NFAT signaling in vertebrate development

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NFATc proteins transduce Ca^{2+} signals to the nucleus and then pair with other proteins on DNA to generate NFAT complexes that activate transcription in response to both electrical and tyrosine kinase signaling. The four NFATc genes arose at the origin of vertebrates, implying that they have evolved for the development of vertebrate-specific functions, such as a complex nervous system, a recombinational immune system, and a vascular system with a complex heart. These speculations are borne out by studies of mice with null mutations in the different family members.

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Current Opinion in Genetics & Development 2001, 11:505–512

0959-437X/01/$ – see front matter

Abbreviations

BNP	b-type natriuretic peptide
CHP	calcineurin homologous protein
CnA	calcineurin A
CnB	calcineurin B
CsA	Cyclosporin A
DSCR	Down syndrome critical region
GSK-3	glycogen-synthase kinase-3
IGF	insulin-like growth factor
IL2	interleukin 2
IP_3	inositol 1,4,5-trisphosphate
IP_3R1	IP_3 receptor, type-1
NFATc	nuclear-factor-of-activated-T-cells cytoplasmic
NMDA	N-methyl-D-aspartate
VSCC	voltage-sensitive calcium channel

Introduction

The genes that encode the cytoplasmic, calcium-sensitive subunits of NFAT transcription complexes arose at the time of the origin of vertebrates and are not found in the genomes of invertebrates [1]. It was probably for this reason that NFAT signaling was not initially recognized as an important developmental pathway. Recent genetic evidence in mice, however, has indicated that these proteins play critical roles in the development of the immune system, the heart and blood vessels as well as the muscular and nervous systems in vertebrates. The latter observations are of particular interest because control of the NFAT pathway is exerted at the subtle interface of electrical and receptor-controlled signaling resulting in greatly expanded regulatory capacity. We will briefly review the complex levels of control of this signaling pathway and then discuss recent data implicating NFAT signaling in development.

