Identification and characterization of binding sites for NF-κB transcription factors in T cells

Seungsoo Lee

July 2011

A thesis submitted for the degree of Doctor of Philosophy of the Australian National University

College of Medicine, Biology and Environment Medical Science Graduate Program The John Curtin School of Medical Research The Australian National University Canberra, Australia
II
Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary education. The data presented in this thesis is my own work and has not been published or written by another person except where due reference has been made in the text.

Seungsoo Lee

July 2011
Statement of Originality

This research contains no material which has been accepted for publication elsewhere or is under consideration for publication elsewhere. The name of any author, society, or publication list is not used as a reference, and no part of this document is to be cited or referred to as having been written by any other person or persons.

Statement of Originality

This research contains no material which has been accepted for publication elsewhere or is under consideration for publication elsewhere. The name of any author, society, or publication list is not used as a reference, and no part of this document is to be cited or referred to as having been written by any other person or persons.

References

1. Smith, J. (2011). An analysis of...
Acknowledgement

Four years have passed since I started my study at the John Curtin School of Medical School at the Australian National. For the last four years, I had to worry about and was distressed by my study. However, I believe that these all things raised me up to more than I can be.

I would like to thank my supervisor Frances Shannon for her consistent advice, encouragement, and patience that she has given me for the last four years. Her support has made me perform research and writing with confidence.

I would like to thank Kristine Hardy for her help and advice on the microarray array. I also have to express my gratitude to Chloe Lim for her time and kind support. Her help was really useful during thesis writing. Thanks to Eloisa Pagler for her fast and kind support for experiments. I would also like to thank all of the past and present members of the Shannon lab for all their advice and support – Jun Wang, Lina Ma, Yan Li, Ian Greaves, Guobing Chen, Yan Xu, Jun Fan, and Karen Bunting.

To Prof. David Tremethick, who is one of my panel members, thanks for his encouragement and attention during my PhD program. I also appreciate his lab members for their kindness and warm hearts.

I also like to thank my good friends Derek and Shobha (who recently moved to Scotland for postdoctor fellowship) for their good friendship and constant attention. I wish I could meet them in Scotland someday. I really appreciate all my house dinner friends - Cavit, Cindy, Rong, Jie-Lian, Vicky, Anggra, and Makoto, who gave me good memories and friendship and made me cheer up when I was exhausted.

A special thank goes to my parents who are looking forward to hearing good news from their son. Although they live far away from me, their encouragement and love gave me energy to perform my study.
Finally, I would like to thank my brothers and sister — Seung-chul, Seung-min, and Seung-hee. Thanks to my best friend Kuk-chun who is looking forward to having a drink when I go back to Korea.

Finishing up this thesis, the words that I wish to say for myself:

“Stay Hungry, Stay Foolish”
Abstract

Transcription of genes is controlled by trans-acting DNA binding transcription factors interacting with cis-regulatory elements containing their cognate binding sites. The interaction between transcription factors and their binding sites has long been studied to understand transcriptional regulatory networks. Initial studies were performed through \textit{in vitro} experiments, which have provided insights into transcription factor-DNA interactions and allowed the identification of regulatory elements for transcription factors of interest. However, these studies provided little information regarding protein-DNA interactions occurring in living cells. Thus, chromatin immunoprecipitation (ChIP) was developed to allow the detection of \textit{in vivo} protein-DNA interactions in living cells. More recently, protein-DNA interactions have been studied using ChIP combined with microarray on a genome-wide scale.

NF-κB family of transcription factors play an important role in the regulation of many genes that are involved in a large number of cellular processes such as immune and inflammatory responses, cell development, cell survival, and cell proliferation.

The main aim of the research reported here was to identify and characterize transcription factor binding sites for the NF-κB transcription factor family members, c-Rel and RelA on a genome-wide scale. Firstly, ChIP assays were developed and used to investigate the kinetics of c-Rel and RelA binding at the known inducible \textit{interleukin-2} (\textit{IL-2}) and \textit{granulocyte-macrophage colony stimulating factor} (\textit{GM-CSF}) proximal promoter regions in EL-4 and primary CD4$^{+}$ T cells. This study showed that the two transcription factors have distinct kinetics of binding in activated T cells, suggesting that they may play distinct roles in the control of gene expression in T cells. Secondly, ChIP was expanded to a genome-wide scale by combining with DNA microarray (ChIP-on-chip), which identified the c-Rel and RelA binding regions across the genome. These binding regions were located not only upstream of the transcription start site (TSS) of genes but also within genes. c-Rel and RelA bound to an overlapping set of regions and while the RelA binding regions were enriched for NF-κB consensus sequences, the c-Rel regions were not. ChIP-on-chip combined with expression profiling data revealed that genes located near c-Rel binding regions were more likely to be responsive to T cell activation and/or c-Rel overexpression compared with the entire gene set on the expression array. However, only a relatively small number of c-Rel-
binding genes were inducible. Validation studies showed that about 31% of the c-Rel binding regions tested bound c-Rel in ChIP-PCR assays and the expression of a small number of these genes was decreased in the absence of c-Rel. Finally, c-Rel binding regions were enriched for IRF transcription factor binding regions and IRF-1 also bound to these regions.

Taken together, these studies demonstrated that two NF-κB proteins have distinct binding profiles in activated T cells and identified binding regions of these transcription factors across the genome. c-Rel target genes were identified and a link between c-Rel and IRF binding regions was elucidated.
# List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin repeats</td>
</tr>
<tr>
<td>AP-1</td>
<td>Transcription factor activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ARRE</td>
<td>Antigen receptor response elements</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BARB</td>
<td>BARbiturate-Inducible Element</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell antigen receptor</td>
</tr>
<tr>
<td>Brg1</td>
<td>Brahma related gene-1</td>
</tr>
<tr>
<td>BRAC</td>
<td>Brachury gene, mesoderm developmental factor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>ChIP-on-chip</td>
<td>Chromatin immunoprecipitation combined with microarray</td>
</tr>
<tr>
<td>ChIP-PET</td>
<td>ChIP-paired end tag</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>ChIP-sequencing</td>
</tr>
<tr>
<td>CIITA</td>
<td>MHC Class II transactivator</td>
</tr>
<tr>
<td>CD28RE</td>
<td>CD28-responsive element</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CN</td>
<td>Phosphatase calcineurin</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>CRFs</td>
<td>Chromatin-remodeling factors</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminal</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal-binding protein</td>
</tr>
<tr>
<td>Cy3</td>
<td>cyanine dye 3</td>
</tr>
<tr>
<td>Cy5</td>
<td>cyanine dye 5</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DH</td>
<td>DNase I hypersensitive</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase I</td>
<td>DNase I hypersensitive</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GMAT</td>
<td>Genome-wide mapping technique</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GTFs</td>
<td>General transcription factors</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H3</td>
<td>Histone 3</td>
</tr>
<tr>
<td>H4</td>
<td>Histone 4</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>HMG</td>
<td>High mobility group protein</td>
</tr>
<tr>
<td>I</td>
<td>Calcium ionophore</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon response factor</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-inducible protein-10</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon response factor</td>
</tr>
<tr>
<td>K</td>
<td>Lysine residue</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MNase</td>
<td>Micrococcal nuclease</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modifier (known as IKKγ)</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor that binds κB enhancers</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>Oct</td>
<td>Octamer-binding transcription factor</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-initiation complex</td>
</tr>
</tbody>
</table>
PBS  Phosphate buffered saline
PMA  Phorbol 12-myristate 13-acetate
PRDF Positive regulatory domain I binding factor
P-TEFb Positive transcription elongation factor b
R Repressor
RHD Rel homology domain
Rho Rhodopsin
RNA Ribonucleic acid
RNA Pol II RNA Polymerase II
rpm Revolutions per minute
RT-PCR Reverse transcriptase PCR
SABE Serial analysis of binding elements
SACO Serial analysis of chromatin occupancy
SD Standard deviation
SDS Sodium dodecyl sulphate
SDS-PAGE Sodium dodecyl sulphate polyacrylamide Gel electrophoresis
SP-1 Specificity protein 1
Srb/Med Srb/Mediator proteins;
STAGE Sequence tag analysis of genomic enrichment
SWI/SNF Switch/sucrose nonfermenting
TAD Transactivation domain
TCR T cell receptor
TE Tris-EDTA
TFBSs Transcription factor binding sites
Th1 T helper 1
Th2 T helper 2
Th17 T helper 17
TNF-β Tumor necrosis factor-beta
Treg Regulatory T cells
TSS Transcription start site
U Unit
UBC Ubiquitin conjugating enzyme
WGA Whole genome amplification
ZEB Zinc finger E-box-binding protein
Publication arising from this study

Table of contents

1 Chapter 1 Introduction......................................................... 1
1.1 Gene regulation in eukaryotes........................................... 3
1.2 Sequence-specific DNA binding transcription proteins.......... 3
    1.2.1 Roles of transcription factors in gene regulation........... 4
    1.2.2 Gene activation............................................... 5
    1.2.3 Gene repression.............................................. 8
    1.2.4 Cis-regulatory element...................................... 10
      1.2.4.1 Promoter regions...................................... 10
      1.2.4.2 Enhancer regions...................................... 11
      1.2.4.3 Silencers............................................... 11
      1.2.4.4 Insulators.............................................. 11
      1.2.4.5 Locus control regions (LCRs)......................... 12
1.3 Genome-wide analysis of transcription factor binding sites... 14
    1.3.1 Protein-DNA interaction studies in vitro................... 14
    1.3.2 A study of protein-DNA interactions using ChIP........... 14
    1.3.3 Identification of transcription factor binding sites using high throughput technique......................................... 15
    1.3.4 Identification of transcription factor sites using ChIP-on-chip... 17
1.4 NF-κB transcription factors........................................... 20
    1.4.1 The structure of the NF-κB family of transcription factors 21
    1.4.2 The NF-κB signalling pathways................................ 26
      1.4.2.1 Classical NF-κB signalling pathway...................... 26
      1.4.2.2 Alternative NF-κB signalling pathway................. 27
    1.4.3 NF-κB in T cell development.................................. 30
      1.4.3.1 The development of T lymphocytes in the thymus........ 30
      1.4.3.2 Development of T cells into different T cell subsets... 30
      1.4.3.3 The role of NF-κB in T cell development............... 33
1.4.4 Role of the NF-κB in immune responses (innate and adaptive immune responses) and inflammation........................... 34
    1.4.4.1 Innate immune response.................................. 34
    1.4.4.2 Adaptive immune response................................ 25
    1.4.4.3 Initiation of T cell activation through T cell receptor and CD28 costimulation......................................... 36
1.4.4.4 NF-κB activation by TCR........................................... 36
1.4.4.5 The role of NF-κB in the adaptive immunity................. 40
1.4.4.6 Inflammatory response........................................... 41
1.4.4.7 The role of RelA in the immune system...................... 41
1.4.5 The role of c-Rel in T lymphocytes.......................... 42
  1.4.5.1 c-Rel in T cell differentiation............................ 43
  1.4.5.2 Roles of c-Rel in T cell activation and in cytokine and cytokine receptor gene expression in T cells........ 44
  1.4.5.3 c-Rel is essential for chromatin remodeling and histone loss at the inducible cytokine genes............. 45
1.5 Project aims...................................................... 47

2 Chapter 2 Materials and Methods.................................. 49
  2.1 Materials.................................................................. 51
    2.1.1 Chemicals....................................................... 51
    2.1.2 Antibiotics..................................................... 51
    2.1.3 Antibodies..................................................... 51
    2.1.4 Oligonucleotides.............................................. 52
    2.1.5 Kits............................................................ 52
    2.1.6 Cell line......................................................... 52
    2.1.7 Mice............................................................ 53
  2.2 Methods................................................................... 53
    2.2.1 Cell culture...................................................... 53
    2.2.2 Primary T cell preparation.................................. 53
    2.2.3 Total RNA isolation......................................... 54
    2.2.4 cDNA synthesis............................................... 55
    2.2.5 Quantitative Real-time PCR.............................. 55
    2.2.6 Nuclear protein extraction.................................. 56
    2.2.7 Bradford assays............................................... 57
    2.2.8 Western blotting assays.................................... 57
    2.2.9 Chromatin immunoprecipitation (ChIP) assay........... 58
    2.2.10 Sequential chromatin immunoprecipitation (Seq-ChIP)....... 59
    2.2.11 Chromatin immunoprecipitation (ChIP) on chip (ChIP-on-chip).............................................. 60
    2.2.12 DNA microarray............................................... 61
3 Chapter 3 Kinetics of transcription factor binding to cytokine gene promoters using chromatin immunoprecipitation in T cells......... 65

3.1 Introduction.................................................. 67
3.2 Results...................................................... 74
  3.2.1 Dynamic binding of transcription factors c-Rel and RelA to the IL-2 and GM-CSF promoter regions in EL-4 T cells............ 74
  3.2.2 Kinetics of binding of transcription factors c-Rel and RelA to the IL-2 and GM-CSF promoters in primary CD4+ T cells...... 81
  3.2.3 AP-1 family c-Jun and ZEB1 bind to the IL-2 and/or GM-CSF proximal promoter region in EL-4 T cells......................... 87
3.3 Discussion.................................................. 92

4 Chapter 4 Identification and characterization of NF-κB (c-Rel and RelA) binding regions using ChIP-on-chip in EL-4 T cells......... 97

4.1 Introduction.................................................. 99
4.2 Results...................................................... 102
  4.2.1 Genome-wide analysis of c-Rel and RelA binding regions using ChIP-on-chip.................................................. 102
  4.2.2 Comparison of ChIP-on-chip replicates using Model-based Analysis of Tiling-arrays (MAT) algorithm.......................... 105
  4.2.3 Identification of enriched regions bound by NF-κB family members c-Rel and RelA using Partek® Genomics Suite™ Software.................................................. 107
  4.2.4 Identification of transcription factor family motifs that are significantly overrepresented in the NF-κB-bound regions..... 108
  4.2.5 Regions bound by c-Rel are also occupied by RelA.............. 112
  4.2.6 Analysis of the genomic location of transcription factor binding regions.................................................. 114
4.2.7 Identification of the nearest genes from transcription factor binding regions.................................................. 118

4.2.7.1 Regulation of expression of gene groups nearest c-Rel and RelA binding regions........................................... 118

4.2.7.2 Effect of c-Rel overexpression on the gene groups nearest c-Rel and RelA binding regions.............................. 122

4.3 Discussion................................................................................................................................................. 125

5 Chapter 5 Verification and characterization of putative c-Rel regulated genes..................................................... 131

5.1 Introduction.................................................................................................................................................. 133

5.2 Results..................................................................................................................................................... 136

5.2.1 Selection of c-Rel binding genes in T cells............................................................................................... 136

5.2.2 Confirmation of the expression of selected genes in EL-4 T cells................................................................. 144

5.2.3 Confirmation of in vivo binding of c-Rel to target genes in EL-4 T cells......................................................... 144

5.2.4 Confirmation of in vivo binding of c-Rel to the putative c-Rel binding genes in primary CD4+ T cells............. 145

5.2.5 Effect of c-Rel in the regulation of genes nearest to c-Rel binding regions following stimulation..................... 151

5.2.6 The relationship between c-Rel and IRF-1 transcription factors in c-Rel binding genes in T cells..................... 156

5.2.6.1 Confirmation of the binding of IRF-1 to c-Rel binding genes in EL-4 T cells................................................. 156

5.2.6.2 Confirmation of in vivo interaction between c-Rel and IRF-1 in T cells....................................................... 157

5.2.6.3 Confirmation of the binding of IRF-1 to c-Rel binding genes in CD4+ T cells............................................. 158

5.3 Discussion................................................................................................................................................... 164

6 Chapter 6 Final discussion............................................................................................................................... 171

6.1 Summary.................................................................................................................................................... 173

6.2 c-Rel and RelA have distinct kinetics of promoter association in T
6.3 c-Rel and RelA binding sites are scattered across the genomic DNA... 175
6.4 c-Rel binding regions are occupied by RelA............................... 177
6.5 Transcription factor and its target genes.................................... 178
6.6 Relationship between the transcription factors c-Rel and IRF-1...... 179
6.7 Conclusions............................................................................ 181

7 References.................................................................................. 183

8 Appendix..................................................................................... 205
Table of contents

List of Figures

Chapter 1
Figure 1.1 Transcription initiation in eukaryotes............................................. 7
Figure 1.2 Repression of basal and activated transcription factors....................... 9
Figure 1.3 Schematic diagram representing the distal regulatory elements.............. 13
Figure 1.4 Overview of ChIP-on-chip procedure.............................................. 19
Figure 1.5 The NF-κB family of transcription factors........................................ 22
Figure 1.6 Dimerization of NF-κB family proteins............................................ 25
Figure 1.7 The NF-κB signalling pathways.................................................... 28
Figure 1.8 T cell development in primary and secondary organs.......................... 32
Figure 1.9 Schematic diagram of T cell activation through TCR/MHC and CD28 costimulatory molecules................................................................. 38
Figure 1.10 NF-κB activation through TCR in T cells........................................ 39

Chapter 3
Figure 3.1 The IL-2 and GM-CSF proximal promoter regions............................ 73
Figure 3.2 Time course of IL-2 mRNA expression in EL-4 T cells......................... 76
Figure 3.3 mRNA expression profiles and nuclear accumulation kinetics of NF-κB c-Rel and RelA in EL-4 T cells.......................................................... 77
Figure 3.4 Kinetics of binding of NF-κB c-Rel and RelA to the IL-2 and GM-CSF promoters in EL-4 T cells................................................................. 79
Figure 3.5 mRNA expression profiles and protein kinetics in primary CD4⁺ T cells................................................................. 83
Figure 3.6 In vivo binding assays of c-Rel and RelA at the IL-2 and GM-CSF promoters in primary CD4⁺ T cells................................................................. 85
Figure 3.7 Recruitment profiles of AP-1 family c-Jun at the IL-2 and GM-CSF promoters................................................................. 89
Figure 3.8 ZEB1 binding profiles at the IL-2 proximal promoter region in EL-4 T cells................................................................. 91

Chapter 4
Figure 4.1 Preparation of DNA for ChIP-on-chip microarray and overview of ChIP-on-chip dat analysis................................................................. 103
Figure 4.2 Visual comparison of ChIP-on-chip signals near the IL-2 gene promoter

Figure 4.3 Identification of regions bound by NF-κB c-Rel and RelA

Figure 4.4 Distribution of distance of c-Rel and RelA binding regions relative to the transcription start site

Chapter 5

Figure 5.1 Putative binding motifs of the NF-κB and IRF transcription factors in c-Rel binding genes

Figure 5.2 NF-κB binding sites at the Gadd45b promoter region and MatInspector™ prediction of putative binding sites of NF-κB and IRF transcription factors within the Gadd45b gene

Figure 5.3 Expression profiles and confirmation of c-Rel binding genes in PMA/I stimulated EL-4 T cells

Figure 5.4 Confirmation of the in vivo binding of c-Rel to target genes in EL-4 T cells

Figure 5.5 In vivo binding assays of c-Rel in primary CD4⁺ T cells following CD3/CD28

Figure 5.6 Effect of c-Rel knockout in the expression of c-Rel binding genes in primary CD4⁺ T cells following PMA/I stimulation

Figure 5.7 Expression profiles of c-Rel binding genes in CD3/CD28 stimulated wild-type and c-Rel⁻/⁻ primary CD4⁺ T cells

Figure 5.8 In vivo binding of IRF-1 to c-Rel binding genes in EL-4 T cells

Figure 5.9 Interaction between c-Rel and IRF-1 transcription factors in T cells

Figure 5.10 In vivo binding assays of IRF-1 in primary CD4⁺ T cells following CD3/CD28

Figure 5.11 MatInspector™ prediction of putative binding sites of IRF and NF-κB transcription factors at the upstream region of the Nek8 gene from the TSS
List of Tables

Chapter 1
Table 1.1 Major phenotypes associated with deletion of NF-κB family members ................................................................. 24

Chapter 4
Table 4.1 Transcription factor family motifs highly enriched in c-Rel and RelA binding sequences .................................................. 111
Table 4.2 Percentage of regions bound by both c-Rel and RelA .................. 113
Table 4.3 Location of NF-κB c-Rel and RelA binding regions across the genomic regions represented on the array .................................. 116
Table 4.4 Percentage of nearest genes to the regions bound by c-Rel and RelA that are affected by PMA/I stimulation ....................... 121
Table 4.5 Percentage of nearest genes in the regions bound by NF-κB c-Rel or RelA that are affected by c-Rel overexpression .................. 124

Chapter 5
Table 5.1 Putative c-Rel binding genes selected by combining ChIP-on-chip and expression profiling data .................................................. 142
Table 5.2 MatInspector™ prediction of putative transcription factor binding sites of NF-κB/c-Rel and IRF in the c-Rel binding genes ............ 143
Table 5.3 Summary of putative c-Rel binding genes containing motifs that responded to PMA/I stimulation in EL-4 T cells and in vivo binding of c-Rel to these genes in EL-4 and primary CD4⁺ T cells .. 150
Table 5.4 Summary of c-Rel binding genes that responded to PMA/I and/or CD3/CD28 stimulation and that were inhibited in c-Rel⁻/⁻ T cells relative to wild-type cells following stimulation ................................. 155
Table 5.5 Summary of c-Rel binding genes bound by IRF-1 in EL-4 and primary CD4⁺ T cells following PMA/I stimulation ....................... 163
Chapter 1

Introduction
Chapter 1

Introduction
1.1 Gene regulation in eukaryotes

Eukaryotic transcriptional regulation is a complex and multistep process. Transcription of the large number of genes is very tightly and precisely regulated by the transcriptional regulatory machinery, with the cis-regulatory elements providing the many binding sequences that are recognized by the trans-acting DNA binding transcription factors. The interaction between cis-regulatory elements and trans-acting factors controls many important nuclear processes in addition to regulation of transcription, including DNA replication, repair, and maintenance of chromosome structure.

In eukaryotes, the genetic information is packaged into highly condensed chromatin which is a higher order structure consisting of DNA, histone proteins, RNA, and other proteins. The compacted chromatin structure has positive properties in that it helps store large amounts of genetic information in the compact space of the cell nucleus. However, this condensed chromatin structure can act as an obstacle and limits access of trans-acting factors to the DNA template (reviewed in Emerson 2002; Bhaumik et al. 2007; Kouzarides 2007; Jiang et al. 2009).

Eukaryotic transcription is initiated by RNA Polymerase II (RNA Pol II) binding to the core promoter or transcription start site (TSS) of a gene. However, RNA Pol II cannot bind to the core promoter alone (reviewed in Patikoglou et al. 1997; Narlikar et al. 2009). Thus, for proper transcription initiation, RNA Pol II needs to interact with many other regulatory proteins such as sequence-specific activators, repressors, mediators (cofactors), and general transcription factors (GTFs). All of these proteins work together to regulate proper and accurate initiation of gene transcription.

1.2 Sequence-specific DNA binding transcription proteins

A great number of sequence-specific DNA binding proteins, including activators and repressors, have been described in eukaryotes. These are involved in many biological processes including transcription, DNA replication, and DNA repair (Dummitt et al. 2006; Chen et al. 2010). Those involved in transcription, known as transcription factors,
have a modular structure and contain specific regions that are responsible for DNA binding, transcriptional activation/repression, protein-protein interaction, and nuclear translocation. Through their DNA binding domains, transcription factors bind to the promoter and distal regulatory regions such as enhancers and/or silencers of the genes they regulate (reviewed in Latchman 1997; Miano et al. 1999; reviewed in Alvarez et al. 2003; reviewed in Kadonaga 2004; Chen et al. 2007). The DNA-binding domain of transcription factors recognizes and binds specific cis-regulatory sequences in the DNA (Narlikar et al. 2006). These regulatory sequences are short DNA sequence (5 to 10 bp) located within the promoter and enhancer (or silencer) regions.

Transcription factors are grouped into families according to their DNA-binding domains such as leucine zipper, helix-turn-helix, and zinc finger (Narlikar et al. 2006). Examples include the NF-κB family composed of five members as described in Chapter 3; the activator protein-1 (AP-1) leucine zipper family consisting of two subgroups, Jun and Fos; the AP-2 families containing a helix-turn-helix motif and comprising five distinct members in mammals (AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ε) (reviewed in Eckert et al. 2005); and the GATA family consisting of six members (GATA1 to 6) containing a conserved zinc finger domain in vertebrates (reviewed in Viger et al. 2008). These family members have structural similarities and conserved DNA-binding domains, however, each member can play distinct roles and regulate distinct sets of genes (reviewed in Kadonaga 2004).

As will be discussed below (Section 1.2.3), transcriptional repressors block the activity of transcriptional activators through a number of mechanisms including inhibiting their binding to DNA, or by directly binding to activators (Latchman 1997; Lee et al. 2000; reviewed in Gaston et al. 2003). Thus, transcription factors maintain a balance of transcription rate by acting as activators and repressors.

1.2.1 Roles of transcription factors in gene regulation

In eukaryotes, there are several thousand transcription factors encoded by the genome. Transcription factors function not only by directly binding to DNA, but also by recruiting transcriptional coactivators and corepressors to the promoter through protein-protein interactions. During transcription, transcription factors interact with RNA Pol II and recruit it to the core promoter (reviewed in Lee et al. 2000; Suhardja et al. 2001;
Gaston et al. 2003; Kadonaga 2004). In addition, by cooperating with cofactors, transcription factors recruit GTFs such as transcription factor II families (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIFH) to the promoter regions. Thus, transcription factors initiate transcription through this basal transcriptional apparatus (reviewed in Lee et al. 2000; Suhardja et al. 2001).

Transcription activators also activate transcription by recruiting to the promoter many enzymes involved in chromatin modifications such as histone modification enzymes and ATP-dependent chromatin-remodeling enzymes. These proteins alter chromatin structure, allowing accessibility of other transcription factors, RNA Pol II, and other factors to the DNA (reviewed in Lee et al. 2000; Gaston et al. 2003; Bernstein et al. 2004). For example, transcription factor Swi5 recruits chromatin remodeling complexes, SWI/SNF and histone acetylase, SAGA to the HO promoter (Cosma et al. 1999). In addition, NF-κB RelA is involved in the recruitment of the Brg1 component of the SWI/SNF chromatin remodeling complex to the GM-CSF promoter in T cells (Holloway et al. 2003). Interestingly, sequence-specific DNA binding transcription factors are also regulated by posttranscriptional modifications including phosphorylation, acetylation, and ubiquitination. For example, p53 is directly acetylated by coactivator p300 which increases DNA-binding activity, and NF-κB is also phosphorylated in the cytoplasm before nuclear accumulation. Furthermore, the transcription factor, cyclic AMP response element binding protein (CREB) is phosphorylated by protein kinase A (Gonzalez et al. 1989; Sharrocks 2000; reviewed in Kadonaga 2004). These modifications often influence the activity of the transcription factors to regulate transcription.

1.2.2 Gene activation

To initiate eukaryotic transcription, RNA Pol II requires the GTFs. These GTFs form a complex with the core promoter which facilitates the binding of RNA Pol II, forming a pre-initiation complex (PIC) (reviewed in Gaston et al. 2003; Green 2005; Narlikar et al. 2009) (Fig 1.1). This PIC formation is stimulated by transcriptional activators (reviewed in Gaston et al. 2003; Green 2005). Activator proteins promote transcription initiation with co-activator proteins which are recruited through protein-protein interactions and function as a bridge between GTFs and activators (reviewed in Hampsey 1998; Gaston et al. 2003; Green 2005). Activator/co-activator proteins can also recruit proteins
involved in chromatin remodeling to the promoter. This results in alterations to chromatin structure into a more accessible state and thereby leads to transcription initiation (reviewed in Hampsey 1998; Gaston et al. 2003; Green 2005).
Figure 1.1 Transcription initiation in eukaryotes

The general transcription factors including transcription factor II (TFIIA), TFIIB, and TFIID bind to the core promoter. In particular, TFIID recognizes the TATA box sequence via a TATA box-binding protein and enables the binding of TFIIIB to the promoter. RNA Pol II forms a complex called a RNA Pol II holoenzyme by interacting with many other factors involved in both positive and negative regulatory responses and in alterations in chromatin structure. These are all recruited to the promoter, forming a PIC which then initiates transcription in response to appropriate signals. Right angled arrow indicates the TSS. Abbreviations: PIC = preinitiation complex; Srb/Med = Srb/Mediator proteins; CRFs = chromatin-remodeling factors; TSS = transcription start site. (Obtained from Gaston et al. 2003)
1.2.3 Gene repression

Repressor proteins can inhibit the transcription of genes by modifying key components of the basal transcription machinery (which consists of RNA Pol II and GTFs) or by blocking the activity of activators/coactivators on transcription (Fig 1.2). In particular, RNA Pol II can be phosphorylated at the C-terminus which hinders PIC assembly and therefore inhibits transcription (Hengartner et al. 1998). In addition, repressor proteins ubiquitinate activators/coactivators which leads to proteasomal degradation (reviewed in Gaston et al. 2003). Transcriptional repression can also occur by histone-modifying proteins such as histone deacetylases (HDACs) and some histone methyltransferases (HMTs) (SUV39H1 and G9a), which deacetylate and methylate histones respectively. These modifications lead to a more highly compacted chromatin structure (reviewed in Gaston et al. 2003; Watanabe et al. 2008) which can result in blocking the accessibility of transcriptional activators and the basal transcriptional machinery to the DNA. In addition, it has been suggested that CpG methylation can bring about transcription inhibition. Because methylated CpG dinucleotides recruit methyl-CpG-binding proteins which promotes the assembly of transcription repressor complexes such as histone deacetylase and histone methylases, changing the chromatin structure and thereby interfering with the binding of transcriptional activators (Gaston et al. 1995; reviewed in Gaston et al. 2003; Maston et al. 2006).
Figure 1.2 Repression of basal and activated transcription factors

(A) RNA Pol II or GTFs is directly targeted by repressors (R) which bring about modification of basal transcription machinery and inhibits its binding to the promoter.

(B) Repressors can inhibit the binding of TFIID (TATA box–binding protein) by competing for the core promoter (R1) or binding directly to TFIID (R2) and blocking binding to the promoter. (C) Repressors block the interaction between TFIID and other GTFs. Right angled arrow indicates the TSS. Abbreviations: R = repressor; TSS = transcription start site; GTFs = general transcription factors.

(Obtained from Gaston et al. 2003)
1.2.4 Cis-regulatory elements

In eukaryotes, protein-coding genes are regulated by short cis-regulatory elements (5 to 12 bp in length) where the binding of regulatory transcription factors occurs (reviewed in Kadonaga 2004; Maston et al. 2006; Tuch et al. 2008). Cis-regulatory elements can occur in distinct regulatory regions such as promoters (core, proximal, and distal) and distal regulatory elements (enhancers, silencers, insulators, and locus control regions) (Fig 1.3). Regulatory regions and elements have also been shown to occur within the genes (reviewed in Delgado et al. 2006; Maston et al. 2006).

1.2.4.1 Promoter regions

Promoters can be divided into three distinct regions: the core, proximal, and distal promoter (reviewed in Delgado et al. 2006). The core promoter plays an important role for transcription initiation as RNA Pol II binds through the GTFs assembling on this region, forming the PIC as described above. The core promoter is adjacent to the transcription start site (TSS) (approximately within 50 bp) and often contains a consensus TATA sequence defined as a TATA box. This TATA box is important in controlling the transcription rate, as well as for the precise initiation of transcription (reviewed in Delgado et al. 2006).

Another important promoter region is defined as a proximal promoter which is immediately upstream from the TSS (usually within 1 to 150 bp) and has many regulatory elements containing multiple binding sites for sequence-specific transcription factors. Upon cell stimulation, a number of regulatory factors bind to the proximal promoter region where they work synergistically to control transcription (reviewed in Patikoglou et al. 1997; Delgado et al. 2006; Maston et al. 2006).

The distal promoter is upstream of the proximal promoter and located between approximately 500 and 1000 bp from the core promoter. It also contains regulatory elements with binding sites for transcription factors. Thus, the distal promoter can regulate gene expression, but has a weaker influence than the proximal promoter (Delgado et al. 2006).
1.2.4.2 Enhancer regions

Enhancers are transcriptional control regions similar to promoter regions in that they contain multiple binding sites for activators and are also cell type specific. However, enhancers have some different properties compared to the promoters. Above all, they can be situated upstream or downstream of the gene and within an intron or even beyond the 3' end of the gene (reviewed in Delgado et al. 2006; Maston et al. 2006). In addition, they can act from several hundred kb either upstream or downstream of the TSS. Thus, enhancers can induce transcription of a gene independently of position, orientation, and distance from the TSS (reviewed in Delgado et al. 2006; Maston et al. 2006; Narlikar et al. 2009). It has been suggested that the DNA between an enhancer and the core promoter can form a loop which allows the activators bound to the enhancer to be located close to the promoter and the TSS (reviewed in Maston et al. 2006; Narlikar et al. 2009).

1.2.4.3 Silencers

Silencers are negative regulatory elements containing binding sites for repressor proteins, which repress gene transcription by inhibiting activator functions as described above (reviewed in Delgado et al. 2006; Maston et al. 2006; Narlikar et al. 2009). As observed in the above regulatory elements, silencers can also function regardless of their distance and orientation from the promoter. Silencers can be found within the intron or in the 3' end of the gene, where they can exist as part of the proximal promoter, a distal enhancer, or an independent distal regulatory module (reviewed in Ogbourne et al. 1998; Maston et al. 2006).

1.2.4.4 Insulators

Unlike other elements, insulators function as a barrier to prevent the spread of repressive chromatin (known as heterochromatin-barrier activity). As a barrier, insulators can protect genes by blocking the transcriptional activity generated from their neighbouring genes. The most important property that insulators have is that they can counteract the interaction between enhancers and promoters, which is referred to as enhancer-blocking activity. The length of insulators is typically less than 3 kb and the function of insulators is dependent on their position, but is orientation independent
(reviewed in West et al. 2005; Delgado et al. 2006; Maston et al. 2006; Narlikar et al. 2009).

1.2.4.5 Locus control regions (LCRs)

LCRs are complex transcriptional regulatory regions containing enhancers, silencers, and insulators. Thus, a large number of transcription factors, coactivators, repressors, and proteins involved in chromatin remodeling bind to these elements (reviewed in Dean 2006; Delgado et al. 2006; Maston et al. 2006; Narlikar et al. 2009). LCRs have some similarities with enhancers in that they regulate the genes from a distance and can be found within the genes they regulate. However, the LCRs are characterized by containing several DNase I hypersensitive sites which induces an open chromatin domain, resulting in gene activation.
Figure 1.3 Schematic diagram representing the distal regulatory elements

Distal regulatory elements which are composed of enhancer, silencer, insulators, and locus control regions play essential roles for activation or repression of transcription.

(Obtained from Maston et al. 2006)
1.3 Genome-wide analysis of transcription factor binding sites

1.3.1 Protein-DNA interaction studies \textit{in vitro}

In higher eukaryotes, gene expression is complex and strictly coordinated. This gene expression is affected by the binding of numerous transcription factors at their binding sites on genomic DNA. Thus, it is essential to investigate the interactions between transcription factors and DNA to understand transcriptional regulatory mechanisms. This has been performed by identifying transcription factor binding sites within genomic DNA (reviewed in Massie et al. 2008; Wu et al. 2008) through \textit{in vitro} binding assays such as electrophoretic mobility shift assay (EMSA) and DNase I footprinting (Jain et al. 1995; Rooney et al. 1995; Serfling et al. 1995; Rothenberg et al. 1996; Tolner et al. 2001). These \textit{in vitro} assays provided insights into transcription factor-DNA interactions and enabled the detection of many regulatory elements and transcription factors of interest. However, \textit{in vitro} binding methods sometimes showed different results compared to those obtained from \textit{in vivo} assays (reviewed in Massie et al. 2008). Furthermore, it is hard to investigate the dynamic kinetics of transcription factor binding as well as the epigenetic mechanisms operating in living cells. Therefore, to understand a detailed and accurate interaction between proteins and DNA, advanced technologies have been developed.

1.3.2 A study of protein-DNA interactions using ChIP

Chromatin immunoprecipitation (ChIP) was developed as a means of examining \textit{in vivo} transcription factor-DNA interactions and has now become the method of choice. ChIP assays involve chemical cross-linking of proteins to genomic DNA in living cells. After cross-linking, genomic DNA is sheared to short fragments, immunoprecipitated with appropriate antibodies, and purified (reviewed in Kim et al. 2006; Kim et al. 2007; reviewed in Hoffman et al. 2009; Pillai et al. 2009). These ChIP fragments can be analysed using several techniques such as Southern blotting and quantitative real-time PCR (Kim et al. 2007; reviewed in Hoffman et al. 2009; Tong et al. 2009). This method has been widely used to identify transcription factor binding sites, histone modifications,
and DNA methylation sites (reviewed in Massie et al. 2008; Furlan-Magaril et al. 2009). For example, in early studies, ChIP assays were used to identify polycomb (Pc) binding sites in the homeotic bithorax complex (BX-C) of Drosophila tissue cells (Orlando et al. 1993). In addition, in a study by Kondo et al. ChIP has been expanded to study histone modifications (Kondo et al. 2003), showing that histone modifications are involved in the silencing of genes including p16, MLH1, and MGMT in cancer cells. Furthermore, ChIP was used for methylation analysis to detect methylated DNA regions across the human genome in colon cancer cells (SW48) (Weber et al. 2005). ChIP was also used in this thesis and in a recently published paper (Wang et al. 2009) to investigate the in vivo binding of transcription factors to inducible cytokine gene promoters in T cells. However, a successful ChIP assay relies on the specificity of the antibody used. In addition, ChIP is helpful in identifying a small number of target sites or known binding sites, but it does not allow the detection of novel binding sites or the investigation of binding sites on a genome-wide scale (reviewed in Kim et al. 2006; Kim et al. 2007; reviewed in Hoffman et al. 2009; Tong et al. 2009). Thus, ChIP has been extended to a genome-wide scale analysis to study all possible transcription factor-DNA interactions on the whole genome.

1.3.3 Identification of transcription factor binding sites using high throughput techniques

Several high throughput techniques using ChIP-enriched DNA have been developed to overcome the limitations of ChIP and to investigate DNA-binding sites on a genome-wide scale. These include ChIP with microarray (ChIP-on-chip) (De Siervi et al. 2009), ChIP-sequencing (ChIP-Seq) (Barski et al. 2007), ChIP-serial analysis of binding elements (SABE) (Chen et al. 2005), ChIP-serial analysis of chromatin occupancy (SACO) (Yochum et al. 2007), ChIP-sequence tag analysis of genomic enrichment (STAGE) (Bhinge et al. 2007), genome-wide mapping technique (GMAT) (Roh et al. 2008), and ChIP-paired end tag (ChIP-PET) (Wei et al. 2006).

SABE, SACO, STAGE, and GMAT are sequence-based and unbiased approaches to sequence short DNA tags. These are technically similar whereby ChIP-enriched DNA is ligated to linkers and this DNA is amplified, digested, concatenated, cloned, and finally sequenced (Impey et al. 2004; Chen et al. 2005; Bhinge et al. 2007; Roh et al. 2008).
ChIP-PET is also an unbiased method, but it differs in that ChIP-enriched DNA is cloned into a plasmid library which is converted into a concatenated PET. This PET is sequenced and the location is mapped to the genome to identify the boundaries of the cloned ChIP fragments (Loh et al. 2006; Wei et al. 2006). ChIP-Seq is currently the most widely used method to map protein-DNA interactions in vivo on a genome scale (Barski et al. 2007; Johnson et al. 2007; Robertson et al. 2007). ChIP-Seq combines ChIP with high throughput massively parallel sequencing and requires a small amount of starting material. In addition, plasmid library preparation and/or hybridization are not necessary (Barski et al. 2007; Johnson et al. 2007; Robertson et al. 2007; reviewed in Massie et al. 2008; Schones et al. 2008).

