Cell Cycle

ISSN: 1538-4101 (Print) 1551-4005 (Online) Journal homepage: https://www.tandfonline.com/loi/kccy20

Transcriptional regulation of the *c-Myc* promoter by NFAT1 involves negative and positive NFAT-responsive elements

Giuliana P. Mognol, Patricia S. de Araujo-Souza, Bruno K. Robbs, Leonardo K. Teixeira & Joao P.B. Viola

To cite this article: Giuliana P. Mognol, Patricia S. de Araujo-Souza, Bruno K. Robbs, Leonardo K. Teixeira & Joao P.B. Viola (2012) Transcriptional regulation of the *c-Myc* promoter by NFAT1 involves negative and positive NFAT-responsive elements, Cell Cycle, 11:5, 1014-1028, DOI: 10.4161/cc.11.5.19518

To link to this article: https://doi.org/10.4161/cc.11.5.19518

Published online: 01 Mar 2012.

Cell Cycle 11:5, 1014-1028; March 1, 2012; © 2012 Landes Bioscience

Transcriptional regulation of the *c-Myc* promoter by NFAT1 involves negative and positive NFAT-responsive elements

Giuliana P. Mognol,[1] Patricia S. de Araujo-Souza,[1,2] Bruno K. Robbs,[1] Leonardo K. Teixeira[1,†] and João P.B. Viola[1,*]

[1]Program of Cellular Biology; Brazilian National Cancer Institute (INCA); Rio de Janeiro, Brazil; [2]Department of Immunobiology; Fluminense Federal University (UFF); Rio de Janeiro, Brazil

[†]Current affiliation: Department of Molecular Biology; The Scripps Research Institute; La Jolla, CA USA

Key words: c-Myc, NFAT, gene expression, transcription factor, gene promoter

A number of physiological processes in both normal and cancer cells are regulated by the proto-oncogene *c-Myc*. Among them, processes such as cell cycle regulation, apoptosis, angiogenesis and metastasis are also controlled by the nuclear factor of activated T cells (NFAT) family of transcription factors. It is already known that NFAT upregulates *c-Myc* expression by binding to an element located in the minimal *c-Myc* promoter. However, the importance of other NFAT sites in the context of the full promoter has not been evaluated. In this work, we demonstrate that the regulation of *c-Myc* by NFAT1 is more complex than previously conceived. In addition to the proximal site, NFAT1 directly binds to distal sites in the *c-Myc* promoter with different affinities. Promoter deletions and site-directed mutagenesis of NFAT binding sites in HEK293T cells suggest that in NFAT1-mediated transactivation, some NFAT elements are negative and dominant, and others are positive and recessive. Furthermore, we demonstrate that cooperation with partner proteins, such as p300, enhances NFAT1-mediated transactivation of the *c-Myc* promoter. At last, the newly identified sites are also responsive to NFAT2 in HEK293T cells. However, in NIH3T3 cells, the regulation mediated by NFAT proteins is not dependent on the known NFAT sites, including the site previously described. Thus, our data suggest that the contribution of NFAT to the regulation of *c-Myc* expression may depend on a balance between the binding to positive and negative NFAT-responsive elements and cooperation with transcriptional cofactors, which may differ according to the context and/or cell type.

Introduction

The proto-oncogene *c-Myc* is a pivotal regulator of a wide variety of biological processes, such as cell growth, biogenesis, metabolism and proliferation, telomere maintenance, apoptosis, angiogenesis, cell migration and invasion.[1-5] Consequently, *c-Myc* is essential for many physiological cell functions, and its deregulation is extremely dangerous. Thus, very tight control of *c-Myc* expression is required, so that it can be activated or repressed rapidly and precisely.[1] The essential role of *c-Myc* in normal cell function is reflected by the finding that *c-Myc* binds to 10–15% of human genes.[2-4] Enhanced and/or constitutive *c-Myc* expression is found in most cancer cells, either through genetic mechanisms or constitutive activation of upstream signaling pathways.[1,2] Moreover, *c-Myc* deregulation is often associated with aggressive, poorly differentiated tumors,[3,5] and even small changes in *c-Myc* levels may have a global impact on cell physiology.[4,6]

Regulation of the *c-Myc* promoter is a very complex and poorly understood process involving redundancy, feedback loops and several cross-regulatory circuits.[1] Hundreds of signals, transcription factors and chromatin components regulate cellular *c-Myc* mRNA levels.[4,6] Furthermore, it has been demonstrated that the proper organization, folding and looping of its promoter are important for normal *c-Myc* regulation.[6] Although gene expression is ultimately regulated at the proximal promoter, where RNA polymerase and the core transcriptional machinery bind, distal regulatory regions have profound effects on the expression of nearly all genes,[7] including *c-Myc*.

The presence of distal regulatory elements is a general feature of NFAT (nuclear factor of activated T cells) target genes.[7-10] The NFAT family of transcription factors encompasses four closely related proteins (NFAT1–4) that are regulated by the Ca^{2+}/calcineurin pathway,[11,12] first described in T lymphocytes as an inducible nuclear factor that could bind and transactivate the *IL-2* promoter.[13] Along with their well-documented role in T lymphocytes, where they control gene expression during cell activation and differentiation,[11,14] NFAT proteins are also expressed in a wide range of cells and tissue types and regulate genes involved in the cell cycle, apoptosis, angiogenesis and metastasis.[14-17] In resting lymphocytes, NFAT is located in the cytoplasm in a hyperphosphorylated, inactive form. Signaling pathways that lead to a rise in intracellular Ca^{2+} levels activate the serine/threonine

*Correspondence to: João P.B. Viola; Email: jpviola@inca.gov.br
Submitted: 08/18/11; Revised: 01/12/12; Accepted: 01/29/12
http://dx.doi.org/10.4161/cc.11.5.19518

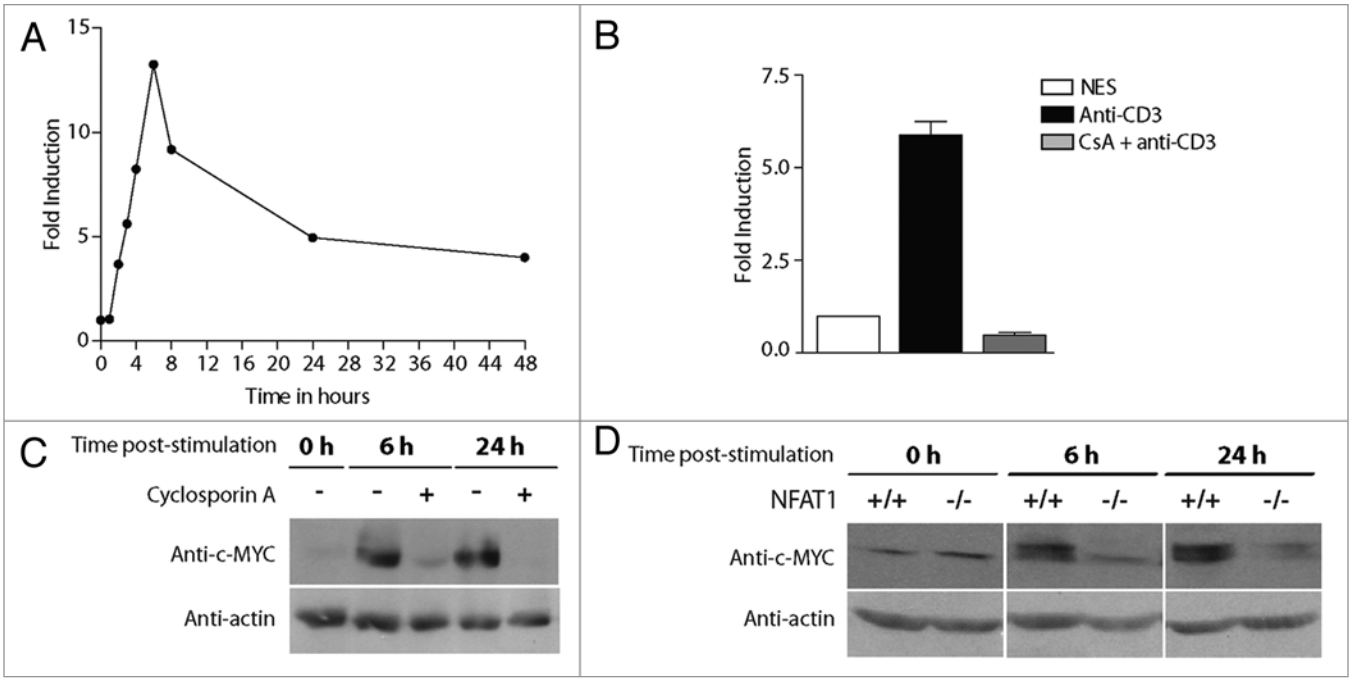

Figure 1. Induction of *c-Myc* expression in response to the Ca^{2+}/Calcineurin/NFAT pathway. *Naïve* C57BL/6 (A–C); NFAT1$^{+/+}$ or NFAT1$^{-/-}$ (D) mice were sacrificed and the lymph nodes were harvested. CD4 T cells were purified and stimulated in vitro with plate-bound anti-CD3 (1 μg/mL). At the indicated time points, total RNA or total protein lysates were obtained, and *c-Myc* levels were analyzed either by RT-PCR (A and B), using HPRT expression as a reference, or by western blot (C and D), using actin as a loading control. (B) Cyclosporin A (CsA, 1 μM) was added 15 min before anti-CD3 stimulation. The results are representative of three independent experiments.

phosphatase calcineurin, which dephosphorylates several serine residues in NFAT proteins, causing a conformational switch that unmasks their nuclear localization sequence and allows their translocation to the nucleus. In the nucleus, NFAT binds to specific DNA-responsive elements defined as (A/T)GGAAA(A/N)(A/T/C) to activate or deactivate transcription either alone or in synergy with a number of other transcriptional regulators.[11,12,14]

Recent studies have convincingly demonstrated that NFAT1/2 regulate *c-Myc* gene expression by binding to an NFAT site located between the two main *c-Myc* promoters (P1 and P2).[18-21] However, these studies did not evaluate whether NFAT binds to other regions in the promoter up to 2.5 kb upstream of the transcription initiation site, where elements essential for *c-Myc* regulation are located. Here, we demonstrate that *c-Myc* is regulated by Ca^{2+}/calcineurin/NFAT1 signaling in mouse T lymphocytes. NFAT1 directly binds to distal sites in the *c-Myc* promoter that have not been previously investigated. We show that while some NFAT elements have a negative effect on NFAT1-mediated transactivation in HEK293T cells, others have a positive effect, and NFAT1 binds to these sites with different affinities. Moreover, regulation of the *c-Myc* promoter by NFAT1 may vary in transactivation assays, depending on whether NFAT1 is expressed alone or in cooperation with nuclear partner proteins, such as p300. Finally, we show that the newly identified sites are also responsive to NFAT2 in HEK293 T cells, but the dominance of sites is apparently different for NFAT1 and NFAT2. However, although both NFAT1 and NFAT2 regulate *c-Myc* expression and transactivation in NIH3T3 cells, this regulation is not dependent on the known NFAT sites, including the previously described site located at the minimal promoter. These results indicate that unidentified sites important in the regulation mediated by NFAT proteins still exist and remain to be identified. Collectively, these data suggest that the extent to which NFAT regulates *c-Myc* expression is more complex than previously conceived and may differ according to the cellular context and/or microenvironment and with the different functions exerted by *c-Myc*.

Results

c-Myc expression in response to TCR stimulation is dependent on the Ca^{2+}/calcineurin/NFAT signaling pathway. As a typical immediate-early gene, *c-Myc* is rapidly induced by mitogens during the entry of cells into the cell cycle (G_0/G_1 transition).[1,4] In T lymphocytes, stimulation via TCR engagement constitutes the main pathway for NFAT activation.[11,12] To evaluate *c-Myc* expression after anti-CD3 stimulation, we first performed quantitative real-time PCR on cDNA generated from naïve CD4 T cells. As shown in **Figure 1A**, *c-Myc* is promptly induced by anti-CD3 stimulation. Moreover, our data agrees with other reports found in the literature, which have shown that *c-Myc* is expressed at low levels in resting cells, rapidly induced after stimulation and then decreased to lower levels that persist throughout the cell cycle.[1]

To investigate a possible correlation between NFAT and *c-Myc* expression, we tested the effect of the immunosuppressant cyclosporin A (CsA), which inhibits calcineurin and, consequently, both NFAT nuclear translocation and activation[12] in stimulated

cells. CsA treatment prevented anti-CD3 stimulation-mediated upregulation of *c-Myc* (**Fig. 1B**), demonstrating the influence of the calcineurin cascade on the regulation of *c-Myc* expression. Importantly, this result was also reflected in *c-Myc* protein levels (**Fig. 1C**). In agreement with our results, others have previously reported that *c-Myc* expression is inhibited by CsA.[19,22,23]

To assess the role of NFAT1 specifically on *c-Myc* expression, we analyzed CD4 T cells from naïve NFAT1$^{+/+}$ or NFAT1$^{-/-}$ mice. We observed that, although there was no difference in *c-Myc* expression in naïve cells from NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice, NFAT1$^{-/-}$ cells had an impairment in the induction of c-MYC protein levels upon anti-CD3 stimulation when compared with NFAT1$^{+/+}$ cells (**Fig. 1D**). Taken together, these results demonstrate an important role for the Ca^{2+}/calcineurin/NFAT1 pathway in *c-Myc* induction by TCR signaling in lymphocytes and suggest that NFAT1 may act as a positive regulator of *c-Myc* gene expression.

