DEK oncoprotein is a novel regulator of NF-B transactivation and DNA damage-induced apoptosis

Shanshan Wan

The University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
Wan, Shanshan, "DEK oncoprotein is a novel regulator of NF-B transactivation and DNA damage-induced apoptosis" (2009). Theses and Dissertations. 1146.
http://utdr.utoledo.edu/theses-dissertations/1146

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
A Dissertation

Entitled

DEK oncoprotein is a novel regulator of NF-κB transactivation and DNA damage-induced apoptosis

by

Shanshan Wan

Submitted as a partial fulfillment of the requirements for

the Doctor of Philosophy degree in Biology

Advisor: Brian P. Ashburner, Ph.D.

College of Graduate Studies

The University of Toledo

August 2009
An abstract of

DEK oncoprotein is a novel regulator of NF-κB transactivation and DNA damage-induced apoptosis

by

Shanshan Wan

Submitted as a partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

The University of Toledo

August 2009

Human DEK has long been implicated in tumorigenesis and auto-immune diseases. DEK is overexpressed in various human tumors and is an autoantigen in many autoimmune diseases. However the contribution of DEK to these human diseases is still unclear. We aimed to better understand the biological function of DEK and its role in regulating NF-κB and apoptosis.

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that activates expression of genes involved in the inflammation, immune responses, cell survival, differentiation, and proliferation. The transcriptional activity of NF-κB is tightly
controlled in multiple levels to ensure the rapid and transient activation of NF-κB. Dynamic association of NF-κB with a number of coactivator and corepressor proteins is crucial for the precise control of NF-κB-regulated gene transcription.

Previously, our lab identified DEK as an NF-κB-interacting protein through a yeast two-hybrid screen using the C-terminal transcription activation domain (TAD) of the p65 subunit of NF-κB as bait. We provided the first demonstration that DEK interacted with the p65 subunit of NF-κB and repressed NF-κB-mediated gene transcription. We also found that DEK associated with the NF-κB-regulated IL-8 and cIAP-2 promoters in uninduced cells and dissociated from the promoters in response to TNFα stimulation. In addition, the expression of NF-κB-regulated Mcp-1 and IκBα genes was elevated in cells isolated from dek−/− mice relative to cells from wild type (WT) mice. Taken together, our data demonstrated DEK as a novel corepressor to negatively regulate NF-κB-mediated gene transcription.

Since DEK has been implicated in facilitating DNA repair and inhibiting apoptosis in response to DNA damage, we also aimed to better understand the cytoprotective role of DEK and its contribution to cancer cell survival. Our data showed increased cell death and enhanced caspase 3 cleavage in DEK−/− MEFs compared to WT MEFs in response to DNA damage induced by doxorubicin. Interestingly, in p53-proficient HCT116 (colon cancer cell line) but not p53-deficient HCT116, ablation of DEK by shRNA led to increased cell death and apoptosis in response to DNA damage. Moreover, DEK loss resulted in decreased p21 expression. These findings indicated that suppression of DEK
sensitized cells to apoptosis which is, at least in part, dependent on the p53-p21 signaling.

In addition to the well-characterized regulation of NF-κB through inducible nuclear translocation and post-translational modifications, various signal transduction pathways also regulate the ability of NF-κB to activate transcription, many of which target transcriptional coactivator and corepressor proteins. Strong evidence has linked p38 activity with NF-κB transactivation. CREB-Binding Protein (CBP) is an important coactivator for NF-κB. We showed that p38 MAP kinase interacted with CBP in vivo in a stimulus-dependent manner and phosphorylated CBP on multiple sites in vitro, and that p38 is recruited to the promoters of NF-κB-regulated gene in a complex with CBP. Here, we proposed that p38 potentiates NF-κB transactivation activity through interacting with and phosphorylating CBP coactivator.
# TABLE OF CONTENTS

ABSTRACT ....................................................................................................................... ii  
DEDICATION ..................................................................................................................... ix  
ACKNOWLEDGMENTS ................................................................................................. x  
LIST OF TABLES ........................................................................................................... xi  
LIST OF FIGURES .......................................................................................................... xii  
ABBREVIATIONS ......................................................................................................... xiv  

1 INTRODUCTION ........................................................................................................... 1  

1.1 Nuclear Factor-kappaB (NF-κB) signaling pathway ........................................... 1  

1.1.1. NF-κB family members .............................................................................. 2  

1.1.2. NF-κB activation pathway ...................................................................... 4  

1.2 Coactivators and corepressors of NF-κB ....................................................... 7  

1.3 DEK oncoprotein .............................................................................................. 9  

1.3.1. Domains and modification of DEK protein ............................................. 9  

1.3.2. Functions of DEK protein .................................................................... 13  

1.4 DNA damage-induced apoptosis and the p53 tumor suppressor protein 16  

1.4.1. DNA damage-induced apoptosis ............................................................ 16  

1.4.2. p53 tumor suppressor .......................................................................... 19  

1.4.3. p21 (WAF1/CIP1/SDI1) ........................................................................ 21  

1.4.4. Cytoprotective roles of p53-p21 signaling ............................................ 22  

1.5 CREB-Binding Protein (CBP) ...................................................................... 24  

v
1.6 p38 MAP kinase........................................................................................................28

1.6.1. Substrates and genes mediated by p38.................................................................29

1.6.2. Biological functions of p38...................................................................................30

1.6.3. Link between NF-κB and p38 MAPK.........................................................32

1.7 Overall objectives: ............................................................................................35

2 MATERIALS AND METHODS ........................................................................36

2.1 Cell lines, plasmids and other materials ...............................................................36

2.2 RNA Interference and stable DEK knockdown cell line .....................................37

2.3 Transient transfection and reporter gene assays ..............................................40

2.4 Caspase-Glo 3/7 Assay .......................................................................................40

2.5 Semi-quantitative reverse transcriptase-PCR (RT-PCR), quantitative
real-time PCR and PCR Array ..............................................................................41

2.6 Coimmunoprecipitation (CoIP) and immunoblotting analysis .........................43

2.7 Cytoplasmic and nuclear extracts .........................................................................44

2.8 Chromatin immunoprecipitation (ChIP) assay and ChIP-ReChIP assay 45

2.9 in vitro binding assays.........................................................................................47

2.10 in vitro kinase assays..........................................................................................47

2.11 Proliferation assay / MTT assay...........................................................................48

2.12 Statistical significance analysis............................................................................48

3 RESULTS.............................................................................................................49

3.1 Part 1: Negative regulation of NF-κB by DEK protein .........................................49
3.2.1 DEK interacts with p65 subunit of NF-κB ........................................... 49
3.2.2 DEK represses NF-κB-mediated transcriptional activation ................. 52
3.2.3 DEK associates with the NF-κB-regulated cIAP2 and IL-8 promoters 55
3.2.4 The absence of DEK enhances expression of NF-κB-regulated genes 58
3.2.5 The absence of DEK enhances the recruitment of p65 and P/CAF to promoters ............................................................................................................ 62
3.2.6 The association of HDAC1, HDAC2 and HDAC3 on NF-κB-regulated promoters in DEK+/+ and DEK−/− MEFs .............................................................. 65
3.2.7 DEK regulates other transcription factors............................................. 68
3.2.8 DEK may regulate a variety of genes involved in NF-κB signaling .... 72
3.2 Part 2: DEK oncoprotein protect cells from apoptosis. ......................75
3.2.1. DEK knockout sensitizes MEFs to DNA damage-induced apoptosis.. 75
3.2.2. DEK knockdown sensitizes HeLa cells to DR-induced death.......... 79
3.2.3. DEK knockdown sensitizes p53+/+ but not p53−/− HCT116 to DNA damage-induced apoptosis ................................................................. 83
3.2.4. DEK knockdown decreases p21 expression in p53+/+ HCT116. ....... 88
3.2.5. DEK knockdown decreases the protein stability of p21 but not p53... 92
3.2.6 DEK may mediate a variety of apoptosis-regulatory genes.............. 95
3.3 Part 3: Regulation of NF-κB activity by p38 MAP Kinases.................98
3.3.1 p38 interacts with CBP in vitro ........................................................... 98
3.3.2 p38 phosphorylates CBP in vitro ..................................................... 99
3.3.3 p38 and CBP interact in vivo ............................................................... 104
3.3.4 p38 is recruited to the NF-κB-regulated promoters in a complex with CBP 107
3.3.5 Inhibition of p38 does not affect the recruitment of p65 and CBP to the promoters of NF-κB-regulated genes ............................................................... 109
3.3.6 Inhibition of p38 selectively regulates the expression of NF-κB-regulated genes in a cell and gene specific manner.................................................... 112

4 DISCUSSION .......................................................................................... 114
4.1 DEK is a novel regulator for NF-κB-mediated transactivation .......... 114
4.2 DEK oncoprotein inhibits DNA damage-induced cell death............. 121
4.3 Regulation of NF-κB transactivation by p38 through CBP coactivator 128

5 REFERENCES ...................................................................................... 135
DEDICATION

This dissertation is dedicated to my parents

Yi, Taoying and Wan, Shaoping

for their sacrifices and love
ACKNOWLEDGMENTS

First of all, I would like to thank my research advisor, Dr. Brian Ashburner, for his guidance and advices in making this dissertation possible. He has always been inspiring when answering my questions and discussing research with me, as well as encouraging me to think independently and thoroughly. The opportunity of conducting exciting research in such a wonderful environment is the greatest experience in my life. I wish to express special thanks to Dr. Douglas Leaman and Dr. Fan Dong for giving me excellent supervision, scientific training and advices. I am also thankful to my other committee members, Dr. Ivana de la Serna and Dr. Kevin Pan, and to Dr. William Taylor for their precious time and suggestions towards my dissertation research.

I wish to acknowledge all the present and past members of the Ashburner and the Leaman labs, Dr. Marta Boeke, Morgan Sammons, Eric Cole, Lindsay Tubbs, Dr. Shawn Roseback, Dr. Da Xu, Tiaannan Chen, Boren Lin, David Velliquette, Kuladeep Reddy Sudini and others for their generous assistance in the lab. Sincere thanks go as well to all of my friends, Haiying Li, Changmeng Cai, Qingquan Liu, Ying Deng, Chenlin Hsieh, Yu Zhan, Shuang Hu, Yalin Qiu and many other people, who helped me in many ways and made my life in Toledo so enjoyable. The department of biological sciences and the University of Toledo are also appreciated for supporting me during my graduate studies.

I wish to extend my deep gratitude to my parents, my sister Zhen Wan and my fiancé Guizhang Zheng for their unyielding support, encouragement, and confidence in me. Their love brightens each day of my life and I can never thank them enough.
LIST OF TABLES

Table 1. Some diseases associated with NF-κB dysregulation ..........................1

Table 2. PCR arrays in NF-κB signaling ..........................................................74

Table 3. PCR arrays in apoptosis pathway .......................................................97
LIST OF FIGURES

Fig. 1. NF-κB family members .......................................................... 3

Fig. 2. The classical NF-κB activation pathway .................................. 6

Fig. 3. Linear representation of the DEK protein .............................. 12

Fig. 4. Common cellular responses to DNA damage .......................... 18

Fig. 5. Representation of the CBP/p300 protein family ..................... 26

Fig. 6. pLKO1 Lentiviral vector used in the production of Lentivirus .... 39

Fig. 7. Coimmunoprecipitation of endogenous DEK and p65 ............ 51

Fig. 8. DEK represses NF-κB-mediated transcriptional activation ....... 54

Fig. 9. DEK associates with the NF-κB-regulated promoters .............. 57

Fig. 10. Increased expression of NF-κB regulated genes in DEK−/− cells ... 60

Fig. 11. Enhanced association of p65 and P/CAF to Mcp-1 and IκBα promoters in the absence of DEK ......................................................... 64

Fig. 12. Association of HDAC1, HDAC2 and HDAC3 on NF-κB-responsive promoters in DEK+/+ and DEK−/− cells ................................. 67

Fig. 13. DEK regulates different transcription factors ........................ 70

Fig. 14. DEK knockout sensitizes cells to DNA damage-induced death .... 76

Fig. 15. DEK knockout sensitizes cells to DNA damage-induced apoptosis .... 78

Fig. 16. DEK knockdown by DEK shRNA in HeLa .......................... 80

Fig. 17. DEK knockdown sensitizes HeLa to DNA damage-induced cell death but not caspase-3 activation .............................................. 82
Fig. 18. DEK knockdown sensitizes p53^{+/+} but not p53^{-/-} HCT116 to DNA damage-induced cell death…………………………………………………………………………………85

Fig. 19. DEK knockdown sensitizes p53^{+/+} but not p53^{-/-} HCT116 to DNA damage-induced apoptosis………………………………………………………………………………86

Fig. 20. DEK knockdown decreases p21 expression in p53^{+/+} HCT116………………90

Fig. 21. DEK knockdown decreases p21 protein stability in p53^{+/+} HCT116……………93

Fig. 22. In vitro binding of p38 and GST-CBP fragments………………………………………101

Fig. 23. Phosphorylation of GST-CBP fragments by p38 in vitro…………………………102

Fig. 24. CBP and p38 interact in coimmunoprecipitation assays……………………………106

Fig. 25. p38 is recruited to promoters through a complex with CBP in ChIP-ReChIP assays…………………………………………………………………………………108

Fig. 26. Inhibition of p38 does not affect the recruitment of p65 and CBP to the promoters in ChIP Assays ………………………………………………………………………111

Fig. 27. The impact of inhibition of p38 activity on gene expression in RT-PCR………..113

Fig. 28. Proposed model of the regulation of NF-κB activity by DEK…………………118

Fig. 29. Proposed model of cytoprotective role of DEK in response to DNA damage 127

Fig. 30. Proposed model of the p38-mediated activation of NF-κB…………………..134
ABBREVIATIONS

AML  Acute myeloid leukemia
Apaf-1  Apoptotic peptidase activating factor 1
AR   Androgen receptor
β-Gal  β-galactosidase
bp    Base pair
BSA  Bovine serum albumin
CBP  CREB Binding Protein
CD   Cluster of differentiation
CDK  Cyclin-dependent kinase
cDNA Complementary deoxyribonucleic acid
CDKI  Cyclin-dependent kinase inhibitors
C/EBP  CAAT/Enhancer Binding Protein
ChIP  Chromatin Immunoprecipitation
CHX  Cycloheximide
Cip1  CDK-interacting protein 1
CK2  Casein kinase II
CREB  cAMP-response element-binding protein
Daxx  death domain associated protein
DBD  DNA binding domain
DHT  5α-dihydrotestosterone
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DR</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblotting</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td><strong>Term</strong></td>
<td><strong>Definition</strong></td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>LXD</td>
<td>LXXLL motifs</td>
</tr>
<tr>
<td>LZ</td>
<td>Leucine zipper</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signaling</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation associated gene 5</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MSK</td>
<td>Mitogen and stress-activated kinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>P53&lt;sup&gt;API1&lt;/sup&gt;</td>
<td>p53-inducible gene 3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P/CAD</td>
<td>p300/CBP associated factor</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PIG3</td>
<td>p53-inducible gene 3</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine-rich domain</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic inducible gene-1</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor interacting protein 1</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SAF</td>
<td>Scaffold attachment factor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β-activated kinase 1</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TNFRI</td>
<td>Type I TNFα receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Type I TNFα receptor associated factor</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole-cell extract</td>
</tr>
<tr>
<td>ZF</td>
<td>Zinc-finger</td>
</tr>
</tbody>
</table>
INTRODUCTION

1.1 Nuclear Factor-kappaB (NF-κB) signaling pathway

NF-κB is a highly conserved and inducible transcription factor responsive to various extracellular stimuli including inflammatory cytokines, growth factors, bacterial and viral infections, and stress. NF-κB controls expression of over 200 genes involved in a broad range of events including inflammation, immune responses, cell survival, apoptosis, differentiation and proliferation (Hayden et al., 2004). Abnormal activation of NF-κB contributes to many human diseases especially cancer and inflammatory diseases (Table 1). Hence, elucidating how NF-κB signaling is regulated in different contexts is important for the development of novel therapeutics for these diseases.

Table 1: Some diseases associated with NF-κB dysregulation:

<table>
<thead>
<tr>
<th>Disease</th>
<th>Disease</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerosis</td>
<td>Diabetes</td>
<td>Stroke</td>
</tr>
<tr>
<td>Asthma</td>
<td>Euthyroid sick syndrome</td>
<td>AIDS</td>
</tr>
<tr>
<td>Arthritis</td>
<td>Inflammatory bowel disease</td>
<td>Cachexia</td>
</tr>
<tr>
<td>Cancer (including Lung, Prostate, and Cervical cancer, Leukemia, and Melanoma)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from review articles (Albert S. Baldwin, 2001; Shishodia et al., 2004)
1.1.1. NF-κB family members

The mammalian NF-κB family consists of five members: p65/RelA (referred to as p65), p52, p50, RelB and c-Rel, which all contain an N-terminal Rel homology domain (RHD) responsible for DNA binding (κB sites), homo- and heterodimerization, interaction with the Inhibitor of κB (IκB) protein and nuclear localization (Fig.1). The p65, RelB, and c-Rel proteins contain a C-terminal transcription activation domain (TAD) necessary for upregulation of gene expression. p50 and p52 are synthesized as large precursors containing five to seven C-terminal ankyrin repeats which are cleaved after processing. The most predominant form of NF-κB is a p50/p65 heterodimer, in which the C-terminal region of p65 contains TAD that can activate gene transcription. In contrast, p50 or p52 homodimers, lacking TADs, bind to NF-κB–responsive promoters and likely repress gene transcription until displaced by TAD-containing NF-κB dimers (Ghosh, 2008). Other forms of NF-κB dimers include p52/c-Rel, p65/c-Rel, p65/p65, p50/p52, p50/RelB, p52/RelB. In addition, a family of ankyrin domain-containing inhibitor proteins IκBs has been identified that keep NF-κB inactive within the cytoplasm. This family includes IκBα, IκBβ, IκBγ, IκBε, IκBξ, bcl-3, p105 and p100, which selectively binds to and inhibits NF-κB dimers (Ghosh, 2008). In particular, IκBα and IκBβ inhibit NF-κB dimers containing p65 or c-Rel; IκBε inhibits specifically p65 homodimer and p65/c-Rel heterodimer; Bcl-3 binds to p50 and p52 homodimers; p100 and p105, precursors of p52 and p50, function to inhibit dimers containing themselves (Ghosh, 2008).
**Fig. 1. NF-κB family members.** The mammalian NF-κB family of transcription factors consists of five members: p65/RelA, p52, p50, RelB and c-Rel. The number of amino acids in each human protein is indicated on the right. Presumed sites of cleavage for p100 (aa 447) and p105 (aa 433) are shown. (RHD) Rel homology domain; (TAD) transactivation domain; (LZ) leucine zipper domain.
1.1.2. NF-κB activation pathway

In the classical NF-κB pathway, the p65/p50 heterodimers are sequestered in the cytoplasm by one of three typical inhibitor proteins: IκBα, IκBβ and IκBε, among which IκBα is the best characterized (Ghosh, 2008). Upon stimulation with extracellular signals such as lipopolysaccharide (LPS) or proinflammatory cytokines IL-1 and TNFα, IκBα is phosphorylated at Ser32 and Ser36 by IκB kinases complex consisting of IKKα, IKKβ and IKKγ (NEMO) (DiDonato et al., 1996). Phosphorylated IκBα undergoes poly-ubiquitination at Lys21 and Lys22 by the E3 SCFβ-TrCP and E2 UbcH5. Ubiquitinated IκBα is then targeted to the 26 S proteasome for degradation (Hayden and Ghosh, 2004). The degradation of IκBα results in the exposure of nuclear localization sequence (NLS) of p65 resulting in the translocation of freed NF-κB to the nucleus. Nuclear NF-κB then binds to its specific consensus site GGGPuNNPyPyCC (κB site, Pu is purine, Py is pyrimidine and N is any base) on NF-κB-responsive promoters to activate gene expression (Gilmore, 1999). Interestingly, NF-κB activates the expression of its own inhibitor IκBα. The resynthesized IκBα binds to NF-κB in the nucleus and translocates NF-κB back to the cytoplasm, creating a negative feedback loop that ensures the transient activation of NF-κB (Baldwin, 1996) (Fig.2).

In the alternative pathway, RelB is sequestered in the cytoplasm through binding to the precursor protein p100 which functions as IκBα. The C-terminal death domain (DD) of p100 functions as a processing inhibitory domain to limit p100 processing in unstimulated cells. The alternative pathway is activated by a variety of inducers
including lymphotoxin (LT), B cell-activating factor (BAFF) and CD40. Binding of these inducers to their receptors activate the NF-κB-inducing kinase (NIK) which then activates IKKα homodimers. IKKα homodimers then phosphorylate p100, leading to its polyubiquitination at Lys855 and subsequent processing into p52 by proteasome. The p52/RelB heterodimers translocate to the nucleus and activate transcription of a subset of NF-κB-regulated genes (Hayden and Ghosh, 2004). Additional mechanisms are also required for controlling NF-κB activation, including the post-translational modification of NF-κB and the dynamic balance of coactivator and corepressor activity (discussed below).
Fig. 2. The classical NF-κB activation pathway. In unstimulated cells, NF-κB is sequestered in cytoplasm by its inhibitor IκBα. Upon stimulation, the IKK complex phosphorylates IκBα which is then be poly-ubiquitinated and degraded by 26 S proteasome. The freed NF-κB translocates to the nucleus and binds to target promoters to activate gene transcription. NF-κB activates the expression of its own inhibitor IκBα, creating a negative feedback.
1.2 Coactivators and corepressors of NF-κB

Gene transcription is tightly controlled by a number of transcriptional coactivator and corepressor proteins that dynamically associate with promoters. Coactivators that enhance NF-κB transcriptional activity include CREB-binding protein (CBP)/p300, p300/CBP associated factor (P/CAF), and members of the steroid receptor coactivator family (SRC1, SRC2 and SRC3). All of the above coactivators contain intrinsic histone acetyltransferase (HAT) activity which acetylates specific lysine residues on the N-terminal tail of core histones, loosening up the chromatin structure and then allowing transcriptional machinery to be recruited to promoter (Gao et al., 2005). Corepressor proteins for NF-κB include the histone deacetylases (HDAC1, HDAC2 and HDAC3), SMRT and NCoR. The HDAC family consists of 17 isoforms grouped into three sub-families: class I HDACs (HDAC1-3 and 8), class II HDACs (HDAC4-7, 9-11), and class III HDACs (sirtuins 1-6) (Rahman et al., 2004). HDAC1-3 are recruited to the promoters of NF-κB–regulated genes, then deacetylate histones to tighten the chromatin structure and reduce the accessibility of promoters to transcription factor binding (Ashburner et al., 2001; Ito et al., 2000; Vanden Berghe et al., 1999). SMRT and NCoR do not contain enzymatic activity, but can trigger the catalytic activity of HDACs (Guenther et al., 2001).

