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NFAT Gene Family in Inflammation and Cancer

M.-G. Pan^{1,*,#}, Y. Xiong^{2,#}, and F. Chen³

¹Division of Oncology and Hematology, Kaiser Permanente Medical Center, Santa Clara, California, USA

²Cardiovascular Institute, Stanford University School of Medicine, Stanford, California, USA

³Division of Nephrology, Washington University School of Medicine, Saint Luis, Missouri, USA

Abstract

Calcineurin-NFAT signaling is critical for numerous aspects of vertebrate function during and after embryonic development. Initially discovered in T cells, the NFAT gene family, consisting of five members, regulates immune system, inflammatory response, angiogenesis, cardiac valve formation, myocardial development, axonal guidance, skeletal muscle development, bone homeostasis, development and metastasis of cancer, and many other biological processes. In this review we will focus on the NFAT literature relevant to the two closely related pathological systems: inflammation and cancer.

Keywords

NFAT; calcineurin; nuclear import; nuclear export; inflammation; cancer

Regulation of calcineurin-NFAT signaling

NFAT (Nuclear Factor of Activated T cell) proteins were first discovered in T-cells as transcriptional activators of interleukin-2 [1, 2], a key regulator of T cell immune response. The immunosuppressant cyclosporine A (CsA) and FK506 (Tacrolimus) inhibit the NFAT pathway to reduce rejection in patients receiving organ transplantation. More than two decades after the discovery, the NFAT gene family is found to play critical roles in many other biological systems of vertebrates.

NFAT proteins are regulated by the phosphatase calcineurin that dephosphorylates NFAT proteins to expose their nuclear localization signals, thus triggering the transport of NFAT proteins from the cytoplasm to the nucleus. Once in the nucleus, NFAT proteins collaborate with other factors to control target gene expression, essential for many biological functions. Calcineurin responds to sustained rise of intracellular calcium level, and NFAT proteins are rapidly imported into or exported from the nucleus, depending on the calcium level and activity of calcineurin. Such mechanism determines that NFAT responds only to a sustained elevation of intracellular calcium level, but not to a transient rise of calcium, to maintain its nuclear presence for the duration needed for transcriptional action. This sets NFAT apart from many regulators that respond to transient calcium signaling [3–6]. Furthermore, the weak DNA binding property of NFAT requires that NFAT partners with other factors to

* Address correspondence to the author at Division of Oncology and Hematology, Kaiser Permanente Medical Center, Santa Clara, CA 95051, USA. Telephone: 408-851-4306. Minggui.Pan@kp.org.

#These authors contribute equally.

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execute transcription regulation [4–6]. For example, NFAT partners with GATA to control heart development, with FOXP3 to regulate immune tolerance, with AP-1 to trigger T cell response, and with MEF to control muscle development [4]. Because of the ability to respond to calcium/calcineurin and to partner with a variety of transcription regulators, NFAT provides a powerful, versatile and delicate tool to control many aspects of the developmental and cellular events.

There are four classic members in the NFAT gene family: NFATc1 (NFATc or NFAT2), NFATc2 (NFATp or NFAT1), NFATc3 (NFAT4), and NFATc4 (NFAT3). In contrast to the classic NFATc proteins, NFAT5 (also known as tonicity enhancer binding protein) does not require calcineurin or a nuclear partner for its activity. In this review, we will focus on the classic NFAT proteins. The classic NFAT protein is composed of a number of functional modules that dictate the protein's phosphorylation, nuclear localization, DNA binding, and transactivation of target genes (Figure 1). The N-terminal region contains regulatory domains, including the casein kinase 1 (CK1) docking site, the transactivation domain (TAD), and a calcineurin (Cn) docking site. The C-terminus contains the DNA-binding Rel Homology Domain (RHD) and an additional calcineurin docking site. In the middle of the protein are several serine-rich domains (SRR, SP1-3) that provide phosphorylation sites for kinases targeting NFAT. Furthermore, the NFAT protein contains two signal sequences that regulate its subcellular localization: the nuclear localization signal sequences (NLS1 and NLS2) and the nuclear export signal (NES).

When NFAT proteins are heavily phosphorylated on the serine residues in the SRR and SP regions, the proteins are confined to the cytoplasm [5, 6]. The activation of cell surface receptors such as T cell receptor (TCR), receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), leads to a signaling cascade (Figure 2) that activates phosphoinositide phospholipase C (PLC) for the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate diacyl glycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃). DAG activates protein kinase C (PKC), whereas IP₃ interacts with its receptor (IP₃R) on the surface of the endoplasmic reticulum (ER). IP₃R activity causes calcium efflux from the ER and subsequent depletion of the ER-stored calcium, thus activating the calcium sensor protein STIM1 [7–9]. STIM1 proteins then form oligomers and move to the junction between ER and the plasma membrane, where the STIM1 oligomers bind to the calcium release-activated calcium (CRAC) channel Orai1, inducing a sustained influx of calcium and the activation of calcineurin. Activated calcineurin then dephosphorylates the cytoplasmic NFAT proteins, causing them to rapidly move into the nucleus [10–13]. The calcineurin activity can be inhibited by the immunosuppressant CsA and FK506, which form CsA–cyclophilin A and FK506–FK506 binding protein 12 (FKBP12) complexes, respectively, to bind and competitively inhibit calcineurin phosphatase activity [14].