NFAT1 binds in vitro to three elements in the *c-Myc* promoter. To identify putative NFAT elements in the *c-Myc* promoter, we performed a bioinformatic analysis using the Genomatix and rVista softwares and found seven possible NFAT binding sites that are conserved between humans and mice (**Fig. 2A**). In general, regions important for gene regulation are evolutionarily conserved, and the probability of a particular putative binding site being functional is higher when it is conserved in two related species compared with a site found in an isolated species.[24] Notably, all the sites we identified are distal to the minimal promoter except for the previously identified +98 bp site (NFATRE 7), which is located within the minimal P2 promoter between P1 and P2 (**Fig. 2B**). Although the distal sites are not part of the basal promoter, they can regulate promoter activity by acting as enhancers or silencers.[25] The core sequence for NFAT binding, 5'-GGA AA-3',[12] was found in the putative NFAT responsive elements (NFATRE) 1, 2, 3, 5, 6 and 7 (**Fig. 2B**). The murine NFATRE 1 and both the human and mouse NFATRE 4 have small but important variations (3'-CTT TT-5' and 5'-GCA AA-3', respectively), suggesting that they are non-ideal sites for NFAT binding.

To characterize the importance of the putative NFAT elements in the regulation of *c-Myc* expression, we performed an electrophoretic mobility shift assay (EMSA) with the recombinant DNA binding domain (DBD) of the NFAT1 protein. As a positive control, this DBD protein was incubated with a probe containing the IL-2 distal NFAT element (**Fig. 2C**). Next, increasing amounts of DBD were incubated with the seven oligonucleotides containing the putative NFAT binding sites found in the mouse *c-Myc* promoter. In fact, NFAT1 bound to three of the oligonucleotides (**Fig. 2C**) that contained NFATREs 2, 5 and 7 and that had a canonical GGAAA element. Of note, the intensity of binding was weaker for NFATRE 7 compared with NFATREs 2 and 5, suggesting that the binding affinity of NFAT1 for this site is lower. Furthermore, we also observed very weak binding of the NFAT1 DBD to the oligonucleotide that contained NFATRE 6. Finally, the DBD of NFAT1 was unable to bind mutated oligonucleotides in which the NFAT-binding sequence GGAAA was substituted with ACTCA, confirming the binding specificity for this site (**Fig. 2D**). These data demonstrate that NFAT1 directly binds to at least three sites in the *c-Myc* promoter.

NFAT1 binds to two distal regions of the *c-Myc* promoter in lymphocytes. Using the chromatin immunoprecipitation assay (ChIP), we next examined the ability of NFAT1 to bind these elements in CD4 T lymphocytes derived from NFAT1$^{+/+}$ or NFAT1$^{-/-}$ mice. Three sets of primers were designed and are listed in **Figure 3A**. As shown in **Figure 3B**, NFAT1 bound to the regions encompassed by primer sets 1 and 2 in wild-type cells after stimulation with PMA plus ionomycin. Primer set 1 encompasses NFATREs 1 and 2, while primer set 2 encompasses NFATREs 5 and 6. Because NFAT1 only bound to NFATREs 2 and 5 in the EMSAs, these data suggest that the interaction observed in the ChIP assay reflect those seen in the EMSAs. Conversely, no interactions were observed within the region covered by primer set 3. This primer set amplifies the region containing the proximal site NFATRE 7, which was previously identified as binding to NFAT1 and NFAT2 in pancreatic cells.[19,21] Interestingly, it has already been shown that NFAT2 binds to this site in B lymphocytes, while NFAT1 does not.[20] Given that the NFATRE 7 localizes to a region that contains overlapping binding sites for several transcription factors, such as ETS1/2, STAT3, E2F, Smad3/4 and MET (**Fig. 3C**), we cannot exclude the possibility that NFAT1 is able to bind to this site at different times or conditions. For example, NFAT binding could be impeded if one of the proteins listed above was bound to this site at the time of analysis. Furthermore, binding to NFATRE 7 might not be detected by ChIP assays, because it may have a low affinity for NFAT1 as we observed in our EMSAs.

Likewise, both NFAT1 and NFAT2 have been shown to associate with the TNFα promoter in ChIP assays when these NFAT proteins were overexpressed in Jurkat cells. However, the physiological association between endogenous NFAT and the TNFα promoter was too weak to further confirm this finding.[26] As a control for the specificity of NFAT1 binding, we also evaluated NFAT1 binding to the IFNγ and IL-4 promoters by ChIP as positive and negative controls, respectively (data not shown).[27] In conclusion, NFAT1 can specifically bind to two distal sites in the *c-Myc* promoter in lymphocytes. However, we were unable to detect NFAT1 binding to the proximal site.

The distal NFAT sites are negative, while the proximal site is a positive element to NFAT1-mediated *c-Myc* promoter transactivation. To determine the functional relevance of NFAT1 to *c-Myc* gene expression, we analyzed the responsiveness of the human *c-Myc* promoter to a constitutively active NFAT1 (CA-NFAT1) protein by luciferase reporter gene assays in HEK293T cells. By using a constitutively active protein, we could minimize the effects of other transcription factors in the promoter regulation. The expression of the CA-NFAT1 protein had a discrete effect on full *c-Myc* promoter (-2,446 bp-Luc) activity when compared with the empty vector; however, a marked increase in promoter activity was observed in a construct that removes more than 1 kb (-1,237 bp-Luc) of the distal promoter, which contained NFATREs 2 and 5. These results suggest that the deleted region has a negative effect on *c-Myc* promoter activity, while a positive element is located in the remaining promoter (**Fig. 4A**). Likewise,

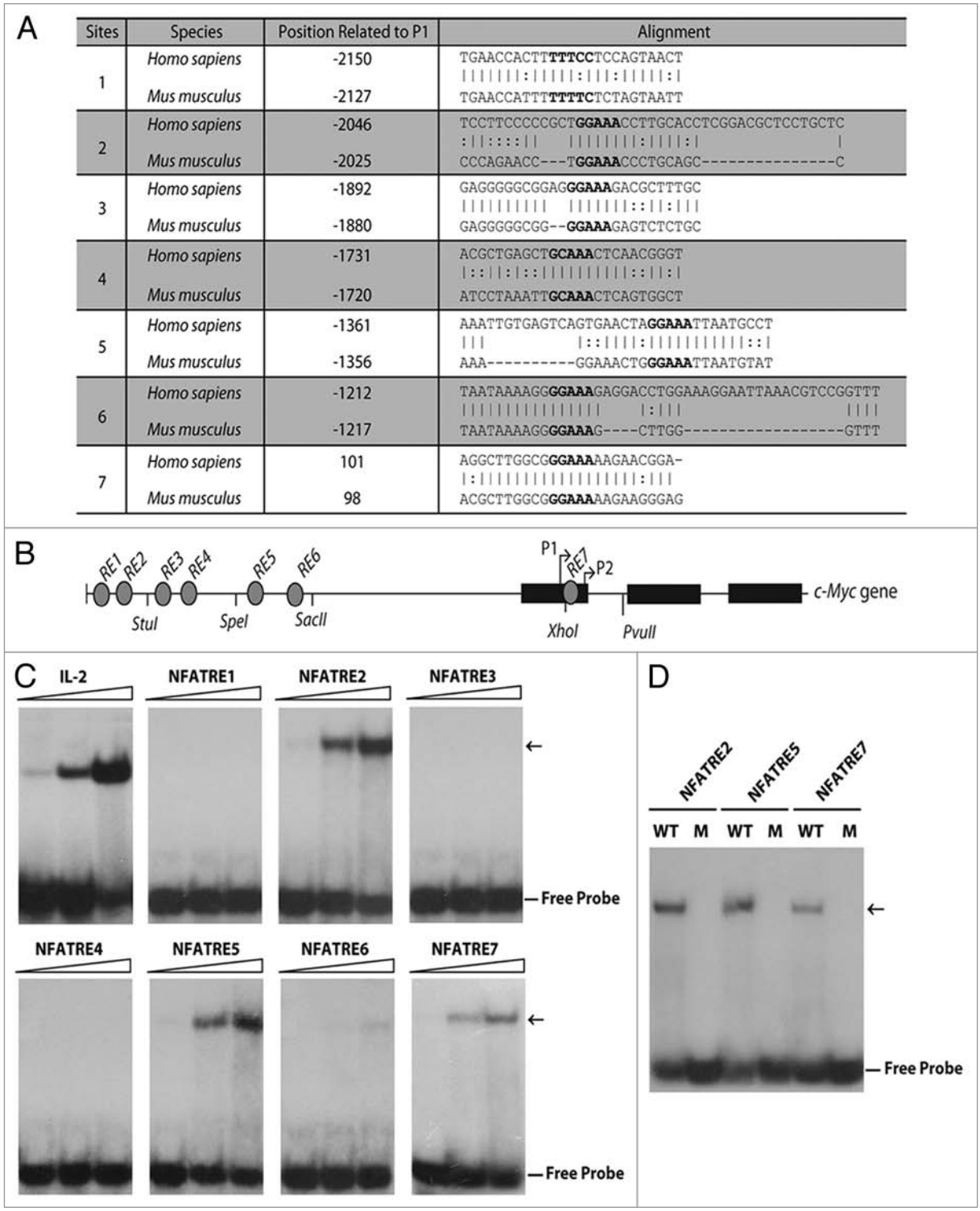

Figure 2. The NFAT1 DNA binding domain (DBD) binds to the c-*Myc* promoter in vitro. The genomic sequences of the human and mouse c-*Myc* promoters were analyzed with rVista and Genomatix transcription factor binding site search softwares. (A) The seven putative NFAT binding sites found by both softwares are shown and aligned. The core NFAT binding sequence is in bold (**GGA AA**). (B) Schematic representation of the *c-Myc* gene. Exons are in black boxes. The major transcription initiation sites (P1 and P2) and the seven putative NFAT elements found by bioinformatic analysis are shown. (C) An oligonucleotide containing the NFAT binding site from the IL-2 promoter or oligos with the putative NFAT binding sites from the human *c-Myc* promoter were end-labeled with [γ^{32}P] ATP and incubated with increasing concentrations of the recombinant DBD of the NFAT1 protein (for the IL-2 oligo: 10 nM, 100 nM and 1 μM and for the *c-Myc* oligos: 100 nM, 500 nM and 1 μM). DNA-protein complexes were analyzed by EMSA and are indicated by an arrow. (D) The core NFAT binding sequence was mutated (GGA AA substituted by ACTCA) in the oligonucleotides of the c-*Myc* promoter that demonstrated binding in (C) and re-analyzed by EMSA. Wild type (WT) and mutated (M) oligos. DBD of NFAT1 protein: 500 nM. All the results are representative of three independent experiments.

