**Review**

# Calcineurin/NFAT signaling in lymphoid malignancies

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**Abstract.** Deregulated calcium signaling is observed at different stages of tumorigenic processes. An important signaling pathway activated in response to calcium involves the protein phosphatase calcineurin and NFAT transcriptional factors. We review here recent data that indicate an important role of the calcineurin/NFAT pathway in lymphoma/leukemogenesis and discuss the potential therapeutic implications of these findings.

**Key words:** Calcineurin — NFAT — Leukemia — Lymphoma

## Introduction

Calcium signaling is used by all cell lineages during development, homeostatic control in adults and responses to a number of physiological and stress signals. Calcium signaling is involved in cell survival/apoptosis, cell cycle progression, differentiation, cross-talk between intracellular compartments (ER, mitochondria), general metabolism, telomerase activity and many others. Not unexpectedly therefore deregulated calcium signaling is observed at different stages of tumorigenic processes (for review see Roderick and Cook 2008), but the effectors of this altered calcium response remains to be fully characterized. We review here recent experimental evidence that support an important role for a signaling pathway involving the protein phosphatase calcineurin and NFAT factors in cell transformation and tumorigenesis.

## The calcineurin/NFAT signaling pathway

Calcium ($Ca^{2+}$)/Calcineurin/NFAT (Nuclear Factor of Activated T-cell) signaling was initially identified in mature T cells as an essential regulator of TCR-induced IL2 gene transcription (McCaffrey et al. 1992; McCaffrey et al. 1993; Shaw et al. 1988). Later studies showed that calcium/calcineurin signaling regulates the expression of a large array of genes including not only cytokines, but also genes encoding proteins involved in signal transduction, transcriptional regulation, survival/apoptosis and cell cycle control (Feske et al. 2001). This signaling pathway is by no means restricted to the immune system and plays major roles in a number of lineages (e.g. nervous system, heart and skeletal muscle, vascular system), thus regulating a wide variety of complex biological processes (Hogan et al. 2003; Wu et al. 2007). Calcineurin (PP2B; PPP3) (Klee et al. 1998) is a unique calcium-calmodulin-dependent serine/threonine protein phosphatase which is ubiquitously expressed. The calcineurin complex is composed of a catalytic subunit A (CnA) and a regulatory subunit B (CnB). In vertebrates, the catalytic subunit is encoded by three distinct genes (*CnAα/PPP3CA, CnAβ/PPP3CB and CnAγ/PPP3CC*). *CnAα* and *CnAβ* are ubiquitously expressed whereas *CnAγ* is specifically expressed in testis (Crabtree 1999; Klee et al. 1998; Rusnak and Mertz 2000). In addition to its catalytic domain (aa 70-328), CnA contains a regulatory domain including a CnB-binding domain (aa 333-390), a calmodulin-binding domain (aa 390-414) and a carboxy terminal autoinhinitory domain. Two different genes have been described in vertebrates encoding the CnB regulatory domain: the *CnB2/PPP3R2* gene is specifically expressed in testis whereas *CnB1/PPP3R1* encodes an ubiquitously expressed protein. Biochemical studies have shown that the heterodimerization of CnB and CnA is absolutely required for calcineurin activity and that both CnB and calmodulin participate in a cooperative fashion to calcineurin activation to narrow threshold of calcium ions in response to cell stimulation (Klee et al. 1998). In accordance, deletion of *CnB1* by homologous recombination results in suppression of calcineurin activity in somatic tissues and early embryonic lethality of *CnB*-deficient mouse embryos due to a failure to organize a normal vaculature (Graef et al. 2001). A major function of cal-

