The Role of NFAT Proteins in

Rag and *NFATc1a* Regulation in Murine Thymus

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Shaoxian Yang

Toronto, Canada

Würzburg, Germany

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Submitted on: _____

Members of the thesis committee:

Chairman:

Examiner: Professor Dr. Edgar Serfling

Examiner: Professor Dr. Erich Buchner

Date of oral examination: _____

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Shaoxian Yang

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To My Dear Mother

In Memory of My Late Father

Table of Contents

1. INTRODUCTION

1.1 Overview of T lymphocyte development in murine thymus

1.1.1 T lymphocytes are derived from blood stem cells

It is well established that in adult, bone marrow hematopoietic stem cells (HSCs) give rise to all types of blood cells including lymphocytes (Kondo et al., 2003). There are three types of HSCs, i.e. long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multi-potent progenitors (MPPs) including common lymphoid precursors (CLPs). LT-HSCs can self-renew virtually for life and can differentiate into ST-HSCs. ST-HSCs have limited self-renewal capacity and have the ability to develop into MPPs. MPPs including CLPs have no self-renewal capacity (Shizuru et al., 2005). CLPs differentiate into T lymphocytes, B lymphocytes, or NK cells depending on the physiological sites where the differentiation occurs (Kondo et al., 1997). CLPs migrate through the bloodstream and seed into the thymus where they eventually differentiate into T lymphocytes (Petrie and Kincade, 2005).

1.1.2 T lymphocytes undergo stepwise development in the thymus

The blood-born CLPs enter the thymus at the cortical medullary junction (CMJ). In the thymus, CLPs develop successively from the double negative (DN) stage through the double positive (DP) stage to the single positive (SP) stage. DN thymocytes express neither CD4 nor CD8 and also undergo stepwise development. CLP-derived DN1 thymocytes ($CD44^+CD25^-$) differentiate into DN2 thymocytes ($CD44^+CD25^+$) which give rise to DN3 thymocytes ($CD44^-CD25^+$). DN3 thymocytes that express pre-T cell

receptors (pre-TCR) will further mature into DN4 thymocytes ($CD44^{-}CD25^{-}$). DN4 thymocytes subsequently differentiate into double positive (DP) thymocytes which express both CD4 and CD8 ($CD4^{+}CD8^{+}$). DP thymocytes bearing T cell receptors (TCR) with low affinity to self MHC-peptide complexes are then matured into either CD4-expressing single positive ($CD4^{+}SP$) or CD8-expressing single positive ($CD8^{+}SP$) thymocytes (Godfrey et al., 1993; Petrie et al., 1990; Taghon et al., 2006; Werlen et al., 2003) (Figure 1.1).

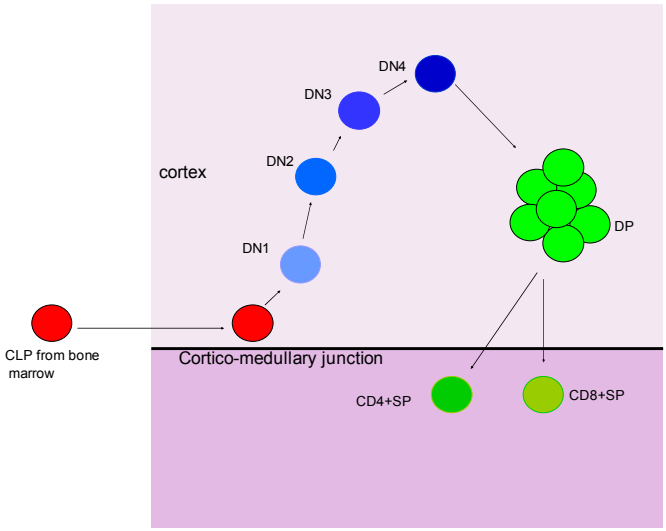

Figure 1.1. T cell development in murine thymus. Blood-born common lymphoid precursors (CLPs) enter the thymus at the cortical-medullary junction (CMJ). In the cortex, CLPs develop successively into double negative 1 (DN1), DN2, DN3, and DN4 cells. DN4 cells then mature into double positive (DP) cells. DP cells migrate into medulla and mature into $CD4^{+}$ and $CD8^{+}$ single positive (SP) cells.

1.2 T cell receptor assembly is essential for thymocyte development

1.2.1 Genes encoding T cell receptors

The pre-TCR consists of a TCRβ chain and an invariant pre-TCRα chain (pTα). The TCR consists of a TCRβ chain and a TCRα chain. There are one variable region (V), one

joining region (J), and one constant region (C) in both TCRβ and TCRα chains (Chien et al., 1984; Hedrick et al., 1984). The V and J regions of TCRβ are encoded by three gene segments, i.e. variable (*V*), diversity (*D*), and joining (*J*) whereas the V and J regions of TCRα are encoded by *V* and *J* segments. The C region of both TCRβ and TCRα is encoded by a single constant (*C*) gene (Davis and Bjorkman, 1988).

The TCRβ genes are organized into the TCRB locus which contains one TCRBV cluster and two DJC clusters. The TCRBV cluster contains 25 *Vβ* segments. Each of the two DJC clusters contains one *Dβ* gene, 6 *Jβ* genes, and one *Cβ* gene (immunogenetics (IMGT) website). Similarly, the TCRα genes are also organized into the TCRA locus which contains one TCRAV cluster and one TCRAJC cluster. The TCRAV cluster contains 100 *Vα* segments while the TCRAJC cluster contains 61 *Jα* segments and one *Cα* gene (IMGT website). All these gene segments are scattered over large tracts of chromosomal DNA (IMGT website).

1.2.2 V(D)J recombination assembles TCR gene segments into functional units

1.2.2.1 Recombination signal sequences (RSSs)

The scattered TCR gene segments are ligated together to form functional genes through a process called V(D)J recombination (Tonegawa, 1983). V(D)J recombination is mediated by recombination signal sequences (RSSs) which flank *V*, *D*, and *J* segments (Tonegawa, 1983). RSSs consist of a nonamer and a heptamer separated by a spacer. The spacer is either a 12 bp (12-RSS) or a 23 bp (23-RSS) non-conserved DNA sequence (Tonegawa, 1983). *V* segments are armed with 23-RSS whereas *J* segments are equipped with 12-RSS.

D segments are sandwiched by both 12-RSS and 23-RSS (Jung and Alt, 2004). V(D)J recombination takes place only between one segment flanked with 23-RSS and one flanked with 12-RSS, a phenomenon called the 12/23 rule (Eastman et al., 1996; Tonegawa, 1983; van Gent et al., 1996).

1.2.2.2 Mechanism of V(D)J recombination

To start V(D)J recombination, one gene segment with 12-RSS and one with 23-RSS are aligned together to form a synapsis. The double strand breaks (DSBs) are then generated between RSS and the coding segments by nicking and hairpin formation steps. The length of the generated coding ends are randomly modified and ligated together by the non-homologous end joining (NHEJ) system. The generated RSS ends are ligated without modifications (Agrawal and Schatz, 1997; Gellert, 2002; Schlissel, 2002) (Figure 1.2). In the TCRB locus, VDJ recombination takes place in DN3 thymocytes through two recombination reactions. The initial recombination is between *Dβ* segments and *Jβ* segments through 23-RSS of a *Dβ* segment and 12-RSS of a *Jβ* segment. Subsequent recombination is between *Vβ* segments and the recombined *DβJβ* fragments through 23-RSS of a *Vβ* segment and the 12-RSS of a *DβJβ* fragment (Jackson and Krangel, 2006). VDJ recombination in the TCRB locus generates a complete coding gene for TCRβ V, D, and J regions. In the TCRA locus, the recombination takes place in DP thymocytes by a one-step reaction. The recombination is between *Vα* segments and *Jα* segments through 23-RSS of a *Vα* segment and 12-RSS of a *Jα* segment (Krangel et al., 2004). VJ recombination in the TCRA locus gives rise to a complete coding gene for TCRα V and J regions.

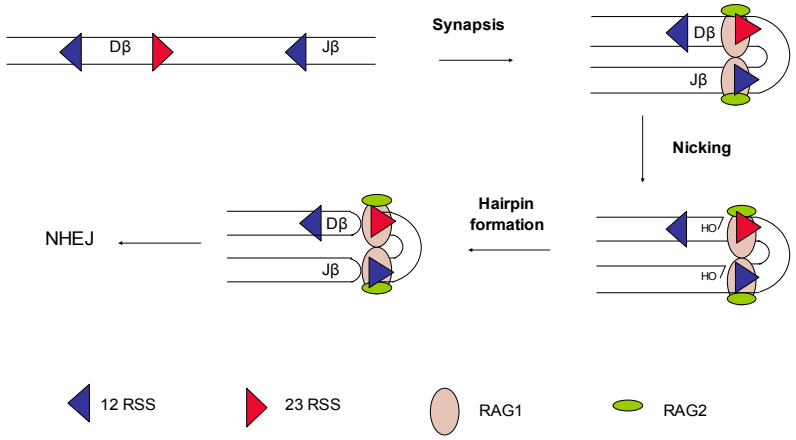

Figure 1.2 Mechanism of V(D)J rearrangement. One Dβ fragment with 23-RSS and one Jβ fragment with 12-RSS form synapsis on RAG1/2 tetramer. For cleavage, a nick is made at the 5' end of RSS, leaving a 3' hydroxyl (OH) on the coding end. Then, the OH group attacks and breaks the opposite DNA strand to produce a hairpin at the coding end, and a blunt at the RSS end. The non-homologous end joining (NHEJ) repair system is recruited to the coding end to complete the NHEJ reaction. The RSS end is rejoined by ligation without modification.

1.2.3 TCRα and TCRβ are essential for T cell development in the thymus

The importance of TCR genes in T cell development is revealed by targeted inactivation of the genes in the mouse germ line. In these genetically engineered mice, thymocyte development is blocked at defined stages (Mombaerts et al., 1992a). In *Tcr*α deficient mice, no mature T lymphocytes are detected in peripheral lymphoid organs, and SP thymocytes are disappeared from the thymus, indicating that the TCRα is essential for DP thymocytes to mature into SP thymocytes (Mombaerts et al., 1992a). In mice with inactivated TCRβ, there are no mature T lymphocytes in the periphery and no DP thymocytes in the thymus. In these mice, the thymocyte development is arrested at the DN3 stage, indicating that the TCRβ is required for DN3 thymocytes to develop into

DN4 thymocytes (Mombaerts et al., 1992a). These results demonstrate that the TCRα and TCRβ play critical roles in T lymphocyte development.

1.3 RAG1 and RAG2 are essential for T cell receptor assembly

1.3.1 Functional domains of RAG1 and RAG2

Both the recombination activating gene 1 (*Rag1*) and recombination activating gene 2 (*Rag2*) are expressed in the thymus with higher expression levels in DN3 thymocytes and DP thymocytes (Wilson et al., 1994). RAG1 is a protein of 1040 amino acid residues and RAG2 is a polypeptide of 527 amino acid residues. Both RAG1 and RAG2 have V(D)J recombinase activity (Oettinger et al., 1990; Schatz et al., 1989). Core RAG1 (residues 384 – 1008) and core RAG2 (residues 1 – 387) are sufficient to cleave double strand DNA (Cuomo and Oettinger, 1994; Sadofsky et al., 1994a; Sadofsky et al., 1993; Sadofsky et al., 1994b). The N-terminal portion of core RAG1 is a nonamer DNA binding region. The central domain of core RAG1 interacts with both heptamer motifs and RAG2. The C-terminal part of the core RAG1 is mainly a self-dimerization domain and part of the core RAG1 active site (De and Rodgers, 2004; Difilippantonio et al., 1996). Core RAG1 contains the active site for the double strand DNA cleavage reaction (Landree et al., 1999). In addition, core RAG1 also participates in hairpin formation (Lu et al., 2006). RAG2 may not participate in the catalytic reaction but plays a regulatory role in V(D)J recombination (De and Rodgers, 2004).

1.3.2 RAG1 and RAG2 initiate V(D)J recombination

RAG1 initiates V(D)J recombination by recognizing and binding to nonamer and heptamer of RSS through its nonamer and heptamer binding sites. Importantly, RAG1 recruits RAG2 to form a stable RAG1-RAG2-RSS complex (Difilippantonio et al., 1996; Hiom and Gellert, 1997) where the RAG1/2 tetramer simultaneously binds to one 12-RSS and one 23-RSS to form a synapsis (Eastman et al., 1996; Mo et al., 1999; van Gent et al., 1996) (Figure 1.2). RAG1 and RAG2 cleave double strand DNA by catalysing hydrolysis and trans-esterification reactions. In the hydrolysis reaction, a nick is made at the 5' end of RSS, leaving a 3' hydroxyl (OH) on the coding end. In the subsequent trans-esterification reaction, the OH group attacks and breaks the opposite DNA strand to produce a hairpin at the coding end and a blunt at the RSS end (McBlane et al., 1995) (Figure 1.2). The DNA double strand break (DSB) generated by RAG1/2 is repaired by the non-homologous end joining (NHEJ) system (Jung and Alt, 2004) which completes the V(D)J recombination reaction.

1.3.3 RAG1 and RAG2 are essential for V(D)J recombination

Both RAG1 and RAG2 play an important role in T cell development as revealed by the gene targeting technology. In *Rag1* or *Rag2* deficient mice, there are no mature lymphocytes in peripheral lymphoid organs, no DP and SP thymocytes in the thymus, and thymocyte development is arrested at the DN3 stage. These phenotypes resemble those observed in *Tcrβ* deficient mice (Mombaerts et al., 1992a). Moreover, there is no TCRβ expression and no V(D)J recombination detected in DN thymocytes (Mombaerts et al., 1992b; Shinkai et al., 1992). These results show that both RAG1 and RAG2 are

required for V(D)J recombination, and that V(D)J recombination is indispensable for TCR assembly.

1.4 NFAT family

1.4.1 Nomenclature of NFAT family members

Nuclear factor of activated T cells (NFAT) family consists of NFATc1 (Northrop et al., 1994; Park et al., 1996), NFATc2 (Hoey et al., 1995; McCaffrey et al., 1993), NFATc3 (Ho et al., 1995; Masuda et al., 1995), NFATc4 (Graef et al., 2001; Hoey et al., 1995), and NFAT5 (López-Rodriguez et al., 1999). The nomenclature of NFAT family recommended by The Gene Nomenclature Committee (HGNC) of The Human Genome Organization (HUGO) is listed in table 1.1 (HUGO website).

Table 1.1 Recommended Nomenclature of NFAT Family

Approved Symbol	Approved Name	Aliases
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	TONEBP, KIAA0827, NFATL1, OREBP, NFATZ, NF-AT5
NFATc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	NF-ATC, NFATc
NFATc2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	NF-ATP, NFATp
NFATc3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	NFAT4, NFATX
NFATc4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	NFAT3

1.4.2 Functions of NFATs in the immune system *in vivo*

NFATc1, NFATc2, and NFATc3 are expressed in the immune system where they are involved in the development of immune system and the regulation of immune responses (Serfling et al., 2000). NFATc1 is involved in regulating lymphocyte proliferation and Th2 cytokine production (Ranger et al., 1998a; Yoshida et al., 1998); NFATc2 overlaps in function with NFATc1 but is also a negative regulator of immune responses (Hodge et al., 1996; Xanthoudakis et al., 1996); NFATc3 plays a role in mouse thymocyte generation and survival (Oukka et al., 1998). Moreover, interaction between NFAT members has a more profound effect on immune responses than the individual NFAT protein does. For example, NFATc1 and NFATc2 together are essential for Th1 and Th2 lymphokine production (Peng et al., 2001) while NFATc2 and NFATc3 together are involved in feedback inhibition of Th2 cytokine production (Ranger et al., 1998b). IL-2 production is a good example to demonstrate the role of NFAT member cooperation in gene regulation. The IL-2 production is not affected in T cells with a single NFAT member deficiency (Oukka et al., 1998; Ranger et al., 1998a; Xanthoudakis et al., 1996; Yoshida et al., 1998). However, the IL-2 production is reduced in T lymphocytes with *Nfatc2* and *Nfatc3* double deficiency, and the IL-2 production is ablated in T lymphocytes with *Nfatc1* and *Nfatc2* double deficiency (Peng et al., 2001; Ranger et al., 1998b), demonstrating the complexity of the NFAT network *in vivo*.

1.4.3 Functional domains of NFATc proteins

The NFAT family shares a Rel/NF-κB similarity domain (RSD) (Ho et al., 1995; López-Rodriguez et al., 1999; Northrop et al., 1994; Serfling et al., 2004). The RSD is about 300

amino acid residues in length with its N-terminus forming a DNA binding loop and C-terminus as a protein interaction domain (Giffin et al., 2003; Jain et al., 1995; Jin et al., 2003; Wolfe et al., 1997). Sequence homology of RSD among NFATc1, NFATc2, NFATc3, and NFATc4 is about 70% while sequence homology of RSD between these four NFAT members and NFAT5 is about 40% (López-Rodriguez et al., 1999). The structure of RSD is highly conserved between NFAT and NF-κB despite the fact that the sequence homology between them is less than 20% (Northrop et al., 1994; Wolfe et al., 1997). The NFAT regulatory region (RR) is next to the N-terminus of RSD (Ho et al., 1995; Luo et al., 1996c; Masuda et al., 1995). The RR of NFATc1-c4 is rich in serine/threonine residues and contains 3 SP motifs SPXXSPXXSPXXXX(D/E)/(D/E)

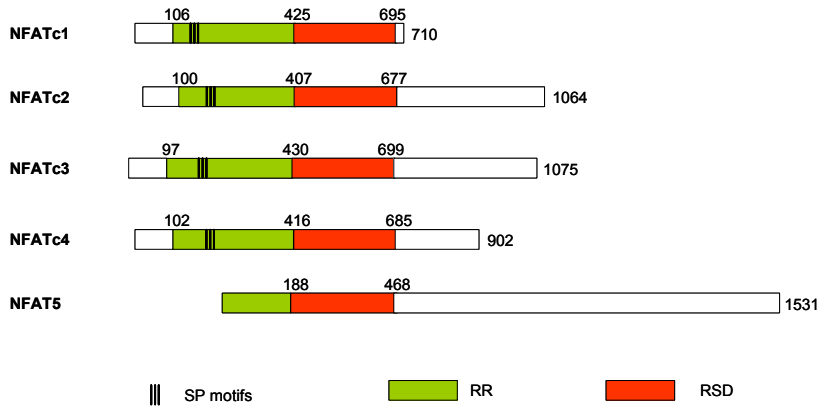

Figure 1.3 Alignment of NFAT family members. NFAT family members have a conserved Rel similarity domain (RSD, red) and a NFAT regulatory region (RR, green). RR of NFATc1-c4 contains three SP motifs (|||) which are absent from NFAT5. Numbers indicate the positions of amino acid residues.

(Ho et al., 1995; Masuda et al., 1995) and 2 SRR motifs (Serfling et al., 2006a). These SP motifs are absent from NFAT5 (López-Rodriguez et al., 1999). RR also contains two calcineurin binding sites that mediate NFAT-calcineurin interactions (Garcia-Cozar et al.,

1998; Shibasaki et al., 2002), two nuclear localization signals (NLS) and two nuclear export signals which mediate NFAT nuclear import and export, respectively (Beals et al., 1997a; Luo et al., 1996c; Zhu and McKeon, 1999; Zhu et al., 1998) (Figure 1.5). In addition, there are trans-activation domains (TADs) at the both N-terminus and C-terminus of NFATc1 and NFATc2. However, these TADs share low degree sequence homology (Chuvpilo et al., 1999; Ho et al., 1995; Luo et al., 1996b; Masuda et al., 1995).

1.4.4 The calcineurin-NFAT signaling pathway

1.4.4.1 Functional domains of calcineurin

Calcineurin (CaN) is a Ca++/calmodulin (CaM) dependent serine/threonine protein phosphatase. Calcineurin is a hetero-dimer consisting of a large catalytic subunit calcineurin A (CnA) and a small calcium-binding regulatory subunit calcineurin B (CnB) (Klee et al., 1998). CnA has a catalytic domain at its N-terminal part and a regulatory domain at its C-terminal part. The regulatory domain consists of a CnB binding subdomain, a CaM binding subdomain, and an auto-inhibitory subdomain (AID) (Hubbard and Klee, 1989).

1.4.4.2 Activation of calcineurin

The AID inhibits catalytic activity of CnA in resting T cells (Hubbard and Klee, 1989). In activated T cells, TCR signaling leads to a release of calcium from the endoplasmic reticulum (Berridge, 1993; Clapham, 1995; Lewis, 2001). The elevated intracellular calcium concentration activates CaM. Subsequently, the activated CaM activates

calcineurin by binding to the CaM binding domain and dissociating the catalytic domain from AID (Ke and Huai, 2003; Klee et al., 1998; Wilkins and Molkentin, 2004).

1.4.4.3 Activation of NFAT factors

1.4.4.3.1 Nuclear translocation is essential for NFAT activation

In T lymphocytes, NFAT is a major enzymatic substrate for calcineurin (Bueno et al., 2002; Chan et al., 2002; Flanagan et al., 1991; Jain et al., 1993; Luo et al., 1996a; Neilson et al., 2004). In resting T cells, NFAT is phosphorylated in an inactive state and located in the cytoplasm. In activated T cells, TCR signaling leads to activation of calcineurin. The activated calcineurin activates NFAT by binding to RR (Beals et al., 1997a; Jain et al., 1993; Loh et al., 1996; Masuda et al., 1997; Shibasaki et al., 2002) and dephosphorylating the phosphoserine residues of RR (Okamura et al., 2000). The dephosphorylation of RR leads to unmasking of NFAT's nuclear import signals and masking of its nuclear export signals which, in turn, leads to NFAT nuclear translocation (Beals et al., 1997; Zhu et al., 1998; Zhu and Mckeon, 1999; Okamura et al., 2000). Persistent TCR signaling is required to maintain calcineurin activity and, therefore, NFAT nuclear localization and activation (Dolmetsch et al., 1997; Dolmetsch et al., 1998; McCaffrey et al., 1993; Northrop et al., 1994; Timmerman et al., 1996).

1.4.4.3.2 MAP kinase signaling pathway is required for NFAT activation

Nuclear localization by itself is essential but not sufficient to activate NFAT. Both calcium and phorbol ester stimuli are required for optimal transactivation activity of NFAT (Amasaki et al., 2002; Garcia-Rodriguez and Rao, 2000; Monticelli and Rao, 2002;

Okamura et al., 2000). Since phorbol ester can activate Ras/Raf/MAPK signaling while calcium activates calcineurin, it has been proposed that Ras/Raf/MAPK signaling pathway and calcineurin synergize to activate NFAT by phosphorylating its trans-activation domain (Avots et al., 1999; Okamura et al., 2000; Round et al., 2007; Woodrow et al., 1993a; Woodrow et al., 1993b).

1.4.4.4 Factors inhibiting NFAT activation

NFAT nuclear localization and activation is counteracted by protein kinases including the casein kinase I, MEKK1, JNK2, p38, GSK3β, and DYRK (Arron et al., 2006; Beals et al., 1997b; Chow and Davis, 2000; Chow et al., 1997; Gwack et al., 2006; Round et al., 2007; Wu et al., 2003; Yang et al., 2002; Zhu et al., 1998). Among these kinases, DYRK plays a more important role in inhibiting NFAT activity because DYRK directly phosphorylates the SP motifs of NFAT and because DYRK primed pre-phosphorylation is required for subsequent phosphorylation of NFAT by GSK3β and casein kinase I (Gwack et al., 2006).

Besides the inhibitory kinases, NFAT activity is inhibited by a SPRIEIT peptide which disrupts calcineurin-NFAT complex formation by competitively binding to calcineurin (Aramburu et al., 1998; Aramburu et al., 1999). NFAT activity is also blocked by cyclosporin A (CsA) or FK506 (Clipstone and Crabtree, 1992; Flanagan et al., 1991) via a different mechanism. CsA forms complexes with cyclophilin (cyclophilin-CsA) while FK506 forms complexes with FKBP (FKBP-FK506). Cyclophilin-CsA and FKBP-FK506 complexes disrupt CnA–CnB dimer formation by competitively occupying the

CnA–CnB interaction interface, respectively (Liu et al., 1991). In addition, NFAT activity is inhibited by an endogenous calcineurin inhibitor Carabin which mediates a negative feedback of TCR signaling (Pan et al., 2007).

