

# Calcineurin/NFAT Coupling Participates in Pathological, but not Physiological, Cardiac Hypertrophy

Benjamin J. Wilkins, Yan-Shan Dai, Orlando F. Bueno, Stephanie A. Parsons, Jian Xu,  
David M. Plank, Fred Jones, Thomas R. Kimball, Jeffery D. Molkentin

**Abstract**—Calcineurin (PP2B) is a calcium/calmodulin-activated, serine-threonine phosphatase that transmits signals to the nucleus through the dephosphorylation and translocation of nuclear factor of activated T cell (NFAT) transcription factors. Whereas calcineurin-NFAT signaling has been implicated in regulating the hypertrophic growth of the myocardium, considerable controversy persists as to its role in maintaining versus initiating hypertrophy, its role in pathological versus physiological hypertrophy, and its role in heart failure. To address these issues, NFAT-luciferase reporter transgenic mice were generated and characterized. These mice showed robust and calcineurin-specific activation in the heart that was inhibited with cyclosporin A. In the adult heart, NFAT-luciferase activity was upregulated in a delayed, but sustained manner throughout eight weeks of pathological cardiac hypertrophy induced by pressure-overload, or more dramatically following myocardial infarction-induced heart failure. In contrast, physiological hypertrophy as produced in two separate models of exercise training failed to show significant calcineurin-NFAT coupling in the heart at multiple time points, despite measurable increases in heart to body weight ratios. Moreover, stimulation of hypertrophy with growth hormone–insulin-like growth factor-1 (GH-IGF-1) failed to activate calcineurin-NFAT signaling in the heart or in culture, despite hypertrophy, activation of Akt, and activation of p70 S6K. *Calcineurin*  $A\beta$  gene–targeted mice also showed a normal hypertrophic response after GH-IGF-1 infusion. Lastly, exercise- or GH-IGF-1–induced cardiac growth failed to show induction of hypertrophic marker gene expression compared with pressure-overloaded animals. Although a direct cause-and-effect relationship between NFAT-luciferase activity and pathological hypertrophy was not proven here, our results support the hypothesis that separable signaling pathways regulate pathological versus physiological hypertrophic growth of the myocardium, with calcineurin-NFAT potentially serving a regulatory role that is more specialized for maladaptive hypertrophy and heart failure. (*Circ Res.* 2004;94:110-118.)

**Key Words:** signaling ■ calcineurin ■ hypertrophy ■ heart failure ■ transcription

Cardiac hypertrophy is defined as an enlargement of the heart associated with an increase in cardiac myocyte cell volume that occurs in response to diverse pathophysiological stimuli such as hypertension, ischemic heart disease, valvular insufficiency, infectious agents, or mutations in sarcomeric genes.<sup>1</sup> Hypertrophic growth of the myocardium is thought to preserve pump function, although prolongation of the hypertrophic state is a leading predictor for the development of arrhythmias, sudden death, and heart failure.<sup>2,3</sup> However, not all forms of cardiac hypertrophy are necessarily pathological, as athletic conditioning can stimulate heart growth without deleterious consequences.<sup>4</sup>

A number of studies have been dedicated to elucidating the molecular mechanisms underlying the hypertrophic growth process of the myocardium.<sup>5,6</sup> One pathway that has received attention is mediated by the calcium/calmodulin-activated protein phosphatase, calcineurin (PP2B). Calcineurin is acti-

vated by sustained elevations in intracellular calcium, which facilitates binding to its primary downstream effector, nuclear factor of activated T cells (NFAT).<sup>7</sup> NFAT transcription factors are normally hyperphosphorylated and sequestered in the cytoplasm, but rapidly translocate to the nucleus after calcineurin-mediated dephosphorylation.<sup>7</sup> Cardiac-specific activation of calcineurin or its downstream effector NFAT are sufficient to induce a robust hypertrophic response in transgenic mice.<sup>8</sup> Furthermore, genetic inhibition of calcineurin or NFAT has shown the pathway to be necessary for a full hypertrophic response in a number of rodent models.<sup>9</sup> Some reports have also shown elevations in calcineurin protein levels or phosphatase activity in failing or hypertrophic human hearts, suggesting that this pathway might regulate pathological remodeling and failure.<sup>10–12</sup>

In contrast to the proposed pathological role for calcineurin-NFAT signaling in the heart, a signaling pathway

Original received May 7, 2003; resubmission received July 24, 2003; revised resubmission received November 14, 2003; accepted November 14, 2003. From the Divisions of Molecular Cardiovascular Biology (B.J.W., Y.-S.D., O.F.B., S.A.P., J.X., D.M.P., J.D.M.) and Cardiology (F.J., T.R.K.), Department of Pediatrics, Children's Hospital Medical Center, Cincinnati, Ohio.

Correspondence to Jeffery D. Molkentin, Division of Molecular Cardiovascular Biology, Department of Pediatrics, Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229-3039. E-mail jeff.molkentin@cchmc.org

© 2004 American Heart Association, Inc.

*Circulation Research* is available at <http://www.circresaha.org>

DOI: 10.1161/01.RES.0000109415.17511.18

initiated by insulin-like growth factor-1 (IGF-1) has been hypothesized to mediate physiological and developmental growth of the myocardium. IGF-1 binds to its receptor on the cell surface leading to the activation of phosphoinositide 3-kinase (PI3K), which in turn promotes activation of Akt through phosphoinositide-dependent protein kinase-1.<sup>13</sup> Akt then facilitates activation of mammalian target of rapamycin (mTOR), leading to p70 S6 kinase activation and augmented protein synthesis.<sup>13</sup> Transgenic mice overexpressing activated or dominant-negative mutants of PI3K in the heart showed larger and smaller hearts, respectively.<sup>14</sup> Lastly, mild overexpression of Akt in the heart showed a more physiological profile of hypertrophic growth.<sup>15–18</sup>

Although IGF-1 signaling has been implicated as a mediator of developmental and adaptive growth of the myocardium, it is not known whether calcineurin-NFAT also functions as a downstream effector of this physiological pathway in the heart, although such regulation was observed in skeletal muscle.<sup>19,20</sup> In addition, the temporal aspects of calcineurin-NFAT signaling in the heart and its activation status during hypertrophy or dilated heart failure remain an area of ongoing controversy.

