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Review

Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development

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Abstract

Calcineurin signaling has been implicated in a broad spectrum of developmental processes in a variety of organ systems. Calcineurin is a calmodulin-dependent, calcium-activated protein phosphatase composed of catalytic and regulatory subunits. The serine/threonine-specific phosphatase functions within a signal transduction pathway that regulates gene expression and biological responses in many developmentally important cell types. Calcineurin signaling was first defined in T lymphocytes as a regulator of nuclear factor of activated T cells (NFAT) transcription factor nuclear translocation and activation. Recent studies have demonstrated the vital nature of calcium/calcineurin/NFAT signaling in cardiovascular and skeletal muscle development in vertebrates. Inhibition, mutation, or forced expression of calcineurin pathway genes result in defects or alterations in cardiomyocyte maturation, heart valve formation, vascular development, skeletal muscle differentiation and fiber-type switching, and cardiac and skeletal muscle hypertrophy. Conserved calcineurin genes are found in invertebrates such as *Drosophila* and *Caenorhabditis elegans*, and genetic studies have demonstrated specific myogenic functions for the phosphatase in their development. The ability to investigate calcineurin signaling pathways in vertebrates and model genetic organisms provides a great potential to more fully comprehend the functions of calcineurin and its interacting genes in heart, blood vessel, and muscle development.

Keywords: Calcineurin; NFAT; Signal transduction; Cardiomyocyte; Hypertrophy; Valvuloseptal development; Vascular development; Skeletal muscle; Fiber-type specialization; *Drosophila*; Interacting genes; Indirect flight muscle; *C. elegans*

Introduction

Calcineurin signaling transduced by nuclear factor of activated T cells (NFAT) activation was first characterized in the immune system (Flanagan et al., 1991; Matilla et al., 1990). Calcineurin (also called protein phosphatase 2B or PP2B) was originally identified as a calcium-regulated enzyme complex composed of calcineurin A (CnA) catalytic and calcineurin B (CnB) regulatory subunits (Fig. 1), and the calcium-binding protein calmodulin (reviewed in Rusnak and Mertz, 2000). Substrates of calcineurin phosphatase activity include most members of the NFAT family of transcription factors. Four NFATc genes, *nfatc1–c4*, have been identified with distinct temporally and spatially regulated expression patterns (reviewed in Pan et al., 1997; Rao et al., 1997). A related rel-like protein, NFAT5, has also been reported with DNA binding specificity and regulatory interactions distinct from NFATc1–c4 (Macian et al., 2001). Loss of specific NFAT isoforms results in cardiovascular, skeletal muscle, cartilage, neuronal, or immune system defects (Bushdid et al., 2003; de la Pompa et al., 1998; Graef et al., 2001a, 2003; Kegley et al., 2001; Peng et al., 2001; Ranger et al., 1998, 2000). Calcineurin and NFATs have also been implicated in the differentiation of bone, cartilage, muscle, skin, and fat in tissue culture experiments (Abbott et al., 1998; Delling et al., 2000; Friday et al., 2000; Ho et al., 1998; Santini et al., 2001; Takayanagi et al., 2002; Tomita et al., 2002). Postnatally, this signaling pathway contributes to normal homeostasis as well as pathological conditions in the skin, cardiovascular system, skeletal muscle, immune cells, and central nervous system (Al-Daraji et al., 2002; Baksh and Burakoff, 2000; Molkentin, 2000; Molkentin et al., 1998). These are many of the same organ systems that require calcineurin/NFAT signaling during embryonic development. Thus, there is accumulating evidence for the widespread utilization of calcineurin/NFAT

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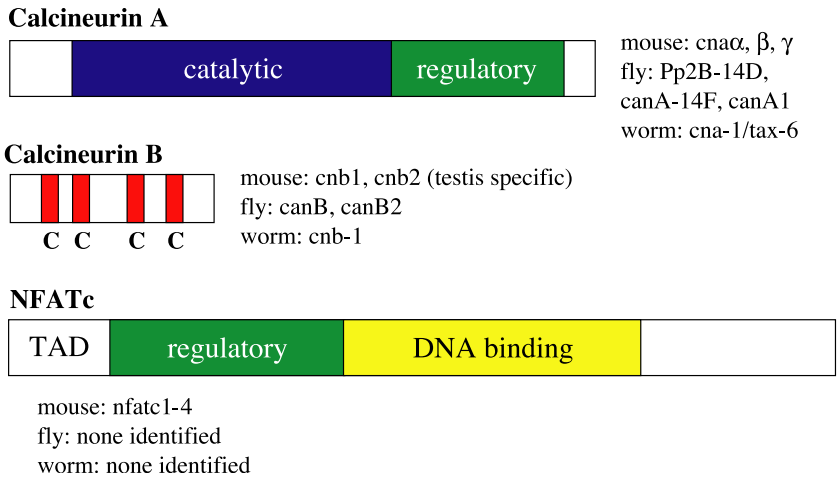

Fig. 1. Calcineurin subunits and NFATc isoforms in mouse, *Drosophila*, and *C. elegans*. Conserved protein domains of calcineurin A, calcineurin B, and NFATc are presented, with known mouse, fly, and worm genes listed to the right. The regulatory part of calcineurin A includes calcineurin B and calmodulin interacting regions and an autoinhibitory domain. The four conserved EF-hand calcium-binding domains (C) of calcineurin B are indicated. Within NFATc, the transactivation domain (TAD) is located at the N-terminus, adjacent to a regulatory domain containing sequences required for calcineurin binding, serine-rich motifs, and nuclear localization signals. The conserved DNA binding domain is of the rel homology class.

signaling mechanisms in a broad spectrum of developmental and disease processes.

Calcineurin dephosphorylates NFATs in response to increased intracellular calcium and regulates gene expression in a variety of calcium-sensitive tissues such as brain, muscle, and lymphocytes (reviewed in Baksh and Burakoff, 2000; Crabtree, 1999; Rusnak and Mertz, 2000). Transcriptional activity of NFATs is dependent on dephosphorylation by calcineurin, which leads to nuclear translocation (Beals et al., 1997a). NFATs can be deactivated and localized in the cytoplasm through phosphorylation by several protein kinases including glycogen synthase kinase-3 (GSK-3), protein kinase A, p38, JNK, and casein kinase (Beals et al., 1997b; Chow et al., 1997; Sheridan et al., 2002; Yang et al., 2002; Zhu et al., 1998). An additional level of regulation of calcineurin activity is through calcineurin interacting proteins including Cabin/Cain and Modulatory Calcineurin-Interacting Protein 1 (MCIP1)/Down syndrome Critical Region 1 (DSCR1) (Rothermel et al., 2003). NFAT transcription factors contain a conserved calcineurin binding domain and a rel homology DNA binding domain (Rao et al., 1997). An NFATc1–c4 consensus binding sequence of (A/T)GGAAA has been defined based on target sequences in cytokine gene regulatory regions (Rao et al., 1997). NFATs bind DNA with low affinity and usually act in conjunction with other transcription factors such as AP-1, MEF2, or GATA4 (Macian et al., 2001; Molkentin et al., 1998; Naya et al., 2000; Rao et al., 1997). During early development, NFATs are involved in establishing anteroposterior polarity in *Xenopus* in conjunction with noncanonical Wnt signaling (Saneyoshi et al., 2002). Thus, the control of NFAT transcriptional regulatory activity in association with other proteins likely represents a nodal point in intersecting signal transduction pathways including calcineurin, MAPK, p38, JNK, and Wnt (Crabtree, 1999; Crabtree and Olson, 2002; Gitler et al., 2003; Saneyoshi et al., 2002).

Highly conserved calcineurin subunit genes are also found in invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Fig. 1), and recent genetic studies have demonstrated pleiotropic functions for calcineurin in fly and worm development (Bandyopadhyay et al., 2002; Gajewski et al., 2003; Kuhara et al., 2002; Sullivan and Rubin, 2002). These include requirements in neural and muscular tissues that are among the organ systems affected in vertebrate embryos with altered calcineurin and NFAT signaling. Given the wide-ranging roles for the phosphatase in diverse animal species and their life processes, we focus this review on the functions of calcineurin signaling and NFAT activation in heart, blood vessel, and muscle formation. As abnormal development or physiological function of these tissues is of clear health relevance, we also address certain disease-related aspects of altering calcineurin/NFAT signal transduction.