1.4.5 Target gene regulation by NFAT factors

1.4.5.1 NFATs mediate immune responses in cooperation with AP-1

NFATs regulate the transcriptional activity of genes by binding to NFAT-binding sites (Rao et al., 1997; Serfling et al., 2000). In most cases, NFAT-dependent genes contain composite NFAT-AP-1 sites consisting of an activation protein-1 (AP-1) binding site adjacent to 3' of a NFAT binding site (Rao et al., 1997). AP-1, a transcription factor consisting of a Fos-Jun dimer, binds to AP-1 site in its target genes (Chiu et al., 1988; Sassone-Corsi et al., 1988; Schuermann et al., 1989). In activated T cells, TCR signaling activates both NFAT and AP-1 which subsequently form NFAT-AP-1 complexes on composite NFAT-AP-1 sites. This NFAT-AP-1-DNA interaction leads to the activation of their target genes (Jain et al., 1993; Jain et al., 1992) (Figure 1.4A).

1.4.5.2 NFATs induce immune anergy without cooperation with AP-1

Mutation of AP-1 interacting residues in NFAT disrupts NFAT-AP-1 complex formation without affecting the DNA binding activity of NFAT (Macián et al., 2000). However, without AP-1 cooperation, binding of NFAT to the NFAT-AP-1 dependent genes doesn't lead to the activation of those genes, instead it induces anergy-related gene expression (Macián et al., 2002; Macián et al., 2000) (Figure 1.4C).

1.4.5.3 NFATs suppress immune responses in cooperation with FOXP3

FOXP3 is a transcription factor which is expressed in regulatory T cells (Tregs). Tregs repress lymphokine production and cell proliferation of CD4+ T cells (Bettelli et al., 2005; Schubert et al., 2001). Similarly, NFAT is also involved in inhibition of immune responses (Xanthoudakis et al., 1996; Hodge et al., 1996; Ranger et al., 1998, Bettelli et al., 2005). NFAT suppresses NFAT-dependent gene activity by forming NFAT-FOXP3 hetero-dimers on composite NFAT-AP-1 sites. In this NFAT-FOXP3-DNA complex, the AP-1 site is occupied by FOXP3 rather than by AP-1 (Wu et al., 2006) (Figure 1.4B). Disruption of FOXP3-NFAT complex formation impaired the function of Tregs (Wu et al., 2006), indicating the importance of interaction between NFAT and FOXP3 in controlling Treg cell activity.

In short, NFATs regulate gene activity by cooperation with other proteins. NFATs activate immune responses in cooperation with AP-1, whereas NFATs suppress immune responses in cooperation with FOXP3, and NFATs induce immune anergy if the proteins described above are unavailable for cooperation. However, the signals that are responsible for these different reactions are not known yet (Rudensky et al., 2006; Serfling et al., 2006b).

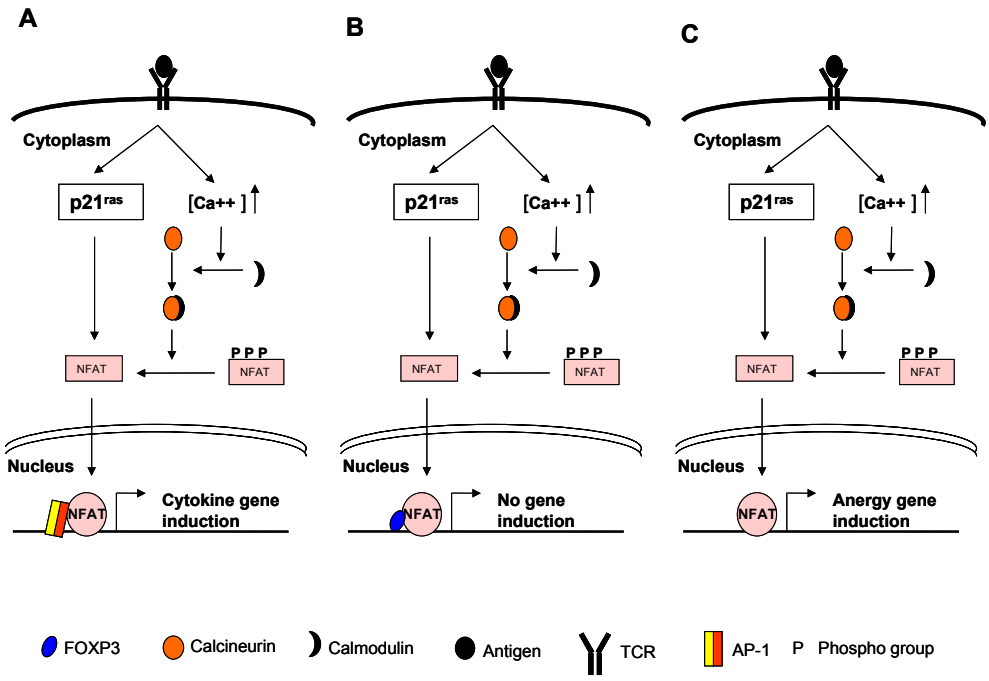

Figure 1.4 NFAT regulates its target gene expression. TCR engagement elevates intracellular calcium concentration which activates calmodulin. The activated calmodulin binds and activated calcineurin which, in turn, binds and dephosphorylates NFAT. The dephosphorylated NFAT is then transported into the nucleus where it regulates its target gene expression together with its DNA binding partners. (A) NFAT-AP-1 interaction on target gene *cis* regulatory elements leads to gene activation while (B) NFAT-FOXP3 interaction on the same *cis* elements represses gene activity. (C) When DNA binding partner is not available, interaction of NFAT with target gene *cis* regulatory elements induces an anergy response.

1.5 Aims of this study

NFATc1, NFATc2, and NFATc3 are expressed in DN thymocytes but the role of pre-TCR signaling in NFAT gene regulation is unknown (Oukka et al., 1998). During DP to SP development, the expression of NFATc1 and NFATc2 is up-regulated whereas the NFATc3 gene expression is down-regulated. However, the signaling events that are responsible for regulating the NFAT gene expression are not clear (Oukka et al., 1998). In the thymus, *Rag* gene expression is down-regulated during positive selection, but the

specific signals that lead to *Rag* gene down-regulation are elusive (Yannoutsos et al., 2004). In this study, we investigated the role of pre-TCR signaling and thymocyte positive selection in NFAT gene expression as well as the role of NFAT in *Rag* gene down-regulation in thymocytes.

2. RESULTS

2.1 NFATs down-regulate the *Rag* gene expression in DP thymocytes

2.1.1 Calcineurin-NFAT signaling is involved in the *Rag* gene down-regulation in murine thymocytes

It has been reported that in the thymus the expression of *Rag1* and *Rag2* genes is down-regulated during positive selection (Kouskoff et al., 1995; Merkenschlager et al., 1997). However, the exact stage where the *Rag* gene down-regulation starts is not known (Kouskoff et al., 1995). In the thymus, the majority of the thymocytes are $TCR^{int}CD69^{-}$ thymocytes which have not experienced positive selection. During positive selection, a small population of $TCR^{int}CD69^{-}$ thymocytes becomes $TCR^{int}CD69^{+}$ thymocytes (Merkenschlager et al., 1997; Yannoutsos et al., 2001). To investigate if the *Rag* gene expression is down-regulating during positive selection, $TCR^{int}CD69^{-}$ thymocytes and $TCR^{int}CD69^{+}$ thymocytes were sorted from mouse total thymocytes and the *Rag* gene expression was measured by quantitative PCR (qPCR) using gene specific primers. Figure 2.1.1.1 shows that the expression of *Rag1* and *Rag2* was approximately 50 fold lower in $TCR^{int}CD69^{+}$ thymocytes compared to that in $TCR^{int}CD69^{-}$ thymocytes (Figure 2.1.1.1, Rag1 and Rag2), demonstrating that the *Rag* gene down-regulation is started in those DP thymocytes which are undergoing positive selection. Because calcium-calcineurin signaling is activated during positive selection of thymocytes (Neilson et al., 2004), we investigated the role of calcium signaling in the down-regulation of the *Rag* genes by determining the effect of ionomycin, an agonist of calcium signaling, on the

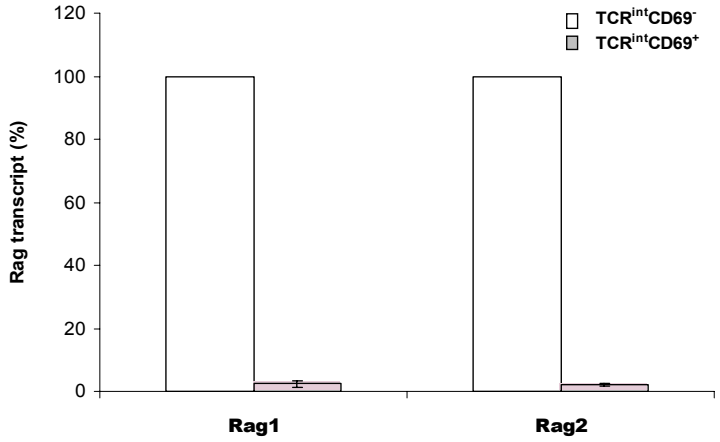

Figure 2.1.1.1. *Rag* gene down-regulation during positive selection. $TCR^{int}CD69^{-}$ and $TCR^{int}CD69^{+}$ thymocytes were electronically sorted from total thymocytes. The expression of *Rag1* (Rag1) and *Rag2* (Rag2) was measured by real time PCR (qPCR) using gene specific primers.

Rag gene expression in thymocytes. Total thymocytes were incubated in the presence or absence of 0.5 μM ionomycin for 2 hours and the *Rag* gene expression was quantified by qPCR. As shown in Figure 2.1.1.2, ionomycin treatment reduced the *Rag1* gene expression about 50 fold and the *Rag2* gene expression about 5 fold (Figure 2.1.1.2A, Rag1 and Rag2), indicating that both *Rag1* and *Rag2* were down-regulated by calcium signaling and that the *Rag1* gene was more sensitive than the *Rag2* gene in response to calcium signaling induced down-regulation. To investigate the possible role of calcineurin-NFAT signaling in the *Rag* gene down-regulation, the immunosuppressant FK506 was used to block calcineurin activity. Total thymocytes were incubated in the presence or absence of 10 nM of FK506 for 1 hour and then continually incubated in the presence of ionomycin for 2 hours. qPCR results showed that the expression of *Rag1* and *Rag2* was higher in the thymocytes pretreated with FK506 relative to that in the thymocytes without FK506 pretreatment (Figure 2.1.1.2B, Rag1 and Rag2), indicating

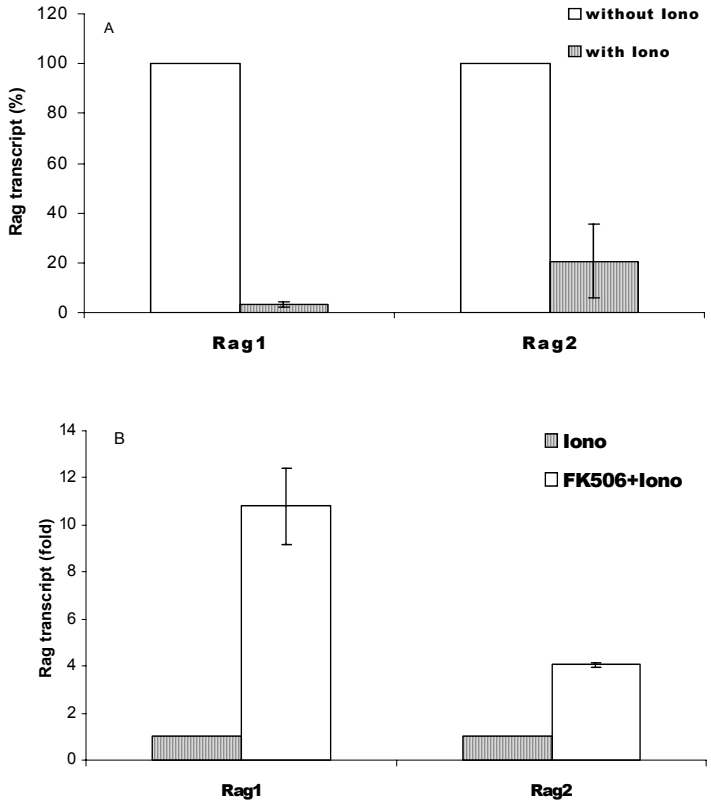

Figure 2.1.1.2. Calcineurin-NFAT signaling in *Rag* gene down-regulation. qPCR of *Rag1* (Rag1) and *Rag2* (Rag2) in total thymocytes (A) treated with 0.5 μM of ionomycin (Iono) for 2 hours and (B) pre-treated with 10 nM FK506 for one hour and then stimulated with ionomycin (FK506+Iono) for two hours.

that the calcineurin-NFAT signaling was involved in down-regulation of both *Rag1* and *Rag2*. Moreover, the *Rag1* gene expression was about 10 fold higher in thymocytes pretreated with FK506 compared to that in thymocytes without FK506 pretreatment (Figure 2.1.1.2B, Rag1) whereas the *Rag2* gene expression was about 4 fold higher in thymocytes pretreated with FK506 relative to that in thymocytes without FK506 pre-treatment (Figure 2.1.1.2B, Rag2). These data indicate again that the *Rag1* gene is more sensitive than the *Rag2* gene in response to the calcineurin-NFAT signaling induced down-regulation.

2.1.2 The *Rag*1 gene promoter contains NFAT binding sites

Figure 2.1.1.2 revealed that both *Rag1* and *Rag2* were regulated by calcineurin-NFAT signaling and that the *Rag1* gene is more tightly controlled by calcineurin-NFAT signaling. It is well established that calcineurin-NFAT signaling regulates gene expression by directly targeting NFAT-binding sites (Serfling et al., 2000). To determine the possible interaction between NFATs and the *Rag1* promoter, we searched the *Rag1* promoter region for potential NFAT-binding sites and identified several consensus NFAT-binding sequence (A/T)GGAAA within the 2 kb 5' region of the *Rag1* promoter. The interaction between NFATs and the potential NFAT-binding sites was validated by electrophoretic mobility shift assay (EMSA). In NFAT EMSAs, purified GST-NFATc2 RSD was incubated with a ^{32}P-labeled double strand oligonucleotide probe, and the reaction was resolved by native polyacrylamid gel electrophoresis (native PAGE) which can detect the free probe and the NFAT protein complex. The results showed that NFATs formed complexes with both NFAT227 and NFAT338 probes (Figure 2.1.2A and B), indicating that both probes contained NFAT-binding sites. NFATs formed hetero-complexes U and L with NFAT338 (Figure 2.1.2B, lanes 1, 2, 3) whereas they formed only an L complex with NFAT227 (Figure 2.1.2B, lanes 4, 5, and 6), indicating that these two probes were different from each other in mediating NFAT complex formation. Moreover, NFATs formed only the U complex with NFAT338 when 1 ng of NFAT protein was included in the reaction (Figure 2.1.2B, lane 1). However, NFAT formed both the U and L complexes with NFAT338 when more than 10 ng of NFAT protein was

included in the reaction (Figure 2.1.2B, lanes 2 and 3), indicating that the U complex has higher binding affinity than the L complex.

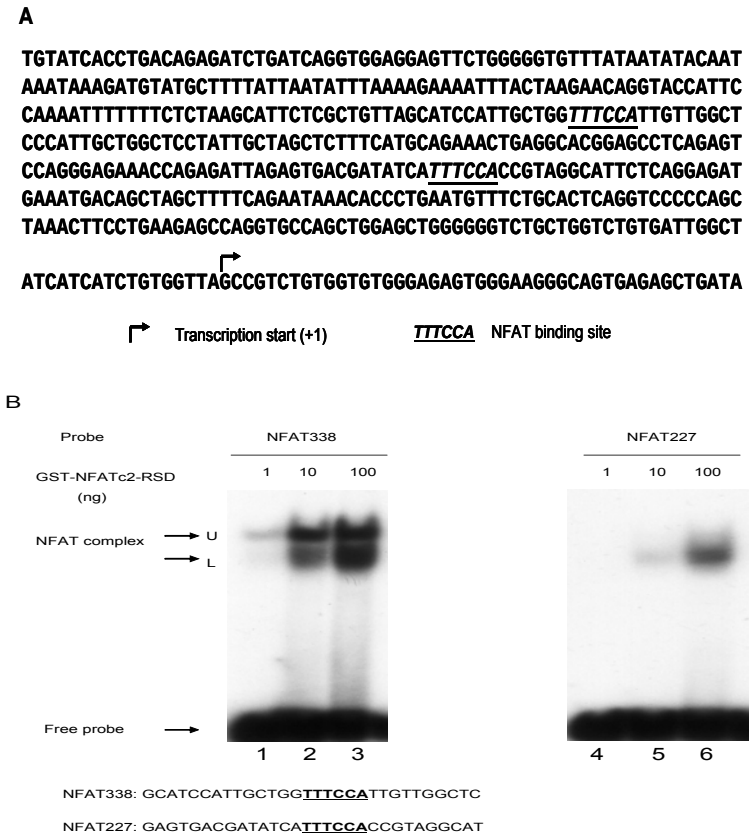

Figure 2.1.2 *Rag1* gene promoter region contains two NFAT binding sites. (A) DNA sequence of *Rag1* promoter. The transcription start site and two NFAT binding sites are indicated. (B) NFATs interact with *Rag1* NFAT probes. Indicated amount of GST-NFATc2 RSD fusion protein was incubated with the ^{32}P labeled NFAT338 and NFAT227 probes, respectively. The reaction was resolved on native PAGE in 1x TBE buffer. The NFAT complexes are indicated. The probe sequences are shown at the bottom.

2.1.3 The role of core GG sequence of the *Rag1* NFAT-binding sites in NFAT complex formation

It is known that, at most binding sites, NFATs form a monomer with the NFAT consensus binding motif (A/T)GGAAA in which the GG core sequence mediates the NFAT-DNA interaction (Chen et al., 1998). However, NFATs can also form dimers with NF-κB-like sites in which the two GG sites mediate a NFAT dimer formation (Giffin et al., 2003; Jin et al., 2003). To characterize the *Rag1* NFAT-binding sites, the GG sequences of NFAT227 and NFAT338 were mutated to CC and the mutant oligos were used in EMSA. The IL-2 NFAT-binding site Puboxd (IL-2) was used as a NFAT

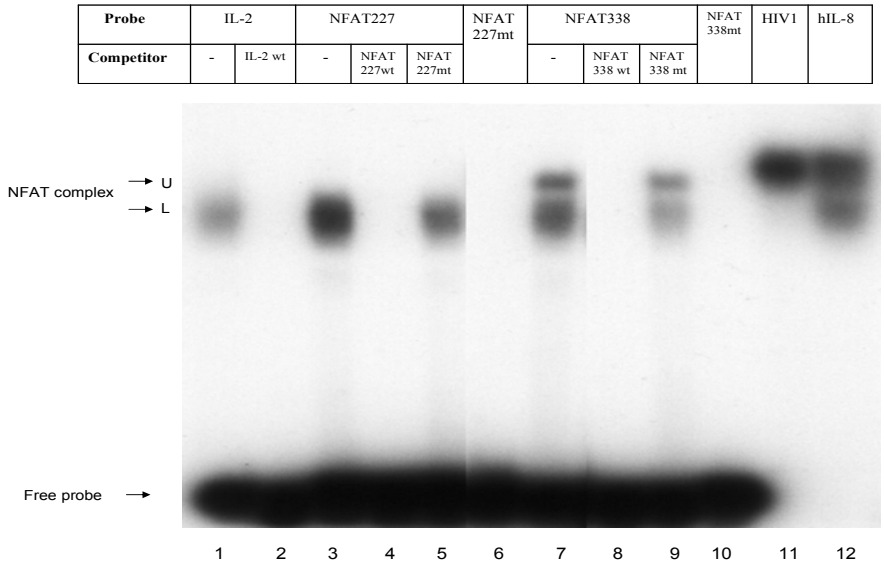

Figure 2.1.3. The role of the core GG sequence of the *Rag1* NFAT binding sites in NFAT complex formation. For EMSA binding assay, 10 ng of GST-NFATc2 RSD was incubated with the indicated NFAT probes. For competition assay, 5 pmol of the indicated oligos were included in the binding reaction. The NFAT complexes are indicated. Wt: wild-type. Mt: mutant.

monomer complex control while the HIV1 NF-κB-like site (HIV1) and the human IL-8 NF-κB-like site (hIL-8) were used as NFAT dimer complex controls (Giffin et al., 2003; Jin et al., 2003). As shown in Figure 2.1.3, the L complex formed between NFAT and NFAT227 is very similar in electrophoretic mobility to the L complex formed between NFAT and IL-2 (Figure 2.1.3, lanes 1 and 3) which is a monomer (Chen et al., 1998), demonstrating that the NFAT-NFAT227 complex is a monomer. The NFAT-NFAT227 complex was disrupted by an excess unlabeled wild-type NFAT227 (Figure 2.1.3, lane 4) but not by the mutant NFAT227 (Figure 2.1.3, lane 5) in which the core GG sequence was mutated to CC. Moreover, NFAT didn't form complex with the mutant NFAT227 (Figure 2.1.3, lane 6). These data indicate that the core GG sequence was essential for NFAT-NFAT227 complex formation. In contrast, NFAT formed the U complex with the HIV1, and the U and L complexes with the hIL-8 NFAT site (Figure 3.1.3, lanes 11 and 12), showing that the U complex is a dimer. The U and L complexes formed between NFAT and NFAT338 are very similar to the complexes formed between NFAT and the hIL-8 NFAT probe (Figure 2.1.3, lanes 7 and 12), indicating that NFAT338 mediated both the NFAT monomer and dimer formation. The NFAT-NFAT338 complexes were disrupted by an excess unlabeled wild-type NFAT338 (Figure 2.1.3, lane 8) but not by the mutant NFAT338 (Figure 2.1.3, lane 9) in which the core GG sequence is mutated to CC. In addition, NFAT didn't form complexes with the mutant NFAT338 probe (Figure 2.1.3, lane 10). These data indicate that the core GG sequence of NFAT338 was essential for NFAT338 to mediate both the NFAT monomer and dimer complex formation.