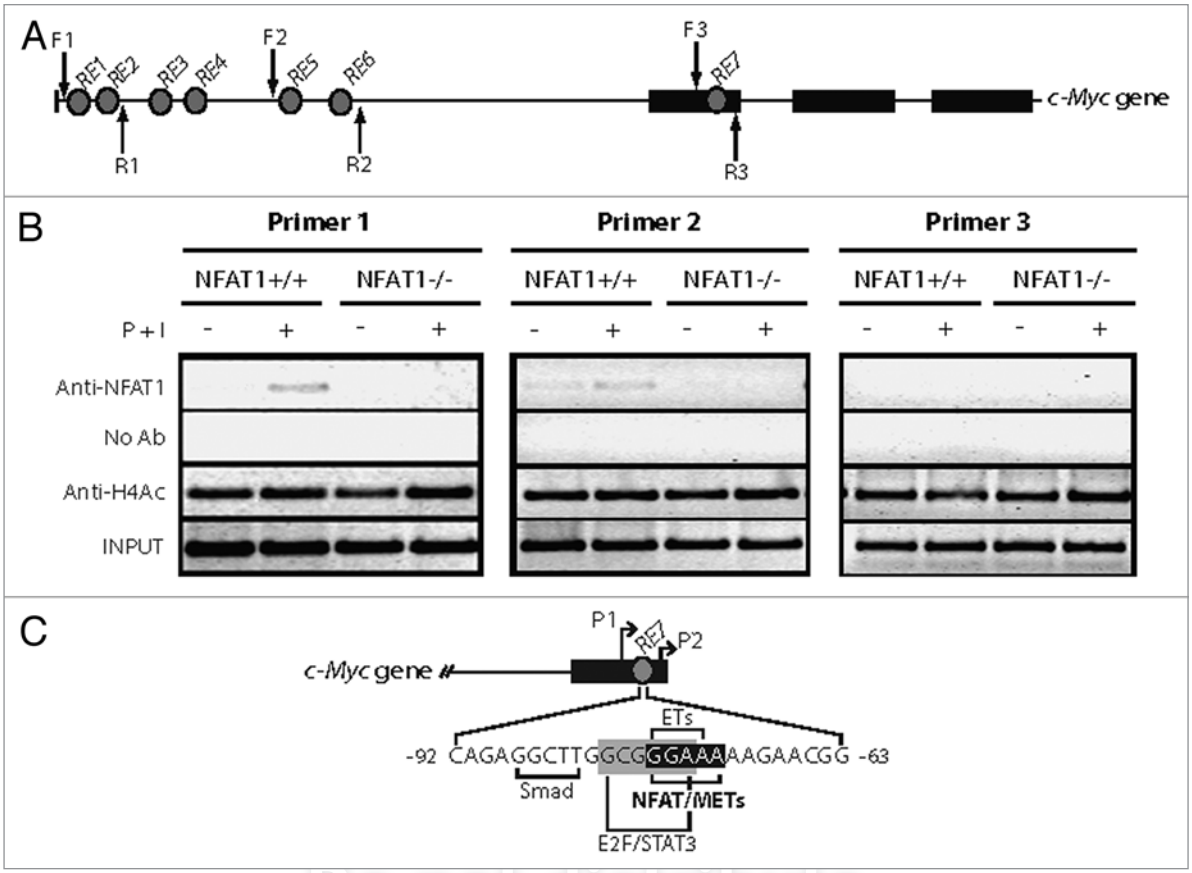

Figure 3. The NFAT1 protein directly binds to the c-*Myc* promoter in CD4 T lymphocytes. (A) Schematic representation of the c-*Myc* gene showing the regions where primers used to evaluate NFAT binding to NFATRE 1/2, 5/6 and 7 annealed. (B and C) CD4 T cells were isolated from *naive* NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice, and primed in vitro with plate-bound anti-CD3 and anti-CD28 for 7 d under Th1 polarizing conditions. Cells were either left unstimulated or stimulated with PMA (10 nM) plus ionomycin (1 μM) for an additional 4 h. Chromatin immunoprecipitation was performed using anti-NFAT1, anti-acetylated forms of histone H4 (anti-H4Ac) antibodies or no antibody as a negative control (No Ab). The DNA was purified and analyzed by PCR with specific primers for *c-Myc* promoter regions containing the NFAT binding sites (B). As a positive control, PCR was done on DNA purified from chromatin before immunoprecipitation (INPUT). (C) The picture shows NFATRE 7 in the *c-Myc* promoter, which overlaps with binding sites for several transcription factors.

analysis of the minimal P2 promoter (-109 bp-Luc), which contains the NFATRE 7 site, showed that CA-NFAT1 could still increase promoter activity, suggesting that NFATRE 7 is a positive element in NFAT1-mediated transactivation.

To specifically evaluate the role of the distal NFAT sites on the regulation of *c-Myc* expression, we performed site-directed mutagenesis of NFATREs 2 and 5 in the context of the full promoter. Mutation of either site alone resulted in a similar effect on *c-Myc* promoter activity as the full promoter (data not shown), but, surprisingly, as with the -1,237 bp-Luc construction, CA-NFAT1 enhanced *c-Myc* promoter activity when NFATREs 2 and 5 were mutated together, indicating that these distal elements function as negative regulators of *c-Myc* expression. The enhanced transactivation observed with the empty vector in this construction is probably caused by preventing the binding of endogenous NFATs or another transcription factor that might bind to these element sites. In contrast, although mutation of the proximal site in the context of the full promoter resulted in an impairment of transactivation compared with the wild-type promoter (compare the white bars), CA-NFAT1 did not change the promoter inducibility when this site was mutated (**Fig. 4A**). These results indicate that the binding of CA-NFAT1 to NFATRE 7 is essential for CA-NFAT1-induced promoter activity.

To confirm the positive role of NFATRE 7 on regulation of *c-Myc* expression, we mutated this element in the context of the minimal promoter. As shown in **Figure 4B**, CA-NFAT1 was unable to induce the *c-Myc* promoter when NFATRE 7 was mutated, providing evidence that this site is a positive NFAT1 element. Taken together, our data suggest that the distal NFAT1 responsive elements are negative regulators, while the proximal site has a positive effect on NFAT1-mediated transactivation. Furthermore, the distal sites seem to play a dominant role in the regulation of the *c-Myc* promoter compared with the proximal site, because CA-NFAT1 only increases promoter activity when the negative distal sites are abrogated.

Mutation of the NFAT-responsive elements suggests that another NFAT1-responsive site is present in the c-*Myc* promoter. To determine whether NFATREs 2, 5 and 7 are the only NFAT-responsive sites in the *c-Myc* promoter, we mutated these three sites together in the context of the full promoter.

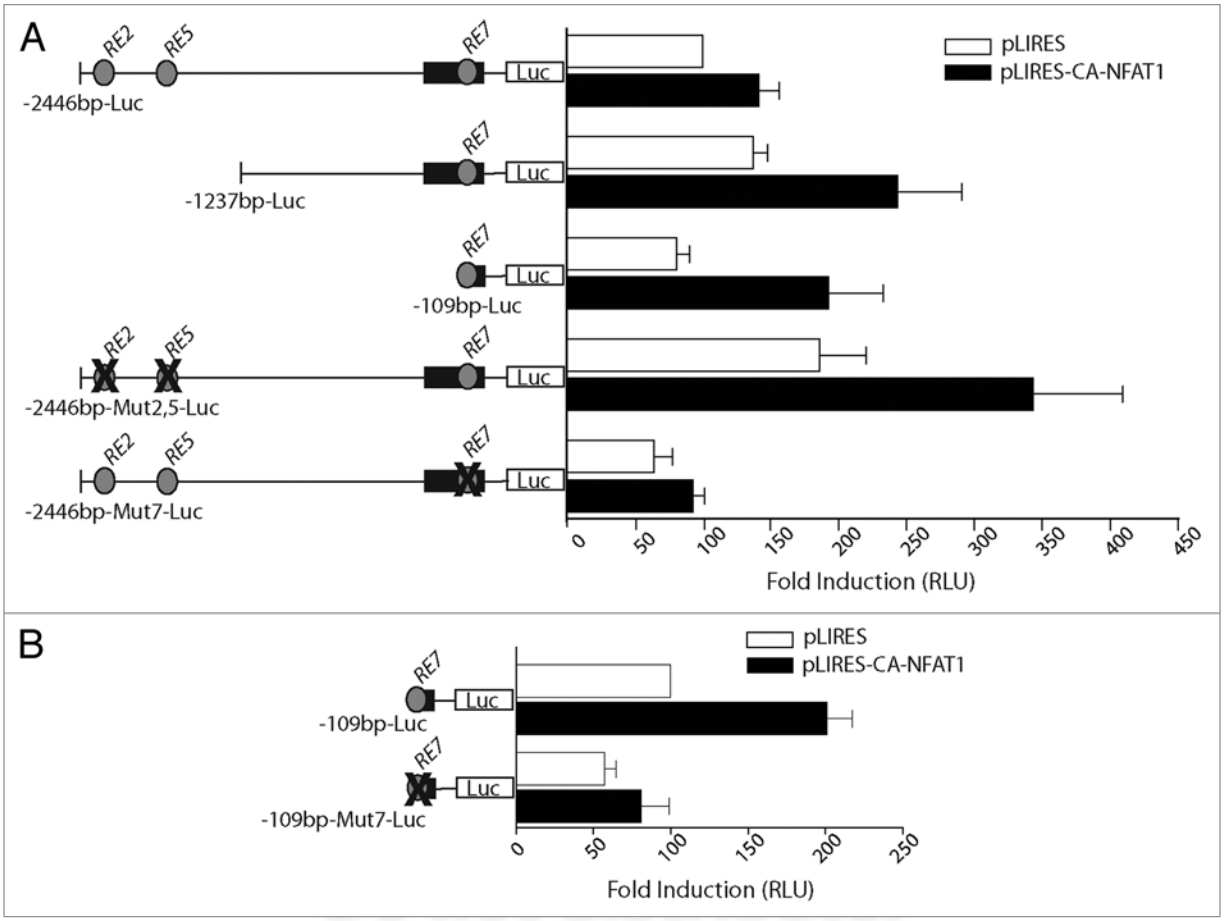

Figure 4. Regulation of the *c-Myc* promoter by NFAT1 involves negative and positive elements. (A) Analysis of NFATREs 2, 5 and 7 in the context of the full promoter and (B) analysis of NFATRE 7 in the context of the minimal P2 promoter. HEK293T cells were co-transfected with the firefly plasmids pBV-luc (0.3 μg), pLIRES or pLIRES-EGFP-CA-NFAT1 (3 μg) and pRL-TK (0.03 μg). After 48 h, the cells were lysed and the firefly luciferase reporter gene was normalized to the renilla vector. The results are representative of at least three independent experiments ± SEM, and the fold induction is relative to the -2,446 bp-Luc construction (pLIRES).

Surprisingly, we observed that CA-NFAT1 still increased the promoter activity of this construct (**Fig. 5A**), suggesting that another functional NFAT1 site might exist in the *c-Myc* promoter or that CA-NFAT1 could bind to sites other than canonical NFAT-responsive sites, as demonstrated for the Nur77 and p21 promoters.[28,29]

To further map the region of the *c-Myc* promoter that is responsive to NFAT1, we used a construct that spans nucleotides -2,446 to -1,237, called FragA-Luc (**Fig. 5B**), which is not under the control of the minimal *c-Myc* promoter. Remarkably, FragA-Luc is highly inducible by CA-NFAT1 (**Fig. 5B**). Because FragA-Luc encompasses the negative NFATREs 2 and 5, these data are at odds with the result for this region in the context of the full promoter (**Fig. 4A**). In fact, FragA-Luc is not under the control of the minimal *c-Myc* promoter, suggesting that the repressive function of NFATREs 2 and 5 may depend on interactions with the basal promoter, which is not present in the FragA-Luc construct. This hypothesis was further supported by the finding that mutation of NFATREs 2 and 5 did not change the responsiveness of FragA-Luc to CA-NFAT1 (**Fig. 5B**), which is in contrast to results with the full promoter carrying these mutations (**Fig. 4A**). Altogether, these data indicate a role for chromatin folding/looping in defining the responsiveness of the *c-Myc* promoter to the distal NFAT1 elements.