Multiple levels of positive and negative controls over NFAT signaling

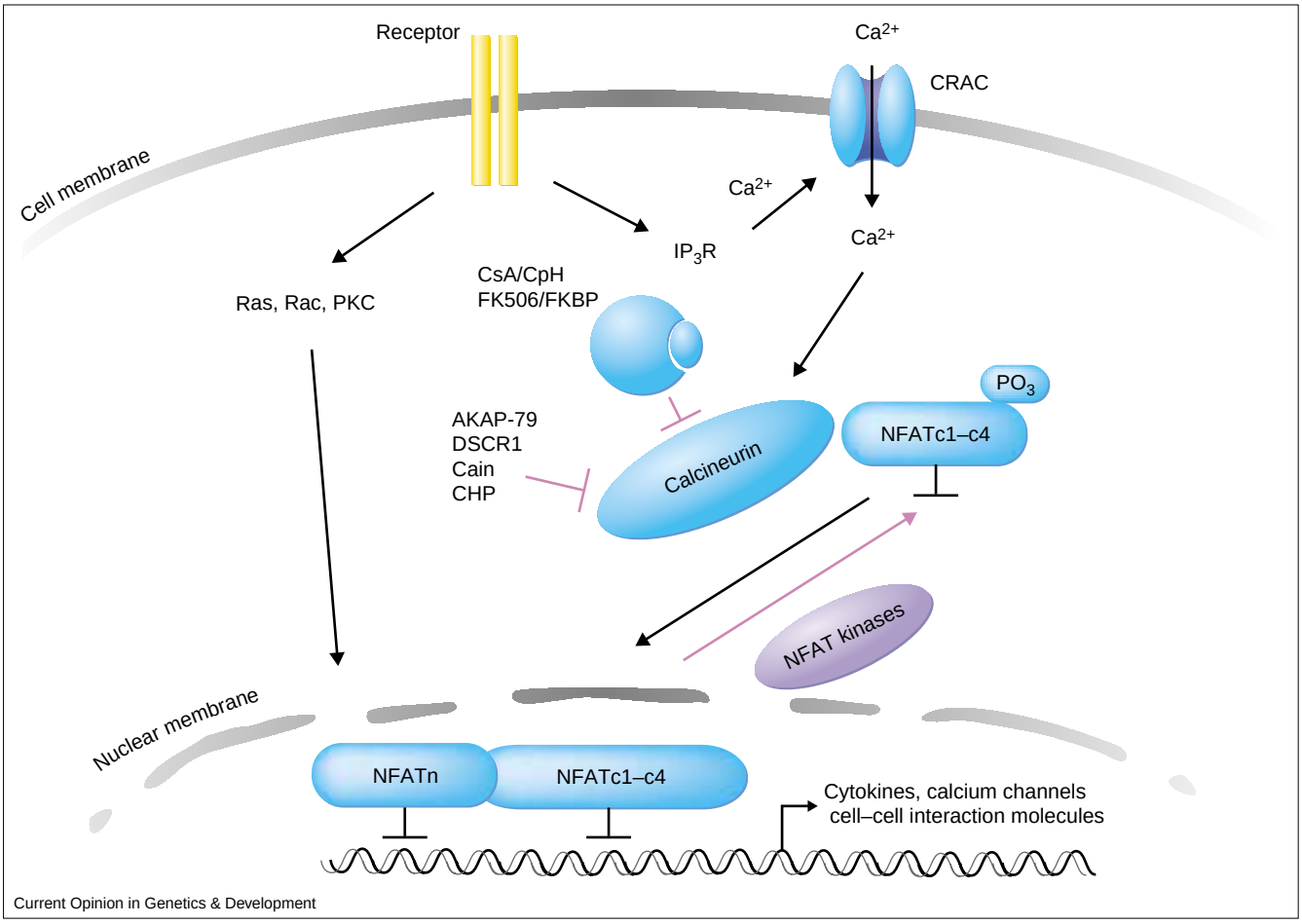
Before reviewing the role of NFAT signaling in development, it is worth pointing out the complex positive and negative feedback controls that modulate this pathway and hence make simple genetic analysis difficult. The NFAT signaling pathway was first described in lymphocytes as a pathway carrying signals from the polymorphic T-cell receptor to genes that coordinate an immune response. The pathway was defined by a strategy of working backward from the regulatory regions of an early activation gene (*IL-2*) to the cell membrane [2,3]. This approach resulted in the delineation of the pathway shown in Figure 1.

Sustained, low-amplitude Ca^{2+} signals lead to the activation of the heterodimeric phosphatase calcineurin [4]. Calcineurin binds directly to NFATc proteins (NFATc1–c4, Hugo Nomenclature Committee http://www.gene.ucl.ac.uk/nomenclature/) through a conserved motif and dephosphorylates serines within the SP repeats and serine rich motifs (SRR and SP-repeats) in the amino-terminus of NFATc family members. This dephosphorylation unmasks nuclear localization sequences in NFATc proteins and triggers their cytoplasmic→nuclear translocation [5]. The immunosupressive drugs CsA and FK506 block the nuclear localization of NFAT transcription complexes by inhibiting calcineurin phosphatase activity. These chemically distinct natural products bind at subnanomolar affinity to intracellular receptors thus forming inhibitory complexes. The resultant drug/protein composite surface binds tightly to calcineurin and prevents substrate access [6,7]. One very intriguing and surprising result published this year was the finding that most Ca^{2+} regulated gene expression in lymphocytes is dependent on calcineurin activity [8••].

NFAT signaling is opposed by at least three negative regulatory mechanisms. The first is exerted by a group of proteins that inhibit calcineurin phosphatase activity. These endogenous inhibitors directly bind to calcineurin and thereby block dephosphorylation of substrates such as NFATc proteins. One class of these inhibitory proteins includes DSCR1 (MCIP1) [9,10], DSCR1L*2* (MCIP*2*) and DSCR1L1 (ZAK-4) [11] which act as competitive inhibitors with nanomolar binding affinity. Cabin (Cain) is a noncompetitive inhibitor of calcineurin activity [12,13]. CHP proteins appear to compete with binding of the regulatory subunit CnB to the catalytic subunit, CnA and thereby inhibit the Ca^{2+}-dependent activation of the phosphatase [14]. The scaffolding protein AKAP is also a calcineurin inhibitor which binds both calcineurin and PKA and may anchor calcineurin at specific sites that allow the protein to engage the proper substrates when activated [15].