These high throughput ChIP-based techniques have been applied to many different studies on a genome scale. For example, ChIP-Seq has been widely used to study histone modifications and nucleosome positioning and to identify transcription factor binding sites (Yuan et al. 2005; Barski et al. 2007; Lee et al. 2007; Mikkelsen et al. 2007; Oszolak et al. 2007; Robertson et al. 2007; Wang et al. 2008). One of the major applications of ChIP-Seq was to map the locations of histone modifications in human CD4+ T and mouse ES cells (Barski et al. 2007; Mikkelsen et al. 2007; Wang et al. 2008). From these studies, histone acetylation and histone methylation locations were mapped on genomic scale. In addition, ChIP-Seq technology has been used to map nucleosome positioning in the human and yeast genomes (Yuan et al. 2005; Lee et al. 2007; Oszolak et al. 2007). These high throughput studies showed that transcript abundance and transcription rate is associated with nucleosomes occupancy. Furthermore, a genome-wide screening of specific binding targets for transcription factor such as STAT1 has been performed using ChIP-Seq in mammalian cells (Robertson et al. 2007). In addition, recently, other sequencing-based methods described above (SACO, SABE, STAGE, and ChIP-PET) have been used for mapping of histone modifications and identification of binding sites of transcription factors such as CREB, p53, STAT1, and RelA in humans and yeast (Impey et al. 2004; Chen et al. 2005; Bhome et al. 2007; Lim et al. 2007). Thus, these high throughput sequencing technologies combined with ChIP have facilitated the determination of the entire spectrum of in vivo binding of many transcription factors. In addition, these advanced methods have shed light on how epigenetic mechanisms are regulated, and how these mechanisms control the expression of the genes in living cells.
1.3.4 Identification of transcription factor sites using ChIP-on-chip

One of the first methods to be developed that could overcome the limitations of ChIP was ChIP-on-chip, which combines ChIP with DNA microarray (Fig 1.4) and is a powerful in vivo method to identify regulatory regions bound by a transcription factor at a genome-wide level. This method has been widely used to identify in vivo DNA-binding sites on a genome-wide scale (Martone et al. 2003; Cawley et al. 2004; Boyer et al. 2005; Phuc Le et al. 2005; Beima et al. 2006; Vigano et al. 2006; Frontini et al. 2009; Song et al. 2009). In ChIP-on-chip, immunoprecipitated genomic DNA and control DNA such as total genomic DNA are amplified and/or reamplified, labeled with fluorescent dyes (Cy3/Cy5), and hybridized to DNA microarrays containing genomic DNA sequences (reviewed in Massie et al. 2008; Gilchrist et al. 2009; Wong et al. 2009). Potential genomic binding sites are considered as enriched regions if they harbour stronger fluorescent signals in the ChIP DNA channel compared with the control DNA sample (reviewed in Massie et al. 2008; Wong et al. 2009). ChIP-on-chip is a high throughput method for localization of target sites of transcription factors. This ChIP-on-chip can be used to interrogate all nonrepeating DNA sequences in the genome and to detect novel binding sites for transcription factors (reviewed in Massie et al. 2008; Wong et al. 2009). However, ChIP-on-chip requires large amounts of starting material for successful hybridization, generates high background noise, and is biased toward regions near the TSS of known genes (reviewed in Wu et al. 2006; Massie et al. 2008; Hoffman et al. 2009). Thus, to overcome these disadvantages, many other methods including ChIP-seq have been developed as described above.

ChIP-on-chip technologies have successfully been used in different systems including human and yeast for identification of genomic binding sites for transcription factors such as GATA1, pRb, MBF, and SBF (Iyer et al. 2001; Horak et al. 2002; Wells et al. 2003). In addition, this thesis used ChIP-on-chip in mouse T cells to identify the genomic target regions for c-Rel and RelA. In addition to identification of in vivo transcription factor binding sites, ChIP-on-chip has been widely applied for other studies. For example, it has been used to investigate the general transcription machinery, histone modifications, and DNA methylation in human, mouse, and yeast systems (Bernstein et al. 2002; Robyr et al. 2002; Harismendy et al. 2003; Roberts et al. 2003; reviewed in Hanlon et al. 2004; Kondo et al. 2004; Moqtaderi et al. 2004; Bernstein et al. 2005; reviewed in Wu et al. 2006; Heintzman et al. 2007; Mo et al. 2010). An early
study by Bernstein et al. showed that histone acetylation (H3 and H4) in promoter regions and dimethylation of H3 (H3K4) in coding regions are linked with transcriptional activation in yeast (Bernstein et al. 2002). An interesting study by Heintzman et al. showed that the active promoters and enhancers share some similar histone modification patterns such as nucleosome depletion and enrichment of histone acetylation, but these transcriptional regulators have distinct chromatin signatures in the human genome. For example, while activated promoters are characterized by enrichment of trimethylated H3K4 and depletion of monomethylated H3K4, enhancers are strongly enriched for monomethylated H3K4 as well as trimethylated H3K4 profiles (Heintzman et al. 2007). Another useful application of ChIP-on-chip performed by Kondo et al. was to identify target genes silenced by histone methylation in cancer cells (Kondo et al. 2004). Furthermore, to investigate the relationship between the binding of transcription factors and their ability to regulate gene expression, ChIP-on-chip followed by validation studies have been performed in mammalian systems. These studies showed that the expression of target genes bound by transcription factors such as T-bet, p63, RelA, and IRF-1 is dependent on cell type or stimulation condition (Beima et al. 2006; Lim et al. 2007; Frontini et al. 2009; Song et al. 2009).
EL-4 T cells were nonstimulated or stimulated with PMA/I for 2 h and 8 h. ChIP assays were performed by cross-linking proteins to genomic DNA followed by sonication to shear genomic DNA. Sonicated DNA fragments were immunoprecipitated with antibodies specific for c-Rel and RelA. Immunocomplexes were then reverse cross-linked followed by DNA purification. After the ChIP experiments, ChIP-DNA was amplified and/or reamplified using WGA (whole genome amplification kit) and purified. Purified genomic DNA was labeled using two different fluorescent dyes (cyanine dyes Cy5 and Cy3), and hybridized to DNA microarrays containing genomic DNA sequences. Total input was used as a control sample. Binding sites having significantly higher fluorescent signals than the control are represented as red spots on the arrays. (Obtained from Acevedo et al. 2007)
NF-κB (nuclear factor of kappa B cells) was first identified in the nucleus of B cells as a factor binding to short sequences of the enhancer of the immunoglobulin kappa (κ) light chain (reviewed in Ghosh et al. 1998; Ahn et al. 2005; Hoffmann et al. 2006). In mammalian cells, the NF-κB family consists of five related members; c-Rel, RelA (p65), RelB, NF-κB1 (p50/precursor p105), and NF-κB2 (p52/precursor p100) (Fig 1.5A). These proteins regulate several hundreds of genes involved in immune and inflammatory responses, cell survival, proliferation, differentiation, and apoptosis (reviewed in Gerondakis et al. 1999; Ahn et al. 2005; Hoffmann et al. 2006; Wu et al. 2008; O'Dea et al. 2009). However, individual NF-κB members play distinct roles in many of these processes (reviewed in Gilmore et al. 2004) (Table 1.1).

In resting cells, NF-κB proteins are sequestered in the cytoplasm of all types of cells in an inactive form by interaction with inhibitory proteins called IκB (Inhibitor of NF-κB) including IκBα, IκBβ, IκBε, IκBγ, and Bcl3 (Ghosh et al. 1998; Li et al. 2002; Gilmore et al. 2004; Ahn et al. 2005; Hoffmann et al. 2006) (Fig 1.5B), preventing the NF-κB transcription factors from entering the nucleus. However, some studies have demonstrated that NF-κB/IκB complexes shuttle continuously between the nucleus and cytoplasm (Johnson et al. 1999; Huang et al. 2000; Birbach et al. 2002), where the dissociation of NF-κB and IκB occurs in the cytoplasm followed by the separate nuclear entrance of NF-κB/IκB complexes (Carlotti et al. 2000; Birbach et al. 2002). In the nucleus, NF-κB and IκB are reassociated and shuttle back to the cytoplasm, mediated by nuclear export sequences in IκB and NF-κB (Harhaj et al. 1999; Carlotti et al. 2000; Birbach et al. 2002; Verma 2004).

Upon stimulation, IκB proteins are phosphorylated by the IKK complex (the catalytic subunits IKKα (IKK1) and IKKβ (IKK2) and the regulatory subunit IKKγ (NEMO)) (Fig 1.5C), ubiquitylated, and degraded by the proteosome machinery. This degradation generates free NF-κB proteins to enter the nucleus and bind to their cognate binding sites to activate target genes. Constitutive or dysregulated NF-κB activation is associated with serious diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD), multiple sclerosis, AIDS, Alzheimer’s disease, cancer, asthma, diabetes, and atherosclerosis (Li et al. 2002; Ahn et al. 2005; Vallabhapurapu et al. 2009; Niederberger et al. 2010).
1.4.1 The structure of the NF-κB family of transcription factors

The NF-κB family of transcription factors share a structurally conserved common 300-amino acid region known as the Rel homology domain (RHD) (reviewed in Li et al. 2002). This domain is responsible for homo- and heterodimerization, nuclear localization, and sequence specific DNA binding (reviewed in O'Dea et al. 2009; Vallabhapurapu et al. 2009). Based on their C-terminal domain structure, NF-κB proteins can be further classified into two groups. The first group includes c-Rel, RelA, and RelB and contains a transactivation domain (TAD) which strongly activates gene transcription (reviewed in Li et al. 2002; Vallabhapurapu et al. 2009). However, RelB is structurally different from c-Rel and RelA in that it contains a leucine-zipper (LZ) motif at its N-terminus, which is also essential for full gene activation function (Dobrzenski et al. 1993). The second group, consisting of p50 and p52 subunits, lacks the TAD and plays a role as transcriptional repressors (reviewed in Li et al. 2002; Vallabhapurapu et al. 2009). Thus, for positive transcriptional activation, these proteins have to interact with other factors, mainly as heterodimers with RelA (reviewed in Vallabhapurapu et al. 2009). In addition, unlike the other NF-κB family members, the p50 and p52 proteins are derived from precursors, p105 and p100 respectively, which contain multiple ankyrin repeats (ANK) at the C-terminus and function as IκB-like proteins (reviewed in Li et al. 2002). The NF-κB family of transcription factors form 15 different homo- or heterodimers. Nine of them, including RelA/p50, RelA/p52, RelA/c-Rel, RelA/RelA, c-Rel/p50, c-Rel/p52, c-Rel/c-Rel, RelB/p50, and RelB/p52 can bind to DNA and activate transcription. Three dimers, including p50/p50, p50/p52, and p52/p52 can bind to DNA, but do not activate transcription. However, RelA/RelB, RelB/c-Rel, and RelB/RelB have not been shown to bind DNA (reviewed in Hoffmann et al. 2006; O'Dea et al. 2009) (Fig 1.6). The most common form of NF-κB is the p50/p65 heterodimer (Zhong et al. 1998; reviewed in Gerondakis et al. 1999). NF-κB dimers bind to specific DNA binding sites 10 bp in length (5'-GGGRNYYCC-3'), where R indicates an unspecified purine, N means nucleotide, and Y is an unspecified pyrimidine (Kunsch et al. 1992; Ghosh et al. 1998; Magne et al. 2006; Lim et al. 2007). c-Rel and RelA can also bind to similar or related DNA sequences as homo- or heterodimers (Kunsch et al. 1992). These NF-κB dimers require the carboxy-terminal transactivation domain for transcriptional activation (reviewed in Gerondakis et al. 1999; Wu et al. 2008).
Figure 1.5 The NF-κB family of transcription factors

(A) All NF-κB members contain the Rel homology domain (RHD) consisting of three domains for DNA binding, dimerization, and nuclear localization (N). Serine phosphorylation sites of RelA are indicated. The endoproteolytic cleavage sites of p105 (amino acid 435) and p100 (amino acid 405) are indicated by arrows. Sequences of inducible phosphorylation sites (DSVCDS and EVKEDSAYGGS) of p105 and p100 are shown at the C-terminus. IKKa-mediated phosphorylation of p105 and p100 leads to a ubiquitination and cleavage to generate active p50 and p52 subunits respectively.

(B) The conserved ankyrin repeats (ANK) are marked. Phosphorylation and ubiquitination sites on IκB proteins are shown at specific serine and lysine residues.

(C) IKK subunits showing major structural and functional domains. Phosphorylation sites are shown at serine residues. Abbreviations: TD, transcriptional activation domains; LZ = leucine zipper domain; GRR = glycine-rich region; ANK = ankyrin
repeats; HLH = helix–loop–helix domain; NBD = NEMO-binding domain; α = α-helical domain; CC1/2 = coiled-coil domains; Z = zinc finger domain; IKK = IκB kinase.

(Obtained from Li et al. 2002; Hayden et al. 2004)
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB1/−</td>
<td>Defects in production of Ab and T-cell proliferative responses. Absence of marginal zone B cells. Defect in Th2 responses. Increased susceptibility to <em>S. pneumoniae</em> and <em>L. monocytogenes</em>. Normal response to <em>E. coli</em> infection and <em>H. influenzae</em> but enhanced resistance to EMCV.</td>
</tr>
<tr>
<td>NF-κB2/−</td>
<td>Disorganized B- and T-cell areas in spleen and lymph nodes associated with absence of marginal zone macrophages and follicular DC. Reduced numbers of B cells and decreased production of antigen-specific Ab. Increased susceptibility to <em>T. gondii</em>, <em>L. monocytogenes</em>, and <em>L. major</em> but normal response to LCMV.</td>
</tr>
<tr>
<td>NF-κB2/−, NF-κB2/−</td>
<td>Increased mortality after birth and developmental defects including osteopetrosis, thymic and lymph node atrophy, and disorganized splenic structure.</td>
</tr>
<tr>
<td>RelA/−</td>
<td>Embryonic lethality at day 15 to 16 of gestation due to widespread apoptosis of liver parenchymal cells mediated by TNF. Required for formation of secondary lymphoid organs.</td>
</tr>
<tr>
<td>RelB/−</td>
<td>Development of lethal T-cell-mediated inflammatory disease. Impaired production of antigen-specific Ab associated with defects in germinal center formation. Lack of marginal zone B cells and thymic and CD8α- DC. Susceptible to <em>L. monocytogenes</em>, LCMV, and <em>T. gondii</em>. Reduced capacity to produce IFN-γ and impaired DTH responses.</td>
</tr>
<tr>
<td>c-Rel/−</td>
<td>Impaired T- and B-cell proliferation and reduced Ab responses. Increased susceptibility to <em>L. major</em> and <em>T. gondii</em>. Memory response to influenza virus is impaired. Decreased production of IL-2, IL-3, IL-12, IFN-γ, and GM-CSF.</td>
</tr>
</tbody>
</table>
Figure 1.6 Dimerization of NF-κB family proteins

Five NF-κB family subunits can form 15 dimers through homo- and heterodimerization. Nine dimers can bind to DNA and act as transcriptional activators (light gray). Three dimers lacking the TAD only bind to DNA without activating gene transcription (medium gray). The remaining three dimers indicated with dark gray are not able to bind to DNA.

(Obtained from O’Dea et al. 2009)
1.4.2 The NF-κB signalling pathways

Because of the importance of the NF-κB transcription factors in the immune system and human diseases, the role of NF-κB proteins together with their distinct signalling pathways has long been studied. NF-κB transcription factors are activated through distinct NF-κB signalling pathways known as classical (canonical) and alternative (non-canonical) signalling pathways (reviewed in Bonizzi et al. 2004; Karin et al. 2005) (Fig 1.7). These pathways perform different roles in the immune system (reviewed in Bonizzi et al. 2004; Hoffmann et al. 2006). The classical NF-κB pathway is essential for regulating innate immunity and inflammation as well as for survival and proliferation of lymphoid cells (B and T cells), while the alternative pathway plays a pivotal role in lymphoid organ development, the adaptive immune system, and dendritic cell (DC) development (reviewed in Bonizzi et al. 2004; Platzer et al. 2004; Hoffmann et al. 2006; Demchenko et al. 2010). In addition, while the classical pathway responds rapidly and is reversible, the alternative pathway is a much slower process than activation of the canonical pathway (reviewed in Dejardin 2006; Hoffmann et al. 2006). Through these pathways, many genes involved in innate and adaptive immune responses and inflammation are regulated by NF-κB (reviewed in Ghosh et al. 1998; Bonizzi et al. 2004; Hayden et al. 2004; Karin et al. 2005; Hoffmann et al. 2006; O’Dea et al. 2009).

1.4.2.1 Classical NF-κB signalling pathway

The classical NF-κB signalling pathway is also called the IKKβ-dependent NF-κB signalling pathway (Fig 1.7A). This pathway is triggered by pro-inflammatory stimuli such as cytokines (tumour-necrosis factor (TNF) and IL-1), lipopolysaccharide (LPS), viruses, Toll-like receptors (TLRs), and antigen receptors (reviewed in Bonizzi et al. 2004; Karin et al. 2005; Hoffmann et al. 2006). This leads to multiple signalling cascades which activate the IKK complex (e.g., MAP/ERK kinase kinase 3 (MEKK3)-mediated activation of the IKK complex) (reviewed in Li et al. 2002; Hayden et al. 2006; Vallabhapurapu et al. 2009).

Upon stimulation, inhibitory protein complexes (IκB proteins) (Fig 1.5B) are phosphorylated at two specific serine sites located at the N-terminus by the activated IKK complex (Fig 1.5C). This phosphorylation triggers polyubiquitination and subsequent proteasomal degradation of IκB inhibitory proteins (reviewed in Bonizzi et al. 2004; Hayden et al. 2004; Hoffmann et al. 2006; Demchenko et al. 2010). As a
consequence, the free NF-κB passes into the nucleus where it binds to its target sites to activate target genes encoding cytokines, chemokines, and enzymes associated with inflammation and innate immunity. This pathway is terminated by IKKα subunit which phosphorylates NF-κB proteins such as c-Rel and RelA. This results in the proteasomal degradation of NF-κB proteins followed by the inhibition of NF-κB-dependent gene activation (reviewed in Karin et al. 2005; Vallabhapurapu et al. 2009). This pathway is very important for innate immunity and multiple innate immune genes are expressed in response to this classical pathway (reviewed in Bonizzi et al. 2004; Karin et al. 2005).

1.4.2.2 Alternative NF-κB signalling pathway

Unlike the classical NF-κB signalling pathway, the alternative NF-κB signalling pathway is IKKβ and IKKγ-independent pathway and IκB does not play a role in this pathway (reviewed in Bonizzi et al. 2004; Karin et al. 2005; Hoffmann et al. 2006; Demchenko et al. 2010). Instead, this pathway is an IKKα homodimer-dependent NF-κB signalling pathway (Fig 1.7B). Compared with the classical signalling pathway, the alternative NF-κB signalling pathway was more recently documented (reviewed in Bonizzi et al. 2004; Brown et al. 2008). The alternative pathway is triggered in response to the TNF family (BAFF), lymphotoxin β signalling, CD40, and CD30 (reviewed in Bonizzi et al. 2004; Karin et al. 2005; Demchenko et al. 2010). In resting cells, p100 (NF-κB2/p52) sequesters other NF-κB proteins such as RelB in the cytoplasm (reviewed in Hoffmann et al. 2006). When IKKα homodimes are activated by NF-κB-inducing kinase (NIK), NF-κB2/p100 is phosphorylated by activated IKKα at the C-terminal and ubiquitinated. This results in the proteasomal degradation of p100 to active p52. As a consequence, p52/RelB dimers accumulate in the nucleus, leading to transcriptional activation of target genes encoding proteins involved in adaptive immunity (reviewed in Bonizzi et al. 2004; Karin et al. 2005; Demchenko et al. 2010). This pathway is also very important for the development of lymphoid organs. Unlike the classical pathway, the alternative NF-κB pathway is slowly processed and results in long-lasting nuclear NF-κB activity (reviewed in Hoffmann et al. 2006; Demchenko et al. 2010).
Figure 1.7 The NF-κB signalling pathways

NF-κB proteins are activated by two different pathways. Two signalling pathways are initiated when cells are activated by a wide variety of stimuli. (A) The classical pathway requires IKK complex consisting of IKKα, IKKβ, and IKKγ (also known as NEMO) which leads to phosphorylation of IkB inhibitors, resulting in their ubiquitination and subsequent proteasome degradation. A number of genes involved in innate response, inflammation, and cell survival are regulated by activated NF-κB proteins.

(B) The alternative pathway is dependent on IKKα homodimers which phosphorylate p100, resulting in degradation of p100 to p52 and nuclear translocation of p52/RelB dimmers. Many genes related to adaptive and lymphoid organ development are activated by this pathway. Abbreviations: GM-CSF = granulocyte-macrophage colony stimulating factor; iNOS = inducible nitric oxide synthase; COX-2 = cyclooxygenase 2;
VCAM-1 = vascular cell adhesion molecule-1; ICAM-1 = intercellular adhesion molecule 1; MCP-1 = monocyte chemotactic protein-1; MIP-1α = macrophage inflammatory protein-1α; RANTES = regulated upon activation, normal T cell expressed and secreted; PLA2 = phospholipase A2; BAFF = B-cell-activating factor belonging to the TNF family; BLC = B-lymphocyte chemoattractant; ELC = Epstein–Barr virus-induced molecule 1 ligand CC chemokine; SLC = secondary lymphoid tissue chemokine; SDF-1 = Super Dimensional Fortress One; RXRA = Retinoid X receptor alpha; PNAd = peripheral node addressins; GlyCAM-1 = glycosylation-dependent cell adhesion molecule-1; IKK = IκB kinase p = phosphorylation.

(Obtained from Bonizzi et al. 2004; Karin et al. 2005)
1.4.3 NF-κB in T cell development

1.4.3.1 The development of T lymphocytes in the thymus

T lymphocytes are derived from lymphoid progenitors in the bone marrow (primary lymphoid organ) and move to the thymus (primary lymphoid organ) through the blood stream (Fabbri et al. 2003; Skapenko et al. 2005; reviewed in Vallabhupurapu et al. 2009). In the thymus, T lymphocytes undergo negative and positive selection steps. During this process, immature CD4<sup>−</sup>CD8<sup>−</sup> (double-negative, DN) thymocytes progress to the CD4<sup>+</sup>CD8<sup>+</sup> (double-positive, DP) thymocytes. These thymocytes differentiate to mature single positive (CD4<sup>+</sup> or CD8<sup>+</sup>) thymocytes (Rincon et al. 1996; Outram et al. 2000) (Fig 1.8A). During this maturation process, only a small percentage of thymocytes (approximately 2%) survive (reviewed in Skapenko et al. 2005). These mature T cells enter the secondary lymphoid organs (lymph node, spleen, and Peyer's patches) through the blood stream (Bonilla et al. 2010), where they are activated by recognizing peptide antigen associated with the major histocompatibility complex (MHC) class I or class II on the antigen presenting cells (APCs) (Rincon et al. 1996).

1.4.3.2 Development of T cells into different T cell subsets

Upon stimulation, naïve T cells are differentiated into T cell subpopulations such as Th helper (Th1 and Th2), Th17, and regulatory T (Treg) cells (Brand 2009; Yi et al. 2009; reviewed in Maniati et al. 2010) (Fig 1.8B). The type of Th cell is dependent on the type of initial stimulus, which then dictates the spectrum of cytokines produced, thus reinforcing differentiation.

CD4<sup>+</sup> T cells are differentiated into Th1 cells producing IL-2, IFN-γ, and TNF-β in response to IL-12. These Th1 cytokines activate macrophages which are important for host defence and fighting against pathogens such as bacteria, resulting in cell-mediated immunity, intracellular defence, and autoimmunity (Cohen et al. 1998; reviewed in O'Garra et al. 2000). On the other hand, Th2 cells producing IL-4, IL-5, and IL-13 under IL-4 inhibit macrophage activity. However, these Th2 cytokines promote B cell proliferation and differentiation into antibody producing plasma cells and induce the isotype switching of B cells from IgM to IgE antibody during a humoral immune response (Cohen et al. 1998; reviewed in O'Garra et al. 2000; Lienhardt et al. 2002; Deo
et al. 2010). Thus, Th1 and Th2 cells play distinct functions by producing different cytokines in the immune system.

Treg cells (CD4⁺CD25⁺Foxp3+regulatory T cells) from CD4⁺ T cells express IL-2Ra (CD25) and transcription factors Foxp3 (foxhead box P3) which is involved in Treg cell development in the thymus (Scheffold et al. 2007; Hogquist et al. 2009; reviewed in Rochman et al. 2009). In response to IL-2 and TGF-β, Treg cells produce three inhibitory cytokines including TGF-β, IL-10, and IL-35 which suppress T cell (Th1) activation, proliferation, and differentiation (Scheffold et al. 2007; Brand 2009; Hogquist et al. 2009; Rochman et al. 2009; reviewed in Maniati et al. 2010). In addition, Treg cells are important for the maintenance of immune cell homeostasis, which is dependent on IL-2 in the peripheral immune tissue (Gupta 2008; reviewed in Malek 2008).

Th17 is a recently discovered subset of CD4⁺ Th cells. TGF-β and IL-6 mediate a differentiation of naive CD4⁺ T cells to Th17 cells which secrete IL-17, IL-21, and IL-22 (reviewed in Maniati et al. 2010). In mice, TGF-β and IL-6 are involved in Th17 cell differentiation, however, in human, the combination of TGF-β with IL-1β, IL-6, IL-21, or IL-23 is associated with Th17 differentiation, suggesting that Th17 cells are developed differently in humans and mice (Acosta-Rodriguez et al. 2007; Evans et al. 2007; van Beelen et al. 2007; Wilson et al. 2007; reviewed in Romagnani 2008). Th17-produced cytokines are concerned with diverse human inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, and asthma (reviewed in Tesmer et al. 2008). Thus, the development of Th cell subsets is orchestrated by different cytokines produced from activated CD4⁺ T cells and cytokines produced from each Th cell play distinct and diverse roles in the immune system.
Figure 1.8 T cell development in primary and secondary organs

(A) T lymphocytes are derived from lymphoid progenitor in the bone marrow (primary lymphoid organ) and migrate to and mature in the thymus (primary lymphoid organ) where T thymocytes are subjected to negative and positive selective steps. Immature CD4⁻CD8⁻ (double-negative, DN) thymocytes progress to the CD4⁺CD8⁺ (double-positive, DP) thymocytes. These thymocytes differentiate into mature CD4⁺ or CD8⁺ (single positive, SP) T cells.

(B) Mature naïve T cells then move to the secondary lymphoid organs (lymph node and spleen), where they are further differentiated into T helper cell subsets (Th1, Th2, Treg, and Th17) under different cytokine conditions. Each Th subset secretes distinct cytokines involved in the immune system. (Obtained from Maniati et al. 2010)
1.4.3.3 The role of NF-κB in T cell development

Many studies investigating the role of NF-κB proteins in T cell development have been performed in knockout and transgenic mouse models. These studies revealed that NF-κB signalling is crucial for the positive and negative selection during thymic T cell development, as the inhibition of the NF-κB pathway by a super-repressor IκB (IκBα(ΔN)) in T cells impaired thymic selection (Mora et al. 2001). However, an interesting study by Jimi et al. showed that NF-κB activation contributes differently to thymic selection (positive/negative) in CD4 and CD8 thymocytes (Jimi et al. 2008), in that NF-κB activation plays a crucial role for thymic selection of CD8 cells, but not for CD4 T cells. More interestingly, NF-κB family subunits play a role at different stages during T cell development (reviewed in Vallabhapurapu et al. 2009). For example, RelB is considered to play a role in the stage of negative selection in the thymus, as the absence of RelB affects the development of antigen-presenting DCs which are involved in this negative selection process (Burkly et al. 1995; reviewed in Barton et al. 2000; Li et al. 2002; Hayden et al. 2006; Vallabhapurapu et al. 2009). In addition, a study using double-mutant mice (p50-/− RelB-/−) showed that that the number of double positive (CD4+CD8+) thymocytes is decreased in double mutation (Weih et al. 1997; reviewed in Liou et al. 2003). However, this double knockout does not block T cell differentiation, thus mature single positive CD4+ and CD8+ thymocytes are detected in the peripheral lymphoid organs (Weih et al. 1997). Although RelB-/− mice display a defect in the hematopoietic system, the absence of RelB is compensated by other NF-κB complexes containing the p50 subunit. An interesting study by Hettmann et al. reported that NF-κB is required for the positive selection, but not the negative selection of CD8+ thymocytes (Hettmann et al. 2000). Another interesting study has been performed in c-rel/RelA double mutants where the number of peripheral CD4+ and CD8+ T cells is significantly decreased (Grossmann et al. 2000; reviewed in Liou et al. 2003). However, the absence of both factors did not display a defect in T cell development (Kontgen et al. 1995; reviewed in Gerondakis et al. 1999; Liou et al. 1999; reviewed in Liou et al. 2003). On the other hand, a recent study demonstrated that Treg development in the thymus is affected by c-Rel-/− alone (Isomura et al. 2009). Further studies revealed that the absence of p50 also does not affect T cell development in mice (Weih et al. 1997; Hilliard et al. 2002; reviewed in Liou et al. 2003). Thus, these results suggested that although the NF-κB family plays a role in thymocyte development and selection, most T cell development is not impaired by the loss of individual NF-κB members,
suggesting that the loss of single individual factors may be compensated by other factors (Boothby et al. 1997; Weih et al. 1997; Hilliard et al. 2002; reviewed in Li et al. 2002; Liou et al. 2003; Vallabhapurapu et al. 2009).

1.4.4 Role of the NF-κB in immune responses (innate and adaptive immune responses) and inflammation

NF-κB proteins play an essential role in the immune system by regulating genes involved in diverse cellular processes including innate and adaptive immune responses, and inflammation (reviewed in Bonizzi et al. 2004; Hoffmann et al. 2006; O'Dea et al. 2009). As mentioned previously, NF-κB is activated by a wide variety of different factors such as proinflammatory cytokines, oxidant free radicals, antigens, or viral and bacterial products (reviewed in Barnes et al. 1997; Bonizzi et al. 2004; Ahn et al. 2005; Karin et al. 2005). The transcription of a large number of target genes encoding cytokines, chemokines, adhesion molecules, and enzymes is activated by the NF-κB family (Fig 1.7) (reviewed in Barnes et al. 1997; Tak et al. 2001; Bonizzi et al. 2004; Karin et al. 2005). These immune mediators play important roles in inflammation and the innate and adaptive immune responses (reviewed in Barnes et al. 1997; Bonizzi et al. 2004).

1.4.4.1 Innate immune response

The classical NF-κB signalling pathway is important for innate immunity. Through this pathway, the NF-κB family regulates genes involved in antimicrobial defence in innate immunity, which is the first host defence immune system and launches an immediate response to invading microorganisms (Wright et al. 2001; reviewed in Li et al. 2002; Hayden et al. 2006; Hoffmann et al. 2006; Nakamura et al. 2007; Phalipon et al. 2007). Thus, the innate immunity is important as an antigen-nonspecific defence mechanism prior to the development of an adaptive immunity (reviewed in Caamano et al. 2002). The NF-κB family is activated by many invaders such as viruses, bacteria, and bacterial products including Mycobacterium tuberculosis, Neisseria gonorrhea, LPS, and Shiga toxin in macrophages and other cell types (Geng et al. 1993; Louise et al. 1997; Naumann et al. 1997; Toossi et al. 1997; reviewed in Caamano et al. 2002; Bonizzi et al. 2004). In this case, many genes encoding chemokines (IL-8), cytokines (interleukin-1
(IL-1), interferon-α (INF-α), and IL-12, and adhesion molecules are regulated by the NF-κB family (reviewed in Caamano et al. 2002; Karin et al. 2005). These immune molecules play an essential role in innate immunity by recruiting inflammatory and phagocytic cells to areas of infection and by activating these cells to kill bacteria (reviewed in Caamano et al. 2002; Bonizzi et al. 2004). The activation of NF-κB by enteroinvasive bacteria also activates several inflammatory mediators such as ICAM-1, IL-8, and TNF-α in human intestinal epithelial cells (Elewaut et al. 1999). In addition to responding to bacteria, NF-κB is activated by the protozoan parasite Trypanosoma cruzi, which induces the production of adhesion molecules in endothelial cells (Huang et al. 1999). It has also been reported that NF-κB is activated by IL-1 released by infection of pulmonary epithelial cells with Mycobacterium tuberculosis (reviewed in Caamano et al. 2002; Bonizzi et al. 2004).

Toll-like receptors (TLRs) also play an important role in the innate immune response by signalling to the NF-κB pathway. TLRs contain N-terminal leucine-rich repeat (LRR) domains which are responsible for recognizing microbial components, and C-terminal intracellular Toll/IL-1 receptor (TIR) domains which recruit TIR domain-containing adaptor proteins to the cytoplasmic domain of TLRs. Thus, the interaction between TLRs and adaptor proteins generates TLR signalling pathways which induce downstream signalling cascades, leading to the activation of canonical NF-κB signalling pathways (reviewed in Takeda et al. 2004; Takeda et al. 2005; Hayden et al. 2006; Kawai et al. 2007; Radhakrishnan et al. 2009).

1.4.4.2 Adaptive immune response

The adaptive immune system is antigen specific and comprised of B and T lymphocytes. The adaptive immune response is initiated by the interaction between APCs and T lymphocytes in the presence of pathogen (reviewed in Wright et al. 2001; Hayden et al. 2006; Bonilla et al. 2010). During infection, a DC captures an antigen in the periphery and enters the secondary lymphoid organs where naïve T lymphocytes recognize the antigen presented by the DC (Faulhaber et al. 2007). This results in T cell activation, proliferation, and differentiation into Th cells. These activated Th cells secrete cytokines that lead to proliferation of B lymphocyte and the differentiation of these cells into antibody-producing plasma cells (Wright et al. 2001; Faulhaber et al. 2007). Unlike the innate immunity which is a rapid response, the adaptive immune response develops slowly, but allows the formation of encountered memory of each pathogen and confers
long-lasting protection against the same pathogen. Thus, the adaptive immunity quickly responds to subsequent infection by the same pathogen (Wright et al. 2001; Lee et al. 2002; reviewed in Li et al. 2002; Hayden et al. 2006; Hahn et al. 2007; Bonilla et al. 2010).

1.4.4.3 Initiation of T cell activation through T cell receptor and CD28 costimulation

During an adaptive immune response, T lymphocytes require two signals to be fully activated (reviewed in Abbas et al. 2000; Wright et al. 2001; Lee et al. 2002; Bonilla et al. 2010) (Fig 1.9). One signal is provided by the engagement of T-cell receptor (TCR) with MHC class II/antigen complexes on APCs. However, T cell activation generated by the TCR alone is insufficient for optimal T cell activation, leading to either no response or clonal anergy (reviewed in Abbas et al. 2000). Thus, secondary signals are required which are provided by the interaction of the CD28 co-receptor expressed by CD4+ T cells with costimulatory molecules (B7-1 (CD80) and B7-2 (CD86)) expressed by the APCs (Bryan et al. 1994; reviewed in Abbas et al. 2000; Beyersdorf et al. 2005; Schulze-Luehrmann et al. 2006). These signals induce a series of intracellular signalling cascades in T cells, which lead to activation of a large number of transcription factors such as NF-κB that are involved in activating genes for a variety of intracellular processes.

1.4.4.4 NF-κB activation by TCR

During the adaptive immune response, the activation of NF-κB is very important as it induces cytokine gene expression, proliferation, and clonal expansion by regulating a number of genes (Himes et al. 1996; Appleman et al. 2002; Lee et al. 2005; reviewed in Hayden et al. 2006). NF-κB is activated by signals derived from antigen-specific T-cell receptors (TCR) (reviewed in Hayden et al. 2006; Rawlings et al. 2006) (Fig 1.10). This signalling pathway leads to the recruitment of tyrosine kinases ZAP-70 to the TCR receptor complex (reviewed in Hayden et al. 2006). This kinase activates downstream molecules such as phosphoinositide 3-kinase (PI3K) (Fruman et al. 2002). In T cells, activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3) and results in the recruitment of the phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane (Chalhoub et al.
2009; David 2010), which is the upstream kinase for protein kinase Cθ (PKCθ). PDK1-associated PKCθ recruits IKK complex (Lee et al. 2005). As described above (Section 1.4.2.1), activated IKK complex phosphorylates NF-κB inhibitory proteins, resulting in nuclear translocation of NF-κB followed by gene transcription.
T cells require two distinct signals to be fully activated. When antigen-presenting cells (APCs) present an antigen coupled with the major histocompatibility complex (MHC) to T cells, the T-cell receptor (TCR) on T cells recognizes the antigen/MHC complex. A secondary signal is provided when the CD28 co-receptor on T cells interacts with costimulatory molecules (B7-1 (CD80) and B7-2 (CD86)) expressed by the APCs.
Figure 1.10 NF-κB activation through TCR in T cells

Signalling through antigen-specific T-cell receptors (TCR) is an important event for the adaptive immune response. This activation through TCR leads to multiple signalling cascades, which activate NF-κB transcription factors. The activation of NF-κB facilitates cytokine gene expression, differentiation, and proliferation. Abbreviations: TCR = T-cell receptor; ZAP70 = Zeta-chain-associated protein kinase 70; PI3K = phosphoinositide 3-kinase; PDK1 = Phosphoinositide-dependent kinase-1; PKCθ = Protein kinase θ; IKK = IκB kinase; NEMO = NF-κB essential modifier (known as IKKγ); Ag = antigen; \( \oplus \) = phosphorylation.
1.4.4.5 The role of NF-κB in the adaptive immunity

The importance of NF-κB in the adaptive immune response has been determined using transgenic mice (Boothby et al. 1997; Ferreira et al. 1999; Das et al. 2001; Mora et al. 2001; reviewed in Caamano et al. 2002; Hilliard et al. 2002). These studies have demonstrated that individual NF-κB members play an important role in the development of adaptive immunity by regulating genes involved in differentiation, activation, survival, B and T cell proliferation, cytokine production, development, and isotype switching (class switching) in B cells (reviewed in Gomez et al. 1997; Caamano et al. 2002; Li et al. 2002; Ahn et al. 2005; Dejardin 2006; Hayden et al. 2006; Phalipon et al. 2007; Schmid et al. 2008). For example, the NF-κB family is involved in the regulation of the expression of costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) (reviewed in Li et al. 2002; Phalipon et al. 2007). Also, the inhibition of NF-κB affected the expression of MHC class II and costimulatory molecules (B7) (Rescigno et al. 1998). A study by Quaaz et al. demonstrated that RelA is required for expression of genes involved in the adaptive immune response such as MHC class I and CD4 ligand (Quaaz et al. 1999). In addition, the differentiation of T helper cells (Th cells) is controlled by NF-κB where IL-18 and IFN-γ that are essential for Th1 cell differentiation are regulated by NF-κB (Kojima et al. 1999; Hilliard et al. 2002; reviewed in Li et al. 2002; Phalipon et al. 2007). In support of this, Balasubramani et al. showed the importance of RelA in the regulation of the interferon-gamma (Ifng) gene in Th1 cells, where induction of Ifng gene was impaired when RelA is deficient in response to TCR or IL-12/IL-18 activation (Balasubramani et al. 2010). c-Rel is an essential factor for Th1 cell differentiation while Th2 cell differentiation is regulated by NF-κB1 (p50) (Das et al 2001; Hilliard et al. 2002). Another similar study reported that RelB is also involved in Th1 differentiation (Corn et al. 2005). Furthermore, the absence of c-Rel causes a defect in T cell proliferation and IL-2 gene expression (Kontgen et al. 1995).

In B cells, the role of NF-κB has also been investigated using knockout mice. These studies showed that RelA is involved in class switching and NF-κB1 is required for B cell proliferation. Greater defects in proliferation and class switching were observed in p50/RelA double knockout mice. In addition, it has been reported that the lack of NF-κB1 and NF-κB2 affected B cell maturation, suggesting that NF-κB is required for the development of B cells. An interesting study showed that c-Rel-deficient mice have a defect in humoral immune response that triggers B cells to proliferate and secrete antibodies to fight against invaders, suggesting that c-Rel plays an important role in
class switching (reviewed in Hayden et al. 2006). Together, these studies showed that the NF-κB transcription factors play essential roles in B and T cells during the adaptive immune responses.

1.4.4.6 Inflammatory response

The NF-κB family plays an important role in inflammatory responses. The activation of NF-κB increases the expression of many inflammatory genes encoding adhesion molecules, chemokines (IL-8), cytokines (IL-1 and IL-6, and TNF-α), and enzymes (COX-2 and iNOS) (reviewed in Tak et al. 2001; Li et al. 2002; Bonizzi et al. 2004; Barnes et al. 2005). These proteins activate and recruit inflammatory cells to the sites of inflammation where they are amplified, leading to inflammatory states (reviewed in Barnes et al. 1997; Bonizzi et al. 2004; Barnes et al. 2005). Therefore, overactivation or dysregulation of NF-κB is associated with diverse inflammatory diseases including rheumatoid arthritis, MS (Multiple sclerosis), psoriasis, asthma, and IBD (Hart et al. 1998; Neurath et al. 1998; reviewed in Li et al. 2002; Ahn et al. 2005). For example, Yang et al. reported that p50-deficient mice could not develop an eosinophilic airway inflammation. Furthermore, in p50-/- mice, the expression of chemokine genes (MIP-1α and MIP-β), which are required for T cell recruitment to inflammation sites, was impaired (Yang et al. 1998). Another pathogenic effect by NF-κB has been reported in c-Rel-/- mice where allergic pulmonary inflammation and airway hyperresponsiveness have been impaired (Donovan et al. 1999). In addition, the overexpression of IκB by blocking NF-κB activity reduced inflammatory response and tissue destruction in rheumatoid synivium (Bondeson et al. 1999). Thus, these studies showed that there is an interaction between the highly activated NF-κB and inflammatory diseases.

