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NFATc2-Mediated Repression of Cyclin-Dependent Kinase 4 Expression

Shairaz Baksh,[1,7] Hans R. Widlund,[1] Ashley A. Frazer-Abel,[3] Jinyan Du,[1] Susan Fosmire,[3] David E. Fisher,[1] James A. DeCaprio,[2] Jaime F. Modiano,[3,4] and Steven J. Burakoff[1,5,6]

[1]Department of Pediatric Oncology
[2]Department of Adult Oncology
The Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts 02115
[3]AMC Cancer Research Center and
Donald Monk Cancer Research Foundation
Denver, Colorado 80214
[4]University of Colorado Cancer Center
Denver, Colorado 80262
[5]Skirball Institute of Biomolecular Medicine
New York University School of Medicine
New York, New York 10016

Summary

The calcineurin-regulated transcription factor, nuclear factor of activated T cells (NFAT), controls many aspects of T cell function. Here, we demonstrate that the calcineurin/NFAT pathway negatively regulates the expression of cyclin-dependent kinase 4 (CDK4). A canonical NFAT binding site was identified and found to be sensitive to calcium signals, FK506/CsA, and histone deacetylase activity and to not require AP-1. Ectopic expression of NFATc2 inhibited the basal activity of the human *CDK4* promoter. Additionally, both calcineurin $A\alpha^{-/-}$ and $NFATc2^{-/-}$ mice had elevated protein levels of CDK4, confirming a negative regulatory role for the calcineurin/NFAT pathway. This pathway may thus regulate the expression of CDK4 at the transcriptional level and control how cells re-enter a resting, nonproliferative state.

Introduction

The activation and proliferation of T lymphocytes is dependent upon the actions of numerous signaling intermediates and transcription factors that control the proliferation and expansion of resting T cells. A key transcription factor, dependent upon calcium transients and the function of calcineurin, is the nuclear factor of activated T cells (NFAT) (Crabtree, 1999). The NFAT family of transcription factors has been demonstrated to play a role in diverse cellular functions ranging from lymphocyte activation and development (Kiani et al., 2000) to cardiac hypertrophy (Molkentin et al., 1998). Four family members have been shown to share significant sequence and functional similarity. NFATc1, c2, and c3 are thought to be immune-specific forms of NFAT, while NFATc4 functions outside of the immune system to regulate cardiac hypertrophy (Molkentin et al., 1998) and hippocampal neuronal signaling (Graef et al., 1999). A fifth family member, NFAT5 (NFATL1, TonEBP), has been recently cloned and demonstrated to be distinct from the other NFAT isoforms (Lopez-Rodriguez et al., 1999; Miyakawa et al., 1999; Trama et al., 2000).

It has been extensively demonstrated that NFATc1, c2, and c3 require the continued activation of calcineurin (Loh et al., 1996) and AP-1 elements (Macian et al., 2001) in order to regulate the production of numerous lymphokine elements. Calcineurin functions to dephosphorylate serine/threonine residues within the amino terminus of NFAT, allowing NFAT to translocate to the nucleus and bind to DNA. Inhibition of the phosphatase activity of calcineurin by FK506 or cyclosporin A (CsA) results in the relocalization of NFAT to the cytosol and loss of the ability to bind to DNA (Kiani et al., 2000). Targeted disruption of NFATc2 (Xanthoudakis et al., 1996), NFATc1 (Ranger et al., 1998), and NFATc3 (Oukka et al., 1998) has confirmed the roles played by NFAT family members in lymphocyte development and proliferation. However, targeted disruption of calcineurin $A\alpha$ did not reveal dramatic changes in overall T cell function and showed only slight impairment in antigen-dependent responses (Zhang et al., 1996). This was unexpected given that calcineurin $A\alpha$ accounts for 70%–80% of the overall phosphatase activity of T cells in the periphery (Jiang et al., 1997). The β form of calcineurin accounts for the remaining 20%–30% of calcineurin activity in the periphery and is thought to be more important in influencing thymocyte activation and negative selection (Hollander et al., 1994; Jiang et al., 1997). The remaining isoform of calcineurin, γ, is testis specific and does not contribute to lymphocyte function (11).

CDK4 functions as an important G0/G1 restriction point kinase to allow cells to leave the resting state and commit to entrance into the cell cycle (Ladha et al., 1998). The main target of CDK4 is the phosphorylation of the retinoblastoma tumor suppressor protein, p110 (pRb). Within resting cells, pRb associates with E2F. However, upon phosphorylation by cyclin dependent kinases, pRb-dependent repression of E2F is relieved, allowing E2F to target S phase-specific genes and commit the cell to enter the cell cycle. The activity of CDK4 is governed by its association with cyclin-dependent kinase inhibitors of the INK4 family, such as p16; by binding to its cyclin counterpart, cyclin D; and by the actions of cyclin-activating kinase (Sherr and Roberts, 1999).

We have previously shown that calcineurin functions to dephosphorylate threonine 172 on CDK4, specifically during the mitotic phase of the cell cycle, resulting in the inactivation of the kinase activity of CDK4 (Baksh et al., 2000). In addition to dephosphorylation-dependent downmodulation of the kinase activity of CDK4, it has also been observed that the expression levels of CDK4 can also be a mechanism of downmodulation (Lucas et

[6]Correspondence: burakoff@saturn.med.nyu.edu
[7]Present address: Signal Transduction Laboratory, Cancer Research UK, London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.

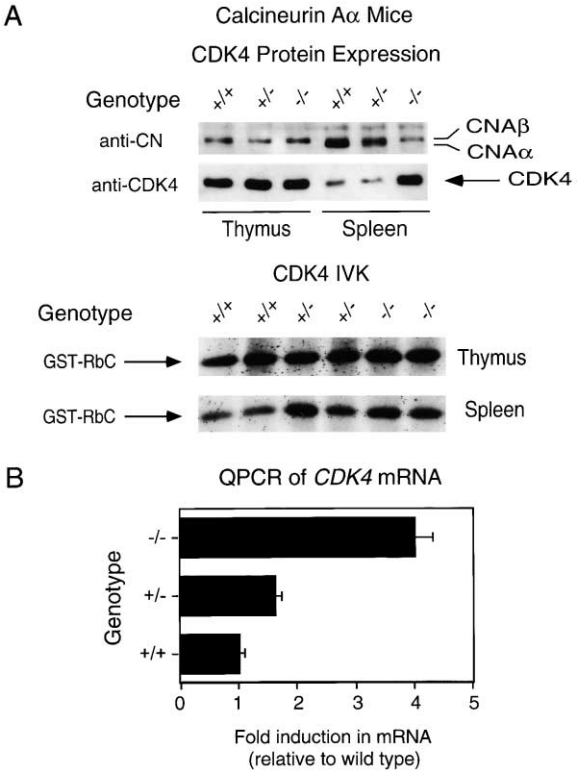

Figure 1. CDK4 Protein Levels and Kinase Activity Are Elevated in Calcineurin $A\alpha^{-/-}$ Mice

(A) Thymocytes and purified peripheral T cells were isolated from splenocytes obtained from calcineurin (CN) $A\alpha^{+/+}$, $A\alpha^{+/-}$, and $A\alpha^{-/-}$ mice. Cells were lysed in kinase buffer, and protein expression was carried out for CDK4. In addition, an in vitro kinase assay was carried out for CDK4 using GST-RbC as the substrate. Calcineurin levels were checked using an antibody from Transduction Laboratories. For CN $A\alpha^{+/+}$, n = 5; for CN $A\alpha^{+/-}$, n = 4; and for CN $A\alpha^{-/-}$, n = 7. (B) Total RNA was isolated, and 2 μg was used in a quantitative PCR analysis (QPCR) for *CDK4* mRNA. Results shown represent mean levels of expression of *CDK4* mRNA that were normalized to GAPDH (n = 5 for +/+ and −/−; n = 2 for +/−).

al., 1995; Modiano et al., 1994). We report here that NFAT can modulate the kinase activity of CDK4 by transcriptionally repressing the *CDK4* promoter and thus regulate CDK4 protein expression. This repression is specific for NFATc2, dependent upon the phosphatase activity of calcineurin, and does not require AP-1 elements. This mechanism of downmodulation defines a negative regulatory mechanism controlled by calcineurin/NFAT to modulate T cell proliferation.

