Aus dem Institut für Pathologie

der Universität Würzburg

Vorstand: Prof. Dr. H.K. Müller-Hermelink

Die Funktionelle Rolle von NFATc1 in der Kontrolle von Leben und Tod von Lymphozyten

Inaugural- Dissertation

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Declaration

I submit the dissertation for Dr. med. I declare that the submitted dissertation was completed by myself and no other. I have not used any sources or materials other than those enclosed.

Moreover, I declare that the dissertation has not been submitted further in this form or any other form, and has not been used to obtain any other equivalent qualification or degree at any other organization.

Additionally, I have not applied for, nor will I attempt to apply for any other degree or qualification in relation to this work.

This work was completed from July 2005 to August 2007 at the Department of molecular Pathology, Institute for Pathology, Bayerische Julius-Maximilians-University, Würzburg, under the supervision of Professor Dr. Edgar Serfling (Faculty of Medicine).

Examiner: Prof. Dr. E. Serfling

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1. Introduction

1.1 The life and death of lymphocytes

B and T lymphocytes are the central cells of the adaptive immune system. To keep the right balance between the life and death of lymphocytes is a key mechanism for maintaining homeostasis of the immune system as well as for maintaining its proper function. Changes in this fine-tuned balance will lead to the generation of autoimmune diseases, to the appearance of leukemias and lymphomas, or to immune deficiencies which affect millions of people worldwide.

1.1.1 Maturation and activation of lymphocytes through crucial signaling pathways

Maturation and activation of lymphocytes are driven by a series of signaling pathways which lead to the activation (or inactivation) of a large number of genes. Lymphocytes derive from lymphocyte progenitor cells which are generated from bone marrow stem cell. These hematopoietic progenitor cells develop in bone marrow (B cells) or in thymus (T cells), and, finally, mature to populations of lymphocytes in peripheral lymphoid tissues, such as in spleen and lymph nodes. The genes which are switched on during development of lymphocytes encode transcription factors, such as the NFAT and NF-kB factors (Kumar et al., 2005; Siebenlist et al., 2005), components of the pre-T and pre-B antigen receptor complexes (Nemazee, 2006; Wang and Clark, 2003), enzymes, such as ZAP-70 and calcineurin (Clipstone and Crabtree, 1992), adapter proteins, such as Grb2 (Peterson et al., 1998), and many other proteins which are predominantly involved in signal transduction in lymphocyte precursor cells (Engel and Murre, 2001). Thereby, lymphocytes transit from one stage to the next, and key events in these differentiation processes are

designated as checkpoints. Naturally occurring mutations, or gene-targeting of such `critical` genes can lead to complete blocks in lymphocyte maturation at certain stages and/or can result in the generation of (human) immunodeficiency, such as in the X-linked severe combined immunodeficiency disease in humans which is due to a natural mutation of IL-2 receptor common γ chain (Noguchi et al., 1993).

Among the events of lymphocyte development, expression of immune T and B cell receptors (TCR, BCR) and of their signal transduction components are crucially involved in the maturation of lymphocytes (Su and Rawlings, 2002). Such lymphocytes expressing functional receptors are “positively” selected to survive, while T and B cells without expression of functional receptors die by apoptosis, such as thymocytes without a functional TCR at the double-positive stage of thymocyte development. These positive and negative selection processes of thymocytes (and B cells) “provide” surviving immune cells with a useful repertoire of receptors. Those cells having low-affinity receptors directed against self antigens proliferate and proceed to mature after positive selection, whereas the cells having high-affinity receptors die by apoptosis by negative selection (Goldrath and Bevan, 1999). Most important, multiple checkpoints ensure that only cells with proper immunoreceptors mature to be effector lymphocytes or, later on, memory cells (Miosge and Zamoyska, 2007; Roth and DeFranco, 1995).

Of the signaling pathways emerging from the TCR and BCR complexes, the NFAT and NF-kB signaling cascades are in the centre of signaling events controlling lymphocyte fate (Crabtree and Olson, 2002; Miosge and Zamoyska, 2007; Wang and Clark, 2003). The members of these two transcription factor families regulate the expression of numerous genes controlling the activation, differentiation and apoptosis of lymphocytes (Bours et al., 1992; Macian, 2005; Serfling et al., 2007). How do they contribute to shape and to activate the immune system? Previous studies indicate that different inputs determine the

way how they are activated. Many other important factors are involved in the NFAT and NF-kB signaling pathways and contribute to the final output. Thus, the MAGUK scaffold protein Dlgh1 was identified to 'orchestrate' TCR signaling which coordinates alternative p38 kinase activation, directing T cell receptor signals towards NFAT but not to NF-kB activation (Round et al., 2007). On the other hand, in inflammated tissues, both NFAT and NF-kB pathways are suppressed by the Foxhead transcription factor Foxd1 (Lin and Peng, 2006).

1.1.2 Apoptosis of lymphocytes: the functional role of Nfatc1

One central event of processes mentioned above is apoptosis. In activation-induced apoptosis (also know as `Activation Induced Cell Death`, AICD) activation is initiated at the receptors of B or T lymphocytes by antigens, and these cells are often ignored by survival signals. AICD involves various signaling pathways – including caspase cascades - and results in death of affected cells and clearance by macrophages. Its morphological features are blebbed membranes, shrunken nuclei and DNA cut to small pieces, but no inflammation of affected cells occurs distinguishing apoptosis from necrosis (Rathmell and Thompson, 2002; Strasser and Bouillet, 2003). Thereby, apoptosis helps to shape the repertoire of B and T cell receptors, to terminate immune responses and to sustain the homeostasis of the immune system.

NFATs play diverse functions in apoptosis. When associated with p300 upon stimulation by ET-1, NFAT factors can exert an anti-apoptotic activity (Kawamura et al., 2004; Van Sant et al., 2007), while in other situations NFATs activate AICD via Fas(CD95) (Holtz-Heppelmann et al., 1998; Latinis et al., 1997; Santini et al., 2001). We have previously observed that NFATc2 and c3 support the apoptosiș of effector T cells (Chuvpilo et al., 2002) and, therefore, exert a pro-apoptotic function in T lymphocytes. In contrast, in these studies

the function of NFATc1/aA, the strongly induced short isoform of NFATc1, remained unclear. The experiments presented in this doctoral thesis contribute to the question which role this particular isoform exerts in the control of AICD in lymphocytes.

1.2 NFATc1: Structure and Function of its Isoforms

1.2.1 NFAT family members

Since the cloning of the first NFAT cDNA in 1993 (McCarthy et al., 1993), five members of family of nuclear factor of activated T cell (NFAT) proteins have been discovered (Macian, 2005; Serfling et al., 2000; Shaw et al., 1988). Their nomenclature and several of their properties are displayed in Table 1-1 (from: http://www.genenames.org/cgi-bin/hgnc_search.pl).

Table 1-1 Nomenclature of NFAT Family by HUGO Gene Nomenclature Committee

Approved Gene Symbol (for human)	Approved Gene Name	Location	Sequence Accession IDs	Aliases
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive factor	16q23.1	AF134870 NM_138714 ENSG00000102908	TONEBP, KIAA0827, NFATL1, OREBP, NFATZ, NF-AT5
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	18q23	U08015 NM_172390 ENSG00000131196	NF-ATC, NFATc
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	20q13.2	U43342, U43341 NM_012340 ENSG00000101096	NF-ATP, NFATp
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	16q22	L41067 NM_004555 ENSG00000072736	NFAT4, NFATX
NFATC4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	14q11.2	BC053855 NM_004554 ENSG00000100968	NFAT3

1.2.2 The structure of the NFATC1 gene and its 6 protein isoforms

The murine and human *Nfatc1* genes consist of 11 exons which are distributed over more than 150 kB DNA. The expression of the *Nfatc1* genes is controlled by two promoters and two polyadenylation (pA) sites and alternative splicing events (Figure 1-1-A)(Serfling et al., 2006). The promoter 1 P1 is located just

in front of exon1 and promoter 2 (P2) in front of exon 2. The polyadenylation site pA1 is located proximal from pA2 which corresponds to the distal polyA site of the gene. P1 and P2 direct the synthesis of three different RNAs, respectively. P1 transcripts start at exon 1 with a corresponding α peptide of 42 amino acids (aa) at its N-terminus while P2 transcripts start at exon 2 with a β peptide of 29 aa (Figure 1-1-B) (Park et al., 1996; Sherman et al., 1999). The length of C-terminal peptide in of A ,B and C isoforms is 19, 128 and 246 aa, respectively (Chuvpilo et al., 1999).

The long isoforms of NFATc1/ βB and βC are the most prominent NFATc1 proteins in resting lymphocytes. Their transcription starts from the P2 promoter, the transcript is spliced from exon 2 to exon 3 and ends at the distal polyadenylation motif pA2. In addition, there is an alternative splicing event at the exon 10 which leads to the generation of the somewhat shorter NFATc1/ßB, compared to NFATc1/ßC. In contrast, transcription of NFATc1/ αA - due to the highly inducible activity of P1 promoter - is strongly induced (up to 15-20 fold) upon stimulation, spliced from exon 1 to exon 2, and ends at the proximal polyadenylation motif pA1 (Chuvpilo et al., 1999). The enriched binding of multiple inducible transcription factors, including NFAT itself, such as NF-κB, CREB, Fos and ATF-2 in P1, but not in P2, contributes to the difference in induction between P1 and P2 activities (Figure 1-1-C).

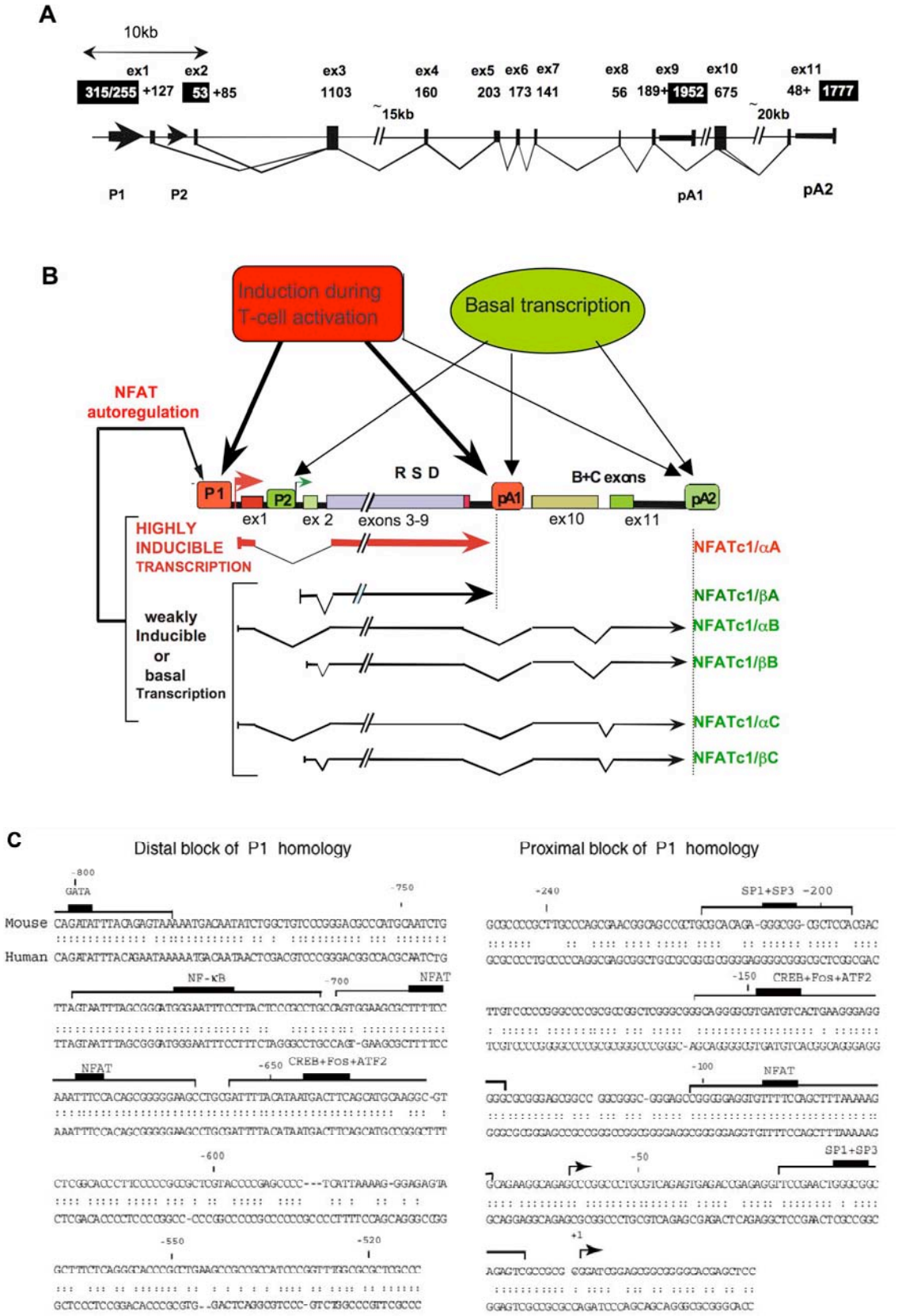


Figure 1- The structure of the *Nfatc1* gene and its expression into 6 RNA isoforms *(from Chuvpilo et al., 2002, modified))*. (A) A scheme of

structure and expression of the Nfatc1 gene. (B) The activity of the constitutive promoter P2 leads to the transcription of exon 2, splicing to exon 3 and the generation of three β isoforms. Upon activation of lymphocytes, the promoter activity switches from P2 to P1, and exon 1 is transcribed and spliced to exon 3, resulting in the generation of α isoforms. Optimal induction conditions facilitate the predominant synthesis of NFATc1/αA (red) by the use of the proximal polyA site pA1. Unlike the distal polyA site pA2, pA1 is not conserved between mouse and human. Thereby NFATc1/αA lacks the C-terminal transactivation domain TAD-B. (C) The binding sites for transcription factors in promoter P1.

1.3 Current problems: NFATc1function and *the goal of this study*

NFATc1-deficient mice bearing a completely disrupted *Nfatc1* gene die of circulatory failure in uterus. Therefore, it is impossible to study NFATc1 function in differentiated organs and cells (de la Pompa et al., 1998). Thus, generation of mice for the conditional knockout of NFATc1 is necessary for the analysis of organ and cell type specific function of NFATc1 (Kondo et al., 2003). However, as I will describe in this thesis, the mouse ES cell knockout strategy is very time consuming and can lead to failures in the germ line transmission of manipulated ES cells. Therefore, in collaboration with Dr. Eisaku Kondo (Univ. Okayama, Japan), we also used in our laboratory an alternative system for studying NFATc1 function, i.e. the system of chicken DT40 B cells (Lahti, 1999). Chicken DT40 B lymphoma cells are a useful system to inactivate eukaryotic genes and, therefore, to generate a loss of function mutations in somatic cells. In these cells, homologous recombination takes place at very high frequencies. In addition, these cells express B cell receptors, and AICD can easily be induced by triggering these cells through their BCRs. Therefore, these cells can be used to knock out candidate genes which are essentially involved in the control of apoptotic processes. By using

cDNA constructs expressing proteins with mutations or deletions, e.g. from human, these cells can also be used to delineate functional domains.

In our study, the murine ES cell gene targeting and DT40 B cell systems were used in parallel to disrupt the *Nfatc1* gene. In particular, we wanted to study the function of individual 6 *Nfatc1* isoforms, and among those the function of highly inducible short isoform *Nfatc1/*αA*.*

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 General materials

96 well qPCR plates,	ABgene
Cell strainer (70µm)	Falcon
Cryotubes 2 ml	Greiner bio-one
Disposable needles, cuvette and syringes	Hartenstein
Glasswares	Schott
Nitrocellulose membrane	Schleicher & Schuell
Parafilm	Hartenstein
Pipette tipps	Eppendorf
Pipettes	Sarstedt
Polypropylene tubes	Greiner bio-one, Nunc
Sterile fiters	Schleicher & Schuell
Tissue culture dishes	Grainer bio-one, Falcon
Tissue culture flasks	Greiner bio-one
Tissue culture plates	Grainer-one, Falcon
Tubes	Eppendorf
Whatmann paper	Schleicher & Schuell
X-ray film	Kodak

2.1.2 Chemicals

2-mercaptoethanol	Carl Roth
2-propanol	Carl Roth
Acetic acid	Carl Roth
Acrylamid solutions	Carl Roth
Agar	Carl Roth
Agarose	Sigma-Aldrich
Ampicillin	Hoechst
APS	Merck
Boric acid	Merck
Bromophenol blue	Merck
ß-mercaptoethanol	Carl Roth
Calcium chloride	Carl Roth
Chloroform	Carl Roth
Coomassie brilliant blue R-250	Roche
Cyclohexamide	Sigma
Cyclosporin	Novartis Pharma

DEPC	Carl Roth
Disodium hydrogen phosphate	Merck
DMEM	Gibco BRL
DMSO	Carl Roth
dNTPs	MBI-Fermentas
DTT	Carl Roth
ECL Chemiluminescence Kit	Amersham
EDTA	Carl Roth
EGTA	Carl Roth
Ethanol	Carl Roth
Ethium bromide	Carl Roth
FCS	Gibco BRL
Formaldehyde	Carl Roth
Gel extraction kit	Qiagen, Genomed
Glutathione sepharose	Sigma-Aldrich
Glycerol	Carl Roth
Glycin	Merck
Hepes	Carl Roth
Hydrochloric acid	Merck
Ionomycin	Aldrich
IPTG	Boehringer-Ingelheim
Isoamyl alcohol	Carl Roth
L-glutamine	Gibco BRL
Leupeptin hydrochloride	Gibco BRL
L-glutamine	Serva
L-glutathione [Mr: 307,3]	Sigma-Aldrich
Lithium chloride [LiCl]	Saliter
Milk powder	Sigma-Aldrich
Low fat skimmed milk	Saliter
Magnesium chloride	Carl Roth
Methanol	Carl Roth
Phenol	Carl Roth
Plasmid DNA purification kit	Genomed
PMSF	Serva
Poly dI·dC	Sigma-Aldrich
Potassium acetate	Carl Roth
Potassium chloride	Sigma-Aldrich
Potassium chloride	Carl Roth
Potassium dihydrogen phosphate	Sigma-Aldrich
Protein A/G sepharose	Santa Cruz
Protein assay solution	Bio-Rad
Protease inhibitor tablet (complete mini)	Santa Cruz
Radioactive nucleotides	Amersham Pharma
RPMI1640	Gibco BRL
SB203580	Calbiochem