There are fourteen phosphorylation sites in the NFAT regulatory domain, and this high number of phosphorylation sites illustrates the importance of phosphorylation and dephosphorylation for controlling NFAT function through nuclear shuttling [15]. Dephosphorylation of serine residues by calcineurin unmasks the nuclear localization signals (NLS) [16], whereas re-phosphorylation of these serine residues masks the NLS sequences and exposes the nuclear export signal (NES). Calcineurin dephosphorylates 13 of the 14 phosphorylation sites of NFAT, thus triggering NFAT to translocate to the nucleus. Calcineurin activity is regulated by several physiological inhibitors, including calcineurin homologous protein (CHP), a CnB homolog [17], DSCR, Down Syndrome's Candidate Region (also called RCAN, regulator of calcineurin) [18–20], DYRK 1 and 2 (dual-specificity tyrosine (Y)-phosphorylation-regulated protein kinase 1 and 2) [20], AKAP79 that binds to CnA subunit [21], and Cabin (CAIN) [22] (Figure 3). To counteract NFAT

dephosphorylation and nuclear localization, several kinases act to phosphorylate NFAT proteins to reduce their nuclear occupancy by either decreasing their nuclear import or increasing their nuclear export. For example, Protein kinase A (PKA) serves as a priming kinase by phosphorylating NFATc1 on Ser²⁶⁹ to prepare its regulatory domain for further phosphorylation by GSK3, and this two-step phosphorylation process is crucial for the efficient nuclear export of NFAT proteins to terminate their transcriptional activity [23, 24]. PKA also phosphorylates NFATc4 on Ser²⁸⁹ to prepare it for forming a complex with 14-3-3 which maintains it in the cytosol in an inactive state, thus also functioning as a maintenance kinase of NFAT [25]. DYRK1 and 2 are priming kinases that phosphorylate NFATc2 on its SP3 region, preparing its subsequent phosphorylation by GSK3 and casein kinase 1 (CK1) on the SRR motif for export [26, 27]. CK1 and MEKK1 phosphorylate NFATc3 to mask its NLS and suppress its nuclear import [28], thus CK1 could serve as an export and a maintenance kinase. JNK phosphorylates NFATc1 and NFATc3 in T cells to facilitate nuclear export [29, 30]. while p38MAPK phosphorylates NFAT c2 and NFATc4 in adipocyte to block its nuclear accumulation [31,32].

NFAT proteins are also regulated by several other mechanisms including sumoylation, proteasome degradation and RNA-protein scaffolding complex [33–35]. Several NFAT review articles over the past few years have covered many areas in details [3–6]. Here we will focus on the literature relevant to NFAT in two pathological aspects that impact human health and are closely related to each other: inflammation and cancer.

NFAT and inflammation

Although the role of NFAT in immune regulation is well established, our knowledge of NFAT in human diseases remains limited. The best clinical knowledge of NFAT resides in the regulation of T cells in organ rejection and the critical importance of cyclosporine and FK506 in preventing rejection. The functions of NFAT in other aspects of human immune or inflammatory diseases are largely unknown.

NFAT and inflammatory bowel disease (IBD)

There are two types of IBD, Crohn's Disease and Ulcerative Colitis, both of which are associated with genetic susceptibility and environmental factors. The mainstay of treatment is the control of bowel inflammation to minimize symptoms through the use of immunosuppressants (such as steroid, azathioprine, and 6-mercaptopurine) and anti-TNF α therapy [36].

NFAT is important in modulating the inflammation of IBD. Genome-wide association studies have identified more than 70 susceptibility loci for IBD, including one residing in the gene that encodes LRRK2 (leucine-rich repeat kinase 2) [37]. LRRK2 inhibits nuclear translocation of NFATc2 by increasing the association of NFATc2 with its negative regulator NRON (Negative non-coding RNA Repressor of NFAT), which is a long non-coding RNA that holds NFATc2 protein in the cytoplasm [37]. In the LRRK2-deficient mice, NFATc2 has higher nuclear occupancy in macrophages, leading to increased activation of NFAT-dependent cytokines that trigger severe colitis [37]. Furthermore, NFATc2-deficient mice are resistant to colitis induced by oxazolone, due to reduced production of IL-6, IL-13, and IL-17 cytokines [38].

NFATc2 may control the transcription of major IBD susceptibility gene NKX2-3 variant rs11190140 [39]. In the intestinal tissues of IBD patients NKX2-3 is expressed in endothelial cells and muscularis mucosa to regulate the expression of endothelin-1 and vascular endothelial growth factor (VEGF) [40], which may contribute to the inflammation and angiogenesis of IBD. NFAT c1, c3 and c4 may also be involved in the pathogenesis of

IBD. The level of nuclear NFATc1 in mononuclear cells of the lamina propria of colon epithelium is increased in patients with ulcerative colitis [41]. NFATc1 activates the expression of TRAIL (a member of the TNF α family) [42], whereas NFATc1 and c4 activate the expression of PTEN [43]—a lipid phosphatase that inhibits mTOR signaling whose activity is enhanced in IBD (44). In addition, NFATc3 inhibits mTOR signaling by activating the expression of its negative regulator REDD1 in human intestinal cells [45]. Further studies of NFAT's role in IBD could be conducted by deleting each individual NFAT gene in the colonic epithelium or inflammatory cells to investigate their precise pathophysiological functions.