1.4.4.7 The role of RelA in the immune system

RelA, another member of NF-κB family, is ubiquitously expressed during the NF-κB activation and can act as a strong activator or repressor for gene expression by recruiting coactivators or corepressors. RelA forms a heterodimer with p50 which is the most abundant form of NF-κB. Furthermore, RelA plays an important role in the immune system as well as in differentiation and proliferation of B and T cells.
RelA plays an important role in the immune response by regulating genes involved in the adaptive immune responses. For example, the activation of H-2 gene, which encodes MHC class I, was inhibited in RelA-deficient mouse embryonic fibroblasts (MEFs) in response to TNF-α and LPS. In addition, the expression of CD40, which is expressed on APCs, was impaired in RelA/-/- in MEFs (Ouaaz et al. 1999; reviewed in Caamano et al. 2002). It has been reported that B cells lacking RelA can proliferate in response to stimulation, but had a defect in isotype (class) switching to IgG3 caused by a decrease of C_H2Y3 (Horwitz et al. 1999). Furthermore, the absence of RelA impaired the expression of chemokine genes such as KC and MIP-2 which are involved in the innate immune response (Ouaaz et al. 1999; reviewed in Caamano et al. 2002). Similarly, it has been reported that RelA is involved in the initiation of the innate immune response.

This study showed that RelA-deficient mice have a defect in the expression of adhesion molecule and chemokine genes such as ICAM-1, KC and MIP-2 (Alcamo et al. 2001). A study by Neurath et al. showed that RelA is associated with chronic inflammatory diseases, showing that deregulated RelA activity is accompanied by increased proinflammatory cytokines such as IL-1 and IL-6 which leads to chronic intestinal inflammation (Neurath et al. 1996; reviewed in Barnes et al. 1997; Weigmann et al. 2009). Thus, like c-Rel, RelA also plays an essential role in the immune system in both B and T cells.

1.4.5 The role of c-Rel in T lymphocytes

c-Rel, a member of the NF-κB transcription factors family, is a protooncogene and the cellular counterpart of the v-Rel oncogene (reviewed in Liou et al. 2003). c-Rel plays a role in both T cell differentiation and activation. This distinct function of c-Rel is not compensated by other NF-κB members (reviewed in Liou et al. 2003; Tian et al. 2009). c-Rel functions as c-Rel/c-Rel homodimers or p50/c-Rel and RelA/c-Rel heterodimers (Lai et al. 1995; Martone et al. 2003; reviewed in Gilmore et al. 2004). These dimers bind to short κB sites (~10 bp) and induce expression of a large number of genes involved in lymphoid cell development, proliferation, and survival (Gilmore et al. 2004; Wei et al. 2008; Tian et al. 2009). In T cells, c-Rel also regulates many genes encoding cytokines and plays an important role in chromatin remodeling and histone loss (Rao et al. 2001; reviewed in Liou et al. 2003; Rao et al. 2003; Brettinham-Moore et al. 2005; Chen et al. 2005; Tian et al. 2009).
1.4.5.1 c-Rel in T cell differentiation

As mentioned previously, in the thymus, T lymphocytes pass through negative and positive selection steps to become mature T cells and then enter the peripheral lymphoid organs where they further develop into Th subsets under different conditions. c-Rel has been shown to be involved in Th1 differentiation, but not Th2 differentiation. c-Rel plays an important role in Th1 cell development by regulating the gene encoding IL-12, which consists of IL-12p35 and IL-12p40 subunits and plays an important role in Th1 cell differentiation (reviewed in Sanjabi et al. 2000; Grumont et al. 2001; Hilliard et al. 2002; Liou et al. 2003; Deng et al. 2010). Thus, c-Rel-deficient mice are impaired in producing IL-12, which results in a defect in Th1 development followed by an inhibition of production of Th1 cytokines (Hilliard et al. 2002). Interestingly, a study by Sanjabi et al. reported that IL-12p40 production was inhibited in c-Rel/-/- macrophages, however, the production of cytokines such as IL-6, IL-1â, and TNF-â was not affected (Sanjabi et al. 2000). In addition, it has been reported that c-Rel inhibited the expression of the IL-12p35, but not IL-12p40 in CD8â DCs (Grumont et al. 2001). These results suggested that c-Rel is a selective regulator of IL-12 and that c-Rel function is cell type dependent. c-Rel also plays a role in Th1 development by regulating IFN-â cytokine production that dominates Th1 differentiation. Deficiency of c-Rel leads to a defect in the production of IFN-â, which results in an increase of Th2 cytokines such as IL-4 because of the cross regulation of Th1 and Th2 differentiation (Hilliard et al. 2002). Thus, c-Rel selectively regulates Th1 differentiation by controlling the expression of both IL-12 and IFN-â (Hilliard et al. 2002; reviewed in Hayden et al. 2006). However, an interesting study performed under Th1-polarizing conditions showed that RelB is required for Th1 differentiation, but not c-Rel where Th1 differentiation and IFN-â production were dramatically defected in RelB/-/- T cells (Corn et al. 2005). However, no consistent defect in Th1 differentiation was observed in c-Rel/-/- T cells, suggesting that the role of c-Rel may be condition dependent in T cell differentiation. Furthermore, in c-Rel/-/- T cells, CD8â T cells failed to differentiate into cytotoxic T cells, though c-Rel deficiency in CD8â T cells is able to be restored by the addition of IL-2 cytokine (Liou et al. 1999). A recent study demonstrated that c-Rel is involved in the development of Treg cells, showing that the number of thymic Treg cells was reduced in c-Rel/-/- mice. In addition, it showed that the reduced population of the peripheral Treg cells in c-Rel/-/- mice is caused by reduced homeostatic proliferation of Treg cells (Isomura et al. 2009).
1.4.5.2 Roles of c-Rel in T cell activation and in cytokine and cytokine receptor gene expression in T cells

T cells are activated through the TCR and CD28 costimulatory molecules and c-Rel responds to signals derived from TCR/CD28 stimulation (Liou et al. 1999). During T cell activation, c-Rel is targeted by CD28-mediated signalling which accelerates activation and nuclear translocation of c-Rel (Bryan et al. 1994; Zhou et al. 2002). This is accompanied by the binding of c-Rel to the promoters containing the CD28 response element (CD28RE) of multiple lymphokines such as interleukin-2 (IL-2), interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon-gamma (IFN-γ) (Bryan et al. 1994; Gerondakis et al. 1996; Liou et al. 1999; Hilliard et al. 2002; Zhou et al. 2002). The binding of c-Rel through CD28 signalling to the CD28RE enhances the production of these cytokines, all of which play an essential role for differentiation and proliferation in T cells (Kontgen et al. 1995; Liou et al. 1999; Hilliard et al. 2002).

c-Rel also plays an important role in the regulation of cytokine and cytokine receptor genes. The role of c-Rel in this event has been studied through c-Rel knockout mouse model (Gerondakis et al. 1996; Liou et al. 1999; Hilliard et al. 2002). A previous study demonstrated that c-Rel is involved in IL-2 gene expression, where the absence of c-Rel significantly decreased IL-2 mRNA expression level following CD3/CD28 stimulation in CD4+ T cells (Rao et al. 2003). In addition, impaired IL-2 production has been observed in CD3 stimulated c-Rel/-/- T cells (Liou et al. 1999). This decrease has also been observed in other cytokine proteins such as IL-3, GM-CSF, and INF-γ where these cytokine proteins were significantly reduced in c-Rel/-/- CD4+ T cells following CD3/CD28 stimulation (Gerondakis et al. 1996; Hilliard et al. 2002). c-Rel is also involved in the interleukin-2 receptor α-chain (IL-2Ra) activation, because a defect in IL-2 gene expression in c-Rel/-/- T cells indirectly impaired the IL-2Ra gene expression following CD3 stimulation. These results suggest that c-Rel is an essential factor for cytokine and cytokine receptor gene expression in T cells and the c-Rel-deficient T cells have a defect in responding to TCR or TCR/CD28 signals. This deficiency of c-Rel cannot be recovered by other NF-κB members, including RelA and p50 responding to T cell signals (Liou et al. 1999).
1.4.5.3 c-Rel is essential for chromatin remodeling and histone loss at the inducible cytokine genes

As previously described, genomic DNA is packaged into higher order chromatin structure. This structure prevents transcription factors from accessing transcription factor binding sites on the promoter, resulting in the inhibition of transcription. Thus, chromatin needs to be remodeled at the promoters of target genes in order for transcription to occur. It has been reported that many factors are involved in alteration of eukaryotic chromatin structure including proteins involved in histone modifications, ATP-dependent chromatin remodeling complexes, histone chaperones, and histone variants (reviewed in Lee et al. 2000; Flaus et al. 2001; Hassan et al. 2001; Turner 2002; reviewed in Clayton et al. 2003; Lusser et al. 2003; reviewed in Nowak et al. 2004; reviewed in Jiang et al. 2009).

In T cells, IL-2 and GM-CSF genes have been used as models to study chromatin remodeling. Chromatin remodeling has been primarily investigated by measuring accessibilities using micrococcal nuclease (MNase), restriction enzymes, DNase I following stimulation at the IL-2 and GM-CSF promoter regions (Rao et al. 2001; Holloway et al. 2003; Rao et al. 2003; Brettingham-Moore et al. 2005). These previous studies revealed that chromatin remodeling following T cell activation is limited to the proximal promoter regions of the IL-2 and GM-CSF genes, and c-Rel plays an essential role in alteration of chromatin structure (Holloway et al. 2003; Rao et al. 2003). Unlike c-Rel, other transcription factors that bind to the promoters of IL-2 and GM-CSF genes such as RelA and NFAT did not appear to alter chromatin structure in T cells (Rao et al. 2001; Brettingham-Moore et al. 2005). Thus, it seems likely that c-Rel may be solely involved in alteration of chromatin structure of IL-2 and GM-CSF. However, interestingly, c-Rel is not required for chromatin remodeling at the IL-12p40 promoter in response to LPS in macrophages (Weinmann et al. 2001), suggesting that the role of c-Rel may be cell type specific or gene specific in a chromatin remodeling event.

Histone proteins including H2A, H2B, H3, and H4 are core components of nucleosomes. Their modifications are significantly concerned with chromatin remodeling and gene expression. A study regarding histone loss has been well documented at the same inducible genes including IL-2 and GM-CSF in T cells (Chen et al. 2005). Histone loss at the IL-2 gene occurs at the proximal promoter regions where an increased chromatin
accessibility has been observed as measured by MNase. However, a region upstream from the TSS (-2 kb) showed a transient increase in histone acetylation, but there was no change in chromatin accessibility and no histone protein loss (Chen et al. 2005). A similar phenomenon has also been observed across the promoter region of the GM-CSF gene (Holloway et al. 2003). This histone loss also occurred at the proximal promoter region where increased chromatin accessibility was observed following stimulation (Holloway et al. 2003; Chen et al. 2005). This histone loss is reversible and requires continuous signals. Further studies showed that histone loss at the IL-2 gene is dependent on c-Rel in primary CD4+ T cells, as this phenomenon has not been observed across the IL-2 gene promoter in c-Rel-/- T cells (Chen et al. 2005). These studies suggested that histone loss is limited to the proximal promoter regions at the inducible genes, and is accompanied by alteration in chromatin structures following stimulation in T cells.
1.5 Project aims

The NF-κB family of transcription factors plays an important role in the regulation of a large number of biological processes such as immune responses, development, cell survival, and proliferation. A large number of genes are targeted and regulated by the NF-κB family and each individual family member appears to have overlapping, but distinct roles in gene regulation. In particular, c-Rel and RelA play an essential role in the immune system and specifically in gene expression in T cells.

One of the intriguing aspects of the NF-κB family, or indeed all families of transcription factors, is the finding that knockout of individual family members leads to very distinct phenotypes while on the other hand they share very similar protein structures and DNA recognition sequences.

Thus, it is important to investigate the binding of NF-κB family members to their target sites to understand their roles in living cells. In addition, to determine the entire spectrum of transcriptional regulatory networks for a specific factor, it is essential to investigate the interaction between a transcription factor and its specific binding sites on a genome-wide scale.

Thus, the overall aim of the project was to determine the genome-wide binding sites for c-Rel and RelA in a T cell model.

Specific aims of this project were

1. Development of a robust ChIP assay for c-Rel and RelA using known target genes in T cells.

2. The use of ChIP-on-chip to identify the genomic target regions for c-Rel and RelA in T cells and the identification of associated genes.

3. Verification and characterization of selected c-Rel binding regions and their associated genes.
The 2'-deoxyribonucleic acid (DNA) is an important role in the regulation of gene expression and is involved in the replication of the genome. A gene mutation or change in sequence can lead to altered function and expression of the protein encoded by the gene. Changes in gene expression can lead to changes in cellular function and may affect the behavior of the cell. DNA replication occurs through a series of steps that involve the synthesis of a new DNA molecule.

- **DNA Synthesis**
  - **DNA Helicase**
  - **ATPase**
  - **RecA**
  - **DNA Polymerase I**
  - **DNA Polymerase III**
  - **DNA Ligase**

- **DNA Repair**
  - **Mismatch Repair**
  - **Nucleotide Excision Repair**
  - **Base Excision Repair**
  - **Double-Strand Break Repair**

- **DNA Replication**
  - **Initiation**
  - **Elongation**
  - **Termination**

- **DNA Packaging**
  - **Histones**
  - **DNA supercoiling**
  - **DNA gyrase**

- **DNA Modification**
  - **Methylation**
  - **Acetylation**
  - **Phosphorylation**

- **DNA Damage**
  - **Radiation**
  - **Chemical agents**
  - **Oxidative stress**

- **DNA Repair systems**
  - **Non-homologous end joining**
  - **Homologous recombination**
  - **Single-strand annealing**

- **DNA Repair enzymes**
  - **Protein kinase**
  - **Poly(ADP-ribose) polymerase**
  - **DNA polymerases**

- **DNA repair mechanisms**
  - **Fanconi anemia**
  - **Ataxia telangiectasia**
  - **Xeroderma pigmentosum**
Chapter 2

Materials and Methods
Chapter 5

Materials and Methods
2.1 Materials

2.1.1 Chemicals

All reagents were of analytical grade, or were of the highest grade obtainable.

2.1.2 Antibiotics

Gentamycin sulphate (MP Biomedical Inc.)
Penicillin (Sigma)

2.1.3 Antibodies

Anti-c-Rel rabbit polyclonal (sc-71, sc-71x) Santa Cruz Biotech
Anti-RelA rabbit polyclonal (sc-372) Santa Cruz Biotech
Anti-c-Fos rabbit polyclonal (sc-52) Santa Cruz Biotech
Anti-c-Jun rabbit polyclonal (sc-1694) Santa Cruz Biotech
Anti-ZEB rabbit polyclonal (sc-25388) Santa Cruz Biotech
Anti-NFATc1 mouse monoclonal (sc-7294) Santa Cruz Biotech
Anti-p50 goat polyclonal (sc-1191, sc-8414) Santa Cruz Biotech
Anti-IRF-1 rabbit polyclonal (sc-497) Santa Cruz Biotech
Anti-IRF-2 rabbit polyclonal (sc-498) Santa Cruz Biotech
Anti-IRF-4 goat polyclonal (sc-6059) Santa Cruz Biotech
Anti-IRF-8 (ICSBP) goat polyclonal (sc-6058) Santa Cruz Biotech
Anti-Sp1 rabbit polyclonal (sc-59) Santa Cruz Biotech
Normal rabbit IgG (sc-2027) Santa Cruz Biotech
HRP-polyclonal Goat Anti-Rabbit (P0448) DAKO Corporation
2.1.4 Oligonucleotides

The oligonucleotides were purchased as Guaranteed Oligos from PROLIGO Australia (Sigma-Aldrich), and used to measure mRNA levels and to amplify genomic DNA isolated by ChIP using quantitative real-time PCR. All primer sets are listed in Appendix I.

Oligonucleotides (primer sets) were designed using OligoPerfect™ Designer (Invitrogen) online software program using the following criteria: annealing temperatures (+/- 60°C), G/C content (40 – 60%), primer length (20 – 27 bp), and the amplified PCR product size (94 – 105 bp). The presence of secondary structure and primer dimers of each primer set were checked using the DNA Calculator (Sigma-Aldrich). To confirm whether a single product was generated, the PCR amplicons were analyzed by dissociation curves (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec; ABI 7900HT Fast Real-time PCR System). Primer efficiencies were determined for some of the primer sets by performing qPCR, using a serial of dilutions of PMA/I stimulated EL-4 T cell line cDNA or genomic DNA by generating standard curves in Microsoft Excel™ (determined by previous lab members). Primer efficiencies were determined from the slope of standard curve using the formula: E=10^(-1/slope) where the slope is the slope of the linear regression of the curve and E represents amplification efficiency.

2.1.5 Kits

GenomePlex® Whole Genome Amplification (WGA2)  
GenomePlex® Whole Genome Reamplification (WGA 3)  
QIAquick® PCR purification kit  
2x SYBR® Green PCR Reaction Mix  
First-strand cDNA Synthesis System  
The GeneChip® WT (Whole Transcript) Terminal Labeling Kit  

2.1.6 Cell lines

EL-4.IL-2 (EL-4) murine T cells were obtained from the American Type Culture Collection (ATCC). Cell line stocks were stored in liquid nitrogen in 5 x 10^6 cells/ml
aliquots in complete RPMI medium containing 45% FCS (foetal calf serum), 9%
DMSO (dimethylsulphoxide).

2.1.7 Mice

Wild-type C57/BL6 and c-Rel-deficient mice were maintained in a pathogen-free
environment in barrier facilities provided by Animal Services Division, JCSMR,
Australian National University (ANU). Experimental mice were used according to the
regulation and approval of the ANU Animal Experimentation Ethics Committee.

2.2 Methods

2.2.1 Cell culture

EL-4 T cells were cultured in RPMI 1640 media containing 10% FCS, 16 μg/ml
gentamycin (Sigma), 120 μg/ml penicillin (Sigma), and 10 mM HEPES. Cells were
stimulated for various times at a density of 1 x 10^6 cells/ml with 10 ng/ml of phorbol
12-myristate 13-acetate (PMA; Sigma) and 1 μM of calcium ionophore (I; Sigma). EL-4
T cells were cultured in a humidified incubator at 37°C in 5% carbon dioxide (Heraeus,
HERAcell).

2.2.2 Primary T cell preparation

C57/BL6 and c-Rel/- mice (5 – 6 weeks old, male) were dissected and spleens were
isolated and placed in incomplete pre-warmed RPMI 1640 media. Spleens were gently
mashed with a syringe plunger, washed with incomplete pre-warmed RPMI 1640 media
in 0.75 μm nylon cell strainers (Becton Dickinson) to isolate single-cell suspension.
Cells were collected in a 50 ml falcon tube and centrifuged (Beckman Allegra™ 6
centrifuge) at 1200 rpm for 10 min at room temperature. Cell pellets were washed twice
by suspending in 1 x cold PBS, then passed through nylon cell strainers, and centrifuged
at the same conditions. Total cells were counted before a second centrifugation and cell
pellets were resuspended in 1x PBS containing 10% BSA and incubated with MACS
CD4⁺ (LT34) MicroBeads (mouse) for 15 min on ice. After incubation, the CD4⁺ T cells were isolated using autoMACS™ Separator (Miltenyi Biotec, Auburn, CA). To measure the purity of isolated cells, cells were stained using antibodies against CD4⁺ T cells and 7-amino-actinomycin D (7-AAD; BD Biosciences) and analysed by flow cytometry. Populations with purity of more than 90% were determined by flow cytometry. Purified CD4⁺ T cells were then stimulated with PMA/I (10 µg/ml) or CD3/CD28 (5 µg/ml) antibodies and cultured in complete MLC medium containing 10% FCS, 4 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 µM 2β-Mercaptoethanol, and PSN (Penicillin-Streptomycin-Neomycin).

### 2.2.3 Total RNA isolation

Total RNA was isolated from nonstimulated or cells stimulated with PMA/I or CD3/CD28 antibodies using TRI reagent (Sigma-Aldrich, St Louis, USA) according to the manufacturer’s guidelines with some modifications. Briefly, 5 × 10⁶ cells were centrifuged (Beckman Allegra X-15R centrifuge) at 1500 rpm for 5 min at 4°C. To isolate total RNA from primary CD4⁺ T cells, cells were centrifuged at 3000 rpm for 10 min. Cell pellets were suspended in 1 ml TRI reagent and incubated for 10 min on ice to allow complete dissociation of nucleoprotein complexes (lysed cells can be stored at -70°C for several weeks). 200 µl of chloroform was added to lysed cells, vortexed vigorously, and incubated on ice for 15 min. Then, the samples were centrifuged at 14000 rpm at 4°C for 15 min. The upper phase was transferred into a new microcentrifuge tube and mixed with an equal volume of isopropanol. The samples were immediately inverted to mix well and stored at -70°C overnight to precipitate the RNA. Samples were then thawed on ice and centrifuged at 14000 rpm for 15 min at 4°C. The RNA pellets were washed with 1 ml of 75% ethanol and centrifuged at the same conditions, and then left to air-dry for 10 min. RNA pellets were resuspended in 50 µl of MilliQ-purified water for EL-4 T cell samples or 30 µl of MilliQ-purified water for CD4⁺ T cell samples. Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies) was used to measure RNA concentration and purity and RNA samples were stored at -70°C until required.
2.2.4 cDNA synthesis

0.5 - 1 μg of total RNA was incubated with DNase I (1 U) (Roche) in a 1 x DNase reaction buffer (20 mM Tris pH 8.3, 50 mM KCl, and 2 mM MgCl₂) at 37°C for 30 min. After DNase I treatment, RNA in a total volume of 15 μl was further incubated at 75°C for 5 min to inhibit DNase I activity. Samples were then reverse transcribed using the First-strand cDNA Synthesis System for Quantitative RT-PCR (Marligen, Biosciences, USA) according to manufacturers' instructions. In brief, DNase I-treated RNA was mixed with 4 μl of 5 x cDNA Synthesis Mix (optimized buffer, magnesium, primers (Oligo(dT)₂₀/random primers, and dNTPs)) and 1 μl of Reverse Transcriptase (50 U/μl) in a 20 μl reaction volume. The reaction mixture was incubated at 22°C for 5 min, then at 42°C for 30 min, and then at 85°C for 5 min to inactivate the enzyme. cDNA was diluted to 1/5 in MilliQ-purified water and stored at -70°C until required.

2.2.5 Quantitative Real-time PCR

SYBR Green Real-time PCR was performed on the ABI 7900HT Fast Real-time PCR System (Perkin Elmer Applied Biosystems) using the 2 x SYBR Green PCR kit. PCR reaction was carried out in a total volume of 10 μl containing 2.5 μl of cDNA or ChIP DNA, 2.5 μl of optimized forward/reverse primer concentrations (a final concentration of 2.5 mM), and 5 μl of 2 x SYBR Green PCR Reaction Mix, according to the manufacturers' guidelines. Primers used for real-time PCR are shown in Appendix I. Real-time PCR reaction was carried out in duplicate under the following conditions: 50°C for 2 min, 95°C for 10 min, 40°C cycles of each: 95°C for 15 sec, 60°C for 1 min.

Real-time PCR for ubiquitin conjugating enzyme (Ubc) reference housekeeping gene was performed in parallel to normalise for the variation in RNA input and cDNA synthesis efficiencies.

For relative quantification of the amount of mRNA expression levels, the fluorescent threshold was set at a value of 0.2, with a baseline level set at the range of 3-15 cycles. To calculate the relative changes in mRNA expression, the comparative Cₙ method, also known as the 2⁻ΔΔCₙ method, was used (Wong et al. 2005). To calculate the relative mRNA levels, cycle threshold (Cₙ) values of the reference housekeeping gene (Cₙ(ref)) (ubc) were subtracted from Cₙ of genes of interest (Cₙ(target)) which
generates ΔΔCt or Ct (target) - Ct (ref). The resulting Ct value is then raised to the power 2 to give the final mRNA levels (2^{ΔΔCt}). To calculate the fold change in mRNA expression levels upon stimulation, the relative mRNA levels in activated cells was normalized to the non-stimulated level (2^{ΔΔCt (activated)} / 2^{ΔΔCt (nonstimulated)}).

To quantify the relative amounts of ChIP DNA, the amount of DNA amplified in no antibody (NA) condition was subtracted from immunoprecipitated DNA (IP) and then normalized to the amount of relative DNA of total Input (TI) (IP-NA/TI). The relative amount of ChIP DNA was quantified by subtracting Ct values of TI DNA from IP (Ct_{IP} - Ct_{TI}) and TI from NA (Ct_{NA} - Ct_{TI}). The resulting Ct values is then raised power 2 to give the final relative amount of ChIP DNA (2^{ΔΔCt (IP-TI)} or 2^{ΔΔCt (NA-TI)}). The results are shown as the mean and standard deviation of three to five independent biological experiments.

2.2.6 Nuclear protein extraction

Nuclei were extracted from nonstimulated or PMA/I-stimulated EL-4 (50 x 10^6 cells) and CD4+ T cells (20 x 10^6 cells). Briefly, cells were harvested by centrifugation (Beckman Allegra X-15R centrifuge) at 1500 rpm for 5 min at 4°C for EL-4 T cells and at 3000 rpm for 10 min for CD4+ T cells. All cells were washed in 30 ml 1 x cold PBS. Cell pellets were resuspended in 1 ml cold buffer A (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 0.1 mM EDTA pH 8.0, 0.1 x EDTA-free protease inhibitors cocktail tablets (Roche Molecular Biochemicals), and 0.5 mM Pefabloc SC). 9 ml of cold buffer A containing 0.5% NP-40 was added to the resuspended cells, mixed well, and incubated on ice for 5 min. Nuclei were collected by centrifugation at 1800 rpm for 5 min at 4°C (and at 3000 rpm for 5 min for CD4+ T cells). Cell nuclei were suspended in 1 ml cold buffer A without NP-40 to wash, transferred into a new microcentrifuge tube, and then centrifuged at 3000 rpm for 5 min at 4°C, twice. Nuclei were resuspended in 100 μl cold buffer C (20 mM HEPES pH 8.0, 400 mM NaCl, 7.5 mM MgCl2, 0.2 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1 mM DTT, 1 x protease inhibitors, and 5 mM Pefabloc SC) and were incubated on ice for 20 min with gentle shaking. Lysed nuclei were centrifuged at 14000 rpm for 15 min at 4°C. The supernatants containing proteins were transferred into a new tube and stored at -70°C until used.
2.2.7 Bradford assays

Protein concentrations of the extracts were determined by the Bradford assay using bovine serum albumin (BSA, New England Biolabs) as a standard. Briefly, a series of dilutions (0.5, 0.25, 0.15, 0.1, and 0.05 mg/ml) of the BSA were prepared. 1 ml of Bio-Rad Protein Assay solution (Bio-Rad) was diluted in 4 ml of MilliQ-purified water (1/5 dilution) and extracted proteins were also diluted in MilliQ-purified water (1/10 dilution). 10 μl of each standard or extracted proteins was mixed with 200 μl of 1/5 diluted Bradford Reagent and 800 μl of MilliQ-purified water in standard cuvette (Sarstedt). Protein concentrations were measured at 595 nm in a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA).

2.2.8 Western blotting assays

Proteins were mixed with 4 x SDS loading buffer (1/4 dilution, Invitrogen) and boiled for 5 min. Denatured proteins were briefly centrifuged and put on ice prior to loading. Samples were loaded on NuPAGE 4-12% Bis-Tris Gels (Invitrogen) with BenchMark Pre-Stained Protein Ladder (Invitrogen) used as a protein molecular weight and size reference. Gel was ran at 120 V for 1.5 h and proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) at 30 V overnight at 4°C in 1 x transfer buffer (3.03 g Tris, 14.4 g glycine, and 200 ml methanol in up to 1 L H₂O) using Mini Trans-Blot™ Cell apparatus (Bio-Rad).

The membrane was briefly rinsed in MilliQ-purified water and incubated in blocking solution (5% skim milk powder dissolved in 1 x PBS) for 1 h, with gentle shaking at room temperature. Primary antibodies were diluted in blocking solution and incubated with the membrane for 1 h at room temperature with rotation on wheel. Anti-IRF-1 was diluted to 1/250 and other antibodies anti-RelA, anti-c-Rel, and anti-Sp1 were diluted to 1/1000 in blocking solution prior to incubation. The membrane was briefly rinsed and washed 3 times in washing buffer (0.3% Tween-20 in 1x PBS) with gentle shaking at room temperature for 10 min each time. Secondary antibody (HRP-polyclonal Goat Anti-Rabbit) was diluted to 1/1000 in blocking solution and incubated with the membrane for 1 h at room temperature with rotation. The membrane was washed 3 times in washing buffer and rinsed briefly in MilliQ-purified water. Proteins were detected using chemiluminescent detection agent (Sigma Aldrich) by mixing 50 μl
luminol (250 mM) and 22 μl coumaric acid (90 mM) in 5 ml of 100 mM Tris-HCl pH 8.5. A luminescent image analyser (Las-1000 Plus, Fuji, Tokyo, Japan) was used to take gel picture and protein bands on the gel were visualized using the Fuji Image Gauge software.

### 2.2.9 Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out as previously described (Chen et al. 2005) with some modifications. Briefly, cells were harvested and proteins were directly cross-linked to DNA by adding 1% formaldehyde final concentration for 15 min at room temperature with shaking. The incubation was stopped by the addition of glycine to a final concentration of 0.125 M followed by shaking for 10 min at room temperature. Cross-linked cells were washed twice with 1 x cold PBS and resuspended in lysis buffer (20 mM TrisHCl pH 8.0, 85 mM KCl, and 0.5% NP40) containing 1 x EDTA-free protease inhibitors for 10 min on ice.

Nuclei were pelleted by centrifugation (Beckman Allegra X-15R centrifuge) at 1500 rpm for 5 min at 4°C and suspended in 1 ml of 1 x cold PBS containing 1 x protease inhibitors. To ensure whether nuclei were extracted, nuclei were mixed with trypan blue (1:1) and counted on the microscope using a counting chamber. Counted nuclei were pelleted by centrifugation (Eppendorf centrifuge 5415R) at 1400 rpm for 5 min at 4°C and further lysed in 250 μl of nuclei buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, and 1% SDS) for 10 min on ice. Nuclear lysates can be stored for several weeks at -70°C.

Nuclear lysates were thawed and sonicated to shear chromatin using the Bioruptor (Diagenode) for 10 min for EL-4 T cells and 15 min for CD4+ T cells on a setting of 30 sec on/off intervals to give a range of DNA fragments from 100 bp to 1 kb in length. To check the sonicated fragment sizes, 20 μl of sonicated lysates were reverse cross-linked with 1 μl of 4 M NaCl at 65°C for 4 h followed by phenol-chloroform extraction and then the extracted DNA fragments were analysed on a 1% agarose gel.

Further experiments were performed by diluting sonicated samples in 1 ml of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) containing 1 x protease inhibitors. Samples were centrifuged at 14000 rpm for 5 min at 4°C and the supernatant collected were pre-cleared with 60 μl of Salmon Sperm DNA/Protein A agarose beads (Upstate Technologies) by incubating for
1.5 h at 4°C with rotation. After incubation, the samples were centrifuged at 1000 rpm for 1 min at 4°C and the supernatant was transferred into a new microcentrifuge tube. 100 μl from supernatants were set aside as total input (TI) and stored at -70°C until required. The remaining samples were evenly divided into no antibody (NA) and antibody (Ab) treatments. NA and Ab were diluted in 750 μl of ChIP dilution buffer and incubated either with 6 μg of antibodies (anti-c-Rel, anti-RelA, anti-c-Jun, anti-IRF-1, anti-ZEB, and anti-IgG) or without antibody as a negative control at 4°C overnight with rotation. After overnight incubation, 60 μl of Salmon Sperm DNA/Protein A agarose beads were added into immunocomplexes followed by incubation for 1.5 h at 4°C. Then, immunocomplexes bound to agarose beads were collected by centrifugation at 1000 rpm for 1 min at 4°C. Agarose beads were sequentially washed with 1 ml of the different washing buffers containing 1 x protease inhibitors including 1 x Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), 1x High Salt Wash Buffer (as for Low Salt Wash Buffer but with 500 mM NaCl instead of 150 mM NaCl), 1 x LiCl Wash Buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), 2 x TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The immunocomplexes were eluted from the agarose beads by adding 2 x 200 μl elution buffer (1% SDS and 0.1 M NaHCO3) for 15 min at room temperature with rotation. 400 μl of eluates were then reverse cross-linked with 20 μl of 4 M NaCl at 65°C overnight (a final concentration of 0.2 M). Reverse cross-linking of TI samples was also performed. Immunoprecipitated DNA was recovered by treatment with an equal volume of Phenol:Chloroform:IAA, pH 7.9 (Ambion), followed by inverting and centrifugation (Eppndorf Centrifuge 5415R) at 14000 rpm for 15 min at room temperature twice. The aqueous phase containing genomic DNA fragments was collected and combined with 2.5 x volume of 100% ethanol, 0.1 x volume of 3M NaOAc pH 5.2, and 1 μl glycogen (Roche), followed by overnight precipitation at -70°C. DNA samples were pelleted by centrifugation at 14000 rpm for 15 min at 4°C and washed with 75% ethanol, followed by air-drying and suspension in 50 μl of MilliQ-purified water. All samples were stored at -20°C until required.

2.2.10 Sequential chromatin immunoprecipitation (Seq-ChIP)

For sequential-ChIP, ChIP experiments were performed as described above but with some modifications (section 2.2.9). In brief, pre-cleared samples were immunoprecipitated with the first antibody anti-c-Rel or anti-IgG as a negative control
overnight at 4°C with rotation. Immunocomplexes were eluted from agarose beads by incubation with 40 μl of 10 mM dithiothreitol (DTT) containing 1 x protease inhibitors for 30 min at room temperature with rotation. Each eluate was then diluted 1:35 in cold ChIP dilution buffer and reimmunoprecipitated with the second antibody anti-IRF-1 (c-Rel to IRF-1 or c-Rel to IgG) or anti-IgG (IgG to IgG) overnight at 4°C with rotation. The immunocomplexes were collected by incubating with 100 μl agarose beads at 4°C for 1.5 h followed by centrifugation at 1000 rpm for 1 min. For reverse sequential ChIP, the order of antibodies was reversed. Thus, samples were immunoprecipitated with anti-IRF-1 or anti-IgG as the first antibody and reimmunoprecipitated with anti-c-Rel (IRF-1 to c-Rel or IRF-1 to IgG) or anti-IgG (IgG to IgG) as the second antibody. All other procedures were performed as above (section 2.2.9) and samples were finally resuspended in 40 to 50 μl of MilliQ-purified water.

2.2.11 Chromatin immunoprecipitation (ChIP) on chip (ChIP-on-chip)

ChIP-on-chip experiments were performed in EL-4 T cells in triplicate. First, ChIP was performed to obtain immunoprecipitated genomic DNA fragments as described above (section 2.2.9) with some modifications. Briefly, nuclei were isolated and lysed before sonication. Chromatin was sheared by sonication for 5 min on a setting of 30 sec on/off intervals to give a range in DNA fragments from 200 bp to 1 kb in length, followed by electrophoresis on 1% agarose gel to check the sonicated fragment sizes. Genomic DNA fragments were immunoprecipitated with 6 μg of anti-c-Rel and anti-RelA. After reverse cross-linking, phenol–chloroform extraction, and ethanol precipitation, genomic DNA fragments were resuspended in 30 μl of MilliQ-purified water.

For microarray, TI, NA, and Ab samples were amplified and reamplified with GenomePlex® Whole Genome Amplification (WGA2) and GenomePlex® Whole Genome Reamplification (WGA 3). In brief, for amplification with WGA2, 20 ng of ChIP DNA was mixed with 2 μl Library Stabilization Solution and 1 μl 1 x Library Preparation Buffer according to manufacturers’ guidelines. The reaction was initiated by incubation at 95°C for 2 min on ice. 1 μl Library Preparation Enzyme was added to the reaction mixture and incubated under the following conditions: 16°C for 20 min, 24°C for 20 min, 37°C for 20 min, and 75°C for 5 min. 15 μl of reaction samples were
then mixed with Master Mix (7.5 μl of 10 x Amplification Master Mix, 45.5 μl of MilliQ-purified water, 5 μl of WGA DNA polymerase, and 2 μl of 4 mM dUTP) in a total volume of 75 μl. Amplification reaction was performed at 95°C for 3 min for initial denaturation, then 14 cycles of 94°C for 15 sec (denature) and 65°C for 5 min (anneal/extend), then 4°C hold. ChIP DNA was loaded on a 1% agarose gel to check amplified ChIP DNA. Amplified ChIP DNA was purified with QIAquick purification kit (QIAGEN) and suspended in 25 ul of elution buffer according to manufacturers' guidelines. Samples were stored at -20°C for several weeks.

Purified ChIP DNA fragments of NA and Ab were further amplified with GenomePlex® Whole Genome Reamplification (WGA 3) to obtain enough materials for microarray. In brief, 20 ng of purified amplified ChIP DNA was mixed with Master Mix (7.5 μl of 10 x Amplification Master Mix, 5 μl of WGA DNA polymerase, 3 μl of 10 mM dNTP Mix, approximately 2.2 μl of 4 mM dNTP, approximately 56 μl of MilliQ-purified water) in a total volume of 75 μl. The reaction was incubated at 95°C for 3 min, then 14 cycles of 94°C for 15 sec and 65°C for 5 min, then 4°C hold. Reamplified ChIP DNA products were purified with QIAquick purification kit (QIAGEN) and suspended in 25 μl MilliQ-purified water. Purified reamplification products were stored at -20°C for several weeks.

2.2.12 DNA microarray

DNA microarray was performed by Dr Kaiman Peng at the Biomolecular Resource Facility (BRF) at the John Curtin School of Medical Research. ChIP-enriched DNA fragments were labeled using the GeneChip® WT (Whole Transcript) Terminal Labeling Kit (Affymetrix) and hybridized to DNA microarrays (Affymetrix Mouse promoter array 1.0R) containing promoter DNA sequences, spanning approximately -10 kb upstream to +2 kb downstream from the transcription start site (TSS). For DNA microarray, ChIP immunoprecipitated (IP) samples from 8 h for c-Rel (c-Rel 8 h) and 2 h for RelA (RelA 2 h) were selected respectively. In addition, an equal mixture of the total input (TI) and mock (no antibody, NA) control samples from the nonstimulation (NS) and the two time points (8 h and 2 h) were combined to make up the TI and mock controls for microarray respectively. Therefore, three biological replicate ChIP-on-chip experiments were performed with these four samples (TI, NA, c-Rel 8 h, and RelA 2 h -
in total 12 samples) (Fig 4.1A). The final amount of DNA used in hybridization was 8 µg for each sample.

2.2.13 Analysis of ChIP-on-chip data using statistical analysis programs

ChIP-on-chip data analysis was carried out by Dr Kristine Hardy at the John Curtin School of Medical Research. After ChIP-on-chip, all raw data were stored into CEL files which contain the signal value of every probe and were analysed by the Model-based Analysis of Tiling-arrays (MAT) algorithm. MAT was used to compare three replicates which generate bar files. The Integrated Genome Browser (IGB, Affymetrix) was used to visualize ChIP-on-chip data from MAT by uploading the resulting bar files into IGB. Partek® Genomics Suite™ (6.4) software was used to normalise the arrays and to compare the replicates using Principal Components Analysis (PCA) (Appendix II). Partek® Genomics Suite™ 6.4 was used to detect highly stringent regions enriched by c-Rel and RelA. ANOVA (Analysis of Variance; Partek® Genomics Suite™ 6.4) was used to identify enriched regions by comparing c-Rel and RelA-enriched regions with controls (total input and no antibody). c-Rel or RelA samples were compared against total input to detect enriched regions compared to total genomic DNA. In addition, to ensure that the regions detected were specific for the antibody used, the samples were compared against no antibody samples. In addition, the Genomatix software program was used to identify nearest genes from transcription factor binding regions, and to detect overrepresented transcription factor family motifs in the sequences bound by c-Rel and RelA.