SDS	Carl Roth
Sephadex G-50	Amersham Pharmacia
Sodium acetate	Merck
Sodium azide	Merck
Sodium carbonate	Carl Roth
Sodium chloride	Carl Roth
Sodium hydrogen phosphate	Merck
Sodium hydroxide	Carl Roth
Sodium pyruvate	Gibco BRL
SP600125	Calbiochem
Streptomycin	Carl Roth
TPA	Sigma Aldrich
Tris	Carl Roth
Triton X-100	Sigma Aldrich
Trizol reagent	Invitrogen
Trypan blue	Gibco BRL
Tween-20	Carl Roth
Western blotting substrate (Lumi-light)	Pierce

2.1.3 Instruments

Autoclave	Stiefenhofer
DNA sequencer 373A	Perkin Elmer
FACSCalibur	BD biosciences
Gel camera	Stratagene
Gel documentation system	Herolab
Gel dryer	H.Hölzel
GenePulser®II electroporation system	Bio-Rad
Haemocytometer	Brand
Heating	Hartenstain
Humidified tissue culture incubator	Nuaire US
Ice machines	Genheimer
Incubator	Mytron
Intensifying screen	DuPont
Labsonic U	B.Braun
Laminar hoods	Heraeus
Light microscope	Olympus
Liquid nitrogen tank	Tec-lab
Microcentrifuge	Eppendorf
Multichannel pipette	Eppendorf
Multi dispenser pipette	Eppendorf
pH meter	Ingold
Phosphorimager	Molecular Dynamics
Power supply	Amersham Pharmacia
Quartz cuvette	Hellma
Real-Time PCR machine	ABI Prism 7000

Refrigerators	Privilege, Bosch, Heraeus
Rotors	Beckman
SDS-PAGE apparatus	BioRad
Shaking incubator	Hartenstein
Spectrophotometer	Amsham Pharmacia
Thermal cycler	MWG
UV lamp (UVT-20M)	Herolab
Varifuge	Hartenstain
Water bath	Hartenstain
Water filtration unit	Milipore
Western blot apparatus	Hoefer, BioRad

2.1.4 Kits

First Strand cDNA Synthesis Kit	MBI Fermentas
NucleoBond®	Macherey-Nagel
LookOut™ Products for Mycoplasma Screening	Sigma
Long PCR enzyme mix	MBI Fermentas

2.1.5 Reagents

DNA size markers	MBI Fermentas
Protein size markers	MBI Fermentas
Restriction enzymes	MBI Fermentas

2.1.6 Antibodies

Anti-mouse NFATc1	polyclonal rabbit	ImmunoGlobe
Anti-mouse NFATc2	polyclonal rabbit	ImmunoGlobe
Anti-mouse NFATc1	ponoclonal rabbit	Alexis Biochemicals
Anti-mouse NF-kB p50	polyclonal rabbit	Santa Cruz Biotechnology
Anti-mouse NF-kB p65	polyclonal rabbit	Santa Cruz Biotechnology
Anti-mouse NF-κB cRel	polyclonal rabbit	Santa Cruz Biotechnology
Anti-mouse Oct-1	polyclonal rabbit	Abcam

2.1.7 Oligonucleotides

The oligonucleotides listed below were synthesized commercially by MWG Biotech. The
lyophilized oligos were dissolved in TE buffer at 100 pmol/µl and stored at – 20^{o} C.

Long-distance PCR for screening of positive ES cell clones:

sense 35406s	CCTAGCCTGGTGCACCGTACCTGCCCAC
antisense neo3872as	CCGCTTCCATTGCTCAGCGGTGCTGTCC

Primers for probe preparation used in Southern blotting:

Probe 1_forward (5´):	5´- TGGGGTGAAAAGTTATTTATTG - 3´
Probe 1_reverse (5´):	5´- TCAGTGAGTCCTAGTGTCGC - 3´

Probe 2_forward (3´): 5´- GCTTATTTGCGGACCATT - 3´
Probe 2_reverse (3´): 5´- CAGCCAACTTTCACCTTTCA - 3´
Probe 3_forward (neo): 5´- TCGCACACATTCCACATCCACC - 3´
Probe 3_reverse (neo): 5´- CGTGCAATCCATCTTGTTCAA - 3´
Probe 4_forward (5´): 5´- GGGTGGATAACCAAATGTGCC - 3´
Probe 4_reverse (5´): 5´- AGCCCTTTCCTGTCTGTCTCA - 3´

ChIP :

CHIP primers for pKC theta promoter:
Direct: 5'-TTGCTCCACCCTCTACGCAGTATG-3'
Reverse: 5'-ACATCAGCTGTCAGAAATGAGAAC-3'
CHIP primers of mouse NFATc1-promoter 1:
Forward: 5'-CGTACAGCAAGCAATCCAGTTCG3-'
Reverse: 5'-GACTGGAGGGAGCTCCCAG-3'

EMSA :

Human PKCθ_NFAT_AP1:
Antisense: 5'-TTG GAG AGT GAG GCA AAA GTT TTC CAA TAT TTT AT-3'
Sense: 5'-ATA AAA TAT TGG AAA ACT TTT GCC TCA CTC TCC AA-3'

Human PKCθ_NFATmut_AP1:
Antisense: 5'-TTG GAG AGT GAG GCA AAA GTT TTG GAA TAT TTT AT-3'
Sense: 5'-ATA AAA TAT TCC AAA ACT TTT GCC TCA CTC TCC AA-3'

Chicken PKCθ_NFAT binding :
Antisense: 5'-TAT TCA GGA TGG AAA TAA TCT TAG GCT TTC AAC TGA-3'
Sense: 5'-TCA GTT GAA AGC CTA AGA TTA TTT CCA TCC TGA ATA-3'

Chicken PKCθ_NFATmutant binding :
Antisense: 5'-TAT TCA GGA TCC AAA TAA TCT TAG GCT TTC AAC TGA-3'
Sense: 5'-TCA GTT GAA AGC CTA AGA TTA TTT GGA TCC TGA ATA-3'

mNFATc1-IgkB_sense 5'-TGCACAGAGGGGACTTTCCGAGAGGC-3'
mNFATc1- IgkB_antisense 5'-GCCTCTCGGAAAGTCCCCTCTGTGCA-3'
mNFATc1-P1kB_ sense 5'-TAGCGGGATGGGAATTTCCTTACTCC-3'
mNFATc1-P1kB_ antisense 5'-GGAGTAAGGAAATTCCCATCCCGCTA-3'
mNF-ATc1-P1kB-mut_sense 5'-TAGCGGGATTTGAATTTCCTTACTCC-3'

mNF-ATc-P1kB-mut_anti 5'-GGAGTAAGGAAATTCAAATCCCGCTA-3'
mPubd-NFATc1-mut_sense 5'-CAAAGACCAAAATTTGTTTCATACAGAAG-3'
mPu-bd-NfATc1-mut_anti 5'-CTTCTGTATGAAACAAATTTTGGTCTTTG-3'
mIL2-Pubd-NFATc1_sense 5'-CAAAGAGGAAAATTTGTTTCATACAGAAG-3'
mIL2-Pubd-NfATc1_anti 5'-CTTCTGTATGAAACAAATTTTCCTCTTTG-3'

2.1.8 Antibiotics

Ampicillin Hoecher

G-418	Invitrogen
Streptomycin	Hoecher
Ganciclovir	Cytovene, Syntex
Ciprofloxacin	Phamcy of Clinical center, Wuerzburg university

2.1.9 Solutions and buffers

Mili-Q grade water was used to prepare all the solutions. The solutions were sterilized by

either autoclaving or filtration.

2.1.9.1 Gernaral solutions

APS solution	1 %
$CaCl_2$	2 M
DTT	1M
EDTA	0.5 M, pH 8.0
EGTA	0.1 M, pH 8.0
Ethanol	70 %
Ethium bromide	10 mg/ml
Glycine	1 M
Hepes	0.5 M, pH 7.9
KCl	1 M
KH_2PO_4	5 mM, pH 7.8
LiCl	8 M
MgCl2	1 M
NaAc	3 M, pH 5.2
NaCl	5 M
NaOH	10 N
Phenol: chloroform: isoamylalcohol	25:24:1
PMSF	0.1 M in 100 % Ethanol
SDS	20 %
SDS	10 %
Tris-HCl	1 M, pH 6.8
Tris-HCl	0.5 M, pH 7.6
Tris-HCl	0.5 M, pH 6.8
Tris-HCl	0.5 M, pH 8.0
Tris-HCl	1.5 M, pH 8.8
Triton X100	10 %

2.1.9.2 Buffers

ChIP buffers

Cell lysis buffer:

Hepes	25 mM, pH 7.9
MgCl2	1.5 mM
KCl	10 mM

NP-40	1 %
PMSF	1 mM

Sonification buffer :

Hepes	50 mM
Nacl	140 mM
EDTA	1 mM
Triton X-100	1 %
Na-deoxycholat	0.1 %
SDS	0.1 %
PMSF	1 mM

High salt buffer:

Hepes	50 mM
Nacl	500 mM
EDTA	1mM
Triton X-100	1 %
Na-deoxycholat	0.1 %
SDS	0.1 %

LiCl buffer:

Tris	20 mM, pH 8.0
EDTA	1 mM
LiCl	250 mM
NP-40	0.5 %
Na-deoxycholat	0.5 %

Elution buffer:

Tris	50 mM, pH 8.0
EDTA	1 mM
SDS	1 %

EMSA buffers

Buffer A :

HEPES	10 mM
KCl	10 mM
EDTA	0.1 mM
EGTA	0.1 mM
DTT	1.0 mM
PMSF	0.5 mM

Buffer C :

HEPES	1mM
NaCl	400 mM
EDTA	1 mM

EGTA	1 mM
DTT	1 mM
PMSF	0.5 mM

5x Binding buffer
For purified proteins:

Hepes	100 mM, pH 7.9
DTT	2 mM
NP-40	0.5 %
BSA	0.5 mg/ml
NaCl	200 mM
Glycerol	20 %

For nuclear extract preparations:

Hepes	100 mM, pH 7.9
DTT	2 mM
NP-40	0.5 %
BSA	0.5 %
Glycerol	20 %

10 x annealing buffer:

Tris-HCl	200 mM, pH 7.6
EDTA	10 mM
NaCl	500 mM

50x TAE buffer:

Tris	242 g
EDTA 0.5 M, pH 8.0	100 ml
Acetic acid	57.1 ml

5x TBE, 1 litre:

Tris base	54 g
Boric acid	27.5 g
EDTA, 0.5 M, pH 8.0	20 ml

$CaCl_2$ (for preparation of competent bacteria with TopF):

$CaCl_2$	60 mM
Pipes	10 mM, pH 7.0
Glycerol	15 %

TE buffer:

Tris-HCl	10 mM, pH 8.0
EDTA	1 mM, pH 8.0

PBS (phosphate-buffered saline), pH 7.4 :

Na_2HPO_4	4.3 mM
KH_2PO_4	1.4 mM
NaCl	137 mM
KCl	2.7 mM

2 X HBS (HEPES buffered saline), pH 7.05:

HEPES	50 mM, pH 7.1
NaCl	280 mM
Na_2HPO_4	1.5 mM

TBS (tris-buffered saline), pH 7.4 :

Tris	25 mM
NaCl	137 mM
KCl	2.7 mM

Western blot blocking and hybridization solution :

TBS, pH 7.4	1x
Tween-20	1 %
Nonfat dried milk	1 %

Western blot washing buffer :

TBS, pH7.4	1x
Tween-20	0.1 %

2 x SDS gel-loading buffer:

Tris-HCl	100 mM, pH 6.8
DTT	200 mM
SDS	4 %
Bromophenol blue	0.2 %
Glycerol	20 %

Western blot transfer buffer:

Glycine	39 mM
Tris base	48 mM
SDS	0.037 %
Methanol	20 %

Coomassie blue solution (1000 ml):

Coomassie brilliant blue R-250	2.5 g
Methanol	450 ml
Acetic acid	100 ml

SDS-PAGE gel destaining solution:

Acetic	10 %

Methanol	30 %

Gel fixing solution:

Acetic acid	10 %
Methanol	10 %

FACS buffer:
PBS pH 7.4

BSA	0.2 %

For Nucleic Acid Transfers

The following solutions are noted in the transfer protocols. All solutions should be prepared with sterile, double-distilled deionized water with high-grade reagents. Filter stock solutions through a cellulose acetate filter with a glass fiber prefilter (e.g., bottle-top filter).

SSC (1x)	(20x)
• 0.15 M NaCl	• 3.0 M NaCl
• 15 mM sodium citrate pH 7.0	• 0.3 M sodium citrate pH 7.0

TBS (1x TurboBlotter Alkaline Transfer Buffer):

• 50 mM Tris	• 3 M NaCl
• 150 mM NaCl (pH 10x stock to 7.5)	• 8 mM NaOH

BLOCKING AGENTS

50 x Denhardt's Solutions:

5g of Ficoll
5g of Polyvinylpyrrolidone
5g of BSA
Adding water to 500 ml

ALKALINE TRANSFER BUFFERS

To make one liter:

Denaturing buffer	Transfer buffer:	Neutralizing buffer 5x:
3 M NaCl, 0.4 M NaOH	3 M NaCl, 8 mM NaOH	1 M phosphate buffer, pH 6.8
175.5 g NaCl	175.5 g NaCl	79.25 g Na2HPO4
16.0 g NaOH	32 g NaOH	60.25 g NaH2PO4•H2O
873.0 ml ddH2O	879.0 ml ddH2	Bring to 1 liter with ddH2O

For isolation of genomic DNA from ES cells in 96 well plates

Required Reagents and Materials:

1. Lysis Buffer for 100 ml:

10 mM Tris-HCl pH 7.5	0.5 ml of 2 M stock

10 mM EDTA	2 ml of 0.5 M stock
10 mM NaCl	0.2 ml of 5 M stock
0.5% Sarcosyl* or SDS w/v	0.5 g / 100 ml
1 mg/ml proteinase K	5ml of 20mg/ml proteinase K (add fresh each time, stored in –80° freezer)

2. Precipitation Buffer for 100 ml (fresh):

150 mM NaCl	3 ml of 5 M stock
100% EtOH	97 ml 100% ETOH (This solution remains slurry)

3. Wash Buffer for 200 ml:

70% EtOH 140 ml 100% EtOH
60 ml ddH2O

4. Resuspension Buffer: for 25 ml:

10 mM Tris-HCl pH 8.0:	0.125 ml of 2 M stock
10 mM EDTA:	0.5 ml of 0.5 M stock

5. PBS without Ca^{2+} and Mg^{2+}

2.1.10 Growth medium

2.1.10.1 Cell culture medium

RPMI1640 plus

FCS	5 %
L-glutamin	2 mM
2-mercaptoethanol	50

2.1.10.2 Bacterial culture medium

LB broth
LB agar (1.5 %)

2.1.10.3 For ES cells:

MEF medium:

components	*ratio*	*volume(ml)*	*volume(ml)*
non amino-acid	1%	5	5.688
pyrutate	1%	5	5.688
B-mercapto	0.10%	0.5	0.5688
FBS	10%	50	56.88
DMEM	87.90%	439.5	500
TOTAL		500	568.83

ES medium:

Components	*ratio*	*volume(ml)*	*stock*
non amino-acid	1%	5	
pyruvate	1%	5	
β-mercapto	0.10%	0.5	
FBS	15%	75	
LIF		0.05	10exp7u/ml
DMEM	82.10%	414.5	

ES cell media with G418 (500ml):

Reagents	*Volume(ml)*
DMEM (with 4500 mg/L glucose, L-GlutaMAXTM, without Na Pyruvate)	431.95
10% FCS	50
ß-Mercaptoethanol (50 mM)	0.5
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Nucleoside stock (100x)	5
107 u/ml LIF (ESGROTM, CHEMICON International Inc)	0.05
G418 (50mg/ml)	2.5
Total	500

ES cell media with G418 and Ganciclovir (500ml):

Reagents	*Volume(ml)*
DMEM (with 4500 mg/L glucose, L-GlutaMAXTM, without Na Pyruvate)	431.45
10% FCS	50
ß-Mercaptoethanol (50 mM)	0.5
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Nucleoside stock (100x)	5
10^7 u/ml LIF (ESGROTM, CHEMICON International Inc)	0.05
2mM ganciclovir (1000 x)	0.5
G418 (50mg/ml)	2.5
Total	500

ES media Cryopreservation Medium:

1. 60% DMEM
2. 20 % FBS
3. 20 % DMSO

2.1.11 Bacterial strains

E.coli strains TopF, DH5

2.1.12 Mammalian cell lines

EL-4	DSMZ (murine thymoma cell line)
Jurkat	DSMZ (human T cell leukemia line)
DT40 chicken B cells: WT, NFATc1$^{-/-/-}$, NFATc1$^{-/-/\alpha A}$, NFATc1$^{-/-/\alpha C}$	Kondo et al.
Embryonic stem (ES) cells:	TC-1 The laboratory of R. Flavell, Yale university,New Haven, USA

2.1.13 Mice

BL6 wild-type	Charles River
Balb/c wild-type	Charles River
C57BL/6	Charles River

2.2 Methods

2.2.1 Bacterial manipulation

2.2.1.1 Storage of bacteria

1 ml of overnight bacterial culture was centrifuged at 5000 rpm for 3 minutes at RT. The collected bacteria were resuspended in 200µl (containing 20% glycerol) and stored at – 70 ^{o}C.

2.2.1.2 E.coli competent cell preparation ($CaCl_2$ method)

A single colony of TopF was inoculated into 50 ml of antibiotics-free LB broth. The bacteria grew at 37 ^{o}C with shaking overnight. Next day, 30 ml of the culture were diluted into 350 ml of antibiotics-free LB broth and shake-cultured at 37 ^{o}C. The OD595 of the culture was monitored every 20-30 minutes until OD595 reached 0.4 - 0.6. The culture was divided into six ice-cold 50 ml Falcon tubes and cooled on ice for 10 minutes. The bacteria were washed once in 20 ml of ice-cold $CaCl_2$ solution. The washed bacteria were then incubated on ice for 30 minutes once in 50 ml of ice-cold $CaCl_2$ solution and once in 4 ml of ice-cold $CaCl_2$ solution. 0.2 ml of competent cells in 1.5 ml Eppendorf tubes was stored at –70 ^{o}C.

2.2.1.3 E.coli competent cell transformation

1 µl of plasmid or no more than 10 µl of a ligation reaction were added into thawed competent cells and incubated on ice for 30 minutes. The competent cells were then heat- shocked at 42^{o} C for 60 seconds and cooled on ice for 2 minutes. 800 µl of antibiotics- free LB broth was added, and the bacteria were shaken-cultured at 37^{o} C for one hour. 100 µl of transformed bacteria were plated onto LB agar plates containing 50 ampicillin and the plates were incubated at 37 ^{o}C for 16 hours.