Gene deletion experiments in mice have shown that NFATc1 and NFATc2 have redundant functions in lymphocytes but are individually indispensable for cytokine production [45, 46]. Mice lacking *Nfatc2* develop lymphoproliferative disorder [47], whereas mice with *Nfatc2* and *c3* double deletion develop spontaneous differentiation of T cells into Th2 cells and excessive production of IgE [48]. Conversely, mice with *Nfatc3* deletion exhibit loss of DP (double positive) cells, likely caused by a failure of immature thymocytes to induce the expression of anti-apoptotic protein Bcl2 during thymic development [49]. In the transgenic mice carrying a constitutively active NFATc1 mutant (NFATc1^{nuc}) a severe global inflammatory response was observed without altering thymocyte development [50]. Despite the above roles of NFAT in immune regulation, it is unclear how NFAT plays into the development of autoimmune diseases in humans. In Figure 4, we outline the unbalanced positive feedback pathway by NFATc1 as a potential mechanism for the run-away autoimmune reaction to its own antigens. In the normal T cells, the ratio of nuclear and cytoplasmic NFATc1 is maintained in a delicate balance in response to TCR stimulation, depending on the level of calcium and calcineurin activity. This regulation is constant and tightly monitored by the several opposing proteins that activate or inhibit calcineurin-NFAT pathway. GSK3 appears to be the central molecule responsible for removing NFATc1 from the nucleus to the cytoplasm, allowing only appropriate duration of NFATc1 in the nuclei of T cells. The perturbation of such tightly regulated feedback loop can lead to uncontrolled NFATc1 activation and subsequent T cell activation, cytokine production, and other serious events.

NFAT in Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE)

RA and SLE are two classic autoimmune diseases challenging to treat. Recent evidence shows that TNF α activates the calcineurin-NFAT pathway in macrophages, which may contribute to the pathogenesis of autoimmune diseases [51]. CsA and FK506 block the activation of calcineurin and TNF α expression in the synoviocytes of patients with RA [52, 53]. TNF α is one of the many pro-inflammatory cytokines implicated in the inflammatory pathology of RA and is stimulated by NFAT activation [50]. Therefore, the dynamic interaction between calcineurin-NFAT and TNF α pathway likely plays an important role in the pathogenesis of RA.

Clinically, CsA has been shown to ameliorate the disease activity of RA [54]. Also, the combination of L-type calcium channel blocker nifedipine and low dose CsA have additive effects on the inhibition on T cell and NFATc1 activation in patients with RA [55]. Moreover, CsA is as effective as intravenous cyclophosphamide in the treatment of proliferative lupus nephritis for preserving renal function and maintaining remission [56]; CsA is also effective for refractory SLE [56]. Other randomized trials and retrospective studies have shown the efficacy of CsA and FK506 in the treatment of lupus nephritis [57–59]. The toxicities of CsA and FK506 have limited their clinical application in RA and SLE. In a mouse model of SLE, dipyridamole (a platelet and calcineurin inhibitor) reduces the disease activity, inhibits T cell and NFAT activation, and blocks the production of CD40 ligand (CD154) and IL-6 [60, 61]. CD40 ligand is a member of TNF family primarily

expressed in the CD4 T cells, important for B cell development, antibody production, and CD8 effector cell function. CD40 ligand is overexpressed in CD4 T cells in patients with SLE and is correlated with the severity of nephritis and NFAT activity [62, 63]. Nuclear NFATc1 activates CD40 ligand in the T cells in our NFATc1^{nuc} transgenic mice [50]. In addition, the T cells of SLE patients show hyper-responsiveness to TCR stimulation with a robust calcium influx and nuclear translocation of NFATc2 which may result in the consequence of increased cytokine production and subsequent tissue and organ damages [64]. The mTOR pathway is involved in the inflammation of joints in RA and its inhibition decreases the invasion of synovial fibroblasts that is a cause of the joint erosion [65]. The interaction between mTOR pathway and NFAT may be an important element in the inflammatory process of RA. The enhanced mTOR activity mediates basal and rephosphorylation of NFATc4 on the serine residues 168 and 170, which removes NFATc4 from the nucleus and terminates its signaling, though it is not clear if NFATc4 is involved in the joint inflammation of RA. ERK5/MEK5 which is involved in the pathogenesis of RA joints, also rephosphorylates the two same residues to export NFATc4 from the nucleus to the cytoplasm [66]. Future studies to examine the nuclear presence of each individual NFAT protein in the synovial cells of the joints of RA patients and of the animal models may provide clearer insight into the role each NFAT may play in the disease process. Quantitative examination of the activity of each individual NFAT in the macrophages of synovial fluid of RA joints can be valuable as well. If specific gene deletion of individual NFAT in the synoviocytes can be established, it would provide enormously valuable models for understanding the role of NFAT in joint diseases including RA.