Results

CDK4 Protein Levels Are Elevated in Calcineurin $A\alpha^{-/-}$ Mice

We have previously demonstrated a distinct role for calcineurin in the regulation of the kinase activity of CDK4 (Baksh et al., 2000). A new functional role for calcineurin in the regulation of CDK4 was observed in vivo using calcineurin $A\alpha^{-/-}$ mice (Figure 1). Calcineurin Aα is predominantly present in the peripheral immune system, whereas the slower migrating β form is predominantly found in the thymus. Calcineurin $A\alpha^{-/-}$ mice consistently had splenomegaly, with a corresponding 2-fold increase in the number of splenic T cells (data not shown). Analysis of the splenic T cells from calcineurin $A\alpha^{-/-}$ mice demonstrated that they had increased CDK4 kinase activity and, surprisingly, higher protein levels of CDK4 (Figure 1A). Importantly, the increases in CDK4 kinase activity and expression levels were not observed in the thymus (Figure 1A), nor was there an increase in the kinase activity and protein expression of CDK6 (data not shown), indicating that the actions of calcineurin Aα were specific for the G0/G1 restriction point kinase, CDK4. In addition, analysis of *CDK4* mRNA indicated that the absence of calcineurin resulted in a 4-fold elevation of the levels of *CDK4* mRNA (Figure 1B), supporting the increased kinase activity and protein levels of CDK4. We propose that the increased numbers of splenic T cells in calcineurin $A\alpha^{-/-}$ mice may have been due to increased CDK4 expression and kinase activity. Calcineurin Aα can, therefore, negatively regulate the levels of CDK4 and control its kinase activity at the level of transcription.

NFATc2-Dependent Repression of the *CDK4* Promoter

Calcineurin can control gene transcription within the immune system by utilizing the NFAT family of transcription factors (Baksh and Burakoff, 2000). We examined the possibility that the calcineurin/NFAT pathway may function to regulate the expression of CDK4. A schematic of the minimal human *CDK4* 5′ flanking sequence is illustrated in Figure 2A. Elements that may be required for basal and inducible CDK4 expression are indicated. This sequence contained an 824 base pair (bp) region of the *CDK4* promoter and 43 bp of the first untranslated exon (−867 bp promoter fragment, F[−867]). A consensus NFAT core binding sequence was identified at +11 from the transcriptional start site of the first untranslated exon of *CDK4*. This site contained a consensus NFAT core binding sequence, TTCCC, but no identifiable AP-1 binding site within the vicinity of the NFAT consensus site. In order to determine the influence of the NFAT family on the regulation of the human *CDK4* promoter, transient reporter assays were carried out in BHK cells, cells that have no detectable levels of the immune-specific isoforms of NFAT (NFATc2 and NFATc3) (data not shown). Ectopic expression of NFATc2 downregulated the activity of F[−867] and fragment B (F[B]) by 52%; fragment A (F[A]) was unaffected, suggesting that F[B] contained the majority of the functional sites for NFAT-dependent transcriptional regulation. The repression observed was specific for the NFATc2 isoform, as no repression was observed with NFATc3 and NFATc4 (Figure 2B), although proteins were expressed at comparable levels (Figure 2C, lanes 1, 2, and 7).

The Ability of NFATc2 to Repress the *CDK4* Promoter Is Dependent upon the Function of Calcineurin

The DNA binding ability of NFATc2 is controlled by the phosphatase activity of calcineurin. A calcineurin binding domain has been characterized within the first 130 amino acids for NFATc2 (Aramburu et al., 1998). Calcineurin functions to dephosphorylate several serine/

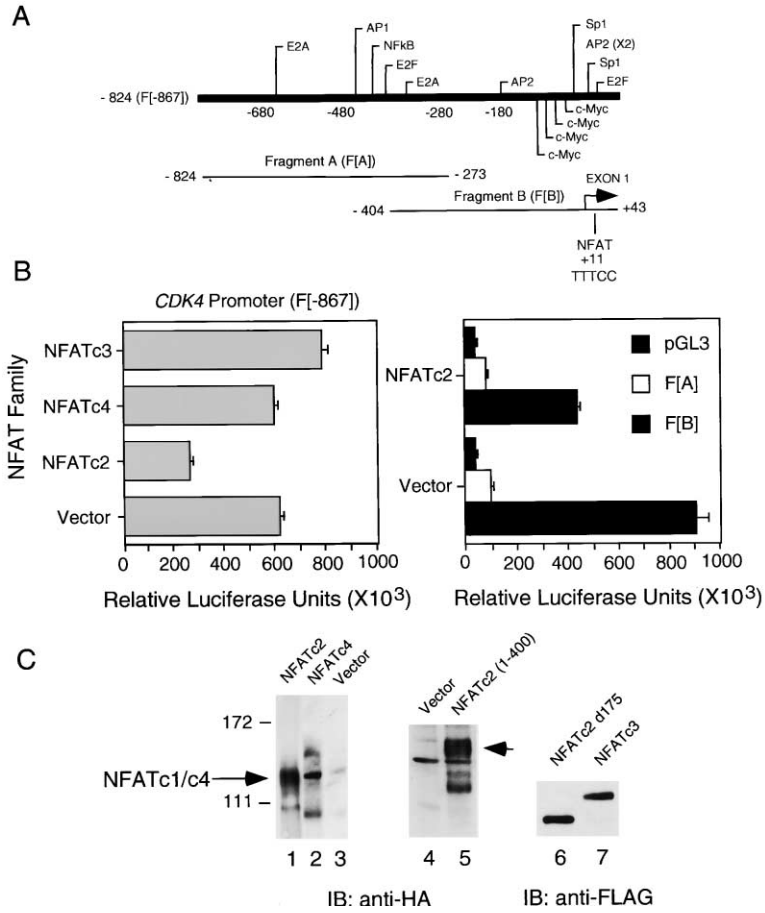

Figure 2. NFATc2-Dependent Repression of the *CDK4* Promoter

(A) Schematic representation of the *CDK4* promoter. Numbers indicate position from the transcriptional start site. The potential NFAT binding site is indicated with the core sequence TTTCC at +11.

(B) BHK cells were transfected with the indicated NFAT expression construct and reporter constructs.

(C) SDS-PAGE was carried out to determine expression of the NFAT constructs. Hematoglutinnin (HA)-NFATc2 (lane 1); HA-NFATc4 (lane 2); vector (pEFTag) (lane 3) (7.5% SDS-PAGE). pEFTag (lane 4); HA-NFATc2 (1–400) is indicated by the arrow (10% SDS-PAGE). FLAG-NFATc2 d175 (lane 6); FLAG-NFATc3 (lane 7) (10% SDS-PAGE). HA-tagged proteins were immunoblotted (IB) using 12CA5 (anti-HA) (0.7 μg/ml), and FLAG-tagged proteins were detected using a rabbit anti-FLAG polyclonal antibody (1:2000) (Affinity Bioreagents Reagents).

threonine residues within the amino terminus of NFATc2, resulting in the exposure of a nuclear localization signal (NLS) and thus translocation to the nucleus (Zhu et al., 1998). We examined the requirement for calcineurin in NFAT-mediated control of the *CDK4* promoter. Ectopic expression of full-length NFATc2 (NFAT WT; Figure 3A, left panel) resulted in a 52% reduction of the basal activity of F[B]. In contrast, a constitutively active NFATc2 mutant with a deletion of the first 175 amino acids, NFATc2 d175, was observed to repress the basal activity of F[B] by ~90% (NFATc2 d175; Figure 3A). The d175 mutant of NFATc2 was found to be localized in the nucleus and did not require calcium or calcineurin activation for its transcriptional activity (Luo et al., 1996), suggesting that the DNA binding domain of NFAT may be sufficient to repress the *CDK4* promoter. Conversely, an NFATc2 mutant lacking the DNA binding domain (NFATc2 1–400; Figure 3A) failed to repress F[B], supporting a role for the DNA binding domain of NFATc2 in the repression of the *CDK4* promoter. The NFATc2-dependent repression of F[B] could be reversed by the addition of FK506 (Figure 3A) and CsA (data not shown). Both FK506 and CsA function to inhibit the phosphatase activity of calcineurin and prevent the dephosphorylation of its substrates. Notably, FK506 reversal of the NFATc2-dependent repression was not complete, restoring only 72% of basal activity of F[B], suggesting that the remaining 28% may be independent of the actions of calcineurin.

In order to confirm the effects of NFATc2, reporter assays were carried out using the bona fide NFAT-responsive element from the *IL-2* promoter, NFAT/AP-1 (Figure 3B). Predictably, transfection with wild-type NFATc2 resulted in a 13-fold elevation in the activity the NFAT/AP-1 reporter from the *IL-2* promoter and a 25-fold elevation in NFAT/AP-1 reporter activity when using the d175 mutant of NFATc2. In contrast, NFATc2 (1–400) was not able to transactivate the NFAT/AP-1 reporter, confirming that the DNA binding domain of NFATc2 was important in its ability to regulate promoter elements. Additionally, FK506 was able to reverse the effects of NFATc2 (Figure 3B).