2.2.2 DNA methods

2.2.2.1 Plasmid DNA purification (NucleoBond® Macherey-Nagel)

1 colony of transformed bacteria was inoculated into 400 ml of ampicillin-containing LB broth and shaken-cultured at 37^{o}C overnight. Next day, the bacteria were collected and resuspended in 15 ml of buffer S1 and lysed by 15 ml of buffer S2 at RT for 5 minutes. The lysate was then neutralized by 15 ml of buffer S3. The clarified supernatant was loaded onto a NucleoBond® Maxi column equilibrated with equilibration buffer and the solution was passed through the column by gravity. The column was washed once with 30 ml of wash buffer and the column-bound DNA was eluted with 15 ml of elution buffer. The eluate was precipitated by 12 ml of 2-propanol. The pellet was washed once with 70% ethanol and dissolved in 200 µl of TE buffer. For mini-preparations, bacteria collected from a 1.5 ml culture were resuspended in 150 µl of buffer S1, lysed in 150 µl of buffer S2, and neutralized in 150 µl buffer S3. The clarified lysate was precipitated in 77% 2-propanol. The precipitated pellet was washed once in 70% ethanol and air-dried. The dried pellet was dissolved in 50 µl TE. 20 µl of miniprep DNA were used for digestion.

2.2.2.2 Determination of DNA/RNA concentration

0.1 ml of DNA/RNA solutions was transferred into a quartz cuvette and the OD260 of the sample was measured. The concentration of DNA/RNA solutions was calculated automatically. For reference, 1 unit of OD260 = 50 µg /ml of double strand DNA (dsDNA) or 40 ug/ml of RNA.

2.2.2.3 Polymerase chain reaction (PCR) of DNA fragments (Fermentas PCR kit)

2x Master-mix（25ul/tube）Fermentas:

1. 0.05 u/μl Taq DNA Polymerase (recombinant)
2. reaction buffer
3. 4 mM $MgCl_2$
4. 0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP).

Primer mix:10ul/tube

Primer chicken PKCθ or mouse IL-2 sense: 0.2uM—1ulx23=23ul stock 10uM

Primer chicken PKCθ or mouse IL-2 sense anti: 0.2uM— 1ulx23=23ul stock 10uM

H_2O: 7ulx23=161μl

Template: 15ul/tube

Template DNA :2.5ul---input control 1ul for EL4, while 7.5ul---input control 1ul for DT40

Fresh Deionized Water: to 15μl H_2O

PCR programme:

Initial denaturation :95℃*3min

Circulation:[denaturation 95℃*40sec—primer annealing 58℃*40s—extending 72℃*70s]*40times,

Final extending :72℃*5min, Store at 4℃ forever

Long distance PCR for screening and temperature cycling:

Step	Temp(°C)	Time(min)	cycling
Initial denaturation step	94	5:00	1 X
Denaturation step	94	0:20	
Annealing and extending step	66	3:00 +0:05	30 X
Final extending step	68	10:00	1 X
End	4	forever	1X

2.2.2.4 Restriction enzyme digestions of DNA

40 μl digestion reaction containing 0.5-1 μg DNA or 20 μl of miniprep DNA,

1x digestion buffer, and 4 µl restriction enzyme were incubated at 37 ^{o}C for 1 hours to overnight. The reaction was resolved on an agarose gel.

2.2.2.5 Agarose gel electrophoresis of DNA

A proper concentration of agarose in 1x TAE buffer was melted and casted in the gel casting mould. 2 µl samples containing 1x loading dye were loaded onto the gel that was then run in 1x TAE buffer at 150 volts for a proper duration. The resolved DNA was visualized under UV- light using a gel camera and recorded using a gel documentation system.

2.2.2.6 DNA fragment extraction from agarose gels (QIAEX II kit)

The cut gel slice containing a DNA fragment was weighed and transferred to a 1.5 ml Eppendorf tube. 3 volumes of buffer QX1 and 1 tenth volume of QIAEXII beads were added to the gel and incubated at 50 ^{o}C for 10 minutes. The beads were collected by centrifugation and washed once with buffer QX1 and once with buffer PE. For DNA elution, the beads were air-dried, resuspended in 50 µl of EB buffer and incubated at 50^{o}C for 5 minutes. The supernatant containing the DNA fragment was then collected, and the beads were discarded. The supernatant was transferred to a fresh Eppendorf tube and stored at -20 ^{o}C.

2.2.2.7 Ligation of DNA fragments

20 µl ligation reaction containing 1x ligation buffer, 100 ng vector, a proper amount of inert DNA (which makes the insert/vector ratio at 5-10 : 1), and 2 µl T4 DNA ligase were incubated at 16 ^{o}C for 4 hours to overnight. The ligation product was transformed into competent bacterial cells.

2.2.2.8 Southern blotting

Separate DNA fragments on a 0.8% agarose gel in TAE buffer overnight. Immerse gels in 0.25 N HCl (500 ml for a 200 ml gel) for 15 min at room

temperature, agitate gently. Denature gel by soaking in denaturation solution (3 M NaCl, 0.4 M NaOH) two times for 30 min each (Shake the dish containing the gel slowly), then soak in transfer buffer (8 mM NaOH, 3 M NaCl) for 15 min. Float nitrocellulose filter membrane in distilled water for 15 min. (If dry spots remain, heat membrane in water briefly until boiling.) Soak membrane in 3 M NaCl, 8 mM NaOH transfer buffer. The membrane should be thoroughly wetted with transfer buffer before using. Set up downward capillary transfers, continue the transfer for 4 h by alkaline buffer transfer. Then, the membrane was ready for hybridization. Prepare probe by random-primed labeling.

High Temperature Hybridization Solution (68°C)

Pre- or hybridization	Solution Stock	Volume(10ml)
2 x Denhardt's solution	50 x	0.4ml
6 x SSC (or SSPE)	20 x	3.0ml
0.5% SDS	10%	0.5ml
1mM CTAB	0.1M	100µl
100 µg/ml denatured DNA (freshly denatured)*	10mg/ml	100µl
dH_2O to 10ml		5.9ml

*Salmon testis DNA (Sigma D-9156), 10.4 mg/ml, boiled 5 min before use, and added freshly each time.

Place the membrane in hybridization bottle with 10 ml (minimal 100 µl/cm^2 filter) of prehybridization buffer (use nylon mesh as a spacer). Incubate for 1–2 hrs at 68° C. Denature probe at 95°C for 5 min, then add probe in hybridization bottle, hybridize at 68° C overnight. Wash membrane three times for at least 15 min, each with at least 125 ml/100 cm^2 of 7x SSPE/0.1–0.5% SDS at room temperature. Then wash membrane twice for at least 15 min each in 1x SSPE/0.5-1.0% SDS at 37° C. Wash finally for 15 min~1 h in 0.1x SSPE/1% SDS at 65° C. Measure the counts coming off the membrane with a Geiger monitor. Stop washing when the counts dropped from 200 to 700 cpm. If the counts were above this, continue washing as outlined below. Be sure to check the counts between each change of wash buffer. After washing, blot filters with filter paper (Whatman 3MM) to remove

most of the excess moisture. Wrap moist blots in plastic wrap prior to autoradiography. Expose filters to Kodak MS film at –70° C with an intensifying screen for 1 day.

2.2.3 Protein methods

2.2.3.1 Expression and purification of GST fusion proteins (Sigma GST fusion purification kit)

One colony of bacteria harboring pGEM-GST-RSD was inoculated to 50 ml of LB broth containing 50 µg/ml ampicillin. The bacteria were shaken-cultured at 37^{o} C overnight. Next day, the culture was transferred to 1500 ml of ampicillin-containing LB broth which was then shaken-cultured at 37 ^{o}C until OD595 incubating the culture at 27 ^{o}C with shaking in the presence of 0.1 mM of IPTG for 2 hours. The collected bacteria were resuspended in 20 ml of cold PBS plus 0.2 ml of Triton X-100. The bacteria suspension was sonicated on ice at full power until the color of the bacteria suspension was changed from yellow to white. The clarified supernatant was mixed with 1.25 ml of 80% Glutathione Sepharose® 4 B beads and incubated at 4 ^{o}C for 2 hours. The beads were washed 4 times with PBS at RT, 5 minutes each, followed by twice washing with 50 mM Tris-HCl, pH 8.0. The beads were eluted with 10 mM glutathione in 50 mM tris-HCl, pH 8.0, at 30 ^{o}C, for 10 minutes. The eluate was stored at -20 ^{o}C.

2.2.3.2 Nuclear extract preparation

Up to 10^8 cells were lysed on ice for 30 minutes in 10 ml of buffer A plus 0.5% NP-40 and then vortexed for 10 seconds. The nuclei were collected and resuspended in buffer C at 10^7 cells/50 µl of buffer C. The nucleus solution was vortexed at 4 ^{o}C for 30 minutes and clarified. The clarified supernatant was transferred to a fresh Eppendorf tube and stored at – 70 ^{o}C.

2.2.3.3 Determination of protein concentration (Bradford method)

Bio-Rad Dye Reagent Concentrate was five times diluted in ddH_2O before use. A BSA standard was prepared at five concentrations. 20 µl of protein sample was mixed with 980 µl of diluted Dye reagent in a 1 ml plastic cuvette and incubated at RT for 5 minutes. The OD595 of the samples were measured in a spectrophotometer. The protein concentrations were calculated according to the BSA protein standard curve.

2.2.3.4 SDS-PAGE electrophoresis of proteins

A 10% separating gel and a 5% stacking gel were casted sequentially in the gel casting mould according to the recipes listed below. 30 µl of protein sample containing 30 µg protein and 1x SDS-PAGE loading dye was heated at 95 ^{o}C for 5 minutes. The samples and the protein size markers were loaded onto the gel, and the electrophoresis was carried out in the 1x SDS-PAGE running buffer at 100V and 35mA until the dye was migrated to the bottom of the gel.

SDS-PAGE gel preparation

Reagent	10% separating volume (ml)	5% stacking volume (ml)
ddH_2O	4.0	3.56
30 % gel stock	3.3	0.67
1.5 M Tris, pH 8.8	2.5	
1.0 M Tris, pH 6.8		0.63
10% SDS	0.1	0.05
10% APS	0.1	0.05
TEMED	0.01	0.005
Total volume	10	5

2.2.3.5 Western blotting and immunodetection

The resolved proteins in the SDS-PAGE gel were transferred onto a nitrocellulose membrane at 100V or 0.35A for one hr. The membrane was then washed once with PBS and then blocked for 30 minutes at RT in

blocking solution. The membrane was then probed with the primary antibody diluted in blocking solution (1: 10000 for anti-NFATc1 antibody) at 4 ^{o}C overnight with gently shaking. Next day, the membrane was washed three times with washing solution followed by incubating with secondary antibody diluted in blocking solution (1:2000 for anti-mouse IgG-HRP conjugate) at 4 ^{o}C for 4 hrs. The membrane was washed five times, 5 minutes each, at RT with washing solution. To reveal the specific protein band, the membrane was rinsed in ECL developing solution and exposed to an X-ray film for a satisfied time. The exposed film was then developed and fixed.

2.2.3.6 Electrophoretic mobility shift assay (EMSA)

2.2.3.6.1 Probe preparation

For oligo annealing, two single strand oligonucleotides of equal concentration were mixed in 20 µl of 1x annealing solution, heated at 95 ^{o}C, and cooled naturally to RT in the heating blocks. For labeling, 10 µl labeling reaction containing 1x PNK buffer, 5pmol (1 µl) of double strand oligo, 1 µl of T4 kinase, 1 µl 10x PNK buffer A and 3-5 µl of ^{32}P γATP 37 ^{o}C for one hours.

2.2.3.6.2 Purification of labeled probes

Purification on a 10% native DNA gel. 10 µl of labeling reaction were loaded onto a 10% native DNA gel which was then run in 0.5x TBE at 240V until dye migrated to one fourth of the gel. The gel was exposed to an X-ray film for 2 minutes. The gel area containing the probe as revealed by the X-ray film was cut and eluted in 200 µl of TE, pH 8.0. The eluted probe was diluted to 6000 cpm/4µl.

Purification on a Sephadex G-50 column. Column was prepared by loading 200 µl of 50% Sephadex G-50 in TE, pH 8.0, into a 200 µl filter tip which

was vertically mounted into a 1.5 ml Eppendorf tube through a cross-cut made on the lid of the tube. The column was centrifuged at 3000 rpm for 3 minutes and flow-through was discarded. 20 µl of labeling reaction was loaded onto the column and centrifuged at 3000 rpm for 3 minutes. The flow-through was diluted to 20.000 cpm/4 µl.

2.2.3.6.3 Binding reaction

A 10 µl binding reaction contains 3.3x binding buffer, 0.7 µl poly dI·dC, 1-4 µl protein, and 1 µl probe. The reaction was incubated at RT for 15 minutes. In competition assays, 0.5 and 5 pmol of double strand oligos (5 pmol/1 µl) were included in the reaction. In super-shift assays, 1µl of antibody was included in the reaction. The total volume in competition and super-shift assays was adjusted to 10 µl. The reaction was resolved on a 5% native PAGE.

2.2.3.6.4 Native gel electrophoresis of protein-DNA complexes

A 5% native PAGE gel was casted according to the recipe listed below in the gel casting mould. 10 µl of binding reaction was loaded onto the gel prerun for 2 hrs at 240V and the electrophoresis was carried out in 0.25X TBE at 10 mA in the cold room until the dye migrated close to the bottom of the gel. The gel was fixed, dried, and exposed to an X-ray film.

Preparation of 60 ml native gel solution

Gel concentration	5%	10%
Reagent	volume (ml)	
ddH2O	48	36.7
30% PAGE	10	20
10x TBE	1.5(x 0.25)	3(x 0.5)
10% APS	0.3	0.1
TEMED	0.1	0.05

2.2.3.7 Chromatin immuno-precipitation (ChIP) assay

2.2.3.7.1 Chromatin preparation

$2x10^8$ cells were fixed on ice or 5 minutes in 1% formaldehyde in 37 ml of RPMI1640. The cells were then lysed on ice for 30 minutes in 20 ml of ChIP lysis buffer and vortexed for 30 seconds. The chromatin was collected, resuspended in 2 ml of sonification buffer and sonicated on ice for 9 cycles, each for 30 seconds at max power, 0.5 duty cycle followed by cooling on ice for 30 seconds. The sheared chromatin was then clarified. The clear chromatin solution was transferred to a 1.5 ml fresh Eppendorf tube. The OD260 of the chromatin solutions was taken and the chromatin solutions were adjusted to equal OD260 units with sonification buffer and stored at -70 ^oC.

2.2.3.7.2 Immuno-precipitation of the sheared chromatin

One ml of chromatin was pre-cleared with 50 µl of salmon sperm DNA/protein A agarose slurry. Chromatin equal to 10^7 lymphocytes in 300 µl was incubated with 3 µl of anti-NFATc1 antibody, or 3 µl of anti-NF-kB p50 or p65 antibodies at 4 ^oC overnight with rotation. Next morning, 30 µl of salmon sperm DNA/protein A agarose slurry was added to each reaction and incubated at 4 ^oC for 2 hours with rotation. The recovered beads were washed sequentially with sonification buffer, high salt solution, LiCl solution, and TE. Twice for each solution, 5 minutes each. The beads were resuspended in 300 µl of elution solution plus 10 µl of 5M NaCl. 30 µl of input chromatin was also diluted to 300 µl with elution solution and 10 µl of 5M NaCl were added. The beads and the input chromatin were incubated at 65 ^oC for overnight and extracted once with PCI(phenol:chloroform:isoamyl alcohol). The water phase was precipitated by 70 % ethanol plus 0.1 M $(NH4)_3Ac$, pH 5.2. The precipitated DNA was washed once with 70% ethanol, and dissolved in 50 µl of TE and stored at – 20 ^oC.

2.2.4 Cell culture methods

2.2.4.1 Storage of cell lines

Chicken DT40 B cells, Jurkat T cells and EL-4 T cells were grown in complete RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and penicillin plus streptomycin. For storage, 5 x 10^6 cells in 1 ml of frozen solution (90% FCS and 10% DMSO) were frozen at -70 ^oC.

2.2.4.2 Maintenance of cell lines

The cell culture work was done under sterile conditions in laminar hoods. All cells were cultured at 37°C (except DT40 cells: 39.5°C) in a humidified tissue culture incubator. To start a cell culture, the frozen cells were thawed in a 37 ^oC water bath and transferred to a cell culture flask containing 20 ml of medium. The cells were splitted twice a week at 1: 5-10 dilutions to maintain the cells at a density of 1- 4 x 10^5 cells /ml.

2.2.4.3 Cell stimulation

In stimulation experiments, 5x 10^7 of cells DT40, Jurkat and EL-4 cells were pre-cultured in 50 ml of complete RPMI1640 at 39.5 ^oC or 37 ^oC for 2 hours. To stimulate the cells, ionomycin (0.5 µM) or TPA (20 ng/ml) + ionomycin (0.5 µM) were mixed with the cells, and the cells were incubated at 39.5 ^oC or 37 ^oC for 4 hours, or H_2O_2 (0.5,1,2,5,10 µM) was mixed with cells for 4, or 8, or 12, or 24 hours. In inhibition experiments, inhibition reagents were added to the desired concentration and the cells were incubated at 37 ^oC for one hour. In ChIP experiments, up to 2x10^8 of thymocytes in 50 ml of complete RPMI1640 were incubated with TPA+ionomycin as described above.

2.2.5 FACS staining and apoptosis analysis

Staining

1. Wash cells twice with cold PBS and then resuspend cells in 1×binding buffer at a concentration of 1 x 10^6cells/ml.

2. Transfer 100 µl of the solution (1 ×10^5cells) to a 5 ml culture tube.

3. Add 5 µl of Annexin V-APC (BDPharmingen^{TM}) and 5 µl of propidium iodide (PI, just before analysis).

4. Gently vortex the cells and incubate for 15 min at RT in the dark.

5. Add 400 µl of 1×binding buffer to each tube. Analyze by flow cytometry within one hour. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

10×Binding Buffer: 0.1 M Hepes (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$. Store at 4 ^{0}C.

The following controls are used to set up compensation and quadrants:

APC emission wavelength is 660 nm and excitation is 650 nm. APC is optimized for FL-4 fluorescence on the FACS Calibur.

1. Unstained cells.

2. Cells stained with Annexin V-APC alone (no PI).

3. Cells stained with PI alone (no Annexin V-APC).

2.2.6 ES cell culture and manipulation

2.2.6.1 Stem cell culture

Two stem cell lines (E14 and TC-1) were adopted in these experiments, both are derived from the 129X1/SvJ mouse strain. These ES cell lines have been proven to generate germ line chimeras with high efficiency. To maintain ES cells integrity, ES cells were carefully expanded and subsequently frozen in liquid nitrogen before screening. All ES cells were grown on MEF feeder layers plus LIF cytokine in high quality medium. This medium prolonged the embryonic phenotype of the cell line. ES cells grew

rapidly, dividing every 18 to 24 h. The cells were kept at relatively high drug concentration: densities to ensure that a high rate of cell division was maintained as this seems to minimize the level of spontaneous differentiation. The cultures were re-fed daily, and subcultured every 2 d. When passaged, it was important to trypsinize the cells well to ensure that a single cell suspension was generated.