The significance of a motif(s) was selected by the Z-score, which is a standard measurement of over representativeness and measures the number of standard deviation from the mean. Z-Score is calculated as \( z = (x - \mu)/S \), where \( x \) means a low score of interest, \( \mu \) means mean, and \( S \) means the standard deviation. A Z-score over 2 (p<0.05) is considered as significant (Apostolico et al. 2005; Ho Sui et al. 2005; Wang et al. 2005; Lutter et al. 2008; van Dijk et al. 2010).

MatInspector™ program within the Genomatix™ Suite software was used to find putative transcription factor binding sites of the NF-κB/c-Rel and IRF transcription
factors in the c-Rel binding region. Genomic sequences of c-Rel binding regions were obtained by comparing regions obtained from ChIP-on-chip analysis data with genomic sequences from the UCSC and Ensembl Genome Browsers.

2.2.14 Statistical analysis

A student’s t-Test was used with a two-tailed distribution to determine whether data obtained from two different groups are significantly different (p≤0.05).
27.1.4 Statistical analysis

A computer program was used with the statistical software to organize the data. The analysis was performed on a cohort of patients with...
Chapter 3

Kinetics of transcription factor binding to cytokine gene promoters using chromatin immunoprecipitation in T cells
Chapter 3

Kinetics of transcription factor binding to cytotoxic gene promoters using chromatin immobilization in T cells
3.1 Introduction

Interleukin-2 (IL-2), which is known as a T cell growth factor, is an important cytokine produced by mature T cells following stimulation. IL-2 plays diverse and important roles in vivo including differentiation, proliferation, and clonal expansion of T cells as well as homeostasis of regulatory T cells (Rothenberg et al. 1996; Bachmann et al. 2007). In addition, IL-2 is known to drive the proliferation of B cells and natural killer (NK) cells (Gaffen et al. 2004). A great number of studies have documented the requirement of the IL-2 gene in T cell activation, development, autoimmunity, and thymic disorders using IL-2-deficient mice (Schorle et al. 1991; Suzuki et al. 1995; Reya et al. 1998; Schimpl et al. 2002). In particular, the absence of IL-2 leads to autoimmune diseases such as inflammatory bowel disease (IBD), type I diabetes, rheumatoid arthritis, and experimental autoimmune encephalomyelitis (EAE) (Sadlack et al. 1993; Horak et al. 1995; Petitto et al. 2000; Eerligh et al. 2004; Pawlik et al. 2005).

The expression of the IL-2 gene is restricted to T cells and is highly regulated by T cell activation (Garrity et al. 1994; Rothenberg et al. 1996; Ward et al. 1998). The IL-2 gene has been used as an ideal model to investigate numerous nuclear mechanisms including inducible gene regulation and the interaction between transcription factors and DNA. To understand the regulatory mechanisms at the IL-2 gene, the promoter region has been extensively investigated using in vitro assays. These studies uncovered a number of binding sites for transcription factors, including the inducible NFAT, NF-κB, and AP-1 transcription factors, and the constitutive factor Oct-1, scattered across the promoter region (Garrity et al. 1994; Rooney et al. 1995; Ward et al. 1998; Murayama et al. 2006). Specifically, a 300 bp region immediately upstream from the transcription start site (TSS) has been defined as the proximal promoter region for IL-2 gene transcription and is regulated by recruiting specific transcription factors (Garrity et al. 1994; Rooney et al. 1995; Murayama et al. 2006). In addition, significant changes in chromatin structure and loss of histone proteins occur at the proximal promoter region of the IL-2 gene following T cell activation (Rao et al. 2001; Chen et al. 2005). This 300 bp region is composed of three elements including the CD28RE and the antigen response elements (ARRE-1 and ARRE-2) (Rooney et al. 1995) (Fig 3.1A). In vivo footprinting studies showed that IL-2 regulatory proteins do not associate with this promoter in resting T cells, but are recruited following activation (Garrity et al. 1994). In addition, each factor binds weakly to its cognate sites because these factors do not bind stably to the
promoter unless all factors are present (Garrity et al. 1994; Ward et al. 1998). The fact that IL-2 regulatory factors coordinately bind to this proximal promoter in T cells has now been demonstrated in a number of ways (Garrity et al. 1994; Ward et al. 1998). For example, c-Rel binds to the CD28RE in response to CD28 signals, but for full activation of IL-2, c-Rel requires the cooperative binding of AP-1 to the adjacent AP-1 site (Shapiro et al. 1997). Similarly a study by Pfeuffer et al. reported that Oct-2 cooperates with AP-1 proteins at the ARRE-1 of the IL-2 proximal promoter where the binding of the two factors generates a synergistic effect in activated EL-4 T cells. While mutation of either the Oct or AP-1 site within the ARRE-1 reduces the IL-2 promoter activity, mutation of both sites abolishes the IL-2 promoter induction (Pfeuffer et al. 1994). Unlike Oct-2, the binding of Oct-1 does not require cooperation with AP-1 at the ARRE-1. Another transcription factor, NFAT, cooperates with AP-1 for binding at the ARRE-1 (de Grazia et al. 1994; Rooney et al. 1995). NFAT also cooperatively associates with the AP-1 family at the distal ARRE-2 of the IL-2 promoter. In particular, Fra-1 and JunB heterodimer are present in the NFAT binding complex where the AP-1 leucine zipper domain interacts with members of the NFAT family (Boise et al. 1993; Erlanson et al. 1996). These previous studies have provided significant insights into understanding the regulatory mechanisms mediated by transcription factors in IL-2 gene expression.

In addition to the IL-2 gene, another well known T cell inducible cytokine gene, granulocyte-macrophage colony stimulating factor (GM-CSF) is involved in proliferation, maturation, and function of hematopoietic cells (reviewed in Gasson 1991). The GM-CSF gene is activated by TCR/CD28 signals in a similar manner to IL-2, and has well-defined proximal promoter and upstream enhancer regions which control gene expression. The proximal promoter region is limited to 100 to 150 bp from the TSS, which contains transcription factor binding sites for NF-κB, Sp1, and NF-κB/c-Rel within the CD28RE and downstream NFAT and AP-1 binding sites (Jenkins et al. 1995; Shannon et al. 1997; Thomas et al. 1997) (Fig 3.1B). Changes in chromatin structure are found to occur across the GM-CSF proximal promoter within the first 200 bp from the TSS upon T cell activation (Holloway et al. 2003). DNase I hypersensitive (DH) sites associated with chromatin remodeling are also observed in the promoter and enhancer of the GM-CSF gene (Cockerill et al. 1999; Holloway et al. 2003; Brettingham-Moore et al. 2008). A mutation in the NF-κB/Sp-1 region within the CD28RE of the GM-CSF promoter inhibits the DH site induction in T cells (Cakouros et al. 2001). Further studies
showed that this NF-κB/Sp-1 region is bound by RelA with p50 as a heterodimer in T cells, but not c-Rel. However, the adjacent NF-κB/c-Rel element within the CD28RE is recognized by both c-Rel and RelA proteins (Himes et al. 1996). Unlike the IL-2 gene, the GM-CSF gene is expressed in a variety of different cells such as macrophages, endothelial cells, fibroblasts, and T cells (reviewed in Gasson 1991), and plays an important role in myeloid cell differentiation and function in response to immune and inflammatory signals (Matsuguchi et al. 1998; Brettinham-Moore et al. 2005). Importantly, dysregulated GM-CSF expression is associated with certain diseases such as myeloblastic leukemia and chronic inflammatory disease (Young et al. 1986; Young et al. 1987; Williamson et al. 1988).

Many activator and repressor proteins including NF-κB, NFAT, AP-1, and ZEB1 are essential factors for the regulation of IL-2 and/or GM-CSF gene expression in T cells. Thus, the role of these regulatory transcription factors in cytokine gene activation, and their cooperative interactions at the cytokine gene promoters, have been well documented and will be discussed below.

As described in Chapter 1, the NF-κB family of transcription factors plays an important role in the regulation of a large number of cellular processes such as innate and adaptive immunity, inflammation, cell development, cell survival, and cell proliferation (reviewed in Li et al. 2002; O'Dea et al. 2009). In particular, NF-κB proteins play critical roles in gene expression and chromatin remodeling of inducible cytokine genes such as IL-2 and GM-CSF in T cells. The absence of c-Rel brings about a decrease in chromatin accessibility at the promoters of both of these genes in activated T cells (Holloway et al. 2003; Rao et al. 2003; Brettinham-Moore et al. 2005; Chen et al. 2005). However, c-Rel is not involved in the recruitment of chromatin modifying complex, p300 histone acetyltransferase (HAT) to the GM-CSF promoters and does not interact with p300 HAT complex as observed in previous studies (personal communication, Karen Bunting). In contrast, RelA recruits p300 HAT complex to and cooperatively activates the GM-CSF promoter (personal communication, Karen Bunting). In addition, a study by Holloway et al. showed that the recruitment of SWI/SNF chromatin remodeling complex, Brg1 to the GM-CSF is RelA-dependent (Holloway et al. 2003). This suggests that c-Rel and RelA may play a distinct function in chromatin remodeling and gene expression events at the IL-2 and GM-CSF in T cells.
The AP-1 family of transcription factors is composed of two subfamilies of inducible transcription factors: Jun, which consists of c-Jun, JunB, and JunD; and Fos, which is composed of c-Fos, FosB, Fra-1, and Fra-2. Like NF-κB, AP-1 also binds to the IL-2 and GM-CSF promoter regions following activation in T cells (Garrity et al. 1994; Jenkins et al. 1995; Rooney et al. 1995; Wang et al. 2009). As described above, AP-1 cooperatively binds with NF-κB to the IL-2 promoter. This cooperative binding between these two transcription factors activates IL-2 gene expression during T cell activation. Thus, reduced binding of AP-1 leads to a defect in IL-2 production in T cells (Jain et al. 1995; Jung et al. 1995; Sundstedt et al. 1998). Furthermore, AP-1 binding sites are adjacent to those of NFAT at the IL-2 and GM-CSF promoters (Fig 3.1), which cooperatively regulates these cytokine genes (Macian et al. 2000; reviewed in Wagner et al. 2005). The AP-1/NFAT complex also cooperatively activates many other cytokine genes such as IL-3, IL-4, IL-5, IL-13, and INF-γ and enhances cytokine production (reviewed in Macian et al. 2001; Wagner et al. 2005; Andrade et al. 2011). This suggests that cooperative interaction between AP-1 and other inducible transcription factors at the cytokine gene promoters is important for cytokine gene activation.

Another transcriptional activator, the nuclear factor of activated T cells (NFAT) family was first found in the human IL-2 promoter in activated T cells as an inducible DNA-binding factor (reviewed in Muller et al. 2010). The NFAT family consists of five members including NFAT1 (NFATc2, NFATp), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3), and NFAT5 (TonEBP) (reviewed in Macian et al. 2001; Muller et al. 2010). Structurally, the NFAT family is similar to the NF-κB family in that it has a similar Rel-homology domain (known as DNA-binding domain) which recognizes similar DNA sequences (reviewed in Nolan 1994; Northrop et al. 1994; Muller et al. 2010). A study by Sica et al. reported that NFAT and NF-κB cooperatively bind to the C3-30 site located at the IFN-γ promoter which increases IFN-γ gene transcription in human Jurkat cells (Sica et al. 1997). NFAT also cooperates with AP-1 forming a stable ternary complex at the ARRE-2 region of the IL-2 promoter. This cooperative binding between NFAT and AP-1 proteins (Fos/Jun) to the NAFT/AP-1 sites is important for the function of cytokine gene promoters (Boise et al. 1993; Jenkins et al. 1995; Sun et al. 1997; Macian et al. 2000; reviewed in Macian et al. 2001). A study by Macian et al. showed that the mutation in the DNA-binding domain of NFAT1 significantly decreases cooperative binding with AP-1 proteins at the IL-2 promoter.
This failure in NFAT/AP-1 cooperation reduces the activity of cytokine gene promoters including IL-2, IL-4, and GM-CSF in Jurkat cells (Macian et al. 2000).

Unlike the three transcriptional activators for the IL-2 and GM-CSF gene transcription described above, ZEB, a zinc finger, E box-binding transcription factor is a negative regulator of gene expression. While NF-κB, AP-1, and NFAT activators are stimulation-dependent, ZEB is expressed in both stimulated and nonstimulated T cells (Wang et al. 2009). Initial studies observed that ZEB binds to the negative regulatory element (NRE-A) of the IL-2 gene promoter at around -100 bp (Williams et al. 1991) (Fig 3.1A). The binding of ZEB to the IL-2 gene promoter region results in the repression of IL-2 gene expression in activated T cells (Yasui et al. 1998; Wang et al. 2009). In agreement with these results, previous studies showed that ZEB makes a complex with C-terminal-binding protein (CtBP), histone deacetylase (HDAC), and histone methyltransferase, which represses IL-2 gene expression in EL-4 T cells (reviewed in Vandewalle et al. 2009; Wang et al. 2009). Thus, it seems likely that ZEB/CtBP/HDAC complexes convert open, active chromatin structure mediated by transcriptional activators such as NF-κB or NFAT proteins to a closed repressive status, leading to gene repression at the IL-2 promoter.

The majority of studies examining the interaction between transcription factors and cis-regulatory elements at the IL-2 and GM-CSF gene promoters have been performed through in vitro DNA binding assays such as electrophoretic mobility shift assay (EMSA) (Garrity et al. 1994; Rooney et al. 1995; Himes et al. 1996). These studies provide little information regarding the in vivo dynamic binding of transcription factors to these gene promoter regions in T cells. Recently, many studies of in vivo DNA binding have been carried out using chromatin immunoprecipitation (ChIP) (Edelstein et al. 2003; Bosisio et al. 2006; Murayama et al. 2006; Wang et al. 2009). ChIP is a powerful technique to investigate the interaction between trans-regulatory elements and cis-regulatory proteins across the promoter and enhancer regions of genes. Using ChIP assays, it is possible to examine the dynamic movements of transcription factor binding to gene promoters in living cells. Thus, in this chapter, ChIP assays were developed to investigate the in vivo binding of transcriptional activator and repressor proteins that were known to bind at least in vitro to the IL-2 and GM-CSF gene promoters. In addition, these transcription factors function on many genes and thus, it would be of interest to map the genome-wide binding sites for these transcription factors. Therefore,
the detailed kinetic studies on well known cytokine genes were used to identify optimal times for performing such genome-wide ChIP-on-chip studies.

The aims were:

♦ To develop the ChIP assays for in vivo transcription factor binding studies and for ChIP-based genome-wide analysis of binding sites of transcription factors in T cells.

♦ To examine the kinetics of binding of specific transcriptional activators and repressors at the inducible IL-2 and GM-CSF proximal promoter regions in primary CD4⁺ and/or EL-4 T cells.

The data presented in this chapter show that:

♦ Different transcriptional activators display distinct kinetics of binding following T cell activation and can differ between gene promoters.

♦ RelA and c-Jun binding appear rapidly and transiently, suggesting that they may play roles in the initiation of transcriptional activation and chromatin remodeling.

♦ c-Rel binding occurs slowly and is prolonged at the promoters, suggesting that it might be associated with maintaining a stable gene transcription and remodeled chromatin state at the promoter.

♦ The ZEB repressor displays a distinct binding profile from the transcriptional activators.
Figure 3.1 The *IL-2* and *GM-CSF* proximal promoter regions

(A) Schematic diagram of the *IL-2* promoter region showing the proximal region containing the known transcription factor binding sites, and the identified control regions (ARRE-1 and 2 and CD28RE). The transcription start site (TSS) is shown as +1. The region adjacent to the TSS is amplified by primer Set B (Appendix I) and used to detect the proximal promoter in ChIP assays. The -2 kb upstream region is amplified by primer Set F (Appendix I) and was used as a negative control region for ChIP assays.

(B) Diagram of the *GM-CSF* promoter showing the the location of the enhancer region which is located at -3 kb and the proximal region within -200 bp from the TSS. The proximal region contains the CD28RE and adjacent binding regions. The CD28RE consisting of binding sites for NF-κB, NF-κB/c-Rel, and Sp1 is shown near -100 bp, which is amplified by primer Set A (Appendix I). The region adjacent to the TSS containing AP-1 and NFAT sites is covered by primer Set J (Appendix I).
3.2 Results

3.2.1 Dynamic binding of transcription factors c-Rel and RelA to the IL-2 and GM-CSF promoter regions in EL-4 T cells

ChIP assays were initially attempted with all of the major transcription factors that were known to bind to the IL-2 and GM-CSF promoters. Accordingly, the in vivo binding of transcription factors including NF-κB (c-Rel and RelA), NFAT1, AP-1 (c-Jun and c-Fos), and ZEB1 proteins were tested in this study. However, antibodies specific for NFAT1, and c-Fos did not provide reliable binding data in these studies (data not shown). Thus, four transcription factors (c-Rel, RelA, c-Jun, and ZEB1) were examined in detailed kinetic experiments in this chapter.

To confirm appropriate activation parameters prior to carrying out ChIP experiments, EL-4 T cells were stimulated with PMA/I for 1, 2, 4, 8, and 16 h. Total RNA was isolated from the cells and IL-2 mRNA levels were measured by real-time PCR as a positive control. As expected from previous studies, IL-2 mRNA showed a transient increase with the peak of expression measured at 4 h poststimulation (Fig 3.2).

To investigate the timing of expression of c-Rel and RelA, which are the two transcription factors at the centre of this study, mRNA expression levels were measured in EL-4 T cells. As shown in Figure 3.3A and B, mRNA expression profiles of c-Rel and RelA were similar in EL-4 T cells, with a peak of expression at 8 h poststimulation and decreasing by 16 h. Interestingly, the largest increase in expression occurred between 0 and 1 h stimulation for c-Rel (Fig 3.3A) while RelA displayed a more gradual rise in expression (Fig 3.3B). RelA mRNA was clearly detected prior to stimulation whereas levels of c-Rel mRNA were barely detectable (Fig 3.3A and B).

In addition, the kinetics of nuclear accumulation of c-Rel and RelA were investigated in T cells. To do this, EL-4 T cells were stimulated as described above. Nuclear extracts were prepared as described in Chapter 2 (Section 2.2.6). As shown in Fig 3.3C, no proteins were detected in the nucleus before stimulation. However, a low level of c-Rel proteins was detected after 1 h of stimulation which gradually increased in the nucleus.
up to 16 h in response to PMA/I activation in EL-4 T cells (Fig 3.3C). RelA appeared as early as 1 h, with a maximum level at 2 h following stimulation, and then levels gradually decreased with low levels remaining at 16 h (Fig 3.3C). These results are in agreement with previous studies suggesting that the accumulation of c-Rel proteins in the nucleus occurs much later than that of RelA (Bryan et al. 1994; Himes et al. 1996).

To examine in vivo binding of c-Rel and RelA to the promoters of the IL-2 and GM-CSF genes in EL-4 T cells, ChIP assays were performed with antibodies against c-Rel and RelA. Although the CD28RE of the IL-2 promoter contains the NF-κB/c-Rel binding site, it proved difficult to design primers to efficiently perform PCR across this region. Therefore, Set B primer amplifying in the first 100 bp upstream from the TSS (Fig 3.1A) was used for ChIP assays since the genomic DNA used in the ChIP assays was on average 500 bp in length. As shown in Figure 3.4A, c-Rel showed a time-dependent increase in binding up to 16 h at the proximal promoter region (Set B) (Fig 3.1A) of the IL-2 gene following stimulation, while no increase in binding of c-Rel was seen at the -2 kb upstream region (Set F) used as a control (Fig 3.1A). In contrast, the binding of RelA was detected as early as 1 h following stimulation and showed a maximum binding at 2 h with a rapid decline thereafter. Once again a low binding level was observed at the control region (Set F) (Fig 3.4B). These results are consistent with those observed in the western blot (Fig 3.3C), suggesting that there may be a connection between nuclear levels of c-Rel and RelA and their in vivo binding in T cells.

The distinct binding profiles of c-Rel and RelA were also observed at the GM-CSF proximal promoter. Quantitative real-time PCR was performed on immunoprecipitated ChIP DNA prepared above using primer sets to amplify the proximal promoter region containing the CD28RR (Set A) (Fig 3.1B). As observed at the IL-2 promoter region, the c-Rel occupation at the GM-CSF promoter was gradually increased up to 16 h following activation (Fig 3.4C). RelA showed a transient increase with a maximum level at 2 h poststimulation at the GM-CSF similar to the IL-2 promoter (Fig 3.4D).

Therefore, these ChIP results revealed that the two NF-κB transcription factors, c-Rel and RelA have distinct kinetics of binding to the IL-2 and GM-CSF proximal promoter regions in EL-4 T cells.
Figure 3.2  Time course of IL-2 mRNA expression in EL-4 T cells

Quantitative real-time PCR was performed on cDNA prepared from nonstimulated (0 h) or PMA/I stimulated EL-4 T cells for the indicated time periods to detect IL-2 mRNA. The results are expressed as the fold change in IL-2 gene expression levels over nonstimulated (NS) cells. To calculate the relative mRNA levels, cycle threshold (Ct) values of the reference housekeeping gene (Ct (ref)) (ubc) were subtracted from Ct values determined for target genes of interest (Ct (target)) which generates ΔΔCt or Ct (target) - Ct (ref). The resulting Ct value is then raised to the power 2 to give the final relative mRNA levels (2^{-ΔΔCt}). To calculate the fold change in mRNA expression levels upon stimulation, the relative mRNA levels in activated cells was normalized to the non-stimulated level (2^{-ΔΔCt (activated)}/2^{-ΔΔCt (nonstimulated)}). The results are shown as the mean and standard deviation (SD) of three independent experiments.
Figure 3.3  mRNA expression profiles and nuclear accumulation kinetics of NF-κB c-Rel and RelA in EL-4 T cells

(A and B) Cells were cultured without or with PMA/I for 1, 2, 4, 8, and 16 h. Relative mRNA levels of c-Rel and RelA were measured by quantitative real-time PCR using specific primer sets. To calculate the relative mRNA levels, C_t values of the reference housekeeping gene (C_t(ref)) (ubc) were subtracted from C_t values determined for target genes of interest (C_t(target)) which generates ΔΔC_t or C_t (target) - C_t (ref). The resulting C_t value is then raised to the power 2 to give the final relative mRNA levels (2^-ΔΔC_t). The results are shown as the mean and standard deviation of three separate experiments.

(C) EL-4 T cells were cultured with or without PMA/I for the indicated time periods. Nuclear protein extracts (20 μg) from each time point were subjected to western blotting using specific antibodies to c-Rel, RelA, and the nuclear protein Sp1 as a loading control.
**IL-2**

**A**
- c-Rel binding activity (Normalized to 11)
- NA
- IP

**B**
- RlA binding activity (Normalized to 11)
- Set F
- Set B

**GM-CSF**

**C**
- c-Rel binding activity (Normalized to 11)

**D**
- RlA binding activity (Normalized to 11)

Time after PMA/I stimulation (h)
Figure 3.4 Kinetics of binding of NF-κB c-Rel and RelA to the \textit{IL-2} and \textit{GM-CSF} promoters in EL-4 T cells

(A and B) Nuclei were extracted from nonstimulated (0 h) or PMA/I stimulated EL-4 T cells for 1, 2, 4, 8, and 16 h. ChIP assays were performed with anti-c-Rel and anti-RelA antibodies. Quantitative real-time PCR was performed on the immunoprecipitated DNA using the \textit{IL-2} primers amplifying near the TSS (Set B) and -2 kb upstream from the TSS (Set F) (Fig 3.1A). The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting \( C_t \) values of TI DNA from IP \( C_t \) values (\( C_{t, IP} - C_{t, TI} \)) and TI from NA (\( C_{t, NA} - C_{t, TI} \)). The resulting \( C_t \) values is then raised power 2 to give the final relative amount of ChIP DNA (\( 2^{-\Delta \Delta C_t (IP-TI)} \) or \( 2^{-\Delta \Delta C_t (NA-TI)} \)). The results are shown as the mean and standard deviation of three to five independent biological experiments.

(C and D) Quantitative real-time PCR was performed on the same ChIP DNA immunoprecipitated with anti-c-Rel and anti-RelA antibodies (A and B), with \textit{GM-CSF} specific primer Set A covering the CD28RR at the \textit{GM-CSF} proximal promoter (Fig 3.1B). The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting \( C_t \) values of TI DNA from IP \( C_t \) values (\( C_{t, IP} - C_{t, TI} \)) and TI from NA (\( C_{t, NA} - C_{t, TI} \)). The resulting \( C_t \) values is then raised power 2 to give the final relative amount of ChIP DNA (\( 2^{-\Delta \Delta C_t (IP-TI)} \) or \( 2^{-\Delta \Delta C_t (NA-TI)} \)). The results are shown as the mean and standard deviation of three independent biological experiments.
3.2.2 Kinetics of binding of transcription factors c-Rel and RelA to the IL-2 and GM-CSF promoters in primary CD4⁺ T cells

To investigate the in vivo binding activity of c-Rel and RelA in primary CD4⁺ T cells isolated from mouse spleens, cells were left nonstimulated or stimulated with PMA/I for the indicated time periods. Total RNA was isolated from these cells to ensure that the cells were appropriately stimulated and IL-2 mRNA levels were measured by real-time PCR as a control (Fig 3.5A). In addition, quantitative real-time PCR was performed to determine mRNA expression profiles of c-Rel and RelA (Fig 3.5B and C). As observed in EL-4 T cells, low levels of c-Rel mRNA was measured in nonstimulated T cells and a rapid increase was observed following stimulation (nearly 10-fold), peaking at 2 h in primary CD4⁺ T cells (Fig 3.5B). Interestingly, RelA mRNA showed a much smaller increase after stimulation (Fig 3.5C). Thus, c-Rel and RelA mRNAs have distinct expression profiles in CD4⁺ T cells.

In order to examine the protein profiles of c-Rel and RelA in the nucleus, nuclei were extracted from primary CD4⁺ T cells isolated from mouse spleens. As observed in EL-4 T cells, c-Rel and RelA proteins were not found in the nucleus of nonstimulated primary CD4⁺ T cells. Higher levels of c-Rel protein were detected at a late time point (8 h) compared to an early time (1 h) poststimulation (Fig 3.5D), while increased RelA proteins were detected as early as 1 h in the nucleus and were reduced at late times (8 h) following T cell activation (Fig 3.5D). These observations suggest that the c-Rel nuclear accumulation occurs slowly and is maintained for longer relative to RelA in the nucleus of both EL-4 and primary CD4⁺ T cells following T cell activation.

To investigate the binding of c-Rel and RelA to the IL-2 and GM-CSF promoter regions in CD4⁺ T cells, in vivo binding assays were performed with antibodies specific for c-Rel and RelA using ChIP. Quantitative real-time PCR was performed on ChIP DNA using primer sets to amplify regions near the TSS, the -2 kb upstream control region of the IL-2 promoter, and the region covering the CD28RR of the GM-CSF promoter. As shown in Figure 3.6A, ChIP analysis revealed that c-Rel binding gradually increased in a time-dependent manner, with a decrease at around 16 h following stimulation. A relatively low binding activity was observed at the upstream control region of the IL-2 promoter (Set F) with no increase following stimulation. As shown in EL-4 T cells, RelA binding occurred rapidly at the proximal promoter region (Set B) in primary CD4⁺
T cells with a maximum level at 2 h and a gradual decrease by 16 h following PMA/I stimulation (Fig 3.6B). Similar profiles were observed at the GM-CSF promoter. In this case, c-Rel binding activity was detected before stimulation and gradually increased up to 4 to 8 h at the proximal promoter (Fig 3.6C), while the maximum binding of RelA was observed at 2 h following stimulation with a gradual decrease by 16 h (Fig 3.6D).

These results show distinct binding patterns for c-Rel and RelA at the cytokine gene promoters in both primary and EL-4 T cells, suggesting that they may play distinct roles in the regulation of the IL-2 and GM-CSF genes.
Figure 3.5 mRNA expression profiles and protein kinetics in primary CD4\(^+\) T cells

(A) IL-2 mRNA expression profile in primary CD4\(^+\) T cells. Quantitative real-time PCR was performed on cDNA prepared from nonstimulated (0 h) or PMA/I stimulated primary CD4\(^+\) T cells isolated from mouse spleens for the indicated time periods. The results are expressed as the fold change in IL-2 gene expression levels over nonstimulated cells. To calculate the relative mRNA levels, cycle threshold (C\(_T\)) values of the reference housekeeping gene (C\(_T\) (ref)) (ubc) were subtracted from C\(_T\) values determined for target genes of interest (C\(_T\) (target)) which generates ΔΔC\(_T\), or C\(_T\) (target) - C\(_T\) (ref). The resulting C\(_T\) value is then raised to the power 2 to give the final relative mRNA levels (2\(^{-ΔΔC_T}\)). To calculate the fold change in mRNA expression levels upon stimulation, the relative mRNA levels in activated cells was normalized to the non-stimulated level (2\(^{-ΔΔC_T\text{(activated)}}\)/2\(^{-ΔΔC_T\text{(nonstimulated)}}\)). The results are shown as the mean and standard deviation (SD) of three independent experiments.

(B and C) Time course of c-Rel and RelA mRNA expression in primary CD4\(^+\) isolated from mouse spleens. Cells were cultured without or with PMA/I for 1, 2, 4, 8, and 16 h. Relative mRNA levels of c-Rel and RelA were measured by quantitative real-time PCR using specific primer sets. To calculate the relative mRNA levels, C\(_T\) values of the reference housekeeping gene (C\(_T\) (ref)) (ubc) were subtracted from C\(_T\) values determined for target genes of interest (C\(_T\) (target)) which generates ΔΔC\(_T\), or C\(_T\) (target) - C\(_T\) (ref). The resulting C\(_T\) value is then raised to the power 2 to give the final relative mRNA levels (2\(^{-ΔΔC_T}\)). The results are shown as the mean and standard deviation of three separate experiments.

(D) Primary CD4\(^+\) T cells were left unstimulated or stimulated with PMA/I for the indicated time periods. Nuclear protein extracts (10 μg) from each time point were subjected to western blotting using antibodies specific for c-Rel, RelA, and Sp1 as a loading control.
IL-2

A

B

Set F  
Set B

RelA binding activity (Normalized to TI)

0.16
0.12
0.08
0.04
0

0  1  2  4  8  16

0.15
0.12
0.09
0.06
0.03
0

0  1  2  4  8  16

GM-CSF

C

D

Time after CD3/CD28 stimulation (h)
Figure 3.6 *In vivo* binding assays of c-Rel and RelA at the *IL-2* and *GM-CSF* promoters in primary CD4^+ T cells

(A and B) ChIP analysis of c-Rel and RelA transcription factor binding at the *IL-2* promoter was performed in primary T cells isolated from mouse spleens. Primary CD4^+ T cells were nonstimulated (0 h) or stimulated for 1, 2, 4, 8, and 16 h with PMA/I followed by nuclear extraction. ChIP DNA fragments were immunoprecipitated with anti-c-Rel and anti-RelA antibodies followed by quantitative real-time PCR with specific primers, amplifying near the TSS (Set B) and the -2 kb upstream region (Set F) upstream of the *IL-2* promoter (Fig 3.1A). The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting C_t values of TI DNA from IP C_t values (C_{t,IP} - C_{t,TI}) and TI from NA (C_{t,NA} - C_{t,TI}). The resulting C_t values is then raised power 2 to give the final relative amount of ChIP DNA (2^(\Delta\Delta C_t (IP-TI)) or 2^(\Delta\Delta C_t (NA-TI))). The results are shown as the mean and standard deviation of three independent biological experiments.

(C and D) Quantitative real-time PCR was performed on the same immunoprecipitated DNA used above (A and B), using a *GM-CSF* primer Set A covering the CD28RR at the *GM-CSF* proximal promoter (Fig 3.1B). The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting C_t values of TI DNA from IP C_t values (C_{t,IP} - C_{t,TI}) and TI from NA (C_{t,NA} - C_{t,TI}). The resulting C_t values is then raised power 2 to give the final relative amount of ChIP DNA (2^(\Delta\Delta C_t (IP-TI)) or 2^(\Delta\Delta C_t (NA-TI))). The results are shown as the mean and standard deviation of two or four independent experiments.
3.2.3 AP-1 family c-Jun and ZEB1 bind to the *IL-2* and/or *GM-CSF* proximal promoter region in EL-4 T cells

In order to investigate the binding kinetics of other transcriptional activators such as the AP-1 family member c-Jun, which is also known to play an important role in *IL-2* gene expression (Jain et al. 1992; Jung et al. 1995; Sundstedt et al. 1998), EL-4 T cells were left unstimulated or stimulated with PMA/I for the indicated time points. As shown in Figure 3.7A, very low binding activity was observed prior to stimulation. However, c-Jun binding activity was significantly increased as early as 1 h following stimulation, peaking at 2 h near the TSS of the *IL-2* gene compared to the upstream control region. These results show a similar profile to that obtained from RelA (Figs 3.4B and 3.6B), suggesting that c-Jun may play similar roles in *IL-2* gene expression as RelA. Interestingly, the occupancy of the *GM-CSF* proximal promoter by c-Jun revealed a completely different behaviour, showing an increase at 1 h but a sustained binding profile up to 16 h poststimulation in EL-4 T cells (Fig 3.7B).

Thus, these results suggest that c-Jun may play a role in the initiation of *IL-2* gene transcription together with RelA in T cells and has different kinetics on the two genes.

To investigate the kinetics of the ZEB1 repressor binding during *IL-2* gene expression, ChIP assays were performed in EL-4 T cells at the indicated time points. Interestingly, strong ZEB1 binding to the *IL-2* proximal promoter region was observed in resting T cells (Fig 3.8) relative to the upstream control region or binding to a control gene *Rho*. This binding remained relatively constant up to 8 h following PMA/I, with a small decrease at 4 h and significantly increased at 16 h poststimulation (two-fold) (Fig 3.8), when RelA and c-Jun bindings had returned to near the baseline levels in EL-4 T cells (Figs 3.4B and 3.7A). This trend was not found either at the -2 kb upstream region or at the *Rho* gene used as a negative control (Fig 3.8).

Taken together, these results suggest that ZEB1 directly binds to the *IL-2* proximal region in both resting and stimulated T cells and plays an opposite role in *IL-2* gene expression. In support of this, ZEB1 represses the *IL-2* promoter by cooperating with corepressors CtBP and HDAC in EL-4 T cells (Wang et al. 2009). However, at the moment, it is not clear why ZEB1 should be maintained at the promoter when a gene is
activated. It is interesting to postulate that ZEB1 repressor may maintain a balance of transcription rate at the IL-2 gene promoter.
IL-2

A

\[ \text{c-Rel binding activity (Normalized to TI)} \]

\[ \begin{align*}
0 & \quad 0.12 \\
1 & \quad 0.09 \\
2 & \quad 0.06 \\
4 & \quad 0.03 \\
8 & \quad 0.00 \\
16 & \quad 0.00 \\
\end{align*} \]

GM-CSF

B

\[ \text{RelA binding activity (Normalized to TI)} \]

\[ \begin{align*}
0 & \quad 0.06 \\
1 & \quad 0.04 \\
2 & \quad 0.02 \\
4 & \quad 0.00 \\
8 & \quad 0.00 \\
16 & \quad 0.00 \\
\end{align*} \]

Time after PMA/I stimulation (h)

Figure 3.7 Recruitment profiles of AP-1 family c-Jun at the IL-2 and GM-CSF promoters

EL-4 T cells were nonstimulated (0 h) or stimulated with PMA/I for the indicated time points followed by nuclear extraction. ChIP DNA was immunoprecipitated with an anti-c-Jun antibody.

(A) Quantitative real-time PCR was performed with specific primers: Set B, which amplifies near the TSS and Set F, which amplifies the -2 kb upstream region of the IL-2 gene promoter used as a negative control (Fig 3.1A).
(B) Quantitative real-time PCR was performed on the same immunoprecipitated DNA used above (A), using a GM-CSF primer Set J (Appendix I) covering the TSS containing AP-1/NFAT sites of the GM-CSF proximal promoter (Fig 3.1B). The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting $C_t$ values of TI DNA from IP $C_t$ values ($C_t^{IP} - C_t^{TI}$) and TI from NA ($C_t^{NA} - C_t^{TI}$). The resulting $C_t$ values is then raised power 2 to give the final relative amount of ChIP DNA ($2^{-\Delta \Delta C_t^{(IP-TI)}}$ or $2^{-\Delta \Delta C_t^{(NA-TI)}}$). The results are shown as the mean and standard deviation of three separate experiments.
Figure 3.8 ZEB1 binding profiles at the IL-2 proximal promoter region in EL-4 T cells

EL-4 T cells were nonstimulated (0 h) or stimulated with PMA/I for the indicated time periods followed by nuclear extraction. Genomic DNA was sheared by sonication and immunoprecipitated with antibodies specific for ZEB1. Quantitative real-time PCR was performed on immunoprecipitated genomic DNA fragments with specific primers amplifying the regions indicated: Set B (near the TSS) and Set F (-2 kb region) (Fig 3.1A). Rho primers were used as a negative control. To quantify the relative amounts of ChIP DNA, Ct values in no antibody (NA) condition was subtracted from the Ct values of immunoprecipitated DNA (IP) and then normalized to the amount of relative DNA of total input (TI) ((Ct_{IP} - Ct_{NA})/Ct_{TI}). The resulting Ct values is the raised power 2 to give the final relative amount of ChIP DNA (2^{-ΔΔCt (IP-NA)/TI}). Significant p-values calculated using paired T tests are shown on the top of the panel. The results are shown as the mean and standard deviation of three separate experiments.
3.3 Discussion

In this chapter, the \textit{in vivo} kinetics of binding of the NF-κB transcription factors, c-Rel and RelA, were investigated at the inducible \textit{IL-2} and \textit{GM-CSF} proximal promoter regions in both EL-4 and primary CD4\textsuperscript{+} T cells. These experiments were carried out: a) to confirm that these transcription factors did indeed bind to these cytokine promoters in T cells \textit{in vivo}; and b) as a prelude to performing ChIP-on-chip experiments to determine genome-wide binding profiles. The most important result of this study is that c-Rel and RelA display distinct kinetics of binding at the \textit{IL-2} and \textit{GM-CSF} gene promoters in both EL-4 and primary CD4\textsuperscript{+} T cells. These results support previous \textit{in vitro} binding studies performed in human T cells, showing that EMSA complexes on the \textit{IL-2} gene were composed mainly of RelA after 1 h of activation while c-Rel was the dominant component at 6 h poststimulation (Himes et al. 1996; Wang et al. 2009). Consistent with the ChIP results, the nuclear accumulation of c-Rel protein was delayed compared with RelA protein, which appeared early and was only transiently maintained in the nucleus following stimulation. Consistent with results shown in this chapter, many previous studies have reported that c-Rel accumulates slowly and is maintained for longer than RelA in the nucleus following stimulation of T cells (Bryan et al. 1994; Himes et al. 1996; Brettingham-Moore et al. 2005), suggesting that they may play distinct roles in the regulation of cytokine gene expression.

NF-κB transcription factors play an important role in modifying chromatin structure and in the regulation of genes by interacting or recruiting other factors (Zhong et al. 1998; Vanden Berghe et al. 1999; Barboric et al. 2001; Zhong et al. 2002; Holloway et al. 2003). For example, RelA directly interacts with the HAT complex, CBP/p300, resulting in a synergistic effect in the activation of RelA-dependent genes (Zhong et al. 1998; Vanden Berghe et al. 1999). A study by Holloway \textit{et al.} demonstrated that RelA is involved in the recruitment of the ATPase component of the SWI/SNF chromatin remodeling complex, Brg1 to the \textit{GM-CSF} promoter (Holloway et al. 2003). Furthermore, RelA promotes the initiation of transcription and transcriptional elongation by recruiting the positive transcription elongation factor b (P-TEFb) which phosphorylates the C-terminal domain of RNA Pol II (Barboric et al. 2001). It has been suggested that RelA alone is insufficient to generate a remodeled chromatin at the \textit{GM-CSF} promoter and is not essential for \textit{IL-2} gene expression (Rao et al. 2003;
Brettingham-Moore et al. 2005). These results imply that RelA may be required for regulating chromatin remodeling and gene expression of the IL-2 and GM-CSF genes by cooperating with other factors involved in these events. In support of this idea, previous studies have suggested that an association between RelA and p300 synergistically activates the GM-CSF gene (personal communication, Karen Bunting). However, given the kinetics of RelA in the nucleus shown in this chapter and in other studies, RelA may be required for the initiation of chromatin remodeling and gene expression at the IL-2 and GM-CSF genes, rather than maintenance of these events in T cells. A study by Rao et al. reported that chromatin remodeling at the IL-2 gene promoter region occurs as early as 1.5 h following stimulation as measured by MNase and restriction enzyme accessibilities (Rao et al. 2001). In addition, MNase accessibility increased significantly at the GM-CSF promoter region after 1 h of stimulation (Holloway et al. 2003). It is also worth noting that RelA appearance at the IL-2 promoter region and histone acetylation at the -2 kb upstream from the TSS occur very early following stimulation (Chen et al. 2005), suggesting that there may be a link between these two events. Thus, this rapid chromatin disruption may be associated with an early binding of RelA to the promoters following activation in T cells.