Calcineurin/NFAT regulation of cardiomyocyte maturation and hypertrophy

Calcineurin signaling and NFAT activation are required for heart development

During embryonic and fetal development, cardiomyocytes become progressively more specialized at molecular, metabolic, morphological, and physiological levels (Rosenthal and Xavier-Neto, 2000; Yutzey and Kirby, 2002). The maturation of cardiomyocytes is fundamental to cardiac function and embryonic viability and includes the atrial or ventricular restricted expression of contractile protein, metabolic, and calcium handling genes (Conway et al., 2003; Gregorio and

Antin, 2000; Lyons, 1994). Among the genes regulated in fetal cardiomyocytes in mice are α- and *β-myosin heavy chain* (*MyHC*), *atrial natiuretic factor* (*ANF*), *brain natiuretic protein* (*BNP*), *myosin light chain 2a (MLC2a*), *phospholamban*, and *SERCA-2* (Franco et al., 1998; Lyons, 1994). Oxidative metabolic genes are likewise up-regulated during the embryonic to fetal transition at midgestation with a shift from glycolytic towards oxidative metabolism and increased mitochondrial function that accompanies increased cardiac demand (Fantel and Person, 2002; Ruiz-Lozano et al., 1998; Shepard et al., 1998). Many of these same contractile protein and metabolic genes are regulated in the adult cardiac hypertrophic response (Fig. 2). The involvement of calcineurin signaling and NFAT activation in the adult cardiac hypertrophic response (see below) led to the investigation of the role of this signal transduction pathway in developmental regulation of cardiac gene expression and function.

Calcineurin signaling and NFAT activation do not appear to be required for the initial events of heart formation. Mouse embryos with targeted mutations of calcineurin subunits or NFAT genes undergo normal heart tube formation and cardiomyocyte differentiation (Graef et al., 2001a; Kayyali et al., 1999; Zhang et al., 1996). Differentiated heart tubes were observed in embryos with mutations in the *cnb1* gene, which has been described as the only CnB isoform expressed in the embryo, making it essential for any calcineurin signal transduction (Graef et al., 2001a). Embryos lacking three of the four NFATc family members, NFATc2–c4, also have

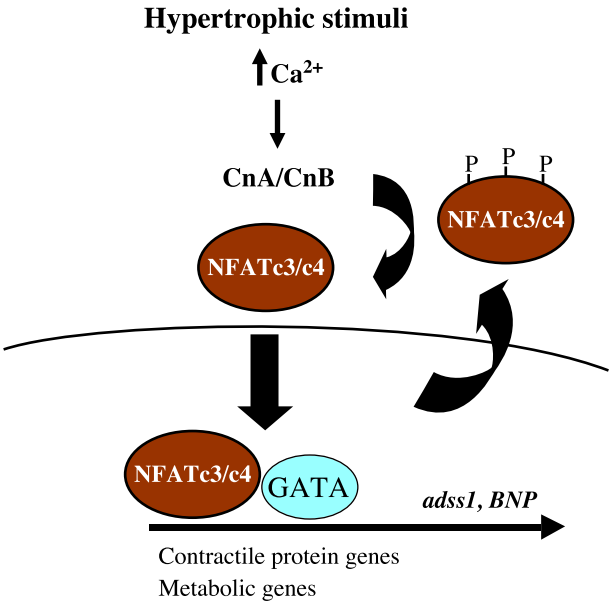

Fig. 2. Calcineurin signaling and NFAT activation in cardiac muscle. In the adult heart, hypertrophic stimuli lead to increased levels of intracellular calcium triggering a cascade of calcineurin activation, NFAT nuclear translocation, and increased gene expression. Target genes of NFATs, functioning with GATA factors, include *adss1* and *BNP*. A similar cascade of calcineurin/NFAT signaling may be acting in the developing heart where NFATc3 and NFATc4 together are required for metabolic activity and myocardial maturation at midgestation.

apparently normal heart tube formation (Graef et al., 2003). In addition, pharmacological inhibition of calcineurin signaling with cyclosporine A (CsA) treatment of early stage mouse or chicken embryos failed to inhibit initial heart tube formation or cardiomyocyte differentiation (Graef et al., 2001a; Liberatore and Yutzey, 2004). Primary diversification of atrial and ventricular cardiomyogenic lineages also was unaffected by calcineurin inhibition in avian embryos. Together, these studies support the hypothesis that calcineurin signaling is not required for the earliest events of heart formation in the vertebrate systems examined.

Targeted mutations of NFAT genes in mice have revealed multiple roles for these transcription factors later in cardiomyocyte maturation and heart chamber formation. At least two NFAT proteins, NFATc3 and NFATc4, are present in the developing myocardium and NFATc1 is expressed in developing heart valves (de la Pompa et al., 1998; Graef et al., 2001a; Ranger et al., 1998). The loss of both NFATc3 and NFATc4 results in embryonic lethality at approximately E10.5, although mice lacking either of the factors are viable (Bushdid et al., 2003; Graef et al., 2001a; Oukka et al., 1998; Wilkins et al., 2002). *nfatc3*$^{-/-}$*nfatc4*$^{-/-}$ embryos presented with cardiovascular abnormalities and heart failure at E10.5, but primary cardiomyocyte differentiation and chamber specification were not affected (Bushdid et al., 2003; Graef et al., 2001a). Nonetheless, the midgestational metabolic transition and mitochondrial function were compromised in the mutant embryonic hearts (Bushdid et al., 2003). Restoring NFAT activity specifically in the heart by transgene expression using the *α-MyHC* promoter prolonged embryonic viability, indicating defective cardiomyocyte maturation was the primary cause of death in the *nfatc3*$^{-/-}$*nfatc4*$^{-/-}$ embryos. Therefore, normal fetal cardiac mitochondrial maintenance and energy metabolism are dependent on NFAT activity, although the precise molecular mechanisms for this dependence are still unknown.

Additional genetic evidence exists for the importance of calcineurin signaling and NFAT activation in cardiomyocytes in mice. In the maturing atria, expression of a dominant-negative NFAT late in fetal development leads to thinning of the myocardium and loss of contractile protein expression (Schubert et al., 2003). Similar hypomorphic development of the ventricular myocardium was observed with the loss of calreticulin, a calcium-binding protein important for intracellular calcium homeostasis (Mesaeli et al., 1999). The embryonic lethality of calreticulin-deficient mice can be rescued with cardiac-specific expression of activated calcineurin, suggesting calcineurin signal transduction regulated by calreticulin is required for maturation of the developing myocardium (Guo et al., 2002). Proteins that interact with calcineurin, including calsarcins and MCIP1/DSCR1, are also developmentally regulated in the mouse embryonic myocardium with early chamber specificity (Casas et al., 2001; Frey et al., 2000; Lange et al., 2004). Together, these studies provide additional evidence for the importance of calcineurin signaling

and NFAT activation in myocardial maturation and function of the developing heart.