The interaction of NF-κB with cofactors is regulated by posttranslational modification. Upon IκBα degradation after stimulation, protein kinase A (PKA) phosphorylates p65 at Ser276, promoting the interaction of p65 with CBP (Zhong et al.,
1998). In addition to PKA, MSK1/2 (mitogen- and stress-activated protein kinase) phosphorylate p65 at Ser276 in the nucleus, and MSK1−/− MSK2−/− cells have diminished p65 transcriptional activity in response to TNFα (Vermeulen et al., 2003). Other p65 phosphorylation events including (1) phosphorylation of p65 at Ser536 by IKKα and IKKβ (Perkins, 2006), (2) phosphorylation of p65 at Ser529 by CK2 (Wang et al., 2000), and (3) phosphorylation of p65 at Ser536 by PKCζ (Duran et al., 2003), may also contribute to the complete p65 activation by facilitating CBP/p300 binding. CBP/p300 and associated HATs acetylate p65 on multiple lysine residues including Lys310. Acetylation of Lys310 enhances the transcriptional activity of p65 (Chen et al., 2004). Phosphorylation of p65 at Ser276 and Ser536 are necessary to recruit CBP/p300 that in turn acetylates p65 (Chen et al., 2005). It is possible that Ser536 phosphorylation alters the binding preference of p65 resulting in decreased interaction with SMRT and HDAC3, and increased interaction with CBP/p300 (Hoberg et al., 2006).

Since CBP/p300 function as coactivators for multiple transcription factors that may have opposite functions, different transcription factors may compete for recruiting CBP/p300. One example is the cross-repression of p53 and NF-κB: Ser1382 and Ser1386 phosphorylation of CBP by IKKα results in increased binding of CBP to NF-κB and decreased binding to p53 (Huang et al., 2007). The focus of our work is to further understand the complicated regulation of NF-κB by coactivators and corepressors, and study new factors that mediate NF-κB transcriptional activity.
1.3 DEK oncoprotein

Human DEK is a highly conserved chromatin-associated protein which was first discovered as DEK-CAN fusion protein in a subset of acute myeloid leukemia (AML). Later, DEK has been implicated in various cancer and autoimmune diseases. The overexpression of DEK in many aggressive human tumors suggests that DEK may function as an oncoprotein (Carro et al., 2006; Sitwala et al., 2003); Whereas the DEK was identified as an autoantigen in patients with autoimmune diseases, indicating a involvement of DEK in immune responses (Fu et al., 1997). However, how DEK contributes to carcinogenesis and autoimmunity is poorly understood. Therefore, we have aimed to better understand the biological functions of DEK, which may shed light on pathogenesis of related diseases.

1.3.1. Domains and modification of DEK protein

The human DEK protein, consisting of 375 amino acids, does not contain any consensus enzymatic domains, but does have intrinsic DNA-binding activity. DEK binds to DNA in a structure-specific, rather than sequence-specific manner, with preference for supercoiled and cruciform DNA, and can introduce positive supercoils into relaxed circular DNA (Kappes et al., 2004b). DEK contains two DNA-binding domains: the central scaffold attachment factors (SAF) motif-containing domain (aa 149-187) and the C-terminal DNA-binding domain (aa 270-350) (Fig. 3) (Kappes et al., 2004b). The central DNA-binding domain containing SAF–box is sufficient to introduce supercoils
into DNA and facilitates the formation of large DNA-protein complexes (intramolecular interaction) (Bohm et al., 2005), whereas the C-terminal DNA-binding domain contributes to the stability of DEK-DNA interactions (Kappes et al., 2004b). In addition to DEK, other proteins containing SAF domain include scaffold attachment factors that is involved in chromatin organization and transcriptional regulation, protein inhibitor of activated STAT (PIAS) which is a transcriptional repressor of several transcription factors, Ku70 which is a DNA repair protein, and poly(ADP-ribose)polymerase that is involved in DNA repair and programmed cell death (Aravind, 2000). Interestingly, DEK has also been implicated in similar functions such as chromatin modulation, transcriptional regulation, DNA repair and apoptosis inhibition (discussed below).

Posttranslational modifications play important roles in regulating DEK DNA binding ability and localization. DEK DNA binding is weakened by casein kinase 2 (CK2)-mediated phosphorylation of DEK (Kappes et al., 2004a), by p/CAF-mediated acetylation of DEK (Cleary et al., 2005) and by PARP1-mediated poly(ADP-ribose)ylation of DEK (Gamble et al., 2007). DEK is a phosphoprotein with most phosphorylation sites mapped to serine and threonine residues in the carboxy-terminal region by mass spectroscopic analyses (Kappes et al., 2004b). DEK can be phosphorylated by CK2 on multiple sites within the C-terminal region, and the binding of phosphorylated DEK to DNA is less stable than that of unmodified DEK (Kappes et al., 2004a). DEK contains 67 lysine residues, 7 of which located in aa 1-70. The antibody specific for acetylated lysine residues can only detect the full-length DEK protein but not a truncated form of DEK (aa
70-375) through immunoprecipitation. Hence, DEK was proposed to be acetylated at one or more of the seven lysine residues within the first 70 N-terminal amino acids (Cleary et al., 2005). Acetylation of DEK not only decreases its affinity for DNA binding, but also drives DEK into interchromatin granule clusters (IGCs), sub-nuclear structures that contain RNA processing factors (Cleary et al., 2005). This may explain why DEK is able to function in multiple pathways that take place in different sub-nuclear compartments. Poly(ADP-ribosyl)ation of DEK by PARP1 is required to displace DEK from a chromatin substrate to allow access of the transcription machinery and to permit transcription (Gamble and Fisher, 2007). Moreover, poly(ADP-ribosyl)ation of DEK during apoptosis results in dissociation of DEK from DNA and its release into the extracellular space, providing a mechanism by which DEK can be presented to the immune system as an autoantigen (F. Kappes, 2008).
Figure 3. Linear representation of the DEK protein. DEK has a nuclear scaffold attachment factor/protein motif (SAF/SAP motif) including residue 149-183; a putative nuclear localization signal including residue 205-221; and three acidic regions including residues 30-49, 228-254, and 300-310. The breakpoint of the chromosomal translocation is residue 350, which produces DEK-CAN fusion protein.
1.3.2. Functions of DEK protein

DEK was first identified in patients with a subtype of acute myeloid leukemia (AML) as part of a fusion protein DEK-CAN resulting from a t(6;9) translocation. DEK-CAN consists of the N-terminal 348 amino acids of DEK and the N-terminal two-thirds (1278 aa) of the nucleoporin CAN (Von Lindern M, 1992). Since then, many functions for DEK have been proposed including chromatin remodeling, DNA supercoiling, DNA repair, RNA splicing, transcriptional regulation, proliferation and apoptosis inhibition.

DEK protein is abundant in the nucleus and is mostly associated with chromatin, whereas about 10% is associated with RNA (Waldmann et al., 2004). DEK is involved in DNA-dependent processes such as transcription regulation and chromatin remodeling, and RNA-dependent processes such as splice site recognition and mRNA processing (Campillos et al., 2003; Soares et al., 2006; Waldmann et al., 2004). DEK binds directly to DNA and can alter the DNA structure by introducing positive supercoils, which may mediate transcriptional activity. For example, DEK appears to interact with the HIV-2 enhancer to suppress viral transcription (Faulkner et al., 2001; Fu et al., 1997). DEK may be also involved in transcriptional repression through interaction with HDAC2 and Daxx, a protein that functions in part as a transcriptional corepressor through its interaction with a number of transcription factors including Pax3 and ETS-1 (Hollenbach et al., 2002). Moreover, the recruitment of DEK to promoters represses the HAT activity of P/CAF and p300 and induces hypoacetylation of histone H3 and H4 (Ko et al., 2006), providing one other mechanism through which DEK represses transcription. Interestingly, DEK also
has a role in transcriptional activation by stimulating the activity of the AP-2α transcription factor (Campillos et al., 2003). Thus, depending on the specific interacting partners, DEK can either negatively or positively regulate gene transcription.

Evidence is accumulating in support of DEK as an oncoprotein. The expression of DEK is upregulated in various human tumors including colon, cervical, breast, bladder, larynx, lung, and stomach cancer, as well as retinoblastomas, lymphomas, sarcomas, and melanomas (Carro et al., 2006; Sitwala et al., 2003). Expression of the dek gene as well as the dek-can fusion gene are controlled by transcription factors NF-Y, YY-1 and E2F, which are all implicated in cellular proliferation and cancer (Carro et al., 2006; Sitwala et al., 2002). Senescence of HeLa cervical cancer cells are accompanied by DEK repression and overexpression of DEK inhibit both senescence and apoptosis in cells infected with high-risk human papilloma virus E7 (Wise-Draper et al., 2006), suggesting a role for DEK in supporting cell proliferation. DEK, in response to genotoxic damage, facilitates DNA repair and protect cells from apoptosis (F. Kappes, 2008), indicating that DEK may contribute to apoptosis inhibition of cancer cells. Most recently, transgenic mouse studies showed reduced murine papilloma formation in DEK knockout mice, providing direct evidences to support the role of DEK in tumorigenesis (Wise-Draper et al., 2009). Moreover, DEK depletion via shRNA resulted in cell death in human tumor in vitro and in vivo (Wise-Draper et al., 2009), presenting DEK as a potential therapeutic target for cancer treatment.

In addition to the oncogenic property, DEK has a potential role in immune
responses. DEK has been identified as an autoantigen in patients with autoimmune diseases such as juvenile rheumatoid arthritis/juvenile idiopathic arthritis (JRA/JIA), systemic lupus erythematosus, and sarcoidosis (Kappes et al., 2004b). However, it is still not clear whether the anti-DEK autoantibodies are a cause or effect of these diseases. One report showed that DEK was actively secreted by activated macrophages in a CK2-dependent manner and that the secretion of DEK was also observed in the synovial fluid samples from patients with JRA. Moreover, they demonstrated that proinflammatory cytokine IL-8 stimulated the secretion of DEK which acted as an extracellular chemoattractant for inflammatory cells including CD8+ T cells, natural killer cells, and neutrophils (Mor-Vaknin et al., 2006). This study suggests a potential link between DEK and inflammation likely through recruiting proinflammatory cells in response to inflammatory signals. Another report showed that, in apoptotic cells, DEK was modified by phosphorylation and poly(ADP-ribosyl)ation, then released from chromatin to the outside of the cell, both in association with oligonucleosomes and in a free form. The released DEK was recognized by DEK-autoantibodies from the synovial fluid of JRA patients. These data provide a second mechanism, besides active secretion, by which DEK was presented to the immune system to generate DEK-autoantibodies in autoimmune diseases (F. Kappes, 2008).

Recently we demonstrated a role for DEK in negatively regulating the transcriptional activity of the p65 subunit of NF-κB (Sammons et al., 2006). Our data indicate that DEK interacts with p65, associates with NF-κB-responsive promoters in vivo, and represses
expression of NF-κB-regulated genes. Our interest is to further understand the mechanism by which DEK negatively regulates NF-κB-mediated transcriptional activation, as well as the role of DEK in mediating cell survival and immune responses.

1.4 DNA damage-induced apoptosis and the p53 tumor suppressor protein

1.4.1. DNA damage-induced apoptosis

Apoptosis is a tightly regulated cell suicide process known as programmed cell death. Apoptosis can be initiated either through the extrinsic pathway mediated by death-receptors or the intrinsic pathway mediated by the mitochondrial pathway. In both pathways, precursors of initiator caspases, i.e. caspase-8, caspase-9, caspase-10, undergo proteolytic auto-processing to generate two subunits that comprise the activated enzyme. The activated initiator caspases then cleave and activate effector caspases, mainly caspase-3, caspase-6 and caspase-7, forming a caspases cascade. Eventually, the effector caspases cleave cellular substrates that lead to biochemical and morphological changes committing cells to apoptosis (Igney et al., 2002). DNA damage-induced apoptosis has been extensively studied due to its significance in protecting the integrity of genomic DNA as well as killing cancer cells. DNA damage can result from environmental and therapeutic genotoxins, reactive oxygen species, or errors in replication (Norbury et al., 2004). DNA damage, through p53-dependent or independent pathways, usually causes cell cycle arrest to assess the damage and activate DNA repair mechanisms.
In some situation, i.e. irreparable damage, apoptosis is induced to eliminate damaged cells and maintain genomic integrity (Fig. 4). p53 tumor suppressor is crucial in governing a cell’s responses to DNA damage (discussed below). Defects in DNA damage-induced apoptosis contribute to tumorigenesis, and to tumor resistance to DNA damage-based therapy (Igney and Krammer, 2002). Understanding the molecular mechanisms of tumor resistance to DNA damage-induced apoptosis will provide insights into tumorigenesis and new therapeutic approaches for cancer treatment.
Fig. 4. Common cellular responses to DNA damage. In response to DNA damage, signal transduction (p53-dependent or independent) leads to cell cycle arrest to assess the damage and activate DNA repair mechanisms. In some situation, i.e. irreparable damage, apoptosis is induced to eliminate damaged cells. Adapted from a review article (Norbury and Zhivotovsky, 2004).
1.4.2. p53 tumor suppressor

The tumor suppressor p53 is critical in maintaining cellular genomic integrity in response to DNA damage through inducing cell cycle arrest to prevent cells from dividing and/or triggering apoptosis to eliminate the damaged cells (Pietsch et al., 2008). Loss of p53-dependent apoptosis plays a major role in tumorigenesis. Indeed, mutations in the p53 gene occur in at least half of all human cancers (Olivier et al., 2002). In undamaged cells, p53 is bound by Mdm2 that acts as an ubiquitin ligase that targets p53 for degradation in a proteasome-dependent manner. DNA damage activates protein kinases that phosphorylate p53 and thereby reduces its binding to Mdm2, resulting in a marked increase in p53 concentration in the cell (White, 1996). Cells with wild-type p53 undergo cell cycle arrest following DNA damage, which is able to allow time for DNA repair. Under circumstances of irreparable damage, wild-type p53 can activate apoptosis. Whereas cells with mutant p53 appear to bypass the G1/S checkpoint and are less likely to undergo apoptosis at similar level of damage (White, 1996). The best-characterized function of p53 is to act as a transcriptional factor that binds to the promoters of target genes and activates expression of genes mediating apoptosis and cell cycle checkpoints. The preferred binding site for p53 is two tandem repeats of the consensus 5’-Pu-Pu-Pu-C-(A/T)-(T/A)-G-Pyr-Pyr-Pyr-3’ (Pu is purine and Pyr is pyrimidine), separated by 0-13 bases (Pietsch et al., 2008). Genes regulated by p53 include those encoding proapoptotic Apaf-1, Bax, caspase-1, caspase-6, DR4, DR5, Fas, p53AIP1, PIG3, PTEN, the BH3-only proteins Noxa and PUMA, cell cycle regulators p21
and GADD45, as well as others (Kannan et al., 2001). After binding to promoters, p53 recruits coactivators such as CBP/p300 and P/CAF as well as general transcription factors to activate gene transcription. The recruited coactivators with HAT activity can also acetylate p53 and influence its transactivation. In particular, acetylation of p53 by P/CAF at Lys320 results in binding of p53 to only certain high affinity binding sites, such as those found at p21, leading to enhanced cell cycle arrest but reduced apoptosis. In contrast, acetylation of p53 at Lys373 by CBP/p300 leads to hyperphosphorylation of p53 N-terminal residues and enhanced ability of p53 to transactivate lower affinity binding sites, such as those of proapoptotic genes Bax, PIG3, and p53\textsuperscript{AIP1} (Knights et al., 2006). Thus, different post-translational modification of p53 may allow p53 to choose between cell cycle arrest and apoptosis in response to various degrees of DNA damage.

There are two p53 homologous proteins, p73 and p63, both of which can regulate particular p53 target genes and induce cell cycle arrest and apoptosis. While p53 is often mutated in human cancers, p63 and p73 mutations rarely occur (Pietsch et al., 2008). Thus, p63 and p73 may complement the tumor suppressive property of p53 or substitute for p53 in cells lack of functioning p53. It is worth noting that p21 can also be activated by DNA damage through p53-independent manner, e.g. through the p53 homologue p73 (Zhu et al., 1998). In the p53-null human osteosarcoma SAOS2 cell line, protein levels of p73 and p21 increased following treatment with anti-cancer agent paclitaxel (Oh et al., 2008). In HeLa cells, p73 associated with p21 promoter and enhanced p21-reporter gene expression (Oh et al., 2008).
1.4.3. p21 (WAF1/CIP1/SDI1)

The cyclin-dependent kinase inhibitor p21WAF1/CIP1/SDI1 (further referred to as p21), induced by both p53-dependent and p53–independent pathways, is a key mediator of cell cycle arrest after DNA damage and may limit tumor growth (Bunz et al., 1998; Chan et al., 2000). On the other hand, p21 may also act as an oncogene, since it inhibits apoptosis and may promote cell proliferation in some tumors (Roninson, 2002). Moreover, p21 is either upregulated or downregulated in various human cancers (Abbas et al., 2009). Therefore, p21 shows both anticancer and procancer properties.

p21 induces cell cycle arrest primarily through binding to and inhibiting cyclin-dependent kinases (CDKs) CDK2 and CDK1 which are required for phosphorylation of Rb and activation of E2F-dependent gene expression allowing cell cycle progression as well as stimulation of replication and DNA synthesis (Zhu et al., 2005). Moreover, p21 interferes with proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase activity. Through binding to PCNA, p21 competes for PCNA binding to DNA polymerase-δ, thereby inhibiting DNA replication and DNA synthesis (Moldovan et al., 2007). Other than inhibiting cell cycle progression, p21 also inhibits apoptosis. Cells lacking p21 failed to arrest in G1, could not maintain a stable G2 arrest, and were more sensitive to apoptosis in response to DNA damage (Chan, Hwang et al. 2000). This p21-induced G1/G2 arrest may protect cells with damaged DNA from apoptosis, and allow DNA repair to proceed. In addition to the anti-apoptotic activities, p21 also exhibits oncogenic activities. For example, p21
can promote the association of cyclin D with CDK4 or CDK6 and the activation of these complexes, supporting tumor growth (Liu et al., 2007). Moreover p21 can act as specific regulator of gene expression. In particular, p21 not only represses the activity of a number of transcription factors including E2F, c-Myc, STAT3, but also stimulates the activity of the transcriptional coactivator p300/CBP histone acetylases and the transcriptional activity of NF-κB which contribute to carcinogenesis (Perkins, 2002; Poole et al., 2004). These studies show that p21 mediates its various functions through different mechanisms. Novel cancer therapeutics targeting the oncogenic properties of p21 but not its tumor suppressor functions is both promising and challenging.

1.4.4. Cytoprotective roles of p53-p21 signaling

Recent reports on p53 and p21 suggest a pro-survival role of p53-p21 signaling in the event of DNA damage. Firstly, while p53 activates pro-apoptotic proteins, it is also able to stimulate expression of DNA-repair proteins such as PCNA, GADD45, and ERCC3 (Kannan et al., 2001). In the absence of GADD45, DNA repair was greatly reduced and cells became sensitized to DNA damage (Smith et al., 1996). Hence, cells lacking p53 would be compromised in DNA repair and be more susceptible to DNA damage-induced death. Secondly, p53 stimulates transcription of p21 which is a cell cycle-dependent kinase inhibitor (CKI) and is responsible, at least in part, for cell cycle arrest induced by p53. DNA damage leads to accumulation of p53 and transcriptional induction of p21 which binds to cyclin-CDK and S cyclin-CDK to inhibit their activities,
thereby blocking entry into S phase (G1 checkpoint). This G1/S arrest may protect cells with damaged DNA from apoptosis, and allow time for DNA repair before DNA replication. It has been reported that the p53-p21-pRb pathway was necessary for protection against apoptosis in response to AAV-induced DNA damage (Garner et al., 2008). When activated by p53-p21 signaling, pRb inhibits the activation of pro-apoptotic protein p84N5 which is activated by DNA damage and able to induce cell death (Garner and Raj, 2008). Normal cells can be prevented from undergoing apoptosis while DNA repair occurs, whereas cells lacking p53-p21 pathway would be more likely to undergo apoptosis in response to DNA damage. Taken together, p53-p21 signaling can not only induce cell death, but can also provide protection to cells with damaged DNA and facilitate DNA repair. This creates a check and balance mechanism governing cell cycle checkpoints, DNA repair and apoptosis.
1.5 CREB-Binding Protein (CBP)

CBP and its homologue p300 are transcriptional coactivators of various sequence-specific transcription factors including CREB, NF-κB, c-Jun, c-Myb, YY1, Myo-D, c-Fos and steroid receptors, which are involved in a wide range of cellular activities, such as DNA repair, cell growth, differentiation and apoptosis (Vo et al., 2001). Their highly conserved domains among species include three zinc finger domains (Z), the CREB-binding domain KIX, the histone acetyltransferase (HAT) domain, the bromodomain which functions as acetyl-lysine binding domain, the N- and C-terminal activation domains (AD), and glutamine-rich domain (Q) which is unique to CBP (Fig. 5). CBP/p300 have two major functions in transcriptional regulation: (1) as a scaffold to form a multiprotein transcriptional complex and (2) as a histone acetyltransferase (HAT) to increase the accessibility of transcriptional complex to promoters (Vo and Goodman, 2001).