## Materials and Methods

### NFAT-Luc Construction and Generation of Transgenic Mice

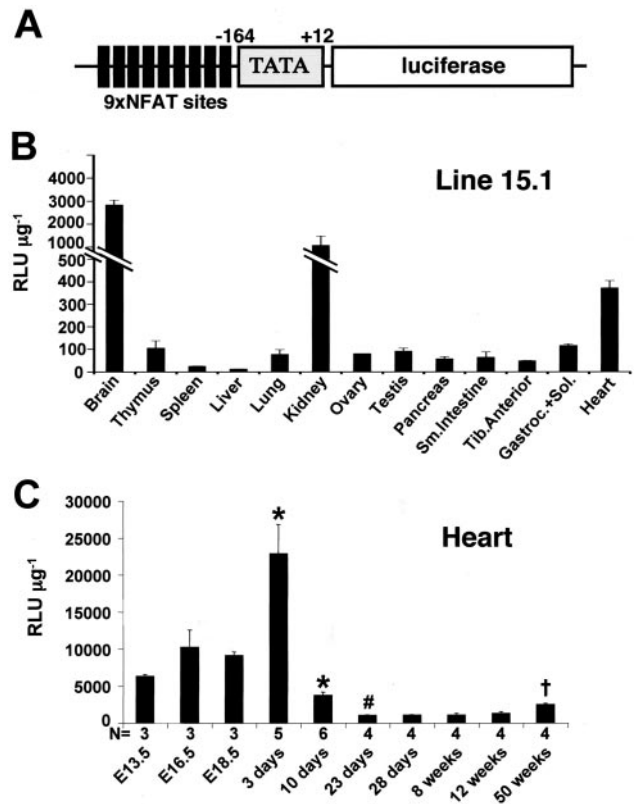
Nine copies of an NFAT binding site from the IL-4 promoter (5'-TGGAAAATT-3') were positioned 5' to a minimal promoter from the  $\alpha$ -myosin heavy chain gene (–164 to +16) and inserted upstream of the luciferase reporter in pGL-3 Basic (Promega) to create NFAT-luc. The NFAT-luciferase transgene was injected into newly fertilized oocytes to generate transgenic mice (FVB/N background), which gave mice that were phenotypically normal. To generate an adenoviral NFAT-luc reporter, the same construct was cloned into the *NotI* sites of pAC-CMVpLpA from which the CMV promoter was removed. Recombinant adenovirus was generated in HEK293 cells using previously described methods.<sup>21</sup> All procedures performed in animals were approved by the Institutional Animal Care and Use Committee.

### Cell Culture

All in vitro experiments utilizing AdNFAT-luc or a similarly designed AdMEF-2-luc were performed in neonatal ventricular myocytes isolated from 1- to 2-day-old rats; infection and culture conditions have been previously reported.<sup>21</sup>

### In Vivo Surgical and Exercise Protocols

Pathological hypertrophy was induced by constriction of the transverse aortic arch.<sup>22</sup> In short, the transverse aortic arch was visualized through a median sternotomy and 7-0 silk ligature was tied around the aorta (27-gauge constriction) between the right brachiocephalic and left common carotid arteries. To generate heart failure, NFAT-luc mice were subjected to permanent ligation of the left anterior descending (LAD) coronary artery for 21 days. Protocols have been previously reported for the exercise training of mice, both by free wheel running<sup>23</sup> and swimming.<sup>24</sup> Voluntary wheel running was performed for 14 days with all mice showing equivalent exercise levels using counters (5 to 7 km a night). For swimming, the first 8 days consisted of a training period that was increased 10 minutes each day until two 90-minute sessions were achieved on the 9th day. Thereafter, groups of mice were swum for as long as 12 additional days (20 days total). Swimming mice were continuously monitored to insure equal exertion. The growth hormone (GH)-IGF-1 infusion model of hypertrophy was previously described.<sup>25</sup> Growth hormone (Nutropin AQ, Genentech) and Long R<sup>3</sup>-IGF-1 (JRH Biosciences)



**Figure 1.** A, Schematic of the reporter construct used to generate NFAT-luciferase transgenic mice. B, Survey of basal NFAT-luciferase tissue activity at 3 weeks of age from transgenic line 15.1, n=3. C, Developmental regulation of basal NFAT-luciferase activity from the heart, with the number of individual samples indicated in the Figure (both males and females were used). \* $P < 0.05$  vs E18.5; # $P < 0.05$  vs 10 days; † $P < 0.05$  vs 12 weeks.

were infused at 4 mg/kg each, twice a day via subcutaneous injection.

### Molecular Analyses

Western blotting was performed as previously described.<sup>26</sup> Luciferase assays from hearts was performed as previously described.<sup>27</sup> Dot blotting for mRNA levels of hypertrophic marker genes was described previously.<sup>8</sup>

### Statistical Analysis

Differences between experimental groups were analyzed by a two-tailed Student's *t* test using Excel software.

## Results

### Construction and Characterization of NFAT-Luciferase Transgenic Mice

The traditional calcineurin enzymatic assay is wrought with both technical and theoretical difficulties (see Discussion). A surrogate measure of calcineurin activity is through analysis of NFAT transcriptional responsiveness. In this study, we generated transgenic mice containing an NFAT binding site-dependent luciferase reporter as a means of examining calcineurin-NFAT signaling in the heart. The transgene includes nine concatamerized high-affinity NFAT binding sites from the IL-4 promoter and a minimal promoter (Figure 1A). Seven founder lines were established, two of which

(lines 15.1 and 15.14) displayed calcineurin-inducible reporter activity in the heart (see next section). Line 15.1 was chosen for all subsequent analyses given its relatively high level of expression in the heart.

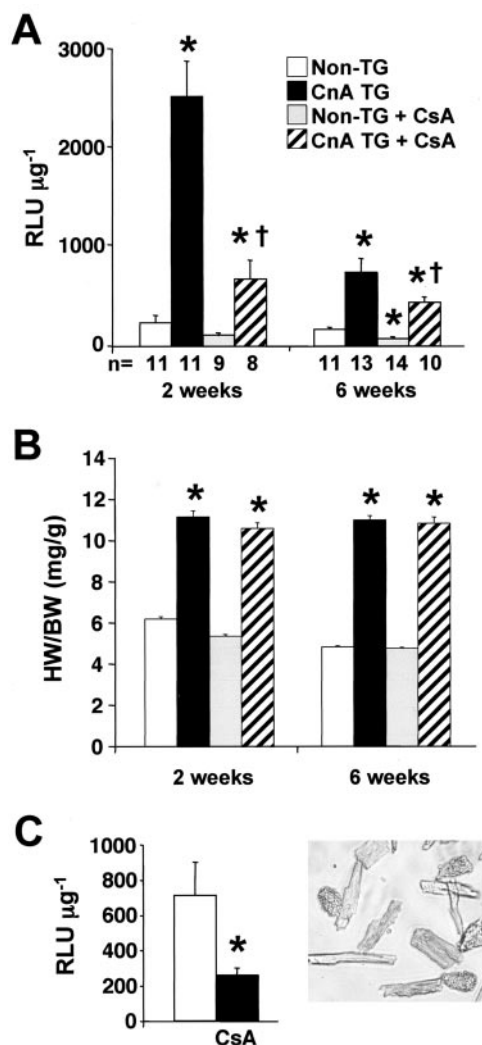
NFAT-luciferase transgenic mice displayed detectable activity in most tissues surveyed at 3 weeks of age, with highest expression occurring in the brain, kidney, and heart, each of which are sites of considerable calcineurin protein expression (Figure 1B). Line 15.14 transgenic mice displayed a very similar profile of basal expression in each of these tissues, albeit with lower absolute activity per microgram of protein (data not shown). NFAT-luciferase activity was assessed from hearts of line 15.1 mice at different developmental times, both pre- and postnatally. Interestingly, NFAT-luciferase activity peaked during developmental maturation of the heart, whereas the adult heart showed relatively lower activity (Figure 1C).