Calcineurin signaling and NFAT activation regulate the adult cardiac hypertrophic response

The importance of calcineurin signaling and NFAT activation in cardiac muscle was first discovered in the hypertrophic response of adult cardiomyocytes (Molkentin et al., 1998). Cardiac hypertrophy is defined as an adaptive change in heart size due to an increase in cardiomyocyte cell volume (reviewed in Molkentin, 2000). Hypertrophic growth occurs in response to several intrinsic and extrinsic stimuli, and while initially beneficial to maintain cardiac contractile performance, sustained hypertrophy can lead to cardiomyopathy and heart failure. Calcium-dependent signaling pathways have been documented in several models of cardiac hypertrophy and calcineurin has been implicated as a central player based on the following experimental evidence. First, the calcineurin inhibitors CsA and FK506 prevent hypertrophy of cardiomyocytes cultured in the presence of hypertrophic agonists such as angiotensin II and phenylephrine (Molkentin et al., 1998). Second, transgenic mice expressing a constitutively activated form of calcineurin A in the heart show a dramatic cardiac enlargement that progresses to heart failure and sudden death. This calcineurin-induced myopathy, and heart disease associated with three other mouse models of cardiac hypertrophy, are prevented by administration of CsA and FK506 (Molkentin et al., 1998; Sussman et al., 1998). Third, forcing the cardiac expression of an activated form of NFATc3 in mice culminates in a hypertrophic heart. Subsequent studies by several investigators have supported the initial observation that activation of the calcineurin/NFAT signal transduction pathway is both necessary and sufficient for this hypertrophic response (reviewed in Crabtree and Olson, 2002; Molkentin, 2000).

A model has been proposed wherein calcineurin transduces the calcium signal generated by sarcomeric dysfunction, mechanical load, or chemical agonists through dephosphorylation and activation of an NFAT transcription factor (Olson and Williams, 2000b; Fig. 2). The nuclear NFAT protein would then cooperate with cardiac expressed transcription factors to initiate the hypertrophic gene expression program. The changes in gene expression that occur with adult cardiac hypertrophy are often considered to be a reactivation of the fetal ventricular gene expression profile (Chien et al., 1993; Sadoshima and Izumo, 1997). These changes include increased *β-MyHC*, *ANF*, and *BNP* expression and decreased *SERCA-2* and *α-MyHC* expression. *BNP* and the cardiac metabolic gene *adenylosuccinate synthetase 1* (*adss1*) are direct transcriptional targets of NFAT, acting in conjunction with GATA4 (Fig. 2; Molkentin et al., 1998; Xia et al., 2000). Thus, the calcineurin/NFAT pathway directly regulates at least some aspects of cardiac muscle remodeling in the adult hypertrophic response in addition to being required for the maturation of cardiomyocytes during development.

NFAT and heart valvuloseptal development

The first evidence of primitive valve formation in the developing vertebrate embryo is the induction of endocardial cushions at the atrioventricular (AV) junction and the outflow tract (OFT). Endocardial cushion formation is initiated in response to signals emanating from the outer myocardial layer that induce the endocardium to undergo an epithelial to mesenchymal transition and invade the intervening cardiac jelly (Eisenberg and Markwald, 1995). The cushions are later remodeled into fibrous valves and membranous/muscular septa that divide the heart into four chambers. While endocardial cushion formation has been studied for many years and growth factors involved in this process identified, relatively little is known about the maturation of the cushions into functional valves (Camenisch et al., 2002; Eisenberg and Markwald, 1995). The remodeling of the mesenchymal endocardial cushion tissue into valves includes the reorganization of the extracellular matrix into highly organized valve leaflets (Icardo and Colvee, 1995; Icardo et al., 1993). Regulatory mechanisms that control the transformation of the cushions into valve leaflets and supporting structures are not well defined. However, the NFATc1 protein is emerging as an essential regulator of this process (Fig. 3).

The requirement for NFAT activity in valvuloseptal development was first demonstrated in *nfatc1* null embryos (de la Pompa et al., 1998; Ranger et al., 1998). Such embryos die due to valvuloseptal defects including hypomorphic semilunar and AV valves, as well as ventricular septal defects. During valve formation, *nfatc1* gene expression is restricted to the endothelial cell layer of the endocardial cushions and nuclear localization of NFATc1 in the AV canal endothelium is observed as early as E10.5. However, endocardial cushion formation and the initial septation of the OFT into aortic and pulmonary channels are apparently normal in *nfatc1* null embryos. Therefore, the developmental lesion(s) caused by *nfatc1* deficiency is likely in the later maturation of the endocardial cushions into valvuloseptal structures. However, the precise molecular nature of these congenital heart abnormalities that result from loss of NFATc1 is not known. In avian embryos, *cnaα* and *cnaβ* are preferentially expressed in the AV canal and OFT endocardial cushion formations, as well as in the maturing AV and semilunar valves (Liberatore and Yutzey, 2004). Together, these studies support the necessity of calcineurin signaling and NFATc1 activation in valvuloseptal development by a yet undefined mechanism.

In adult human pulmonary valve endothelial cells, nuclear localization of activated NFATc1 and cell proliferation are induced with increased vascular endothelial growth factor (VEGF) signaling (Johnson et al., 2003). VEGF activation of NFATc1 is inhibited by CsA treatment, suggesting that VEGF functions through calcineurin activation. The requirement for

calcineurin in the VEGF signaling pathway has also been observed in vascular endothelial cells (see below). There is preliminary evidence that adult valve disease recapitulates at least some aspects of embryonic valve development (Johnson et al., 2003; Paranya et al., 2001). Therefore, VEGF signaling mediated by NFATc1 may also regulate endothelial cell proliferation during embryonic valvuloseptal development. Support for this regulatory interaction is provided by studies in genetically altered mice demonstrating that gain or loss of VEGF activity leads to embryonic death as a result of cardiovascular malformations including valvuloseptal defects (Carmeliet et al., 1996; Dor et al., 2001; Ferrara et al., 1996; Miquerol et al., 2000). However, the relationship between VEGF signaling and NFAT activation during valvuloseptal development remains to be determined.

The signal transduction pathways that regulate NFATc1 activation in the developing heart valves also likely intersect with neurofibromin (NF1) and MAPK signaling (Gitler et al., 2003). *NF1* encodes a tumor suppressor gene associated with neurofibromatosis type 1, a genetic disorder characterized by tumors and cardiovascular defects (Bollag et al., 1996; Friedman et al., 2002). Endothelial-specific loss of NF1, a Ras inhibitor, results in increased MAPK phosphorylation and enlarged endocardial cushions (Gitler et al., 2003; Lakkis and Epstein, 1998). Premature nuclear localization of NFATc1 also was observed in endocardial cells of $NF1^{-/-}$ embryos and in endothelial cells expressing constitutively active Ras (Gitler et al., 2003). In contrast, decreased nuclear localization of NFATc1 in endocardial endothelial cells was observed in mouse embryos lacking connexin45 (Cx45), which die of heart failure at approximately E10 (Kumai et al., 2000). The mechanism by which loss of Cx45 leads to reduced NFATc1 nuclear localization has not been identified, but VEGF has been demonstrated to activate connexin gene expression in neonatal cardiomyocytes (Pimentel et al., 2002). Together, these studies suggest that NFATc1 nuclear localization and transcriptional activity in endocardial endothelial cells are regulated by intersecting signal transduction pathways (Fig. 3).

The transcriptional targets for NFATc1 in the AV canal and OFT have not been thoroughly described. Recently, *endothelin-1* has been identified as a direct downstream target of NFATc1 and GATA5 in cultured TC13 endothelial cells (Nemer and Nemer, 2002). An additional target of NFATc1 in AV and OFT endothelial cells appears to be the *dscr1/mcip1* gene (Lange et al., 2004; Rothermel et al., 2000; Yang et al., 2000). DSCR1 is expressed in the AV and OFT valve endothelial cells and is enriched in the forming septa of the mouse heart, which is consistent with the often severe valvuloseptal abnormalities associated with Down syndrome/trisomy 21 (Freeman et al., 1998; Lange et al., 2004). The alternative exon 4 promoter of *dscr1/mcip1* is responsive to calcineurin activity via a dense cluster of conserved NFAT consensus binding sequences (Yang et al., 2000). This *dscr1/mcip1* NFAT-rich regulatory region is specifically expressed in the AV valve endothelial cells in transgenic mice and the expression of the *dscr1/mcip1* NFAT-rich regulatory region is dependent on NFATc1 (Lange et al., 2004). The function of *dscr1/mcip1* in valve development is not known, but the NFATc1-dependent expression of the *dscr1/mcip1* calcineurin response element in valve primordial endothelial cells is further support for the importance of calcineurin/NFAT signal transduction during valvuloseptal development.