CBP and p300 can interact with the basal transcription factors TBP and TFIIIB (Kwok et al., 1994), and form a complex with RNA polymerase II (Cho et al., 1998). These interactions occur through the N- and C-terminal AD containing zinc finger domains (Fig. 5). In addition, CBP and p300 can bind to diverse transcription factors such as NF-κB, c-Jun, and p53 through the zinc finger, KIX, and Q domains (Vo and Goodman, 2001). Take NF-κB as an example, the N-terminus of CBP (amino acid 1-771) containing a zinc finger domain as well as the C-terminus of CBP (aa 1892-2441) containing Q domain interacts with the C-terminus of p65 (aa 286-551) containing the TAD (Gerritsen et al.,
The interactions between CBP/p300 and transcription factor can be phosphorylation-dependent. The CBP-CREB interaction is dependent on the cAMP-dependent protein kinase A (PKA)-phosphorylated form of CREB (Lundblad JR, 1995). The interaction of CBP with p65 is mediated by posttranslational modification of both CBP and p65, e.g. the phosphorylation of p65 by PKA and MSK1 on S276 promotes the association of p65 with CBP (Zhong et al., 1998). Taken together, CBP and p300 function as bridge or scaffold between basal transcription machinery and upstream transcription factors in order to stabilize the transcriptional complex.

CBP/p300 also possesses intrinsic histone acetyltransferase (HAT) activity, which acetylates lysine (K) residues on the histone tails to remodel chromatin structure and increase the accessibility of transcription factors and basal transcriptional machinery to the DNA template (Kalkhoven, 2004). The CBP/p300 preferred sites of acetylation are K12 and K15 in histone H2B, K14 and K18 in histone H3, and K5 and K8 in histone H4 (Schiltz et al., 1999). In addition to the HAT activity, CBP/p300 can also acetylate a number of nonhistone proteins like transcription factors to alter their activities. For example, CBP/p300 acetylates p53 at K373 and thereby stimulates p53 DNA binding and transcriptional activities (Gu et al., 1997). CBP/p300 or other associated HATs may acetylate p65 at Lys310 to enhance p65 transcriptional activity, in part through increasing the interaction of CBP and p65 (Chen and Greene, 2004).
**Fig. 5. Linear representation of the CBP/p300 protein family.** The highly conserved domains of CBP/p300 between species were indicated. Shown are three zinc finger domains (Z), the CREB-binding domain (KIX), the bromodomain (Bromo), the HAT domain, the N- and C-terminal activation domains (AD), and the glutamine-rich domain (Q, unique to CBP).
Interestingly, CBP/p300 contributes to various and even opposed cellular processes. As mentioned earlier, diverse transcription factors may compete with each other to interact with limited amounts of CBP/p300 in the cell. One example is the functional antagonism between NF-κB and p53 through competition for CBP/p300. NF-κB generally promotes cell growth and survival, whereas p53 promotes apoptosis. Therefore, binding of CBP/p300 to NF-κB or p53 may determine whether a cell undergoes proliferation or apoptosis. One group proposed that IKKα-mediated phosphorylation of CBP at Ser1382 and Ser1386 switches the binding preference of CBP from p53 to NF-κB (Huang et al., 2007). Phosphorylation of CBP and p300 is a critical regulation for their functions. Other than IKKα, the proposed kinases that phosphorylate CBP/p300 and mediate their HAT activity include p42/44 MAPK (Janknecht et al., 1996), cyclin E/Cdk2 (Ait-Si-Ali et al., 1998; Perkins et al., 1997), and PKA (Xu et al., 1998). Cyclin E/Cdk2 was shown to represses p300 coactivator function (Perkins et al., 1997) but increase the intrinsic CBP HAT activity, potentially activating expression of S-phase genes (Ait-Si-Ali et al., 1998). The phosphorylation of p300 at S89 by PKCζ in vivo reduces its HAT activity (Yuan et al., 2002). The phosphorylation of CBP is required for the PKA-mediated activation of the transcription factor Pit-1 (Xu et al., 1998). MAPK-mediated phosphorylation of CBP enhances the transactivation potential of CBP to induce expression of the c-Fos proto-oncogene (Janknecht and Nordheim, 1996). However, the phosphorylation sites in CBP/p300 have not been completely mapped, and how specific phosphorylation regulates CBP/p300 in various contexts requires further study.
CBP is an important coactivator of NF-κB and interacts with the p65 subunit of NF-κB (Gerritsen et al., 1997). The interaction between p65 and CBP is regulated by post-translational modification of both CBP and p65 (Vo and Goodman, 2001). Our preliminary data showed that the p38 MAP kinase interacts with and phosphorylates CBP \textit{in vitro}. Therefore, part of the work described in this dissertation is focused on understanding how p38 regulates CBP and NF-κB activity.

1.6 p38 MAP kinase

The mitogen-activated protein kinase (MAPK) pathways are involved in important cellular processes including cell growth, differentiation, and apoptosis. The MAP kinases are activated through phosphorylation cascades in which a mitogen-activated protein kinase kinase kinase (MAKPKKK) phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK (Dhillon et al., 2007). Three distinct MAPK pathways have been described in mammalian cells: the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs (Dhillon et al., 2007). In general, the ERK pathway is activated by mutagenic and proliferative stimuli, whereas JNK and p38 pathways are stimulated by environmental stresses (UV, heat, osmotic shock) and inflammatory cytokines (TNFα and IL-1).

p38α MAPK was first identified as a major phosphoprotein activated by lipopolysaccharide (LPS) (Han et al., 1994) and as a target of a pyridinyl imidazole drug that blocked the production of cytokines TNFα and IL-1 (Lee et al., 1994). The p38 MAP
kinases are a family of serine/threonine protein kinases that includes four isoforms: p38α/β/γ/δ, all of which contain Thr-Gly-Tyr (TGY) phosphorylation motif in their activation loop (Dhillon et al., 2007; Kumar et al., 2003; Perkins et al., 1997). This motif is phosphorylated by the MAP2Ks, MKK3 and MKK6, specifically on Thr180 and Tyr182 of p38α; MKK3 and MKK6 in turn are phosphorylated and activated by various MAPKKKs induced by environmental stresses and inflammatory responses (Zarubin et al., 2005). p38 was shown to be present in both the cytoplasm and the nucleus, and activated p38 MAPKs translocate from the cytosol to the nucleus where they phosphorylate various downstream targets, including transcription factors and other kinases (Roux et al., 2004).

1.6.1. Substrates and genes mediated by p38

The first identified substrates of p38 was the MAP kinase-activated protein kinase 2 (MK2) (Rouse et al., 1994), which activate various downstream targets including small heat shock protein 27 (HSP27) (Rouse et al., 1994); transcription factors including cAMP response element-binding protein (CREB), ATF1 (Tan et al., 1996) and serum response factor (SRF) (Heidenreich et al., 1999); as well as tristetraprolin (TTP) which is known to destabilize mRNA, suggesting a role of p38 pathway in mRNA stability (Mahtani et al., 2001). Mitogen- and stress-activated protein kinase-1 (MSK1) is another substrate for both p38 and ERK, and may phosphorylate and activate CREB in response to stress and growth factor (Deak et al., 1998). About half of p38 substrates are transcription factors involved in a variety of actions. Examples include activating transcription factor (ATF) 1,
2 & 6, SRF accessory protein (Sap1) (Janknecht et al., 1997), CHOP (involved in differentiation and apoptosis) (Wang et al., 1996), tumor suppressor p53 (in response to UV) (Huang et al., 1999), myocyte-enhancer factor 2C (MEF2C) (involved in LPS-induced c-Jun expression) (Han et al., 1997), NFATp (del Arco et al., 2000), as well as high mobility group-box protein 1 (HBP1) that has been linked to G1 cell cycle arrest (Yee et al., 2004). These diverse substrates indicate that the p38 MAPK pathway has broad functions.

By utilizing small molecule p38 inhibitors (e.g. SB203580) and dominant active mutants of MKK3 and 6, numerous genes regulated by the p38 pathway have been identified, including genes encoding cytokines, transcription factors, and cell surface receptors (Zarubin and Han, 2005). In addition to transcriptional regulation, the p38 pathway has been linked to post-transcriptional regulation of inflammatory genes. For example, p38 inhibitors blocked protein synthesis of TNFα and IL-1β while their mRNA levels showed little or no change (Kontoyiannis et al., 2001). Moreover, MK2 knockout mice had impaired protein synthesis of TNFα with unchanged TNFα mRNA levels, suggesting that p38 may act through MK2 to activate TNFα mRNA translation (Kontoyiannis et al., 1999).

1.6.2. Biological functions of p38

p38 MAPK has been strongly linked with inflammatory responses. p38 activation is important for the production of TNFα, IL-1β, IL6, and other cytokines during
inflammatory responses, and several p38 inhibitors (e.g. SB203580) have anti-inflammatory effects (Kumar et al., 2003). In addition, p38 plays a role in the proliferation and differentiation of immune cells. For example, p38 is required for CD40-induced proliferation of B lymphocytes (Craxton et al., 1998) and for synthesis of granulocyte macrophage colony stimulating factor (GM-CSF), a hematopoietic growth factor (Beyaert R, 1996).

The p38 pathway is also involved in regulation of apoptosis, cell survival, and differentiation, but the effects seem to be cell type and stimulus-dependent. For example, Nerve growth factor (NGF) withdrawal from rat PC-12 cells led to sustained activation of JNK and p38 which is critical for induction of apoptosis in these cells (Xia et al., 1995). The differentiation of PC12 cells into neurons and SKT6 cells into hemoglobinized cells requires p38 (Morooka et al., 1998; Nagata et al., 1998). In muscle, differentiation of myoblasts into myotubules requires both PI3-kinase and p38 signaling, proposing a cross-talk model for these two pathways (Li et al., 2000). Also, p38 has been linked with cell cycle control. p38 is activated when mammalian cells are arrested in M phase by nocodazole indicating a function of p38 in the spindle assembly checkpoint (Takenaka et al., 1998). As mentioned earlier, p38 is involved in G1 arrest through its substrate HBP1 (Yee et al., 2004). Expression of p38 or MEKK3 (a MAPKKK that activates p38) induces G1 arrest and reverses oncogenic Ras-mediated transformation in NIH 3T3 cells through down-regulation of cyclin D1 (Ellinger-Ziegelbauer et al., 1999; Molnar et al., 1997). In addition, p38 has a role in senescence: Ras-induced premature senescence of primary
human fibroblasts requires sequential activation of the ERK and p38 pathways (Wang et al., 2002) indicating a function for p38 in negative regulation of cell growth. It is worth noting that p38 signaling is not an isolated event, but acts cooperatively with other MAPKs and other signaling pathways to exert distinct effects in different contexts.

1.6.3. **Link between NF-κB and p38 MAPK**

NF-κB and p38 MAPK can be activated by many of the same proinflammatory cytokines and strong evidence indicates that p38 plays a role in NF-κB activation. Blocking p38 by its inhibitor SB80 diminishes TNFα-induced and CD40-induced NF-κB-dependent reporter gene expression, but does not affect the DNA binding of NF-κB (Beyaert R, 1996; Chae et al., 2001; Craxton et al., 1998). Moreover, blocking p38 inhibits oncogenic Ras-induced as well as IL-1β- and Akt-induced transactivation of Gal4-p65 fusion protein, which contains the C-terminal 30 amino acid transactivation domain (TAD) of p65 (aa519-551) fused to the DNA binding domain (DBD) of the yeast transcription factor Gal4 (Madrid et al., 2001; Norris et al., 1999). Akt is a serine/threonine kinase involved in activation of NF-κB in response to TNF, Ras, and IL-1β (Madrid et al., 2001). These data demonstrate that p38 or its substrate is involved in activation of NF-κB, at least in part through modulating the p65 TAD, but the mechanism is not understood.

Expression of some NF-κB target genes may depend on p38 for chromatin modification. A subset of NF-κB-responsive promoters has constitutive accessibility for
NF-κB, thus can recruit NF-κB dimers immediately after their nuclear translocation. In contrast, other promoters (e.g. IL-6 promoter) require stimulus-dependent chromatin structure remodeling to expose NF-κB-binding site (Saccani et al., 2001). One group proposed that a subset of NF-κB-responsive promoters underwent p38α-dependent histone H3 phosphorylation at Ser10. Ser10 phosphorylation promoted subsequent H3 acetylation at Lys14 correlating with increased expression of several inflammatory cytokines and chemokines (Saccani S, 2002). Thus, these p38-dependent histone H3 modifications may mark selective promoters for NF-κB recruitment and subsequent transcriptional activation. Another group showed that MSK1, a downstream target of p38 and ERK MAPKs, phosphorylates p65 at serine 276 in the RHD in response to TNFα, leading to stimulation of particular NF-κB-mediated genes (Vermeulen et al., 2003). Furthermore, in response to LPS, NF-κB and p38 cooperate to induce transcription of two antiapoptotic genes, Pai-2 and Bfl-1/A1 to maintain macrophage survival. The direct targets for p38 in this pathway that induces Pai-2 are MSK1/2, which in turn activate transcription factor CREB (Park et al., 2005). These data demonstrate different mechanisms by which the p38 pathway induces expression of specific NF-κB-regulated genes, however the question of how p38 mediates the transactivation function of NF-κB has not been fully answered.

Based on the fact that IL-1β and Akt requires not only p38 but also coactivator CBP/p300 and phosphorylation of Ser529 and Ser536 of p65 for efficient stimulation of p65 transcriptional activity (Madrid et al., 2001) and the possibility that phosphorylation
of p65 at Ser529 and Ser536 facilitate the interaction of CBP and p65 TAD, we hypothesize that p38 may mediate the activity of the p65 TAD through CBP. Our data demonstrate that p38 interacts with and phosphorylates CBP \textit{in vitro}. Part of this dissertation will focus on assessing the role of p38 in mediating NF-κB activation through targeting the CBP coactivator.
1.7 Overall objectives:

The objectives of this dissertation research have been to address the functions of the novel oncoprotein DEK in mediating NF-κB and apoptosis signalings, as well as to better understand the control of NF-κB activation by p38 MAP kinase. The specific aims of these studies have been:

(1) To characterize the role of DEK on NF-κB mediated transcriptional activation
(2) To study the mechanism by which DEK mediates NF-κB activity
(3) To determine the function of DEK in DNA damage-induced apoptosis
(4) To study the mechanism by which DEK inhibits apoptosis
(5) To confirm the p38-CBP interaction and phosphorylation of CBP by p38
(6) To assess the role of p38 in mediating NF-κB transactivation through CBP
2 MATERIALS AND METHODS

2.1 Cell lines, plasmids and other materials

HeLa human cervical carcinoma cell line, p53\(^{+/+}\) and p53\(^{-/-}\) HCT116 human colorectal carcinoma cell line, human embryonic kidney (HEK) 293 cell line, HEK 293 cells containing SV40 Large T-antigen (293T) cell line, Immortalized DEK\(^{-/-}\) and DEK\(^{+/+}\) mouse embryonic fibroblasts (MEFs) were grown in Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% penicillin/streptomycin (P/S). Jurkat human T lymphoma cell line were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% P/S. Mouse embryonic fibroblasts (MEFs) from DEK\(^{-/-}\) and DEK\(^{+/+}\) mouse were kindly provided by Dr. Gerard Grosveld (St. Jude Children’s Research Hospital).

3xκB-luc, 5xGAL4-luc, CMV-p65, GAL4-p65 have been described previously (Ashburner et al., 2001). The DEK expression plasmid (FLAG-DEK) was kindly provided by Dr. D. Markovitz (University of Michigan). The GAL4-AR (N terminus) was kindly provided by Dr. L. Shemshedini (University of Toledo). The GAL4-p53 was kindly provided by Dr. A. Baldwin (University of Toledo). pcDNA-CBP was kindly provided by Dr. R. Kwok (University of Michigan).
Antibodies used are: anti-p65 (sc-372), anti-RNA polymerase II (H-224, sc-9001), anti-CBP (C-20, sc-538), anti-Ικβα (sc-371), anti-p53 (sc-126), anti-p21, anti-p38 (N-20, sc-728), anti-myc (9E10, sc-40), anti-PCAF (E-8, sc-13124), anti-Histone H3 (sc-10809), anti-HDAC1 (sc-7872), anti-HDAC2 (sc-7899) and anti-HDAC3 (sc-11417) antibodies from Santa Cruz Biotechnology; anti-caspase3 (CAT-9662), antibodies from Cell Signaling; anti-flag-M2 (CAT-F3165) from Sigma-Aldrich; anti-p73 (5B1288, CAT-IMG-259A) from Imgenex; anti-DEK (CAT-610948) from BD Transduction Laboratories; anti-β-actin (CAT-ab6276) from Abcam.

Recombinant human tumor necrosis factor alpha (TNFα) (Biosource, Camarillo, CA) was used at a final concentration of 10 ng/ml. Doxorubicin (DR) (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 200 ng/ml or as indicated. TSA was used as a final concentration of 0.2 μM. Puromycin (Mediatech Inc.) was used at a final concentration of 1 μg/ml. Polybrene (Chemicon) was used at a final concentration of 8 μg/ml.

2.2 RNA Interference and stable DEK knockdown cell line

The lentiviral plasmids expressing short hairpin RNAs (shRNAs) directed against human DEK were purchased from Open Biosystems (Huntsville, AL). The five individual short hairpin DNAs are cloned in pLKO.1 lentiviral vector (Fig. 6) and are able to produce shRNA targeting different sequence in the DEK mRNAs. The identifications of the five different shRNA constructs are: TRCN0000013103, TRCN0000013104,
TRCN0000013105, TRCN0000013106 and TRCN0000013107. HEK 293T cells were seeded on 10-cm dishes and cultured in DMEM supplemented with 10% FBS. The lentiviruses containing shRNA were produced by co-transfecting HEK293T cells with the individual shRNA construct (1 μg) along with the packaging plasmids (RRE 0.9 μg, RSV 0.9 μg and VSVG 0.1 μg), using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer’s recommendation. Eighteen hours post-transfection, medium was replaced with DMEM containing 30% FBS and 1% P/S. Lentivirus-containing medium was harvested at 24 and 48 hours later, then pooled and centrifuged to obtain the viral supernatant. HeLa, p53+/+ and p53−/− HCT116 cells were seeded on 6 cm plates with 3x10⁵ cells per plate. After overnight incubation, cells were infected twice for a total of 48 h with the viral supernatant in the presence of 8 μg/ml of polybrene, and selected in 1 μg/ml puromycin 48 hours post-infection. A control vector, PLKO.1_PURO, coding for the puromycin resistance gene and no shRNA, was used as a negative control.
**Fig. 6. pLKO1 Lentiviral vector used in the production of Lentivirus.** The shRNA constructs consisting of optimal hairpin DNA (a 21 base stem and a 8 base loop) were cloned into the pLKO1 vector. Figure adapted from OpenBiosystems (http://www.openbiosystems.com/RNAi/shRNALibraries/TRCLibraryDetails/).
2.3 Transient transfection and reporter gene assays

Transient transfections were performed using Fugene 6 (Roche Biochemicals, Indianapolis, IN) or TransIT-LT1 (Mirus, Madison, WI) by following the manufacturer’s instructions. For reporter gene assays, cells were plated in 24-well dishes and cotransfected with 100 ng of indicated luciferase reporter plasmids, β-galactosidase reporter plasmids (pCMV-LacZ) and/or indicated expression plasmids. 48 hours post-transfection, cells were lysed with 100 μl M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL), and incubated at room temperature for 10 minutes with gentle shaking followed by spinning at 4°C for 5 minutes at full speed. 50 μl of cell lysate was assayed for luciferase activity using LMax luminometer (Molecular Devices, Sunnyvale, CA). Data were normalized to β-Galactosidase (β-Gal) activity assayed. For the β-Gal assay, 5 μl of cell lysate was diluted in 45 μl of M-PER buffer and then mixed with 50 μl 2xβ-Gal buffer (200mM sodium phosphate buffer, 2mM MgCl₂, 100mM β-mercaptoethanol, 1.33 mg/ml ONPG). After incubation at 37°C for 30 minutes to 3 hours, absorbance at 420nm was measured by Vmax kinetic microplate reader (Molecular Devices). All experiments were performed a minimum of three times in triplicate.

2.4 Caspase-Glo 3/7 Assay

Cells were seeded in 96-well plate at 15,000 cells per well, and untreated or treated with Doxorubicin (DR) for indicated time and concentration. Then, 100 μl of Caspase-Glo 3/7 Reagent was added in each well and mixed on a plate shaker for 30
seconds. After incubation at room temperature for 50 minutes, luminescence that is proportional to Caspase 3/7 activity was measured by LMax luminometer (Molecular Devices, Sunnyvale, CA). Data were blank subtracted and normalized to the untreated control sample as recommended by the manufacture (Promega, Madison, WI). Data were presented as relative luciferase units.

