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Topical Review

Calcineurin and cardiac hypertrophy: Where have we been? Where are we going?

Benjamin J. Wilkins *† and Jeffery D. Molkentin *

** Division of Molecular Cardiovascular Biology, Department of Pediatrics, Children's Hospital Medical Center, Cincinnati, OH and † Physician Scientist Training Program, Molecular and Developmental Biology Graduate Program, University of Cincinnati College of Medicine, Cincinnati, OH, USA*

The heart is a dynamic organ capable of adapting its size and architecture in response to alterations in workload associated with developmental maturation, physiological stimulation and pathological diseases. Such alterations in heart size typically result from the hypertrophic growth of individual myocytes, but not myocyte cellular proliferation. In recent years, a great deal of investigation has gone toward elucidating the molecular signalling machinery that underlies the hypertrophic response and manner in which increased cardiac load promotes alterations in gene expression. To this end, the Ca^{2+}–calmodulin-activated phosphatase calcineurin has been proposed as a necessary component of the multi-pathway hypertrophy program in the heart. Despite initial controversy over this hypothesis due to disparate results from pharmacological inhibitory studies in animal models of hypertrophy, compelling data from genetic models with calcineurin inhibition now exist. This review will summarize many of these studies and will attempt to address a number of unanswered issues. In particular, specific downstream mediators of calcineurin signalling will be discussed, as well as the need to identify calcineurin's temporal activation profile, transcriptional targets and cross-communication with other reactive signalling pathways in the heart. Finally, we will present evidence suggesting that calcineurin, as a Ca^{2+}-responsive enzyme, may function as an internal load sensor in cardiac myocytes, matching output demands to hypertrophic growth.

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Corresponding author J. D. Molkentin: Division of Molecular Cardiovascular Biology, Department of Pediatrics, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA. Email: molkj0@chmcc.org

Cardiac hypertrophy is defined by an increase in heart size and/or myofibrillar volume without a change in myocyte number, which occurs in response to both physiological and pathophysiological stimulation. Hypertrophy allows the myocardium to adapt functional performance to alterations in workload associated with developmental maturation, physiological challenge, or injury. While cardiac hypertrophy is typically viewed as a compensatory response that normalizes ventricular wall stress, sustained hypertrophy is correlated with an increase in both the incidence of and mortality from cardiovascular disease (Levy *et al.* 1990), and often is a first step in the progression to congestive heart failure. Cardiac hypertrophy is also a risk factor for arrhythmia and sudden cardiac death due to prolongation of the myocyte action potential (Kääb *et al.* 1998). While the clinical consequences of cardiac hypertrophy have been known for some time, only recently have significant inroads been made into our understanding of the molecular underpinnings of this response. A large body of literature has emerged describing the intracellular signalling pathways that transduce hypertrophic stimulation into alterations in gene expression, which include the mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), PI3K–Akt and calcineurin–nuclear factor of activated T-cells (NFAT) (McKinsey & Olson, 1999; Steinberg, 2000; Molkentin & Dorn, 2001).

The Ca^{2+}–calmodulin-activated phosphatase calcineurin and its downstream transcriptional effector NFAT have been implicated as critical transducers of the hypertrophic response that uniquely link alterations in intracellular calcium handling in a myocyte to the hypertrophic growth response. The initial description of calcineurin and NFAT as hypertrophic transducers involved transgenic overexpression of each factor in the heart, which promoted a dramatic hypertrophy response that quickly transitioned to heart failure and death in the mouse (Molkentin *et al.* 1998). However, controversy quickly followed as pharmacological studies employing the calcineurin inhibitors cyclosporin A or FK506 in different rodent models of heart disease produced largely equivocal results (Olson & Molkentin, 1999; Molkentin, 2000). More recently, data generated by loss-of-function approaches in genetically modified mouse models have revitalized the hypothesis that calcineurin and NFAT function as critical transducers of the hypertrophic response. This review will largely focus on recent studies using genetic manipulations in

the mouse to address the importance of calcineurin signalling in hypertrophy, and will conclude with a discussion of important questions that remain to be answered.