### NFAT-Luciferase Reporter Activity Is Calcineurin Responsive

To verify the specificity of the NFAT-luciferase reporter, these mice were crossed with activated calcineurin transgenic mice, as well as treated with the calcineurin-specific inhibitor cyclosporin A (CsA). At 2 and 6 weeks of age, the activated calcineurin transgene, which is expressed only within myocytes, produced a 6- to 10-fold increase in NFAT reporter activity in the heart that was inhibited after 36 hours of CsA administration (Figure 2A). The calcineurin transgene produced a 2-fold increase in heart weight to body weight ratios, and this increase was not affected by short CsA treatment, indicating that reporter activity likely reflects cardiac calcineurin signaling and not the hypertrophic state of the heart (Figure 2B). Adult cardiac myocytes were also purified after enzymatic disassociation, from mice previously treated with or without CsA, as a means of further verifying that myocytes themselves can express the transgene and that this expression is calcineurin-regulated (Figure 2C).

### NFAT-Luciferase Activity in Pathological Hypertrophy

Acute pressure overload is typically used as a means of inducing a pathological profile of cardiac hypertrophic growth and ventricular remodeling.<sup>5,6</sup> Whereas initiation of pressure overload-induced hypertrophy (first 2 days) is associated with immediate early gene activation, less is known of the signaling factors or genes that sustain the long-term hypertrophic growth of the myocardium and its transition to dilated failure. To address these issues, NFAT-luciferase transgenic mice were subjected to transverse aortic constriction (TAC) for various lengths of time, followed by analysis of cardiac luciferase activity. Interestingly, cardiac calcineurin-NFAT activity was not increased within 24 hours, suggesting a delay in the initiation phase of this pathway (Figure 3A). However, by day 2 and thereafter, NFAT-luciferase activity was elevated by 2- to 3-fold for up to 8 weeks (Figure 3A). This activation profile, combined with the fact that NFAT reporter activity is elevated before the onset of definable hypertrophy (Figures 3B and 3C), suggests that calcineurin-NFAT signaling functions as a delayed, but



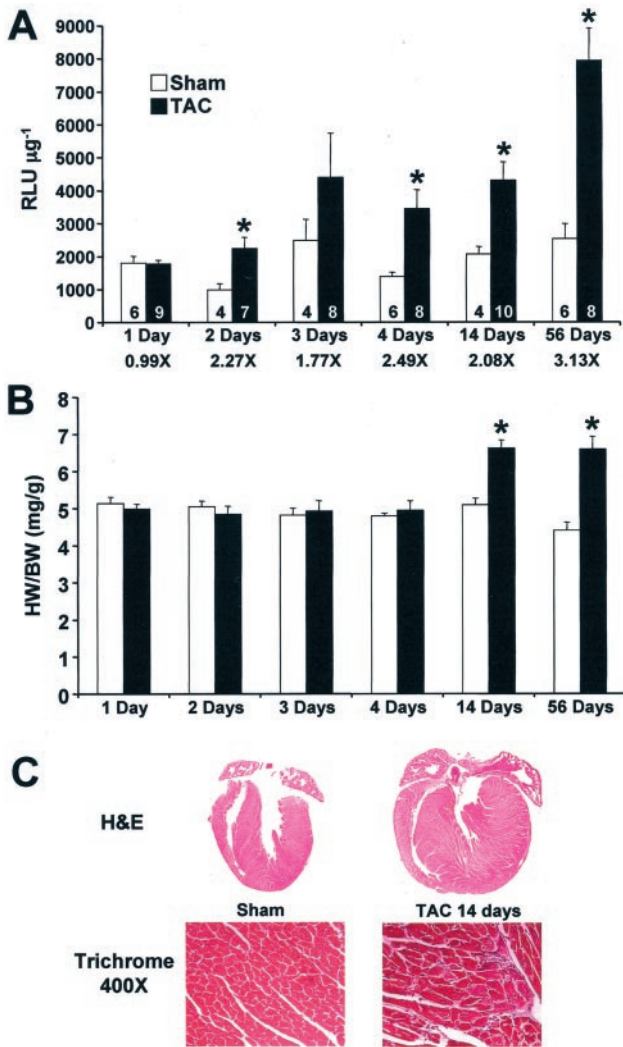
**Figure 2.** NFAT-luciferase transgenic mice display cardiac reporter activity that is responsive to calcineurin activation and inhibition. A, Hearts from NFAT-luciferase mice (line 15.1) were assayed at 2 and 6 weeks of age in the presence or absence of a cardiac-restricted, activated calcineurin transgene (CnA TG) or acute treatment with CsA (CsA, 20 mg  $\cdot$  kg<sup>-1</sup> every 12 hours for 36 hours). Number of mice is shown in the Figure (n) (both males and females were used). B, Corresponding heart weight to body weight measures for groups in A. \* $P < 0.05$  vs Non-TG; † $P < 0.05$  vs CnA TG. C, Cardiomyocytes were purified from adult hearts (light micrograph of purity is shown) from mice either untreated or treated with CsA for 2 days. Luciferase activity from isolated myocytes is shown (\* $P < 0.05$  vs untreated).

sustained signal for pathological hypertrophy. These data are similar to our previous study that used a calmodulin immunoprecipitation technique as a means of measuring calcineurin activation in the heart. Lim et al<sup>28</sup> demonstrated a 2-day delay in calcineurin-calmodulin association after aortic banding in the rat, but after this time activation was maintained for at least 6 weeks.

### Calcineurin-NFAT Signaling Is Upregulated in Failing Hearts

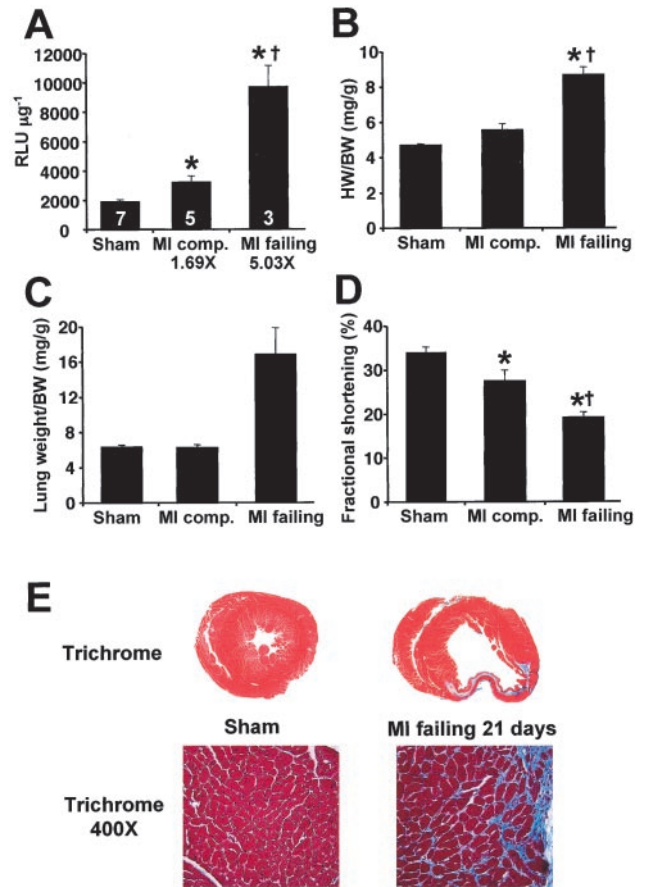
Although pathological hypertrophy has many potential etiologies, a common clinical endpoint is congestive heart failure. Previous reports have shown elevated calcineurin activity in