The second negative regulatory mechanism is the rapid regulated export of the NFATc family members from the nucleus by kinases such as GSK-3 [16–18]. Rephosphorylation of NFATc proteins leads to rapid export of NFATc family members from the nucleus and

Figure 1



Signal transduction by Ca^{2+}, calcineurin, and NFAT. A rise in intracellular Ca^{2+} following receptor stimulation leads to activation of the Ca^{2+}/calmodulin-regulated serine/threonine phosphatase calcineurin. Calcineurin directly dephosphorylates NFATc proteins, which rapidly translocate to the nucleus upon dephosphorylation. Calcineurin activity is controlled by endogenous inhibitory proteins such as DSCR1, AKAP-79, Cain or CHP and can be inhibited by the immunosupressive drugs CsA and FK506. The term NFAT-kinases is used for a group of kinases such as GSK-3 that oppose calcineurin by rephosphorylation of NFATc proteins. Ras, Rac or PKC signaling must be coincident with the Ca^{2+}/calcineurin signaling to assemble the NFAT transcriptional complex. NFATn is used to indicate tissue-specific transcription factors that act together with NFATc to activate transcription.

termination of transcription. Preliminary data indicate that GSK-3 in the nucleus might be controlled independently from GSK-3 in the cytoplasm (K Stankunas, GR Crabtree, unpublished data).

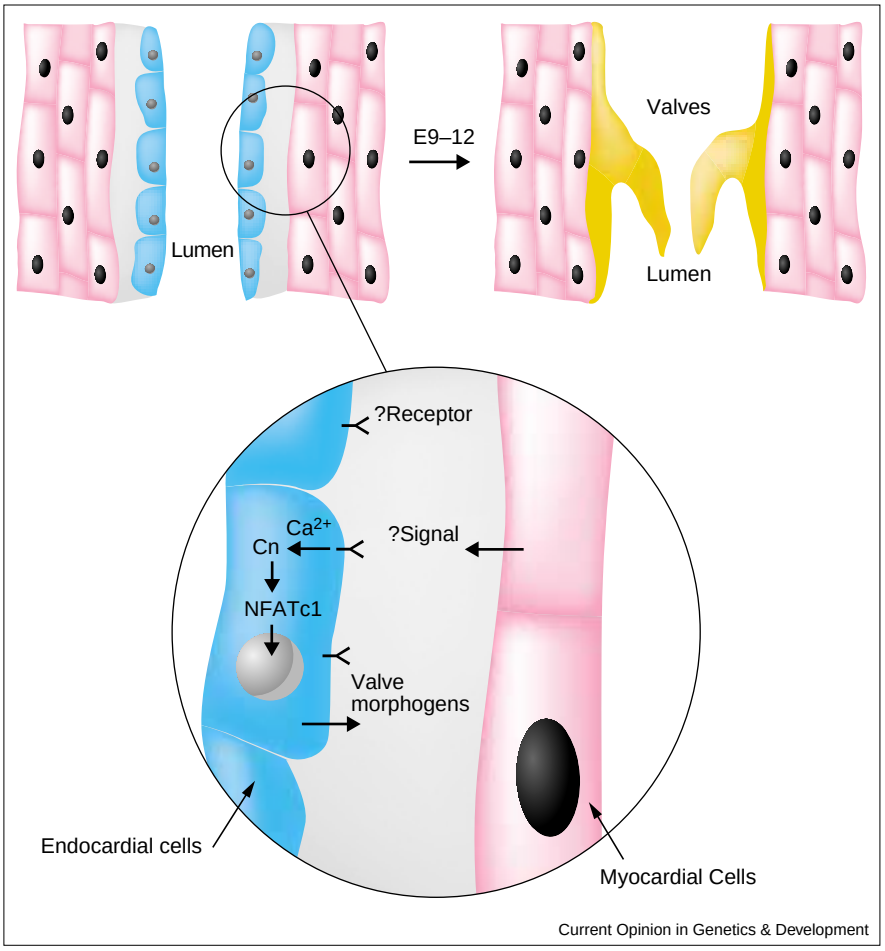
The third mechanism of negative inhibition involves the transcriptional activation of the *DSCR1* (*MCIP*) gene by NFAT signaling and the modulation of forward signaling by the accumulation of a calcineurin inhibitor [19•].