2.5 Semi-quantitative reverse transcriptase-PCR (RT-PCR), quantitative real-time PCR and PCR Array

Total cellular RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. 2 μg of total cellular RNA was reverse transcribed into cDNA by Random Hexamer Primer and M-MLV reverse transcriptase (Promega, Madison, WI) in a total volume of 20 μl with 1 hour incubation at 37 °C followed by 5 minutes incubation at 85°C. 2 μl of the reverse transcribed cDNA was then subjected to PCR amplification with gene-specific primers and MasterMix (5 PRIME, Gaithersburg, MD) in a total reaction volume of 25 μl. The transcripts of genes in different samples were quantitated against the transcript of the house-keeping gene GAPDH. PCR products were resolved on 1% agarose gels, visualized by ethidium bromide staining under UV light and images were captured with a digital camera.

For real time-PCR, 2 μl of the reverse transcribed cDNA was used as the template in a total reaction volume of 25 μl containing final concentrations of 1x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and 600 nM of each forward and reverse primers.
Real-time PCR was performed on the Bio-Rad iCycler with a two-step cycling program as follows: 1 cycle of 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The threshold cycle (Ct) data were read using the Bio-Rad iCycler. Expression levels were normalized to β-actin and presented as relative expression.

RT-PCR and real time-PCR were carried out using primer pairs to amplify the cDNA region of human cIAP2 (Forward 5′-ATGCTTTTGCTGTGATGGTG-3’, Reverse 5′-TGAAACTTGACGGATGAACTCC-3’), human IL8 (Forward 5′-CTCTCTTTGGCAGCCTTCTC-3’, Reverse 5′-AATTTCTGTGTGGGCGAG-3’), mouse IκBα (Forward 5′-GCCTGGACTCCATGAAAGAC-3’, Reverse 5′-CTTCCATGGTCAGTGCTTTT), mouse MCP1 (Forward 5′-CCCCAGTCACCTGCTGTTAT-3’, Reverse 5′-TGGAATCCTGAAGCTTC-3’), mouse p21 (Forward 5′-CCTCTTGAGCTCACTCCCATGAAAGAC-3’, Reverse 5′-CTTCCATGGTCAGTGCTTTT), mouse GAPDH (Forward 5′-CGACCACTTTGTCAAGCCTCA-3’, Reverse 5′-AGGGMAATCAGTGCTGGTG-3’), mouse β-Actin (Forward 5′-AGGGGAGATTCACTGCTGGTG-3’, Reverse 5′-TGTATGAGGTTTGGTCTCCCT-3’).

For PCR arrays (SuperArray Biosciences), 96-well plates containing real-time PCR primers for mouse genes involved in either NF-κB signaling or apoptosis signaling were used. WT MEFs and DEK-/- MEFs were treated with TNFα (10 ng/ml 1, 2, and 6 hrs) or Doxorubicin (200 ng/ml 6, 12 and 24 hrs), and RNA was prepared using TRIzol reagent. 1.5 μg of RNA was reverse transcribed into cDNA by Random Hexamer Primer and
M-MLV reverse transcriptase in a total volume of 20 μl with 1 hour incubation at 37 °C followed by 5 minutes incubation at 95 °C. 91 μl of ddH2O was added to 20 μl of cDNA reaction to dilute cDNA. Real-time PCR master mix was prepared by mixing 1325 μl of 2xSYBR Green Supermix, 1214 μl of ddH2O and 111 μl of diluted cDNA. 25 μl of the real-time PCR master mix was added to each well of the PCR Array 96-well plate.

Real-time PCR was performed on the Bio-Rad iCycler with a two-step cycling program as follows: 1 cycle of 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The threshold cycle for each well was calculated using the instrument’s software (Bio-Rad) and analyzed with Web-Based PCR Array Data Analysis software (SuperArray Biosciences).

### 2.6 Coimmunoprecipitation (CoIP) and immunoblotting analysis

For CoIP, the cells were lysed in cell lysis buffer (50 mM TRIS, pH 7.5; 85 mM KCl; 0.5% NP-40 with protease inhibitors) for 10 minutes on ice, then the intact nuclei fraction was pelleted by centrifugation at 3,300 rpm for 5 minutes. The nuclei was lysed in nuclear lysis buffer (50 mM TRIS, pH 7.5; 200 mM KCl; 1% Triton X-100; 0.5% NP-40 10 mM EDTA with protease inhibitors) for 10 minutes on ice. The soluble nuclear extract was recovered by centrifugation at 16,000 rpm for 15 minutes. Protein concentration determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of nuclear extracts were subjected to immunoprecipitation using indicated antibodies and Protein A/G-agarose (Santa Cruz Biotechnology). The
immunoprecipitated complexes were washed 4 times with 500 μl of nuclear lysis buffer and resuspended in 15 μl of 2X SDS-PAGE sample buffer. Immunoprecipitated samples or cell extracts were boiled for 3 minutes and resolved by 10% SDS-PAGE before transfer to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked in 1XTBST with 5% milk and incubated with the appropriate antibodies. Proteins were visualized by anti-rabbit IgG-HRP, anti-mouse IgG-HRP or anti-Goat IgG-HRP (Promega) and Chemiluminescence reagent (Pierce, Rockford, IL) followed by exposure to film.

2.7 Cytoplasmic and nuclear extracts

Cells were washed with phosphate-buffered saline (PBS), harvested and resuspended in cytoplasmic lysis buffer (10 mM Hepes pH 7.6, 60 mM KCl, 0.3% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 μg/ml of aprotinin, leupeptin, and pepstatin), and lysed on ice for 5 minutes. After centrifugation at 1200 rpm for 4 min at 4°C, supernatant was removed to a new eppendorf tube and spun again at full speed for 15 minutes. Pellets were washed once in 100 ml of cytoplasmic lysis buffer without NP-40 and spun at 1200 rpm for 4 minutes at 4°C. The nuclear pellets were resuspended in nuclear lysis buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 2.5 μg/ml of aprotinin, leupeptin, and pepstatin), and lysed on ice for 15 minutes. Both cytoplasmic and nuclear extracts were centrifuged at 12,000 rpm for 20 min. The resulting supernatants were collected as cytoplasmic and nuclear extracts respectively. A final concentration of 400 mM NaCl and
20% glycerol were added to cytoplasmic extracts. Both cytoplasmic and nuclear extracts
were assayed for protein concentration using Bio-Rad protein assay dye, and stored and -
80°C

2.8 Chromatin immunoprecipitation (ChIP) assay and ChIP-ReChIP assay

Cellular proteins and DNA were cross-linked by adding formaldehyde at a final
concentration of 1% to the growth medium for 10 min at 37 °C. Cells were washed twice
with ice cold PBS and harvested by scraping. Cells were lysed first in cell lysis buffer (50
mM Tris-HCl pH 8.0, 85 mM KCl, 0.5% NP40 and protease inhibitors) for 10 minutes on
ice. After centrifugation at 3300 g for 5 min at 4 °C, the pellets of nuclei were lysed in
nuclear lysis buffer (1% SDS, 10 mM EDTA, and 50mM Tris pH 8.0 with protease
inhibitors) for 10 minutes on ice. Lysates were sonicated to shear chromatin DNA to
about 500 bp fragments using a Sonic Dismembranator Model 500 (Fisher Scientific).
1/20 of each nuclear lysate was kept for input and the rest was precleared with protein
A-agarose or protein A/G-agarose beads and rabbit or mouse nonspecific IgG (Santa Cruz
Biotechnology, Santa Cruz, CA) for 1 hour. Lysates were then diluted in half by adding
equal volume of dilution buffer (0.01% SDS, 1.1% Triton-X 100, 167 mM NaCl, 16.7 mM
Tris-HCl pH 8.1), and immunoprecipitated using specific antibodies with rocking
overnight at 4 °C followed by incubation with protein A-agarose or protein A/G-agarose
for 1h at 4 °C with rocking. Immunoprecipitated complexes were washed once in low salt
buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 150 mM NaCl),
once in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 500 mM NaCl), once in LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0), and twice in TE buffer (20 mM Tris and 2 mM EDTA). Precipitated chromatin DNA was eluted with freshly prepared elution buffer (1% SDS, 1% NaHCO₃), and the Na⁺ concentration was adjusted to 200 mM by adding NaCl, followed by incubation at 65 °C overnight to reverse cross-links. DNA was purified using Wizard SV Gel and PCR clean-up System (Promega, Madison, WI), and 1/10 of the DNA was used to amplify the indicated promoter region. Semi-quantitative PCR was carried out using primer pairs to amplify the promoter regions of human IL8 (Forward 5’-GGGCCATCAGTTGCAAATC-3’, Reverse 5’-TTCCCTCCGTTGTTTCTTC3’), human cIAP2 (Forward 5’-GTGTGTTGTTATTACCGC-3’, Reverse 5’-AGCAAGGACAAGGCCAGTCT-3’), human GAPDH (Forward 5’-AGCGCAGGCTCAAGACCTT-3’, Reverse 5’-AAGAAGATGCGGCTGACTGT-3’), mouse IkBa (Forward 5’-CGCTAAGAGCCAGGCCTCAG-3’, Reverse 5’-CAGCTGGCTGAAACATGGC-3’), mouse MCP-1 (Forward 5’-CACCCATTACATCTCTTCC-3’, Reverse 5’-TGTTTCCCTCTCACTTCTCTGTC-3’).

Amplified DNA was visualized by agarose gel electrophoresis using SYBR Gold (Invitrogen/Molecular Probes). Images were captured using Kodak EAS290 Digital Camera.

For ChIP-ReChIP assay, single ChIP complexes were eluted with 30 μl 10 mM DTT by incubating for 30 min at 37 °C with agitation. The eluted complexes were then diluted...
20 times with Re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 7.5) and subjected to the second immunoprecipitation, and the following steps of Re-ChIP was carried out as the same as ChIP described above.

2.9 **in vitro** binding assays

Various GST-CBP fragments were purified from bacteria and immobilized on Glutathione-sepharose beads. $^{35}$S-labeled p38 proteins were made by *in vitro* transcription/translation in the presence of $^{35}$S-Methionine. Then $^{35}$S-labeled p38 were mixed with GST or GST-CBP fragments immobilized on beads. After incubation with rotation, beads were collected by centrifugation and washed 3 times with binding buffer. Then the beads were boiled in 2xSDS loading dye and the eluted proteins were separated on SDS-PAGE. $^{35}$S-labeled p38 coprecipitated with GST-CBP fragment were visualized by autoradiography. Coomassie blue stained gel visualized the total protein level of GST-CBP fragments as the loading control.

2.10 **in vitro** kinase assays

Various GST-CBP fragments were purified from bacteria and immobilized on Glutathione-sepharose beads, then incubated with activated p38 (purchased from Millipore) in the presence of $\gamma^{32}$P-ATP with rotation. After incubation, the reaction was stopped by adding 2xSDS loading dye and then boiled. Eluted proteins were separated on SDS-PAGE. Autoradiography was performed to detect the GST-CBP fragments that
were phosphorylated by activated p38 and thus strongly labeled by \(^{32}\)P. Coomassie blue stained gel visualized the total protein level of GST-CBP fragments as the loading control.

2.11 Proliferation assay / MTT assay

20,000 cells were seeded in 24-well plate with 500 µl DMEM with 10% FBS. After 24 hrs incubation, cells were treated with or without Doxorubicin (DR) for the indicated concentrations and times. MTT (Sigma, CAT# M 5655) assay was performed by adding 50 µl MTT (5 mg/ml in PBS) into each well. After 3 hrs incubation at 37 °C, 500 µl MTT lysis buffer (0.1N HCl, 10% Triton X-100 Plus in isopropanal) was added into each well followed by pipetting up and down for several times. After 30 minutes shaking at room temperature, 100 µl of the solution from each well were transferred to clear bottom 96-well plate to measure the 570 nm absorbance by microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). Data is expressed as percentage of cell viability using the data from treated wells divided by that from untreated control wells on the same day.

2.12 Statistical significance analysis

Statistical significance was calculated by Student’s t-test. Microsoft Excel Data Analysis Tool was used to perform Student’s t-test (two sample assuming equal variants) and generate the p-value. If the p-value is smaller than 0.05, the difference between two samples was judged to be statistically significant.
3 RESULTS

3.1 Part 1: Negative regulation of NF-κB by DEK protein

DEK was identified as an NF-κB-interacting protein in a yeast two-hybrid screen using the C-terminal TAD of p65 as the bait suggesting that DEK might be a regulatory factor of NF-κB (Vogel N.L., MS Thesis, 2004). Hence, we aimed to determine the role of DEK in NF-κB-mediated transcriptional activation.

3.2.1 DEK interacts with p65 subunit of NF-κB

The NF-κB subunit p65 C-terminal transactivation domain (TAD) contains three LXXLL (where L is Leucine and X is any amino acid) motifs that functions as protein-protein interaction domains in other proteins (Heery et al., 1997). Therefore, coactivators or corepressors are likely to associate with p65 through these LXXLL motifs.

To search proteins that interact with NF-κB, the C-terminal TAD of p65 was used as the bait in cytoplasmic yeast two-hybrid screen, and DEK was identified as a p65 TAD-interacting protein (Vogel N.L., MS Thesis, 2004).

To confirm the DEK-p65 interaction, co-immunoprecipitations (co-IPs) were performed to study the interaction between endogenous DEK and p65. HeLa cells were untreated or treated with TNFα (10ng/ml) for the indicated times, and nuclear extracts
were immunoprecipitated with a DEK antibody. In untreated cells, a low level of p65 was co-precipitated with DEK (Fig. 7, upper panel, lane 1). After 20 min TNFα treatment, DEK and p65 interaction was greatly enhanced; whereas at 120 min TNFα treatment, DEK and p65 interaction was decreased compared to the 20 min, but still greater than in UT cells (Fig. 7, upper panel, lanes 1-3), despite a similar level of nuclear p65 in 20 and 120 min treatment (Fig. 7, middle panel, lanes 2-3). Thus, DEK and p65 interaction is induced quickly after TNFα treatment, and then decreased after 2 hours. This decrease is possibly due to the association of p65 with coactivators or with IκBα, both of which may displace DEK. The above data confirmed our two-hybrid results and showed that DEK and p65 interact in the nucleus in vivo.
Fig. 7. Coimmunoprecipitation of endogenous DEK and p65.

Upper panel, nuclear extracts from HeLa cells that were either untreated or treated with TNFα (10 ng/ml) for the indicated times were immunoprecipitated with an antibody specific for DEK. The immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-p65 antibody. Lower two panels, nuclear extracts were subject to immunoblotting for anti-p65 and anti-DEK antibodies to detect nuclear p65 and DEK as input control.
3.2.2 DEK represses NF-κB-mediated transcriptional activation

Since DEK may positively or negatively regulate specific transcription factor activity, we asked whether DEK affects NF-κB-mediated transactivation. Luciferase assays were performed using a NF-κB-dependent κB luciferase reporter gene (κB-Luc) plasmid which is an artificial reporter plasmid derived from the MHC I enhancer with four κB binding sites. HeLa cells were transiently transfected with the κB-Luc plasmid and the indicated plasmids. Transfection of increasing amount of Flag-DEK repressed p65-induced κB-Luc reporter gene expression in a dose dependent manner (Fig. 8A, lanes 3-5) indicating that DEK suppresses the exogenous p65-mediated transactivation. To address whether overexpression of DEK also represses the ability of endogenous NF-κB to activate transcription, we utilized κB-Luc reporter plasmid to monitor the TNFα-induced NF-κB transactivation. Transfection with 25ng and 50ng of Flag-DEK plasmid resulted in a 50% and 90% reduction, respectively, of TNFα-induced κB-Luc reporter gene expression (Fig. 8B, black bars). These data reveal that DEK negatively regulates NF-κB-mediated reporter gene expression.

Yeast-two hybrid data show that DEK interacts with the C-terminal TAD of the p65 subunit. To determine if DEK represses NF-κB activity through the p65 TAD, we used a plasmid with C-terminal p65 TAD (amino acid 313-551) fused to GAL4 DNA binding domain (G4-DBD) in a reporter gene assays. Expression of the G4-p65 TAD induced the expression of 5XGAL4-luc-reporter gene over 500 fold (Fig. 8C, lane 2 v. 1). Cotransfection of Flag-DEK resulted in reduction of G4-p65 TAD-induced reporter gene
expression in a dose dependent manner (Fig. 8C, lanes 3-5). This DEK-mediated repression was specific to the p65 TAD since no effect was seen when DEK was cotransfected with G4-DBD (Fig. 8C, lanes 6-8). Thus, DEK represses NF-κB transcriptional activity through the p65 transactivation domain (TAD).
Fig. 8. DEK represses NF-κB-mediated transcriptional activation.

HeLa cells were transiently transfected with the κB-luc reporter plasmid (A and B) or 5X GAL4-luc reporter plasmid (C) and the indicated plasmids. Cells were harvested 48 hr after transfection and assayed for luciferase activity (A and C). (B) 40 hr after transfection, the cells were either left untreated or treated with TNFα (10 ng/ml) for 6 hr, then extracts were assayed for luciferase activity. (A and C) For CMV-F-DEK, Lanes 3, 4, 5, 7 and 8 were transfected with 10, 25, 50, 25, and 50 ng of plasmid respectively. Luciferase activities were normalized to total protein assayed and expressed as the average RLU/μg of extract +/- standard deviation of a representative experiment performed in triplicate.
3.2.3 DEK associates with the NF-κB-regulated cIAP2 and IL-8 promoters

Previous work has shown that DEK associates with chromatin. To see if DEK binds to promoters of NF-κB-regulated genes, we performed chromatin immunoprecipitation (ChIP) assays for two NF-κB-regulated genes: cIAP2 and IL-8. Interestingly, DEK associated with both promoters in untreated HeLa cells, and remained associated at the early TNFα time points (15 min and/or 30 min) (Fig. 9A, lanes 1-3 & 6-7). DEK began to dissociate from the promoters by 60 min (cIAP2) or 30 min (IL-8) TNFα treatment (Fig. 9A, lanes 4-5 & 8-10). As expected, after TNFα treatment, the amount of p65 and RNA polymerase II bound to both promoters were enhanced, peaking at 60 min time point, then reduced back to basal levels after 120 min (Fig. 9A, lanes 1-5 & 6-10). The dissociation of DEK from these promoters correlates well with the enhanced binding of p65 and RNA polymerase II to these promoters. As negative controls, neither DEK nor p65 were associated with the constitutively expressed GAPDH promoter (Fig. 9A, lanes 11-15), and no promoters were precipitated with non-specific IgG antibodies (Fig. 9A).

In addition, to correlate the dissociation of DEK from promoters with expression of these genes in response to TNFα, gene expression was analyzed by real-time PCR. Interestingly, as DEK began to dissociate from both promoters at 30 min, expression of both genes gradually increased (Fig. 9A&B). When DEK binding is at its lowest level at 60 min, gene expression is maximal (Fig. 9A&B). Data so far indicate that DEK binds to cIAP2 and IL-8 promoters at a high level in untreated cells and low level in TNFα-treated cells. Upon TNFα treatment, DEK dissociates from these promoters, and this
dissociation correlates well with increased gene expression. Taken together, these data reveal that DEK associates with promoters of NF-κB-regulated genes (but not the constitutively expressed GAPDH) and further support that DEK functions as a repressor for NF-κB-dependent transactivation.
**Fig. 9. DEK associates with the promoters of NF-κB-regulated genes.**

(A) ChIP assays. HeLa cells were untreated or treated with TNFα for indicated times. Nuclear extracts were used for immunoprecipitation with p65, DEK, RNA pol II and rabbit IgG antibodies; Coimmunoprecipitated DNA was subjected PCR using primers specific to the cIAP2, IL-8 and GAPDH promoters; Amplified DNA was then visualized by agarose gel electrophoresis. (B) Real-time PCR analysis of the cIAP2 and IL-8 genes in response to TNFα. HeLa cells were either untreated or treated with TNFα for the indicated times and total RNA was reverse transcribed, and subjected to real-time PCR in triplicate. Expression of cIAP2 and IL-8 was normalized to β-Actin, and data expressed as fold activation relative to the untreated sample.
3.2.4 The absence of DEK enhances expression of NF-κB-regulated genes

To further confirm the repressive effect of DEK on NF-κB-dependent transcriptional activation, we used immortalized mouse embryonic fibroblasts (MEFs) from dek−/− knockout mice and those from wild type (WT) control littermates. Wild type and DEK−/− MEFs were transiently transfected with the κB-luc reporter plasmid. About 36 hours after the transfection, cells were either left untreated or treated with TNFα (10 ng/ml) for 6 hrs, then harvested and assayed for luciferase activity. When comparing the untreated DEK−/− to WT MEFs, the DEK knockout leaded to a 5.6-fold increase in basal reporter gene expression (Fig. 10A, left, black bar v. white bar). When comparing the TNFα-treated DEK−/− to WT MEFs, the absence of DEK resulted in a 5.5-fold increase of TNFα-induced NF-κB transactivation activity (Fig. 10A, right, black bar v. white bar). These data are consistent with our observation that DEK represses the ability of NF-κB to activate transcription.