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modulin is to relieve the intramolecular inhibition of CnA catalytic domain by its carboxyterminal inhibitor domain in response to calcium. Thus, CnA proteolytic fragments or experimentally engineered mutants lacking the calmodulin binding and autoinhibitory domains show calcium-independent catalytic activity (Klee et al. 1998). Under physiological settings, engagement of cell surface receptors, such as antigen receptors of mature T and B cells, the Fc receptors in monocytes, natural killer cells and mast cells, a number of tyrosine kinase receptors and G protein-coupled receptors leads to the activation of phospholipase C (PLC-γ, PLCβ). Thus, activated PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to produce two second messengers, namely diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (InsP3). Binding of InsP3 to the InsP3R calcium channel located on the endoplasmic reticulum (ER) membrane induces the release of calcium from internal stores. Store depletion triggers in turn the opening of store-operated calcium-release-activated calcium channels (CRAC, e.g. the ORAI/STIM complex, (Oh-hora and Rao 2008)) in the plasma membrane increasing levels of intracellular calcium ($Ca^{2+}{}_i$). Finally, influx of extracellular calcium leads to the calcium-calmodulin dependent activation of calcineurin and the dephosphorylation of its substrates, including NFAT transcription factors. Dephosphorylation of NFAT by activated calcineurin allows their translocation to the nucleus, where in cooperation with other transcriptional partners they regulate the expression of a number of genes (for review see Macian 2005). NFAT transcription factors form a family of 5 members: 4 calcium signaling responsive members including NFATc1 (also known as NFATc or NFAT2), NFATc2 (also known as NFATp or NFAT1), NFATc3 (also known as NFATx or NFAT4) , NFATc4 (also known as NFAT3). NFAT5, which is ubiquitously expressed and unresponsive to calcium/calcineurin activation, is activated by osmotic stress (Lopez-Rodriguez et al. 2001; Miyakawa et al. 1999). NFAT5 is the founding member of the NFAT family that emerged during evolution from the REL/NFκB gene superfamily of transcription factors since it is the only NFAT family member found to be expressed in invertebrates (Lopez-Rodriguez et al. 2001; Stroud et al. 2002). NFATc1, NFATc2 and NFATc3 are expressed in the lymphoid lineage where they are critically involved in the development, differentiation and function of multiple T-and B-cell subsets (Macian 2005). Each gene can be expressed as a number of alternatively spliced forms encoding different N-or C- terminal sequences and with distinct functions (Chuvpilo et al. 1999; Imamura et al. 1998; Luo et al. 1996; Park et al. 1996). NFATc1-c4 share a similar global modular structure, including N-terminal and C-terminal activation domains, a central Rel-homology (RHR) domain highly conserved among family members that mediates DNA binding, a moderately conserved regulatory domain (also known as the NFAT-homology region or NHR) containing 12-14 serine phosphorylation residues located in serine rich regions, a nuclear localization signal (NLS) and calcineurin docking sites centered over two critical motifs referred to as PxIxIT and LxVP respectively (Aramburu et al. 1998; Martinez-Martinez et al. 2006). In resting cells, NFATc1-c3 are located in the cytosol in a fully phosphorylated, inactive conformation that masks their NLS and inhibits their DNA binding activity. Calcium/calcineurin-induced activation leads to NFAT dephosphorylation, inducing a conformational switch that unmasks their NLS, allowing their nuclear translocation, DNA binding activity and interaction with co-regulators required for transcriptional activation (Okamura et al. 2000). To regulate transcription of their many target genes, NFAT proteins bind DNA either as monomer to specific A/TGGAA motifs, or as dimers to NFκB-like response elements, or in cooperation with other transcriptional partners activated by other signaling pathways (e.g. the AP1 complex in response to MAPK activation) on composite DNA binding sites (for review see Macian 2005). Regulation of NFAT factors activity through the regulation of their phosphorylation state has to be tightly regulated to ensure a controlled and balanced activation of the calcium/calcineurin signaling pathway (Muller et al. 2009). Several kinases are implicated in the maintenance of NFAT hyperphosphorylation in resting cells and in their nuclear re-phosphorylation after activation, including casein kinase 1, glycogen synthase kinase 3 (GSK3), JUN kinase 1 (JNK1) and DYRK protein kinases (for review see Arron et al. 2006; Gwack et al. 2006; Macian 2005). Although classic NFAT members are all phosphorylated in their NHR, several NFATs are subjected to other types of regulatory modifications including other phosphorylation events outside the regulatory domain, ubiquitination, sumoylation that modulate their activity, stability or subcellular localization (for review see Macian 2005; Nayak et al. 2009; Terui et al. 2004; Yoeli-Lerner et al. 2005). Although many mouse genetic studies have clearly demonstrated that NFATc1-c4 are critical effectors of calcineurin in several developmental processes (for review see Macian 2005), the best characterized function is their role in the immune response. Mouse genetic studies have revealed an intricate level of complexity between family members. For example in the lymphoid lineage, NFAT proteins can display either specific, redundant or antagonist functions (for review see Macian 2005). The calcineurin/NFAT signaling module is important at specific steps (preTCR signaling; TCR-mediated positive selection of antigen specific self MHC-restricted T cells) in thymocytes development as illustrated by the phenotype of the conditional deletion of mouse *CnB1* (Neilson et al. 2004) and of the single and combined knockout of *NFATc1-3* (Cante-Barrett et al. 2007; Hodge et al. 1996; Oukka et al. 1998; Peng et al. 2001; Ranger et al. 1998; Rengarajan et al. 2002; Yoshida