Calcineurin as a sufficient and necessary mediator of cardiac hypertrophy

Calcineurin was initially described as a Ca^{2+}-activated, cyclosporin A (CsA)/FK506-inhibited phosphatase that, upon activation, catalysed the dephosphorylation and nuclear accumulation of cytoplasmic NFAT transcription factors (Flanagan *et al.* 1991; Clipstone & Crabtree, 1992; Shibasaki *et al.* 1996) (Fig. 1). In the heart, nuclear NFATs bind to the transcription factor GATA-4 and activate transcription of hypertrophic genes. Cardiac overexpression of the constitutively active calcineurin catalytic subunit or a constitutively nuclear NFATc4 mutant protein each induced massive cardiac hypertrophy that quickly transitioned to heart failure and death (Molkentin *et al.* 1998).

Since that initial report, numerous studies have employed CsA or FK506 to determine the necessity of calcineurin signalling in multiple models/experimental systems of hypertrophy. At present count, 22 studies support the hypothesis that calcineurin functions as an important hypertrophic transducing factor in cardiac myocytes (18 *in vivo* and 4 *in vitro*) (Mende *et al.* 1998; Molkentin *et al.* 1998; Sussman *et al.* 1998; Meguro *et al.* 1999; Shimoyama *et al.* 1999; Hill *et al.* 2000; Kato *et al.* 2000; Lim *et al.* 2000*a*,*b*; Murat *et al.* 2000; Sakata *et al.* 2000; Shimoyama *et al.* 2000; Xia *et al.* 2000; Goldspink *et al.* 2001; Wang *et al.* 2001*a*). In addition, three reports demonstrated electrophysiological changes in two models of hypertrophy that were reversible with CsA administration (Wang *et al.* 2001*a*,*b*; Yatani *et al.* 2001). However, five studies utilizing these calcineurin inhibitory agents failed to identify a significant attenuation of cardiac hypertrophy in similar rodent models (Luo *et al.* 1998; Muller *et al.* 1998; Zhang *et al.* 1999; Ding *et al.* 1999; Fatkin *et al.* 2000). It is likely that experimental variables such as drug dosing and the specifics of each animal model underlie these differing accounts. However, another important issue that should be considered is the specificity of CsA or FK506. For example, high doses of CsA can alter sarcoplasmic reticulum Ca^{2+} release through the ryanodine receptor, or through a non-specific leakage from the sarcoplasmic reticulum itself (Park *et al.* 1999; Janssen *et al.* 2000). CsA also inhibits the Na^+,K^+-ATPase promoting neurotoxicity and nephrotoxicity, which could lead to a secondary increase in blood pressure and increased load on the heart (reviewed in Klee *et al.* 1998). Finally, CsA can antagonize apoptosis in certain cell types by binding to mitochondrial cyclophilin D and inhibiting the mitochondrial permeability transition, an effect that is independent of calcineurin activity (Nazareth *et al.* 1991; Griffiths & Halestrap, 1993, 1995; Halestrap *et al.* 1997; Lemasters *et al.* 1997; Molkentin, 2001).