In addition, NFAT signaling is probably also subject to at least one level of positive feedback control. Many receptors respond to stimulation by generating the second messenger IP_3. IP_3 triggers the release of Ca^{2+} from the endoplasmic reticulum by binding to the IP_3 receptor, thereby causing the rapid influx of extracellular Ca^{2+} via calcium release-activated calcium (CRAC) channels. In addition, Ca^{2+} release from IP_3-sensitive stores can cooperate with Ca^{2+} influx via voltage-gated and receptor-operated calcium channels [20]. In neurons, activation of NFAT signaling leads to induction of the *IP_3R1* gene and thus provides a positive feedback mechanism that could alter the amplitude or spatial organization of Ca^{2+} signals. These levels of regulatory control are summarized in Figure 1 and provide an explanation for some of the unanticipated results of disruption of genes within the pathway. They also point to the need for new genetic approaches if we are to understand the mechanistic aspects of this pathway *in vivo*.

DNA binding by NFATc proteins is quite weak and therefore NFATc family members probably never act alone but rather need a partner protein, NFATn, to bind to DNA at physiologic concentrations [21–23]. Thus, cooperative

Figure 2

Morphogenesis of the heart valves appears to involve an NFATc-dependent cross talk between endocardium and local mycocardium. The blue cells in the upper left hand corner are the NFATc1-expressing endocardium that will be transformed into components of a heart valve. The signal appears to come from the myocardium (pink cells) and is likely to be transmitted through the gray cardiac jelly to interact with receptors that are on the endocardial cells leading to calcineurin activation and the nuclear import of NFATc1. A few days later, valves (yellow) appear as a result of this developmental inductive event.

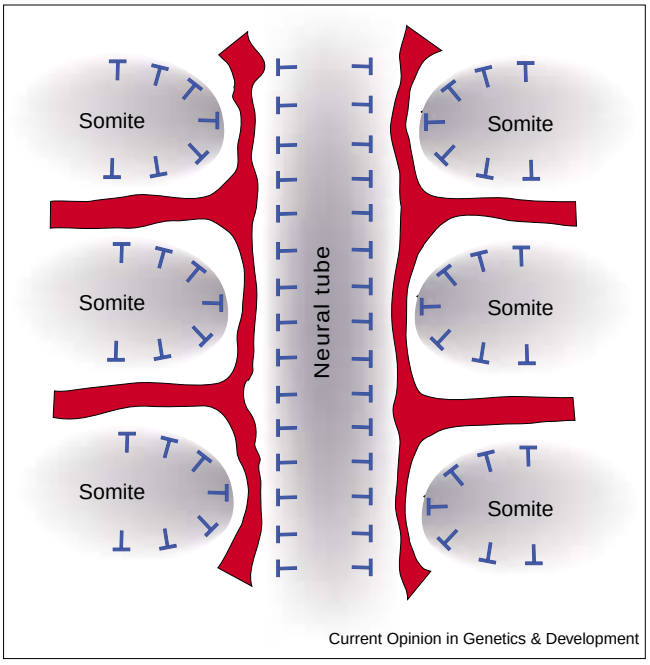


binding of NFATc proteins with diverse NFATn proteins, such as AP-1, GATA, cMAF and MEF2 family members makes Ca^{2+} signaling dependent on coincident activation of other signaling pathways and as such contributes to the activation of the NFAT pathway by a variety of upstream signals in many cell types and the control of transcription of very diverse target genes [3,24]. NFAT signaling thus acts as a signal integrator and coincidence detector rather than as a master-control pathway.

