NFATc2 Is a Necessary Mediator of Calcineurin-dependent Cardiac Hypertrophy and Heart Failure

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One major intracellular signaling pathway involved in heart failure employs the phosphatase calcineurin and its downstream transcriptional effector nuclear factor of activated T-cells (NFAT). In vivo evidence for the involvement of NFAT factors in heart failure development is still ill defined. Here we reveal that nfatc2 transcripts outnumber those from other nfat genes in the unstimulated heart by severalfold. Transgenic mice with activated calcineurin in the postnatal myocardium crossbred with nfatc2-null mice revealed a significant abrogation of calcineurin-provoked cardiac growth, indicating that NFATc2 plays an important role downstream of calcineurin and validates the original hypothesis that calcineurin mediates myocyte hypertrophy through activation of NFAT transcription factors. In the absence of NFATc2, a clear protection against the geometrical, functional, and molecular deterioration of the myocardiun following biomechanical stress was also evident. In contrast, physiological cardiac enlargement in response to voluntary exercise training was not affected in nfatc2-null mice. Combined, these results reveal a major role for the NFATc2 transcription factor in pathologic cardiac remodeling and heart failure.

Heart failure, or the inability of the heart to meet hemodynamic demands, represents the end stage of various forms of cardiac disease. In the Western world, the prevalence and incidence of heart failure are increasing steadily, and heart failure is now the leading cause of hospitalization in the elderly. The leading cause of heart failure is left ventricular hypertrophy, defined as an increase in heart size without a change in myocyte number, because chronic hypertrophied hearts remodel and dilate (1, 2). Conversely, not all forms of cardiac hypertrophy are necessarily pathological, as athletic conditioning can stimulate heart growth without deleterious consequences (3). Hence, a better understanding of the mechanisms underlying pathological versus adaptive hypertrophic growth is key to develop preventative measures and therapeutics for heart failure patients (4).

Gain- and loss-of-function studies in genetically altered mice and cultured cardiomyocytes have demonstrated the sufficiency and necessity of calcineurin to regulate pathological cardiac hypertrophy (5–12). In contrast, in vivo confirmation about the involvement of its direct downstream transcriptional effectors in the heart is still incompletely resolved. Calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT)2 transcription factor family (13), allowing NFAT to translocate to the nucleus where it cooperates with other transcription factors to regulate calcineurin-responsive target genes. The ventricular cardiomyocyte contains all four calcineurin-sensitive NFATc isoforms, NFATc1 (NFATc), NFATc2 (NFATp), NFATc3 (NFAT4), and NFATc4 (NFAT3) (14, 15), and expression of dominant-negative forms of NFAT virtually abolishes calcineurin-mediated hypertrophy in cultured cardiomyocytes (14, 16). In vivo, however, nfatc4-null mice harboring a cardiac specific calcineurin transgene did not display a compromise of cardiac hypertrophy and heart failure (15). nfatc3-null mice are only very partially deficient in their ability to undergo cardiac hypertrophy and display no improvement on hypertrophic marker gene expression or cardiac dysfunction in response to calcineurin activation (15). Combined, a vast disparity exists between in vivo and in vitro studies concerning the involvement of NFAT factors in cardiac hypertrophy.

Here we provide evidence that NFATc2 mRNA levels are the most abundantly expressed in the heart among all NFAT isoforms. In line, nfatc2-deficient mice harboring a calcineurin transgene or subjected to pressure overload are substantially compromised in their ability to undergo cardiac hypertrophy. Moreover, at 8 weeks after pressure overload, echocardiography indicated marked left ventricular dilation and loss of systolic function in wild-type mice, whereas nfatc2-null mice displayed a prominent reduction in myofiber hypertrophy, preservation of left ventricular geometry and contractility,
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reduced fibrosis, and a diminished hypertrophic gene program. Remarkably, nfatc2-null mice were not compromised in their ability to undergo athletic cardiac enlargement. Taken together, these findings reveal a main role for NFATc2 downstream of calcineurin signaling in pathological cardiac remodeling.

EXPERIMENTAL PROCEDURES

Mice—αMHC-calcineurin transgenic mice, described previously (5) and generously provided by Eric N. Olson, were cross-bred with mice harboring a nfatc2 null mutation (17) and generously provided by Laurie Glimcher.