Since calcium signals control the actions of calcineurin, we investigated the effects of calcium signals on the basal activity of F[B]. Addition of the calcium ionophore, A23187, induced the repression of the *CDK4* promoter in Jurkat cells (Figure 3C) in a time-dependent manner starting at 10–15 hr and was sustained for greater than 20 hr. Calcineurin activation and NFATc2 translocation occurs within the first 15 min of T cell activation, paralleling calcium transients (Dolmetsch et al., 1997); however, during this time frame there is only a minor effect of ionophore treatment on the basal activity of F[B] (Figure 3C). This delay suggests that the calcium-dependent downmodulation of *CDK4* promoter activity may be dependent upon a specific stage in the cell cycle or that there may be a requirement for the production of a specific factor to cooperate with NFAT

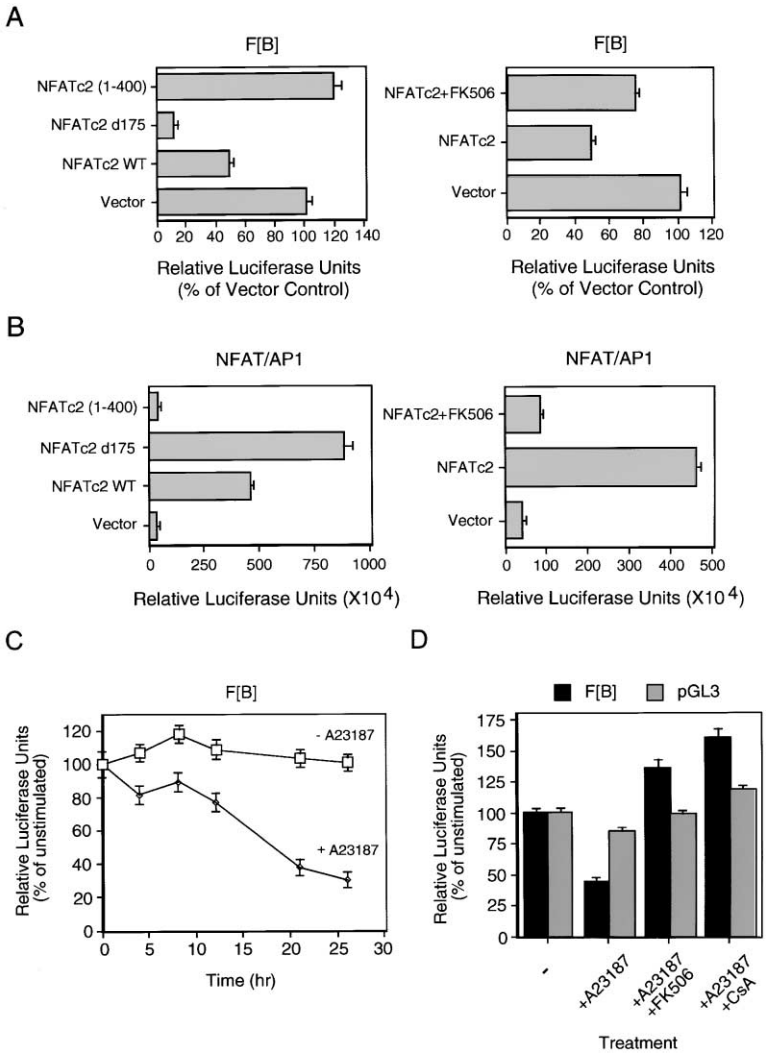

Figure 3. NFATc2-Dependent Repression of Fragment B of the *CDK4* Promoter Requires Calcineurin Activation and Calcium Signals

(A and B) BHK cells were transfected with the indicated NFAT expression vectors with (A) *CDK4* promoter fragment B (F[B]), and (B) NFAT/AP-1 reporter construct (1 μg) from the *IL-2* promoter.

(C) F[B] was transfected in Jurkat cells. Cells were left unstimulated (−A23187) or were stimulated with 2 μM ionophore (+A23187) 20 hr post transfection. The cells were harvested at the indicated times post stimulation.

(D) Jurkat cells were transfected with F[B]. At 20 hr post transfection, cells were left unstimulated or were stimulated with 2 μM A23187 for 20 hr in the absence (+A23187) or presence of 100 nM FK506 (+ A23187+FK506) or 2 μM CsA (+A23187+CsA).

in the downmodulation of the *CDK4* promoter. To confirm the requirement for NFATc2 in the calcium-induced repression of F[B], FK506 or CsA was used to inhibit the activity of calcineurin and modulate the nuclear localization of NFATc2. FK506 or CsA treatment resulted in the reversal of the repressive effects of A23187 to levels above the control reporter vector (pGL3) (Figure 3D). FK506 and CsA did not modulate the levels of pGL3 (Figure 3D), nor did the addition of phorbol myristate acid (PMA) affect the basal activity of F[B] (data not shown). Taken together, these data suggest that NFATc2 functions to repress the *CDK4* promoter by utilizing calcium signals and calcineurin.

A Functional NFAT Response Element Is Located Immediately Downstream of the 5′ Untranslated Region of Exon 1

NFAT binding sequences are present within the promoters of numerous cytokine elements and surface receptors. The core sequence of an NFAT response element, GGAAA, is characteristic of a number of NFAT binding sites within the *IL-2*, *IL-4*, *IL-3*, *GM-CSF*, *Fas Ligand* (Kel et al., 1999), and *TRAIL* promoters (Wang et al., 2000). In the majority of these promoters, multiple NFAT binding sites are present that coordinate transcriptional control of the promoter. Electromobility shift assays (EMSA) were carried out to determine binding characteristics of the NFAT site at +11. A purified histidine-tagged NFATc2 DNA binding domain fusion protein (NFATc2 DBD) was used in an EMSA analysis and was observed to bind to an oligonucleotide containing the NFAT binding site within F[B] of the *CDK4* promoter (Figure 4A, lane 1). Stoichiometrically, this site bound more NFATc2 DBD than an NFAT site from the *IL-2* promoter (Figure 4A, compare lanes 1 and 7). For both NFAT sites, the mutation of the core of the NFAT binding sequence resulted in the complete loss of NFATc2 DBD interaction with the oligonucleotide (Figure 4A, lanes 2 and 8). In addition, the interaction of the NFATc2 DBD with the labeled NFAT(CDK4) oligonucleotide can be competed with the corresponding unlabeled wild-type oligonucleotide (Figure 4A, lanes 3 and 10). Furthermore, the binding of the NFATc2 DBD to the NFAT site on the *CDK4* promoter can be competed with the NFAT(IL-2) wild-type oligonucleotide (Figure 4A, lane 5), indicating that the binding characteristics of NFATc2 DBD to

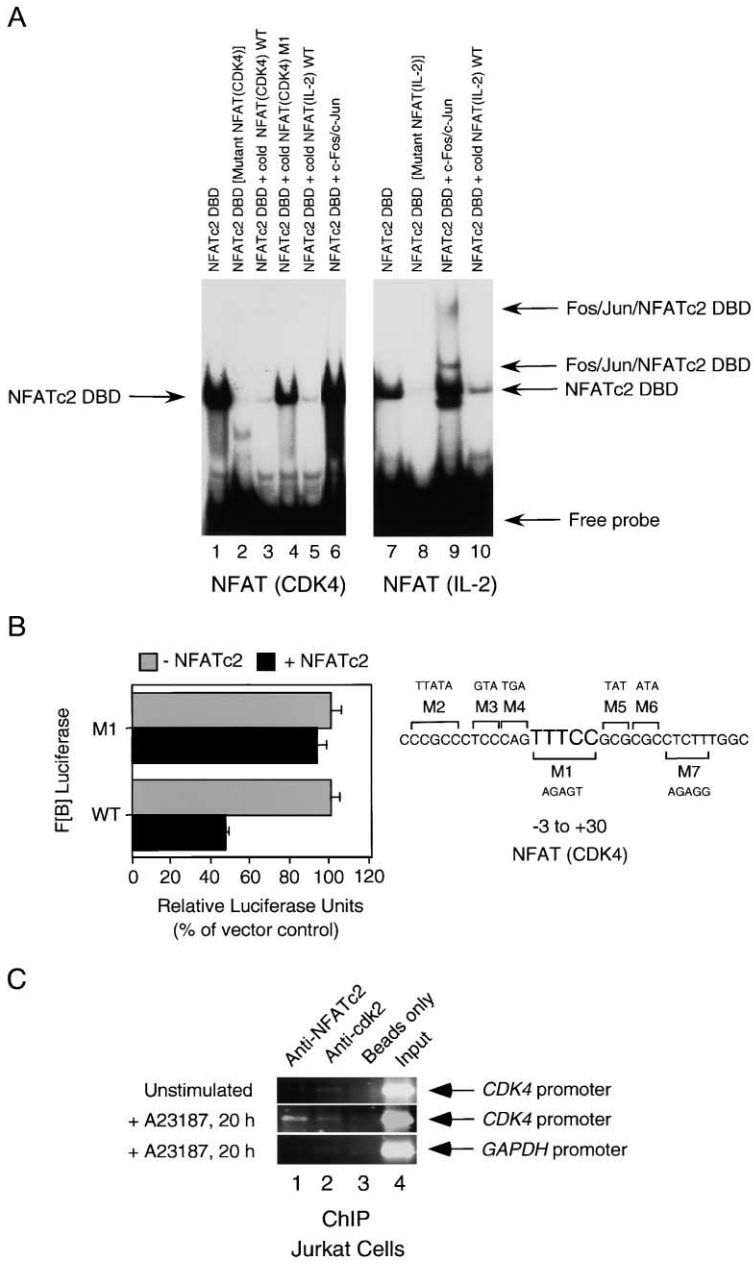

Figure 4. Characterization of the NFAT Binding Site within Fragment B

(A) EMSA was carried out on a 4% nondenaturing acrylamide gel. For NFAT (CDK4) oligonucleotide, 7 nM of NFATc2 DBD was used; for NFAT (IL-2) oligonucleotide, 21 nM of NFATc2 DBD was used. For oligonucleotide competition experiments, a 100-fold excess was used. Lanes 1–6, oligonucleotide for NFAT site on the *CDK4* promoter [NFAT(CDK4)]; lanes 7–10, oligonucleotide for NFAT site on the *IL-2* promoter [NFAT(IL-2)]; lane 2, M1 mutant of the NFAT(CDK4) oligonucleotide (a change of TTTCC→AGAGT) (see [B]); lane 8, mutant oligonucleotide of the NFAT binding site of the *IL-2* promoter (a change of GGA→TTC).