Genetic inhibition of calcineurin

To avoid many of the complications arising from the use of CsA and FK506 as calcineurin inhibitors, recent work has involved the use of genetic models with inhibited calcineurin activity. This has been accomplished by either overexpressing naturally occurring inhibitors of calcineurin (so-called 'calcipressins'), through dominant-negative strategies, or more recently through gene targeting. The first such approach involved overexpression of the calcineurin inhibitory domain from two proteins, Cabin1/Cain and AKAP79. Cain is a ubiquitously expressed 230 kDa protein containing a C-terminal domain that acts as a non-competitive inhibitor of calcineurin, while AKAP79 is a scaffolding protein that docks calcineurin, PKA and PKC. Using a recombinant adenoviral approach to infect rat neonatal cardiomyocytes, Taigen *et al.* (2000) demonstrated that the calcineurin inhibitory domain from either Cain or AKAP79 blocked *in vitro* hypertrophy in response to phenylephrine (PE), angiotensin II (AngII) and 1% serum. These studies were then extended to the *in vivo* setting by the creation of Cain and AKAP79 transgenic mice (De Windt *et al.* 2001). Low-copy Cain or AKAP79 mice displayed

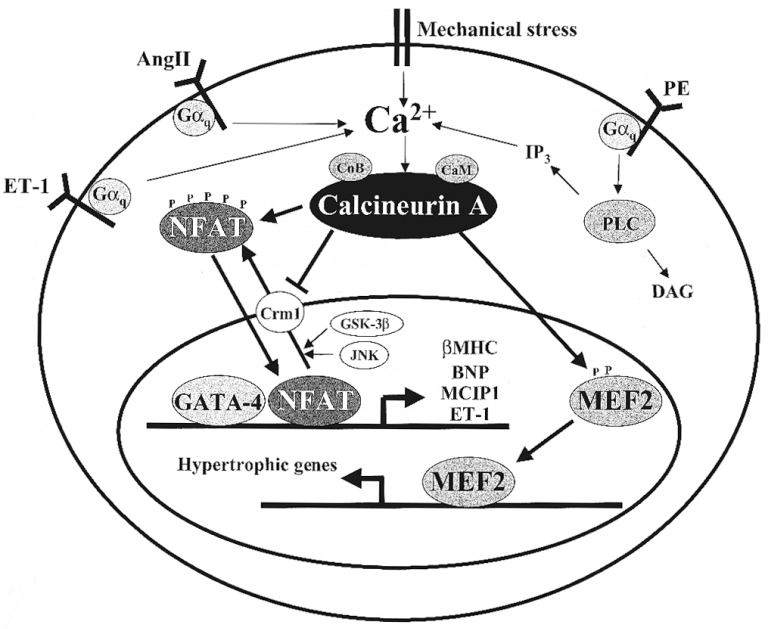

Figure 1. Calcineurin signalling pathways in cardiac myocytes

Activation of $G\alpha_q$-coupled or mechanical stretch receptors leads to an elevation of intracellular Ca^{2+} and activation of the calmodulin-regulated phosphatase, calcineurin. Calcineurin activation causes nuclear localization of NFAT transcription factors by direct dephosphorylation as well as inhibition of the exportin Crm1 (Zhu & McKeon, 1999). Calcineurin also directly activates nuclear MEF2 factors and probably has other, unknown effectors. These factors, along with GATA-4 and other partners, cooperatively activate transcription of the hypertrophic gene programme. Reductions in $[Ca^{2+}]_i$ cause inactivation of calcineurin, de-stimulation of MEF2, and Crm1-mediated nuclear export of NFATs catalysed by NFAT kinases such as GSK-3β and JNK.

Table 1. Summary of genetic models of calcineurin inhibition subjected to hypertrophic stimuli

Study	Model	Stimulus	% Increase (− inhibitor)	% Increase (+ inhibitor)	Relative % decrease
Bueno *et al.* 2002	CnAβ −/− mouse	AngII, 14 days	14	−6.5	146
		Iso, 14 days	33	8	75
		AAC, 14 days	39	13	67
Hill *et al.* 2002	MCIP1 TG mouse	TAC, 21 days	70	40	43
De Windt *et al.* 2001	ΔCain TG mouse	Iso, 14 days	24	12	50
		AAC, 14 days	22	5	79
	ΔAKAP TG mouse	Iso, 14 days	24	12	50
		AAC, 14 days	22	14	38
	AdCain infuse rat	TAC, 7 days	27	16	40
Rothermel *et al.* 2001	MCIP1 TG mouse	CnA TG, 12 weeks	129	39	70
		Iso, 7 days	23	9	59
		E xercise, 28 days	29	12	58
Zou *et al.* 2001*a*	dnCnA TG mouse	AAC, 21 days	58	26	56