It has been reported that c-Rel is essential for chromatin remodeling and gene expression at the IL-2 and GM-CSF genes in T cells (Rao et al. 2003; Brettingham-Moore et al. 2005; Chen et al. 2005). In the absence of c-Rel, chromatin accessibility was reduced across these gene promoters and gene expression was impaired in T cells (Rao et al. 2003; Brettingham-Moore et al. 2005; Chen et al. 2005). However, unlike RelA, it does not seem likely that c-Rel directly recruits the p300 HAT complex to change chromatin structure at the IL-2 and GM-CSF promoters, because in previous studies, a synergistic effect between c-Rel and p300 was not observed at the GM-CSF promoter and there was no physical interaction detected between these two factors (personal communication, Karen Bunting). Interestingly, it has been reported that a combination of c-Rel and p300 enhances the IL-12p40 promoter activity relative to c-Rel or p300 alone (Sun et al. 2004), suggesting that a synergistic effect between c-Rel and p300 may be gene or cell-specific.

It is of interest to note that c-Rel and RelA showed distinct kinetics of accumulation and binding in the nucleus of T cells. These results suggest that the entry of c-Rel and RelA to the nucleus is controlled differently, even though they are both members of the NF-
κB transcription factor family. It is possible that different T cell signals may be involved in activation of these transcription factors. Previous studies demonstrated that RelA is bound by both IκBα and IκBβ while c-Rel is only associated with IκBβ inhibitory molecule in T cells, which is not targeted by TCR-initiated signals, hence allowing only RelA induction in T cells (Tam et al. 2001; Banerjee et al. 2005). As suggested in previous studies, new protein synthesis may be associated with nuclear accumulation of c-Rel (Venkataraman et al. 1995; Rao et al. 2003; Brettingham-Moore et al. 2005). One report suggested that c-Rel/IκBβ complexes are altered to c-Rel/IκBα by proinflammatory cytokines such as IL-1 and TNF-α, which is then easily degraded by TCR-mediated signalling pathways, allowing rapid translocation of c-Rel to the nucleus followed by activation of cytokine genes such as IL-2 (Banerjee et al. 2005). Thus, these mechanisms may be associated with the late response of c-Rel to T cell signals and slower c-Rel accumulation in the nucleus compared to RelA in T cells.

The ChIP results presented here showed a slow and gradual increase in the binding profile of c-Rel across the IL-2 and GM-CSF gene promoters following stimulation. Other studies have reported that c-Rel is more stably maintained in the nucleus relative to other transcription factors such as RelA after stimulus removal, suggesting that this stable c-Rel may be associated with the maintenance of a stable chromatin remodeled status in T cells (Brettingham-Moore et al. 2005). Chromatin remodeling is maintained up to 16 and 24 h at the IL-2 and GM-CSF promoters in response to T cell activation respectively (Rao et al. 2001; Brettingham-Moore et al. 2005). Thus, the gradual and late appearance of c-Rel to the nucleus observed in these studies may be associated with a stable maintenance of remodeled chromatin until expression of the IL-2 and GM-CSF genes is complete. Further experiments to study how c-Rel maintains a modifying chromatin structure across the IL-2 and GM-CSF promoters would be of interest. It is interesting to speculate that c-Rel may block reformation of a nucleosome across the promoter region during gene expression.

In summary, this chapter showed that different transcriptional activators have distinct kinetics of binding at the inducible IL-2 and GM-CSF promoters following stimulation in T cells. Different binding profiles may be associated with distinct functions in the regulation of target genes. The translocation of c-Rel and RelA to the nucleus does not occur at the same time, suggesting that they may be affected by distinct intracellular
signalling pathways. Therefore, these different mechanisms may lead to nonoverlapped or distinct roles for c-Rel and RelA in gene regulation in T cells.
Chapter 4

Identification and characterization of NF-kB (c-Rel and RelA) binding regions using ChIP-on-chip in EL-4 T cells
Chapter 4

Identification and Characterization of NF-κB (κB-Ret and RelA) Binding Regions Using Chip-on-Chip in EL-4 T Cells
4.1 Introduction

Chromatin immunoprecipitation (ChIP) is a powerful technique to study in vivo binding of proteins to specific regions of the genome, allowing the examination of the protein-DNA interactions that occur in living cells. However, the ChIP method is limited to investigating a small number of genes and/or known binding sites and does not allow the identification of novel binding sites (reviewed in Buck et al. 2004; Schreiber et al. 2006; Tong et al. 2009). But when ChIP is combined with genome-wide approaches and computational analysis, this method becomes a powerful tool to study the entire genomic spectrum of binding sites for a transcription factor of interest. Thus, the identification of transcription factor binding sites (TFBSs) occupied by specific transcription factors through genome-wide location approaches has become attractive, and indeed essential, for more extensive investigation of transcription regulatory patterns, gene regulatory networks, and genomic regulatory regions (Long et al. 2004; Tong et al. 2009). For these reasons, a genome-wide survey of in vivo interactions between DNA and transcription factors using a method known as ChIP-on-chip has become widely performed (Horak et al. 2002; Lee et al. 2002; Beima et al. 2006; De Siervi et al. 2009; Frontini et al. 2009; Song et al. 2009). ChIP-on-chip combines chromatin immunoprecipitation with DNA microarray technology in which ChIP DNA is amplified and/or reamplified, subsequently fluorescently labelled and hybridized to DNA microarrays containing genomic (or promoter) DNA sequences. In addition to ChIP-on-chip, many other ChIP-based high throughput techniques have been developed to investigate the location of TFBSs on a genome-wide scale including ChIP-sequencing (ChIP-seq), ChIP-serial analysis of binding elements (SABE), ChIP-serial analysis of chromatin occupancy (SACO), ChIP-sequence tag analysis of genomic enrichment (STAGE), genome-wide mapping technique (GMAT), and ChIP-paired end tag (ChIP-PET) (Chen et al. 2005; Wei et al. 2006; Barski et al. 2007; Bhinge et al. 2007; Yochum et al. 2007; Roh et al. 2008).

Chromatin immunoprecipitation combined with microarray (ChIP-on-chip) has been extensively used to investigate transcriptional regulatory networks and to identify TFBSs in yeast (Iyer et al. 2001; Lee et al. 2002). In addition, these approaches have been successfully applied to mammalian cells for the identification of target DNA binding sites of many transcription regulatory factors such as GATA-1, E2F, and pRb
(Horak et al. 2002; Weinmann et al. 2002; Wells et al. 2003). In addition to searching for TFBSs, ChIP-on-chip has been used for many other studies. For example, a microarray-based location analysis of binding sites of components of the yeast transcription machinery such as RNA polymerase III and associated factors (TFIIB and TFIIIC) has been performed across the yeast genome (Harismendy et al. 2003; Moqtaderi et al. 2004). In addition, it has been used to investigate components of the epigenome such as the identification of genes silenced by H3K9 methylation (Kondo et al. 2004). The investigation of the function of the histone deacetylase family, including Rpd3 and Hda1, has also been performed on a genome-wide scale (Robyr et al. 2002).

These microarray-based studies brought an innovative development in the survey of in vivo protein-DNA interactions, allowing the high throughput exploration of genome-wide transcription regulatory networks in living cells. The identification of TFBSs is often the first step in predicting the target genes of transcription factors on a genome-wide scale. Thus, ChIP-on-chip permits not only identification of a great number of known and novel binding sites of transcription factors on genomic DNA, but when combined with computational analysis can identify the nearest genes to these binding sites, implicating the transcription factor in their regulation. For this reason, the ChIP assay developed and described in Chapter 3 was combined with DNA microarray to perform a genome-wide location analysis of binding sites of c-Rel and RelA. Computational analysis was then used for the identification of the nearest genes to these binding sites that potentially could be regulated by c-Rel and RelA. In order to determine the likelihood of the genes being a target of the transcription factor, it is necessary to investigate the interaction between the binding of the transcription factor and the regulation of the nearest genes. Thus, ChIP-on-chip was combined with available expression profiling to investigate whether the nearest genes are directly regulated by the transcription factor, and these findings are presented in this chapter.

The aims of the study presented in this chapter were:

♦ To identify genomic regions bound by the c-Rel and RelA transcription factors.

♦ To investigate the nearest genes to the c-Rel and RelA binding regions and combine these data with available expression profiling data to determine groups of c-Rel or RelA target genes.
To find transcription factor family motifs overrepresented in the sequences bound by the c-Rel and RelA transcription factors.

The data presented in this chapter show that:

- c-Rel and RelA have overlapping but distinct genomic location profiles and their binding regions are not limited to proximal promoter regions of genes.

- A small number of nearest genes to c-Rel and RelA binding regions are inducible in T cells and/or are c-Rel-dependent in T cells.

- IRF transcription factor family motif(s) are significantly enriched in the genomic regions bound by c-Rel and RelA, suggesting a functional interaction between the two transcription factor families.
4.2 Results

4.2.1 Genome-wide analysis of c-Rel and RelA binding regions using ChIP-on-chip

Using ChIP-on-chip, genome-wide location analysis of regions bound by c-Rel and RelA was performed in EL-4 murine T cells that were nonstimulated or stimulated with PMA/I for 2 h and 8 h. Since c-Rel displayed the strongest binding to the interleukin-2 (IL-2) promoter at 8 h after stimulation, while the highest binding of RelA was observed at 2 h (Chapter 3), these two time points were used to carry out the genome-wide survey for c-Rel and RelA binding sites respectively. Thus, immunoprecipitated samples from 8 h for c-Rel and 2 h for RelA were used for DNA microarray respectively. In addition, samples from total input and mock immunoprecipitation were used as controls and were an equal mixture from the nonstimulation and two time points (Fig 4.1A). Three biological replicates were performed for ChIP-on-chip experiments. ChIP-on-chip data were sequentially analyzed using several software programs including the Model-based Analysis of Tiling-arrays (MAT), Integrated Genome Browser (IGB, Affymetrix), Partek® Genomics Suite™ 6.4 (ANOVA and Principal Components Analysis (PCA) (Appendix II)), Genomatix software, and UCSC Genome Browser (Fig 4.1B).
Figure 4.1 Preparation of DNA for ChIP-on-chip microarray and overview of ChIP-on-chip data analysis

(A) Chromatin immunoprecipitation (ChIP) was performed with nonstimulated (NS, 0 h) or stimulated EL-4 T cells for 2 h and 8 h with PMA/I. ChIP immunoprecipitated samples from 8 h for c-Rel (c-Rel 8 h) and 2 h for RelA (RelA 2 h) were chosen for DNA microarray respectively. The total input and no antibody samples of an equal mixture of the NS and the two time points were used as controls respectively. Each sample was amplified and/or reamplified and DNA microarray was performed in triplicate with these four amplified samples (12 samples in total). (B) All raw data from the three ChIP-on-chip replicates were stored in CEL files and analyzed by the Model-based Analysis of Tiling-arrays (MAT) algorithm. MAT generates bar files which were used for visualisation on the Integrated Genome Browser (IGB). CEL files were also imported into the Partek® Genomics Suite™ 6.4 software and ANOVA (Analysis of Variance) was used to identify enriched regions by comparing c-Rel and RelA-enriched
regions with controls (Total Input and No Antibody). Motif analysis was performed by inputting significantly enriched regions detected by the Partek software into the Genomatix software program to identify strongly overrepresented transcription factor family motif(s). The Genomatix software was also used to detect genes nearest to the regions bound by c-Rel and RelA. Principal Components Analysis (PCA) was used to normalize and to compare the replicates in the Partek® Genomics Suite™ 6.4 software.
Comparison of ChIP-on-chip replicates using Model-based Analysis of Tiling-arrays (MAT) algorithm

To compare the three different replicates, the MAT algorithm (Johnson et al. 2006) was used which utilises the multiple readings within sliding windows of base pairs to obtain a probability score of the signal strength from single arrays. Using the matched total input sample as the control, the individual c-Rel, RelA and no antibody replicates were analysed in MAT using the NCBI version 36 (mm8) build of the mouse genome. The following parameters were used: a bandwidth of 200 bp (half of the average ChIP fragment length), maximum gap 150 bp (maximum distance between positive probes), minimum number of significant probes (at least 7), and a p value cut-off of 0.01. MAT generated a bar file which contained a MAT score for each probe and was used for visualizing enriched signals in the IGB software.

To visualize data from MAT, the resulting bar files were imported into the IGB software and the three ChIP-on-chip replicates were compared. Binding at the inducible *IL-2* gene promoter region was examined to visualize the ChIP-on-chip replicates since ChIP-PCR has clearly shown this gene promoter bound both c-Rel and RelA. As shown in Figure 4.2, two out of the three replicates showed signals at the *IL-2* promoter region for both c-Rel and RelA, matching the ChIP-PCR data. The average of the three replicates displayed stronger signals for both c-Rel and RelA compared with the no antibody average signals again supporting the identification of the *IL-2* promoter as a binding region for c-Rel and RelA. Interestingly, the RelA signal was stronger than that of the c-Rel signal at the *IL-2* promoter; in particular, the average signal for RelA was much stronger than that for c-Rel and no antibody averages.

Taken together, these results showed that c-Rel and RelA binding was enriched at the *IL-2* promoter region, supporting the ChIP results shown in Chapter 3 and providing evidence that the ChIP-on-chip approach had worked.
c-Rel
1st
2nd
3rd
Combination

RelA
1st
2nd
3rd
Combination

No Antibody
1st
2nd
3rd
Combination

y-axis scale = Minimum: 0; Maximum: 0.8
y-axis: log10 scale

Figure 4.2 Visual comparison of ChIP-on-chip signals near the IL-2 gene promoter

Antibodies specifics for c-Rel and RelA were used for chromatin immunoprecipitation of genomic DNA followed by fluorescent labeling and hybridization on DNA microarrays (Affymetrix Mouse promoter array 1.0R). ChIP-on-chip data was analysed by the Model-based Analysis of Tiling-arrays (MAT). To visualize data from MAT, the resulting bar files were input into the IGB software. Signals from three ChIP-on-chip replicates are shown at the IL-2 gene promoter region used as an example. Signals from no antibody were used as a control to compare with those obtained from c-Rel and RelA. Signals shown within the boxes marked by “combination” are the average of the three independent replicates. The blue arrow indicates the direction of transcription. Signals are shown with the same y-axis scale for each plot (minimum zero (0) and maximum 0.8). Data on the y-axis are presented in log10 scale and the x-axis shows genome positions in base pairs. The number in green (37, 317, 423) means the base pair location on chromosome 3.
4.2.3 Identification of enriched regions bound by NF-κB family members c-Rel and RelA using Partek® Genomics Suite™ software

To further characterise regions enriched by c-Rel and RelA, 12 CEL files, which contain raw data from three independent experiments including controls (total input and no antibody) were analyzed by the Partek® Genomics Suite™ 6.4 software. Bound regions were identified using the following parameters: minimum number of probes in a region (at least 7), 0.1 probe not in mean (fraction of the lowest 10% and highest 10% of the probes excluded from calculation of mean (average)), and a maximum bandwidth of 500 bp (average ChIP fragment length). Analysis of Variance (ANOVA) was used to identify enriched regions by comparing c-Rel or RelA-enriched regions with controls (total input and no antibody). c-Rel or RelA samples were compared against total input to detect enriched regions compared to total genomic DNA. In addition, to ensure that the regions detected were specific for the antibody used, the samples were compared against no antibody samples. A total of 16104 and 16377 enriched regions were detected at a p-value threshold of 0.05 by comparing c-Rel against total input (c-Rel vs TI) and c-Rel against no antibody (c-Rel vs NA) respectively (Fig 4.3A). In addition, 15452 and 17479 enriched regions were uncovered through RelA vs TI and RelA vs NA comparisons respectively under the same conditions (p<0.05) (Fig 4.3B). To identify highly enriched regions, the overlap of the two comparisons was used. A total of 1552 (average length: 340 bp) and 2463 (average length: 410 bp) significant regions were detected for c-Rel and RelA respectively (p<0.05) (Fig 4.3A and B). If a higher stringency p-value of less 0.01 was used, then 81 of the 1552 c-Rel enriched regions and 276 out of the 2463 regions enriched by RelA were identified (Fig 4.3C). These highly enriched regions were used for further studies such as motif analysis.
4.2.4 Identification of transcription factor family motifs that are significantly overrepresented in the NF-κB-bound regions

Short DNA sequences bound by transcription factors are referred to as motifs. Motif-analysis was carried out to determine if the identified regions were enriched for NF-κB motifs and to identify other motifs that might be enriched in those regions. The factors that bound to these motifs could potentially cooperate with each other in gene regulation. By inputting the 81 c-Rel and 276 RelA highly significant regions detected by Partek® Genomics Suite™ 6.4 software (Section 4.2.3 and Fig 4.3C) into the Genomatix software program, significantly overrepresented transcription factor family motif(s) were detected in the sequences bound by c-Rel and RelA. Overrepresented motifs with a Z-score over 2 (equivalent to p<0.05) were selected (van Dijk et al. 2010).

As shown in Table 4.1, 13 and 14 overrepresented motifs with a Z-score of 2 or greater were found in the c-Rel and RelA regions respectively. Surprisingly, NF-κB transcription factor family motif(s) was classified as an underrepresented transcription factor motif(s) in c-Rel binding regions (Z-score = -0.63) (Table 4.1). This was unexpected as c-Rel is a member of the NF-κB family and is known to bind to NF-κB motif(s). However, NF-κB was the most highly overrepresented motif(s) in the regions bound by RelA (Z-score = 6.51). The interferon response factor (IRF) family motif(s) was the most highly overrepresented motif in the sequences bound by c-Rel (Z-score = 4) and was also overrepresented in the RelA bound regions (Z-score = 3.97) (Table 4.1). These results suggest that there may be a relationship between NF-κB and IRF transcription factors. In support of this, many previous studies have demonstrated that NF-κB and IRF transcription factors act synergistically in the regulation of gene expression (Drew et al. 1995; Neish et al. 1995; Saura et al. 1999; Azimi et al. 2000; Sgarbanti et al. 2008). The interaction between these two transcription factors will be explored in more detail in Chapter 5. In addition to IRF motif(s), other motifs including Positive regulatory domain 1 binding factor (V$PRDF or V$PRD1), Barbiturate-inducible element (V$BARB), and Brachyury gene, mesoderm developmental factor (V$BRAC) were also enriched in both c-Rel and RelA binding regions.

PRD1, which is well known as a repressor, is involved in immune function by regulating the expression of MHC-II gene expression that is controlled by the MHC Class II transactivator (CIITA) which in turn is regulated by the myeloid cell-specific promoter, CIITAP1. The binding of PRD1 to the CIITAP1 promoter suppresses CIITA
gene transcription, resulting in a decrease in the CIITA activator and in the silencing of MHC-II expression in mature dendritic cells (DCs). The C14ap1 promoter contains binding sites for NF-κB and IRFs adjacent to a PRD1 binding site where NF-κB and IRFs are essential for full activation of C14ap1 (Ghosh et al. 2001; Smith et al. 2011). A study using computational analysis showed that NF-κB binding sites are adjacent to BARB motifs at the 5’ upstream region of the CXCR1 chemokine receptor gene which is involved in the innate immune response (Leyva-Baca et al. 2008; Yin et al. 2010). Thus, PRD1 and BABR factors seem likely to be relevant to NF-κB and immune response. On the other hand, BRAC, which is a member of the T-box family transcription factor, is essential for posterior mesoderm formation in vertebrates (Martin et al. 2008). It has been reported that murine Brachyury gene protein has weak sequence similarities with NF-κB proteins including DNA-binding and dimerization motifs (Perrin-Schmitt 1992). Thus, BRAC may be less relevant to T cell immune response or NF-κB, but further investigation may be warranted.

Taken together, these results suggested that NF-κB motifs are enriched in the RelA binding regions, but not c-Rel regions. However, IRF motifs are enriched in both, suggesting that there may be an association between NF-κB and IRF transcription factors.
Figure 4.3 Identification of regions bound by NF-κB c-Rel and RelA

Analysis of Variance (ANOVA) in the Partek® Genomics Suite™ was used to identify enriched regions which are displaced in venn diagrams. Enriched regions were identified by comparing c-Rel (A) and RelA (B) -enriched regions with controls (Total Input (TI) and No Antibody (NA)) (p-value < 0.05). The set of regions in common between these two comparisons were chosen for further analysis. Common regions were subgrouped by a high (p<0.01) and less (p<0.05) stringent p-value threshold (C).
Table 4.1 Transcription factor family motifs highly enriched in c-Rel and RelA binding sequences

<table>
<thead>
<tr>
<th>Motifs enriched by the NF-κB transcription factors</th>
<th>c-Rel</th>
<th>RelA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription Factor Families</strong></td>
<td><strong>Z-Score</strong></td>
<td><strong>Transcription Factor Families</strong></td>
</tr>
<tr>
<td>VSIRFF</td>
<td>4</td>
<td>VSNFκB</td>
</tr>
<tr>
<td>V5BARB</td>
<td>3.92</td>
<td>V$\alpha$P1F</td>
</tr>
<tr>
<td>VSACBL</td>
<td>3.5</td>
<td>VSIRFF</td>
</tr>
<tr>
<td>V5PRDF</td>
<td>3.12</td>
<td>V5PRDF</td>
</tr>
<tr>
<td>V5FξκB</td>
<td>2.98</td>
<td>V5HAML</td>
</tr>
<tr>
<td>V5CEBP</td>
<td>2.28</td>
<td>V5MYBL</td>
</tr>
<tr>
<td>V5KSRSh</td>
<td>2.28</td>
<td>V5RREB</td>
</tr>
<tr>
<td>V5H5NF1</td>
<td>2.2</td>
<td>V5BARB</td>
</tr>
<tr>
<td>V5BRAC</td>
<td>2.16</td>
<td>V5κKRS</td>
</tr>
<tr>
<td>V5CHOP</td>
<td>2.13</td>
<td>V5κRS</td>
</tr>
<tr>
<td>V5EIVII</td>
<td>2.05</td>
<td>ORFκRE</td>
</tr>
<tr>
<td>V5BPTF</td>
<td>2.02</td>
<td>V5κXRF</td>
</tr>
<tr>
<td>V5MYT1</td>
<td>2.01</td>
<td>V5κXRF</td>
</tr>
<tr>
<td>V5NFκB</td>
<td>-0.63</td>
<td>V5κXRF</td>
</tr>
</tbody>
</table>

a. Transcription factor family motif(s) enriched in the sequences bound by c-Rel and RelA.

b. Transcription factor family motifs are distinguished according to the Z-score; a Z-score over 2 (p<0.05) is statistically significant; underrepresented NF-κB motif(s) (-) in the c-Rel binding regions are shown. V$\$ indicates the abbreviation for the vertebrate matrix group and O$\$ means the general core promoter elements as used in genomatix software suite.
It has been reported that RelA binding sequences are also recognized by c-Rel as they often form heterodimers, and therefore those sites can be cooccupied by both subunits (Martone et al. 2003). For example, in vitro EMSA has previously shown that c-Rel binding sequences are also bound by p65/p50 heterodimers at the GBP-1 promoter following stimulation with IL-1β and TNF-α (Naschberger et al. 2004). In addition, a study by Himes et al. showed that the CD28RE of the GM-CSF and IL-2 gene promoters are bound by both c-Rel and RelA transcription factors (Himes et al. 1996). Similarly, c-Rel and RelA bind to the CD28RE of the IL-2 promoter by forming a heterodimer which activates the CD28 enhancer synergistically (Lai et al. 1995). These studies suggest that the two transcription factors may cooperate in the regulation of gene expression. Therefore, the overlap between the c-Rel binding regions and RelA binding regions was examined. Interestingly, 25% (p<0.01) or 31% (p<0.05) of regions bound by c-Rel were also bound by RelA (Table 4.2). Thus, these results imply that at least a quarter of c-Rel bound regions observed in this chapter may be coregulated by both transcription factors. On the other hand, this implies that over 60% or 70% of the regions bound by the c-Rel subunit were not bound by RelA, suggesting that the c-Rel and RelA transcription factors may not act together in the regulation of many genes. It may be possible that the regions bound by c-Rel alone could be coregulated by other transcription factors or other NF-κB family members, since gene transcription is rarely regulated by an individual transcription factor (reviewed in Qiu 2003; Narlikar et al. 2009).

These results suggest that c-Rel and RelA may coregulate a subset of genes, but many c-Rel binding regions are not occupied by RelA in T cells, implying that each factor may play both independent and overlapping roles in the gene regulatory network of T cells.
Table 4.2 Percentage of regions bound by both c-Rel and RelA

<table>
<thead>
<tr>
<th>Cut-off (p-value)</th>
<th>Transcription factor</th>
<th>% bound by RelA</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01</td>
<td></td>
<td>25 %</td>
</tr>
<tr>
<td></td>
<td>c-Rel</td>
<td></td>
</tr>
<tr>
<td>&lt;0.05</td>
<td></td>
<td>31 %</td>
</tr>
</tbody>
</table>

a. Lower (p<0.05) and higher (p<0.01) stringency threshold p-values used to classify regions bound by both transcription factors.

b. Percentage of regions bound by RelA in the regions bound by c-Rel.
4.2.6 Analysis of the genomic location of transcription factor binding regions

It has been demonstrated that transcription factor binding sites are not limited to the promoter but scattered across the genomic DNA (Martone et al. 2003). Therefore, the location of the transcription factor binding regions of c-Rel and RelA detected by Partek® Genomics software was analyzed using the Genomatix software program. Among c-Rel binding regions, over half of the regions (53% or 60%) were detected upstream from the TSS. Similarly a high proportion (over 50%) of regions bound by RelA were also found upstream from the TSS. In addition, nearly 10% or 20% of c-Rel and RelA binding regions respectively classified with a high stringent cut-off (p<0.01) are present spanning the TSS (Table 4.3). These results indicate that most important regulatory regions occupied by NF-κB proteins are primarily concentrated upstream or near the promoter regions in EL-4 T cells. Strikingly, a significant portion (between 20% and 30%) of the bound regions are located within genes. It is interesting to note that a small proportion (≤ 5%) of binding regions are present in the genomic DNA beyond the 3’ ends of the genes (Table 4.3). Therefore, these results are consistent with previous studies demonstrating that many TFBSs exist within genes such as in introns, exons, or beyond the 3’ ends of the genes (Martone et al. 2003; Cawley et al. 2004; Carroll et al. 2006; Lim et al. 2007). However, it should be noted that the microarrays used in this chapter were biased to the promoter and upstream regions of genes. The arrays used span approximately -10 kb upstream to +2 kb downstream of the TSS. Thus, they do not cover the whole genome and to get a clearer picture of the whole genome, it would be necessary to perform ChIP-seq.

Further analysis using the Wilcoxon Rank Sum test showed a significantly difference in distribution between the binding regions for c-Rel and RelA relative to the TSS (p-value = 0.002440) (Fig 4.4). A box and whisker plot was used to represent the distribution of binding regions relative to the TSS. As shown in Figure 4.4, c-Rel bound regions were on average distributed further upstream from the TSS compared with the RelA bound regions.

Taken together, these results suggested that many TFBSs are not only present at the promoter regions but also lie within transcribed regions. These results suggest that binding regions within the gene may also play a role (Schjerven et al. 2001; Guo et al.
2003), and the function of transcription factors may not be limited to the promoter or upstream regions for gene regulation. Furthermore, c-Rel may be able to function at a greater distance from the TSS.

<table>
<thead>
<tr>
<th>Table 4:</th>
<th>Transcription Factors</th>
<th>TSS</th>
<th>Upstream Region</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB</td>
<td>TATAA</td>
<td>0</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>c-Rel</td>
<td></td>
<td>0</td>
<td>30%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>40%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>50%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 4.3 Location of NF-κB c-Rel and RelA binding regions across the genomic regions represented on the array

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Cut-off (p-value)</th>
<th>No of regions</th>
<th>Upstream of TSS</th>
<th>Spanning TSS</th>
<th>Within gene</th>
<th>3'end</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Rel</td>
<td>0.01</td>
<td>81</td>
<td>53.1%</td>
<td>9.9%</td>
<td>33.3%</td>
<td>3.7%</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>1552</td>
<td>60.0%</td>
<td>8.3%</td>
<td>26.4%</td>
<td>5.3%</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>276</td>
<td>56.5%</td>
<td>19.6%</td>
<td>22.8%</td>
<td>1.1%</td>
</tr>
<tr>
<td>RelA</td>
<td>0.05</td>
<td>2463</td>
<td>57.3%</td>
<td>10.2%</td>
<td>29.5%</td>
<td>2.9%</td>
</tr>
</tbody>
</table>

The genomic tiling assays used in this study were limited to -10 kb upstream to +2 kb downstream from the TSS.

a. Statistical p-values (high stringency (p<0.01) and lower stringency (p<0.05)) used for classification of regions bound by the c-Rel and RelA transcription factors.

b. The number of regions within the bound group by p-value (p<0.01 or p<0.05).

c. The percentage of c-Rel and RelA-bound regions detected at the upstream region from the TSS (up to -10 kb) or spanning the TSS.

d. The proportion of regions bound by c-Rel and RelA located within genes (exons and introns) or in the genomic DNA beyond the 3’ ends of the genes.
Figure 4.4 Distribution of distance of c-Rel and RelA binding regions relative to the transcription start site

The visual comparison of c-Rel and RelA binding regions showing the distribution relative to the transcription start site (TSS) which is indicated as zero. c-Rel binding regions are concentrated further upstream from the TSS compared with the RelA bound regions. A box and whisker plot was used to display the distribution of distances of binding regions to the TSS. Thick black lines (*) in the centre of the box indicates the median. The upper quartile (median of the upper half) (+) and the lower quartile (median of the lower half) (-) are represented by vertical blue lines respectively. The upper extreme (the highest value in the data set) (+) and the lower extreme (the lowest value in the data set) (-) are shown as red lines respectively. Two square boxes (red and yellow) contain both quartiles and the median respectively. Whiskers are indicated by dotted lines and connect the two extreme points to the box. The Wilcoxon Rank Sum test was used to compare distance between the binding regions to the TSS for c-Rel and RelA (W = 1802887, p-value = 0.002440).
4.2.7 Identification of the nearest genes from transcription factor binding regions

The identification of the TFBSs is important as it subsequently permits the identification of the nearest genes from regions bound by the transcription factor of interest. The identification of nearest genes that could be potentially regulated by c-Rel and RelA was performed in the Genomatix software program, using the 1552 and 2463 significantly enriched regions (p-value <0.05) detected by Partek (Section 4.2.3 and Fig 4.3). Through this approach, the nearest gene for each binding region was identified in the p<0.01 or p<0.05 group. 81 and 276 genes for c-Rel and RelA respectively were identified in the high stringent group (p<0.01) (Appendix III), and 1471 and 2187 genes for c-Rel and RelA respectively were identified in the less stringent groups (p<0.05) (data not shown).

4.2.7.1 Regulation of expression of gene groups nearest c-Rel and RelA binding regions

To investigate whether genes close to the TFBSs are directly regulated by and/or dependent on the transcription factor of interest, ChIP-on-chip has been combined with expression profiling (Friedman et al. 2004; Phuc Le et al. 2005; Song et al. 2009). Thus, in this study, the nearest gene groups were investigated to examine whether these genes responded to PMA/I stimulation in EL-4 T cells. For this experiment, firstly, 1552 and 2463 nearest genes (p<0.05) (Section 4.2.7) and the gene set (21882) represented on the whole expression array were compared. A total of 1161 and 1909 of the c-Rel and RelA nearest genes were present on the expression array respectively (p<0.05) (Table 4.4). Among them, 58 and 217 genes were classified in the high stringency group (p<0.01) respectively (Table 4.4).

The proportion of these genes groups that were responsive to PMA/I stimulation was determined and compared with the overall proportion of responsive genes on the array. This analysis was performed in EL-4 T cells stimulated with PMA/I for 30 min, 2 h (referred to as early) (Bunting et al. 2007), and 6 h (referred to as late) (Brettyngham-Moore et al. 2008) (Gene sets identified from expression microarray were previously generated in the Shannon lab). As shown in Table 4.4, of the 58 (p<0.01) nearest genes to c-Rel binding regions, 13.8% were significantly upregulated in response to early
stimulation compared with 4% of all the genes on the array. In addition, 22% of the same group of genes was highly induced after 6 h of stimulation compared with 7.8% of all the genes on the array. Similarly, among the 217 (p<0.01) nearest genes to regions bound by RelA, a significantly higher percentage (20% and 24% respectively) were upregulated in early and late stimulated samples compared with those upregulated on the whole array (4.1% and 7.8% respectively). In addition, a more significant percentage (about 2 to 3-fold) of both the less stringent groups (p<0.05) of nearest genes was induced compared with the whole array following early and late stimulation. In spite of this significant enrichment in the nearest gene groups compared with the entire array, the actual number of nearest genes that were upregulated by PMA/I stimulation in EL-4 T cells was small. Furthermore, nearest genes to RelA binding regions were more affected than those of c-Rel. For example, among the 217 nearest genes (p<0.01) from regions bound by RelA, about 20% (early) and 24% (late) of genes were induced by PMA/I stimulation, while 13.8% (early) and 22.4% (late) of genes nearest to c-Rel binding regions were upregulated in response to stimulation. In addition, in the less stringent groups (p<0.05), 13.3% (early) and 18.5% (late) of nearest genes from RelA binding regions were upregulated in response to early and late PMA/I stimulation, while 7.9% (early) and 15.2% (late) of nearest c-Rel binding genes were induced following stimulation. Interestingly, the percentage of upregulated nearest genes to RelA binding regions was similar after early and late stimulation, while a more significant percentage of genes nearest to c-Rel binding regions were affected after late stimulation (6 h). This may be associated with the timing of nuclear entry, given c-Rel comes into the nucleus later than RelA (Chapter 3).

In addition to the investigation of genes induced by PMA/I stimulation, those that were repressed by stimulation were also investigated. Interestingly, among the 58 genes (p<0.01) nearest to c-Rel binding regions, a lower percentage (1.7% and 6.9%) compared with the array (4.4% and 8.4% respectively) were downregulated in response to early (30 min or 2 h) and late (6 h) stimulation. In addition, among the 217 genes (p<0.01) nearest to RelA binding regions, a small percentage of genes (6%) were repressed compared with the array (4.4%) following early PMA/I stimulation. However, after late stimulation (6 h), a significant percentage of genes (12.4%) were downregulated compared with that of the array (8.4%). Furthermore, in both the less stringent groups (p<0.05), a significant percentage of nearest genes were downregulated in response to early and late stimulation compared with the whole array. Thus, c-Rel
and RelA may play a role in repression of nearest genes, but this may not be as significant as their role in gene activation.

Overall, a significantly greater proportion of the selected gene groups were responsive to stimulation compared with the entire gene set on the array, but only a small number of genes are up- or downregulated in response to PMA/I stimulation.
Table 4.4 Percentage of nearest genes to the regions bound by c-Rel and RelA that are affected by PMA/I stimulation

<table>
<thead>
<tr>
<th>Cut-off (p-value)</th>
<th>Transcriptor factor</th>
<th>a No of genes on expression array</th>
<th>Upregulated by early Stimulation (30 m or 2 h)</th>
<th>Upregulated by late stimulation (6 h)</th>
<th>Downregulated by early Stimulation (30 m or 2 h)</th>
<th>Downregulated by late stimulation (6 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>c-Rel</td>
<td>58</td>
<td>13.8%*</td>
<td>22.4%**</td>
<td>1.7%</td>
<td>6.9%</td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>1161</td>
<td>7.9%**</td>
<td>15.2%**</td>
<td>5.8%*</td>
<td>10.8%*</td>
</tr>
<tr>
<td>0.01</td>
<td>RelA</td>
<td>217</td>
<td>19.8%**</td>
<td>24.4%**</td>
<td>6.0%</td>
<td>12.4%*</td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>1909</td>
<td>13.3%**</td>
<td>18.5%**</td>
<td>6.6%**</td>
<td>13.3%**</td>
</tr>
<tr>
<td>Whole expression array (unique)</td>
<td>21882</td>
<td>4.1%</td>
<td>7.8%</td>
<td>4.4%</td>
<td>8.4%</td>
<td></td>
</tr>
</tbody>
</table>

a. Number of nearest genes that were represented on the expression profiling arrays.
b. The percentage of the gene groups that were up- or downregulated by PMA/I at the indicated times. The significance of the enrichment relative to the percentage of all genes on the expression array (blue) that were up- or downregulated by PMA/I stimulation is indicated by either a single star (*, p<0.05) or a double star (**, p<0.01).
4.2.7.2 Effect of c-Rel overexpression on the gene groups nearest c-Rel and RelA binding regions

To identify whether nearest genes are affected by c-Rel overexpression, nearest genes described in Section 4.2.7.1 were compared with gene sets identified from expression profiling of EL-4 T cells where c-Rel was overexpressed (Bunting et al. 2007) (These gene sets were previously identified in the Shannon lab). This study was performed by transfecting a c-Rel expressing vector into EL-4 T cells with an empty vector as a control and stimulating with PMA/I for 30 min and 2 h.

As shown in Table 4.5, of the 58 (p<0.01) nearest genes from c-Rel binding regions, 8.6% were induced by c-Rel overexpression compared with 4.2% of genes on the array in the absence or presence of PMA/I stimulation. Interestingly, 6.9% of nearest genes (over 3-fold) were significantly upregulated compared with 2% of genes on the array in the absence of stimulation. In addition, in the less stringent group (p<0.05), 3.4% and 6.8% were induced by c-Rel overexpression respectively compared with the array (2% and 4.2%) regardless of stimulation. Further study showed that among the 217 (p<0.01) in the high stringent group, 6.5% and 11.5% (over 2 to 3-fold) of nearest genes from regions bound by RelA were significantly upregulated by c-Rel overexpression compared with those of whole array (2% and 4.2%) in non- or PMA/I stimulated EL-4 T cells respectively. In addition, in the less stringent group (1909) (p<0.05), 5.4% and 9.6% were significantly induced by c-Rel overexpression respectively compared with the array (2% and 4.2%) with or without stimulation. However, only small number of these genes were upregulated by c-Rel overexpression regardless of stimulation in EL-4 T cells. In addition, nearest genes (p<0.01 and p<0.05) from RelA binding regions were more affected than those from c-Rel binding regions by c-Rel overexpression regardless of stimulation in EL-4 T cells.

In addition to the analysis of upregulated nearest genes by c-Rel overexpression, genes downregulated by c-Rel overexpression were also examined. As shown in Table 4.5, either no or a low fraction (0% to 4%) of nearest genes in both the high stringent groups (p<0.01) compared with the array (1.2% to 3.1%) were affected in the absence or presence of stimulation. A significant percentage (2% and 5.5%) of genes nearest to RelA binding regions in the less stringent group were affected by c-Rel overexpression.
compared with the array (1.2% to 3.1%). However, only few genes were affected by c-Rel overexpression.