NFAT signaling in lymphoid development

Although the NFAT signaling pathway was first defined in lymphocytes, ironically it has been most difficult to study the role of individual components in these cells because of the high degree of genetic redundancy at each level of the pathway. Lymphoid cells express all four family members. Although all family members are able to interact with the same core DNA-binding element, it now appears that they have at least some degree of specificity in DNA binding, which may be a product of their associated nuclear partners. Deletion of NFATc2 results in minor defects in lymphoid development and a mild paradoxical hyperproliferation of lymphocytes, which is likely the result of the complex positive and negative feedback mechanisms mentioned above [25,26]. Insight into the lymphoid roles of NFATc1 has been difficult because of its early essential role in heart valve development (see below) but, using RAG-deficient blastocyst complementation, NFATc1 was found to play a role in the proliferative expansion of immature thymocytes as well as of peripheral, mature T- and B-lymphocytes [27,28]. Recently, *NFATc1/c2* double mutant T-lymphocytes were found to pass through early thymic development and have profound defects in cytokine production and differentiation, whereas double mutant B-lymphocytes were hyperactive [29•]. These cells were derived from RAG-deficient mice in which the immune system was reconstituted with fetal liver cells from *NFATc1/c2* double-mutant embryos. In contrast, *NFATc2/c3* mice are viable but develop a severe lymphoproliferative disorder with dramatic increase of cytokines and IgE levels [30]; this results in the development of inflammatory symptoms resembling a severe allergic response. At this point, it is not clear whether these hyperallergic responses reflect early developmental roles for NFATc2 and NFATc3 or a role in mature T-cell responses.

Figure 3



NFAT signaling at E8.5 patterns the mammalian vascular system (red). Local calcineurin/NFAT signaling in the somites and neural tubes leads to the production of an inhibitory signal (blocking symbol) that prevents aberrant branching of vessels into the areas where NFATc4 is highly expressed.

NFAT signaling is essential for the morphogenesis of vertebrate heart valves

NFATc1-deficient mice have defects in the formationof heart valves and the interventricular septum [31,32] that resemble congenital heart defects seen in humans. Congenital heart valve and septal defects occur in ~1% of the human population, making it one of the most common serious congenital defects. Despite their vital importance, very little is known about the molecular mechanisms governing valve development.

The heart valve forms by a unique developmental process in which endothelial cells delaminate from the endocardial layer and are transformed into mesenchymal cells that accumulate in the endocardial cushion area. The cushion then undergoes a complex series of morphogenic steps that lead to the formation of the valve. The mitral and tricuspid valves also have to connect with papillary muscles that provide precise control of apposition of the valve leaflets to prevent regurgitation. *NFATc1*-expressing cells are initially distributed over the entire endocardium but by E11.5 *NFATc1* expression becomes restricted to regions of the future heart valves (Figure 2). It is unknown at present whether *NFATc1*-expressing endocardial cells migrate to the valve primordia or whether specific signals regulate the restricted expression of *NFATc1*. The nuclear localization of NFATc1 is reversed rapidly by CsA treatment, indicating that calcineurin is essential for NFATc1 function in the nucleus. Mice with mutations in *calcineurin B* also show a failure of nuclear localization of NFATc1, providing genetic evidence that Ca^{2+}/calcineurin signaling is indeed essential for NFAT localization [33•], as first discovered in lymphocytes by biochemical reconstitution. But what regulates Ca^{2+} and calcineurin at the sites where cardiac valves will eventually form? The observation that NFATc1 is directed to the nucleus of heart valve precursor cells by a signaling process makes it possible to look for upstream regulators of NFATc1 localization in this essential process.