2.1.4 The usage of NFAT227 and NFAT338 sites by different NFAT members

It has been shown that different NFAT members have distinct NFAT site preferences (Ho et al., 1995). The possible preferential usage of NFAT227 and NFAT338 by different NFAT members was investigated by super-shift assay. Because NFATc1, NFATc2, and NFATc3 are expressed in the thymus (Oukka et al., 1998), nuclear protein extracts prepared from total thymocytes were used in the super-shift assays. The nuclear extract was incubated with the *Rag1* promoter NFAT probes in the presence or absence of anti-NFAT antibodies, and the antibody super-shifted NFAT complex was detected by native PAGE. The super-shift results showed that the nuclear extract prepared from the total

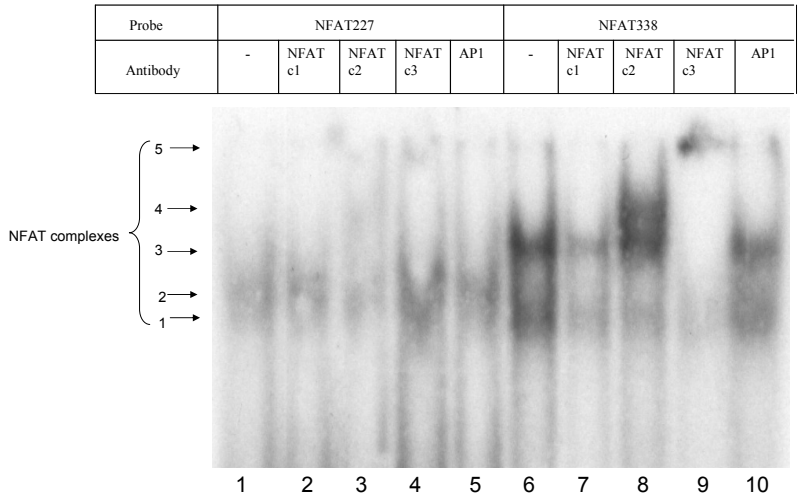

Figure 2.1.4 Usage of *Rag1* promoter NFAT binding sites by NFAT members. For EMSA binding assay, 2 ug of nuclear protein extract prepared from untreated total thymocytes was incubated with the indicated probes. For supershift assays, 1 ul of the indicated antibodies was included in the binding reaction. The NFAT complexes are indicated.

thymocytes formed a single NFAT complex with NFAT227 (Figure 2.1.4, lane 1, complex 2). This NFAT complex was shifted by anti-NFATc2 antibody but not shifted

by either anti-NFATc1 antibody or anti-NFATc3 antibody (Figure 2.1.4, lanes 2, 3, and 4, complex 4), demonstrating that the NFAT-NFAT227 complex contained NFATc2 but didn't contain either NFATc1 or NFATc3. In contrast, the same nuclear protein extract formed two NFAT complexes with NFAT338 (Figure 3.1.4, lane 6, complexes 1 and 3). The lower complex (complex 1) was shifted by anti-NFATc2 antibody while the upper complex (complex 3) was shifted by anti-NFATc3 antibody (Figure 3.1.4, lane 8 and lane 9, complexes 4 and 5, respectively). However, neither complex 1 nor complex 3 was shifted by an anti-NFATc1 antibody (Figure 3.1.4, lane 7), pointing out that in non-induced thymocytes the NFAT-NFAT338 complexes contained both NFATc2 and NFATc3 but not NFATc1. In addition, both the NFAT-NFAT227 and NFAT-NFAT338 complexes were not shifted by anti-AP-1 antibody (Figure 3.1.4, lanes 5 and 10), indicating that the NFAT-NFAT-227 and NFAT-NFAT338 complexes contained no AP-1 proteins. Together, these data show that NFATc3 formed complexes only with NFAT338 while NFATc2 formed complexes with both the NFAT227 and NFAT338 sites.

2.1.5 Both NFATc2 and NFATc3 are recruited to the *Rag1* promoter during the down-regulation of *Rag* genes

Data described above revealed that NFATs interact with the *Rag1* promoter *in vitro*. Since gene regulation is mainly conducted by recruiting sequence-specific DNA binding factors to the corresponding cis-elements (Kadonaga, 2004; Ptashne and Gann, 1997), the *in vivo* recruitment of NFAT to the *Rag1* promoter during the *Rag* gene down-regulation was investigated by chromatin immuno-precipitation (ChIP) assays (Wells and Farnham,

2002). Total thymocytes were incubated with 20 ng/ml of TPA+0.5 μM of ionomycin for desired times. One portion of the thymocytes was used for the *Rag1* gene expression assay. Another portion was fixed, and the formaldehyde cross-linked chromatin was immuno-precipitated by anti-NFAT antibodies. The genomic DNA released from the immuno-precipitated chromatin was assayed by PCR using primers covering the *Rag1* promoter region. The RT-PCR results showed that after TPA-ionomycin treatment, the *Rag1* gene expression was rapidly down-regulated within 30-60 min, and the down-regulation was slowed down thereafter (Figure 2.1.5A). The ChIP assay results showed that NFATc2 was associated with the *Rag1* promoter region in 30 min and the association between NFATc2 and the *Rag1* promoter was decreased after one hour (Figure 2.1.5B, αNFATc2, lanes 1, 2, 3, and 4). Similarly, NFATc3 association with the *Rag1* promoter increased within 30 minutes, and this association was disappeared thereafter (Figure 2.1.5B, αNFATc3, lanes 1, 2, 3, and 4). These data indicate that both NFATc2 and NFATc3 were recruited to the *Rag1* gene promoter during *Rag1* gene down-regulation. In contrast, NFATc1 was not detected in the *Rag1* promoter region during the first 30 min during the *Rag* gene down-regulation (Figure 2.1.5B, αNFATc1, lanes 1, 2, 3, and 4), indicating that NFATc1 was not recruited to the *Rag1* gene promoter during *Rag1* gene down-regulation.

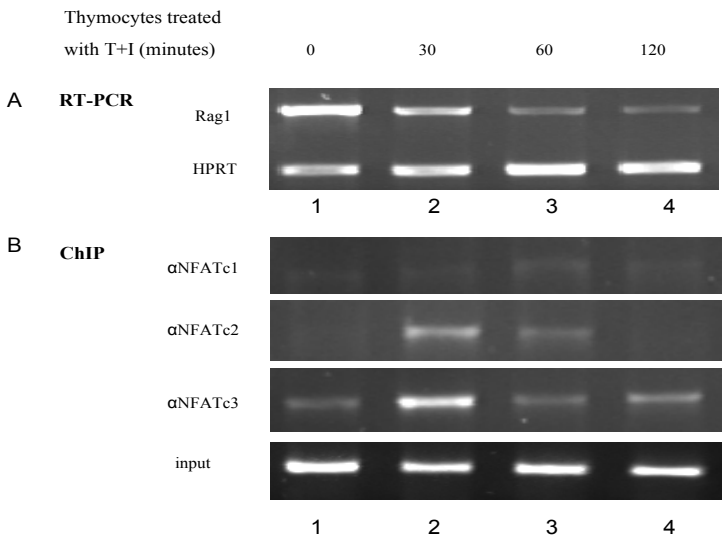

Figure 2.1.5 *In vivo* interaction between NFATs and the *Rag1* promoter during *Rag* gene down-regulation. (A) RT-PCR of *Rag1* (Rag1) gene expression in total thymocytes treated with TPA (20 ng/ml) plus ionomycin (0.5 μM) (T+I) for the indicated times. *Hprt* was co-amplified with *Rag1* and used as loading control (HPRT). (B) ChIP assay of total thymocytes treated as in (A). Chromatin was immuno-precipitated by the indicated antibodies. The antibody enriched genomic DNA was assayed by PCR using primers covering *Rag1* gene promoter. 10% of input chromatin was used as loading control (input).

2.1.6 Effect of a single NFAT gene deficiency on the down-regulation of *Rag* genes

Figure 2.1.5 suggests that both NFATc2 and NFATc3 could be involved in the *Rag1* gene down-regulation. The possible role of individual NFAT members in the *Rag* gene down-regulation was investigated by studying the *Rag* gene expression in wild-type and NFATc2 or NFATc3 deficient thymocytes stimulated with 0.5 μM of ionomycin for 2 hours. As shown in Figure 2.1.6, there was no difference in the *Rag1* gene expression between NFATc2 deficient thymocytes treated with ionomycin and wild-type thymocytes with the same treatment (Figure 2.1.6A, lanes wt+iono and $C2^{-/-}$+iono). However, the *Rag1* gene expression was about 1.5 fold higher in the NFATc3 deficient thymocytes

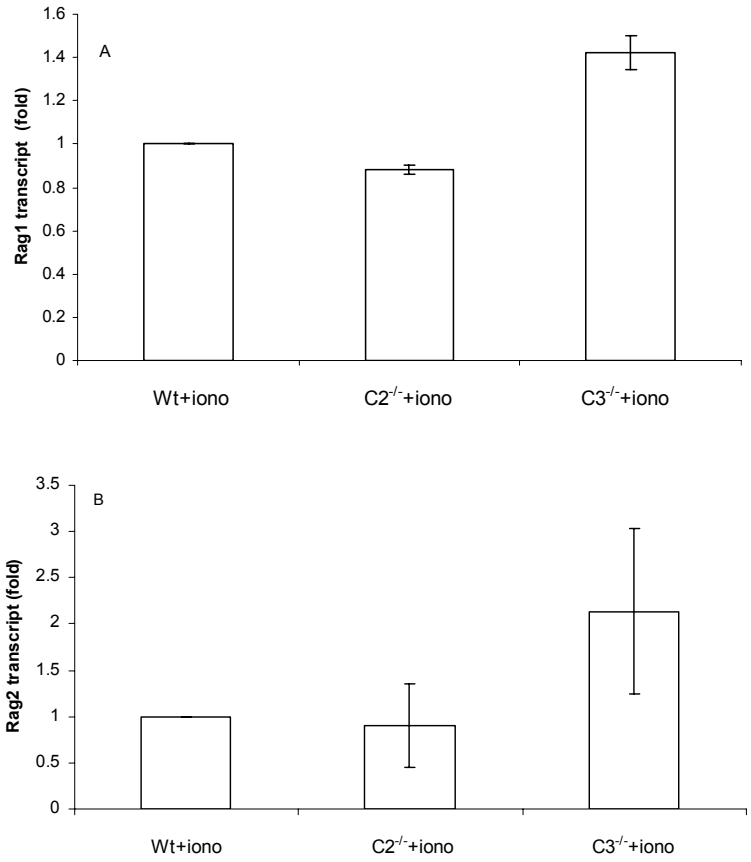

Figure 2.1.6 Effect of a single *Nfat* gene deficiency on *Rag* gene down-regulation. qPCR of (A) the *Rag1* gene expression and (B) the *Rag2* gene expression in the indicated total thymocytes treated with 0.5 μM of ionomycin (iono) for 2 hours. Wt: wild-type. $C2^{-/-}$: NFATc2 deficient. $C3^{-/-}$: NFATc3 deficient.

treated with ionomycin relative to that in the wild-type thymocytes with the same treatment (Figure 2.1.6A, lanes wt+iono and $C3^{-/-}$+iono). Similarly, there was no difference in the *Rag2* gene expression between NFATc2 deficient thymocytes treated with ionomyin and wild-type thymocytes with the same treatment (Figure 2.1.6B, lanes wt+iono and $C2^{-/-}$+iono). However, the *Rag2* gene expression was about 2 fold higher in the NFATc3 deficient thymocytes treated with ionomycin compared to that in the wild-type thymocytes with the same treatment (Figure 2.1.6B, lanes wt+iono and $C3^{-/-}$+iono).

These results indicate that NFATc2 deficiency had no effect on the down-regulation of the *Rag1* and *Rag2* genes whereas NFATc3 deficiency had a weak positive effect on the down-regulation of the *Rag1* and *Rag2* genes.

2.1.7 NFATc2 and NFATc3 double deficiency attenuates ionomycin induced *Rag* gene down-regulation

To investigate the possible interaction between NFATc2 and NFATc3 in the *Rag* gene down-regulation, we investigated the *Rag* gene expression in both NFATc2 and NFATc3 double deficient (DKO) thymocytes and wild-type thymocytes stimulated with 0.5 μM of ionomycin for 2 hours. As shown in Figure 2.1.7, there was no difference in the expression of *Rag1* and *Rag2* between wild-type and DKO thymocytes when the thymocytes were left untreated (Figure 2.1.7A, Rag1 and Rag2). However, when the thymocytes were treated with ionomycin, the *Rag1* gene expression was about 6 fold higher and the *Rag2* gene expression was about 2.5 fold higher in the DKO thymocytes compared to that in the wild-type thymocytes (Figure 2.1.7B, Rag1 and Rag2). These results indicate that the inactivation of both NFATc2 and NFATc3 in thymocytes attenuated ionomycin induced *Rag* gene down-regulation, and that NFAT had a stronger down-regulation effect on the *Rag1* gene than the *Rag2* gene.

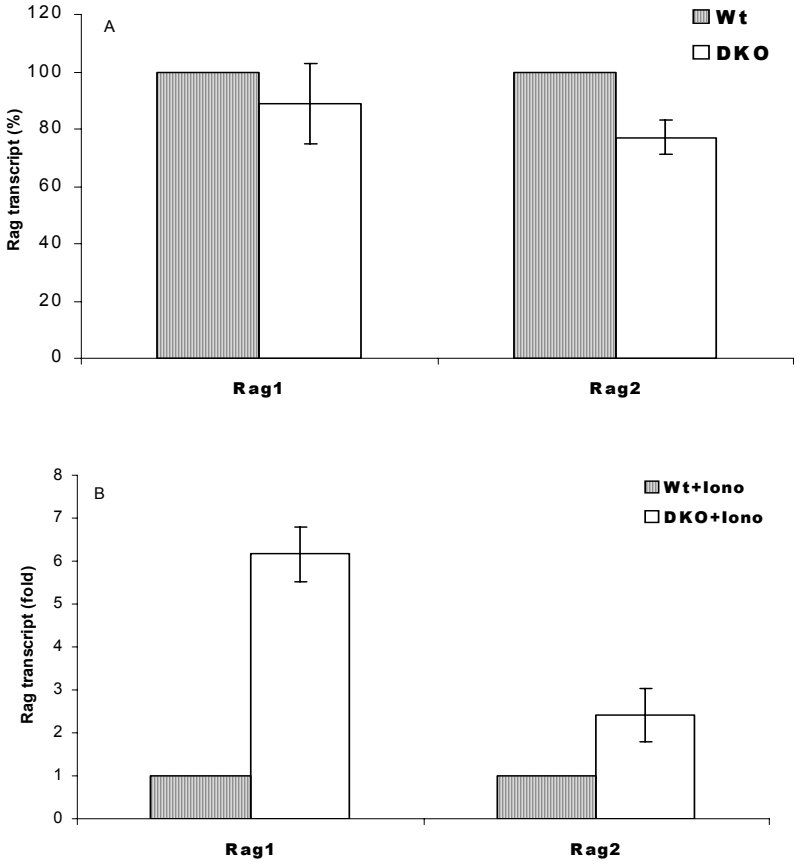

Figure 2.1.7 Effect of *Nfatc2* and *Nfatc3* double deficiency on *Rag* gene expression. qPCR of *Rag1* and *Rag2* in wild-type (Wt) and NFATc2 and NFATc2 double deficient (DKO) total thymocytes (A) without treatment and (B) treated with 0.5 μM ionomycin (Iono) for 2 hours.

2.1.8 Pre-TCR signaling up-regulates *Rag1* gene expression in DN thymocytes

Data described above suggest that NFATs play an important role in calcium signaling induced *Rag* gene down-regulation in DP thymocytes. Next, we investigated the role of NFAT in *Rag* gene expression in DN thymocytes by quantitatively comparing the *Rag1* gene expression in DN cell line SCIET27 with that in DN cell line SCB29. SCIET27 is a SCID thymocyte-derived pre-T cell line that has no pre-TCR expression, while SCB29 is derived from SCIET27 which has been stably transfected with TCRβ gene and expressing

pre-TCR (Groettrup et al., 1992; Groettrup et al., 1993). Since NFAT is activated in SCB29 cells by pre-TCR signaling (Aifantis et al., 2001), SCB29 is a good model for investigating the effect of NFAT on the *Rag* gene expression in DN thymocytes. As shown in Figure 2.1.8, the *Rag1* gene expression in SCB29 cells was about 7 fold higher relative to that in SCIET27 cells, demonstrating that pre-TCR expression in SCB29 cells up-regulated the *Rag1* gene expression, and that NFAT activation didn't down-regulate the *Rag1* gene expression in SCB29 cells.

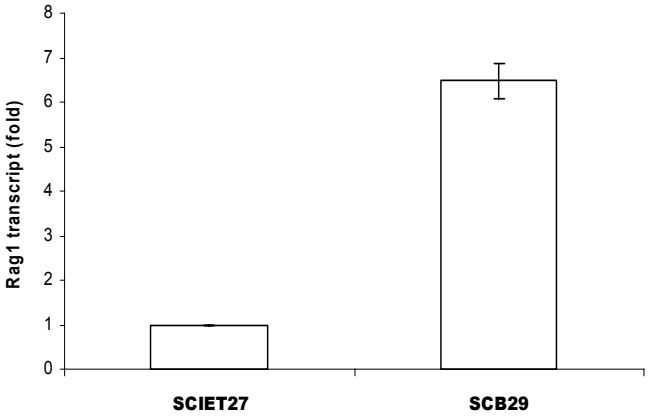

Figure 2.1.8 Effect of pre-TCR expression on *Rag1* expression in DN cells. *Rag1* gene expression in pre-TCR$^-$ (SCIET27) and pre-TCR$^+$ (SCB29) cells was measured by qPCR using gene specifc primers.

2.1.9 Over-expressed NFATs enhance calcium signaling induced *Rag1* gene down-regulation in DP thymocytes

VL3-3M2 is a DP thymoma cell line in which the *Rag1* gene is expressed whereas the *Rag2* gene expression is not detected (Groves et al., 1995). To confirm that calcium-calcineurin signaling plays a role in the *Rag* gene down-regulation in VL3-3M2 cells, we

quantified the *Rag1* gene expression in VL3-3M2 cells treated with 0.5 μM of ionomycin for 2 hours. Figure 2.1.9.1A shows that ionomycin treatment reduced the *Rag1* gene

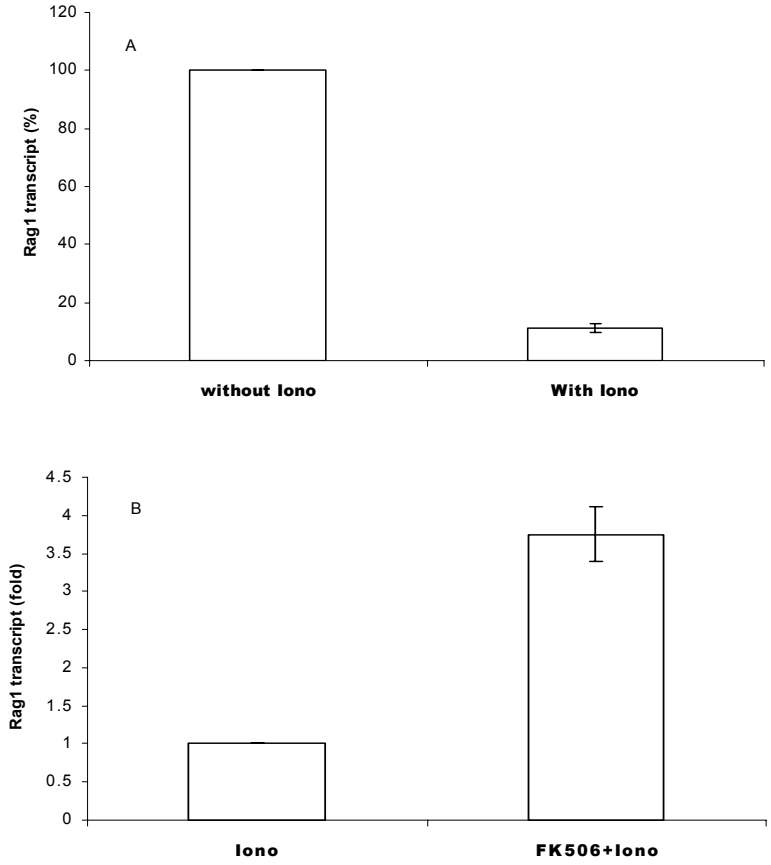

Figure 2.1.9.1 Calcineurin-NFAT signaling down-regulates *Rag1* gene expression in DP VL3-3M2 cells. qPCR of *Rag1* gene expression in (A) VL3-3M2 cells treated with 0.5 μM of ionomycin (Iono) for 2 hours and (B) VL3-3M2 cells were incubated for one hour in the presence or absence of 10 nM FK506 (FK506) and then stimulated

expression about 10 fold in VL3-3M2 cells. To confirm that calcineurin-NFAT signaling is involved in down-regulating the *Rag1* gene expression in the cell line, the *Rag1* gene expression was measured in the cells incubated in the presence or absence of 10 nM of FK506 for one hour and then stimulated with 0.5 μM of ionomycin for 2 hours. As shown in Figure 2.1.9.1B, the *Rag1* gene expression was about 4 fold higher in the cells

pretreated with FK506 relative to that in the cells without FK506 pretreatment. These results demonstrate that the calcineurin-NFAT signaling pathway was involved in the *Rag1* gene down-regulation in VL3-3M2 cells. To further demonstrate the role of individual NFAT members in the *Rag1* gene down-regulation, we took advantage of constitutively active version of NFAT (caNFAT). caNFAT is constitutively localized in the nucleus of cells and this nuclear localization can not be blocked by calcineurin inhibitors (Amasaki et al., 2002; Monticelli and Rao, 2002; Okamura et al., 2000). Importantly, in the presence of the calcineurin inhibitor, the over-expressed caNFAT can be activated but the endogenous NFAT can not. In such DP VL3-3M2 cells, the effect of over-expressed caNFATc2 or caNFATc3 on the *Rag1* gene expression can be determined. For this purpose, stable clones were generated from VL3-3M2 cells transfected with caNFAT expression vectors. Cap21 cells harbor the construct pEGZ-HA-caNFATc2, Cax27 cells harbor the construct pMSCV-neo-HA-caNFATc3, and CloneA cells contain control plasmid pMSCV-neo. The nuclear localization of HA-tagged NFAT in Cap21 and Cax27 cells was confirmed by Western blotting using anti-HA antibody (Figure 2.1.9.2).

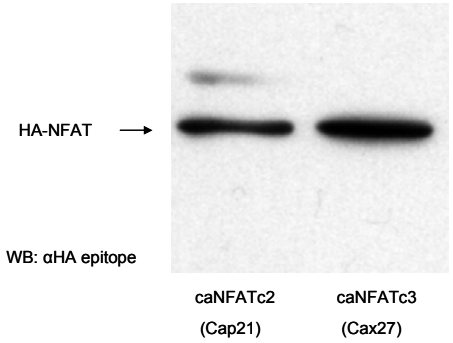

Figure 2.1.9.2 Expression of HA-caNFATc2 and HA-caNFATc3 in DP cells. Western blot of nuclear extracts prepared from caNFATc2 expression clone Cap21 (Cap21) and caNFATc3 expression clone Cax27 (Cax27) was detected by anti-HA antibody and the secondary antibody-HRP conjugate. The specific band was revealed by the enhanced chemiluminescnce system (ECL) as indicated.

To investigate the effect of over-expressed caNFAT on *Rag1* gene expression, the VL3-3M2 derived cells were incubated in the presence of 10 nM of FK506 for one hour and then stimulated with 0.5 μM of ionomycin for two hours. The *Rag1* gene expression was measured by qPCR. Figure 2.1.9.3A shows that there was no difference in the *Rag1* gene expression between Cap21 and Cax27 cells and CloneA cells when the cells were left untreated. However, when these cells were treated with FK506 plus ionomycin the *Rag1* gene expression was about 2.5 fold lower in the Cap21 cells and about 2 fold lower in the Cax27 cells compared to that in the CloneA cells (Figure 2.1.9.3B). These data indicate that over-expression of either caNFATc2 or caNFATc3 in DP cells enhanced the ionomycin induced *Rag1* gene down-regulation.

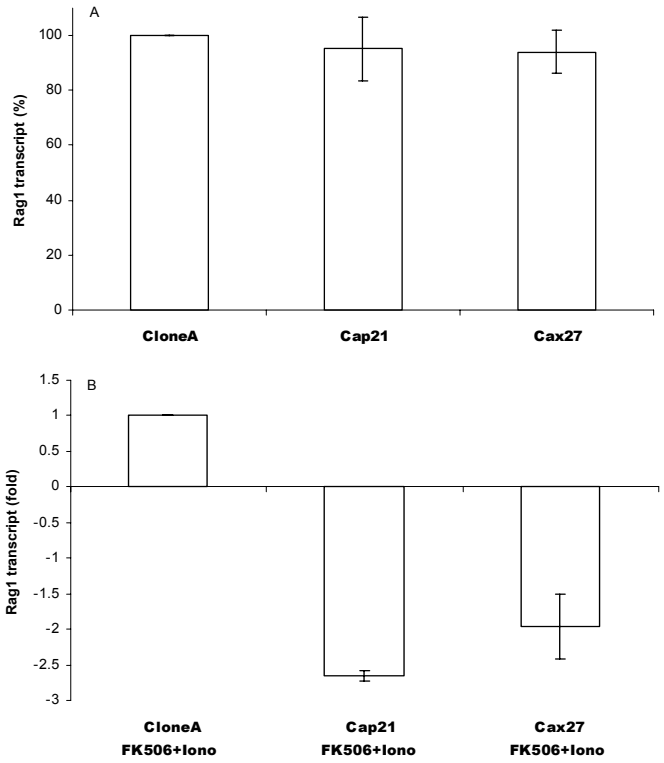

Figure 2.1.9.3 Over-expressed NFATs enhance calcium signaling induced *Rag1* gene down-regulation in DP cells. qPCR of *Rag1* gene expression in the indicated cells (A) without treatment and (B) treated with 10 nM of FK506 for one hour and then with 0.5 μM of ionomycin for 2 hours.