2.2.6.2 Preparation of MEFs

Fresh MEFs were isolated from mouse embryos. MEFs were cultured until passage 3, then frozen in liquid nitrogen as stock. Since MEFs have a limited life span in culture after 3 passages, they can be used to support ES growth.

2.2.6.3 Preparation of targeting vector DNA , Cre recombinase encoding DNA and electroporation

A large scale preparation of gene targeting vector DNA was prepared and purified by the Maxprep kit (QIAGEN). 100 µg of targeting construct DNA was digested with an enzyme to linearize. 0.5 µl of digested DNA were fractionated on an agarose gel to control that the vector DNA was completely digested. The DNA was extracted 2X with phenol/chloroform, afterwards 0.15 volumes of 2M NaOAc and 2 volumes EtOH were added for precipitation. The DNA was pelleted, and then washed with 70% EtOH. The DNA was resuspended in 100 µl sterile H_20 or TE in a laminar flow hood. Check 0.5 µl DNA on an agarose gel, and determine DNA concentration.

Preparation of ES cells for transformation and electroporation: Approximately 6 x 10^7 ES cells (~3 confluent 10 cm plates) were needed for one electroporation. Split one plate of subconfluent ES cells into four dishes such that the next day

the cells are ready to be split again. Fed the cells in the morning, as the cells were plated more confluent than usual. The cells were ready for electroporation after a few hours. Trypsinize the cells: remove the medium and rinse 2 times briefly with 10 ml of calcium-free PBS (37°C), add 3 ml of fresh trypsin-EDTA and incubate for 5 min at 37°C. Digested cells were checked under the microscope to see if the colonies looked like grapes (not single cell). Then the cells were pipetted gently in trypsin-EDTA to break up the clumps, after that add 7 ml medium and pipette gently up and down again. The cells were pelleted for 5 min at 270g. Aspirate the supernatant and resuspend the cells in 50ml of PBS. Count cells in hemocytometer. The cells were centrifuged and resuspended to a final concentration of 2.0~2.5 x 10^7 cells/1ml PBS. Transfer 0.8 ml of cell suspension into a 4 mm electroporation cuvette (gap width 0.4 cm) with 25~40 µg DNA (1 µg/µl of linearized and purified DNA) as below: Bio-Rad electroporator (Gene Pulser II System, Bio-Rad, Inc., CA) -- 250V, 500µF. Disconnect the resistance and use the capacity extender to achieve these settings. Monitor time constants, which was ~7 seconds. Let cuvettes sit on ice for 20 min and plate onto three 10-cm feeder dishes in regular ES media (without G418).

2.2.6.4 Drug selection and cell culture

The day after electroporation there should be some floating dead cells but most cells should sit down on the feeder cells. Start selection 24 h after the electroporation with ES medium containing selection drug (250 µg /ml G418). After about 6-8 d of selection, individual drug-resistant colonies should have appeared and be large enough to pick and subcloned for screening.

2.2.6.5 Picking of ES cells and 96-well plate culture

Wash plate with PBS. Pick the ES colonies when they were easily seen

microscopically but have not yet begun to show any signs of differentiation. This was usually after ~7-9 d under selection, but some variability was not unusual. Pick colonies into a 96 well plate (U shaped wells) with 35 µl of trypsin / well. Pick 24 colonies and incubate at 37 ^{o}C for 10 min. Used a multichannel pipette to break up colonies. Transfer directly into a 96-well plate with feeders and 150 µl of ES medium (without selection drug). Fed every day with ES medium containing selection drug (250 µg /ml G418). Repeat this step on second and third picking days, use the original plates of electroporated cells. If the colonies have not grown to ~80% confluence in two to three days, trypsinize the cells using the following procedure: Aspirate the medium and wash the cells with PBS 2 times. Culture for another 3 -5 d.

2.2.6.6 Freezing in 96-well plates

Working one row at a time using a multichannel pipette. Change the medium 2-3 h prior freezing. After 2 days, cells were ready (~80% confluence) to be frozen. Wash with PBS, add 35 µl of trypsin, incubate at 37 ^{o}C for 10 min, and add 65 µl of ES medium. Use a multichannel pipette to disperse cells. Transfer 65 µl into replica plate (U shaped wells) containing 65 µl of cold 2 x ES freezing medium. Wrap with parafilm, place in a styrofoam box with dry ice for 20 min, and transfer to -80 ^{o}C. After removing cells for freezing, add 150 µl of ES medium to left 35 µl cells. Grow cells without feeder for 3 - 5 d till the medium became yellow. Split into 2 - 4 x 96 well plates for southern blotting and PCR assays.

2.2.6.7 96-well plate DNA extraction

When cells were confluent, aspirate the medium from each well on the plate and wash twice with PBS. Add 50 µl of lysis buffer to each well. Dampen some paper towels and place in the bottom of a tupperware container.

Place the plates on paper towels and seal the lid of the tupperware container. Incubate the plates (with lids on) in this sealed humid container in a 56°C incubator overnight. Add 120 µl of fresh precipitation buffer to each well; leave at RT for 4 h to allow the DNA to precipitate, avoiding vibration. DNA precipitates were visible against a black background or web-like DNA fibers were seen under a low power microscope. Remove lid, take five layers of paper towel and pre-wet one side by 70% EtOH, then put the wetted-side on plate over open wells. Invert plate together with paper towels slowly and keep it on bench for 30 seconds. Lift the plate and blot away liquid. The precipitated DNA remained attached to the bottom of wells. Wash the precipitated DNA by carefully adding 200 µl of wash buffer 3 times, keep at room temperature for 10 min for each washing. Each time remove alcohol by inversion of plate on paper towels. At this point, DNA could be stored in 70% ethanol in a parafilm sealed plate at –20 °C (for four to five weeks) until proceeded with diagnostic restriction enzyme digestion and PCR assays (or southern blotting). Add 60 dH_2O, leave at RT overnight. Mix up a sample for a final volume of 40 µl . Check on a 0.8 % agarose mini-gel, and if not completely digested, add 10u/per well restriction enzyme mix again. Add 4-5 µl of loading buffer to each well, electrophorese the digested DNA on an agarose gel at 20 V, overnight. For PCR, use 1~2 µl of DNA sample. To run a nested-PCR, external PCR: 25~30 cycles were run and internal PCR: 15~25 cycles. The expected yield of DNA from each plate well was about 10 µg.

3. Results

3.1 Creation of Murine Embryonic Stem Cells Containing a Targeted NFATc1 Allele for its Conditional Inactivation in Mice

3.1.1 The DNA construct for targeting the NFATc1 P1 promoter

To delete conditionally the promoter P1 of *Nfatc*1 gene, a targeting vector was constructed by Dr. Jiming Liu using the pGL3-basic vector. It was sequenced to ensure its fidelity (which is shown in Figure 3-1). The DNA of targeting vector was amplified upon transformation, and the recombinant DNA of targeting vector was proven to be correct by digestion with Acc65I (Figure 3-1-B).

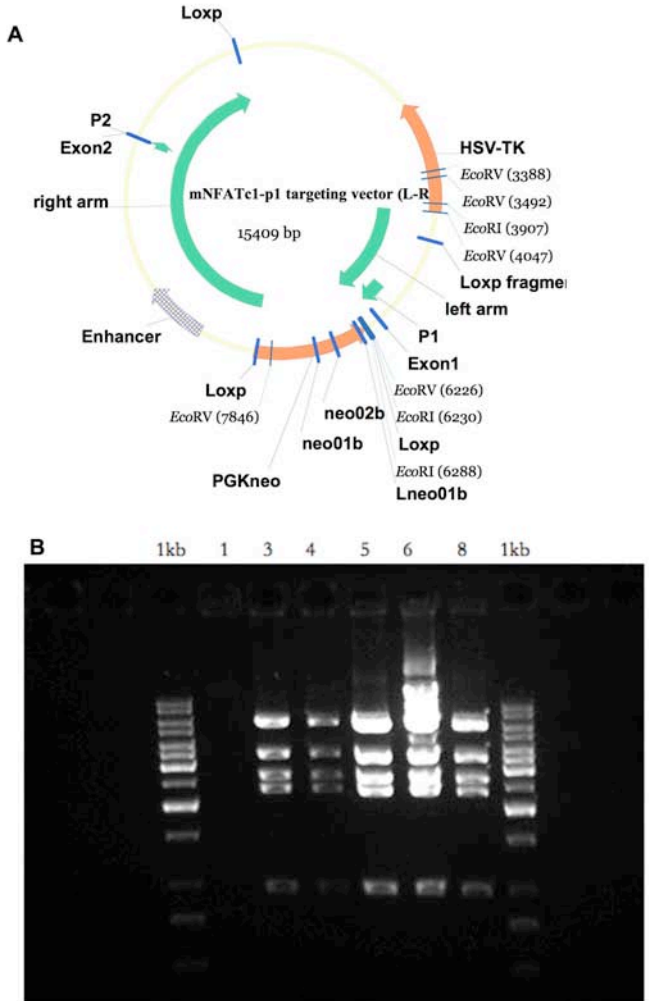


Figure 3-1 Targeting vector for the conditional deletion of the *Nfatc1* promoter P1 . (A) Scheme of the targeting vector. The pGL3-basic vector was used for

constructing the targeting vector. Serial clonings were done to incorporate the following DNA fragments: HSV-TK (herpes simplex virus thymidine kinase) gene for negative selection; the left DNA “arm” for site-specific recombination, containing a loxp sequence after subcloning, as well as exon 1 and promoter 1 DNA; a PGK-neomycin resistance gene (for positive selection) flanked by Cre recombinase recognition (loxP) sequences; and the right “arm” for site specific recombination containing the enhancer and exon 2, and another loxp sequence. (B) Digestion with the Acc65I enzyme showing the predicted fragments (5873+3527+2721+2296+996=15409) which is in line with the expected outcome. Electrophoresis on a 1% agarose gel at 80 V for 100 min: Positive colonies of *E. coli* carrying the targeting vector are shown in lanes 3,4,5,6 and 8.

3.1.2 Electroporation of targeting vector DNA and double selection of recombinant ES cells

To mutate the endogenous *Nfatc1* gene in ES cell, the purified DNA of targeting vector was electroporated into TC-1 ES cells (a gift from the laboratory of R. Flavell, Yale University, New Haven, USA). During double selection with ganciclovir and G418, the quality of feeder layer cells is of crucial importance. On radiated feeder layers, the ES cells grew well, and they maintained an undifferentiated state (Figure 3-2-B,C,G), while with overcrowded feeder layers or without feeder layer, they grew slowly (Figure 3-2-A,D) or differentiated into many branches (Figure 3-2-E). Interestingly, contrary to previous observations (Cohen et al., 2006), in our hands the use of antibiotics such as ampicillin, in addition to selection drugs, facilitated the differentiation of ES cells (Figure 3-2-F,G). Furthermore, it is compulsory to avoid contamination with mycoplasms which can be found in many culture media and, therefore, established cell lines. Such a contamination is deleterious for maintaining pluripotency which is necessary for the faithful germ line transmission of ES cells upon injection into murine blastocysts (Cobo et al., 2007).

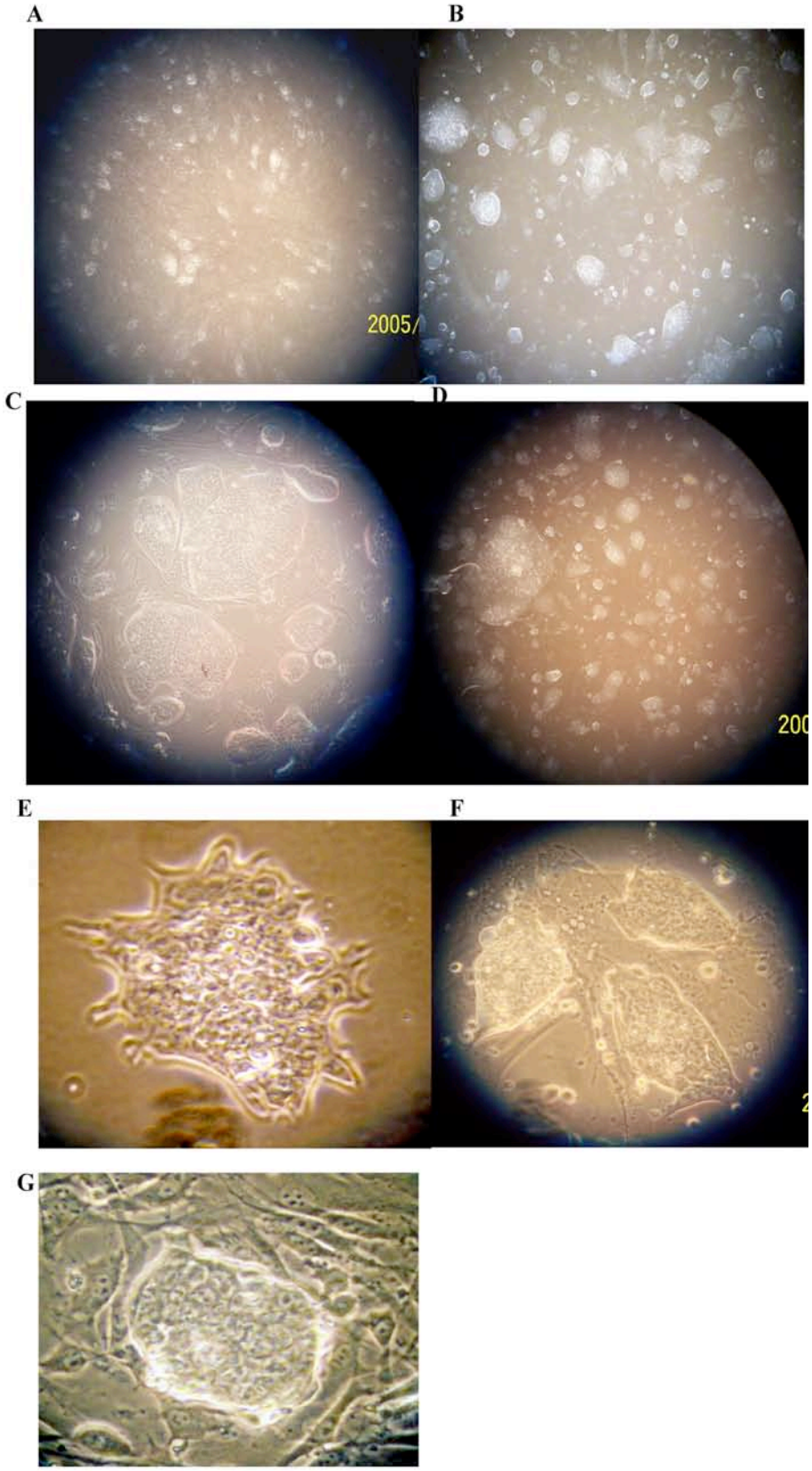


Figure 3-2 Morphology of murine TC-1 ES cells under different culture conditions, as it can be seen by light microscopy.

(A) ES cell growth on feeder cells which were not radiated. (B and C) Growth on

feeder cells which were radiated, magnification:10x and 40x. (D) ES cells without MEF feeder cells. (E) Differentiated ES cells growing without feeder layer and with ciprofloxacin. (F) Slightly differentiated ES cells growing on a feeder layer and ciprofloxacin. (G) “Normal” ES cells with feeder layer but without ciprofloxacin.

3.1.3 Preliminary screening of ES cells for site-specific recombination by long distance PCR

To screen the neo-resistant T1-C ES cells for site-specific integration of the targeting vector, among the 600 colonies which we have picked according to standard procedures (Figure 3-2-C), long distance PCR assays were performed using as template the genomic DNA from the above picked clones (Loukianov et al., 1997). 10 positive clones were obtained (Figure 3-3).

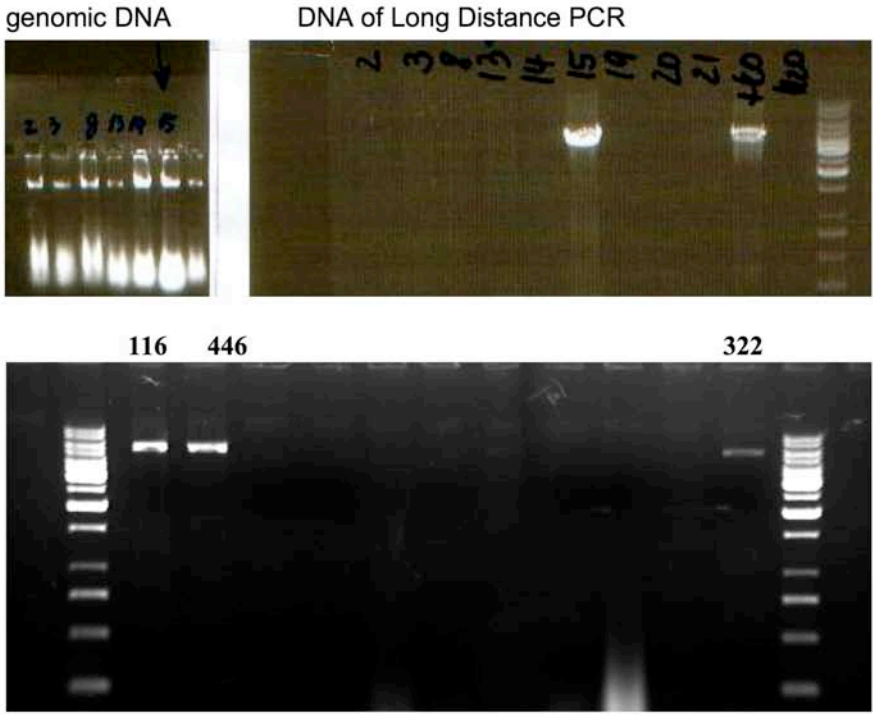


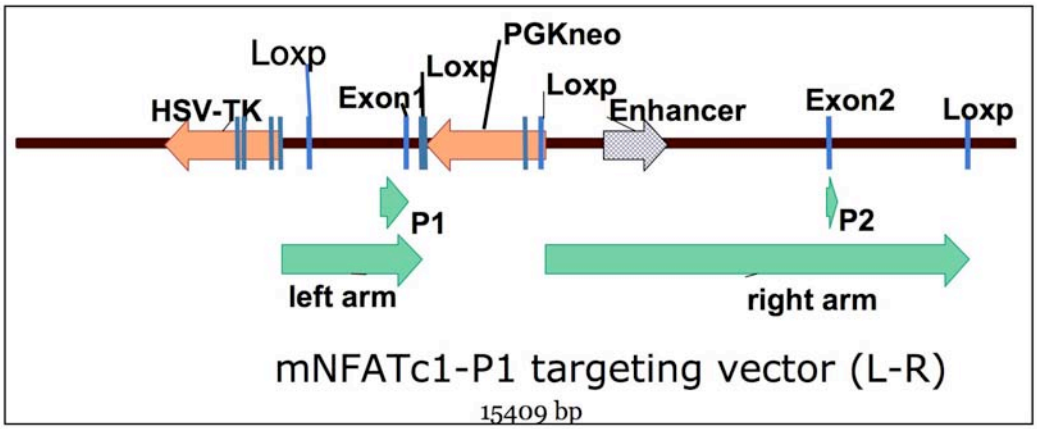
Figure 3-3 Positive ES cell colonies screened by long distance PCR after double selection. The predicted positive PCR fragment has to be 4 kb in length. 4 positive clones are shown here corresponding to the colonies no. 15, 116, 322 and 446.