Next, we used constructs with sequential deletions of FragA-Luc to identify the region responsive to CA-NFAT1 (**Fig. 5C**). This region was located between nucleotides 891–1,219 of FragA-Luc in the vicinity of NFATRE 5. Analysis of this specific region revealed three other putative NFAT elements (GGA AA) besides NFATRE 6. Additionally, five putative SP-1 binding sites are located in this region. It is possible that NFAT1 could bind to these sites, since NFAT1 has been shown to indirectly regulate the p21 promoter through interactions with Sp1/Sp3 proteins.[29] Thus, our data suggest that NFAT1 binds directly or indirectly to an element between nucleotides 891–1,219 of FragA-Luc (-1,555 to -1,227 bp relative to P2) to induce *c-Myc* promoter activity.

p300 enhances NFAT1-mediated transactivation of the full *c-Myc* promoter. It is well-established that chromatin remodeling is a critical factor in *c-Myc* promoter regulation,[1,30,31] and strong evidences have pointed NFAT as a transcription factor involved in this process due to its ability to recruit chromatin remodeling

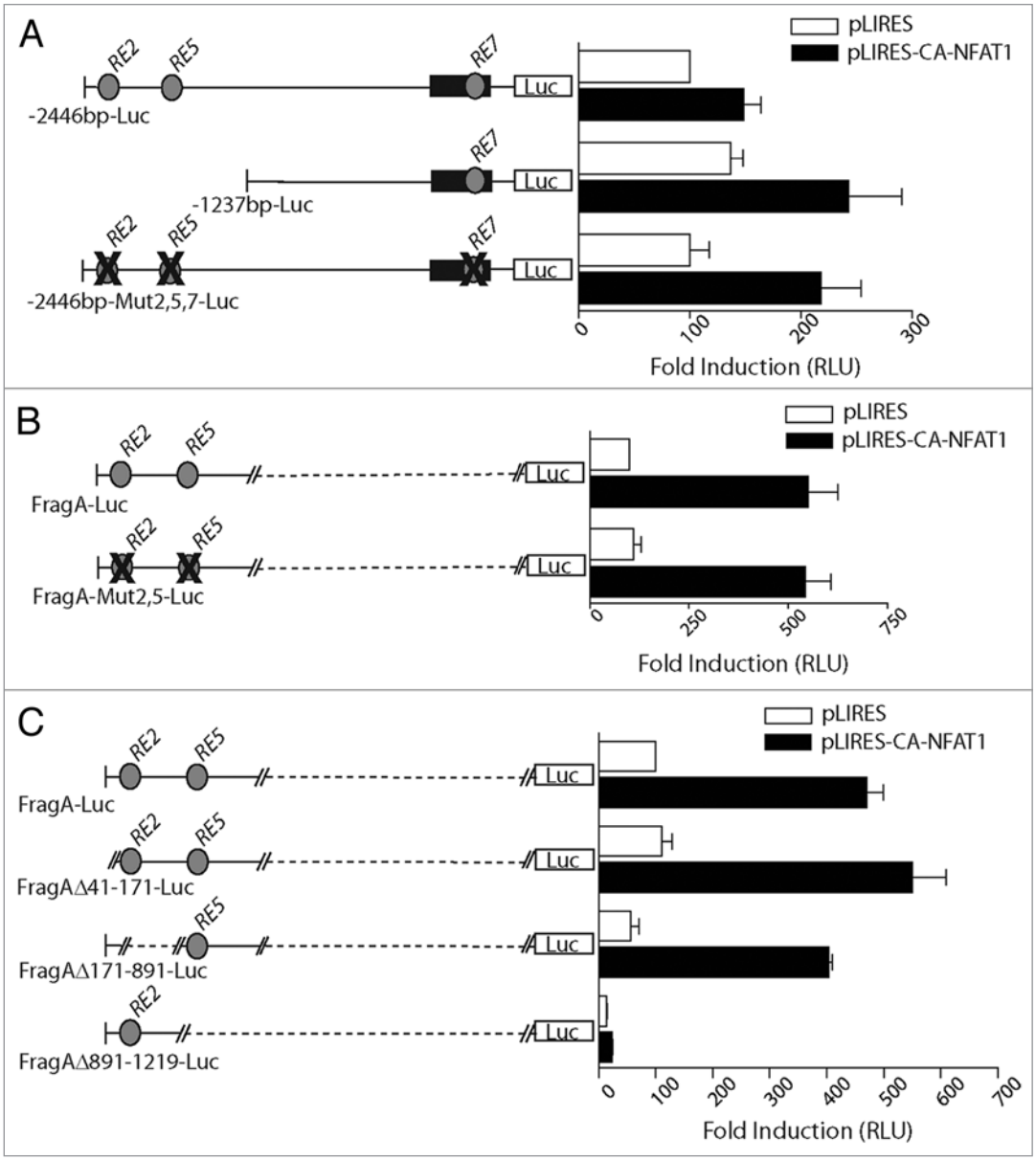

Figure 5. Identification of another element responsive to NFAT1 in the *c-Myc* promoter. HEK293T cells were co-transfected with the firefly plasmids pBV-luc (0.3 μg), pLIRES or pLIRES-EGFP-CA-NFAT1 (3 μg) and pRL-TK (0.03 μg). After 48 h, the cells were lysed and the firefly luciferase reporter gene was normalized to the renilla vector. The fold induction is relative to the -2,446 bp-Luc (pLIRES) (A) or to the FragA-Luc construction (pLIRES) (B) and (C). The results are representative of three independent experiments ± SEM.

proteins and histone acetyltransferases to chromatin.[10,20,32-34] Although we have seen that the distal NFATREs are negative and dominant over the proximal sites in CA-NFAT1-mediated transactivation of the *c-Myc* promoter, we also showed that CD4 T lymphocytes from NFAT1$^{+/+}$ mice express higher levels of *c-Myc* when compared with lymphocytes from NFAT1$^{-/-}$ mice (**Fig. 1D**). These results suggest that a partner protein collaborates with NFAT1 to induce *c-Myc* expression. In line with this, p300 is a transcriptional coactivator that links chromatin remodeling with transcription,[35] and NFAT has been previously shown to interact with p300 to induce promoter activity.[28,33,34,36] Thus, we investigated the influence of p300 on NFAT1-mediated transactivation of the *c-Myc* promoter. For this purpose, we transfected the full *c-Myc* promoter along with p300 into HEK293T cells. As shown in **Figure 6A**, p300 alone represses the *c-Myc* promoter, which is in agreement with previous results.[37,38] Conversely, expression of both p300 and CA-NFAT1, in contrast to CA-NFAT1 alone, increased *c-Myc* promoter activity (**Fig. 6B**). This finding could explain why wild-type CD4 T cells express more *c-Myc* compared with NFAT1$^{-/-}$ cells and reinforces the importance of partner proteins in NFAT protein-mediated transcriptional regulation, as has been described for several other promoters.[7,11,12] Thus, we showed that NFAT1 may collaborate with p300 to induce *c-Myc* transcription and suggest that the response mediated by NFAT1

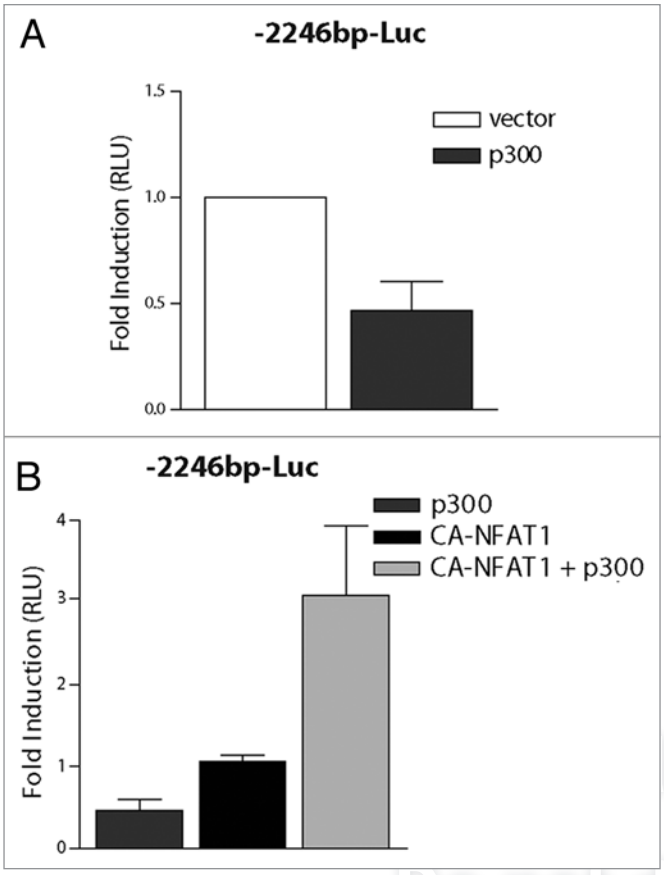

Figure 6. p300 enhances transactivation of the *c-Myc* promoter mediated by NFAT1. HEK293T cells were co-transfected with the plasmid -2446 bp-Luc (0.1 μg), pLIRES-EGFP-CA-NFAT1 or empty vector (1 μg), pCMVb-p300-HA or empty vector (5 μg) and pRL-TK (0.01 μg). After 48 h, the cells were lysed and the firefly luciferase reporter gene was normalized to the renilla vector. The results are representative of three independent experiments ± SEM and the fold induction is relative to the -2,446 bp-Luc construction (pLIRES).

is also dependent on other transcription factors with which it associates.

The distal NFAT sites are negative but not dominant to NFAT2-mediated *c-Myc* promoter transactivation. Although all NFAT members share a highly conserved DNA binding domain (DBD) that confers to them a common DNA binding specificity,[12] it has become clear that NFAT1 and NFAT2 proteins could exert both redundant and opposite functions on cell physiology.[11,12,14,39] To address whether NFAT1 and NFAT2 could differentially affect the NFATRE here evaluated, we performed gene reporter assays with the same constructions of *c-Myc* promoter used in **Figure** 4. HEK293T cells were transfected with the empty vector or with a plasmid encoding CA-NFAT2 (**Fig.** 7). Different from the observed with CA-NFAT1, expression of CA-NFAT2 alone transactivated the full *c-Myc* promoter (**Fig.** 7**A**). Furthermore, the extent of promoter induction remained practically the same with the constructions -1,237 bp-Luc, -109 bp-Luc and even with the -2,446 bp-Mut7-Luc construct, a result that was unexpected, because the NFATRE 7 was described as a NFAT2-positive element. As mutation of this site did not abolish the transactivation mediated by CA-NFAT2, this result indicates that the responsiveness of full *c-Myc* promoter by CA-NFAT2 is determined by other sites that remain to be identified. Despite that, the positive role for NFATRE 7 was confirmed with the mutation of this site in the context of minimal promoter (**Fig.** 7**B**) as shown for CA-NFAT1 (**Fig.** 4**B**), showing that NFATRE 7 is the only NFAT-responsive site located at the minimal promoter in HEK293T cells.

Relative to the mutation of the distal NFATRE in the context of full *c-Myc* promoter, we observed that the disruption of NFATRE 2 and 5 increased both the transactivation of the empty vector as the CA-NFAT2 (**Fig.** 7**A**). These data are in agreement with the result obtained with NFAT1 (**Fig.** 4**A**), but even though the distal sites apparently have a negative role for NFAT2 transactivation, they are not dominant over the other NFATREs as showed for NFAT1. Thus, these results indicate that CA-NFAT2 is able to transactivate the *c-Myc* human promoter through an element that remains to be described, and that the NFATRE 7 is not the preponderant positive site responsive to CA-NFAT2. Furthermore, these data also demonstrated that the newly identified NFATRE 2 and 5 are negative but not dominant for the transactivation mediated by CA-NFAT2.

The positive regulation of *c-Myc* promoter by both NFAT1 and NFAT2 in NIH3T3 cells is independent of the known NFATRE. We have previously shown in NIH3T3 cells that both NFAT1 and NFAT2 differentially regulate aspects of cell transformation, cell cycle and apoptosis.[39] As both proteins regulate *c-Myc* expression, we were interested in evaluating their role on *c-Myc* promoter in this cell type, with regards to the NFAT sites herein evaluated. For this purpose, we stabilized NIH3T3 cells with the vector pLIRES-EGFP empty or coding for NFAT1 or NFAT2 wild-type proteins. As shown in **Figure 8A**, the expression of both NFAT1 and NFAT2 proteins increased *c-Myc* expression levels after stimulation with PMA plus ionomycin. In accordance, both proteins equally upregulated the full *c-Myc* promoter (**Fig. 8B**) and the construction -1,237 bp-Luc after stimulation. Both NFATs also induced the minimal promoter (-109 bp-Luc) with the higher value achieved for NFAT1. Interestingly, while the mutation on NFATRE 2 and 5 led to a modest increase in the transactivation mediated by NFAT1, it led to a slight reduction in the transactivation of NFAT2. Lastly, the mutation of NFATRE 7 did not reduce neither the transactivation of the full promoter (-2,446 bp-Mut7-Luc) nor of the minimal promoter (-109 bp-Mut7-Luc) mediated by both NFAT1 and NFAT2.

Thus, in NIH3T3 cells, both NFAT proteins transactivate the full *c-Myc* promoter, but the known NFATRE 2, 5 and 7 do not have an important role in this regulation. We did not find relevant differences between the transactivation mediated by NFAT1 and NFAT2 that could infer a differential regulation of *c-Myc* promoter by individual NFAT proteins. Moreover, our results clearly indicate that another important positive NFAT element exists within the minimal *c-Myc* promoter, a result that was not observed in HEK293T cells. Therefore, these data indicate that the usage of different NFATRE must depend on the cellular type.

