Calcineurin: a central controller of signalling in eukaryotes

Workshop on the Calcium/Calcineurin/NFAT Pathway: Regulation and Function

José Aramburu¹⁺, Joseph Heitman²⁺⁺ & Gerald R. Crabtree³⁺⁺⁺

¹Universitat Pompeu Fabra, Barcelona, Spain, ²Duke University Medical Center and Howard Hughes Medical Institute, Durham, North Carolina, USA, and ³Stanford University, Howard Hughes Medical Institute, Stanford, California, USA

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Introduction

Calcineurin is a serine- and threonine-specific protein phosphatase that is conserved in all eukaryotes and is unique among phosphatases for its ability to sense Ca^{2+} through its activation by calmodulin. Identified and characterized in pioneering work by the Claude Klee and Philip Cohen laboratories in the late 1970s, calcineurin catapulted to centre stage when the groups of Stuart Schreiber and Irving Weissman discovered that it is the target of the immunosuppressants cyclosporin A and FK506. In the same year, the laboratory of Gerald Crabtree showed that cyclosporin blocks the nuclear import of the nuclear factor of activated T cell (NFAT) proteins and in 1993, the group of Anjana Rao showed that these proteins are dephosphorylated by calcineurin. These findings revealed a central pathway that coupled calcineurin to transcriptional regulation (Fig 1). Since then, calcineurin and NFAT proteins have been shown to participate in signalling cascades that govern the development and function of the immune, nervous, cardiovascular and musculoskeletal systems. Parallel advances made in microbial systems, including model yeasts and pathogenic fungi, have revealed that the basic mechanisms of action of calcineurin are conserved from unicellular to multicellular eukaryotes.

The nomenclature for the calcineurin-dependent cyclosporin-sensitive cytoplasmic subunits of NFAT transcriptional complexes (NFATc proteins) used here is that used by HUGO and GenBank: NFATc1 (c1) = NFATc = NFAT2; NFATc2 (c2) = NFATp = NFAT1; NFATc3 (c3) = NFATx = NFAT4; NFATc4 (c4) = NFAT3 (see HGNC Gene Family Nomenclature online at: <http://www.gene.ucl.ac.uk/nomenclature/genefamily/NFAT/NFAT.shtml>).

Calcineurin: from yeast to mammals

The meeting began with C. Klee (Bethesda, MD, USA), who reviewed the now classical studies that showed that calcineurin comprises three subunits: the catalytic A and regulatory B subunits, and calmodulin. Calcineurin B is essential for enzyme activity, drug binding and activation by calmodulin. Calmodulin

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¹Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Carrer Dr Aiguader 80, 08003 Barcelona, Spain

²Department of Molecular Genetics and Microbiology, 322 CARL Building, Research Drive, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710, USA

³Stanford University, Howard Hughes Medical Institute, Beckman Center, 279 Campus Drive, Stanford, California 94305-5323, USA

*Corresponding author. Tel: +34 93 542 2893; Fax: +34 93 542 2802;

E-mail: jaramburu@imim.es

**Corresponding author. Tel: +1 919 684 2824; Fax: +1 919 684 5458;

E-mail: heitm001@duke.edu

+++Corresponding author. Tel: +1 650 723 8391; Fax: +1 650 723 5158;

E-mail: crabtree@cmgm.stanford.edu

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Fig 1 | Signal integration and coincidence detection by assembly of NFATc transcriptional complexes in the nucleus. A range of signalling pathways are integrated in the nucleus by the assembly of NFATc transcriptional complexes from calcineurin-dependent NFATc subunits and inducible nuclear partners (NFATn), which regulate the affinity and specificity of NFATc DNA binding and ensure transcriptional activation in response to two signals. This mechanism allows the complexes to behave as coincidence detectors and signal integrators. CK1, casein kinase 1; Csp1, calcipressin 1; DSCR1, Down syndrome critical region 1; Erk, extracellular-signal-regulated kinase; GSK3, glycogen synthase kinase 3; MCIP1, myocyte-enriched calcineurin interacting protein 1; NFAT, nuclear factor of activated T cells; PKA, protein kinase A; PKC, protein kinase C; RCN1, regulator of calcineurin 1.

activates the catalytic subunit A by displacing an autoinhibitory domain (AID) from the active site of the enzyme and also through a poorly understood AID-independent mechanism. The active site contains $\text{Fe}^{2+}/\text{Zn}^{2+}$ metal ions and the oxidation of Fe^{2+} to Fe^{3+} inactivates the enzyme. Under aerobic conditions, superoxide dismutase (SOD) protects the active-site metal ions from oxidation. At the meeting, Klee supported the hypothesis that the AID or calcineurin-binding proteins, such as the calcipressins, might not merely keep calcineurin in an inactive state but also protect the active-site metals from oxidation. Further insights into calcineurin function, and its interactions with both substrates and endogenous and exogenous inhibitors, require biochemical and genetic approaches.

