kesavan et al. 2004); T-cells isolated from $Mekk4^{-/-}$ mice have reduced IL-18-stimulated p38 MAPK activation (Chi et al. 2004); and macrophages isolated from $Tpl2^{-/-}$ mice have reduced LPS-stimulated ERK activation (Dumitru et al. 2000). Therefore, the effects of MAP3K on MAPK activation appear to be cell-type and stimulus specific. Interestingly, four different MAP3K have all been implicated in TNFα signaling (Sathyanarayana et al. 2002; Takaesu et al. 2003; Tobiume et al. 2001; Yang et al. 2000) However, the relevant contribution of each individual MAP3K remains unclear.

TNFα activates several signaling pathways and mediates a balance between life and death (Wajant et al. 2003; Varfolomeev and Ashkenazi 2004). TNFα binds to the TNF receptor type 1 (TNF-R1). Upon ligand binding, the silencer of death domain (SODD) repressor protein dissociates from the TNF-R1 receptor and recruits the TNF-R1- associated death domain (TRADD) protein through direct interaction between TNF-R1 and the death domain of TRADD.

TRADD serves as a molecular scaffold, binding the adaptor proteins Fas-associated death domain protein (FADD), TNFR-1- associated factor-2 (TRAF2), and receptor interacting protein kinase-1 (RIP1). The FADD adaptor protein signals the caspase-8 pathway to stimulate apoptosis, while the TRAF2 and RIP1 adaptor proteins activate the NF-κB pathway to stimulate cell survival. In addition, TRAF2 and RIP1 also mediate JNK- and p38 MAPK- dependent gene transcription through interaction with GCK and MAP3K. Recently, gene targeted disruption studies of *Traf2* and *Rip1* indicate that TRAF2 and RIP1 selectively mediate JNK and p38 MAPK activation, respectively (Lee et al. 2003; Yeh et al. 1997).

MEF isolated from the MAP3K *Ask1*$^{-/-}$ mice are resistant to TNFα-induced apoptosis (Tobiume et al. 2001). This resistance is due to the loss of sustained JNK activation, which is dependent on the generation of reactive oxygen species. In contrast, MEF isolated from the MAP3K *Mekk3*$^{-/-}$ mice have increased sensitivity to TNFα-induced apoptosis (Yang et al. 2000). MEKK3 contributes to TNFα-stimulated NF-κB activation. MEKK3 binds to RIP1 and phosphorylates IκB kinases *in vitro*, leading to the subsequent phosphorylation and degradation of IκB and the activation of NF-κB. MEKK3 also contributes to TNFα-stimulated p38 MAPK activation through it association with RIP1 (Lee et al. 2003). These studies of MAP3K gene disruptions suggest a distinct role for individual MAP3K in TNFα signaling.

Interestingly, studies using chemical inhibitors or siRNA have suggested a role for two other MAP3K in the TNFα signaling pathway. First, transfection of *Tak1* siRNA inhibits JNK, p38 MAPK, and NF-κB activation in response to TNFα, IL-1, and LPS stimulation (Chen and Goeddel 2002; Ishitani et al. 2003; Takaesu et al. 2003). TAK1 activates the MAPK pathways through its association with TRAF2 through a TAK1-TAB2/3-TRAF2 complex (Takaesu et al. 2003; Ishitani et al. 2003). However, since TRAF2-deficient cells are not implicated in TNFα-

stimulated p38 MAPK activation and RIP1-deficient cells are not implicated in TNFα-stimulated JNK activation (Lee et al. 2003; Yeh et al. 1997), it is not obvious how a MAP3K could commonly activate JNK, p38 MAPK, and NF-κB. Interestingly, studies using the MLK chemical inhibitor CEP-11004 indicate that MLK3 selectively inhibits TNFα-stimulated JNK activation (Sathyanarayana et al. 2002). However, transfection of *Mlk3* siRNA has also been shown to inhibit multiple pathways in response to TNFα (Chadee and Kyriakis 2004). To date, no targeted gene disruption study of *Mlk3* has been reported and the role of MLK3 in TNFα signaling remains unclear.

MLK3 appears to play a role in other signaling pathways as well. Overexpression of MLK3 activates the ERK, JNK, and p38 MAPK pathways and MLK3 phosphorylates MEK1, MKK3, MKK4, MKK6, and MKK7 *in vitro* (Shen et al. 2003; Gallo and Johnson 2002). This MLK3 activation of MAPK may be mediated by various stimuli. For example, chemical inhibition suppresses MLK3 activation of JNK in response to ceramide (Sathyanarayana et al. 2002). Inhibition of MLK3 by siRNA also reduces PDGF-stimulated ERK activation, EGF-stimulated ERK and JNK activation, and T-cell co-stimulated activation of ERK, JNK, and p38 MAPK (Chadee and Kyriakis 2004). Finally, MLK3 may activate the NF-κB pathway in response to T-cell co-stimulation. (Hehner et al. 2000). MLK3 phosphorylates IκB kinases *in vitro*, leading to the subsequent phosphorylation and degradation of IκB and the activation of NF-κB.

Interestingly, recent RNAi-based studies suggest that MLK3 is required for serum-stimulated cell proliferation and cell migration. Serum-stimulated cell proliferation was reduced by siRNA-inhibition of MLK3 (Chadee and Kyriakis 2004). In addition, depletion of MLK3 by siRNA increased cell sensitivity to taxol (Swenson et al. 2003). This suggests that MLK3 may

be important for mitosis and cell migration, processes where microtubule regulation is essential.

Previous studies also suggest essential roles for MLK family members in JNK activation in neurons [reviewed in (Wang et al. 2004; Gallo and Johnson 2002)]. Overexpression of MLK2, MLK3, or DLK induces apoptosis in differentiated PC12 cells and neurons, and inhibition of MLK with CEP-1347 suppresses apoptosis in these cells upon NGF withdrawal (Xu et al. 2001; Putcha et al. 2003; Maroney et al. 2001; Mata et al. 1996; Harris et al. 2002a; Harris et al. 2002b). Recent studies also demonstrate that overexpressed DLK disrupts neural cell migration and telencephalon morphogenesis (Hirai et al. 2002). These data suggest essential roles for the MLK protein kinases in developing and differentiated neurons.

Gene targeted disruption studies of the MAP2K and the MAP3K will be useful in determining their physiological role in MAPK cascades. In my thesis work, I used molecular, biochemical, and genetic approaches to examine how MAP2K and MAP3K mediate signaling specificity and to define their role in the JNK and p38 MAPK pathways. Specifically, I examined the regulation of JNK and p38 MAPK by investigating: (1) the docking interactions between MAPK and MAP2K; (2) the differential activation of MAPK by MAP2K; and (3) the selective involvement of MLK MAP3K in MAPK activation. Together, these data bring insight into the regulation and function of stress-activated MAPK signal transduction pathways.

CHAPTER II

MOLECULAR DETERMINANTS THAT MEDIATE

SELECTIVE ACTIVATION OF p38 MAPK ISOFORMS

Summary

The p38 mitogen-activated protein kinase (MAPK) group is represented by four isoforms in mammals (p38α, p38β2, p38γ and p38δ). These p38 MAPK isoforms appear to mediate distinct functions *in vivo* due, in part, to differences in substrate phosphorylation by individual p38 MAPK and also to selective activation by MAPK kinases (MAP2K). My data demonstrates that specificity can be caused by the selective formation of functional complexes between the MAP2K and different p38 MAPK. The formation of these complexes requires the presence of a MAPK docking site in the NH_2-terminus of the MAP2K. This process provides a mechanism that enables the selective activation of p38 MAPK in response to activated MAP2K.

This study was done in collaboration with Herve Enslen during the early part of my thesis work. I generated the MKK3/MKK6 chimeras and contributed to data in Figure II.1. Herve Enslen contributed to data in Figures II.2 - II.5.

Introduction

The p38 group of mitogen-activated protein kinases (MAPK) is activated by treatment of cells with pro-inflammatory cytokines and by exposure to environmental stress (Cohen 1997). One important function of the p38 signaling pathway appears to be the regulation of cytokine expression (Lee et al. 1999).

Molecular cloning studies have led to the identification of four p38 isoforms: p38α (also known as SAPK2a), p38β2 (SAPK2b), p38γ (SAPK3) and p38δ (SAPK4). The p38α and p38β MAPK are 60% identical to p38γ and p38δ MAPK, indicating that these protein kinases represent related, but distinct, MAPK sub-groups (Cohen 1997). One sub-group (p38α and p38bβ) is inhibited by a class of pyridinyl imidazole drugs, while the other sub-group (p38γ and p38δ) is insensitive to these drugs (Lee et al. 1999). These p38 MAPK phosphorylate both a common group of substrates and distinct substrates (Cohen 1997), and they can be selectively activated by some extracellular stimuli (Wilk-Blaszczak et al. 1998; Conrad et al. 1999). This suggests that they may exert distinct biological actions. Indeed, studies of HeLa cells indicate that while p38α induces apoptosis, p38β2 promotes cell survival (Nemoto et al. 1998). These studies suggest that different p38 isoforms have overlapping, but also distinct physiological roles.

Specificity of p38 MAPK signaling has also been reported in studies of the MAPK kinases (MAP2K) that activate the p38 MAPK isoforms. Two genes that encode p38-specific MAP2K have been described: *Mkk3* (Derijard et al. 1995) and *Mkk6* (Han et al. 1996; Moriguchi et al. 1996; Raingeaud et al. 1996; Stein et al. 1996). Targeted gene disruption studies in mice have demonstrated non-redundant functions of the *Mkk3* and *Mkk6* genes (Lu et al. 1999; Wysk

et al. 1999). Furthermore, expression of activated forms of the MKK3 and MKK6 protein kinases cause different biological responses in cardiac myocytes (Wang et al. 1998). These actions are altered by co-expression of specific p38 isoforms; thus, p38α induces apoptosis while p38β2 promotes a hypertrophic response in cardiac myocytes (Wang et al. 1998). This signaling specificity is likely to be important for the generation of appropriate biological responses by the p38 MAPK pathway.

The mechanism that accounts for signaling specificity by the p38 MAPK pathway is not understood. Previously it has been demonstrated that MKK6 is a common activator of p38α, p38β2, p38γ and p38δ MAPK, while MKK3 activates only p38α, p38γ and p38δ MAPK (Jiang et al. 1996; Cuenda et al. 1997; Goedert et al. 1997; Jiang et al. 1997; Wang et al. 1997; Enslen et al. 1998). This selectivity of p38 MAPK activation by MKK3 and MKK6 may contribute to the specificity of signal transduction by the p38 MAPK pathway.

The purpose of the study reported here was to examine the specificity of activation of p38 MAPK isoforms by MKK3 and MKK6. I show that the specificity of p38 MAPK signaling can be mediated by the selective docking interactions between the MAP2K and p38 MAPK. These observations provide insight into the mechanism of p38 MAPK activation.

Materials and Methods

Plasmids. The p38, MKK3, and MKK6 expression vectors have been described (Enslen et al. 1998). Point mutations and chimeric constructs were prepared using standard techniques.

Protein kinase isolation. Cells were solubilized in Triton lysis buffer [20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin and leupeptin] and centrifuged at 15,000 x g for 15 min at 4°C. Epitope-tagged protein kinases were immunoprecipitated by incubation (4 hr) with the M2 Flag monoclonal antibody (Sigma) bound to Protein G-Sepharose (Amersham Pharmacia Biotech). The Sepharose beads were collected by centrifugation, washed twice with TLB, and twice with kinase assay buffer (25 mM HEPES pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). The immunoprecipitation of endogenous p38α was performed similarly except that an anti-p38 rabbit polyclonal antibody bound to Protein A-Sepharose (Sigma Chemical Co.) was employed (Raingeaud et al. 1995). In some assays, the immunoprecipitated epitope-tagged protein kinases were eluted by incubation with 0.1 mg per ml of Flag synthetic peptide at 30°C (20 min).

Protein kinase assays. Protein kinase immunoprecipitates were used for kinase assays. The reactions were initiated by the addition of substrate protein (1 μg) and 50 μM [γ-^{32}P]ATP (10 Ci/mmol) and the reactions were terminated after 20 min at 30 °C by addition of Laemmli sample buffer. Assays of p38 kinase activity were performed using GST-ATF2 as the substrate

(Raingeaud et al. 1995). Substrate phosphorylation was examined after SDS-PAGE by autoradiography and Phosphorimager analysis (Molecular Dynamics Inc.).

Peptide competition assays. Peptide competition experiments were performed using bacterially expressed GST-p38 bound to GSH-Sepharose and pre-incubated with synthetic peptides for 2 hr at 4°C. The recombinant p38 was phosphorylated by MKK3b or MKK6 (isolated by elution from immunoprecipitates prepared from transfected COS7 cells) by incubation (20 min at 30°C) in kinase buffer with 50 μM ATP. The immobilized p38 was then washed four times with kinase buffer and the p38 activity was measured in kinase assay with GST-ATF2 (1 μg) and 50 μM [γ-^{32}P]ATP (10Ci/mmol). The reaction was terminated after 20 min at 30°C and the phosphorylated ATF2 examined by SDS-PAGE, detected by autoradiography, and quantitated by Phosphorimager analysis.

Binding assays. GST-tagged MKK proteins were isolated from transfected COS7 cells using glutathione (GSH)-Sepharose (Amersham Pharmacia Biotech) in TLB for (7 hr at 4°C). The beads were washed five times with TLB and the presence of bound co-transfected p38α or p38β2 was examined by immunoblot analysis.

Results

Role of the NH$_2$-terminal region of p38 MAP2K in the selective activation of p38β2 MAPK

MKK3 and MKK6 selectively activate different p38 isoforms (Enslen et al. 1998). To identify the molecular determinants responsible for this selectivity, I constructed chimeric protein kinases using MKK3 and MKK6 sequences (Figure II.1a). Constitutively activated MAP2K were constructed by replacing the sites of activating phosphorylation with Glu. These chimeric kinases caused similar activation of p38α in co-transfection assays (Figure II.1b). In contrast, differences were detected in assays using p38β2 (Figure II.1c). As expected MKK6, but not MKK3, caused p38β2 activation. However, replacement of the NH$_2$-terminal region of MKK6 with sequences derived from MKK3 blocked the ability of MKK6 to activate p38β2 (Figure II.1c). Conversely, chimeras of MKK3 with the NH$_2$-terminal region of MKK6 were able to activate p38β2 (Figure II.1c). Residues 1-18 of MKK6 were sufficient, but a larger activation of p38β2 was observed when MKK6 residues 1-82 were fused to the NH$_2$-terminus of MKK3. These data indicated that the NH$_2$-terminal region of MKK3 and MKK6 regulates substrate specificity (Figure II.1c), but not activity (Figure II.1b).

This conclusion was confirmed by studies of an alternative form of MKK3 (MKK3b) (Moriguchi et al. 1996; Han et al. 1997) which contains an additional 29 amino acids fused to the NH$_2$-terminus of MKK3. I found that while MKK3 and MKK3b both activated p38α (Figure II.1b), only MKK3b caused activation of p38β2 (Figure II.1c). Together, these data identify a role for the NH$_2$-terminal region of MKK3 and MKK6 in the determination of substrate specificity.

The NH$_2$-terminal region of MAP2K is required for binding to p38α and p38β2 MAPK

The analysis of MAP2K chimeras indicated that the first 18 amino acids of MKK6 contains a region that can confer the ability to activate p38β2 on MKK3 (Figure II.1c). Alignment of the sequences of MKK3, MKK3b, and MKK6 indicates that this 18 amino acid sequence present in the NH$_2$-terminal region of MKK6 is absent in MKK3, but is conserved in MKK3b (Figure II.2a). This region contains several basic amino acids and also a sequence motif that has been previously identified as a MAPK docking site (Holland and Cooper 1999). The NH$_2$-terminal region of MKK3b and MKK6 conforms to the consensus sequence for this type of MAPK docking site (-Lys/Arg-Xaa$_3$-Leu/Ile-Xaa-Leu/Ile-). The NH$_2$-terminal specificity determining region of MKK3b and MKK6 may therefore function as a p38 docking site.

To test this hypothesis, binding of p38α and p38β2 to MKK3 and MKK6 was examined in co-precipitation assays using extracts prepared from transfected COS7 cells (Figure II.2). The p38α and p38β2 MAPK co-precipitated with MKK6 and MKK3b, but not with MKK3. Interestingly, more p38β2 than p38α was observed to co-precipitate with MKK3b and MKK6. To test whether the putative docking site located in the NH$_2$-terminal region of MKK6 and MKK3b was required for binding to p38, the effect of mutations of the conserved Leu-Xaa-Ile motif on the interaction with p38 MAPK was examined. Mutational removal of the Leu-Xaa-Ile motif (MKK3Δ and MKK6Δ) reduced the co-precipitation of p38α and p38β2 to MKK6 and MKK3b. Furthermore, fusion of the NH$_2$-terminal region of MKK6 (containing the putative MAPK docking site to MKK3) allowed co-precipitation of MKK3 with p38α and p38β2. Fusion of MKK6 residues 1-18 was sufficient for co-precipitation of p38α and p38β2, but a larger amount of co-precipitation was detected when MKK6 residues 1-82 were fused to MKK3.

These data indicate that a p38 docking site is present in the NH_2-terminal region of MKK3b and MKK6, but not MKK3.

Binding to MKK3b and MKK6 potentiates p38 MAPK activation

There is a strong correlation between MAP2K binding to p38 MAPK and the activation of p38α and p38β2. For example, MKK3b and MKK6 (but not MKK3) bind and activate p38β2. Similarly, p38α (which is activated less potently by MKK3 than by MKK3b and MKK6), binds to MKK3b and MKK6, but not to MKK3. To examine the relationship between MAP2K binding and p38 activation, the effect of mutations that inhibit MAP2K binding on p38 activation was examined (Figure II.3). Mutational removal of the MAPK docking site (Leu-Xaa-Ile) reduced the binding of MKK3b and MKK6 to p38α and p38β2 (Figure II.2). The same mutation also reduced p38α activation caused by MKK3b and MKK6 (Figure II.3b, c). Furthermore, like MKK3, these mutant MKK3b and MKK6 proteins were unable to activate p38β2 (Figure II.3d, e). These data indicate that binding to MKK3b and MKK6 potentiates p38α activation and is required for p38β2 activation.

To confirm the conclusion that MAP2K binding increases p38 activation, the effect of activated MKK3 and MKK6 on the activation of endogenous p38α was examined in COS7 cells. It has been previously reported that MKK6 (but not MKK3) potently activates endogenous p38α (Raingeaud et al. 1996). Here, it is also demonstrated that MKK3b, like MKK6, strongly activates endogenous p38α MAPK (Figure II.4). Disruption of the MAPK docking site located in the NH_2-terminal region of MKK3b and MKK6 reduced the activation of endogenous p38α. Furthermore, fusion of the NH_2-terminal region of MKK6 (containing the putative MAPK docking site to MKK3) allowed MKK3 to activate endogenous p38α. These data suggest that

the binding of MKK3b and MKK6 to p38 is a determinant of p38α activation *in vivo*. This requirement for a binding interaction can be compensated by overexpression of p38α, but not by overexpression of p38β2 (Figure II.1).

The NH₂-terminal MAPK docking site is likely to contribute to p38 activation. To test this hypothesis, competition analyses using synthetic peptides corresponding to the MAPK docking sites of MKK3b was performed (Figure II.5). The effect of wild-type peptides was compared with the effect of peptides in which the Leu-Xaa-Ile motif was replaced with Gly-Xaa-Gly (Figure II.5a). If binding to the NH₂-terminal docking site was required for p38 activation, a synthetic peptide containing this docking site should function as an inhibitor of p38 activation. Indeed, peptides derived from MKK3b (Figure II.5b) caused inhibition of p38α activation by MKK3b and MKK6, but not by MKK3, which lacks the MAPK docking site (Figure II.5b). In contrast, mutant synthetic peptides lacking the MAPK docking site caused no change in p38α activation. Inhibition of p38β2 activation by wild-type synthetic peptides, but not by mutated synthetic peptides, was detected in experiments using MKK3b (Figure II.5c, d) and MKK6 (Figure II.5e, f). However, the inhibitory effect of the peptides on p38β2 activation was larger than on p38α, confirming the previous observation (Figure II.3) that binding is required for the activation of p38β2 *in vitro*, but serves only to potentiate the activation of p38α.

Taken together, these data indicate that binding of p38 to a docking site in the NH₂-terminal region of MAPKK contributes to p38 activation.

Discussion

MAPK regulate a wide array of biological functions, therefore mechanisms must exist to achieve signaling specificity and to ensure the correct biological response to extracellular stimulation. The complexity of each MAPK pathway provides multiple levels where specificity may be determined. Two simple mechanisms that can achieve signaling specificity are: 1) selective activation of different MAPK; and 2) distinct MAPK substrate specificities. Such differences in signaling specificity exist not only between the major groups of MAPK, but also between individual members of a single group of MAPK.

The substrate specificity of MAPK depends, in part, on binding interactions between the MAPK and its substrates (Ip and Davis 1998; Holland and Cooper 1999). Here I report that a p38 docking domain in p38-specific MAP2K contributes to the selective activation of p38 isoforms. For example, p38β2 is not activated by MAP2K isoforms that lack a docking site (e.g. MKK3), but can be activated by MAP2K with a docking site (e.g. MKK6). These data indicate that the selective activation of p38 by MAP2K requires molecular determinants present in the MAP2K.

MAPK docking domains

Two genes encode proteins that act as specific activators of p38, *Mkk3* and *Mkk6* (Derijard et al. 1995; Han et al. 1996; Moriguchi et al. 1996; Raingeaud et al. 1996; Stein et al. 1996). Targeted gene disruption studies in mice have demonstrated non-redundant functions of the *Mkk3* and *Mkk6* genes (Lu et al. 1999; Wysk et al. 1999), indicating that the MKK3 and MKK6 protein kinases have distinct biological functions.

MKK3 activates the isoforms p38α, p38γ and p38δ, but not p38β2. However, both MKK6 and MKK3b (a variant form of MKK3 which has an additional 29 amino acids fused to the NH$_2$-terminus of MKK3) activate the four p38 isoforms p38α, p38β2, p38γ and p38δ. In this study, I have identified a p38 MAP kinase docking site within the NH$_2$-terminal region of MKK3b and MKK6. These sequences are highly conserved and are required for p38β2 activation by these two enzymes. MKK3, which lacks the docking site, does not activate p38β2. Fusion of the p38 docking site of MKK6 to the NH$_2$-terminus of MKK3 allows activation of p38β2. Furthermore, synthetic peptides based on the primary sequence of the docking sites of MKK3b and MKK6 inhibit the activation of p38β2. These data indicate that the binding of p38β2 to an NH$_2$-terminal region of the MAPKK is necessary for p38β2 activation. In contrast, binding to p38α is not a requirement for activation by MKK3b and MKK6 *in vitro*. However, the binding interaction does serve to potentiate p38α activation. These data provide an explanation for the selective activation of p38α, but not p38β2, by MKK3.

A similar role for a docking mechanism has been described in the yeast *S. cerevisiae* where a high affinity interaction between a MAP2K (Ste7p) and MAPK (Kss1p and Fus3p) is required for MAPK activation (Bardwell et al. 1996). This interaction depends on a MAPK docking site present in the NH$_2$-terminus of Ste7p. The sequence of the MAPK docking site of Ste7p is related to that identified in MKK3b and MKK6. These sequences are similar to the previously reported MAPK docking site consensus sequence found in MAPK substrates: -Arg/Lys-Xaa$_3$-Leu/Ile-Xaa-Leu/Ile- (Yang et al. 1998a; Yang et al. 1998b; Holland and Cooper 1999). Interestingly, the NH$_2$-terminal regions of MEK1 (32 amino acids), MKK4 (43 amino acids) and MKK7 (73 amino acids) have been demonstrated to be important for binding and activation of ERK and JNK (Fukuda et al. 1997; Xia et al. 1998; Tournier et al. 1999; Xu et al. 1999).

Sequences similar to the MAPK docking sites of MKK3b and MKK6 are present in the NH_2-terminal regions of MEK1, MKK4, and MKK7. Further studies are required to delineate the MAPK docking site with the NH_2-terminus of MEK1, MKK4, and MKK7.

MAPK docking sites are present in MAP2K, MAPK substrates and MAPK phosphatases

Short peptide sequences that bind p38α and p38β2 have been identified in the transcription factors MEF2A and MEF2C (Yang et al. 1999). These domains are necessary for efficient phosphorylation and activation of MEF2A and MEF2C by p38α and p38β2 (Yang et al. 1999). Similar MAPK binding motifs have been characterized in several MAPK substrates, including c-Jun (Hibi et al. 1993), JunB (Gupta et al. 1996; Kallunki et al. 1996), Elk-1 (Yang et al. 1998a; Yang et al. 1998b), NFAT4 (Chow et al. 1997), ATF-2 (Gupta et al. 1995; Livingstone et al. 1995). In general, these docking sites conform to the general consensus sequence: -Arg/Lys-Xaa-Xaa-Xaa-Xaa-Leu/Ile-Xaa-Leu/Ile- (Yang et al. 1998a; Yang et al. 1998b; Holland and Cooper 1999). Binding sites for MAPK have also been identified in phosphatases that regulate MAPK activation (Camps et al. 1998; Pulido et al. 1998; Oh-hora et al. 1999). These considerations indicate that MAPK docking sites are located on the kinases that activate MAPK (MAP2K), on phosphatases that inactivate MAPK, and on MAPK substrates.

The observation that MAPK can bind related sequences in both their substrates and enzymes that regulate MAPK activity (MAP2K and phosphatases) is intriguing. Is it possible that the regulatory enzymes and substrates compete for binding to MAPK? This hypothesis predicts that substrate phosphorylation by MAPK would only be observed following release of the activated MAPK from MAP2K. Indeed, evidence in favor of this hypothesis has been reported in studies of yeast MAPK signaling pathways. For example, the *S. cerevisiae* MAPK

Kss1p is not able to phosphorylate exogenous substrates when bound to the MAP2K Kss1p

(Bardwell et al. 1996). Similar models for binding and release of activated MAPK in mammals

have been reported for JNK activation by MKK4 (Xia et al. 1998) and ERK activation by

MEK1 (Fukuda et al. 1997).

Conclusions

In this study, protein regions that determine the specificity of p38 MAPK activation by

MAP2K were identified. This study has established the presence of p38 docking sites on

MKK3b and MKK6 by mutational analysis and peptide competition analysis. Differences in

the interaction of activators with individual p38 MAPK isoforms may contribute to the

specificity of p38 MAPK signaling *in vivo*.

The MAPK docking sites present in MAP2K appear to be targeted during infection by

some pathogens. The lethal factor (LF) of anthrax lethal toxin (the major cause of death in

animals infected with anthrax) is a protease that cleaves the ERK docking site of MEK1 and

MEK2, causing decreased activity towards ERK (Duesbery et al. 1998). Loss of the ERK

docking site may account for the effect of LF to block activation of ERK by MEK1 and MEK2

in vivo (Duesbery et al. 1998). Interestingly, sequences similar to the LF cleavage site in MEK1

and MEK2 are present in the NH_2-terminus of MKK3b and MKK6. Cleavage of MKK3b and

MKK6 by LF at these sites would remove the p38 docking domain and would therefore be

predicted to prevent binding and activation of p38β2 and markedly decrease activation of p38α.

Indeed, LF was recently reported to inhibit p38 MAPK signaling in macrophages (Pellizzari et al.

1999).

Figure II.1. Identification of a domain required for activation of p38β2 by MAP kinase kinases.

(A) Schematic representation of MKK3, MKK3b, MKK6 and chimeras. MKK6 is shown in white, MKK3 in black and the NH₂-terminal extension of MKK3b is grey. In the chimeras, domains from MKK3 are shown in black and domains from MKK6 in white.

(B and C) Epitope-tagged p38α (B) or epitope-tagged p38β2 (C) were immunoprecipitated from COS7 cells co-transfected with an empty vector (Control) or activated MAPKK. The activated MAPKK were constructed by replacing the two sites of activating phosphorylation with Glu residues. Immunecomplex kinase assays were performed to measure p38 MAP kinase activity using ATF2 as the substrate. The expression of MAPKK and p38 was examined by immunoblot analysis (lower panel). The rate of ATF2 phosphorylation was quantitated by Phosphorimager analysis and is presented as relative protein kinase activity.
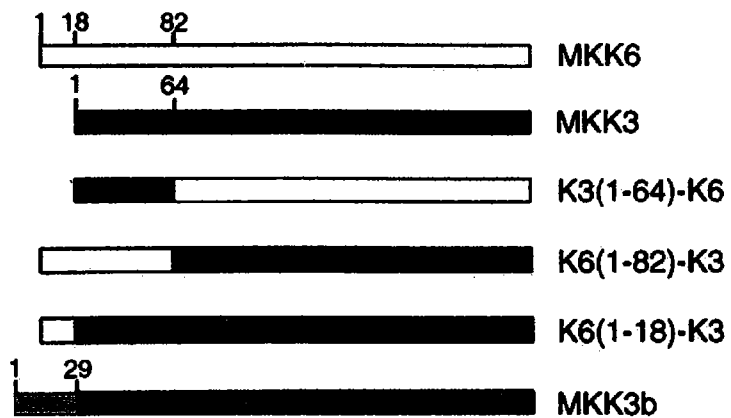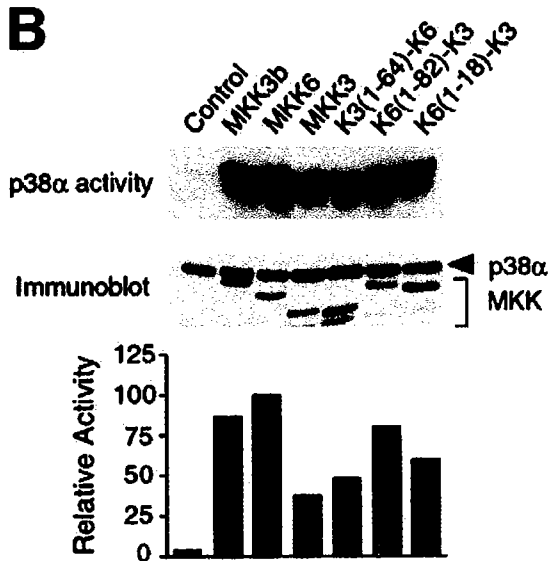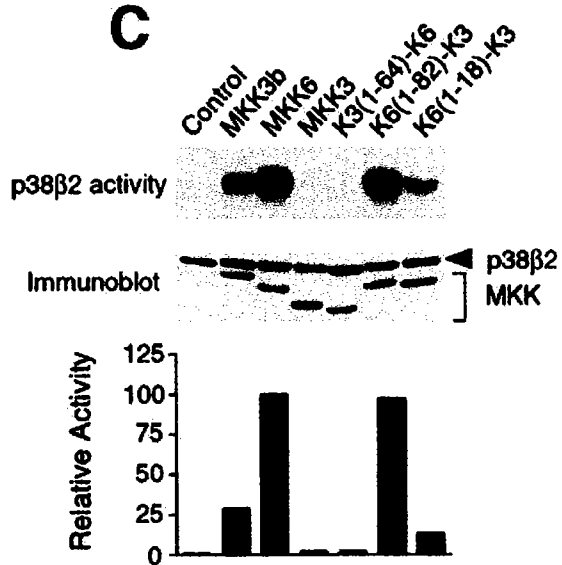
Figure II.2. Binding of p38α and p38β2 to MAP kinase kinases.

(A) Primary sequence of the NH₂-terminal domain of MKK3, MKK3b, MKK6 and deletion mutants (D) are aligned. Residues that are identical to MKK3b are indicated with a period (.). The residues of MKK3b (LRI) and MKK6 (LKI) deleted in MKK3bΔ and MKK6Δ are indicated in bold. The deleted residues are indicated with a dash (-). Basic residues are indicated by asterisks.

(B) Activated GST-tagged MKK3, K6(1-18)-K3, or K6(1-82)-K3 were co-transfected with an empty vector (Control), Flag-tagged p38α or Flag-tagged p38β2 in COS7 cells. The activated MKK were constructed by replacing the two sites of activating phosphorylation with Glu residues. Protein expression was monitored by immunoblot analysis of cell extracts. The GST-MKK fusion proteins were isolated from the cell extracts by incubation with GSH-Sepharose. The co-precipitation of p38α and p38β2 with the MKK was examined by immunoblot analysis with an antibody to the Flag epitope.

(C and D) The interaction of Flag-tagged p38α and p38β2 to GST-tagged activated MKK3, MKK3b and MKK3bΔ (C) or MKK6 and MKK6Δ (D) co-expressed in COS7 cells was examined using the methods described in panel (B). The activated MKK were constructed by replacing the two sites of activating phosphorylation with Glu residues.

A

Docking Domain

```
MKK3b   ¹MESPASSQPASMPQSKGKSKRKKDLRISCMS³²
MKK3bΔ  ·······················---····
MKK3                                 ··
MKK6    .S.....-..NPG.K.PKEA
MKK6Δ   .S.....-..NPG---PKEA
```
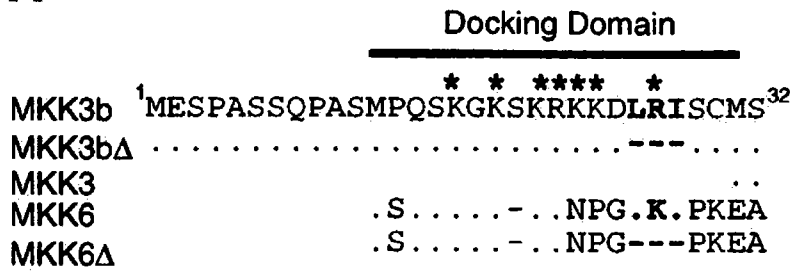
B
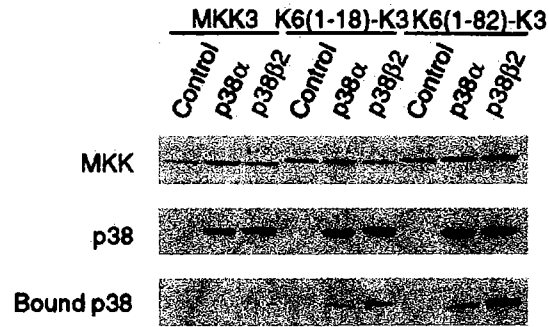
C

D

Figure II.3. Regulation of p38α and p38β2 by MAP kinase kinases.

(A) The primary sequence of the NH$_2$-terminal region of MKK3, MKK3b and MKK3bΔ *(left panel)* and of MKK6, K6(1-18)-K3 and MKK6Δ *(right panel)* are aligned.

(B - E) Flag-tagged p38α (B and C) or Flag-tagged p38β2 (D and E) were co-transfected together with activated MKK3, MKK3b and MKK3bΔ (B and D) or activated MKK6, K6(1-18)-K3 and MKK6Δ (C and E) in COS7 cells. The activated MKK were constructed by replacing the two sites of activating phosphorylation with Glu residues. The expression of p38 and MKK was examined by immunoblot analysis of cell lysates. The protein kinase activity of p38α and p38β2 was measured in immunecomplex kinase assays using ATF2 as the substrate. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography and was quantitated by Phosphorimager analysis. The p38 activity is presented as relative protein kinase.

A

MKK3b ^{14}QSKGKSRKKD**LRI**SCMSKPPA^{35} MKK6 ^{1}MSQSKGKKRNPG**LKI**PKEAFEQ^{22}
MKK3bΔ   . . . . **- - -** . . . .   K6(1-18)-K3   . . . . MSKPP
MKK3   . . . . . .   MKK6Δ   . . . . **- - -** . . . . . .

B
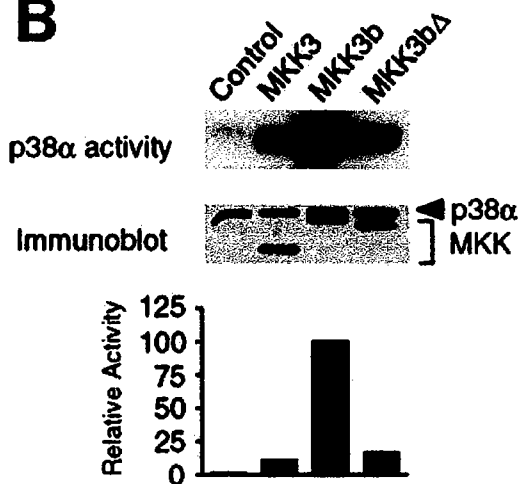
C

D

E
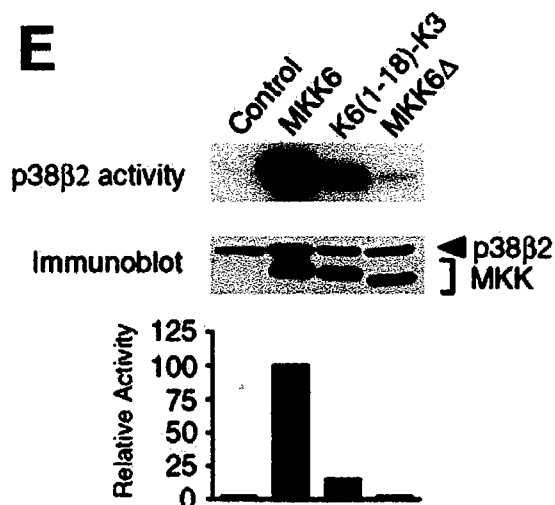
Figure II.4. Regulation of endogenous p38α activity by MAP kinase kinases.