(B) Analysis of mutants to NFAT(CDK4) in BHK cells. A schematic of the location of mutants to NFAT(CDK4) is shown in the right panel; residues above mutants (M1–M7) denote changed nucleotides. Left panel, corresponding reporter analysis of M1 mutant of NFAT(CDK4). Mutations were made within the context of F[B] (WT). All experiments were performed at least four times.

(C) 2% agarose gel of a chromatin immunoprecipitation on Jurkat cells. The sample ("Input") was divided into three and immunoprecipitated using 10 μg of anti-NFATc2 (Upstate Biotechnology), anti-cdk2 (Santa Cruz Biotechnology) (control antibody), or with beads alone ("Beads only").

NFAT(CDK4) may be similar to an NFAT binding site on the *IL-2* promoter.

NFAT binding sequences have been described as composite binding sequences, requiring a second element, such as MEF2 (Youn et al., 2000), GATA2 (Musaro et al., 1999; Semsarian et al., 1999), GATA4 (Molkentin et al., 1998), or, more commonly, AP-1 (Kel et al., 1999), in order to tether NFAT to its binding site. The primary sequence surrounding the NFAT site at +11 lacks a defined recognition sequence for MEF2, GATA2, or GATA4 elements. We investigated AP-1 binding by the addition of c-Fos and c-Jun to the binding mixture of NFATc2 DBD and the NFAT(CDK4) oligonucleotide (Figure 4A, lane 6). This did not result in the appearance of slower migrating complexes of c-Fos/c-Jun/NFATc2 DBD, indicating that the binding of NFAT to the NFAT binding site on F[B] may not require AP-1 elements in order to regulate the expression of the *CDK4* promoter. In contrast, the addition of c-Fos and c-Jun to the binding mixture of NFATc2 DBD and the NFAT(IL-2) oligonucleotide resulted in the appearance of slower migrating complexes containing c-Fos/c-Jun/NFATc2 DBD (Figure 4A, lane 9).

Mutational analysis of NFATc2 binding to the *CDK4* promoter was carried out within the context of the F[B] reporter. Mutation of the core sequence (M1) (Figure 4B, left panel), but not other mutations (M2, M5–M7) (data not shown), resulted in the loss of NFATc2-dependent transcriptional repression, suggesting that TTTCC was required to coordinate the binding of NFATc2 to F[B]. Additionally, mutations M3 and M4 also resulted in the substantial loss of NFATc2-dependent repression (data

not shown), suggesting that NFATc2 may require a factor that binds immediately 5′ to the core NFAT binding sequence in order to repress the *CDK4* promoter.

In order to further confirm the binding of NFATc2 to the promoter of *CDK4*, chromatin immunoprecipitation was carried out to observe an in vivo association of NFATc2 with the *CDK4* promoter. Jurkat cells were left unstimulated or were stimulated with the calcium ionophore, A23187 (for 20 hr); the resultant nuclear fractions were immunoprecipitated using specific antibodies to NFATc2, and the associated DNA was purified out (Figure 4C). Using specific primers to the *CDK4* promoter, a 400 bp PCR product was obtained from Jurkat cells stimulated for 20 hr with A23187 (Figure 4C, lane 1), but not from unstimulated cells (Figure 4C, lane 1) nor from cells stimulated with A23187 for 6 hr (data not shown). This association was specific for NFATc2 since no PCR product was obtained when using a nonspecific antibody (anti-cdk2), when using beads alone (Figure 4C, "Beads only"), or when using primers to an unrelated promoter, such as GAPDH. We speculate that NFAT can transcriptionally regulate the promoter during the later stages of T cell activation where calcium transients are still at a high level due to the presence of the I_{CRAC} channels (Parekh and Penner, 1997), functioning to maintain NFATc2 within the nucleus. As T cells return to the quiescent state or unstimulated state, calcium levels subside and NFATc2 looses its association with the *CDK4* promoter (Figure 4C, unstimulated) and relocalizes to the cytosol.

NFATc2 May Downmodulate the Expression of CDK4 in Peripheral Blood Lymphocytes as Cells Exit the Cell Cycle

To examine the functional significance of NFATc2 regulation of the *CDK4* promoter, human peripheral blood lymphoctyes (PBLs) were isolated, rendered competent, and stimulated with IL-2 to promote progression to the S phase in the presence and absence of the calcineurin inhibiting drug CsA (Figure 5A). CDK4 protein was present at 21 hr post IL-2 stimulation and was sustained until 72 hr. As previously demonstrated (Lucas et al., 1995; Modiano et al., 1994), the levels of CDK4 declined by 88 hr as cells possibly exit the cell cycle (Figure 5A). In the presence of CsA, levels of CDK4 accumulated to the same extent in the absence of CsA; however, the downmodulation was not observed by 88 hr (Figure 5A, right panel), suggesting a role for the calcineurin/NFAT pathway in the downmodulation of the protein levels of CDK4 as cells re-enter into quiescence. The presence of CsA did not alter CDK6 levels (Figure 5A, bottom panel), confirming the specificity in the role played by calcineurin/NFATc2 in regulating protein expression levels of CDK4. The addition of a calcineurin-inhibiting drug, FK506, also resulted in an increase in *CDK4* mRNA (but not *IL-2* mRNA) (Figure 5B), further confirming that the calcineurin/NFATc2 pathway may negatively regulate the transcription of *CDK4*. We have observed similar results with another calcineurin inhibiting drug, CsA (data not shown).

Additional in vivo evidence for NFATc2 regulation of the *CDK4* promoter was obtained by carrying out chromatin immunoprecipitations (ChIP) on PHA-stimulated

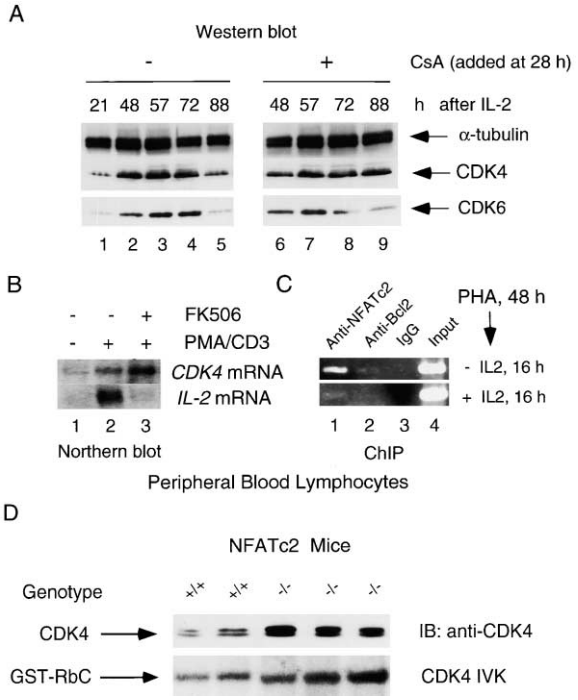

Figure 5. The Expression of CDK4 Is Dependent upon the Activity of Calcineurin and the Function of NFATc2

(A) PBLs were stimulated with PMA/A23187 for 20 min at 37°C to achieve competency (Modiano et al., 1994). The cells were then washed three times with 1× PBS and then stimulated with 20 U/ml human recombinant IL-2 and harvested at the indicated times. At 28 hr, 2 μM CsA was added to lanes 6–9, and the cells were then harvested at the indicated times post IL-2 stimulation. The CDK4 and CDK6 expression profile was performed with α-tubulin (ICN) expression carried out to check for protein loading.

(B) PBLs were cultured without stimulation or were stimulated with PMA and anti-CD3 (50 ng/ml) for 6 hr in the absence or presence of a 30 min preincubation with 100 nM FK506 as indicated. Northern blotting was carried out using full-length *CDK4* and *IL-2* cDNA as respective probes.