Calcineurin/NFAT regulation of vascular development

Blood vessel formation occurs throughout embryonic development to meet the circulatory needs of the different organ systems. This process has been described as occurring by vasculogenesis followed by angiogenesis. Vasculogenesis is the initial formation of a vascular plexus of differentiated endothelial cells and angiogenesis or angiogenic remodeling is the fusion and patterning of the endothelial cells in the plexus to form larger vessels (Sato and Loughna, 2002; Yancopoulos et al., 2000). Genetic studies in zebrafish and mice have identified several growth factors and signaling molecules involved in primary vasculogenesis and angiogenic remodeling during embryonic blood vessel development (Rossant and Howard, 2002; Weinstein, 1999). Among these are VEGF, which is involved in vasculogenesis and angiogenesis, as well as fibroblast growth factor (FGF), angiopoietin, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and ephrin family members (Poole et al., 2001; Rossant and Howard, 2002). While many of the ligands and receptors required for vascular development have been identified, less is known about how the intracellular signal transduction pathways intersect with transcriptional regulatory factors. Initial studies in mouse and chicken embryos, as well as in cultured endothelial cells, provide evidence for a role for calcineurin signaling and NFAT activation in the regulation of embryonic vascular development (Fig. 3).

The roles for calcineurin signaling in early embryonic vascular development were examined in mice with targeted mutations of calcineurin or NFATc genes. In either *cnb1* or *nfatc3/nfatc4* mutant embryos, endothelial differentiation and primary vascular plexus formation appeared normal. The *cnb1* mutant embryos do not develop beyond E9.5 and exhibit defects in angiogenesis, apparent in the lack of fusion and remodeling of the vascular plexus into larger vessels (Graef et al., 2001a). Neither *cnaα* nor *cnaβ* mutant mice has been reported to have vascular patterning defects and null animals survive into adulthood (Bueno et al., 2002; Zhang et al., 1996). Increased vascular plexus formation was also observed in *nfatc3/nfatc4* null embryos that survive to E10.5. However, restoration of NFAT activity specifically in the heart prolongs the viability of the *nfatc3/nfatc4* null embryos and restores apparently normal vascular development at E10.5 (Bushdid et al., 2003). The restoration of vascular development by cardiac-specific expression of

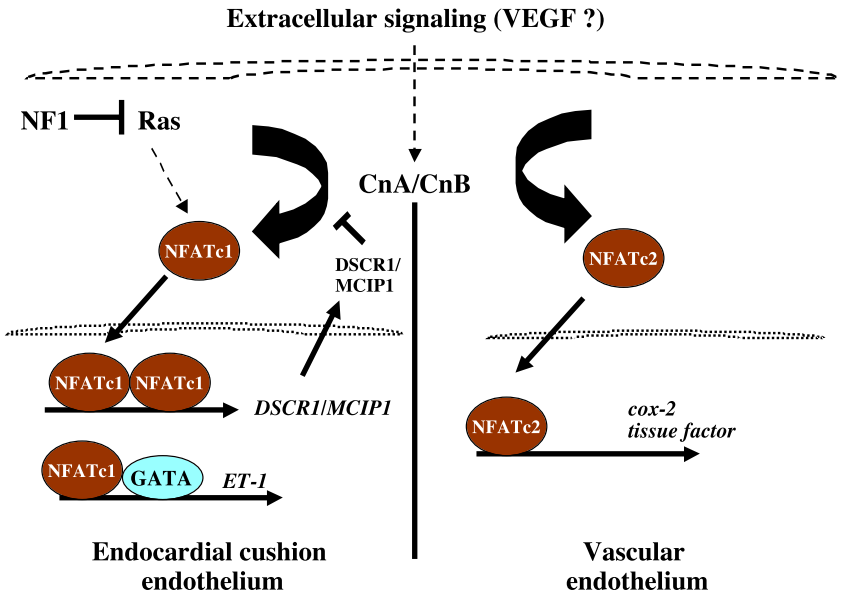

Fig. 3. Calcineurin signaling and NFAT activation in embryonic endocardial and vascular endothelial cells. Calcineurin/NFAT signaling pathways are shown for primitive valve endothelial cells on the left and vascular endothelial cells on the right. In both systems, extracellular signaling can induce calcineurin activation and NFATc nuclear translocation. VEGF, possibly induced by hypoxia, has been implicated as an activator of calcineurin signaling in both valve and vascular endothelial cells. In the developing heart valves, NFATc1 nuclear translocation is enhanced by NF1 inhibition of Ras activity. Transcriptional targets of NFATs in these tissues include the calcineurin interacting protein gene *dscr1/mcip1* and the *endothelin-1* gene. In vascular endothelial cells, activated NFATc2 is a transcriptional regulator of *cox-2* and *tissue factor* gene expression.

NFAT was evidence that the defects in angiogenic remodeling are secondary to cardiac defects. Still other NFATs expressed in the developing vasculature, such as NFATc2, could mediate calcineurin signal transduction in the regulation of angiogenic remodeling in the developing vasculature from E9 to E11 (Bushdid et al., 2003; Graef et al., 2001a). Further studies are necessary to dissect the precise calcineurin/NFAT signaling events required for angiogenic remodeling and blood vessel formation.

Studies of mouse and chicken embryos treated with CsA provide additional evidence for the importance of calcineurin signaling in early blood vessel development. Targeted mutagenesis of the *cnb1* gene affects the entire embryo, making it difficult to pinpoint the precise temporal and spatial requirements for calcineurin signaling in the cardiovascular system. Transient inhibition of calcineurin activity with embryonic administration of CsA was used to examine the temporal requirements for this signaling pathway. In mice, CsA treatment from E7.5 to E8.5 leads to defects in vascular remodeling similar to those observed in the *cnb1* mutant embryos (Graef et al., 2001a). These defects were not observed with earlier or later CsA treatments. In avian embryos, CsA administration during comparable stages also leads to vascular patterning defects. Together, these studies support the early requirements of calcineurin signaling to potentiate blood vessel development. The spatial requirements and primary vs. secondary effects of calcineurin inhibition on angiogenic remodeling were examined in avian embryos with local applications of CsA. CsA infiltrated agar plugs were placed directly on the developing vascular bed or near the developing heart. Direct application of CsA to the remodeling vessels did not prevent angiogenic remodeling, whereas administration of CsA near the developing heart inhibited blood vessel formation (Liberatore and Yutzey, 2004). These studies support secondary effects of compromised heart function as a result of altered calcineurin signaling on blood vessel formation observed in the *nfatc3/nfatc4* mutant mice. Together, these studies underscore the complexity of the temporal and spatial requirements for calcineurin/NFAT signaling in the developing heart and vasculature.

Comparison of embryos with alterations in either calcineurin/NFAT function or VEGF signaling suggests these pathways may intersect during vascular development. Studies in mice and chicken embryos indicate that VEGF signaling is necessary and sufficient for the remodeling of the primary capillary plexus into larger blood vessels during the initial stages of vascular development (Argraves et al., 2002; Carmeliet et al., 1996; Drake and Little, 1995; Ferrara et al., 1996; Flamme et al., 1995). This sensitivity period is consistent with the temporal and spatial requirements for calcineurin/NFAT signaling in early blood vessel remodeling (Graef et al., 2001a; Liberatore and Yutzey, 2004). Intersection of these signaling pathways has already been demonstrated in human umbilical vein endothelial cells (HUVEC) and adult pulmonary valve endothelial cells where VEGF induces calcineurin signaling and NFAT activation (Armesilla et al., 1999; Hernandez et al., 2001; Johnson et al., 2003). In HUVECs, VEGF-induced angiogenesis and *cyclooxygenase-2* (*cox-2*) gene activation is inhibited by CsA treatment (Hernandez et al., 2001). VEGF induces NFAT binding

activity and NFAT sites in the *cox-2* promoter are required for trans-activation by NFATs and induction by VEGF (Fig. 3). VEGF signaling also induces *tissue factor* gene expression in HUVECs, which is mediated by NFAT trans-activation of binding sites in the *tissue factor* promoter (Armesilla et al., 1999). These studies identified NFATc2 as the primary mediator of VEGF signal transduction in the HUVEC system (Armesilla et al., 1999; Hernandez et al., 2001). Further studies are necessary to determine if VEGF function in primary embryonic vasculature formation requires calcineurin/NFAT signaling and to identify the specific NFAT family members involved.