NFAT and glomerulosclerosis

Glomerulus of the kidneys is a filtration unit consisting of endothelial cells, mesangial cells, podocytes, and the glomerular basement membrane. Disruption of this highly specialized structure can lead to many types of glomerular pathologies, including nephrotic syndrome and glomerular sclerosis with the risk of progressing to renal failure. Many autoimmune diseases, especially SLE, involve kidneys as a common disease manifestation and cause secondary nephritis. Steroid and other immunosuppressive drugs remain the standard of care for immune-related glomerular diseases and many forms of idiopathic nephrotic syndromes.

Idiopathic focal segmental glomerulosclerosis (FSGS) occurs in both adults and children. Several cytokines, including TNF α , TGF- β 1, and IL-10, have been implicated in its pathogenesis [67]. IL-10 gene promoter polymorphism has been shown to be associated with FSGS [68]. Treatment with the inhibitors of calcineurin-NFAT pathway (CsA, FK506) ameliorates proteinuria and improves symptoms in these patients [69].

Contrary to the CRAC channels essential for T cell response and to the L-type channels critical in the central nervous system, the canonical transient receptor potential channel (TRPC6) responds to store-induced calcium influx and is important in regulating renal podocyte function. Gain-of-function mutations of TRPC6 cause human hereditary autosomal dominant FSGS, and overexpressing TRPC6 in the podocytes causes FSGS in mice [70, 71]. Such gain-of-function TRPC6 leads to increased intracellular calcium influx and enhanced NFAT signaling [72], suggesting that hyperactive NFAT may be causal for FSGS. Indeed, when constitutively nuclear NFATc1 (NFATc1^{nuc}) was expressed in the murine podocytes, mice developed proteinuria and FSGS similar to the phenotypes observed the patients with TRPC6 gain-of-function mutation [73]. These studies thus implicate a crucial role of NFATc1 in a non-immune associated kidney disorder and suggest that NFAT activation may be a key intermediate step in the pathogenesis of mutant TRPC6-mediated FSGS.

Angiotensin II (AT-II) is implicated in kidney injuries in patients with uncontrolled hypertension, diabetes, and other diseases that can lead to renal failure. Drugs that block the

AT-II pathway have been the milestones of treatment for hypertension and heart failure. AT-II was recently found to activate TRPC6 expression in podocytes both *in vitro* and *in vivo* via the NFAT signaling pathway, possibly forming a positive feedback loop in a disease condition in which AT-II level is increased [74]. CsA inhibits TRPC6 expression, while the constitutively active NFATc1^{nuc} stimulates TRPC6 expression [69, 73]. Certain NFATc1-activated-cytokines are likely involved in this AT-II-mediated kidney injury. CAML, a calcium-modulating cyclophilin ligand mediates the AT-II activation of NFATc1 [75]. These findings suggest that specific inhibition of NFAT activity may be effective in ameliorating the progression of certain secondary renal insufficiency caused by diabetes, hypertension and other primary diseases. CsA and FK506 cannot accomplish such task due to its toxicities of triggering kidney vessel spasm and hypertension. Clinically effective specific NFAT inhibitor is still lacking.

NFATc1 and global inflammation

In addition to its critical role in a wide range of T and B cell functions, NFAT signaling regulates the function of other hematopoietic cells including dendritic cells [76], megakaryocytes [77], and osteoblasts [78]. NFAT pathway plays an important role in innate immunity and is activated by LPS (lipopolysaccharide) via CD14 to cause apoptosis of terminally differentiated dendritic cells, and by doing so, to help maintain self-tolerance and prevent autoimmunity [76]. The broad range of functions exerted by NFAT molecules predict that deregulation of NFAT expression can be associated with severe immune and inflammatory disorders.

The transgenic mice that express constitutively nuclear NFATc1 (NFATc1^{nuc}) in T cells show a wide range of pathology involving multiple organs due to severe global inflammation [50, 79]. These mice only have 1/7 increased nuclear level of NFATc1 in the T cells yet display some of the most severe inflammatory pathology. In an unstimulated state, the T cells of mutant mice produce Th1 and Th2 cytokines (IL-4, IL-5, IL-2, interferon-gamma, TNF- α , IgG2a) by several folds higher than the wild-type T cells. However, when stimulated with TCR ligation, the mutant T cells generates cytokines by 10–100 folds higher than the control, accompanied by increased CD25-, CD69-, and CD40L-positive cells. This nuclear NFATc1 triggers a positive feedback loop that leads to the destabilization of T cell regulation and to the hypersensitivity of T cells in response to external stimulation. The hyperactive feedback loop is associated with many autoimmune diseases, including SLE. The mutant NFATc1^{nuc} mice show immune cell infiltrations to the lungs, liver, kidneys, muscle, and joints. The serology titers for autoimmune disease are elevated in the NFATc1^{nuc} mice and the glomeruli contain immune complex deposits. In Figure 4 we illustrate the unopposed positive feedback loop triggered by the constitutively active NFATc1 mutant that represents a possible mechanism for immune-related inflammatory diseases such as SLE and others. Such a destabilized positive feedback mechanism may also be a key pathogenic trigger of certain variants of FSGS.