**Figure 3.** Pressure overload leads to delayed and sustained NFAT activation. A, Cardiac NFAT-luciferase reporter activity as assessed after the indicated days of TAC in 8-week-old mice (both sexes). Number of mice used in each group and fold increase are shown. B, Corresponding heart weight to body weight ratios for groups in A. \* $P < 0.05$  vs time-matched sham. C, Representative gross and microscopic Masson's trichrome-stained histological heart sections from sham or TAC mice after 14 days (blue area represents fibrosis).

ventricular tissue from failing human hearts,<sup>10–12</sup> yet others have not seen an association.<sup>29,30</sup> In this study, NFAT-luciferase transgenic mice were subject to permanent left anterior descending coronary artery (LAD) occlusion, creating a myocardial infarct (MI) model of heart failure. Twenty-one days afterward, these mice showed substantial loss of left ventricular tissue, scarring, and interstitial fibrosis (Figure 4E). Assessment of heart and lung weight to body weight ratios and fractional shortening measured by echocardiography showed two distinct phenotypes of mice: those with overt failure ( $n=3$ ) and those that were less severely affected ( $n=5$ ) (Figures 4B through 4D). Ventricular tissue was harvested from the noninfarcted area for assessment of NFAT-luciferase activity. Compared with sham-operated mice, non-failing hearts showed a 1.7-fold increase in cardiac NFAT activity, similar to mice undergoing hypertrophy in response



**Figure 4.** Failing hearts demonstrate an exaggerated increase in NFAT reporter activity. A, Cardiac NFAT-luciferase reporter activity from sham, nonfailing, and failing myocardial-infarcted (MI) mice of both sexes (8 weeks of age). B and C, Heart- and lung weight to body weight ratios for the indicated groups of mice. D, Echocardiographic assessment of fractional shortening. \* $P < 0.05$  vs sham; † $P < 0.05$  vs MI compensated. E, Representative gross and microscopic Masson's trichrome-stained histological heart sections from sham and MI mice 21 days afterward from a noninfarcted portion of the myocardium (blue area represents fibrosis).

to pressure overload (Figure 4A). In contrast, severely failing hearts displayed a significantly higher level of NFAT-luciferase activity (5-fold) compared with sham controls (Figure 4A). These results indicate that the magnitude of calcineurin-NFAT signaling correlates with the severity of the underlying pathological condition.

### Exercise-Induced Hypertrophy Fails to Upregulate NFAT-Luc Reporter Activity

The data described in the previous sections suggest that calcineurin-NFAT signaling is associated with growth and remodeling of the myocardium after pathological stimulation. However, the role that calcineurin-NFAT signaling plays in mediating adaptive or physiological growth of the myocardium is less defined. In this study, separate cohorts of NFAT-luciferase reporter transgenic mice were exercised using either voluntary free wheel running or swimming for various lengths of time. All mice analyzed underwent a similar degree of exercise stimulation (similar number of wheel revolutions

or similar degree of exertion during swimming). Interestingly, neither swimming nor wheel running produced an increase in NFAT-luciferase reporter activity in the heart (Figure 5A). In fact, mice subjected to swimming exercise actually showed a reproducible decrease in cardiac NFAT activity between days 3 and 14 of exercise. Activity was not increased after 1 day of swimming, and by 20 days of swimming, activity was similar to rested controls (Figure 5A). All hearts were collected from animals for analysis 4 hours after their last swim, although we also analyzed hearts collected 15 minutes and 24 hours afterward in the event that the maximal transcriptional response occurred before or after 4 hours (Figure 5A). Fourteen days of running and 14 and 20 days of swimming produced a significant increase in heart to body weight ratios compared with sedentary controls with no signs of myocardial interstitial fibrosis (Figures 5B and 5E, and data not shown). The two exercise protocols used in this study did not effect overall body weight in each cohort, nor was there a difference in the hypertrophy response between males and females after 14 days of swimming (Figures 5C and 5D). Collectively, the results presented suggest that adaptive or physiological growth of the myocardium does not invoke significant calcineurin-NFAT signaling. However, mice subjected to swimming for 20 days showed significant upregulation in Akt phosphorylation in the heart, suggesting that a "physiological-like" growth response pathway was activated (Figure 5F).

### Calcineurin-NFAT/MEF2 Signaling Is Not Regulated by IGF-1–Akt Signaling

That mice undergoing swimming showed Akt activation is consistent with the observation that competitive male athletes have elevated IGF-1 levels in the heart.<sup>31</sup> To more carefully evaluate the potential association between IGF-1–PI3K–Akt signaling and calcineurin-NFAT signaling an NFAT-luciferase reporter adenovirus was generated for *in vitro* studies in cultured cardiomyocytes (AdNFAT-luc). To verify the specificity of this reporter, cultured neonatal rat cardiomyocytes were infected with AdNFAT-luc alone or in combination with viruses encoding activated calcineurin (Ad $\Delta$ CnA) or activated NFATc3 (Ad $\Delta$ NFAT). Coinfection with Ad $\Delta$ CnA or Ad $\Delta$ NFAT resulted in a 200- and 30-fold activation of NFAT-luciferase activity, respectively (Figure 6A). The activity induced by Ad $\Delta$ CnA coinfection, but not Ad $\Delta$ NFAT, was completely inhibited with CsA, further verifying specificity (Figure 6A). Cultured cardiomyocytes infected with the NFAT-luc reporter were also subjected to serum stimulation to examine the time course of activation. Similar to the NFAT-luciferase reporter transgenic mice, a somewhat delayed profile of activation was observed in culture, such that maximal activity was not observed for 24 to 36 hours (Figure 6B) (see Discussion).