Activated epitope-tagged MKK3b, MKK3, K6(1-18)-K3 and K6(1-82)-K3 (A) or MKK3b,

MKK3bΔ, MKK6, MKK6Δ and MKK3 (B) were expressed in COS7 cells. The activated MKK

were constructed by replacing the two sites of activating phosphorylation with Glu residues. The

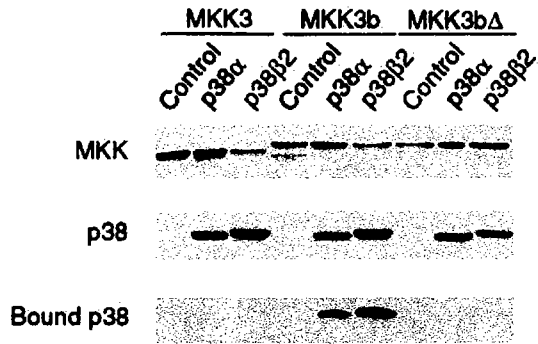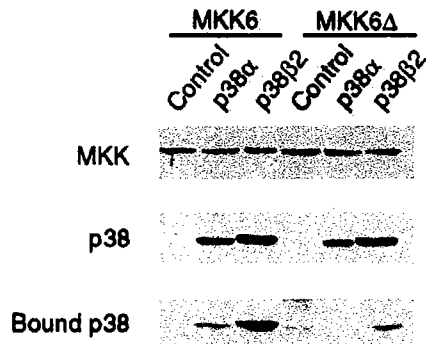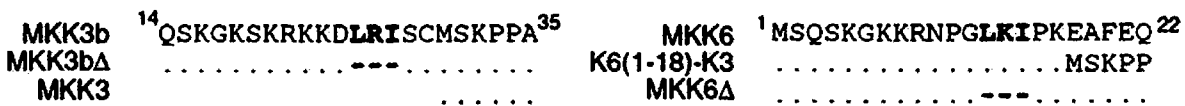epitope-tagged MKK expression and endogenous p38α was examined by immunoblot analysis of

cell lysates. The activity of the endogenous p38α was measured in an immunecomplex kinase

assays using ATF2 as the substrate. The phosphorylated ATF2 was detected after SDS-PAGE by

autoradiography and was quantitated by Phosphorimager analysis. The p38 activity is presented

as relative protein kinase activity.
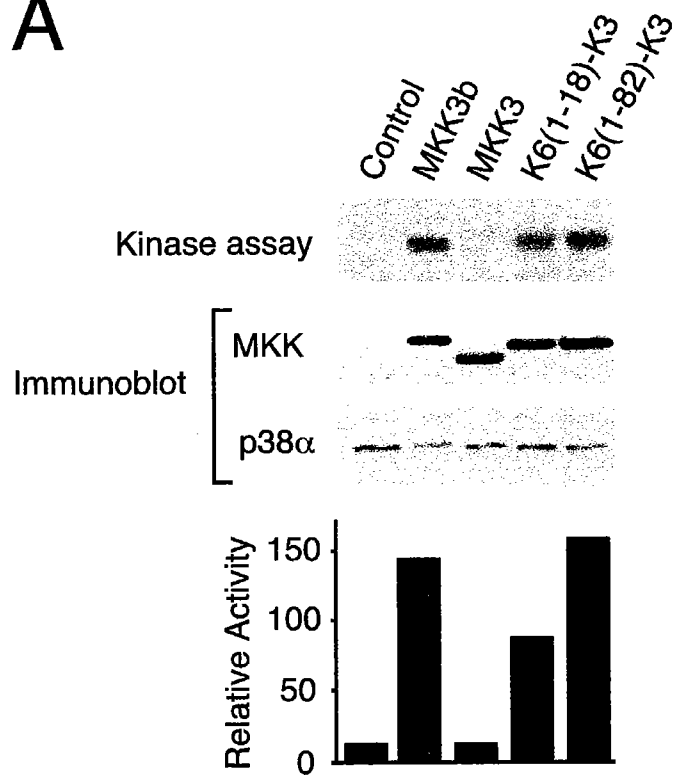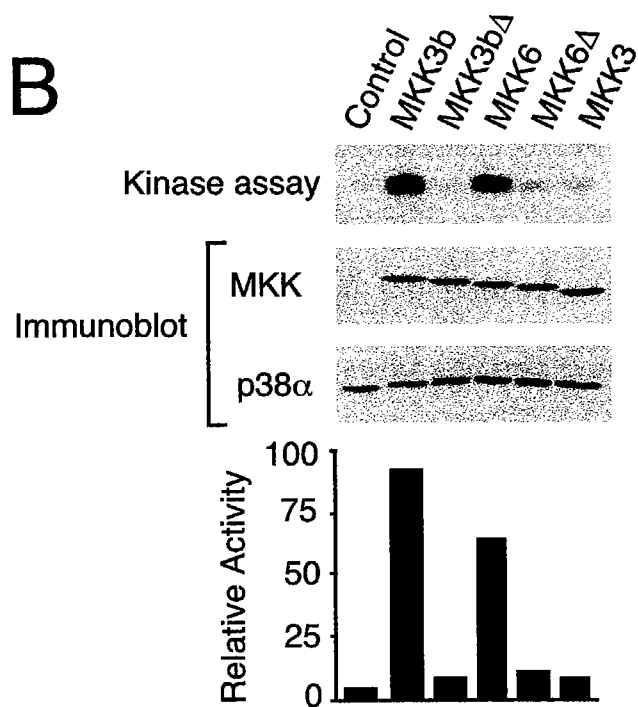
Figure II.5. Inhibition of p38β2 activity by peptide competition.

(A) The primary sequence of synthetic peptides corresponding to the native (wt-pep) or mutated (gly-pep) NH_2-terminal region of MKK3b are shown. The mutated peptide was prepared by replacing the residues of MKK3b (LRI) indicated in bold, with Gly.

(B) Purified bacterially expressed GST-p38α was bound to GSH Sepharose and incubated with 100 μM wild-type or mutated MKK3b peptide. Purified activated MKK3, MKK3b, or MKK6 were incubated with the immobilized GST-p38α in kinase buffer with ATP for 20 min. The GST-p38α was washed with kinase buffer and the p38α activity was measured using ATF2 and [γ-^{32}P]ATP as the substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography and was quantitated by Phosphorimager analysis. The p38α activity is presented as relative protein kinase activity.
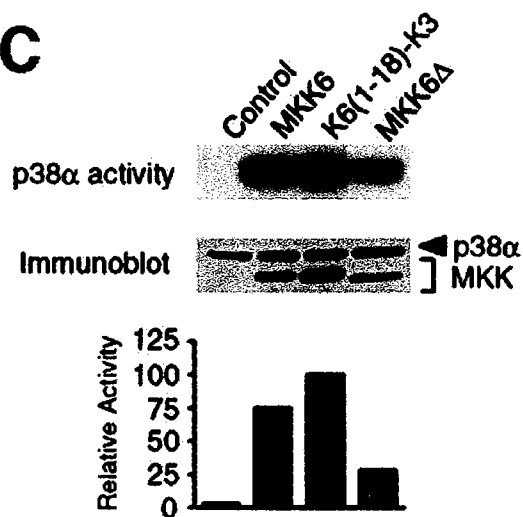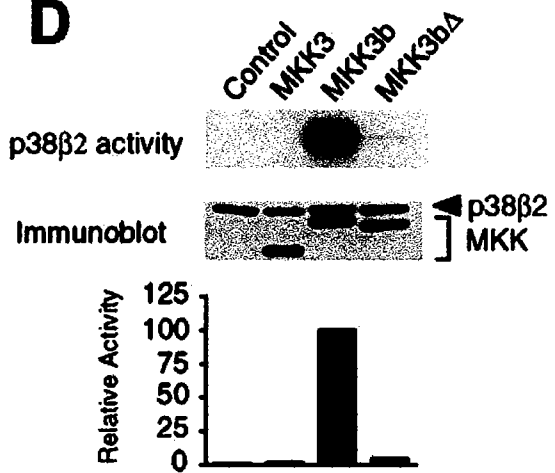
(C - F) Purified bacterially expressed GST-p38β2 was bound to GSH Sepharose and incubated with increasing concentration of the wild-type (C and E) or mutated (D and F) MKK3b peptide. Purified activated MKK3b (C and D) or MKK6 (E and F) were incubated with the immobilized GST-p38β2 in kinase buffer with ATP for 20 min. The GST-p38β2 activity was measured as described in (B).

A

MKK3b [wt-pep PQSKGKSKRKKD**LRI**SCMSKPPAPN
gly-pep . . . .**G.G**. . . . .

B

C

D

E

F
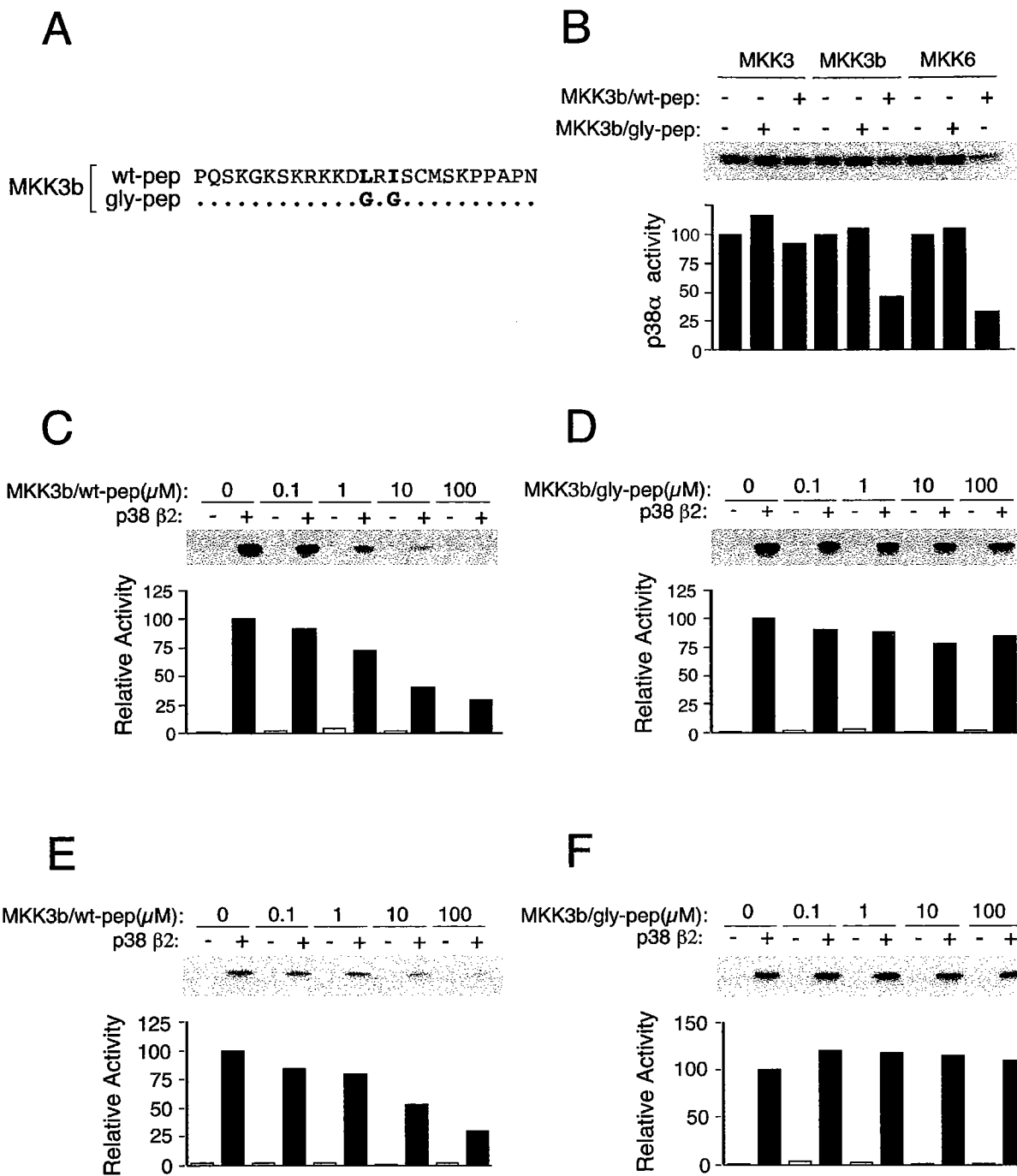
CHAPTER III

MECHANISM OF p38 MAPK ACTIVATION *in vivo*

Summary

The p38 mitogen-activated protein kinase (MAPK) is activated *in vitro* by three different protein kinases: MKK3; MKK4; and MKK6. To examine the relative roles of these protein kinases in the mechanism of p38 MAP kinase activation *in vivo*, I examined the effect of disruption of the murine *Mkk3*, *Mkk4*, and *Mkk6* genes on the p38 MAPK signaling pathway. I show that MKK3 and MKK6 are essential for tumor necrosis factor-stimulated p38 MAPK activation. In contrast, ultraviolet radiation-stimulated p38 MAPK activation was mediated by MKK3, MKK4, and MKK6. Loss of p38 MAPK activation in the mutant cells was associated with defects in growth arrest and increased tumorigenesis. These data indicate that p38 MAPK is regulated by the coordinated and selective actions of three different protein kinases in response to cytokines and exposure to environmental stress.

The *Mkk3*$^{-/-}$ *Mkk6*$^{-/-}$ mice and fibroblasts were generated in collaboration with Nobuyuki Tanaka in Dr. Richard Flavell's laboratory at the Yale University School of Medicine. I initially characterized the *Mkk3*$^{-/-}$ *Mkk6*$^{-/-}$ fibroblasts. Nobuyuki Tanaka contributed to data in Figure III.1, Nyaya Kelkar contributed to data in Figure III.4, Anja Jaeschke contributed to data in Figure III.8, and Juan-Jose Ventura contributed to data in Figure III.10a, c, d, e. Nyaya Kelkar also contributed to data in Figure III.7; however, I independently obtained similar results during the initial characterization.

Introduction

Several groups of mitogen-activated protein kinase (MAPK) signal transduction pathways have been identified in mammals, including extracellular signal-regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK. Each of these groups of MAPK is activated by dual phosphorylation on Thr and Tyr within a tripeptide motif (Thr-Xaa-Tyr) located within the activation loop of the MAPK. This phosphorylation is mediated by seven MAPK kinases (MAP2K) that have specificity for individual MAPK isoforms. Thus, ERK1 and ERK2 are activated by MEK1 and MEK2, ERK5 is activated by MEK5, JNK is activated by MKK4 and MKK7, and p38 MAPK is activated by MKK3 and MKK6 (Schaeffer and Weber 1999; Kyriakis and Avruch 2001). These MAP2K and MAPK can create independent signaling modules that may function in parallel.

The mechanism that accounts for the specificity of MAP2K to activate individual MAPK isoforms is mediated, in part, by an interaction between an NH_2-terminal region located on the MAP2K and a docking site located on the MAPK (Bardwell et al. 1996; Enslen and Davis 2001). Recently, structural insight into the mechanism of interaction between a MAP2K and a MAPK has been achieved by X-ray crystallography (Chang et al. 2002). This analysis demonstrated that there is a direct interaction of the NH_2-terminal region of the MAP2K with a docking groove present on the surface of the MAPK distant from the catalytic active site (Weston and Davis 2002). A second determinant of MAP2K specificity is the structure of the MAPK activation loop that contains the Thr-Xaa-Tyr dual phosphorylation motif (Enslen et al. 2000). The specificity of these interactions mediates, in part, the ability of an individual MAP2K to activate a particular MAPK selectively.

It is interesting that mammalian MAPK signaling modules include more than one MAP2K because in yeast only a single MAP2K appears to activate each MAPK. The role of this pathway complexity in mammals is unclear. However, it may be significant that individual yeast MAPK isoforms are activated by only a limited group of extracellular stimuli, but mammalian MAPK isoforms are activated by a wide array of extracellular stimuli. It is therefore possible that the employment of more than one MAP2K for the activation of a specific mammalian MAPK may contribute to the ability of these signaling modules to respond to multiple stimuli.

Studies of the ERK1/2 signaling module demonstrate that MEK1 has an essential function in placental formation during embryonic development, but the role of MEK1 to activate ERK1/2 appears to be largely redundant with MEK2 (Giroux et al. 1999). In contrast, studies of the JNK signaling module have demonstrated non-redundant functions of both MKK4 and MKK7 in JNK activation. Although MKK4 and MKK7 phosphorylate JNK on both Thr and Tyr residues, MKK4 preferentially phosphorylates JNK on Tyr, while MKK7 preferentially phosphorylates JNK on Thr (Lawler et al. 1998; Tournier et al. 2001; Wada et al. 2001). Since dual phosphorylation on Thr and Tyr is required for full activation of JNK (Derijard et al. 1994), these data suggest that MKK4 and MKK7 may cooperate to activate JNK. Strong support for this conclusion has been obtained from studies of *Mkk4* and *Mkk7* gene disruption. Ultraviolet (UV) radiation causes activation of both MKK4 and MKK7 (Tournier et al. 1999) and loss-of-function mutations in either *Mkk4* or *Mkk7* cause reduced UV-stimulated JNK activation (Nishina et al. 1997; Yang et al. 1997; Ganiatsas et al. 1998; Tournier et al. 2001; Wada et al. 2001; Kishimoto et al. 2003). Significantly, compound mutations of both *Mkk4* and *Mkk7* eliminated the ability of UV radiation to activate JNK, indicating that these MAP2K isoforms are essential for JNK activation (Tournier et al. 2001).

The observation that MKK4 and MKK7 have non-redundant functions in the activation of JNK suggests that other MAP2K may also have specialized functions in mammalian MAPK signaling modules. The purpose of the study reported here was to examine the role of MAP2K isoforms in the activation of p38 MAPK. Previous studies have identified the isoforms MKK3 and MKK6 as specific activators of p38 MAPK (Derijard et al. 1995; Raingeaud et al. 1996). However, *in vitro* studies suggest that MKK4, an activator of JNK, may also contribute to p38 MAPK activation (Derijard et al. 1995; Lin et al. 1995). Furthermore, it has also been reported that some cell surface receptors, including tumor necrosis factor (TNF) receptors, may activate p38 MAPK by a MAP2K-independent mechanism (Ge et al. 2002; Ge et al. 2003). My approach was to examine the effect of loss-of-function mutations in the murine *Mkk3*, *Mkk4*, and *Mkk6* genes. I show that all three MAP2K isoforms can contribute to p38 MAPK activation and that the repertoire of MAP2K isoforms that cause p38 MAPK activation *in vivo* depends upon the specific stimulus that is examined. Loss of p38 MAPK regulation in the mutant cells causes defects in growth arrest and increased tumorigenesis.

Materials and Methods

Mice. *Mkk3* [^-/-] mice (Wysk et al. 1999) (Lu et al. 1999), *Mkk4* [^-/-] mice (Yang et al. 1997), and

Mkk6 [^-/-] mice (Tanaka et al. 2002) have been described. *Mkk3* [^-/-] *Mkk6* [^-/-] mice were obtained by

interbreeding *Mkk3* [^-/-] and *Mkk6* [^-/-] mice. Tumor assays were performed using 12 week old male

athymic nude mice (Charles River) by subcutaneous injection of 1×10^6 fibroblasts. All animals

were housed in a facility accredited by the American Association for Laboratory Animal Care

(AALAC) and the animal studies were approved by the Institutional Animal Care and Use

Committee (IACUC) of the University of Massachusetts.

Cell culture. Murine embryo fibroblasts were isolated and cultured in Dulbecco's modified

Eagle's medium supplemented with 10 % fetal calf serum (Invitrogen). The fibroblasts were

immortalized using the SV40 large T antigen expression vector p321-T and the Fugene reagent.

In vitro fibroblast proliferation assays were performed by staining with crystal violet (Tournier et

al. 2001). Dominant-negative JNK was expressed in cells in retroviral transduction experiments

(Ventura et al. 2003).

siRNA studies. Fibroblasts were transfected with double stranded RNA (Dharmacon Research)

targeting the sequence AATGCGGAGTAGTGATTGCCC using Lipofectamine 2000

(Invitrogen) following the manufacturer's recommendations (Elbashir et al. 2001). The double-

stranded siRNA was designed to selectively suppress expression of mouse MKK4. Control

experiments were performed using double stranded RNA targeting the sequence

AAACATGCAGAAAATGCTGTT that suppresses the expression of luciferase (Elbashir et al.

2001). The cells were examined 48 hr post-transfection.

Biochemical assays. Cells were lysed in Triton lysis buffer containing 20 mM Tris (pH 7.4), 1%

Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1mM

sodium orthovanadate, 1 mM phenymethylsulfonyl fluoride, and 10 μg/ml of aprotinin and

leupeptin. Extracts (50 μg protein) were examined by protein immunoblot analysis by probing

with antibodies to JNK (PharMingen), phospho-JNK (Cell Signaling), p38 MAPK (Santa Cruz),

phospho-p38 MAPK (Cell Signaling), ERK1/2 (Santa Cruz), phospho-ERK (Cell Signaling),

MKK3 (Pharmingen), phospho-MKK3/6 (Cell Signaling), MKK4 (Santa Cruz), MKK6

(Stressgen), MKK7 (Pharmingen), Rb (Pharmingen), hypo-pRb (Pharmingen) and α-tubulin

(Sigma). Immunecomplexes were detected by enhanced chemiluminescence (NEN). MAP

kinase activity was measured by *in vitro* kinase assays (Raingeaud et al. 1995). Ribonuclease

protection assays were performed using reagents obtained from Pharmingen.

Results

Targeted disruption of Mkk3 and Mkk6

We have previously reported phenotypes of mice with targeted disruptions of the *Mkk3* and *Mkk6* genes (Wysk et al. 1999; Tanaka et al. 2002; Lu et al. 1999). The *Mkk3* $^{-/-}$ and *Mkk6* $^{-/-}$ mice were viable with no obvious developmental abnormalities. Mice with compound mutations in *Mkk3* and *Mkk6* were created by breeding these mutant mice. Since *Mkk3* and *Mkk6* are both located on mouse chromosome 11, mice were screened for the presence of a chromosome containing disruptions of both *Mkk3* and *Mkk6*. Subsequent breeding resulted in the generation of mice with compound mutations in *Mkk3* and *Mkk6*. Mice lacking expression of both MKK3 and MKK6 were not viable. The *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ embryos died during mid-gestation at E11.0 - E11.5. Major defects in the formation of the placenta and deficiencies in the development of the embryonic vasculature were observed (Figure III.1). The mutant mice appeared to be developmentally delayed and exhibited symptoms of severe anoxia. This phenotype of *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ mice resembles that previously described for *p38 MAPK* $^{-/-}$ embryos (Adams et al. 2000; Allen et al. 2000; Tamura et al. 2000). The similar embryonic phenotype of these mice is consistent with the known role of MKK3 and MKK6 to selectively activate p38 MAPK (Raingeaud et al. 1996). Together, these data indicate that MKK3 and MKK6 serve redundant roles that are essential for survival.

To characterize the effects of MKK3 and MKK6 deficiency biochemically, fibroblasts were isolated from wild-type and mutant embryos. Immunoblot analysis demonstrated that *Mkk3* $^{-/-}$ mice did not express MKK3, that *Mkk6* $^{-/-}$ cells did not express MKK6, and that *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ cells did not express MKK3 or MKK6 (Figure III.2a). In contrast, these cells expressed

similar amounts of JNK, p38 MAPK, MKK4, and MKK7 (Figure III.2a). These data indicated

that defects in the expression of MKK3 and MKK6 did not cause marked changes in the

expression of other components of stress-activated MAP kinase pathways. Phase contrast

microscopy demonstrated that the wild-type, $Mkk3^{-/-}$, $Mkk6^{-/-}$, and $Mkk3^{-/-}$ $Mkk6^{-/-}$ cells

displayed the typical flattened appearance of embryonic fibroblasts (Figure III.2b). The rate of

proliferation of these cells in medium supplemented with 10% fetal calf serum was similar,

although the $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts were found to reach a slightly higher saturation density

compared with wild-type fibroblasts (Figure III.2c).

MKK3 and MKK6 are essential for cytokine-stimulated p38 MAPK activation

To examine the role of MKK3 and MKK6 in p38 MAPK activation *in vivo*, I investigated

the effect of TNFα on wild-type and mutant fibroblasts by immunoblot analysis using antibodies

that bind phosphorylated and activated MAPK and MAPKK. Control studies using wild-type

cells demonstrated that TNFα caused an increase in JNK, ERK, and p38 MAPK activation

(Figure III.3) (Chen et al. 2001; Ventura et al. 2003) and a more moderate activation of both

MKK3 and MKK6 (Figure III.3). *Mkk6* gene disruption did not cause obvious changes in

MAPK activation in cells treated with TNFα. In contrast, *Mkk3* gene disruption caused reduced

activation of p38 MAPK, but did not alter JNK activation. $Mkk3^{-/-}$ cells also exhibited a slight

decrease in ERK activation, however the mechanism of MKK3-mediated ERK activation

remains unclear. *Mkk3* gene disruption may down-regulate p38 MAPK activation and disrupt

signaling cross-talk between the MAPK pathways (Xia et al. 1995; Hall and Davis 2002).

Interestingly, compound mutant cells lacking both MKK3 and MKK6 were severely

defective in TNFα-stimulated p38 MAPK activation (Figure III.3). This defect in TNFα-

stimulated p38 MAPK activation in *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ cells was confirmed by *in vitro* kinase assays (Figure III.4). *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ cells also exhibited similar defects in p38 MAPK activation in response to IL-1 cytokine stimulation (Figure III.5). Together, these data indicate that MKK3 and MKK6 serve redundant, but essential, roles in the cytokine-stimulated activation of p38 MAPK.

MKK3 and MKK6 contribute to p38 MAPK activation caused by UV radiation

MKK3 and MKK6 are essential for cytokine-stimulated p38 MAPK activation (Figures III.3 - 5). To investigate whether MKK3 and MKK6 are required for other types of stimuli that activate p38 MAPK, I examined the effect of an environmental stress, UV radiation. Control studies using immunoblot analysis with phospho-specific antibodies demonstrated that exposure of wild-type cells to UV radiation caused increased activation of the MAP2K MKK3, MKK4, and MKK6 (Figure III.6) and caused increased activation of ERK, JNK, and p38 MAPK (Figure III.6). Deficiency of MKK3 or MKK6 did not cause marked defects in p38 MAPK activation. Strikingly, UV radiation also caused p38 MAP kinase activation in *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ cells, although the extent of p38 MAPK activation was diminished compared with wild-type cells (Figure III.6). This observation was confirmed by using *in vitro* kinase assays (Figures III.5, III.7). Together, these data indicate that although MKK3 and MKK6 contribute to p38 MAPK activation in cells exposed to UV radiation, these protein kinases are not essential for UV-stimulated p38 MAPK activation (Figures III.5 - 7). This observation markedly contrasts with the essential role of MKK3 and MKK6 in cytokine-stimulated p38 MAPK activation (Figures III.3 - 5).

MKK4 contributes to UV-stimulated p38 MAPK activation

The observation that $Mkk3^{-/-}$ $Mkk6^{-/-}$ cells can respond to UV radiation with increased

p38 MAPK activation demonstrated that a mechanism must exist in UV-stimulated cells to

activate p38 MAPK in the absence of MKK3 and MKK6. Previous studies have indicated two

possible mechanisms. First, p38 MAPK may be activated by a MAP2K-independent

mechanism. For example, it has been reported that the TAB1 adapter protein may cause p38

MAPK activation in the absence of MAP2K involvement (Ge et al. 2002; Ge et al. 2003). This

TAB1-dependent (and MAP2K-independent) mechanism has been implicated in p38 MAPK

activation caused by TNFα (Ge et al. 2002; Ge et al. 2003). However, since I now show that

TNFα-stimulated p38 MAPK requires MKK3 and MKK6 (Figure III.3 - 4), the conclusion that

TNFα causes MAP2K-independent activation of p38 MAP kinase (Ge et al. 2002; Ge et al.

2003) can be questioned. Since TAB1 has not been implicated in p38 MAPK activation caused

by UV radiation (Ge et al. 2002; Ge et al. 2003), it is unlikely that TAB1 contributes to the UV-

stimulated activation of p38 MAPK observed in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.5 - 7). A

second possible mechanism that may account for MKK3 and MKK6-independent activation of

p38 MAPK is that these protein kinases may not be the only MAP2K that can activate p38

MAPK. For example, in vitro studies indicate that MKK4, an established activator of JNK, may

also activate p38 MAPK (Derijard et al. 1995; Lin et al. 1995).

To test the role of MKK4 in the UV-stimulated activation of p38 MAPK, p38 MAPK

activation was compared in wild-type and $Mkk4^{-/-}$ fibroblasts. As expected (Nishina et al. 1997;

Yang et al. 1997a; Ganiatsas et al. 1998; Tournier et al. 2001), $Mkk4$ gene disruption caused

decreased activation of JNK following exposure to UV or TNFα (Fig. 5A). In contrast, the loss

of MKK4 expression caused no marked decrease in p38 MAPK activation in response to UV or

TNFα (Figure III.8). These data confirm the conclusion that MKK4 has a non-redundant role in the activation of JNK and demonstrate that MKK4 has either no role, or a redundant role, in the activation of p38 MAPK.

To test whether MKK4 may have a redundant role in the activation of p38 MAPK, the effect of MKK4 loss-of-function in wild-type and $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts was examined. Attempts to construct triple knockout mice ($Mkk3^{-/-}$ $Mkk4^{-/-}$ $Mkk6^{-/-}$) by breeding were not successful, in part, because all three genes are linked on mouse chromosome 11. Therefore an alternative approach was employed to test the role of MKK4 in p38 MAPK activation using siRNA (Figure III.8c). Decreased expression of MKK4 caused by siRNA caused no change in UV-stimulated activation of p38 MAPK in wild-type cells, but strongly suppressed p38 MAPK activation in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.8c). Similarly, inhibition of MKK4 with dominant-negative JNK caused little change in UV-stimulated p38 MAPK activation in wild-type cells, but inhibited p38 MAPK activation in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.9). Together, these data indicate that MKK4 serves a role that is redundant with MKK3 and MKK6 in the activation of p38 MAPK in cells exposed to UV radiation.

Altered p38 MAPK regulation causes defects in growth arrest and increased tumorigenesis

It is been proposed that the p38 MAPK pathway regulates growth arrest (Bulavin et al. 2002a). Since $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts exhibit severe defects in p38 MAPK regulation, I investigated whether these cells might have altered proliferative responses. Control studies demonstrated that although p38 MAPK activation was markedly reduced in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.2a), the rate of proliferation of wild-type and $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts was similar (Figure III.2c). However, differences in proliferation between these cells were

observed following serum starvation (Figure III.10b). Wild-type cells cultured in serum-free medium were not observed to proliferate. In contrast, the $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts were found to have increased proliferation potential in serum-free medium (Figure III.10b).

To biochemically characterize the difference between the wild-type and $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts, Rb phosphorylation (a hallmark of G1/S progression) was examined in serum-starved wild-type and mutant fibroblasts. Serum starvation caused Rb dephosphorylation in wild-type fibroblasts, but not in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.10d). Cyclin-dependent protein kinases are thought to represent the major group of Rb kinases *in vivo*. Therefore cyclin expression was examined in the wild-type and $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts. Ribonuclease protection assays demonstrated that serum starvation caused decreased expression of D-type cyclins in wild-type cells (Figure III.10c). In contrast, the expression of D-type cyclins was maintained in serum-starved $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts. Immunoblot analysis confirmed that D-type cyclins were selectively expressed in serum-starved $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts, but not in the serum-starved wild-type fibroblasts (Figure III.10d). This deregulated expression of D-type cyclins in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts is consistent with the established role of p38 MAP kinase as an inhibitor of cyclin D gene expression (Lavoie et al. 1996).

The p38 MAP kinase pathway has also been implicated in the regulation of c-Jun gene expression (Han et al. 1997; Hazzalin et al. 1997). Defects in the p38 MAPK pathway in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts could therefore cause disrupted c-Jun expression and consequently altered cellular proliferation. Therefore *c-Jun* mRNA expression was examined in wild-type and $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.10e). Contrary to expectations, c-Jun expression was increased in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts. These data indicate that while the MKK3/6 pathway may contribute to the regulation of c-Jun expression, this role of MKK3/6 can be compensated by the

function of other signal transduction pathways in fibroblasts. Indeed, previous studies have demonstrated that c-Jun expression is regulated by multiple functionally redundant signaling pathways (Chiariello et al. 2000).

Together, these data indicate that $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts exhibit a deregulated cell cycle associated with a failure to growth arrest in serum-free medium and altered expression of D-type cyclins and c-Jun (Figure III.10). Consistent with this conclusion, a dramatic increase in tumor burden was observed when SV40 large T antigen immortalized $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (compared with wild-type cells) were injected subcutaneously in athymic nude mice (Figure III.11).

Discussion

The protein kinases MKK3 and MKK6 have been reported to specifically activate p38 MAP kinase (Derijard et al. 1995; Raingeaud et al. 1996). Consequently, these MAP2K have been considered to be critical for p38 MAPK activation *in vivo* (Schaeffer and Weber 1999; Kyriakis and Avruch 2001). However, biochemical studies indicate that MKK4, an activator of JNK, can also activate p38 MAPK *in vitro* (Derijard et al. 1995; Lin et al. 1995). The physiological significance of this observation has been questioned. Indeed, the role of MKK4 in p38 MAPK activation *in vivo* is controversial because studies of $Mkk4^{-/-}$ mice demonstrate major defects in JNK activation without obvious changes in p38 MAPK activation (Nishina et al. 1997; Yang et al. 1997a). Furthermore, the proposed dual function of MKK4 to activate two separate groups of MAPK (JNK and p38 MAPK) is unprecedented.

In this study I have examined the role of MKK3, MKK4, and MKK6 using targeted gene disruption in mice. I show that all three MAPKK isoforms contribute to the activation of p38 MAP kinase in cells exposed to UV radiation. These data confirm the importance of MKK3 and MKK6 in p38 MAP kinase activation. In addition, I demonstrate that MKK4 also contributes to p38 MAP kinase activation in cells exposed to UV radiation. This function of MKK4 was not detected in previous studies because the role of MKK4 in fibroblasts exposed to UV radiation is redundant with MKK3 and MKK6. Nevertheless, MKK4 is established by these data to be an activator of two different groups of MAPK: JNK and p38 MAPK.

MKK3 and MKK6 are essential for cytokine-stimulated p38 MAPK activation

TNFα causes p38 MAP kinase activation in wild-type fibroblasts, but not in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts (Figure III.3 - 4). This observation demonstrates that TNFα causes p38 MAPK activation in a MAPKK-dependent manner. However, a significant question relates to the specificity of this response involving MKK3 and MKK6. Why is there no discernable contribution of MKK4 to TNFα-stimulated p38 MAPK activation? One contributing factor may be the observation that TNFα activates MKK3, MKK6, and MKK7, but does not activate MKK4 (Tournier et al. 1999; Tournier et al. 2001). However, disruption of the $Mkk4$ gene does cause reduced TNFα-stimulated JNK activity, indicating that basally active MKK4 is required for maximal TNFα -stimulated JNK activation (Tournier et al. 2001). The effectiveness of MKK4 to activate JNK under these conditions may be accounted by the observation that MKK7 primarily phosphorylates JNK on Thr[180], while MKK4 primarily phosphorylates JNK on Tyr[182]. Interestingly, phosphoThr[180]-JNK is the preferred substrate for MKK4 compared with non-phosphorylated JNK (Lawler et al. 1998). The low Km of phosphoThr[180]-JNK as a substrate for Tyr phosphorylation by MKK4 most likely accounts for the ability of basally active MKK4 to participate in TNFα-stimulated JNK activation.

The mechanism of MKK4 activation of p38 MAPK is markedly different from the activation of JNK. MKK4 preferentially phosphorylates JNK on Tyr (Lawler et al. 1998), but phosphorylates p38 MAPK equally on Thr and Tyr (Enslen et al. 2000). Similarly, p38 MAPK is phosphorylated on both Thr and Tyr by MKK3 and MKK6 (Enslen et al. 2000). The absence of preferential Thr or Tyr phosphorylation of p38 MAPK may contribute to the lack of a role for MKK4 in TNFα-stimulated p38 MAPK activation.

Activation of p38 MAP kinase by MAP2K-independent mechanisms.