K. Cunningham (Baltimore, MD, USA) presented an update on the *Saccharomyces cerevisiae* calcipressin homologue Rcn1. This protein was a founding member of the calcipressin family of calcineurin-binding proteins, which are homologous proteins that were first described as endogenous calcineurin inhibitors and are co-expressed with calcineurin in all organisms. Subsequent studies revealed that *rcn1* mutants do not show increased expression

of calcineurin-dependent genes, indicating that Rcn1 does not simply repress calcineurin. Calcipressins are calcineurin substrates and contain the amino-acid sequence SPPxSPP, similar to NFATc proteins. The Rcn1 motif is phosphorylated in the first serine by the yeast glycogen-synthase kinase 3 (GSK3) homologue Mck1 after the phosphorylation of the second serine by a priming kinase. The GSK3 phosphorylation site, but not the priming site, can be dephosphorylated by calcineurin (Hilioti *et al*, 2004). The phosphorylation state of calcipressins regulates their binding to calcineurin and their half-life, and seems to be crucial for their positive and negative modulation of calcineurin (Genesca *et al*, 2003). Cunningham presented an attractive model in which Rcn1 regulates calcineurin by allosteric mechanisms similar to the regulation of protein phosphatase 1, which is structurally related to calcineurin, by inhibitor 2.

M. Cyert (Stanford, CA, USA) presented studies on the roles of calcineurin in *S. cerevisiae*. Several stresses and environmental stimuli activate calcineurin and its downstream transcription factor Crz1, which in turn induces a range of genes including *rcn1*. Crz1 has no mammalian counterpart but contains a calcineurin docking site (amino-acid sequence PIISIQ) and a serine-rich region (SRR) that is also found in vertebrate NFATc proteins. In stressed cells, calcineurin activates the nuclear import of Crz1 by docking at the PIISIQ site and dephosphorylating the SRR motif. Hrr25, which is a casein kinase I (CK1) homologue, phosphorylates Crz1 and inhibits nuclear import, setting the activation threshold for target genes (Kafadar *et al*, 2003). Interestingly, CK1 and CK2 phosphorylate mammalian NFATc proteins and oppose calcineurin-induced translocation and activation. Crz1 is still phosphorylated in *hrr25* mutants, and protein kinase A also acts on Crz1 *in vitro*. Cyert proposed a model by which nutrient sensing and stress responses are coordinated through pathways that converge on Crz1. Calcineurin and *crz1* mutants share many phenotypes, but the loss of calcineurin is more severe. This indicates that calcineurin has additional targets, such as Hph2, which, together with its homologue Hph1, is localized to the endoplasmic reticulum and functions in calcineurin-dependent pathways that mediate pH stress responses. Whether Hph1 homologues are conserved calcineurin targets in mammals is not known.

Despite the established role of calcineurin in the immune response, the use of calcineurin by pathogenic fungi to invade their host has not been well characterized. J. Heitman (Durham, NC, USA) presented studies on the antifungal action of cyclosporin and FK506, and the role of calcineurin in fungal virulence. Cyclosporin and FK506 are produced by soil microorganisms and show intrinsic antimicrobial properties, so that competing microbes are inhibited. Heitman described their mechanisms of antifungal action against two human pathogens: *Cryptococcus neoformans* and *Candida albicans*. Cyclophilin A, FKBP12 and calcineurin homologues mediate cyclosporin and FK506 toxicity, and were validated as drug targets. A new synergistic drug interaction between the antifungal drug fluconazole and FK506 in *C. albicans* is attributable to calcineurin inhibition by FK506. Calcineurin is essential for viability in *C. albicans* only when the membrane is perturbed by ergosterol-biosynthesis inhibitors, which is an observation that could be used in the development of potential therapeutics. Heitman described how calcineurin promotes the virulence of *C. neoformans* and *C. albicans* through distinct mechanisms: in *C. neoformans*, calcineurin is

required for growth at 37 °C, whereas in *C. albicans* it is required for survival in the serum (Blankenship *et al*, 2003). These studies reveal signalling pathways that are conserved from unicellular to multicellular eukaryotes, and underscore how related we are despite roughly one billion years of divergence from our last common ancestor.