Aortic Banding and Angiotensin II Infusions—Angiotensin II infusion with Alzet 2002 mini osmotic pumps was performed as described previously (8). Transverse aortic (TAC) banding or sham surgery was performed in nfatc2+/+ or nfatc2−/− mice. The aorta was subjected to a defined, 27-gauge constriction between the first and second truncus of the aortic arch as described in detail previously (18). Pressure gradients between the proximal and distal sites of the transverse aortic constriction were determined by Doppler echocardiography (19) or invasive pressure measurements (18).

Transtrhastoracic Echocardiography—Cardiac remodeling and function were serially assessed at 2, 4, 6, and 8 weeks after TAC surgery by noninvasive echocardiography using a Hewlett-Packard Sonos 5500 instrument (Hewlett-Packard), 15-MHz transducer (15-6L linear probe, Philips Medical Systems) as described in detail previously (19).

Immunolabeling, Immunohistochemistry, and Immunofluorescence Microscopy—Hearts were harvested in diastole and perfusion-fixed with 4% paraformaldehyde and embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin, Sirius red, or fluorescent isothiocyanate-labeled wheat germ agglutinin (WGA). Slides were visualized using a Nikon Eclipse E600 microscope and a Zeiss Axiovert 135 for immunofluorescence. Cell surface areas were determined using SPOT imaging software (Diagnostic Instruments). Sections were immunolabeled with the following: Mac3 (1:30, Pharmingen) to detect macrophages; CD31 monoclonal antibody (1:50, Pharmingen) to detect capillaries, and CD45 (1:30, Pharmingen) to detect leukocytes.

Quantitative RT-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen). One μg of RNA was used as template for Superscript reverse transcriptase II (Promega) using indicated primer combinations (primer sequences available upon request). For real time RT-PCR, the Bio-Rad iCycler (Bio-Rad) and SYBR Green was used as described in detail previously (20) and as described in detail in the supplemental Expanded Methods.

Cage-wheel Exercise—Male nfatc2−/− and nfatc2+/+ mice were subjected to voluntary cage wheel exercise (21). Briefly, individual animals were individually housed in a cage equipped with an 11.5-cm-diameter running wheel with a 5.0-cm-wide running surface equipped with a digital magnetic counter activated by wheel rotation. Daily exercise values for time and distance run were recorded for individual exercised animals throughout the duration of the exercise period (4 weeks).

Statistical Analysis—The results are presented as means ± S.E. Statistical analyses were performed using INSTAT 3.0 software (GraphPad) and consisted of analysis of variance, followed by Tukey’s post-test when group differences were detected at the 5% significance level or the Student t test when two experimental groups were compared. Statistical significance was accepted at a p value <0.05.

RESULTS

NFATc2 Is the Most Abundant Isoform in the Mouse Heart—Recently, we demonstrated that all four calcineurin-regulated members of the NFAT family (NFATc1–c4) exist in cardiomyocytes (14, 15). Members of the NFAT transcription factor family are expressed as in multiple spliced transcripts (22–24). We analyzed the relative abundance of NFAT (splice) transcripts using quantitative RT-PCR, because commercially available antibodies against NFAT (splice) isoforms are qualitatively weak and unsuitable to provide relative NFAT isoform protein quantities. We found that transcripts for nfatc2 are the most abundant in excitable tissues such as brain, soleus muscle, and heart (Fig. 1a).