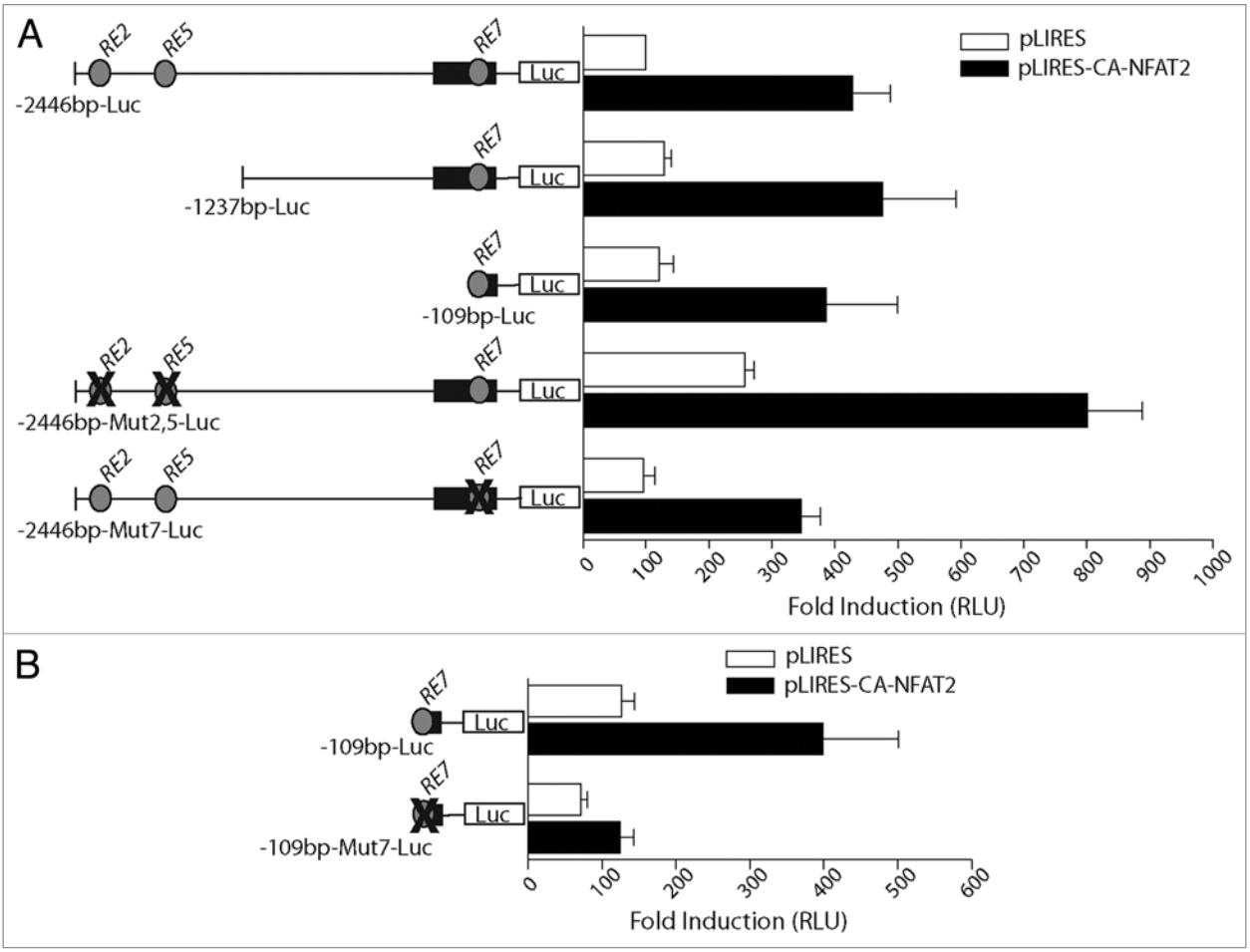

Figure 7. Regulation of the *c-Myc* promoter by CA-NFAT2 is preponderantly by positive elements. HEK293T cells were co-transfected with the firefly plasmids pBV-luc (0.3 μg), pLIRES or pLIRES-EGFP-CA-NFAT2 (3 μg) and pRL-CMV (0.03 μg). After 48 h, the cells were lysed and the firefly luciferase reporter gene was normalized to the renilla vector. (A) Analysis of NFATREs 2, 5 and 7 in the context of the full promoter and (B) analysis of NFATRE 7 in the context of the minimal P2 promoter. The results are representative of three independent experiments ± SEM, and the fold induction is relative to the -2,446 bp-Luc construction (pLIRES).

Discussion

In this work, we have further characterized the transcriptional regulation of the *c-Myc* promoter by the transcription factor NFAT1. Previous studies have linked the upregulation of *c-Myc* with the NFAT binding site located in its minimal promoter (referred to here as NFATRE 7), induced by either NFAT2[18-21] or NFAT1.[19,21] Nonetheless, distal regions of the *c-Myc* promoter are pivotal to the tight regulation of *c-Myc* expression and are commonly abrogated as a result of genetic mechanisms, such as breaks and proviral insertions, which are frequently associated with carcinogenesis.[1,40-42] In the *c-Myc* promoter, euchromatin and heterochromatin are separated by MINE (*c-Myc* insulator element), which functions as an efficient insulator, located approximately 2.5 kb upstream of the *c-Myc* transcription initiation site.[43] All these observations prompted us to evaluate the importance of NFAT1 in this 2.5 kb context.

We first demonstrated that the induction of *c-Myc* expression is dependent on the Ca^{2+}/calcineurin/NFAT signaling pathway in CD4 T cells upon anti-CD3 stimulation (**Fig. 1**). NFAT1 and NFAT2 are the prevalent NFAT members expressed in peripheral T lymphocytes,[12] and we used CD4 T cells from NFAT1$^{-/-}$ mice to specifically evaluate the role of NFAT1. The absence of NFAT1 in these cells was associated with decreased levels of *c-Myc*, pointing to NFAT1 as a positive regulator of *c-Myc* expression. Thereafter, our EMSA results demonstrated that NFAT1 is capable of binding to three of the seven putative NFAT sites in the *c-Myc* promoter that were identified by bioinformatic analysis, with higher affinity for NFATREs 2 and 5 compared with the previously identified NFATRE 7 (**Fig. 2**). We also verified a weak interaction between NFAT1 and NFATRE 6 that was not further examined here. Binding of NFAT1 to the distal sites was further confirmed by ChIP assays (**Fig. 3**). This group of results clearly demonstrates that NFAT1 is able to bind to the *c-Myc* promoter and suggests a direct role for NFAT1 in regulation of *c-Myc* expression, including binding of NFAT to distal elements in the *c-Myc* promoter.

Our data from transactivation assays in HEK293T cells characterized the distal NFAT sites as negative regulators of

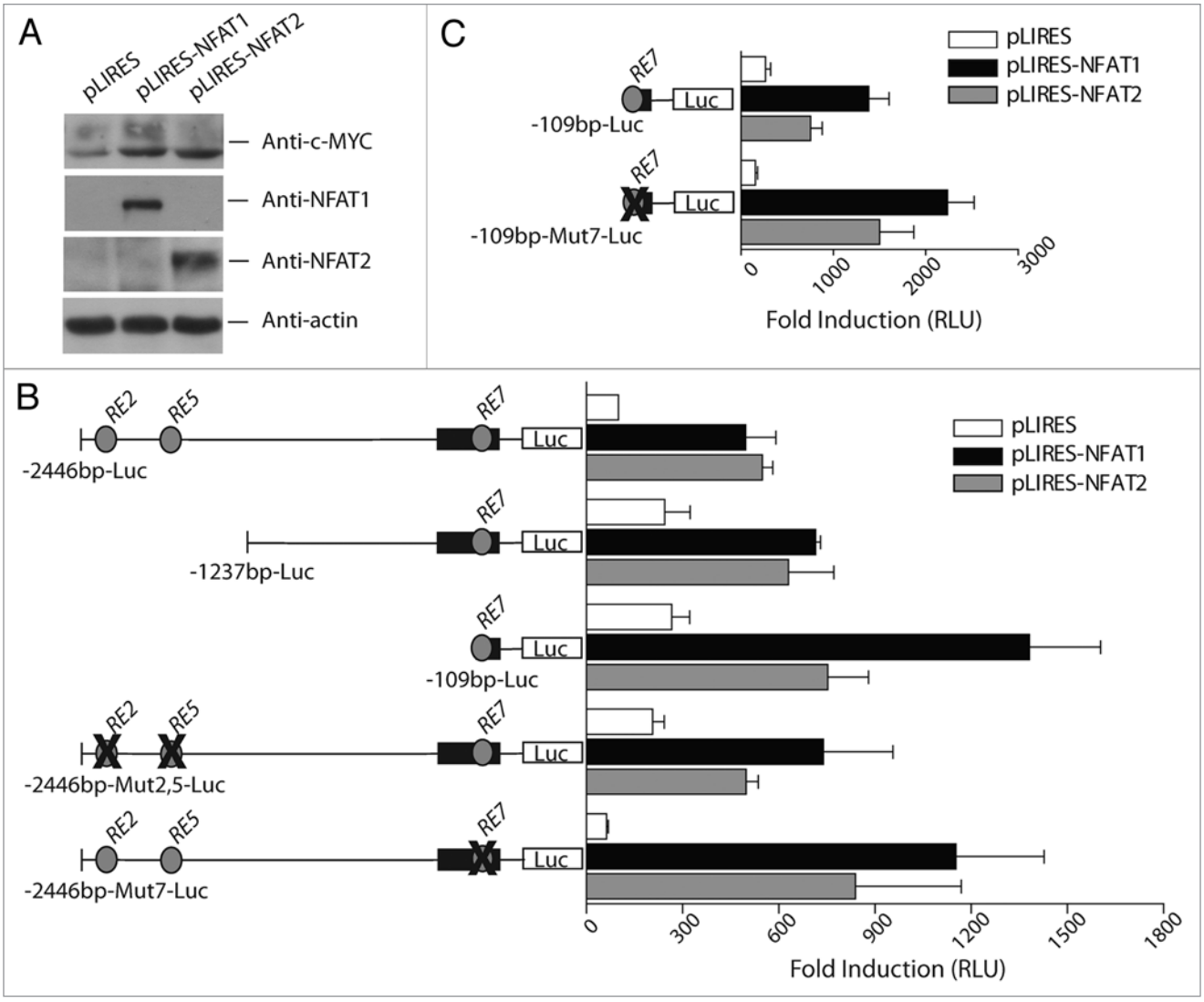

Figure 8. The regulation of *c-Myc* promoter by NFAT proteins in NIH3T3 cells is not dependent on the known NFATRE. NIH3T3 cells were stably with the retroviral vector pLIRES-EGFP empty or coding for NFAT1 or NFAT2 wild type proteins. (A) Cells were seeded on plates for 24 h and then stimulated with PMA (20 nM) plus ionomycin (2 μM). After additional 24 h, whole cell extracts were recovery and western blot performed. (B and C) The stabilized NIH3T3 cells were co-transfected with the firefly plasmids pBV-luc (10 μg) and pRL-TK (1 μg). After 24 h, cells were stimulated with PMA (20 nM) and ionomycin (2 μM) for 6 h, when cells were lysed. The firefly luciferase reporter gene was normalized to the renilla vector of cells stabilized with the pLIRES empty vector. The results are representative of at least three independent experiments ± SEM, and the fold induction is relative to the -2,446 bp-Luc construction (pLIRES).

NFAT1-mediated transcription (**Fig. 4A**). Moreover, mutational analyses of the distal NFAT elements in the context of the full promoter strongly suggested that regulation through these negative elements is dominant over regulation via the positive proximal site, because CA-NFAT1 only enhances the activity of the *c-Myc* promoter when the distal sites are abrogated (**Fig. 4A**). Unexpectedly, even when all three elements were mutated, CA-NFAT1 still enhanced the transcriptional activity of the *c-Myc* promoter, suggesting the presence of another recessive and positive NFAT1 site (**Fig. 5A**). Thus, the region was further mapped between nucleotides -1,555 and -1,227 relative to P2, in the vicinity of NFATRE 5 (**Fig. 5C**). These transactivation assays identified several CA-NFAT1-responsive elements in the *c-Myc* promoter, supporting a model in which a functional hierarchy of regulatory NFAT elements exists. NFAT1 binds with greater affinity to some of the NFAT-responsive elements, as demonstrated for the negative distal sites, but when these elements are abrogated or become inaccessible, NFAT1 can bind to lower affinity sites, such as NFATRE 7, to positively regulate this promoter. However, this dominance may vary according to the presence of partner proteins and cell types. Furthermore, we cannot discard the possibility of a kinetics on the occupancy of the different NFAT sites in the *c-Myc* promoter, where the binding of NFAT1 to the distal sites might occur later after cell activation, leading a late downregulation of *c-Myc* to the resting levels.