3.1.4 Confirmation of positive clones by Southern blot assays

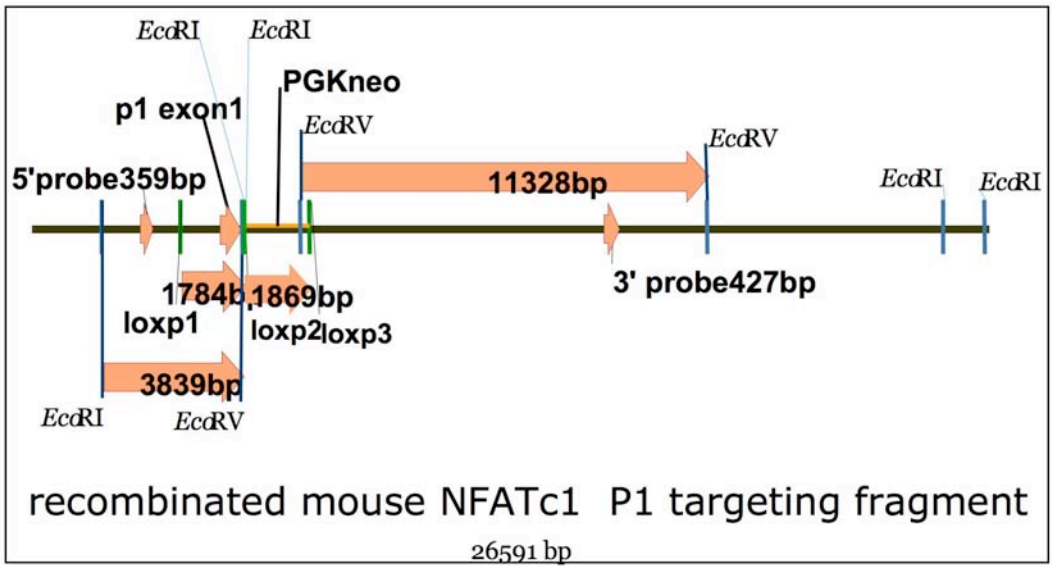
To ensure the intactness of targeted *Nfatc1* locus containing all functionally important components, such as the loxP sequences, before injecting positive ES cells into blastocysts, Southern blot assays were done using DNA from positive ES cell clones. The probe for hybridization was synthesized using as

template DNA from a BAC (bacterial artificial chromosome) bearing the murine *Nfatc1* gene. The NFATc1

A



B



C

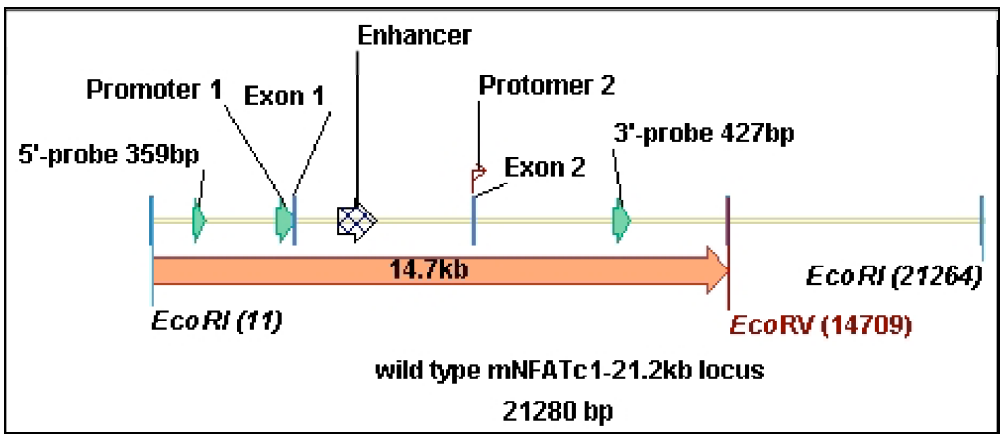


Figure 3-4 Targeting vector for the deletion of the murine *Nfatc1* promoter 1 and the structure of the targeted locus (obtained from Jiming Liu).
(A) Several cloning steps were done (by Jiming Liu) to incorporate the following components into the pGL3-Basic vector for constructing the targeting vector: HSV-TK (herpes simplex virus thymidine kinase gene), the negative selection gene, left arm for site-specific recombination with an introduced loxp sequence, exon1 and promoter 1, PGKneo (positive selection gene)-the neomycin resistance gene flanked by Cre recombinase recognition (loxP) sequences, then the right arm for recombination with enhancer and exon 2, and another loxp sequence. (B) The targeted *Nfatc1* locus in the mouse genome after successful recombination. (C) The wild type locus of themurine *Nfatc1* gene indicating where the probes are located and the length of DNA fragments.

5' (left) probe spanning 359 bp is located upstream the first loxp site, while the 3' (right) one spanning 427 bp in the right arm and downstream the third loxp site (Figure 3-4-B). Their hybridized genomic fragments obtained by EcoRI and EcoRV digestion are displayed (Table 3-1). Finally, 6 positive clones were identified: the clones No.8, 10, 15, 322, 446 and 560 (Figure 3-5-A and results not shown)

Table 2-1 Probes for Southern blot hybridization and their corresponding segments of genomic DNA in both wild type (WT) and mutated (promoter 1 – P1) ES cells upon cutting by EcoRI and EcoRV.

	5'Probe	*3'Probe*
WT	14.7kb	14.7kb
P1	3.8kb	11.3kb
Enzyme	EcoRI + EcoRV	EcoRI + EcoRV

3.1.5 Cre-recombinase expression in ES cells carrying a mutated *Nfatc1* gene

To test the efficiency of the Cre system to cut the targeted locus, a vector expressing Cre recombinase was electroporated into the mutated ES cell clone No.15. The Cre recombinase was expressed and the PGKneo gene was removed. The ES cells were found to die in G418 media without PGKneo gene

whereas ES cells with unremoved PGKneo survived, which was proven by Southern blot hybridization (Figure 3-4,5-B,C). Taken together, the targeting vector and the corresponding ES cell clones for the conditional deletion of the *Nfatc1* P1 promoter were proven to be intact for the conditional deletion and, therefore, suitable for the inactivation of P1 and P1-directed transcription.

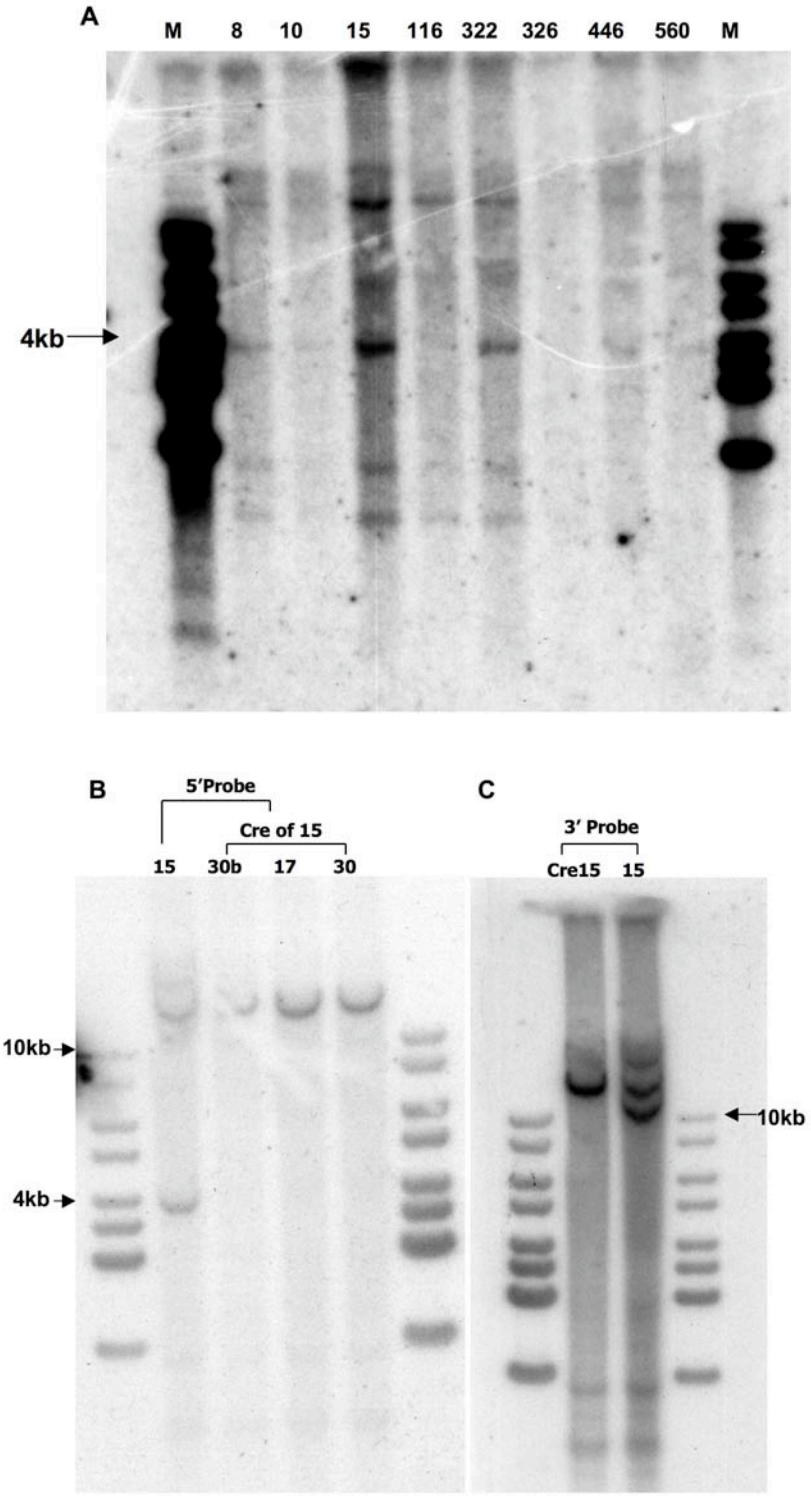


Figure 3-5 Confirmation of positive ES clones by Southern blot and

Cre recombinase treatment of mutated ES cells.

Note the appearance of a rapidly moving band of 3.8 kb from the mutated allele of the *Nfatc1* gene and a slowly migrating band of 14.7 kb from the wild type allele hybridized with a probe for the left arm (A and B). Bands of 11.3 kb and 14.7 kb appeared upon hybridization with the right probe (as shown in C, lane 15). When Cre recombinase was expressed in the positive ES cells No. 15, the PGKneo fragment flanked by loxp sites was removed. The removal of bands of 4 kb (B) and 11.3 kb (C) upon Cre recombinase expression indicated the intactness of the targeted allele which is efficiently cut by Cre.

3.1.6 Chimeras

To get mutated mice carrying mutated *Nfatc1* alleles for the conditional deletion of its promoters, the positive ES cell lines shown above were injected into murine blastocysts. But no germ line was detected in spite of extended efforts and dozens of chimeras (Figure 3-6) for unknown reason.

Figure 3-6 Chimeras generated from ES cells carrying a targeted *Nfatc1* allele (black dots) in a host mouse (brown).

3.2 Analysis of NFATc1 Function in Chicken B cell Line DT40

To explore NFATc1 function in a different system, we established collaboration with Dr. Eisaku Kondo (University of Okayama, Japan) for the inactivation of NFATc1 in the chicken B cell line DT40. This cell line represents an effective alternative system for gene disruption experiments in somatic cells (Winding and Berchtold, 2001). In addition, DT40 B cells provide a versatile system to study molecular mechanisms controlling immunoreceptor-mediated apoptosis.

3.2.1 Disruption of the *Nfatc1* gene in chicken DT40 B lymphocytes and re-expression of human *NFATc1/αA* or *αC*

To identify the function of different isoforms of *Nfatc1*, the DT40 B system has been used to inactivate the chicken endogenous *Nfatc1* gene. Upon inactivation of endogenous *Nfatc1* gene by Eisaku Kondo, he introduced plasmids encoding the human *Nfatc1* isoforms *Nfatc1*/αA and *Nfatc1*/αC into NFATc1$^{-/-/-}$ DT40 cells to study the function of individual NFATc1 isoforms. To disrupt the endogenous *Nfatc1* gene three rounds of inactivation had to be performed to destroy the three *Nfatc1* alleles which are present in DT40 cells (Figure 3-7-A). By Southern blot hybridizations it was shown that indeed the three *Nfatc1* alleles were inactivated, and by PCR and Western blots assays it was demonstrated that no NFATc1 protein was synthesized (Kondo et al., in prep.). Afterwards, upon transfection of expression vectors encoding the human *Nfatc1/αA* or *Nfatc1/aC* proteins these proteins were faithfully expressed in NFATc1$^{-/-/-}$ DT40 cells (Figure 3-7-B). These approaches and models provide the opportunity to study the function of individual NFATc1 isoforms, especially of NFATc1/αA wich differs remarkably from the other isoforms by its short C terminal peptide (Figure 1-1).

A

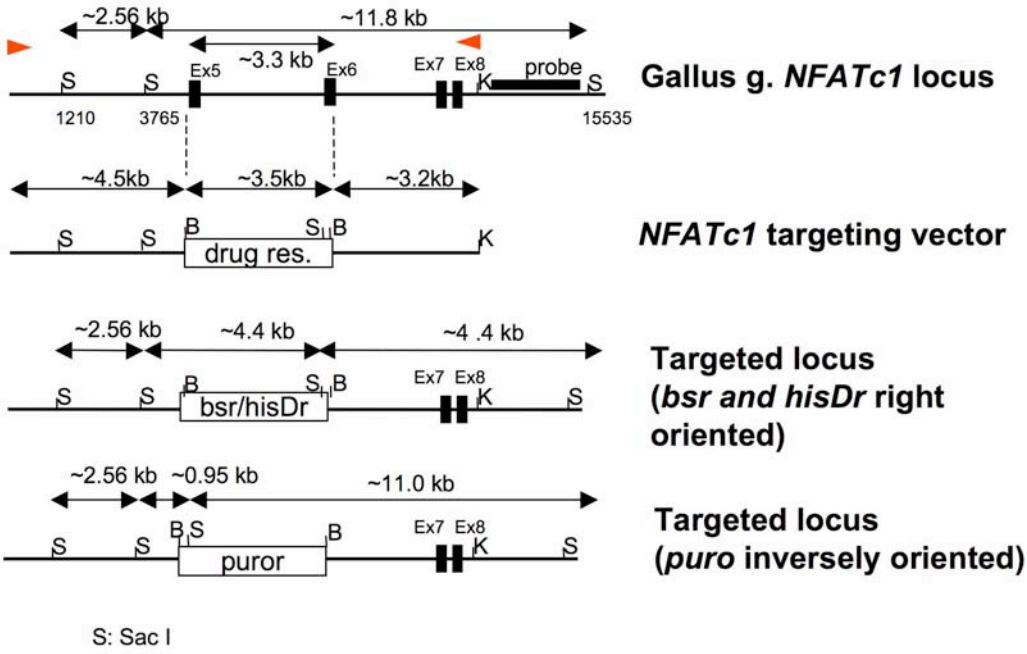


Figure 3-7-A Inactivation of the chicken *Nfatc1* gene in DT40 B cells: Scheme of targeting strategy (from Kondo et al., 2007, in preparation).
Above, the locus of chromosomal chicken *Nfatc1* gene is depicted including exons 5, 6, 7 and 8. Restriction sites are indicated for cleavage by Sac I (S), Bam HI (B) and Kpn I (K). The probe which was used for the Southern blot is shown by a black bar. Targeting vectors were constructed by the assembly of a resistance gene (blasticidin S, bsr; histidinol D, hisDr; puromycin, puror) with two chromosomal *Nfatc1* DNA segments of 4.5 kb and 3.2 kb, respectively. Site-specific recombination resulted in the appearance of three targeted loci containing either the *bsr* and *hisDr* genes in the orientation of transcription, or the *puror* gene in the reversed orientation.

B

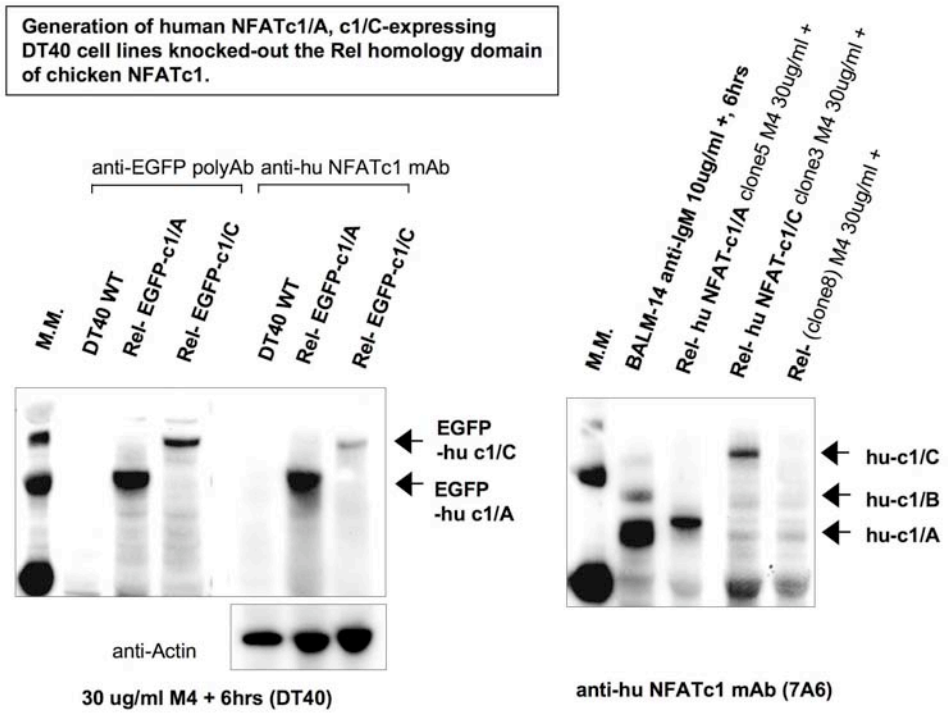


Figure 3-7-B Re-expression of human *Nfatc1/αA* or *Nfatc1/αC in* NFATc1$^{-/-}$ DT40 cells (from Kondo et al., 2007, in prep.).
Expression of human chimeric *Nfatc1/αA or Nfatc1/aC*- EGFP (left part of B) or HA-tagged fusion proteins (right part of B) in NFATc1$^{-/-}$ DT40 cells was shown by Western blot.