To assess the impact of the absence of DEK on NF-κB-regulated gene expression, real-time PCR analysis was performed to compare the expression of two NF-κB-regulated genes, MCP-1 and IκBα, between DEK−/− MEFs and WT MEFs. The basal expression of both genes was elevated in DEK−/− MEFs compared to WT MEFs (Fig.10B). For MCP-1, gene expression was greatly enhanced in the DEK−/− MEFs compared to WT MEFs after TNFα induction (Fig.10B, upper graph). For IκBα, after 15 min and 30 min TNFα treatment, IκBα gene expression was increased about 2-fold higher in DEK−/− MEFs compared to WT MEFs (Fig. 9B, lower graph). However, at the later time points 60 and 58
120 min, there was no difference between DEK<sup>-/-</sup> MEFs and WT MEFs (Fig. 10B, lower part).

In agreement with the increased gene expression, the IκBα protein level was enhanced in the absence of DEK (Fig. 10C). IκBα is the inhibitor of NF-κB and can also be induced by NF-κB, forming a negative feedback loop to limit NF-κB activity. Immunoblotting analysis showed a typical IκBα degradation and resynthesis in response to TNFα treatment in WT MEFs (Fig. 10C, lanes 1-5). In untreated DEK<sup>-/-</sup> MEFs, a basal level of IκBα protein was detected and was similar to that in WT MEFs (Fig. 10C, lane 6 v. 1). However, the resynthesized IκBα protein level in DEK<sup>-/-</sup> MEFs was consistently greater at all time points compared to that in WT MEFs (Fig. 10C, lanes 7-10 v. 2-5). These data correlate well with the increased mRNA level of IκBα observed by real-time PCR in DEK<sup>-/-</sup> MEFs (Fig. 9B). No detectable difference in β-actin protein level between DEK<sup>-/-</sup> MEFs and WT MEFs was observed, indicating that DEK-mediated repression had some promoter specificity. Taken together, the data in Figure 10 support a role of DEK in repressing both basal and TNFα-induced NF-κB-mediated gene expression.
Fig. 10. Increased expression of NF-κB regulated genes in the absence of DEK.

(A) Reporter gene assay. DEK−/− or WT MEFs were transiently transfected with κB-luc plasmid. 40 hrs post-transfection the cells were treated with TNFα (10 ng/ml) for 6 hrs and extracts were assayed for luciferase activity. Data was normalized to total protein assayed and is expressed as average fold activation compared to the wild type untreated +/- st. dev.

(B) Real-time PCR analysis. WT and DEK−/− MEFs were either untreated or treated with TNFα (10 ng/ml) for the indicated times. Total RNA was harvested, reverse transcribed, and subjected to real-time PCR analysis in triplicate. Expression Mcp-1 and IκBα was normalized to β-Actin and data expressed as fold activation relative to the untreated sample.

(C) Immunoblotting analysis. WT and DEK−/− MEFs were either untreated or treated with TNFα (10 ng/ml) for the indicated times. Whole cell extracts (25 μg/lane) from WT and DEK−/− MEFs showed increased TNFα-induced IκBα protein level in the DEK−/− cells compared to WT cells.
3.2.5 The absence of DEK enhances the recruitment of p65 and P/CAF to promoters

To assess the mechanism of DEK-mediated repression of NF-κB tranactivation, ChIP assays were performed on DEK\(^{\text{−/−}}\) MEFS and WT MEFs to determine if the absence of DEK affects binding of p65 to the promoters of the MCP-1 and IκB\(\alpha\) genes. The cells were untreated or treated with TNF\(\alpha\) (10 ng/ml) for the indicated times. In DEK\(^{+/+}\) (WT) MEFs, a typical p65 binding pattern to promoters of both MCP-1 (Fig. 11, lanes 1-5) and IκB\(\alpha\) (Fig. 11, lanes 11-15) was observed, with peak binding at 30 min, followed by a gradual decrease (Fig.11, upper panel). Interestingly, in DEK\(^{\text{−/−}}\) MEFs, TNF\(\alpha\)-induced recruitment of p65 to both promoters was enhanced and prolonged (Fig.11, upper panel, lanes 6-10 v. 1-5 and lanes 16-20 v. 11-15). Moreover, a previous study showed that P/CAF-mediated acetylation of DEK resulted in decreased affinity of DEK for DNA (Cleary et al., 2005), indicating a functional interaction between P/CAF and DEK. We performed ChIP assays to determine if DEK loss affected the recruitment of the P/CAF coactivator to the MCP-1 and IκB\(\alpha\) promoters. In WT MEFs, the recruitment of P/CAF to the MCP-1 promoter was inducible by TNF\(\alpha\) with peak binding at 60 and 120 minutes (Fig. 11, lanes 1-5). In the absence of DEK, P/CAF is recruited to the promoter at earlier times and at greater levels, correlating with the enhanced recruitment of p65 to the promoter in DEK\(^{\text{−/−}}\) MEFs compared to WT MEFs (Fig.11, middle panel, lanes 6-10 v. 1-5). Similarly, there was also enhanced association of P/CAF with the IκB\(\alpha\) promoter (Fig.11, middle panel, lanes 16-20 v. 11-15). This enhanced recruitment of both p65 and P/CAF to these promoters correlates well with the increased expression of the MCP-1 and IκB\(\alpha\) genes.
genes in DEK⁺⁻ MEFs observed from real time PCR (Fig. 10B). These data further support our hypothesis that DEK functions as a transcriptional corepressor protein for NF-κB-mediated transcription.
Fig. 11. Enhanced association of p65 and P/CAF to Mcp-1 and IkBα promoters in the absence of DEK.

ChIP assays were performed on wild type and DEK<sup>−/−</sup> MEFs untreated or treated with TNFα for the indicated times. After crosslinking, DNA-protein complexes were immunoprecipitated with p65 or P/CAF antibodies (1μg). Co-immunoprecipitated DNA was subjected to PCR amplification using primers specific to the mouse Mcp-1 or IkBα promoters. PCR products were visualized by agarose gel electrophoresis. Each ChIP was performed a minimum of three times with similar results.
3.2.6 The association of HDAC1, HDAC2 and HDAC3 on NF-κB-regulated promoters in DEK\(^{+/+}\) and DEK\(^{-/-}\) MEFs

Since DEK does not contain any enzymatic domains, DEK may exert its repressive function in part through recruiting other corepressors to promoters. A common mechanism of transcriptional repression is the promoter-specific recruitment of histone deacetylases (HDAC) that results in the compaction of chromatin structure and reduces the accessibility of the transcriptional machinery to promoters. The class I HDACs, HDAC1, 2 and 3 are recruited to the promoters of NF-κB–regulated genes and are involved in the repression of NF-κB transcriptional activity (Ashburner et al., 2001; Ito et al., 2000; Vanden Berghe et al., 1999). Moreover, DEK can associate with the Daxx and HDAC2 corepressors (Hollenbach et al., 2002) suggesting a link between DEK and HDACs.

To examine if DEK modulates the recruitment of HDAC2 and other Class I HDACs: HDAC1 and HDAC3 to NF-κB-responsive promoters, chromatin immunoprecipitation (ChIP) assays were performed in DEK\(^{-/-}\) and wild type (WT) MEFs. In WT MEFs, HDAC1, HDAC2 and HDAC3 are recruited to both mIκB\(\alpha\) and mMCP-1 promoters (Fig. 12, lanes 1-5 and 11-15). In DEK\(^{-/-}\) MEFs, the absence of DEK does not change the recruitment of HDAC1 (Fig.12, lanes 6-10 and 16-20, 1\(^{st}\) and 3\(^{rd}\) row), but slightly enhances the recruitment of HDAC2 and HDAC3 to both promoters, compared to that in WT MEFs (Fig.12, lanes 6-10 v. 1-5 and 16-20 v. 11-15, 2\(^{nd}\) row). This enhanced recruitment of HDAC2 and HDAC3 may be caused by the increased association of p65 to the promoters observed in DEK\(^{-/-}\) MEFs (Fig.11). HDAC2 does not interacts with NF-κB
directly but can be recruited to NF-κB through HDAC1 that interacts with p65 subunit of NF-κB directly (Ashburner et al., 2001). Since HDAC2 represses both basal and TNFα-induced expression of NF-κB-regulated genes (Ashburner et al., 2001), HDAC2 and HDAC3 may be recruited to promoters together with NF-κB and coactivators to coordinately regulate gene expression. However, because DEK does not affect the recruitment of HDAC1 to these promoters, it is still not clear how DEK modulates the recruitment of HDAC2. These data show that DEK does not help recruiting HDAC1, HDAC2 and HDAC3 to the promoters. But this does not exclude the possibility that DEK requires the HDAC enzyme activity to exert its repression effects.
Fig. 12. Association of HDAC1, HDAC2 and HDAC3 on NF-κB-responsive promoters in DEK^{+/+} and DEK^{-/-} cells. ChIP assays were performed on DEK^{+/+} and DEK^{-/-} MEFs either untreated or treated with TNFα (10ng/ml) for the indicated times. After crosslinking and sonication, DNA and protein complexes were immunoprecipitated with HDAC1, HDAC2 or HDAC3 antibodies (1μg). Co-immunoprecipitated DNA was subjected to PCR using primers specific to the mouse Mcp-1 or IκBα promoters. PCR products were visualized by agarose gel electrophoresis.
3.2.7 DEK regulates other transcription factors

To determine whether the ability of DEK to repress transcription is specific to NF-κB or if DEK is also able to regulate the activity of other transcription factors, transient transfection reporter gene assays were performed using luciferase-reporter genes driven by the IFNβ (IFNβ-prom-luc), androgen receptor (PSA-luc), p53 (p53-luc), and Stat5B (IRF-luc). The results showed that DEK was able to repress transiently transfected AR- and p53-driven reporter gene expression in a concentration-dependent manner (Fig. 13A and 13B, lanes 3-5 v. lane 2), and modestly repress Stat5B-driven reporter gene expression at the highest concentration of transfected DEK plasmid (Fig. 13C, lane 5 v. lane 2). In addition, we also tested if DEK could repress the ability of Gal4-p53 and Gal4-AR(N-terminus) fusion proteins to activate transcription. Cotransfection of DEK with Gal4-AR or Gal4-p53 resulted in a strong repression of Gal4-AR- or Gal4-53-mediated transcriptional activation of 5XGal4 luc reporter gene (Fig. 13 D&E, lanes 3-4 v. 2). Thus, similar to the data shown in Fig. 13A and B, DEK can also repress the ability of these heterologous Gal4 fusion proteins to activate transcription, further supporting a role for DEK in repressing transcription.

IFNβ induces expression of IFN-stimulated genes (ISGs) which plays a vital role in innate and adaptive immune responses (Meurs et al., 2007). RNA helicase MDA5, product of Melanoma differentiation associated gene 5 and homolog of RIG-I, can recruit adaptor protein IPS-1/MAVS, and subsequently activate transcription factors including NF-κB and IRF3 which induce IFNβ expression (Meurs and Breiman, 2007). To
determine whether DEK mediates IFNβ production, we transiently transfected MDA5- or MAVS-expression plasmids into 293T cells to induce the IFNβ-promoter-luc reporter gene expression. Overexpression of MDA5 or MAVS elevated the IFNβ-promoter-luc reporter gene expression over 400 and 700 fold respectively (Fig. 13F lanes 5-7, 11-13). However, overexpression of DEK failed to suppress MDA5- or MAVS-induced IFNβ-promoter-Luc reporter gene expression (Fig. 13F, lanes 8-9 v. 5-7 and 14-16 v. 11-13) suggesting that DEK does not regulate the ability of NF-κB and IRF3 to stimulate IFNβ expression. Taken together, these data indicate that DEK may function as transcriptional repressor for transcription factors besides NF-κB including AR, p53, and Stat5B, and that the transcriptional repression function of DEK is specific for particular genes.
Fig. 13. DEK regulates different transcription factors.

(A-C) HeLa cells were transiently transfected with PSA-luc (A), p53-luc (B), and IRF-luc (C) and AR- (A), p53- (B), and Stat5B- (C) expression plasmids as indicated. Lanes 3, 4, and 5 were cotransfected with 10, 25 and 50 ng of DEK-expression plasmid respectively. Cells were harvested 48 hr after transfection and extracts were assayed for luciferase activity. (D-E) HeLa cells were transfected with a GAL4-p53 fusion (D) or a GAL4-AR(N-terminus) (E) fusion protein and lanes 3 and 4 were cotransfected with 25 and 50 ng of DEK-expression plasmid respectively. Cell extracts were assayed for luciferase activity. (A-E) Luciferase values were normalized to total amount of protein assayed and expressed as average RLU/μg of extract +/- st. dev. All experiments were performed in triplicate and performed three times with similar results.

(F) 293T cells were transiently transfected with IFNβ-prom-luc and MDA5- or MAVS-expression plasmids as indicated. Lanes 5-7, 8-10, 11-13 and 14-16 were transfected with 10, 25 and 50 ng of DEK-expression plasmid respectively. Cells were harvested 48 hr after transfection and extracts were assayed for luciferase activity. Luciferase activity was normalized to β-Gal activity assayed and expressed as changes relative to the cells transfected with IFNβ-prom-luc plasmid only.
3.2.8 DEK may regulate a variety of genes involved in NF-κB signaling

Our data showed that DEK represses NF-κB-mediated transcriptional activation and that both basal and TNFα-induced expression of two NF-κB-regulated genes MCP-1 and IκBα are enhanced in the absence of DEK (Fig.10B). We wanted to expand our knowledge on the impact of DEK knockout on other genes involved in NF-κB signaling. Hence a PCR gene expression array (SuperArray Bioscience) analyzing the expression of 84 genes involved in NF-κB signaling was performed. The PCR array is a set of optimized real-time PCR primer assays on a 96-well plate and includes control genes and a focused panel of genes involved in specific signaling or events (e.g. NF-κB signaling). DEK−/− and DEK+/+ MEFs were treated with TNFα (10 ng/ml) for 1, 2 and 6 hours. Then the same MEFs with different treatment time were collected and combined. RNA and cDNA from these DEK−/− and DEK+/+ MEFs were prepared. cDNA templates were mixed with SYBR-PCR master mix, and aliquoted to each well of the 96-well plate of PCR arrays containing primers for mouse NF-κB signaling as well as control primers, and real-time PCR was performed. Using the provided analysis spreadsheet (SuperArray Bioscience), changes in expression of 84 genes between DEK−/− and DEK+/+ MEFs were calculated. The data from this PCR array show that expression of 20 genes were increased by at least 2-fold, whereas expression of 10 genes were decreased by at least 50% in the DEK−/− MEFs when compared to DEK+/+ MEFs (Table 2). Among the listed thirteen NF-κB-responsive genes that are affected by the absence of DEK, 11 genes were upregulated and 2 genes were downregulated in DEK−/− MEFs, supporting our hypothesis that the function of DEK
is more likely to repress expression of NF-κB-regulated genes. Genes changed in the absence of DEK include those encoding inflammatory cytokines, transcription factors, kinases, ligands and receptors, as well as others involved in immune responses and NF-κB signaling, suggesting that DEK regulates a broad range of genes involved in NF-κB signaling either directly or indirectly.
<table>
<thead>
<tr>
<th>Genes upregulated or downregulated in DEK&lt;sup&gt;−/−&lt;/sup&gt; MEFs compared to DEK&lt;sup&gt;+/+&lt;/sup&gt; MEFs in response to TNFα</th>
<th>Data expressed as fold change of gene expression in DEK&lt;sup&gt;−/−&lt;/sup&gt; MEFs relative to WT MEFs.</th>
<th>* indicates NF-κB-responsive genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated genes in DEK&lt;sup&gt;−/−&lt;/sup&gt; MEFs</strong></td>
<td><strong>Down-regulated genes in DEK&lt;sup&gt;−/−&lt;/sup&gt; MEFs</strong></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cytokines</td>
<td>*GM-Csf</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td>*IL-6</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>*TNF</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>IL-1α</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>*IFN-γ</td>
<td>2.3</td>
</tr>
<tr>
<td>Immune responses</td>
<td>*C3</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Myd88</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>*Ccl2</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>TLR1</td>
<td>2.1</td>
</tr>
<tr>
<td>Transcription factor</td>
<td>Fos</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Jun</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>*NFκB2</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Egr1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>*IRF1</td>
<td>2.0</td>
</tr>
<tr>
<td>Kinases</td>
<td>Ripk2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligands and receptors</td>
<td>*Cd40</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others involved in NF-κB</td>
<td>*Icam1</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>Nod1</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>*Bcl3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**Table 2. PCR arrays in NF-κB signaling.** Genes upregulated or downregulated in DEK<sup>−/−</sup> MEFs compared to DEK<sup>+/+</sup> MEFs in response to TNFα. Data expressed as fold change of gene expression in DEK<sup>−/−</sup> MEFs relative to WT MEFs. * indicates NF-κB-responsive genes.
3.2 Part 2: DEK oncoprotein protect cells from apoptosis.

DEK has been linked to cell death protection, and overexpression of DEK has been found in several types of tumors (Sitwala et al., 2003). Moreover, knockdown of DEK in tumor cells and in human primary fibroblasts results in increased p53-dependent apoptosis (Wise-Draper et al., 2006), and sensitizes cells to DNA-damage reagents (F. Kappes, 2008), suggesting a role for DEK in protecting cells from apoptosis. We aimed to better understand the cytoprotective role of DEK and its contribution to cancer cell survival.

3.2.1. DEK knockout sensitizes MEFs to DNA damage-induced apoptosis.

To determine if DEK is involved in cell death protection in response to a DNA damage, we treated DEK^{+/+} and DEK^{-/-} MEFs with DNA damage-inducing anti-cancer drug doxorubicin (DR) and examined the cell death. MEFs were treated with DR for 0 to 3 days, and exposed to MTT reagent for colorimetric detection of cellular metabolic activity proportional to the number of living cells as a measurement of cell survival. MTT assay showed that the absence of DEK resulted in decreased cell viability at all DR time points, day 1, day2, and day3, while the greatest difference seen on day 1, suggesting that the absence of DEK sensitizes cells to DR-induced death (Fig. 14). Hence, DEK acts to protect cells from DNA damage-induced death.
Fig. 14. DEK knockout sensitizes cells to DNA damage-induced death. MTT assay.

MEFs were seeded in 24-well plate, and either untreated or treated with doxorubicin (DR 200 ng/ml) for 1, 2, or 3 days in triplicate. Cells were exposed to MTT reagent (as described in methods and materials) for colorimetric detection of viable cells. Data is expressed as percentage of cell viability (DR-treated cells divided by untreated control cells with the same incubation time).
In order to determine whether DEK knockout would also affects cell apoptosis, we performed immunoblotting to detect the cleaved (activated) form of caspase-3, which is an indicator of apoptosis. MEFs were treated with DR (60 or 100 ng/ml) for the indicated times. In response to DR, DEK\(^{-/-}\) MEFs showed a higher level of cleaved caspase-3 compared to DEK\(^{+/+}\) MEFs, which was true for all DR-treatment concentration and time points (Fig. 15A, compare right panel to left panel). Immunoblotting was performed to verify DEK knockout in DEK\(^{-/-}\) MEFs (Fig. 15B). To further confirm the immunoblotting data, DR-induced caspase-3 activity was measured by monitoring the cleavage of a colorimetric caspase-3 substrate (Ac-DEVD-pNA) using the CaspACE Assay System Clolorimetric (Promega). Consistent with the immunoblotting data, DEK\(^{-/-}\) MEFs showed almost 3-fold and 7-fold increase of caspase-3 activity when compared to WT MEFs after a 4 and 8 hour DR-treatment respectively (Fig. 15C). Taken together, these data show that DEK\(^{-/-}\) MEFs are more sensitive to doxorubicin-induced apoptosis, suggesting that DEK protects cells from DNA damage-induced apoptosis.
Fig. 15. DEK knockout sensitizes cells to DNA damage-induced apoptosis.

(A) DEK\(^{+/+}\) and DEK\(^{-/-}\) MEFs were treated with doxorubicin (60 or 100 ng/ml) for the indicated times. WCE were prepared for immunoblotting with the caspase-3 antibody. The blots were stripped and reprobed with the β-actin antibody as a loading control. (B) Immunoblotting with the DEK antibody showed the absence of DEK protein in DEK\(^{-/-}\) MEFs. Immunoblotting with β-actin antibody showed equal loading. (C) Caspase-3 activity assay using WCE (40 µg of protein) form WT and DEK\(^{-/-}\) MEFs treated with DR (60ng/ml) for the indicated times. SA = Specific activity of caspase-3 (pmol pNA/h/µg)
3.2.2. DEK knockdown sensitizes HeLa cells to DR-induced death.