2.1.10 NFATs interact with the Rag anti-silencer element (ASE) during *Rag* gene down-regulation

It has been established that the active Rag anti-silencer element (ASE) is required to maintain the expression of both *Rag1* and *Rag2* in DP thymocytes, and that inactivation of ASE activity leads to down-regulation of both *Rag1* and *Rag2* in DP thymocytes (Yannoutsos et al., 2004; Yu et al., 1999). The involvement of calcineurin-NFAT signaling in down-regulation of both *Rag1* and *Rag2* in DP thymocytes prompted us to investigate the possible *in vivo* interaction between NFAT and ASE by ChIP assays. The *Rag* gene expression was down-regulated by treating the total thymocytes with

TPA+ionomycin for different times. The chromatin prepared from these thymocytes was used for chromatin immuno-precipitation by NFAT specific antibodies. The genomic DNA released from the immuno-precipitated chromatin was assayed by PCR using ASE specific primers. Since there are many potential NFAT-binding sequences in the 7.6 kb genomic ASE DNA fragment (Yannoutsos et al., 2004; GeneBank Accession Number AY215076), PCR primers were designed to cover each region which contains the potential NFAT-binding sequences. These primers were used in ChIP assays to locate the regions which could be targeted by NFAT during the *Rag* gene down-regulation. Initial ChIP experiments identified that only the region ASEd, which spans 4800 bp to 5401bp of ASE, was targeted by NFAT, the interaction between NFAT and the ASEd was investigated in the ChIP experiments thereafter. As shown in Figure 2.1.10.1, after

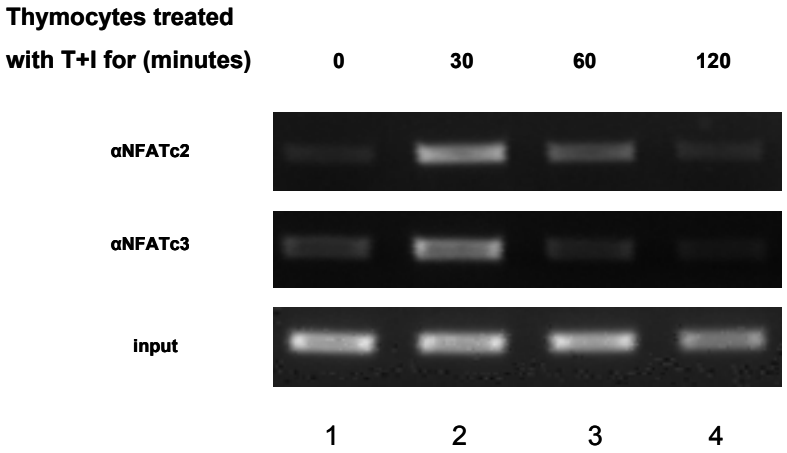

Figure 2.1.10.1. *In vivo* interaction of NFATs with Rag ASE during *Rag* gene down-regulation. ChIP assay of wild-type total thymocytes treated with TPA (20 ng/ml) + ionomycin (0.5 μM) (T+I) for the indicated times. Chromatin was immuno-precipitated by the indicated antibodies. 10% of chromatin input was used as loading control (input).

treatment with TPA+ionomycin, NFATc2 was associated with the ASEd in 30 minutes and was dissociated to a large extent from the ASEd after 60 minutes (Figure 2.1.10.1, αNFATc2, lanes 1, 2, 3, and 4). Similarly, NFATc3 was associated with the ASEd in 30 minutes and was dissociated from the ASEd after 60 minutes (Figure 2.1.10.1, αNFATc2, lanes 1, 2, 3, and 4). These data indicate that both NFATc2 and NFATc3 were recruited to the Rag ASE during the *Rag* gene down-regulation.

There are three NFAT-binding sequences within the ASEd region (Figure 2.1.10.2A). EMSAs were used to validate the NFAT binding sites. Figure 2.1.10.2B shows that NFAT formed complexes with both ASEd2 and ASEd3 probes (Figure 2.1.10.2B, lanes 2 and 5), indicating that both probes contain NFAT binding sites. The complexes formed between NFAT and probes ASEd2 and ASEd3 were the same as the complexes formed between NFAT and the IL-2 NFAT site (Figure 2.1.10.2B, lanes 1, 2, and 5), demonstrating that both ASEd2 and ASEd3 mediated the NFAT monomer formation. In competition experiments, the NFAT-ASEd2 complexes were disrupted by an excess unlabeled wild-type ASEd2 but were not disrupted by the mutant ASEd2 in which the GG site was mutated to CC (Figure 2.1.10.2B, lanes 3 and 4), demonstrating that the GG sequence of probe ASEd2 is essential to mediate the NFAT-ASEd2 complex formation. Similarly, the NFAT-ASEd3 complexes were disrupted by an excess unlabeled wild-type ASEd3 (Figure 2.1.10.2B, lane 6), however, the complexes were almost completely disrupted by an excess ASEd3mutA in which the GG site of TTGGAAA was mutated to CC (Figure 2.1.10.2B, lane 7), indicating that the ASEd3 probe contained more than one NFAT binding sites. Although ASEd3mutB, in which the GG site of TGGAA sequence

was mutated to CC, did disrupt the NFAT-ASEd3 complexes (Figure 2.1.10.2B, lane 8), the NFAT-ASEd3 complexes were not disrupted by an excess ASEd3mutAB (Figure 2.1.10.2B, lane 9) in which the GG site of TGGAA and the GG site of TTGGAAA were simultaneously mutated to CC, demonstrating that both TTGGAAA and TGGAA sequences in probe ASEd3 mediated NFAT-ASEd3 complex formation.

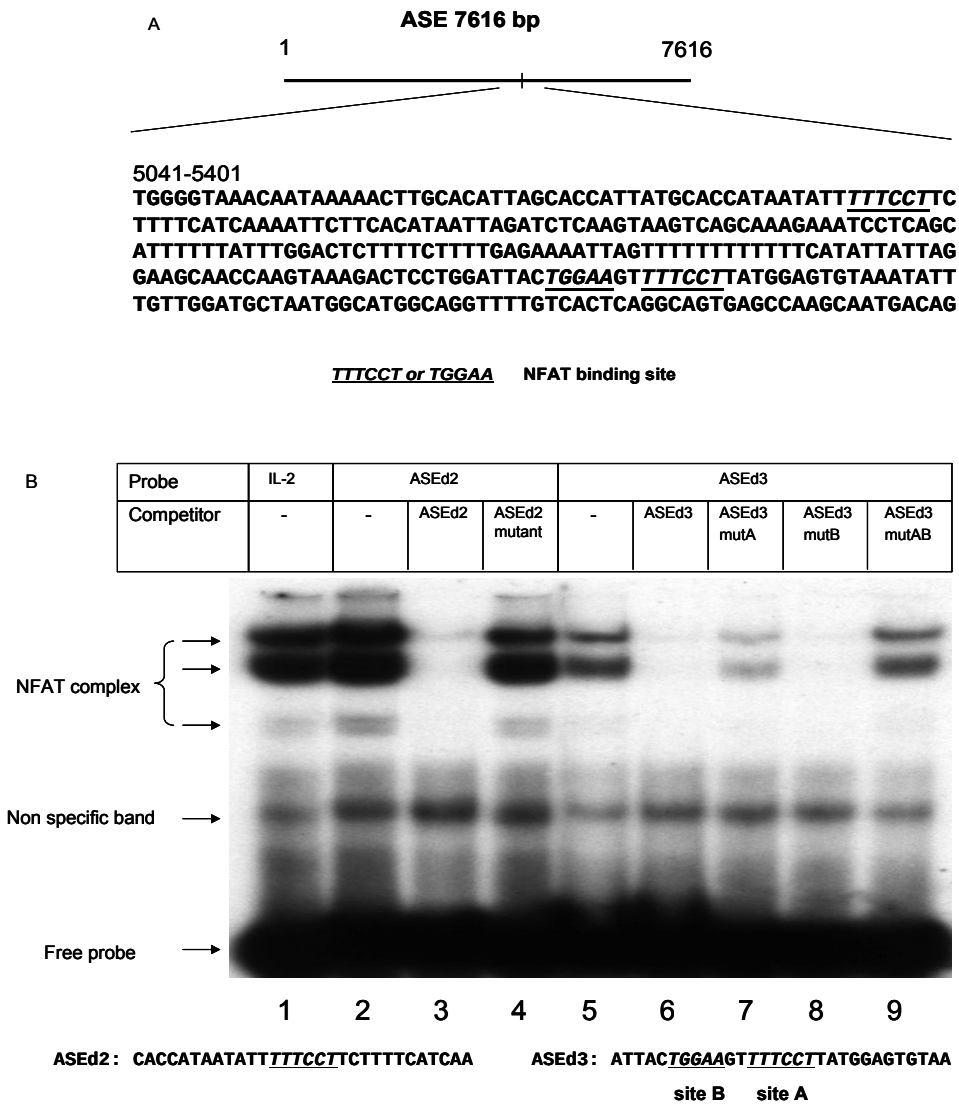

Figure 2.1.10.2. The Rag ASE contains three NFAT binding sites. (A) Sequence of ASEd region with the NFAT binding sites indicated. (B) EMSA. 10 ng of GST-NFATc2 RSD was incubated with the indicated ASEd probes. For competition, 5 pmol of indicated oligos were included in the binding reaction. The NFAT complexes are indicated. The probe sequences are shown at the bottom with NFAT binding sites indicated.

2.2 *Nfatc1α* activation in murine thymocytes

2.2.1 *Nfatc1*, *Nfatc2*, and *Nfatc3* are regulated differentially by pre-TCR signaling in DN thymocytes

Nfatc1, *Nfatc2,* and *Nfatc3* are expressed in DN thymocytes (Oukka et al., 1998). To investigate the effect of pre-TCR signaling on the expression of *Nfatc1*, *Nfatc2,* and *Nfactc3*, we quantitatively compared the *Nfat* gene expression in pre-TCR$^-$ SCIET27 cells with that in pre-TCR$^+$ SCB29 cells. As shown in Figure 2.2.1, there was no difference in the *Nfatc3* expression between SCB29 cells and SCIET27 cells (Figure 2.2.1, NFATc3), indicating that pre-TCR expression had no effect on *Nfatc3* expression. However, the *Nfatc1* expression was about 2 fold higher and *Nfatc2* expression was about 5 fold higher in SCB29 cells relative to that in SCIET27 cells (Figure 2.2.1, NFATc1 and NFATc2), indicating that pre-TCR expression up-regulated the expression of both *Nfatc1* and *Nfatc2* in SCB29 cells.

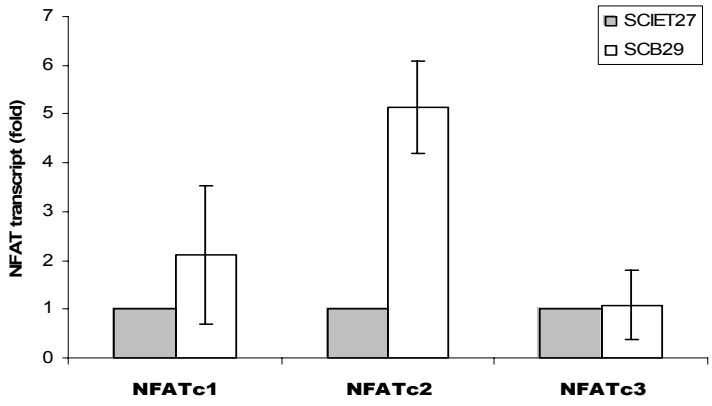

Figure 2.2.1. *Nfat* gene expression is differentially regulated by pre-TCR signaling. qPCR of *Nfatc1*(NFATc1), *Nfatc2*(NFATc2), and *Nfatc3*(NFATc3) was carried out in RNA prepared from SCIET27 and SCB29 cells using gene specific primers.

2.2.2 Pre-TCR signaling activates *Nfatc1α* expression in DN thymocytes

The *Nfatc1* gene has two promoters, designated as P1 and P2. The P1 promoter directs the *Nfatc1α* transcription while the P2 promoter the *Nfatc1β* transcription (Chuvpilo et al., 2002). The finding that TCR signaling induces *Nfatc1α* gene expression in peripheral T cells (Chuvpilo et al., 2002; Sherman et al., 1999) prompted us to investigate the effect of pre-TCR signaling on the *Nfatc1* isoform induction in DN thymocytes. To determine if pre-TCR signaling is involved in the induction of *Nfatc1α* and *Nfatc1β*, the expression of *Nfatc1α* and *Nfatc1β* in SCIET27 and SCB29 cells was measured by qPCR. Figure 2.2.2 shows that there was no difference in the *Nfatc1β* expression between SCIET27 and SCB29 cells (Figure 2.2.2, NFATc1β). However, the *Nfatc1α* expression was about 70 fold higher in SCB29 cells compared to that in SCIET27 cells (Figure 2.2.2, NFATc1α), indicating that pre-TCR signaling selectively activated the *Nfatc1α* gene expression in SCB29 cells.

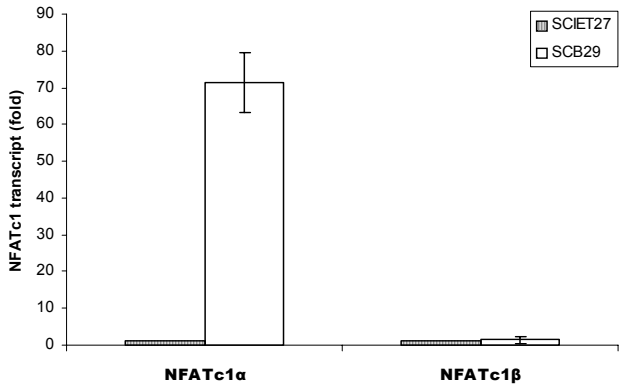

Figure 2.2.2. *Nfatc1α* gene is activated by pre-TCR signaling. qPCR of *Nfatc1α* (NFATc1α) and *Nfatc1β* (NFATc1β) was carried out using NFATc1 isoform specific primers in RNA prepared from SCIET27 and SCB29 cells.

2.2.3 Calcineurin signaling is not involved in *Nfatc1α* gene activation in SCB29 cells

Next, we investigated the role of NFATs in pre-TCR signaling induced *Nfatc1α* gene expression in DN thymocytes. We determined the effect of calcineurin inhibitor CsA on *Nfatc1α* expression in SCB29 cells. SCB29 cells were left untreated or treated with 160 nM of CsA for 6 hours. The *Nfatc1α* expression was quantified by qPCR. As shown in Figure 2.2.3, there was no difference in the *Nfatc1α* expression in SCB29 cells treated with CsA and SCB29 cells left untreated, indicating that calcineurin-NFAT signaling was not involved in *Nfatc1α* activation in SCB29 cells.

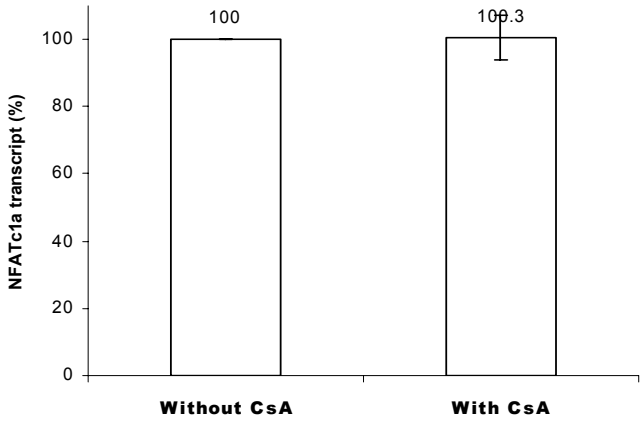

Figure 2.2.3. Blockade of calcineurin signaling doesn't affect *Nfatc1α* expression in SCB29 cells. qPCR of *Nfatc1α* expression was carried out in RNA prepared from SCB29 cells incubated for 6 hours in the presence or absence of 160 nM of cyclosporin A (CsA).

2.2.4 MEK-ERK and JNK signaling pathways have opposing effects on *Nfatc1α* expression in SCB29 cells

Pre-TCR signaling activates the MAPK signaling pathway in DN thymocytes (Xi et al., 2006). The MAPK signaling pathway consists of ERK, JNK, and p38 protein kinases

(Johnson and Lapadat, 2002). The MEK-ERK signaling pathway can be blocked by the MEK inhibitor U0126 (Favata et al., 1998), the JNK signaling pathway can be blocked by the JNK inhibitor SP600125 (Bennett et al., 2001), and the p38 signaling pathway can be blocked by the p38 inhibitor SB203580 (Barančik et al., 2001). To determine the role of the MAPK signaling pathway in the *Nfatc1α* gene expression in DN thymocytes, we investigated the effects of the MAPK inhibitors on *Nfatc1α* gene expression in SCB29 cells. SCB29 cells were incubated with 40 μM of U0126, 50 μM of SP600125, or 25 μM of SB203580 for 6 hours. Since these MAPK inhibitors are dissolved in DMSO, the control cells were incubated with DMSO for 6 hours. The *Nfatc1α* gene expression was measured by qPCR. As shown in Figure 2.2.4, there was no difference in the *Nfatc1α* expression between the cells treated with the 38 inhibitor SB203580 and the cells treated with DMSO alone (Figure 2.2.4, lanes 1 and 3), indicating that the blockade of p38 signaling pathway had no effect on the *Nfatc1α* expression in SCB29 cells. However, the *Nfatc1α* expression was reduced about 6 fold in the cells treated with the MEK inhibitor U0126 compared to that in the DMSO treated cells (Figure 2.2.4, lanes 1 and 2), indicating that the blockade of MEK-ERK signaling down-regulated *Nfatc1α* expression in SCB29 cells. In contrast, the *Nfatc1α* expression was increased about 8 fold in the cells treated with the JNK inhibitor SP600125 compared to that in the DMSO treated cells (Figure 2.2.4, lanes 1 and 4), indicating that the blockade of JNK signaling up-regulated *Nfatc1α* expression in SCB29 cells. These data suggest that MEK-ERK signaling pathway up-regulates whereas JNK signaling pathway suppresses *Nfatc1α* expression in SCB29 cells.

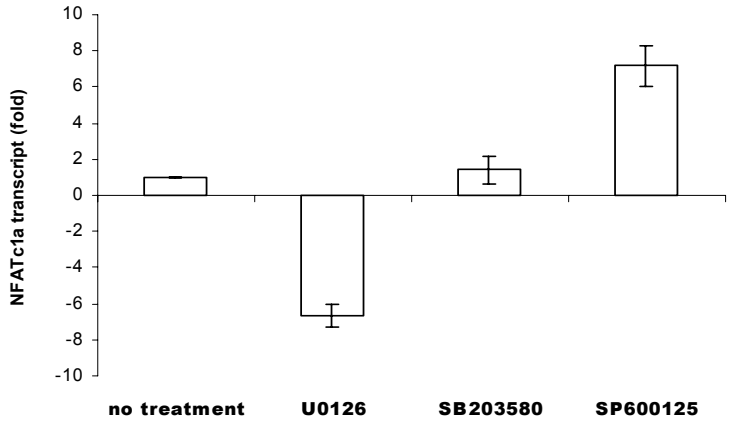

Figure 2.2.4. MEK-ERK and JNK signaling pathways regulate *Nfatc1α* expression in SCB29 cells. SCB29 cells were left untreated or treated with 40 μM of U0126, 50 μM of SP600125, and 25 μM of SB203580, respectively, in the presence of equal amounts of DMSO for 6 hours. *Nfatc1α* gene expression was measured by qPCR in RNA prepared from the cells.

2.2.5 Positive selection differentially regulates NFAT member expression

Although it is known that the expression of *Nfatc1* and *Nfatc2* is up-regulated and the expression of *Nfatc3* is down-regulated during DP thymocytes to SP thymocytes maturation (Oukka et al., 1998), the signaling which is responsible for the regulated NFAT gene expression is unknown. During DP to SP transition, the critical checkpoint is positive selection (Werlen et al., 2003). During positive selection, $TCR^{int}CD69^{-}$ thymocytes become $TCR^{int}CD69^{+}$ thymocytes (Merkenschlager et al., 1997; Yannoutsos et al., 2001). To determine the possible role of positive selection in *Nfat* gene expression, we quantitatively compared the expression of *Nfatc1*, *Nfatc2*, and *Nfatc3* in $TCR^{int}CD69^{-}$ thymocytes with that in $TCR^{int}CD69^{+}$ thymocytes. Figure 2.2.5 shows that the *Nfatc1* expression was increased about 2.5 fold and the *Nfatc2* expression was increased about 3.0 fold in the $TCR^{int}CD69^{+}$ thymocytes compared to that in the $TCR^{int}CD69^{-}$ thymocytes (Figure 2.2.5, NFATc1 and NFATc2), indicating that positive selection up-regulated both

Nfatc1 and *Nfatc2* expression. However, the *Nfatc3* gene expression was decreased by 50% in the $TCR^{int}CD69^{+}$ thymocytes relative to that in the $TCR^{int}CD69^{-}$ thymocytes

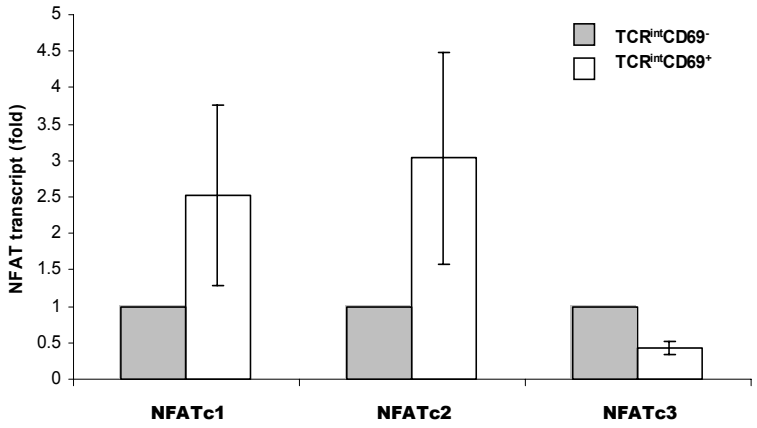

Figure 2.2.5 Positive selection regulates *Nfat* gene expression. *Nfatc1, Nfatc2,* and *Nfatc3* gene expression was measured in electronically sorted $TCR^{int}CD69^{-}$ and $TCR^{int}CD69^{+}$ thymocytes by qPCR using gene specifc primers.

(Figure 2.2.5, NFATc3), indicating that positive selection down-regulated *Nfatc3* expression. These data suggest that positive selection had different effects on *Nfatc1, Nfatc2,* and *Nfatc3* gene expression in DP thymocytes.

2.2.6 Positive selection activates *Nfatc1α* expression

To further determine the role of positive selection on the induction of *Nfatc1* promoters P1 and P2, we compared the expression of *Nfatc1α* and *Nfatc1β* in $TCR^{int}CD69^{-}$ thymocytes with that in $TCR^{int}CD69^{+}$ thymocytes. Figure 2.2.6 shows that there was no difference in the *Nfatc1β* expression between $TCR^{int}CD69^{-}$ and $TCR^{int}CD69^{+}$ thymocytes (Figure 2.2.6, NFATcβ), indicating that positive selection didn't regulate *Nfatc1β* gene expression. However, the *Nfatc1α* expression was increased about 40 fold in the

$TCR^{int}CD69^{+}$ thymocytes compared to that in the $TCR^{int}CD69^{-}$ thymocytes (Figure 2.2.6, NFATc1α), indicating that positive selection activates *Nfatc1α* expression. Collectively, these data indicate that positive selection selectively activated *Nfatc1α* expression.