Relative percent decrease for all models was determined to be statistically significant in the original publications. All data represent percent increase and decrease in heart-to-body weight ratio, except Bueno *et al.* which utilizes heart weight-to-tibia length ratio. Abbreviations: TG, transgenic; AngII, angiotensin II; Iso, isoproterenol; AAC, abdominal aortic constriction; CnA, calcineurin transgenic; TAC, transverse aortic constriction.

normal heart function at baseline and an impaired hypertrophic response to abdominal aortic constriction and angiotensin II infusion. In addition, rats subjected to *in vivo* cardiac gene transfer with the Cain-expressing adenovirus demonstrated a significant resistance to hypertrophy induced by transverse aortic constriction as compared to controls subjected to an equal transaortic pressure gradient (De Windt *et al.* 2001) (Table 1).

A second approach used to genetically inhibit calcineurin involved transgenic overexpression of the calcineurin inhibitory protein MCIP1 (myocyte-enriched calcineurin-interacting protein-1) in the heart. MCIP1 was first identified as DSCR1 (Down syndrome critical region-1), a member of a family of calcineurin inhibitors conserved from yeast to humans (Görlach *et al.* 2000). MCIP1 is highly enriched in cardiac and slow skeletal muscle and is overexpressed in Down syndrome (potentially lending to the cognitive and cardiac defects observed in the syndrome) (Fuentes *et al.* 2000; Rothermel *et al.* 2000; Casas *et al.* 2001). Interestingly, MCIP1 expression is transcriptionally regulated by calcineurin and NFAT, such that the third intron of the human *MCIP1* gene contains a cluster of 15 consensus NFAT binding sites, making it the first known feedback regulator of calcineurin activity (Yang *et al.* 2000). In accordance with these functions, cardiac-specific MCIP1 overexpressing transgenic mice demonstrated impaired hypertrophy to isoproterenol infusion, treadmill running, pressure overload, and the activated calcineurin catalytic transgene (Rothermel *et al.* 2001; Hill *et al.* 2002) (Table 1).

The third transgenic approach used to inhibit cardiac calcineurin activity employed a dominant-negative mutant of calcineurin (Zou *et al.* 2001*a*). Dominant negative calcineurin expressing transgenic mice had normal hearts at baseline, but demonstrated an attenuation of cardiac hypertrophy induced by abdominal aortic banding (Table 1). Finally, calcineurin Aβ gene targeted mice were also recently generated and shown to have reduced cardiac hypertrophy in response to aortic banding, isoproterenol infusion, and angiotensin II infusion (Bueno *et al.* 2002) (Table 1) (see paragraph below). Taken together, four separate transgenic approaches (Cain, AKAP79, MCIP1 and dnCnA) and one gene targeted mouse model (CnAβ nulls) have demonstrated reduced cardiac hypertrophy to a wide variety of acute stimuli *in vivo*, unequivocally implicating calcineurin as an important signalling constituent in the heart.