My studies of fibroblasts have not revealed a role for a MAPKK-independent mechanism of p38 MAPK activation. However, it is possible that such mechanisms of p38 MAPK activation may exist in other cell types. Similarly, MAPKK-independent mechanisms of p38 MAPK activation may be present in fibroblasts exposed to specific stimuli. Recent studies have established that the adapter protein TAB1 represents an example of a mechanism of MAPKK-independent activation of p38 MAPK (Ge et al. 2002). TAB1 binds and activates TAK1, a MAP3K that can activate both the JNK and p38 MAPK pathways. However, TAB1 also binds p38 MAPK and causes MAPKK-independent activation by causing p38 MAPK autophosphorylation and activation. Evidence that this function of TAB1 is independent of TAK1 has been obtained from the identification of the splice variant TAB1b that does not bind TAK1, but does bind and activate p38 MAPK (Ge et al. 2003). The MAPKK-independent activation of p38 MAPK caused by TAB1 has been proposed to regulate the basal activity of p38 MAPK and to contribute to the activation of p38 MAPK by cell surface receptors, including TNF receptors (Ge et al. 2002; Ge et al. 2003). The observation that TNFα does not activate p38 MAPK in $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts suggests that TAB1 does not contribute to TNFα-stimulated p38 MAPK activation in this cell type. Further studies are required to determine the physiological context of TAB1-mediated p38 MAPK activation. Importantly, the recent description of $Tab1^{-/-}$ mice, which die during early embryogenesis with cardiovascular and lung dysmorphogenesis, will facilitate this analysis (Komatsu et al. 2002).

The MKK3/6 pathway regulates the cell cycle

The p38 MAPK pathway has been reported to inhibit cell cycle progression by at least three different mechanisms (Bulavin et al. 2002a). First, p38 MAP kinase inhibits the expression of D-type cyclins (Lavoie et al. 1996). Second, p38 MAPK can phosphorylate and inhibit Cdc25B and Cdc25C, two protein phosphatases that activate cyclin-dependent protein kinase activity (Bulavin et al. 2001). Third, p38 MAP kinase phosphorylates the p53 tumor suppressor on two activating sites in the NH_2-terminal region (Ser-33 and Ser-46) and causes p53-dependent growth arrest (Bulavin et al. 1999; Sanchez-Prieto et al. 2000). Together, these targets of the p38 MAP kinase pathway (cyclin D, Cdc25, and p53) may cooperate to arrest the cell cycle. This finding suggests that defects in p38 MAPK function may contribute to cell cycle defects and increased tumorigenesis. Indeed, the *Ppm1D* gene (which encodes a phosphatase that inhibits p38 MAPK) is amplified in many human tumors (Bulavin et al. 2002a). Inactivation of p38 MAPK by gene targeting in mice or by overexpression of PPM1D dramatically increases tumorigenesis (Bulavin et al. 2002a). Similarly, in this study I found that decreased p38 MAPK activity caused by compound mutations of *Mkk3* and *Mkk6* causes growth arrest defects (Figure III.10) and increased tumorigenesis (Figure III.11).

Conclusions

The results of this study indicate that p38 MAPK is regulated by MKK3, MKK4, and MKK6. The MKK3 and MKK6 protein kinases are specific activators of p38 MAPK. In contrast, MKK4 represents a site of integration of stress-activated MAPK pathways because it can activate both JNK and p38 MAPK. The repertoire of protein kinases that contribute to p38 MAPK activation depends upon the specific stimulus that is examined. Thus, exposure of cells

to TNF causes p38 MAPK activation by a mechanism that requires MKK3 and MKK6. In contrast MKK3, MKK4, and MKK6 contribute to p38 MAPK activation caused by UV radiation. This role of MKK4 in p38 MAPK activation in fibroblasts is largely redundant with MKK3 and MKK6.

The contribution of MKK3, MKK4, and MKK6 to p38 MAPK activation may depend upon the cell type that is examined. For example, differences in the expression of MKK3 and MKK6 account for the observation that T cell receptor-mediated p38 MAPK activation is selectively defective in $Mkk6^{-/-}$ thymocytes and $Mkk3^{-/-}$ CD4$^+$ peripheral T cells, respectively (Tanaka et al. 2002; Lu et al. 1999). Similarly, MKK4 may be an important activator of p38 MAPK in cells with low levels of MKK3 and MKK6.

The observation that p38 MAPK is activated by three different protein kinases suggests that this pathway represents a site of signal integration during the response of cells to cytokines, growth factors, and environmental stimuli. This is likely to be biologically significant because of the role of p38 MAPK in the regulation of cell cycle progression (Bulavin et al. 2002a). Indeed, defects in the p38 MAPK pathway are associated with tumorigenesis (Bulavin et al. 2002b).

Figure III.1. Characterization of *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ mice.

(A) The morphology of wild-type and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ embryos at E11.0 is illustrated. The mutant embryos are anaemic and hypovascular.

(B) The morphology of the placenta of wild-type and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ embryos at E11.0 is illustrated (ventral view). The placenta of the mutant embryos is pale with decreased vascularization.

(C) Sections of the placenta from wild-type and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ embryos at E10.5 were stained with Hematoxylin and Eosin (H&E). The developing labyrinth and spongiotrophoblast layers are markedly decreased in the mutant compared to the wild-type. Key: Ch, chorionic plate; la, labyrinth; sp, spongiotrophoblast; ma, maternal decidual tissue; and Gi, trophoblast giant cells.

A

B

C
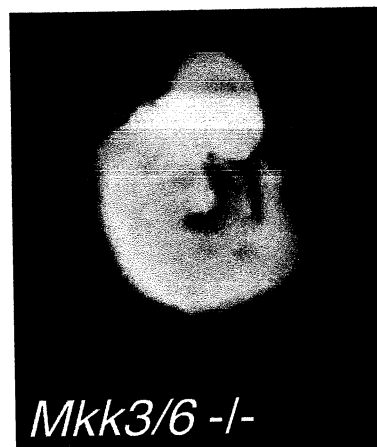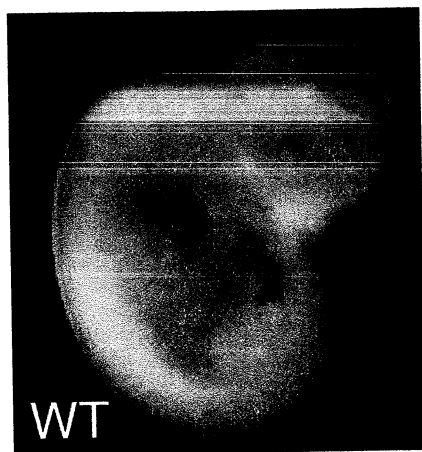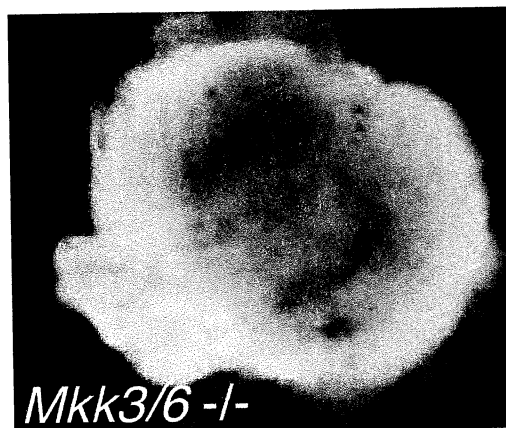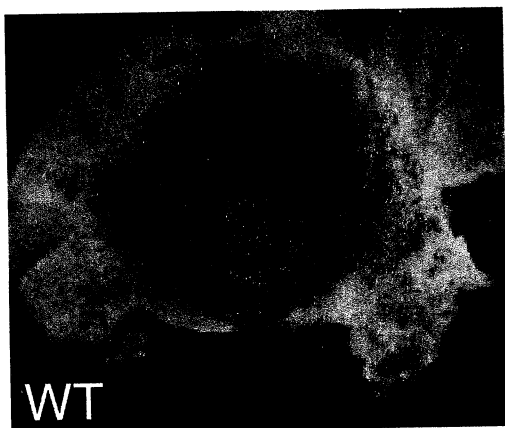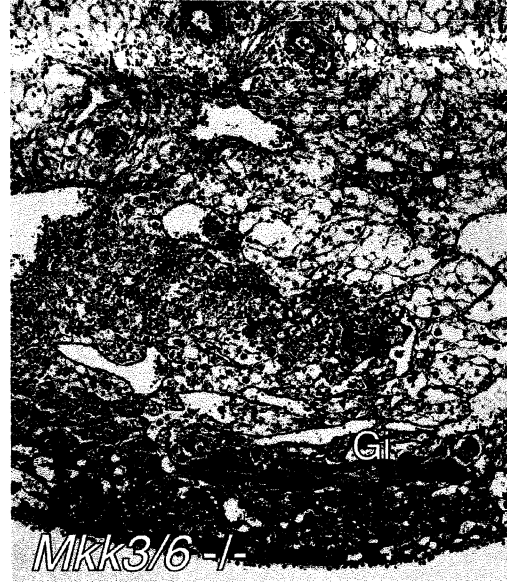
Figure III.2. Isolation of fibroblasts from MKK3/MKK6-deficient mice.

(A) Wild-type (WT), *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were cultured *in vitro*. Extracts prepared from these cells were examined by immunoblot analysis using antibodies to JNK, ERK, p38 MAPK, MKK3, MKK4, MKK6, MKK7 and α-tubulin.

(B) Cultures of WT, *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were examined by phase contrast microscopy.

(C) The proliferation of WT, *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts cultured in medium supplemented with 10% fetal calf serum was examined. The relative cell number was measured by staining with crystal violet (OD at 550nm). The normalized data presented are the mean of triplicate determinations and are representative of three independent experiments.
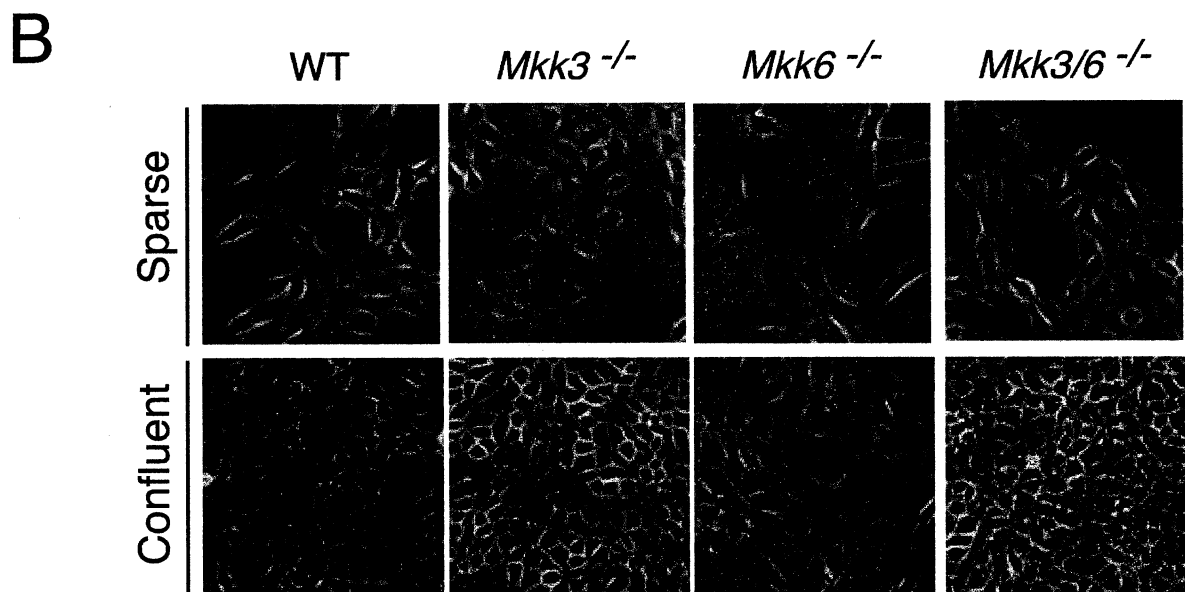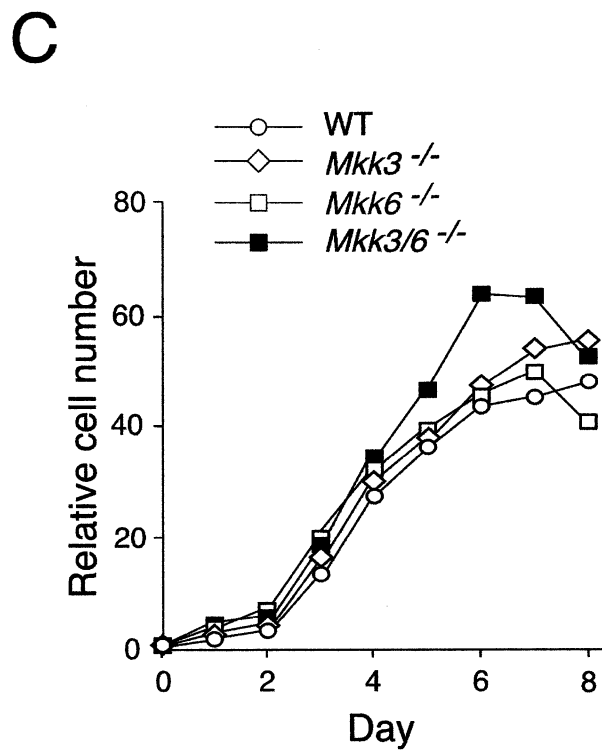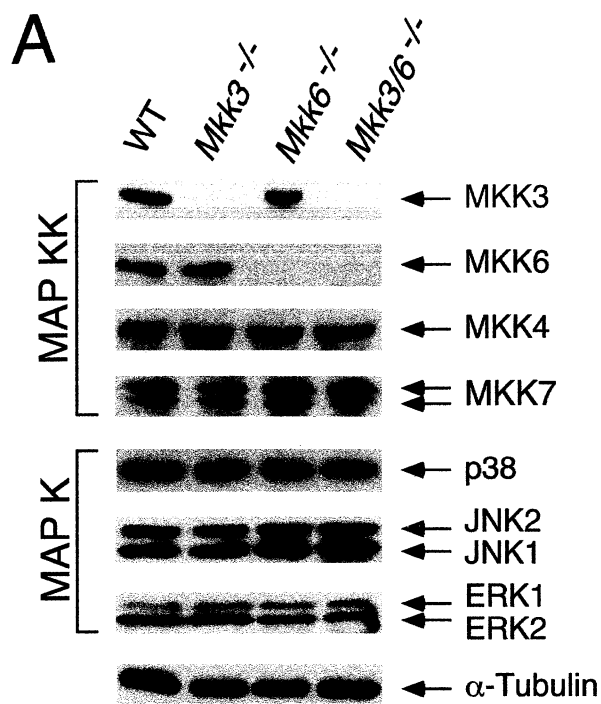
Figure III.3. Targeted disruption of *Mkk3* and *Mkk6* prevents phosphorylation of p38 MAPK by Tumor Necrosis Factor.

Wild-type (WT), *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were treated without and with 10 ng/ml TNFα (10 min). Extracts prepared from these cells were examined by immunoblot analysis using antibodies to phospho-MKK3/6 (P-MKK3, P-MKK4, P-MKK6), MKK3, MKK4, MKK6, phospho-p38 MAPK (P-p38), p38 MAPK, phospho-JNK (P-JNK), JNK, phospho-ERK (P-ERK), ERK, and α-tubulin. The phospho-MKK3/6 antibody binds activated MKK3 and MKK4 and can also bind more weakly to activated MKK6. Two exposures of the phospho-MKK3/6 immunoblot are presented to show activated MKK3, MKK4, and MKK6.

Figure III.4. Targeted disruption of *Mkk3* and *Mkk6* prevents activation of p38 MAPK by Tumor Necrosis Factor.

Wild-type (WT), *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were treated without and with 10 ng/ml TNFα (10 min). The activation of p38 MAPK was examined by *in vitro* kinase assays using ATF2 as the substrate.
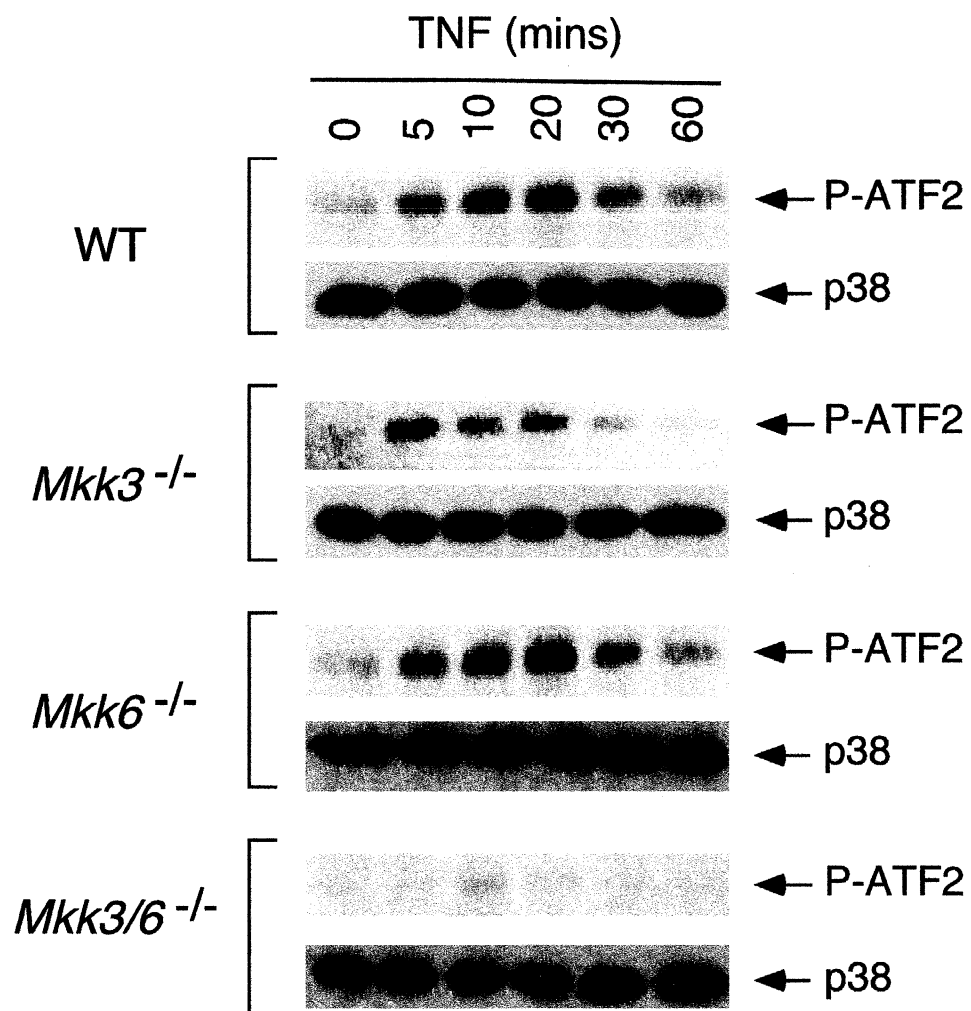
Figure III.5. Targeted disruption of *Mkk3* and *Mkk6* prevents activation of p38 MAPK by Interleukin-1.

Wild-type (WT) and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were treated without and with 10 ng/ml IL-1 (10 min) or 60 J/m^2 of UV radiation. The activation of p38 MAPK was examined by *in vitro* kinase assays using ATF2 as the substrate.

A
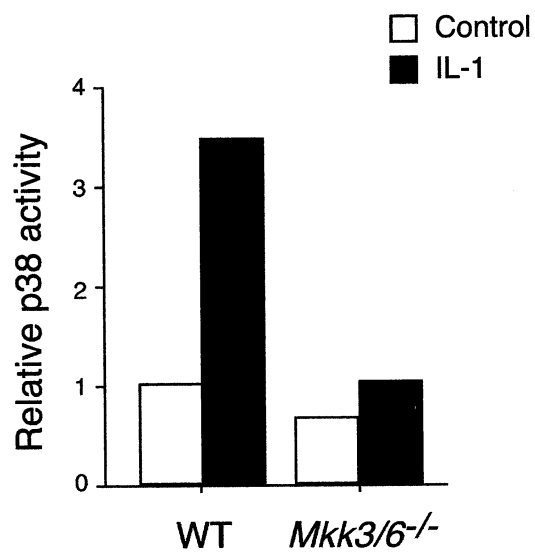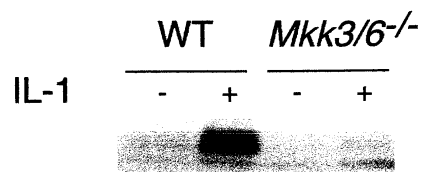
B
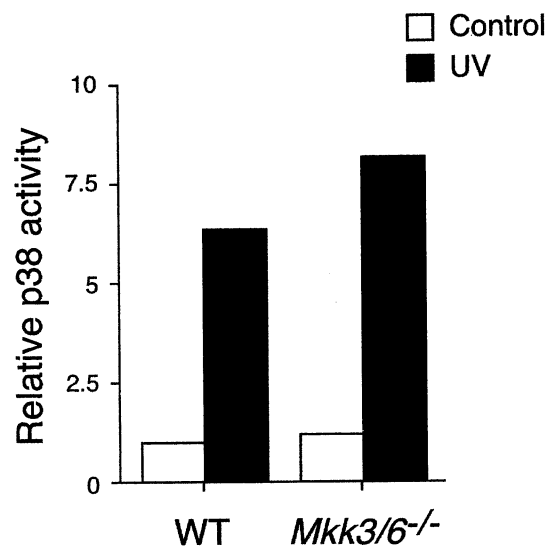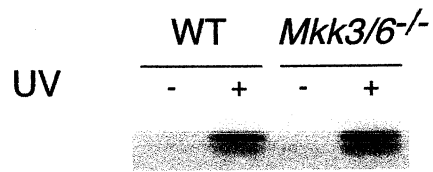
Figure III.6. Targeted disruption of *Mkk3* and *Mkk6* does not prevent UV-stimulated phosphorylation of p38 MAPK.

Wild-type (WT), *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were treated without and with 60 J/m^2 UV radiation and incubated (30 min). Extracts prepared from these cells were examined by immunoblot analysis using antibodies to phospho-MKK3/6, MKK3, MKK4, MKK6, phospho-p38 MAPK, p38 MAPK, phospho-JNK, JNK, phospho-ERK, ERK, and α-tubulin.
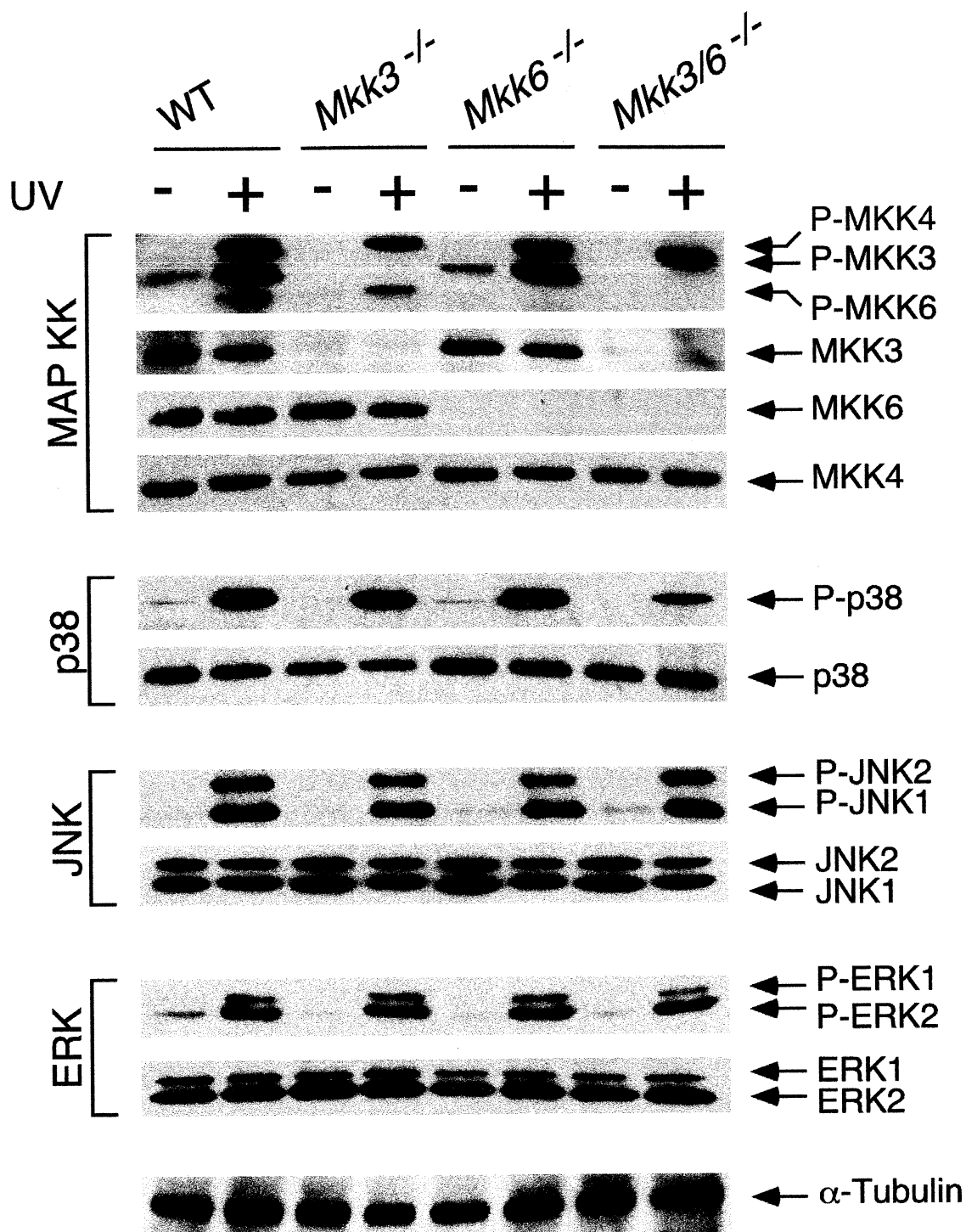
Figure III.7. Targeted disruption of *Mkk3* and *Mkk6* does not prevent UV-stimulated activation of p38 MAPK.

Wild-type (WT), *Mkk3* $^{-/-}$, *Mkk6* $^{-/-}$, and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were treated without and with 60 J/m^2 UV radiation and incubated (30 min). The activation of p38 MAPK was examined by *in vitro* kinase assays using ATF2 as the substrate.

**Figure III.8. MKK4-deficiency causes decreased activation of JNK and p38 MAPK.**

(A - B) WT and *Mkk4* $^{-/-}$ fibroblasts were treated without and with 10 ng/ml TNFα (15 min) or

60J/m^2 UV (30 min). The expression of JNK (A) and p38 MAPK (B) were examined by

immunoblot analysis (*upper panels*). Activated JNK (P-JNK) and p38 (P-p38) were detected by

immunoblot analysis (*middle panels*). JNK and p38 MAPK activity was also measured with an

in vitro kinase assay using c-Jun and ATF2 as substrates, respectively (*lower panels*). The

amount of phosphorylated c-Jun and ATF2 were quantitated by Phosphorimager analysis

(Molecular Dynamics). The data are presented in arbitrary units.

(C) WT and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts were transfected with siRNA duplexes targeting MKK4

(+ siRNA) or luciferase (- siRNA). The cells were treated without and with 60 J/m^2 UV (30 min)

at 48 hr post-transfection. The expression of MKK4, p38 MAPK, and activated p38 MAPK (P-

p38) was examined by immunoblot analysis. p38 MAPK activity was examined in an *in vitro*

kinase assay using the substrate ATF2. The amount of phosphorylated ATF2 was quantitated by

Phosphorimager analysis.
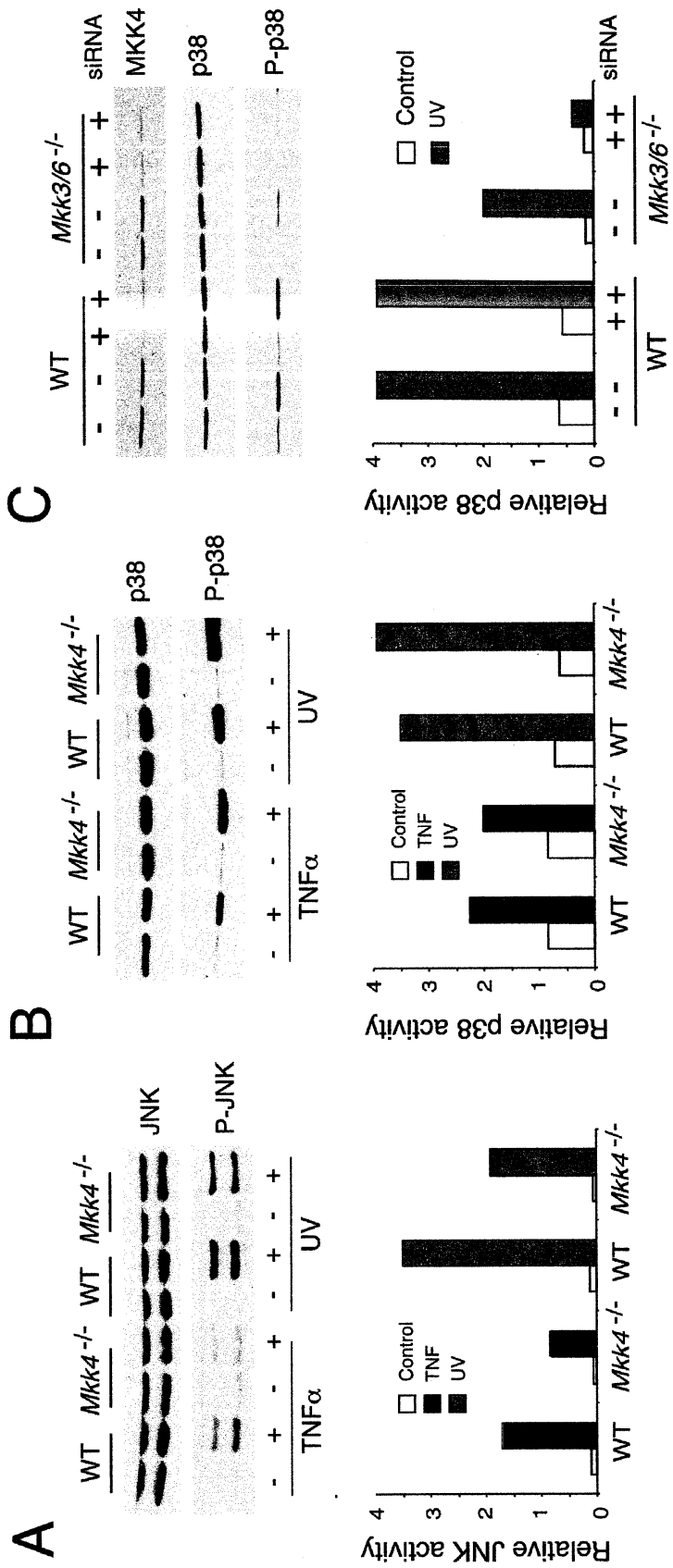
Figure III.9. Inhibition of MKK4 decreases UV-stimulated p38 MAPK activation.

WT and *Mkk3* $^{-/-}$*Mkk6* $^{-/-}$ fibroblasts were retrovirally infected without and with $T^{180}A$, $Y^{182}F$ JNK1 (dnJNK1). Infected cells were treated without or with 60 J/m^2 UV for 30 min. The expression and activation of p38 MAPK and JNK were examined by immunoblot analysis. Activated p38 (P-p38) and p38 MAPK were detected by immunoblot analysis *(upper panels)*. Activated JNK (P-JNK), dnJNK1, and JNK were detected by immunoblot analysis *(lower panels)*.
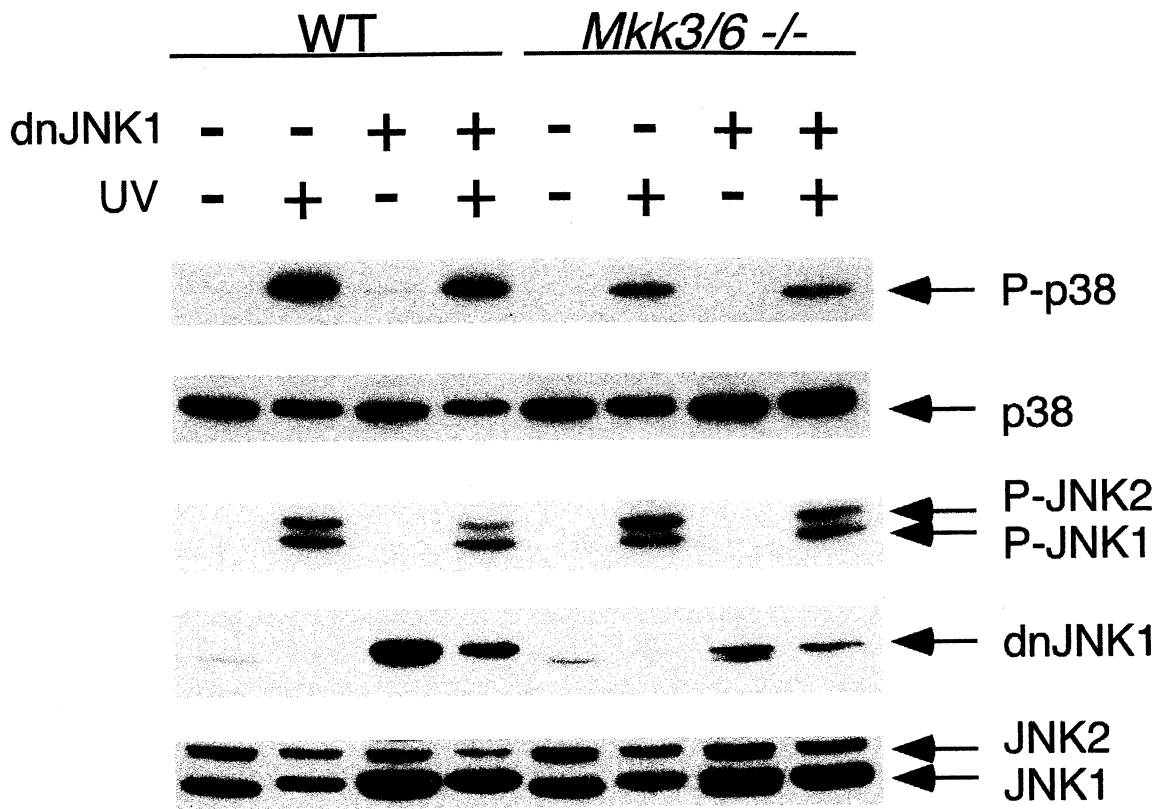
Figure III.10. *Mkk3 $^{-/-}$ Mkk6 $^{-/-}$* **fibroblasts exhibit defects in growth arrest.**

(A) WT and *Mkk3 $^{-/-}$ Mkk6 $^{-/-}$* fibroblasts were serum starved (24 hr). The effect of addition of 10% serum (30 min) to the serum starved cells is presented. The expression of p38 MAPK and activated p38 MAPK (P-p38) was examined by immunoblot analysis.

(B) WT, *Mkk3 $^{-/-}$*, *Mkk6 $^{-/-}$*, and *Mkk3 $^{-/-}$ Mkk6 $^{-/-}$* fibroblasts were cultured in different concentrations of fetal calf serum (7 days). The relative cell number was measured by staining with crystal violet (OD at 550nm). The normalized data presented are the mean of triplicate determinations and are representative of three independent experiments.

(C) Cyclin and L32 mRNA expression in WT and *Mkk3 $^{-/-}$ Mkk6 $^{-/-}$* fibroblasts was examined in a ribonuclease protection assay. Cells growing in 10% fetal calf serum were compared with cells cultured (24 hr) in serum-free medium.

(D) Extracts prepared from WT and *Mkk3 $^{-/-}$ Mkk6 $^{-/-}$* fibroblasts were examined by immunoblot analysis using antibodies to cyclin D1, cyclin D2, Rb (detects Rb and phospho-Rb), hypo-pRb (detects hypo-phosphorylated Rb), and α-tubulin. Cells growing in 10% fetal calf serum was compared with cells cultured (24 hr) in serum-free medium.

(E) c-Jun, JunB, and JunD mRNA expression in WT and *Mkk3 $^{-/-}$ Mkk6 $^{-/-}$* fibroblasts incubated without and with 10 ng/ml TNFα or 10% serum (24 hrs) was examined in a ribonuclease protection assay.