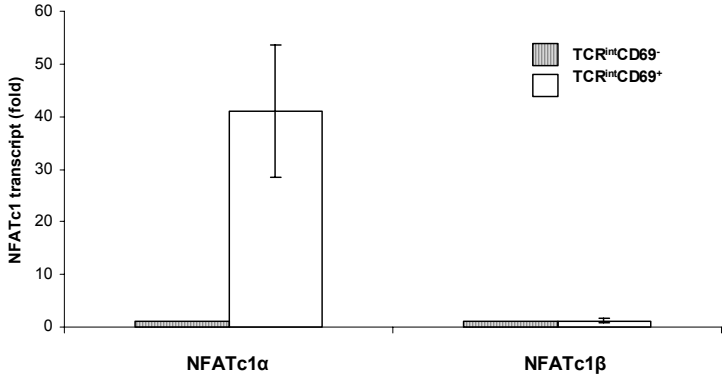

Figure 2.2.6. Positive selection activates *Nfatc1α* expression. *Nfatc1α* and *Nfatc1β* gene expression was measured in electronically sorted $TCR^{int}CD69^{-}$ and $TCR^{int}CD69^{+}$ thymocytes by qPCR using NFATc1 isoform specific primers.

2.2.7 Calcineurin activity is indispensable for *Nfatc1α* activation in DP thymocytes

Next, we investigated the possible role of NFAT in the *Nfatc1α* activation in DP thymocytes by determining the effect of CsA on the TPA+ionomycin induced *Nfatc1α* gene expression in total thymocytes. Total thymocytes were pretreated with 160 nM of CsA for one hour and then stimulated with TPA (20 ng/ml) + ionomycin (0.5 μM) for 2 hours. The *Nfatc1α* gene expression was quantified by qPCR. Figure 2.2.7 shows thatTPA+ionomycin treatment increased *Nfatc1α* expression approximately 35 fold (Figure 2.2.7, treatment and TPA+Iono), indicating that TPA+ionomycin activated *Nfatc1α* expression in DP thymocytes. However, TPA+ionomycin treatment failed to increase *Nfatc1α* expression in the thymocytes pretreated with CsA for one hour (Figure

2.2.7, no treatment and CsA+TAP+Iono), indicating that the blockade of calcineurin-NFAT signaling ablated the TPA+ionomycin induced *Nfatc1α* expression. These data suggest that the calcineurin-NFAT signaling pathway was indispensable for *Nfatc1α* activation in DP thymocytes.

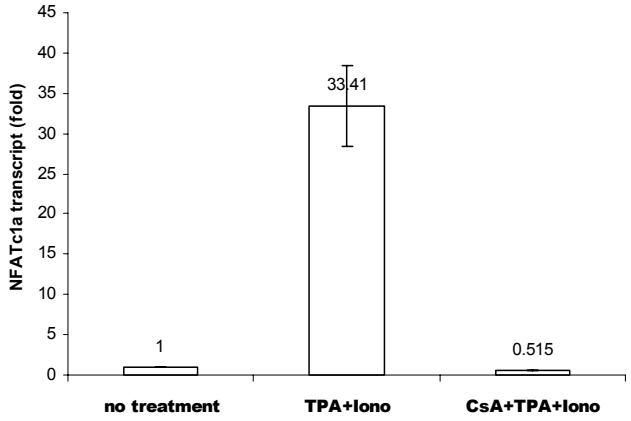

Figure 2.2.7. CsA blocks *Nfatc1α* activation in DP cells. qPCR of *Nfatc1α* expression in total thymocytes stimulated with TPA+ionomycin (TPA+Iono) or pre-treated with CsA and then stimulated with TPA+ionomycin (CsA+TPA+Iono) or simply incubated with medium (no treatment).

2.2.8 Both NFATc2 and NFATc3 are not required for *Nfatc1α* activation in DP thymocytes

Figure 2.2.7 revealed that calcineurin-NFAT activity was required for *Nfatc1α* activation in DP thymocytes. To determine which NFAT member is essential for the *Nfatc1α* activation in the thymocytes, we investigate the *Nfatc1α* expression during positive selection in NFATc2 and NFATc3 double deficient (DKO) thymocytes. To do so, $TCR^{int}CD69^{+}$ thymocytes and $TCR^{int}CD69^{-}$ thymocytes were sorted from DKO total thymocytes and the *Nfatc1α* expression in these cells was quantified by qPCR. As shown in Figure 2.2.8, the *Nfatc1α* expression was increased about 20 fold in the DKO

$TCR^{int}CD69^{+}$ thymocytes compared to that in the DKO $TCR^{int}CD69^{-}$ thymocytes, indicating that positive selection activated *Nfatc1α* expression in DKO thymocytes. This result indicates that both NFATc2 and NFATc3 were dispensable for *Nfatc1α* activation during positive selection and implies that only NFATc1 was required for the autoregulation of *Nfatc1α* activation in DP thymocytes.

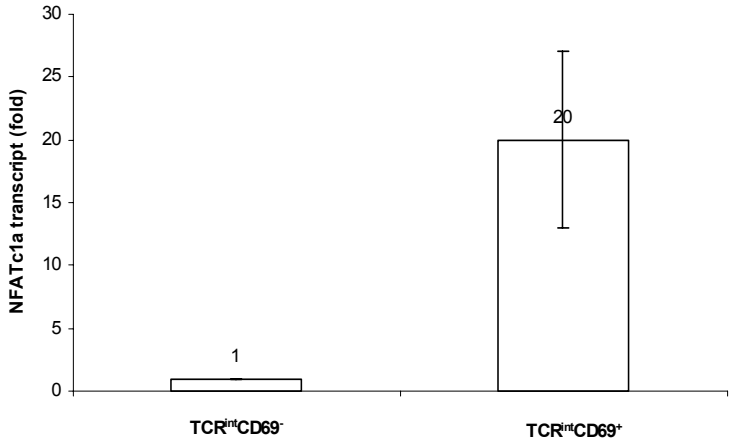

Figure 2.2.8. NFATc2 and NFATc3 are not required for *Nfatc1α* activation during positive selection. qPCR of *Nfatc1α* expression in $TCR^{int}CD69^{-}$ and $TCR^{int}CD69^{+}$ cells electronically sorted from total thymocytes of *Nfatc2* and *Nfatc3* double deficient mice.

2.2.9 De novo protein synthesis is required for the optimal *Nfatc1α* activation in DP thymocytes

It has been shown that the transcription of *Nfatc1α* is auto-regulated in peripheral T cells (Chuvpilo et al., 2002; Serfling et al., 2006a). Figures 2.2.6, 2.2.7, and 2.2.8 revealed that NFATc1 activated *Nfatc1α* gene expression in DP thymocytes, suggesting that *Nfatc1α* could also be auto-regulated in DP thymocytes. According to the auto-regulation model, protein synthesis is not required for initial *Nfatc1α* gene expression but is required for

optimal *Nfatc1α* gene expression through positive feedback regulation. To test if newly synthesized NFAT is required for *Nfatc1α* activation, total thymocytes were incubated in the presence of the protein synthesis inhibitor cyclohexamide (CHX) (Covert et al., 2005) at 10 μg/ml for 1 hour and then stimulated with TPA+ionomycin for two hours. The *Nfatc1α* gene expression was quantified by qPCR. Figure 2.2.9 shows that *Nfatc1α* expression was approximately 62 fold higher in cells treated with TPA+ionomycin compared to that in the cells left untreated (Figure 2.2.9, no treatment and T+I). However, *Nfatc1α* expression was about 11 fold higher in the cells pre-treated with CHX and then stimulated with TPA+ionomycin relative to that in the cells left untreated (Figure 2.2.9, no treatment and CHX+T+I), indicating that the blockade of protein synthesis reduced *Nfatc1α* expression by 82%. These data suggest that de novo NFATc1 synthesis was required for the optimal activation of *Nfatc1α* in DP thymocytes.

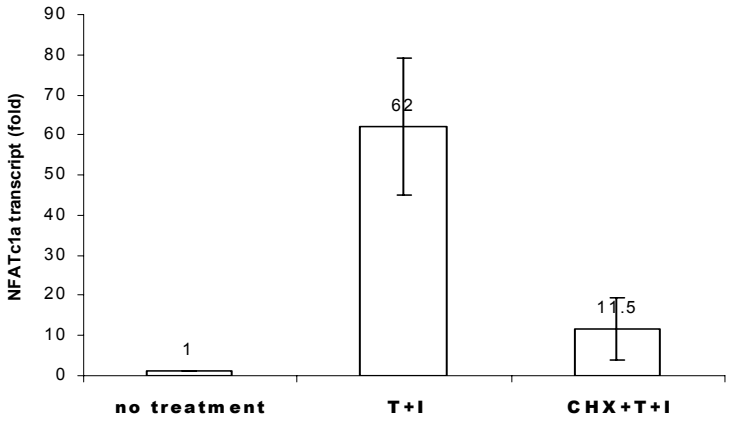

Figure 2.2.9. *Nfatc1α* activation is compromised by cyclohexamide. qPCR of *Nfatc1α* in total thymocytes treated as indicated. T+I: cells were treated with TPA+ionomycin for 2 hours. CHX+T+I: cells were treated with 10 μg/ml of cyclohexamide (CHX) for 1 hour and then stimulated with TPA+ionomycin for two hours. No treatment: cells were incubated in medium.

2.2.10 Calcium signaling is not sufficient for the optimal activation of *Nfatc1α* in DP thymocytes

To determine if calcium signaling is sufficient to activate *Nfatc1α* gene expression in DP thymocytes, we determined the effect of ionomycin on the *Nfatc1α* gene expression in total thymocytes. Total thymocytes were left untreated or treated with 0.5 μM of ionomycin for 2 hours. The *Nfatc1α* gene expression was measured by qPCR. Figure 2.2.10 shows that the *Nfatc1α* expression was increased about 3.5 fold in the cells treated with ionomycin compared to that in the untreated total thymocytes (Figure 2.2.10, no treatment and Iono). In comparison, the *Nfatc1α* expression was increased about 33 fold in the cells treated with TPA+ionomycin compared to that in the cells left untreated (Figure 2.2.10, no treatment and TPA+Iono). These data suggest that ionomycin had a weak effect on the *Nfatc1α* gene activation and that both TPA and ionomycin stimuli were needed to achieve an optimal *Nfatc1α* gene activation in DP thymocytes.

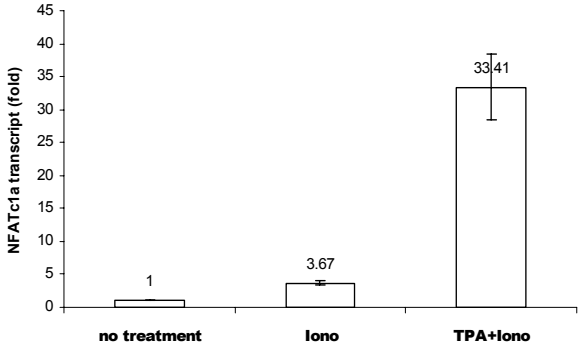

Figure 2.2.10. calcium signaling up-regulates *Nfatc1α* expression. qPCR of *Nfatc1α* expression in total thymocytes with the indicated treatment. Iono: incubated with 0.5 μM ionomycin for 2 hours. TPA+Iono: incubated with TPA+ionomycin for 2 hours. No treatment: incubated in medium for 2 hours.

2.2.11 The MAP kinase signaling pathways up-regulate *Nfatc1α* expression in DP thymocytes

Figure 2.2.7 and Figure 2.2.10 showed that calcineurin-NFAT signaling was essential but not sufficient to achieve an optimal *Nfatc1α* gene expression in DP thymocytes. These data also suggest that other signaling pathways were required for the optimal *Nfatc1α* gene activation in DP thymocytes. The activation of both MAPK signaling pathway (Daniels et al., 2006; Neilson et al., 2004) and *Nfatc1α* gene expression (Figure 2.2.6) during positive selection prompted us to investigate the possible role of the MAPK signaling pathway in *Nfatc1α* gene expression in DP thymocytes. To determine the role of MAP Kinases in *Nfatc1α* gene expression, total thymocytes were pre-treated with the MAPK inhibitors for one hour at concentrations described before and then stimulated for two hours with TPA+ionomycin. The *Nfatc1a* gene expression was measured by qPCR. As shown in Figure 2.2.11, the MEK-ERK inhibitor U0126 treatment reduced *Nfatc1α* expression by 66% (Figure 2.2.11, lanes 1 and 2), the p38 inhibitor SB203580 treatment reduced *Nfatc1α* expression by 50% (Figure 2.2.11, lanes 1 and 3), and the JNK inhibitor SP600125 treatment reduced *Nfatc1α* expression by 27% (Figure 2.2.11, lanes 1 and 4). These data suggest that all three MAP kinases were involved in the *Nfatc1α* gene activation in DP thymocytes. The role of the MEK-ERK signaling pathway appeared to be more important than the p38 signaling pathway which seemed more important than the JNK signaling pathway in *Nfatc1α* activation.

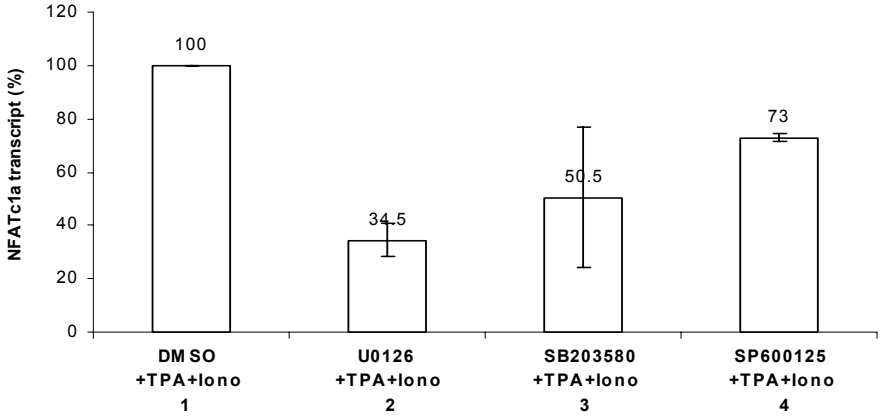

Figure 2.2.11 MAP kinase signaling pathways enhance *Nfatc1α* expression. qPCR of *Nfatc1α* expression in total thymocytes with the indicated treatment. Cells were incubated with medium (DMSO), 40 μM of U0126, 50 μM of SP600125, or 25 μM of SB203580 for one hour and then were stimulated with TPA (20 ng/ml) + ionomycin (0.5 μM) for two hours. Equal concentrations of DMSO were included in all experiments.

3. DISCUSSION

3.1 The *Rag* gene regulation in the thymus

3.1.1 The *Rag* genes are regulated differentially in DN thymocytes and in DP thymocytes

In mouse thymus, both *Rag1* and *Rag2* transcripts can be detected in the cortex but not in the medulla (Boehm et al., 1991; Brandle et al., 1992; Turka et al., 1991). Moreover, the *Rag* gene expression levels differ from stage to stage in developing thymocytes (Wilson et al., 1994). During thymocyte development, the basal level of *Rag* gene expression is detected in DN1 and DN2 thymocytes. Once DN2 thymocytes develop into DN3 thymocytes, the *Rag* gene expression is up-regulated about 10 fold (Taghon et al., 2006). After this surge, the *Rag* gene expression is down-regulated to its basal level at the DN4 stage. Upon DN thymocytes develop into DP thymocytes, the expression of both *Rag1* and *Rag2* is re-induced and the *Rag* gene expression reaches its highest level that is about 10 fold higher than that in DN3 thymocytes (Wilson et al., 1994; Yannoutsos et al., 2004). Following this second surge, the *Rag* gene expression is down-regulated again to its basal level in SP thymocytes and shut down completely in peripheral T cells (Wilson et al., 1994). The concordant expression of the *Rag1* and *Rag2* genes suggests that both genes share very similar regulation mechanisms.

In the Rag locus, the convergent *Rag1* and *Rag2* genes are separated by a 7 kb inter-genic region (IR) (Oettinger et al., 1990). The IR contains a Rag silencer which is located directly 3' of the *Rag1* gene (Yannoutsos et al., 2004). The silencer is required for down-regulating *Rag* gene expression in DP thymocytes but is ineffective in DN thymocytes (Yannoutsos et al., 2004). In addition, there is a T cell specific Rag locus regulatory region located upstream of the *Rag2* promoter (Yannoutsos et al., 2001; Yu et al., 1999). This region contains an anti-silencer element (ASE) that is located about 70 kb upstream of the *Rag2* promoter (Yannoutsos et al., 2004). The ASE is indispensable for the *Rag* gene re-induction in DP stage but dispensable for the *Rag* gene activation in DN3 stage (Yannoutsos et al., 2001; Yannoutsos et al., 2004). It has been proposed that upon DN4 thymocytes develop into DP thymocytes, the ASE is activated by so far unidentified signals. The activated ASE activates the *Rag* gene expression in DP thymocytes by counter-acting the Rag silencer activity through a long range interaction. During positive selection, the ASE is inactivated and the Rag silencer becomes active. The active Rag silencer suppresses the *Rag* gene expression in SP thymocytes (Yannoutsos et al., 2004) (Figure 3.1.1). This model suggests that the *Rag* genes are regulated differentially in DN thymocytes and in DP thymocytes. Our results that the *Rag1* and *Rag2* genes are down-regulated in DP thymocytes by TCR signaling (Figure 2.1.1.1) whereas the *Rag1* gene is up-regulated in SCB29 cells by pre-TCR signaling (Figure 2.1.8) support the notion that the *Rag* genes are regulated differentially in DN thymocytes and in DP thymocytes.

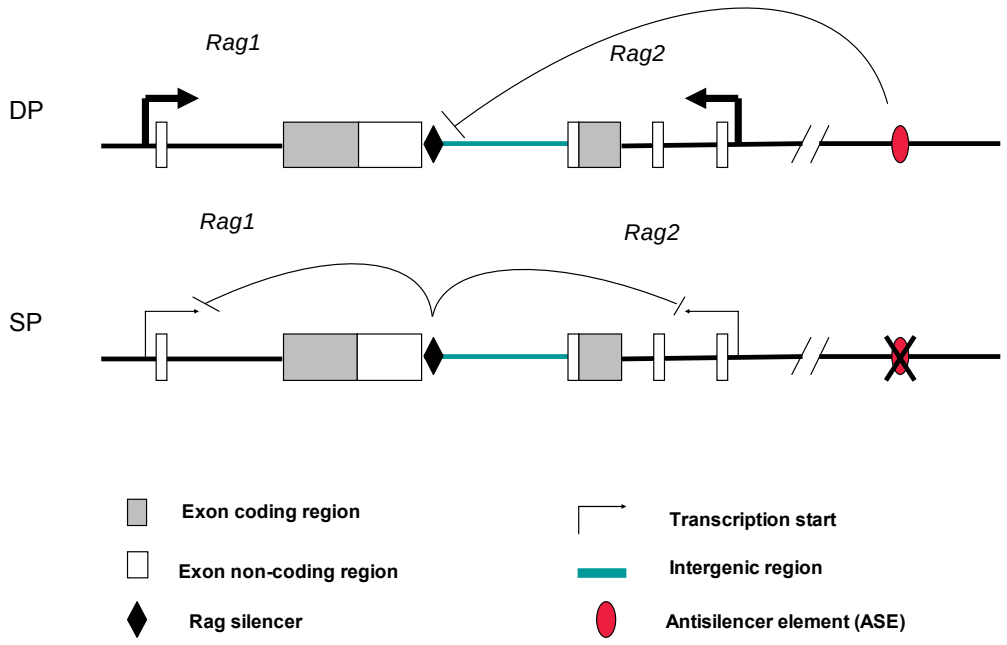

Figure 3.1.1 Long range interactions of *cis*-regulatory elements control *Rag* gene expression in the thymus. The active Rag anti-silencer element (ASE) activates *Rag* gene expression in DP cells by suppressing Rag silencer activity. In SP cells, the ASE is inactivated and the Rag silencer becomes active. The active Rag silencer keeps *Rag* gene expression at base level probably by suppressing the promoter activity (Yannoutsos et al., 2004).

3.1.2 Signaling pathways mediating *Rag* gene down-regulation in DP thymocytes

The *Rag* gene expression is down-regulated *in vivo* by TCR engagement in DP thymocytes (Brandle et al., 1992). In an *in vitro* system, the *Rag* gene down-regulation can be induced by TCR cross-linking of DP thymocytes (Turka et al., 1991) or by activating downstream TCR signaling by TPA+ionomycin stimulation of DP thymocytes (Menetski and Gellert, 1990; Turka et al., 1991). However, the exact signals that mediate the *Rag* gene down-regulation remain elusive (Nagaoka et al., 2000; Yannoutsos et al., 2004). On the other hand, during positive selection of thymocytes, the expression of both *Rag1* and *Rag2* genes is down-regulated and both MEK-ERK signaling and calcineurin-NFAT signaling are activated (Kouskoff et al., 1995; Neilson et al., 2004). We asked the

question: what is the possible connection between the signaling pathways activated by positive selection and the *Rag* gene down-regulation? We found that ionomycin, a Ca++ agonist, induced the down-regulation of both *Rag1* and *Rag2* genes (Figure 2.1.2.2A) which was attenuated by the calcineurin inhibitor FK506 in DP thymocytes (Figure 2.1.2.2B). We also found that ionomycin induced *Rag* gene down-regulation was attenuated in *Nfatc2* and *Nfatc3* double deficient thymocytes (Figure 2.1.7B), and that over-expressed NFAT enhanced the ionomycin induced *Rag* gene down-regulation in DP thymocytes (Figure 2.1.9.3). These results demonstrate that calcineurin-NFAT signaling mediates calcium signaling induced *Rag* gene down-regulation in DP thymocytes.

The identification that calcineurin-NFAT signaling participates in the *Rag* gene down-regulation in DP thymocytes prompted us to investigate if the MEK-ERK signaling pathway is also involved in the *Rag* gene down-regulation in DP thymocytes. We found that the blockade of MEK-ERK signaling by the MEK inhibitor U0126 blocked TPA+ionomycin induced *Rag1* gene down-regulation in DP thymocytes (Figure 3.1.2). One hour of pretreatment of total thymocytes with 10 μM and 20 μM U0126 didn't affect TPA+ionomycin induced *Rag1* gene down-regulation (Figure 3.1.2, lanes 1, 2, 4, and 5). However, pretreatment of total thymocytes with 40 μM of U0126 attenuated TPA+ionomycin induced *Rag1* gene down-regulation (Figure 3.1.2, lane 6). When total thymocytes were pretreated with 80 μM of U0126, TPA+ionomycin induced *Rag1* down-regulation was blocked to a large extent (Figure 3.1.2, lane 7), indicating that MEK-ERK signaling mediated TPA+ionomycin induced *Rag* gene down-regulation in DP thymocytes. As a control, 80 μM of U0126 treatment of total thymocytes didn't affect

Rag1 gene expression (Figure 3.1.2, lanes 1 and 3), indicating that MEK-ERK signals were not involved in *Rag* gene expression in resting thymocytes. These data suggest that MEK-ERK signaling pathway could play a role in TCR signaling induced *Rag* gene down-regulation in DP thymocytes. Since both MEK-ERK and calcineurin-NFAT signals are induced during thymic positive selection, it could be that TCR signaling induced *Rag* gene down-regulation during positive selection is mediated through both the MEK-ERK signaling pathway and the calcineurin-NFAT signaling pathway.