Mediators of calcineurin hypertrophic signalling

While the transgenic approaches discussed above have provided a convincing data set, a number of critical questions remain unanswered. For example, the identity of the downstream transcriptional effectors that mediate calcineurin-induced hypertrophy *in vivo* remains uncharacterised. In addition, the molecular identity of the calcineurin isoform that regulates the hypertrophic response is uncertain (three calcineurin catalytic genes have been identified: *CnA*α, *CnA*β, and *CnA*γ). Our laboratory has attempted to address each of these issues using gene-targeted mice. Previous *in vitro* studies indicated that of the three CnA isoforms, only CnAα and CnAβ are present in cardiomyocytes, and interestingly, CnAβ mRNA and protein levels are upregulated in cardiac myocytes undergoing hypertrophy (Taigen *et al.* 2000). Accordingly, the *CnA*β gene was recently targeted in embryonic stem cells and used to generate homozygous null mice. *CnA*β null mice are viable, fertile and overtly normal. *CnA*β null mice had reduced cardiac calcineurin activity and demonstrated impaired hypertrophy in response to AngII infusion, Iso infusion, or abdominal aortic constriction (Bueno *et al.* 2002) (Table 1). These data not only further extend the transgenic approaches

discussed above, but they more specifically implicate the *CnAβ* gene in regulating the hypertrophic response.

Finally, the downstream transcriptional mechanisms whereby calcineurin might function *in vivo* remain largely uncharacterised. However, both NFAT and MEF2 transcriptional regulators are directly regulated by calcineurin, suggesting obvious candidates for genetic analysis in the heart. The NFAT family consists of five members, four of which (NFATc1–c4) are partitioned between the cytoplasm and nucleus by calcineurin (Rao *et al.* 1997). Most of the isoforms are expressed in several tissues at different times in development (Crabtree, 1999). For example, NFATc1–c2 are expressed in T lymphocytes where they regulate mature immune function, while NFATc3 tends to be more highly expressed in developing immune cells (Oukka *et al.* 1998). While analysis of mRNA levels suggests that multiple NFAT factors are expressed in the heart (Hoey *et al.* 1995), the lack of good antibodies and the relatively low abundance of NFAT proteins has made it difficult to correlate mRNA expression with protein expression. Despite definitive data identifying which NFAT protein isoforms are present in the heart, gene targeted mice have been described for NFATc1–c4, which might provide additional insight as to the necessary regulators downstream of calcineurin. Indeed, recent investigation has suggested that the *NFATc3* gene plays an important role in mediating the cardiac hypertrophic response downstream of calcineurin (B. J. Wilkins & J. D. Molkentin, unpublished observations). Consistent with this observation, NFATc3 protein, but not NFATc4 protein, is detected in the adult myocardium. While it is uncertain if NFATc1 and/or NFATc2 also participate in calcineurin-induced cardiac hypertrophy, a more pervasive genetic analysis employing *NFATc1* null mice or simultaneous disruptions of multiple *NFAT* genes is not possible given embryonic lethality (de la Pompa *et al.* 1998; Ranger *et al.* 1998; Graef *et al.* 2001). Future work along these lines will most likely require cardiac-specific or conditional inactivation of these factors.

The MEF2 family of transcriptional regulators has also been implicated in calcineurin-mediated signalling in a variety of cell types. Specifically, a cardiac-restricted, constitutively active calcineurin transgene was shown to activate expression of a MEF2 reporter construct *in vivo* (Passier *et al.* 2000). Studies in skeletal muscle have shown that calcineurin is able to dephosphorylate and directly bind to MEF2A, to synergize with MEF2A–D-dependent transcription, and to strongly activate a MEF2 reporter transgene (Wu *et al.* 2000, 2001). In T lymphocytes, NFATc2 acts as a synergistic coactivator of MEF2D and facilitates recruitment of the general coactivator p300 to target promoters (Youn *et al.* 2000). Finally, studies in cerebellar neurons have shown that calcineurin directly dephosphorylates and enhances the DNA binding activity of MEF2A (Mao & Wiedmann, 1999). Given that MEF2C is also a regulator of embryonic and postnatal cardiac growth (Lin *et al.* 1997; Kolodziejczyk *et al.* 1999), it will be of great interest to determine the role of MEF2 factors as downstream effectors of the hypertrophic response.