(C) 2% agarose gel of chromatin immunoprecipitation analysis carried out on PHA-stimulated PBLs for 48 hr followed by no addition (−IL2) or the addition (+IL2) of IL-2 for 16 hr. ChIP analysis was carried out with 10 μg of rabbit polyclonal NFATc2, rabbit anti-Bcl-2 (a nonspecific antibody), or rabbit IgG control (Upstate Biotechnology).

(D) Splenic T cells were isolated from NFATc2 wild-type and NFATc2$^{-/-}$ mice, and protein expression was carried out for CDK4. In addition, an in vitro kinase assay was carried out for CDK4 using GST-RbC as the substrate. A representation of two NFATc2$^{+/+}$ mice and three NFATc2$^{-/-}$ mice is shown (analysis of three additional NFATc2$^{+/+}$ and NFATc2$^{-/-}$ mice yielded similar results).

PBLs that were allowed to proliferate or return to the resting state (Figure 5C). ChIP analysis using a specific NFATc2 antibody demonstrated that NFATc2 binding to the promoter was obtained as cells were allowed to re-enter the resting state (Figure 5C, "−IL2"); if cells were allowed to proliferate in the presence of IL-2 (Figure 5C, "+IL2"), no interaction with the *CDK4* promoter was obtained, and thus, NFATc2 was not required to downmodulate the expression of CDK4. FACS analysis confirmed that PBLs returned to the resting state in the absence of IL-2 (data not shown). These observations demonstrate that the calcineurin/NFATc2 pathway may

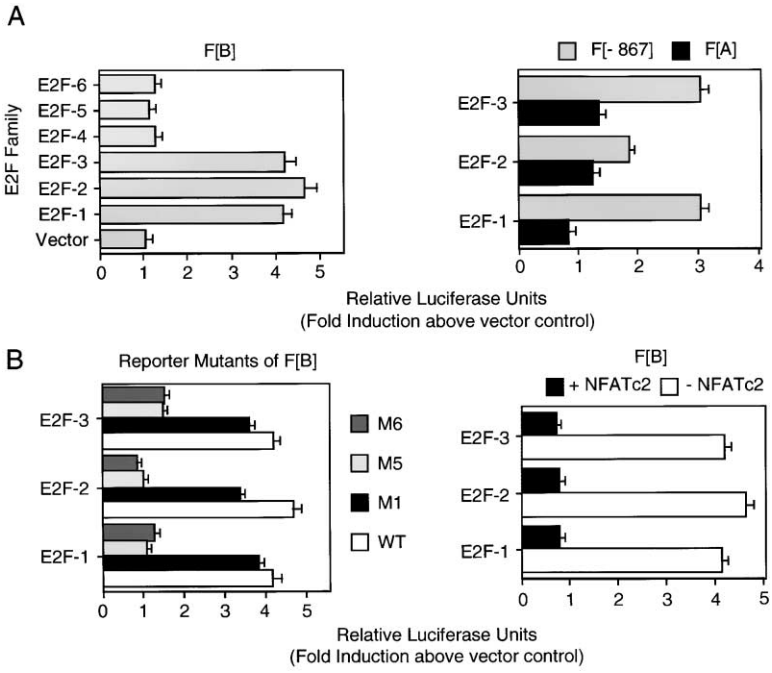

Figure 6. NFATc2 Can Downmodulate the Activity of the *CDK4* Promoter by Displacing E2F

(A) BHK cells were transfected with 0.5 μg of the indicated E2F expression constructs. Total DNA for the transfection was made to 4 μg with the E2F empty vector (pRCCMV).
(B) Left panel: E2F-1, E2F-2, or E2F-3 was coexpressed in BHK cells with reporter constructs containing wild-type F[B] (WT) or F[B] mutants M1, M5, and M6 (see Figure 4B); right panel: BHK cells transfected with the indicated E2F expression constructs in the absence (−NFATc2) or presence of 4 μg NFATc2 (+NFATc2).

function to downregulate the levels of CDK4 as cells exit the cell cycle and return to the resting state. It is at this stage that we observe the interaction of NFATc2 with the *CDK4* promoter, providing a mechanism for the downmodulation of CDK4 levels observed in Figure 5A.

NFATc2$^{-/-}$ Mice Have Elevated Expression and Kinase Activity of CDK4

To confirm the negative regulatory role of NFATc2 in the regulation of the expression levels of *CDK4*, we carried out an analysis of CDK4 protein levels and kinase activity in NFATc2$^{-/-}$ mice (Figure 5D). NFATc2$^{-/-}$ mice have been previously demonstrated to have an enhanced inflammatory response, hyperproliferation, and splenomegaly (Xanthoudakis et al., 1996). CDK4 protein levels and kinase activity were elevated in majority of the splenic T cells in NFATc2$^{-/-}$ mice (Figure 5D), supporting a negative regulatory role for the calcineurin/NFATc2 pathway in modulating the expression levels of CDK4. We did not observe changes in CDK6 kinase activity or protein expression in NFATc2$^{-/-}$ mice (data not shown), suggesting a specific role for NFATc2 in the control of CDK4 expression.

E2F Transactivation of the CDK4 Promoter Is Repressed by NFATc2 via Recruitment of Histone Deacetylase Activity

The E2F family of transcription factors is composed of two distantly related subfamilies, E2F and DP (Black and Azizkhan-Clifford, 1999), that regulate numerous S phase genes (such as dihydrofolate reductase, cyclin E, thymidine kinase, and DNA polymerase α). E2F-1, E2F-2, and E2F-3 are associated with pRb, functioning to prevent E2F from binding to its promoter. E2F-4 and E2F-5 are thought to be in association with the Rb-related proteins, p130 and p107 (Verona et al., 1997). A putative E2F binding site was found to be present immediately 3′ to the core NFAT(CDK4) binding sequence (CGCG) (Figure 4B). We therefore investigated whether E2F family members could possibly regulate the *CDK4* promoter in cooperation with NFATc2. Transient transfection of E2F family members in BHK cells demonstrated that E2F-1, E2F-2, and E2F-3 can transactivate F[B] 4- to 5-fold above basal activity (Figure 6A, left panel). The ability to transactivate the promoter was localized to F[B] of the *CDK4* promoter (Figure 6A, right panel) and required the E2F core sequence, CGCG, in order to bind to the promoter (Figure 6B, left panel). Unexpectedly, coexpression with NFATc2 inhibited the ability of E2F-1, E2F-2, and E2F-3 to transactivate F[B] (Figure 6B, right panel). In vivo promoter analysis via ChIP confirmed that E2F-1 (but not E2F-4 or an unrelated transcription factor, Sp3) can specifically bind to the *CDK4* promoter and that this association is lost under conditions that stimulate NFATc2 interaction with the promoter (Figure 7A, "+A23187, 20 hr"). Furthermore, in vitro DNA binding analysis of the ability of E2F-1, E2F-2, or E2F-3 to bind to the NFAT(CDK4) oligonucleotide mutant M1 (defective in NFATc2 binding) revealed that E2F binding was dramatically enhanced when compared to E2F binding to the wild-type NFAT(CDK4) oligonucleotide (data not shown). We propose that E2F can bind to the E2F site in F[B], but binding may be optimal in the absence of NFATc2.

E2F-dependent activation has also been demonstrated to be regulated by histone deacetylase (HDAC) activity by the recuitment of HDAC activity by pRb (Brehm and Kouzarides, 1999; Luo et al., 1998). Specifically, the recruitment of HDAC1 results in the deacetylation of histone H3 and thus suggests a critical role for HDAC1 in pRb-dependent repression of E2F transcriptional activity (Luo et al., 1998). In light of our data suggesting that NFATc2 functions to repress the expression of CDK4, we utilized ChIP analysis to look at the histone