nfat genes can have redundant, overlapping functions in distinct organs. To analyze whether auto-amplification of nfat isoforms may exist in the heart, we quantified their transcripts in hearts from wild-type mice and transgenic mice harboring a constitutively active mutant of calcineurin under control of the Myh6 promoter (MHC-CnA), leading to a profound hypertrophy response in juvenile mice and fulminant heart failure at adulthood (5, 25). The results indicate that nfat transcript distribution remains relatively similar, except for slight increases...
Gravimetrical, histological, functional, and molecular analysis of calcineurin-transgenic mice crossed into a nfatc2-null background. a, representative gross morphology and hematoxylin and eosin-stained four-chamber view of hearts dissected from 3-week-old mice of indicated genotypes, demonstrating a profound rescue of cardiac enlargement by nfatc2 ablation downstream of calcineurin activation (bar, 5 mm). b, representative histological images of hearts from mice with genotypes indicated under each genotype bar, demonstrating a profound rescue of cardiac enlargement by nfatc2 ablation downstream of calcineurin activation (bar, 0.2 mm). Hematoxylin and eosin (H&E)-stained images reveal remarkable myocyte hypertrophy, myofiber disarray, and cellular infiltrates (arrowheads) in MHC-CnA/nfatc2+/+ mice, whereas MHC-CnA/nfatc2−/− mice are largely protected against these structural alterations. Sirius red staining indicates massive interstitial and perivascular fibrosis in hearts of MHC-CnA/nfatc2+/+ mice, which is attenuated in MHC-CnA/nfatc2−/− mice. Wheat germ agglutinin staining reveals a significant increase in cardiomyocyte size in MHC-CnA/nfatc2+/+ mice compared with unstimulated hearts (Fig. 1 b). c, representative M-mode images of hearts from mice with genotypes indicated under each genotype bar, demonstrating a profound rescue of cardiac enlargement by nfatc2 ablation downstream of calcineurin activation (bar, 5 mm). d, quantification of myofiber cross-sectional area from indicated genotypes shows significant attenuation of myocyte hypertrophy in MHC-CnA/nfatc2+/+ mice compared with MHC-CnA/nfatc2−/− mice (n = 3 per group). d, quantification of myofiber cross-sectional area from indicated genotypes shows significant attenuation of myocyte hypertrophy in MHC-CnA/nfatc2+/+ mice compared with MHC-CnA/nfatc2−/− mice (n = 3 per group). d, quantification of myofiber cross-sectional area from indicated genotypes shows significant attenuation of myocyte hypertrophy in MHC-CnA/nfatc2+/+ mice compared with MHC-CnA/nfatc2−/− mice (n = 5 per group). e, real time PCR analysis for transcript abundance in hearts from mice with genotypes indicated under each genotype bar, demonstrating a profound rescue of cardiac enlargement by nfatc2 ablation downstream of calcineurin activation (bar, 0.2 mm). f, bar graph representations of fractional shortening (FS) and LVID at systole, indicating protection against functional and geometrical deterioration after TAC compared with nfatc2+/+ mice (n = 4–6 per group). h and i, real time PCR analysis for rcan1.4 (h) and hypertrophic markers (i), all of which were increased in MHC-CnA/nfatc2+/+ mice and repressed in MHC-CnA/nfatc2−/− mice (n = 3–5 per group). * p < 0.05; ** p < 0.01.

NFATc2 Is Required for Calcineurin-induced Cardiac Hypertrophy—The transcriptional mechanisms whereby calcineurin initiates or maintains pathological hypertrophy in vivo are still ill defined. To determine the relevance of the relative abundance of nfatc2 transcripts downstream of calcineurin signaling in the postnatal heart, we crossedbred nfatc2-null mice with MHC-CnA mice. At 3 weeks of age, nfatc2+/+ and nfatc2−/− mice displayed comparable gross morphology and equal HW/BW ratios, a standardized measure of cardiac hypertrophy (5.6 ± 0.4 and 6.2 ± 0.4 mg/g, respectively). In contrast, MHC-CnA/nfatc2+/+ mice displayed grossly enlarged atrial and ventricular chambers, biventricular dilation, and a tripling of the HW/BW ratio (16.9 ± 0.6 mg/g; Fig. 2, a and c). Remarkably, MHC-CnA mice harboring a null mutation for the nfatc2...
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gene displayed a visible reduction in cardiac enlargement (11.5 ± 0.6 mg/g; Fig. 2b), which constitutes a decrease of 53% in HW/BW ratios compared with MHC-CnA/tnfatc2+/−/− mice. Body weights were not different between the four experimental cohorts (12.4 ± 1.0, 12.6 ± 0.9, 13.2 ± 0.9, and 12.0 ± 0.3 g, not significant for tnfatc2+/+, tnfatc2−/−, MHC-CnA/tnfatc2+/−/−, and MHC-CnA/tnfatc2−/− mice, respectively).

Histopathological analysis from hematoxylin and eosin-, Sirius red-, and wheat germ agglutinin (WGA)-stained cardiac sections revealed cardiomyocyte hypertrophy, myocyte disarray, mild invasion of inflammatory infiltrates, and extensive areas of interstitial and perivascular fibrosis were evident in MHC-CnA/tnfatc2+/+ hearts, whereas MHC-CnA/tnfatc2−/− mice did not display these abnormalities (Fig. 2b). As a more quantitative evaluation of individual myofiber hypertrophy, myofibril cross-sectional areas were quantified from WGA-stained sections. tnfatc2+/+ and tnfatc2−/− mice had similar myofiber cross-sectional areas, whereas MHC-CnA/tnfatc2+/++ mice had significantly increased individual myofibril size (Fig. 2d). In contrast, a 47% reduction was observed in MHC-CnA mice lacking tnfatc2. These data confirm that loss of tnfatc2 attenuates calcineurin-induced cardiac hypertrophy.