While previous work has reasonably established that calcineurin activation is necessary for hypertrophy, the exact timing of activation is still a matter of debate. Four reports indicate that calcineurin activity is increased in failing human hearts as compared to normal samples, but activity is increased still further in compensated, non-failing hypertrophy (Lim & Molkentin, 1999, 2000; Tsao *et al.* 2000; Haq *et al.* 2001). NFAT and MEF2-driven reporter constructs used *in vitro* and *in vivo* should allow investigators to temporally track calcineurin activation under different stimuli that are known to activate hypertrophy through different pathways.

Calcineurin cross-talk with other hypertrophic signalling pathways

A common feature of reactive hypertrophic signalling in cardiac myocytes is that multiple pathways cross-talk with one another to orchestrate a productive response. With respect to calcineurin signalling, its activation is associated with the activation of certain PKC isoforms, c-Jun N-terminal kinase (JNK), and Akt (De Windt *et al.* 2000*a*,*b*). Conversely, β-adrenergic-mediated activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signalling and endothelin-1 transcription is blocked by calcineurin inhibition (Morimoto *et al.* 2001; Zou *et al.* 2001*b*). In addition, ras signalling activates NFAT-mediated transcription (Ichida & Finkel, 2001), JNK signalling antagonizes NFAT nuclear accumulation (Chow *et al.* 1997), and GSK-3β directly phosphorylates NFAT factors preventing nuclear accumulation, DNA binding, and calcineurin-mediated hypertrophy (Graef *et al.* 1999; Haq *et al.* 2000; Neal & Clipstone, 2001; Antos *et al.* 2002). Each of these descriptions suggests cross-talk whereby calcineurin–NFAT-signalling is 'fine-tuned' or coordinated within a larger context of signalling during the hypertrophic response.

Role of calcineurin in physiological and developmental hypertrophy

While a convincing data set has emerged implicating calcineurin as a regulator of pathophysiological hypertrophy, less is understood of calcineurin's role in potentially regulating physiological growth of the heart. Indeed, it is often speculated that pathophysiological and physiological hypertrophy utilize similar signalling pathways, although the timing and degree of signalling probably regulate the ultimate phenotype of each response. By definition, physiological hypertrophy generally refers to the clinical phenomenon known as an 'athletic' heart. Physiological hypertrophy is induced by regular exercise training that promotes myocyte growth changes in the heart that are reversible and do not progress to decompensation (Oakley, 2001). For example, professional football (soccer) players were suggested to have physiological hypertrophy associated with increased myocardial insulin-like growth factor-1 (IGF-1) and myocardial sympathetic activation (Serneri *et al.* 2001).

Circumstantial data suggest a potential role for calcineurin signalling in regulating physiological hypertrophy. *In vivo* calcineurin inhibition by MCIP1 overexpression attenuates

exercise-induced hypertrophy (Rothermel *et al.* 2001). In additon, athletic hypertrophy is associated with increased IGF-1 production, which was previously shown to cause skeletal muscle hypertrophy through a calcineurin–NFAT–GATA pathway in cultured myoblasts (Musarò *et al.* 1999; Semsarian *et al.* 1999). Future studies will need to address how hypertrophic signalling pathways are differentially modulated in these two general forms of hypertrophy, and in particular, what role calcineurin activation plays in each.