A

B

C

D

E
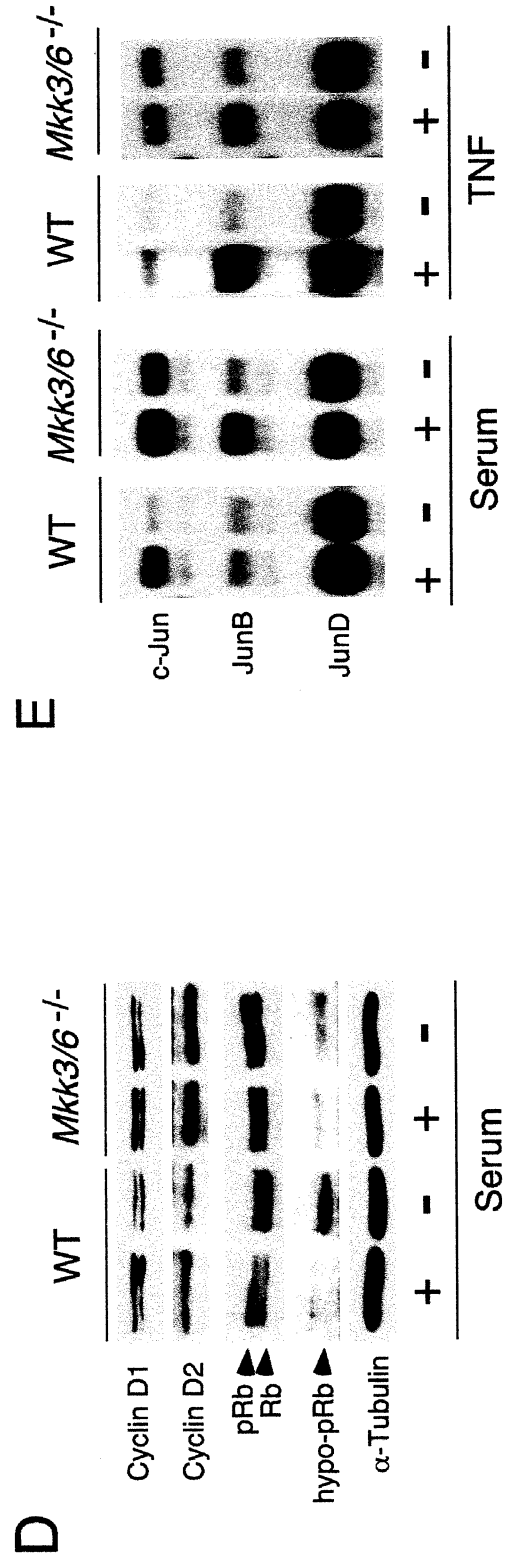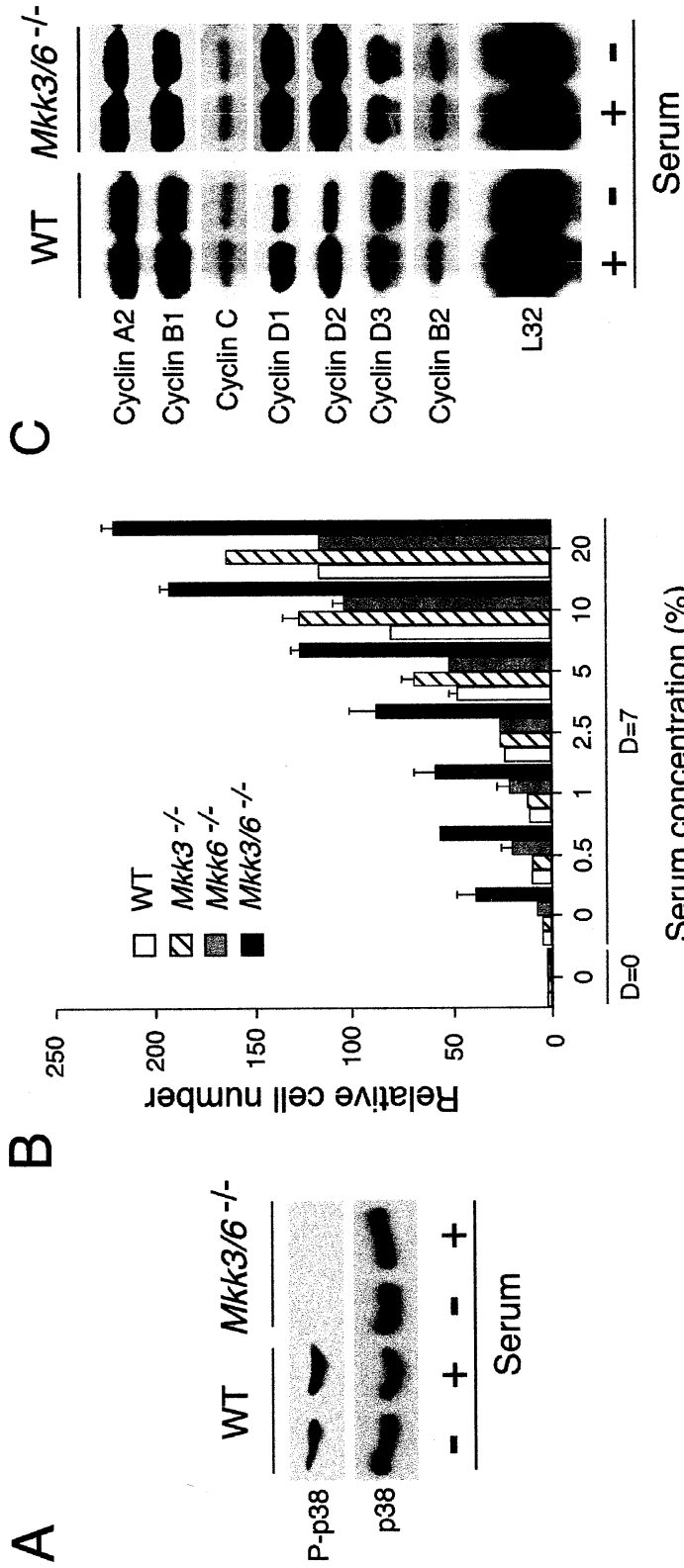
Figure III.11. Compound disruption of *Mkk3* and *Mkk6* causes increased tumorigenesis.

WT and *Mkk3* $^{-/-}$ *Mkk6* $^{-/-}$ fibroblasts immortalized with SV40 large T antigen were injected subcutaneously in athymic nude mice. Representative mice with tumors are illustrated. The mice were euthanized and the tumors were fixed and processed for histological analysis. Sections of the tumors stained with H & E are shown. The tumor volume was measured and is presented graphically (mean – SD; n = 5).

WT   Mkk3/6 $^{-/-}$
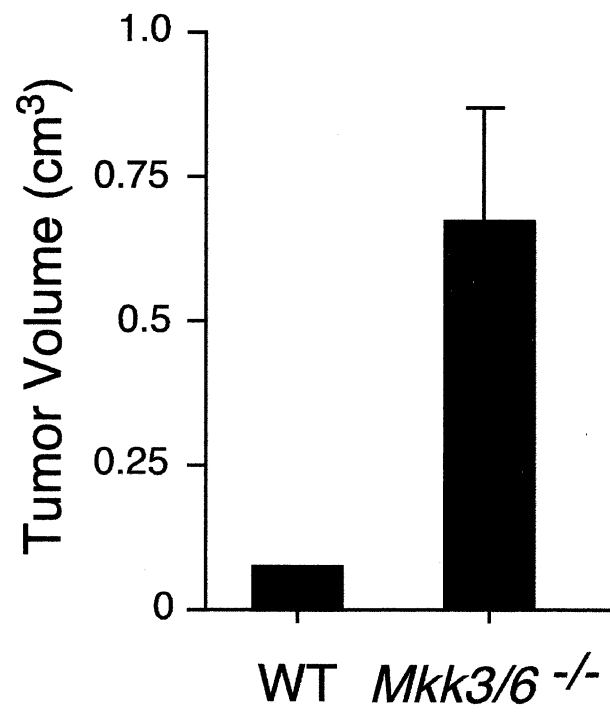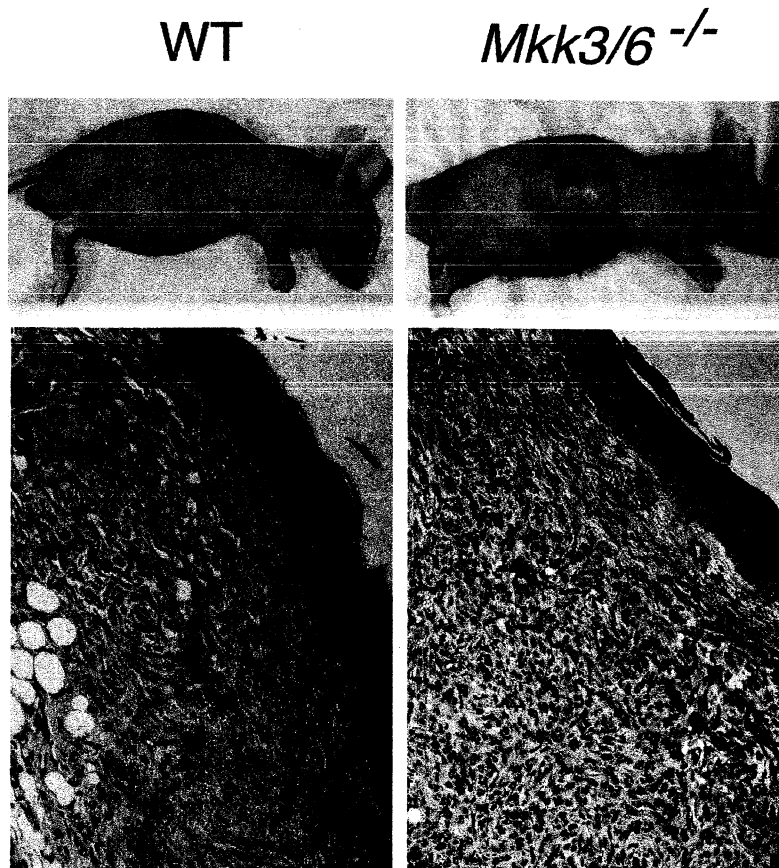
CHAPTER IV

ROLE OF MLK3 IN THE REGULATION OF MAPK

SIGNALING CASCADES

Summary

Mixed-lineage protein kinase 3 (MLK3) is a member of the MAP kinase kinase kinase (MAP3K) group that has been implicated in multiple signaling cascades. The effect of targeted disruption of the murine *Mlk3* gene was examined. MLK3-deficiency caused a selective reduction in tumor necrosis factor-α (TNFα) stimulated activation of the c-Jun NH$_2$-terminal kinase (JNK). These data demonstrate that MLK3 is a component of the TNFα signaling pathway that activates JNK.

The *Mlk3$^{-/-}$* mice were generated in collaboration with Dr. Richard Flavell's laboratory at the Yale University School of Medicine. I prepared MEF from the WT, *Mlk3$^{-/+}$*, and *Mlk3$^{-/-}$* mice and initially characterized these fibroblasts. Anja Jaeschke contributed to the immunofluorescence data in Figure IV.3A. Juan-Jose Ventura contributed to data in Figure IV.4A, C, D. Tamara Barrett, Judith Reilly, and Vicky Benoit assisted with the mouse breeding and genotyping.

Introduction

The mechanism of JNK activation caused by TNFα is incompletely understood. It is established that JNK is activated by dual phosphorylation on the T-loop within the motif Thr-Pro-Tyr (Derijard et al. 1994). This phosphorylation is mediated by the actions of two different MAP kinase kinases (MAP2K) MKK4 and MKK7 (Tournier et al. 2001). These MAP2K can be activated by MAP kinase kinase kinases (MAP3K), but the identity of the relevant TNFα-stimulated MAP3K is unclear.

Many transfection studies of MAP3K have been reported that employ over-expression and dominant-negative approaches (Widmann et al. 1999). In general, these studies have not provided useful information in studies of JNK signaling because of the promiscuous function of MAP3K in both gain-of-function and dominant-negative experiments. In contrast, studies using mouse knockouts have provided rigorous evidence for roles of individual MAP3K. Thus, MEKK1 is required for TGFβ/activin-stimulated JNK activation (Zhang et al. 2003), MEKK2 is required for FGF-stimulated JNK activation (Kesavan et al. 2004), MEKK3 contributes to TNFα-stimulated p38 MAPK activation (Lee et al. 2003), MEKK4 is required for IL-18 stimulated p38 MAPK activation (Chi et al. 2004), and Tpl-2 is required for LPS-stimulated ERK activation (Dumitru et al. 2000).

Although progress towards understanding the role of MAP3K has been achieved, the mechanism of JNK activation in response to TNFα remains unclear. Three MAP3K have been implicated in TNFα-stimulated JNK activation. First, ASK1 is thought to be involved in the late phase of JNK activation in response to TNFα, most likely as a result of the generation of reactive oxygen species (Tobiume et al. 2001). The immediate activation of JNK caused by TNFα may

be mediated by TAK1 and/or by one or more members of the mixed-lineage protein kinase (MLK) family. MLK protein kinases may be selectively involved in TNFα-stimulated JNK activation (Sathyanarayana et al. 2002), while TAK1 is implicated as a common TNFα-stimulated activator of JNK, p38 MAPK, and NF-κB (Ishitani et al. 2003; Takaesu et al. 2003). Since *Traf2* $^{-/-}$ fibroblasts exhibit defects in TNFα-stimulated JNK and NF-κB (but not p38 MAPK) and *Rip1* $^{-/-}$ fibroblasts exhibit defects in p38 MAPK and NF-κB (but not JNK) (Lee et al. 2003; Yeh et al. 1997), it is not obvious how a common MAP3K could be responsible for the TNFα-stimulated activation of JNK, p38 MAPK, and NF-κB. For this reason, I considered the possibility that a MLK protein kinase might contribute to TNFα-stimulated JNK activation.

There are three sub-groups of mixed-lineage protein kinases (Gallo and Johnson 2002). The MLK group, which consists of MLK1, MLK2, MLK3, and MLK4, share similar structural domains, including an SH3 domain and a Crib motif that binds Cdc42 and Rac1. The DLK group (DLK and LZK) is structurally distinct and lacks the SH3 and Crib sequences. The third group of protein kinases consists of a single member (ZAK) that is distinctive because of the presence of a sterile-α motif (SAM). Many of these protein kinases are expressed in only a limited number of tissues; for example, MLK1 is expressed in epithelial cells and DLK is expressed in neurons (Gallo and Johnson 2002). However, one member of this gene family is ubiquitously expressed, consistent with a possible role as a mediator of TNFα signaling in many tissues — MLK3.

This possibility is consistent with previous studies showing that TNFα activates MLK3 (Sathyanarayana et al. 2002), that TNFα and MLK protein kinases (Tournier et al. 2001) can selectively activate MKK7 (Hirai et al. 1998; Merritt et al. 1999), and that a small molecule MLK inhibitor can inhibit TNFα-stimulated JNK activation (Sathyanarayana et al. 2003).

However, recent RNAi-based studies have suggested that MLK3 is critically required for cellular proliferation and is essential for the activation of multiple MAP kinase signaling pathways in response to a broad range of stimuli (Chadee and Kyriakis 2004; Swenson et al. 2003). The role of MLK3 in signaling and the specific relevance of MLK3 to TNFα signal transduction are therefore unclear.

The purpose of this study was to test the role of MLK3 in TNFα-stimulated JNK activation. My approach was to examine the effect of targeted disruption of the *Mlk3* gene. I report that MLK3-deficiency causes a selective defect in TNFα-stimulated JNK activation and that MLK3 is not required for cellular proliferation. Surprisingly, MLK3-mediated JNK activation negatively regulated the activation of p38 MAPK. This negative cross-talk was observed in response to TNFα stimulation and during *in vitro* adipogenesis. Together, these data suggest that MLK3 plays a non-redundant role in mediating the specific activation of JNK.

Materials and Methods

Mice. Mouse strain 129/SvJ genomic clones of the Mlk3 gene were isolated by PCR and sub-cloned into the vector pCR™II (Invitrogen). A targeting vector designed to disrupt the *Mlk3* gene (Figure IV.1a) was constructed using standard techniques. ES cells were electroporated with this vector and selected with 200 μg/ml G418 (Invitrogen) and 2 μM gangcyclovir (Syntex). Twelve *Mlk3*$^{-/+}$ ES cell clones were identified by Southern blot analysis and two were injected into C57BL/6J blastocysts to create chimeric mice that transmitted the disrupted Mlk3 allele through the germ-line. The mice were backcrossed ten generations to the C57BL/6J strain (Jackson Laboratories). Homozygous *Mlk3*$^{-/-}$ mice were obtained by crossing heterozygous *Mlk3*$^{-/+}$ animals. Histological analysis of the mice was performed using tissue fixed in 10% formalin for 24 hr, dehydrated, and embedded in paraffin. Sections (4 μm) were cut and stained with Harris hematoxylin (Sigma) and eosin (Sigma). The mice were housed in a facility accredited by the American Association for Laboratory Animal Care (AALAC). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

Genotype analysis. The genotype at the *Mlk3* locus was examined by Southern blot analysis of Nco1 restricted genomic DNA by probing with a random-primed ^{32}P-labeled probe (529 bp) that was isolated by PCR using an *Mlk3* cDNA as the template and the primers 5'-CTCCGAAGGCAACAGCAGCTTATGCCA-3' and 5'-CACACGCCGACCAGCCAGGGCCCGGCT-3'. The wild-type (140 bp) and disrupted (275 bp) alleles of Mlk3 were also detected by PCR amplification of genomic DNA using the primers

5'- AAGCGGAGCAAACTCCGAGCAAG-3', 5'- AAAGGCTAAACCAGAACTCAAG-3' and

5'- GTAGAAGGTGGCGCGAAGGG-3'.

Cell culture. Primary murine embryo fibroblasts (MEF) were isolated and cultured in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum

(Invitrogen). All experiments were performed using MEF between passage 2 and passage 5.

Similar data were obtained in experiments using independently isolated MEF cultures.

Proliferation assays were performed by staining with crystal violet (Tournier et al. 2000).

Adipocyte differentiation assays (Green and Kehinde 1975; Picard et al. 2002) were performed

by culturing 2-day post-confluent cells in medium supplemented with 10 μg/ml insulin (Sigma),

0.5 mM isomethylbutyl-1-xanthine (IBMX, Sigma), and 1 μg/ml dexamethasone (Sigma). The

medium was replaced after 72 hr with fresh medium supplemented with 10 μg/ml insulin and 1

μM Troglitizone (Calbiochem). The accumulation of fat droplets within the cytoplasm was

detected by staining the cells with Oil-Red-O (VWR).

Migration and invasion assays. Boyden chamber assays were performed using 2.5×10^4 cells in

0.5ml DMEM placed in each insert of a 24 multi-well plate (BIOCOAT®, Becton Dickinson).

Migration and invasion assays were performed without and with Matrigel®, respectively, by

incubating the cells at 37 °C (16 hr). The inserts were placed in methanol (-20 °C) and stained

with 4'-6'- diamino-2-phenylindole (DAPI, Vector Laboratories). The cells were visualized with

an Axioplan 2 microscope and a MicroImager CCD camera (Carl Zeiss).

Immunofluorescence analysis. Cells were grown on coverslips and fixed at -20 °C in methanol (5 min) and in acetone (2 min). The coverslips were washed in PBS, incubated (30 min at 22 °C) in BPT buffer (3% BSA/PBS, 0.2% Tween-20), and stained with a mouse monoclonal antibody to α-tubulin (Sigma) in BPT buffer (60 min at 22 °C). Immunecomplexes were visualized using a FITC-conjugated goat anti-mouse Ig secondary antibody (Jackson ImmunoResearch). Slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Inc). Fluorescence microscopy was performed using a Ziess inverted microscope (Axiovert M200) with a 10x objective (N.A. 0.30), a CCD camera (Zeiss Axiocam), and image acquisition software (Zeiss Axiovision).

Immunoblot analysis. Cell extracts were prepared using Triton lysis buffer [20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin and leupeptin]. Extracts (50 μg of protein) were examined by protein immunoblot analysis by probing with antibodies to JNK (Pharmingen), phospho-JNK (Cell Signaling), p38 MAPK (Santa Cruz), phospho-p38 MAPK (Cell Signaling), ERK1/2 (Santa Cruz), MLK3 (Cell Signaling), IκBα (Cell Signaling), C/EBPα (Santa Cruz), phospho-Thr-222/226-C/EBPα (Cell Signaling), C/EBPβ (Santa Cruz), phospho-Thr-235-C/EBPβ (Cell Signaling), and α-tubulin (Sigma). Immunocomplexes were detected by enhanced chemiluminescence (NEN).

Protein kinase assays. The activity of JNK, ERK, and p38 MAP kinase was measured using *in vitro* kinase assays using the substrates cJun, myelin basic protein, and ATF2, respectively (Whitmarsh and Davis 2001).

RNase protection assays. Total RNA (5 μg) was examined using the "Multi-probe RNase protection assay" (Pharmingen) with the template sets mFos/Jun, mCR-4, and mCK-3b following the manufacturer's recommendations. The products were separated on a 5% sequencing gel, detected by autoradiography, and quantitated by Phosphorimager analysis (Molecular Dynamics).

Results

Targeted disruption of *Mlk3*

I constructed a targeting vector to disrupt the *Mlk3* gene. This vector was designed to replace the kinase domain, exons 2 - 5 with a Neo^R cassette (Figure IV.1a). The vector was linearized and electroporated into embryonic stem (ES) cells to obtain homologous recombination within the *Mlk3* gene. Twelve ES cell clones with the correctly targeted *Mlk3* gene were identified by Southern blot analysis. Two of these clones were injected into C57BL/6J blastocysts to create male chimeric mice that were bred to obtain germ-line transmission of the disrupted *Mlk3* allele. The mice were backcrossed to the C57BL/6J strain background. Genomic DNA isolated from the progeny obtained from crossing *Mlk3*$^{-/+}$ mice was examined by PCR and Southern blot analysis to identify wild-type, *Mlk3*$^{-/+}$, and *Mlk3*$^{-/-}$ littermates (Figure IV.1b, c). The number of wild-type, heterozygous, and homozygous knockout mice obtained from these crosses conform to the expected Mendelian inheritance. Immunoblot analysis demonstrated that the level of MLK3 expression was reduced in *Mlk3*$^{-/+}$ mice and was absent in *Mlk3*$^{-/-}$ mice (Figure IV.1d).

MLK3-deficient mice were found to be viable, had a normal life-span, and were not found to be tumor-prone compared with wild-type mice. The *Mlk3*$^{-/-}$ mice appeared to be morphologically normal, but displayed a slight defect along the dorsal midline (Figure IV.2a). Histological analysis indicated that MLK3-deficiency reduced the amount of dorsal epidermal tissue (Figure IV.2b). The mechanism for this epidermal defect remains unclear; however, defects in the dorsal epidermis are frequently associated with neural tube disclosure in mice and dorsal disclosure in *Drosophilia* (Davis 2000; Weston and Davis 2002). Recent reports indicate

that *slipper*, a dMLK, is required for dorsal closure during embryogenesis (Stronach and Perrimon 2002; Sathyanarayana et al. 2003). This is consistent with the finding that epidermal defects are observed along the dorsal midline of $Mlk3^{-/-}$ mice.

MLK3 is not essential for cellular proliferation

It has been established in previous RNAi-based studies that MLK3 is essential for serum-stimulated cell proliferation (Chadee and Kyriakis 2004). This conclusion appears to be inconsistent with the finding that $Mlk3^{-/-}$ mice are viable. To directly investigate whether MLK3 is required for proliferation, I isolated primary murine embryo fibroblasts (MEF) from wild-type and $Mlk3^{-/-}$ E13.5 embryos. Phase contrast microscopy indicated that the morphology of the wild-type and $Mlk3^{-/-}$ MEF was similar in sparse cultures and that both groups of MEF exhibited contact growth inhibition in confluent cultures (Figure IV.3a). Measurement of proliferation during culture in medium supplemented with 10% fetal bovine serum for 8 days demonstrated no differences between wild-type and $Mlk3^{-/-}$ MEF (Figure IV.3b). Similarly, no differences between wild-type and $Mlk3^{-/-}$ MEF were detected in experiments using serum concentrations ranging between 0% and 20% (Figure IV.3c). The regulated expression of members of the AP-1 family of transcription factors is implicated in cell proliferation and these genes represent potential targets of MLK3 signaling; however, comparison of wild-type and $Mlk3^{-/-}$ MEF indicated no differences in AP-1 mRNA expression (Figure IV.4a). Together, these data indicate that MLK3 is not essential for MEF proliferation.

A previous RNAi-based study has indicated that MLK3 is also essential for the regulation of microtubule dynamics (Swenson et al. 2003). Defective microtubule regulation would be expected to cause defects in mitosis and cell migration (Swenson et al. 2003). Comparison of

wild-type and *Mlk3* $^{-/-}$ MEF did not indicate a requirement for MLK3 in proliferation (Figure IV.3b, c). I therefore investigated potential defects in cell migration caused by MLK3-deficiency. Boyden chamber assays using an imposed serum gradient (0 - 10%) demonstrated no differences in serum-induced chemotaxis between wild-type and *Mlk3* $^{-/-}$ MEF (Figure IV.3d). Similarly, no differences between the ability of wild-type and *Mlk3* $^{-/-}$ MEF to invade a Matrigel® layer were observed (Figure IV.3d). These data suggest that MLK3-deficiency does not cause major microtubule defects. Indeed, immunofluorescence microscopy using a monoclonal antibody to α-tubulin indicated the presence of similar microtubule networks in wild-type and *Mlk3* $^{-/-}$ MEF (Figure IV.3a).

MLK3-deficiency causes a selective defect in TNFα-stimulated JNK activation

It is established that the MLK3 protein kinase is activated when cells are exposed to TNFα, and pharmacological studies using the drug CEP-11004 have implicated MLK3 in TNFα-stimulated JNK activation (Sathyanarayana et al. 2002). I therefore examined the ability of TNFα to activate JNK in wild-type and *Mlk3* $^{-/-}$ MEF with an *in vitro* protein kinase assay using cJun as the substrate. This analysis demonstrated that TNFα caused a rapid and transient increase in JNK activity in wild-type MEF and that the extent of JNK activation was suppressed (but not eliminated) in *Mlk3* $^{-/-}$ MEF (Figure IV.5a). The effect of MLK3-deficiency to suppress JNK regulation by TNFα was also observed in experiments in which the JNK activation state was monitored by immunoblot analysis with an antibody that binds dual (Thr and Tyr) phosphorylated JNK (Figure IV.6a). The defect in JNK activation was not a consequence of decreased TNF receptor expression because wild-type and *Mlk3* $^{-/-}$ MEF expressed similar amounts of TNF-R1 and TNF-R2 (Figure IV.4c). To confirm that the reduced TNFα-stimulated

JNK activation observed in *Mlk3*^{-/-} MEF was caused by MLK3-deficiency, I performed

complementation analysis (Figure IV.7). Together, these data demonstrate that MLK3

contributes to TNFα-stimulated JNK activation. However, MLK3 was not essential for TNFα-

stimulated JNK activity.

The requirement of MLK3 for maximal JNK activation appears to be selective for TNFα.

Thus, no defect in IL-1 stimulated JNK activation was detected in *Mlk3*^{-/-} MEF (Figure IV.6b).

Similarly, MLK3-deficiency did not cause defects in JNK activation when the MEF were

exposed to several environmental stresses, including ultraviolet radiation, osmotic stress,

anisomycin, or ceramide activation (Figure IV.8). Furthermore, MLK3-deficiency did not cause

defects in growth factor-stimulated JNK activation, including EGF, PDGF, and FGF activation

(Figure IV.9). The selective (and partial) defect in TNFα-stimulated JNK activation observed in

MLK3-deficient fibroblasts markedly contrasts with previous RNAi-based studies that have

implicated an essential role of MLK3 in JNK activation caused by multiple stimuli (Chadee and

Kyriakis 2004).

MLK3-mediated JNK activation negatively regulates p38 MAPK

TNFα causes the activation of several signal transduction pathways, including JNK,

ERK, p38 MAPK, and NF-κB. Gene disruption experiments demonstrate that MLK3 is required

for maximal TNFα-stimulated JNK activation (Figure IV.5; Figure IV.6). It is possible that

MLK3 may also play a role in the TNFα-stimulated activation of ERK, p38 MAPK, and NF-κB.

Indeed, previous studies have implicated MLK3 in ERK activation (Chadee and Kyriakis 2004),

p38 MAPK activation (Rana et al. 1996; Chadee and Kyriakis 2004), and NF-κB activation

(Hehner et al. 2000). Control studies demonstrated that TNFα-stimulated IκBα degradation and

the activation of ERK and p38 MAPK was not reduced in MLK3-deficient MEF (Figure IV.4b; Figure IV.5b, c). An unexpected discovery was that TNFα-stimulated p38 MAPK activity was increased in $Mlk3^{-/-}$ MEF (Figure IV.5b). This increased TNFα-stimulated p38 MAPK activation was confirmed by immunoblot analysis using an antibody to phospho-p38 MAPK (Figure IV.6a) and was not observed in cells treated with IL-1 (Figure IV.6b). Since JNK can suppress p38 MAPK activation and JNK-deficient cells exhibit increased TNFα-stimulated p38 MAPK signaling (Morton et al. 2004), it is likely that the increased TNFα-stimulated p38 MAPK activation is related to the observed decrease in TNFα-stimulated JNK activation (Figure IV.6a). This increased TNFα-stimulated p38 MAPK activation is consistent with the finding that MLK3-deficient MEF exhibit greater TNFα-stimulated expression of IL-6, a p38 MAPK target, than wild-type MEF (Figure IV.4d).

Altered MAPK regulation causes increased adipogenic potential

The increased p38 MAPK activation and decreased JNK activation observed in $Mlk3^{-/-}$ MEF is likely to be physiologically significant. It has been reported that JNK activation inhibits adipogenesis, by phosphorylating and inactivating the pro-adipogenic transcription factor PPARγ (Camp et al. 1999). Conversely, p38 MAPK activation promotes adipogenesis, by increasing the expression and phosphorylation of C/EBP transcription factors (Engelman et al. 1998; Zhang et al. 2004b). I therefore investigated whether altered MAP kinase activation might contribute to an effect of MLK3-deficiency during adipose differentiation. Wild-type and $Mlk3^{-/-}$ MEF were differentiated *in vitro* to adipocytes and the expression and phosphorylation of JNK, p38 MAPK, and ERK were examined by immunoblot analysis (Figure IV.10a). During differentiation, JNK phosphorylation was decreased in the $Mlk3^{-/-}$ MEF. Conversely, p38 MAPK phosphorylation

was slightly increased in the $Mlk3^{-/-}$ MEF. No differences in the expression or phosphorylation of ERK were observed. It has been reported that MAP kinases target transcription factors involved in adipogenesis (Engelman et al. 1998; Camp et al. 1999; Hu et al. 1996). I therefore examined the expression and phosphorylation of C/EBPα and C/EBPβ during adipose differentiation (Figure IV.10b). Expression and phosphorylation of C/EBPα was increased in the $Mlk3^{-/-}$ MEF at early times during differentiation. Similarly, an increase of expression and phosphorylation of C/EBPβ was also observed at early times during differentiation. These data indicate that MLK3-deficiency can alter MAP kinase and transcription factor activation during adipogenesis.

This suggests that MLK3-deficiency may increase adipose differentiation *in vitro*. To directly investigate whether MLK3-deficiency can increase adipocyte differentiation *in vitro*, I examined the morphology of differentiated wild-type and $Mlk3^{-/-}$ MEF by phase contrast microscopy and by staining accumulated fat droplets with Oil-Red-O. Indeed, examining the morphology of wild-type and $Mlk3^{-/-}$ MEF indicated an increase of adipocyte differentiation in the $Mlk3^{-/-}$ MEF (Figure IV.11a). To confirm that the increased adipocyte differentiation observed in the $Mlk3^{-/-}$ MEF was caused by MLK3-deficiency, I performed complementation analysis (Figure IV.11b). Expression of MLK3 was found to decrease the number of adipocytes in the differentiated MLK3-deficient MEF. In addition, $Mlk3^{-/-}$ MEF showed an increase in differentiated adipocytes in both the presence and absence of insulin and the PPARγ ligand troglitizone as compared to wild-type MEF (Figure IV.11c). Together, these data demonstrate that MLK3-deficiency increases the adipogenic potential of primary MEF.

Discussion

My analysis of *Mlk3*$^{-/-}$ mice indicates that MLK3 functions as component of the TNFα

signaling pathway that causes JNK activation in primary MEF. Previous studies using RNAi-

mediated gene suppression have suggested a more general role of MLK3 as an essential protein

kinase for the activation of multiple MAPK signaling pathways in response to a broad array of

extracellular stimuli (Chadee and Kyriakis 2004). In addition, RNAi-based studies have

suggested that MLK3 is critical for cell proliferation (Chadee and Kyriakis 2004) and normal

microtubule function (Swenson et al. 2003). Analysis of *Mlk3*$^{-/-}$ MEF do not support these

conclusions. Since my biochemical analysis were restricted to MEF, it is possible that the

divergent conclusions between these studies reflect differences in the role of MLK3 between cell

types. Nevertheless, it is difficult to reconcile the conclusion that MLK3 is critical for

proliferation and has multiple essential signaling roles with the finding that *Mlk3*$^{-/-}$ mice are

viable and healthy. A second possible explanation of the more restricted phenotype observed in

my study is that a greater degree of compensation may have occurred in the MLK3 knockout

mice from a MLK family member compared with *in vitro* studies with RNAi. A number of other

potential explanations also exist, including the possibility that the phenotype caused by a

reduction of MLK3 expression is greater than that caused by the elimination of MLK3

expression (Editorial 2003).

MLK3 is a component of the TNFα signaling pathway that activates JNK

TNFα binds to the TNF-R1 receptor to activate several signaling pathways, mediating a

balance between life and death (Wajant et al. 2003). TNFα can activate the caspase-8 pathway

to stimulate apoptosis, or promote cell survival by activating NF-κB. In addition, TNFα can mediate JNK- and p38 MAPK- dependent gene transcription through the interaction of the TNF adaptor proteins, TRAF2 and RIP1, with MAP3K. In this study, I demonstrate that the MAP3K, MLK3, has a non-redundant role in TNFα signaling (Figure IV.4 - 7). MLK3 mediated the immediate activation of JNK in response to TNFα and was required for maximal TNFα-stimulated JNK activation. In addition, I demonstrated that MLK3 is not required for TNFα-stimulated activation of p38 MAPK and NF-κB. These data indicate that MLK3 selectively regulates TNFα-stimulated JNK activity.

Although MLK3-deficient MEF exhibit a selective reduction in TNFα-stimulated JNK activation, a low level of TNFα-induced JNK activation was observed in $Mlk3^{-/-}$ MEF (Figure IV.5a ; Figure IV.6a). One key question for future studies is the identity of the MAP3K that mediates the effects of TNFα on JNK activation in MLK3-deficient cells. A plausible hypothesis is that this protein kinase activity represents another member of the MLK group, but other MAP3K may contribute to TNFα-stimulated JNK activation. A second key question relates to the mechanism by which TNFα activates MLK3. The adapter protein TRAF2 is required for TNFα-stimulated JNK activation (Yeh et al. 1997), but whether TRAF2 might regulate MLK3 directly or indirectly is unclear. Analysis of the regulatory relationship between TRAF2 and MLK3 represents a critical goal for future experiments.

Interestingly, TNFα-stimulated activation of p38 MAPK is increased in $Mlk3^{-/-}$ MEF (Figure IV.5b; Figure IV.6a). Recent studies of JNK-deficient cells demonstrate increased p38 MAPK activation in response to TNFα (Morton et al. 2004). Conversely, decreased p38 MAPK activity increases JNK activation during macrophage differentiation (Hall and Davis 2002).

These data suggest that negative cross-talk exists between the JNK and the p38 MAPK pathways.

A JNK-dependent mechanism may cause the observed increase in TNFα-stimulated p38 MAPK activation in MLK3-deficienct MEF. Since the immediate activation of JNK and p38 MAPK is altered in these cells, the mechanism probably does not require gene regulation. One plausible hypothesis is that this MAPK activity depends on the activation of JNK-dependent phosphatases. For example, MAP kinase phosphatases (MKP) are dual-specific phosphatases that regulate the ERK, JNK, and p38 MAPK pathways (Camps et al. 2000; Theodosiou and Ashworth 2002). Several MKP, such as MKP5 and MKP7, appear to preferentially dephosphorylate JNK and p38 MAPK (Theodosiou et al. 1999; Tanoue et al. 1999; Tanoue et al. 2001; Matsuguchi et al. 2001). The activity of these phosphatases may be reduced in MLK3-deficient cells and de-repress p38 MAPK. A second explanation relies on the presence of a negative feedback loop. JNK may down-regulate its activity by inactivating MAP3K (Phelan et al. 2001; Vacratsis et al. 2002). The lack of MAP3K down-regulation may promote p38 MAPK activation by channeling the activated MAP3K to other adaptor proteins. Recently, gene targeted disruption studies indicate that RIP1 is essential for TNFα-stimulated p38 MAPK activation (Lee et al. 2003). Further studies are, thus, required to analyze the regulatory relationship between RIP1, MLK3, and other MAP3K.

The JNK and the p38 MAPK pathways regulate adipogenesis

Adipogenesis is a physiological process that is differentially regulated by MAP kinases (Rosen and Spiegelman 2000; Rosen et al. 2000). For example, JNK activation inhibits the pro-adipogenic transcription factor PPARγ (Adams et al. 1997; Camp et al. 1999). Conversely, p38

MAPK activation promotes adipogenesis, in part, by increasing the transcriptional activation of C/EBP (Engelman et al. 1998; Engelman et al. 1999). This suggests that adipogenesis is a paradigm in which reciprocal alterations in JNK and p38 MAPK activity may have biological consequences. In this study, I show that MLK3-deficiency increases the adipogenic potential of primary MEF (Figure IV.10 - IV.11). MLK3-deficiency altered MAPK activation and increased the expression and phosphorylation of the pro-adipogenic transcription factors C/EBPα and C/EBPβ. In addition, MLK3-deficiency increased adipocyte differentiation *in vitro*. These data suggest that MLK3 plays a role in regulating adipogenesis.