These data show that the overexpression of c-Rel upregulates a significantly greater proportion of the genes nearest to c-Rel or RelA binding regions compared with all the gene set tested. However, a large number of the nearest gene groups are not affected by c-Rel overexpression in T cells.
Table 4.5 Percentage of nearest genes in the regions bound by NF-κB c-Rel or RelA that are affected by c-Rel overexpression

<table>
<thead>
<tr>
<th>Cut-off (p-value)</th>
<th>Transcription factor</th>
<th>No of genes on expression array</th>
<th>Upregulated by c-Rel o/e (with or without stimulation)</th>
<th>Upregulated by c-Rel o/e (nonstimulation)</th>
<th>Downregulated by c-Rel o/e (with or without stimulation)</th>
<th>Downregulated by c-Rel o/e (nonstimulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>c-Rel</td>
<td>58</td>
<td>8.6%</td>
<td>6.5%**</td>
<td>3.4%</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>1161</td>
<td>6.8%**</td>
<td>3.4%**</td>
<td>4.4%**</td>
<td>1.3%</td>
</tr>
<tr>
<td>0.01</td>
<td>RelA</td>
<td>217</td>
<td>11.5%**</td>
<td>6.5%**</td>
<td>4.1%</td>
<td>0.5%</td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>1909</td>
<td>9.6%**</td>
<td>5.4%**</td>
<td>5.5%**</td>
<td>2.0%*</td>
</tr>
<tr>
<td>Whole expression array (unique)</td>
<td>21882</td>
<td></td>
<td>4.2%</td>
<td>2.0%</td>
<td>3.1%</td>
<td>1.2%</td>
</tr>
</tbody>
</table>

a. Number of nearest genes that were represented on the expression profiling arrays.
b. The percentage of the gene groups in (a) that were up- or downregulated by c-Rel overexpression (o/e) at the indicated times. The significance of the enrichment relative to the percentage of all genes on the expression array (blue) that were up- or downregulated by c-Rel overexpression is indicated by either a single star (*, p<0.05) or a double star (**, p<0.01).
4.3 Discussion

In this chapter, a genome-wide analysis was performed for the identification and characterization of genomic regions bound by the NF-κB transcription factors c-Rel and RelA in PMA/I stimulated EL-4 T cells. Subsequently, nearest genes were identified using computational analysis and the regulation of those gene sets during T cell activation or following c-Rel overexpression was investigated. The major findings presented in this chapter are: i) While c-Rel and RelA binding regions are concentrated at the promoter or upstream region from the TSS, some sites are also found within the transcribed regions; ii) At least a quarter of regions identified in the present study are bound by both transcription factors; iii) A significantly greater proportion of genes nearest the binding regions are induced by PMA/I stimulation and/or c-Rel overexpression compared with all genes on the expression array; and finally, iv) IRF family motifs are significantly enriched in the regions bound by c-Rel and RelA, suggesting that there may be an important functional interaction between IRF and NF-κB transcription factors.

It has been suggested that the cis-regulatory regions including the promoter and enhancer regions, which have been considered as important regions to understand the transcriptional regulatory mechanisms, contain most transcription factor binding sites (Zhang 1998). For these reasons, many previous ChIP-on-chip assays have focused on the promoter regions (Odom et al. 2004; Beima et al. 2006; Song et al. 2009). Consistent with these previous studies, ChIP-on-chip results described in this chapter uncovered that over 50% of transcription factor binding sites of c-Rel and RelA are located at the upstream region from the TSS. In addition, some binding regions span the TSS. Therefore, these findings indicate that the promoter or upstream region from the TSS contain many important regulatory binding regions for transcription factors.

On the other hand, many previous studies have also demonstrated that the binding sites of transcription factors are not only limited to the promoter regions, but also lie within genes including intron and exon regions, or in the genomic DNA beyond the 3' ends of the genes (Martone et al. 2003; Cawley et al. 2004; Lim et al. 2007). Similarly, a quarter of the binding regions for c-Rel and RelA were found to exist within genes. Therefore, these results suggest that transcription factor binding regions located within genes act as
potential regulatory elements. Thus, it seems likely that transcription factors also play important roles not only at cis-regulatory regions such as promoters or enhancers, but in many different regions across the whole genomic DNA. It is worth considering why a significant number of binding regions for transcription factors lie within transcribed regions and in particular within introns. Guo et al. demonstrated that an intron situated within the Mn-SOD regulatory region plays an important role in epigenetic regulation including chromatin structural changes as well as histone acetylation by interacting with transcription factors such as p65 (Guo et al. 2003). It should be noted that the arrays used are biased and only cover on average -10 kb upstream and +2 kb downstream of the TSS. Thus, large regions of the genome were not covered and the results might be quite different if whole genome arrays or ChIP-seq were used.

ChIP-on-chip analysis in this chapter demonstrated that about a quarter of c-Rel binding regions are also occupied by the RelA subunit, suggesting that these regions may be regulated by both transcription factors. Using EMSA, it has been demonstrated that NF-κB p65/p50 heterodimers bind to the c-Rel binding site of the GBP-1 promoter in response to IL-1β and TNF-α (Naschberger et al. 2004). In addition, a study by Martone et al. suggested that RelA binding regions contain c-Rel binding motifs (GGGA/GNTTCC) where RelA and c-Rel bind by forming a heterodimer (Martone et al. 2003). Furthermore, the NF-κB/c-Rel site located within the CD28RE of the GM-CSF proximal promoter is also bound by both transcription factors (Himes et al. 1996). Similarly, it has been reported that the binding of a c-Rel and RelA heterodimer leads to a synergistic effect in the CD28RE enhancer activity at the IL-2 promoter (Lai et al. 1995). Thus, these results suggest that c-Rel binding regions could be coregulated by both transcription factors.

However, about 70% of c-Rel binding regions were not cooccupied by RelA. It is possible that these c-Rel binding regions may be coregulated by other transcription factors or other NF-κB family members, since gene regulation mediated by a single transcription factor is not common (Qiu 2003; Narlikar et al. 2009). It has been suggested that NF-κB family members cooperate in gene regulation and that the recruitment of multiple subunits of the NF-κB family increases RNA Polymerase II occupancy, which induces a stronger gene expression relative to a single transcription factor (Schreiber et al. 2006). In addition, as mentioned in Chapter 1, c-Rel can form homodimers with itself (c-Rel/c-Rel) or heterodimers with other NF-κB family
members (c-Rel/p50 and c-Rel/p52), which can bind DNA and activate gene transcription (Chapter 1). However, only two transcription factors were investigated in this chapter, hence, it may be valuable to investigate whether other NF-κB members could occupy the c-Rel binding regions. Another possibility is related to the fact that maximum binding of c-Rel and RelA were observed at different time points following stimulation. For example, Himes et al. reported that while RelA maximally bound to the IL-2 promoter as early as 1 h following stimulation, the strongest binding of c-Rel was observed at 6 h of activation in human T cells (Himes et al. 1996). In addition, in this chapter, two different time points were selected for c-Rel (8 h) and RelA (2 h) ChIP-on-chip respectively, because the time for maximum binding of c-Rel was different from that of RelA. Thus, this different timing of c-Rel and RelA binding may explain why 70% of regions were bound by c-Rel alone. These data may also be explained in part by a study showing that c-Rel homodimers have much higher affinity for a broad range of NF-κB recognition sites than RelA homodimers (Sanjabi et al. 2005).

In this chapter, a significant percentage of nearest genes to c-Rel and RelA binding regions are induced by PMA/I stimulation and c-Rel overexpression in EL-4 T cells compared with the whole expression array. However, this percentage represents a relatively small number of genes. These phenomena have also been observed in many previous studies where the expression of a large number of genes bound by NF-κB proteins has been unchanged in response to activation. For example, a study by Martone et al. showed that a number of RelA target genes were not up- and downregulated in response to TNF-α in human cells (Martone et al. 2003). Another interesting study to find direct target genes of a transcription factor has been performed using ChIP-PET combined with expression profiling in a human cell line. This study showed that among 259 RelA target genes represented on the arrays, only 20% were upregulated while 4% were downregulated following LPS treatment (Lim et al. 2007). Similar results have also been observed with other transcription factors such as p63, where a significant correlation between p63 binding and p63-dependent transcriptional activity was observed, but only a small percentage of genes were up- (14% to 27%) or downregulated (12% to 15%) in human cells (Yang et al. 2006). Thus, considering these results, it is possible that many target genes may be cell type or stimulation specific, or transcription factors can bind to certain genes or regions without affecting gene transcription (Lim et al. 2007).
Motif analysis was performed by searching for highly overrepresented motif(s) in the regions bound by c-Rel and RelA. Interestingly, the IRF binding motif(s) was a highly overrepresented motif in both c-Rel and RelA binding regions. In particular, it was the most highly represented motif in c-Rel binding regions. It is known that NF-κB family members interact with IRF transcription factors forming transcriptional complexes, consequently leading to synergistic effects (Sgarbanti et al. 2008). Earlier studies have shown that NF-κB and IRF-1 motifs are adjacent to each other and play a crucial role in the activation of the IL-15 gene promoter by cooperating as transcriptional activators (Azimi et al. 2000). In another study, Naschberger et al. reported that the guanylate-binding protein-1 (GBP-1) gene promoter contains binding sites for NF-κB c-Rel and the interferon-stimulated response element (ISRE), and that c-Rel cooperates in the regulation of GBP-1 gene expression with the ISRE/IRF complex in response to inflammatory cytokines, such as TNF-α and IL-1β (Naschberger et al. 2004). In a recent study, Sgarbanti et al. reported that NF-κB and IRF transcription factors form a functional complex at the κB sites of the long terminal repeat (LTR) enhancer region of human immunodeficiency virus type 1 (HIV-1), which leads to full HIV-1 LTR transcription activity in T cells (Sgarbanti et al. 2008). Therefore, the motif analysis data shown in this chapter, together with other studies, implies that there may be an interaction between IRF and c-Rel transcription factors which could lead to a cooperative effect for gene activation.

In addition to IRF motifs, other motifs including V$PRD1$, V$SARB$, and V$BRAC$ motifs were also overrepresented in both regions bound by c-Rel and RelA. In particular, the PRD1 binding site is adjacent to NF-κB and IRF binding sites at the CIIApI promoter of the CIITA gene (Smith et al. 2011). However, PRD1 recruits histone methyltransferase G9a to the CIIApI promoter and represses the CIITA gene activation in mature dendritic cells (DCs), while NF-κB and IRF act as activators for CIITA gene expression (Smith et al. 2011). Thus, as these factors were found in the overrepresented motifs together, it could be possible to speculate that they may function together on more than one gene as activators and a repressor by maintaining a transcriptional balance.

Interestingly, the motif analysis presented in this chapter showed that the NF-κB transcription factor motif(s) are underrepresented in c-Rel binding regions, but they are the most highly represented in the regions bound by RelA. This result was unexpected
because c-Rel is one of NF-κB family members and also binds to NF-κB motif(s). However, at the moment, it is not clear why the NF-κB family motif(s) is not highly enriched in c-Rel binding regions. It is interesting to speculate that most NF-κB motifs may not be significantly overrepresented at late time that c-Rel binding was measured. Instead, they may be highly enriched at an earlier time point.

Overall, in this chapter, genome-wide binding sites for c-Rel and RelA and their nearest genes that could be possibly targeted by these factors were identified using genome-wide location analysis. This data is of value in order to understand the genome-wide regulatory networks mediated by these particular factors. ChIP-on-chip does not show evidence that these target genes are directly regulated by the transcription factor hence validation experiments are necessary. This study will be further explored in the next chapter.
Chapter 5

Verification and characterization of putative c-Rel regulated genes
Chapter 5

Verification and Characterization of putative c-Rel regulated genes
5.1 Introduction

In the experiments described in Chapter 4, ChIP-on-chip was used to identify transcription factor binding regions for c-Rel and RelA across the genome. Combining the ChIP-on-chip data with computational analysis and expression profiling data available from previous work in the laboratory identified a small subset of genes that were putative c-Rel target genes.

Many studies have used ChIP assays on a genome-wide scale analysis in order to identify and characterize the binding sites of transcription factors of interest and their target genes in yeast, mouse, and human (Ren et al. 2000; Lee et al. 2002; Mao et al. 2003; Martone et al. 2003; Wells et al. 2003; Friedman et al. 2004; Odom et al. 2004). However, ChIP-on-chip based binding data do not prove the importance of a transcription factor for regulation of target genes. Therefore, follow-up validation studies are essential to verify the ability of a transcription factor to regulate target genes. A study by Beima et al. identified many potential target genes bound by the T-bet transcription factor, and confirmed that mRNA expression levels of some of these genes including Cxcr3, Ccl3, Ifnγ, and Stat1, were directly upregulated by T-bet overexpression in EL-4 T cells. In addition, Cxcr2 gene expression was dramatically decreased in T-bet/- T cells (Beima et al. 2006). Similar validation studies were also performed to confirm whether potential target genes of the p63 transcription factor were directly bound by p63 in human keratinocyte (HaCaT) cells. Expression of most of the selected genes was induced by overexpression of the ΔNp63α isoform after CaCl2 treatment (Vigano et al. 2006). Recently, validation experiments also have been performed for interferon response factor (IRF) bound target genes and their gene expression profiles. This study showed that most of the selected target genes (19/21) are bound by IRF-1, and half of them are induced by IRF-1 overexpression or IFN-γ activation in human breast cancer cells (Frontini et al. 2009). Thus, these studies suggest that the binding of a transcription factor to target genes is associated with their regulation. However, these validation studies have also uncovered that not all transcription factor binding results in the induction or repression of target genes. In addition, the expression profiles of selected target genes depend on the cell type or on stimulation conditions (Martone et al. 2003; Beima et al. 2006; Lim et al. 2007; Frontini et al. 2009).
The IRF family of transcription factors plays an important role in immunity, oncogenesis, development, and differentiation (reviewed in Tamura et al. 2008; Frontini et al. 2009). In mammalian systems, the IRF family of transcription factors consists of nine members including IRF-1, IRF-2, IRF3, IRF4 (LSIRF/Pip), IRF5, IRF6, IRF7, IRF8 (ICSBP), and IRF9 (ISGF3γ). Each IRF protein contains a conserved helix-turn-helix DNA binding domain at the N-terminus composed of 120 amino acids. This domain is responsible for binding to the interferon-stimulated responsive element (ISRE) which is similar to the IRF-E consensus sequence, while the less conserved C-terminus is responsible for protein-protein interaction (Sgarbanti et al. 2008; reviewed in Tamura et al. 2008). Among IRF family members, IRF-1 has attracted significant attention because of its various roles in immune cells such as the development of CD8+ T and NK cells and Th1 cell differentiation (reviewed in Tamura et al. 2008). In particular, the interaction between NF-κB and IRF-1 transcription factors has been well studied, as the cooperative interaction of these transcription factors leads to synergistic activation of many genes, such as major histocompatibility class I (MHC-I), vascular cell adhesion molecule 1 (VCAM1), Interleukin-15 (IL-15), guanylate-binding protein-1 (GBPI), and human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) (Drew et al. 1995; Neish et al. 1995; Azimi et al. 2000; Naschberger et al. 2004; Sgarbanti et al. 2008).

To validate the genome-wide analysis, several putative c-Rel binding genes that were affected by PMA/I stimulation (6 h) and/or by c-Rel knockout were selected by combining ChIP-on-chip with available expression profiling analysis data. Verification studies were performed by investigating whether these genes were directly bound and regulated by c-Rel. In addition, the motif analysis described in Chapter 4 which identified IRF motifs enriched within the c-Rel binding regions was further investigated.
Thus, the main aims of the study presented in this chapter were:

♦ To confirm the in vivo binding of c-Rel to selected binding regions in T cells.

♦ To verify whether the expression of genes nearest to the c-Rel binding regions were directly affected by c-Rel.

♦ To investigate the interaction between c-Rel and IRF-1 transcription factors.

Data represented in this chapter show that:

♦ c-Rel binding to target regions was confirmed for a subset of the regions tested.

♦ The expression of a subset of c-Rel binding genes was responsive to T cell activation and reduced in c-Rel⁻/⁻ T cells.

♦ IRF-1 binds to several target genes that also contain c-Rel binding regions.
5.2 Results

5.2.1 Selection of c-Rel binding genes in T cells

To select a gene set for verification, the regions were first filtered to remove those less than 60 bp as those were less likely to represent the detection of real c-Rel binding regions because the overlap between the no antibody and total input regions was not large. 81 putative c-Rel binding genes (p<0.01) (Section 4.2.7) were compared with available gene sets identified from expression profiling (cDNA microarray) performed in EL-4 T cells (Brettingham-Moore et al. 2008) to investigate whether c-Rel binding genes were also upregulated by PMA/I stimulation (6 h). Through this approach, 12 genes (Table 5.1A) were selected using the parameters: i) region bound by c-Rel was at least 60 bp in length (average length: 491 bp) (p<0.01), and ii) genes responded to PMA/I stimulation (6 h) (>1.4-fold change) in EL-4 T cells. Similarly, four c-Rel binding genes (Table 5.1B) were chosen by comparing the 81 c-Rel binding genes (p<0.01) with available gene sets identified from expression microarray (cDNA microarray) that were affected by PMA/I stimulation (6 h) and by c-Rel knockout in wild-type and c-Rel−/− CD4+ T cells (Chen et al; unpublished data). These genes were selected with the following parameters: i) region bound by c-Rel was at least 60 bp in length (average length: 491 bp) (p<0.01), ii) c-Rel-bound regions were located within 5 kb from the TSS of the gene, and iii) genes responded to PMA/I stimulation (>1.4-fold change) in primary CD4+ T cells. These gene sets identified from expression profiling were previously generated in the Shannon lab.

The regions containing sequences bound by c-Rel identified from ChIP-on-chip analysis were mapped by comparing with genomic sequences from the UCSC and Ensembl Genome Browsers. For amplification of c-Rel binding regions, specific primer sets were designed within the above mentioned regions (Appendix I). As shown in Figure 5.1, eight out of 15 c-Rel bound regions were observed within the transcribed regions of those genes (all were in intronic regions) (Fig 5.1A), whereas seven of the regions were detected upstream from the transcription start site (Fig 5.1B). Further studies showed that each of the c-Rel binding genes had motif(s) containing binding sequences for NF-κB/c-Rel and/or IRF transcription factors except for Tlk2 (Fig 5.1, Tables 5.1 to 5.3).
It has been reported that the *Gadd45b* gene has three κB sites in the promoter region (Figs 5.1 and 5.2) (Jin et al. 2002). However, the current ChIP-on-chip data did not find evidence of binding to the upstream regions of the *Gadd45b* gene. Instead, a c-Rel binding region was identified within the gene (Figs 5.1 and 5.2). Thus, to confirm previous studies and to investigate whether c-Rel binds to both the promoter and downstream regions of the *Gadd45b*, both regions were included in the verification studies.

These 16 genes were investigated to confirm microarray analysis data by measuring their gene expression and/or *in vivo* binding of c-Rel in EL-4 and primary CD4⁺ T cells.
Figure 5.1 Putative binding motifs of the NF-κB and IRF transcription factors in c-Rel binding genes

Schematic representation of the upstream and downstream regions from the transcription start site (TSS), containing putative transcription factor binding motifs in c-Rel binding genes. The regions amplified by specific primer sets are presented by a short red bar. Three κB motifs of located at the Gadd45b gene promoter (Gadd45bP) are indicated with triple diamonds (♦️) (Jin et al 2002). NF-κB, c-Rel, and IRF binding motif(s) are indicated with single circle (●), diamond (♦️), and triangle (▲) respectively. The regions bound by c-Rel in the putative c-Rel binding genes are indicated by line (→). The TSS (+) is indicated with a curved arrow. The location of each motif was confirmed by comparing genomic sequences obtained from the UCSC and Ensembl Genome Browsers with regions obtained from the ChIP-on-chip analysis data.
**Forward primer**

AGCTTGGGAA AGGCTAGGGA CTCTCCGGGG ACAGCGGAGGG GATTCGACCC AGGCTCTCCC 60

GAAAGTTCAG GCCAGCCTCT CGGCGCTGAAA ACCCCCGCCGC CGGCTGCCTG AGGCGGCGCTG 120

CGGGGAATCG AGGAGAGAAG CTCTTGTGCT TTTTTTTTTTT TTTTTTTTTTT TTTTTTTTTTT 180

CTCTCTAGAG CTCTCTCTCT AGAGCTCTCT GCCTTTTTCTA GCTGTGCGCG CTGCTGCGGT 240

TCACGCTCTC CCAAGCCCTGT ACCCCACCGT GGGGCCGCCTG GAGCTCCGAG CTCCGCCCCTT 300

TCCATCTCCA GCGCAATCTCA GCGGGGATA GCTGAGGCTT TTGGCATCTA CCAATGGGTTG 360

GAAAGCGCAT GCCCTCACTGT GCCAGCGCTCC CACCCCGGAGA GTCATAATAA AGGCTGCGAG 420

CGCCCGGCCT CTCATCCGCG GCAGAACCTGT GGCTGCTGCTT GATCTGCTGT TCCTGTGATT 480

AATTTTGAGG GGGATTTTTC AATCTTCTTTT TACCCCTACT TTTTTCTTTG GGAAGGGGAG 540

TCCACCGCC TCGGAGAGGC CTCGAGACAC TCTGTTGCGA CGGAGAGGT TTTTTGCTCT 600

TTGAGTTCGT ATCTGGACTT GTATTTTGGCT TTGGGAGGAT TCCGGTGAGG GTCGGGCTG 660

GAGTGTAAGC CATCATCGAC CCTGAGGAGAG CTGTTGGCGA CGGCAACGCG GTTCTGAGAG 720

TGAGTACACAG AGAGGCGSGAG GTCTCTCGAC CGCCCGGCGGT AGGTCTGCGC GGGCTGCTAC 780

GGCTAATCTC CCAATTTGCG CTTTTAGGAT GCAGCCGGTG ACTGCCGCCTC TGAGAGCGCT 840

GCTGAGTGGCC GCCGACGCCT AGGATCGCCT CACGGTGAGG GTGTAAGGAG CGCCCAAAC 900

**Reverse primer**

GATGAAAAGGG TGAGTCAGCA CCCCCCTTTC TCAGCGGCTGG GTGAGGGGGCC CCGCCTCGATG 960

CAGCGGGGGCT CCAACATCCC TATAGGGTCT GCAGCTTTTG GGGACTTTCC CAGCGCTCTC 1020

**Forward primer**

GTTTTTTGGG AGGTTGGGAGC GACATTTCCTC AGAGAATTTT TTTTTGGGAG GAGGAAGAG 1080

**Reverse primer**

IRF-4

AGTCGCTGCA GAACCCTGAG TCACGAGATGT TGAGGTTTTG TGGTTTTTTGT TTTGCAAAAGG 1140

CAACCACTGG AACTTTGGGC TATGTGCTTC CTCCCCACCC GCCATCAGTA TTTGGCATAAGT 1200

CGGGCAACCC AGCAACCGCGT CAGTACTCGG GCTGGGTTGGC GTTTCTTATA TACACTCTTG 1260

ATGACAGGATG GTCTTGTGCC ACCCTTCCGG CCTGCGGGTTC CTGGAAGCTA TACCCCCCTA 1320

TCCACCACCG TGCAAGGGTA CACCGCGCTCC TGTTCTGAGG ACGGCCTGAC ACTCTCTTCC 1380

TCCCTGAGGG ACCCGGACAG CTGTTGTCTGG TGCCCTCTGG CCATAGACGA AGAAGAGGAG 1440

GATGATAATCG CTGTGCACTGC TCAACTCACTG TGATGCCAGT GCTTCCTGCTG CGACAAATGAC 1500

ATTGCATCG TCCGGGTATAC AGGCATGAG AGGTGTGCAG AGCTCCGCTGG GGAGGGCGCG 1560

GAGACATTGG GCACAAACCGA AGCCCGAGAC CTGCCACTGCC 1600
Figure 5.2 NF-κB binding sites at the Gadd45b promoter region and MatInspector™ prediction of putative binding sites of NF-κB and IRF transcription factors within the Gadd45b gene

Three NF-κB binding sites (κB-1, κB-1, and κB-3) were derived from previous studies (Jin et al. 2002). Putative NF-κB binding sequences marked by blue are shown between the primer sets. Two putative IRF binding sites (IRF-4 and ISRE) are marked in red. All primer sets are indicated by green. Transcription start site (TSS) is indicated by a right angled arrow. ATG indicates the translation start codon. Upstream and downstream sequences are obtained from the UCSC and Ensembl Genome Browsers by comparing with regions obtained from ChIP-on-chip analysis data.
Table 5.1 Putative c-Rel binding genes selected by combining ChIP-on-chip and expression profiling data

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Name</th>
<th>Motif(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadd45b</td>
<td>growth arrest and DNA-damage-inducible 45 beta gene</td>
<td>NFκB (2), IRF (2)</td>
</tr>
<tr>
<td>Taf1503</td>
<td>tumor necrosis factor, alpha-induced protein 3 gene</td>
<td>NFκxB</td>
</tr>
<tr>
<td>Bcl10</td>
<td>B-cell leukemia/lymphoma 10 gene</td>
<td>NFκB (2), IRF (1)</td>
</tr>
<tr>
<td>Cdc14a</td>
<td>CDC14 cell division cycle 14 homolog A (S. cerevisiae) gene</td>
<td>IRF (2)</td>
</tr>
<tr>
<td>Tbk2</td>
<td>turred-like kinase 2 (Arabidopsis) gene</td>
<td></td>
</tr>
<tr>
<td>Pou2/f1</td>
<td>POU domain, class 2, transcription factor 2 gene</td>
<td>NFκB (2)</td>
</tr>
<tr>
<td>Nek8</td>
<td>NIMA (never in mitosis gene a)-related expressed kinase 8 gene</td>
<td>NFκB (3), IRF (3)</td>
</tr>
<tr>
<td>Trip4</td>
<td>thyroid hormone receptor interactor 4 gene</td>
<td>IRF (3)</td>
</tr>
<tr>
<td>Snai2</td>
<td>snail homolog 2 (Drosophila) gene</td>
<td>IRF (4)</td>
</tr>
<tr>
<td>Ervr11</td>
<td>ERO1-like (S. cerevisiae) gene</td>
<td>IRF (5)</td>
</tr>
<tr>
<td>281045706Rik</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>1700113020Rik</td>
<td>RIKEN cDNA 1700113020 gene</td>
<td>NFκB, IRF (3)</td>
</tr>
</tbody>
</table>

b. Transcription factor family motif(s) that were identified from regions bound by c-Rel. Figures in parenthesis refer the number of motifs.

(A) Genes affected by PMA/I stimulation in EL-4 T. NA = not available. (B) Genes affected by both PMA/I and c-Rel knockout in CD4^+ T cells.
Table 5.2 *MatInspector™* prediction of putative transcription factor binding sites of NF-κB/c-Rel and IRF in the c-Rel binding genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Strand(^a)</th>
<th>NF-κB/c-Rel binding sequence</th>
<th>Strand(^b)</th>
<th>IRF binding sequence(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadd45b</td>
<td>+</td>
<td>ggGGGACTTcccag</td>
<td>-</td>
<td>cccceAAAACEgagaceccttg (IRF4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AggtgaagatGAAACTttg (ISRE)</td>
</tr>
<tr>
<td>Tnfaip3</td>
<td>+</td>
<td>aagTTGGAActccccaa</td>
<td>-</td>
<td>tggatccAGAAAACctaaac (IRF1)</td>
</tr>
<tr>
<td>Cede94</td>
<td>+</td>
<td>tggaggTTTCCCCcct</td>
<td>-</td>
<td>aacaaataAGAAAatiaac (IRF4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cccceAAAAGgagaccttg (IRF4)</td>
</tr>
<tr>
<td>Bel10</td>
<td>-</td>
<td>tggcgggaaTTTCCagg</td>
<td>-</td>
<td>actgaaaacGAAAtatggc (ISRE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gatataanAGAAAgcaatt (IRF1)</td>
</tr>
<tr>
<td>Nek8</td>
<td>-</td>
<td>etgagggTTTCCaggg</td>
<td>+</td>
<td>agtGAAATggagagacggtagata (IRF7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cceAAAAAACgagagcttg (IRF4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ctaggaanAGAGacgtg (IRF3)</td>
</tr>
<tr>
<td>Pou2f2</td>
<td>+</td>
<td>ggaGGGACcgcceccgc</td>
<td>ggGGGAccttcctcct</td>
<td>cccceAAAGacaaactaagcag (IRF4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>taacaaatgAAAttacagg (IRF1)</td>
</tr>
<tr>
<td>Pigb</td>
<td>-</td>
<td>cgggAAActccacgcgc</td>
<td>ggGGGAccttcctcct</td>
<td>ttagaAGAAAtgagagctact (IRF7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gaagaagaAGAAAagagaga (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gagaagaAGAAAagagagag (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gagaAGAAATgagaagaaaatgag (IRF7)</td>
</tr>
<tr>
<td>Snai2</td>
<td>-</td>
<td>tggcgggaaTTTCCagg</td>
<td>-</td>
<td>taataaGAAAtgagagctact (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gaagaagaAGAAAagagaga (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gagaagaAGAAAagagagag (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gagaAGAAATgagaagaaaatgag (IRF7)</td>
</tr>
<tr>
<td>Cde14a</td>
<td>+</td>
<td>ggaGGGACcgcceccgc</td>
<td>ggGGGAccttcctcct</td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ttagaAGAAAtgagagctact (IRF3)</td>
</tr>
<tr>
<td>2810457106Rik</td>
<td>-</td>
<td>gcaggagaaaGCAAtgagacec (ISRE)</td>
<td></td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
</tr>
<tr>
<td>Ero11</td>
<td>+</td>
<td>aaaaGAAAtcgaacccctctag</td>
<td>ggGGGAccttcctcct</td>
<td>ttagaAGAAAtgagagctact (IRF3)</td>
</tr>
<tr>
<td>Fbxw8</td>
<td>+</td>
<td>tggaggtAGAAAAccctaaac (IRF4)</td>
<td></td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
</tr>
<tr>
<td>Haver2</td>
<td>-</td>
<td>ttagaAGAAAtgagagctact (IRF4)</td>
<td></td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
</tr>
<tr>
<td>Trip4</td>
<td>+</td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
<td></td>
<td>aaaaatttAAAGATgtctctaa (IRF3)</td>
</tr>
</tbody>
</table>

\(^a\) forward (+) and/or reverse strand (-); \(^b\) IRF binding sequences and their IRF family members, capital letter means core sequence. Red indicates a high conservation in the matrix.
5.2.2 Confirmation of the expression of selected genes in EL-4 T cells

To confirm microarray analysis mentioned above (Section 5.2.1), firstly, mRNA expression levels of 12 c-Rel binding genes (Table 5.1A) that were upregulated by 6 h of stimulation in EL-4 T cells were measured in nonstimulated or PMA/I stimulated EL-4 T cells by quantitative real-time PCR (Fig 5.3). In agreement with microarray analysis data, all 12 c-Rel binding genes responded to PMA/I stimulation showing an increase in mRNA expression levels following 6 h of stimulation (Fig 5.3). Three genes including Tnfaip3, Erol1, and Tlk2 revealed a continuous increase to 12 h poststimulation, while other genes such as Gadd45b, Bcl10, Cdc14a, Pou2f2, Nek8, Trip4, Snai2, 2810457106Rik, and 1700123O20Rik showed a transient increase, peaking at 6 h with a decrease at 12 h following stimulation (Fig 5.3).

Thus, expression profiling results were confirmed on an individual gene level by quantitative real-time PCR (Table 5.3).

5.2.3 Confirmation of *in vivo* binding of c-Rel to target genes in EL-4 T cells

To verify the ChIP-on-chip data presented in Chapter 4, the detection of c-Rel binding to the selected genes (Section 5.2.1) was carried out using ChIP assays in EL-4 T cells nonstimulated or stimulated with PMA/I for 8 h. Since EL-4 T cells were stimulated for 8 h to perform ChIP-on-chip experiments (Section 4.2.1), *in vivo* binding assays were also carried out at this time point. ChIP assays were performed on 16 of the putative c-Rel binding genes (Table 5.1).

In agreement with previous results, c-Rel binding was observed at the Gadd45b promoter region after 8 h of stimulation in EL-4 T cells (Fig 5.4). However, a stronger binding activity (about 4-fold) was observed at the c-Rel binding region located within the gene. In addition, strong c-Rel binding activity was observed for two other genes in the gene set; Ccdc94 and Nek8, and a much lower level of binding was observed at Bcl10 and Tnfaip3 genes (Fig 5.4). Much weaker binding was observed at four genes
with or without κB site, including the Pou2f2, Snai2, Pigb, and Cdc14a relative to control binding activity at 8 h of stimulation. However, other genes were not bound by c-Rel at this time point (data not shown).

These results confirmed the binding of c-Rel to 5 of the 16 genes (31%) following stimulation (Table 5.3). It is interesting to note that all the regions where strong binding was confirmed contain NF-κB motifs and 4 out of 5 regions contain c-Rel binding motifs (Table 5.3). Furthermore, these results suggest that the binding of c-Rel occurs both within the gene as well as at the promoter.

5.2.4 Confirmation of in vivo binding of c-Rel to the putative c-Rel binding genes in primary CD4+ T cells

To further address whether c-Rel binds to putative c-Rel binding genes, in vivo ChIP assays were performed in wild-type and c-Rel−/− CD4+ T cells following 8 h of CD3/CD28 stimulation. In vivo binding assays were also performed on the selected 16 genes (Table 5.1). As shown in Figure 5.5, c-Rel bound to two genes, Gadd45b and Nek8 following stimulation in wild-type CD4+ T cells with little or no binding observed in c-Rel−/− T cells. This result confirms that the signal observed in wild-type CD4+ T cells is indeed a result of c-Rel binding. c-Rel also bound to the three genes Tnfaip3, Cede94, and Bcl10, with again no binding above baseline levels observed in c-Rel−/− T cells (Fig 5.5). These are clearly consistent with results observed in EL-4 T cells (Fig 5.4). However, other genes were not bound by c-Rel at this time point (data not shown). Thus, these results suggest that the binding of c-Rel to these genes is associated with the presence of NF-κB sites and in particular c-Rel sites, supporting the microarray analysis data showing that c-Rel binds to putative c-Rel binding genes in activated CD4+ T cells (Table 5.3).
Figure 5.3 Expression profiles and confirmation of c-Rel binding genes in PMA/I stimulated EL-4 T cells

EL-4 T cells were nonstimulated (0 h) or stimulated with PMA/I for 6 h and 12 h. Total RNA was reverse transcribed into cDNA followed by quantitative real-time PCR analysis using gene specific primer sets. To calculate the relative mRNA levels, \( C_t \) values of the reference housekeeping gene (\( C_t \) (ref)) (\( ubc \)) were subtracted from \( C_t \) values determined for target genes of interest (\( C_t \) (target)) which generates \( \Delta \Delta C_t \), or \( C_t \) (target) - \( C_t \) (ref). The resulting \( C_t \) value is then raised to the power 2 to give the final relative mRNA levels (\( 2^{-\Delta \Delta C_t} \)). The results are shown as the mean and standard deviation (SD) of three independent experiments.
Figure 5.4 Confirmation of the \textit{in vivo} binding of c-Rel to target genes in EL-4 T cells

Nuclei were isolated from EL-4 T cells nonstimulated (0 h) or stimulated with PMA/I for 8 h. ChIP assays were carried out with an anti-c-Rel antibody. Quantitative real-time PCR was performed on the immunoprecipitated DNA with specific primer sets, amplifying the regions bound by the c-Rel transcription factor. The graph is represented by comparing c-Rel binding to \textit{Rho} (■) relative to c-Rel binding to the gene of interest (■). \textit{Rho} was used as a negative binding control. The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting \( C_t \) values of TI DNA from IP \( C_t \) values (\( C_t^{IP} - C_t^{TI} \)) and TI from NA (\( C_t^{NA} - C_t^{TI} \)). The resulting \( C_t \) values is then raised power 2 to give the final relative amount of ChIP DNA (\( 2^{-\Delta\Delta CT (IP-TI)} \) or \( 2^{-\Delta\Delta CT (NA-TI)} \)). The results are shown as the mean and standard deviation of three to five independent biological experiments. Pro and Int mean the \textit{Gadd45b} promoter and Intron respectively. The \textbf{red} dotted line indicates a base-line (0.03), where the values below the line is considered as no binding of c-Rel to target genes. The results are shown as the mean and standard deviation (SD) of three independent experiments.
Figure 5.5 *In vivo* binding assays of c-Rel in primary CD4^+^ T cells following CD3/CD28 stimulation (h)

Wild-type or c-Rel^/-^ CD4^+^ T cells were isolated from mouse spleens followed by nonstimulation (0 h) or stimulation for 8 h with CD3/CD28 antibodies. Nuclei were extracted from the cells. Genomic DNA was sheared by sonication and immunoprecipitated with antibodies specific for c-Rel. Quantitative real-time PCR was performed on immunoprecipitated genomic DNA fragments with specific primer sets amplifying the regions bound by c-Rel. The graph is represented as c-Rel binding to *Rho* (■) relative to c-Rel binding to the gene of interest (■). To quantify the relative amounts of ChIP DNA, C\textsubscript{T} values in no antibody (NA) condition was subtracted from the C\textsubscript{T} values of immunoprecipitated DNA (IP) and then normalized to the amount of relative DNA of total input (TI) (C\textsubscript{IP} - C\textsubscript{NA})/C\textsubscript{T}. The resulting C\textsubscript{T} values is the raised power 2 to give the final relative amount of ChIP DNA (2^ΔΔC\textsubscript{T}((IP-NA)/TI)). *Rho* was used as a negative binding control. The red dotted line indicates a base-line (0.01), where the graph below the line is considered as no binding of c-Rel to target genes. The result is presented from two independent experiments.
Table 5.3 Summary of putative c-Rel binding genes containing motifs that responded to PMA/I stimulation in EL-4 T cells and in vivo binding of c-Rel to these genes in EL-4 and primary CD4⁺ T cells

<table>
<thead>
<tr>
<th>Genes</th>
<th>Motifs¹</th>
<th>mRNA expression levels³</th>
<th>ChIP²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NF-κB</td>
<td>c-Rel</td>
<td>IRF</td>
</tr>
<tr>
<td>Gadd45b</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Taf15p3</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ero1l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2810457H06Rik</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pox2f2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rctl0</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Nek8</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Trip4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tlk2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sna12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc14a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1700123O2005k</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccnd94</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pigh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fbx58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haver2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a = Transcription factor binding motifs present (+).
b = Genes marked by arrow (↑) show an increase in mRNA expression levels in response to PMA/I stimulation (6 h) in EL-4 T cells.
c = Genes bound by c-Rel at 8 h of PMA/I stimulation in EL-4 and CD4⁺ T cells: strong (***); low (**); weak (*) binding; and no binding or data not shown (-).
5.2.5 Effect of c-Rel in the regulation of genes nearest to c-Rel binding regions following stimulation

To investigate whether the expression of the putative c-Rel binding genes is directly affected by c-Rel, mRNA expression levels of 16 genes described in Section 5.2.1 (Table 5.1) were measured in wild-type and c-Rel/− T cells. Firstly, in order to confirm that CD4⁺ T cells were appropriately stimulated and that the gene expression of c-Rel and IL-2 used as control genes was inhibited in c-Rel/− T cells, wild-type and c-Rel deficient CD4⁺ T cells isolated from mouse spleens were nonstimulated or stimulated with PMA/I for 6 h and 12 h. As expected, c-Rel mRNA levels were completely absent in c-Rel/− T cells relative to wild-type T cells following PMA/I stimulation, and mRNA levels of the IL-2 were decreased in c-Rel/− cells by 12 h poststimulation (Appendix IV A). In addition, no c-Rel gene expression was observed in c-Rel/− T cells following stimulation with CD3/CD28 antibodies, and IL-2 mRNA levels were decreased at 6 h following stimulation (Appendix IV B).

To investigate whether the expression of the c-Rel binding genes was affected by c-Rel deletion, primary CD4⁺ T cells isolated from wild-type and c-Rel/− mouse spleens were not stimulated or were stimulated with PMA/I as described above. Of the 16 genes, only the five genes (Gadd45b, Tnfaip3, Ccdc94, Bcl10, and Nek8) that were bound by c-Rel in EL-4 T cells or CD4⁺ cells (Figs 5.4 and 5.5) were investigated. As shown in Figure 5.6, Gadd45b and Tnfaip3 strongly responded to PMA/I stimulation while Ccdc94 and Bcl10 genes weakly responded to stimulation and Nek8 did not respond to stimulation. Among the five genes, only two genes; Gadd45b (p-value = 0.04) and Tnfaip3 (p-value = 0.034) were inhibited in c-Rel/− T cells at 6 h following stimulation (Fig 5.6 and Table 5.4), suggesting a partial dependence on c-Rel. This decrease was also observed at 12 h poststimulation. However, no effect was observed on Ccdc94, Bcl10, and Nek8 in c-Rel/− T cells (Fig 5.6 and Table 5.4).