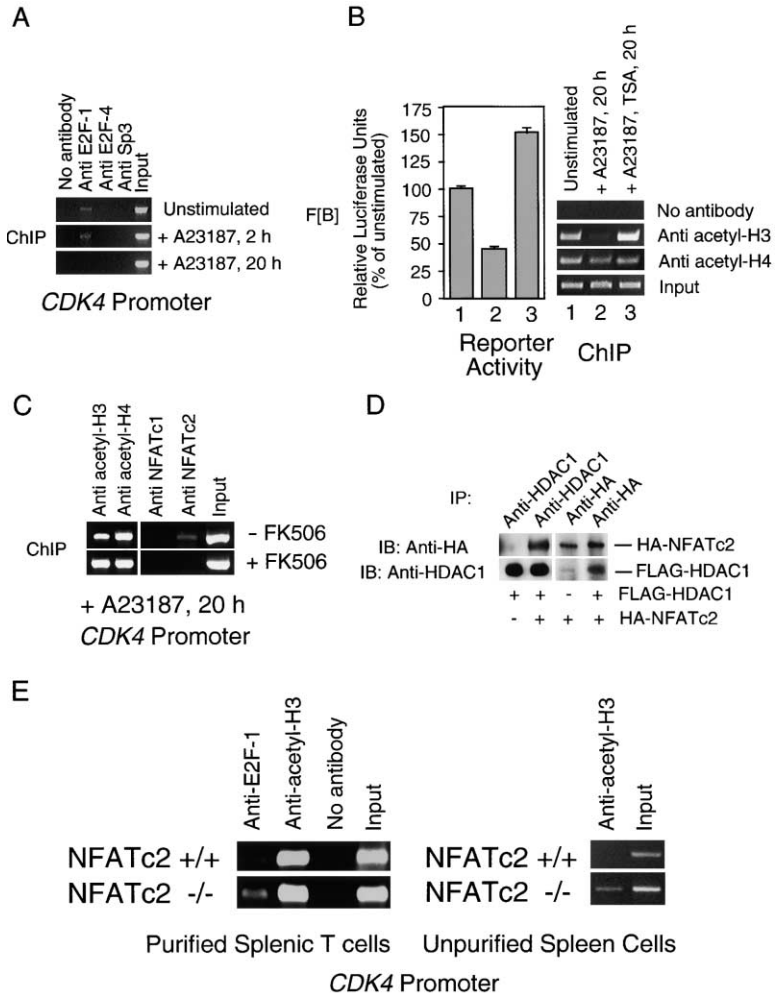

Figure 7. NFATc2-Dependent Negative Regulation of the *CDK4* Promoter May Involve the Recruitment of Histone Deacetylase Activity

(A) ChIP analysis was carried out in Jurkat cells under conditions indicated and with the labeled antibodies.

(B) Left panel: Jurkat cells transfected with 1 μg of F[B]. Twenty hours post transfection, cells were treated with either 2 μM calcium ionophore A23187 or with 300 nM of the histone deacetylase inhibitor TSA, as indicated. Cells were harvested 20 hr post stimulation, and reporter activity was determined. Right panel: ChIP analysis carried out in Jurkat cells under conditions described in the left panel.

(C) ChIP analysis was carried out in Jurkat cells under conditions indicated and with the labeled antibodies. All experiments were carried out at least three times.

(D) Cells were transfected with the indicated DNA. Fourty-eight hours post transfection, cells were harvested, lysed, and immunoprecipitated (IP) with the indicated antibodies and immunoblotted (IB).

(E) Left panel: purified splenic T cells isolated from NFATc2$^{+/+}$ and NFATc2$^{-/-}$ mice (n = 8); right panel: red cell lysed splenocytes isolated from NFATc2$^{+/+}$ and NFATc2$^{-/-}$ mice (n = 4); both populations were stimulated with Con A (5 μg/ml) for 48 hr followed by a change to media without Con A to allow to enter back into quiescence. Cells were harvested 16 hr later, and ChIP analysis was carried out using the indicated antibodies. Resultant DNA was amplified using specific primers to the human *CDK4* promoter (A–C) or to the mouse *CDK4* promoter (E) and was visualized on a 2% agarose gel.

H3 and H4 acetylation status of the *CDK4* promoter under conditions that maximize NFATc2-dependent repression of the *CDK4* promoter. Calcium-dependent repression of the *CDK4* promoter, by treatment of cells with A23187, resulted in a significant decrease in the acetylation of histone H3 (Figure 7B), indicative of chromatin-mediated transcriptional repression. The addition of the specific and irreversible HDAC inhibitor, trichostatin A (TSA), resulted in the enhancement of the acetylation of histone H3 to levels above what is present in unstimulated Jurkat cells (Figure 7B). Interestingly, histone H4 acetylation levels did not change, suggesting a specific role for histone H3 deacetylation in the repression of the *CDK4* promoter. Furthermore, transient reporter assays in Jurkat cells confirmed the importance of an HDAC-mediated pathway in regulating the activity of the *CDK4* promoter, since the addition of TSA potently reversed the ionophore-induced repression to levels 60% above basal activity (Figure 7B, left panel). Similarly, the inhibition of the calcineurin/NFATc2 pathway by the addition of FK506 was observed to enhance the H3 acetylation status of the *CDK4* promoter and inhibit NFATc2 binding in Jurkat cells (Figure 7C), supporting a role for both the calcineurin/NFATc2 pathway and the requirement for HDAC activity in the regulation of the *CDK4* promoter.

NFATc2 recruitment of an HDAC activity was further confirmed by immunoprecipitation experiments demonstrating that NFATc2 can associate with HDAC1 (Figure 7D) and thus bring an HDAC activity to the *CDK4* promoter. In addition, in purified splenic T cells that were allowed to proliferate and return to quiescence, we observed a dramatic enhancement of the binding of E2F-1 and an increase in the H3 acetylation status of the *CDK4* promoter in NFATc2$^{-/-}$ splenic T cells when compared to wild-type controls (Figure 7E, left panel). Histone H3 acetylation levels were also elevated in unpurified splenocytes from NFATc2$^{-/-}$ mice (Figure 7E, right panel), indicating that the mechanisms that may control CDK4 expression are not restricted to T lymphocytes. These observations again suggest that calcium-dependent transcriptional control of the *CDK4* promoter may be dependent upon both the calcium sensitive transcription factor, NFATc2, and the recruitment of an HDAC activity that may regulate histone H3-specific deacetylation, resulting in transcriptional repression of the *CDK4* promoter.

Discussion

In summary, our results demonstrate a negative regulatory role of the calcineurin/NFATc2 pathway in the con-

trol of the expression levels of an important G0/G1 cyclin-dependent kinase, CDK4. CDK4 controls how cells enter into and are committed to progress through the cell cycle. The calcineurin/NFATc2 pathway appears to be very specific for regulating CDK4 protein expression, since we did not observe changes in the expression profiles of CDK6 in calcineurin $A\alpha^{-/-}$, $NFATc2^{-/-}$ mice nor in human peripheral blood lymphocytes treated with CsA. NFATc2 can bind to a site immediately downstream of the transcriptional start site and may function to downmodulate the activity of the promoter. The ability of NFATc2 to negatively regulate the *CDK4* promoter is unique; NFATc2 does not require AP-1 elements and requires only calcium-dependent signals in order to stabilize the association with the *CDK4* promoter. NFATc2 is normally thought to be a positive regulator of transcription by upregulating the expression levels of numerous cytokine receptors and noncytokine receptors (Hodge et al., 1996). However, a negative role for NFATc2 has been reported in adult mice missing NFATc2 (Ranger et al., 2000). The lack of NFATc2 resulted in increased expression of cartilage markers, type II and type X collagen, markers important in the development of chondrogenesis. Furthermore, $NFATc2^{-/-}$ mice have an enhanced inflammatory response in an in vivo model of allergic inflammation (Xanthoudakis et al., 1996) and splenomegaly (Hodge et al., 1996). It was speculated that the increased splenocyte numbers were due to decreased expression of key apoptotic mediators, such as CD95. An alternate explanation for the increased numbers of splenic T cells may be related to the role carried out by NFATc2 in the regulation of the expression of CDK4. In the absence of NFATc2 (in $NFATc2^{-/-}$ mice), an increase in proliferation may arise from the lack of downmodulation of CDK4 levels, resulting in an increased entry of T cells into the cell cycle and, hence, an enhanced immune response.

We propose that NFATc2 functions to negatively regulate the transcription of the *CDK4* promoter by displacing E2F family members and possibly to modulate the acetylation status of the core histone H3 by recruitment of an HDAC. We demonstrate that under conditions that maximize promoter repression (ionophore treatment for 20 hr), we observed a reduction in the acetylation of histone H3 within the *CDK4* promoter (Figure 7B). This was reversed by the addition of TSA, an HDAC inhibitor, and with FK506/CsA, an inhibitor of NFAT transcriptional activity (Figure 7C). Furthermore, we can demonstrate a direct association between HDAC1 and NFATc2 (Figure 7D) and propose that NFATc2-dependent repression of the CDK4 promoter may involve HDAC activity by the recruitment of an HDAC family member by NFATc2. NFATc2, therefore, does not cooperate with E2F, but may oppose its transactivation of the *CDK4* promoter. We postulate that E2F may function to stimulate the expression of CDK4 as cells enter the cell cycle; however, NFATc2 functions to downmodulate the activity of the *CDK4* promoter as cells return to the quiescent state by displacing E2F from the promoter and thus negatively regulates the expression of CDK4.

Acetylation/deacetylation of histones is a well characterized posttranslational modification that the cell employs to tightly control transcription. Both histone H3 and H4 acetylation are associated with transcriptional control mechanisms, allowing *trans*-acting factors to associate with the cognate DNA binding sites and allow transcription to occur (Roth et al., 2001; Wolffe and Guschin, 2000). Conversely, histone deacetylases reverse histone acetylation, resulting in a more compact chromatin environment that is transcriptionally repressive. We speculate that the calcium-dependent repression of the *CDK4* promoter may involve the recruitment of HDAC (possibly via its interaction with NFATc2) to downmodulate the E2F-dependent activation of the promoter in order to regulate the expression of CDK4 as cells return to the resting state. This role of NFATc2 may offer an alternative explanation for the hyperproliferation observed in $NFATc2^{-/-}$ mice and the increased risk of lymphomas in patients treated with CsA or FK506 for a prolonged period post transplant.