et al. 1998). Calcineurin/NFAT signaling plays a critical role in peripheral T cell activation following TCR engagement. Under these conditions, NFATc1 and NFATc2 play redundant roles in activating expression of several cytokine genes (Peng et al. 2001) while NFATc2 and NFATc3 function in a redundant way to negatively control peripheral T cells homeostasis (Ranger et al. 1998; Xanthoudakis et al. 1996). The crucial role of the calcineurin/NFAT signaling pathway in T cell activation downstream of TCR engagement responsible for the initiation of a productive immune response is highlighted by its sensitivity to calcineurin inhibitors such as FK506 (Tacrolimus) and Cyclosporin A (CsA). Both FK506 and CsA are extensively used in human medicine as immunosuppressive agents to improve allograft survival and to treat auto-immune diseases. These calcineurin inhibitors act through their binding to distinct intracellular receptors (Monticelli and Rao 2002) FKBP12 and cyclophilinA, respectively to inhibit access of substrates to calcineurin catalytic site (for review see Macian 2005). Although both FK506 and CsA inhibit other but distinct signaling pathways, their common inhibitory activity of calcineurin make them valuable pharmacological tools to study calcium/calcineurin/NFAT signaling pathway.

## Oncogenic potential of the calcineurin/NFAT signaling pathway in hematologic disorders

Although critically involved in many aspects of normal T cells survival, proliferation and activation, the direct implication of calcineurin and/or its downstream NFAT targets in lymphomagenesis and cancer in general even if suspected for a long time has only been recently reported (Buchholz and Ellenrieder 2007; Medyouf and Ghysdael 2008). Since NFAT proteins are essential effectors of calcineurin in the control of a broad spectrum of genes in many different cell lineages which are critical for proliferation, growth, differentiation, migration and survival, processes commonly deregulated in cancer cells, it is easy to speculate an oncogenic potential for NFAT transcription factors. As some NFAT members are involved in the positive or negative regulation of cell cycle components, it is tempting to speculate that deregulation of their expression could play a role in cellular transformation. In line with this hypothesis, Clipstone and colleagues have shown that enforced expression of a constitutively nuclear and transcriptionally active NFATc1 mutant (caNFATc1, made by substitution of the serine residues of the NHR by alanine) in the 3T3L1 preadipocyte cell line impaired terminal differentiation into adipocytes and induced the acquisition of a transformed phenotype (Neal and Clipstone 2003). More recently, enforced expression of similarly mutated, constitutively active mutants of NFATc1 (short isoform) and NFATc2 (long isoform) was shown to induce distinct phenotypes in NIH 3T3 cells. Both of these caNFAT proteins are constitutively localized to the nucleus, bind DNA with high affinity and activate endogenous NFAT target genes (Monticelli and Rao 2002; Neal and Clipstone 2003; Okamura et al. 2000). Whereas enforced expression of the short caNFATc1 isoform led to increased cell proliferation and induction of cellular transformation as previously described in 3T3L1 cells (Neal and Clipstone 2003), expression of a long isoform of caNFATc2 induced cell cycle arrest and apoptosis (Robbs et al. 2008). Furthermore, enforced expression of caNFATc2 interfered with Ras-and caNFATc1-induced transformation of NIH3T3 cells, suggesting that NFATc2 long isoform may act as a tumor suppressor gene whereas the NFATc1 short isoform may function as an oncogene (Robbs et al. 2008). Genetics studies have clearly proved that in the immune system different NFAT factors can play either specific, redundant or antagonist function regarding normal T cell development (Macian 2005), these results highlight the notion that different members and splicing-dependent isoforms of the NFAT family can play different roles in tumor development (Robbs et al. 2008). The proposed tumor suppressive function of NFATc2 must however be highly context or signal dependent as NFATc2 knockout mice develop and live normally and, except for the fact that the differentiation defect observed in cartilage differentiation in these animals is associated with the emergence of chondrosarcoma (Ranger et al. 2000), these mice are not particularly tumor-prone. Interestingly, NFATc2-deficient mice are more susceptible to carcinogen-induced tumorigenesis (Robbs et al. 2008). Whether this reflects a cell-autonomous requirement for NFATc2 loss-of-function intrinsic to the tumor cells or results from impaired function in cells in the tumor environment or results from a defective tumor immune surveillance in NFATc2-/- mice remains to be addressed. Albeit involvement of calcineurin and/or NFAT proteins has been proposed in several solid tumors, in particular breast carcinoma and pancreatic cancer (for review see Buchholz and Ellenrieder 2007; Medyouf and Ghysdael 2008) we will mainly focus here on their cell-autonomous implication in hematologic malignancies. A few reports have described gain-of-function mutation in *CnA* in T or B lymphoma derived cell lines, but it is unknown whether these mutations were part of the dominant clone in the primary tumors from which these cell lines were derived. In the EL4 murine T lymphoma cells, a missense mutation changed an evolutionary conserved aspartic acid to asparagine within the autoinhibitory domain of the *CnAα* gene (Fruman et al. 1995). This substitution leads to the generation of a mutant CnA*α* hypersensitive to calcium signaling which affects normal signal transduction pathways in EL4 T-lymphoma cells (Fruman et al. 1995). A differential proteomic screen performed in a squirrel monkey-derived B-lymphoma (SML) cell line resulted in