NFAT and cancer

The evidence is accumulating that NFAT genes are involved in the development and metastasis of cancer. It is speculated that the low rate of cancer incidence (except a slightly increased risk of leukemia) in Down Syndrome patients may be related to the 1.5 fold increase in the gene dosage of DSCR (Down Syndrome Critical Region) and DYRK1 [20, 80], which synergistically inhibit the calcineurin/NFAT signaling. How NFAT genes are involved in cancer development and metastasis is far from being understood. There is also evidence that NFAT genes can be tumor suppressive as well. No viral homolog of NFAT has been identified. However, a gene translocation involving NFATc2 was recently identified in four cases of a variant of Ewing's sarcoma (an aggressive sarcoma that occurs

most commonly in pediatric population), in which EWSR1 was fused to the N-terminally truncated and C-terminally intact active domain of NFATc2 [81]. The role of NFATc2 in Ewing's sarcoma, an aggressive sarcoma that most commonly occurs in pediatric population, is not clear and remains to be investigated. Although NFAT proteins are involved in inflammatory and immune responses, there is no evidence at the present time that NFAT is involved in inflammation-associated malignancies, unlike that of NF- κ B [82, 83]. Although CsA inhibits cell growth in some cell lines, its clinical application in cancer therapy is not expected due to its immunosuppression and other toxicities. In Table 1, we have summarized the literature showing NFAT's impact on cancer development, cell proliferation, and drug resistance.

NFAT's cross talk with other major oncogenic pathways

A constitutively active and nuclear NFATc1 transforms a preadipocyte cell line via activation of the JAK-Stat3 pathway, and the transformed cells are capable of forming tumors in athymic mice [84, 85]. This is the first laboratory evidence that NFAT is involved in promoting cellular transformation. NFATc1 induces the expression of TNF α and cyclooxygenase 2 (COX2). COX2 is implicated in the progression and angiogenesis of several cancers and has been shown in many studies to mediate the oncogenic effect of NFAT [86]. For example, NFATc4 mediates COX2 expression and the transformation of the C141 epidermal cells induced by TNF α [87]. In a malignant melanoma cell line, the BRAF-MEK-ERK pathway activates NFATc1 and c3 which in turn directly activates COX2 [88]. In endometrial adenocarcinoma, NFAT mediates prostaglandin-induced expression of chemokine CXCL8 and cytokine Interleukin-11 (IL-11) [89, 90]. NFAT signaling is activated by TRPC channel to maintain high proliferation rate in prostate cancer cells [91]. In NFATc2-deficient mice, less tumor masses grow in the lungs with the inoculation of malignant melanoma cells compared to the wild-type mice [92]. In addition, NFATc2-deficient mice have different cytokine profile in the lung tissues surrounding the tumor masses in the lungs [92]. These findings suggest that COX2, certain chemokines and cytokines are downstream of NFAT to create an environment for promoting cancer growth.

NFATc1 is overexpressed and activated in many pancreatic cancers. Also, NFATc1 activates c-Myc, a potent proto-oncogene, in pancreatic cancer cells. Inhibiting NFAT activity with CsA or knock-down of NFATc1 by siRNA attenuates c-Myc expression, cell growth, and cell cycle progression [93]. NFAT proteins can bind to a serum responsive element within the c-Myc promoter to initiate a p300-dependent histone acetylation which creates a permissive chromosomal environment for recruiting Ets-like gene 1 (ELK-1) to maximally activate c-Myc [94]. Remarkably, NFAT signaling is found to control the switch of TGF- β pathway from a repressor in the early stage cancer to a promoter of cell proliferation at the advanced stage. TGF- β stimulates a 3–5 fold increase in NFATc1 and c2 expression and also triggers NFAT accumulation in the nucleus, displacing Smad3 repressor from the gene promoter of c-Myc and thus activating c-Myc transcription [95]. Knockdown of NFATc1 or c2 by siRNA in two pancreatic cell lines partially restores the cell growth inhibitory effect of TGF- β [95]. These findings are interesting because NFATc1 is not physiologically expressed in pancreatic cells. How NFATc1 is activated in pancreatic cancer cells will be an important question to investigate.

A recent study using synthetic lethal screen with a shRNA library shows that the activation of NFATc1 is associated with the resistance to imatinib therapy in chronic myeloid leukemia (CML) [96]. The Wnt receptor FZD-8 is also identified in the same lethal synthetic screening, suggesting that the non-canonical Wnt/Ca²⁺/NFAT pathway supports the survival of CML cells and their resistance to the tyrosine kinase inhibitor (TKI) therapy. IL-4 appears to be the main target gene of this Wnt/Ca²⁺/NFAT pathway because IL-4 down-regulation renders CML cells sensitive to TKI. Furthermore, down-regulating NFATc1 with RNAi or

blocking calcineurin-NFAT pathway with CsA sensitizes CML cells to the BCR-ABL kinase inhibitor dasatinib. This is reminiscent of our study in which the expression of NFATc1^{nuc} in osteoblasts results in coordinated expression of the proteins that underlie the positive and negative regulation of Wnt-Frizzled pathway [78]. NFAT and Wnt pathways may therefore reciprocally regulate each other in certain cells and tissues for coordinating their effects on cell growth and differentiation.