Experimental Procedures

Calcineurin $A\alpha^{-/-}$ and $NFATc2^{-/-}$ Mice

Calcineurin $A\alpha^{-/-}$ mice (Zhang et al., 1996) and $NFATc2^{-/-}$ mice (Xanthoudakis et al., 1996) were generated as previously described. The thymus and spleen of wild-type, hetereozygous, and homozygous knockout mice were analyzed at 8–9 weeks of age for calcineurin $A\alpha$, and at 12–14 weeks of age for $NFATc2^{-/-}$. Calcineurin $A\alpha^{-/-}$ mice were genotyped using specific primers to calcineurin $A\alpha$, and $NFATc2^{-/-}$ mice by Western blotting with an NFATc2 antibody (Pharmingen). Wild-type controls were matched for age and sex, and all mice were housed under sterile conditions.

Isolation of Thymic, Splenic, and Peripheral Blood Lymphocytes

Thymus was removed from mice and processed as described elsewhere (Hollander et al., 1994). Spleen and peripheral blood lymphocytes were similarly treated and purified by applying the cellular suspension to a nylon wool column, and 2×10^7 cells were used for CDK4 kinase assay. FACS analysis to characterize surface expression and determine purity of the eluted suspension was carried out using antibodies to CD4 and CD3 (data not shown). More than 90% of the eluted cells were CD3+ T cells. For isolation of T cells from peripheral blood, a similar protocol was used as described above for splenic T cells.

Cells Lines and DNA Transfections

Jurkat cells were grown in 10% FCS/RPMI. Transfections were carried out using electroporation with settings of 800 μF, low ohm, and 250V. Cells (1×10^7/500 μl) were transfected with 10 μg of DNA. Stimulations were carried out (as indicated) with 2 μM calcium ionophore, A23187 (Calbiochem), or 2 μg/ml phorbol ester (PMA) in 24 well costar plates at 37°C. Baby hamster kidney (BHK) cells (Frank McKeon, Harvard Medical School) were grown in 10% FCS/DMEM. Transfections were carried out on 70% confluent cells using a 1:1.5 ratio of DNA to Fugene6 (Roche). Cells were then harvested 48 hr post transfection. For all NFAT expression constructs, 4 μg of DNA was transfected with the reporter construct. Inhibition of calcineurin activity was carried out by the addition of 100 nM FK506 or 2 μM cyclosporin A (Calbiochem) for 20 hr at 16–20 hr post transfection at 37°C.

CDK4 and CDK6 Kinase Assays

In vitro kinase assays (IVK) were performed for CDK4 and CDK6 as described elsewhere (Baksh et al., 2000) using the carboxy-terminal residues of pRb fused to GST as substrate (GST-RbC; amino acids 772–928 of pRb) (Boston Biologicals). For Western blot analysis, 1/30 of the immunoprecipitation sample was run on a 10% gel, transferred to polyvinylidene difluoride (PVDF), immunoblotted with the indicated antibody, and developed by the enhanced chemiluminescence (ECL) system (Amersham).

Cloning of the Human *CDK4* Promoter

We used the PromoterFinder method (Human PromoterFinder DNA walking kit, Clontech) to amplify the genomic *CDK4* 5′ flanking re-

gion (Genebank accession number AY034380). This method uses human genomic DNA samples that have each been cut with one of five different restriction enzymes (EcoRV, ScaI, DraI, PvuII, SspI) and ligated with adaptors that prevent amplification in the 3′ direction. A series of primers spanning the 5′ untranslated region of the first exon of *CDK4* was used, between nucleotides 9 and 258. Each primer also contained the HindIII restriction site, 5′-aaagctt. We initially used the following primers 5′-TCCCATAGGCACCGACAC CAATTTC-3′, 5′-CCCATAGGCACCGACACCAATTTCAG-3′, and 5′-CGAGAGGTGGCCATTCTCAGATCAAGGG-3′, encompassing nucleotides 282–258, 281–256, and 241–214, respectively. These primers generated various products that were used as templates for reamplification with nested primers encompassing the sequence from nucleotide 43 to nucleotide 21, 5′-aaagcttATGTGACCAGCTG CCAAAGACGC-3′, from nucleotide 61 to nucleotide 37, 5′-CTCACC CCCACCCTACCATGTGAC-3′, and from nucleotide 32 to nucleotide 9, 5′-TAGACACAGGCCGCAAGCTAGAGA-3′. Products of 2120 bp and 867 bp generated from the primer sequence spanning nucleotides 43 to 21 showed identical banding patterns to a full-length human *CDK4* cDNA probe on Southern blotting using DNA digested with BamHI and HindIII. The sequence of these products showed that the distal 867 bases of the 2120 bp product were identical to the sequence of the shorter product. Therefore, each of these fragments, incorporating the 43 bp of untranslated exon 1 sequence, was then cloned into the pGL3-luciferase vector (Promega) using XhoI/HindIII. Both fragments maintained basal activity when transiently transfected into Jurkat T cells, but only the 867 bp fragment (F[−867]) showed inducible activity when the cells were stimulated with anti-CD3, PMA, PHA, or combinations thereof, and it was used to represent the full-length *CDK4* promoter.

Fragment A (F[A]) was generated by cleavage at the NcoI site, and fragment B (F[B]) by cleavage at the KpnI site. Each fragment was cloned into the pGL3-luciferase vector as described above. F[B] mutants (M1–M7) were made using the Stratagene Quickchange Kit using complimentary oligonucleotides containing the mutations of interest. For mutations to the NFAT(CDK4) (mutational changes are underlined), forward primers were: M1, 5′-CCCGCCCTCCCAGA GAGTGCGCGCCTCTTTGGC-3′; M5, 5′-CCCGCCCTCCAGTTTCC TATCGCCTCTTTGGC-3′; M6, 5′-CCCGCCCTCCCAGTTTCCGCGA TACTCTTTGGC-3′. For all of these mutations, the template for the PCR was pGL3-F[B].

Reporter Assay Analysis

The phRL null expression vector (Promega) encoding the renilla firefly luciferase gene under the control of a constitutive promoter was used to monitor transfection efficiencies and as the normalization plasmid. BHK cells were transiently transfected with the following amounts of reporter constructs: F[−867], F[B], and mutants of F[B], 150 ng; F[A], 1 μg; internal control luciferase was phRL null, 30 ng. For Jurkat cells, 1 μg of F[B] and 3 μg of F[A] were used with 60 ng of phRL null. Cellular pellets (containing $\sim 2 \times 10^6$ cells) were washed once with 1× PBS and then frozen as a dry pellet until lysis. Reporter activity levels were determined using the Promega Dual Luciferase Kit as per manufacturers instructions. Relative luciferase units were obtained by normalizing the luciferase activity obtained for the reporter luciferase with that of renilla luciferase. Individual experiments were carried out at least four times.

EMSA

NFAT EMSA was carried using a modified protocol of Macian et al. (2000). Briefly, the DNA binding domain (DBD) of NFATc2 (398–694) and full-length AP-1 elements (c-Fos and c-Jun) were purified as 6× histidine-tagged proteins using the TALON affinity matrix (Clontech). Purification was carried out under reducing conditions in the presence of 5 mM dithiotrietol (DTT), and the resultant purified sample was stored in 10 mM HEPES, 120 mM NaCl, 5 mM DTT. Oligonucleotide duplexes were generated by labeling at 37°C with [γ-^{32}P]dCTP and 12 U of Klenow (Roche). For all labeled duplexes, labeling was initiated by the addition of a primer (CCGATCA) that binds to a common 3′ sequence (TGATCGG) on the oligonucleotide probes (see below). Duplexes were then purified using G-50 Sephadex columns (Roche). The following oligonucleotide duplexes were used (NFAT core sequences are in bold; changed residues are underlined and in lower case; 3′ Klenow initiating sequence are in italics): NFAT(IL-2), CGCCC AAGA**GGAAA**ATTTGTTTCATA*TGATCGG*; NFAT(IL-2)Mutant, CGCCCAAGctt**AAA**ATTTGTTTCATA*TGATCGG*; NFAT(CDK4), CCCGCCCTCCCAG**TTTCC**GCGCGCCTCTTTGGC-*TGATCGG*; NFAT(CDK4)M1, CCCGCCCTCCCAGagagtGCGCGCCTCTTTGGC-*TGATCGG*.

For EMSA, the reaction mixture contained the following: DBD protein (7 nM DBD NFATc2 and/or 160 nM c-Fos/c-Jun), 4000 cpm/μl of radiolabeled duplex; 10 mM HEPES; 125 mM NaCl; 20% glycerol; 0.04 μg/ml poly(dI:dC); 1.6 mg/ml BSA, 0.5 mM DTT in 20 μl volume. Incubation was carried out at room temperature for 30 min and then separated using a 4% nondenaturing acrylamide gel. The gel was prerun for 30 min in 0.25× TBE and then run for 2 hr in 0.25× TBE. The gel was rinsed for 30 s in 10% acetic acid/10% methanol, washed with 500 ml of distilled water, and dried onto Whatman filter paper and autoradiographed.