Relative to the partner proteins, we observed collaboration between NFAT1 and p300 to induce *c-Myc* promoter activity. The inhibition of the *c-Myc* promoter by p300 (**Fig. 6A**) was

previously related to prevent the premature exit from G_0/G_1.[37] The authors hypothesized that when cells are activated by mitogens, other transcription factors that are not activated in the resting state can bind p300 and stimulate *c-Myc* expression. We suggest that NFAT1 is one of these factors, given that the expression of p300 together with a constitutively active NFAT1 protein, which would physiologically require activation, enhances the activity of the *c-Myc* promoter (**Fig. 6B**). The p300-CA-NFAT1 interaction could facilitate the recruitment of transcriptional cofactors and RNA polymerase to NFAT-dependent promoters. Likewise, p300 may acetylate NFAT1 and increase its DNA binding affinity, as reported for several transcription factors, such as p53 and E2F.[35,44] All of these possibilities could explain the increase in promoter transactivation by p300-NFAT1 and explain the higher *c-Myc* expression observed in NFAT1$^{+/+}$ lymphocytes compared with NFAT1$^{-/-}$ lymphocytes (**Fig. 1D**). Notably, the p300-NFAT1 cooperation bypasses the negative regulation of the *c-Myc* promoter exerted by NFATREs 2 and 5 in HEK293T cells. As protein-protein interactions contribute to the stability of the cooperative NFAT-AP-1 complex,[7] the NFAT1-p300 interaction could increase the affinity of NFAT1 for the positive NFATRE 7 and/or other positive NFAT elements in the distal promoter. NFAT proteins are weak transactivators and are usually associated with partner proteins to regulate transcription.[32] Apparently, this also occurs during regulation of the *c-Myc* promoter by NFAT.

The regulation of the *c-Myc* promoter is highly context-dependent. The same factors that exert a dominant influence on *c-Myc* transcription in one biological setting or cell line may be impotent under other circumstances.[1] In fact, a stimulus that upregulates *c-Myc* in one condition may downregulate it in another.[1] Target gene regulation by NFAT1 has also been described as cell type-specific at the *Cox-2* and *TNF*α promoters.[45] While two NFAT sites are necessary for complete *Cox-2* promoter transactivation in Jurkat cells,[46] only the proximal site seems to be important in colon carcinomas[47] and endothelial cells.[16] The dominance or the differential use of NFATRE was clearly evidenced in our assays. In HEK293T cells, although CA-NFAT2 also regulated the negative distal sites, these elements were not dominant as seen for CA-NFAT1 (**Fig. 7A**). Moreover, the known positive NFATRE 7 was only important in the context of the minimal promoter (**Fig. 7B**). The regulation mediated by CA-NFAT2 occurs preponderantly by a positive NFAT element that remains to be described, possibly the same element that is located between nucleotides -1,555 and -1,227 relative to P2, which is recessive and responsive to CA-NFAT1 (**Fig. 5C**). By contrast, in NIH3T3 cells, neither mutation of distal sites nor of the proximal site inhibited the transactivation mediated by both NFAT1 and NFAT2 (**Fig. 8B**), supporting the existence of other important NFAT sites. The NFATRE 7 is not essential for the NFAT-mediated transactivation of the minimal promoter (**Fig. 8C**), suggesting the presence of other NFAT-responsive sites within the minimal promoter, which does not seem relevant in HEK293T cells. Additional experiments will be necessary to identify the other NFAT sites that are present in the *c-Myc* promoter, to determine in which context the different NFAT sites are used and to understand the molecular mechanisms involved in this process.

NFATRE 2 is located in a region called MINE that contains both insulator and heterochromatin barrier activity. Additionally, the MINE region also contains a repressor activity.[43] Originally, the CTCF protein was identified as a repressor of the *c-Myc* promoter.[48] However, Gombert et al. have shown that CTCF binds constitutively to the *c-Myc* promoter independent of the level of *c-Myc* transcription, therefore suggesting that CTCF is not directly involved in repression of *c-Myc* expression. Interestingly, NFATRE 2 and the distal CTCF binding site are separated by 86 nucleotides, and both elements are present within the 640 bp fragment of the MINE region, which has been shown to decrease the transcriptional activity of the Neo^r gene.[43] Moreover, a loop between the CTCF site at the 5' boundary, near NFATRE 2, and another CTCF element located immediately downstream of the *c-Myc* promoter start site has been observed in repressed cells.[30] Thus, binding of NFAT to the NFATRE 2 might contribute to the repressive phenotype exerted by the MINE region. The mechanisms by which P2 is repressed require histone deacetylase (HDAC) activities,[49] and NFAT1 can recruit HDACs, as has been shown for the repression of the CDK4 promoter.[15] NFATRE 5 is located between nucleotides -1,690 and -1,054 relative to P1 in a fragment that has been predicted to form a curved structure by computer modeling.[50] Curved structures are found near elements that are important for transcriptional regulation and have the potential to influence chromatin structure by positioning nucleosome core particles through interaction with histones and other chromosomal proteins.[50] Thus, the binding of NFAT to two different elements in the distal *c-Myc* promoter can result in bending of the promoter and interactions with other proteins that then influence gene transcription. In fact, the flexible structure of the NFAT DBD allows several surfaces to be available for interactions with different transcriptional partners on DNA, such as activator protein-1 (AP-1),[11,12] Maf, ICER, GATA, EGR, Oct, the nuclear receptor PPAR-γ[7,11] and p300,[28,33,34,36] thereby allowing NFAT to integrate Ca^{2+} signaling with many other signaling pathways.[11]

Together, our results show that regulation of the *c-Myc* promoter by NFAT is more complex than previously conceived. In addition to the induction of *c-Myc* expression driven by NFATRE 7, which is located at the minimal promoter, NFAT also binds to at least two other elements in the distal promoter. The negative NFAT-responsive elements identified here could be contributing to the control of *c-Myc* expression by modulating the use of positive regulatory sites or by preventing aberrant activation of *c-Myc,* which could result in deleterious consequences, such as tumorigenesis. Indeed, both *c-Myc* and NFAT are overexpressed in leukemias, lymphomas, pancreatic, colon and breast cancers.[5,17,18,51-54] Furthermore, NFAT controls critical processes during carcinogenesis, including angiogenesis,[16] cell migration and invasion.[16,17,55,56] Moreover, some pathways that regulate the activity of the NFAT proteins are deregulated in cancer and contribute to both tumor progression[17,57,58] and *c-Myc* activation.[1,59,60] These include the WNT pathway, which influences the metastatic behavior of human breast cancer cells,[57] the

α6β4 integrin signaling pathway, which is deregulated in breast cancer,[17] and the cytokine TNFα, which is involved in invasion and metastasis.[58] Thus, constitutive activation of the Ca^{2+}/calcineurin pathway along with the removal/abrogation of the negative NFATREs 2 and 5 may facilitate binding of NFAT to the positive NFAT-responsive sites and consequently enhance *c-Myc* promoter activity during carcinogenesis.

Materials and Methods

Animals and cell culture. C57BL/6, NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice were maintained at the Brazilian National Cancer Institute animal facility. NFAT1$^{+/+}$ and NFAT1$^{-/-}$ (C57BL/6 x 129/Sv) mice were generated as previously described in reference 61. 8–12-week-old mice were used following the "Principals of laboratory animal care" (NIH publication No. 85-23, revised in 1985). CD4 T cells were purified (>90% purity) by negative selection according to the manufacturer's instructions (Dynal Mouse CD4 Negative Isolation Kit, Invitrogen) from inguinal, brachial, axillary and superficial cervical lymph nodes of 8–12-week-old mice. All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin-streptomycin, essential and nonessential amino acids, sodium pyruvate, vitamins, 10 mM HEPES and 2-mercaptoethanol in 5% CO_2 at 37°C.

Plasmids. The human *c-Myc* reporter constructs were a gift from Dr. Bert Vogelstein (Del-1 and Del-2)[62] or were obtained from Addgene (Del-6)[63] and were renamed relative to the P2 transcription initiation site as -2,446 bp-Luc, -1,237 bp-Luc and -109 bp-Luc, respectively. All these constructions span up to +334 bp of the *c-Myc* promoter. FragA[62] and pCMVb-p300-HA were also acquired from Addgene. FragA is referred here as FragA-Luc and spans from -2,446 bp to -1,237 bp of the *c-Myc* promoter. pRL-TK and pRL-CMV were acquired from Promega, and pLIRES-EGFP, pLIRES-EGFP-CA-NFAT1, pLIRES-EGFP-CA-NFAT2α, pLIRES-NFAT1 and pLIRES-NFAT2α have been previously described in references 39 and 64.

Construction of the *c-Myc* promoter reporters. NFAT1 binding site mutants were generated from the -2,446 bp-Luc or -109 bp-Luc reporter constructs as indicated in the figures using the Gene Tailor™ Site-Directed Mutagenesis System (Invitrogen). For site-directed mutagenesis, the forward primers (IDT) were as follows: MutNFATRE 2: 5'-TGT CCT TCC CCC GCT **ACT CA**C CTT GCA CCT CG-3'; MutNFATRE 5: 5'-TGA GTC AGT GAA CTA **ACT CA**T TAA TGC CTG G-3' and MutNFATRE 7: 5'-CTC AGA GGC TTG GCG **ACT CA**A AGA ACG GAG-3' and their complementary strands. The mutated NFAT consensus-binding site is in bold (GGA AA substituted for **ACT CA**). To generate FragA-Mut2,5-Luc, the *SpeI-SacII* fragment of -2,446 bp-Mut2,5-Luc was cloned into the same restriction sites in the FragA-Luc vector. The FragAΔ41–171-Luc, FragAΔ171–891-Luc and FragAΔ891–1,219-Luc vectors were obtained by deletion of the indicated regions from the FragA-Luc plasmid with the restriction enzymes *AgeI-PstI*, PstI-*SpeI* and *SpeI-SacII*, respectively.

Real-time PCR. Freshly isolated CD4 T lymphocytes were stimulated in vitro with plate-bound anti-CD3 (1 μg/mL). At the indicated time points, the total cellular RNA was isolated using Trizol LS Reagent (Invitrogen), and the cDNA synthesis was performed using the oligo(dT) primer (Superscript™ III Reverse Transcriptase, Invitrogen) according to the manufacturer's instructions. When used, cyclosporine A (1 μM; LC Laboratories®) was added 15 min before anti-CD3 stimulation. Synthesized cDNA was subjected to quantitative RT-PCR for the detection of *c-Myc* gene transcripts. Amplification was performed in a 7500 Real-Time PCR System (Applied Biosciences) using the following cycling program: 95°C for 10 min and 40 cycles of 95°C for 15 sec followed by 60°C for 60 sec. Relative expression levels of *c-Myc* were normalized to the endogenous reference HPRT using the Equation $2^{-\Delta\Delta CT}$. The primers and probes were purchased from Applied Biosystems (*c-Myc*: Mm00487804_m1; HPRT: Mm00446968_m1). The expression values are relative to *c-Myc* amplification in unstimulated cells.

Western blot. Total protein lysates from freshly isolated CD4 T lymphocytes or from stabilized NIH3T3 cells were resolved on 8% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Total protein was collected from 2 x 10^6 CD4 T cells at the indicated times after stimulation with plate-bound anti-CD3 (1 μg/mL) in cell lysis buffer (40 mM Tris pH 7.5, 60 mM sodium pyrophosphate, 10 mM EDTA and 5% SDS) and then incubated at 100°C for 15 min. When used, cyclosporine A (1 μM, LC Laboratories®) was added 15 min before anti-CD3 stimulation. For NIH3T3 cells, 2 x 10^5 cells were seeded in a 10 cm^2 plate with 10 mL of DMEM 5% FBS. Twenty-four hours later, cells were stimulated with PMA (20 nM) and ionomycin (2 μM) for additional 24 h, when total protein was collected. 150 μg of protein were used in western blot. The following antibodies were used: NFAT1 polyclonal antibody anti-67.1; NFAT2 monoclonal antibody 7A6 (Santa Cruz), *c-Myc* polyclonal antibody (N-262, Santa Cruz or D84C12, Cell Signaling) and actin monoclonal antibody (A3853, Sigma). Immunodetection was performed with ECL western blotting detection kit (GE Healthcare).

Bioinformatic analysis. The genomic sequences from the *Homo sapiens* and *Mus musculus c-Myc* promoters (up to -3 kb from the first exon) were obtained from the Ensembl Genome Browser (www.ensembl.org) and aligned with the Clustal W program (www.ebi.ac.uk/clustalw). Then, the sequences were submitted for analysis using two transcription factor binding sites search softwares, rVista (http://rvista.decode.org) and Genomatix (www.genomatix.de/products/MatInspector).