Dephosphorylated and aberrantly activated NFATc1 and c2 can be found in many human and mouse lymphomas, including a Notch- and a TEL-JAK-induced mouse model of acute T-lymphoblastic leukemia [97, 98]. Treatment with CsA inhibits leukemia cell growth, induces apoptosis and prolongs mouse survival [98]. NFATc1 is found to be constitutively active in large B-cell lymphoma where it cooperated with NF- κ B to activate the expression of CD40 ligand and to maintain lymphoma cell survival [99]. Since NFATc1 is normally expressed in many lymphoid tissues, its activation in lymphoma and leukemia may be viewed as tumor cells taking advantage of an inherent pathway for growth. The unexpected finding of ectopic activation of NFATc1 in pancreatic cancers suggests that NFAT activation may contribute to a much wider set of tumors originated from cells that have no physiological expression of these proteins [94–98]. In large B cell lymphoma, ectopically expressed NFATc1 also recruits chromatin remodeling complex proteins Brg-1 and Brm to the promoter of its target genes, including c-Myc [100]. This is interesting as *Brg1* is considered a tumor suppressor gene, which is mutated in many malignancies with deletion of one copy of *Brg1* causing epithelial cancer in mice [101, 102] though evidence is accumulating that Brg1 can have an opposite role by suppressing p53 to promote cell growth in certain cellular context [103]. In our preliminary study in 150 human invasive breast cancer cases, we find that NFATc1 is expressed in 22% of the cases, and its expression correlates with that of Brg1 and Brm [104]. These data suggest that there are additional roles for NFAT genes in the chromatin remodeling for regulating cell growth and differentiation that remain to be explored.

NFAT in metastasis and angiogenesis

In addition to enhancing cell growth and proliferation, NFAT has been shown to play roles in tumor cell migration that is intimately linked to tumor invasion and metastasis. NFATc2 and NFAT5 are expressed in invasive human ductal breast carcinomas. In both breast and colon cancer cell lines, NFATc2 and NFAT5 promote cell migration, and their expression correlates with that of $\alpha 6\beta 4$ integrin, which promotes cancer metastasis. Interestingly, $\alpha 6\beta 4$ integrin activates NFAT5 transcription [105]. Further more, Akt promotes E3 ubiquitin ligase HDM2-mediated degradation of NFATc2 to block breast cancer mobility and invasion [106]. There is also evidence that NFATc2 promotes breast cancer cell invasion through upregulation of Cox2 [107]. NFATc2 can bind to the promoter of glypican-6 (GPC6) and directly regulates its transcription to promote invasive migration of breast cancer cells [108]. Also in breast cancer cells, mutations in casein kinase 1 epsilon (CK1 ϵ) activates NFAT pathway through the non-canonical Wnt signaling to reduce cell adhesion and to enhance cell migration [109]. The potential roles of NFAT in tumor invasion are not limited to breast cancer cells. In Notch-driven glioblastoma cells, hypoxia induces the expression of the calcium channel protein TRPC6, thus activating Ca²⁺ entry and NFAT to enhance tumor cell invasiveness [110]. The interactions between Notch, Wnt and NFAT pathways in cancer development will be interesting to explore further.

Angiogenesis has been the target for cancer drug development over the past two decades. The role of calcineurin-NFAT signaling in angiogenesis was first revealed in the double *NFATc3/c4* knockout mice and in the *calcineurin B (Cnb1)* knockout mice [111]. Mice with deletion of *Cnb1* or both *NFATc3/c4* genes died at midgestation due to disorganized vasculature, caused by ectopic expression of VEGF (vascular endothelial growth factor) that

is normally suppressed by NFAT. Calcineurin-NFAT signaling plays an important role in tumor vasculature as well. NFAT controls the angiogenic functions of SFRP2 (secreted frizzled-related protein 2). A modulator of Wnt signaling, SFRP2 is expressed in the vasculature of a wide range of tumors, including approximately 85% of human breast carcinomas [112]. Inhibition of NFATc3 reduces the SFRP2-stimulated angiogenesis *in vitro*, and inhibition of calcineurin with FK506 also blocks SFRP2-stimulated angiogenesis and angiosarcoma growth [113]. In a MMTV-neu breast cancer transgenic mouse model, FK506 treatment results in the reduction of tumor microvascular density and tumor growth rate [114]. However, the involvement of calcineurin-NFAT signaling in tumor vasculature appears to be a complex one. Although one isoform of the endogenous calcineurin inhibitor DSCR1 (DSCR1.Ex4) blocks angiogenesis through suppressing calcineurin-NFAT signaling, the other isoform DSCR.Ex1 appears to promote angiogenesis. In the study by Ryeom et al., deletion of both DSCR1 isoforms in mice causes hyperactivation of calcineurin-NFAT signaling and premature endothelial apoptosis, leading to inhibition of tumor angiogenesis [115]. Treatment of these mutant mice with the calcineurin inhibitor CsA rescues the endothelial defects and restored tumor growth. On the other hand, Baek et al. show that modest increase of DSCR1 expression from an extra transgenic copy of DSCR1 contributes to deficient tumor angiogenesis due to calcineurin inhibition, resulting in significant suppression of tumor growth [80]. Results from these studies suggest that the exact effects of calcineurin-NFAT are context dependent, and the relative strength of the signals may dictate the phenotypic outcome. Furthermore, DSCR1 is induced by VEGF in the endothelial cells to suppress tissue factor (TF), E-selectin, and Cox2 expression. Knockdown of DSCR1 attenuates NFAT activity and reduces the expression of these genes [116]. The paradoxical interaction is also observed between NFAT and VEGF signaling pathways. Though NFAT suppresses VEGF for guiding vascular formation, VEGF can induce NFAT transcriptional activity in HUVEC (human umbilical vein endothelial cells) to upregulate the expression of TF [117]. Using CsA to suppress calcineurin-NFAT inhibits VEGF-induced Cox2 expression in endothelial cells and angiogenesis [118]. Zaichuk et al. has proposed that NFAT balances its effect on angiogenesis by using c-FLIP to modulate endothelial cell apoptosis [119]. NFAT signaling induces the expression of c-FLIP, a caspase-8 inhibitor, while pigment epithelial derived factor (PEDF) recruits JNK kinases to phosphorylate and confine NFATc2 to the cytoplasm to inhibit angiogenesis. Therefore, NFAT targets different array of genes in different cellular and tissue context to regulate vessel growth. Dysregulation of NFAT signaling in human and animal malignancies can thus affect tumor angiogenesis.