3.2.2 Expression of human NFATc1/αA or human NFATc1/αC and apoptosis induction

Upon activation of the BCR by anti-IgM Ab, the induction of apoptosis was checked by FACS. When no NFATc1 isoform was expressed, apoptosis increased by 30% upon Ab stimulation. When NFATc1/αA was expressed, the apoptosis increased only slightly – or stayed the same - whereas expression of NFATc1/αC led to an increase to 23% compared to NFATc1$^{-/-}$ DT40 cells, and it increased 11 fold compared to cells expressing NFATc1/αA. This indicates that NFATc1/αA protects DT40 B cells against apoptosis while NFATc1/αC appears to enforce apoptosis (Figure 3-8).

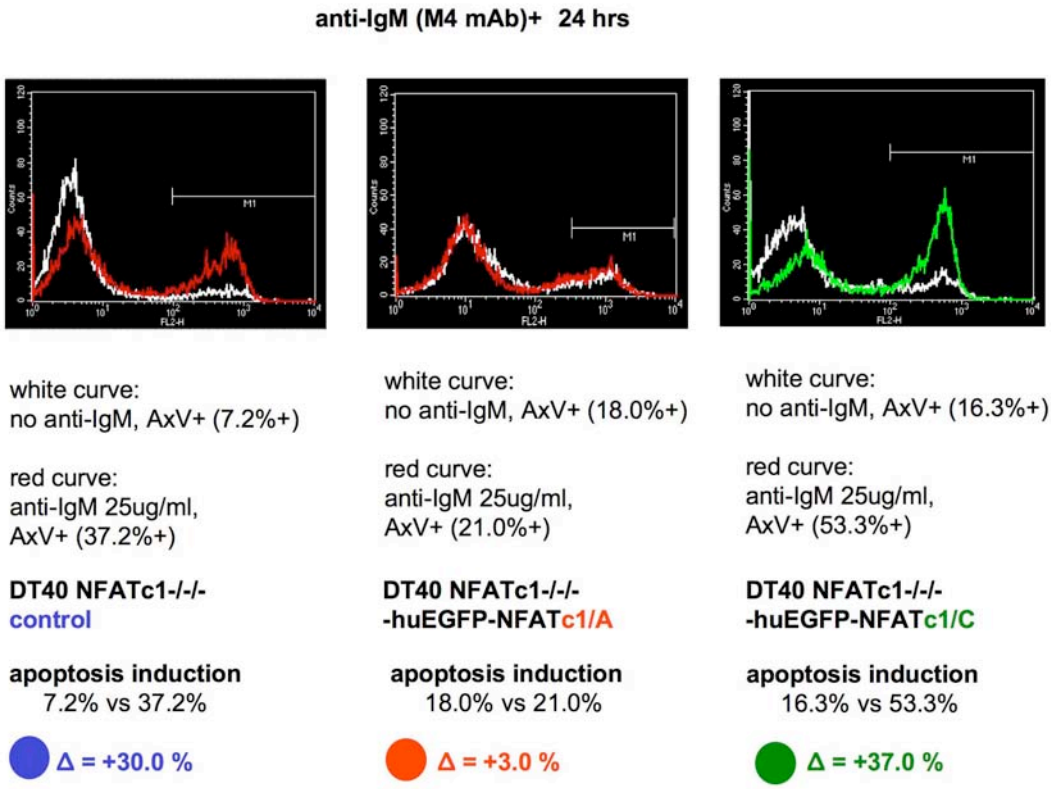


Figure 3-8 NFATc1/αA protects cells from apoptosis while NFATc1/αC exaggerates apoptosis, which is initiated by anti-IgM Ab directed against the BCR (from Kondo et al., 2007, in prep.)**.**
The apoptosis induction of NFATc1-deficient DT40 cells increased 30% (left panel); whereas expression of human NFATc1/αA rescued NFATc1-deficient DT40 cells from apoptosis (middle column). Moreover, expression of NFATc1 /αC increased apoptosis up to 37% (right column) upon activation of BCR.

Next, we measured apoptosis induction in these manipulated DT40 cells by H_2O_2. It was astonishing to see that induction of apoptosis by H_2O_2 resulted in effects on apoptosis for NFATc1/αA and NFATc1/αC that were exactly inverse (Figure 3-9) compared with anti-IgM Ab stimulation. H_2O_2 exerts a number of diverse effects which, maybe, affect many physiological processes and signaling pathways (Iwata et al., 1997; Kim et al., 2005; Velez-Pardo et al., 2002), and lead to these surprising results.

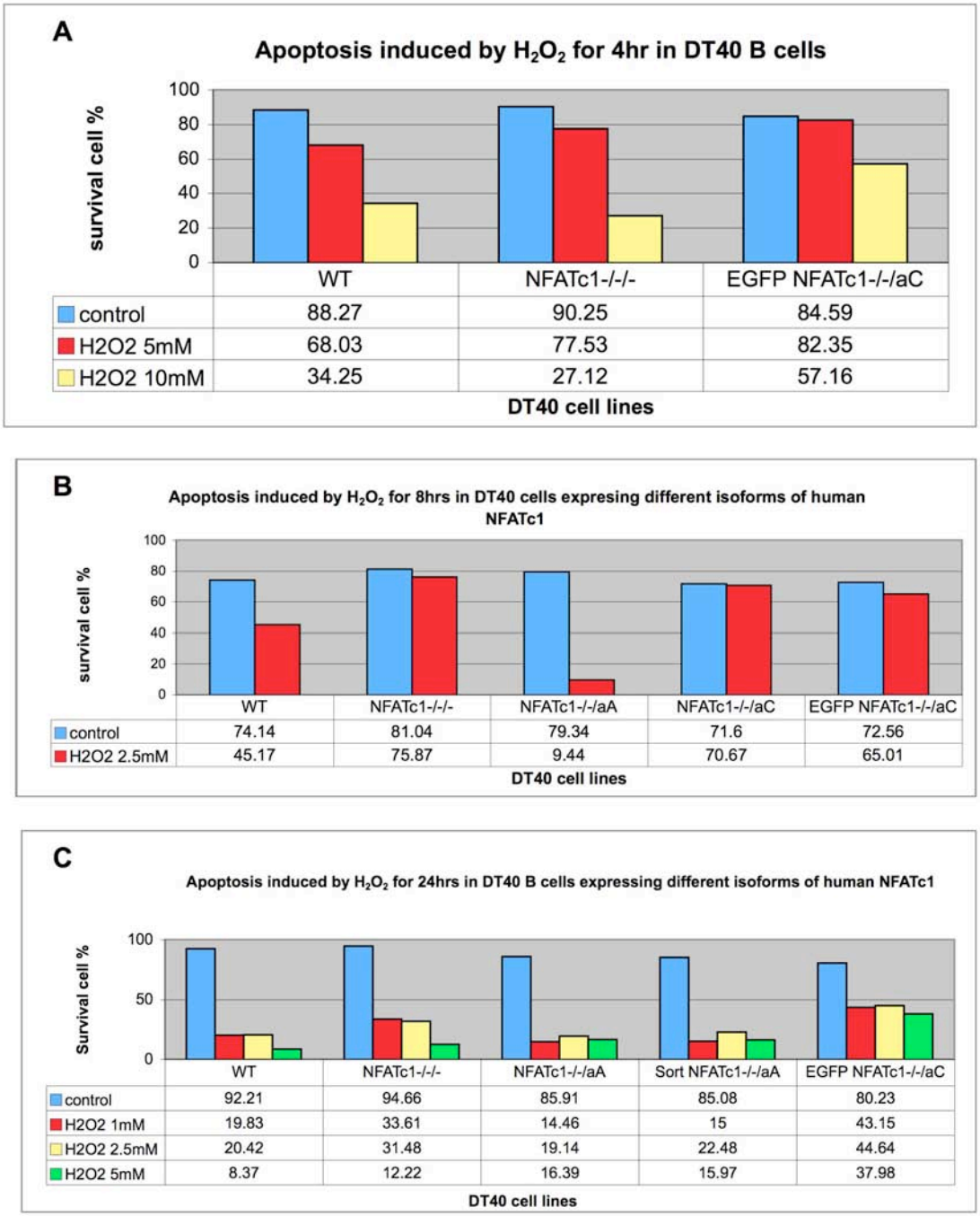


Figure 3-9 H_2O_2 treatment led to inverse effects on apoptosis induction of DT40 cells with protection of cells expressing the long isoform NFATc1/αC while exaggerating apoptosis of cells expressing the short isoform NFATc1/αA.
(A) Treatment for short time but with high concentrations of H_2O_2. (B, C) Treatment for relatively longer time but with low concentrations of H_2O_2.

3.2.3 The *pkc-θ* promoter is a NFATc1/αA target

The IL-2 promoter is well known to be bound and controlled by NFAT transcription factors (Jain et al., 1995; Serfling et al., 1995). To set a positive

control, the human short isoform NFATc1/αA expressed in transfected DT40 cells was checked to bind to a probe from the IL-2 promoter. Electrophoretic Mobility Shift Assays (EMSA) (Figure 3-10) were repeated 3 times using nuclear proteins from the WT, NFATc1$^{-/-/-}$ and NFATc1$^{-/-/\alpha A}$ DT40 cell lines. The murine IL-2 promoter's distal NFAT binding-site was used as probe labeled by P^{32}-ATP. It formed complexes with the *hNfatc1/αA* protein while no such complexes were formed using protein from DT40 WT and NFATc1$^{-/-/-}$ cells (Figure 3-10, lanes 1-4). Such a complex formation is much more stronger (Figure 3-10, lane 5) upon induction of NFATc1 with TPA+ionomycin than that without induction (Figure 3-10, lane 6), and it can be disrupted by an unlabeled excess of murine IL2-NFAT oligonucleotides (Figure 3-10, lane 7 and 8) but not by mutated ones (Figure 3-10, lane 9 and 10). Furthermore, when a monoclonal Ab of NFATc1 was added, a larger, slowly migrating complex was formed leading to a super-shift (Figure 3-10, lane 11). So, this experiment clearly shows that NFATc1/αA binds to the IL-2 promoter and controls its activity.

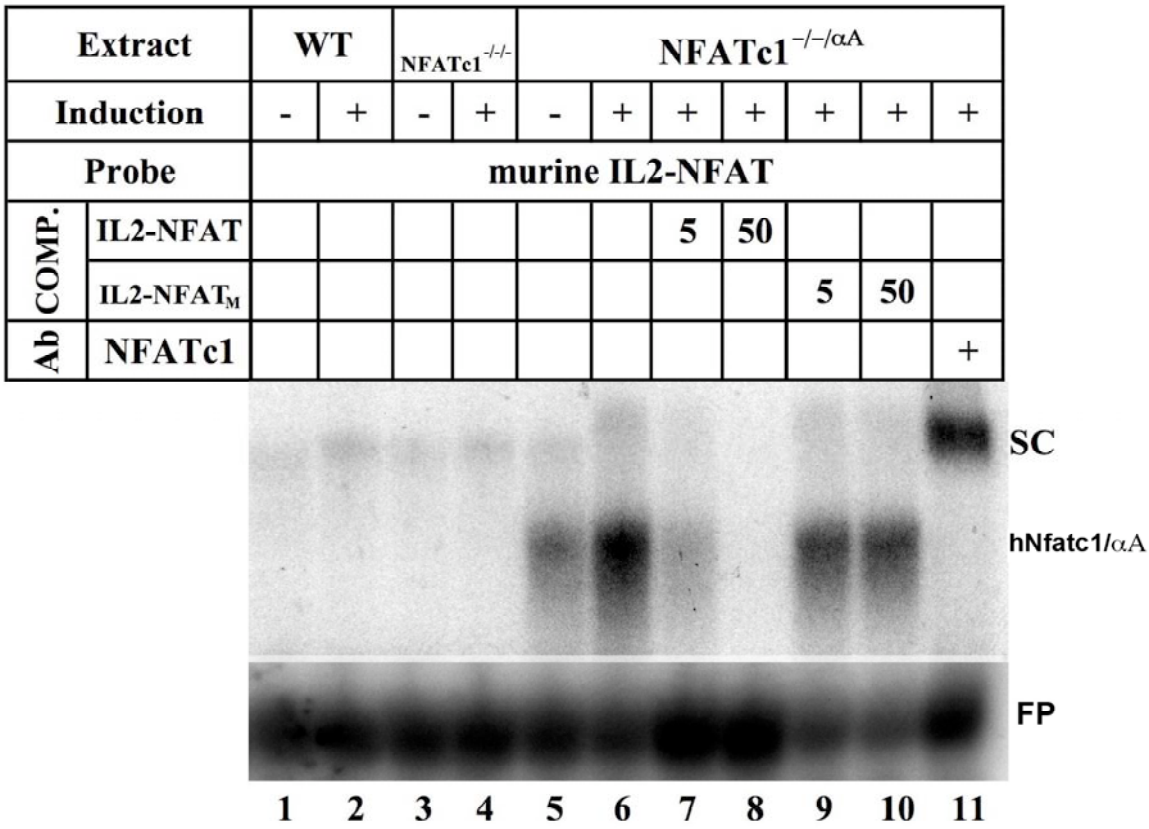


Figure 3-10 Binding of NFATc1/αA in nuclear proteins from *Nfatc1*$^{-/-/\alpha A}$ DT40 cells to the distal NFAT-site of the IL-2 promoter.

In lanes 5-11, protein extracts from DT40 B cells in which human NFATc1/αA was expressed. The cells in Lanes 6-11 were induced with TPA+ionomycin for 4 hrs while in lane 5 the cells remained untreated. To demonstrate NFATc1 in the major complex formed, a monoclonal antibody raised against NFATc1 was added to the EMSA assay. For competition, a five or fifty fold excess of unlabeled wild type (lanes 7 and 8) or mutated oligonucleotide (9 and 10) of murine IL2-NFAT/AP-1 site was added into the incubation mixture. Lanes 1-4: Controls of untransfected DT40 cells lacking the formation of NFATc1/αA complexes.

SC: super-shift complex; FP: free probe; IL2-NFAT$_M$: IL2-NFAT mutant.

DNA microarray studies have shown that in *NFATc1*$^{-/-/\alpha A}$ DT40 B cells expressing ectopically human NFATc1/αA the *pkc-θ* gene is several fold stronger expressed as in wild type cells (E. Kondo et al., unpublished). *PKC-θ* is crucial for the life and death of lymphocytes (Altman and Villalba, 2002; Krappmann et al., 2001), and its promoter is conserved during evolution. As shown in Figure 3-11-A, the *pkc-θ* promoter harbors a typical composite NFAT/AP-1 binding motif around nucleotide position -330. To test whether the *pkc-θ* promoter is really a target of NFATc1/αA *in vitro*, EMSA experiments

were done with nuclear protein from DT40 NFATc1$^{-/-/\alpha A}$ cells, and from EL-4 T cells as controls. Both types of cells were induced with T+I for 4 hrs. The conserved NFAT sequence motif of chicken *pkc-θ* promoter was labeled as probe. It formed one unique band (the first, faster running complex) with nuclear protein from DT40 NFAT$^{-/-/\alpha A}$ cells, while one additional band (the second complex) - in addition to the previous one - was found in EL-4 T cells. The first complex was super-shifted by use of a monoclonal Ab raised against NFATc1 (Figure 3-11-B, lane 4 and 5), or was disrupted by a polyclonal Ab against NFATc1 (Figure 3-11-B, lane 17). Moreover, it was disassembled by an excess of NFAT-binding oligonucleotides from either the IL-2 promoter (Figure 3-11-B, lanes 8 and 9), and the human (Figure 3-11-B, lanes 10 and11) or *chicken pkc-θ* promoters (Figure 3-11-B, lanes 12 and 13), but remained unchanged in competitions using mutated oligonucleotides of *chicken PKC-θ* promoter (Figure 3-11-B, lanes 14 and 15). In addition, it remained unaffected by unrelated Abs raised against Oct1 (Figure 3-11-B, lane 3) or RelA/p65 (Figure 3-11-B, lanes 6 and 7). All these findings showed that the protein of the complex corresponds to NFATc1/αA which bound to the promoter of pkc-θ (and IL-2). The protein of the second complex detected in EL-4 cell protein extracts corresponds to NFATc2 since this complex was found to be disrupted by a polyclonal Ab raised against NFATc2 (Figure 3-11-B, lane 18). So, our results demonstrate the binding of NFATc1/αA to the pkc-θ promoter *in vitro.*

To confirm that NFATc1/αA binds to the *pkc-θ* promoter *in vivo*, chromatin immuno-precipitation experiments (CHIP) were done with DT40 WT, NFATc1$^{-/-}$ and NFATc1$^{-/-/\alpha A}$ cells. Cross-linked and sheared chromatin from these cells was immunoprecipitated using a human NFATc1 Ab after induction of cells with T+I. Upon precipitation and DNA extraction, fragments of pkc-θ promoter DNA were amplified by PCR with specific primers recognizing the *pkc-θ* promoter. These PCR amplifications resulted in fragments of the expected

length of 320 bp (Figure 3-11-C). This demonstrates that human NFATc1/αA expressed in DT40 cells binds to the *PKC-θ* promoter *in vivo.*

Figure 3-11 The *pkc-θ* promoter is an NFATc1/αA target

(A) A highly conserved composite NFAT+AP-1 sequence motif is located within the promoter region of chicken and human *pkc-θ* genes. The core-sequences of NFAT and AP-1 binding motifs are depicted in bold face letters.

(B) Detection of NFATc1/αA binding to the NFAT site from the chicken *pkc-θ* promoter in EMSAs using nuclear protein extracts from NFATc1$^{-/-/\alpha A}$ DT40 B cells and EL-4 T cells induced by TPA+ionomycin for 4 hrs. An oligonucleotide probe of NFAT site (see Fig. 2-11-A) was incubated with nuclear proteins from NFATc1$^{-/-/\alpha A}$ DT40 B cells (lanes 2-15) or EL-4 cells (16-18) in the absence (lanes 2 and 16) or presence of Abs specific for Oct1 (lane 3), NFATc1 (lanes 4,5 and 17), NFATc2 (lane 18), p65/RelA (lane 6) or c-Rel (lane 7), or with a 5 (lanes 8, 10, 12 and 14) and 50 fold excess (lanes 9, 11, 13 and 15) of distal NFAT site of murine IL-2 promoter (lanes 8 and 9), of a WT NFAT+AP-1 site from the human *pkc-θ* promoter(lanes 10 and 11), or of a WT (lanes 12 and 13) or mutated version (lanes 14 and 15) NFAT site from the chicken *pkc-θ* promoter. In lane 1, the probe alone was fractionated.