The transcription factor myocyte enhancer factor 2 (MEF-2) is also activated by calcineurin. Infection of neonatal cardiomyocytes with a MEF-2–dependent luciferase reporter adenovirus also demonstrated responsiveness to activated calcineurin and partial inhibition with CsA, suggesting that analysis of MEF2 could function as an additional surrogate for calcineurin activation (Figure 6C). Using these

two reporters, IGF-1 treatment for 24 hours had no significant effect on activity, despite inducing Akt phosphorylation (Figures 6D and 6E). Moreover, overexpression of Akt with a recombinant adenovirus did not affect either NFAT or MEF-2 reporter activity, in contrast to the massive increase observed with Ad $\Delta$ CnA coinfection (Figures 6A and 6C). By comparison, serum stimulation induced an approximate 5-fold activation of both reporters (Figure 6C). These results suggest that IGF-1–Akt signaling does not activate calcineurin-NFAT signaling, further suggesting a specialization in signaling.

To extend the results observed *in vitro*, an *in vivo* model of IGF-1 signaling was performed in the NFAT-luciferase reporter mice. Ross and colleagues previously reported that 14 days of GH/IGF-1 infusion produced a myocardial growth response.<sup>25</sup> NFAT-luciferase mice were injected two times a day with GH/IGF-1 (each at 4 mg/kg), resulting in a 35% increase in body weight, whereas vehicle-injected littermate reporter mice showed only a 3% increase over this time (Figure 7A). Analysis of heart-to-tibia-length ratios showed that GH/IGF-1 injection augmented heart growth, although NFAT-luciferase activity was not altered (Figure 7A). GH/IGF-1 infusion produced robust activation of both Akt and p70 S6K in the hearts of injected mice, further validating the effectiveness of this protocol (Figure 7B). Collectively, these results indicate that cardiac growth driven by IGF-1–Akt signaling does not utilize calcineurin-NFAT.

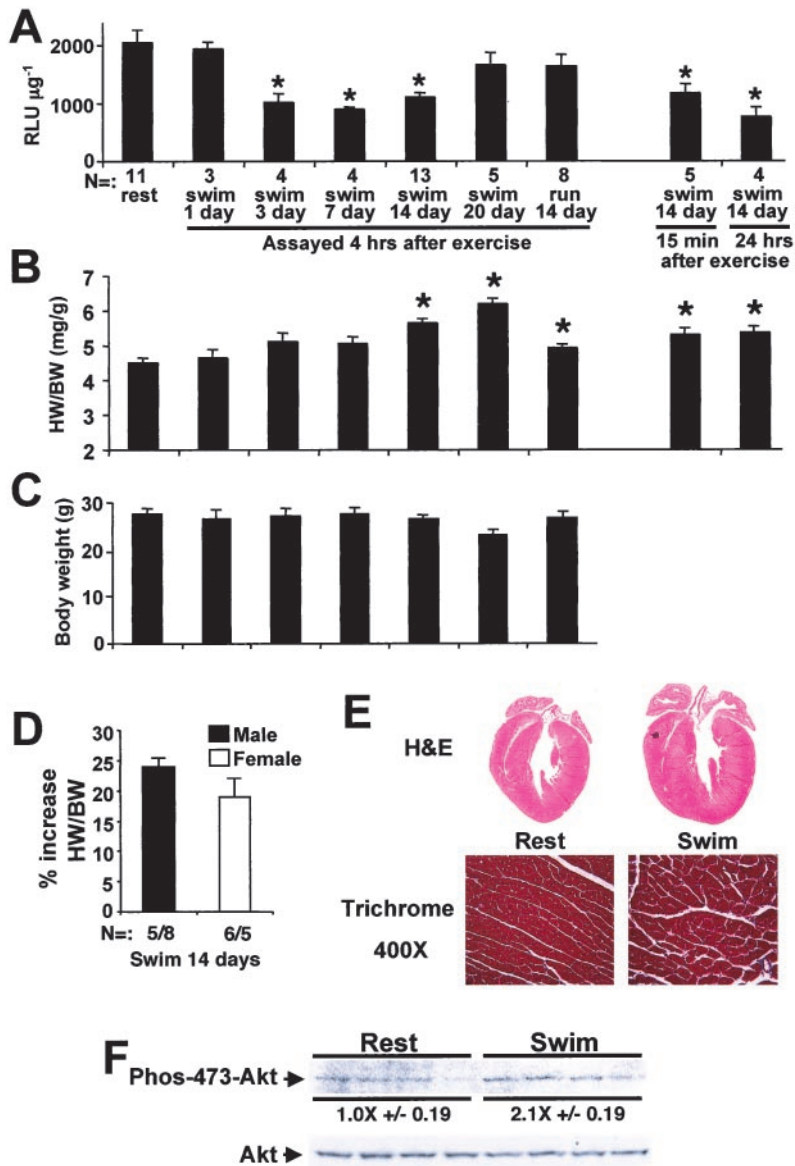
These results suggest that IGF-1–Akt signaling may induce a different molecular program compared with a pathological stimulus such as pressure overload. To more directly address this interpretation, molecular markers of cardiac hypertrophy were analyzed by mRNA dot blotting. The data show that TAC stimulation for 14 days induced expression of  $\beta$ -myosin heavy chain ( $\beta$ -MHC), atrial natriuretic factor (ANF), b-type natriuretic peptide (BNP), and skeletal  $\alpha$ -actin in the heart. In contrast, mice subjected to 20 days of swimming or 14 days of GH/IGF-1 infusion had no induction of these same marker genes in the heart, despite Akt and p70 S6K activation (Figure 7C). These results support the hypothesis that different molecular pathways underlie pathological versus physiological cardiac hypertrophy.

Finally, *calcineurin A $\beta$*  gene–targeted mice were analyzed for their ability to hypertrophy after GH/IGF-1 infusion. Previously, *calcineurin A $\beta$* -null mice were shown to have a substantial defect in their ability to undergo cardiac hypertrophy after a pathological stimulus due to pressure overload.<sup>32</sup> However, GH/IGF-1 infusion induced the same relative level of cardiac hypertrophy between *calcineurin A $\beta$* -null mice and their strain-matched wild-type controls (Figure 7D). These results indicate that GH/IGF-1 stimulation does not depend on optimal calcineurin activity for the induction of cardiac hypertrophy.