Quantitative Polymerase Chain Reaction Analysis

Total RNA was isolated from mouse spleens using the tissue RNA isolation kit from Roche. Purity was tested by A260/280 ratios and analysis by gel electrophoresis. For quantitative polymerase chain reaction analysis (QPCR) on calcineurin Aα mice, murine *CDK4* primers used were as follows (accession number NM_009870): forward (1–21), 5′-ATGGCTGCCACTCGATATGAA-3′; reverse (70–52), 5′-GGGCTTTGTACACCGTCCC-3′; and murine *CDK4* probe (23–46), 5′-CCGTGGCTGAAATTGGTGTCGGTG-3′. Each reaction (total volume of 25 μl and assayed in triplicate) was set up using 100 ng of RNA containing 0.2 μM primers and 0.05 μM probe; MuRT and RNase inhibitor without the addition of uracil N-glycosylase (UNG) in the PCR master mix was also used. PCR reaction cycles (using Taqman one-step RT-PCR) were 48°C for 30 min, 95°C for 10 min (for one cycle), 95°C for 15 s, and 60°C for 1 min (repeated for 40 cycles). The data were acquired with the ABI PRISM 7700 Sequence Detection System and analyzed with Sequence Detection System Software version 1.7. *CDK4* mRNA levels were normalized to GAPDH levels.

ChIP

ChIP was carried out using the Upstate Biotechnolgy ChIP assay kit. Briefly, cells were crosslinked by adding 1/10th volume of buffered formaldehyde solution (11% formaldehyde, 0.1 M NaCl, 1 mM EDTA [pH 8.0], 50 mM HEPES [pH 7.4]) to the media at room temperature for 20 min with gentle mixing. The nuclear fraction was isolated using a Dounce homogenizer, sonicated to shear genomic chromatin, clarified by centrifugation (14,000 × g, 10 min), and then diluted 10-fold in ChIP buffer, precleared with protein A beads, and immunoprecipitated with 10 μg of the appropriate antibodies. Following immunoprecipitation and immobilization of immunocomplexes, proteinase K digestion was allowed to proceed at 65°C overnight to reverse the formaldehyde crosslinks. Associated DNA was purified by phenol/chloroform extraction (containing 0.6 M sodium acetate [pH 8.0]) followed by ethanol precipitation. PCR was carried out on the resulting DNA using specific primers to the promoter region of human *CDK4* (forward, 5′-GTGGACCGAAAAGGTGACAGGATC-3′ [from −434 to −414]; and reverse, 5′-GGGCGGGGCGAACGCCGG ACGTTC-3′ [from −20 to +4]) or mouse *CDK4* (forward, 5′-CAGCG CAAAGTCAAGGGGTCACGTGG-3′; and reverse, 5′-GGCTAAGAG CTCTGGAGGCCCTT-3′).

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References

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.

Baksh, S., and Burakoff, S.J. (2000). The role of calcineurin in lymphocyte activation. Semin. Immunol. *12*, 405–415.

Baksh, S., DeCaprio, J.A., and Burakoff, S.J. (2000). Calcineurin regulation of the mammalian G0/G1 checkpoint element, cyclin dependent kinase 4. Oncogene *19*, 2820–2827.

Black, A.R., and Azizkhan-Clifford, J. (1999). Regulation of E2F: a family of transcription factors involved in proliferation control. Gene *237*, 281–302.

Brehm, A., and Kouzarides, T. (1999). Retinoblastoma protein meets chromatin. Trends Biochem. Sci. *24*, 142–145.

Crabtree, G.R. (1999). Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. Cell *96*, 611–614.

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.

Graef, I.A., Mermelstein, P.G., Stankunas, K., Neilson, J.R., Deisseroth, K., Tsien, R.W., and Crabtree, G.R. (1999). L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. Nature *401*, 703–708.

Hodge, M.R., Ranger, A.M., de la Brousse, C.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.

Hollander, G.A., Fruman, D.A., Bierer, B.E., and Burakoff, S.J. (1994). Disruption of T cell development and repertoire selection by calcineurin inhibition in vivo. Transplantation *58*, 1037–1043.

Jiang, H., Xiong, F., Kong, S., Ogawa, T., Kobayashi, M., and Liu, J.O. (1997). Distinct tissue and cellular distribution of two major isoforms of calcineurin. Mol. Immunol. *34*, 663–669.

Kel, A., Kel-Margoulis, O., Babenko, V., and Wingender, E. (1999). Recognition of NFATp/AP-1 composite elements within genes induced upon the activation of immune cells. J. Mol. Biol. *288*, 353–376.

Kiani, A., Rao, A., and Aramburu, J. (2000). Manipulating immune responses with immunosuppressive agents that target NFAT. Immunity *12*, 359–372.

Ladha, M.H., Lee, K.Y., Upton, T.M., Reed, M.F., and Ewen, M.E. (1998). Regulation of exit from quiescence by p27 and cyclin D1-CDK4. Mol. Cell. Biol. *18*, 6605–6615.

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.

Lopez-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. USA *96*, 7214–7219.

Lucas, J.J., Szepesi, A., Domenico, J., Tordai, A., Terada, N., and Gelfand, E.W. (1995). Differential regulation of the synthesis and activity of the major cyclin-dependent kinases, p34cdc2, p33cdk2, and p34cdk4, during cell cycle entry and progression in normal human T lymphocytes. J. Cell. Physiol. *165*, 406–416.

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

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463–473.

Macian, F., Lopez-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.

Macian, F., Lopez-Rodriguez, C., and Rao, A. (2001). Partners in transcription: NFAT and AP-1. Oncogene *20*, 2476–2489.

Miyakawa, H., Woo, S.K., Dahl, S.C., Handler, J.S., and Kwon, H.M. (1999). Tonicity-responsive enhancer binding protein, a rel-like protein that stimulates transcription in response to hypertonicity. Proc. Natl. Acad. Sci. USA *96*, 2538–2542.

Modiano, J.F., Domenico, J., Szepesi, A., Lucas, J.J., and Gelfand, E.W. (1994). Differential requirements for interleukin-2 distinguish the expression and activity of the cyclin-dependent kinases Cdk4 and Cdk2 in human T cells. J. Biol. Chem. *269*, 32972–32978.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., and Olson, E.N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell *93*, 215–228.

Musaro, A., McCullagh, K.J., Naya, F.J., Olson, E.N., and Rosenthal, N. (1999). IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. Nature *400*, 581–585.

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.

Parekh, A.B., and Penner, R. (1997). Store depletion and calcium influx. Physiol. Rev. *77*, 901–930.

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

Ranger, A.M., Gerstenfeld, L.C., Wang, J., Kon, T., Bae, H., Gravallese, E.M., Glimcher, M.J., and Glimcher, L.H. (2000). The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. J. Exp. Med. *191*, 9–22.

Roth, S.Y., Denu, J.M., and Allis, C.D. (2001). Histone acetyltransferases. Annu. Rev. Biochem. *70*, 81–120.

Semsarian, C., Wu, M.J., Ju, Y.K., Marciniec, T., Yeoh, T., Allen, D.G., Harvey, R.P., and Graham, R.M. (1999). Skeletal muscle hypertrophy is mediated by a Ca2+-dependent calcineurin signalling pathway. Nature *400*, 576–581.

Sherr, C.J., and Roberts, J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501–1512.

Trama, J., Lu, Q., Hawley, R.G., and Ho, S.N. (2000). The NFAT-related protein NFATL1 (TonEBP/NFAT5) is induced upon T cell activation in a calcineurin-dependent manner. J. Immunol. *165*, 4884–4894.

Verona, R., Moberg, K., Estes, S., Starz, M., Vernon, J.P., and Lees, J.A. (1997). E2F activity is regulated by cell cycle-dependent changes in subcellular localization. Mol. Cell. Biol. *17*, 7268–7282.

Wang, Q., Ji, Y., Wang, X., and Evers, B.M. (2000). Isolation and molecular characterization of the 5′-upstream region of the human TRAIL gene. Biochem. Biophys. Res. Commun. *276*, 466–471.

Wolffe, A.P., and Guschin, D. (2000). Review: chromatin structural features and targets that regulate transcription. J. Struct. Biol. *129*, 102–122.

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.

Youn, H.D., Chatila, T.A., and Liu, J.O. (2000). Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. EMBO J. *19*, 4323–4331.

Zhang, B.W., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, F.W., Wiederrecht, G., Cryan, J., O'Neill, E.A., Seidman, C.E., et al. (1996). T cell responses in calcineurin A alpha-deficient mice. J. Exp. Med. *183*, 413–420.

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.