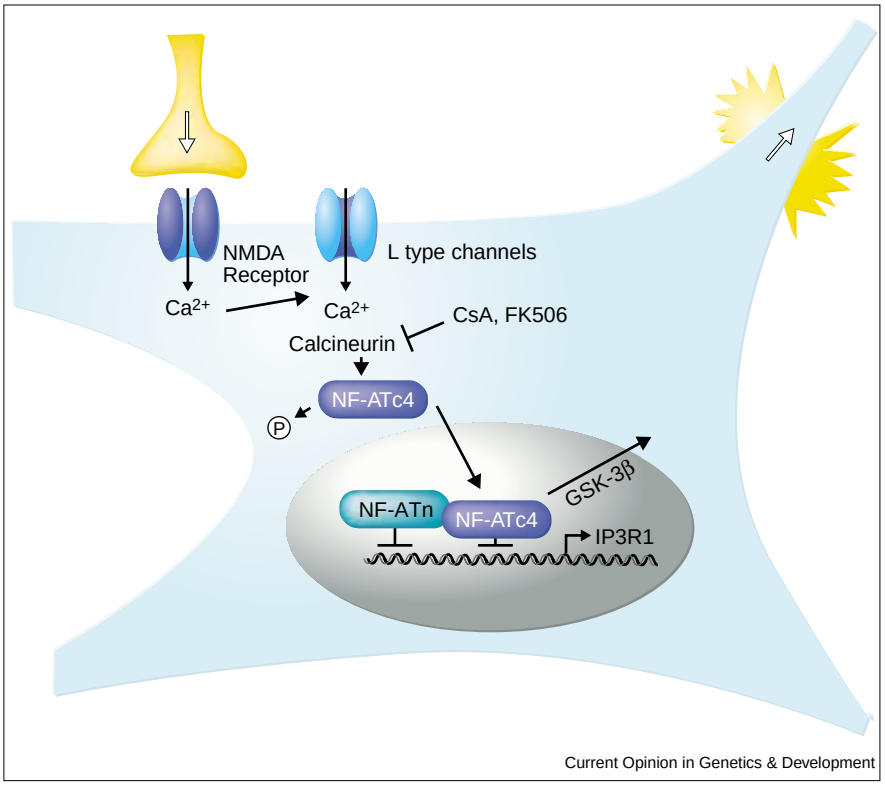
One of the most exciting developments in signaling in recent years has been the discovery of the unusual role of the connexins [34] — transmembrane proteins that form gap junctions between cells, allowing the passage of ions and other second messengers between cells. These gap junctions synchronize connected cells both electrically and biochemically, thereby coordinating intercellular communication. Mutation of *connexin-45* results in defects in endocardial cushion formation that resemble those of NFATc1 null mice [35••]. In connexin-45-deficient mice, NFATc1 is cytoplasmic, indicating that a potential role of connexin-45 is to allow Ca^{2+} signals to be transmitted within a sheet of synchronized valvular precursor cells. This is also underscored by the earlier finding that NFATc1 is preferentially nuclear in endocardial cells that are adjacent to each other. The developmental implications of this are profound, in that it allows a morphogenic Ca^{2+} signal to spread very rapidly through a population of cells. However, these exciting findings raise as many questions as they answer. For example, which receptor initiates the Ca^{2+} signal? Previous work has shown that Ca^{2+} signals are constrained to ~$1/10^{th}$ a cell diameter by Ca^{2+} buffering molecules. How is it that the Ca^{2+} signal is transmitted between cells? What is the time course of the movement of the hypothetical intercellular Ca^{2+} wave and how does this timing regulate valvular morphogenesis?

The role of NFATc3/c4 signaling in the organization of the vascular system

Early in embryonic life, NFATc3 and c4 function to organize the peripheral vascular system. During the first stage of vascular development, VEGF signaling directs the differentiation of endothelial cells from mesodermally derived precursors and the assembly of a relatively uniform network of endothelial cells. This is followed by remodeling of these primary vessels into the anatomically highly stereotyped hierarchical network of mature vessels composed of endothelial cells and perivascular supporting cells — i.e. angiogenesis. Mice with null mutations in both NFATc3 and c4, but neither alone, show both disorganization of growing vessels and a failure to fully assemble mature vessels resulting in death at ~E11.5 [33•].

A similar defect is seen in mice bearing a mutation in the regulatory subunit of calcineurin, CnB [33•]. This mutation prevents the interaction of CnB with CnA and hence specifically interferes with the Ca^{2+}-dependent activation

Figure 4



Interplay of dephosphorylation and phosphorylation in the regulation of NF-ATc4 in hippocampal neurons. Synaptic transmission driven by NMDA-receptors leads to Ca^{2+} influx via L-type voltage-gated Ca^{2+} channels and activation of calcineurin. Calcineurin dephosphorylates NFATc4, thereby triggering its cytoplasmic→nuclear translocation. GSK-3β acts in the nucleus of hippocampal neurons to re-phosphorylate NF-ATc4, leading to nuclear export of NFATc4 and termination of NFAT-dependent transcription. NFATn denotes an unidentified nuclear partner protein (distinct from AP-1) that co-operates with NFATc4 in the activation of IP_3R1 transcription.