Since DEK is overexpressed in various cancer cell lines, we were interested in determining whether DEK also protects cancer cells from DNA damage-induced death. We therefore generated stable DEK knockdown using lentiviral shRNA delivery system in cancer cell lines including the cervical carcinoma cell line HeLa and the colon carcinoma cell line HCT116. DEK shRNA plasmid and packaging plasmids were cotransfected into HEK293T cells to produce lentiviral particles containing DEK shRNA. Then the lentiviral supernatants were transduced into target cells. After transduction, cells were treated with puromycin (1μg/ml) to select for stable DEK knockdown cell pools (Fig.16A). Non-silencing Vector shRNA plasmid was transduced as a control. Five different DEK shRNA plasmids were used to individually knockdown DEK. DEK protein levels were significantly decreased in HeLa, most effectively by shRNA-B10 (Fig. 16B, lanes 1-5 v. 6). The DEK mRNA levels were also reduced in HeLa transduced with shRNA-B10 (Fig. 16C). Hence, we used this shRNA-B10 generated stable DEK knockdown as a model to study the role of DEK in cancer cell lines.
Fig. 16. DEK knockdown by DEK shRNA in HeLa. (A) Procedures for generating stable DEK knockdown by lentiviral delivered shRNA. (B) Immunoblotting analysis probing for DEK antibody showed that DEK protein level was knocked down by five different DEK shRNA. Immunoblotting for β-Actin showed equal loading. (C) RT-PCR showed reduced DEK mRNA level by DEK shRNA (B10). RT-PCR for β-Actin showed equal loading.
To assess the impact of DEK on cell survival in response to doxorubicin, we analyzed the cell viability in control and DEK knockdown (KD) HeLa cells in MTT assays. The HeLa DEK KD cells showed fewer viable cells compared to control HeLa throughout the 3-day DR-treatment (Fig. 17A), suggesting that the loss of DEK sensitized HeLa cells to DNA damage-induced cell death. However, immunoblotting analysis for caspase-3 showed similar levels of cleaved caspase-3 in the HeLa DEK KD cells compared to control HeLa cells (Fig. 17B, lanes 2-3 v. 5-6). Thus, DEK knockdown sensitize HeLa cells to DNA damage-induced death but had no influence on caspase-3 cleavage, an indicator of apoptosis. It is worth noting that HeLa cells are highly resistant to DNA damage-induced apoptosis partly due to low levels of wild-type p53 which is degraded by the HPV type 18 E6 oncoprotein (Olivier et al., 2002). These data suggest that DEK may protect cervical cancer cells from DNA damage-induced death through pathways independent of p53 and caspase-3 activation.
Fig. 17. DEK knockdown sensitizes HeLa to DNA damage-induced cell death but not caspase-3 activation. (A) MTT assay. Vector control or DEK knockdown (DEK KD) HeLa stable cell lines were treated with doxorubicin (DR 200 ng/ml) for 0 to 3 days, and exposed to MTT reagent for colorimetric detection of viable cells. (B) Immunoblotting analysis. Control and DEK KD HeLa cells were untreated or treated with DR (200 ng/ml) for 18 and 26 hrs. Whole cell extracts were prepared and subjected to immunoblotting for caspase-3 antibody and β-actin antibody. The blots were stripped and reprobed with the DEK antibody showing DEK knockdown.
3.2.3. DEK knockdown sensitizes p53\(^{+/+}\) but not p53\(^{-/-}\) HCT116 to DNA damage-induced apoptosis.

Since p53 is an extensively studied tumor suppressor that is activated by DNA damage, we wanted to determine if the protective function of DEK is dependent on p53. We obtained p53\(^{+/+}\) and p53\(^{-/-}\) HCT116 cells to examine the impact of DEK knockdown on DNA damage-induced death of colon cancer cells in the presence or absence of p53. Stable knockdown of DEK in both p53\(^{+/+}\) and p53\(^{-/-}\) HCT116 were generated using lentiviral delivered DEK shRNA-B10 (Fig. 18A). Cell death induced by doxorubicin was analyzed in control and DEK knockdown cells by MTT assays (Fig. 18B). In p53\(^{+/+}\) HCT116, DEK loss resulted in a decrease in viable cells in response to DR compared to control cells (Fig. 18B, left panel); whereas in p53\(^{-/-}\) HCT116, DEK KD cells showed similar cell viability as that in control cells throughout the 3-day DR treatment (Fig. 18B, right panel). Thus, the ablation of DEK increased the sensitivity of p53\(^{+/+}\) HCT116 but not p53\(^{-/-}\) HCT116 to DNA damage-induced cell death, suggesting that the cytoprotective function of DEK is at least partially dependent on the p53 pathway.

Since DEK affects the DNA damage-induced cell death of p53\(^{+/+}\) but not p53\(^{-/-}\) HCT116 cells, we wanted to further assess if DEK protects these cells from apoptosis. To monitor the DNA damage-induced apoptosis, immunoblotting analysis probing for caspase-3 was performed. DEK knockdown in p53\(^{+/+}\) HCT116 cells resulted in higher level of DR-induced caspase-3 cleavage compared to vector control cells (Fig. 19A, lanes 5-6 v. 2-3). Whereas in p53\(^{-/-}\) HCT116 cells, DEK knockdown caused only minor
increase of DR-induced caspase-3 cleavage compared to control cells (Fig. 19A, lanes 11-12 v. 8-9). As a second approach to examine caspase-3 activation, we performed caspase-3/7 activity assays, in which the cleavage of a luminogenic caspase-3/7 substrate DEVD-afc was monitored. The amount of luminescent product is representative of the amount of active caspase-3/7, an indicator of apoptosis. Caspase-3/7 activity assay revealed that DEK loss increased the activation of caspase-3/7 in p53^{+/+} HCT116 cells (Fig. 19B, stripped bars v. gray bars) at untreated as well as at the 12 and 16 hours DR-treated time points (Fig. 19B). In the absence of p53, DEK KD caused only a small increase of caspase-3/7 activity compared to control p53^{-/-} HCT116 cells (Fig. 19B, black bars v. white bars). These results correlate well with the cleaved caspase-3 results observed from immunoblotting analysis (Fig. 19A). Taken together, these data show that DEK knockdown sensitizes p53^{+/+} HCT116 to DNA damage-induced apoptosis but had only minor effect on p53^{-/-} HCT116, suggesting that DEK may protect HCT116 from DNA damage-induced apoptosis better in the presence of p53 than in the absence of p53.
**Fig. 18.** DEK knockdown sensitizes p53\(^{+/+}\) but not p53\(^{-/-}\) HCT116 to DNA damage-induced cell death.

**A.** Immunoblotting analysis probing with DEK antibody showed that DEK protein level was knocked down by DEK shRNA-B10 in both p53\(^{+/+}\) or p53\(^{-/-}\) HCT116 cells. Immunoblotting for β-Actin showed equal loading. **B.** MTT assay. p53\(^{+/+}\) or p53\(^{-/-}\) HCT116 with Vector control or DEK knockdown (DEK KD) were untreated or treated with doxorubicin (DR 200 ng/ml) for 1 to 3 days in triplicate, then exposed to MTT reagent for colorimetric detection of viable cells. Data is expressed as percentage of cell viability (DR-treated cells divided by untreated control cells with the same incubation time).
A.

<table>
<thead>
<tr>
<th></th>
<th>HCT116 p53+/+</th>
<th>HCT116 p53/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vector</td>
<td>DEK shRNA</td>
</tr>
<tr>
<td>Doxorubicin (200ng/ml)</td>
<td>UT 16 24</td>
<td>UT 16 24</td>
</tr>
<tr>
<td>caspase3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30sec exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cleaved caspase3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3min exposure</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

caspase-3/7 activity

![Graph showing caspase-3/7 activity](image)
Fig. 19. DEK knockdown sensitizes p53^{+/+} but not p53^{-/-} HCT116 to DNA damage-induced apoptosis.

(A) Immunoblotting analysis. p53^{-/+} or p53^{-/-} HCT116 cells with Vector control or DEK KD were untreated or treated with doxorubicin (DR 200 ng/ml) for the indicated times. 40 μg of whole cell extracts (WCE) were prepared for immunoblotting with caspase-3 antibody. 1st and 2nd panel showed results with 30 seconds and 3 minutes film exposure respectively.

(B) Caspase-3/7 activity assay. Same cells as above were untreated or treated with DR (200 ng/ml) for the indicated times. Caspase-3/7 activity was measured by luminescent signal of substrate DEVD-afc cleavage. Measurements were performed in triplicate. Error bars indicate standard deviations. * indicates statistical significance with \( P<0.02; \) ** indicates statistical significance with \( P<0.01. \) Statistical significance was calculated by T-test using Microsoft Excel Data Analysis Tool. For p53^{+/+} HCT116 cells with DEK KD compared to Vec control, \( P \) values are 0.0005 for UT, 0.003 for DR 12 hrs and DR 16 hrs; For p53^{-/-} HCT116 cells with DEK KD compared to Vec control, \( P \) values are 0.002 for UT, 0.015 for DR 12 hrs, and 0.012 for DR 16 hrs.
3.2.4. **DEK knockdown decreases p21 expression in p53^{+/+} HCT116.**

DNA damage causes accumulation of p53 which induces expression of p21, a key mediator of cell cycle arrest (Bunz et al., 1998). Since DEK loss sensitizes p53^{+/+} HCT116 cells to DNA damage-induced apoptosis, we asked whether DEK knockdown (KD) could inhibit apoptosis through modulating p53-p21 pathway. Immunoblotting analyses showed that doxorubicin treatment induced expression of p53 and p21 proteins in p53^{+/+} HCT116 (Fig. 20A, 2\textsuperscript{nd} and 3\textsuperscript{rd} panel, lanes 1-3), whereas no p53 or p21 was detected in p53^{-/-} HCT116 (Fig. 20A, 2\textsuperscript{nd} and 3\textsuperscript{rd} panel, lanes 7-12). The ablation of DEK in p53^{+/+} HCT116 cells resulted in decreased DR-induced p53 and p21 protein levels compared to control cells (Fig. 20A, 2\textsuperscript{nd} and 3\textsuperscript{rd} panel, lanes 5-6 v. 2-3) suggesting that DEK may promote p53 and p21 protein expression.

To address whether the reduced protein level is due to changes of transcription level, quantitative real-time PCR was performed to monitor the endogenous p21 mRNA level. Consistent with the reduced p21 protein level, real-time PCR revealed a more than 40% decrease of DR-induced p21 mRNA level in DEK-KD p53^{+/+} HCT116 cells compared to control cells (Fig. 20B, stripped bar v. grey bar). In p53^{-/-} HCT116 cells, DEK knockdown did not affect p21 mRNA levels (Fig. 20B, black bar v. white bar). Interestingly, in unstimulated p53^{+/+} HCT116 cells, the absence of DEK resulted in a 1.6-fold increase of p21 mRNA level compared to control (Fig. 20B, stripped bar v. grey bar), while no p21 protein was detected in untreated cells (Fig. 20A lanes 1 and 4). It is possible that DEK represses p21 expression under normal condition, but promotes p21
expression when DNA damage has occurred. This hypothesis requires further evaluation. In addition, DEK knockdown decreased the protein levels of p73, a homolog of p53, in both p53\(^{+/+}\) and p53\(^{-/-}\) HCT116 (Fig. 20A, 1st panel, lanes 5-6 v. 2-3 and 11-12 v. 8-9), suggesting that the reduced p21 in DEK knockdown cells may be caused by decreased p53 and/or p73. In p53\(^{-/-}\) HCT116 cells, p21 mRNA levels were increased about 4 fold by doxorubicin, indicating that p21 expression can be induced by regulators other than p53, although the induced p21 mRNA levels were much less than that in p53\(^{+/+}\) HCT116 (Fig. 20B). Immunoblotting with DEK antibody showed successful knockdown of DEK, whereas immunoblotting with β-actin antibody showed equal loading of proteins (Fig. 20A, bottom two panels).
### A.

<table>
<thead>
<tr>
<th>Doxorubicin (200ng/ml)</th>
<th>HCT116 p53+/+</th>
<th>HCT116 p53-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>Vector</td>
<td>DEK shRNA</td>
</tr>
<tr>
<td>16</td>
<td>UT</td>
<td>16</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fold activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>p73</td>
</tr>
<tr>
<td>p53</td>
</tr>
<tr>
<td>p21</td>
</tr>
<tr>
<td>DEK</td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
</tbody>
</table>

### B.

**Real-Time PCR - p21**

- **UT**: p53+/+ Vec 1.0, p53+/+ DEKKD 1.6, p53-/- Vec 0.3, p53-/- DEKKD 0.3
- **DR-12h**: p53+/+ Vec 1.1, p53+/+ DEKKD 1.0, p53-/- Vec 1.0, p53-/- DEKKD 8.9

- UT: p53+/+ Vec 1.0, p53+/+ DEKKD 1.6
- DR-12h: p53+/+ Vec 1.0, p53+/+ DEKKD 8.9

- UT: p53-/- Vec 0.3, p53-/- DEKKD 0.3
- DR-12h: p53-/- Vec 1.0, p53-/- DEKKD 8.9

* * *
**Fig. 20. DEK knockdown decreases p21 expression in p53²⁺/⁺ HCT116.**

**(A)** Immunoblotting analysis with same samples as in Fig.19A. 10 μg of proteins were used for immunoblotting with p21, p73, or β-actin antibodies. The membrane was stripped and reprobed with p53 antibody, and then reprobed with DEK antibody. Lower bands on p53 and DEK membrane are β-actin bands not stripped completely.

**(B)** Real-time PCR. p53²⁺/⁺ and p53⁻⁻ HCT116 cells with DEK-KD or vector control were left untreated or treated with doxorubicin (DR, 200 ng/ml) for 12 hrs. Real-time PCR was performed in triplicate. Expression of p21 was normalized to β-Actin and the data expressed as fold activation relative to the untreated p53⁻⁻ HCT116 control sample. * indicates statistical significance with $P<0.02$; ** indicates statistical significance with $P<0.01$. Statistical significance was calculated by T-test using Microsoft Excel Data Analysis Tool. For p53²⁺/⁺ HCT116 cells with DEK-KD, $P$ values are 0.004 for UT, and 0.011 for DR 12 hrs compared to control cells.
3.2.5. DEK knockdown decreases the protein stability of p21 but not p53.

The ablation of DEK decreased p21 expression which likely results from reduced p53 protein levels (Fig. 20A). Since DEK knockdown reduced p53 protein but not p53 mRNA (data not shown), we sought to assess the impact of DEK loss on p53 protein stability. DEK knockdown (KD) and vector control p53\(^{+/+}\) HCT116 cells were pretreated with the protein synthesis inhibitor cycloheximide (CHX) for 1 hour, and then were further treated with doxorubicin (DR) for 3 to 24 hours. p53 protein levels declined over the time course in response to CHX and DR in the control cells (Fig. 21A, top panel, lanes 1-6). A similar decrease in p53 protein levels was observed in the DEK-KD cells (Fig. 21, top panel, lanes 7-12). Quantification of p53 band intensity also showed similar p53 protein levels between DEK-KD and control cells throughout the time course (Fig. 21B, stripped bars v. grey bars) indicating that DEK does not affect the p53 protein stability. Hence the reduced p53 observed in DEK-KD cells is not due to changes in transcription or protein stability. Interestingly, in control cells, p21 protein levels were increased after 3 hours DR treatment then declined after 6 hours and longer time points (Fig. 21A, 2\(^{nd}\) panel, lanes 1-6). In contrast, p21 proteins were not detected at any time points in DEK-KD cells (Fig. 21A, 2\(^{nd}\) panel, lanes 7-12) suggesting that p21 protein is more stable in the presence of DEK. Therefore, the decreased p21 protein levels in DEK-KD cells is likely due to declined p21 mRNA levels and/or reduced p21 protein stability (Fig. 20B). Immunoblotting analyses with DEK and \(\beta\)-Actin antibodies showed the absence of DEK in DEK-KD cells and equal loading of proteins (Fig. 21, 3\(^{rd}\) panel and bottom panel).
A. p53 protein levels

B. p53 protein levels
Fig. 21. DEK knockdown decreases p21 protein stability in p53\(^{+/+}\) HCT116.

(A) Immunoblotting (IB) analyses. DEK knockdown and control p53\(^{+/+}\) HCT116 cells were pretreated with cycloheximide (CHX, 100 \(\mu\)g/ml) for 1 hour, then treated with doxorubicin (DR, 200 ng/ml) for the indicated times. 40 \(\mu\)g of whole cell extracts were used for immunoblotting with p21 antibody. 10 \(\mu\)g of the same whole cell extracts were used for immunoblotting with p53 antibody, then the membrane was stripped and reprobed with DEK or \(\beta\)-Actin antibodies.

(B) Quantification of p53 IB bands. The intensity of p53 protein bands from the above IB was quantified using Kodak Molecular Imaging Software and normalized to the intensity of \(\beta\)-actin protein bands. The data were expressed as relative intensity compared to the untreated p53\(^{+/+}\) HCT116 vector control cells (set as 1.0).
3.2.6 DEK may mediate a variety of apoptosis-regulatory genes.

Knowing that DEK inhibits DNA damage-induced apoptosis through mediating p21 gene expression, we wanted to identify other genes that are affected by DEK during apoptosis. Hence, we compared expression of 84 apoptosis-regulatory genes between DEK\(^{-/-}\) and WT MEFs using Apoptosis PCR Arrays (SuperArray Bioscience) which is a set of real-time PCR primer assays on 96-well plate for a focused panel of genes involved in apoptosis signaling. DEK\(^{-/-}\) and DEK\(^{+/+}\) MEFs were treated with doxorubicin (200 ng/ml) for 6, 12 and 24 hours to trigger apoptosis signaling. Then the same MEFs with different treatment time were collected and combined. RNA and cDNA from these DEK\(^{-/-}\) and DEK\(^{+/+}\) MEFs were prepared. cDNA templates were mixed with SYBR-PCR master mix, and subject to real-time PCR on the 96-well plate of PCR arrays containing primers for mouse apoptosis signaling. Using the provided analysis spreadsheet (SuperArray Bioscience), changes in expression of 84 genes between DEK\(^{-/-}\) and DEK\(^{+/+}\) MEFs were calculated. PCR array shows 16 genes over-expressed (almost or more than 2-fold) and 5 gene under-expressed (lower than 50%) in DEK\(^{-/-}\) MEFs when compared to DEK\(^{+/+}\) MEFs (Table 3). The absence of DEK resulted in enhanced mRNA levels of caspases-1, -4, -6, -7, and -12 which may contribute to increased cell death and apoptosis observed in DEK\(^{-/-}\) MEFs. Interestingly, several pro-apoptotic genes and anti-apoptotic genes were either upregulated or downregulated in DEK\(^{-/-}\) MEFs compared to WT MEFs, some of which have opposite effects on cell fate. Further evaluation will be needed in order to determine which of these genes directly mediates the cytoprotective function of
DEK. All of the 6 genes listed that are responsive to NF-κB were upregulated at least 2 fold in DEK−/− MEFs, consistent with our data that DEK functions as repressor for the expression of NF-κB-mediated genes. How DEK mediates NF-κB-regulated genes in response to apoptosis stimuli and whether this contributes to cell survival are under further evaluation. Overall, our data suggest that DEK may up- or down-regulate a variety of genes regulating apoptosis directly or indirectly, coordinately controlling the cell fate under stress.
### Table 3. PCR arrays in apoptosis pathway.

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Fold</th>
<th>Function</th>
<th>Gene</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor suppressor</td>
<td>p73</td>
<td>41.1</td>
<td>Tumor suppressor</td>
<td>Dapk1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>p63</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bnip3L</td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cidea</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiapoptosis</td>
<td>*clAP</td>
<td>3.2</td>
<td>Antiapoptosis</td>
<td>Tsc22d3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>*Bcl-xl</td>
<td>2.4</td>
<td></td>
<td>Akt1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>*xlAP</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pak7</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase Family</td>
<td>*Casp4</td>
<td>16.7</td>
<td>Caspase Family</td>
<td>Casp14</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Casp12</td>
<td>8.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Casp1</td>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Casp6</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Casp7</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligands and receptors</td>
<td>*Traf1</td>
<td>4.5</td>
<td>Ligands and receptors</td>
<td>Tnfrsf11b</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Cd70</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Traf2</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* NF-κB-responsive genes

16 genes up-regulated and 5 genes down-regulated in DEK<sup>−/−</sup> MEFs compared to DEK<sup>+/+</sup> MEFs in response to doxorubicin (200 ng/ml). Data expressed as fold change of gene expression in DEK<sup>−/−</sup> MEFs relative to WT MEFs. * indicates NF-κB-responsive genes.
3.3 Part 3: Regulation of NF-κB activity by p38 MAP Kinases

It has been reported that p38 MAP kinase (further referred to as p38) plays a role in the ability of certain stimuli (e.g. LPS, IL-1 and TNFα) to activate NF-κB transcriptional activity (Beyaert R, 1996; Han et al., 1997). The exact mechanism that p38 uses to activate NF-κB is not fully understood. Blocking p38 by its inhibitor SB80 reduces the ability of Gal4-p65 fusion protein (aa519-551 of p65, containing the TAD) to activate transcription, but does not affect p65 nuclear translocation and DNA binding (Norris and Baldwin, 1999). Moreover no direct association between p38 and p65 has been detected (B. Ashburner, unpublished data), indicating that p38 modulates NF-κB through an indirect mechanism. Since the CBP coactivators directly bind to p65 (aa 286-551 containing TAD) and enhances p65-mediated transcription, we sought to determine whether p38 targeted CBP to stimulate NF-κB transactivation.

3.3.1 p38 interacts with CBP in vitro

To determine if CBP is a substrate of p38, we first tested if CBP interacts with p38 using in vitro binding assays. Various GST-CBP fragments were purified from bacteria and immobilized on Glutathione-sepharose beads. Linear representation of CBP and GST-CBP fragments was illustrated (Fig.22A). 35S-labeled p38 protein was synthesized using in vitro transcription/translation in the presence of 35S-Methionine. The 35S-labeled p38 was incubated with immobilized GST or GST-CBP fragments. After 30 min incubation, the beads were washed, resuspended in 2xSDS loading dye, and eluted
proteins were separated by SDS-PAGE. The gel was stained with Gel-code blue, dried, and exposed to film for autoradiography. Results from this experiment show that p38 only interacts with aa251-450 of CBP, a region of CBP that includes a zinc finger domain (Fig. 22B, upper panel, lane 2). Other CBP fragments or GST did not interact with p38 (Fig. 22B, upper panel, lanes 1 and 3-7). p38 input was used as a positive control (Fig. 22B, lane 8). Coomassie blue stained gel visualized the total protein level of GST-CBP fragments in various size as the loading control (Fig. 22B, lower panel). These data indicate that p38 directly interacts with the CBP N-terminal Zinc finger domain in vitro.