the expression of a truncated, constitutively active form of CnA (Gross et al. 2004). In both instances, the consequences of these mutations on the cell line maintenance *in vitro* and tumorigenic activity *in vivo* have not been assesed. Moreover, large-scale retroviral insertional mutagenesis screens carried out in lymphoproliferative disorders have identified two cases of retroviral insertions in genes encoding NFAT family members, including NFATc2 and NFAT5 (Suzuki et al. 2002). In B-cell chronic lymphocytic leukemia (B-CLL), persistent nuclear localization and constitutive activation of NFATc2 (Schuh et al. 1996) were shown to be responsible, in cooperation with STAT6, to the high expression of CD23 on the surface of B-CLL cells, thus contributing to the pathogenesis of this disease (Kneitz et al. 2002). Importantly, immunohistological analysis of NFATc1 expression and subcellular localization has been performed in a large panel of Non-Hodgkin B and T-cell lymphoma (Marafioti et al. 2005). Although NFATc1 expression was not detected in classical Hodgkin's lymphoma (cHL) and plasma cell proliferations, NFATc1 was expressed in a majority of aggressive B-cell lymphomas cases, with nuclear localization of NFATc1 found in a subset of these cases (Akimzhanov et al. 2008; Marafioti et al. 2005). Suppressed expression of NFATc1 in human cHL and anaplastic large cell lymphomas (ALCLs), which both are lymphoma entities with immunoreceptor signaling, was recently explained by NFATc1 transcriptional silencing through hypermethylation of the NFATc1 P1 promoter (Akimzhanov et al. 2008). NFATc1 nuclear localization or dephosphorylation of both NFATc1 and NFATc2 were found in primary tumor samples and cell lines derived from aggressive B and T- cell lymphoma patient (Medyouf et al. 2007; Pham et al. 2005). Moreover treatment of these cell-lines with CsA triggered cell cycle inhibition and induced apoptosis. In vitro studies performed on diffuse large B cell lymphoma (DLBCL) derived cell lines have shown that NFATc1 together with NFκB are constitutively activated and cooperatively regulate the expression of target genes important for proliferation and cell survival (Fu et al. 2006; Pham et al. 2005). Of particular interest, Ford and colleagues have proposed that expression of the genes encoding CD154 and BLys, two ligands belonging to the TNFα superfamily, is synergistically regulated by activation of NFAT and NFκB (Fu et al. 2006; Pham et al. 2005), thus possibly regulating survival and proliferation of malignant B cells. A recent transcriptomic analysis of angioimmunoblastic T-cell lymphoma (AITL) also provided evidence for NFATc1 overexpression in this pathology (de Leval et al. 2007). Regarding the implication of the calcineurin/NFAT pathway in *in vivo* mouse models of human T-ALL/lymphoma, persistent activation of calcineurin/NFAT signaling was observed in primary leukemic cells and shown to be independent of preTCR/TCR signaling, but to depend upon their maintenance in their *in vivo* context (Medyouf et al. 2007). Short-term treatment of leukemic mice with