Tumor suppression by NFAT

Different NFAT proteins appear to have distinct roles in the development of cancer. Although NFATc1 has been consistently found to be pro-transformation and oncogenic, constitutively active NFATc2 was found to induce cell cycle arrest and apoptosis in NIH3T3 fibroblasts [120]. NFATc3 expression is repressed in T cell lymphomas induced by the murine lymphomagenic virus SL3-3, whereas mice with the germline deletion of NFATc3 develop SL3-3 virus-induced lymphoma in shorter latency and with higher frequency than wild-type and NFATc2-deficient mice. These studies suggest a tumor suppressor role for NFATc3 [121]. In breast cancer cells, NFATc2 inhibits Stat5-dependent gene expression and appears to be inversely related to cancer progression [122]. The context-dependent mechanism of tumor suppression by NFAT remains to be further understood. It is possible that the differential combination of downstream target genes of NFAT determines growth versus differentiation, setting an intracellular or intercellular environment for responding to external oncogenic stimuli.

In summary, we have reviewed the recent literature of NFAT concerning two closely connected pathological processes: inflammation and cancer. The literature of NFAT in inflammation is more extensive than that of NFAT in cancer. Studies in these areas will continue to expand and hopefully lead to therapeutic intervention targeting the NFAT pathway.

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ABBREVIATIONS

NFAT	Nuclear factor of activated T cell
NFATc	Cytoplasmic NFAT
NFATn	Nuclear NFAT (NFATc partner)
Cn	Calcineurin
CsA	Cyclosporine A
FKBP	FK binding protein
NES	Nuclear export signal
NLS	Nuclear localization signal
RANKL	Receptor activator of NF- κ B ligand
NF-κB	Nuclear factor of κ B promoter
CRAC channel	Calcium release-activated calcium channel
RTK	Receptor tyrosine kinase
GPGR	G-protein coupled receptor
Stat	Signal transducers and activators of transcription

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Figure 1.

Sketch of NFAT Structural Features. The structure is based on NFATc1 which contains an N-terminal transactivation domain (TAD), an N-terminal Casein Kinase 1 (CK1) docking site, two calcineurin (Cn) docking domains (one in the N-terminus and another in the C-terminus), one Serine-Rich Region (SRR), three SP repeat motifs, two Nuclear Localization Signal (NLS) sequences, one Nuclear Export Signal (NES), and a Rel Homology Domain (RHD) at the C-terminus.

Figure 2.

Activation of the calcineurin-NFAT pathway. Upon receptor activation (TCR, RTKs, GPCRs, etc.), phospholipase C (PLC) is activated. Activated PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacyl glycerol (DAG). IP3 binds to IP3R on the surface of the endoplasmic reticulum (ER), resulting in a brief efflux of calcium from the ER to the cytoplasm, transiently raising the calcium level in the cytoplasm but depleting the calcium store in the ER. The depletion of store calcium causes the calcium sensor STIM on the ER to form oligomers and translocate to the ER-plasma membrane junction where they bind to and activate Orai (CRAC channel) on the plasma membrane. Orai forms a tetramer channel and opens a sustained oscillating low amplitude influx of calcium that binds to the calcium sensor calmodulin (CAM) in the cytoplasm, forming a calcium/calmodulin/calcineurin complex, and activating calcineurin [7–13]. Calcineurin has a catalytic (CnA) and a regulatory subunit (CnB). Activated CnA dephosphorylates NFAT proteins and unmasks their NLSs, leading to their nuclear translocation [7–13].

Figure 3.