3.3.2 p38 phosphorylates CBP in vitro

Since an interaction between p38 and CBP was detected in vitro, we next performed in vitro kinase assays to determine if p38 can phosphorylate CBP. Various GST-CBP fragments were purified from bacteria and immobilized on Glutathione- sepharose beads, then incubated with activated p38 (purchased from Millipore) in the presence of γ-32P-ATP. The reaction was stopped by adding 2xSDS loading dye. The samples were boiled and proteins were separated on SDS-PAGE gel. Autoradiography showed that four GST-CBP fragments, aa2-250, aa251-450, aa451-682 and aa683-900, were strongly labeled by 32P and thus phosphorylated by activated p38 (Fig. 23A, upper panel, lanes 1-4). Coomassie blue stained gel visualized the total protein level of GST-CBP fragments as the loading control (Fig. 23A, lower panel). In vitro kinase assay data suggest that p38 may phosphorylate CBP on multiple sites.
The NetPhos 2.0 program was used to predict potential serine and threonine phosphorylation sites within CBP. Based on the specific consensus sites phosphorylated by p38: serine/threonine followed by proline (S/T-P), we identified several potential p38-mediated phosphorylation sites within CBP. GST-CBP fragments 1, 2, 3 and 4 have seven, two, one and four potential sites respectively (Fig. 23A, numbers on top). It is noteworthy that, GST-CBP fragment 3 (aa451-682) has only one potential site (Thr614) yet had the strongest phosphorylation in the in vitro kinase assay (Fig. 23A, lane 3), indicating that T614 may be phosphorylated by p38 in vivo. Other than T614, we also selected potential phosphorylation sites Ser19, S92, S246 and S275 for further study.

To verify if S19, S92, S275, and T614 are p38 phosphorylation sites on CBP, these four sites were mutated into alanine individually in the GST-CBP fragments. In vitro kinase assays were performed as described above. All four GST-CBP mutant fragments showed reduced p38-mediated phosphorylation to a different degree when compared to the respective wild type fragments (Fig. 23B). S19A resulted in a greater reduction of phosphorylation than S92A for GST-CBP aa2-250 fragment (Fig. 23B, upper panel, lanes 2-3 v. 1). S275A decreased the phosphorylation level of GST-CBP aa251-451 fragment (Fig. 23B, upper panel, lanes 5 v. 4). T614A mutant lost the phosphorylation completely, indicating the potential importance of the site T614 for p38-mediated phosphorylation of CBP (Fig. 23B, upper panel, lanes 6 v. 7). Coomassie blue stained gel showed the total level of GST-CBP fragments as a control (Fig. 20B, lower panel). These data show that p38 may phosphorylate CBP on multiple sites including T614, S19, S92 and S275.
**Fig. 22. In vitro binding of p38 and GST-CBP fragments**

(A) Linear representation of CBP and GST-CBP fragments. (B) In vitro binding assay. GST-CBP fusion proteins purified from bacteria were immobilized on Glutathione-sepharose beads, then incubated with in vitro transcribed/translated p38 in the presence of $^{35}$S-met. After incubation, beads were washed, and boiled in 2xSDS loading dye. Eluted proteins were separated by SDS-PAGE. The gel was stained in coomassie blue to visualize GST-CBP proteins. Then the gel was dried and exposed to film to visualize $^{35}$S-labeled p38.
A. Potential p38 phosphorylation sites

<table>
<thead>
<tr>
<th>GST-CBP fragments</th>
<th>7</th>
<th>2</th>
<th>1</th>
<th>4</th>
<th>9</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-CBP 2-250</td>
<td>250</td>
<td>450</td>
<td>682</td>
<td>900</td>
<td>1100</td>
<td>1231</td>
</tr>
</tbody>
</table>

32P-labeled GST-CBP fragments

Autoradiography

Coomassie stained gel

B. GST-CBP

<table>
<thead>
<tr>
<th>GST-CBP 2-250</th>
<th>GST-CBP 251-451</th>
<th>GST-CBP 451-682</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>S19A</td>
<td>S92A</td>
</tr>
<tr>
<td>WT</td>
<td>S275A</td>
<td>T614A</td>
</tr>
<tr>
<td>WT</td>
<td>GST</td>
<td></td>
</tr>
</tbody>
</table>

 Autoradiography

1 2 3 4 5 6 7 8

(Eric Cole and Brian Ashburner)
Fig. 23. Phosphorylation of GST-CBP fragments by p38 in vitro

(A) *In vitro* kinase assay. Purified GST-CBP fusion proteins from bacteria were immobilized on Glutathione-sepharose beads, and then incubated with activated p38 in the presence of $\gamma^{32}$P-ATP. The reaction was stopped by adding 2xSDS loading dye. The samples were boiled and proteins were separated on SDS-PAGE gel. The gel was stained in coomassie blue to visualize GST-CBP proteins. Then the gel was dried and exposed to film to visualize $^{32}$P-labeled phosphorylated GST-CBP fragments.

(B) Phosphorylation of GST-CBP point mutation fragments by p38 *in vitro*. GST-CBP point mutation fragments were generated using mutagenesis kit and verified by sequencing. *In vitro* kinase assays were performed as described above.
3.3.3 p38 and CBP interact in vivo.

In vitro binding assays showed that p38 directly interacts with GST-CBP N-terminal (aa 251-450) fragment within the zinc finger domain (Fig. 22). Since CBP can also bind to NF-κB through zinc finger domain (Vo and Goodman, 2001), p38, CBP and NF-κB may form a ternary complex. To test this hypothesis and confirm the p38-CBP-NF-κB interaction in vivo, co-immunoprecipitation (co-IP) assays were performed. HeLa cells were treated with TNFα to activate both NF-κB and p38. Nuclear extracts were then prepared for immunoprecipitation using a p38 antibody. The precipitated p38 associated complexes were separated by SDS-PAGE and immunoblotted for CBP, p65 and p38 (Fig. 24A, lanes 1-4). In untreated cells, only low levels of nuclear CBP and p65 were coprecipitated with p38 (Fig. 24A, lane 1). After TNFα stimulation, higher levels of nuclear CBP and p65 were coprecipitated with p38 (Fig. 24A, lane 2-4), indicating that the p38 associates with CBP and p65 in vivo and that p38-CBP-p65 interaction is inducible by TNFα. Whole cell extracts were prepared for immunoblotting analysis as input control showing equal protein levels of CBP, p65 and p38 in each lane (Fig. 24 A, lanes 5-8).

Knowing that endogenous CBP can be coprecipitated by endogenous p38 in vivo, we tried reciprocal coIP using anti-CBP antibody for immunoprecipitation, then immunoblotting for p38. However, we did not successfully detect p38 or p65 coprecipitated with CBP (data not shown). This is possibly due to problems of the CBP antibody which could not specifically pull down enough CBP associating complexes for coIP. To further confirm the interaction between p38 and CBP, we transfected cells with
Flag-CBP (full length) plasmid and performed coIP using Flag antibody. 293T cells were transfected with Flag-CBP or vector control plasmids, and 24 hrs posttransfection cells were either untreated or treated with TNFα (10 ng/ml) for 30 and 60 min. Nuclear extracts were prepared for immunoprecipitation using Flag antibody. Immunoprecipitated complexes were subject to immunoblotting using Flag, p65, and p38 antibodies. Equal amounts of Flag-CBP were precipitated (Fig. 24B, upper panel, lane 1-3). In untreated cells, a low level of p65 was coprecipitated with Flag-CBP, whereas no p38 was coprecipitated with Flag-CBP (Fig. 24B, middle panel, lane 1). In response to TNFα treatment, p38 coprecipitated with Flag-CBP at the 30 minute time point and the interaction between p38 and Flag-CBP was reduced after the 60 minute treatment (Fig. 24B, lower panel, lane 2-3). Meanwhile, the amount of p65 that interacted with Flag-CBP increased after the 30 and 60 minute TNFα treatment (Fig. 21B, middle panel, lane 2-3). These data illustrates that p38 interacts with overexpressed CBP and that the p38-CBP interaction is transient and inducible by TNFα. No p38 band was observed from vector control transfected cells (Fig. 24B, lower panel, lane 4), indicating the specificity of p38-CBP interaction. Vector control cells showed low levels of CBP and p65 bands which were probably nonspecific bands. The right panels (Fig. 24B, lanes 5-8) showed the input nuclear extracts used for the immunoprecipitation with elevated nuclear p65 after TNFα treatment and similar amounts of nuclear Flag-CBP and nuclear p38 at all time points. Overall, coIP results suggest that an interaction between CBP and p38 occurs in the nucleus.
Fig. 24. CBP and p38 interact in coimmunoprecipitation assays.

(A) HeLa cells were treated with TNFα (10 ng/ml) for the indicated times, then nuclear extracts were prepared for immunoprecipitation with p38 antibodies. (B) 293T cells were transfected with Flag-CBP plasmids (7 μg). 24 hrs post-transfection, nuclear extracts were prepared for immunoprecipitation with flag antibody. (A,B) The immunoprecipitated protein complexes were then subjected to immunoblotting analysis probing for p38, p65, and CBP (A) or flag (B) antibodies. Whole cell extracts were prepared for immunoblotting analysis probing for p38, p65, and CBP (A) or flag (B) antibodies as input control.
3.3.4 p38 is recruited to the NF-κB-regulated promoters in a complex with CBP

Since p38 interacts with CBP in the nucleus in response to TNFα (Fig. 24) and CBP is recruited to promoters upon stimulation, we wanted to examine whether p38 is recruited to the promoters of NF-κB-regulated genes in a complex with CBP. ChIP-ReChIP assays were performed to assess the binding of p38-CBP complexes to promoters in vivo. HeLa cells were either untreated or treated with TNFα for the indicated times and cellular proteins and DNA were crosslinked. Nuclear extracts were prepared and subjected to the first chromatin-immunoprecipitation (ChIP) with a CBP antibody. Then the CBP associating complexes were eluted and subjected to the second ChIP with a p38 antibody. Chromatin DNA associated with the CBP-p38 complexes was eluted and used to amplify the promoter regions of the cIAP2 and IL-8 genes. In untreated cells, no association of CBP-p38 complex to either promoter was detected (Fig. 25, upper panel, lanes 1 and 4). In contrast, TNFα stimulation greatly induced the binding of CBP-p38 complex to both promoters (Fig. 25, upper panel, lanes 2-3 and 5-6). This enhanced association of CBP-p38 complex is likely due to increased interaction between p38 and CBP and/or increased binding of CBP to the promoters upon stimulation. Lower panels showed equal amounts of input chromatin DNA as a control (Fig. 25). ChIP-ReChIP results revealed that p38 is recruited to promoters of NF-κB-regulated genes in a complex with CBP, providing the possibility that p38 regulates NF-κB transactivation function through an interaction with CBP which brings p38 to the promoters to modulate transcription factor and/or cofactor functions.
**ChIP-ReChIP**: 1st ChIP: CBP → 2nd ChIP: p38

<table>
<thead>
<tr>
<th></th>
<th>cIAP2</th>
<th></th>
<th>IL-8</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td></td>
<td></td>
<td>UT</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>CBP - p38 complex</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td>min.</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 25. p38 is recruited to NF-κB-regulated promoters through a complex with CBP.**

HeLa cells were either untreated or treated with TNFα (10 ng/ml) for the indicated times. After crosslinking and sonication, DNA and protein complexes were subject to ChIP-ReChIP experiment in which an antibody against CBP was used for the first chromatin-immunoprecipitation (ChIP). Complexes were eluted from the beads and a second ChIP (ReChIP) was performed using an antibody specific to p38. The ReChIP complexes were eluted from the beads, cross links reversed, and the recovered DNA was used in PCR reactions to amplify the promoter regions of the NF-κB-regulated genes cIAP2 and IL-8. PCR products were visualized by agarose gel electrophoresis.
3.3.5 Inhibition of p38 does not affect the recruitment of p65 and CBP to the promoters of NF-κB-regulated genes

CBP functions as a scaffold protein to stabilize the multi-protein transcription complex, consisting of coactivators, inducible and general transcription factors (Vo and Goodman, 2001). Since p38 phosphorylates CBP in vitro and p38 is found associated with promoters in a complex with CBP in vivo, we asked whether p38 enhances the recruitment of CBP and other components of the transcription complex to promoters. ChIP assays were performed to assess the recruitment of p38, CBP, and p65 to NF-κB-responsive promoters. HeLa cells were pretreated with the p38 inhibitor SB80 (5 μM) or DMSO as negative control for 2 hours, and then untreated or treated with TNFα (10 ng/ml) for the indicated times (Fig. 26). Nuclear extracts were immunoprecipitated by specific antibodies for p38, CBP, and p65. Precipitated DNA was amplified by PCR using primers specific for the IL-8, and cIAP2 promoters. In DMSO treated control cells, a typical pattern of recruitment of p65 and CBP to both promoters was observed in response to TNFα (Fig. 26, lanes 6-10) with peak binding occurring at 30 minutes (Fig. 26, lane 8) and a gradual decrease at 60 and 120 minutes (Fig. 26, lanes 9-10). The association of p38 with the IL-8 promoter slightly increased, peaking at 30 minutes (Fig. 26, top panel, lanes 6-10), whereas p38 bound to cIAP2 promoters in untreated and 15 minute treated cells, dissociated at 30 minutes and then reappeared on the promoters at 60 and 120 minutes (Fig. 26, 4th panel, lanes 6-10). Inhibition of p38 enzymatic activity by SB80 did not change the recruitment of CBP and p65 to both promoters (Fig. 26, lanes 1-5).
v. lanes 6-10), but reduced the association of p38 to IL-8 promoters at all time points (Fig. 26, top panel, compare lanes 1-5 to lanes 6-10) and to cIAP2 promoters at UT and 15 minutes time points (Fig. 26, 4th panel, lanes 1-2 v. lanes 6-7). These data suggest that the enzymatic activity of p38 is not required for the recruitment of CBP and p65 to promoters of NF-κB-responsive genes, but may be required for the association of p38 itself to promoters.
Fig. 26. Inhibition of p38 does not affect the recruitment of p65 and CBP to the promoters of NF-κB-regulated genes IL8 and cIAP2.

HeLa cells were pretreated with 5 μM SB80 or with an equal volume of DMSO (control) for 2 hr and then treated with TNFα for the indicated times. Cells were cross linked with formaldehyde, DNA sheared by sonication, and extracts were used for immunoprecipitations with antibodies against p38, CBP, or p65. The recovered DNA was subjected to PCR using primers specific for the promoters of NF-κB-regulated genes IL-8 and cIAP2.
3.3.6 Inhibition of p38 selectively regulates the expression of NF-κB-regulated genes in a cell and gene specific manner.

It has been well established that p38 activity is important for stimulating NF-κB activity. For example, in response to LPS, NF-κB and p38 cooperate to induce transcription of two antiapoptotic genes, Pai-2 and Bfl-1/A1 to maintain macrophage survival through p38-mediated phosphorylation of MSK1/2 (Park et al., 2005). To address whether inhibition of p38 diminishes expression of NF-κB-regulated genes in response to TNFα and LPS, RT-PCR was performed to monitor the expression of IL-8, MCP-1, cIAP2 and IκBα. HeLa and U937 cells were pretreated without or with SB80 (5 μM) for 2 hours then untreated or treated with TNFα (10 ng/ml) and LPS (100 ng/ml) to activate both p38 and NF-κB. U937 is a human leukemia cell line that has a high level of CBP and p38 expression. In control HeLa cells, TNFα treatment induced the expression of IL-8, MCP-1, cIAP2 and IκBα as expected (Fig. 27, left panel, lane 1-2). Blocking the enzymatic activity of p38 by SB80 had no impact on the TNFα-induced expression of all four NF-κB-responsive genes (Fig. 27, left panel, lanes 3-4 v. lanes 1-2). In control U937 cells, both TNFα and LPS treatment elevated the expression of IL-8, MCP-1, cIAP2, and IκBα (Fig. 27, right panel, lanes 6, 8 v. lane 5). Inhibition of p38 activity resulted in reduced expression of IL-8 and MCP-1 in response to TNFα and LPS, but did not affect the expression of cIAP2 and IκBα (Fig. 27, right panel, lanes 7 v. 6 and lane 9 v. 8). The above results indicated that p38 selectively induces expression of particular NF-κB-mediated genes and its impact varies in different cells.
Fig. 27. The impact of inhibition of p38 activity on gene expression in RT-PCR.

HeLa cells were untreated or pretreated with SB80 (5 μM) and then treated with or without TNFα for 30 minutes. U937 cells were untreated or pretreated with SB80 (5 μM) and then untreated or treated with TNFα (10 ng/ml) for 30 minutes or with LPS (100 ng/ml) for 60 minutes. Total cellular RNA was prepared and reverse transcribed into cDNA. 2 μl of the cDNA was then subjected to PCR amplification with primers specific for IL-8, MCP-1, cIAP2, IκBα and GAPDH. PCR products were visualized by agarose gel electrophoresis. White arrow indicates reduced gene expression responded to SB80.
4 DISCUSSION

4.1 DEK is a novel regulator for NF-κB-mediated transactivation

Human DEK has long been implicated in tumorigenesis and auto-immune diseases (Waldmann et al., 2004). Proposed functions for DEK include a role in: chromatin remodeling, DNA supercoiling, DNA repair, RNA splicing, apoptosis inhibition, and transcription regulation (both as transcriptional stimulator and transcriptional repressor) (F. Kappes, 2008; Waldmann et al., 2004). However, how these various DEK functions contribute to cancer and autoimmune diseases is not understood. Hence, we aimed to better understand the biological function of DEK and its link to cell survival and immune responses.

Previously, we identified DEK as an NF-κB-interacting protein in a cytoplasmic yeast two-hybrid screen using the C-terminal transactivation domain (TAD) (aa 313-551) of p65 subunit of NF-κB as the bait protein (Vögel, MS thesis, 2004). Our subsequent data confirmed that DEK interacted with the p65 subunit of NF-κB by coimmunoprecipitations of endogenous DEK and p65. Transient overexpression of DEK resulted in dose-dependent repression of NF-κB-mediated reporter gene expression indicating that DEK negatively regulates NF-κB-mediated transcriptional activation. In addition, DEK also regulates the activity of other transcription factors including AR, p53,
and STAT5B in transient transfection reporter gene assays; however, DEK does not affect the NF-κB- and IRF-regulated expression of IFNβ-promoter-luc reporter gene, suggesting that DEK is regulator for a subset of genes and transcription factors. Because DEK binds to DNA in a structure-specific, with preference for supercoiled and cruciform DNA, rather than in a sequence-specific manner (Kappes et al., 2004b), how DEK specifically regulates transcription factors and expression of genes is still not clear. Our data indicate that DEK may be recruited to the promoters of NF-κB-regulated genes through the direct interaction with p65. It is possible that DEK is recruited to particular promoters through its interaction with transcription factors and/or cofactors and that the specificity of DEK function can be resulted from various interplay among DEK, transcription factors, and/or cofactors.

To further confirm the repressive function of DEK for NF-κB-mediated transcription, we studied the effects of DEK loss using MEFs isolated from DEK knockout mice or from wild type (WT) littermates. Quantitative real-time PCR (QRT-PCR) showed that expression of the NF-κB-regulated Mcp-1 and IκBα genes were increased in DEK−/− MEFs relative to WT MEFs, supporting our hypothesis that DEK functions as a corepressor to negatively regulate NF-κB-mediated gene transcription. Additionally, we performed PCR arrays to compare expression of genes involved in the NF-κB pathway between DEK−/− MEFs and WT MEFs in response to TNF stimulation. These results showed that DEK regulates a variety of genes involved in NF-κB signaling either directly or indirectly. Moreover, the DEK knockout mice have an interesting phenotype. The
DEK knockout mice cleared a sublethal dose of HKx31 flu virus much quicker than their wild type littermates, and survived a lethal dose that kills all the wild type mice. Overall, DEK knockout mice appear to have an enhanced immune response to viral infection (unpublished data, Mientjes and Grosveld). Given that NF-κB is vital for innate immune responses and that DEK represses NF-κB transactivation, we hypothesize that DEK loss may results in increased expression of NF-κB-mediated genes leading to enhanced immune responses observed in DEK knockout mice. The possible role of DEK in suppressing immune responses through repressing NF-κB will be an interesting area to study in the future.

Using Chromatin Immunoprecipitation (ChIP) assays, we demonstrated that DEK associated with the NF-κB-regulated IL-8 and cIAP-2 promoters in uninduced cells, while basal nuclear p65 associated with these promoters. Thus, DEK may function to repress the ability of the basal nuclear NF-κB to activate transcription and to prevent inappropriate transcription of genes, such as those encoding proinflammatory cytokines, in the absence of an activating signal. Upon TNFα stimulation, DEK dissociated from these promoters as binding of the p65 to these promoters increased. At the early TNFα time points, a low level of DEK remains associated with the promoters and may function to maintain a low level of expression of these genes as shown in real-time PCR. At the later time points most of the DEK dissociates from the promoters correlating with the maximal recruitment of p65 and coactivator protein P/CAF. Consistent with the ChIP data, real-time PCR analysis showed that maximal expression of these genes was detected when lowest level of
DEK was associated with the promoters. These data suggested that DEK dynamically regulates NF-κB-mediated gene expression through its association and dissociation with the promoters under unstimulated and TNFα-stimulated status.