calcineurin inhibitors (CsA or FK506) leads to inactivation of calcineurin/NFAT signaling (NFAT rephosphorylation), inhibition of cell cycle progression and induction of apoptosis in leukemic cells. These combined cellular effects led to severe inhibition of tumor load in treated animals and their increased survival (Medyouf et al. 2007). Pharmacological treatment of diseased mice with these compounds is likely to inhibit calcineurin in non tumor cells of the leukemic niche, possibly contributing this way to leukemia regression. For example, recent evidence shows that the calcineurin/NFAT signaling pathway is involved in tumor angiogenesis, acting downstream of the VEGFR and being negatively controlled by the DSCR1/calcipressin inhibitors of calcineurin (Ryeom et al. 2008). Ectopic expression of a constitutively activated calcineurin mutant in leukemic cells was shown to enhance leukemic cells aggressiveness *in vivo*, suggesting an intrinsic role for calcineurin in leukemic cells (Medyouf et al. 2007). Taken together these observations provide clear evidence that aberrant activation of the calcineurin/NFAT signaling module plays a critical role in the pathogenesis of hematologic disorders. It remains to be seen whether NFAT are effectors of calcineurin in leukemic cells, whether different NFAT play similar or distinct roles and whether their activity in different leukemias/lymphomas involves the deregulation of expression of cytokines, cytokine receptors, cell cycle components or components of the apoptosis machinery. In some settings, acute activation of the calcineurin/NFAT pathway has been shown to have a negative impact on tumor cell maintenance. For example, in type I Burkitt's lymphoma (BL) cells, it has been reported that B cell antigen receptor (BCR) *in vitro* cross-linking with an anti-IgM antibody leads to endogenous calcineurin activation and nuclear translocation of NFATc2 triggering lymphoma cells apoptosis probably through the induction of Nur77 (Kondo et al. 2003). Additionally, NFATc3 has been proposed to function as a tumor suppressor for the development of murine T-cell lymphomas (Glud et al. 2005). Analysis of T-cell lymphoma induced by the murine lymphomagenic SL3-3 retrovirus identified proviral integration within the *Nfatc3* locus that specifically repressed NFATc3 expression. Moreover, NFATc3-deficient mice infected with the murine SL3-3 retrovirus developed T-cell lymphoma with accelerated tumor onset as compared to wild type mice or NFATc2-deficient mice (Glud et al. 2005). It appears therefore that an appropriate balance in calcineurin/NFAT signaling can have either pro-or anti-oncogenic properties. This might be true both in signaling pathways intrinsic to tumor cells or to cells of their supportive stroma. It is thus critical to identify in specific leukemias and lymphomas the signaling cascades that function upstream of the calcineurin/NFAT signaling module and to delineate (i) how similar these pathways are in different tumor types; (ii) at what step

of disease progression they actually impinge upon the oncogenic process.