To examine the impact of tnfatc2 ablation on calcineurin-induced hemodynamic dysfunction, all cohorts were subjected to serial two-dimensional and M-mode echocardiography at 4 weeks of age. Representative images of M-mode recordings are displayed in Fig. 2c. An increase in left ventricular internal diameter (LVID) and a proportional decrease in systolic contractility (FS) were evident in the MHC-CnA/tnfatc2−/− mice, whereas these parameters were clearly improved in MHC-CnA/tnfatc2+/− animals (Fig. 2f and g).

Transcript abundance of the exon 4 splice isoform of rcan1 (regulator of calcineurin-1) may reflect a quantitative measure of total NFAT activity downstream of calcineurin in the heart (12). Transcripts for rcan1.4 were substantially up-regulated in MHC-CnA/tnfatc2+/+ mice and reduced to 50% in MHC-CnA/tnfatc2−/− hearts (Fig. 2h). Likewise, reactivation of fetal gene expression is a hallmark of pathological hypertrophy and heart failure, and therefore the expression levels of a number of fetal genes were determined. Transcripts for nppa (atrial natriuretic factor), nppb (brain natriuretic peptide), and myh7 (β-myosin heavy chain) were substantially repressed upon tnfatc2 deletion (Fig. 2i). In conclusion, these results indicate that loss of tnfatc2 led to a significant reduction of all major calcineurin-induced structural alterations in the myocardium.

Tnfatc2-deficient mice display modest splenomegaly, hyperproliferation of T- and B-cells, and dysregulated interleukin-4 production (17, 26). To exclude the possibility that the observed cardiac phenotype was indirectly related to the relative immunodeficiency because of loss of NFATc2, we analyzed histological sections of hearts from the experimental groups for macrophages (MAC3) and leukocytes (CD45). Cardiac sections of tnfatc2-null mice showed no increase in numbers of macrophages and infiltrated leukocytes (supplemental Figure). Likewise, NFATc2 was shown to promote angiogenesis by regulating c-Flip expression (27). To ascertain that tnfatc2 ablation did not influence the cardiac phenotype by dysregulating the myocardial angiogenic potential, we analyzed capillary densities in cardiac sections by staining with CD31 (supplemental Figure). We did not observe a difference in capillary density in cardiac sections of tnfatc2-null mice, MHC-CnA transgenic mice, or wild-type mice. These results indicate that tnfatc2 deficiency produces a fundamental deficit in the ability of calcineurin to execute a full myocyte hypertrophy response.

NFATc2 Deficiency Compromises Pathophysiologic Cardiac Hypertrophy—To determine whether NFATc2 also regulates hypertrophy in response to more physiologic stimuli apart from transgenic stimuli, continuous angiotensin II infusion was performed. Vehicle-treated tnfatc2+/+ and tnfatc2−/− mice displayed similar HW/BW ratios (4.1 ± 0.1 and 4.3 ± 0.1 mg/g, respectively). In response to angiotensin II, tnfatc2-null mice still developed some degree of hypertrophy, although this was significantly blunted compared with the response displayed by tnfatc2+/+ mice (Fig. 3a and b).

Next, TAC banding was performed, a surgical technique where the aorta was partially constricted for 1 week to mimic chronic hypertensive disease in humans. To validate that the surgical procedure produced equal pressure gradients in all experimental groups, transcarotid pressures were measured invasively (Fig. 3c). Sham-operated tnfatc2+/+ and tnfatc2−/− mice displayed similar HW/BW ratios (4.7 ± 0.1 and 5.3 ± 0.1 mg/g, respectively). In response to TAC, tnfatc2-null mice still developed some degree of hypertrophy, although this was significantly blunted compared with the response displayed by tnfatc2+/+ mice. This was further reflected in HW/BW ratios (6.0 ± 0.1 and 6.9 ± 0.2 mg/g, respectively; Fig. 3d), indicating that ablation of one single nfat isoform was sufficient to abrogate the early cardiac growth response by 68% in response to hemodynamic loading. Body weights were not different between the groups (28.3 ± 0.3, 25.4 ± 2.3, 27.8 ± 0.6, and 26.3 ± 1.2 g, not significant, for tnfatc2+/+ and tnfatc2−/− sham and tnfatc2+/+ and tnfatc2−/− TAC, respectively).