## Discussion

### Advantages of an *In Vivo* Reporter System

In recent years, a number of groups have constructed and characterized transgenic mice carrying reporter elements as a way of directly assessing biological activity *in vivo*. Reporter



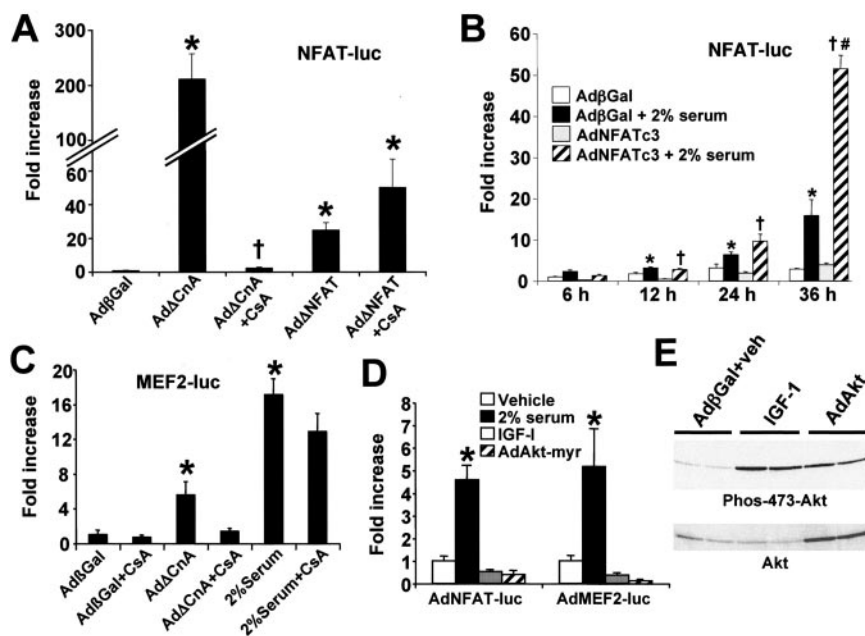
**Figure 5.** Exercise-induced hypertrophy fails to upregulate NFAT-luciferase reporter activity. A, Cardiac NFAT reporter activity in mice subjected to 14 days of free-wheel running or various time points of swimming (males and females, beginning at 8 weeks of age, were used). B, Corresponding heart weight to body weight ratios for mice in A. \* $P < 0.05$  vs respective sedentary control. C, Averaged body weights for the rested and exercised groups shown in A. D, Male and female mice showed the same relative increase in heart weight to body weight ratios after 14 days of swimming (n=rested/exercised). E, Representative gross and microscopic Masson's trichrome-stained histological heart sections from rested or exercised mice. F, Western blotting for phosphorylated Akt or total Akt protein from the indicated heart extracts. Fold increase in phosphorylated Akt is shown.

transgenic mice have been characterized for the transcription factors myocyte enhancer factor-2 (MEF2),<sup>33</sup> estrogen receptor,<sup>27</sup> NF $\kappa$ B,<sup>34</sup> and LEF/TCF.<sup>35</sup> These systems allow for the determination of the spatiotemporal activity of transcription factors or their upstream signaling effectors in a quantitative manner. By comparison, calcineurin-NFAT activity has been traditionally measured with an enzymatic assay from protein lysates. However, the enzymatic phosphatase assay is plagued by both technical and theoretical shortcomings. For example, calcineurin activity is extremely sensitive to oxidation, and commonly used lysis buffers lack reducing agents. The calcineurin assay requires addition of calcium and saturating levels of calmodulin, which essentially nullifies any differences in activity due to calmodulin association. Lastly, it is still unclear how measurement of calcineurin activity from protein lysates reflects *in vivo* activity, if at all. An alternative method for measuring calcineurin activity consists of calmodulin immunoprecipitation followed by

Western blotting for calcineurin protein association.<sup>28</sup> Although this later assay has revealed data similar to that reported with the NFAT-luciferase reporter transgene in this study, it is also less than ideal.

Analysis of NFAT subcellular distribution or transcriptional activity is arguably the most reliable means of assessing calcineurin activity, especially given the intimate relationship between these two factors.<sup>7</sup> However, the activation profile of NFAT is also modulated by counter-acting kinases such as c-Jun N-terminal kinases, p38, protein kinases A, glycogen synthase kinase 3 $\beta$ , and casein kinase.<sup>7,9</sup> These considerations suggest that although the NFAT-luciferase reporter mice can serve as a calcineurin assay surrogate in the heart, the effects of other signaling pathways likely modulate the magnitude and duration of the read-out. Finally, whereas the NFAT-luciferase reporter transgene reveals important correlative data suggesting a role for this pathway in various disease states of the heart, it does not prove a direct cause-and-effect relationship.





**Figure 6.** AdNFAT- and AdMEF2-luciferase reporter activity in neonatal cardiomyocyte cultures. **A**, NFAT-luciferase reporter activity from AdNFAT-luc cultures, at baseline (AdβGal) or after infection with AdΔCnA or AdΔNFATc3, in the presence or absence of CsA, 500 ng · mL<sup>-1</sup>. \**P* < 0.05 vs AdβGal; †*P* < 0.05 vs AdΔCnA. Results represent triplicate measures from each of 2 independent experiments. Cells were analyzed 24 hours after infection. **B**, Time course of NFAT-luciferase reporter activation in neonatal cardiomyocytes stimulated with 2% fetal calf serum in the presence or absence of infected AdNFATc3 (full-length). \**P* < 0.05 vs AdβGal; †*P* < 0.05 vs AdNFATc3; #*P* < 0.05 vs AdβGal + 2% serum. Results represent triplicate measures from each of 2 independent experiments. **C**, AdMEF2-luciferase reporter-infected cardiomyocytes assayed at baseline (AdβGal), with CsA, with AdΔCnA, or with 2% fetal bovine serum. \**P* < 0.05 vs AdβGal. **D**, NFAT- and MEF2-luciferase activity in response to 2% fetal bovine serum, IGF-1, or AdAkt coinfection. **E**, Effectiveness of IGF-1 treatment or AdAkt infection was demonstrated by Western blotting for phosphorylated Akt (right panels).

### Immediate Versus Delayed Activation of Calcineurin in Pathological Hypertrophy

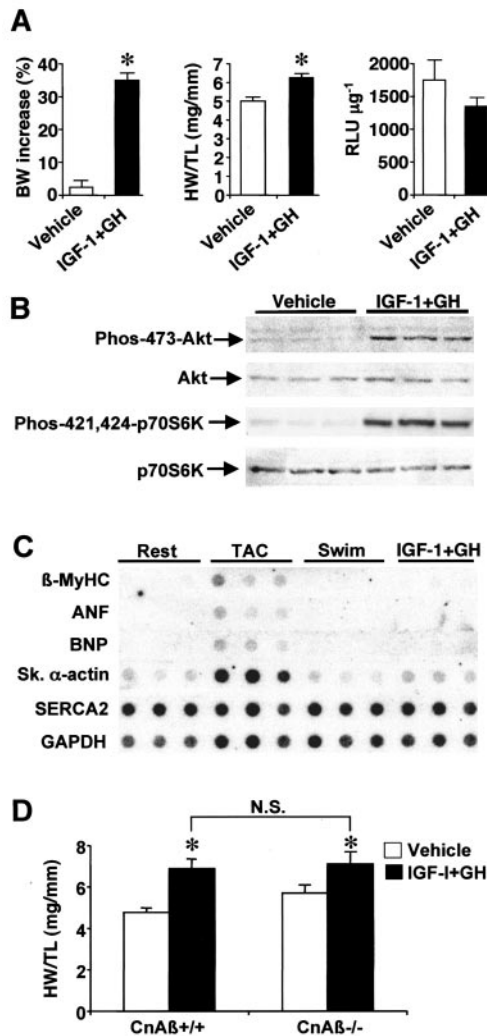
Our data showing continued activation of the NFAT-luc reporter throughout pressure-overload hypertrophy and infarct-induced failure strengthen the hypothesis that calcineurin-NFAT functions to maintain the hypertrophic profile of the heart. This hypothesis is also supported by the fact that hypertrophied and failing hearts have altered intracellular Ca<sup>2+</sup> handling,<sup>36</sup> potentially serving as part of the stimulus for calcineurin-NFAT activation. However, it is uncertain why significant calcineurin-NFAT activation is not observed until 24 to 36 hours after stimulation *in vitro* and *in vivo*. This is in contrast to *in vitro* reports using calcium ionophores, which stimulate NFAT translocation within 10 minutes.<sup>37</sup> One possible explanation lies in the mechanisms whereby NFAT factors are phosphorylated by the kinases discussed earlier, directly antagonizing effects of calcineurin. It is conceivable that, although NFATs initially accumulate in the nucleus immediately after an appropriate calcium signal, the actual transcriptional response is integrated over substantially longer periods of time that require maintained presence of NFAT. Indeed, many of the stimuli that activate calcineurin in cardiac myocytes also activate mitogen-activated protein kinases (MAPKs) and PKA, which would temporarily antagonize NFAT nuclear accumulation. However, it is possible that these kinase-dependent signaling pathways are partially desensitized within 1 to 2 days, thus permitting significant NFAT nuclear accumulation thereafter.