In the rodent heart, myocytes exit the cell cycle within the first week of postnatal development, yet the heart enlarges substantially afterwards through a process referred to as developmental hypertrophy (Claycomb, 1977). As with physiological hypertrophy induced by exercise training, developmental hypertrophy is also probably regulated by adaptive haemodynamic load, implicating a similar array of signal transduction factors. That calcineurin might regulate developmental hypertrophy, in part, is suggested by a number of observations. First, *MEF2C* gene targeted mice die during embryonic development with a hypoplastic right ventricle (Lin *et al.* 1997). Second, targeted disruption of the calcium regulatory genes *calreticulin* and *connexin45* each resulted in embryonic lethality that was associated with defective NFAT-mediated transcription in the heart (Mesaeli *et al.* 1999; Kumai *et al.* 2000). Third, deletion of *NFATc1* in the mouse resulted in defective cardiac valve formation and embryonic lethality (de la Pompa *et al.* 1998; Ranger *et al.* 1998). In the postnatal heart, two studies have suggested a role for calcineurin in regulating hypertrophic maturation. First, high copy number Cain and AKAP79 transgenic mice each perished during with the first 2 weeks of neonatal development with severely atrophic hearts, although low copy number lines were viable (De Windt *et al.* 2001). Second, while MCIP-1 transgenic mice survive successfully through neonatal development, adult heart size was 5–10 % smaller than wild-types, suggesting an attenuation in developmental hypertrophy (Rothermel *et al.* 2001). Collectively, these observations suggest that calcineurin transduces, in part, the signals that control physiological and developmental growth of the myocardium.

Is hypertrophy a beneficial response?

Cardiac hypertrophy is generally assumed to be a necessary compensation to injury or increased workload that benefits the heart in the short term. However, a few recent studies have questioned this assumption and demonstrated that acute injury or pressure overload can be dissociated from the hypertrophic response without leading to decompensation. Specifically, analysis of mice with inhibited $G\alpha_q$, adrenergic, or calcineurin signalling during acute pressure overload stimulation revealed normal cardiac function in conditions of increased wall stress without decompensation (Hill *et al.* 2000; Esposito *et al.* 2002; Hill *et al.* 2002). However, an early study showed that while calcineurin inhibition reduced pressure overload-induced hypertrophy in mice subjected to TAC, it also resulted in increased decompensation and mortality (Meguro *et al.* 1999). Given these disparate results, more studies are necessary to resolve the question of how to modulate the hypertrophic response for the greatest clinical benefit (Force *et al.* 1999).

Calcineurin activation and function in an unstimulated heart.

While calcineurin probably transduces physiological and pathophysiological growth responses in the heart, its functional role at baseline in an unstimulated heart is unknown. It has been suggested that calcineurin is inactive in an unstimulated heart given the observation that dominant negative calcineurin expressing transgenic mice have similar calcineurin phosphatase activity compared to wild-type controls (Zou *et al.* 2001*a*). However, calcineurin is also known to directly sense intracellular calcium levels, which constantly cycle in contracting myocytes. The calcium transient itself may regulate, in part, basal calcineurin activity as a mechanism for adapting cardiac load or inotropy with hypertrophic signalling. Indeed, the steady-state size of the heart directly responds to haemodynamic load in a continuous fashion. For example, it is well established that heart size closely parallels body size (haemodynamic load) in rats and mice and probably all mammals (Goodman *et al.* 1984; Ernsberger *et al.* 1996). By a similar mechanism, unloading of the heart results in severe atrophy such that 50 % of cardiac mass is lost after only 14 days of unloading in the rat (Klein *et al.* 1990). In other cell types, the nature and duration of calcium increase directly modify calcineurin activity in a graded fashion. For example, calcineurin/GSK-3β signalling in T lymphocytes responds to intracellular Ca^{2+} levels to monitor the duration of antigen receptor occupancy, which activates immune cells in response to antigen 'load' (Neilson *et al.* 2001). Finally, cardiac myocytes isolated from adult mice treated with CsA demonstrated a 10 % reduction in size compared with wild-type controls (Wang *et al.* 2001*b*). Collectively, these observations suggest that calcineurin participates in regulating the homeostatic size of the heart in response to load-associated alterations in calcium handling. To definitively address this hypothesis, complete inactivation of calcineurin activity in the heart is required, especially since CsA and FK506 only partially block calcineurin activity. While the complete disruption of all calcineurin activity results in early embryonic lethality (Graef *et al.* 2001), tools now exist to conditionally inactivate calcineurin specifically within the heart to directly address this intriguing hypothesis (Sohal *et al.* 2001; Zeng *et al.* 2001).

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