The mechanism of MLK3-mediated adipogenesis, however, remains unclear. Adipocyte differentiation may be enhanced by an increase in C/EBPα and C/EBPβ phosphorylation by p38 MAPK (Engelman et al. 1998). In addition, up-regulated p38 MAPK may phosphorylate the transcription factors, CREB and ATF1, and increase C/EBPβ expression (Zhang et al. 2004b). Conversely, adipocyte differentiation may be enhanced by a decrease in PPARγ phosphorylation by JNK inhibition (Camp et al. 1999). Other transcription factors involved in adipose differentiation, such as C/EBPδ, may also be affected by MLK3-deficiency.

Interestingly, the increased C/EBPβ activation observed in *Mlk3* [-/-] MEF during adipose differentiation may partially explain the increased IL-6 expression observed in *Mlk3* [-/-] MEF following TNFα stimulation (Figure IV.4d). TNFα-stimulated IL-6 expression is dependent on the activation of p38 MAPK (Beyaert et al. 1996; Wysk et al. 1999). In addition, IL-6 expression can also be regulated by the transcriptional activation of C/EBPβ (Akira et al. 1990; Nakajima et al. 1993). This suggests that in TNFα-stimulated *Mlk3* [-/-] MEF, increased p38 MAPK activation may cause increased C/EBPβ activation, resulting in increased IL-6 expression.

Although MLK3-deficiency caused increased adipogenesis in MEF cultures *in vitro*, analysis of *Mlk3* $^{-/-}$ mice fed standard chow *ad libitum* did not reveal increased adiposity *in vivo*. Further studies of these mice are required to determine whether MLK3-deficiency affects adiposity *in vivo*; for example, if the mice are environmentally challenged on a high fat diet.

Conclusions

In this study, I have examined the role of MLK3 in the TNFα signaling pathway. My approach was to examine the effect of targeted disruption of the *Mlk3* gene. A consensus conclusion from my MLK3 knockout analysis (Figure IV.1 - IV.9) and previously reported studies (Sathyanarayana et al. 2002; Swenson et al. 2003; Chadee and Kyriakis 2004) is that the limited role of MLK3 that I observe in TNFα signaling represents a non-redundant function of MLK3. My analysis does not exclude the possibility that MLK3 may also have additional functions that are redundant with other members of the MAP3K family.

I also observe that MLK3-mediated JNK activation can negatively regulate TNFα-stimulated p38 MAPK activity. This suggests that the JNK and the p38 MAPK pathways can coordinate their actions in response to specific stimuli. Regulating MAPK activation is likely to be biologically significant, because of the opposing roles these MAPK play in adipogenesis. Indeed, altered JNK and p38 MAPK regulation are associated with changes in adipogenic potential (Figure IV.10 - IV.11).

In conclusion, I report that the MLK3 protein kinase contributes to TNFα-stimulated JNK activation. This identification of a role for MLK3 fills an important gap in our understanding of the mechanism of JNK activation caused by TNFα.

Figure IV.1. Disruption of the *Mlk3* gene in mice.

(A) The strategy employed to disrupt the *Mlk3* gene by homologous recombination is illustrated. The structure of the *Mlk3* gene, the targeting vector, and the disrupted *Mlk3* gene are shown. Restriction enzyme sites are indicated: B (*BamH*I), Nc (*Nco*I), Nt (*Not*I), R (*EcoR*I), X (*Xba*I). The probe used for Southern analysis and the expected results of *Nco*I restriction digestion are illustrated.

(B,C) Genomic DNA isolated from $Mlk3^{+/+}$, $Mlk3^{-/+}$, and $Mlk3^{-/-}$ mice was examined by PCR (B) and Southern blot (C) analysis.

(D) Protein extracts prepared from MEF isolated from $Mlk3^{+/+}$, $Mlk3^{-/+}$, and $Mlk3^{-/-}$ mice were examined by immunoblot analysis using antibodies to MLK3 and α-tubulin.

A

Mlk3 locus
(Mlk3 +)

12.0 kb

Nco digestion

Targeting
vector

pBS

Homologous
Recombination

Mutated Mlk3
locus (Mlk3 −)

5.4 kb

Nco digestion

cDNA
probe

B

+/+ −/+ −/−

— 275 bp

— 140 bp

C

+/+ −/+ −/−

— 12.0 kb

— 5.4 kb

D

+/+ −/+ −/−

MLK3

α-Tubulin
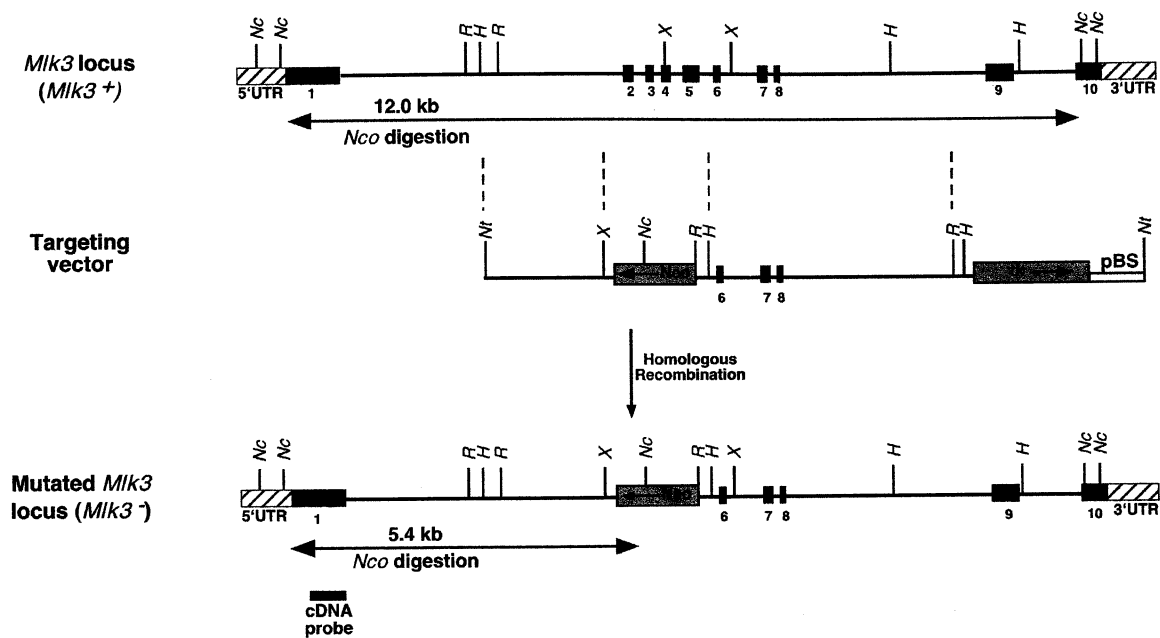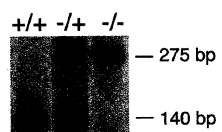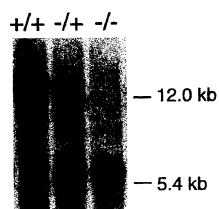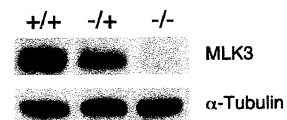
Figure IV.2. MLK-deficiency cause epidermal defects along the dorsal midline.

(A) *Mlk3*$^{-/-}$ mice are viable. WT and *Mlk3*$^{-/-}$ mice were bred and four day old littermates were examined and photographed. The *Mlk3*$^{-/-}$ mice appear morphologically normal, but exhibit a dorsal midline defect (red arrow).

(B) Histological examination of the WT and *Mlk3*$^{-/-}$ mice. Sections of the dorsal epidermis and underlying tissue were stained with hematoxylin and eosin (H & E). Images of low and high magnification are shown. The red arrow indicates the loss of epidermal tissue along the dorsal midline of the *Mlk3*$^{-/-}$ mice.

A

WT Mlk3⁻/⁻

B

Neck

Hind brain

WT Mlk3⁻/⁻
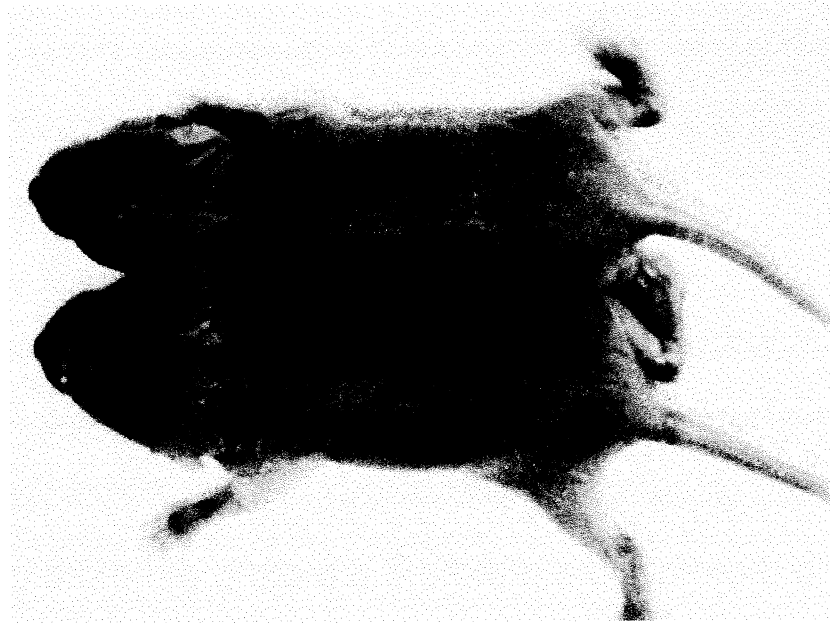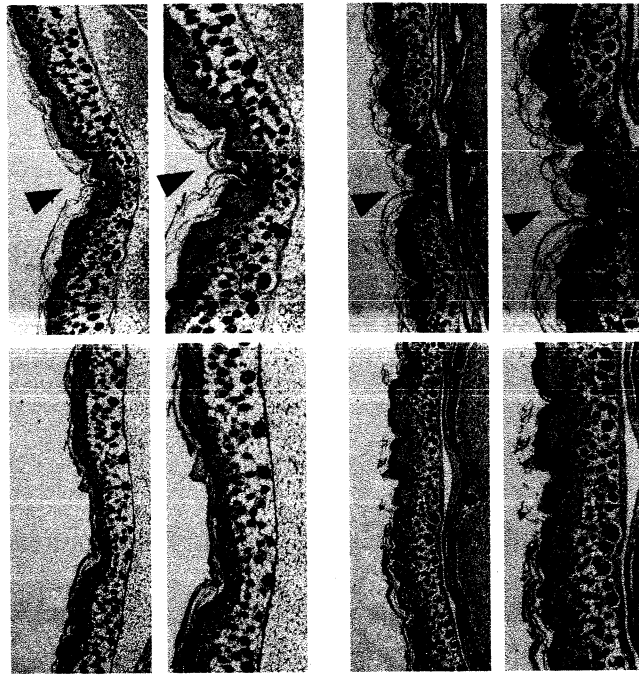
Figure IV.3. Characterization of primary MEF isolated from wild-type and *Mlk3*^{-/-} mice.

(A) The morphology of sparse and confluent cultures of WT and *Mlk3*^{-/-} MEF was examined by phase contrast microscopy (*upper panels*). The presence of microtubules (green) in WT and *Mlk3*^{-/-} MEF was examined by immunofluorescence microscopy (*lower panels*). DNA in the nucleus was stained with DAPI (blue).

(B) Cells (2.5 x 10^4) were incubated in 11 mm wells with medium supplemented with 10% fetal calf serum. Relative cell number was measured by staining with crystal violet. The proliferation of WT and *Mlk3*^{-/-} MEF during culture for 8 days was examined. The data shown represent the mean – SD (n = 3).

(C) The effect of serum concentration on the saturation growth density of WT and *Mlk3*^{-/-} MEF was examined. Cells (2.5 x 10^4) were incubated in 11 mm wells with medium supplemented with different concentrations of serum for 7 days. Relative cell number was measured by staining with crystal violet. The data shown represent the mean – SD (n = 3).

(D) Boyden chamber assays were performed to measure chemotaxis (cell migration) from serum free medium to medium supplemented with 10% serum. Invasion assays were performed in similar experiments using Matrigel®. The cells were stained with 4'-6'- diamino-2-phenylindole (DAPI) and visualized by fluorescence microscopy. The relative cell migration and invasion was quantitated by counting the number of cells (mean – SD; n = 3). The migration and invasion by WT and *Mlk3*^{-/-} MEF is shown.

117
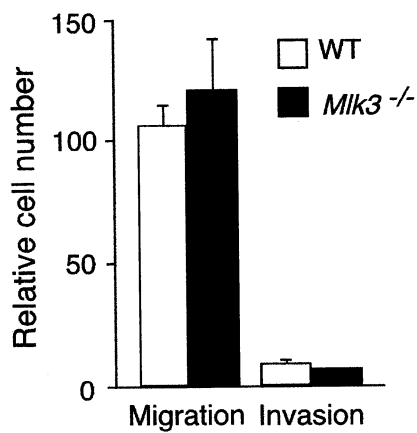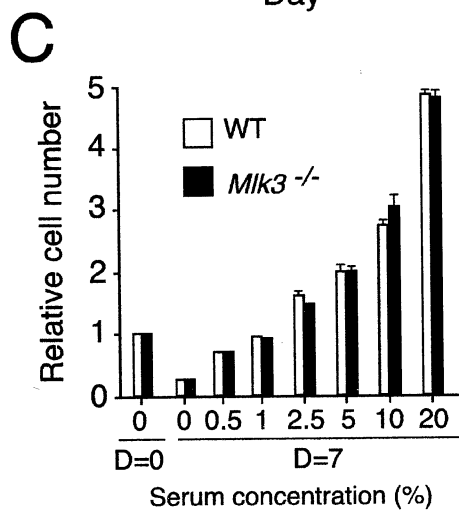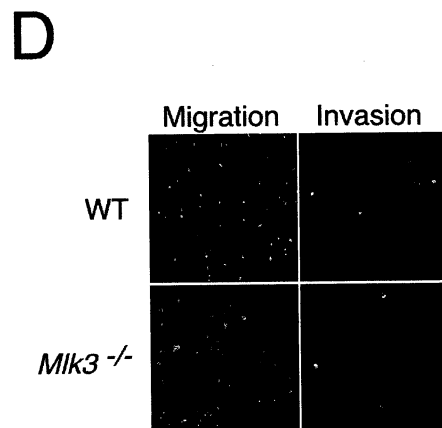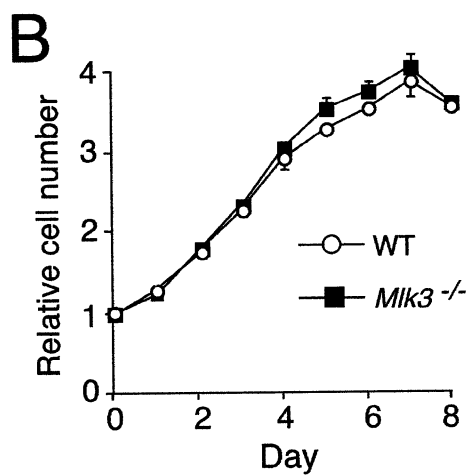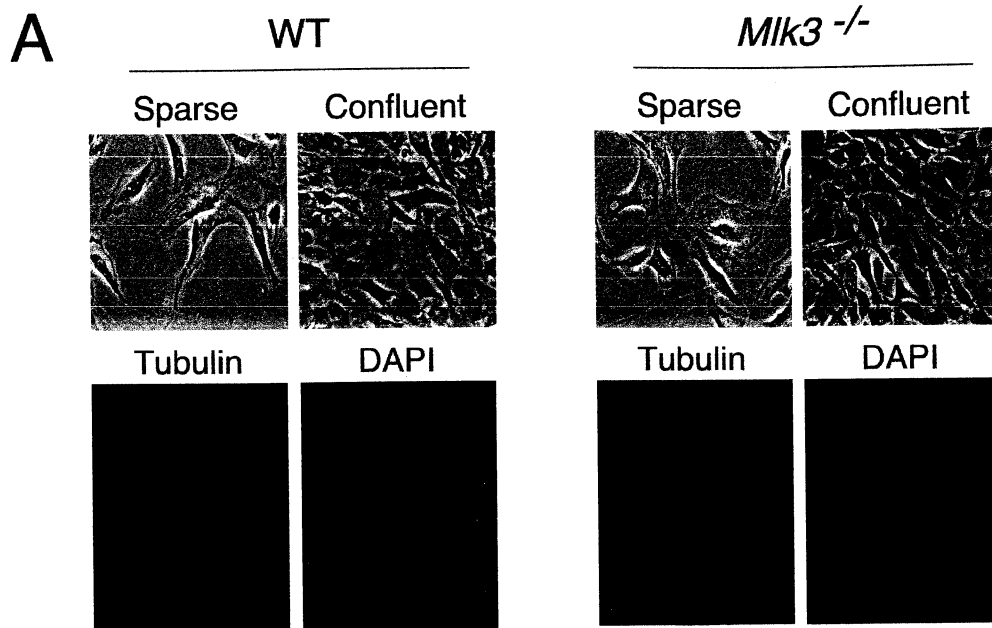
Figure IV.4. Effect of MLK3-deficiency on gene transcription.

(A) Effect of MLK3-deficiency on AP-1. The expression of several genes that encode proteins that can form AP-1 transcription factors by exponentially growing WT and $Mlk3^{-/-}$ MEF was examined in a ribonuclease protection assay to detect mRNA. The expression of ribosomal protein L32 mRNA was examined in control experiments.

(B) Effect of MLK3-deficiency on NF-κB. WT and $Mlk3^{-/-}$ MEF were incubated with TNF (10 ng/ml) and the degradation of IκBα was examined by immunoblot analysis. Control experiments were performed by probing immunoblots with an antibody to α-tubulin.

(C) Effect of MLK3-deficiency on TNF-R. The expression of TNF-R1 and TNF-R2 was examined in a ribonuclease protection assay to measure the amount of TNF receptor mRNA in WT and $Mlk3^{-/-}$ MEF. Control experiments were performed by measuring the amount of ribosomal protein L32 mRNA. The data were quantitated by Phosphorimager analysis and the relative TNF receptor expression was calculated as the ratio of TNF receptor mRNA and L32 mRNA.

(D) Effect of MLK3-deficiency on IL-6. WT and $Mlk3^{-/-}$ MEF were incubated with TNF (10 ng/ml) and the amount of IL-6 and L32 mRNA was examined in a ribonuclease protection assay. The data were quantitated by Phosphorimager analysis and the relative IL-6 mRNA expression was calculated as the ratio of IL-6 mRNA and L32 mRNA.
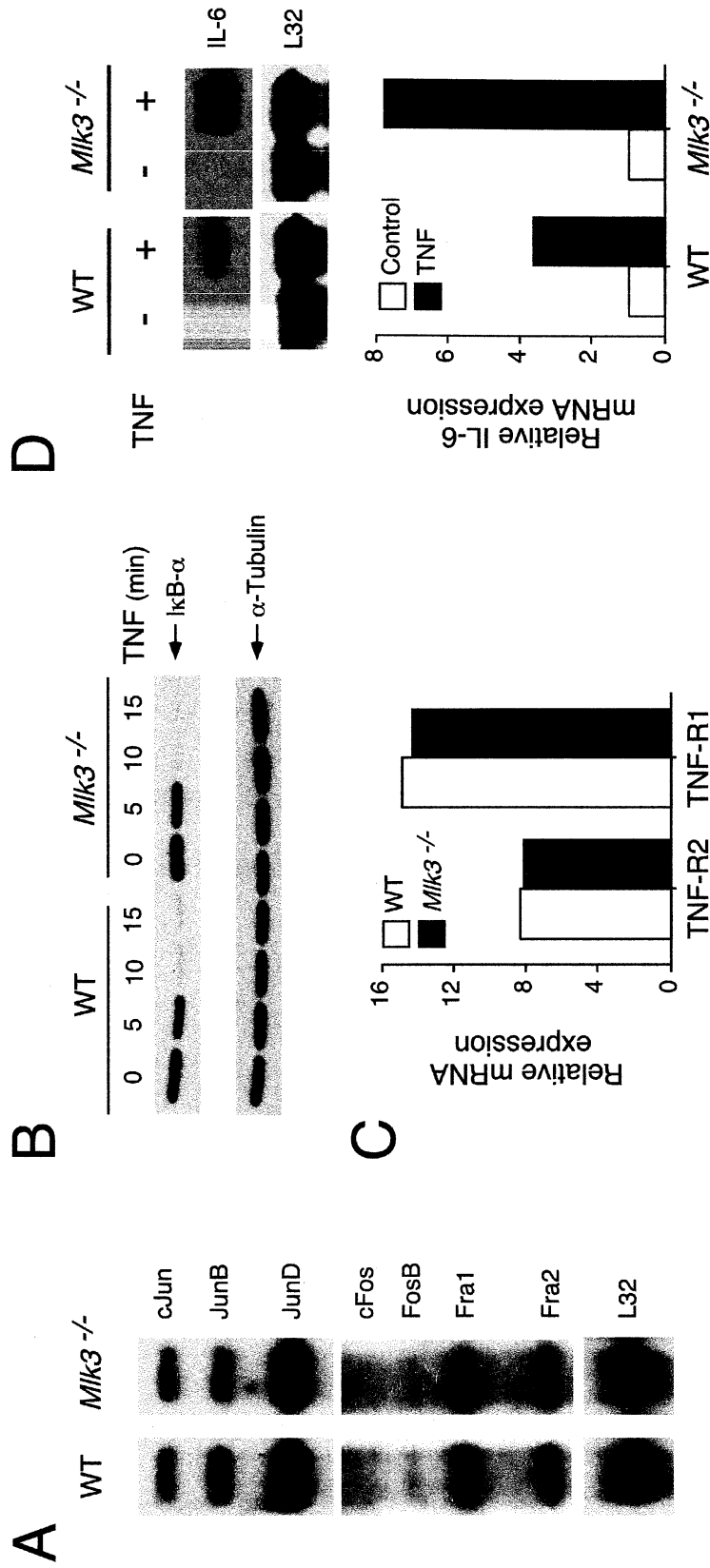
Figure IV.5. MLK3 is required for maximal TNF-stimulated JNK activation.

(A) WT and *Mlk3* $^{-/-}$ MEF were incubated with TNF (10 ng/ml) for different periods of time before the cells were harvested to prepare protein extracts. JNK activity was measured with an *in vitro* kinase assay using the substrate cJun. The phosphorylation of cJun was detected by autoradiography and was quantitated by Phosphorimager analysis. The amount of JNK in each assay was examined by immunoblot analysis.

(B) The TNF-stimulated activation of p38 MAP kinase was examined in an *in vitro* kinase assay using the substrate ATF2.

(C) The TNF-stimulated activation of ERK was examined in an *in vitro* kinase assay using myelin basic protein (MBP) as the substrate.
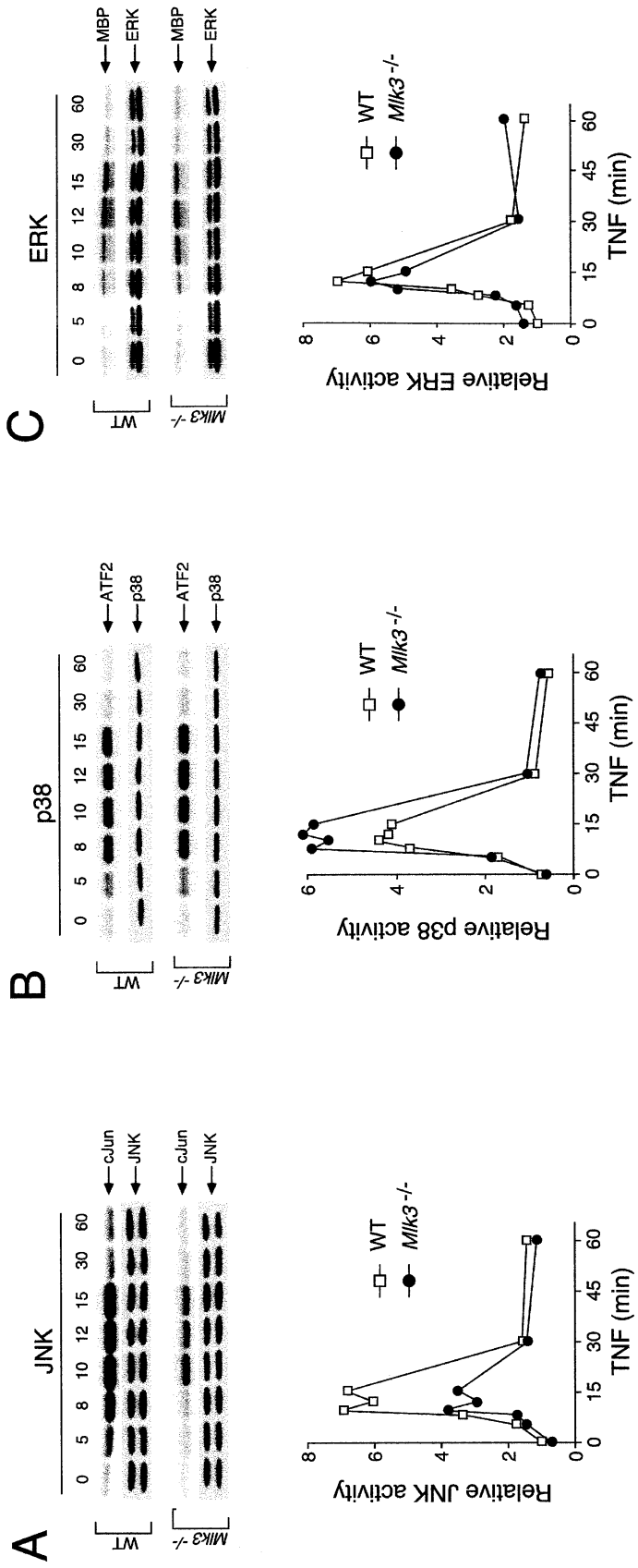
Figure IV.6. Maximal TNF-stimulated JNK phosphorylation on Thr and Tyr requires MLK3.

(A) WT and *Mlk3*$^{-/-}$ MEF were incubated with TNF (10 ng/ml) and the activation of JNK and p38 MAPK was examined by immunoblot analysis using antibodies to phospho-JNK and phospho-p38. The amount of MAPK in each assay was examined by probing immunoblots with antibodies to JNK and p38 MAPK.

(B) The effect of IL-1 (10 ng/ml) on JNK and p38 MAPK activation in WT and *Mlk3*$^{-/-}$ MEF was examined by immunoblot analysis.
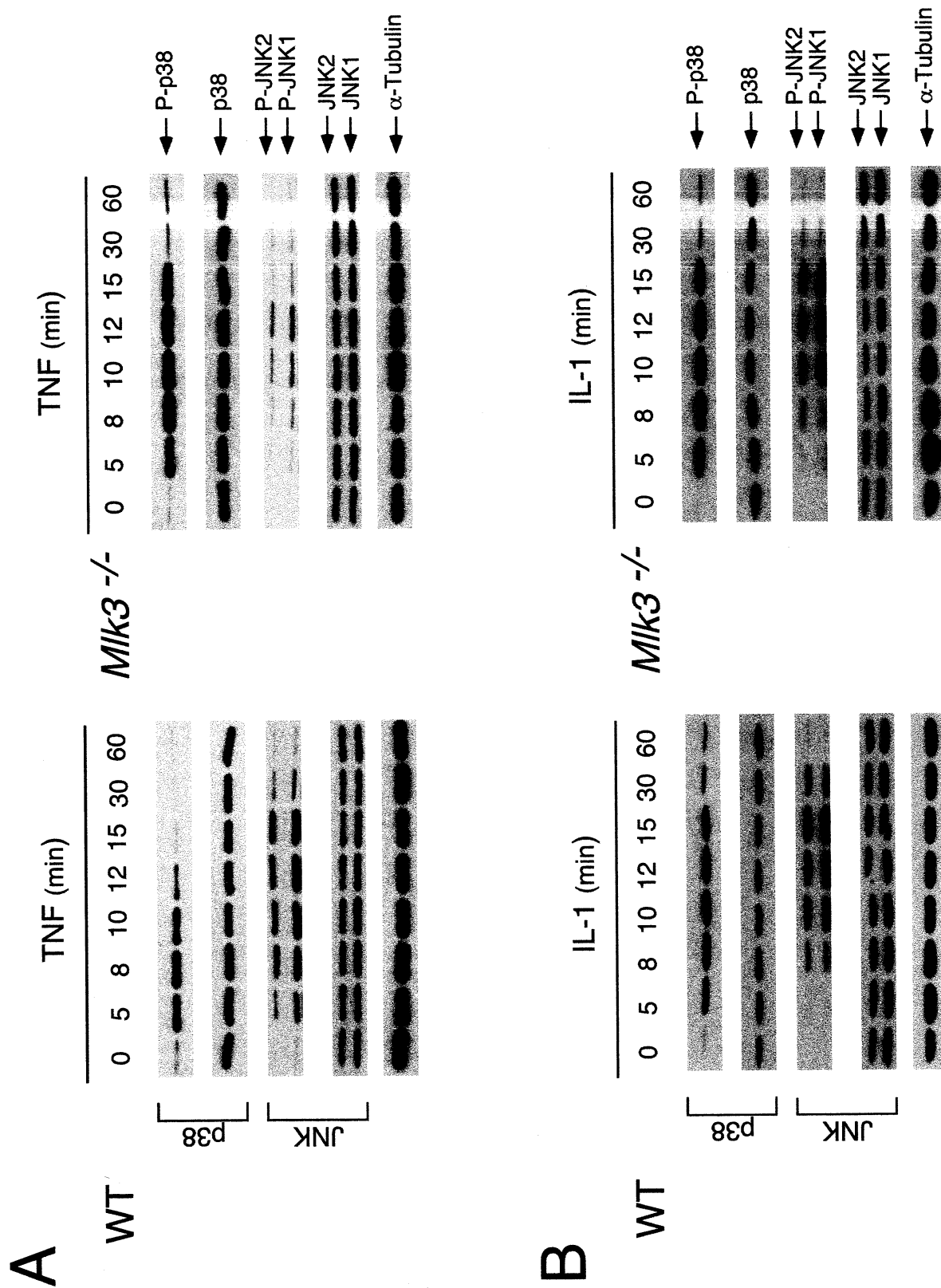
Figure IV.7. Complementation analysis demonstrated that the expression of MLK3 increases TNF-stimulated JNK activation in MLK3-deficient MEF.

MEF were incubated without and with 10 ng/ml TNF (10 min). Protein extracts were investigated by probing western blots with antibodies to JNK, MLK3, and α-tubulin. JNK activity was measured in an immunecomplex kinase assay with cJun as the substrate. The incorporation of ^{32}P-phosphate into the substrate was detected by autoradiography and was quantitated by Phosphorimager analysis.
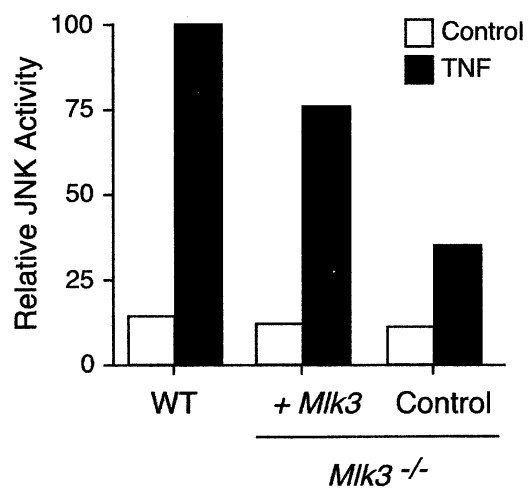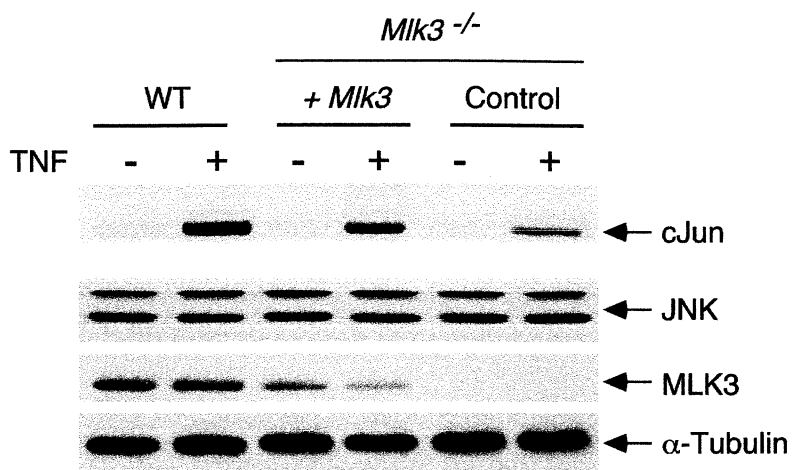
Figure IV.8. MLK3 is not required for JNK or p38 MAPK activation caused by stress.

(A) WT and $Mlk3^{-/-}$ MEF were exposed to UV (50 J/m^2) and the activation of JNK and p38 MAPK was examined by immunoblot analysis using antibodies to phospho-JNK and phospho-p38. The amount of MAPK in each assay was examined by probing immunoblots with antibodies to JNK and p38 MAPK.

(B) The effect of osmotic stress (300 mM sorbitol) on JNK and p38 MAPK activation in WT and $Mlk3^{-/-}$ MEF was examined by immunoblot analysis.

(C) The effect of anisomycin (1 μg/ml) on JNK and p38 MAPK activation in WT and $Mlk3^{-/-}$ MEF was examined by immunoblot analysis.

(D) The effect of ceramide (10 μM) on JNK and p38 MAPK activation in WT and $Mlk3^{-/-}$ MEF was examined by immunoblot analysis.

128
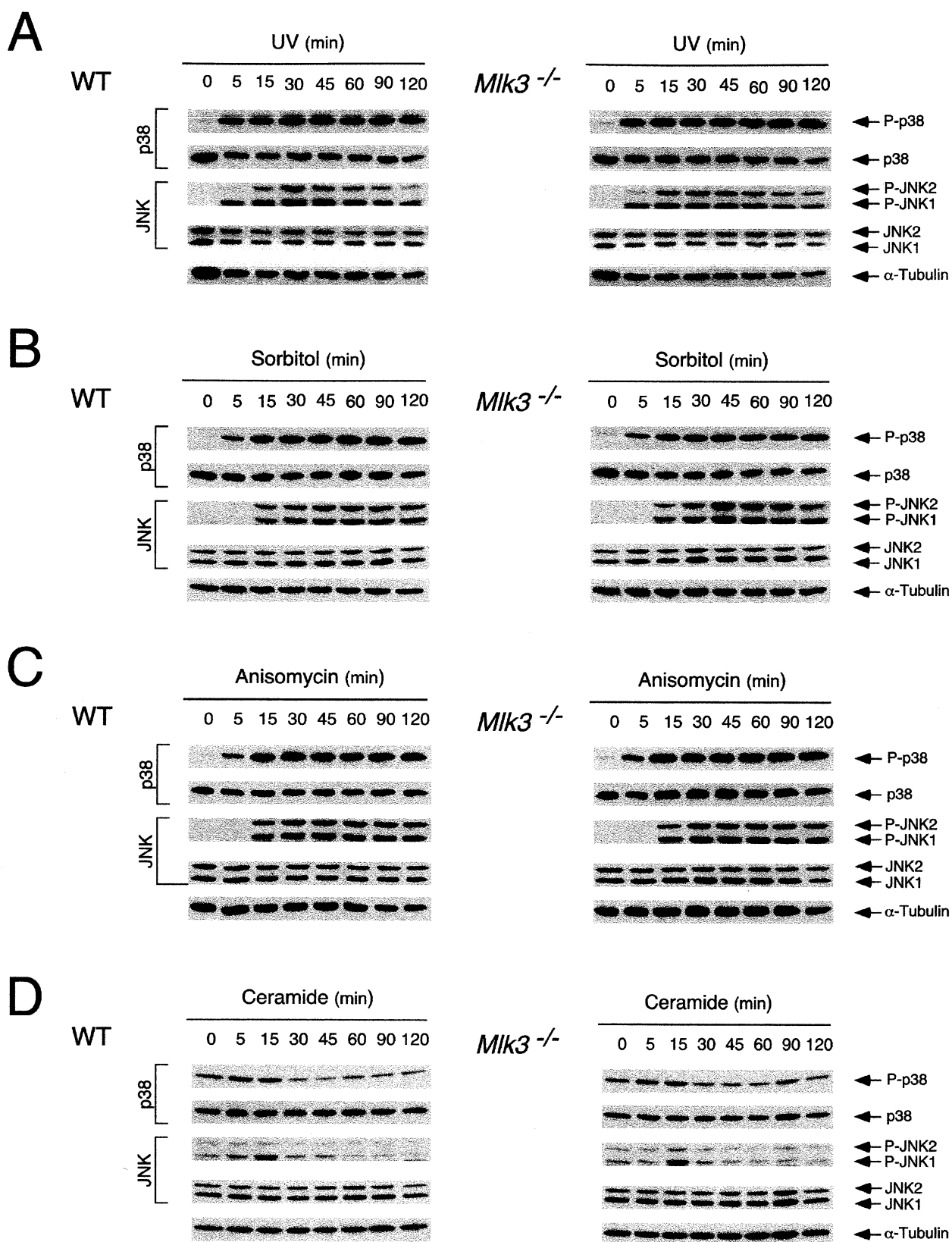
Figure IV.9. MLK3 is not required for JNK or p38 MAPK activation caused by growth factors.