Hematoxylin and eosin- and Sirius red-stained cardiac histological sections did not show any signs of histopathology in tnfatc2+/+ and tnfatc2−/− sham-operated mice. In contrast, cardiomyocyte hypertrophy, myocyte disarray, mild invasion of inflammatory infiltrates, and extensive areas of interstitial and perivascular fibrosis were evident in pressure-overloaded tnfatc2+/+ hearts, whereas tnfatc2−/− mice displayed these abnormalities in a much milder form in response to TAC (Fig. 3, e and f). Myofibril cross-sectional areas were quantified from WGA-stained histological sections. tnfatc2+/+ and tnfatc2−/− sham-operated mice had similar myofiber cross-sectional areas (232 ± 7 and 256 ± 4 mm², respectively), whereas pressure-overloaded tnfatc2−/− mice had significantly decreased individual myofibril size compared with tnfatc2+/+ mice after TAC surgery (434 ± 10 and 588 ± 14 mm², respectively). These data confirm that loss of tnfatc2 reduced pressure overload-induced myofibril hypertrophy (Fig. 3g).

Reactivation of fetal gene expression is a hallmark of pathological hypertrophy and heart failure, and therefore the expression levels of a number of fetal genes were determined. Transcripts for nppa (atrial natriuretic factor), nppb (brain natriuretic peptide), and myh7 (β-myosin heavy chain) were substantially repressed upon pressure overload in tnfatc2-null mice compared with wild-type controls (Fig. 3g). Collectively,
FIGURE 3. Nfatc2 ablation attenuates agonist-induced and pressure overload-induced cardiac hypertrophy. a, representative gross morphology of hearts dissected from mice of indicated genotypes continuously infused with angiotensin II (AngII) or vehicle, demonstrating a profound rescue of cardiac enlargement by nfatc2 ablation (bar, 5 mm). b, heart weight to body weight (HW/BW) ratios of indicated genotypes show a decreased hypertrophic response for nfatc2−/− hearts compared with wild-type hearts after 2 weeks of vehicle or angiotensin II infusion (n = 6 per group). c, pressure gradients across the proximal and distal transverse aorta were measured invasively to validate the TAC procedure. d, HW/BW ratios of indicated genotypes subjected to sham or TAC surgery show a decreased hypertrophic response for nfatc2−/− hearts compared with wild-type hearts after 1 week of TAC (n = 6 per group). e, hematoxylin and eosin (H&E), Sirius red, and WGA staining indicates an increase in myocyte hypertrophy, myofiber disarray, cellular infiltrates (arrowheads), accumulation of interstitial and perivascular fibrosis, and increased myofiber cross-sectional areas in nfatc2−/− mice subjected to TAC compared with sham-operated genotypes, whereas this was attenuated in nfatc2−/− mice subjected to TAC. f, quantification of myofiber cross-sectional areas from WGA-stained sections of indicated genotypes (n = 3 per genotype, with 100 fibers counted per animal). g, real time PCR analysis for hypertrophic markers, all of which were increased in nfatc2−/− TAC mice and repressed in nfatc2−/− mice subjected to TAC (n = 3 per group). N.S., not significant; *, p < 0.05; **, p < 0.01.
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these results demonstrate a clear defect in the structural and molecular program of pathological cardiac hypertrophy in the absence of nfatc2.

NFATc2 Deficiency Ameliorates Heart Failure—To test whether sustained attenuation of pressure-overload hypertrophy ameliorates cardiac function and ensuing heart failure development in the absence of nfatc2, we performed TAC on nfatc2+/+ and nfatc2−/− mice for 8 weeks. To ensure equal loading conditions on all experimental groups, pressure gradients were measured noninvasively (Fig. 4a). At 8 weeks, gross morphology showed no differences between sham-operated nfatc2+/+ and nfatc2−/− mice (HW/BW ratios of 4.1 ± 0.2 and 4.4 ± 0.2 mg/g, respectively, not significant; see Fig. 4, b and c). In contrast, substantial cardiac enlargement was evident in nfatc2+/+ mice at 8 weeks after TAC surgery, whereas nfatc2−/− mice had visibly smaller hearts (Fig. 4, b and c). This was further reflected by HW/BW ratios (6.2 ± 0.3 and 5.8 ± 0.2 mg/g, respectively, p < 0.05; see Fig. 4c), indicating that nfatc2−/− mice displayed a sustained reduction in cardiac hypertrophy over longer periods of pressure overload. Hema-toxylin and eosin staining showed no myocyte disarray or infiltration of inflammatory cells in both sham groups. Pressure overloaded nfatc2−/− hearts showed less myocyte disarray and infiltration in sections compared with pressure-overloaded nfatc2+/+ hearts (Fig. 4g). Sirius red staining of hearts demonstrated a profound reduction in fibrosis in pressure-overloaded nfatc2−/− hearts compared with pressure overloaded nfatc2+/+ hearts (Fig. 4g).