Calcineurin/NFAT control of skeletal muscle development and differentiation

Requirement of NFAT proteins for skeletal muscle development

The complex interplay of signaling systems and myogenic regulatory genes in the formation of vertebrate skeletal muscle has been the subject of comprehensive reviews (Bailey et al., 2001; Buckingham, 2001) and thus will not be addressed herein. The process of skeletal muscle formation occurs developmentally in two phases as sets of primary myofibers are initially generated, followed by a second wave of myofiber formation. Both myofiber groups undergo growth through myotube fusion with additional myoblasts and patterning within the developing muscle. NFAT proteins show different functions in various aspects of this developmental process.

Cultured human skeletal muscle cells express NFATc1, NFATc2, and NFATc3 in three distinct stages of differentiation: myoblasts, nascent myotubes, and mature myotubes (Abbott et al., 1998). The proteins reside predominantly in the cytoplasm under standard conditions, but when cultured in the presence of the calcium ionophore ionomycin, individual isoforms undergo an induced nuclear translocation at specific stages of muscle differentiation. That is, NFATc3 moves into myoblast nuclei, NFATc2 translocates into nascent myotube nuclei, and NFATc1 shuttles into nascent and mature myotube nuclei. Forcing calcineurin expression in C2C12 myoblasts by adenovirus-mediated gene transfer also induces nuclear translocation of NFATc3, but not NFATc1 and NFATc2 (Delling et al., 2000). Together, these preferential translocation events are suggestive of NFAT isoform specificity in the regulation of skeletal muscle gene expression and development.

In support of this notion, an analysis of *nfatc2* and *nfatc3* knockout mice demonstrated distinct skeletal muscle defects in the two mutants as compared to each other and wild-type controls. In *nfatc3*$^{-/-}$ animals, reduced muscle masses were observed due to a decrease in the number of myofibers of both the slow and fast types (Kegley et al., 2001). Defects in muscle formation were traced back to early stages of myogenesis ongoing within the embryo, specifically a decrease in the total number of primary myofibers. Since the size and organization of formed myofibers appeared normal, and such myofibers supported a conventional secondary myogenesis, it was concluded that NFATc3 served a specialized role in primary myogenesis. Such a distinct function for this isoform would occur if NFATc3 were active only in embryonic myoblasts that contribute to primary myofiber formation, but not in fetal myoblasts that are essential for secondary myofiber production (Kegley et al., 2001).

nfatc2 knockout mice present a different type of muscle abnormality, that being reduced muscle size due to a defect

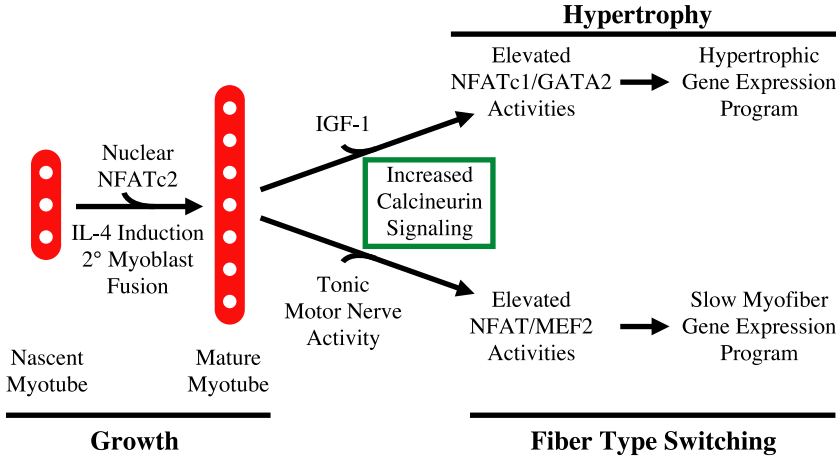

Fig. 4. Calcineurin and NFAT functions in skeletal myotube formation, myocyte hypertrophy, and myofiber-type switching. A key event in skeletal muscle growth is the nuclear translocation of NFATc2 in the nascent myotube and IL-4 growth factor secretion, which promotes a second phase of myoblast fusion to form the mature myotube. Further molecular or electrical inputs can result in dramatic alterations of skeletal muscle phenotype. Both the IGF-1 growth factor and tonic motor nerve activity increase calcineurin-mediated signaling. The former leads to enhanced NFATc1 and GATA2 activities resulting in skeletal muscle hypertrophy, while the latter culminates in elevated NFAT and MEF2 activities leading to myofiber-type switching. Adapted in part from Horsley et al., 2003.

in skeletal muscle growth (Horsley et al., 2001). Phenotypic analyses revealed a normal formation of nascent myofibers, but these myofibers contained a limited number of nuclei due to a lack of further growth-promoting myoblast fusion. This phenotype, and the selective calcium-induced nuclear translocation of NFATc2 in nascent myotubes of differentiating muscle cells in culture (Abbott et al., 1998), are consistent with a novel function for NFATc2 in the regulation of myofiber and myoblast fusion, leading to the growth of multinucleated muscle tubes. This could occur through NFATc2 regulation of a cell surface protein within myofibers, which mediates cell fusion with neighboring differentiating cells, or NFATc2 regulation of a myofiber secreted factor that attracts myoblasts to the growing syncytial muscle (Horsley et al., 2001). Recent studies strongly support the latter mechanism as the cytokine IL-4, expressed under the control of NFATc2, is secreted by nascent myotubes and attracts IL-4α receptor positive myoblasts, promoting further cell fusion and mature myotube formation (Horsley et al., 2003; Fig. 4). Taken together, the NFAT localization studies and loss-of-function analyses point to a differential contribution of NFAT isoforms to the control of skeletal muscle development. As the NFATc2 and NFATc3 phenotypes appear unique when compared to those observed in mice with disruptions of other myogenic factor genes, NFAT protein activation likely represents a crucial facet of this programmed developmental process.

Calcineurin activity is required for skeletal muscle differentiation

A role for calcineurin in the terminal differentiation of skeletal myocytes was initially suggested using cultured cells and an in vivo muscle regeneration assay. Treating human skeletal muscle cells with CsA prevented biochemical and morphological differentiation of such cells, while allowing normal proliferative properties (Abbott et al., 1998). In mice, induction of a limb muscle injury is normally followed by repair 12–14 days after damage. However, in CsA-treated animals, such injured muscles fail to heal and are largely devoid of regenerated myofibers. A direct involvement of calcineurin in the differentiation process was demonstrated in C2C12 and Sol8 myoblasts (Delling et al., 2000). Activity of the phosphatase is increased during myogenic differentiation of C2C12 cells and forced expression of activated calcineurin in both C2C12 and Sol8 myoblasts enhances myotube formation. In contrast, expressing the calcineurin inhibitory protein Cain in C2C12 cells culminated in decreased calcineurin activity and the attenuation of myogenic differentiation. As noted previously, activated calcineurin expression also results in a specific nuclear translocation of NFATc3 within the cells. Together, these findings implicate calcineurin signaling and NFATc3 activation in the regulation of skeletal muscle cell differentiation and myotube formation. Such in vitro results are consistent with the muscle phenotype of *nfatc3* knockout mice, where mutant animals have muscles of reduced size due to defects in primary myogenesis (Kegley et al., 2001).