(A) WT and *Mlk3 $^{-/-}$* MEF were exposed to PDGF (20 ng/ml) and the activation of JNK and p38 MAPK was examined by immunoblot analysis using antibodies to phospho-JNK and phospho-p38. The amount of MAPK in each assay was examined by probing immunoblots with antibodies to JNK and p38 MAPK.

(B) The effect of EGF (50 ng/ml) on JNK and p38 MAPK activation in WT and *Mlk3 $^{-/-}$* MEF was examined by immunoblot analysis.

(C) The effect of FGF (25 ng/ml) on JNK and p38 MAPK activation in WT and *Mlk3 $^{-/-}$* MEF was examined by immunoblot analysis.
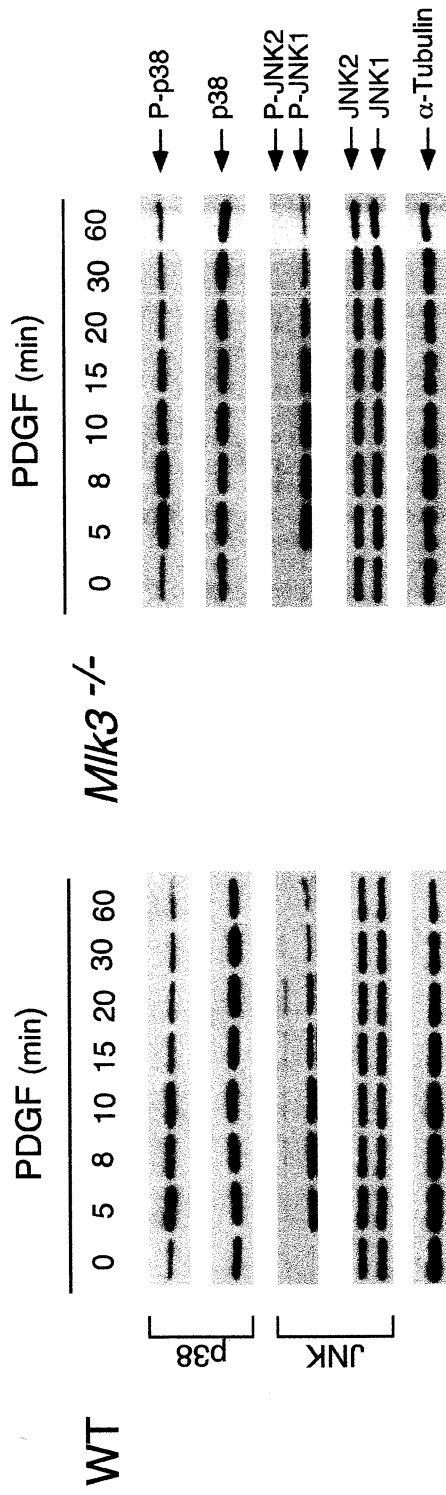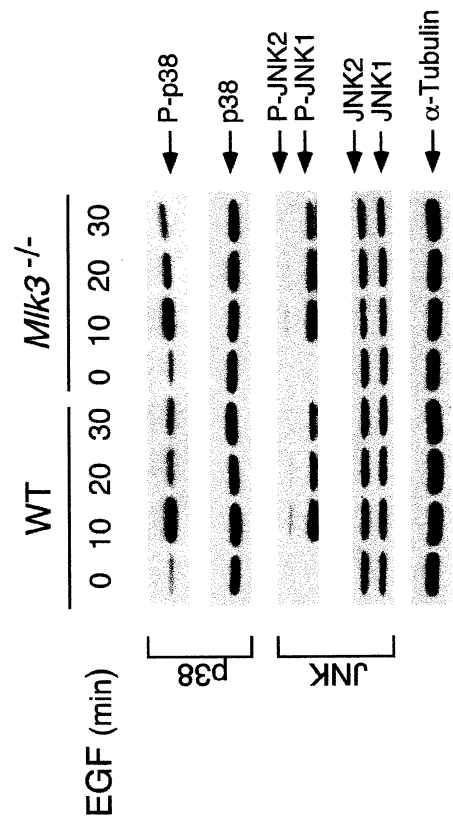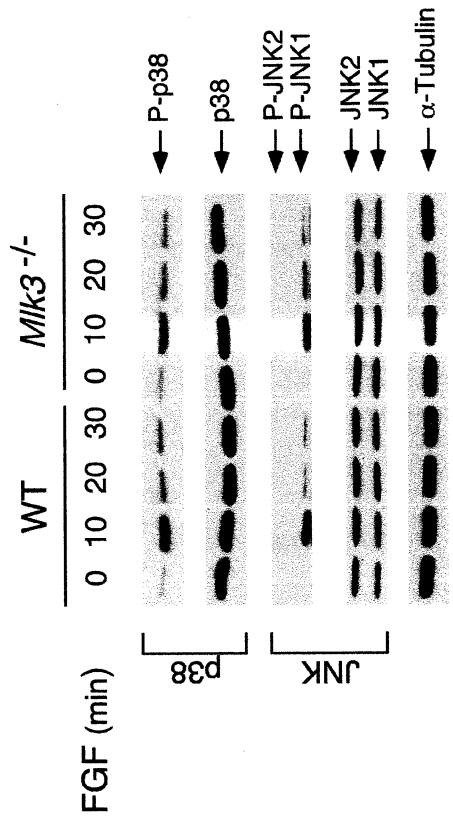
Figure IV.10. MLK3-deficiency alters MAP kinase and transcription factor activation during adipocyte differentiation.

(A) MLK3-deficiency alters MAPK phosphorylation during adipose differentiation. The activation of p38 MAPK, JNK, and ERK during *in vitro* differentiation of MEF to adipocytes was examined by immunoblot analysis.

(B) MLK3-deficiency increases the expression and phosphorylation of C/EBP transcription factors. The phosphorylation of C/EBPα and C/EBPβ during adipose differentiation was examined by immunoblot analysis using antibodies to phospho-C/EBPα (Ser-222/226) and phospho-C/EBPβ (Thr-235). The amount of C/EBP in each assay was examined by probing immunoblots with antibodies to C/EBPα and C/EBPβ.
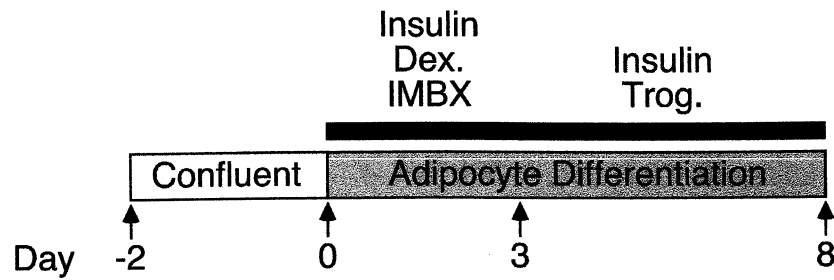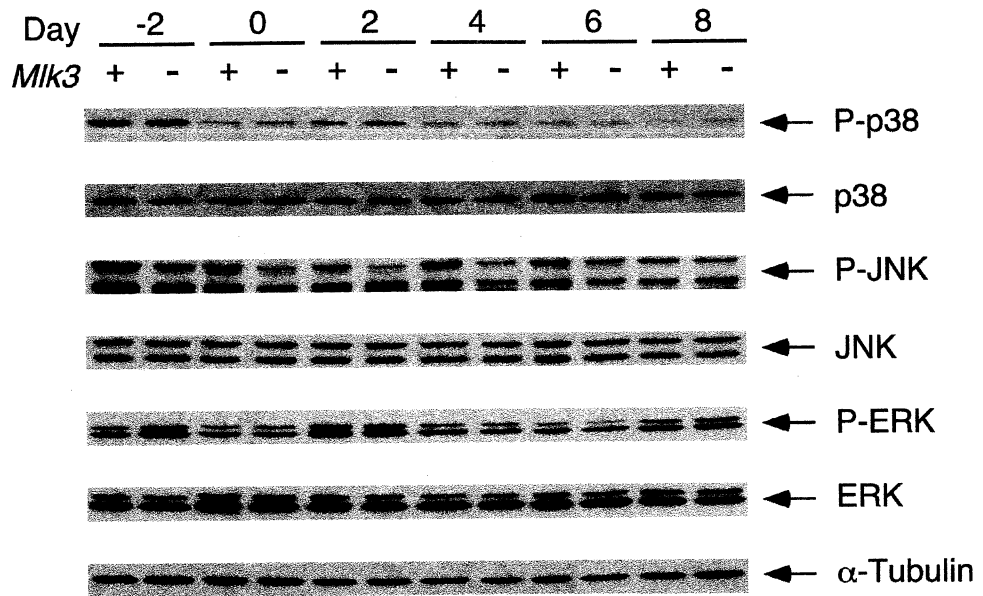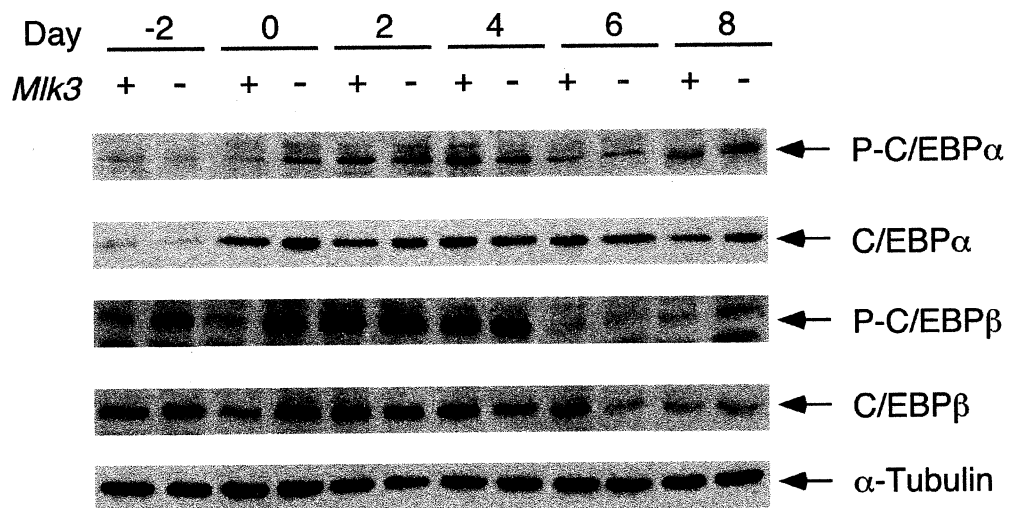
Figure IV.11. MLK3-deficiency increases adipocyte differentiation *in vitro*.

(A) The morphology of WT and $Mlk3^{-/-}$ MEF before differentiation (Control) and following adipocyte differentiation (8 days) was examined by phase contrast microscopy and by staining accumulated fat droplets with Oil-Red-O.

(B) The number of adipocytes observed following *in vitro* differentiation of WT, $Mlk3^{-/+}$, and $Mlk3^{-/-}$ MEF was counted. The data are presented as the relative number of adipocytes (mean SD; n = 3).

(C) The effect of the presence or absence of insulin and troglitizone during the final 5 differentiation days of WT and $Mlk3^{-/-}$ MEF was examined. The data are presented as the relative number of adipocytes (mean SD; n = 3).

133

A

Insulin
Trog.

Confluent | Adipocyte Differentiation

Day   -2   0   3   8

Insulin
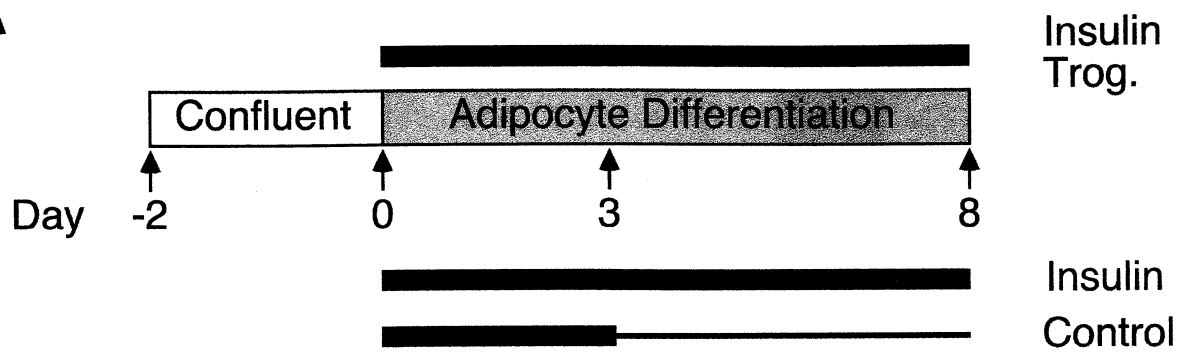
Control

WT   Mlk3 -/-
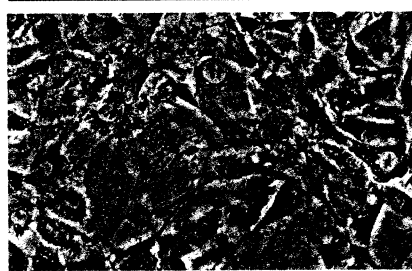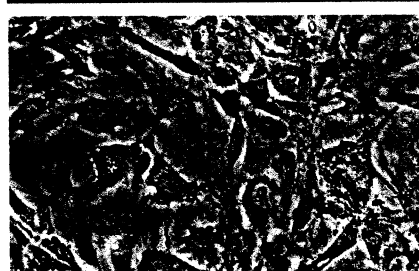
Control
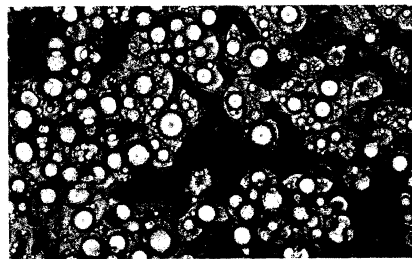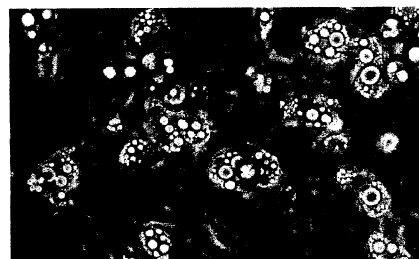
Differentiated
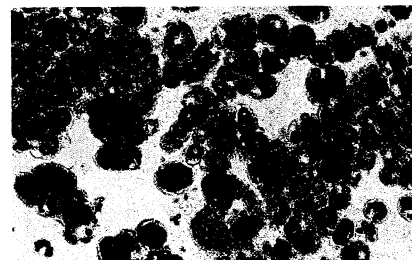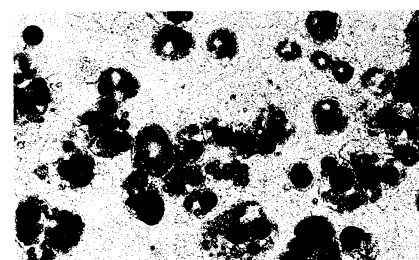
Oil-Red-O

B
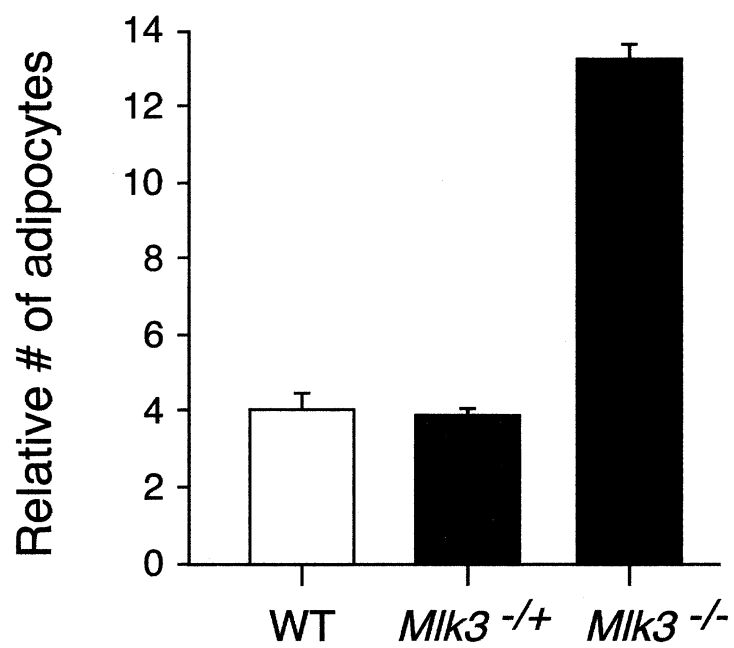
C
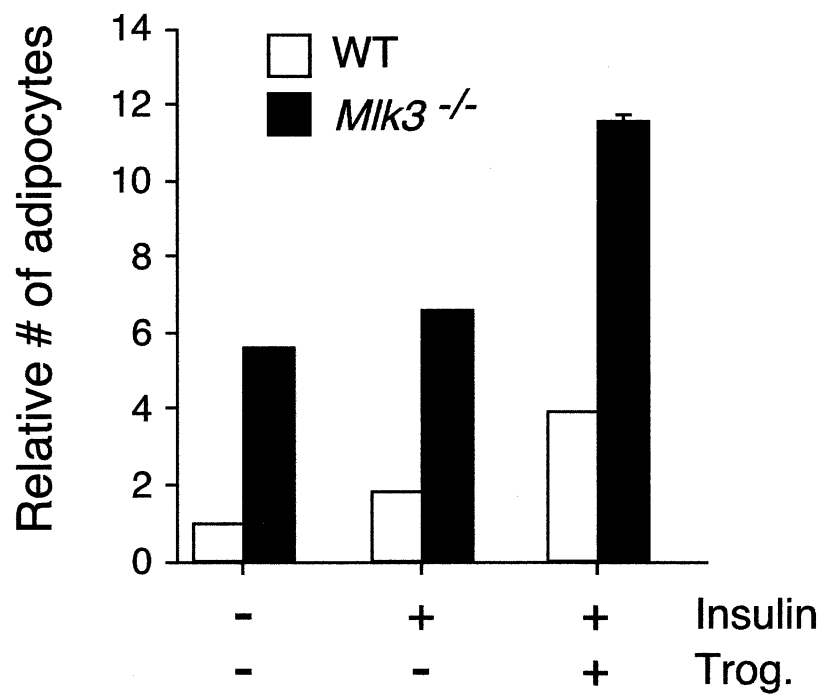
CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Due to the large number of protein kinases involved in MAPK cascades, regulation of the MAPK pathway is needed in order to elicit an appropriate response. My thesis work examined several ways of mediating MAPK signaling specificity using molecular and biochemical techniques and animal models. Specifically, I examined the regulation of MAP kinases by investigating: (1) the docking interactions between MAPK and MAP2K (Chapter II); (2) the differential activation of MAPK by MAP2K (Chapter III); and (3) the selective involvement of MLK MAP3K (Chapter IV and Appendix). In addition, I analyzed what effect MAPK signaling specificity has on biological function, particularly cell proliferation and adipocyte differentiation (Chapter III and Chapter IV). To conclude this dissertation, I will summarize the results below and discuss possible future directions.

Regulation of MAPK by docking interactions

Docking domains are present in many proteins that interact with MAPK to regulate signaling specificity. In Chapter II, I identified a p38 MAPK docking site, a D domain, in the NH_2-terminal region of the MKK3b and MKK6 (Figure V.1). Binding of MKK3b or MKK6 to p38 MAPK increases p38 MAPK activation. My data demonstrates for the first time a specific interaction between a MAP2K and the p38 MAP kinase. Recent studies using X-ray crystallography and mass spectrometry provide structural details on p38α - pepMKK3b interactions (Weston and Davis 2002; Chang et al. 2002; Lee et al. 2004). These studies

demonstrate that the hydrophobic residues in the MKK3b D domain directly interact with hydrophobic residues in a COOH-terminal docking groove of p38α MAPK.

The formation of MAP2K - p38 MAPK complexes appears to have functional significance because D domain - p38 MAPK interactions increase p38α MAPK activation and are necessary for p38β MAPK activation (Figure II.1, II.3). Interestingly, p38γ MAPK has a slightly different CD motif than p38α MAPK (Tanoue et al. 2000) and is only weakly activated by MKK6 (Alonso et al. 2000). This suggests that docking interactions may contribute to the selective activation of other p38 MAPK isoforms. Future studies should focus on determining whether D domain - MAPK interactions contribute to the activation of p38γ and p38δ MAPK.

Docking interactions may also contribute to the selective activation of the MAP2K. For example, the MAP2K, MKK4, contains a NH_2-terminal MAPK binding site that mediates JNK activation (Xia et al. 1998). In Chapter III, I demonstrate that MKK4 also activates the p38 MAPK pathway. Future studies should focus on characterizing this proposed docking site and analyzing the affect that this docking interaction has on p38 MAPK activation. Together, these studies will provide further insight into how docking interactions modulate p38 MAPK signaling specificity. These docking interactions may have biological significance. For example, the anthrax lethal factor is a protease that destroys the D domains of MAP2K and inhibits MAP kinase activation during pathogenic infection (Duesbery et al. 1998).

Figure V.1. The NH$_2$-terminal regions of the MAP2K MKK3b and MKK6 contain a p38 MAPK docking site.

The D domain, a p38 MAPK docking site, is identified in the NH$_2$-terminal regions of MKK3b and MKK6. Binding of MKK3b or MKK6 to p38 MAPK increases p38 MAPK activation. The formation of the MAP2K - p38 MAPK complexes appears to have functional significance because D domain - p38 MAPK interactions increase p38α MAPK activation and are necessary for p38β MAPK activation. This is the first demonstration of a specific interaction between MAP2K and the p38 MAP kinase.

P Thr 211
P Ser207
MAP2K
Thr 180
Tyr 182 P
β3
A

P Thr 211
P Ser 207
MAP2K
Active site
D-domain
MAPK docking
Thr 180
Tyr 182
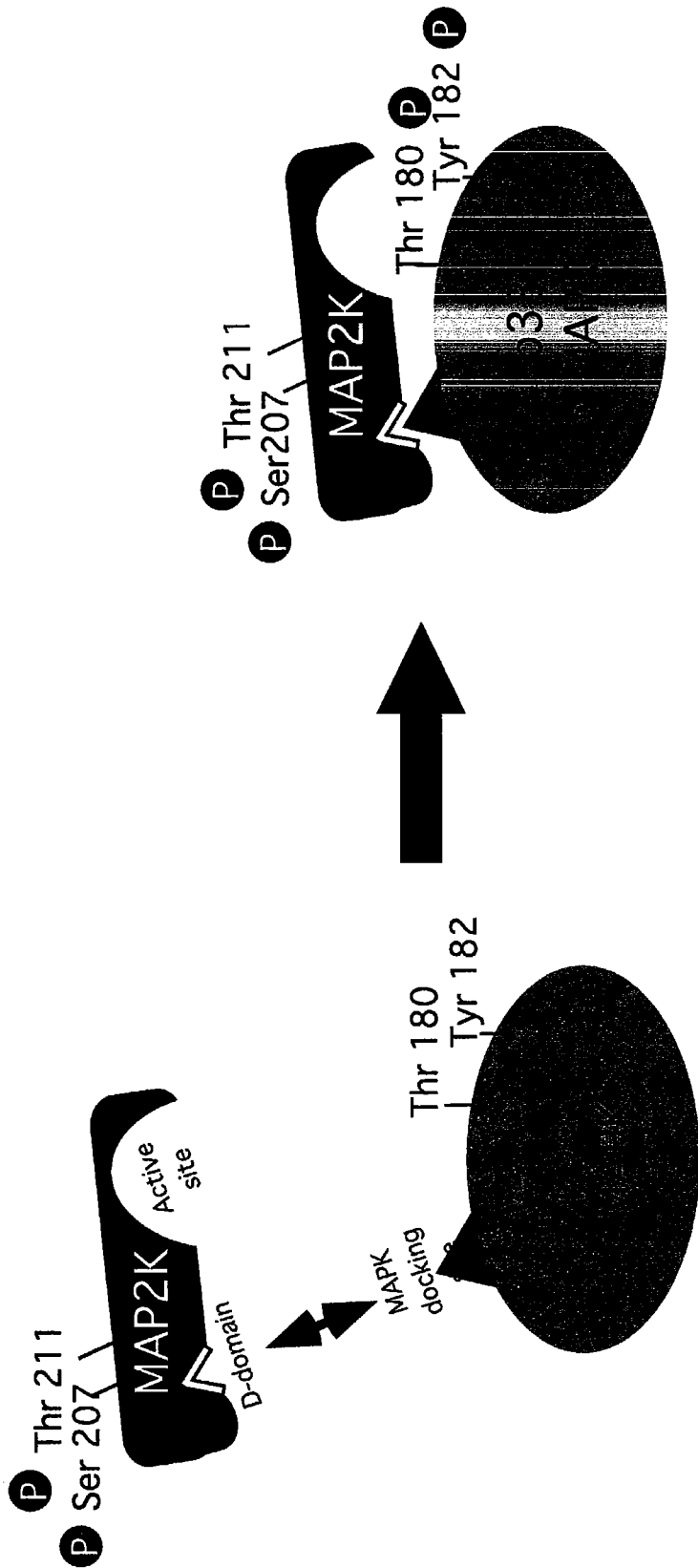
Regulation of MAPK by MAP2K

MAP2K differentially activate the MAP kinases in order to regulate signaling specificity. In Chapter III, I demonstrate that the p38 MAP kinase is activated *in vivo* by three different MAP2K: MKK3, MKK4, and MKK6 (Figure V.2). I demonstrate that MKK3 and MKK6 are essential for TNFα-stimulated activation of p38 MAPK. In contrast, MKK3, MKK4, and MKK6 all contribute to p38 MAPK activation in response to UV radiation. This suggests that the mechanism of p38 MAPK activation depends on the specific stimulus examined. In addition, MKK3 and MKK6 are selective activators of the p38 MAP kinase; they do not activate JNK. MKK4, however, is the first MAP2K reported to activate two separate groups of MAPK. Thus, MKK4 may represent a site of signal integration that coordinates overall MAP kinase activation in response to growth factors, cytokines, and environmental stress.

MKK4-deficiency caused no significant difference in p38 MAPK activation in wild-type or $Mkk4^{-/-}$ MEF (Tournier et al. 2001) (Figure III.8). However, MKK4-deficiency suppressed p38 MAPK activation in $Mkk3^{-/-} Mkk6^{-/-}$ MEF (Figure III.8; Figure III.9). Together these data suggest that MKK4 displays a redundant role in p38 MAPK activation under the conditions examined. The relevant contribution of MKK3, MKK4, and MKK6 in p38 MAPK activation may therefore depend on the cell environment and the cell-type. For example, different levels of MAP2K kinase activity may affect p38 MAPK activation. Basal unphosphorylated MKK6 activates p38α MAPK, while constitutively active MKK6 activates both p38α and p38γ MAPK (Alonso et al. 2000). This preferential MAPK activation appears to depend on different stimuli as well. Unphosphorylated MKK4 affects MAPK activation in response to TNFα, while phosphorylated MKK4 affects MAPK activation in response to UV (Tournier et al. 2001).

In addition, the level of MAP2K expression may differentially affect p38 MAPK activation. Peripheral T-cell p38 MAPK activity is primarily dependent on MKK3, while thymocyte p38 MAPK activity is primarily dependent on MKK6 (Tanaka et al. 2002). This suggests that the relevant contribution of MAP2K in p38 MAPK activation is also cell-type specific. Interestingly, $Mkk4^{-/-}$ mice are early embryonic lethal due to liver apoptosis (Yang et al. 1997; Ganiatsas et al. 1998; Nishina et al. 1999). This lethality may be due, in part, to defective p38 MAPK activity during hepatogenesis, for a recent study proposed a role for p38 MAPK activation in hepatocyte proliferation and liver development (Awad et al. 2000).

Understanding the relevant contribution of MAP2K to p38 MAPK activation represents a critical goal for future experiments. Since this contribution may depend on the cell-type and the cell environment, future experiments should focus on context dependent effects of MKK3, MKK4, and MKK6. Specifically, MKK3, MKK4, and MKK6 may have non-redundant roles in T-cells and hepatocytes and may affect p38 MAPK activation in response to different stimuli. In addition, MKK3, MKK4, and MKK6 may selectively activate particular p38 MAPK isoforms. In a preliminary study of UV-stimulated fibroblasts, MKK4 and MKK6 appear to activate one p38 MAPK isoform (p38α), while MKK3 appears to activate two isoforms of p38 MAPK (p38α and possibly p38β). Future experiments should focus on this observation and provide insight into the mechanism of total p38 MAPK activation *in vivo*. Together, results of these questions will advance our understanding of the individual roles MKK3, MKK4, and MKK6 in p38 MAPK activation.

Figure V.2. The p38 MAP kinase is activated *in vivo* by three different MAP2K: MKK3, MKK4, and MKK6.

MKK3, MKK4, and MKK6 contribute to p38 MAPK activation in response to UV radiation. In contrast, MKK3 and MKK6 are essential for TNFα-stimulated activation of p38 MAPK. This suggests that the mechanism of p38 MAPK activation depends on the specific stimulus examined. In addition, MKK3 and MKK6 are selective activators of the p38 MAP kinase; they do not activate JNK. MKK4, however, is the first MAP2K reported to activate two separate groups of MAPK and may represent a site of signal integration to coordinate MAPK activation.
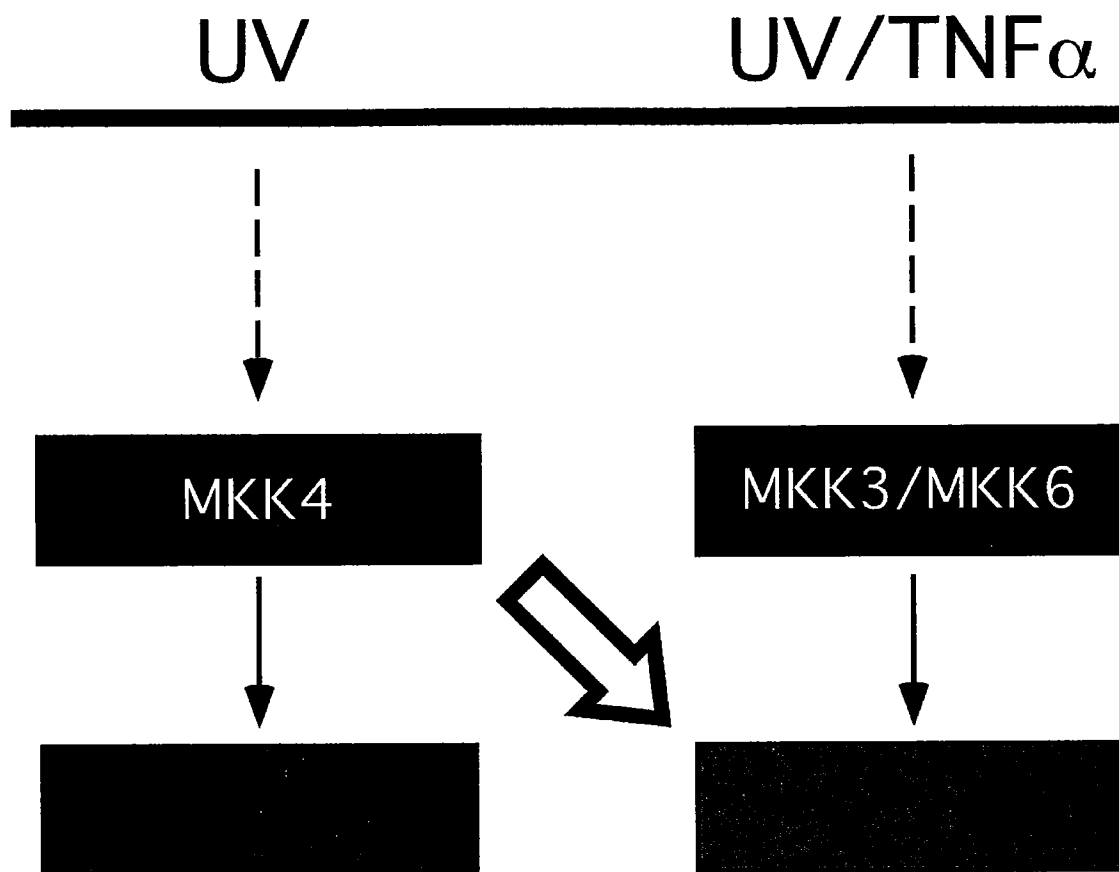
Regulation of MAPK by MAP3K

MAP3K selectively activate different MAP kinases to regulate signaling specificity. In Chapter IV, I demonstrate that the MLK group of MAP3K selectively regulates the JNK pathway. Specifically, I show that the mixed-lineage protein kinase 3 (MLK3) is a component of the TNFα signaling pathway that activates JNK (Figure V.3). MLK3 mediates the immediate activation of JNK in response to TNFα. In addition, MLK3-deficiency causes a selective reduction in TNFα-stimulated JNK activation in primary MEF. This suggests that MLK3 is required for maximal TNFα-stimulated JNK activation. In contrast, MLK3 is not required for TNFα-stimulated activation of NF-κB and p38 MAPK. Together, these data suggest that MLK3 is selectively involved in TNFα-stimulated JNK activation. Indeed, my studies demonstrate that MLK3 has a non-redundant role in TNFα signaling and represents a point of divergence to mediate distinct MAP kinase activation.

The *Mlk3* gene is the first MLK MAP3K family member reported to be targeted by gene disruption in mice. MEF isolated from *Mlk3*$^{-/-}$ mice exhibit a decrease of early TNFα-stimulated JNK activation (Figure IV.5; Figure IV.6). Although this JNK activation is suppressed in the MLK3-deficient MEF, a low level of JNK activity still remains. This observation suggests that a second MAP3K may contribute to TNFα-stimulated JNK activation in MLK3-deficient cells. The identity of this MAP3K is not clear, but several MAP3K have been previously implicated in the TNFα signaling pathway. Two MAP3K appear to have specific roles in TNFα signaling and probably do not contribute to the low level of JNK activity. ASK1 contributes to the late phase of JNK activation, while MEKK3 mediates p38 MAPK activation by directly interacting with RIP1 (Lee et al. 2003; Tobiume et al. 2001). In contrast, TAK1 appears to play a general role in TNFα signaling and may contribute to JNK activation in *Mlk3*$^{-/-}$ MEF. TAK1 has been

implicated in the immediate activation of JNK through its association with TRAF2 (Ishitani et al.

2003; Takaesu et al. 2003). A second explanation is that the low level of JNK activity is

mediated by an unidentified MAP3K, particularly a member of the MLK family. This idea is

consistent with the fact that functional redundancy exists among MLK, as MLK2 or DLK also

mediates JNK activation (Gallo and Johnson 2002).

Interestingly, TNFα-stimulated activation of p38 MAPK is increased in the $Mlk3^{-/-}$ MEF

(Figure IV.5; Figure IV.6). Recent studies of JNK-deficient cells also demonstrate increased p38

MAPK activation in response to TNFα (Morton et al. 2004). In contrast, decreased p38 MAPK

activation caused an increase in JNK activity during macrophage differentiation (Hall and Davis

2002). Together, these data suggest that negative cross-talk exists between the JNK and the p38

MAPK pathways. The cross-talk that I observed may be mediated by TNF receptor adaptor

proteins that bind different MAP3K. For example, the TRAF2 adapter protein is required for

TNFα-stimulated JNK activation (Yeh et al. 1997), however, whether TRAF2 directly or

indirectly activates MLK3 remains unclear. Conversely, the RIP1 adaptor protein is essential for

TNFα-stimulated p38 MAPK activation (Lee et al. 2003), but whether MLK3 directly or

indirectly inhibits RIP1-mediated p38 MAPK activation has not been determined. Thus,

understanding the mechanism of MLK3 activation is critical for understanding the regulation of

TNFα-activated MAP kinases.

Future experiments should therefore focus on understanding the relevant contribution of

MAP3K in TNFα-stimulated MAPK activation. Specifically, the combined effect of a

MLK3/MAP3K-deficiency on JNK and p38 MAPK activation should be examined. Mice with

compound mutations in $Mlk3$ and MAPK3K genes ($Mlk2$, Dlk, $Tak1$, $Ask1$, $Mekk3$), or siRNA

inhibition of MAPK3K genes in $Mlk3^{-/-}$ MEF may be two approaches used to analyze TNFα-

stimulated MAPK activation. A second goal should be to understand the mechanism of TNFα-stimulated MLK3 activation. Analysis of the regulatory relationship between TRAF2, RIP1, and MLK3 may provide insight into the role MAP3K have in mediating TNFα signaling specificity.

Figure V.3. MLK3 is a component of the TNF signaling pathway that activates JNK.