Calcineurin and NFAT in lymphocyte function

A. Rao (Boston, MA, USA) showed that NFATc2 can induce distinct transcriptional programmes that lead to T-cell activation or anergy (characterized by unresponsiveness to subsequent antigenic stimulation) depending on whether its transcriptional partner AP-1 (Fos/Jun) is available (activation) or not (anergy). Rao reported that the induction of anergy by calcineurin/NFATc2 involved the upregulation of the E3 ubiquitin ligases Itch, Cbl-b and Grail. Furthermore, she showed that *Itch*^{-/-} and *Cbl-b*^{-/-} mice develop autoimmune disease and that their T cells are resistant to ionomycin-induced anergy. Correlating with enhanced levels of ubiquitin ligases, phospholipase Cγ1, Lck and RasGAP were downregulated through protein degradation in anergic T cells, resulting in impaired calcium mobilization and the formation of ineffective immunological synapses (Heissmeyer *et al*, 2004). F. McKeon (Boston, MA, USA) discussed the function of T cells in mice that lack the calcipressin Csp1 (Ryeom *et al*, 2003). *Csp1*^{-/-} cells showed poor proliferation and Th1 differentiation owing to enhanced expression of the Fas ligand (FasL) and accelerated cell death. Reducing calcineurin activity with low concentrations of cyclosporin A or blocking FasL with antibodies could rescue proliferation and interferon-γ production in *Csp1*^{-/-} lymphocytes.

Programmed cell death might be differentially regulated by specific NFATc proteins. E. Serfling (Wuerzburg, Germany) showed that T cells that lack NFATc2, or both NFATc2 and NFATc3, are more resistant to CD3-induced cell death than wild-type lymphocytes, whereas overexpression of NFATc2 and the NFATc1 isoform C, but not A, enhanced apoptosis (Chuvpilo *et al*, 2002). Serfling also showed that NFATc1 is repressed in human Hodgkin lymphoma and diffuse large B-cell lymphomas owing to DNA hypermethylation and histone deacetylation in its P1 promoter region, which indicates that suppression of NFATc1 might help tumour progression. The role of individual NFATc proteins in tumour progression and apoptosis might depend on the cellular context, as indicated by the contrast between the repression of NFATc1 in lymphomas and the ability of constitutively active NFATc1 to transform fibroblasts (Neal & Clipstone, 2003). J. Liu (Baltimore, MD, USA) discussed the induction of the proapoptotic protein Nur77 in thymocytes by the coordinated activation of myocyte-enhancer factor 2D (MEF2D) through Ca²⁺-activated calmodulin-dependent kinase II (CaMKII) and the interaction of MEF2D with calcineurin-activated NFATc2. These results indicate that binding of NFATc to the Nur77 promoter might not be required for its activation and favour a model in which calcineurin-activated NFATc2, by associating with MEF2D, would enhance its transcriptional capacity on the Nur77 promoter. NFATc proteins might also regulate immunological memory. M. Rincón (Burlington, VT, USA) reported that memory CD4⁺ T cells contain higher levels of NFATc1 and c2 than naive CD4⁺ T cells, and NFATc-mediated transcription is more rapidly induced in memory cells in response to T-cell receptor ligation. In addition, interleukin-2 (*IL-2*) gene expression requires NFATc-mediated transcription in memory CD4⁺ T cells, but not in naive cells.