(C) *In vivo* interaction of NFATc1/αA protein with the chicken *pkc-θ* promoter. ChIP assay of WT, NFATc1$^{-/-/-}$ or NFATc1$^{-/-/aA}$ DT-40 B cells treated with TPA (20 ng/ml) + ionomycin (0.5 µM) (T+I) for 4 hrs was done. Chromatin was immunoprecipitated by a polyclonal Ab raised against NFATc1. 10% of chromatin input was used as loading control (input). Unrelated Abs (GST 0.4ug/ul) immunoprecipitation was used as negative control. The predicted fragment of PCR should be 320bps. MM: molecular marker, 100bp ladder.

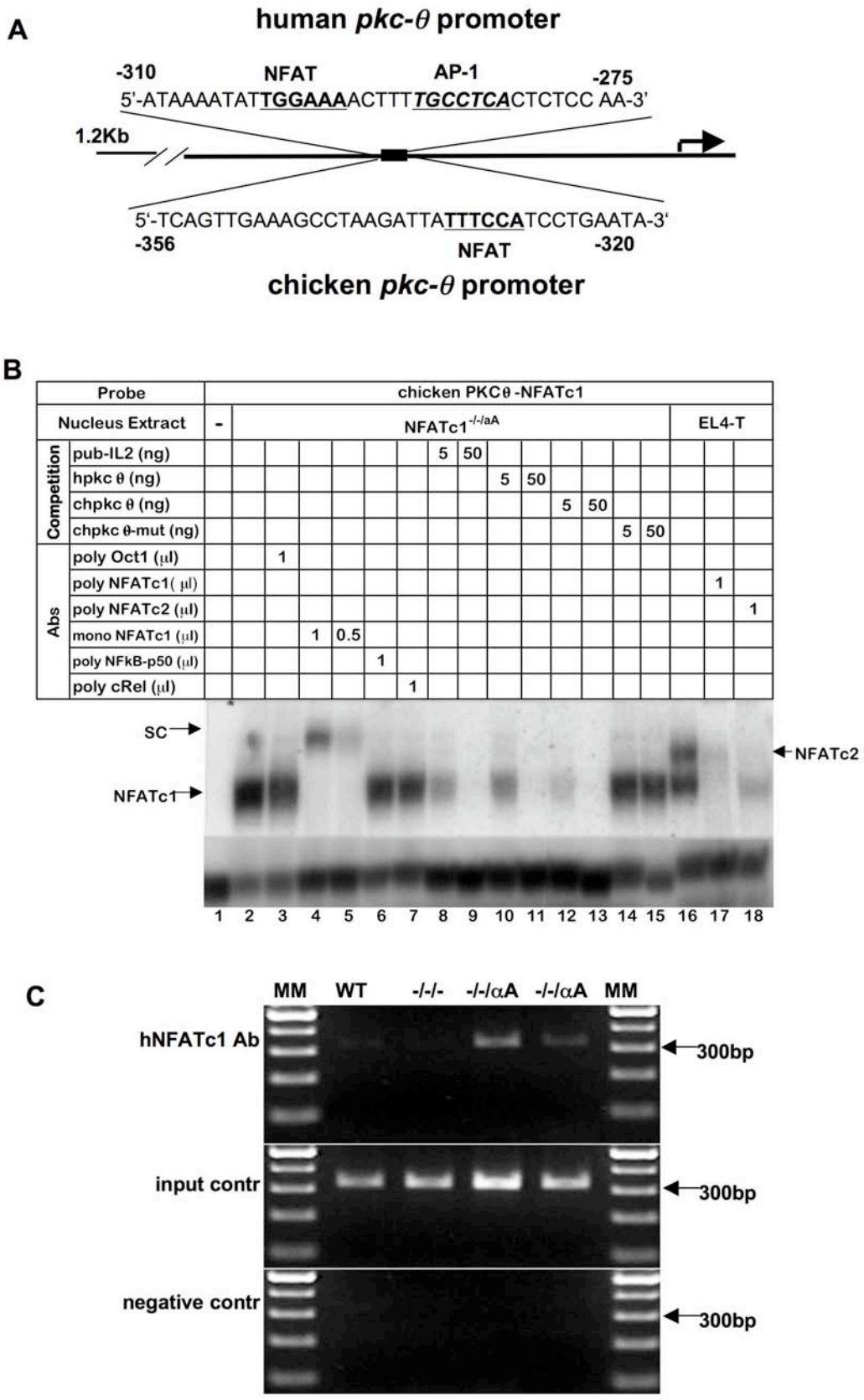

A
human pkc-θ promoter
-310
NFAT
AP-1
-275
5'-ATAAAATATTGGAAAACTTTTGCCTCACTCTCC AA-3'
1.2Kb
5'-TCAGTTGAAAGCCTAAGATTATTTCCATCCTGAATA-3'
-356
NFAT
-320
chicken pkc-θ promoter
B
Probe
chicken PKCθ -NFATc1
Nucleus Extract
-
NFATc1 -/-/aA
EL4-T
Competition
pub-IL2 (ng)
hpkc θ (ng)
chpkc θ (ng)
chpkc θ-mut (ng)
Abs
poly Oct1 (μl)
poly NFATc1(μl)
poly NFATc2 (μl)
mono NFATc1 (μl)
poly NFkB-p50 (μl)
poly cRel (μl)
SC
NFATc1
NFATc2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
MM
WT
-/-/-
-/-/αA
-/-/αA
MM
hNFATc1 Ab
input contr
negative contr
300bp
300bp
300bp

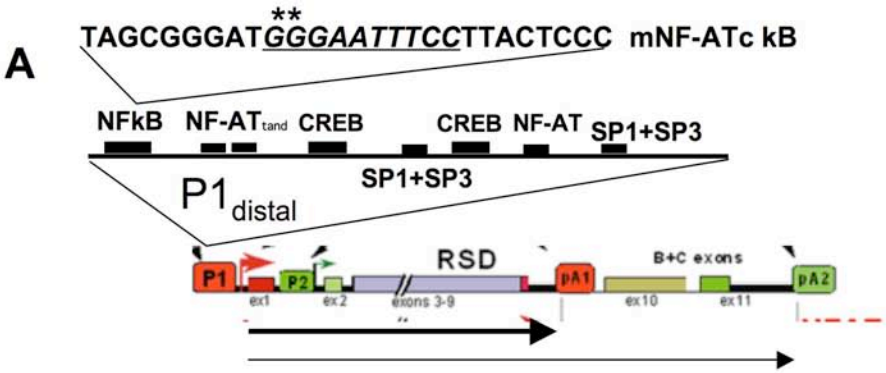
Figure 3-11

3.2.4 NFATc1/αA cooperates with NF-κB to regulate transcription of Nfatc1 in lymphocytes

Activation of NF-κB induced by T cell receptor costimulation is mediated by PKC-θ (Coudronniere et al., 2000). While PKC-θ is an NFATc1 target (shown in 3.2.3), we asked whether NF-κB is another target of NFATc1/αA. Or do these two transcription factors have reciprocal interactions? Several binding sites for transcription factors, as for NF-κB, NF-AT and CREB, are located within the NFATc1 promoter P1 (Figure 3-12-A)(Chuvpilo et al., 2002). To test whether NF-κB interacts indeed with the NFATc1 P1 promoter *in vitro*, EMSA experiments were done using nuclear extract from Jurkat T cells. The oligonucleotids named P1- κB from the *Nfatc1* promoter P1 and the one named Ig- κB corresponding to a consensus κB from the immunoglobulin kappa light chain gene enhancer, carrying binding sites for NF-κB protein, were used as probes. These probes formed complexes with nuclear protein of Jurkat cells, especially after induction of cells with T+I (Figure 3-12-B, C). T+I treatment of cells induces activation of *Nfatc1* gene directly. So, the increased activity of NF-κB was indicated by much stronger complex formation whether with P1-κB or with Ig-κB, as a consequence of induction of Nfatc1, suggesting a cross-talk between these two transcription factors. Such complexes were disassembled by competition with an excess of cold oligonucleotides of P1-κB or Ig-κB (Figures 2-12-B, C, lanes 8,9, 12 and 13), but not by mutated ones (Figures 2-12-B and C, lanes 10 and 11). Abs raised against NFATc1 and NFATc2 and oligonucleotides from the IL-2 promoter did not affect the complex formation. It is astonishing that Abs raised against NF-κB generated super-shift complex with the probe Ig-κB (Figure 3-12-C, lanes 16 and 17), whereas a very poor supershif was observed with the complex from the probe P1-kB (Figure 3-12-B, lanes 16 and 17). Taken together the reciprocal interaction between NFATc1/αA and NF-κB was demonstrated *in vitro*.

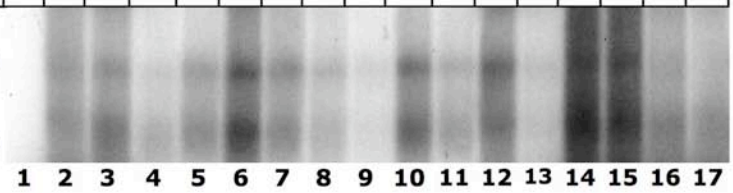
To confirm the binding of NF-κB protein to the *Nfatc1* promoter P1 *in vivo*, CHIP assays were performed 3 times (Figure 3-12-D). EL-4 cells remained

uninduced or were induced by TPA+ionomycin for 1 or 4 hrs. After shearing and immuno-precipitation with the Abs raised against NF-κB p50 or p65 the nuclear DNA was prepared. The bound DNA was amplified by primers matching the *Nfatc1* promoter P1. The length of the fragments of the *Nfatc1* promoter P1 from the PCR was consistent with the expected 500 bps, which indicates the two NF-κB proteins contribute to the complex formation. The binding activity of NF-κB p50 was stronger upon induction for 1 hr while the activity of p65 was stronger upon induction for 4 hrs. The negative control showed that no P1-DNA was detected in complexes immunoprecipitated by the unrelated Ab: GST. These data demonstrate that NF-κB can bind to the NFATc1 P1-promoter *in vivo*. They suggest and further prove that NF-κB contributes to the induction of the NFATc1 P1 promoter upon activation of T cells.



B

Nuclear Extract		-	Jurkat														
Induction			-	TPA+Ionomycin 4hrs													
Probe		mouse NFATc1-P1κB															
COMPETITION	Pu-b_d				5	50											
	Pu-b_d mut						5	50									
	P1-κB								5	50							
	P1-κB mut										5	50					
	Ig-κB												5	50			
Ab	NFATc														c1	c2	
	NF-κB																p50 p65



C

Nuclear Extract		-	Jurkat														
Induction			-	TPA+Ionomycin 4hrs													
Probe		mouse NFATc1-Igκ B															
COMPETITION	Pu-b_d				5	50											
	Pu-b_d mut						5	50									
	P1-κB								5	50							
	P1-κBmut										5	50					
	Ig-κB												5	50			
Ab	NFATc														c1	c2	
	NF-κB																p50 p65

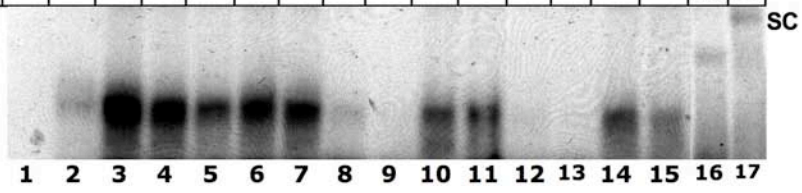


Figure 3-12-A, B, C

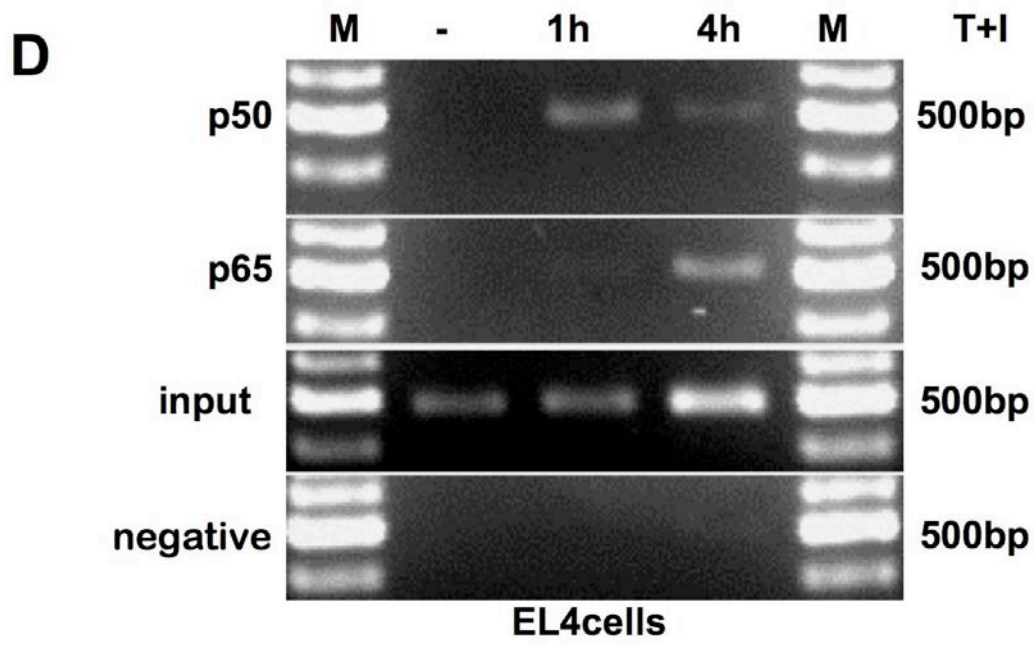


Figure 3-12 NF-κB factors contribute to induction of *Nfatc1* P1 promoter
(A) A highly conserved binding site and its sequence for the binding of NF-κB, followed by other transcription factor binding sites, in the Nfatc1 Promoter 1.
(B) Detection of NF-κB binding to the *Nfatc1* P1 promoter in induced or uninduced Jurkat T cells with EMSA and band-shift. There are two kinds complexes, of which the fast migrating may be from individual isoform protein of NF-κB while the slower one from isoform combination. The complexes were much stronger upon induction by TPA+ionomycin for 4 hrs (lane 3) than without induction (lane 2). Such complexes were disrupted by polyclonal Ab raised against NF–κB (Lanes 16 and 17), or by an excess of oligonucleotides of P1-κB (lanes 8 and 9) or Ig–κB (lanes 12 and 13), but remained unchanged when incubated with Abs against NFATc1 or NFATc 2, or with mutated P1-κB oligos (lanes 10 and 11), or with wild type (Pu-b_d, lanes 4 and 5) or mutated (Pu-b_d mut, lanes 6 and 7) oligonucleotides from the IL-2 promoter.
(C) Detection of NF-κB binding to immunoglobulin kappa light chain gene enhancer in induced or uninduced Jurkat T cells by EMSA. The result presented is similar to those in (B) except that when the Ab against NF-κB was applied, larger complexes were formed and super-shifted (Lane 16 and 17), and no double complexes.
(D) *In vivo* interaction of NF-κB protein with the murine *Nfatc1* Promoter P1. ChIP assay of EL-4 T cells treated with TPA (20 ng/ml) + ionomycin (0.5 μM) (T+I) for 4hrs. Chromatin was immuno-precipitated by antibodies raised against p50 & p65. The predicted fragment of PCR should be 500bps.
P1-κB: oligonucleotides from *Nfatc1* Promoter 1; Ig-κB: oligonucleotides from consensus immunoglobulin kappa light chain gene enhancer, both harboring binding sites for NF-κB protein

4. Discussion

The central position of NFATc1 as a signaling integrator (Macian, 2005; Serfling et al., 2007) in controlling the life and death of lymphocytes prompted us to explore the function of six individual NFATc1 isoforms which show distinct differences in their spatial and temporal expression in lymphocytes. We found that the short isoform NFATc1/αA can protect B cells against apoptosis upon stimulation of cells through the BCR complex. And signaling pathways controlling NFATc1/αA and/or NF-κB activation play an important role in apoptosis control. What are the molecular mechanisms controlling NFATc1/αA and/or NF-κB activation in delaying or preventing apoptosis?

4.1 The role of NFATc1 factors in the apoptosis induction of lymphocytes

4.1.1 NFATc1/αA protects lymphocytes against apoptosis

Previous studies have shown that NFAT factors contribute to survival of B cells (Fu et al., 2006), myocardiocytes (Pu et al., 2003), and osteoblasts (Van Sant et al., 2007). What are the mechanisms, which are controlled by the survival NFATc protein? Here, we have identified the short isoform NFAT1/αA as an anti-apoptotic factor in mature lymphocytes whereas the long isoform NFAT1/αC acts as a pro-apoptotic factor upon stimulation of BCR or TCR complexes (Figure 3-8). Upon stimulation of DT40 B cells with αIgM Ab, NFATc1/αA (and NFATc1/αC) was found to be translocated into the nucleus, thereby stimulating the expression of IL-2, a main factor of proliferation. However, numerous other genes are also activated by NFATc1/αA (and NFATc1/αC), including the Fas Ligand (FasL) gene. Previously, it has been shown that in mice double-deficient for NFATc2+c3, no FasL is formed upon stimulation, which might explain the strong defect in AICD induction of lymphocytes from such mice. These and further experimental data led us to conclude that – in contrast to NFATc1/αA - NFATc2+c3 exert a pro-apoptotic

function (Chuvpilo et al., 2002).

NFATc1 is a general integrator of signaling pathways and regulates the expression of many genes (Macian, 2005; Serfling et al., 2007), but individual NFATc1 isoforms appear to interact with different partners to initiate and to coordinate different gene expression programs. In normal B-cells, low grade malignant B lymphoma or B-cell non-Hodgkin lymphoma (NHL-B) cells, NFATc1 coordinates with NF-kB a B-lymphocyte stimulation (BLyS) pathway leading to survival and proliferation of B cells(Do et al., 2000; Fu et al., 2006). Two binding sites for NFAT and one for NF-kB factors were identified within the BLyS promoter. Abnormal BLyS signaling and NF-κB activity form a positive feedback loop which seems to be associated with lymphoma cell survival and proliferation (Fu et al., 2006). In cardiomyocytes, NFATc factors interact with numerous other transcription factors to balance between pro-apoptotic and anti-apoptotic activities initiated by calcineurin activation (Asada et al., 1998; Jayaraman and Marks, 2000; Lotem et al., 1999; Wang et al., 1999; Zhao et al., 1995). Such partners are AP-1, MEF2, and GATA4, each of which is integrated into its own cascades to which NFATc factors contribute (Pu et al., 2003).