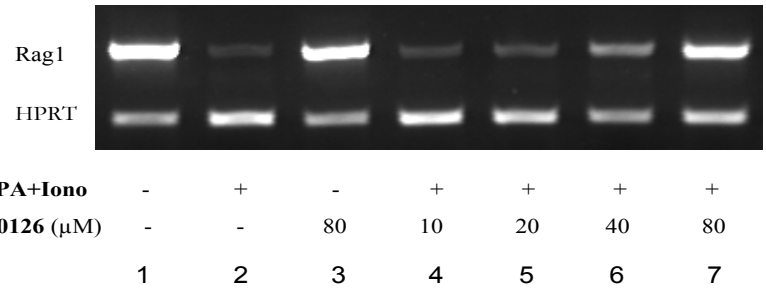

Figure 3.1.2. U0126 blocks TPA+ionomycin induced *Rag1* gene down-regulation in thymocytes. Total thymocytes were incubated in the absence or presence of U0126 at the indicated concentrations for one hour and then incubated for 2 more hours in the presence or absence of TPA (20 ng/ml)+ionomycin (0.5 μM) (TPA+Iono). *Rag1* (Rag1) expression was assayed by RT-PCR. *Hprt* (HPRT) was co-amplified and used as loading control.

To date, it is unclear what signaling mediates *Rag* gene down-regulation in DN thymocytes. It has been proposed that pre-TCR signaling could down-regulate the *Rag* gene expression in DN thymocytes (Jackson and Krangel, 2006) and that calcineurin-NFAT signaling could play a role in down-regulating *Rag1* gene expression in DN thymocytes (Patra et al., 2006). Because (1) pre-TCR signaling is turned on and NFAT is activated in SCB29 cells (Aifantis et al., 2001) and (2) *Rag1* gene expression was found

to be up-regulated in SCB29 cells (Figure 2.1.8), it is unlikely that pre-TCR signaling or calcineurin-NFAT signaling down-regulates the *Rag* gene expression this DN cell line.

3.1.3 The role of NFATs in *Rag* gene down-regulation

The expression of both *Rag1* and *Rag2* was down-regulated by the calcineurin-NFAT signaling (Figures 2.1.1.2B and 2.1.7B), suggesting that calcineurin-NFAT signaling coordinately regulates the expression of both the *Rag1* gene and the *Rag2* gene. This coordinate regulation requires a common mechanism through which NFAT can regulate the expression of both *Rag1* and *Rag2*. In the Rag locus, ASE coordinately regulates the expression of *Rag1* and *Rag2* because its activation is required for the expression of both *Rag1* and *Rag2* in DP thymocytes and its inactivation leads to down-regulation of the *Rag* genes in SP thymocytes (Yannoutsos et al., 2004). During the *Rag* gene down-regulation, both NFATc2 and NFATc3 were recruited to ASE (Figures 2.1.10.1). Therefore, it is likely that calcineurin-NFAT signaling down-regulates the expression of the *Rag1* and *Rag2* genes in DP thymocytes, at least in part, by suppressing the ASE activity.

Although the calcineurin-NFAT signaling mediated the down-regulation of both *Rag1* and *Rag2* in DP thymocytes, the *Rag1* gene expression was more effectively down-regulated by ionomycin than the *Rag2* gene (Figure 2.1.1.2A). This ionomycin induced gene down-regulation was found to be attenuated by FK506 more efficiently in the *Rag1* gene than in the *Rag2* gene (Figure 2.1.1.2B), and the ionomycin induced gene down-regulation in DP thymocytes is more effectively attenuated by NFATc2 and NFATc3

double deficiency in the *Rag1* gene than in the *Rag2* gene (Figure 2.1.7B). These results suggest that the calcineurin-NFAT differentially regulates the expression of *Rag1* and *Rag2* genes in DP thymocytes and that calcineurin-NFAT signaling has a stronger effect on the *Rag1* gene down-regulation than on the *Rag2* gene down-regulation. Since both NFATc2 and NFATc3 were recruited to the *Rag1* promoter during *Rag* gene down-regulation (Figure 2.1.5), it could be that the recruitment of NFATc2 and NFATc3 to the *Rag1* promoter confer the *Rag1* gene greater sensitivity in response to calcineurin-NFAT signaling induced gene down-regulation.

3.1.4 The interaction of NFATs with the *Rag1* promoter

The interaction of two and more NFAT members with promoters has more profound effects on immune responses than the individual NFAT member does (Peng et al., 2001; Ranger et al., 1998b). For example, IL-2 expression is not impaired in T cells when either NFATc1 or NFATc2 is inactivated (Hodge et al., 1996; Ranger et al., 1998a; Xanthoudakis et al., 1996; Yoshida et al., 1998). However, IL-2 production is ablated in NFATc1 and NFATc2 double deficient T cells (Peng et al., 2001). In contrast, IL-4 gene expression is not affected in T cells when either NFATc2 or NFATc3 is inactivated (Hodge et al., 1996; Oukka et al., 1998). However, IL-4 is extremely over-produced in NFATc2 and NFATc3 double deficient T cells (Ranger et al., 1998). These mouse models establish that a synergistic interaction between NFAT members is desirable in conferring efficient gene regulation. In case of *Rag* gene regulation, although inactivation of NFATc3 had a weak attenuation effect on the ionomycin induced *Rag* gene down-regulation in DP thymocytes, the inactivation of NFATc2 had no effect on ionomycin

induced *Rag* gene down-regulation (Figure 2.1.6). However, inactivation of both NFATc2 and NFATc3 in thymocytes led to an apparent attenuation of ionomycin induced *Rag* gene down-regulation in DP thymocytes (Figure 2.1.7B). These results further support the notion that a synergistic activity between NFAT members is important in regulating immune responses *in vivo*.

3.1.5 Binding site selectivity by NFAT members

Each NFAT member has a different binding affinity to a unique NFAT site (Rao et al., 1997). In the absence of AP-1, NFATc2 has the highest binding affinity to both the distal IL-2 promoter NFAT site and the proximal IL-4 promoter NFAT site, whereas NFATc3 has the lowest binding affinity to these NFAT sites. The binding affinity of NFATc1 to these NFAT sites is between that of NFATc2 and NFATc3. However, in the presence of AP-1, all these NFAT members show very similar binding affinities to the distal IL-2 promoter NFAT site (Hoey et al., 1995). These data demonstrate the importance of AP-1 in determining the binding site specificity in NFAT-AP-1 complexes. In addition, when over-expressed in non-lymphocytes, NFATc1, NFATc2, and NFATc3 have very similar binding affinities to the distal IL-2 NFAT site. However, NFATc1 has a very low binding affinity to the IL-4 NFAT site compared to NFATc2 and NFATc3 (Ho et al., 1995). These data demonstrate the NFAT site selectivity of different NFAT members. Moreover, in nuclear protein extracts prepared from T cells activated by TPA+ionomycin, NFATc2 had a strong binding affinity to the *Egr3* NFAT site whereas NFATc1 showed no binding activity at all to the same site (Rengarajan et al., 2000). All these data establish that different NFAT members have their distinct NFAT site preferences. The reason for this

distinct site usage by different NFAT members is unknown (Rao et al., 1997). We found that, in protein extracts prepared from untreated thymocytes, NFATc2 bound to NFAT227 but neither NFATc1 nor NFATc3 bound to this same site (Figure 2.1.4) whereas the NFATc2 and NFATc3 bound to NFAT338 but NFATc1 did not (Figure 2.1.4). Neither NFAT-NFAT227 complex nor NFAT-NFAT338 complexes contained AP-1 binding partner (Figure 2.1.4). These data provide further evidence that NFAT members have their binding site selectivity if AP-1 protein is not present in the NFAT containing complex.

3.1.6 NFAT dimerization

NFATs form a monomer on the distal IL-2 NFAT site because the NFAT site has one GG sequence to interact with one NFAT molecule whereas NFATs form a dimer on NF-κB-like sites because the NF-κB-like site bears two GG nucleotides, each of which can interact with one NFAT molecule (Chen et al., 1998; Giffin et al., 2003; Jin et al., 2003; Zhou et al., 1998). We found that NFAT formed a dimer with NFAT338, and mutation of a single GG site in NFAT338 is sufficient to disrupt NFAT dimer formation, demonstrating that NFAT338 is a very unique NFAT binding site in which one GG site mediates NFAT dimer formation.

3.2 *Nfatc1a* gene regulation in the murine thymus

3.2.1 The gene expression of NFAT members is differentially regulated during thymocyte development

Nfatc1, *Nfatc2*, and *Nfatc3* are expressed in DN3 thymocytes (Figure 2.2.1). We found that pre-TCR signaling up-regulated *Nfatc1* and *Nfatc2* gene expression but had no effect on *Nfatc3* gene expression (Figure 2.2.1). During positive selection of thymocytes, the expression of both *Nfatc1* and *Nfatc2* was up-regulated but the *Nfatc3* expression was down-regulated (Figure 2.2.5). These data demonstrate that pre-TCR signaling and TCR signaling differentially regulate individual NFAT member expression during thymocyte development.

3.2.2 *Nfatc1α* activation during thymocyte development

Nfatc1 transcription is controlled by two promoters P1 and P2. The P1 promoter directs α isoform mRNA synthesis whereas the P2 promoter directs β isoform mRNA synthesis (Chuvpilo et al., 2002; Serfling et al., 2006a). In DN thymocytes, pre-TCR signaling activated *Nfatc1α* expression but not *Nfatc1β* expression (Figure 2.2.2), and in DP thymocytes, TCR signaling also activated *Nfatc1α* but not *Nfatc1β* (Figure 2.2.6). These data indicate that *Nfatc1α* is activated by both pre-TCR signaling and TCR signaling during thymocyte development. Because both pre-TCR signaling and TCR signaling are critical for thymocytes to survive (Aifantis et al., 2006; Werlen et al., 2003), it seems that during thymocyte development, *Nfatc1* gene activation may be related to thymocyte

survival or may be involved in an anti-apoptosis mechanism as proposed by Chuvpilo et al. (2002).

3.2.3 Signaling pathways regulating *Nfatc1a* expression in DN thymocytes

Pre-TCR initiated signaling is essential for the survival, proliferation and differentiation of DN thymocytes (Aifantis et al., 2006). Pre-TCR is a ligand-free cell receptor which could transduce signals in a ligand-independent manner (Irving et al., 1998). Pre-TCR expression was sufficient to activate *Nfatc1a* gene expression in DN thymocytes without any extra stimulation (Figure 2.2.2) which indicates that pre-TCR initiates autonomous signaling. During DN cell development, pre-TCR signaling activates both MER-ERK signaling and JNK signaling pathways (Crompton et al., 1996; Michie et al., 1999; Murga and Barber, 2002). We found that MER-ERK signaling activated *Nfatc1a* expression in DN thymocytes (Figure 2.2.4) whereas JNK signaling suppressed *Nfatc1a* expression in DN thymocytes (Figure 2.2.4), demonstrating opposing effects of these two signaling pathways on *Nfatc1a* expression in DN thymocytes. Although pre-TCR signaling activates p38 signaling and calcineurin-NFAT signaling pathways (Aifantis et al., 2001; Murga and Barber 2002), we found that these two signaling pathways were not involved in *Nfatc1a* gene expression in DN thymocytes (Figures 2.2.3 and 2.2.4). It appears that it is the balance between MEK-ERK signaling pathway and JNK signaling pathway that plays an important role in maintaining *Nfatc1a* expression level in DN thymocytes.

3.2.4 Autoregulation of *Nfatc1a* during thymic positive selection in DP thymocytes

During thymocyte development, DP thymocytes with low affinity to self peptide-MHC complexes are rescued from apoptosis by positive selection (Werlen et al; 2003). Positive selection activates TCR signaling (Daniels et al., 2006) and we found that *Nfatc1a* gene expression was activated in DP thymocytes during positive selection (Figures 2.2.6 and 2.2.7), suggesting that TCR signaling could activate *Nfatc1a* gene expression in DP thymocytes. Since NFAT was essential for *Nfatc1a* gene activation in DP thymocytes (Figure 2.2.7) and both NFATc2 and NFATc3 were not involved in *Nfatc1a* gene activation during positive selection (Figure 2.2.8), NFATc1 is the only NFAT protein which is required to mediate the TCR signaling for the induction of *Nfatc1a* gene expression in DP thymocytes. Moreover, de novo NFATc1 but not pre-existing NFATc1 was required for optimal *Nfatc1* gene activation (Figure 2.2.9), indicating that NFAT positive feedback regulation played a crucial role in achieving optimal *Nfatc1* gene expression in DP thymocytes. These results establish that during positive selection, the pre-existing NFATc1 initiates *Nfatc1a* gene expression and the subsequently expressed NFATc1 mediates positive feedback through which *Nfatc1a* gene is activated to its optimal expression in DP thymocytes.

3.2.5 The role of MAPK signaling pathway in *Nfatc1a* gene activation

Although NFATc1 was essential for its own gene activation, calcineurin signaling alone was not sufficient to achieve an optimal *Nfatc1a* gene expression in DP thymocytes (Figure 2.2.10). The optimal *Nfatc1a* gene expression was achieved by combined stimulation of TPA and ionomycin (Figure 2.2.10), demonstrating the importance of a

synergistic interaction between the signaling activated by TPA and the signaling activated by ionomycin in *Nfatc1a* gene activation. TPA activated MAPK signaling pathway is critical in activating the NFAT trans-activation domain (Avots et al., 1999; Okamura et al., 2000; Round et al., 2007; Woodrow et al., 1993a; Woodrow et al., 1993b). TCR signaling activates the MAPK signaling pathway during positive selection (Werlen et al., 2003; Daniels et al., 2006) and we found that the MAPK signaling enhanced *Nfatc1a* gene expression in DP thymocytes (Figure 2.2.11), suggesting that the MAPK signaling plays a role in mediating TCR signaling induced *Nfatc1a* gene activation in DP thymocytes. It is likely that during positive selection, calcineurin transports pre-existing NFATc1 into the nucleus where calcineurin together with the MAPK signaling activates NFATc1 trans-activation activity. The activated pre-existing NFATc1 initiates *Nfatc1* gene expression. In the presence of calcineurin and the MAPK signaling, the newly expressed NFATc1 is activated *in situ*. The activated newly expressed NFATc1 activates its own gene expression through NFATc1 mediated positive feedback regulation.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 General materials

96 well qPCR plates,	ABgene
Absolute QPCR seal	ABgene
Cell strainer (70 µm)	Falcon
Cryotubes 2 ml	Greiner bio-one
Disposable needles, cuvette and syringes	Hartenstein
Glasswares	Schott
Nitrocellulose membrane	Schleicher & Schuell
Parafilm	Hartenstein
Pipette tips	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

4.1.2 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
Calcium chloride	Carl Roth
Chloroform	Carl Roth
Coomassie brilliant blue R-250	Roche
Cyclohexamide	Sigma
Cyclosporin A	Novartis Pharma
DEPC	Carl Roth
Disodium hydrogen phosphate	Merck
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
FK506	Sigma
Formaldehyde	Carl Roth
Gel extraction kit	Qiagen, Genomed
Glutathione sepharose	Sigma-Aldrich
Glycerol	Carl Roth
Glycin	Merck
Hepes	Carl Roth
Hydrochloric acid	Merck
Ionomycin	Sigma-Aldrich
IPTG	Boehringer-Ingelheim
Isoamyl alcohol	Carl Roth
L-glutamine	Gibco BRL
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
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 orthovanadate	Fluka
Sodium pyruvate	Gibco BRL
SP600125	Calbiochem
Streptomycin	Hoechst

TaqDyePrimer sequencing kit	Perkin Elmer
TEMED	Carl Roth
TPA	Sigma Aldrich
Trichloroacetic acid	Sigma Aldrich
Tris	Carl Roth
Triton X-100	Sigma Aldrich
Trizol reagent	Invitrogen
Trypan blue	Gibco BRL
Tween-20	Carl Roth
U0126	Calbiochem

4.1.3 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 blocks	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
Microcentrifuge	Eppendorf
pH meter	Ingold
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
Varifuge 3.0R	Haraeus
Vortexer	Hartenstain
Water bath	Hartenstain
Water filtration unit	Milipore
Western blot apparatus	Hoefer, BioRad

4.1.4 Kits

First Strand cDNA Synthesis Kit	MBI Fermentas
NucleoBond®	Macherey-Nagel
QIAEXII	Qiagen
QuantiTect™SYBR® Green PCR	Qiagen

4.1.5 Reagents

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

4.1.6 Antibodies

Anti-HA epitope monoclonal clone HA.11	BabCO
Anti-mouse CD69-PE	BD Pharmingen
Anti-mouse IgG-HRP	Amersham Pharmacia
Anti-mouse NFATc1 polyclonal rabbit	ImmunoGlobe
Anti-mouse NFATc2 polyclonal rabbit	ImmunoGlobe
Anti-mouse NFATc3 polyclonal rabbit	Santa Cruz
Anti-mouse TCRβ-biotin	BD Pharmingen
Streptavidin-PE	BD Pharmingen

4.1.7 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° C.

RT-PCR		
RAG1F	CCAAGCTGCAGACATTCTAGCACTC	
RAG1R	CTGGATCCGGAAAATCCTGGCAATG	
HPRTF	GCT GGT GAA AAG GAC CTC TC	
HPRTR	CAC AGG ACT AGA ACA CCT GC	
qPCR		
qGAPDHF	ATCACTGCCACCCAGAAGAC	qGAPDHF/ qGAPDHR Ef = 1.0
qGAPDHR	CACATTGGGGGTAGGAACAC	
qRAG2F	CCTGGCTTGGCCGAAAGGATTCA	qRAG2F/qRAG2R Ef=0.85
qRAG2R	CTTCCTGCTTGTGGATGTGAAA	
qRAG1F	GCTATCTCTGTGGCATCGAGTG	qRAG1F/qRAG1R Ef=0.91
qRAG1R	GGTGTTGAATTTCATCGGGTG	
qNFATc1Ex1F	CGGGAGCGGAGAAACTTTGC	qNFATc1Ex1F/qNFATc1Ex3R Ef=1.0
qNFATc1Ex2F	AGGACCCGGAGTTCGACTTC	qNFATc1Ex2F/qNFATc1Ex3R Ef=0.95

qNFATc1Ex3R CAGGGTCGAGGTGACACTAG
ChIP
ChIP-ASE-DF TAATGAGTCTGCCATTCACCAAGC
ChIP-ASE-DR GGAAAACTTCCAGTAATCCAGGAGTC
ChIP-RAG1F GCATCCATTGCTGGTTTCCATTGTTGGCTC
ChIP-RAG1R AGTCTAAGCTTCAAGAGTTACCTTGCTCCACAG
EMSA
ASE-D2F CACCATAATATTTTTCCTTCTTTTCATCAA
ASE-D2R TTGATGAAAAGAAGGAAAAATATTATGGTG
mASE-D2F CACCATAATATTTTTGGTTCTTTTCATCAA
mASE-D2R TTGATGAAAAGAACCAAAAATATTATGGTG
ASE-D3F ATTACTGGAAGTTTTCCTTATGGAGTGTAA
ASE-D3R TTACACTCCATAAGGAAAACTTCCAGTAAT
mASE-D3F2 ATTACTCCAAGTTTTCCTTATGGAGTGTAA
mASE-D3R2 TTACACTCCATAAGGAAAACTTGGAGTAAT
mASE-D3F3 ATTACTCCAAGTTTTGGTTATGGAGTGTAA
mASE-D3R3 TTACACTCCATAACCAAAACTTGGAGTAAT
Pu-boxdf CCAAAGAGGAAAATTTGTTTCATACAGAA
Pu-Boxdr TTCTGTATGAAACAAATTTTCCTCTTTGG
227m ATGCCTACGGTGGAAATGATATCGTCACTC
227p GAGTGACGATATCATTTCCACCGTAGGCAT
m227m ATGCCTACGGTCCAAATGATATCGTCACTC
m227p GAGTGACGATATCATTTGGACCGTAGGCAT
338m GAGCCAACAATGGAAACCAGCAATGGATGC
338p GCATCCATTGCTGGTTTCCATTGTTGGCTC
m338m GAGCCAACAATCCAAACCAGCAATGGATGC
m338p GCATCCATTGCTGGTTTGGATTGTTGGCTC
HIV-1f AATGGGGACTTTCCAAA
HIV-1r TTTGGA AAGTCCCCATT
hIL-8f TTGAGGAATTTCCATT
hIL-8r AATGGAAATTCCTCAA

The primers listed below were purchased from Qiagen.

Primer	Catalog number
Nfatc1	QT00167692
Nfatc2	QT00136647
Nfatc3	QT00176218

4.1.8 Antibiotics

Ampicillin	Hoecher
G-418	Invitrogen
Streptomycin	Hoecher
Zeocin	Invitrogen

4.1.9 Solutions and buffers

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

4.1.9.1 Gernaral solutions

APS solution	1 %
$CaCl_2$	2 M
DTT	1 M
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
$MgCl_2$	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 %

X-gal 20 mg/ml

4.1.9.2 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

Sonication 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	1 mM
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	20 mM
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
Tween-20 1 %
Nonfat dried milk 1 %

Western blot washing buffer
TBS pH7.4
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 %

4.1.10 Growth medium

4.1.10.1 Cell culture medium

RPMI1640 plus

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

4.1.10.2 Bacterial culture medium

LB broth

LB agar (1.5 %)

4.1.11 Bacterial strains

E.coli strains TopF, DH5α, and XL-1 blue were purchased from Stratagene.

4.1.12 Mammalian cell lines

VL3-3M2	Dr.Guidos, University of Toronto

4.1.13 Mice

BL6 wild-type	Charles River
Balb/c wild-type	Charles River
Balb/c NFATc2 deficient mice	Generated in this Lab
Balb/c NFATc2 deficient mice	Dr. Glimcher, Harvard Medical School
Balb/c NFATc3 deficient mice	Dr. Glimcher, Harvard Medical School

4.1.14 Plasmids

pGEM-GST-RSD	Constructed in this Lab
pME-HA-caNFATx1	Dr. Miyatake, Tokyo Metropolitan Institute of Medical Science
pMSCV-neo	Clonetech
pEGZ-HA-caNFATc2	Constructed in this Lab
pMSCV-neo-caNFATx1	Constructed in this Lab

4.2 Methods

4.2.1 Bacterial manipulation

4.2.1.1 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 200 µl of bacterial storage solution (80% medium and 20% glycerol) and stored at – 70 ^{o}C.

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

A single colony of TopF was inoculated into 50 ml of antibiotics-free LB broth, grew at 37 ^{o}C with shaking overnight. Next day, 30 ml of the culture was 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.

4.2.1.3 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 shake-cultured at 37^{o} C for one hour. 100 µl of transformed bacteria were plated onto LB agar plates containing 50 µg/ml ampicillin and the plates were incubated at 37 ^{o}C for 16 hours.

4.2.2 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 shake-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 transfered into a quartz cuvette and the OD260 of the sample was taken. 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.

4.2.2.3 Polymerase chain reaction (PCR) of DNA fragments (Fermantas PCR kit)

50 µl PCR reaction containing 1x PCR buffer, 200 µM dNTP, 40 pmol primer each, 1 U Taq DNA polymerase, and DNA template was run 30 cycles in the MWG thermal cycler at 94 ^{o}C for 30 seconds, 60 ^{o}C for 45 seconds, and 72 ^{o}C for 45 seconds. The PCR products were resolved on an agarose gel. For ChIP assays, 1µl of DNA was used for one PCR reaction with primers ChIP-ASE-DF and ChIP-ASE-DR for ASE ChIP and primers ChIP-RAG1F and ChIP-RAG1R for the *Rag1* promoter ChIP. For RT-PCR, cDNA was adjusted to equal concentrations and 2 µl cDNA was used for one PCR reaction with the primers RAG1F and RAG1R for *Rag1* and HPRTF and HPRTR for *Hprt*.

4.2.2.4 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 was incubated at 37 ^{o}C for 1 hours to overnight. The reaction was resolved on an agarose gel.

4.2.2.5 Agarose gel electrophoresis of DNA

A proper concentration of agarose in 1x TAE buffer was melted and casted in the gel casting mould. 0.5 µg/ml of EtBr was included in the gel. DNA markers and DNA 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 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 Ependorf 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 transfered to a fresh Eppendorf tube and stored at -20 ^{o}C.