of CnA catalytic activity, resulting in hyperphosphorylation and cytoplasmic localization of NFATc proteins. Calcineurin's activity towards all of its substrates is expected to be inhibited by the introduced mutation but the phenotype of the NFATc3/c4 null mice and the CnB mice is so similar that it appears that the major function of calcineurin in early development is to dephosphorylate NFATc3 and c4. Remarkably, administration of the calcineurin inhibitors, cyclosporin A or FK506 to pregnant mice phenocopies the developmental defect of the NFATc3/c4 and CnB mutants. This observation permitted an experiment that underlines the power of small molecules in deciphering complex development phenotypes. CsA administration to pregnant females was used to produce brief pulses of CnA inhibition during development and it was demonstrated that calcineurin/NFAT signaling was only required between E7.5 and E8.5. At E 8.5, NFATc4 is expressed most prominently in the tissues surrounding vessels rather than the newly differentiated endothelial cells. When signals transmitted by calcineurin/NFATc3/c4 are missing, the newly formed vessels lose guidance cues and begin to invade the somites and the neural tube where NFATc3 and c4 are expressed at the highest levels (Figure 3). A vascular patterning defect reminiscent of the one seen in the NFATc3/c4 null and CnB mutant mice has been described in ephrinB2 as well as EphB2/EphB3 mutant embryos [36,37], but not in other knockout animals with angiogenic defects. Interestingly, VEGF is overproduced in *NFATc3/c4* double mutants, CnB mutants and CsA-treated embryos. The most parsimonious explanation of these observations is that NFAT signaling in the somites and neural tube either represses the expression of molecules that promote vascular growth, such as VEGF, or that NFAT signaling induces anti-angiogenic molecules that prevent the formation of aberrant sprouts into to the neural tube and somites. Additional experiments will be necessary to distinguish these possible mechanisms.

NFAT signaling may function concurrently in the endothelium as well as surrounding tissues. Recent work has shown that NFATc1 is expressed in endothelial cells and is regulated by VEGF [38]. The same group also demonstrated that VEGF induces vascular growth in adult mice is blocked by systemic administration of CsA [39•]. Although VEGF-dependent endothelial differentiation is normal in the NFATc3/c4 and CnB mutant mice, it is possible that NFAT mediates only a subset of specific outcomes of VEGF signaling. Such observations raise the possibility that NFAT signaling is critical, in both the cells producing the signal and the cells responding to it. If this is correct, a number of critical questions arise. What are the receptors that regulate NFAT in cooperating cell types? Are the same NFATc family members used in both cell types? Does the use of the same pathway to carry out responses in both tissues have some particular advantage in coordinating the responses of the two communicating tissues? Do the NFAT-dependent genes focus on regulating the cooperating cell types or do they also control other as

yet unknown processes that must be brought into play to form and guide a vessel along stereotypic paths? The discovery that NFAT signaling is critical for angiogenesis will provide a starting point that will be useful in defining the molecules critical for understanding the interplay of signals leading to the development of an organized vascular system.

The role of NFAT in the development of cardiac and skeletal muscle hypertrophy

Hypertrophic growth of cardiac and skeletal muscle, in response to mechanical overload and a variety of other stimuli, has long been known to be dependent on Ca^{2+}. It has been discovered only recently, however, that some of these responses are mediated by calcineurin/NFAT signaling. As the role of this pathway in muscle development and growth has been the topic of several excellent recent reviews [40,41•,42•], we will only highlight a few major points. Transgenic overexpression of constitutively active calcineurin or truncated, nuclear NFATc4 leads to severe cardiac hypertrophy. Nuclear NFATc4 associates with the zinc-finger protein GATA-4 and they synergistically activate fetal cardiac genes such as *BNP* [43]. Furthermore, in several models of cardiac hypertrophy, the hypertrophic response is blocked by treatment with either FK506 or CsA [40,42•]. Similarly, it has been shown that calcineurin/NFATc1 mediate hypertrophic growth of skeletal muscle in response to IGF-1 [44,45]. Moreover, IGF-1 stimulation of skeletal myocytes induces the association of NFATc1 with GATA-2, indicating that cardiac and skeletal myocytes utilize similar effectors to regulate hypertrophic growth.