To examine the impact of nfatc2 ablation on pressure overload-induced hemodynamic behavior, all cohorts were subjected to serial two-dimensional and M-mode echocardiography at 2, 4, 6, and 8 weeks after TAC. Representative images of M-mode recordings at 4 and 8 weeks are displayed in Fig. 4d. Four weeks after TAC, an increase in LVID (Fig. 4, d and e) and a proportional decrease in systolic contractility (FS) were evident in the nfatc2+/+ mice subjected to pressure overload (Fig. 4, d and f), in contrast to nfatc2-null animals. At 8 weeks after TAC, a thickening of the posterior wall in diastole, further increases in LVID, and progressive decreases in FS were visible in nfatc2+/+ mice, indicative of progressive left ventricular dilation and heart failure (Fig. 4e and Table 1). nfatc2-deficient mice displayed a significant reduction of these geometrical and functional deteriorations (Fig. 4, d–f, and Table 1). Taken together, these results indicate that nfatc2 deficiency not only protects the heart from pathological hypertrophy but also efficiently counteracts myocardial functional deterioration following biomechanical stress.

NFATc2 Ablation Does Not Affect Physiological Cardiac Hypertrophy—One vexing question relates to whether genetically distinct molecular mechanisms are employed to achieve pathological versus athletic cardiac enlargement, because the latter form of cardiac growth does not provoke hemodynamic demise or predisposes to heart failure. To this end, we chose voluntary running-wheel exercise (21) as a model to stimulate physiological cardiac hypertrophy in cohorts of nfatc2+/+ and nfatc2−/− mice. After 4 weeks of voluntary wheel exercise, nfatc2−/− mice were able to generate a cardiac growth response identical to that observed in nfatc2+/+ mice as evidenced by their HW/BW ratios (Fig. 5, b and c). As expected, exercised nfatc2+/+ or nfatc2−/− mice did not display any evidence of histopathology despite a 40% increase in heart weight. Our results indicate that calcineurin-NFAT signaling is not activated after voluntary wheel running, given that the hypertrophy response was not rescued in nfatc2 null mice.

DISCUSSION

Functional Hierarchy among Cardiac NFAT Isoforms in Cardiac Pathology—One unanticipated finding of this study is the relative high abundance of the NFATc2 isoform in cardiac muscle. Calcineurin-regulated members of the NFAT family (NFATc1–c4) are encoded by four separate genes and expressed as multiple spliced transcripts in rodents and human (13, 22–24). Recently, we demonstrated the existence of proteins for all four NFATc isoforms in cardiomyocytes (14, 15). Here, we analyzed the relative abundance of NFAT (splice) transcripts, because most commercially available antibodies proved ineffective to quantify the relative abundance of the low levels of NFAT proteins in the adult heart (15).

Here we show that mRNAs for nfatc4 and nfatc1 are relatively less abundant in the heart. Indeed, nfatc4-null mice harboring a cardiac specific calcineurin transgene did not display a compromise of cardiac hypertrophy and heart failure (15). In contrast, transcripts for nfatc3 and nfatc2 are relatively most abundant in the heart, with the latter still present at several -fold more than those for nfatc3. nfatc3-null mice are also partially deficient in their ability to undergo cardiac hypertrophy (15). In this study, we show that nfatc2-null mice display abrogation of calcineurin-provoked cardiac growth and a clear protection against the geometrical, functional, and molecular deterioration of the myocardium following hemodynamic loading. The combined findings imply predominant roles for nfatc2 and nfatc3, in the execution of cardiac remodeling and heart failure downstream of calcineurin. The collective findings would also suggest that mice deficient for both nfatc2 and nfatc3 might display an even more complete inhibition of calcineurin-mediated cardiac hypertrophy and heart failure. Conversely, given previous findings with nfatc4-null mice (15) and the very low transcripts levels for nfatc1 and nfatc4 we detected in this study, the combined observations also suggest that latter nfat isoforms have very little impact on calcineurin-dependent hypertrophy (15).