Electrophoretic mobility shift assay (EMSA). The NFAT1 DNA binding domain (DBD) recombinant protein was expressed as previously described in reference 65. The DBD was then purified under native conditions with Ni-NTA spin columns according to the manufacturer's instructions (Qiagen). The proteins were eluted in 50 mM Tris-Cl pH 8.0; 100 mM NaCl and 300 mM imidazole. Oligonucleotide duplexes (5.0 μg of each oligo) were generated by denaturation for 10 min at 95°C in hybridization buffer (10 mM Tris pH 7.5, 50 mM NaCl) followed by overnight hybridization at room temperature. Oligonucleotides (50 ng) were labeled for 1 h at 37°C with T4

polynucleotide kinase (10 U; New England Biolabs) and 50 μCi [γ^{32}P] dATP (Amersham Biosciences). Probes were then purified with MicroSpin™ G-25 columns (Amersham Biosciences). Increasing amounts of purified NFAT1 DBD protein (100 nM; 500 nM and 1 μM) were incubated with the indicated labeled oligonucleotides (20,000 counts/min) and 0.2 μg/reaction of poly(dI:dC) (Amersham Biosciences) in a total volume of 20 μL of binding buffer (10 mM HEPES pH 7.0, 125 mM NaCl, 10% glycerol, 0.25 mM DTT, 0.8 mg/mL BSA) for 20 min at room temperature. DNA-protein complexes were separated by electrophoresis under non-denaturating conditions on a 4% polyacrylamide gel in 1x TBE buffer. The gel was pre-run for 1 h at 100 V, and samples were run for an additional hour at 200 V. The gel was dried onto Whatman filter paper and analyzed by autoradiography. The following oligonucleotides were used: IL-2:[66] 5'-GCC CAA AGA **GGA AA**A TTT GTT TCA TAC AG; NFATRE 1: 5'-TGA ACC ATT T**TT TTC** TCT AGT AAT T-3'; NFATRE 2: 5'-CCC AGA ACC T**GG AAA** CCC TGC AGC C-3'; NFATRE 3: 5'-GAG GGG GCG G**GG AAA** GAG TCT CTG C-3'; NFATRE 4: 5'-ATC CTA AAT T**GC AAA** CTC AGT GGC T-3'; NFATRE 5: 5'-AAA GGA AAC TG**G GAA A**TT AAT GTA T-3'; NFATRE 6: 5'-TAA TAA AAG **GGG AAA** GCT TGG GTT T-3' and NFATRE 7: 5'-ACG CTT GGC G**GG AAA** AAG AAG GGA G-3'. For mutated oligos, the nucleotides **GGA AA** (the NFAT consensus binding site) were replaced by **ACT CA**.

Chromatin immunoprecipitation assay (ChIP). ChIP was performed as described in reference 27. Briefly, freshly isolated CD4 T cells were isolated by magnetic bead selection from total lymph nodes of NFAT1$^{+/+}$ and NFAT1$^{-/-}$ mice (Dynal). The cells (2 x 10^6 cells/well) were stimulated in vitro with plate-bound anti-CD3 antibody (2C11; 0.25 μg/mL) and anti-CD28 antibody (37.51; 1 μg/mL) for seven days under Th1 polarizing conditions [IL-12 (10 ng/mL), anti-IL-4 (10 μg/mL) and IL-2 (20 U/mL)]. Then, cells were washed, rested for 2 h and either left unstimulated or restimulated in vitro with PMA (10 nM) and ionomycin (1 μM) for an additional four hours. Immunoprecipitation was performed overnight at 4°C with a set of NFAT1-specific antibodies (anti-67.1 and anti-T2B1; 15 μg and 50 μg, respectively) or an anti-acetyl-histone H4 antibody (5 μg; Upstate Biotechnology). DNA was purified by phenol-chloroform extraction and resuspended in 100 μL of TE buffer. PCR was performed with 5 μL of the immunoprecipitated DNA. PCR conditions were as follows: 3 min at 95°C, followed by 28 (primers 1 and 2 of *c-Myc* promoter) or 26 (primer 3 of *c-Myc* promoter, IFNγ and IL-4) cycles of 30 sec at 95°C, 30 sec at 48°C (IL-4 and IFNγ), 55°C (primer 2 of *c-Myc* promoter), 61°C (primer 1 of *c-Myc* promoter) or 62°C (primer 3 of *c-Myc* promoter) and 1 min at 72°C, completed by 8 min at 72°C. The following primers were used: for the *c-Myc* promoter: primer set 1, 5'-CCC CAT CCA CAA CTA GGG CTC TG-3' and 5'-AAC GAG GGC GGA GGT CGG ATG G (252-bp product); primer set 2, 5'-AGG AGT GAA TTG CCA ACC CAG A-3' and 5'-GGA TTG TAC AGA ATG CAC AGC GT 3' (282-bp product); primer set 3, 5'-AGC TGC CGG GTC CGA CTC GCC TCA-3' and 5'-ACA CGG CTC TTC CAA CCG TCC GCT CA 3' (205-bp product); primers for the IFNγ promoter, 5'-GCT CTG TGG ATG AGA AAT-3' and 5'-AAG ATG GTG ACA GAT AGG-3' (250-bp product); and primers for the IL-4 promoter, 5'-AAG ATT AGT CTG AAA GGC C-3' and 5'-TCA AGA GAT GCT AAC AAT GC-3' (290-bp product) (IDT). As a loading control, the same PCR was performed directly on input DNA purified from chromatin before immunoprecipitation. PCR products were resolved on 1.5% agarose gels and visualized with ethidium bromide staining (0.5 μg/mL).

NIH3T3 cells stably expressing NFAT1 or NFAT2 proteins. BD EcoPack2 ecotropic packing cell line (BD Biosciences) was transiently transfected with retroviral vectors pLIRES-EGFP empty or coding for NFAT1 or NFAT2α murine cDNAs by calcium phosphate precipitation. Cell-free virus-containing supernatant was collected 48 h after transfection and added 1:1 with fresh medium to NIH3T3 cells. The supernatant was supplemented with 8 μg/ml polybrene (Fluka) and immediately used for spin-infection (2 x 45 min at 400 g, at room temperature) of 2.5 x 10^4 NIH3T3 cells. Infected cells were incubated at 37°C for further 24 h, supplemented with fresh media and selected for G418 resistance (1,500 μg/ml, Invitrogen) 14 d more.

Reporter gene assays. HEK293T cells (1.5 x 10^5 cells/well) were seeded in 6-well tissue culture dishes. Twenty-four hours later, cells were co-transfected with luciferase firefly reporter plasmids, pLIRES-EGFP; pLIRES-EGFP-CA-NFAT1 or pLIRES-EGFP-CA-NFAT2α and a renilla plasmid (pRL-TK or pRL-CMV) by calcium phosphate precipitation. When indicated, a fourth plasmid, pCMVb-p300-HA, was also co-transfected. The following day, cells were washed twice with 1x PBS and cultured in 2 mL of DMEM without serum. The cells were incubated for an additional 24 h and lysed for 15 min at room temperature with 100 μL of passive lysis buffer (Promega). The extracts were analyzed in a Veritas™ Microplate Luminometer (Turner Biosystems) using a Dual-Luciferase Reporter Assay System (Promega) as directed by the manufacturer. Luciferase activities were expressed as relative light units, and the firefly luciferase reporter gene was normalized to the renilla vector. The average values of the tested constructs were normalized to the activities of the empty vectors.

After confirmation of NFAT1 of NFAT2 protein expression by western blot, 2.5 x 10^4 stabilized NIH3T3 cells were seeded in 6-well tissue culture dishes. Twenty-four hours later, cells were co-transfected by calcium phosphate precipitation method with luciferase firefly reporter plasmids and a renilla plasmid (pRL-TK). The following day, cells were washed twice with 1x PBS and cultured in 2 mL of DMEM 5% FBS. The cells were incubated overnight and stimulated with PMA (20 nM) and ionomycin (2 μM) for 6 h. The lyses and luciferase reading were performed as described for HEK293T cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

We are especially grateful to Dr. A. Rao for kindly providing the NFAT1 reagents. This work was supported by grants to J.P.B.V. from the ICGEB (CRP/BRA09-01), CNPq (478780/2010-9), FAPERJ (102.357/2009) and INCT-Cancer (573806/2008-0 and 170.026/2008). G.P.M. was supported by a FAPERJ fellowship and B.K.R. was supported by a CAPES/PNPD fellowship.