4.2.2.7 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 was incubated at 16 ^{o}C for 4 hours to overnight. The ligation product was transformed into competent bacterial cells.

4.2.2.8 Construction of pMSCV-neo-HA-caNFATx1

A 3.4 kb DNA fragment digested from pME-HA-caNFATx1 by EcoRI and XhoI was ligated to pMSCV-neo EcoRI-XhoI site to generate pMSCV-neo-HA-caNFATx1. The positive clones were screened by restriction enzyme digestion.

4.2.3 RNA methods

4.2.3.1 Total RNA isolation (Trizol method)

Up to $1x10^7$ cells were homogenized in 1 ml of TRI reagent and left at RT for 10 minutes. The phases were separated by adding 0.2 ml of chloroform and centrifugation. The 0.5 ml of water phase was transferred to a fresh tube and mixed with 0.5 ml of 2-propanol. The precipitate was collected by centrifugation. The pellet was washed once with 1 ml of 70% ethanol, air-dried, and dissolved in 20 µl of DEPC treated ddH_2O.

4.2.3.2 First strand cDNA synthesis (Fermentas first strand cDNA synthesis kit)

11 ul solution containing 0.5- 1.0 µg of RNA and 1 µl of random primers was denatured at 70 ^oC for 5 minutes and quenched on ice for 5 minutes. 4 µl of 5x reaction buffer, 2 µl of 10 mM dNTPs, 1 µl of RNase inhibitor, and 2 µl of reverse transcriptase were added and mixed well. The reaction was incubated at RT for 5 minutes and then at 37 ^oC for 60 minutes. The cDNA synthesized was diluted to 5 ng/ µl of input RNA with TE buffer and stored at -20 ºC.

4.2.3.3 Quantitative PCR (qPCR, Qiagen SYBR method)

Master mix of qPCR reaction was prepared as listed below. 23 µl of master mix was mixed with 2 µl of cDNA in a well of 96 well qPCR plates. All qPCR reactions were done in duplicate. PCR was run for 40 cycles at 94 ºC for 15 seconds followed by at 60 ºC for 1 minute in TaqMAN. Primers used for *Gapdh*, *Nfatc1a*, *Nfatc1b,* and *Rag1* quantification were diluted to 5pmol/µl/each in TE buffer. *Rag2* primers were diluted to 20 pmol//µl/each. Commercial primers (NFATc1, NFATc2, and NFATc3) were used according to manufacturer's instruction.

Recipe for preparing master mix for qPCR	
2x SYBR	12.5 µl
Primer mix	2.5 µl
ddH_2O	8 µl

The qPCR data were used to calculate the threshold cycle (C_t) by ABI Prism 7000 SDS software. The relative gene expression levels were calculated from the C_t by Microsoft Excel according to the formula described below (Pfaffl, 2001). *Gapdh* was used as reference gene.

Formula for calculating the relative gene expression levels

$$\text{ratio} = \frac{(\mathbf{E}_{\text{target}})^{\Delta Ct(\text{control-sample})}}{(\mathbf{E}_{\text{ref}})^{\Delta Ct\,(\text{control-sample})}}$$

where ($\mathbf{E}_{\text{target}}$): amplification efficiency of target gene primers
($\mathbf{E}_{\text{ref}}$): amplification efficiency of reference gene primers

4.2.4 Protein methods

4.2.4.1 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 shake-cultured at 37^{o} C overnight. Next day, the culture was transferred to 1500 ml of ampicillin-containing LB broth which was then shake-cultured at 37 ^{o}C until OD595 ≈ 0.5 - 1.5. The protein was expressed by 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 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.

4.2.4.2 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.

4.2.4.3 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.

4.2.4.4 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		
	10% separating	5% stacking
Reagent	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

4.2.4.5 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 hour. 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: 2000 for anti-HA 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 hours. 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.

4.2.4.6 Electrophoretic mobility shift assay (EMSA)

4.2.4.6.1 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, 1 pmol of double strand oligo, 1 µl of T4 kinase, and 3-5 µl of ^{32}P γATP was incubated at 37 ^{o}C for two hours. The reaction was stopped by 1 µl of 0.5 M EDTA, pH 8.0.

4.2.4.6.2 Purification of labeled probes

Purification on a 10% native DNA gel. 20 µl of labeling reaction were loaded onto a 10 % native DNA gel which was then run in 0.5x TBE at 150V 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.

4.2.4.6.3 Binding reaction

A 20 µl binding reaction contains 1x binding buffer, 2 µl poly dI·dC, 4 µl protein, and 4 µl probe. The reaction was incubated on ice for 30 minutes. In competition assays, 5 pmol of double strand oligo (5 pmol/1 µl) was 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 20 µl. The reaction was resolved on a 6% native PAGE.

4.2.4.6.4 Native gel electrophoresis of protein-DNA complexes

A 6% native PAGE gel was casted according to the recipe listed below in the gel casting mould. 20 µl of binding reaction was loaded onto the gel and the electrophoresis was carried out in 0.5X TBE at 200V 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 10 ml native gel		
Gel concentration	6%	10%
Reagent	volume (ml)	
ddH_2O	7.4	6.4

40% gel stock	1.5	2.5
5x TBE	1	1
10% APS	0.1	0.1
TEMED	0.01	0.01

4.2.4.7 Chromatin immuno-precipitation (ChIP) assay (Wells and Farnham, 2002)

4.2.4.7.1 Chromatin preparation

$2x10^8$ thymocytes 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 sonication 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 transfered to a 1.5 ml fresh Eppendor tube. The OD260 of the chromatin solutions was taken and the chromatin solutions were adjusted to equal OD260 units with sonication buffer and stored at -70 ^oC.

4.2.4.7.2 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 thymocytes in 300 µl was incubated with 2 µl of anti-NFATc1 antibody, 2 µl of anti-NFATc2 antibody, or 4 µl of anti-NFATc3 antibody 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 sonication 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 ^{o}C for 5 hours and extracted once with PCI. The water phase was precipitated by 70 % Ethanol plus 0.1 M NaAc, pH 5.2. The precipitated DNA was washed once with 70% ethanol, and dissolved in 50 µl of TE and stored at – 20 ^{o}C.

4.2.5 Cell culture methods

4.2.5.1 Storage of cell lines

VL3-3M2 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 ^{o}C.

4.2.5.2 Maintenance of cell lines

The cell culture work was done under sterile conditions in laminar hoods. All cells were cultured at 37°C in a humidified tissue culture incubator. To start a cell culture, the frozen cells were thawed in a 37 ^{o}C 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.

4.2.5.3 Electroporation of VL3-3M2 cells (Trinh et al., 2001)

The day before experiment, VL3-3M2 cells were seeded at 2 x 10^5 /ml. The next day, 1x 10^7 cells were collected and resuspended in 0.4 ml of electroporation solution (complete RPMI1640 containing 16.6% FCS) and transferred to an electroporation cuvette. The cuvette was then cooled on ice for 10 minutes. 50 µg of plasmid DNA were added to the

cells and the cells were electroporated at 975 μF and 300V. After electroporation, the cells were left at RT for 10 minutes and then transferred to 2 ml of culture medium in a 6 well cell culture plate and incubated at 37 ^{o}C.

4.2.5.4 Cloning of stably transfected cells

24 hours after electroporation, the cells were supplemented with 600 μg/ml of G418 for pMSCV-neo plasmid selection or 3 mg/ml of zeocin for EGZ plasmid selection. Seven days later, the survival cells were counted and diluted to 10 cells /ml. 0.1 ml of cells was transfered to each well of 96 well plates. The plates were cultured until colonies can be seen under light microscope. 20 clones from each cell cloning experiment were screened for desired protein expression by Western blotting. The positive clones were amplified and frozen in frozen solution at -70 ^{o}C.

4.2.5.5 Mouse thymocyte preparation

4-8 week-old mice were used in the experiments. A mouse was killed by neck dislocation and the skin sterilized by 70% ethanol. The chest was opened using standard surgical operation. The thymus was taken carefully from the chest to avoid damaging the large blood vessels. The thymuses were kept in 3 ml of complete RPMI1640 either at RT or on ice. Single cell suspensions were obtained by forcing the thymus pass through a 40 μm cell strainer.

4.2.5.6 Thymocyte staining

Up to 10^8 thymocytes in 3 ml of cold FACS buffer were mixed with 10 µl of anti-mouse TCRβ-biotin and incubated on ice for 30 minutes. The cells were washed once in 30 ml of cold FACS buffer and resuspended in 3 ml of cold FACS buffer. 8 µl of streptavidin-PE and 50 µl of anti-mouse CD69-PE were mixed with the cells and incubated on ice for 30 minutes. The cells were washed twice in 30 ml of cold FACS buffer and resuspended in 4 ml of cold FASC buffer. The stained cells were sorted on FACSCalibur in the Institute of Virology and Immunology, University of Würzburg, Würzburg, operated by Dipl. Biol. Lindon.

4.2.5.7 Cell stimulation

In stimulation experiments, 1x 10^7 of thymocytes in 2 ml of complete RPMI1640 or 5x 10^6 of VL3-3M2 cells in 10 ml of complete RPMI 1640 were pre-cultured at 37 ^oC for 30 minutes. 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 37 ^oC for 2 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 20 ml of complete RPMI1640 were incubated with TPA+ionomycin as described above.

5. SUMMARY

In this thesis we have investigated the effect of NFAT (Nuclear Factor of Activated T Cell) transcription factors on the expression of *Rag*-(Recombination Activating Genes) genes in murine thymus. The protein products of *Rag* genes, RAG1 and RAG2, are critical for the recombination and generation of the TCR (T Cell Receptor) repertoire during thymocyte development, and their expression can be suppressed by the activity of NFAT factors.

In thymus, the expression of *Rag1* and *Rag2* genes is induced at the double-negative (DN, $CD4^-8^-$) 3 stage, down-regulated at the DN4 stage, re-induced at the double-positive (DP, $CD4^+8^+$) stage, and suppressed again at the single-positive (SP, $CD4^+8^-$ or $CD4^-8^+$) stage. Although it is known that TCR signaling suppresses the expression of *Rag1* and *Rag2* at the SP stage, the signals that mediate the *Rag* gene down-reulation remain elusive. Here we report that both the calcineurin-NFAT-signaling and MAPKinase signaling pathways, which are activated by TCR signaling during positive selection, mediate the *Rag* gene down-regulation in DP thymocytes. The calcineurin-NFAT pathway suppresses both the *Rag1* and the *Rag2* gene expression. This pathway has a stronger suppressive effect on the *Rag1* than the *Rag2* gene. A synergistic activity between the two NFAT factors NFATc2 and NFATc3 is essential for calcineurin-NFAT signaling to efficiently suppress the *Rag* gene expression in DP thymocytes. It is likely that the calcineurin-NFAT signaling down-regulates *Rag* gene expression by suppressing both the Rag anti-silencer element (ASE) activity and the *Rag* promoter activity. Similarly, MEK-ERK signaling of MAPK signaling pathway mediates the *Rag* gene suppression in DP thymocytes although the mechanism through which MEK-ERK

mediates the *Rag* gene down-regulation has to be elucidated. In DN thymocytes, it appears that neither the calcineurin-NFAT signaling nor MAPK signaling is involved in the *Rag* gene down-regulation. However, a role for these two signaling pathways in the *Rag* gene up-regulation in DN thymocytes is not excluded.

In DN thymocytes, pre-TCR signaling stimulates the expression both *Nfatc1* and *Nfatc2* genes but has no effect on *Nfatc3* gene expression. In DN thymocytes, pre-TCR signaling activates *Nfatc1α* expression but not *Nfatc1ß* expression, i.e. the two promoters controling *Nfatc1* gene xpression are differently controled by pre-TCR signals. *Nfatc1α* gene expression in DN thymocytes is mainly regulated by the MAPK signaling pathway because activation of *Nfatc1α* is mediated by MEK-ERK signaling but opposed by JNK signaling. Calcineuirn-NFAT and p38 signaling pathways are not involved in *Nfatc1α* promoter regulation in DN thymocytes. In DP thymocytes, TCR signaling up-regulates *Nfatc1* and *Nfatc2* expression but down-regulates *Nfatc3* expression. In DP thymocytes, TCR signaling activates *Nfatc1α* expression. The activation of *Nfatc1α* in DP thymocytes is mediated by NFATc1, but not or to a less degree by NFATc2 and NFATc3. MEK-ERK, JNK, and p38 signaling pathways are involved in *Nfatc1α* gene activation in DP thymocytes, probably by activating NFAT trans-activation activity. All these findings illustrate that in thymocytes the expression of NFAT transcription factors – which are essential for thymic development - is controled at multiple levels.

Zusammenfassung

Wir haben in den experimentellen Arbeiten zu dieser Dissertation den Effekt der NFAT (`Nuclear Factor of Activated T Cell`)-Transkriptionsfaktoren auf die Expression der Rag (`Recombination Activating`)-Gene im Thymus der Maus untersucht. Die Proteine der beiden *Rag*-Gene, RAG1 und RAG2, sind entscheidend für die Bildung des TCR (`T Zell-Rezeptor`)-Repertoires, und ihre Expression wird durch die NFATs supprimiert.

Während der Thymozyten-Entwicklung wird die Expression der *Rag1*- und *Rag2*-*Gene* in DN (`double negative`, $CD4^-8^-$) 3-Thymozyten induziert, in DN4-Thymozyten „herunterreguliert“, re-induziert in DP (`double positive`, $CD4^+8^+$)-Thymozyten und supprimiert in SP (`single positive`, $CD4^+8^-$ oder $CD4^-8^+$) Thymozyten. Obwohl bekannt ist, dass der TCR-Signalweg die Expression von *Rag1* und *Rag2* in SP-Thymozyten supprimiert, sind die daran beteiligten Signale weitgehend unbekannt. In der vorliegenden Arbeit wurde gezeigt, dass sowohl der Calcineurin-NFAT-Signalweg als auch der MAP Kinase-Signalweg die *Rag*-Gen- „Herunterregulierung“ in DP-Thymozyten vermitteln. Beide Wege werden über TCR-Signale während der positiven Selektion aktiviert. Der Calcineurin-NFAT-Signalweg supprimiert die Genexpression von *Rag1* und *Rag2*, wobei *Rag1* stärker betroffen ist. Dabei ist eine synergistische Aktiviät zwischen den beiden NFAT-Transkriptionsfaktoren NFATc2 und NFATc3 im Calcineurin-NFAT-Signalweg notwendig, um die *Rag* Genexpression in DP Thymozyten „herunterzuregulieren“. Der Calcineurin-NFAT-Signalweg reguliert offensichtlich die *Rag*-Genexpression durch eine Unterdrückung der *Rag* `anti-silencer-element`-(ASE)-Aktivität und der *Rag*-Promotoraktivität. Auch die MEK-ERK-Signalkaskade des MAPK-Signalwegs ist an der Suppression des *Rag*- Gens in DP Thymozyten beteiligt,

wobei der Mechanismus zu untersuchen bleibt. In DN-Thymozyten hingegen scheinen weder der Calcineurin-NFAT-Signalweg noch der MAPK-Signalweg an der Hemmung der *Rag*-Genaktivität beteiligt zu sein. Eine Beteiligung dieser beiden Signalwege bei der *Rag*-Gen-Aktivierung in DN-Thymozyten kann hingegen nicht ausgeschlossen werden.

In DN-Thymozyten regulieren Prä-TCR-Signale eine stärkere Expression der beiden NFAT-Faktoren *Nfatc1* und *Nfatc2*, wohingegen *Nfatc3* unbeeinflusst bleibt. In DN-Thymozyten aktivieren die Prä-TCR-Signale die Expression von *Nfatc1α*, aber nicht von *Nfatc1ß*. Die *Nfatc1α* Genexpression wird vermutlich hauptsächlich über den MAPK-Signalweg reguliert, da eine Aktivierung von *Nfatc1α* über MEK-ERK Signale vermittelt wird und JNK Signale gegensätzlich wirken. Der Calcineurin-NFAT- und der p38-Signalweg spielen keine Rolle bei der Regulation von *Nfatc1α* in DN-Thymozyten. In DP-Thymozyten erfolgt durch TCR-Signale eine Aktivierung der *Nfatc1*- und *Nfatc2*- sowie eine Represson der *Nfatc3*-Genexpression. In DP-Thymozyten aktivieren die TCR-Signale die *Nfatc1α* Expression. Die Aktivierung von *Nfatc1α* in DP Thymozyten wird über NFATc1 autoreguliert. NFATc2 und NFATc3 sind daran wenig oder gar nicht beteiligt. Hingegen sind MEK-ERK-, JNK- und p38-Signalwege bei der *Nfatc1α*–Genaktivierung in DP-Thymozyten - wahrscheinlich durch die Aktivierung der NFAT Transaktivierungsaktivität - beteiligt. All diese Daten zeigen, dass die NFAT-Transkriptonsfaktoren einer komplexen Regulation in Thymozyten unterzogen sind, deren Entwicklung sie andererseits – wie z.B. durch die Suppression der *Rag*-Gene – maßgeblich beeinflussen.

6. REFERENCES

Agrawal, A., and Schatz, D. G. (1997). RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. Cell *89*, 43-53.

Aifantis, I., Gounari, F., Scorrano, L., Borowski, C., and von Boehmer, H. (2001). Constitutive pre-TCR signaling promotes differentiation through Ca2+ mobilization and activation of NF-kappaB and NFAT. Nat Immunol *2*, 403-409.

Amasaki, Y., Adachi, S., Ishida, Y., Iwata, M., Arai, N., Arai, K., and Miyatake, S. (2002). A constitutively nuclear form of NFATx shows efficient transactivation activity and induces differentiation of CD4(+)CD8(+) T cells. J Biol Chem *277*, 25640-25648.

Aramburu, J., Garcia-Cozar, F., Raghavan, A., Okamura, H., Rao, A., and Hogan, P. G. (1998). Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol Cell *1*, 627-637.

Aramburu, J., Yaffe, M. B., López-Rodriguez, C., Cantley, L. C., Hogan, P. G., and Rao, A. (1999). Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science *285*, 2129-2133.

Arron, J. R., Winslow, M. M., Polleri, A., Chang, C. P., Wu, H., Gao, X., Neilson, J. R., Chen, L., Heit, J. J., Kim, S. K.*, et al.* (2006). NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature *441*, 595-600.

Avots, A., Buttmann, M., Chuvpilo, S., Escher, C., Smola, U., Bannister, A. J., Rapp, U. R., Kouzarides, T., and Serfling, E. (1999). CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. Immunity *10*, 515-524.

Barančik, M., Bohacova, V., Kvackajova, J., Hudecova, S., Krizanova, O., and Breier, A. (2001). SB203580, a specific inhibitor of p38-MAPK pathway, is a new reversal agent of P-glycoprotein-mediated multidrug resistance. Eur J Pharm Sci *14*, 29-36.

Beals, C. R., Clipstone, N. A., Ho, S. N., and Crabtree, G. R. (1997a). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev *11*, 824-834.

Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997b). Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science *275*, 1930-1934.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y.*, et al.* (2001). SP600125, an

anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A *98*, 13681-13686.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. Nature *361*, 315-325.

Bettelli, E., Dastrange, M., and Oukka, M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. Proc Natl Acad Sci U S A *102*, 5138-5143.

Boehm, T., Gonzalez-Sarmiento, R., Kennedy, M., and Rabbitts, T. H. (1991). A simple technique for generating probes for RNA in situ hybridization: an adjunct to genome mapping exemplified by the RAG-1/RAG-2 gene cluster. Proc Natl Acad Sci U S A *88*, 3927-3931.

Brandle, D., Muller, C., Rulicke, T., Hengartner, H., and Pircher, H. (1992). Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression. Proc Natl Acad Sci U S A *89*, 9529-9533.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet *27*, 68-73.

Bueno, O. F., Brandt, E. B., Rothenberg, M. E., and Molkentin, J. D. (2002). Defective T cell development and function in calcineurin A beta -deficient mice. Proc Natl Acad Sci U S A *99*, 9398-9403.

Chan, V. S., Wong, C., and Ohashi, P. S. (2002). Calcineurin Aalpha plays an exclusive role in TCR signaling in mature but not in immature T cells. Eur J Immunol *32*, 1223-1229.

Chen, L., Glover, J. N., Hogan, P. G., Rao, A., and Harrison, S. C. (1998). Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. Nature *392*, 42-48.

Chien, Y., Becker, D. M., Lindsten, T., Okamura, M., Cohen, D. I., and Davis, M. M. (1984). A third type of murine T-cell receptor gene. Nature *312*, 31-35.

Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell *54*, 541-552.

Chow, C. W., and Davis, R. J. (2000). Integration of calcium and cyclic AMP signaling pathways by 14-3-3. Mol Cell Biol *20*, 702-712.

Chow, C. W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R. J. (1997). Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. Science *278*, 1638-1641.

Chuvpilo, S., Jankevics, E., Tyrsin, D., Akimzhanov, A., Moroz, D., Jha, M. K., Schulze-Luehrmann, J., Santner-Nanan, B., Feoktistova, E., Konig, T.*, et al.* (2002). Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. Immunity *16*, 881-895.

Chuvpilo, S., Zimmer, M., Kerstan, A., Glockner, J., Avots, A., Escher, C., Fischer, C., Inashkina, I., Jankevics, E., Berberich-Siebelt, F.*, et al.* (1999). Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells. Immunity *10*, 261-269.

Clapham, D. E. (1995). Intracellular calcium. Replenishing the stores. Nature *375*, 634-635.

Clipstone, N. A., and Crabtree, G. R. (1992). Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature *357*, 695-697.

Covert, M. W., Leung, T. H., Gaston, J. E., and Baltimore, D. (2005). Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science *309*, 1854-1857.

Crompton, T., Gilmour, K. C., and Owen, M. J. (1996). The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. Cell *86*, 243-251.

Cuomo, C. A., and Oettinger, M. A. (1994). Analysis of regions of RAG-2 important for V(D)J recombination. Nucleic Acids Res *22*, 1810-1814.

Daniels, M. A., Teixeiro, E., Gill, J., Hausmann, B., Roubaty, D., Holmberg, K., Werlen, G., Hollander, G. A., Gascoigne, N. R., and Palmer, E. (2006). Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. Nature *444*, 724-729.

Davis, M. M., and Bjorkman, P. J. (1988). T-cell antigen receptor genes and T-cell recognition. Nature *334*, 395-402.

De, P., and Rodgers, K. K. (2004). Putting the pieces together: identification and characterization of structural domains in the V(D)J recombination protein RAG1. Immunol Rev *200*, 70-82.

Difilippantonio, M. J., McMahan, C. J., Eastman, Q. M., Spanopoulou, E., and Schatz, D. G. (1996). RAG1 mediates signal sequence recognition and recruitment of RAG2 in V(D)J recombination. Cell *87*, 253-262.

Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997). Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature *386*, 855-858.

Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature *392*, 933-936.

Eastman, Q. M., Leu, T. M., and Schatz, D. G. (1996). Obeying V(D)J rules in vitro. Immunol Today *17*, 205.

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F.*, et al.* (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem *273*, 18623-18632.

Flanagan, W. M., Corthesy, B., Bram, R. J., and Crabtree, G. R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature *352*, 803-807.

Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330-336.

Garcia-Cozar, F. J., Okamura, H., Aramburu, J. F., Shaw, K. T., Pelletier, L., Showalter, R., Villafranca, E., and Rao, A. (1998). Two-site interaction of nuclear factor of activated T cells with activated calcineurin. J Biol Chem *273*, 23877-23883.

Garcia-Rodriguez, C., and Rao, A. (2000). Requirement for integration of phorbol 12-myristate 13-acetate and calcium pathways is preserved in the transactivation domain of NFAT1. Eur J Immunol *30*, 2432-2436.

Gellert, M. (2002). V(D)J recombination: RAG proteins, repair factors, and regulation. Annu Rev Biochem *71*, 101-132.