In this study, we have identified NF-kB factors as “strong partners” for the NFATc1 short isoform NFATc1/αA upon induction of apoptosis in lymphocytes (Figure2-12). But for the H2O2-mediated apoptosis induction in DT40 B cells, the short isoform NFATc1/aA showed a pro-apoptotic activity while the long isoform NFATc1/aC acted in an anti-apoptotic manner. It is likely that differential factors and signaling pathways cause these surprising findings, such as FasL and MEK/ERK signaling (Devadas et al., 2002; Liu et al., 2006), protein kinase D (Zhang et al., 2005), the expression of the bcl-2 gene (Mao et al., 2001) and further events which might be needed to identify the synergy between partners of NFATc1 and corresponding signaling pathways. Our data appear to be consistent with published results that NFATc1 is a `moderate transcription factor` which needs other partners to facilitate its transcriptional function. Moreover, it is just this limitation of transcriptional capacity which leaves much

more `space` for other factors to join in and to lead to diverse consequences. So, it is not only one individual transcription factor to decide the final event but the net effect of involved transcription factors and other co-factors.

4.1.2 NFATc1/αA cooperates with NF-kB to determine the fate of lymphocytes upon stimulation through immunoreceptors.

NF-kB is a crucial transcription factor for lymphocyte activation, development, maturation, and apoptosis (Bours et al., 1992; Voll and Ghosh, 1999; Voll et al., 2000), and – if de-regulated – for lymphoma genesis (Patra et al., 2004). Upon stimulation of BCR or TCR complexes, the member of PKC family, PKCθ, is activated and then - via phosphorylation - stimulates IKK/NF-kB (Arendt et al., 2002) and IP3/Ca^{2+}/calcineurin/NFAT(Pfeifhofer et al., 2003) signaling pathways, i.e. two signaling pathways which were not known to "cooperate". Structurally, NF-κB and NFATc factors share homologous domains which are conserved between human and mouse, and – in the case of their similar DNA binding domains – lead to the binding to promoters of several common targeting genes. Thus, NFAT and NF-kB can recognize and control the expression of a set of target genes (Badran BM et al., 2002; Macian, 2005; Serfling et al., 2004). In this study, the PKCθ promoter was detected as a target of NFAT1/αA *in vitro* and *in vivo* (E. Kondo et al., unpublished). Thus, in addition to auto-regulation of NFATc1 gene (Asagiri et al., 2005; Chuvpilo et al., 2002), the PKCθ and NFAT1/αA form a positive feedback loop, and – as a result - the activation of NFATc1 becomes amplified. Consequently, NF-kB - as a putative downstream substrate of NFAT - is also amplified. In addition, activated calcineurin can also increase the activity of NF-κB via several other mechanisms, such as by its immediate effect on the phosphorylation and degration of IκBα, thereby leading to increased levels of active nuclear NF-κB (Jain et al., 1995). And in this study, the NFATc1 promoter was shown to harbor binding sites for NF-κB to which NF-kB can bind *in vitro* and *in vivo* (Figure 3-12). As a consequence, the transcriptional

activity of Nfatc*1* was up-regulated by NF-κB (E. Kondo et al., unpublished).

Interleukin 2 (IL-2) is an important lymphokine which is required for the processes of T cell activation, proliferation, clonal expansion and differentiation (Jain et al., 1995). Its promoter is one prominent target for NFAT transcription factors. The IL-2 promoter harbors two high-affinity and several low-affinity NFAT-binding sites, and several binding sites for NF-κB (Kang et al., 1992; Zhang and Nabel, 1994). In addition, several other factors, such as for AP-1, early growth response factors (EGR) and octamer binding factors (Oct) can bind to and control the IL-2 promoter (Macian, 2005; Serfling et al., 1995). These transcription factors can form composite complexes together with their targeting promoter sequence (Macian, 2005).

In summary, these two transcription factors, NFATs and NF-κBs, synergize via multiple interactions leading to the stimulation of early upstream initiator PKCθ and of downstream target gene promoters, such as the IL-2 promoter (Figure 4-1). But the direct reciprocal transcriptional up-regulation between NF-κB and NFATc1 remains to be elucidated. The collaboration between these two signaling pathways together drives cells to proliferate and to be resistant against AICD, which is in line with the NF-κB's pro-survivable properties (Serfling et al., 2004; Voll et al., 2000). We further speculate that upon stimulation, NFATc1, as a balancer between life and death of lymphocytes, can form and shape the platform for its partner, i.e. of other transcription factor(s) as executor(s). However, how these partners of NFATc1 are selected, how they control the decision between life or death of lymphocytes are unknown and deserve further experimental work.

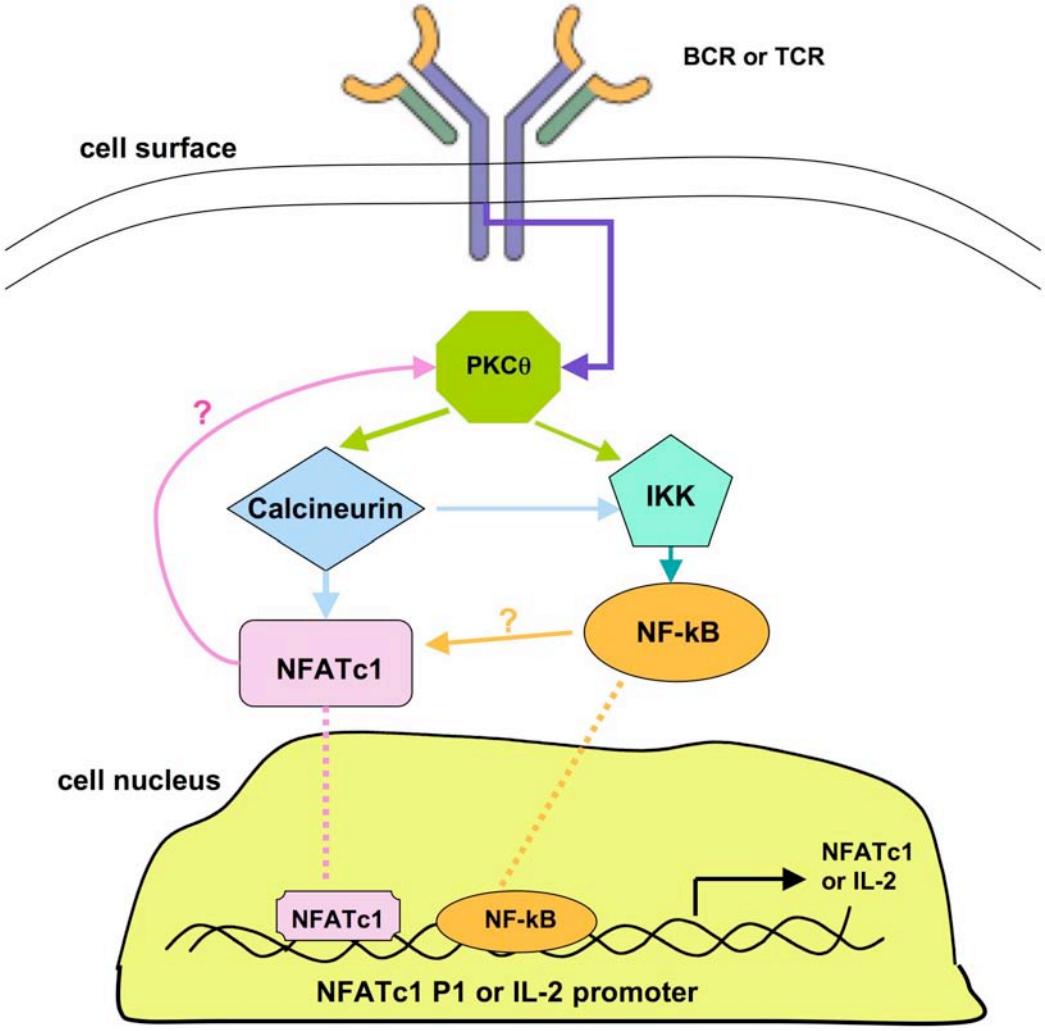


Figure 4-1 Nfatc1/αA cooperates with NF-κB to regulate gene expression in lymphocytes

4.2 Experimental models for the conditional disruption of the *Nfatc1* gene

To conditionally delete NFATc1 *in vivo*, we modified ES cells (Figure 3-5, 6) by electroporation with a targeting vector for the site-specific integration into the *Nfatc1* gene (Figure 3-4). The targeting vector was constructed by Dr. Jiming Liu using conventional DNA cloning strategies. In future, we will simplify and shorten the process of building targeting constructs by using a new strategy using ET recombination (Angrand et al., 1999). Such recombination ensures a rapid creation of vectors carrying mutations flanked by short synthesized homologous arms of the targeting gene and the corresponding BAC DNA in E. coli, thereby avoiding the tedious work of multiple rounds of digestion, ligation, transformation and DNA sequencing. In our targeting vectors, the P1 and P2 promoters of *Nfatc1* are flanked by loxp sites which allow the deletion of these DNA segments by Cre recombinase treatment *in vitro* and *in vivo* in the wild type locus of one allele (Fig 2-5 B and C). Unfortunately, in our experiments such a targeted Nfatc1 allele (Fig 2-6) could not be transmitted to the germ line. Currently, we do not know the reason for this failure. It could be due to the loss of pluripotency of ES cells which we used, or our handling of the ES cells. An emerging technique called laser-assisted injection may be a powerful solution to circumvent this dilemma (Poueymirou et al., 2007). It not only enforces the production of germ line transmission, but also makes possible early phenotype analysis with F0 chimeras which derived from ES cells with 90% germ line transmission.

Other choices also affect the germ line transmission. Thus, contaminations with bacteria, fungi, yeast or mycoplasma can also block germ line transmission (Cobo et al., 2007).

4.3 The significance of this study

In this study, the inducible short isoform of NFATc1, NFATc1/αA, was found to protect lymphocytes against apoptosis whereas the long isoform, NFATc1/αC, appears to facilitate apoptosis. NFATc1/αA and NF-κB were found to cross-talk in the transcriptional upregulation of their target genes, such as the IL-2 gene and – controled by a positive feed-back loop- the *Nfatc1* gene itself, at multiple steps upon induction of apoptosis. While the pro-apoptotic mechanism of NFATc1`s long isoform(s) remains unclear, its corresponding "death partners" are worth for further studies. From previous investigations, the transcription factor Egr might be one promising candidate of such "death partners" (Rengarajan et al., 2000).

Apoptosis determines the involved lymphocytes to live or to die. The elucidation of functional roles of NFATc1`s short or long isoforms in the control of apoptosis of lymphocytes helps to understand apoptosis regulation, and thereby, the fate of lymphocytes. This is a key mechanism for the generation of autoimmunity, immune deficiencies, leukemias and lymphomas, which affect millions of people all over the world. Moreover, the cross-talk pattern – i.e. how NFATc1`s isoforms and their partners synergize - also indicates a valuable approach to study how the individual NFATc1 isoforms (and other NFATc members) cross-talk with their transcriptional partners in other cells or organs at the molecular level, such as in cardiomyocytes during heart failure (Diedrichs et al., 2004; Phoon et al., 2004). Since the different NFATc1 isoforms have distinct functions, it will be necessary to develop individual Abs raised against these isoforms for basic research and, in a next step, for therapeutic purposes. So, the main finding of our work, i.e. the anti-apoptotic role of NFATc1/αA and its cross-talk with NF-κB, confirmed former studies of this laboratory and prompted us to go further**.**

Summary

Functional Role of NFATc1 in the Control of Life and Death of Lymphocytes

Wen Chen

B and T lymphocytes are the central cells of the adaptive immune system. Among the events of lymphocyte development, the expression of immune T and B cell receptors (TCR, BCR) and of their signal transduction components are crucially involved in the maturation of lymphocytes. Of the signaling pathways emerging from the TCR and BCR complexes, the NFAT and NF-kB signaling cascades upon apoptosis induction are in the centre of signaling events which direct lymphocyte fate. NFATs play diverse functions in apoptosis. We have previously observed that NFATc2 and c3 support the apoptosis of effector T cells, and, therefore, exert a pro-apoptotic function in T lymphocytes. In contrast, in these studies the function of NFATc1/αA remained unclear. In this study, murine ES cells and DT40 B cells were used in parallel to disrupt the *Nfatc1* gene and to study the function of individual 6 *Nfatc1* isoforms, especially the function of highly inducible NFATc1/αA.

We found that the short isoform NFATc1/αA protects DT40 B cells against apoptosis while the long isoform NFATc1/αC appears to enforce apoptosis. DNA microarray studies have shown that in *NFATc1*$^{-/-/\alpha A}$ DT40 B cells expressing ectopically human NFATc1/αA, the *pkc-θ* gene is several fold

stronger expressed as in wild type cells. Our results of EMSA (Electrophoretic Mobility Shift Assays) and ChIP (chromatin immuno-precipitation) experiments demonstrated the binding of NFATc1/αA to the pkc-θ promoter *in vitro and in vivo.* NF-κB was also found to bind to the NFATc1 P1-promoter *in vitro* and *in vivo*. These data suggest and further prove that NF-κB contributes to the induction of the NFATc1 P1 promoter upon activation of T cells. So, NFATc1/αA and NF-κB were found to cross-talk in the transcriptional upregulation of their target genes, such as the IL-2 gene and the *Nfatc1* gene itself, at multiple steps upon induction of apoptosis. While the pro-apoptotic mechanism of NFATc1`s long isoform(s) remains unclear, its corresponding "death partners" are worth further studies. The elucidation of functional roles of NFATc1`s short or long isoforms in the control of apoptosis of lymphocytes helps to understand apoptosis regulation, and thereby, the fate of lymphocytes.

Zusammenfassung

Die Funktionelle Rolle von NFATc1 in der Kontrolle von Leben und Tod von Lymphozyten

Wen Chen

Die B und T Lymphozyten sind von zentraler Bedeutung für das adaptive Immunsystem. Während der Lymphozytenentwicklung ist die Expression von T und B Zellrezeptoren (TCR, BCR) und ihrer Signalstransduktionskomponenten im Reifungsprozeß der Lymphozyten von entscheidender Bedeutung. Von den vom TCR und BCR Komplex ausgehenden Signalwegen sind die NFAT und die NF-κB Signalskaskaden, mit der Kontrolle der Apoptoseinduktion, zentrale Ereignisse des Lymphozytenschicksals. NFAT´s spielen unterschiedliche Rollen in der Apoptose. In vorausgehenden Experimenten konnten wir zeigen, daß NFATc2 und c3 die Apoptose von Effektor T Zellen unterstützen, und somit weisen sie eine pro-apoptotische Funktion in T Lymphozyten auf. Die Funktion von NFATc1/aA blieb jedoch ungeklärt. In der vorliegenden Studie wurden ES Zellen von der Maus und DT40 B Zellen vom Huhn verwendet, um paralell das *Nfatc1* auszuknocken und die Rolle der einzelnen 6 *Nfatc1* Isoformen zu studieren, insbesorndere die Funktion des stark induzierbaren NFATc1/αA.

Wir konnten feststellen, daß die kurze Isoform NFATc1/αA DT40 B Zellen gegen Apoptose schützt, während die lange Isoform NFATc1/αC scheinbar die Apoptose verstärkt. DNA Microarray Studien haben gezeigt, daß NFATc1$^{-/-/\alpha A}$ DT40 B Zellen, die humanes NFATc1/αA ektopisch exprimieren, das *pkc–θ* Gen deutlich stärker exprimieren als in Wildtyp Zellen. Unsere Ergebnisse von EMSA und ChIP Experimenten demonstrieren die Bindung von NFATc1/αA an den *pkc-θ* Promoter *in vitro* und *in vivo*. Für NF-κB wurde eine Bindung am *Nfatc1* P1 Promoter *in vitro* und *in vivo* gezeigt. Dies lässt vermuten, daß NF-

κB eine Rolle bei der Induktion am *Nfatc1* P1 Promoter nach der Aktivierung der T Zelle spielt.

Es wurde herausgefunden, daß nach Induktion der Apoptose, NFATc1/αA und NF-κB „cross-talk“ an unterschiedlichen Stellen in der transkriptionellen Hochregulierung ihrer Zielgene, wie z.b. dem IL-2 Gen und dem *Nfatc*1 Gen selbst. Weil die pro-apoptotischen Mechanismen der lange(n) Isoform(en) von NFATc1 unklar bleiben, sollten die korrespondierenden „death partners“ in weiteren Studien untersucht werden. Eine Klärung der funktionellen Rollen der NFATc1 Isoformen in der Kontrolle der Apotose in Lymphozyten wird helfen, die Regulation der Apotose, und damit auch das Schicksal der Lymphozyten, zu verstehen.

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Abbreviations

µg	microgram
µl	microliter
µm	micrometer
aa	amino acid
AID	auto-inhibitory domain
AP-1	activation protein-1
APS	ammonium persulfate
BSA	bovine serum albumin
CaM	calmodulin
CaN	calcineurin
CD	cluster of differentiation
ChIP	chromatin immuno-precipitation
CHX	cyclohexamide
CLP	common lymphoid precursor
CnA	calcineurin A
CnB	calcineurin B
CPM	counts per minute
CREB	cAMP response element binding
CsA	cyclosporin A
Ct	threshold cycle
DEPC	diethylpyrocarbonate
DKO	double knockout
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DP	double positive
DSBs	double strand breaks
ECL	enhanced chemical lumiscence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycotetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FKBP	FK506 binding protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK3	glycogen synthase-3 kinase
GST	glutathione-S-transferase
HGNC	the gene nomenclature committee of the human genome organization
HIV	human immunodeficiency virus

HPRT	hypoxanthine phosphoribosyltransferase
HSCs	hematopoietic stem cells
HUGO	the human genome organization
IL	interleukin
IMGT	immunogenetics
IPTG	isopropyl-ß-D-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
LT-HSCs	long-term HSCs
mA	milliampere
MAPK	mitogen-activated protein kinase
MEK	MAP kinase/ERK kinase
MEKK	MEK kinase
mg	milligram
MHC	major histocompatibility complex
mm	millimeter
mM	millimolar
MPP	multi-potent progenitor
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T cells
ng	nanogram
RR	NFAT regulatory region
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCI	phenol:chloroform:isoamyl alcohol
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
poly(dI·dC)	poly(deoxyinosine·deoxycytosine)
pre-TCR	pre-T cell receptor
qPCR	quantitative PCR
RNA	ribonucleic acid
rpm	revolution per minute
RSD	Rel/NF-κB similarity domain
RSSs	recombination signal sequences
RT	room temperature; reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SP	single positive
TAD	trans-activation domain
TBS	tris-buffered saline
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TPA	12-O-tetradecanoyl phorbol 13-acetate

Treg	regulatory T cell
WT	wild-type
ZAP-70	ζ-associated protein of 70 kD

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Dedicated

to

My Dear Father

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