References

1. Wierstra I, Alves J. The c-myc promoter: still MysterY and challenge. Adv Cancer Res 2008; 99:113-333; PMID:18037408; http://dx.doi.org/10.1016/S0065-230X(07)99004-1.
2. Evan GI, Christophorou M, Lawlor EA, Ringshausen I, Prescott J, Dansen T, et al. Oncogene-dependent tumor suppression: using the dark side of the force for cancer therapy. Cold Spring Harb Symp Quant Biol 2005; 70:263-73; PMID:16869762; http://dx.doi.org/10.1101/sqb.2005.70.054.
3. Zeller KI, Zhao X, Lee CW, Chiu KP, Yao F, Yustein JT, et al. Global mapping of c-Myc binding sites and target gene networks in human B cells. Proc Natl Acad Sci USA 2006; 103:17834-9; PMID:17093053; http://dx.doi.org/10.1073/pnas.0604129103.
4. Levens D. How the c-myc promoter works and why it sometimes does not. J Natl Cancer Inst Monogr 2008:41-3; PMID:18648001; http://dx.doi.org/10.1093/jncimonographs/lgn004.
5. Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 2006; 16:318-30; PMID:16934487; http://dx.doi.org/10.1016/j.semcancer.2006.07.015.
6. Levens D. You Don't Muck with MYC. Genes Cancer 2010; 1:547-54; PMID:20882108; http://dx.doi.org/10.1177/1947601910377492.
7. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin and NFAT. Genes Dev 2003; 17:2205-32; PMID:12975316; http://dx.doi.org/10.1101/gad.1102703.
8. Avni O, Lee D, Macian F, Szabo SJ, Glimcher LH, Rao AT. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. Nat Immunol 2002; 3:643-51; PMID:12055628.
9. Bert AG, Johnson BV, Baxter EW, Cockerill PN. A modular enhancer is differentially regulated by GATA and NFAT elements that direct different tissue-specific patterns of nucleosome positioning and inducible chromatin remodeling. Mol Cell Biol 2007; 27:2870-85; PMID:17283044; http://dx.doi.org/10.1128/MCB.02323-.
10. Johnson BV, Bert AG, Ryan GR, Condina A, Cockerill PN. Granulocyte-macrophage colony-stimulating factor enhancer activation requires cooperation between NFAT and AP-1 elements and is associated with extensive nucleosome reorganization. Mol Cell Biol 2004; 24:7914-30; PMID:15340054; http://dx.doi.org/10.1128/MCB.24.18.7914-30.2004.
11. Macian F. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 2005; 5:472-84; PMID:15928679; http://dx.doi.org/10.1038/nri1632.
12. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 1997; 15:707-47; PMID:9143705; http://dx.doi.org/10.1146/annurev.immunol.15.1.707.
13. Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. Identification of a putative regulator of early T cell activation genes. Science 1988; 241:202-5; PMID:3260404; http://dx.doi.org/10.1126/science.3260404.
14. Viola JP, Carvalho LD, Fonseca BP, Teixeira LK. NFAT transcription factors: from cell cycle to tumor development. Braz J Med Biol Res 2005; 38:335-44; PMID:15761612; http://dx.doi.org/10.1590/S0100-879X2005000300003.
15. Baksh S, Widlund HR, Frazer-Abel AA, Du J, Fosmire S, Fisher DE, et al. NFATc2-mediated repression of cyclin-dependent kinase 4 expression. Mol Cell 2002; 10:1071-81; PMID:12453415; http://dx.doi.org/10.1016/S1097-2765(02)00701-3.
16. Hernández GL, Volpert OV, Iñiguez MA, Lorenzo E, Martínez-Martínez S, Grau R, et al. Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. J Exp Med 2001; 193:607-20; PMID:11238591; http://dx.doi.org/10.1084/jem.193.5.607.
17. Jauliac S, López-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nat Cell Biol 2002; 4:540-4; PMID:12080349; http://dx.doi.org/10.1038/ncb816.
18. Buchholz M, Schatz A, Wagner M, Michl P, Linhart T, Adler G, et al. Overexpression of c-myc in pancreatic cancer caused by ectopic activation of NFATc1 and the Ca^{2+}/calcineurin signaling pathway. EMBO J 2006; 25:3714-24; PMID:16874304; http://dx.doi.org/10.1038/sj.emboj.7601246.
19. Köenig A, Linhart T, Schlengemann K, Reutlinger K, Wegele J, Adler G, et al. NFAT-induced histone acetylation relay switch promotes c-Myc-dependent growth in pancreatic cancer cells. Gastroenterology 2010; 138:1189-99; PMID:19900447; http://dx.doi.org/10.1053/j.gastro.2009.10.045.
20. Pham LV, Tamayo AT, Li C, Bueso-Ramos C, Ford RJ. An epigenetic chromatin remodeling role for NFATc1 in transcriptional regulation of growth and survival genes in diffuse large B-cell lymphomas. Blood 2010; 116:3899-906; PMID:20664054; http://dx.doi.org/10.1182/blood-2009-12-257378.
21. Singh G, Singh SK, König A, Reutlinger K, Nye MD, Adhikary T, et al. Sequential activation of NFAT and c-Myc transcription factors mediates the TGFbeta switch from a suppressor to a promoter of cancer cell proliferation. J Biol Chem 2010; 285:27241-50; PMID:20516082; http://dx.doi.org/10.1074/jbc.M110.100438.
22. Furue M, Katz SI, Kawakami Y, Kawakami T. Coordinate expression of src family protooncogenes in T cell activation and its modulation by cyclosporine. J Immunol 1990; 144:736-9; PMID:2104905.
23. Golay J, Cusmano G, Introna M. Independent regulation of c-myc, B-myb and c-myb gene expression by inducers and inhibitors of proliferation in human B lymphocytes. J Immunol 1992; 149:300-8; PMID:1376749.
24. Nardone J, Lee DU, Ansel KM, Rao A. Bioinformatics for the 'bench biologist': how to find regulatory regions in genomic DNA. Nat Immunol 2004; 5:768-74; PMID:15282556; http://dx.doi.org/10.1038/ni0804-768.
25. Lee TI, Young RA. Transcription of eukaryotic protein-coding genes. Annu Rev Genet 2000; 34:77-137; PMID:11092823; http://dx.doi.org/10.1146/annurev.genet.34.1.77.
26. Kaminuma O, Kitamura F, Kitamura N, Hiroi T, Miyoshi H, Miyawaki A, et al. Differential contribution of NFATc2 and NFATc1 to TNFalpha gene expression in T cells. J Immunol 2008; 180:319-26; PMID:18097033.
27. Carvalho LD, Teixeira LK, Carrossini N, Caldeira AT, Ansel KM, Rao A, et al. The NFAT1 transcription factor is a repressor of cyclin A2 gene expression. Cell Cycle 2007; 6:1789-95; PMID:17637565; http://dx.doi.org/10.4161/cc.6.14.4473.
28. Youn HD, Chatila TA, Liu JO. Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. EMBO J 2000; 19:4323-31; PMID:10944115; http://dx.doi.org/10.1093/emboj/19.16.4323.
29. Santini MP, Talora C, Seki T, Bolgan L, Dotto GP. Cross talk among calcineurin, Sp1/Sp3 and NFAT in control of p21(WAF1/CIP1) expression in keratinocyte differentiation. Proc Natl Acad Sci USA 2001; 98:9575-80; PMID:11493684; http://dx.doi.org/10.1073/pnas.161299698.
30. Liu J, Kouzine F, Nie Z, Chung HJ, Elisha-Feil Z, Weber A, et al. The FUSE/FBP/FIR/TFIIH system is a molecular machine programming a pulse of c-myc expression. EMBO J 2006; 25:2119-30; PMID:16628215; http://dx.doi.org/10.1038/sj.emboj.7601101.
31. Nagl NG Jr, Wang X, Patsialou A, Van Scoy M, Moran E. Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell cycle control. EMBO J 2007; 26:752-63; PMID:17255939; http://dx.doi.org/10.1038/sj.emboj.7601541.
32. Cockerill PN. NFAT is well placed to direct both enhancer looping and domain-wide models of enhancer function. Sci Signal 2008; 1:15; PMID:18385038; http://dx.doi.org/10.1126/stke.113pe15.
33. García-Rodríguez C, Rao A. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). J Exp Med 1998; 187:2031-6; PMID:9625762; http://dx.doi.org/10.1084/jem.187.12.2031.
34. Liu C, Lu J, Tan J, Li L, Huang B. Human interleukin-5 expression is synergistically regulated by histone acetyltransferase CBP/p300 and transcription factors C/EBP, NF-AT and AP-1. Cytokine 2004; 27:93-100; PMID:15271374; http://dx.doi.org/10.1016/j.cyto.2004.02.003.
35. Chan HM, La Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J Cell Sci 2001; 114:2363-73; PMID:11559745.
36. Avots A, Buttmann M, Chuvpilo S, Escher C, Smola U, Bannister AJ, et al. CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. Immunity 1999; 10:515-24; PMID:10367897; http://dx.doi.org/10.1016/S1074-7613(00)80051-5.
37. Sankar N, Baluchamy S, Kadeppagari RK, Singhal G, Weitzman S, Thimmapaya B. p300 provides a corepressor function by cooperating with YY1 and HDAC3 to repress c-Myc. Oncogene 2008; 27:5717-28; PMID:18542060; http://dx.doi.org/10.1038/onc.2008.181.
38. Baluchamy S, Rajabi HN, Thimmapaya R, Navaraj A, Thimmapaya B. Repression of c-Myc and inhibition of G_1 exit in cells conditionally overexpressing p300 that is not dependent on its histone acetyltransferase activity. Proc Natl Acad Sci USA 2003; 100:9524-9; PMID:12883011; http://dx.doi.org/10.1073/pnas.1633700100.
39. Robbs BK, Cruz AL, Werneck MB, Mognol GP, Viola JP. Dual roles for NFAT transcription factor genes as oncogenes and tumor suppressors. Mol Cell Biol 2008; 28:7168-81; PMID:18809576; http://dx.doi.org/10.1128/MCB.00256-08.
40. Remmers EF, Yang JQ, Marcu KB. A negative transcriptional control element located upstream of the murine c-myc gene. EMBO J 1986; 5:899-904; PMID:3013622.

41. Dudley JP, Mertz JA, Rajan L, Lozano M, Broussard DR. What retroviruses teach us about the involvement of c-Myc in leukemias and lymphomas. Leukemia 2002; 16:1086-98; PMID:12040439; http://dx.doi.org/10.1038/sj.leu.2402451.
42. Nielsen AA, Sørensen AB, Schmidt J, Pedersen FS. Analysis of wild-type and mutant SL3-3 murine leukemia virus insertions in the c-myc promoter during lymphomagenesis reveals target site hot spots, virus-dependent patterns and frequent error-prone gap repair. J Virol 2005; 79:67-78; PMID:15596802; http://dx.doi.org/10.1128/JVI.79.1.67-78.2005.
43. Gombert WM, Farris SD, Rubio ED, Morey-Rosler KM, Schubach WH, Krumm A. The c-myc insulator element and matrix attachment regions define the c-myc chromosomal domain. Mol Cell Biol 2003; 23:9338-48; PMID:14645543; http://dx.doi.org/10.1128/MCB.23.24.9338-48.2003.
44. Iyer NG, Ozdag H, Caldas C. p300/CBP and cancer. Oncogene 2004; 23:4225-31; PMID:15156177; http://dx.doi.org/10.1038/sj.onc.1207118.
45. Tsai EY, Jain J, Pesavento PA, Rao A, Goldfeld AE. Tumor necrosis factoralpha gene regulation in activated T cells involves ATF-2/Jun and NFATp. Mol Cell Biol 1996; 16:459-67; PMID:8552071.
46. Iñiguez MA, Martinez-Martinez S, Punzón C, Redondo JM, Fresno M. An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. J Biol Chem 2000; 275:23627-35; PMID:10816557; http://dx.doi.org/10.1074/jbc.M001381200.
47. Duque J, Fresno M, Iñiguez MA. Expression and function of the nuclear factor of activated T cells in colon carcinoma cells: involvement in the regulation of cyclooxygenase-2. J Biol Chem 2005; 280:8686-93; PMID:15632146; http://dx.doi.org/10.1074/jbc.M413076200.
48. Filippova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, et al. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. Mol Cell Biol 1996; 16:2802-13; PMID:8649389.
49. Albert T, Wells J, Funk JO, Pullner A, Raschke EE, Stelzer G, et al. The chromatin structure of the dual c-myc promoter P1/P2 is regulated by separate elements. J Biol Chem 2001; 276:20482-90; PMID:11279041; http://dx.doi.org/10.1074/jbc.M100265200.
50. Kumar S, Leffak M. DNA topology of the ordered chromatin domain 5' to the human c-myc gene. Nucleic Acids Res 1989; 17:2819-33; PMID:2717410; http://dx.doi.org/10.1093/nar/17.7.2819.
51. Arvanitis C, Felsher DW. Conditional transgenic models define how MYC initiates and maintains tumorigenesis. Semin Cancer Biol 2006; 16:313-7; PMID:16935001; http://dx.doi.org/10.1016/j.semcancer.2006.07.012.
52. Wu CH, van Riggelen J, Yetil A, Fan AC, Bachireddy P, Felsher DW. Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation. Proc Natl Acad Sci USA 2007; 104:13028-33; PMID:17664422; http://dx.doi.org/10.1073/pnas.0701953104.
53. Marafioti T, Pozzobon M, Hansmann ML, Ventura R, Pileri SA, Roberton H, et al. The NFATc1 transcription factor is widely expressed in white cells and translocates from the cytoplasm to the nucleus in a subset of human lymphomas. Br J Haematol 2005; 128:333-42; PMID:15667535; http://dx.doi.org/10.1111/j.1365-2141.2004.05313.x.
54. Medyouf H, Ghysdael J. The calcineurin/NFAT signaling pathway: a novel therapeutic target in leukemia and solid tumors. Cell Cycle 2008; 7:297-303; PMID:18235236; http://dx.doi.org/10.4161/cc.7.3.5357.
55. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell 2005; 20:539-50; PMID:16307918; http://dx.doi.org/10.1016/j.molcel.2005.10.033.
56. Yiu GK, Toker A. NFAT induces breast cancer cell invasion by promoting the induction of cyclooxygenase-2. J Biol Chem 2006; 281:12210-7; PMID:16505480; http://dx.doi.org/10.1074/jbc.M600184200.
57. Dejmek J, Säfholm A, Kamp Nielsen C, Andersson T, Leandersson K. Wnt-5a/Ca^{2+}-induced NFAT activity is counteracted by Wnt-5a/Yes-Cdc42-casein kinase 1alpha signaling in human mammary epithelial cells. Mol Cell Biol 2006; 26:6024-36; PMID:16880514; http://dx.doi.org/10.1128/MCB.02354-05.
58. Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, et al. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. Cancer Res 2006; 66:11565-70; PMID:17178846; http://dx.doi.org/10.1158/0008-5472.CAN-06-2540.
59. Han Y, San-Marina S, Liu J, Minden MD. Transcriptional activation of c-myc proto-oncogene by WT1 protein. Oncogene 2004; 23:6933-41; PMID:15286719; http://dx.doi.org/10.1038/sj.onc.1207609.
60. Dydensborg AB, Teller IC, Groulx JF, Basora N, Paré F, Herring E, et al. Integrin alpha6Bbeta4 inhibits colon cancer cell proliferation and c-Myc activity. BMC Cancer 2009; 9:223; PMID:19586553; http://dx.doi.org/10.1186/1471-2407-9-223.
61. Xanthoudakis S, Viola JP, Shaw KT, Luo C, Wallace JD, Bozza PT, et al. An enhanced immune response in mice lacking the transcription factor NFAT1. Science 1996; 272:892-5; PMID:8629027; http://dx.doi.org/10.1126/science.272.5263.892.
62. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science 1998; 281:1509-12; PMID:9727977; http://dx.doi.org/10.1126/science.281.5382.1509.
63. Chen CR, Kang Y, Massagué J. Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. Proc Natl Acad Sci USA 2001; 98:992-9; PMID:11158583; http://dx.doi.org/10.1073/pnas.98.3.992.
64. Teixeira LK, Fonseca BP, Vieira-de-Abreu A, Barboza BA, Robbs BK, Bozza PT, et al. IFN-gamma production by $CD8^+$ T cells depends on NFAT1 transcription factor and regulates Th differentiation. J Immunol 2005; 175:5931-9; PMID:16237086.
65. Jain J, Burgeon E, Badalian TM, Hogan PG, Rao A. A similar DNA binding motif in NFAT family proteins and the Rel homology region. J Biol Chem 1995; 270:4138-45; PMID:7876165; http://dx.doi.org/10.1074/jbc.270.8.4138.
66. Jain J, McCaffrey PG, Valge-Archer VE, Rao A. Nuclear factor of activated T cells contains Fos and Jun. Nature 1992; 356:801-4; PMID:1533441; http://dx.doi.org/10.1038/356801a0.