MLK3 mediates the immediate activation of JNK in response to TNFα and is required for

maximal TNFα-stimulated JNK activation. A low level of JNK activity still remains in MLK3-

deficient MEF, suggesting that a second MAP3K may contribute to TNFα-stimulated JNK

activation in MLK3-deficient cells. In contrast, MLK3 is not required for TNFα-stimulated

activation of NF-κB and p38 MAPK. These data demonstrate that MLK3 is selectively involved

in TNFα-stimulated JNK activation. Interestingly, TNFα-stimulated activation of p38 MAPK is

increased in MLK3-deficient MEF, suggesting that negative cross-talk exists between the JNK

and the p38 MAPK pathways.
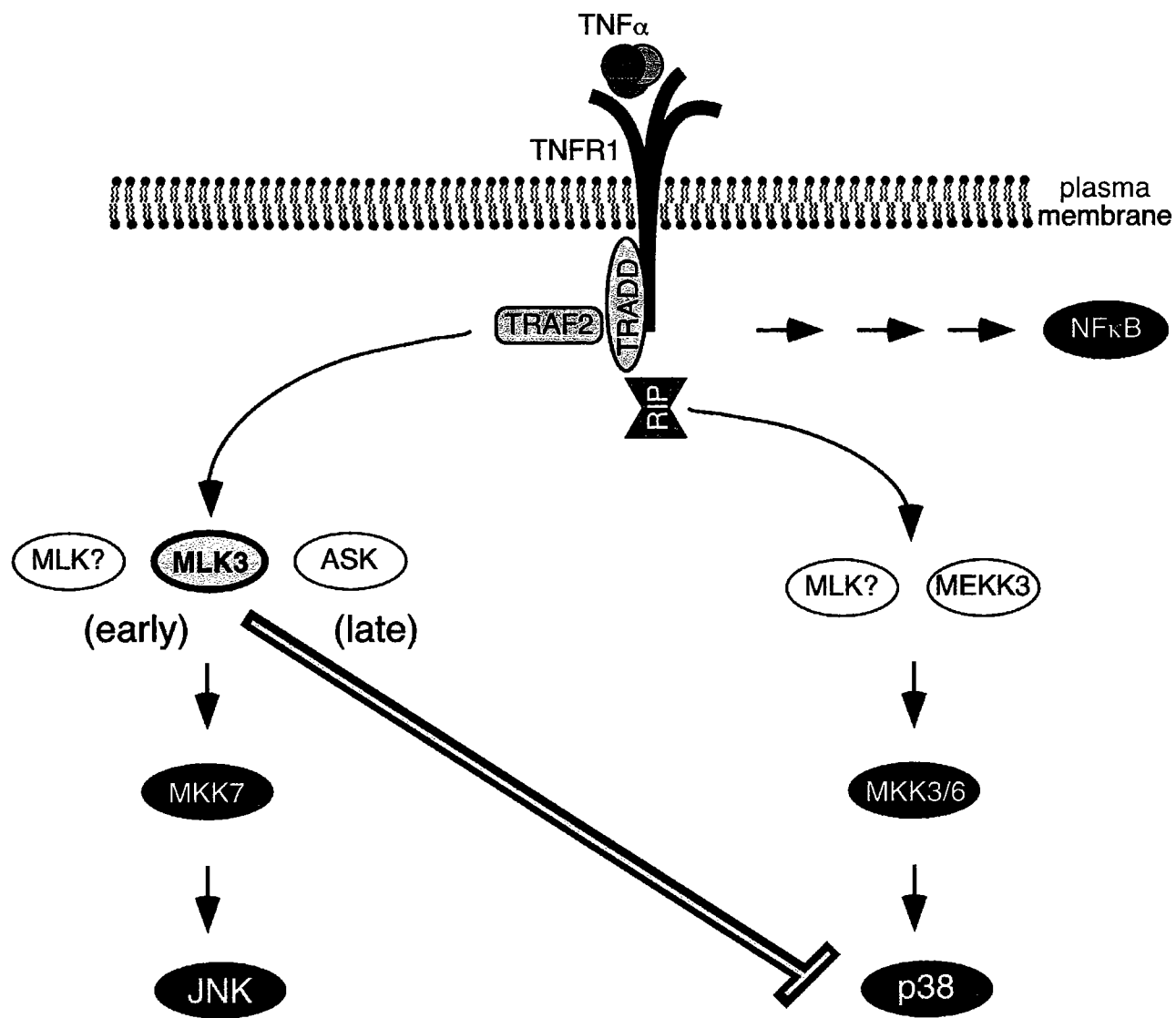
MAP kinases and cell cycle progression

Cellular growth is a physiological process that is differentially regulated by MAP kinases. Recent reviews have focused on the role of p38 MAPK in the cell cycle (Ambrosino and Nebreda 2001; Bulavin et al. 2002; Yee et al. 2004). The p38 MAPK negatively regulates the G1/S and G2/M transitions, depending on the cell-type and the stimulus examined. For example, p38 MAPK inhibits cyclin D expression upon serum starvation (G1/S arrest), but inactivates the Cdc25B phosphatase following UV stimulation (G2/M arrest). The p38 MAPK also phosphorylates p53 and p21CIP to inhibit both G1/S and G2/M progression in response to DNA damage. This suggests that loss of p38 MAPK regulation, due to MKK3/MKK6-deficiency, may alter cell proliferation. Indeed, my data demonstrates that MKK3/MKK6-deficiency causes serum-stimulated G1/S growth defects (Figure III.2B-C; Figure III.10). $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts lose contact-growth-inhibition and fail to growth arrest in serum-free medium. Serum-starved $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts also have increased expression of cyclin D and reduced hypo-phosphorylated Rb. Interestingly, a recent report suggests that p38 MAPK phosphorylates the transcriptional repressor HBP1 and provides a mechanism to inhibit cyclin D expression (Xiu et al. 2003). Together, these data indicate a role for MKK3/MKK6 in inhibiting cell proliferation.

The inhibitory role of MKK3/MKK6 in cell proliferation suggests that MKK3/MKK6 may function as tumor suppressors. Indeed, $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts increase tumorgenesis *in vivo* (Figure III.11). Preliminary studies suggest that MKK3 contributes to the G1/S growth arrest and increased tumorgenesis (Figure III.2B-C; Figure III.10B). The $Mkk3^{-/-}$ fibroblasts exhibit reduced contact-growth-inhibition and promote tumor growth *in vivo*. However, these effects are less dramatic than those observed in the $Mkk3^{-/-}$ $Mkk6^{-/-}$ fibroblasts, suggesting

cooperation between the MAP2K. In addition, MKK4 is also implicated in growth arrest, tumorgenesis, and metastasis suppression (Molnar et al. 1997; Teng et al. 1997; Yamada et al. 2002). Future experiments should therefore focus on understanding the individual roles of MKK3, MKK4, and MKK6 in cell cycle progression and tumorgenesis.

MAP kinases and adipogenesis

Adipogenesis is a physiological process that involves the cooperation of many signaling pathways. Recent reviews have focused on the role of MAPK and the regulation of adipocyte differentiation (Rosen and Spiegelman 2000; Rosen et al. 2000; Lane et al. 1999; Rosen 2002; Camp et al. 2002). The JNK MAPK negatively regulates adipogenesis, by phosphorylating and inhibiting the transcription factor PPARγ. In contrast, the p38 MAPK phosphorylates members of the C/EBP transcription factor family to promote adipocyte differentiation. This suggests that negative cross-talk exists between the JNK and the p38 MAPK pathways during adipose differentiation. Thus, a MLK3-deficiency may disrupt the MAPK cross-talk and affect adipogenesis. Indeed, I show that MLK3-deficiency increases the adipogenic potential of primary MEF (Figure IV.10; Figure IV.11). JNK activity is decreased, while p38 MAPK activity is modestly increased in $Mlk3^{-/-}$ MEF. These MEF also exhibit increased expression and phosphorylation of C/EBPα and C/EBPβ. Furthermore, MLK3-deficiency increases adipocyte differentiation from MEF *in vitro*. Together, these data suggest that MLK3-mediated MAPK activation plays a role in the regulation of adipogenesis. However, the precise mechanism of regulation remains unclear. Interestingly, a recent report indicates that C/EBPβ expression is increased by the p38-dependent transcription factors CREB, ATF1, and ATF2 during adipose differentiation (Zhang et al. 2004). Other transcription factors, such as PPARγ and C/EBPδ, may

also be affected by MLK3-deficiency and provide a mechanism for MLK3-mediated regulation of adipocyte differentiation.

My observation that MLK3-deficiency increases adipogenesis *in vitro* suggests that *Mlk3*$^{-/-}$ mice may have altered adiposity *in vivo*. Preliminary studies suggest that *Mlk3*$^{-/-}$ mice fed a standard chow diet do not exhibit increased adiposity *in vivo*. Future studies should therefore examine *Mlk3*$^{-/-}$ mice that are environmentally challenged. For example, *Mlk3*$^{-/-}$ mice should be fed a high fat diet or exposed to cold temperature. Metabolic effects of MLK3-deficiency should be determined. In addition, MAPK signaling should also be examined in the adipose tissues. Together, these data will advance our understanding of the role of MAPK in adipogenesis and metabolic homeostasis.

Conclusion

In conclusion, the work presented in this dissertation focuses on understanding the regulation and function of stress-activated MAPK signal transduction pathways. I used molecular and biochemical techniques to examine how MAP2K and MAP3K mediate signaling specificity and to define their role in the MAPK cascade. In addition, I used gene targeted disruption strategies to determine the *in vivo* role of MAP2K and MAP3K in MAPK activation. Specifically, I examined the regulation and function of the JNK and the p38 MAPK pathways by analyzing: (1) docking interactions between p38 MAPK and MKK3 or MKK6 (Chapter II); (2) the differential activation of p38 MAPK by MKK3, MKK4, and MKK6 (Chapter III); and (3) the selective involvement of MLK in JNK and p38 MAPK activation (Chapter IV and Appendix). I also examined the role of MKK3 and MKK6 in cell proliferation and the role of MLK3 in adipocyte differentiation (Chapter III and Chapter IV). Together, these data provide insight into the regulation and function of MAPK signal transduction cascades. Understanding these signaling pathways is essential for understanding and treating human diseases.

APPENDIX

THE MIXED-LINEAGE KINASE DLK IS ESSENTIAL

FOR EARLY EMBRYONIC DEVELOPMENT

Summary

The DLK protein kinase is a member of the mixed-lineage protein kinase group of MAP3K that is implicated in the regulation of the JNK pathway in neurons. To examine the function of DLK, I used a targeted gene disruption strategy in embryonic stem cells to create mice with a germ-line mutation of the *Dlk* gene. In this Appendix, I demonstrate that *Dlk*$^{-/-}$ mice are not viable and this mutation causes an unexpected early lethal phenotype. The mechanism of the requirement of DLK for viability is not established, but DLK is expressed in the trophoblast giant cells of the placenta, and may therefore play a role in embryonic development.

The *Dlk*$^{-/-}$ mice were generated in collaboration with Dr. Richard Flavell's laboratory at the Yale University School of Medicine. I constructed the targeting vector and initially characterized the embryonic lethality. Claire Weston and Beth Doran genotyped the E18.5 embryos in Figure A.2 and contributed to the *in situ* hybridization data in Figure A.3. Tamara Barrett, Judith Reilly, and Vicky Benoit assisted with the mouse breeding and genotyping.

Introduction

The mechanism of JNK activation by MAP3K in neurons is incompletely understood. It is established that JNK activation is mediated by two different MAP kinase kinases (MAP2K), MKK4 and MKK7, which are activated by various MAP kinase kinase kinases (MAP3K) (Davis 2000). However, the identity of relevant MAP3K in MAPK activation in neurons remains unclear.

The dual-leucine-zipper-bearing kinase (DLK/ZPK/MUK) is a member of the mixed-lineage kinase group of MAP3K that activates the JNK pathway (Holzman et al. 1994; Reddy and Pleasure 1994; Hirai et al. 1996). Mixed-lineage kinases share a common protein kinase domain, followed by a leucine zipper region that mediates homodimerization and activation of these proteins (Gallo and Johnson 2002). The DLK protein kinase is closely related to the leucine-zipper kinase (LZK), while other members of the mixed-lineage kinase family (MLK1, MLK2, MLK3, MLK4 and ZAK) are more distinct. DLK and LZK share the characteristic MLK kinase domain, but in contrast to the other MLK family members, DLK and LZK contain two leucine-zipper motifs. The structure of DLK also includes a proline rich region of unknown function in the COOH-terminus. Biochemical studies have showed that DLK can selectively activate MKK7, an activator of JNK (Merritt et al. 1999), and that DLK and MKK7 can bind to the JIP scaffold proteins to regulate JNK activation (Morrison and Davis 2003).

Many of the mixed-lineage kinases are expressed in a limited number of tissues (Gallo and Johnson 2002). Previous studies have shown that DLK is abundantly expressed in the embryonic and adult brain, including developing cells of the telencephalon and differentiated cells of the neural cortex (Holzman et al. 1994; Blouin et al. 1996; Nadeau et al. 1997). This

expression is consistent with a possible role for DLK as a mediator of JNK activation in neurons. This possibility is consistent with recent studies showing that overexpressed DLK disrupts neural cell migration and telencephalon morphogenesis (Hirai et al. 2002), and that overexpressed DLK induces neuronal apoptosis (Xu et al. 2001). However, DLK is also expressed in differentiating epithelial cells and regenerating hepatocytes, suggesting multiple roles for DLK during development (Nadeau et al. 1997; Douziech et al. 1998). The role of DLK in JNK signaling and the relevance of DLK in MAPK activation in neurons therefore remain unclear.

The purpose of this study was to investigate the biological function of the DLK protein kinase. My approach was to use a targeted gene disruption strategy to examine the effect of DLK-deficiency in mice. I report that DLK is required for early embryonic development and suggest that DLK plays an essential role in JNK activation and MAP kinase regulation.

Materials and Methods

Mice. A 129/SvJ strain *Dlk* genomic clone was isolated from a BAC library by hybridization analysis using a probe derived from a *Dlk* cDNA. A targeting vector designed to disrupt the *Dlk* gene (Figure A.1a) was constructed using standard techniques. ES cells (strain 129svev) were electroporated with this vector and selected with 200 μg/ml G418 (Invitrogen) and 2 μM gangcyclovir (Syntex). Two *Dlk^{-/+}* ES cell clones identified by Southern blot analysis were injected into C57BL/6J blastocysts to create chimeric mice. The chimeric mice were used to obtain germ-line transmission of the disrupted *Dlk* allele and the mice were backcrossed for ten generations to the C57BL/6J strain (Jackson Laboratories). The mice were housed in a facility accredited by the American Association for Laboratory Animal Care (AALAC). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

Genotype analysis. The genotype of mice and embryos was examined by Southern blot analysis of *Xba*I or EcoRV (5' probe) and *Bam*HI (3' probe) digested genomic DNA using 5' probe primers 5'-GAATAGTACGTCTCTTGGAGTCAAGT-3' and 5'-TGGAGGCAGGAGGATCAAACATTCAA-3' and 3' probe primers 5'-GAACAGCAAACCACGAAATCG-3' and 5'-TACTTCTTCCCGCCACTCTG-3' (Figure A.1). The wild-type (260bp) and disrupted (160 bp) *Dlk* alleles were also detected by PCR analysis using the primers 5'-CCTGGTACACATTCTGTGTCC-3', 5'-CCTACAAAGATGTAGATTCCTC-3', and 5'-GTGGAATGTGTGCGAGGCCA -3'.

In situ hybridization. Embryos from timed matings were fixed in 10% formalin for 24 hr,

dehydrated, and embedded in paraffin for sectioning (4 μm). Digoxigenin-labeled anti-sense

oligoprobes were generated as follows: DLK: 5'-TTCAAGGAAGCCACTGCCAC-3', 5'-

CTTTCGCATAGAAGCCTCAC-3', 5'-TTCCCACACCCCAGATGATG-3', 5'-

ACCTTCTTCACAGCTACCTC-3', 5'-CAGGTAATTCATGCCACCAG-3', 5'-

TGCTCTTGTCACTCAGCTCC-3'. Corresponding sense oligoprobes were also prepared.

Oligoprobes were hybridized overnight at 37 °C in a humid chamber. The signal was enhanced

using a Tyramide Amplification System (Biogenex) followed by incubation (15 min) with

streptavidin-conjugated horseradish peroxidase (Biogenex). The amplified product was

developed with 3,3'-diaminobenzidine (Vector Laboratory), and counterstained briefly with

Mayer's hematoxylin (Sigma).

Results and Discussion

Disruption of the murine *Dlk* gene

I constructed a targeting vector to disrupt the murine *Dlk* gene. This vector was designed to replace exons 2 - 6 with a Neo^R cassette (Figure A.1a). This region of the *Dlk* gene includes the translational initiation codon and the kinase domain. The vector was linearized and electroporated into embryonic stem (ES) cells to obtain homologous recombination within the *Dlk* gene. Four *Dlk*$^{-/+}$ ES cell clones were identified by Southern blot analysis. Two of these clones were injected into C57BL/6J blastocysts to create male chimeric mice that were bred to obtain germ-line transmission of the disrupted *Dlk* allele. The mice were backcrossed to the C57BL/6J strain background for ten generations. The *Dlk*$^{-/+}$ mice were found to be viable, fertile, and had a normal life-span.

DLK is essential for early embryonic development

Genomic DNA isolated from the progeny obtained from crossing *Dlk*$^{-/+}$ mice was examined by PCR and Southern blot analysis to identify wild-type, *Dlk*$^{-/+}$, and *Dlk*$^{-/-}$ mice (Figure A.1b, c). However, the number of wild-type, heterozygous, and homozygous knockout littermates obtained from these crosses did not conform to the expected Mendelian inheritance. Indeed, no *Dlk*$^{-/-}$ mice were detected (Figure A.2). This observation suggests that homozygous deletion of *Dlk* may cause embryonic lethality. This is consistent with previous studies demonstrating DLK expression in developing mouse embryos from E11 onwards (Holzman et al. 1994; Nadeau et al. 1997). To directly investigate whether DLK-deficiency causes embryonic lethality, I examined the genotypes of embryos obtained from timed matings of *Dlk*$^{-/+}$ mice.

Analysis of E9.5, E16.5, and E18.5 embryos demonstrated a non-Mendelian ratio of wild-type, *Dlk*$^{-/+}$, and *Dlk*$^{-/-}$ embryos (Figure A.2). Because no *Dlk*$^{-/-}$ embryos were detected at or after E9.5, these data indicate that DLK is essential for early embryonic development.

DLK is expressed in the trophoblast giant cells of the placenta

The *Dlk*$^{-/-}$ embryos are not viable and this mutation causes a lethal phenotype. To further investigate the spatial and temporal pattern of DLK expression during embryonic development, *Dlk* expression was examined in wild-type embryos by *in situ* hybridization. Analysis of E14.5 embryonic brains indicate a high level of *Dlk* expression in the developing neural cortex (Figure A.3a). This is consistent with previous reports that examine *Dlk* expression in embryonic and adult neural tissues (Holzman et al. 1994; Blouin et al. 1996; Nadeau et al. 1997). Unexpectedly, a low level of *Dlk* expression was also detected in the placenta of wild-type embryos. Analysis of E12.5 embryonic placentas indicate that DLK is expressed in the trophoblast giant cells of the placenta (Figure A.3b). Since trophoblast giant cells contribute to the viability of the embryo (Rossant and Cross 2001), DLK may play an essential role in these cells during early embryonic development.

Discussion

In this study, I report that the DLK protein kinase is essential for embryonic development. Analysis of DLK-deficient mice indicates that DLK is required for embryonic viability prior to E9.5 (Figure A.2). In addition, DLK is expressed in the trophoblast giant cells of the placenta (Figure A.3b). Together, these data suggest that DLK plays a role in the development of the early embryo. The mechanism of DLK-mediated embryonic morphogenesis

remains to be established. Future experiments will therefore focus on analyzing E3.5 cultured blastocysts. Specifically, the effect of DLK-deficiency on early trophoblast development will be examined.

My analysis of DLK-deficient mice does not exclude the possibility that DLK may be required for additional functions. Previous studies suggest that DLK plays a role in JNK activation during neuronal apoptosis, neural cell migration, and telencephalon morphogenesis (Xu et al. 2001; Hirai et al. 2002). In addition, previous reports suggest a role for DLK in JNK activation in differentiating epithelial cells and ocular development (Nadeau et al. 1997; Cai et al. 2002; Weston et al. 2003; Weston et al. 2004). Together, these data implicate DLK in multiple roles in JNK signaling during embryogenesis. Future experiments will thus focus on understanding the role of DLK-mediated MAPK activation in developing and differentiated neurons. The $Dlk^{-/+}$ ES cells will be used to obtain $Dlk^{-/-}$ ES cells and differentiated into neurons to examine the effect of DLK-deficiency on JNK activation in neural tissues. The role of DLK in MAPK activation in neurons may represent a non-redundant function of DLK in developing and differentiated cells. Alternatively, DLK may have additional roles that are redundant with other MLK family members.

In conclusion, I report that the DLK protein kinase is required for early embryonic development. This identification of a function for DLK suggests essential roles for the mixed-lineage protein kinases in JNK activation and MAP kinase regulation during development.

Figure A.1. Targeted disruption of the murine *Dlk* gene.

(A) The strategy employed to disrupt the *Dlk* gene is illustrated. The structure of the wild-type *Dlk* gene and the targeting vector are shown. Homologous recombination in ES cells results in the replacement of *Dlk* exons 2 - 6 with a Neo^R cassette. Restriction enzyme sites are indicated: B (*Bam*HI), H (*Hind*III), N (*Not*I), RV (*Eco*RV), X (*Xba*I), Xh (*Xho*I). The *Xba*I, *Eco*RV, and *Bam*HI digestions of genomic DNA result in restriction fragments that can be used to distinguish between the wild-type and disrupted *Dlk* alleles by Southern blot analysis using 5' and 3' probes.

(B) DNA isolated from the tails of wild-type and *Dlk*$^{-/+}$ mice was restricted with *Bam*H1 and examined by Southern blot analysis using a 3' probe. The wild-type and disrupted *Dlk* alleles are indicated.

(C) Tail DNA was examined by PCR using primers that detect the wild-type (260 bp) and disrupted (160 bp) *Dlk* alleles.

A

Dlk locus
(Dlk+)

X — B — RV — RV — B — X — H — X — H — X H — B — B — B — B — RV

5'UTR 1   2   3 4   5 6   7 8 9 10 11   12 13   14   3'UTR

9.7 kb
EcoRV digestion

5.2 kb
Xbal digestion

2.1 kb
BamHI digestion

Targeting vector

N — RV — X — B — RV — H — RV — X — pBS — N

Homologous Recombination

Mutated Dlk
locus (Dlk-)

X — B — RV — RV — X — RV — B — H — RV — B — B — B — RV

5'UTR 1   7 8 9 10 11   12 13   14   3'UTR

2.9 kb
EcoRV digestion

4.8 kb
Xbal digestion

3.0 kb
BamHI digestion

DNA 5'probe

DNA 3'probe

B

+/+ +/-

— 3.0 kb

— 2.1 kb

C

+/+ +/-

— 260 bp

— 160 bp

Figure A.2. DLK is essential for early embryonic development.

Genomic DNA isolated from the progeny obtained from crossing $Dlk^{-/+}$ mice was examined by

PCR and Southern blot analysis. The number of wild-type, $Dlk^{-/+}$, and $Dlk^{-/-}$ littermates are

indicated.

Age	# of mice	+/+	-/+	-/-
E9.5	26	8	18	0
E16.5	24	5	19	0
E18.5	22	7	15	0
Weaned	274	71	203	0

Figure A.3. DLK is expressed in the trophoblast giant cells of the placenta.

(A) *Dlk* expression was examined by *in situ* hybrization using anti-sense probes (Magnification, x40). Sections prepared from the brain of wild-type E14.5 embryos indicated high levels of *Dlk* expression in the developing neural cortex. Control experiments were performed using sense probes.

(B) *Dlk* expression was examined by *in situ* hybrization using anti-sense probes (Magnification, x10 and x40). Low levels of *Dlk* expression was detected in the trophoblast giant cells of the placenta of wild-type E12.5 embryos. Control experiments were performed using sense probes.

A

DLK   Control
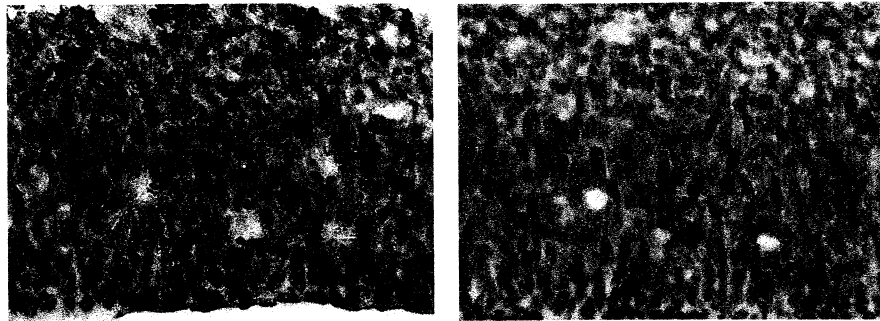
E 14.5 Cortex

B

DLK   Control

Trophoblast giant cells

Decidual reaction

Trophoblast giant cells
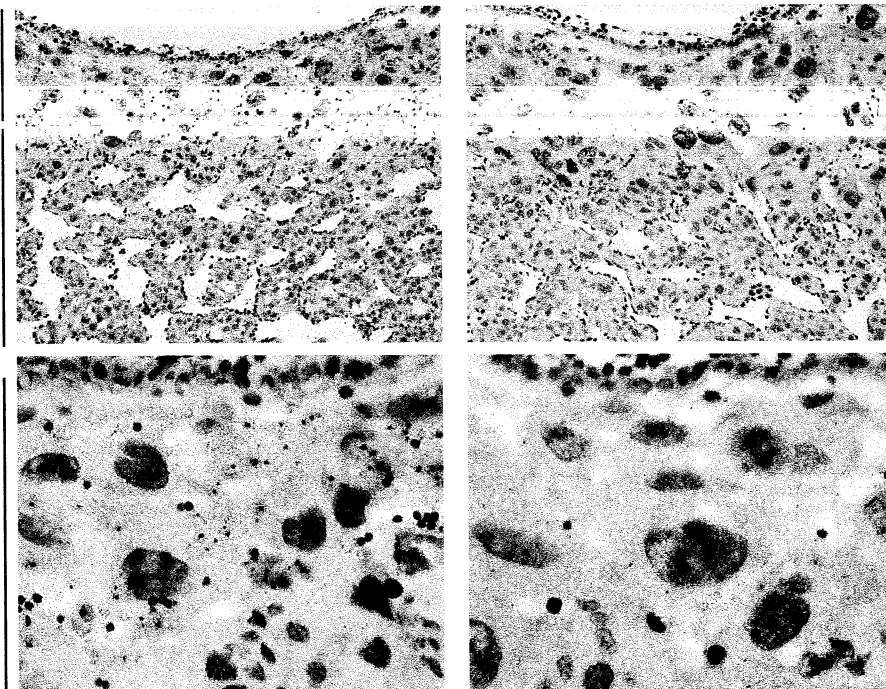
E 12.5 Placenta

REFERENCES

Adams, M., Reginato, M.J., Shao, D., Lazar, M.A., and Chatterjee, V.K. 1997. Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J Biol Chem* **272**: 5128-32.

Adams, R.H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R., and Nebreda, A.R. 2000. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* **6**: 109-16.

Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J., and Ronai, Z. 1999. Regulation of JNK signaling by GSTp. *Embo J* **18**: 1321-34.

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *Embo J* **9**: 1897-906.

Allen, M., Svensson, L., Roach, M., Hambor, J., McNeish, J., and Gabel, C.A. 2000. Deficiency of the stress kinase p38alpha results in embryonic lethality: characterization of the kinase dependence of stress responses of enzyme-deficient embryonic stem cells. *J Exp Med* **191**: 859-70.

Alonso, G., Ambrosino, C., Jones, M., and Nebreda, A.R. 2000. Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J Biol Chem* **275**: 40641-8.

Ambrosino, C. and Nebreda, A.R. 2001. Cell cycle regulation by p38 MAP kinases. *Biol Cell* **93**: 47-51.

Awad, M.M., Enslen, H., Boylan, J.M., Davis, R.J., and Gruppuso, P.A. 2000. Growth regulation via p38 mitogen-activated protein kinase in developing liver. *J Biol Chem* **275**: 38716-21.

Bardwell, L., Cook, J.G., Chang, E.C., Cairns, B.R., and Thorner, J. 1996. Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. *Mol Cell Biol* **16**: 3637-50.

Barsyte-Lovejoy, D., Galanis, A., and Sharrocks, A.D. 2002. Specificity determinants in MAPK signaling to transcription factors. *J Biol Chem* **277**: 9896-903.

Baud, V., Liu, Z.G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. 1999. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev* **13**: 1297-308.

Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J.C., Haegeman, G., Cohen, P., Fiers, W. 1996. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *Embo J* **15**: 1914-23.

Blouin, R., Beaudoin, J., Bergeron, P., Nadeau, A., and Grondin, G. 1996. Cell-specific expression of the ZPK gene in adult mouse tissues. *DNA Cell Biol* **15**: 631-42.

Bock, B.C., Vacratsis, P.O., Qamirani, E., and Gallo, K.A. 2000. Cdc42-induced activation of the mixed-lineage kinase SPRK in vivo. Requirement of the Cdc42/Rac interactive binding motif and changes in phosphorylation. *J Biol Chem* **275**: 14231-41.

Bowman, A.B., Kamal, A., Ritchings, B.W., Philp, A.V., McGrail, M., Gindhart, J.G., and Goldstein, L.S. 2000. Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein. *Cell* **103**: 583-94.

Brancho, D., Tanaka, N., Jaeschke, A., Ventura, J.J., Kelkar, N., Tanaka, Y., Kyuuma, M., Takeshita, T., Flavell, R.A., Davis, R.J. 2003. Mechanism of p38 MAP kinase activation *in vivo*. *Genes Dev* **17**: 1969-78.

Brancho, D., Ventura, J.J., Jaeschke, A., Doran, B., Flavell, R.A., Davis, R.J. Role of MLK3 in the regulation of MAP kinase signaling cascades. *Mol Cell Biol* In press.

Buchsbaum, R.J., Connolly, B.A., and Feig, L.A. 2002. Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade. *Mol Cell Biol* **22**: 4073-85.

Bulavin, D.V., Amundson, S.A., and Fornace, A.J. 2002a. p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony. *Curr Opin Genet Dev* **12**: 92-7.

Bulavin, D.V., Demidov, O.N., Saito, S., Kauraniemi, P., Phillips, C., Amundson, S.A., Ambrosino, C., Sauter, G., Nebreda, A.R., Anderson, C.W., Kallioniemi, A., Fornace, A.J., Jr., and Appella, E. 2002b. Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. *Nat Genet* **31**: 210-5.

Bulavin, D.V., Higashimoto, Y., Popoff, I.J., Gaarde, W.A., Basrur, V., Potapova, O., Appella, E., and Fornace, A.J., Jr. 2001. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* **411**: 102-7.

Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace, A.J., Jr. 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *Embo J* **18**: 6845-54.

Cai, Y., Lechner, M.S., Nihalani, D., Prindle, M.J., Holzman, L.B., and Dressler, G.R. 2002. Phosphorylation of Pax2 by the c-Jun N-terminal kinase and enhanced Pax2-dependent transcription activation. *J Biol Chem* **277**: 1217-22.

Camp, H.S., Ren, D., and Leff, T. 2002. Adipogenesis and fat-cell function in obesity and diabetes. *Trends Mol Med* **8**: 442-7.

Camp, H.S., Tafuri, S.R., and Leff, T. 1999. c-Jun N-terminal kinase phosphorylates peroxisome proliferator-activated receptor-gamma1 and negatively regulates its transcriptional activity. *Endocrinology* **140**: 392-7.

Camps, M., Nichols, A., and Arkinstall, S. 2000. Dual specificity phosphatases: a gene family for control of MAP kinase function. *Faseb J* **14**: 6-16.

Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., and Arkinstall, S. 1998. Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science* **280**: 1262-5.

Chadee, D.N. and Kyriakis, J.M. 2004. MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. *Nat Cell Biol*.

Chang, C.I., Xu, B.E., Akella, R., Cobb, M.H., and Goldsmith, E.J. 2002. Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. *Mol Cell* **9**: 1241-9.

Chen, G. and Goeddel, D.V. 2002. TNF-R1 signaling: a beautiful pathway. *Science* **296**: 1634-5.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., and Cobb, M.H. 2001. MAP kinases. *Chem Rev* **101**: 2449-76.

Chi, H., Lu, B., Takekawa, M., Davis, R.J., and Flavell, R.A. 2004. GADD45beta/GADD45gamma and MEKK4 comprise a genetic pathway mediating STAT4-independent IFNgamma production in T cells. *Embo J* **23**: 1576-86.

Chiariello, M., Marinissen, M.J., and Gutkind, J.S. 2000. Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. *Mol Cell Biol* **20**: 1747-58.

Chow, C.W., Dong, C., Flavell, R.A., and Davis, R.J. 2000. c-Jun NH(2)-terminal kinase inhibits targeting of the protein phosphatase calcineurin to NFATc1. *Mol Cell Biol* **20**: 5227-34.

Chow, C.W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R.J. 1997. Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. *Science* **278**: 1638-41.

Clark, A.R., Dean, J.L., and Saklatvala, J. 2003. Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. *FEBS Lett* **546**: 37-44.

Cobb, M.H. 1999. MAP kinase pathways. *Prog Biophys Mol Biol* **71**: 479-500.

Cohen, P. 1997. The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends in Cell Biology* **7**: 353-361.

Conrad, P.W., Rust, R.T., Han, J., Millhorn, D.E., and Beitner-Johnson, D. 1999. Selective activation of p38alpha and p38gamma by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. *J Biol Chem* **274**: 23570-6.

Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**: 1137-46.

Cuenda, A., Cohen, P., Buee-Scherrer, V., and Goedert, M. 1997. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *Embo J* **16**: 295-305.

Dai, T., Rubie, E., Franklin, C.C., Kraft, A., Gillespie, D.A., Avruch, J., Kyriakis, J.M., and Woodgett, J.R. 1995. Stress-activated protein kinases bind directly to the delta domain of c- Jun in resting cells: implications for repression of c-Jun function. *Oncogene* **10**: 849-55.

Davis, R.J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell* **103**: 239-52.

Deak, M., Clifton, A.D., Lucocq, L.M., and Alessi, D.R. 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *Embo J* **17**: 4426-41.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**: 1025-37.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I.H., Han, J., Ulevitch, R.J., and Davis, R.J. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* **267**: 682-5.

Dickens, M., Rogers, J.S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J.R., Greenberg, M.E., Sawyers, C.L., and Davis, R.J. 1997. A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science* **277**: 693-6.

Dong, C., Yang, D.D., Tournier, C., Whitmarsh, A.J., Xu, J., Davis, R.J., and Flavell, R.A. 2000. JNK is required for effector T-cell function but not for T-cell activation. *Nature* **405**: 91-4.

Dong, C., Yang, D.D., Wysk, M., Whitmarsh, A.J., Davis, R.J., and Flavell, R.A. 1998. Defective T cell differentiation in the absence of Jnk1. *Science* **282**: 2092-5.

Dorow, D.S., Devereux, L., Dietzsch, E., and De Kretser, T. 1993. Identification of a new family of human epithelial protein kinases containing two leucine/isoleucine-zipper domains. *Eur J Biochem* **213**: 701-10.

Douziech, M., Grondin, G., Loranger, A., Marceau, N., and Blouin, R. 1998. Zonal induction of mixed lineage kinase ZPK/DLK/MUK gene expression in regenerating mouse liver. *Biochem Biophys Res Commun* **249**: 927-32.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K.R., Copeland, T.D., Ahn, N.G., Oskarsson, M.K., Fukasawa, K., Paull, K.D., and Vande Woude, G.F. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **280**: 734-7.

Dumitru, C.D., Ceci, J.D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J.H., Patriotis, C., Jenkins, N.A., Copeland, N.G., Kollias, G., and Tsichlis, P.N. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* **103**: 1071-83.

Editorial. 2003. Whither RNAi? *Nat Cell Biol* **5**: 489-90.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494-8.

Engelman, J.A., Berg, A.H., Lewis, R.Y., Lin, A., Lisanti, M.P., and Scherer, P.E. 1999. Constitutively active mitogen-activated protein kinase kinase 6 (MKK6) or salicylate induces spontaneous 3T3-L1 adipogenesis. *J Biol Chem* **274**: 35630-8.

Engelman, J.A., Lisanti, M.P., and Scherer, P.E. 1998. Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J Biol Chem* **273**: 32111-20.

Enslen, H., Brancho, D.M., and Davis, R.J. 2000. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. *Embo J* **19**: 1301-11.

Enslen, H. and Davis, R.J. 2001. Regulation of MAP kinases by docking domains. *Biol Cell* **93**: 5-14.

Enslen, H., Raingeaud, J., and Davis, R.J. 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J Biol Chem* **273**: 1741-8.

Fanger, G.R., Gerwins, P., Widmann, C., Jarpe, M.B., and Johnson, G.L. 1997. MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev* **7**: 67-74.

Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. 1994. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**: 1039-49.

Fuchs, S.Y., Fried, V.A., and Ronai, Z. 1998. Stress-activated kinases regulate protein stability. *Oncogene* **17**: 1483-90.

Fukuda, M., Gotoh, Y., and Nishida, E. 1997. Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *Embo J* **16**: 1901-8.