M. Fresno (Madrid, Spain) presented insights into the regulation of NFATc by mechanisms other than calcineurin and inhibitory kinases. He showed that the MAP3 kinase Cot and protein kinase C-zeta (PKC-ζ) regulate the transcriptional activity of the amino-terminal domain of NFATc2 when it is fused to a heterologous DNA-binding domain (GAL4). Fresno also showed that Cot potentiated PKC-ζ-mediated phosphorylation of the amino-terminal domain of NFATc2. Cot and PKC-ζ-mediated activation of NFATc2 are calcium independent and insensitive to calcineurin inhibitors. Interestingly, Cot kinase and PKC-ζ can activate the MEK1/Erk pathway, which, as shown by J. Molkentin (Cincinnati, OH, USA), might activate calcineurin under constant calcium conditions in cardiac cells. These and other studies presented at the meeting underscored the ability of individual NFATc proteins to execute distinct transcriptional programmes by integrating inputs from other pathways and establishing interactions with cell-specific and/or inducible transcription factors. In this context, J. Aramburu (Barcelona, Spain) presented a potential regulation of NFATc proteins by NFAT5, which is also known as tonicity responsive enhancer-binding protein (TonEBP). NFAT5 lacks the calcineurin-interaction motifs that are characteristic of NFATc proteins, but is induced in T cells by calcineurin activation. Collaborating with C. López-Rodríguez (Barcelona, Spain) and Rao, Aramburu showed that NFAT5 overexpression potentiated the dephosphorylation of NFATc2 by calcineurin, and that transfected NFAT5 could co-immunoprecipitate calcineurin and NFATc2 in 293 cells. Further studies are needed to address the role of NFAT5 in calcineurin–NFATc pathways.

NFATc signalling in angiogenesis

Although the calcineurin–NFATc signalling pathway was first defined in lymphocytes in the late 1980s and early 1990s, it is now clear that it has far more extensive roles. J.M. Redondo (Madrid, Spain) reported that vascular endothelial growth factor (VEGF) controls the activity of NFATc proteins in endothelial cells, and that the inhibition of calcineurin in cultured primary endothelial cells prevents VEGF-induced migration and angiogenesis (Fig 2). NFATc regulates angiogenesis in part by activating the COX2 gene (Hernandez *et al*, 2001), which is essential for the production of prostaglandins and thromboxanes (Fig 2). Inhibition of NFATc-dependent angiogenesis might be useful for the treatment of retinal deterioration in diabetes and the vascular invasion of tumours. By contrast, studies by I. Graef (Stanford, CA, USA) on mice with mutations in *NFATc3*, *NFATc4* and calcineurin B1 (Graef *et al*, 2001) showed that calcineurin–NFATc signalling inhibits VEGF expression by non-endothelial cells. In mice, calcineurin and NFATc3 and c4 are also required in the surrounding somatic cells to block VEGF production in the neural tube and somites to limit vascular invasion. Therefore, a pathway of angiogenesis that is dependent on NFATc signalling is emerging in which NFATc functions both downstream and upstream of VEGF in different tissues (Fig 2).

The development and function of the nervous system

Graef and colleagues showed that brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) induced the nuclear translocation of endogenous NFATc4 and NFATc-dependent transcription in embryonic cortical neurons. Neurons from mice bearing a combination of *NFATc2*, *NFATc3* and *NFATc4* mutations were

Fig 2 | Reciprocal NFATc2, c3 and c4 signalling in angiogenesis and endothelial migration. Local signals lead to the activation of NFATc3 and c4, and the inhibition of VEGF production (Graef *et al*, 2001) as well as, possibly, the synthesis of anti-angiogenic factors. Later in development, VEGF signalling requires NFATc2 in endothelial cells to activate COX2 and to lead to the migration of endothelial cells (Hernandez *et al*, 2001). Cn, calcineurin; COX2, cyclo-oxygenase 2; NFAT, nuclear factor of activated T cells; VEGF, vascular endothelial growth factor.

unable to respond to neurotrophins with axonal outgrowth, which led to a failure of most axonal connections in the mice although neurotrophin-induced survival was not affected. These defects seem to be related to a failure to transcribe genes that control the axonal cytoskeleton and rapid axonal extension in response to guidance signals. Graef also reported that mice lacking NFATc2, c3 and c4 have a complete defect of midline crossing of the commissural neurons owing to their inability to increase axonal outgrowth in response to netrins. Therefore, axonal extension in response to both netrins and neurotrophins requires NFATc signalling. The idea that rates of axonal extension are regulated is in contrast to the prevailing view that they are specified at the time of neural commitment. Accelerated axonal outgrowth in response to guidance signals might allow axons to meet developmental 'windows of opportunity' for the formation of certain neuronal morphologies.

P. Mermelstein (Minneapolis, MN, USA) reported that neurotrophins also activate endogenous NFATc4 in postnatal rat CA3-CA1 hippocampal neurons. Earlier work had indicated that neurotrophins are important for long-term adaptive responses in the brain by inducing activity-dependent plasticity. Mermelstein showed that NFATc-dependent transcription of the inositol 1,4,5-triphosphate receptor (IP3R1) required PKC activation in addition to the calcium stimulus, which implicates NFATc in a feedback loop in which induction of the *IP3R1* gene would lead to more efficient responses to Ca²⁺ and modulation of synaptic strength.