Calcineurin/NFAT control of skeletal muscle hypertrophy and fiber-type specialization

Genetic control of skeletal muscle hypertrophy

While hypertrophic growth of cardiac muscle may ultimately be harmful and potentially life threatening, analogous growth of skeletal muscle in response to aging or disease may be of clear benefit. The growth of skeletal muscle due to prolonged use is a well-established physiological response, yet the molecular pathways that regulate such an adaptation have been poorly understood. Recent studies have implicated calcineurin as an important regulator of this cellular growth response. Specifically, a mouse model has been established wherein the plantaris muscle of the hind limb is subjected to functional overload in vivo, resulting in a doubling of muscle mass and individual fiber sizes, and increased muscle strength within a few weeks (Dunn et al., 1999). However, when identical overloading conditions were used with mice administered CsA or FK506, such animals failed to undergo muscle compensation and enlargement. This result suggested a critical role for calcineurin activity in the overload induced hypertrophy, but the study did not address the function of an NFAT protein as a downstream effector of calcineurin signaling.

Insulin-like growth factors (IGFs) are known to be potent inducers of skeletal muscle growth and hypertrophy (reviewed in Florini et al., 1991). A role for calcineurin in IGF-1-induced skeletal muscle hypertrophy has been demonstrated by two groups using myogenic cell cultures. Transfecting IGF-1 into postmitotic rat L6E9 skeletal myocytes results in an induction of *cna* gene transcripts and CnA nuclear localization in hypertrophic myocytes (Musaro et al., 1999). Similarly, introducing an activated form of calcineurin into these cells phenocopies the effects of IGF-1, while dominant-negative calcineurin expression or CsA treatment prevents myocyte differentiation and hypertrophy. Mechanistically, it was proposed that IGF-1 promotes hypertrophy through the induction of calcineurin-mediated signaling and the activation of the GATA2 transcription factor. GATA2 associates with calcineurin and a dephosphorylated form of NFATc1 in some nuclei, culminating in the activation of the myocyte hypertrophic gene expression program (Fig. 4). Independent studies using C2C12 skeletal muscle cells showed that transfection with IGF-1, or treatment with a combination of insulin and dexamethasone, resulted in hypertrophy of differentiated myotubes (Semsarian et al., 1999). Calcineurin activation was demonstrated in these cells, as well as inducement of NFATc1 nuclear translocation. Consistent with the activation of the phosphatase, treatment of C2C12 cells with CsA blocked the hypertrophic response to IGF-1 expression or duo hormone

treatment. To summarize, these two studies have generated important insights into the control of skeletal muscle hypertrophy by the calcineurin/NFATc1 signaling pathway.

It should be noted, however, that the equivalence of the calcineurin regulatory paradigm between skeletal and cardiac muscle hypertrophy is likely more complex than originally proposed. For example, expression of activated calcineurin in skeletal muscles of mice does not cause hypertrophy as seen in the heart (Naya et al., 2000). Additional studies showed that CsA had either no effect (Dupont-Versteegden et al., 2002) or differential effects (Mitchell et al., 2002) on the maintenance of muscle mass in animals exposed to atrophy-inducing stimuli. Other findings suggested calcineurin did not mediate IGF-1-induced skeletal muscle hypertrophy, rather the Akt/mTOR pathway served as the critical regulator of this myotube growth process (Bodine et al., 2001; Pallafacchina et al., 2002; Rommel et al., 2001). Thus, while it is clear that calcineurin/NFAT signaling plays some role in the hypertrophic response of skeletal muscle, the precise function of this pathway must be further investigated and deciphered.

Calcineurin and muscle fiber-type specialization

Skeletal muscle fibers can generally be classified as slow or fast based on inherent metabolic and contractile properties, dependent on the degree of motor nerve stimulation and the corresponding gene programs expressed within (reviewed in Hughes, 1998; Olson and Williams, 2000a,b). Slow twitch fibers are subject to tonic motor neuron activity, undergo sustained contractile events, and maintain high concentrations of intracellular calcium. These fibers are predominantly oxidative and express slow contractile protein isoforms and metabolic enzymes that are highly efficient in converting energy into contractile work. In contrast, fast twitch fibers are exposed to sporadic motor neuron input, exhibit bursts of contractile activity, and maintain relatively low concentrations of intracellular calcium, with transient high-level spikes occurring during neuromuscular activity. Such fast fibers are glycolytic in nature and express contractile protein isoforms and enzymes that are essential for infrequent, robust bursts of contractile work. Understanding the regulatory pathways that control specialization and inter-conversion of these myofiber types would be of potential benefit to both healthy individuals engaged in exercise training and patients challenged by various myopathies or physical inactivity.

Several lines of evidence have demonstrated the importance of calcineurin signaling in the control of skeletal muscle fiber-type specialization. First, forcing the expression of an activated form of calcineurin in C2C12 cell cultures resulted in promoter activation of several slow myofiber-specific genes, including *MyHC*, *troponin I*, and *myoglobin* (Chin et al., 1998; Delling et al., 2000). Calcineurin-dependent regulation of the latter two was implicated to be through the combinatorial function of NFAT and MEF2 class transcription factors (Chin et al., 1998). In contrast, the promoter for the fast myofiber *muscle creatine kinase* (*MCK*) gene failed to be activated under comparable transfection conditions. Second, studies in whole animals have confirmed the ability of calcineurin to activate the slow fiber gene expression program. Targeted mutagenesis of *cnaα* and *cnaβ* in mice leads to a reduction in oxidative/slow fibers in several muscles (Parsons et al., 2003). Conversely, transgenic mice expressing activated calcineurin under the control of the *MCK* enhancer exhibited increased numbers of slow-type myofibers (Naya et al., 2000). Likewise, injection of a recombinant adenovirus expressing activated calcineurin into the gastrocnemius muscle of neonatal rats led to an enhanced expression of the slow *MyHC* isoform in normally fast expressing areas, coincident with the accumulation of activated calcineurin protein (Delling et al., 2000). Third, studies involving CsA have yielded complementary data in support of calcineurin's role in muscle remodeling in vivo. Specifically, intraperitoneal administration of the calcineurin inhibitor promoted a pronounced slow to fast fiber transformation within soleus muscles of treated rats (Chin et al., 1998). Also, the fast to slow fiber conversions normally observed in overloaded mice failed to occur when such animals were administered CsA (Dunn et al., 1999).

Taken together, these findings are consistent with a model that explains how motor nerve activity controls specific muscle gene expression programs, culminating in slow versus fast fiber types (Chin et al., 1998). In slow fibers, tonic motor nerve activity results in sustained levels of intracellular calcium sufficient to induce calcineurin signaling and NFAT activation. Nuclear NFAT proteins would then work combinatorially with MEF2 (and other) transcription factors to regulate slow fiber-expressed genes (Fig. 4). In fast fibers, phasic motor nerve activity is insufficient to maintain calcium concentrations that are required for calcineurin activation and NFAT nuclear translocation. Subsequently, genes encoding fast fiber-type protein isoforms would be transcribed as they are independent of NFAT regulation. Based on these regulatory controls, selective induction or inhibition of calcineurin phosphatase activity would result in altered gene expression programs and corresponding muscle fiber-type conversions.

In addition to NFAT proteins, there is emerging evidence for MEF2 family members being direct targets of calcineurin activity in skeletal muscles. Using transgenic mice harboring a putative MEF2-dependent reporter gene (Naya et al., 1999), calcineurin was shown to be essential for the functional activation of MEF2 in the reprogramming of myofiber-specific gene expression during the physiological adaptation of exercised muscles (Wu et al., 2001). The phosphatase can physically interact with MEF2 so as to remove phosphate groups, with the hypo-phosphorylated factor showing enhanced transcriptional activation properties. The effect in actively contracting muscles is the up-regulation of previously repressed MEF2 target genes, leading to a transition from the resting IIb fiber type to

the contractile type I myofiber. A separate study showed that calcineurin can mediate the dephosphorylation of NFATc1, MEF2A, and MEF2D proteins to an extent that is dependent on the degree of muscle nerve activity and resulting intracellular calcium levels (Dunn et al., 2001). Such a finding is consistent with the aforementioned model wherein slow muscle fibers are conducive to calcineurin activation, since they receive a tonic motor nerve input that sustains elevated calcium concentrations (Chin et al., 1998).