Galanis, A., Yang, S.H., and Sharrocks, A.D. 2001. Selective targeting of MAPKs to the ETS

domain transcription factor SAP-1. *J Biol Chem* **276**: 965-73.

Gallo, K.A. and Johnson, G.L. 2002. Mixed-lineage kinase control of JNK and p38 MAPK

pathways. *Nat Rev Mol Cell Biol* **3**: 663-72.

Gallo, K.A., Mark, M.R., Scadden, D.T., Wang, Z., Gu, Q., and Godowski, P.J. 1994.

Identification and characterization of SPRK, a novel src-homology 3 domain-containing

proline-rich kinase with serine/threonine kinase activity. *J Biol Chem* **269**: 15092-100.

Ganiatsas, S., Kwee, L., Fujiwara, Y., Perkins, A., Ikeda, T., Labow, M.A., and Zon, L.I. 1998.

SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and

leads to abnormal hepatogenesis. *Proc Natl Acad Sci U S A* **95**: 6881-6.

Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R.J., Luo, Y., and Han, J. 2002.

MAPKK-independent activation of p38alpha mediated by TAB1-dependent

autophosphorylation of p38alpha. *Science* **295**: 1291-4.

Ge, B., Xiong, X., Jing, Q., Mosley, J.L., Filose, A., Bian, D., Huang, S., and Han, J. 2003.

TAB1beta (transforming growth factor-beta-activated protein kinase 1-binding protein

1beta), a novel splicing variant of TAB1 that interacts with p38alpha but not TAK1. *J

Biol Chem* **278**: 2286-93. Epub 2002 Nov 11.

Giroux, S., Tremblay, M., Bernard, D., Cardin-Girard, J.F., Aubry, S., Larouche, L., Rousseau,

S., Huot, J., Landry, J., Jeannotte, L., and Charron, J. 1999. Embryonic death of Mek1-

deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of

the placenta. *Curr Biol* **9**: 369-72.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R., and Cohen, P. 1997. Activation of the novel

stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by

SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *Embo J* **16**: 3563-71.

Green, H. and Kehinde, O. 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* **5**: 19-27.

Gupta, S., Barrett, T., Whitmarsh, A.J., Cavanagh, J., Sluss, H.K., Derijard, B., and Davis, R.J. 1996. Selective interaction of JNK protein kinase isoforms with transcription factors. *Embo J* **15**: 2760-70.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**: 389-93.

Hall, J.P. and Davis, R.J. 2002. Inhibition of the p38 pathway upregulates macrophage JNK and ERK activities, and the ERK, JNK, and p38 MAP kinase pathways are reprogrammed during differentiation of the murine myeloid M1 cell line. *J Cell Biochem* **86**: 1-11.

Han, J., Lee, J.D., Bibbs, L., and Ulevitch, R.J. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**: 808-11.

Han, J., Lee, J.D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R.J. 1996. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J Biol Chem* **271**: 2886-91.

Han, J., Lee, J.D., Tobias, P.S., and Ulevitch, R.J. 1993. Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. *J Biol Chem* **268**: 25009-14.

Han, J., Wang, X., Jiang, Y., Ulevitch, R.J., and Lin, S. 1997. Identification and characterization of a predominant isoform of human MKK3. *FEBS Lett* **403**: 19-22.

Harris, C., Maroney, A.C., and Johnson, E.M., Jr. 2002a. Identification of JNK-dependent and -independent components of cerebellar granule neuron apoptosis. *J Neurochem* **83**: 992-1001.

Harris, C.A., Deshmukh, M., Tsui-Pierchala, B., Maroney, A.C., and Johnson, E.M., Jr. 2002b. Inhibition of the c-Jun N-terminal kinase signaling pathway by the mixed lineage kinase inhibitor CEP-1347 (KT7515) preserves metabolism and growth of trophic factor-deprived neurons. *J Neurosci* **22**: 103-13.

Hazzalin, C.A., Cuenda, A., Cano, E., Cohen, P., and Mahadevan, L.C. 1997. Effects of the inhibition of p38/RK MAP kinase on induction of five fos and jun genes by diverse stimuli. *Oncogene* **15**: 2321-31.

Hehner, S.P., Hofmann, T.G., Ushmorov, A., Dienz, O., Wing-Lan Leung, I., Lassam, N., Scheidereit, C., Droge, W., and Schmitz, M.L. 2000. Mixed-lineage kinase 3 delivers CD3/CD28-derived signals into the IkappaB kinase complex. *Mol Cell Biol* **20**: 2556-68.

Heo, Y.S., Kim, S.K., Seo, C.I., Kim, Y.K., Sung, B.J., Lee, H.S., Lee, J.I., Park, S.Y., Kim, J.H., Hwang, K.Y., Hyun, Y.L., Jeon, Y.H., Ro, S., Cho, J.M., Lee, T.G., and Yang, C.H. 2004. Structural basis for the selective inhibition of JNK1 by the scaffolding protein JIP1 and SP600125. *Embo J* **23:** 2185-95.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* **7**: 2135-48.

Hirai, S., Izawa, M., Osada, S., Spyrou, G., and Ohno, S. 1996. Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. *Oncogene* **12**: 641-50.

Hirai, S., Kawaguchi, A., Hirasawa, R., Baba, M., Ohnishi, T., and Ohno, S. 2002. MAPK-upstream protein kinase (MUK) regulates the radial migration of immature neurons in telencephalon of mouse embryo. *Development* **129**: 4483-95.

Hirai, S., Noda, K., Moriguchi, T., Nishida, E., Yamashita, A., Deyama, T., Fukuyama, K., and Ohno, S. 1998. Differential activation of two JNK activators, MKK7 and SEK1, by MKN28-derived nonreceptor serine/threonine kinase/mixed lineage kinase 2. *J Biol Chem* **273**: 7406-12.

Holland, P.M. and Cooper, J.A. 1999. Protein modification: docking sites for kinases. *Curr Biol* **9**: R329-31.

Holzman, L.B., Merritt, S.E., and Fan, G. 1994. Identification, molecular cloning, and characterization of dual leucine zipper bearing kinase. A novel serine/threonine protein kinase that defines a second subfamily of mixed lineage kinases. *J Biol Chem* **269**: 30808-17.

Hu, E., Kim, J.B., Sarraf, P., and Spiegelman, B.M. 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science* **274**: 2100-3.

Ikeda, A., Masaki, M., Kozutsumi, Y., Oka, S., and Kawasaki, T. 2001. Identification and characterization of functional domains in a mixed lineage kinase LZK. *FEBS Lett* **488**: 190-5.

Ip, Y.T. and Davis, R.J. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr Opin Cell Biol* **10**: 205-19.

Ishitani, T., Takaesu, G., Ninomiya-Tsuji, J., Shibuya, H., Gaynor, R.B., and Matsumoto, K. 2003. Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. *Embo J* **22**: 6277-88.

Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K.I. 1999. JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. *Mol Cell Biol* **19**: 7539-48.

Jacobs, D., Glossip, D., Xing, H., Muslin, A.J., and Kornfeld, K. 1999. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev* **13**: 163-75.

Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J.A., Lin, S., and Han, J. 1996. Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem* **271**: 17920-6.

Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R.J., and Han, J. 1997. Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J Biol Chem* **272**: 30122-8.

Kallunki, T., Deng, T., Hibi, M., and Karin, M. 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* **87**: 929-39.

Kelkar, N., Delmotte, M.H., Weston, C.R., Barrett, T., Sheppard, B.J., Flavell, R.A., and Davis, R.J. 2003. Morphogenesis of the telencephalic commissure requires scaffold protein JNK-interacting protein 3 (JIP3). *Proc Natl Acad Sci U S A* **100**: 9843-8.

Kesavan, K., Lobel-Rice, K., Sun, W., Lapadat, R., Webb, S., Johnson, G.L., and Garrington, T.P. 2004. MEKK2 regulates the coordinate activation of ERK5 and JNK in response to FGF-2 in fibroblasts. *J Cell Physiol* **199**: 140-8.

Kiefer, F., Tibbles, L.A., Anafi, M., Janssen, A., Zanke, B.W., Lassam, N., Pawson, T., Woodgett, J.R., and Iscove, N.N. 1996. HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. *Embo J* **15**: 7013-25.

Kishimoto, H., Nakagawa, K., Watanabe, T., Kitagawa, D., Momose, H., Seo, J., Nishitai, G., Shimizu, N., Ohata, S., Tanemura, S., Asaka, S., Goto, T., Fukushi, H., Yoshida, H., Suzuki, A., Sasaki, T., Wada, T., Penninger, J.M., Nishina, H., and Katada, T. 2003. Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells. *J Biol Chem* **278**: 16595-601. Epub 2003 Mar 06.

Komatsu, Y., Shibuya, H., Takeda, N., Ninomiya-Tsuji, J., Yasui, T., Miyado, K., Sekimoto, T., Ueno, N., Matsumoto, K., and Yamada, G. 2002. Targeted disruption of the Tab1 gene causes embryonic lethality and defects in cardiovascular and lung morphogenesis. *Mech Dev* **119**: 239-49.

Kuan, C.Y., Yang, D.D., Samanta Roy, D.R., Davis, R.J., Rakic, P., and Flavell, R.A. 1999. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22**: 667-76.

Kumar, S., Boehm, J., and Lee, J.C. 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov* **2**: 717-26.

Kyriakis, J.M. 1999. Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Expr* **7**: 217-31.

Kyriakis, J.M. and Avruch, J. 1990. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J Biol Chem* **265**: 17355-63.

Kyriakis, J.M. and Avruch, J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* **81**: 807-69.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369**: 156-60.

Lamb, J.A., Ventura, J.J., Hess, P., Flavell, R.A., and Davis, R.J. 2003. JunD mediates survival signaling by the JNK signal transduction pathway. *Mol Cell* **11**: 1479-89.

Lane, M.D., Tang, Q.Q., and Jiang, M.S. 1999. Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem Biophys Res Commun* **266**: 677-83.

Lavoie, J.N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271**: 20608-16.

Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. 1998. Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. *Curr Biol* **8**: 1387-90.

Lee, C.M., Onesime, D., Reddy, C.D., Dhanasekaran, N., and Reddy, E.P. 2002. JLP: A scaffolding protein that tethers JNK/p38MAPK signaling modules and transcription factors. *Proc Natl Acad Sci U S A* **99**: 14189-94.

Lee, J.C., Kassis, S., Kumar, S., Badger, A., and Adams, J.L. 1999. p38 mitogen-activated protein kinase inhibitors--mechanisms and therapeutic potentials. *Pharmacol Ther* **82**: 389-97.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.R., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L., and Young, P.R. 1994.

A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **327**: 739-746.

Lee, T., Hoofnagle, A.N., Kabuyama, Y., Stroud, J., Min, X., Goldsmith, E.J., Chen, L., Resing, K.A., and Ahn, N.G. 2004. Docking motif interactions in MAP kinases revealed by hydrogen exchange mass spectrometry. *Mol Cell* **14**: 43-55.

Lee, T.H., Huang, Q., Oikemus, S., Shank, J., Ventura, J.J., Cusson, N., Vaillancourt, R.R., Su, B., Davis, R.J., and Kelliher, M.A. 2003. The death domain kinase RIP1 is essential for tumor necrosis factor alpha signaling to p38 mitogen-activated protein kinase. *Mol Cell Biol* **23**: 8377-85.

Leung, I.W. and Lassam, N. 1998. Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3. *J Biol Chem* **273**: 32408-15.

Lin, A., Minden, A., Martinetto, H., Claret, F.X., Lange-Carter, C., Mercurio, F., Johnson, G.L., and Karin, M. 1995. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science* **268**: 286-90.

Ling, P., Yao, Z., Meyer, C.F., Wang, X.S., Oehrl, W., Feller, S.M., and Tan, T.H. 1999. Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. *Mol Cell Biol* **19**: 1359-68.

Livingstone, C., Patel, G., and Jones, N. 1995. ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *Embo J* **14**: 1785-97.

Lu, H.T., Yang, D.D., Wysk, M., Gatti, E., Mellman, I., Davis, R.J., and Flavell, R.A. 1999. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *Embo J* **18**: 1845-57.

Manning, A.M. and Davis, R.J. 2003. Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* **2**: 554-65.

Maroney, A.C., Finn, J.P., Connors, T.J., Durkin, J.T., Angeles, T., Gessner, G., Xu, Z., Meyer, S.L., Savage, M.J., Greene, L.A., Scott, R.W., and Vaught, J.L. 2001. Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. *J Biol Chem* **276**: 25302-8.

Mata, M., Merritt, S.E., Fan, G., Yu, G.G., and Holzman, L.B. 1996. Characterization of dual leucine zipper-bearing kinase, a mixed lineage kinase present in synaptic terminals whose phosphorylation state is regulated by membrane depolarization via calcineurin. *J Biol Chem* **271**: 16888-96.

Matsuguchi, T., Musikacharoen, T., Johnson, T.R., Kraft, A.S., and Yoshikai, Y. 2001. A novel mitogen-activated protein kinase phosphatase is an important negative regulator of lipopolysaccharide-mediated c-Jun N-terminal kinase activation in mouse macrophage cell lines. *Mol Cell Biol* **21**: 6999-7009.

Merritt, S.E., Mata, M., Nihalani, D., Zhu, C., Hu, X., and Holzman, L.B. 1999. The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate. *J Biol Chem* **274**: 10195-202.

Minden, A., Lin, A., Claret, F.X., Abo, A., and Karin, M. 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**: 1147-57.

Molnar, A., Theodoras, A.M., Zon, L.I., and Kyriakis, J.M. 1997. Cdc42Hs, but not Rac1, inhibits serum-stimulated cell cycle progression at G1/S through a mechanism requiring p38/RK. *J Biol Chem* **272**: 13229-35.

Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. 1996a. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem* **271**: 13675-9.

Moriguchi, T., Toyoshima, F., Gotoh, Y., Iwamatsu, A., Irie, K., Mori, E., Kuroyanagi, N., Hagiwara, M., Matsumoto, K., and Nishida, E. 1996b. Purification and identification of a major activator for p38 from osmotically shocked cells. Activation of mitogen-activated protein kinase kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H2O2. *J Biol Chem* **271**: 26981-8.

Morrison, D.K. and Davis, R.J. 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Annu Rev Cell Dev Biol* **19**: 91-118.

Morton, S., Davis, R.J., and Cohen, P. 2004. Signalling pathways involved in multisite phosphorylation of the transcription factor ATF-2. *FEBS Lett* **572**: 177-83.

Mudgett, J.S., Ding, J., Guh-Siesel, L., Chartrain, N.A., Yang, L., Gopal, S., and Shen, M.M. 2000. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. *Proc Natl Acad Sci U S A* **97**: 10454-9.

Musti, A.M., Treier, M., and Bohmann, D. 1997. Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science* **275**: 400-2.

Nadeau, A., Grondin, G., and Blouin, R. 1997. In situ hybridization analysis of ZPK gene expression during murine embryogenesis. *J Histochem Cytochem* **45**: 107-18.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein

kinase cascade is essential for transcription factor NF-IL6. *Proc Natl Acad Sci U S A* **90**: 2207-11.

Nebreda, A.R. and Porras, A. 2000. p38 MAP kinases: beyond the stress response. *Trends Biochem Sci* **25**: 257-60.

Nemoto, S., Xiang, J., Huang, S., and Lin, A. 1998. Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase. *J Biol Chem* **273**: 16415-20.

New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L.J., Kato, Y., Parry, G.C., and Han, J. 1998. PRAK, a novel protein kinase regulated by the p38 MAP kinase. *Embo J* **17**: 3372-84.

Nihalani, D., Merritt, S., and Holzman, L.B. 2000. Identification of structural and functional domains in mixed lineage kinase dual leucine zipper-bearing kinase required for complex formation and stress-activated protein kinase activation. *J Biol Chem* **275**: 7273-9.

Nihalani, D., Meyer, D., Pajni, S., and Holzman, L.B. 2001. Mixed lineage kinase-dependent JNK activation is governed by interactions of scaffold protein JIP with MAPK module components. *Embo J* **20**: 3447-58.

Nihalani, D., Wong, H.N., and Holzman, L.B. 2003. Recruitment of JNK to JIP1 and JNK-dependent JIP1 phosphorylation regulates JNK module dynamics and activation. *J Biol Chem* **278**: 28694-702.

Nishina, H., Fischer, K.D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E.A., Bernstein, A., Mak, T.W., Woodgett, J.R., and Penninger, J.M. 1997. Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. *Nature* **385**: 350-3.

Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., De la Pompa, J.L., Furlonger, K., Paige, C., Hui, C., Fischer, K.D., Kishimoto, H., Iwatsubo, T., Katada, T., Woodgett, J.R., and

Penninger, J.M. 1999. Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. *Development* **126**: 505-16.

Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. 1998. ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell* **2**: 389-95.

Oh-hora, M., Ogata, M., Mori, Y., Adachi, M., Imai, K., Kosugi, A., and Hamaoka, T. 1999. Direct suppression of TCR-mediated activation of extracellular signal-regulated kinase by leukocyte protein tyrosine phosphatase, a tyrosine-specific phosphatase. *J Immunol* **163**: 1282-8.

Ono, K. and Han, J. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal* **12**: 1-13.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* **22**: 153-83.

Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M., and Montecucco, C. 1999. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNgamma-induced release of NO and TNFalpha. *FEBS Lett* **462**: 199-204.

Phelan, D.R., Price, G., Liu, Y.F., and Dorow, D.S. 2001. Activated JNK phosphorylates the c-terminal domain of MLK2 that is required for MLK2-induced apoptosis. *J Biol Chem* **276**: 10801-10.

Picard, F., Gehin, M., Annicotte, J., Rocchi, S., Champy, M.F., O'Malley, B.W., Chambon, P., and Auwerx, J. 2002. SRC-1 and TIF2 control energy balance between white and brown adipose tissues. *Cell* **111**: 931-41.

Porter, A.C., Fanger, G.R., and Vaillancourt, R.R. 1999. Signal transduction pathways regulated by arsenate and arsenite. *Oncogene* **18**: 7794-802.

Pulido, R., Zuniga, A., and Ullrich, A. 1998. PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *Embo J* **17**: 7337-50.

Pulverer, B.J., Kyriakis, J.M., Avruch, J., Nikolakaki, E., and Woodgett, J.R. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature* **353**: 670-4.

Putcha, G.V., Le, S., Frank, S., Besirli, C.G., Clark, K., Chu, B., Alix, S., Youle, R.J., LaMarche, A., Maroney, A.C., and Johnson, E.M., Jr. 2003. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* **38**: 899-914.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., and Davis, R.J. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* **270**: 7420-6.

Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B., and Davis, R.J. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* **16**: 1247-55.

Rana, A., Gallo, K., Godowski, P., Hirai, S., Ohno, S., Zon, L., Kyriakis, J.M., and Avruch, J. 1996. The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. *J Biol Chem* **271**: 19025-8.

Reddy, U.R. and Pleasure, D. 1994. Cloning of a novel putative protein kinase having a leucine zipper domain from human brain. *Biochem Biophys Res Commun* **202**: 613-20.

Rosen, E.D. 2002. Molecular mechanisms of adipocyte differentiation. *Ann Endocrinol (Paris)* **63**: 79-82.

Rosen, E.D. and Spiegelman, B.M. 2000. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* **16**: 145-71.

Rosen, E.D., Walkey, C.J., Puigserver, P., and Spiegelman, B.M. 2000. Transcriptional regulation of adipogenesis. *Genes Dev* **14**: 1293-307.

Rossant, J. and Cross, J.C. 2001. Placental development: lessons from mouse mutants. *Nat Rev Genet* **2**: 538-48.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A.R. 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**: 1027-37.

Sabapathy, K., Hu, Y., Kallunki, T., Schreiber, M., David, J.P., Jochum, W., Wagner, E.F., and Karin, M. 1999. JNK2 is required for efficient T-cell activation and apoptosis but not for normal lymphocyte development. *Curr Biol* **9**: 116-25.

Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M., and Zon, L.I. 1994. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature* **372**: 794-798.

Sanchez-Prieto, R., Rojas, J.M., Taya, Y., and Gutkind, J.S. 2000. A role for the p38 mitogen-acitvated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. *Cancer Res* **60**: 2464-72.

Sathyanarayana, P., Barthwal, M.K., Kundu, C.N., Lane, M.E., Bergmann, A., Tzivion, G., and Rana, A. 2002. Activation of the Drosophila MLK by ceramide reveals TNF-alpha and ceramide as agonists of mammalian MLK3. *Mol Cell* **10**: 1527-33.

Sathyanarayana, P., Barthwal, M.K., Lane, M.E., Acevedo, S.F., Skoulakis, E.M., Bergmann, A., and Rana, A. 2003. Drosophila mixed lineage kinase/slipper, a missing biochemical link in Drosophila JNK signaling. *Biochim Biophys Acta* **1640**: 77-84.

Savinainen, A., Garcia, E.P., Dorow, D., Marshall, J., and Liu, Y.F. 2001. Kainate receptor activation induces mixed lineage kinase-mediated cellular signaling cascades via post-synaptic density protein 95. *J Biol Chem* **276**: 11382-6.

Schaeffer, H.J. and Weber, M.J. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* **19**: 2435-44.

Schonhoff, C.M., Bulseco, D.A., Brancho, D.M., Parada, L.F., Ross, A.H. 2001. The Ras-ERK pathway is required for the induction of neuronal nitric oxide synthase in differentiating PC12 cells. *J. Neurochem.* **78**: 631-9.

Schoorlemmer, J. and Goldfarb, M. 2001. Fibroblast growth factor homologous factors are intracellular signaling proteins. *Curr Biol* **11**: 793-7.

Schoorlemmer, J. and Goldfarb, M. 2002. Fibroblast growth factor homologous factors and the islet brain-2 scaffold protein regulate activation of a stress-activated protein kinase. *J Biol Chem* **277**: 49111-9.

Sharrocks, A.D., Yang, S.H., and Galanis, A. 2000. Docking domains and substrate-specificity determination for MAP kinases. *Trends Biochem Sci* **25**: 448-53.

Shen, Y.H., Godlewski, J., Zhu, J., Sathyanarayana, P., Leaner, V., Birrer, M.J., Rana, A., and Tzivion, G. 2003. Cross-talk between JNK/SAPK and ERK/MAPK pathways: sustained

activation of JNK blocks ERK activation by mitogenic factors. *J Biol Chem* **278**: 26715-21.

Shi, C.S., Leonardi, A., Kyriakis, J., Siebenlist, U., and Kehrl, J.H. 1999. TNF-mediated activation of the stress-activated protein kinase pathway: TNF receptor-associated factor 2 recruits and activates germinal center kinase related. *J Immunol* **163**: 3279-85.

Smeal, T., Binetruy, B., Mercola, D.A., Birrer, M., and Karin, M. 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* **354**: 494-6.

Stein, B., Brady, H., Yang, M.X., Young, D.B., and Barbosa, M.S. 1996. Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. *J Biol Chem* **271**: 11427-33.

Stronach, B. and Perrimon, N. 2002. Activation of the JNK pathway during dorsal closure in Drosophila requires the mixed lineage kinase, slipper. *Genes Dev* **16**: 377-87.

Su, Y.C., Han, J., Xu, S., Cobb, M., and Skolnik, E.Y. 1997. NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. *Embo J* **16**: 1279-90.

Swenson, K.I., Winkler, K.E., and Means, A.R. 2003. A new identity for MLK3 as an NIMA-related, cell cycle-regulated kinase that is localized near centrosomes and influences microtubule organization. *Mol Biol Cell* **14**: 156-72.

Takaesu, G., Surabhi, R.M., Park, K.J., Ninomiya-Tsuji, J., Matsumoto, K., and Gaynor, R.B. 2003. TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *J Mol Biol* **326**: 105-15.

Tamura, K., Sudo, T., Senftleben, U., Dadak, A.M., Johnson, R., and Karin, M. 2000. Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* **102**: 221-31.

Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P., and Comb, M.J. 1996. FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *Embo J* **15**: 4629-42.

Tanaka, N., Kamanaka, M., Enslen, H., Dong, C., Wysk, M., Davis, R.J., and Flavell, R.A. 2002. Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. *EMBO Rep* **3**: 785-91.

Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat Cell Biol* **2**: 110-6.

Tanoue, T., Maeda, R., Adachi, M., and Nishida, E. 2001a. Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. *Embo J* **20**: 466-79.

Tanoue, T., Moriguchi, T., and Nishida, E. 1999. Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *J Biol Chem* **274**: 19949-56.

Tanoue, T. and Nishida, E. 2003. Molecular recognitions in the MAP kinase cascades. *Cell Signal* **15**: 455-62.

Tanoue, T., Yamamoto, T., Maeda, R., and Nishida, E. 2001b. A Novel MAPK phosphatase MKP-7 acts preferentially on JNK/SAPK and p38 alpha and beta MAPKs. *J Biol Chem* **276**: 26629-39.

Teng, D.H., Perry, W.L., 3rd, Hogan, J.K., Baumgard, M., Bell, R., Berry, S., Davis, T., Frank, D., Frye, C., Hattier, T., Hu, R., Jammulapati, S., Janecki, T., Leavitt, A., Mitchell, J.T.,

Pero, R., Sexton, D., Schroeder, M., Su, P.H., Swedlund, B., Kyriakis, J.M., Avruch, J., Bartel, P., Wong, A.K., Tavtigian, S.V., and et al. 1997. Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. *Cancer Res* **57**: 4177-82.

Teramoto, H., Coso, O.A., Miyata, H., Igishi, T., Miki, T., and Gutkind, J.S. 1996. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. *J Biol Chem* **271**: 27225-8.

Theodosiou, A. and Ashworth, A. 2002. MAP kinase phosphatases. *Genome Biol* **3**: REVIEWS3009.

Theodosiou, A., Smith, A., Gillieron, C., Arkinstall, S., and Ashworth, A. 1999. MKP5, a new member of the MAP kinase phosphatase family, which selectively dephosphorylates stress-activated kinases. *Oncogene* **18**: 6981-8.

Tibbles, L.A., Ing, Y.L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J.R., and Lassam, N.J. 1996. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *Embo J* **15**: 7026-35.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. 2001. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* **2**: 222-8.

Tournier, C., Dong, C., Turner, T.K., Jones, S.N., Flavell, R.A., and Davis, R.J. 2001. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev* **15**: 1419-26.

Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimnual, A., Bar-Sagi, D., Jones, S.N.,
Flavell, R.A., and Davis, R.J. 2000. Requirement of JNK for stress-induced activation of
the cytochrome c-mediated death pathway. *Science* **288**: 870-4.

Tournier, C., Whitmarsh, A.J., Cavanagh, J., Barrett, T., and Davis, R.J. 1997. Mitogen-activated
protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. *Proc Natl Acad
Sci U S A* **94**: 7337-42.

Tournier, C., Whitmarsh, A.J., Cavanagh, J., Barrett, T., and Davis, R.J. 1999. The MKK7 gene
encodes a group of c-Jun NH2-terminal kinase kinases. *Mol Cell Biol* **19**: 1569-81.

Vacratsis, P.O. and Gallo, K.A. 2000. Zipper-mediated oligomerization of the mixed lineage
kinase SPRK/MLK-3 is not required for its activation by the GTPase cdc 42 but Is
necessary for its activation of the JNK pathway. Monomeric SPRK L410P does not
catalyze the activating phosphorylation of Thr258 of murine MITOGEN-ACTIVATED
protein kinase kinase 4. *J Biol Chem* **275**: 27893-900.

Vacratsis, P.O., Phinney, B.S., Gage, D.A., and Gallo, K.A. 2002. Identification of in vivo
phosphorylation sites of MLK3 by mass spectrometry and phosphopeptide mapping.
Biochemistry **41**: 5613-24.

Varfolomeev, E.E. and Ashkenazi, A. 2004. Tumor necrosis factor: an apoptosis JuNKie? *Cell*
116: 491-7.

Ventura, J.J., Kennedy, N.J., Lamb, J.A., Flavell, R.A., and Davis, R.J. 2003. c-Jun NH(2)-
terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. *Mol Cell
Biol* **23**: 2871-82.

Ventura, J.J., Cogswell, P., Flavell, R.A., Baldwin Jr., A.S., and Davis, R.J. 2004. JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. *Genes Dev* **18**: 2905-15.

Verhey, K.J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B.J., Rapoport, T.A., and Margolis, B. 2001. Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. *J Cell Biol* **152**: 959-70.

Wada, T., Nakagawa, K., Watanabe, T., Nishitai, G., Seo, J., Kishimoto, H., Kitagawa, D., Sasaki, T., Penninger, J.M., Nishina, H., and Katada, T. 2001. Impaired synergistic activation of stress-activated protein kinase SAPK/JNK in mouse embryonic stem cells lacking SEK1/MKK4: different contribution of SEK2/MKK7 isoforms to the synergistic activation. *J Biol Chem* **276**: 30892-7. Epub 2001 Jun 19.

Wajant, H., Pfizenmaier, K., and Scheurich, P. 2003. Tumor necrosis factor signaling. *Cell Death Differ* **10**: 45-65.

Wang, L.H., Besirli, C.G., and Johnson, E.M., Jr. 2004. Mixed-lineage kinases: a target for the prevention of neurodegeneration. *Annu Rev Pharmacol Toxicol* **44**: 451-74.

Wang, X.S., Diener, K., Manthey, C.L., Wang, S., Rosenzweig, B., Bray, J., Delaney, J., Cole, C.N., Chan-Hui, P.Y., Mantlo, N., Lichenstein, H.S., Zukowski, M., and Yao, Z. 1997. Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem* **272**: 23668-74.

Wang, Y., Huang, S., Sah, V.P., Ross, J., Jr., Brown, J.H., Han, J., and Chien, K.R. 1998. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem* **273**: 2161-8.

Waskiewicz, A.J., Flynn, A., Proud, C.G., and Cooper, J.A. 1997. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *Embo J* **16**: 1909-20.

Waskiewicz, A.J., Johnson, J.C., Penn, B., Mahalingam, M., Kimball, S.R., and Cooper, J.A. 1999. Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. *Mol Cell Biol* **19**: 1871-80.

Weston, C.R., Brancho, D., Xu, P., Doran, B., Goad, M.E., Flavell, R.A., and Davis, R.J. The mixed-lineage protein kinase DLK is essential for early embryonic development. *In preparation.*

Weston, C.R. and Davis, R.J. 2002. The JNK signal transduction pathway. *Curr Opin Genet Dev* **12**: 14-21.

Weston, C.R., Lambright, D.G., and Davis, R.J. 2002. Signal transduction. MAP kinase signaling specificity. *Science* **296**: 2345-7.

Weston, C.R., Wong, A., Hall, J.P., Goad, M.E., Flavell, R.A., and Davis, R.J. 2003. JNK initiates a cytokine cascade that causes Pax2 expression and closure of the optic fissure. *Genes Dev* **17**: 1271-80.

Weston, C.R., Wong, A., Hall, J.P., Goad, M.E., Flavell, R.A., and Davis, R.J. 2004. The c-Jun NH2-terminal kinase is essential for epidermal growth factor expression during epidermal morphogenesis. *Proc Natl Acad Sci U S A* **101**: 14114-9.

Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R.J. 1998. A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science* **281**: 1671-4.

Whitmarsh, A.J. and Davis, R.J. 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* **74**: 589-607.

Whitmarsh, A.J. and Davis, R.J. 1998. Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. *Trends Biochem Sci* **23**: 481-5.

Whitmarsh, A.J. and Davis, R.J. 2001a. Analyzing JNK and p38 mitogen-activated protein kinase activity. *Methods Enzymol* **332**: 319-36.

Whitmarsh, A.J. and Davis, R.J. 2001b. Signal transduction by target-derived neurotrophins. *Nat Neurosci* **4**: 963-4.

Whitmarsh, A.J., Shore, P., Sharrocks, A.D., and Davis, R.J. 1995. Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**: 403-7.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* **79**: 143-80.

Wilk-Blaszczak, M.A., Stein, B., Xu, S., Barbosa, M.S., Cobb, M.H., and Belardetti, F. 1998. The mitogen-activated protein kinase p38-2 is necessary for the inhibition of N-type calcium current by bradykinin. *J Neurosci* **18**: 112-8.

Wysk, M., Yang, D.D., Lu, H.T., Flavell, R.A., and Davis, R.J. 1999. Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. *Proc Natl Acad Sci U S A* **96**: 3763-8.

Xia, Y., Wu, Z., Su, B., Murray, B., and Karin, M. 1998. JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. *Genes Dev* **12**: 3369-81.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**: 1326-31.

Xiu, M., Kim, J., Sampson, E., Huang, C.Y., Davis, R.J., Paulson, K.E., and Yee, A.S. 2003. The transcriptional repressor HBP1 is a target of the p38 mitogen-activated protein kinase pathway in cell cycle regulation. *Mol Cell Biol* **23**: 8890-901.

Xu, B., Wilsbacher, J.L., Collisson, T., and Cobb, M.H. 1999. The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 in vitro and ERK activation in vivo. *J Biol Chem* **274**: 34029-35.

Xu, Z., Maroney, A.C., Dobrzanski, P., Kukekov, N.V., and Greene, L.A. 2001. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol* **21**: 4713-24.

Yamada, S.D., Hickson, J.A., Hrobowski, Y., Vander Griend, D.J., Benson, D., Montag, A., Karrison, T., Huo, D., Rutgers, J., Adams, S., and Rinker-Schaeffer, C.W. 2002. Mitogen-activated protein kinase kinase 4 (MKK4) acts as a metastasis suppressor gene in human ovarian carcinoma. *Cancer Res* **62**: 6717-23.

Yang, D., Tournier, C., Wysk, M., Lu, H.T., Xu, J., Davis, R.J., and Flavell, R.A. 1997a. Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. *Proc Natl Acad Sci U S A* **94**: 3004-9.

Yang, D.D., Conze, D., Whitmarsh, A.J., Barrett, T., Davis, R.J., Rincon, M., and Flavell, R.A. 1998a. Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. *Immunity* **9**: 575-85.

Yang, D.D., Kuan, C.Y., Whitmarsh, A.J., Rincon, M., Zheng, T.S., Davis, R.J., Rakic, P., and Flavell, R.A. 1997b. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389**: 865-70.

Yang, J., Boerm, M., McCarty, M., Bucana, C., Fidler, I.J., Zhuang, Y., and Su, B. 2000. Mekk3 is essential for early embryonic cardiovascular development. *Nat Genet* **24**: 309-13.

Yang, S.H., Galanis, A., and Sharrocks, A.D. 1999. Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol Cell Biol* **19**: 4028-38.

Yang, S.H., Whitmarsh, A.J., Davis, R.J., and Sharrocks, A.D. 1998b. Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. *Embo J* **17**: 1740-9.

Yang, S.H., Yates, P.R., Whitmarsh, A.J., Davis, R.J., and Sharrocks, A.D. 1998c. The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. *Mol Cell Biol* **18**: 710-20.

Yasuda, J., Whitmarsh, A.J., Cavanagh, J., Sharma, M., and Davis, R.J. 1999. The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol Cell Biol* **19**: 7245-54.

Yee, A.S., Paulson, E.K., McDevitt, M.A., Rieger-Christ, K., Summerhayes, I., Berasi, S.P., Kim, J., Huang, C.Y., and Zhang, X. 2004. The HBP1 transcriptional repressor and the p38 MAP kinase: unlikely partners in G1 regulation and tumor suppression. *Gene* **336**: 1-13.

Yeh, W.C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J.L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rothe, M., Goeddel, D.V., and Mak, T.W. 1997. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* **7**: 715-25.

Yuasa, T., Ohno, S., Kehrl, J.H., and Kyriakis, J.M. 1998. Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase

kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38. *J Biol Chem* **273**: 22681-92.

Yujiri, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsu, Y., Clarke, P., Tyler, K., Oka, Y., Fanger, G.R., Henson, P., and Johnson, G.L. 2000. MEK kinase 1 gene disruption alters cell migration and c-Jun NH2-terminal kinase regulation but does not cause a measurable defect in NF-kappa B activation. *Proc Natl Acad Sci U S A* **97**: 7272-7.

Zhang, H. and Gallo, K.A. 2001. Autoinhibition of mixed lineage kinase 3 through its Src homology 3 domain. *J Biol Chem* **276**: 45598-603.

Zhang, H., Wu, W., Du, Y., Santos, S.J., Conrad, S.E., Watson, J.T., Grammatikakis, N., and Gallo, K.A. 2004a. Hsp90/p50cdc37 is required for mixed-lineage kinase (MLK) 3 signaling. *J Biol Chem* **279**: 19457-63.

Zhang, J.W., Klemm, D.J., Vinson, C., and Lane, M.D. 2004b. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. *J Biol Chem* **279**: 4471-8.

Zhang, L., Wang, W., Hayashi, Y., Jester, J.V., Birk, D.E., Gao, M., Liu, C.Y., Kao, W.W., Karin, M., and Xia, Y. 2003. A role for MEK kinase 1 in TGF-beta/activin-induced epithelium movement and embryonic eyelid closure. *Embo J* **22**: 4443-54.

Zhong, J. and Kyriakis, J.M. 2004. Germinal center kinase is required for optimal Jun N-terminal kinase activation by toll-like receptor agonists and is regulated by the ubiquitin proteasome system and agonist-induced, TRAF6-dependent stabilization. *Mol Cell Biol* **24**: 9165-75.