Giffin, M. J., Stroud, J. C., Bates, D. L., von Koenig, K. D., Hardin, J., and Chen, L. (2003). Structure of NFAT1 bound as a dimer to the HIV-1 LTR kappa B element. Nat Struct Biol *10*, 800-806.

Godfrey, D. I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol *150*, 4244-4252.

Graef, I. A., Chen, F., Chen, L., Kuo, A., and Crabtree, G. R. (2001). Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell *105*, 863-875.

Groettrup, M., Baron, A., Griffiths, G., Palacios, R., and von Boehmer, H. (1992). T cell receptor (TCR) beta chain homodimers on the surface of immature but not mature alpha, gamma, delta chain deficient T cell lines. Embo J *11*, 2735-2745.

Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M. J., Hayday, A. C., and von Boehmer, H. (1993). A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. Cell *75*, 283-294.

Groves, T., Katis, P., Madden, Z., Manickam, K., Ramsden, D., Wu, G., and Guidos, C. J. (1995). In vitro maturation of clonal CD4+CD8+ cell lines in response to TCR engagement. J Immunol *154*, 5011-5022.

Gwack, Y., Sharma, S., Nardone, J., Tanasa, B., Iuga, A., Srikanth, S., Okamura, H., Bolton, D., Feske, S., Hogan, P. G., and Rao, A. (2006). A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. Nature *441*, 646-650.

Hedrick, S. M., Cohen, D. I., Nielsen, E. A., and Davis, M. M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature *308*, 149-153.

HGNT website http://www.gene.ucl.ac.uk/nomenclature/)

Hiom, K., and Gellert, M. (1997). A stable RAG1-RAG2-DNA complex that is active in V(D)J cleavage. Cell *88*, 65-72.

Ho, S. N., Thomas, D. J., Timmerman, L. A., Li, X., Francke, U., and Crabtree, G. R. (1995). NFATc3, a lymphoid-specific NFATc family member that is calcium-regulated and exhibits distinct DNA binding specificity. J Biol Chem *270*, 19898-19907.

Hodge, M. R., Ranger, A. M., Charles de la Brousse, F., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1996). Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. Immunity *4*, 397-405.

Hoey, T., Sun, Y. L., Williamson, K., and Xu, X. (1995). Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. Immunity *2*, 461-472.

Hori, S., Takahashi, T., and Sakaguchi, S. (2003). Control of autoimmunity by naturally arising regulatory CD4+ T cells. Adv Immunol *81*, 331-371.

Hubbard, M. J., and Klee, C. B. (1989). Functional domain structure of calcineurin A: mapping by limited proteolysis. Biochemistry *28*, 1868-1874.

IMGT website http://imgt.cines.fr:8081/GeneInfo/index.html

Irving, B. A., Alt, F. W., and Killeen, N. (1998). Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. Science *280*, 905-908.

Jackson, A. M., and Krangel, M. S. (2006). Turning T-cell receptor beta recombination on and off: more questions than answers. Immunol Rev *209*, 129-141.

Jain, J., Burgeon, E., Badalian, T. M., Hogan, P. G., and Rao, A. (1995). A similar DNA-binding motif in NFAT family proteins and the Rel homology region. J Biol Chem *270*, 4138-4145.

Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature *365*, 352-355.

Jain, J., Valge-Archer, V. E., Sinskey, A. J., and Rao, A. (1992). The AP-1 site at -150 bp, but not the NF-kappa B site, is likely to represent the major target of protein kinase C in the interleukin 2 promoter. J Exp Med *175*, 853-862.

Jin, L., Sliz, P., Chen, L., Macián, F., Rao, A., Hogan, P. G., and Harrison, S. C. (2003). An asymmetric NFAT1 dimer on a pseudo-palindromic kappa B-like DNA site. Nat Struct Biol *10*, 807-811.

Johnson, G. L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298*, 1911-1912.

Jung, D., and Alt, F. W. (2004). Unraveling V(D)J recombination; insights into gene regulation. Cell *116*, 299-311.

Kadonaga, J. T. (2004). Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell *116*, 247-257.

Ke, H., and Huai, Q. (2003). Structures of calcineurin and its complexes with immunophilins-immunosuppressants. Biochem Biophys Res Commun *311*, 1095-1102.
Klee, C. B., Ren, H., and Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J Biol Chem *273*, 13367-13370.

Kondo, M., Wagers, A. J., Manz, M. G., Prohaska, S. S., Scherer, D. C., Beilhack, G. F., Shizuru, J. A., and Weissman, I. L. (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol *21*, 759-806.

Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Kouskoff, V., Vonesch, J. L., Benoist, C., and Mathis, D. (1995). The influence of positive selection on RAG expression in thymocytes. Eur J Immunol *25*, 54-58.

Krangel, M. S., Carabana, J., Abbarategui, I., Schlimgen, R., and Hawwari, A. (2004). Enforcing order within a complex locus: current perspectives on the control of V(D)J recombination at the murine T-cell receptor alpha/delta locus. Immunol Rev *200*, 224-232.

Landree, M. A., Wibbenmeyer, J. A., and Roth, D. B. (1999). Mutational analysis of RAG1 and RAG2 identifies three catalytic amino acids in RAG1 critical for both cleavage steps of V(D)J recombination. Genes Dev *13*, 3059-3069.

Lewis, R. S. (2001). Calcium signaling mechanisms in T lymphocytes. Annu Rev Immunol *19*, 497-521.

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807-815.

Loh, C., Shaw, K. T., Carew, J., Viola, J. P., Luo, C., Perrino, B. A., and Rao, A. (1996). Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. J Biol Chem *271*, 10884-10891.

López-Rodriguez, C., Aramburu, J., Rakeman, A. S., and Rao, A. (1999). NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. Proc Natl Acad Sci U S A *96*, 7214-7219.

Lu, C. P., Sandoval, H., Brandt, V. L., Rice, P. A., and Roth, D. B. (2006). Amino acid residues in Rag1 crucial for DNA hairpin formation. Nat Struct Mol Biol *13*, 1010-1015.

Luo, C., Burgeon, E., Carew, J. A., McCaffrey, P. G., Badalian, T. M., Lane, W. S., Hogan, P. G., and Rao, A. (1996a). Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. Mol Cell Biol *16*, 3955-3966.

Luo, C., Burgeon, E., and Rao, A. (1996b). Mechanisms of transactivation by nuclear factor of activated T cells-1. J Exp Med *184*, 141-147.

Luo, C., Shaw, K. T., Raghavan, A., Aramburu, J., Garcia-Cozar, F., Perrino, B. A., Hogan, P. G., and Rao, A. (1996c). Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. Proc Natl Acad Sci U S A *93*, 8907-8912.

Macián, F., Garcia-Cozar, F., Im, S. H., Horton, H. F., Byrne, M. C., and Rao, A. (2002). Transcriptional mechanisms underlying lymphocyte tolerance. Cell *109*, 719-731.

Macián, F., Garcia-Rodriguez, C., and Rao, A. (2000). Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. Embo J *19*, 4783-4795.

Masuda, E. S., Liu, J., Imamura, R., Imai, S. I., Arai, K. I., and Arai, N. (1997). Control of NFATx1 nuclear translocation by a calcineurin-regulated inhibitory domain. Mol Cell Biol *17*, 2066-2075.

Masuda, E. S., Naito, Y., Tokumitsu, H., Campbell, D., Saito, F., Hannum, C., Arai, K., and Arai, N. (1995). NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. Mol Cell Biol *15*, 2697-2706.

McBlane, J. F., van Gent, D. C., Ramsden, D. A., Romeo, C., Cuomo, C. A., Gellert, M., and Oettinger, M. A. (1995). Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell *83*, 387-395.

McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., and et al. (1993). Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science *262*, 750-754.

Menetski, J. P., and Gellert, M. (1990). V(D)J recombination activity in lymphoid cell lines is increased by agents that elevate cAMP. Proc Natl Acad Sci U S A *87*, 9324-9328.

Merkenschlager, M., Graf, D., Lovatt, M., Bommhardt, U., Zamoyska, R., and Fisher, A. G. (1997). How many thymocytes audition for selection? J Exp Med *186*, 1149-1158.

Michie, A. M., Trop, S., Wiest, D. L., and Zuniga-Pflucker, J. C. (1999). Extracellular signal-regulated kinase (ERK) activation by the pre-T cell receptor in developing thymocytes in vivo. J Exp Med *190*, 1647-1656.

Mo, X., Bailin, T., and Sadofsky, M. J. (1999). RAG1 and RAG2 cooperate in specific binding to the recombination signal sequence in vitro. J Biol Chem *274*, 7025-7031.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., and et al. (1992a). Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. Nature *360*, 225-231.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. (1992b). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869-877.

Monticelli, S., and Rao, A. (2002). NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription. Eur J Immunol *32*, 2971-2978.

Murga, C., and Barber, D. F. (2002). Molecular mechanisms of pre-T cell receptor-induced survival. J Biol Chem *277*, 39156-39162.

Nagaoka, H., Yu, W., and Nussenzweig, M. C. (2000). Regulation of RAG expression in developing lymphocytes. Curr Opin Immunol *12*, 187-190.

Neilson, J. R., Winslow, M. M., Hur, E. M., and Crabtree, G. R. (2004). Calcineurin B1 is essential for positive but not negative selection during thymocyte development. Immunity *20*, 255-266.

Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994). NF-AT components define a family of transcription factors targeted in T-cell activation. Nature *369*, 497-502.

Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science *248*, 1517-1523.

Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J. P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P. G., and Rao, A. (2000). Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol Cell *6*, 539-550.

Oukka, M., Ho, I. C., de la Brousse, F. C., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1998). The transcription factor NFAT4 is involved in the generation and survival of T cells. Immunity *9*, 295-304.

Pan, F., Sun, L., Kardian, D. B., Whartenby, K. A., Pardoll, D. M., and Liu, J. O. (2007). Feedback inhibition of calcineurin and Ras by a dual inhibitory protein Carabin. Nature *445*, 433-436.

Park, J., Takeuchi, A., and Sharma, S. (1996). Characterization of a new isoform of the NFAT (nuclear factor of activated T cells) gene family member NFATc. J Biol Chem *271*, 20914-20921.

Patra, A. K., Drewes, T., Engelmann, S., Chuvpilo, S., Kishi, H., Hunig, T., Serfling, E., and Bommhardt, U. H. (2006). PKB rescues calcineurin/NFAT-induced arrest of Rag expression and pre-T cell differentiation. J Immunol *177*, 4567-4576.

Peng, S. L., Gerth, A. J., Ranger, A. M., and Glimcher, L. H. (2001). NFATc1 and NFATc2 together control both T and B cell activation and differentiation. Immunity *14*, 13-20.

Petrie, H. T., Hugo, P., Scollay, R., and Shortman, K. (1990). Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. J Exp Med *172*, 1583-1588.

Petrie, H. T., and Kincade, P. W. (2005). Many roads, one destination for T cell progenitors. J Exp Med *202*, 11-13.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29*, e45.

Ptashne, M., and Gann, A. (1997). Transcriptional activation by recruitment. Nature *386*, 569-577.

Ranger, A. M., Hodge, M. R., Gravallese, E. M., Oukka, M., Davidson, L., Alt, F. W., de la Brousse, F. C., Hoey, T., Grusby, M., and Glimcher, L. H. (1998a). Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. Immunity *8*, 125-134.

Ranger, A. M., Oukka, M., Rengarajan, J., and Glimcher, L. H. (1998b). Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. Immunity *9*, 627-635.

Rao, A., Luo, C., and Hogan, P. G. (1997). Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol *15*, 707-747.

Rengarajan, J., Mittelstadt, P. R., Mages, H. W., Gerth, A. J., Kroczek, R. A., Ashwell, J. D., and Glimcher, L. H. (2000). Sequential involvement of NFAT and Egr transcription factors in FasL regulation. Immunity *12*, 293-300.

Round, J. L., Humphries, L. A., Tomassian, T., Mittelstadt, P., Zhang, M., and Miceli, M. C. (2007). Scaffold protein Dlgh1 coordinates alternative p38 kinase activation, directing T cell receptor signals toward NFAT but not NF-kappaB transcription factors. Nat Immunol *8*, 154-161.

Rudensky, A. Y., Gavin, M., and Zheng, Y. (2006). FOXP3 and NFAT: partners in tolerance. Cell *126*, 253-256.

Sadofsky, M. J., Hesse, J. E., and Gellert, M. (1994a). Definition of a core region of RAG-2 that is functional in V(D)J recombination. Nucleic Acids Res *22*, 1805-1809.

Sadofsky, M. J., Hesse, J. E., McBlane, J. F., and Gellert, M. (1993). Expression and V(D)J recombination activity of mutated RAG-1 proteins. Nucleic Acids Res *21*, 5644-5650.

Sadofsky, M. J., Hesse, J. E., McBlane, J. F., and Gellert, M. (1994b). Expression and V(D)J recombination activity of mutated RAG-1 proteins. Nucleic Acids Res *22*, 550.

Sassone-Corsi, P., Ransone, L. J., Lamph, W. W., and Verma, I. M. (1988). Direct interaction between fos and jun nuclear oncoproteins: role of the 'leucine zipper' domain. Nature *336*, 692-695.

Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989). The V(D)J recombination activating gene, RAG-1. Cell *59*, 1035-1048.

Schlissel, M. S. (2002). Does artemis end the hunt for the hairpin-opening activity in V(D)J recombination? Cell *109*, 1-4.

Schubert, L. A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S. F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem *276*, 37672-37679.

Schuermann, M., Neuberg, M., Hunter, J. B., Jenuwein, T., Ryseck, R. P., Bravo, R., and Muller, R. (1989). The leucine repeat motif in Fos protein mediates complex formation with Jun/AP-1 and is required for transformation. Cell *56*, 507-516.

Serfling, E., Berberich-Siebelt, F., Avots, A., Chuvpilo, S., Klein-Hessling, S., Jha, M. K., Kondo, E., Pagel, P., Schulze-Luehrmann, J., and Palmetshofer, A. (2004). NFAT and NF-kappaB factors-the distant relatives. Int J Biochem Cell Biol *36*, 1166-1170.

Serfling, E., Berberich-Siebelt, F., Chuvpilo, S., Jankevics, E., Klein-Hessling, S., Twardzik, T., and Avots, A. (2000). The role of NF-AT transcription factors in T cell activation and differentiation. Biochim Biophys Acta *1498*, 1-18.

Serfling, E., Chuvpilo, S., Liu, J., Hofer, T., and Palmetshofer, A. (2006a). NFATc1 autoregulation: a crucial step for cell-fate determination. Trends Immunol *27*, 461-469.

Serfling, E., Klein-Hessling, S., Palmetshofer, A., Bopp, T., Stassen, M., and Schmitt, E. (2006b). NFAT transcription factors in control of peripheral T cell tolerance. Eur J Immunol *36*, 2837-2843.

Sherman, M. A., Powell, D. R., Weiss, D. L., and Brown, M. A. (1999). NF-ATc isoforms are differentially expressed and regulated in murine T and mast cells. J Immunol *162*, 2820-2828.

Shibasaki, F., Hallin, U., and Uchino, H. (2002). Calcineurin as a multifunctional regulator. J Biochem (Tokyo) *131*, 1-15.

Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and et al. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855-867.

Shizuru, J. A., Negrin, R. S., and Weissman, I. L. (2005). Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. Annu Rev Med *56*, 509-538.

Taghon, T., Yui, M. A., Pant, R., Diamond, R. A., and Rothenberg, E. V. (2006). Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. Immunity *24*, 53-64.

Timmerman, L. A., Clipstone, N. A., Ho, S. N., Northrop, J. P., and Crabtree, G. R. (1996). Rapid shuttling of NF-AT in discrimination of Ca2+ signals and immunosuppression. Nature *383*, 837-840.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature *302*, 575-581.

Trinh, L. A., Ferrini, R., Cobb, B. S., Weinmann, A. S., Hahm, K., Ernst, P., Garraway, I. P., Merkenschlager, M., and Smale, S. T. (2001). Down-regulation of TDT transcription in CD4(+)CD8(+) thymocytes by Ikaros proteins in direct competition with an Ets activator. Genes Dev *15*, 1817-1832.

Turka, L. A., Schatz, D. G., Oettinger, M. A., Chun, J. J., Gorka, C., Lee, K., McCormack, W. T., and Thompson, C. B. (1991). Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross-linking. Science *253*, 778-781.

van Gent, D. C., Ramsden, D. A., and Gellert, M. (1996). The RAG1 and RAG2 proteins establish the 12/23 rule in V(D)J recombination. Cell *85*, 107-113.

Wells, J., and Farnham, P. J. (2002). Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. Methods *26*, 48-56.

Werlen, G., Hausmann, B., Naeher, D., and Palmer, E. (2003). Signaling life and death in the thymus: timing is everything. Science *299*, 1859-1863.

Wilkins, B. J., and Molkentin, J. D. (2004). Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun *322*, 1178-1191.

Wilson, A., Held, W., and MacDonald, H. R. (1994). Two waves of recombinase gene expression in developing thymocytes. J Exp Med *179*, 1355-1360.

Wolfe, S. A., Zhou, P., Dotsch, V., Chen, L., You, A., Ho, S. N., Crabtree, G. R., Wagner, G., and Verdine, G. L. (1997). Unusual Rel-like architecture in the DNA-binding domain of the transcription factor NFATc. Nature *385*, 172-176.

Woodrow, M., Clipstone, N. A., and Cantrell, D. (1993a). p21ras and calcineurin synergize to regulate the nuclear factor of activated T cells. J Exp Med *178*, 1517-1522.

Woodrow, M. A., Rayter, S., Downward, J., and Cantrell, D. A. (1993b). p21ras function is important for T cell antigen receptor and protein kinase C regulation of nuclear factor of activated T cells. J Immunol *150*, 3853-3861.

Wu, C. C., Hsu, S. C., Shih, H. M., and Lai, M. Z. (2003). Nuclear factor of activated T cells c is a target of p38 mitogen-activated protein kinase in T cells. Mol Cell Biol *23*, 6442-6454.

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A. D., Stroud, J. C., Bates, D. L., Guo, L., Han, A., Ziegler, S. F*., et al.* (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell *126*, 375-387.

Xanthoudakis, S., Viola, J. P., Shaw, K. T., Luo, C., Wallace, J. D., Bozza, P. T., Luk, D. C., Curran, T., and Rao, A. (1996). An enhanced immune response in mice lacking the transcription factor NFAT1. Science *272*, 892-895.

Xi, H., Schwartz, R., Engel, I., Murre, C., and Kersh, G. J. (2006). Interplay between RORgammat, Egr3, and E proteins controls proliferation in response to pre-TCR signals. Immunity *24*, 813-826.

Yang, T. T., Xiong, Q., Enslen, H., Davis, R. J., and Chow, C. W. (2002). Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. Mol Cell Biol *22*, 3892-3904.

Yannoutsos, N., Barreto, V., Misulovin, Z., Gazumyan, A., Yu, W., Rajewsky, N., Peixoto, B. R., Eisenreich, T., and Nussenzweig, M. C. (2004). A cis element in the recombination activating gene locus regulates gene expression by counteracting a distant silencer. Nat Immunol *5*, 443-450.

Yannoutsos, N., Wilson, P., Yu, W., Chen, H. T., Nussenzweig, A., Petrie, H., and Nussenzweig, M. C. (2001). The role of recombination activating gene (RAG) reinduction in thymocyte development in vivo. J Exp Med *194*, 471-480.

Yoshida, H., Nishina, H., Takimoto, H., Marengere, L. E., Wakeham, A. C., Bouchard, D., Kong, Y. Y., Ohteki, T., Shahinian, A., Bachmann, M*., et al.* (1998). The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. Immunity *8*, 115-124.

Yu, W., Misulovin, Z., Suh, H., Hardy, R. R., Jankovic, M., Yannoutsos, N., and Nussenzweig, M. C. (1999). Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5' of RAG2. Science *285*, 1080-1084.

Zhou, P., Sun, L. J., Dotsch, V., Wagner, G., and Verdine, G. L. (1998). Solution structure of the core NFATC1/DNA complex. Cell *92*, 687-696.

Zhu, J., and McKeon, F. (1999). NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature *398*, 256-260.

Zhu, J., Shibasaki, F., Price, R., Guillemot, J. C., Yano, T., Dotsch, V., Wagner, G., Ferrara, P., and McKeon, F. (1998). Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. Cell *93*, 851-861.

7. ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometer
aa	amino acid
AID	auto-inhibitory domain
AP-1	activation protein-1
APS	ammonium persulfate
ASE	anti-silencer element
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	count per minute
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
DYRKs	dual-specificity tyrosine-phosphorylation regulated kinases
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
NF-κB	nuclear factor κB
ng	nanogram
NHEJ	non-homologous end joining
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
pTα	pre-TCRα chain
qPCR	quantitative PCR
RAG1/2	recombination activating gene 1 or 2
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
ST-HSCs	short-term HSCs
TAD	trans-activation domain
TBS	tris-buffered saline
TCR	T cell receptor
TEMED	tetramethylethylenediamine

Th	helper T cell
TPA	12-O-tetradecanoyl phorbol 13-acetate
Treg	regulatory T cell
WT	wild-type

8. CURRICULUM VITAE

Name: Shaoxian Yang
Sex: male
Citizenship: Canadian
Date of Birth: October 10, 1960
Phone: (0931)20147495 (lab)
(0931)20388318 (home)
Mailing Address: SWH2, Apt.606, Straubmuhlweg 0 Nr. 97078 Würzburg, Germany
E-mail: yangshaoxian@hotmail.com

Education
August 2002–March 2007, Ph.D student in Immunology, Institute of Pathology, University of Würzburg, Würzburg, Germany.
M.Sc. in Microbiology and Immunology, Academy of Military Medical Sciences, Beijing, China (September 1987).
M.D. Enshi Medical College, Enshi, China (March 1982).

Work Experience
April 1998-July 2002, Ontario Cancer Institute, University of Toronto, Canada, Research Technician.

January 1995-December 1997, Memorial University of Newfoundland Faculty of Medicine, St. John's, Canada, Research technician.

October 1991-December 1994, Hyper Pharmaceutical Company, Beijing, China, Scientist.

September 1987-September 1991, Institute of Pharmacology and Toxicology, Beijing, China, Assistant Research Fellow.

September 1984-August 1987, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, China, M.Sc. student.

April 1982-Augst 1984, Department of Internal Medicine, Qianjiang Hospital, Qianjiang, China, Physician.

Publications
Yang, S., and Serfling, E. (2007). The role of NFAT in *Rag* gene down-regulation in the murine thymocytes. Manuscript in preparation

Tang, Y., Yang, S., Gariepy, J., Scollard, DA., Reilly, RM.(2007). Construction and Evaluation of the Tumor Imaging Properties of ^{123}I-Labeled Recombinant and Enzymatically Generated Fab Fragments of the TAG-72 Monoclonal Antibody CC49. Bioconjug Chem. ASAP Web Release Date: 16-Mar-2007; DOI: 10.1021/bc060260r

Brokx, RD., Revers, L., Zhang, Q., Yang, S., Mal, TK., Ikura, M., Gariepy, J. (2003). Nuclear magnetic resonance-based dissection of a glycosyltransferase specificity for the mucin MUC1 tandem repeat. Biochemistry. 42:13817-25.

Wang, J., Reilly, R., Chen, P., Yang, S., Bray, M., Gariépy, J., Chan, C., and Sandhu, J.(2002). Fusion of the CH1 domain of IgG1 to epidermal growth factor (EGF) prolongs its retention in the blood but does not increase tumor uptake. Cancer Biother Radiopharm.17: 665-672.

Gariepy, J., Singh, D., Kawamura, K., and Yang, S.(2000). Identification of short peptide ligands that bind specifically to the breast tumor-associated MUC1 epitope. American Association for Cancer Research 91st Annual Meeting. March 2000, San Francisco, USA.

Gariepy, J., and Yang, S. (1999). Muc1 ligands. WO 0077031.

Yang, S., Lockwood, A., Hollett, P., Ford, R. and Kao, K. (1998). Over-expression of a novel Xenopus Rel gene mRNA induces tumors in early embryos. J. Biol. Chem. 273:13746-13752.