A surprise reported by G. Crabtree (Stanford, CA, USA) was the dedication of calcineurin to NFATc-dependent transcription at early points in development. The neural outgrowth and vascular developmental phenotype of mice that lack calcineurin B1 (and therefore have no calcineurin activity) was essentially identical to mice that lack NFATc2, c3 and c4. Furthermore, transcript-array studies revealed similar target genes in these mice, and cyclosporin A produced phenotypic and gene-expression changes similar to those seen in mice mutant for either calcineurin B1 or *NFATc2*, *NFATc3* and *NFATc4*.

Calcineurin and NFATc proteins are crucial for the function of cortical and hippocampal neurons in which synaptic activity results in Ca²⁺ influx through L-type channels and N-methyl-D-aspartate (NMDA) receptors (Graef *et al*, 1999). Signalling through calcineurin is likely to be crucial for memory (Winder *et al*, 1998; Malleret *et al*, 2001; Zeng *et al*, 2001) and might have a role in human schizophrenia (Miyakawa *et al*, 2003). Clearly additional studies are needed, and conditional knockout of *NFATc3* and *NFATc4* in the cortex and hippocampus will be essential to establish a role for NFATc in learning and memory. The crucial role of NFATc signalling in the development, morphogenesis and function of the nervous system should be a fruitful area of research in coming years and might unveil new insights into the basis of schizophrenia and other disorders.

NFATc signalling in cardiovascular function

Myocardial hypertrophy is important in several forms of cardiovascular disease, and J.D. Molkentin and E. Olson (Dallas, TX, USA) showed that calcineurin and NFATc4 are essential to induce hypertrophy in response to stress (Molkentin *et al*, 1998). These observations led Olson to screen chemical libraries to identify molecules that could modify the hypertrophic response. Using an NFATc-based reporter assay, Olson and collaborators at Myogen, Inc. (Westminster, CO, USA) identified molecules that modulate steps upstream of myocyte-enriched calcineurin-interacting protein (MCIP1) to control the actions of calcineurin and NFATc complexes. Such molecules might be modulators of therapeutic processes, including myocardial stress responses, autoimmunity, neural regeneration, osteoporosis, tumour angiogenesis and pathological retinal angiogenesis.

Olson also reported that nuclear exclusion of the class II histone deacetylases (HDACs) was crucial for the function of MEF2 in myocardial growth and development. Nuclear exclusion is the result of class II HDAC phosphorylation. Olson and colleagues have mapped the two crucial phosphorylation sites in class II HDACs and reported a preliminary characterization of the kinase that controls these sites. The kinase recognizes CaMK sites, and seems to be responsive to aortic banding and calcineurin activation. Preliminary results on knockouts of the class II HDACs were also reported. *HDAC5* and *HDAC9* seem to be redundant, in that mutations in either gene alone do not give rise to a change in phenotype, but when combined with a calcineurin transgene both mutations cause enhanced myocardial hypertrophy. It is likely that these HDACs collaborate with either MEF2 or NFATc family members in stress-induced hypertrophy.

NFATc seems to be selectively involved in pathological, but not physiological, hypertrophy. B. Wilkins (Cincinnati, OH, USA) used transgenic mice carrying an NFATc-dependent luciferase reporter to show that induction of pathological hypertrophy (pressure

overload) was calcineurin dependent and preceded by NFATc activation. However, stimulation of hypertrophy through exercise, or with insulin-like growth factor 1 (IGF1), neither activated NFATc nor induced genes characteristic of pathological hypertrophy. Consistent with this, calcineurin $A\beta^{-/-}$ mice developed less pathological hypertrophy but responded normally to IGF1 (Wilkins *et al*, 2004).

A controversial topic was that of calcineurin regulation by means other than calcium. Molkentin presented data showing that Erk kinase could phosphorylate and activate calcineurin, as measured by NFATc translocation and activity. Several groups have reported that different NFATc family members can be inhibited by kinases, such as GSK3, PKA, p38 and CK1. These observations open up the possibility of the phosphorylation of calcineurin as a regulatory mechanism in calcineurin–NFATc signalling.