Overall, these results showed that the expression of the Gadd45b and Tnfaip3 genes is partially dependent on the presence of c-Rel in PMA/I-stimulated T cells. However, considering other genes did not show significant changes in c-Rel/− T cells, it seems likely that the transcriptional regulation of these genes by c-Rel may be dependent on conditions such as cell type or stimulation regime.
To investigate whether the expression of the above five genes are affected by different stimulus conditions, primary CD4+ T cells were stimulated with CD3/CD28 antibodies followed by measurement of mRNA levels using quantitative real-time PCR assays. In this case, Gadd45b strongly responded to CD3/CD28 antibodies, and the three other genes; Bcl10, Nek8, and Ccdc94 weakly responded following stimulation, but Tnfaip3 gene was not responsive (Fig 5.7 and Table 5.4). Among these five genes, the three genes; Gadd45b (p-value = 0.026), Tnfaip3 (p-value = 0.025), and Bcl10 (p-value = 0.018) were affected in c-Rel-deficient CD4+ T cells poststimulation relative to wild-type while there was no effect on Nek8 and Ccdc94 genes (Fig 5.7 and Table 5.4). Interestingly, mRNA expression levels for Tnfaip3 were also decreased in nonstimulated c-Rel/- T cells (Fig 5.7). This decrease in Tnfaip3 expression levels could be associated with the reduction in the Treg cell population in c-Rel/- CD4+ T cells (Isomura et al. 2009).

Taken together, these results validated that selected putative c-Rel binding genes are affected by c-Rel, but the effects of c-Rel/- on gene expression are generally weak.
Figure 5.6 Effect of c-Rel knockout in the expression of c-Rel binding genes in primary CD4$^+$ T cells following PMA/I stimulation

Relative comparison of mRNA levels of c-Rel binding genes in the presence and absence of c-Rel in CD4$^+$ T cells. Primary CD4$^+$ T cells were isolated from wild-type (■) and c-Rel/-- (●) mouse spleens. Cells were nonstimulated (0 h) or stimulated with PMA/I for 6 h and 12 h. cDNA was prepared and analysed by quantitative real-time PCR assays using primer sets. To calculate the relative mRNA levels, C$_i$ values of the reference housekeeping gene (C$_i$(ref)) (ubc) were subtracted from C$_i$ values determined for target genes of interest (C$_i$(target)) which generates ΔΔC$_i$ or C$_i$(target) - C$_i$(ref). The resulting C$_i$ value is then raised to the power 2 to give the final relative mRNA levels ($2^{\Delta \Delta C_i}$). The results are shown as the mean and standard deviation (SD) of three independent experiments. Significant p-values were calculated using paired T tests and are indicated on the top of the panel.
Comparison of mRNA expression levels of c-Rel binding genes in wild-type (■) and c-Rel−/− (■) CD4⁺ T cells. Primary CD4⁺ T cells were isolated from mouse spleens followed by nonstimulation (0 h) or stimulation with CD3/CD28 antibodies for the times indicated. Total RNA was prepared and cDNA was analysed by quantitative real-time PCR assays using specific primer sets. To calculate the relative mRNA levels, \( C_t \) values of the reference housekeeping gene (\( C_t \) (ref)) (ubc) were subtracted from \( C_t \) values determined for target genes of interest (\( C_t \) (target)) which generates \( \Delta\Delta C_t \) or \( C_t \) (target) - \( C_t \) (ref). The resulting \( C_t \) value is then raised to the power 2 to give the final relative mRNA levels (\( 2^{-\Delta\Delta C_t} \)). The results are shown as the mean and standard deviation (SD) of three independent experiments. Significant p-values calculated using paired T tests are shown.
Table 5.4 Summary of c-Rel binding genes that responded to PMA/I and/or CD3/CD28 stimulation and that were inhibited in c-Rel/-/ T cells relative to wild-type cells following stimulation

<table>
<thead>
<tr>
<th>Genes</th>
<th>Motifs</th>
<th>mRNA expression levels</th>
<th>Cmp</th>
<th>mRNA expression levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EL-4</td>
<td>CD4</td>
<td>PMA/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6 h)</td>
<td>(8 h)</td>
<td>(6 h)</td>
</tr>
<tr>
<td>Gadd45b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tnfalp3</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ero1l</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2818457I06Rik</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P62f2</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Bcel10</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Nck8</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Tripp1</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Tlk2</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sna12</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cdc14a</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1700123020Rik</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cdc94</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pigb</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Fbxw8</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hasvr2</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Genes responded to PMA/I and CD3/CD28 stimulation: strongly responding genes (▲); weakly responding genes (†); no response (†); genes inhibited (+++) and not inhibited (Δ) in c-Rel/-/ T cells following stimulation, no data (--).
The relationship between c-Rel and IRF-1 transcription factors in c-Rel binding genes in T cells

It has been well documented that the binding sites of NF-κB and IRF transcription factors are adjacent to each other and the cooperation of two factors leads to active gene expression (Drew et al. 1995; Neish et al. 1995; Saura et al. 1999; Azimi et al. 2000; Naschberger et al. 2004; Kollet et al. 2006; Sgarbanti et al. 2008). In support of this, IRF transcription factor family motifs were the most highly overrepresented in the sequences bound by c-Rel (Section 4.2.4). In addition, as shown in Figure 5.1 and Table 5.1, NF-κB/c-Rel and IRF motifs were detected in the putative c-Rel binding genes. These results, together with previous studies, suggested that the relationship between NF-κB and IRF sites could be important and that there may be an interaction between c-Rel and IRF transcription factors. Thus, to examine the interaction between these two transcription factors, in vivo binding assays were performed in EL-4 and CD4+ T cells. Several IRF antibodies (anti-IRF-1, anti-IRF-2, anti-IRF-4, and anti-IRF-8) were tested to find which antibodies would work in ChIP assays. ChIP assays were performed in EL-4 T cells stimulated with PMA/I for 4 h. While the binding of IRF-1 was significantly increased at this time point on target genes following stimulation, IRF-2 and IRF-4 did not show a significant difference in non- and PMA/I stimulated EL-4 T cells. Interestingly, IRF-8 showed a decreased binding pattern following stimulation (Appendix V). Thus, further in vivo binding studies were performed with anti-IRF-1.

Confirmation of the binding of IRF-1 to c-Rel binding genes in EL-4 T cells

To investigate where IRF-1 binds to putative c-Rel binding genes, in vivo binding assays were performed on five c-Rel-binding genes (Gadd45b, Ccde94, Tnfaip3, Nek8, and Bcl10). ChIP DNA was amplified with specific primer sets used for c-Rel binding assays (Figs 5.4 and 5.5). As shown in Figure 5.8, IRF-1 binds to selected c-Rel binding genes including Gadd45b and Ccde94 containing IRF motifs relative to control after stimulation. In agreement with these results, MatInspector™ program within the Genomatix™ Suite software showed the ISRE which is also bound by IRF-1 (Naschberger et al. 2004) and IRF-1 binding sequences in c-Rel binding regions of Gadd45b and Ccde94 genes respectively (Table 5.2). Interestingly, an increased binding
was also observed in *Tnfaip3* in which an IRF motif(s) was not detected (Fig 5.1 and Table 5.1). *MatInspector*™ program also did not find any putative binding site for IRF-1 in *Tnfaip3*. Instead, a binding site (CTGAGAAACCCATCCCCCTGTT (-)) for IRF-4 was observed near the c-Rel binding site. Although *Nek8* and *Bcl10* genes have IRF motifs, only a low level of binding was observed at 4 h of stimulation. In the case of *Bcl10*, putative ISRE and IRF-1 sites were observed (Table 5.2), but the binding was slightly decreased following 4 h of stimulation. Thus, it is possible that the strong IRF-1 binding to these genes may occur much earlier than the time measured in this experiment, or other IRF family members may be recruited to these genes at this time point.

Overall, these results showed that IRF-1 also binds to c-Rel binding genes following stimulation in T cells (Table 5.5).

### 5.2.6.2 Confirmation of in vivo interaction between c-Rel and IRF-1 in T cells

To address *in vivo* interactions between c-Rel and IRF-1, sequential ChIP assays (c-Rel to IRF-1) were carried out on the above five genes. For this experiment, EL-4 T cells were nonstimulated or stimulated with PMA/I for 4 h. Sheared genomic DNA fragments were initially immunoprecipitated with anti-c-Rel and sequentially, protein/genomic DNA complexes were immunoprecipitated with a second antibody specific for IRF-1. As controls, sequential ChIP was performed with anti-c-Rel followed by anti-IgG (c-Rel to IgG) and with IgG followed by IgG (IgG to IgG). Single ChIP assays with anti-c-Rel antibody were used as reference to show that c-Rel binding normally occurs at 4 h of stimulation in EL-4 T cells (Fig 5.9). As shown in Figure 5.9, the binding in sequential ChIP (c-Rel to IRF-1) increased in three genes, *Gadd45b*, *Ccdc94*, and *Nek8* relative to controls examined following stimulation. However, very small binding activity was observed in the other two genes, *Tnfaip3* and *Bcl10* (Table 5.5). Reverse sequential ChIP assays (IRF-1 to c-Rel) were also performed with anti-IRF-1 followed by c-Rel as a second antibody, together with controls (IRF-1 to IgG and IgG to IgG) to determine whether these factors interact in the opposite direction (data not shown). However, when the order of antibodies was reversed, the binding was not significantly different relative to controls and also binding activity was very low. It seems likely that IRF-1 binding may be c-Rel-independent or the relationship between the two proteins may be a partial co-occupancy as suggested in previous studies (Geisberg et al. 2004). However,
to better interpret these results, more controls where c-Rel shows a high signals but not IRF-1 signals would be needed.

5.2.6.3 Confirmation of the binding of IRF-1 to c-Rel binding genes in CD4+ T cells

To address whether IRF-1 binding to c-Rel binding genes is c-Rel dependent or if the binding of two transcription factors occurs separately in these genes, in vivo ChIP assays were performed in wild-type and c-Rel−/− CD4+ T cells following 8 h of CD3/CD28 stimulation. As shown in Fig 5.10, IRF-1 bound to two genes Nek8 and Gadd45b following 8 h of stimulation in wild-type cells (Fig 5.10). In contrast, the binding at the Bcl10, Tnfaip3, and Ccdeb9 genes was similar to that of nonstimulated cells at 8 h of stimulation in wild-type CD4+ T cells (data not shown), suggesting that IRF-1 binding may occur much earlier than 8 h of stimulation. Interestingly, in c-Rel−/− T cells, IRF-1 binding was inhibited in the Nek8 gene, suggesting that IRF-1 binding may be dependent on c-Rel in the Nek8 gene. However, this inhibition of IRF-1 binding was not observed in Gadd45b gene in c-Rel−/− CD4+ T cells relative to that of wild-type cells (Table 5.5).

Thus, these results suggest that IRF-1 also binds to c-Rel binding genes in CD4+ T cells, but IRF-1 binding to the Nek8 gene may be dependent on the c-Rel subunit.
Figure 5.8  In vivo binding of IRF-1 to c-Rel binding genes in EL-4 T cells

ChIP assays were performed with anti-IRF-1 using EL-4 T cells nonstimulated (0 h) or stimulated for 4 h with PMA/I. Quantitative real-time PCR was performed with specific primer sets used for c-Rel binding assays (Figs 5.4 and 5.5) on the immunoprecipitated genomic DNA fragments. The graph is represented by comparing IRF-1 binding to Rho ( ■ ) relative to IRF-1 binding to the gene of interest ( ■ ). Rho was used as a negative binding control. The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting C_t values of TI DNA from IP C_t values (C_{t IP} - C_{t TI}) and TI from NA (C_{t NA} - C_{t TI}). The resulting C_t values is then raised power 2 to give the final relative amount of ChIP DNA (2^{-ΔΔCt (IP-TI)} or 2^{-ΔΔCt (NA-TI)}). The results are shown as the mean and standard deviation of three to five independent biological experiments. The red dotted line indicates a base-line (0.02), where the graph below the line is considered as no binding of IRF-1 to target genes. The results are shown as the mean and standard deviation (SD) of three independent experiments.
Figure 5.9 Interaction between c-Rel and IRF-1 transcription factors in T cells

Nuclei were extracted from EL-4 T cells nonstimulated (0 h) or PMA/I stimulated for 4 h. Forward sequential ChIP assays were performed with antibodies specific for c-Rel, IRF-1, and IgG as a negative control. Genomic DNA was first immunoprecipitated with anti-c-Rel and anti-IgG respectively and then eluted DNA/protein complex were immunoprecipitated with anti-IRF-1 (c-Rel to IRF-1) and anti-IgG (c-Rel to IgG) or IgG to IgG as second antibodies. Quantitative real-time PCR was performed on the immunoprecipitated DNA with specific primer sets. The graph is represented by comparing the binding activity of c-Rel/IRF-1 (c-Rel to IRF-1) to those from controls (c-Rel to IgG or IgG to IgG). Rho was used as negative binding control. Single ChIP assays were performed with anti-c-Rel antibody. The graph is represented by comparing c-Rel binding to Rho to c-Rel binding to the gene of interest. To quantify the relative amounts of ChIP DNA, C_t values in no antibody (NA) condition was subtracted from the C_t values of immunoprecipitated DNA (IP) and then normalized to the amount of relative DNA of total input (TI) ((C_t^{IP} - C_t^{NA})/C_t^{TI}). The resulting C_t values is the raised power 2 to give the final relative amount of ChIP DNA (2^-ΔΔCt ((IP-NA)/TI)). The red dotted line indicates a base-line (0.02 and 0.2 respectively), where the graph below the line is considered as no binding of IRF-1 to target genes. The results are shown as the mean and standard deviation (SD) of three independent experiments.
Figure 5.10 *In vivo* binding assays of IRF-1 in primary CD4⁺ T cells following CD3/CD28

Wild-type or *c-Rel/-/-* CD4⁺ T cells were isolated from mouse spleens followed by nonstimulation (0 h) or stimulation for 8 h with CD3/CD28 antibodies. Nuclei were extracted from the above cells. Genomic DNA was sheared by sonication and immunoprecipitated with antibodies specific for IRF-1. Quantitative real-time PCR was performed on immunoprecipitated genomic DNA fragments with specific primer sets used for IRF-1 binding assays (Fig 5.8) amplifying the regions bound by c-Rel. The graph is represented as IRF-1 binding to *Rho* (■) relative to IRF-1 binding to the gene of interest (□). To quantify the relative amounts of ChIP DNA, *C*ₜ values in no antibody (NA) condition was subtracted from the *C*ₜ values of immunoprecipitated DNA (IP) and then normalized to the amount of relative DNA of total input (TI) ((Cₜ⁰⁻ Cₜ⁰[Na])/Cₜ⁰[TI]). The resulting *C*ₜ values is the raised power 2 to give the final relative amount of ChIP DNA (2⁻ΔΔCₜ(Δ(Δ(Δ(Δ(TI-NA)))). *Rho* was used as a negative binding control. The red dotted line indicates a base-line (0.05), where the graph below the line is considered as no binding of IRF-1 to the target genes. The result is presented from two independent experiments.
Table 5.5 Summary of c-Rel binding genes bound by IRF-1 in EL-4 and primary CD4+ T cells following PMA/I stimulation

<table>
<thead>
<tr>
<th>Genes</th>
<th>Motifs*</th>
<th>mRNA expression levels</th>
<th>ChIPa</th>
<th>mRNA expression levels</th>
<th>ChIP</th>
<th>Sequential ChIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NF-κB</td>
<td>c-Rel</td>
<td>IRF</td>
<td>EL-4</td>
<td>EL-4</td>
<td>CD4+</td>
</tr>
<tr>
<td></td>
<td>PMA/I</td>
<td>(6 h)</td>
<td>(6 h)</td>
<td>(6 h)</td>
<td>(8 h)</td>
<td>(8 h)</td>
</tr>
<tr>
<td>Gadd45b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tufip3</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evt1l</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010457I06Rik</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fos2f2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nic2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tri4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tkl2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sna12</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc14a</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1701130020Rik</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celda94</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigh</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phx4</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haver2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genes that were bound by IRF-1 at 4 h of PMA/I stimulation in EL-4 T cells: low (••); weak binding (•); no data (—). Genes bound by c-Rel following CD3/CD28 stimulation in wild-type CD4+ T cells: strong (◦); no binding (/); no data (—). Genes bound by both c-Rel/IRF-1 transcription factors following PMA/I stimulation in EL-4 T cells: low (x); very low (x); no binding or no data (—).
5.3 Discussion

The key aims of this chapter were to validate the ChIP-on-chip results and microarray analysis data presented in Chapter 4. The confirmation experiments showed that 31% of the selected regions were bound by c-Rel in EL-4 and CD4+ T cells following T cell stimulation using ChIP-PCR. In addition, two of c-Rel binding genes may be partially dependent on c-Rel following stimulation. IRF-1 also binds to this subgroup of c-Rel binding genes and the binding of IRF-1 to the Nek8 gene may be c-Rel-dependent.

To understand the gene expression mechanisms mediated by transcription factors, ChIP-on-chip has been performed to identify binding regions and genes nearest to these binding regions (Chapter 4). A very large number of transcription factor binding sites and their nearest genes have been found through this approach. However, not all genes/regions identified by these genome-wide approaches are actually bound by a transcription factor. A false positive rate of binding has previously been observed and thus validation experiments are essential for ChIP-on-chip data analysis. Many studies have performed validation experiments to confirm authentic binding of transcription factor to target sites using ChIP (Vigano et al. 2006; Frontini et al. 2009; Margolin et al. 2009). In this chapter, 16 genes selected by combining ChIP-on-chip and available expression profiling data were investigated. The validation experiments using ChIP confirmed that 31% (5 out of 16 genes) of these genes are bound by c-Rel in vivo in EL-4 T cells. Interestingly, all five genes where binding was validated have NF-κB motifs, and four out of these five genes have c-Rel specific motifs containing core sequences (TTCC). In addition, the binding of c-Rel was stronger to three out of these four genes. Studies in c-Rel/- T cells confirmed that the signal is indeed from c-Rel binding and that these genes also bind c-Rel in CD4+ T cells. However, no or little binding was observed in genes harbouring no κB motifs (Fig 5.4). Thus, these results suggest that the binding of c-Rel may be associated with the presence of NF-κB motifs, and the stronger binding is associated with the presence of c-Rel motifs.

The binding of a transcription factor to the target gene and/or the region of a gene suggests that the gene will potentially be regulated by the transcription factor. However, the transcription of target genes is not always associated with the binding of transcription factors (Phuc Le et al. 2005; Beima et al. 2006; Song et al. 2009). For this
reason, gene expression profiles of transcription factor target genes have been investigated using quantitative real-time PCR (Martone et al. 2003; Bernstein et al. 2004; Kurdistani et al. 2004; Phuc Le et al. 2005; Beima et al. 2006; Vigano et al. 2006; Frontini et al. 2009; Song et al. 2009). Five c-Rel binding genes were tested to investigate whether these genes are directly affected by c-Rel deletion. However, even though these genes were bound by c-Rel following stimulation, not all the genes were affected by c-Rel deletion. Only two genes (Gadd45b and Tnfaip3) were partially affected in c-Rel/- T cells following PMA/I and CD3CD28 stimulation, while the expression of Ccdc94 and Nek8 genes was not affected by c-Rel deletion under the same stimulation conditions tested and the effects of c-Rel deletion on Bcl10 gene expression were dependent on the stimulation conditions. These data suggest that even though c-Rel binds to these genes following stimulation, the effects of c-Rel deletion on expression of these genes is subtle. This c-Rel redundancy has also been observed in a previous study (Bunting et al. 2007) where only small effects of c-Rel deletion on expression of genes were observed following stimulation. In this study, among 15 or 20 genes that were identified as c-Rel-affected by microarray analysis, only small number of genes (~30%) showed a reduced expression level in c-Rel/- T cells. Thus, it is possible that there is redundancy with other NF-κB family members. Therefore, to examine this c-Rel redundancy, it could be useful to overexpress c-Rel or combine c-Rel/- with the deletion of other family members.

This similar phenomenon has also been commonly observed in many ChIP-on-chip studies (Martone et al. 2003; Beima et al. 2006; Lim et al. 2007; Frontini et al. 2009). These studies reported that transcription factors bind to many genes, but some target genes either are not affected under the conditions tested, or are regulated differently in response to different stimulation conditions, or are cell type specific (Martone et al. 2003; Beima et al. 2006; Vigano et al. 2006; Frontini et al. 2009). For example, the T-bet transcription factor bound to numerous target genes, but many of them were not affected by either T-bet overexpression or in T-bet/- B cells (Beima et al. 2006). In particular, three genes; Cxcr3, Rad51, and Ccl3, showed a cell type dependent expression in T-bet/- B or T cells. Another study showed that selected p63 target genes vary in expression profiles in human cells (HaCaT). Thus, some of them were transiently induced or repressed, while some genes were not affected or changed under the same condition (Vigano et al. 2006). This similar phenomenon has also been observed in human breast cancer cells (H3396) where many genes were bound by IRF-1
and only a subset were affected after IFN-γ treatment. However, some of them did not respond or were unchanged under the same condition (Frontini et al. 2009).

The effect of c-Rel deficiency was observed on three genes; Gadd45b, Tnfaip3, and Bcl10. Interestingly, the mRNA expression levels of Gadd45b and Tnfaip3 were decreased in c-Rel/-/- T cells regardless of the stimulus conditions examined. These results suggest that the binding of c-Rel may be associated with their expression. As demonstrated in many previous studies, Gadd45b and Tnfaip3 contain κB sites and the mutation of these sites prevents the binding of NF-κB proteins, which abolishes the transcriptional activation of these genes (Krikos et al. 1992; Jin et al. 2002; Papa et al. 2004; Liuwantara et al. 2006). In addition, Yeh et al. reported that NF-κB activation is associated with Bcl10 gene upregulation in human breast cancer cells (Yeh et al. 2006). Thus, considering these results with others, Gadd45b, Tnfaip3, and Bcl10 may be c-Rel-dependent genes.

Another interesting observation is that the binding sites identified for these three genes were located downstream from the TSS (intronic region), suggesting that these intronic sites may also be important for their gene expression in T cells. In support of this, Schjerven et al. demonstrated that an intronic region containing a NF-κB binding site is required for full activation of the human polymeric Ig receptor (pIgR/SC) gene where NF-κB cooperates with the DNA elements located in the proximal promoter for full activation of the pIgR/SC gene (Schjerven et al. 2001). Intronic regions are also associated with chromatin remodeling and histone acetylation (Guo et al. 2003). An intron of the manganous superoxide dismutase (Mn-SOD) housing a TNF-responsive element (TNFRE) contains a NF-κB binding site and the binding of NF-κB to this region leads to histone acetylation as well as an alternation in chromatin structure by recruiting histone acetyltransferase complexes such as CBP/p300 (Guo et al. 2003). Considering these results, it is possible that NF-κB binding sites identified within the intronic region may also be involved in activation of the c-Rel binding genes.

The physical interaction or cooperation between NF-κB and IRF transcription factors has long been studied, as NF-κB/IRF complexes have been shown to mediate synergistic effects in the regulation of many genes such as MHC1, VCAM1, IP-10, IL-15, GBP1, and HIV-1 LTR (Drew et al. 1995; Neish et al. 1995; Ohmori et al. 1995; Azimi et al. 2000; Naschberger et al. 2004; Sgarbanti et al. 2008). Motif analysis (Section
4.2.4) suggested a potential interaction between two transcription factors with binding sites of these transcription factors detected in the putative c-Rel binding regions. Thus, it was worth investigating whether c-Rel and IRF-1 may have some dependence in terms of binding. ChIP assays showed that IRF-1 also binds to c-Rel binding regions that were also bound by c-Rel in T cells. However, little evidence was observed that there was a co-dependency for binding, except for Nek8. Knockout experiment showed that IRF-1 did not bind to the Nek8 in c-Rel/- T cells, suggesting that IRF-1 binding may be c-Rel-dependent. This NF-κB dependent binding has also been reported in human Jurkat cells, where mutation in NF-κB sites prevented the formation of the NF-κB/IRF-1 complex, resulting in a decrease of the HIV-1 LTR transcriptional activation (Sgarbanti et al. 2008). However, these c-Rel/- experiments need to be repeated to verify the data.

In this chapter, to investigate the interaction between two transcription factors, sequential ChIP was performed using two antibodies. ChIP-PCR showed a signal when ChIP was performed with c-Rel followed by IRF-1, but when the order of antibodies was reversed, the binding signal was not significant. Thus, these sequential ChIP assays could not prove evidence of interaction between the two transcription factors. To better interpret the interaction between these two factors, more controls such as a gene with high c-Rel signal and no IRF-1 signal would be necessary. Considering the results from the MatInspector\textsuperscript{TM} program showing that putative IRF binding sites within the five genes bound by c-Rel (Gadd45b, Tnfaip3, Bcl10, Ccdc94, and Nek8) are adjacent to those of NF-κB without overlapping, they may bind independently to target sites. As suggested in previous studies, this interaction seems like a partial co-occupancy, namely, the binding of both proteins is independent (Geisberg et al. 2004). The most interesting finding was that only the binding of IRF-1 to the Nek8 gene is c-Rel dependent. The MatInspector\textsuperscript{TM} program represented several putative binding sites for IRF proteins (IRF3, IRF4, and IRF7), but not IRF-1 in the c-Rel binding region of the Nek8. In addition, these binding sites are nonoverlapped with NF-κB sites respectively (see Fig 5.11 and Table 5.2). Thus, it remains to be determined how the binding of IRF-1 to the Nek8 gene is affected in c-Rel/- T cells. One possibility is that IRF-1 may bind to the target site indirectly through a c-Rel subunit.

In summary, the binding of c-Rel to a number of regions was validated using ChIP-PCR. It was observed that these regions all contained NF-κB and/or c-Rel binding motifs thus
implying a high false positive rate among regions that did not have NF-κB motifs. Small effects of c-Rel deletion on the expression of these genes were observed, implying that there may be significant redundancy in the function of c-Rel. While IRF motifs were highly enriched in c-Rel binding regions and IRF-1 was shown to bind to many of these regions, a functional interaction remains to be proven.
GCGGGGCAGG GACAAAAAT AAACAACTCT TCTCTGGCCC ACTGAGAAAT AGGAAGGGT 60

TTAGCTAAGG ACCACTCTCT GAAATCTGAC ATTTTATAAC TCCCTTAGAG CTGCAGGGCGT 120
Forward primer

CATGAAGAAG TCAAGATTTC ATTCATCTCTG GTGAAACCAC TAGCAGCGAT ACCAAACCAC 180
Reverse primer

CCACTATTTCC TGGACAGAAG CATAGAAACCT TCTTGCGGTT TCCCTCTACC GGCACGCTAT 240

NF-κB p50

AAGTTTTGAG TAAAGCTCCG AGTCCCCCCTA CGGCAGCGGT TGGTGCGGAT TCTGAGAAAC 300

CGAGAGCAGT GGTGCTAGTA GGTGTTCAAA CCAAGAGACC AGTCATGTA TCTTCAGGT 360

IRF-3

GAATCGATTGC TCACCAACC CTGGGTTTGCGG TCCCTCTACC GGCACGCTAT GAATAACGC 420

NF-κB c-Rel

CGCTCCTCTC CCCACACAGA CCCCCTCGGT TTTGGGTGT TATACTTTAC GTGCCAATCA 480

IRF-4

TGAGAATCCC TTTATCTTTCC TGAAGTAGTG CCAACTCGTC CCGGACAGTG GGGACTGATT 540

CGATCTTCGC ATTCCACTG CATTGACACT CTGAAAGAAC ACTCTTTGTT TTTTGCCCAGG 600

IRF-7

CAACATAAGT GGGGCGCTTTA TCGCTCGAGT CTAACTACCT ATGCCCAAGT CAGGCGGTGT 660

CGATGGGTGCT GTAGCGGCTC TGAAGACTGA GGGACTCGTC AAGAAACCCA GAACGACTAT 720

TCTCTGATCC CTCGGGTTGAA AACACGACT CAGCTCTAGG GTTGAGTTCA AAGTCAGAGA 780

AAGAGCCGG ACTCGACAGA CGCCCCCGTT TCGCGGGGTC CGCCCTGCAA AAGTTTTACA 840

GCAAGGAGGA GGAGCTCCCA GATGGGAGGG TCCGCGACAG CCGCCGACCG GGGATTCCAGA 900

AGCGTAACTG AGAAATGAGA TGGAGAAGTA CGAGCGGATC CGAAGGTGGA GAGAGGTTGC 960
Figure 5.11 *MatInspector*™ prediction of putative binding sites of IRF and NF-κB transcription factors at the upstream region of the *Nek8* gene from the TSS

Two NF-κB binding sequences marked by blue are shown between the primer sets (green) and the IRF binding sequences. Three IRF binding sequences located near the NF-κB binding sites are indicated by red. The transcription start site (TSS) is indicated by a right angled arrow. ATG indicates the translation start codon. Upstream sequences were obtained from the UCSC and Ensembl Genome Browsers by comparing with regions obtained from ChIP-on-chip analysis data.
Chapter 6

Final discussion
Chapter 6

Preliminary Discussion
6.1 Summary

The main aim of this thesis was to determine the genomic binding sites of the NF-κB c-Rel and RelA proteins in a T cell model using molecular and computational approaches. The work performed in this study involved the determination of the kinetics of in vivo binding of c-Rel and RelA to specific genes using ChIP, followed by the identification and characterization of binding regions of these transcription factors using a genome-wide location analysis approach. Potential target genes for c-Rel were identified by combining the location analysis data with expression profiling data from PMA/I stimulated and/or c-Rel/- T cells. Both binding and expression data were verified for a small number of genes. Finally, a possible interaction between c-Rel and IRF-1 transcription factors was investigated using conventional ChIP and sequential ChIP assays. Thus, the results described in this study will facilitate a better understanding of the distinct roles of NF-κB proteins and the relationship between NF-κB and gene expression in T cells.

6.2 c-Rel and RelA have distinct kinetics of promoter association in T cells

As described previously, c-Rel and RelA are members of the NF-κB family of transcription factors and are structurally similar. However, even if they are closely related, they play different roles in T cells (Banerjee et al. 2005). Many previous studies have reported that the time frame of nuclear accumulation of c-Rel and RelA is distinct (Bryan et al. 1994; Venkataraman et al. 1995; Himes et al. 1996; Rao et al. 2003). These studies have showed that the accumulation of RelA is fast and transient, while c-Rel accumulation is relatively late compared with that of RelA and is stably maintained for longer, even after stimulus withdrawal, in the nucleus relative to RelA or other transcription factors (Bryan et al. 1994; Rao et al. 2003; Brettingham-Moore et al. 2005). A fast accumulation of RelA has also been observed at gene promoters such as IkBa and MIP-2 (Saccani et al. 2001). Thus, the translocation of c-Rel and RelA to the nucleus does not occur at the same time and c-Rel may be stably maintained in the nucleus of T cells. In support of this, Chapter 3 of the study presented here showed that c-Rel and RelA have different protein kinetics in the nucleus of activated T cells. Furthermore, in
vivo binding assays using ChIP also showed distinct kinetics of binding of c-Rel and RelA to the IL-2 and GM-CSF gene promoters (Chapter 3). This difference may lead to distinct roles of these individual NF-κB proteins in controlling the expression of these genes.

Many studies have shown that RelA is associated with HAT complex, p300/CBP, which acts as a bridge between transcription factors and the basal transcriptional machinery and plays an important role in chromatin remodeling (reviewed in Giles et al. 1998; Zhong et al. 1998; Vanden Berghe et al. 1999; reviewed in Roth et al. 2001; Kadonaga 2004). The interaction between RelA and p300/CBP leads to an increased transcriptional activation of RelA-dependent genes (Gerritsen et al. 1997; Perkins et al. 1997; Zhong et al. 1998; Sheppard et al. 1999). In addition, it has been shown that RelA interacts with p300 which results in a synergistic activation of the GM-CSF promoter in human T cells (personal communication; Karen Bunting). Further studies have shown that Brg1, which is a key component of the ATP-dependent chromatin remodeling SWI/SNF complex, is recruited to the promoter of GM-CSF by RelA in T cells (Holloway et al. 2003). Thus, the rapid appearance of RelA may recruit coactivators involved in histone modification to the promoter region which leads to chromatin remodeling and increased gene expression. It is also worth speculating that this rapid RelA appearance in the nucleus may be relevant to the timing of histone acetylation which occurs very early at -2 kb upstream from the TSS of the IL-2 gene following stimulation (Chen et al. 2005). Thus, RelA may be required for the initial processes of the above events at the IL-2 and GM-CSF gene promoters.

As reported previously, c-Rel plays a nonredundant role for chromatin remodeling and histone loss at the IL-2 or GM-CSF promoter region (Rao et al. 2003; Chen et al. 2005). However, c-Rel may not directly interact with p300 to change chromatin structure and to activate gene expression at the IL-2 and GM-CSF promoters because previous studies did not find evidence for interaction or for synergistic function between the two factors at the GM-CSF gene promoter following stimulation (personal communication, Karen Bunting). However, an interesting study by Sun et al. showed that the interaction of c-Rel and p300 synergistically increased the IL-12p40 promoter activity in 293 T human embryonic kidney epithelial cells (Sun et al. 2004), suggesting that the function of c-Rel may be gene or cell type specific. Thus, these results imply that a gradual and slow binding of c-Rel to inducible gene promoters may be associated with the stable
maintenance of chromatin remodeling and transcriptional activation of the IL-2 and GM-CSF genes, rather than the initiation of these events in T cells. However, at this stage, it is not clear how c-Rel carries out this function, and further experiments are required. It is possible to speculate that this stable existence of c-Rel in the nucleus may allow chromatin remodeling to be maintained for a longer time at the IL-2 (12 h) and GM-CSF (24 h) promoters following activation in T cells. Taken together, these results suggest that c-Rel and RelA may play distinct roles in the regulation of cytokine genes in T cells.

6.3 c-Rel and RelA binding sites are scattered across the genomic DNA

Many genome-wide analyses for identification of transcription factor binding sites have been primarily focused on promoter regions (Friedman et al. 2004; Odom et al. 2004; Laganier et al. 2005; Beima et al. 2006; Cheng et al. 2006; Pillai et al. 2009). However, as previously reported, many binding sites for transcription factor and other factors involved in the regulation of gene expression have been observed not only at the promoter or upstream region from the TSS but also within other regions such as exons, introns, or at some distance from the gene (Martone et al. 2003; Cawley et al. 2004; Euskirchen et al. 2004; Hartman et al. 2005; Vigano et al. 2006; Lim et al. 2007). In a study by Martone et al. the binding sites for NF-κB RelA have also been observed in coding and non-coding regions across the human genome in human HeLa cells (Martone et al. 2003). In addition, a recent unbiased study using ChIP-PET performed in THP-1 human monocytic cells showed that a high proportion of RelA binding sites (38%) are located within introns and in other regions including exons and 3' region (within 100 kb from the transcriptional end point) (Lim et al. 2007). In agreement with these studies, in Chapter 4 of this study at least, a quarter of binding sites for c-Rel and RelA were present within genes (transcribed regions). In addition, few binding sites were observed in the genomic DNA beyond the 3' end of the genes. Using ChIP assays, it has been confirmed that binding sites are located within intronic regions (Chapter 5). These findings imply that the binding sites for the NF-κB transcription factors are not limited only to the promoter or upstream region from the TSS, but probably lie across the entire genome.
In this study (Chapter 4), over half of the binding sites of c-Rel and RelA were detected at the promoter or upstream from the TSS in EL-4 T cells. A study by Martone et al. showed that nearly a third of NF-κB RelA binding regions are located within 5 kb upstream of the 5’ ATG in human HeLa cells (Martone et al. 2003). In addition, another recent study performed in human monocytic cells showed that a quarter of RelA binding regions (26%) were observed in the proximal upstream promoter region (Lim et al. 2007). These results imply that the upstream or promoter regions are important for the function of NF-κB in gene regulation. The results observed in this study, together with other studies, imply that NF-κB binding sites are scattered within the coding and non-coding regions of the genome. However, the majority of c-Rel and RelA binding sites are primarily located in the upstream or close to the promoter regions in EL-4 T cells. However, it should be noted that the DNA microarray used in the study described here does not cover the whole mouse genome. It is biased toward regions near the TSS of known genes as it only covers from -10 kb upstream to +2 kb downstream of RefSeq genes from the University of California Santa Cruz (UCSC) database (Xia et al. 2009). Thus, an unbiased method such as ChIP-Seq would reveal a better view of the distribution of binding sites for c-Rel and RelA scattered across the whole genome.

In a study by Guo et al. the importance of the intronic region that bind NF-κB proteins has been well documented. This study showed that chromatin remodeling and histone acetylation occur in response to a tumor necrosis factor (TNF) at a TNF-responsive element (TNFRE), containing binding sites for NF-κB binding within an intron of the Mn-SOD gene. These changes rely on the binding of RelA to the TNFRE which is accompanied by the induction of the Mn-SOD gene (Guo et al. 2003). Another study has also observed an NF-κB site within the intronic enhancer of the human polymeric Ig receptor/secretory component (pIgR/SC) gene, where NF-κB is required for full TNF-α mediated activation of the pIgR/SC gene (Schjerven et al. 2001). In agreement with these findings, in the study presented here several regions bound by c-Rel were observed within introns of target genes and were confirmed by ChIP assays (Chapter 5). However, direct evidence that c-Rel binding to these intronic regions is involved in gene expression of the target genes has not been shown in this study and would require further investigation. Considering the results described in this study and those of others, intronic regions may play important roles for the regulation of target genes.
6.4 c-Rel binding regions are occupied by RelA

In this study (Chapter 4), at least a quarter of c-Rel bound regions were also occupied by the RelA subunit. A study by Martone et al. suggested that over 20% of RelA bound sequences contain specific c-Rel recognition sequences, although no experimental evidence was provided, suggesting that RelA binds to these sequences by forming a heterodimer with c-Rel. Similarly, an in vitro study showed that a p65/p50 heterodimer binds to the c-Rel site (GGAAATCCCC) of the GBP-1 promoter in response to IL-1β and TNF-α in human umbilical vein endothelial cells (HUVEC) (Naschberger et al. 2004). These observations imply that many c-Rel binding regions may also be recognized by RelA and that c-Rel and RelA may coregulate many regions.

However, a high proportion (approximately 70%) of regions bound by c-Rel were not recognized by RelA in current study (Chapter 4). Given that a single transcription factor rarely regulates gene expression (reviewed in Qiu 2003; Narlikar et al. 2009), it could be possible that these regions bound by c-Rel alone may be coregulated by other NF-κB members by forming heterodimers with c-Rel. As described in Chapter 1, c-Rel can form several homo- or heterodimers with itself or other NF-κB family members, which can bind to DNA and activate gene expression (reviewed in Hoffmann et al. 2006; O’Dea et al. 2009). This implies that c-Rel binding regions may be corecognized by other homo- or heterodimers containing c-Rel and other NF-κB families. As only RelA has so far been investigated, it will be necessary to investigate whether c-Rel binding regions are cooccupied by other NF-κB members in T cells. Another influencing factor may be the different time frame of c-Rel and RelA binding in T cells. As previously described in Chapter 4, two different time points (8 h and 2 h) were chosen respectively for ChIP-on-chip experiment because the maximum binding of c-Rel was observed at 8 h after activation at the IL-2 promoter region, while RelA peaked at 2 h following stimulation (Chapter 3). Thus, when c-Rel is maximally bound to the promoter, RelA has returned to the basal levels. Another possibility may be associated with the affinity of c-Rel for NF-κB recognition sites. As reported previously homodimers of c-Rel can bind to a broad range of NF-κB sites with much higher affinities than RelA homodimers (Sanjabi et al. 2005). Thus, these differences in binding kinetics and in binding affinity may explain why most of the c-Rel binding regions (70%) were only occupied by c-Rel.
6.5 Transcription factor and its target genes

ChIP-on-chip has been widely performed to identify transcription factor binding sites as well as genes targeted by transcription factors of interest on a genome-wide scale in different species including yeast, mouse, and human (Ren et al. 2000; Lee et al. 2002; Mao et al. 2003; Martone et al. 2003; Wells et al. 2003; Friedman et al. 2004; Odom et al. 2004). However, ChIP-on-chip does not provide evidence on whether the transcription factor of interest is important for the regulation of target genes (reviewed in Kirmizis et al. 2004; Phuc Le et al. 2005; Beima et al. 2006; Song et al. 2009). Thus, it is necessary to confirm the relationship between transcription factor binding and its functional effect on gene regulation.