Myogenic functions of calcineurin in model genetic systems

Drosophila calcineurin genes

Calcineurin subunit genes are conserved among animal species, with the *Drosophila* genome containing three *cna* and two *cnb* genes that are highly homologous to their vertebrate relatives (Fig. 1). Historically, *canA1* and *canB* were isolated from a *Drosophila* embryonic cDNA library by low stringency hybridization using human gene probes (Guerini et al., 1992). The fly CnA1 protein is 73% identical to human CnAα and CnAβ, with catalytic motifs almost completely conserved. *Drosophila* CnB is 88% identical to human CnB1 and CnB2, with strong amino acid conservation within the four EF-hand calcium-binding motifs. *canB2*, the second calcineurin regulatory gene of *Drosophila*, was discovered during the molecular analysis of the *cinnabar* region of chromosome 2 (Warren et al., 1996). Its encoded product is 98% identical to fly and 88% identical to human CnB proteins. As for the two other *cna* genes, *Pp2B-14D* was isolated from a *Drosophila* eye disc cDNA library (Brown et al., 1994) and *canA-14F* was discovered through the genome sequencing project (Adams et al., 2000). Proteins encoded by either of these genes are 73–78% identical to various human protein phosphatase 2B isoforms, with near perfect conservation within their catalytic domains. Therefore, all fly calcineurin proteins are predicted to function comparably to their vertebrate counterparts.

Given these strong sequence identities, it made sense to investigate the possible conservation of the calcineurin/NFAT pathway in *Drosophila* and determine what functions this signaling cassette might assume during fly development and life processes. NFAT proteins contain a DNA-binding domain of the rel class and comparative evolutionary studies, based on the recent sequencing of numerous animal genomes, has led to the categorization of Rel family proteins (Graef et al., 2001b). Surprisingly, while the fly genome encodes proteins like Dorsal, Dif, and Relish that are related to the vertebrate transforming protein Rel and NFκB family, calcium-responsive Rel proteins such as the NFAT group are present only in vertebrates. That is, while certain *Drosophila* genes may encode the conserved DNA-binding domain, they do not possess sequences encoding a calcium/calcineurin-responsive domain that defines the calcium-dependent NFAT proteins. Thus, given the strong conservation of calcineurin genes, and the absence of NFATc proteins from the fly, a challenging question arises as to which proteins might be effectors of calcineurin signaling in *Drosophila*. One potential target for this pathway is noted below.

Screens for calcineurin interacting loci in Drosophila

Focused genetic screens can serve as powerful vehicles to identify genes that function within defined developmental processes and complex regulatory hierarchies. Considering the involvement of calcineurin in several critical myogenic events in vertebrates, and the potential to use genetic means to identify new calcineurin interacting loci, Gajewski et al. (2003) expressed an activated form of mouse CnA in *Drosophila* muscles. For reasons yet unknown, such expression resulted in arrested animal development around pupal day 1. Based on this occurrence, an F2 deficiency screen was undertaken to search for chromosome intervals that harbored genetic suppressors of the induced phenotype. Animals hemizygous for seven distinct regions of *Drosophila* chromosomes 2 and 3 were able to suppress the calcineurin-induced phenotype to adult viability. The strongest suppressing deficiency corresponded to a deletion of the 42B03–43E18 region of chromosome 2, with the *canB2* gene mapping at 43E16. A P-element induced mutation of *canB2* by itself was able to prevent the lethality caused by activated calcineurin expression. This suppression of phenotype demonstrated a normal dosage and function of the calcineurin regulatory gene was needed for the adverse effects of calcineurin on animal development and viability.

Another chromosome region identified in the screen contained the *D-mef2* gene at 46C and preliminary tests using gene mutations suggested this muscle differentiation factor contributed to the suppression activity of interval 3 (K. Gajewski and R.A. Schulz, unpublished data). Extensive information has been generated on *D-mef2*, which is the sole *mef2* family member in the fly. D-MEF2 is expressed in all differentiated muscles and their precursors during embryogenesis, and adepithelial cells that serve as precursors for adult thoracic muscles (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). Genetic studies have shown *D-mef2* is required for the proper differentiation of body wall, heart, and visceral muscles during embryogenesis and correct formation of dorsal longitudinal indirect flight muscles (DLM) in the adult (Bour et al., 1995; Cripps and Olson, 1998; Lilly et al., 1995; Ranganayakulu et al., 1995). Given the known activation of a vertebrate MEF2 protein by calcineurin in skeletal muscles of exercised mice (Wu et al., 2001), further biochemical and genetic studies may prove fruitful in identifying D-MEF2 as a direct transcriptional effector of calcineurin signaling in *Drosophila*. Likewise, the analysis of other suppressor genes within the remaining five intervals should provide additional insights into those proteins required for calcineurin function during muscle formation.

Sullivan and Rubin (2002) completed a more extensive search for calcineurin interacting genes by screening for dominant modifiers of a rough eye phenotype induced by activated calcineurin expression. Four enhancing and five suppressing complementation groups were identified in these studies, with a major conclusion being that calcineurin functions with specific proteins to antagonize epidermal growth factor receptor/Ras signaling in the eye imaginal disc. *canB2* was shown to be one of the suppressor groups, demonstrating the shared importance of this calcineurin regulatory gene in the eye and muscle developmental assays. The remaining suppressor groups identified in the eye screen map to different locations than the suppressing intervals uncovered in the muscle screen, indicating calcineurin activity requires several other to be defined cell-specific factors in addition to *canB2*.

canB2 function is required for indirect flight muscle formation in Drosophila

An analysis of strong *canB2* mutations showed the gene is essential for normal *Drosophila* development, with mutants dying at a late larval/early pupal stage (Sullivan and Rubin, 2002). However, genetic combinations involving weaker alleles result in adult escapers that are flightless with wings positioned at abnormal angles (Gajewski et al., 2003). This phenotype was suggestive of irregularities among certain indirect flight muscle (IFM) groups in the thorax. The events required for DLM formation in wild-type animals are outlined in Fig. 5. DLM develop from three pairs of larval oblique muscles that fail to histolyze during metamorphosis within the pupal period (Fernandes and Keshishian, 1996; Fernandes et al., 1991). These persistent muscles split into six pairs that serve as a framework for DLM formation, with muscle growth occurring due to the ordered fusion of Twist-expressing adult myoblasts onto the templates. When formed, the six central DLM pairs and seven flanking pairs of dorsal ventral indirect flight muscles (DVM) fill the adult thorax.

The analysis of IFM structure in *canB2* mutant pupae and adults revealed two reproducible phenotypes (Gajewski et al., 2003). In animals that were able to eclose as adults, abnormalities were observed in the DLM pattern with most muscle pairs absent from anterior thoracic sections and disorganized muscle masses present in posterior sections. It was concluded that this phenotype was not a result of the lack of larval muscle templates or initial muscle formation, but due to the displacement of most DLM to the posterior of the thorax. Presumably, sufficient IFM integrity exists in these animals so as to facilitate movement out of the pupal case. A more severe phenotype was observed when IFM formation was followed in living pupae using a sensitive *MHC-GFP* transgene muscle marker (Chen and Olson, 2002). In animals that progressed only to the pharate adult stage, a complete retraction of all IFM to a posterior thoracic position was observed (Fig. 5). It is likely that such animals are unable to eclose due to thoracic compression and/or insufficient IFM contraction, resulting in lethality.

IFM retraction in *canB2* animals is reminiscent of the hypercontracted IFM phenotype found in certain *myosin heavy chain* (Kronert et al., 1995) and *troponin I* (Kronert et al., 1999) gene mutants. It is possible that calcineurin activity is required for the modification of a myogenic transcription factor, whose function is needed for the activation (or repression) of specific IFM contractile protein genes. Genetic and molecular analyses should help to identify the transcriptional regulator that serves as the target of calcineurin phosphatase activity and the muscle structural genes controlled by such a factor. Consistent with the muscle phenotypes observed in mutant pupae and adults, *canB2* and the *cna* gene *Pp2B-14D* are expressed in forming IFM, as well as in the central nervous system (Gajewski et al., 2003). In contrast, calcineurin subunit genes are not expressed in developing embryonic muscles, indicating a clear specificity in calcineurin's myogenic function during *Drosophila* development.