Regulation of the calcineurin-NFAT pathway. Calcium influx activates calcineurin. Calcineurin is negatively regulated by several endogenous inhibitors including the calcineurin homologous protein (CHP), a CnB homolog [17], DSCR, Down Syndrome's Candidate Region (also called RCAN, regulator of calcineurin) [18–20], DYRK 1 and 2 (dual-specificity tyrosine (Y)-phosphorylation-regulated protein kinase 1 and 2) [20], AKAP79 that binds to the CnA subunit [21], and Cabin (CAIN) [22]. The immunosuppressant CsA and FK506 form CsA–cyclophilin A (Cy) and FK506–FKBP complexes, respectively, to bind and competitively inhibit calcineurin phosphatase activity [14]. NFAT is negatively regulated by several kinases including Protein kinase A (PKA) that serves as a priming kinase to phosphorylate NFATc1 to prepare its regulatory domain for further phosphorylation by GSK3 [23,24]. PKA also functions as a maintenance kinase to phosphorylates NFATc4 for forming a complex with 14-3-3 and maintain it in the cytosol in an inactive state [25]. DYRK1 and 2 are priming kinases that phosphorylate NFATc2 on its SP3 region and prepare its subsequent phosphorylation by GSK3 and casein kinase 1 (CK1) on the SRR motif for export [26,27]. CK1 and MEKK1 phosphorylate NFATc3 to mask its NLS and suppress its nuclear import [28], while JNK and p38MAPK serve as export kinases to phosphorylate NFAT for facilitating nuclear export [29–32]. NFATc1 can autoamplify its own transcription and also stimulate the transcription of NFATc2 [50]. NFAT proteins partner with other transcription factors to stimulate the expression of their target genes.

Figure 4.

NFATc1^{nuc} provokes a positive feedback loop. Because NFATc1^{nuc} is not phosphorylable by GSK3 and other NFAT kinases, it remains in the nucleus constitutively and not exportable, therefore bypassing the critical step of negative regulation by GSK3 and other kinases for removing it back to the cytoplasm. This small 1/7th of physiologic level of NFATc1 tips the balance in response to the receptor occupancy and initiates an unopposed positive feedback mechanism. NFATc1 activates it own transcription, though the newly synthesized NFATc1 proteins are subjected to the regulation by NFAT kinases, the signal coming from the hyperactivated TCR, continuously send the activated NFATc1 to the nucleus, causing the imbalance of the immune regulation [50, 78, 79].

Figure 5.

Figure 5A. NFATc1 as part of the non-canonical Wnt-Frizzled pathway. Wnt-Frizzled pathway is implicated in many malignancies, most notably colorectal cancer in which Wnt activates its canonical β -catenin-TCF pathway for signal transduction and nuclear transcription. In CML cells, non-canonical Wnt pathway activates NFATc1 pathway, causing activation of IL-4, leading to the resistance to TKI therapy [96].

Figure 5B. NFAT promotes cancer metastasis and angiogenesis. In many cancer cell types, NFAT can activate COX2, c-Myc, Wnt, Frizzled, SFRP2 and others to cause increased cell migration, metastasis, and angiogenesis. This NFAT action is context dependent and is responsive to the external stimuli such as the activation of RTKs, integrin, and Wnt pathway (also see Table 1).

Table 1

Summary of NFAT regulation in cancer

NFAT Subtype	Cell/Tissue type	Tumor/Phenotype	Mechanism	Ref
NFATc1	3T3-L1 fibroblasts	Transformed phenotype	Stimulating c-Myc expression and activating JAK-Stat pathway	84 85
NFATc4	Cl41 epidermal cells	Transformed phenotype	Enhanced Cox2 expression	87
NFATc1 and c3	A375, CHL-1 and WM266-4	Human melanoma cell lines	Upregulation of NFATc1 and c3 by oncogenic BRAF mutation via the MEK/ERK signaling	88
NFAT	Endometrial adenocarcinoma	Increased cell growth	Upregulating CXCL8 and Interleukin-11	89 90
NFAT	LNCaP prostate cancer cell line	Enhanced proliferation	TRPC6-mediated Ca^{2+} influx activates NFAT promoter	91
NFATc2	NFATc2 ^{-/-} mice	Lower transplanted melanoma growth	Changing cytokine profile and reducing expression of TGF- β	92
NFATc1	Panc-1, S2-028, IMIM-PC	Pancreatic cancer cell lines	Stimulating c-Myc expression, recruiting Elk to c-Myc promoter	93 94
NFATc1 and c2	Panc-1, PaTu8988t, HT-29	Pancreatic and colon cancer lines	Displacing Smad3 repressor complex on the c-Myc promoter to activate c-Myc expression	95
NFATc1	CML cell line	Increased resistance to imatinib	Wnt/ Ca^{2+} /NFAT pathway mediates resistance to imatinib	96
NFATc1	LBCL-MS cells	Human B-cell lymphoma	Enhanced resistance to apoptosis by stimulating expression of CD154 ligand and BLYS	99
NFATc2 NFAT5	Human ductal breast carcinomas	Enhanced invasiveness of human breast carcinoma	Mediating $\alpha_6\beta_4$ integrin signaling and stimulating Cox2 expression	105 107
NFATc2	Breast carcinoma cell line	Enhanced invasive migration phenotype	Activating JNK and p38MAPK via GPC6 and Wnt5A signaling	109
NFATc3	angiosarcoma	Stimulates angiogenesis	NFATc3 inhibition reduces SFRP2-mediated angiogenesis	113
NFATc3	MMTV-Neu cells	Suppresses angiogenesis	NFATc3 mediates SFRP2 stimulated angiogenesis	114
NFATc2 NFATc1	NIH3T3 NIH3T3	Tumor suppressive Oncogenic	Induces cell cycle arrest, apoptosis Stimulates cell proliferation	120 120
NFATc3	Murine T cell lymphoma	Suppresses cell growth	Inactivated by murine lymphomagenic virus SL3-3	121
NFATc2	Breast cancer		Inhibits Stat-5 activity	122