Calcineurin and NFATc signalling in skeletal muscle

N. Rosenthal (Rome, Italy) discussed the role of calcineurin–NFATc signalling in muscle regeneration. Transgenic mice that express *IGF1* have enhanced muscle mass and fail to accumulate fat. The *IGF1* transgene induces a switch in calcineurin expression from the cytosolic and less active $A\beta 2$ isoform to the nuclear and more active $A\beta 1$. This transgene also prevents muscle degeneration in the amyotrophic lateral sclerosis model in which a mutated *SOD* is expressed in neurons in transgenic mice. This group also found that the calcineurin $A\alpha$ transgenic muscle regenerates faster after cardiotoxin injection. Although it is not known which NFATc family member, or perhaps other calcineurin substrate, is involved, studies by G. Pavlath (Atlanta, GA, USA) and by S. Schiaffino (Padova, Italy) indicate that these effects might be mediated by NFATc1 or c2.

Pavlath and colleagues also reported a remarkable role of NFATc2 in controlling myoblast fusion. Skeletal muscle fibres are syncytia formed from the fusion of mononucleated myoblasts. Myoblast fusion increases the number of nuclei in the myofibre and allows the enlargement of muscle cells. Pavlath reported that prostaglandin F₂ (PGF₂) and two analogues require NFATc2 to augment muscle cell size *in vitro*. This observation indicates that, in addition to the many receptors and ion channels that regulate NFATc-dependent transcription, prostaglandins are also important activators. These findings led them to look for genes that are dependent on PGF₂ and NFATc2. *IL-4* was one such gene, which, remarkably, was found to control the fusion of myoblasts. This surprising result indicates a new role for IL-4, which was previously thought to be confined to immune functions.

NFATc1/c4: frequency-response sensors in neurons/muscle

Studies in hippocampal neurons have indicated that NFATc4 nuclear localization is sensitive to the frequency of depolarization, with increased physiological stimuli promoting localization to the nucleus. Schiaffino reported that NFATc transcriptional activity, as determined by NFATc-dependent reporters, is decreased after denervation of slow muscle and is increased by stimulation of denervated muscle with low frequency (20 Hz), but not high frequency (100 Hz), stimuli. Nuclear translocation of NFATc1 is also induced in fast muscle by low frequency, but not high frequency, stimulation. These observations raise the question of how NFATc1 in muscle cells and NFATc4 in neurons sense the frequency of electrical depolarization.

Calcineurin function in flies and worms

Although *Drosophila* and *C. elegans* do not have an NFATc family member, they do have calcineurin. R. Schulz (Houston, TX, USA) reported that calcineurin is essential for *Drosophila* development because flies that are mutated in the calcineurin B2 gene die at the late larval or early pupal stage. Weaker alleles show abnormalities in the development of indirect flight-muscle cells, in which calcineurin B2 is expressed. These abnormalities resemble certain troponin I or myosin heavy-chain mutant flies, which indicates that calcineurin might regulate these molecules. Alternatively, calcineurin might regulate a transcription factor that has SP regions like NFATc proteins in mammals or similar to Crz1 in yeast. However, SP repeats are abundant in transcription factors and simple searches might not uncover a potential calcineurin-regulated factor. Additional genetic screens are likely to determine whether a transcription factor lies downstream of calcineurin and whether it is similar to the NFATc family. In *C. elegans*, calcineurin seems to function by controlling sensitivity to odorants, so that worms lacking calcineurin are hypersensitive (Kuhara *et al*, 2002). In addition, defects in movement are seen in worms that are defective in calcineurin function, which indicates that it could have a role in either motor neuron or muscle development. Again, worms do not have NFATc proteins; therefore, further work will be necessary to define the genes that mediate these effects.

Future directions

The meeting highlighted several important areas for future studies. First, the realization that calcipressins can inhibit or activate calcineurin depending on their phosphorylation state gives rise to exciting questions about the role of calcipressins and the mechanisms involved. Second, the regulation of calcineurin and NFATc proteins by signals other than calcium raises the possibility that these proteins can sense a wider range of cues and act on additional pathways than previously thought. Third, it will be necessary to define the basis of specificity of the transcriptional activity of endogenous NFAT proteins and the identity of proteins that are contained in NFAT transcriptional complexes in different cells, developmental stages and physiopathological situations. As calcineurin and NFAT proteins are found to be involved in an increasing number of biological processes, improving our understanding of these issues will be crucial for the development of pharmacological modulators of these proteins that might open up new therapeutic horizons.

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