Calcineurin expression and function in the nematode C. elegans

While calcineurin subunits are encoded by multiple genes in vertebrates and *Drosophila*, the *C. elegans* genome contains a single gene for each of the highly conserved proteins (Fig. 1). Worm CnA is encoded by the *cna-1/tax-6* gene and possesses a 77% overall amino acid identity to a human isoform (Kuhara et al., 2002). *tax-6* was initially discovered as a gene required as a negative regulator of multiple signaling pathways in sensory neurons. Expression studies showed this catalytic subunit is present in diverse tissues and cell types including sensory neurons, interneurons, body wall muscle, vulval muscle, and spermatheca (Bandyopadhyay et al., 2002; Kuhara et al., 2002). CnB is encoded by the *cnb-1* locus and exhibits 80% identity with human and *Drosophila* proteins (Bandyopadhyay et al., 2002). Enhancer-GFP expression and protein immunostaining approaches confirmed the co-expression of CnB with CnA in hypodermal tissue, neurons, muscle, and the male germline.

Consistent with the diverse expression of the *tax-6* and *cnb-1* genes, phenotypic analyses identified cuticle defects, small body size, decreased brood size, locomotion abnormalities, and egg-laying defects in the calcineurin gene mutants. The latter two phenotypes are consistent with the requirement of phosphatase activity in body wall and vulval muscles. Excitingly, double mutants of *cnb-1* and *unc-43(gf)*, which encodes a gain-of-function mutation of the CaMKII protein kinase, show a synergistic severity of animal movement and egg-laying defects (Bandyopadhyay et al., 2002). Such results suggest that calcineurin and CaMKII have opposing functions in these physiological processes and highlight the potential of *C. elegans* as a

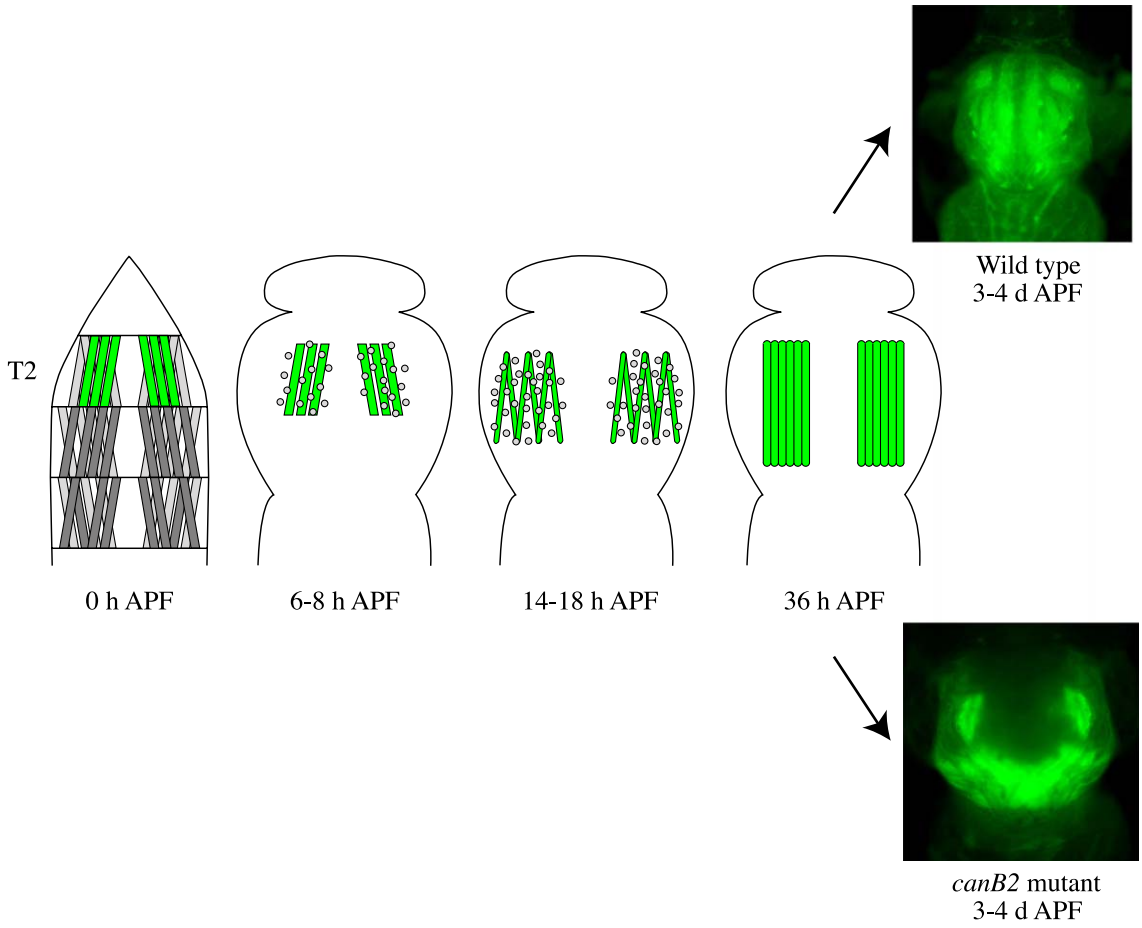

Fig. 5. Requirement of the *canB2* gene for IFM formation in *Drosophila*. During normal development, three pairs of body wall muscles within the second thoracic segment (T2) of third instar larvae fail to histolyze and persist into the pupal stage as templates for DLM growth. At specific times after puparium formation (APF), the muscles split into six scaffold pairs and myoblasts fuse with these templates, generating six sets of DLM that eventually occupy most of the adult thorax. While the initial steps of IFM formation appear normal in *canB2* mutant animals, such muscles become abnormally organized and displaced to a posterior thoracic position, likely due to hypercontractility of the muscle sets. Adapted in part from Fernandes and Keshishian (1996) and Gajewski et al. (2003).

model organism to unravel complex genetic aspects of calcineurin signaling.

It is noteworthy that *nfatc* homologues are absent from the nematode genome (Graef et al., 2001b). Thus, as in *Drosophila*, the effectors of calcineurin signaling remain to be identified. Screens for genetic modifiers of worm muscle phenotypes are likely to generate needed information as to the targets of calcineurin activity. It is plausible that as in vertebrates, MEF2 proteins may serve as tissue-specific end points of calcineurin signaling in both worms and flies. Unraveling the composition of such calcineurin regulatory pathways should provide fundamental insights into the role of the phosphatase in basic cellular and physiological processes associated with muscle formation and function. The absence of *nfatc* genes from invertebrates may also speak to an evolutionary aspect of calcineurin signaling. That is, with the increased complexity of vertebrate tissue and organ development, calcium-dependent NFAT proteins may have been recruited into this signal transduction network to provide additional levels of biological regulation in calcineurin-mediated events.

Conclusions and perspectives

It is clear that through innovative genetic and physiological approaches, much has been learned in recent years about the importance of calcineurin signaling and NFAT activation in the regulation of cardiovascular and skeletal muscle development in vertebrate animals. With the emergence of technological advancements for studying individual genes or gene networks within select tissues and cell types, even greater knowledge of the intricacies of calcineurin signaling should be forthcoming. These predicted advances in our understanding of regulatory interactions will be especially important for those developmental processes that when altered or aberrant, result in human health risks or pathologies. The demonstration of the functional requirement of invertebrate calcineurin genes in fundamen-

tal processes of muscle formation likewise constitutes important progress and the use of highly sensitive genetic methods in these model organisms should allow for the identification and analysis of calcineurin interacting loci unattainable by most other experimental approaches. Collectively, the future looks quite promising to use combined developmental and genetic approaches to more fully comprehend calcineurin signaling, its targets, and its regulators in heart, vasculature, and muscle development and disease.

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