## Therapeutic targeting of calcineurin/NFAT signaling

Since the NFAT activation process can be mainly divided into five steps: upstream events, calcineurin activation, NFAT dephosphorylation, NFAT nuclear translocation, NFAT target gene deregulation and cellular response, therapeutic intervention at each specific step can be envisaged to modulate the activity of this signaling module in tumorigenesis. The most potent and well characterized calcineurin inhibitors, CsA and FK506, have proved their therapeutic efficiency in pre-clinical models of T-cell leukemia/lymphoma (Medyouf et al. 2007). Importantly, therapeutic benefit of CsA has been shown in a small cohort of AITL patients (Advani et al. 2007) and larger clinical trials are ongoing to confirm these preliminary findings. Also therapeutic benefit is observed following treatment of myelodysplastic syndromes (MDS) with CsA, in particular in patients with refractory anemia and refractory anemia with excess blasts (Chen et al. 2007). However, both CsA and FK506 display off-target and severe toxic side effects ranging from neurotoxicity, nephrotoxicity, hypertension and gastrointestinal disturbances (for review see Dumont 2000). They also function as efficient immunosuppressants and their use in remission induction protocols may thus compromise tumor immunosurveillance mechanisms. Other synthetic or natural calcineurin inhibitors were identified or synthetized after the discovery of CsA and FK506. For example, a FK506 analog (designated as L-732, 531) (Dumont 2000), and a CsA analog (named $ISA_{TX}247$) (for review see Aspeslet et al. 2001; Lee and Park 2006; Stalder et al. 2003) displaying a similar or even higher efficiency with a reduced nephrotoxicity have been generated. Attempts to find novel calcineurin inhibitors through large-scale screens of chemical libraries have identified small molecules that block either NFAT activation or NFAT nuclear translocation, but turned out not to inhibit calcineurin itself but the activity of upstream calcium channels (Venkatesh et al. 2004). The interaction between calcineurin and NFAT factors and other substrates occurs at the level of two motifs, highly conserved among NFAT family members and centered over the PxIxIT and LxVP motifs in the NHR regulatory domain. Pharmacological compounds or synthetic peptide inhibitors spanning the PxIxIT motif have been identified in high throughput screens and shown to efficiently prevent NFAT dephosphorylation and nuclear translocation without affecting calcineurin activity (Aramburu et al. 1999; Kang et al. 2005; Lee and Park 2006; Noguchi et al. 2004; Roehrl et al. 2004). Designing more selective inhibitors targeting the physical interaction of calcineurin with its substrates is clearly an option to specifically target calcineurin signaling (Kang et al. 2005). Furthermore, several endogenous proteins that inhibit calcineurin activity have been identified (for review see Macian 2005) and appear to be potential targets for NFAT inhibition (for review see Lee and Park 2006). For example, therapeutic inhibition of calcipressin may prove to be an alternative or synergistic approach to existing anti-angiogenic therapies (Ryeom et al. 2008).

The most promising advance towards specificity is to bring to light the respective roles of calcineurin downstream targets that mediate its oncogenic or anti-oncogenic activity in different human malignancies, both in the tumor themselves and in supporting cells of the microenvironment. Available evidence shows that NFAT transcription factors are mediators of calcineurin in different cancers (Buchholz et al. 2006; Jauliac et al. 2002; Pham et al. 2005). It is however possible that NFAT factors are not the only targets of calcineurin in leukemogenesis as calcineurin can dephosphorylate other effectors possibly relevant to its oncogenic properties (see for example Huang et al. 2008)

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