A possible role for NFAT signaling in brain development

The topographic pattern of some developing neural circuits is fine-tuned by use-dependent modifications in connectivity, and therefore requires neural activity. This process is thought to occur during the first few weeks of life and is best studied in the development of the visual system [46,47]. Needless to say, the underlying molecular events during this period are of utmost importance and the subject of intense investigation. One of these molecular events is the induction of the *IP_3R1* gene. Expression of *IP_3R1* increases during the time of synaptogenesis in the central nervous system and axonogenesis in the peripheral nervous system [48,49]. The regulation of IP_3R1 expression may therefore have interesting implications for developmental plasticity. IP_3R1-induced Ca^{2+} signals have been shown to cooperate with Ca^{2+} influx via voltage-gated and receptor-operated calcium channels to amplify and shape the intracellular Ca^{2+} signal [20]. In addition, the induction of long-term depression in the cerebellum and hippocampus has been shown to dependent on Ca^{2+} release from IP_3-sensitive stores [50,51]. This raises the possibility that this channel could be involved in a regulatory feedback loop that regulates synaptic strength and thereby influences the refinement of functional connections during development. The expression of IP_3R1 in cultured neonatal neurons is regulated by Ca^{2+} influx through L-type voltage-sensitive calcium channels (VSCCs) and NMDA (N-methyl-D-aspartate) receptors at the transcriptional level and this induction requires calcineurin activity [52•]. Calcineurin has been shown to have important roles in neuronal function [53] and has been implicated in plasticity in the mature nervous system [54,55]. In general, these functions of calcineurin have been thought to be independent of transcription but recent evidence indicates that NFATc family members may have critical roles in neuronal gene transcription in response to electrical activity [56]. The activation of NFAT-dependent transcription in neurons requires Ca^{2+} influx via L-type VSCCs and NMDA receptors. NFAT transcriptional complexes bind to the *IP_3R1* promoter at several sites and hence is likely to be one of the transcription factors that regulate activity-dependent expression of this receptor during development. In addition, mice with mutations in *NFATc4* or double mutant for the *NFATc2* and *c4* genes have reduced levels of *IP_3R1* expression in the brain (IA Graef, unpublished observation).

These observations raise a number of questions. Does the Ca^{2+}/calcineurin/NFATc4 pathway, in response to neural activity, indeed initiate a positive feedback loop by inducing the *IP_3R1* gene? Does the newly formed IP_3R1, in turn, enhance Ca^{2+} signaling initiated by L-type VSCCs and NMDA receptors in a synapse-specific manner? Although local regulation of Ca^{2+} beneath a synapse would be an attractive way to regulate its efficacy, such a mechanism would require that the newly induced IP_3R1 be targeted to the active synapse. Finally, to what degree do developmental plasticity and plasticity in the mature nervous system share molecular mechanisms and is the Ca^{2+}/calcineurin/NFATc4 pathway involved in both?

Conclusions and perspectives

NFATc proteins are major regulators of Ca^{2+}-dependent gene transcription in many cells types and progress in understanding their role in development has been greatly accelerated in recent years. Although much has been added to our knowledge about the role of NFAT-dependent gene transcription, many questions remain unanswered. It will be important to identify additional *cis*- and *trans*- regulatory elements that dictate transcriptional specificity and cross-regulation of this gene family. The identification of nuclear partner proteins involved in NFAT-dependent transcription will be critical to testing the hypothesis that that combinatorial association of the different NFAT subunits underlies the biologic specificity of this pathway. We also anticipate exciting progress as more upstream receptors that regulate gene transcription via NFAT proteins and NFAT-dependent target genes are identified. A major challenge will be to understand how different signals are integrated at the level of NFAT-dependent gene regulation and how they contribute to developmental processes *in vivo*. Analysis of the full spectrum of NFAT function, both developmental and adult, will require conditional inactivation of the components of this pathway.

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