A recent report has shown that P/CAF acetylates DEK resulting in decreased DNA binding affinity of DEK (Cleary et al., 2005). Since P/CAF is an important coactivator for NF-κB, we wanted to see if DEK affects the recruitment of P/CAF to NF-κB-regulated promoters. Our results show that the absence of DEK enhanced the recruitment of both p65 and P/CAF to NF-κB-regulated promoters. Therefore, it is possible that the recruitment of P/CAF to promoters by activated NF-κB leads to the subsequent acetylation of DEK and its dissociation from promoters. Our proposed model of the regulation of NF-κB by DEK is as follows: under normal physiological conditions, DEK interacts with basal nuclear NF-κB through transactivation (TAD) domain of p65 and blocks transcriptional-activation. Upon stimulation by TNFα, cytoplasmic NF-κB translocates to the nucleus and recruits the CBP and P/CAF coactivators to the promoters. Once P/CAF is recruited to the promoters, it may acetylate DEK and cause DEK to dissociate from the promoters, allowing for optimized NF-κB-mediated transcriptional activation (Fig.28).
Fig. 28.  Proposed model of the regulation of NF-κB activity by DEK

Under normal physiological conditions, DEK interacts with basal nuclear p65 TAD and represses transcription. Upon stimulation by TNFα, cytoplasmic NF-κB translocates to the nucleus and recruits coactivators CBP and P/CAF to the promoters. Recruited P/CAF may acetylate DEK causing it to dissociate from the promoters and allowing expression of NF-κB-responsive genes.
It is noteworthy that the absence of DEK enhances expression of NF-κB-regulated genes not only in untreated cells but in TNFα-treated cells (Fig. 10A), suggesting that DEK also represses TNFα-induced NF-κB transactivation. Indeed ChIP assays showed a low but detectable level of DEK remains associated with promoters of NF-κB-responsive genes after TNFα stimulation. In fact, studies from other groups and ours showed that several corepressors including HDAC1, HDAC2, HDAC3, SMRT, and N-CoR remain associated with the promoters of IκBα and/or MCP-1 in response to TNFα (Yamagoe et al., 2003). Therefore, it appears that various corepressors are associated with promoters even under transcriptional activation conditions, implying that these corepressors possibly function to maintain a proper level of transcription during activation. It is likely that appropriate activation of NF-κB-mediated transcription requires a dynamic balance between coactivators and corepressors: in the resting state, corepressors are the major factor associated with promoters to block transcription; whereas in the stimulated state, more coactivators become associated with the promoters and displace most of the corepressors in order to enhance transcription. However, some corepressors may still remain associated with the promoters under activating conditions to counteract the activity of coactivators and to prevent over-induction of transcription.

The precise mechanism through which DEK represses NF-κB-mediated transcription is still not known. Our data indicates that DEK may function through a direct interaction with p65 transactivation domain (TAD). DEK may mask the transactivation domain of p65, preventing p65 from recruiting coactivators that are essential for NF-κB-dependent
gene transcription. It is also possible that DEK represses transcription through other mechanisms including direct binding to promoters and recruiting other corepressors. One report demonstrated that the transcriptional repression by Daxx is mediated through its association with core histones, HDAC II and DEK (Hollenbach et al., 2002), suggesting that DEK, without any known enzymatic domain, may cooperate with corepressor HDACs and Daxx to suppress transcription. Our ChIP assays for HDACs showed that the absence of DEK did not change the association of HDAC1 and HDAC3 to NF-κB-responsive promoters, and resulted in a modest increase of HDAC2 recruitment which could not explain the enhanced NF-κB-mediated transcription. However, these results did not rule out the possibility that DEK requires the enzymatic activities of HDACs to exert its function. Another report showed that DEK is able to repress the histone acetyltransferase activity of the P/CAF and p300 through a direct interaction with these coactivators resulting in decreased acetylation of histone H3 and H4 (Cleary et al., 2005). Given the essential role of P/CAF and p300/CBP for NF-κB-mediated transcription, DEK may repress NF-κB activity by repressing the HAT activities of the P/CAF and p300 coactivators.
4.2 DEK oncoprotein inhibits DNA damage-induced cell death

DEK has been implicated in oncogenesis, DNA repair, and apoptosis inhibition, and DEK expression is up-regulated in many solid human tumors (Waldmann et al., 2004). Moreover, DEK knockout mice showed reduced murine papilloma formation (Wise-Draper et al., 2009), supporting the function of DEK as an oncoprotein promoting tumorigenesis. Therefore, DEK oncoprotein is a potential therapeutic target for treatment of cancer. We aimed to better understand the mechanism through which DEK contributes to cell survival and tumorigenesis.

Our published data demonstrate that DEK interacts with the p65 subunit of NF-κB and can repress NF-κB-mediated gene transcription. In particular, the expression of cIAP-2 anti-apoptotic gene is upregulated in DEK\(^{-/-}\) MEFs. Moreover DEK associates with the cIAP-2 promoter, and dissociates from this promoter after TNF\(\alpha\) stimulation. Based on the fact that NF-κB plays an important role in inhibiting cell death by activating expression of anti-apoptotic genes (Hayden and Ghosh, 2004), we would predict that DEK represses NF-κB-regulated anti-apoptotic genes, and thus promotes apoptosis. However, this is not the case. Our data showed increased cell death and enhanced caspase-3 cleavage in DEK\(^{-/-}\) MEFs compared to WT MEFs in response to DNA damage induced by doxorubicin indicating that the DEK oncoprotein protects cells from apoptosis. DEK represses NF-κB in response to TNF\(\alpha\) and inhibits apoptosis in response to DNA damage suggesting that DEK may exert distinct function in response to different stimuli or cell events. Another possibility is that DEK represses both NF-κB and pro-apoptotic
pathways, whereas in response to DNA damaging agents such as doxorubicin, DEK may have a stronger repressive effect on proapoptotic pathways than on anti-apoptotic pathways leading to apoptosis inhibition. Since doxorubicin is a strong inducer of both apoptosis and NF-κB, whether or not DEK regulates NF-κB signaling in response to apoptotic stimuli and whether this inhibits or promotes cell survival will be an interesting subject to study. The involvement of NF-κB signaling in the cytoprotective role of DEK is the focus of ongoing experiments in our lab.

To examine whether DEK also protects cancer cells from death, stable DEK knockdown in three different cancer cell lines were generated using lentiviral delivered shRNA targeting DEK. DEK knockdown sensitized HeLa cells to DNA damage-induced cell death, but did not affect the caspase-3 activation. HeLa cells are highly resistant to DNA damage-induced apoptosis due to the inactivation of the p53 tumor suppressor by HPV E6 oncoprotein, which is different from mouse embryonic fibroblasts with functioning p53. Hence DEK may protect cervical cancer cells from death through pathways independent of p53 and caspase-3 activation. Since p53 is a well characterized tumor suppressor that can be activated by DNA damage, we asked whether DEK inhibits DNA damage-induced apoptosis through mediating p53 signaling. Stable DEK knockdown in both p53+/+ and p53−/− HCT116 (colon cancer cell line) were generated. Interestingly, in p53-proficient HCT116, DEK knockdown by shRNA leads to increased cell death and increased caspase-3 cleavage in response to DNA damage compared to control cells. However, in p53-deficient HCT116, DEK knockdown does not greatly
affect the DNA damage-induced apoptosis. These findings suggest that suppression of DEK sensitizes cells to apoptosis that is at least in part dependent on the p53 pathway. In addition, DEK not only protects p53-proficient HCT116 cells and mouse embryonic fibroblasts (MEFs) from apoptosis, but also protects HeLa cells lacking active p53 from DNA damage-induced death. One group proposed that DEK, in response to genotoxic damage, may help repair DNA strand breaks and thus protects HeLa cells from apoptosis (F. Kappes, 2008). Hence, DEK can suppress apoptosis through multiple mechanisms and the pathways involved may vary in different cancers.

Treatment of cells with DNA damaging agent leads to accumulation of p53 and transcriptional induction of p21 which causes cell cycle arrest. To further study the mechanism by which DEK protects p53+/+ HCT116 cells from apoptosis, we assessed the impact of DEK loss on p53 and its target p21. DEK knockdown cells showed reduced p53, p73 and p21 protein levels, suggesting that DEK inhibits apoptosis through modulating p53/p73-p21 pathway. The ablation of DEK resulted in decreased mRNA and protein expression of p21 which is a key mediator of cell cycle arrest. Moreover, p21 protein is more stable in the presence of DEK. Therefore, the decreased p21 protein levels in DEK KD cells is likely due to both declined p21 mRNA levels and reduced p21 protein stability. In DNA damaged cells, p21 causes cell cycle arrest probably allowing more time for the DNA repair machinery to exert their functions. Therefore, it is possible that DEK protects cells from DNA damage-induced apoptosis through increasing p21 expression and facilitating DNA repair process. Moreover, there is some
evidence showing that p21 has tumor-promoting activities through inhibiting apoptosis and that the degradation of p21 can sensitize HCT116 to radiotherapy-induced cell death (Abbas and Dutta, 2009; Tian et al., 2000). This supports our model that DEK loss sensitizes colon cancer cells to apoptosis through reducing p21 protein levels.

To determine the mechanism by which DEK promotes p21 expression, we assessed the impact of DEK on p53. Previously, studies from another group and ours showed that overexpressed DEK negatively regulates expression of a p53-responsive reporter gene (Sammons et al., 2006; Wise-Draper et al., 2006). However, this does not explain our observation that DEK promotes the expression of p21 in vivo. The function of DEK in vivo may be different from that in vitro and the activity of DEK may vary among different cells and contexts. We found that DEK knockdown caused decreased p53 and p73 protein levels in response to DNA damage. Thus, the decreased p21 mRNA level is likely due to reduced p53/p73 protein levels. However, DEK loss did not affect the p53 mRNA level or the p53 protein stability. Hence the reduced p53 observed in DEK KD cells is not due to changes of mRNA or protein stability, but other unknown mechanisms. In undamaged cells, p53 is bound by Mdm2, an ubiquitin ligase, which targets p53 for degradation by the proteasome, whereas DNA damage leads to phosphorylation of p53 which releases p53 from binding to Mdm2, resulting in a marked increase in p53 protein level in the cell (White, 1996). One possibility is that DEK elevates p53 protein levels through promoting the phosphorylation of p53 and its dissociation from Mdm2.
p53 is a well characterized tumor suppressor that activates expression of genes that encode proteins involved in cell cycle regulation and apoptosis. How could DEK inhibit apoptosis through regulating the p53 tumor suppressor? Recent reports had shown that signaling from p53 can elicit protection to cells. Firstly, p53 activates transcription of p21 which causes cell cycle arrest, thereby allowing cells time for DNA repair. Secondly, while p53 activates pro-apoptotic proteins, it is also able to stimulate expression of DNA-repair proteins such as PCNA, GADD45 and ERCC3 (Kannan et al., 2001) suggesting that p53 promotes DNA repair. Thus, cells lacking p53 would be compromised in DNA repair and would be more likely to undergo apoptosis in response to DNA damage. Moreover, p53-p21 signaling activates pRb that inhibits the pro-apoptotic protein p84N5 which is able to induce cell death (Garner and Raj, 2008), showing another level of cell-protection from p53 signaling. Taken together, p53-p21 signaling can not only induce cell death, but also provide protection to cells with damaged DNA and facilitate DNA repair. DEK may utilize the cytoprotective functions of p53 and p21 to inhibit DNA damage-induced apoptosis.

Overall, our data show that DEK is required for apoptosis protection in response to DNA damage. We propose a working model in which DEK protects cells from apoptosis through mediating the p53-p21 signaling and DNA repair (Fig. 29). Anticancer drug-induced DNA damage activates both p53-dependent and p53-independent pathways. In p53-proficient cells, p21 is induced by p53, and DEK promotes and/or maintain the p21 expression levels. Induction of p21 leads to cell cycle arrest allowing the cell time for
DNA repair. Additionally, DEK facilitates repair of DNA breaks providing another level of protection for cells after DNA damage. On the other hand, the absence of DEK results in decreased p21 and increased sensitivity of p53-proficient cells to DNA damage-induced apoptosis. In p53-deficient HCT116, p53 loss leads to decreased expression of a broad range of pro-apoptotic factors, thus cells become more resistant to DNA damage-induced apoptosis. Since little p21 is expressed in p53-deficient HCT116, DEK loss is unable to induce apoptosis through mediating p21. Overall, our findings suggest that DEK protects cells from apoptosis through the p53-p21 pathway and that targeting the DEK oncoprotein may be a novel approach to sensitize cancer cells to chemotherapy.
Fig. 29. Proposed model of cytoprotective role of DEK in response to DNA damage.

Anticancer drug-induced DNA damage activates both p53-dependent and p53-independent pathways. In p53-proficient cells, p21 is induced by p53, and DEK promotes the expression of p21. Induction of p21 leads to cell cycle arrest allowing the cell time for DNA repair. DEK also facilitates repair of DNA breaks. Both functions of DEK protect cells from DNA damage-induced apoptosis.
4.3 Regulation of NF-κB transactivation by p38 through CBP coactivator

p38 MAP Kinase activity is critical for immune and inflammatory responses and can be activated by proinflammatory cytokines, chemokines, and bacterial lipopolysaccharide which also activate NF-κB (Roux and Blenis, 2004). Strong evidence links p38 activity with NF-κB activation. Moreover, blocking p38 inhibits oncogenic Ras-induced as well as IL-1β- and Akt-induced transactivation of Gal4-p65-TAD fusion protein, suggesting that p38 regulates NF-κB at least in part through mediating the p65 TAD (Madrid et al., 2001; Norris and Baldwin, 1999). p38 does not interact with or phosphorylate p65; therefore it is likely that p38 targets some intermediate protein that is then able to enhance the NF-κB activity. One possibility is through the MSK1 protein, which is a substrate of p38 and also can activate the CREB transcription factor in response to stress and growth factor (Deak et al., 1998). Upon stimulation by TNFα, p38 phosphorylates MSK1, which then phosphorylates p65 on serine 276 within the N-terminal Rel homology domain (RHD), leading to induction of expression of NF-κB-mediated genes (Vermeulen et al., 2003). However, this does not explain how p38 affects the transactivation activity of Gal4-p65-TAD fusion protein that only contains C-terminal TAD (aa519-551) of p65.

The CBP coactivator interacts directly with the p65-TAD and is required for NF-κB-dependent gene expression (Gerritsen et al., 1997). The interaction between CBP and p65 is at least in part dependent on phosphorylation of p65 (Chen et al., 2005), indicating an important role for phosphorylation in mediating this interaction. Recent evidence has also indicated the phosphorylation of CBP plays a role in regulating the
interaction of CBP with p65 and p53 (Huang et al., 2007). We propose a hypothesis that p38 may mediate NF-κB activity through phosphorylation of the CBP coactivator, which may explain how p38 potentiates Gal4-p65-TAD-driven gene expression. Here, we demonstrated that p38 interacts with CBP in vitro and in vivo and that p38 can phosphorylate a GST-CBP fusion protein on multiple sites in vitro, suggesting that p38 may regulate NF-κB through phosphorylation of CBP.

In vitro binding assays show that p38 interacts with CBP in the region of aa251-451 which includes a zinc finger domain, a common protein-protein interaction domain. Moreover in vitro kinase assays reveal that p38 phosphorylates CBP on multiple fragments. Four potential p38 phosphorylation sites were identified: S19, S92, S275 and T614 through point mutation and in vitro kinase assays. Interestingly, GST-CBP fragment aa451-682 has only one potential p38 phosphorylation site (Thr614) yet showed the strongest phosphorylation by p38 (Fig. 23A, lane 3). Moreover, T614A mutated fragment lost the phosphorylation completely (Fig. 23B, upper panel, lane 6-7), indicating that T614 may be important for p38-mediated phosphorylation of CBP in vitro. Given the above data and the proximity of p38-interacting domain (aa251-451) and the p38-phosphorylation site T614 on CBP, T614 is likely to be phosphorylated by p38 in vivo.

We aimed to better understand the mechanism by which p38 regulates NF-κB transactivation function through targeting CBP. We confirmed the p38-CBP interaction in vivo through coimmunoprecipitations (coIPs). Firstly, we demonstrated interactions between endogenous CBP and p38 using p38 antibody for IP (Fig. 21A). Then, we
overexpressed Flag-CBP in 293T cells and used Flag antibody for IP. The data showed that Flag-CBP interacted with p38 in the nucleus, and this interaction was transient and inducible by TNFα (Fig. 24B). p65 was also coprecipitated with endogenous p38 and overexpressed Flag-CBP, suggesting that p38-CBP-p65 may form a ternary complex in the nucleus following NF-κB nuclear translocation and p38 activation in response to TNFα. Given the fact that MAP kinase activation is often transient, p38 may interact with CBP in a short time to phosphorylate CBP upon stimulation and then rapidly dissociate. Moreover, ChIP-ReChIP assays reveal that p38 is recruited to the promoters of NF-κB regulated genes in a complex with CBP following TNFα stimulation (Fig. 25). The CBP coactivator associates with promoters through a direct interaction with the p65 TAD and functions as a scaffold to stabilize the multiprotein transcriptional complex (Vo and Goodman, 2001). Thus, p38 may be recruited to promoters through an interaction with CBP and may facilitate the association of members of the transcriptional complex.

To address whether p38 enhances the recruitment of transcriptional complex to promoters, we studied the impact of blocking p38 on the association of CBP and p65 with the promoters of NF-κB regulated genes. Inhibition of p38 activity by SB80 reduced the binding of p38 itself to the promoters, but failed to influence the recruitment of CBP and p65 to the promoters (Fig. 23). Thus, p38 enzymatic activity seems to facilitate the association of p38 with the promoters, which is in a complex with CBP, suggesting that p38-mediated phosphorylation of CBP is likely to strengthen the interaction between p38 and CBP. Although p38 did not affect the binding of CBP and p65 to the promoters, this
does not exclude the possibility that the phosphorylation of CBP by p38 may potentiate the interaction of CBP with other proteins and help stabilize the transcriptional complex. It is also likely that p38-mediated phosphorylation of CBP enhances CBP HAT activity leading to enhanced accessibility of transcriptional complex to promoters. Previous reports showed that the HAT activity of CBP and p300 is upregulated through phosphorylation by p42/44 MAPK, cdk2 or PKA in vitro, whereas phosphorylation of p300 at S89 by PKCζ in vivo reduces its HAT activity (Yuan et al., 2002). Thus, phosphorylation of CBP may induce a conformational change of CBP that either positively or negatively regulates its HAT activity. The exact impact of p38-mediated phosphorylation of CBP on CBP activity and its association with transcriptional complex is still unclear.

Strong evidence indicates that p38 is involved in NF-κB signaling, and that p38 indirectly modulates the p65-TAD-mediated transcriptional activation (Madrid et al., 2001; Norris and Baldwin, 1999). To examine the effect of inhibition of p38 on the expression of endogenous NF-κB-regulated genes, we performed RT-PCR on cells that were pretreated with SB80 or untreated. Following the SB80 treatment, NF-κB was activated by treatment with TNFα (HeLa) or TNFα or LPS (U937). Inhibition of p38 activity decreased the expression of IL-8 and MCP-1 but not cIAP2 and IκBα in U937 cells, whereas blocking p38 had no influence on the TNFα-induced expression of all four genes in HeLa cells (Fig. 27). Therefore, p38 induces transcription of a subset of NF-κB-regulated genes in a cell type dependent manner. U937, a human leukemia cell line, has higher level of p38 expression than in HeLa cells, probably resulting in more
activated p38 to induce expression of particular genes. p38 plays an essential role in the production of TNFα, IL-1β, IL6, and other cytokines during inflammatory responses (Kumar et al., 2003). Consistently, our data suggest that in U937 p38 potentiates the expression of IL-8 and MCP-1, both of which are proinflammatory cytokines responsive to NF-κB. The mechanism by which p38 specifically regulates expression of particular genes is not clear. Future work in understanding how p38 and NF-κB cooperatively and selectively induce the production of proinflammatory cytokines will shed light on the pathology of diseases such as rheumatoid arthritis and inflammatory bowel disease.

The mechanism through which p38 mediates NF-κB activation are starting to emerge. One group proposed that mitogen and stress-activated kinase 1 (MSK1), a downstream target of p38, phosphorylates p65 at S276, leading to stimulation of particular NF-κB-mediated genes (Vermeulen et al., 2003). Another group showed that p38, through MSK1, mediates the phosphorylation of S10 of Histone H3 within the promoters of NF-κB-regulated genes, thus stimulating NF-κB transactivation function (Vermeulen et al., 2003). Based on our findings, we proposed a model of p38-mediated NF-κB activation, in which p38 targets two intermediate proteins: MSK1 and CBP. In response to stimuli such as TNFα and LPS that activate both p38 and NF-κB, p38 activates MSK1 which in turn phosphorylates p65 at S276 within the RHD. In the mean time, p38 also phosphorylates CBP at T614 and/or other sites which may increase the HAT activity of CBP. CBP directly interacts with the transactivation domain of p65 and promotes p65-activated transcription (Gerritsen et al., 1997). The phosphorylation of p65 and CBP
may enhance the coactivator activity of CBP, the interaction between p65 and CBP, as well as their association with other members of the transcriptional complex, potentiating expression of particular NF-κB-regulated genes.
Fig. 30. Proposed model of the p38-mediated activation of NF-κB

Upon stimulation, p38 activates MSK1 which in turn phosphorylates p65 at S276 within the RHD. p38 may also phosphorylate CBP at T614 and/or other sites. The phosphorylation of CBP and p65 may enhance the HAT activity of CBP and/or increase the interaction among CBP, p65, and other proteins in the transcriptional complex.
5 REFERENCES


135
arrest after DNA damage. Science 282, 1497-1501.


domains of the ubiquitous chromatin protein DEK. Molecular and Cellular Biology 24, 6000-6010.


144


Zarubin, T., and Han, J. H. (2005). Activation and signaling of the p38 MAP kinase