In the study presented here, although a significant percentage of genes nearest to c-Rel or RelA binding regions were affected by PMA/I and c-Rel overexpression compared with the whole expression array, only a small number of genes were up- or downregulated (Chapter 4), suggesting that many of these genes do not respond to PMA/I stimulation and/or are c-Rel-independent. Similar results have been reported in LPS-stimulated THP-1 human monocytic cells using ChIP-PET (Lim et al. 2007), where only 20% and 4% of nearest RelA genes were induced or repressed respectively. A similar phenomenon has also been observed in human HeLa cells where a high proportion of genes bound by RelA (about 38%) located adjacent to or within genes were not expressed in response to TNF-α (Martone et al. 2003). Similarly, only a small percentage of genes bound by transcription factor p63 were also upregulated (12% to 15%) or downregulated (14% to 27) following stimulation in human cervical carcinoma (ME180) cells (Yang et al. 2006). Given the series of results shown in this study and others, a single transcription factor may not be sufficient to alter the expression of a gene. Thus, in addition to the binding of individual transcription factor, other factors or signals may be required for gene activation. In agreement with this, it has been reported that the Mn-SOD gene induction by a tumor necrosis factor (TNF) requires two transcription factors, RelA and Sp1 in mouse NIH3T3 cells. The absence of RelA or Sp1 did not induce Mn-SOD activation after treatment of TNF (Guo et al. 2003). In addition, some binding sites targeted by transcription factor may not be relevant to transcriptional changes (Lim et al. 2007). Furthermore, it could be possible that target
genes may be expressed in different cell types or controlled under different conditions (Martone et al. 2003; Beima et al. 2006; Frontini et al. 2009).

In the current study, validation studies showed that expression profiles of c-Rel binding genes vary in CD4+ T cells. However, only a small number of c-Rel binding genes including Gadd45b, Tnfap3, and Bcl110 were affected in c-Rel/-/- T cells in response to PMA/I and/or CD3/CD28 (Chapter 5). A similar phenomenon has also been observed in many ChIP-on-chip studies (Martone et al. 2003; Beima et al. 2006; Vigano et al. 2006; Frontini et al. 2009). For example, a study by Beima et al. reported that the expression of a number of target genes of the transcription factor T-bet was not affected by T-bet overexpression or by the absence of T-bet in B and T cells following stimulation. In addition, the gene expression of several genes including Cxcr3, Ccl3, and Ifny was decreased in T-bet/-/- CD4+ T cells, but they were not affected in T-bet/-/- CD8+ T cells. Moreover, two genes, Stat1 and Il2Rb were not affected under the same conditions (Beima et al. 2006), suggesting a cell type specific activation. In addition, a similar study performed in human breast cancer cells showed that some IRF-1 target genes were upregulated after IFN-γ stimulation, but a number of genes were transiently expressed, repressed, or not affected following stimulation (Frontini et al. 2009). Vigano et al. reported that target genes bound by p63 are differently regulated in human HaCaT cells where some genes were strongly activated, repressed, or unchanged under the same condition (Vigano et al. 2006). Thus, these results suggest that expression profiles of target genes bound by a transcription factor could vary according to cell type or stimuli condition.

6.6 Relationship between the transcription factors c-Rel and IRF-1

The interaction between NF-κB and IRF-1 transcription factors has been well documented in previous studies (Drew et al. 1995; Neish et al. 1995; Azimi et al. 2000; Naschberger et al. 2004; Kollet et al. 2006). These studies demonstrated that binding sites of these transcription factors are adjacent and cooperate with each other, leading to synergistic effects on gene transcription. A study by Sgarbanti et al. suggested that NF-κB p50/p65 heterodimers and IRF-1 form a complex at the long terminal repeat (LTR)
enhancer of the *human immunodeficiency virus type 1* (*HIV-1*) containing κB sites and this complex induces full HIV-1 LTR transcription (Sgarbanti et al. 2008). The relationship between c-Rel and IRF-1 has also been reported at the *p35* proximal promoter region where c-Rel and IRF-1 independently bind to the proximal promoter region and overexpression of the two transcription factors significantly increased the promoter activity. In addition, c-Rel alone did not activate the *p35* promoter without activated IRF-1 (Kollet et al. 2006), which is reminiscent of findings described in this study showing that certain c-Rel binding genes were not altered in *c-Rel/-/-* T cells (Chapter 5), suggesting that other transcription factors may be required for gene transcription. An interesting study by Saura *et al.* suggested that although the binding sites for NF-κB and IRF-1 are not adjacent to each other (over 850 bp apart) at the *inducible nitric oxide synthase* (*iNOS*) gene promoter, DNA bending promotes the physical interaction of NF-κB and IRF-1 which generates a synergistic induction of the *iNOS* gene (Saura et al. 1999). Thus, these previous studies imply that NF-κB and IRF-1 induce gene expression through a cooperative physical interaction regardless of distance.

In the study presented here, the relationship between NF-κB and IRF transcription factors has been detected by motif analysis where IRF transcription factor family motifs were observed as highly overrepresented motifs in the sequences bound by c-Rel and RelA (Chapter 4). These findings suggest a potential interaction between NF-κB and IRF transcription factors and support the previous studies. *In vivo* binding assays using forward sequential ChIP experiments showed an interaction between c-Rel and IRF-1 transcription factors at the c-Rel target genes. However, reverse sequential ChIP results did not show a distinct relationship between the two transcription factors (Chapter 5). Thus, to better prove the interaction between two transcription factors, more controls such as a gene with high c-Rel signal, but no IRF-1 signal would be required. As suggested previously, this interaction may be a partial co-occupancy, namely, the binding of both transcription factors is independent (Geisberg et al. 2004), suggesting that binding sites may not be overlapped at the c-Rel binding genes (Drew et al. 1995; Saura et al. 1999; Azimi et al. 2000; Naschberger et al. 2004). In support of this, analysis of the binding sites of c-Rel and IRF-1 transcription factors using the *MatInspector*™ program showed that putative NF-κB and IRF-1 binding sites are adjacent to each other with no overlap at the nearest c-Rel binding genes (see Figs 5.2 and 5.11).
Further ChIP assays performed in CD4⁺ T cells showed that IRF-1 binds to c-Rel binding genes including Nek8 and Gadd45b that were also bound by c-Rel following stimulation (Chapter 5). However, no binding activity was observed in three other genes including Bck10, Tnfaip3, and Ccdo94. It seems likely that the binding of IRF-1 to these genes may occur much earlier than measured. It is also possible that these genes may be bound by other IRF members, as the MatInspector™ program showed that these genes have binding sites for other IRF proteins. Interestingly, IRF-1 binding to Nek8 was c-Rel-dependent in T cells. In the study presented here, whether c-Rel and IRF-1 cooperate and generate a strong activation of nearest genes has not been investigated. However, considering the results described in this study together with other studies (Saura et al. 1999; Azimi et al. 2000), they may potentially cooperate and function together regardless of distance in the regulation of c-Rel binding genes selected.

6.7 Conclusions

The findings described in this study demonstrated that the NF-κB family members c-Rel and RelA have distinct kinetics of binding at the IL-2 and GM-CSF promoter regions in the nucleus of activated T cells, implying that they may play nonoverlapping roles in cytokine gene regulation in T cells. Using ChIP-on-chip analysis, this study showed that the binding sites of c-Rel and RelA are present not only within both promoter and upstream regions but also within genes, implying that both regions may be important for gene regulation. ChIP-on-chip combined with expression profiling data showed that some nearest genes to c-Rel and RelA binding regions may be activated under different conditions. Validation studies showed that only a small number of c-Rel binding genes are affected by the absence of c-Rel in activated T cells, suggesting the effects of c-Rel deletion in the regulation of the nearest c-Rel binding genes are small. Finally, it is possible that c-Rel and IRF-1 may potentially cooperate and enhance gene expression of c-Rel binding genes. However, further studies are required to test this possibility. The findings described in this study would be helpful in terms of understanding not only the role of NF-κB proteins in the regulation of genes activated in T cells, but also genome-wide regulatory networks mediated by the NF-κB family.
References


identified by CpG island arrays shows that Max is essential for Myc-dependent repression." Curr Biol 13(10): 882-886.


Appendix
<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Oligonucleotide sequences</td>
<td>207</td>
</tr>
<tr>
<td>II</td>
<td>Comparison of triplicate ChIP-on-chip experiments using PCA</td>
<td>211</td>
</tr>
<tr>
<td>III</td>
<td>Nearest genes to NF-κB c-Rel and RelA binding regions</td>
<td>212</td>
</tr>
<tr>
<td>IV</td>
<td>mRNA expression of the c-Rel and IL-2 genes in CD4⁺ T cells</td>
<td>216</td>
</tr>
<tr>
<td>V</td>
<td><em>In vivo</em> binding of IRF transcription factors to c-Rel binding genes in T cells</td>
<td>217</td>
</tr>
</tbody>
</table>
## Oligonucleotides used for quantitative real-time PCR

### cDNA (mRNA) amplification

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 For</td>
<td>5'-CCTGAGCAGGATGGAGAATTACA</td>
</tr>
<tr>
<td>IL-2 Rev</td>
<td>5'-TCCAGAACATGGCCGCAGAG</td>
</tr>
<tr>
<td>IRF-1 For</td>
<td>5'-CTTATCGCTGGGTGGCCCTTG</td>
</tr>
<tr>
<td>IRF-1 Rev</td>
<td>5'-CTCATTTAGTGCAATTGTCTCC</td>
</tr>
<tr>
<td>cRel For</td>
<td>5'-TTACCAAGAATGCCCCAGTC</td>
</tr>
<tr>
<td>cRel Rev</td>
<td>5'-AGGCCCTTCTAGGAATGGAA</td>
</tr>
<tr>
<td>ReLA For</td>
<td>5'-TTCCCAATGGTCTCTCAGGA</td>
</tr>
<tr>
<td>ReLA Rev</td>
<td>5'-GTGCGTGCGAGCACCCTTAGGA</td>
</tr>
<tr>
<td>UBC For</td>
<td>5'-AAGAGAATCCACAAAGGAAATTGAG</td>
</tr>
<tr>
<td>UBC Rev</td>
<td>5'-CAACAGGACCTGCATGCAACTCG</td>
</tr>
<tr>
<td>1700123O20Rik For</td>
<td>5'-CACCTGAACTGCCAGGGTAGTG</td>
</tr>
<tr>
<td>1700123O20Rik Rev</td>
<td>5'-CGGGTCTCGCTTTGTAGTG</td>
</tr>
<tr>
<td>Nek8 For</td>
<td>5'-GTGCCCGAGGAATGACTGTTG</td>
</tr>
<tr>
<td>Nek8 Rev</td>
<td>5'-GAGCTTTGGGACAGGGACTTGG</td>
</tr>
<tr>
<td>Gadd45b For</td>
<td>5'-CTTGTGCTCCTCCTGGCATAG</td>
</tr>
<tr>
<td>Gadd45b Rev</td>
<td>5'-GAGACGATGTCAATGTCTATTGAGC</td>
</tr>
<tr>
<td>Bcl10 For</td>
<td>5'-GTGAAGCATGGTGAGCAGCGAGG</td>
</tr>
<tr>
<td>Bcl10 Rev</td>
<td>5'-GCAGTAAGACACGGATGGGAC</td>
</tr>
<tr>
<td>Trip4 For</td>
<td>5'-GTGGCTGGAGCTTAGGAATTC</td>
</tr>
<tr>
<td>Trip4 Rev</td>
<td>5'-CTGTGGAGAGGGAGCTTCAACATC</td>
</tr>
<tr>
<td>Tlk2 For</td>
<td>5'-GTGGCAATGTTCATTCCGTCAG</td>
</tr>
<tr>
<td>Tlk2 Rev</td>
<td>5'-CCAGGCATCTCGAATTCCGAC</td>
</tr>
<tr>
<td>Pou2f2 For</td>
<td>5'-CTCTCCCTCGTCTCTTCATCTTC</td>
</tr>
<tr>
<td>Pou2f2 Rev</td>
<td>5'-CTCTCACTCAGCCTTGAGGACC</td>
</tr>
<tr>
<td>Tnfaip3 For</td>
<td>5'-GTCACAGCAGCCATGACCATCAG</td>
</tr>
<tr>
<td>Tnfaip3 Rev</td>
<td>5'-GCAATAGCTTCAGGCTGACCATG</td>
</tr>
<tr>
<td>Snai2 For</td>
<td>5'-GTTTCAAGAGAGAGAGAGATCTGCC</td>
</tr>
<tr>
<td>Snai2 Rev</td>
<td>5'-CAGGTAGCACAACCAGGGATAC</td>
</tr>
<tr>
<td>Genomic DNA and ChIP DNA amplification</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Name</strong></td>
<td><strong>Primer sequence</strong>^a^</td>
</tr>
<tr>
<td>IL-2SetB For</td>
<td>5'-ACAGGTTAGACTCTTTGAAAAATATGTGA</td>
</tr>
<tr>
<td>IL-2SetB Rev</td>
<td>5'-CATGGGAGGCAATTATATACTGTTAATG</td>
</tr>
<tr>
<td>IL-2SetF For</td>
<td>5'-CATGCAAGGTAAAAATATCTTCTTCT</td>
</tr>
<tr>
<td>IL-2SetF Rev</td>
<td>5'-GCCTAAAAGTCTCTCAAAAGAACAGA</td>
</tr>
<tr>
<td>GM-CSFSetA For</td>
<td>5'-GCCTGACAACCTGGGGAAG</td>
</tr>
<tr>
<td>GM-CSFSetA Rev</td>
<td>5'-TGATTAATGGTGACCACAAGACTC</td>
</tr>
</tbody>
</table>
GM-CSF Set J For 5'-GAGTTCTGTGGTGGTACACCTTTACAA
GM-CSF Set J Rev 5'-GAGGACCAGGCTTTTCTTGA
Gadd45b Pro For 5'-ACAGGCGAGGGGATCCAGCA
Gadd45b Pro Rev 5'-ATTCCCCGGCAGCCGGCTAC
Gadd45b Int For 5'-CAAACTGATGAATGTGTTAGTC
Gadd45b Int Rev 5'-AGCCTCATTAGGGATGTGGAGG
Tnfaip3 For 5'-CGTGCTTTGAGATTCGACACT
Tnfaip3 Rev 5'-AAGTGGAGTCCCAACAAAGCAG
Ero1l For 5'-CTGTGGAGAGGGGAAGTATC
Ero1l Rev 5'-GAGTACCAGCCTGTCATTTTC
2810457106 Rik For 5'-CTGGGACTTCTGCAAAAAACGT
2810457106 Rik Rev 5'-GTTGGCAAGGAGCCAGCAAGG
Pou2f2 For 5'-CAGGAACCGTGGAGAATCAAG
Pou2f2 Rev 5'-GTGGAGGGCTAAGTCAGAGAC
Bcl10 For 5'-CCGGTAACTCGTCTTACTTAGC
Bcl10 Rev 5'-GGGTCTGAAGCACAACATCATCTTC
Nek8 For 5'-GTITGTTAGCTAAGGACCAC
Nek8 Rev 5'-CGACCAGGATGAATGAAATCC
Trip4 For 5'-CAGCATATAAGATTATCTGAGAAGAAACC
Trip4 Rev 5'-GTTTCTAAGGCCATATTAAAG
Tlk2 For 5'-CTCAGTCTCCAAGTATTGGA
Tlk2 Rev 5'-CTCCTCAGTGGAGCAGATGG
Snai2 For 5'-GTGGCTGTGCTCCAAGAAGAC
Snai2 Rev 5'-CACAAGTGAAATACCTAGGCC
Cdc14a For 5'-GGCCTGATGTTATTATTGTCC
Cdc14a Rev 5'-CTTTACATCCATGGCCTTAC
Ccde94 For 5'-GATGTTGTTGGTCACCTCAGTTC
Ccde94 Rev 5'-CCAGGAAAACCACATGATG
Pigb For 5'-GCACACTGGGAAAAGCAAAC
Pigb Rev 5'-GCTAAGGTTCAAGAACTGCTTTG
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fbxw8 For</td>
<td>5'-CTCTTTCTCGGCCTCAATTG</td>
</tr>
<tr>
<td>Fbxw8 Rev</td>
<td>5'-CTCTTCTGACCTAACTCTTAAC</td>
</tr>
<tr>
<td>Havcr2 For</td>
<td>5'-GATGCGAGATTCTGTGTTTTAAC</td>
</tr>
<tr>
<td>Havcr2 Rev</td>
<td>5'-GGCTCTGTTTGTGAGGCACCTCAG</td>
</tr>
<tr>
<td>Rho For</td>
<td>5'-ATATCTCGCGGATGCTGAAT</td>
</tr>
<tr>
<td>Rho Rev</td>
<td>5'-GACAGAGACCAAGGCTGCTT</td>
</tr>
</tbody>
</table>

*a* = Forward (For) and Reverse (Rev) primer sequences used for quantitative real-time PCR. Pro = promoter; Int = intron. All oligonucleotide sequences used in this thesis for gene expression and ChIP assays were designed based on the mouse genomic sequence.
Appendix II  Comparison of triplicate ChIP-on-chip experiments using PCA

Partek® Genomics Suite™ (6.4) software was used to normalise the arrays and to compare the replicates using Principal Components Analysis (PCA). Arrays were background corrected using robust multichip average (RMA) subtraction with the signal adjusted for probe sequence and the arrays were quintile normalised. They were mapped against the mm8 version of the genome. The first replicate (1st) was less similar compared to the second and third replicates (2nd and 3rd).
## Appendix III Nearest genes to NF-κB c-Rel and RelA binding regions

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Distance (bp)</th>
<th>symbol</th>
<th>c-Rel binding genes</th>
<th>Description*</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr13</td>
<td>1090</td>
<td>Naf7</td>
<td>nuclear proteins 7 gene</td>
<td></td>
</tr>
<tr>
<td>chr3</td>
<td>1809</td>
<td>Bcl10</td>
<td>B-cell leukemia/lymphoma 10 gene</td>
<td></td>
</tr>
<tr>
<td>chr6</td>
<td>5002</td>
<td>Kr3δB</td>
<td>killer cell lectin-like receptor, subfamily A, member 8 gene</td>
<td></td>
</tr>
<tr>
<td>chr6</td>
<td>2688</td>
<td>Ddx47</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 47 gene</td>
<td></td>
</tr>
<tr>
<td>chr3</td>
<td>8481</td>
<td>P2ry23</td>
<td>purinergic receptor P2Y5, G-protein coupled 12 gene</td>
<td></td>
</tr>
<tr>
<td>chr16</td>
<td>5039</td>
<td>Osmr</td>
<td>osteostatin M receptor gene</td>
<td></td>
</tr>
<tr>
<td>chr10</td>
<td>1168</td>
<td>G6de45Bb</td>
<td>growth arrest and DNA-damage-inducible 45 beta gene</td>
<td></td>
</tr>
<tr>
<td>chr13</td>
<td>714</td>
<td>ASK3008244X12</td>
<td>RIKEN cDNA ASK3008244X12 gene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1452</td>
<td>Kerat</td>
<td>keratin 40 gene</td>
<td></td>
</tr>
<tr>
<td>chr9</td>
<td>970</td>
<td>Oftrf96</td>
<td>olfactory receptor 96 gene</td>
<td></td>
</tr>
<tr>
<td>chr5</td>
<td>1483</td>
<td>Adams3</td>
<td>a disintegrin-like and metalloendopeptidase (reprolysin-type) with thrombospondin type 1 motif, 3 gene</td>
<td></td>
</tr>
<tr>
<td>chr14</td>
<td>2374</td>
<td>Sfnoh1</td>
<td>scavenger with four orth domains 1 gene</td>
<td></td>
</tr>
<tr>
<td>chr4</td>
<td>6134</td>
<td>AS034710H0Tik</td>
<td>RIKEN cDNA AS034710H0Tik gene</td>
<td></td>
</tr>
<tr>
<td>chr1</td>
<td>1177</td>
<td>Creb1</td>
<td>cAMP responsive element binding protein 1 gene</td>
<td></td>
</tr>
<tr>
<td>chr11</td>
<td>1016</td>
<td>Tbk2</td>
<td>wounded-kinase 2 (Archaeplastida) gene</td>
<td></td>
</tr>
<tr>
<td>chr9</td>
<td>529</td>
<td>Thd4</td>
<td>thrombospondin, type I, domain containing 4 gene</td>
<td></td>
</tr>
<tr>
<td>chr7</td>
<td>348</td>
<td>Egr1</td>
<td>EGF-like module containing, medul-like, hormone receptor-like sequence 1 gene</td>
<td></td>
</tr>
<tr>
<td>chr9</td>
<td>1634</td>
<td>Trp4</td>
<td>thyroid hormone receptor interactor 4 gene</td>
<td></td>
</tr>
<tr>
<td>chr15</td>
<td>1214</td>
<td>Sla</td>
<td>src-like adaptor gene</td>
<td></td>
</tr>
<tr>
<td>chr7</td>
<td>631</td>
<td>Elkd2</td>
<td>E12-domain containing 2 gene</td>
<td></td>
</tr>
<tr>
<td>chr12</td>
<td>264</td>
<td>Blk2</td>
<td>btryrophoblast-like 2 gene</td>
<td></td>
</tr>
<tr>
<td>chr11</td>
<td>1282</td>
<td>C63004017O21Rik</td>
<td>RIKEN cDNA C63004017O21 gene</td>
<td></td>
</tr>
<tr>
<td>chr17</td>
<td>1746</td>
<td>Shhfd2</td>
<td>SFTJ domain containing 2 gene</td>
<td></td>
</tr>
<tr>
<td>chr14</td>
<td>1999</td>
<td>Ewrl1</td>
<td>ERO1-like (S. cerevisiae) gene</td>
<td></td>
</tr>
<tr>
<td>chr5</td>
<td>1412</td>
<td>Fbxw8</td>
<td>F-box and WD-40 domain protein 8 gene</td>
<td></td>
</tr>
<tr>
<td>chr14</td>
<td>1044</td>
<td>Kdm3l</td>
<td>histone 1 gene</td>
<td></td>
</tr>
<tr>
<td>chr5</td>
<td>1081</td>
<td>Podc57</td>
<td>protocadherin 7 gene</td>
<td></td>
</tr>
</tbody>
</table>

| chr6       | 2894         | Nbs1   | nibrin gene |             |
| chr11      | 135          | Tuba8  | transmembrane channel-like gene family 8 gene |             |
| chr12      | 2230         | Cdc46Aa | CDC46 cell division cycle 4 homolog A (S. cerevisiae) gene |             |
| chr7       | 639          | Pou2f2 | POU domain, class 2, transcription factor 2 gene |             |
| chr11      | 16089        | 4932427E13Rik | RIKEN cDNA 4932427E13 gene |             |
| chr14      | 4117         | 1700123D20Rik | RIKEN cDNA 1700123D20 gene |             |
| chr2       | 1595         | Traf2  | TNF receptor-associated factor 2 gene |             |
| chr15      | 457          | Atph1b1 | ATPase, Na+/K++transporter, beta 2 polypeptide gene |             |
| chr2       | 223          | Cenph  | centromere protein B gene |             |
| chr7       | 59           | Nek8   | NIMA (never in mitosis a)-related expressed kinase 8 gene |             |
| chr2       | 581          | Hsf2   | intracellular transport 53 homolog (Chlamydomonas) gene |             |
| chr16      | 1708         | Atp13b5 | ATPase type 13A5 gene |             |
| chr18      | 948          | Dctd2  | DCT domain containing 2 gene |             |
| chr1       | 5540         | Zfhx1p | zinc finger protein 185 gene |             |
| chr19      | 961          | Imp3a  | insulator (myn)-like 4-microphthalmus 2 gene |             |
| chr12      | 466          | D230037D09Rik | RIKEN cDNA D230037D09 gene |             |
| chr16      | 2533         | Smad2  | small homolog 2 (Drosophila) gene |             |
| chr16      | 2597         | Rtn4r  | reticulon 4 receptor gene |             |
| chr16      | 2511         | Hira   | histone cell cycle regulation defective homolog A (S. cerevisiae) gene |             |
| chr19      | 875          | Cdf5   | CDS antigen gene |             |
| chr8       | 3165         | Aipt1  | adenosine phosphotransferase gene |             |
| chr19      | 3493         | Oftrf1436 | olfactory receptor 1436 gene |             |
| chr10      | 2297         | Plcg1  | phospholipase C alpha 1 gene |             |
| chr6       | 169          | Aarbip5 | ADP-ribosylation factor-like 5 interacting protein 5 gene |             |
| chr10      | 852          | Tsn1p3 | tumor necrosis factor, alpha-induced protein 3 gene |             |
| chr6       | 2123         | Me31a  | meiosis 1, alpha gene |             |
| chr6       | 1871         | Add2   | adipin 2 (beta) gene |             |
| chr19      | 1145         | 6030453H10Rik | family with sequence similarity 17B, member A gene |             |
| chr11      | 1019         | Wd7b   | WD repeat domain 79 gene |             |
| chr9       | 2251         | Arox1  | AXIN1 up-regulated 1 gene |             |
| chr5       | 2382         | 4921502G13Rik | RIKEN cDNA 4921502G13 gene |             |
| chr19      | 2251         | 1110967D12Rik | RIKEN cDNA 1110967D12 gene |             |

| chr6       | 15249        | AK014430 |                     |             |
| chr5       | 1677         | EG656340 |                     | N/A         |
| chr9       | 471          | 231900A05Rik |                     | N/A         |
| chr14      | 1516         | AK146699 |                     | N/A         |
| chr5       | 2397         | Scoeln |                     | N/A         |
| chr17      | 148          | AK164689 |                     | N/A         |
| chr6       | 227          | 261209C06Rik |                     | N/A         |
| chr11      | 1294         | LOC544760 |                     | N/A         |
| chr17      | 478          | AK051183 |                     | N/A         |
| chr11      | 103514       | AK038310 |                     | N/A         |
| chr8       | 3518         | LOC209541 |                     | N/A         |
| chr9       | 2340         | 28104475D06Rik |                     | N/A         |
| chr9       | 780          | AK040330 |                     | N/A         |
| chr7       | 1392         | AK132602 |                     | N/A         |
| chr19      | 831          | LOC383425 |                     | N/A         |
| chr19      | 295          | LOC383425 |                     | N/A         |
| chr13      | 2512         | EG332721 |                     | N/A         |
| chr6       | 753          | EG233588 |                     | N/A         |
| chr8       | 1469         | EG666442 |                     | N/A         |
| chr8       | 311          | EG425475 |                     | N/A         |
| chr16      | 997          | LOC635911 |                     | N/A         |
| chr11      | 21935        | AK045016 |                     | N/A         |

---

a. Distance from the transcription start site (TSS); b. full name of genes was obtained from the Ensembl Genome Browser; #N/A = not available.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4930597D18Rb</td>
<td>RIKEN cDNA 4930597D18Rb gene</td>
</tr>
<tr>
<td>361</td>
<td>SFT2a domain containing 1 gene</td>
</tr>
<tr>
<td>36</td>
<td>RXR</td>
</tr>
<tr>
<td>375</td>
<td>Ake1</td>
</tr>
<tr>
<td>46a</td>
<td>Protein tyrosine phosphatase 4a gene</td>
</tr>
<tr>
<td>46a</td>
<td>GRP3</td>
</tr>
<tr>
<td>2673</td>
<td>C10B3.6A3 gene</td>
</tr>
<tr>
<td>2209</td>
<td>ATPase, H+ transporting, lysosomal 9b subunit C gene</td>
</tr>
<tr>
<td>498</td>
<td>Thc1d22a</td>
</tr>
<tr>
<td>3331</td>
<td>Synaptyctin 4 gene</td>
</tr>
<tr>
<td>15391</td>
<td>C10I0.160 gene</td>
</tr>
<tr>
<td>2274</td>
<td>Smc-like with four mb domains 1 gene</td>
</tr>
<tr>
<td>1872</td>
<td>Ikf1</td>
</tr>
<tr>
<td>974</td>
<td>Ikf1</td>
</tr>
<tr>
<td>1286</td>
<td>KDEL</td>
</tr>
<tr>
<td>1872</td>
<td>Spct</td>
</tr>
<tr>
<td>4830</td>
<td>Trpl1</td>
</tr>
<tr>
<td>3702</td>
<td>Trpl1</td>
</tr>
<tr>
<td>507</td>
<td>Acm13</td>
</tr>
<tr>
<td>5686</td>
<td>Gadd45b</td>
</tr>
<tr>
<td>40</td>
<td>Rps7a</td>
</tr>
<tr>
<td>1371</td>
<td>Sph2b</td>
</tr>
<tr>
<td>1623</td>
<td>ALC1- terminal like gene</td>
</tr>
<tr>
<td>2342</td>
<td>Asp31a</td>
</tr>
<tr>
<td>1249</td>
<td>Egal1</td>
</tr>
<tr>
<td>1878</td>
<td>Id3</td>
</tr>
<tr>
<td>6226</td>
<td>Smad3</td>
</tr>
<tr>
<td>1095</td>
<td>Ca2+</td>
</tr>
<tr>
<td>3618</td>
<td>Rasal2</td>
</tr>
<tr>
<td>40</td>
<td>Rps7a</td>
</tr>
<tr>
<td>1571</td>
<td>Sph2a</td>
</tr>
<tr>
<td>1623</td>
<td>ALC1- terminal like gene</td>
</tr>
<tr>
<td>2342</td>
<td>Asp31a</td>
</tr>
<tr>
<td>1249</td>
<td>Egal1</td>
</tr>
<tr>
<td>1878</td>
<td>Id3</td>
</tr>
<tr>
<td>6226</td>
<td>Smad3</td>
</tr>
<tr>
<td>1095</td>
<td>Ca2+</td>
</tr>
<tr>
<td>3618</td>
<td>Rasal2</td>
</tr>
<tr>
<td>324</td>
<td>hsa2</td>
</tr>
<tr>
<td>361</td>
<td>hsa6</td>
</tr>
<tr>
<td>1095</td>
<td>Vars</td>
</tr>
<tr>
<td>1999</td>
<td>ERO1-like (S. cerevisiae) gene</td>
</tr>
<tr>
<td>4391</td>
<td>Lta</td>
</tr>
<tr>
<td>487</td>
<td>Mc16</td>
</tr>
<tr>
<td>3742</td>
<td>Wnt6</td>
</tr>
<tr>
<td>1654</td>
<td>Fubh1</td>
</tr>
<tr>
<td>553</td>
<td>Trn35</td>
</tr>
<tr>
<td>1078</td>
<td>Oroc1</td>
</tr>
<tr>
<td>3139</td>
<td>Pch7</td>
</tr>
<tr>
<td>2426</td>
<td>Lif</td>
</tr>
<tr>
<td>880</td>
<td>SK2b3</td>
</tr>
<tr>
<td>62</td>
<td>Anap5</td>
</tr>
<tr>
<td>2685</td>
<td>Rtk61</td>
</tr>
<tr>
<td>2994</td>
<td>Adher</td>
</tr>
<tr>
<td>1639</td>
<td>Tita8</td>
</tr>
<tr>
<td>1333</td>
<td>Orc1c2</td>
</tr>
<tr>
<td>3007</td>
<td>Dpy21f</td>
</tr>
<tr>
<td>958</td>
<td>I1900421N15Rk</td>
</tr>
<tr>
<td>131</td>
<td>I1900421N15Rk</td>
</tr>
<tr>
<td>3496</td>
<td>Intu</td>
</tr>
<tr>
<td>410</td>
<td>49334230B9Rk</td>
</tr>
<tr>
<td>1316</td>
<td>Cth34</td>
</tr>
<tr>
<td>137</td>
<td>Pkl4</td>
</tr>
<tr>
<td>1717</td>
<td>I1900421N15Rk</td>
</tr>
<tr>
<td>2568</td>
<td>Nch1</td>
</tr>
<tr>
<td>72</td>
<td>Mec1</td>
</tr>
<tr>
<td>1245</td>
<td>Ctd1</td>
</tr>
<tr>
<td>9</td>
<td>Pol2a</td>
</tr>
<tr>
<td>1878</td>
<td>Cdh</td>
</tr>
<tr>
<td>1307</td>
<td>Dspa2</td>
</tr>
<tr>
<td>1884</td>
<td>Pfx1</td>
</tr>
<tr>
<td>185</td>
<td>Ska7a</td>
</tr>
<tr>
<td>1722</td>
<td>Pipc4</td>
</tr>
<tr>
<td>36</td>
<td>Pipc4</td>
</tr>
<tr>
<td>1186</td>
<td>Pipc4</td>
</tr>
<tr>
<td>1372</td>
<td>Fbpl1</td>
</tr>
<tr>
<td>1183</td>
<td>A3br</td>
</tr>
<tr>
<td>2778</td>
<td>Bipl1</td>
</tr>
<tr>
<td>314</td>
<td>Tend1</td>
</tr>
<tr>
<td>2143</td>
<td>Tct16</td>
</tr>
<tr>
<td>1538</td>
<td>I1900421N15Rk</td>
</tr>
<tr>
<td>2632</td>
<td>Sra1</td>
</tr>
<tr>
<td>707</td>
<td>Zbtb6</td>
</tr>
<tr>
<td>230</td>
<td>Pppl19</td>
</tr>
<tr>
<td>200</td>
<td>Masp3h</td>
</tr>
<tr>
<td>975</td>
<td>I1900421N15Rk</td>
</tr>
<tr>
<td>1075</td>
<td>Rpl10</td>
</tr>
<tr>
<td>1348</td>
<td>Pupc2</td>
</tr>
<tr>
<td>771</td>
<td>Apm2</td>
</tr>
<tr>
<td>25010</td>
<td>Cek1e3</td>
</tr>
<tr>
<td>181</td>
<td>Deta4</td>
</tr>
<tr>
<td>311</td>
<td>C163090P16Rk</td>
</tr>
<tr>
<td>2609</td>
<td>Nfac1</td>
</tr>
<tr>
<td>831</td>
<td>Nfac1</td>
</tr>
<tr>
<td>4949</td>
<td>Mefv</td>
</tr>
<tr>
<td>2345</td>
<td>Aob2</td>
</tr>
<tr>
<td>268</td>
<td>Snai2</td>
</tr>
<tr>
<td>2351</td>
<td>Hira</td>
</tr>
<tr>
<td>67</td>
<td>Hef1</td>
</tr>
<tr>
<td>3185</td>
<td>Aeg1</td>
</tr>
</tbody>
</table>

The table lists various genes and their descriptions, with some gene names being repeated due to formatting issues. The genes are likely related to the functions of proteins involved in cellular processes such as signal transduction, metabolism, and transcription. The table format helps organize these genetic information for better readability.
196 chr19 546 Castolf uillary neurotrophic factor gene
197 chr0 2403 Zhp376 nine finger proline (CX32 type) 276 gene
198 chr10 3473 Jmege1e junamaji domain containing 1C gene
199 chr9 1365 Im5p2 interferon regulatory factor 2 binding protein 2 gene
200 chr10 1600 Twilp3 tumor necrosis factor, alpha-induced protein 3 gene
201 chr10 1328 Shil5d3 nolute carrier family 55, member D3 gene
202 chr2 932 Gati2 glutathione s-transferase, theta 2 gene
203 chr8 76 Rxd Ras-related associated with diabetes gene
204 chr8 195 Nh4l2 nuclear factor of kappa light polypeptide gene enhancer in b-cells 1, p49/p100 gene
205 chr8 32387 Anopheles DOT1-like, histone H3 methyltransferase (S. cerevisiae) gene
206 chr1 2766 Dorn1
207 chr11 4442 OTMUSIG00000005491 N/A
208 chr11 181 OTMUSIG00000005491 N/A
209 chr7 3056 Gmn1560 N/A
210 chr9 3198 Scodin N/A
211 chr9 3397 Scodin N/A
212 chr9 3132 Scodin N/A
213 chr7 311 DTVars 28e N/A
214 chr15 97 2810451A06Rik N/A
215 chr12 8298 2810002204Rik N/A
216 chr11 774 LOC382523 N/A
217 chr15 454 ENSMUSG0000053931 N/A
218 chr14 4679 4939054L18Rik N/A
219 chr10 288 AK038773 N/A
220 chr1 156 Brg15 N/A
221 chr12 21998 AK046229 N/A
222 chr11 4679 X33328 N/A
223 chr2 7790 AK035661 N/A
224 chr6 869 BC020002 N/A
225 chr19 311 LOC751195 N/A
226 chr13 42987 AK033626 N/A
227 chr6 2649 Gm1066 N/A
228 chr16 908 4632174520Rik N/A
229 chr4 579 D2366766 N/A
230 chr18 577 1500015A7Rik N/A
231 chr17 88 AK146669 N/A
232 chr11 868 W679 N/A
233 chr14 71990 AK038994 N/A
234 chr5 954 F1198 N/A
235 chr19 230 AK156531 N/A
236 chr19 720 AK156531 N/A
237 chr3 292 2810014188Rik N/A
238 chr9 2275 BS1218114R5Rik N/A
239 chr17 54 AK155805 N/A
240 chr6 2997 S383657010Rik N/A
241 chr6 2719 18103333M9Rik N/A
242 chr7 2529 Trps3s N/A
243 chr5 25 AK146625 N/A
244 chr3 1048 AK090733 N/A
245 chr6 3639 LOC67645 N/A
246 chr16 16237 AK134430 N/A
247 chr19 954 AK145157 N/A
248 chr14 601 933812088Rik N/A
249 chr9 2188 Axad1 N/A
250 chr19 902 LOC382425 N/A
251 chr5 1382 Tum2h12a N/A
252 chr13 115163 A201380M9Rik N/A
253 chr7 204 AK042522 N/A
254 chr19 1096 AK089894 N/A
255 chr7 429 AY070869 N/A
256 chr6 2222 Terc-1 N/A
257 chr10 459 Ups1 N/A
258 chr17 3456 AK078570 N/A
259 chr14 36514 Gm1482 N/A
260 chr18 71 Sts1p1 N/A
261 chr4 1750 2331007A19Rik N/A
262 chr9 1777 640301L24Rik N/A
263 chr12 1184 S3831234A9Rik N/A
264 chr9 111732 AK038885 N/A
265 chr18 558 AK023255 N/A
266 chr15 1368 Hde7a N/A
267 chr3 899 Nola1 N/A
268 chr9 1157 Edg5 N/A
269 chr9 2861 Edg5 N/A
270 chr5 1655 Zp5469 N/A
271 chr17 790 Notc2 N/A
272 chr15 991 AK155479 N/A
273 chr10 2125 Tmem1 N/A
274 chr7 294 ECG45575 N/A
275 chrX 3193 LOC436231 N/A
276 chrX 6409 46083 LOC436231 N/A

a. Distance from the transcription start site (TSS); b. full name of genes was obtained from the Ensembl Genome Browser; #N/A = not available.
Appendix IV mRNA expression of the c-Rel and IL-2 genes in CD4⁺ T cells

Primary CD4⁺ T cells isolated from wild-type (■) and c-Rel/-/- (□) mouse spleen were nonstimulated or cells were stimulated with PMA/I (A) and with CD3/CD28 antibodies (B) for the time points indicated. cDNA was prepared from the cells and analysed by quantitative real-time PCR using primer sets. To calculate the relative mRNA levels, Cₜ values of the reference housekeeping gene (Cₜ (ref)) (ubc) were subtracted from Cₜ values determined for target genes of interest (Cₜ (target)) which generates ΔΔCₜ or Cₜ (target) - Cₜ (ref). The resulting Cₜ value is then raised to the power 2 to give the final relative mRNA levels (2^ΔΔCₜ). The results are shown as the mean and standard deviation (SD) of three or four independent experiments.
Appendix V *In vivo* binding of IRF transcription factors to c-Rel binding genes in T cells

Nuclei were isolated from EL-4 T cells nonstimulated or stimulated with PMA/I for 4 h. ChIP assays were carried out with anti-IRF-1, anti-IRF-2, anti-IRF-4, and anti-IRF-8. Quantitative real-time PCR was performed on the immunoprecipitated DNA with specific primer sets, amplifying the regions bound by c-Rel. The graph shows ChIP signal for immunoprecipitated (IP) DNA and no antibody (NA) control signal relative to the total input (TI). Thus, the relative amount of ChIP DNA was quantified by subtracting C_t values of TI DNA from IP C_t values (C_t^{IP} - C_t^{TI}) and TI from NA (C_t^{NA} - C_t^{TI}). The resulting C_t values is then raised power 2 to give the final relative amount of ChIP DNA ($2^{-\Delta\Delta C_t}$ (IP-TI) or $2^{-\Delta\Delta C_t}$ (NA-TI)). The results are presented from a single experiment.