### Role of Calcineurin-NFAT in Pathological Versus Physiological Hypertrophy

In most models of pathological hypertrophy studied to date, inhibition of calcineurin-NFAT signaling has yielded either a reduction in the hypertrophic response and/or a delay in the

progression from hypertrophy to heart failure.<sup>9,38</sup> The data presented in this study extend this paradigm to demonstrate that calcineurin-NFAT signaling is activated in a sustained manner during both TAC-induced pressure overload and myocardial infarction-induced heart failure. However, very little is presently known regarding the role of calcineurin-NFAT signaling in regulating physiological hypertrophy or adaptive growth of the myocardium. Our results indicate that calcineurin-NFAT is not activated after either voluntary wheel-running or swimming, despite the observation of significant cardiac hypertrophy. In fact, swimming exercise even produced a significant and reproducible reduction in NFAT-luciferase activity in the heart at certain time points. Also of note, direct infusion of GH-IGF-1, which is thought to underlie adaptive or physiological growth, had no effect on NFAT-luciferase activity in the heart or in cultured cardiomyocytes. Indeed, a recent report from McMullen et al<sup>39</sup> has extended a convincing data set suggesting that IGF-1-dependent signaling through PI3K serves as a critical determinant of physiological but not pathological cardiac hypertrophy.

In contrast to our results, Eto et al<sup>40</sup> reported that calcineurin enzymatic activity was significantly increased in rat hearts subjected to voluntary exercise training, and that CsA blocked hypertrophy in this rat model. However, as discussed earlier, sole reliance on the enzymatic phosphatase assay from heart extracts is problematic. Rothermel et al<sup>23</sup> reported a reduction in exercise-induced cardiac hypertrophy in transgenic mice expressing the calcineurin inhibitory protein MCIP1 (muscle-enriched calcineurin-interacting protein-1). Although NFAT activation is exquisitely sensitive to calcineurin signaling, it is possible that calcineurin activity is partitioned between effectors based on the magnitude or duration of the stimulus. Indeed, calcineurin signaling has been previously shown to underlie exercise-induced activa-



**Figure 7.** GH-IGF-1 infusion in NFAT-luciferase reporter transgenic mice. **A**, Fourteen days of GH-IGF-1 injections induced a significant increase in total body weight (BW), heart weight to tibia length (HW/TL), but not relative luciferase activity (RLU) in the hearts of 8-week-old male mice. \* $P < 0.05$  vs vehicle treated. **B**, Cardiac Western blot analysis from GH-IGF-1-treated mice for phospho-Akt, total Akt levels, phospho-p70 S6K, and total p70 S6K. **C**, Dot blot analysis of cardiac RNA levels from mice treated/subjected to the indicated stimulus. **D**, Heart weight to tibia lengths in CnA $\beta^{+/+}$  ( $n=6$ ) and CnA $\beta^{-/-}$  ( $n=6$ ) mice treated with GH-IGF-1 for 14 days, or vehicle-treated ( $n=6$  each). \* $P < 0.05$  vs vehicle treated.

tion of a putative MEF2- $\beta$ -galactosidase reporter in skeletal muscle, although activity in the heart was not reported.<sup>41</sup> Although the results obtained with MEF2- and NFAT-dependent reporter mice are seemingly at odds, it is possible that exercise-induced hypertrophy results in a more transient alteration in calcium handling, either through inotropic drive or indirectly through IGF-1 signaling, such that calcineurin, MEF2, and NFAT might show different activation properties. In contrast, pathological hypertrophy is typically characterized by a continuous stimulus that may evoke a more sustained calcineurin response, hence mobilizing NFAT factors. However, we cannot formally disregard the possibility that calcineurin-NFAT signaling may still play some un-

known role in regulating the physiological hypertrophy program of the myocardium, potentially through a transcriptional regulatory partner such as AP-1 or GATA4. It is also possible that the NFAT-luciferase reporter line described in this study might be subject, in part, to nonspecific regulation through another factor that recognizes the multimerized NFAT site. However, levels of endogenous cardiac MCIP1 mRNA were not altered in mice subjected to swimming or GH-IGF-1 infusion (data not shown). Because MCIP1 expression is also directly regulated by NFAT activation in the heart, this result further supports the hypothesis that calcineurin-NFAT are not primary mediators of the physiological growth response.

Finally, the data presented in this study suggest that calcineurin inhibition would be desirable in potentially treating certain forms of maladaptive hypertrophy. However, given the relatively toxic profile of existing calcineurin inhibitory drugs, novel therapeutic agents would be needed. Also of concern, inhibition of calcineurin signaling may render the heart more susceptible to apoptotic cell death because this pathway has been previously implicated in the survival of cardiac myocytes after hypoxic stimuli, as extensively detailed previously.<sup>42</sup>

### Acknowledgments

This work and J.D.M. were supported by the NIH. B.J.W. was supported by a University of Cincinnati MD/PhD scholar award and the Albert J. Ryan Foundation. S.A.P. was supported by NIH training grant 5T32 HL07382.

### References

1. Lorell BH, Carabello BA. Left ventricular hypertrophy: pathogenesis, detection, and prognosis. *Circulation*. 2000;102:470–479.
2. Ho KK, Levy D, Kannel WB, Pinsky JL. The epidemiology of heart failure: the Framingham study. *J Am Coll Cardiol*. 1993;22:6–13.
3. Lloyd-Jones DM, Larson MG, Leip EP, Beiser A, D'Agostino RB, Kannel WB, Murabito JM, Vasan RS, Benjamin EJ, Levy D. Lifetime risk for developing congestive heart failure: the Framingham Heart Study. *Circulation*. 2002;106:3068–3072.
4. Oakley D. General cardiology: the athlete's heart. *Heart*. 2001;86:722–726.
5. Molkentin JD, Dorn GW 2nd. Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev Physiol*. 2001;63:391–426.
6. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45–79.
7. Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. *Cell*. 2002;109(suppl):S67–S79.
8. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 1998;93:215–228.
9. Wilkins BJ, Molkentin JD. Calcineurin and cardiac hypertrophy: where have we been? Where are we going? *J Physiol*. 2002;541:1–8.
10. Ritter O, Hack S, Schuh K, Rothlein N, Perrot A, Osterziel KJ, Schulte HD, Neyes L. Calcineurin in human heart hypertrophy. *Circulation*. 2002;105:2265–2269.
11. Lim HW, Molkentin JD. Calcineurin and human heart failure. *Nat Med*. 1999;5:246–247.
12. Haq S, Choukroun G, Lim H, Tymitz KM, del Monte F, Gwathmey J, Grazette L, Michael A, Hajjar R, Force T, Molkentin JD. Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. *Circulation*. 2001;103:670–677.
13. Kozma SC, Thomas G. Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K. *Bio Essays*. 2002;24:65–71.
14. Shioi T, Kang PM, Douglas PS, Hampe J, Yballe CM, Lawitts J, Cantley LC, Izumo S. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO J*. 2000;19:2537–2548.
15. Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC, Izumo S. Akt/protein kinase B promotes organ growth in transgenic mice. *Mol Cell Biol*. 2002;22:2799–2809.



16. Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C, Latronico MV, Napoli C, Sadoshima J, Croce CM, Ross J Jr. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc Natl Acad Sci U S A*. 2002;99:12333–12338.
17. Shiojima I, Yefremashvili M, Luo Z, Kureishi Y, Takahashi A, Tao J, Rosenzweig A, Kahn CR, Abel ED, Walsh K. Akt signaling mediates postnatal heart growth in response to insulin and nutritional status. *J Biol Chem*. 2002;277:37670–37677.
18. Matsui T, Li L, Wu JC, Cook SA, Nagoshi T, Picard MH, Liao R, Rosenzweig A. Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. *J Biol Chem*. 2002;277:22896–22901.
19. Musaró A, McCullagh KJA, Naya FJ, Olson EN, Rosenthal N. IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature*. 1999;400:581–585.
20. Semsarian C, Wu M-J, Ju Y-K, Marciniak T, Yeoh T, Allen DG, Harvey RP, Graham RM. Skeletal muscle hypertrophy is mediated by a  $\text{Ca}^{2+}$ -dependent calcineurin signaling pathway. *Nature*. 1999;400:576–580.
21. Taigen T, De Windt LJ, Lim HW, Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A*. 2000;97:1196–1201.
22. Hill JA, Karimi M, Kutschke W, Davison RL, Zimmerman K, Wang Z, Kerber RE, Weiss RM. Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. *Circulation*. 2000;101:2863–2869.
23. Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN, Williams RS. Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A*. 2001;98:3328–3333.
24. NHLBI Program for Genomic Applications Beth Israel Deaconess Medical Center, 2003. Available at: <http://www.cardiogenomics.org>. Accessed November 14, 2003.
25. Tanaka N, Ryoke T, Hongo M, Mao L, Rockman HA, Clark RG, Ross J Jr. Effects of growth hormone and IGF-I on cardiac hypertrophy and gene expression in mice. *Am J Physiol*. 1998;275:H393–H399.
26. De Windt LJ, Lim HW, Haq S, Force T, Molkentin JD. Calcineurin promotes protein kinase C and c-Jun NH2-terminal kinase activation in the heart: cross-talk between cardiac hypertrophic signaling pathways. *J Biol Chem*. 2000;275:13571–13579.
27. Ciana P, Di Luccio G, Belcredito S, Pollio G, Vegeto E, Tatangelo L, Tiverton C, Maggi A. Engineering of a mouse for the in vivo profiling of estrogen receptor activity. *Mol Endocrinol*. 2001;15:1104–1113.
28. Lim HW, De Windt LJ, Steinberg L, Taigen T, Witt SA, Kimball TR, Molkentin JD. Calcineurin expression, activation, and function in cardiac pressure-overload hypertrophy. *Circulation*. 2000;101:2431–2437.
29. Luo Z, Shyu KG, Gualberto A, Walsh K. Calcineurin inhibitors and cardiac hypertrophy. *Nat Med*. 1998;4:1092–1093.
30. Tsao L, Neville C, Musaro A, McCullagh KJ, Rosenthal N. Revisiting calcineurin and human heart failure. *Nat Med*. 2000;6:2–3.
31. Neri Serneri GG, Boddi M, Modesti PA, Cecioni I, Coppo M, Padeletti L, Michelucci A, Colella A, Galanti G. Increased cardiac sympathetic activity and insulin-like growth factor-I formation are associated with physiological hypertrophy in athletes. *Circ Res*. 2001;89:977–982.
32. Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, Lorenz JN, Molkentin JD. Impaired cardiac hypertrophic response in calcineurin A $\beta$ -deficient mice. *Proc Natl Acad Sci U S A*. 2002;99:4586–4591.
33. Naya FJ, Wu C, Richardson JA, Overbeek P, Olson EN. Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. *Development*. 1999;126:2045–2052.
34. Schmidt-Ullrich R, Memet S, Lilienbaum A, Feuillard J, Rapheal M, Israel A. NF- $\kappa$ B activity in transgenic mice: developmental regulation and tissue specificity. *Development*. 1996;122:2117–2128.
35. DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development*. 1999;126:4557–4568.
36. Balke CW, Shorofsky SR. Alterations in calcium handling in cardiac hypertrophy and heart failure. *Cardiovasc Res*. 1998;37:290–299.
37. Shibasaki F, Price ER, Milan D, McKeon F. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature*. 1996;382:370–373.
38. Molkentin JD. Calcineurin and beyond: cardiac hypertrophic signaling. *Circ Res*. 2000;87:731–738.
39. McMullen JR, Shioi T, Zhang L, Tarnavski O, Sherwood MC, Kang PM, Izumo S. Phosphoinositide 3-kinase (p110 $\alpha$ ) plays a critical role for the induction of physiological, but not pathological, cardiac hypertrophy. *Proc Natl Acad Sci U S A*. 2003;100:12355–12360.
40. Eto Y, Yonekura K, Sonoda M, Arai N, Sata M, Sugiura S, Takenaka K, Gualberto A, Hixon ML, Wagner MW, Aoyagi T. Calcineurin is activated in rat hearts with physiological left ventricular hypertrophy induced by voluntary exercise training. *Circulation*. 2000;101:2134–2137.
41. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. *EMBO J*. 2001;20:6414–6423.
42. De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW II, Kitsis RN, Molkentin JD. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: an apoptosis-independent model of dilated heart failure. *Circ Res*. 2000;86:255–263.