NFAT proteins can have redundant, overlapping functions in distinct organs. Indeed, NFATc1 and NFATc2 are involved in an autoregulatory mechanism controlling bone homeostasis by inducing transcription of nfatc1 by NFAT through its promoter region (28). We found that in the heart NFAT transcript distribution remains relatively similar, except for slight increases in NFATc1.1, NFATc3.1, and NFATc3.2 mRNA, and a relative decrease in NFATc4 mRNA compared with unstimulated hearts. The functional ramifications of this transcript redistribution remain unknown. Collectively, the data indicate that nfatc2 transcripts outnumber those from other nfat genes in the heart by severalfold and that mild auto-amplification loops involving nfatc1 and nfatc3 exist following calcineurin activation.
**FIGURE 4.** Nfatc2 deficiency prevents pressure overload-induced heart failure. 

a, pressure gradients across the proximal and distal transverse aorta were measured noninvasively to validate the TAC procedure. 
b, representative gross morphology of hearts dissected from mice of indicated genotypes subjected to 8 weeks of TAC, indicating profound rescue of cardiac enlargement by nfatc2 deletion (bar, 5 mm). 
c, heart weight to body weight (HW/BW) ratios of indicated genotypes subjected to sham or TAC surgery show a decreased hypertrophy response for nfatc2−/− hearts compared with wild-type hearts after 8 weeks of TAC (n = 6–10 per group). 
d, representative M-mode images of sham or TAC nfatc2+/+ and nfatc2−/− mice at 4 and 8 weeks indicates progressive dilation and loss of contractile behavior in nfatc2+/+ mice, which was substantially attenuated in nfatc2 null mice. 

e and f, bar graph representations of fractional shortening (FS) and left ventricular internal diameter at systole (LVIDs), indicating protection against functional and geometrical deterioration after TAC compared with nfatc2+/+ mice (n = 6–10 per group). 
g, hematoxylin and eosin (H&E), Sirius red, and WGA staining indicates an increase in myocyte hypertrophy, myofiber disarray, cellular infiltrates (arrowheads), accumulation of interstitial and perivascular fibrosis, and increased myofiber cross-sectional areas in nfatc2+/+ mice subjected to 8 weeks of TAC compared with sham-operated genotypes, whereas this was attenuated in nfatc2−/− mice subjected to TAC. 
h, quantification of myofiber cross-sectional areas from WGA-stained sections of indicated genotypes (n = 3 per genotype, with 100 fibers counted per animal). N.S., not significant; *, p < 0.05; **, p < 0.01.
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NFAT Signaling Is Genetically Restricted to Pathological Cardiac Growth and Maladaptive in Nature—Classical conceptualization has it that left ventricular hypertrophy would start as an adaptive, beneficial response to normalize wall stress to either altered mechanical loading conditions (e.g. resulting from valvular disease or chronic hypertension) or decreased performance because of loss of contractile units (e.g. after ischemic heart loss), and only later acquires maladaptive characteristics. Following this interpretation, increased wall thickness serves as the means to restore wall stress in line with the law of Laplace (29).

Recent insights have demanded a more nuanced interpretation of this phenomenon of “compensatory hypertrophy” and the absolute need to restore wall stress to prevent hemodynamic demise (12, 30, 31). First, ventricular hypertrophy is demonstrably a risk factor for cardiovascular mortality in humans (32). Second, beyond just increased mass, the specific long term transcriptional responses to increased load entail a myriad of quantitative and qualitative changes in cardiac gene expression that are reminiscent of fetal cardiac myocytes. In patients with cardiac failure, functional improvement related to treatment with β-blockers is correlated with beneficial changes in myocardial gene expression, most prominently exemplified by a correction in the mRNA expression level of the β-MHC gene (33). In this study we noted a pronounced decrease in β-MHC gene expression in nfatc2-null mice compared with their wild-type counterparts after hemodynamic loading. Conclusively, Laplace’s Law, although conceptually sound, does not take into account the qualitative alterations of the wall, and only incompletely explains the phenotypic particulars of heart enlargement.

In most models of pathological hypertrophy studied to date, inhibition of the calcineurin/NFAT axis has yielded either a reduction in the hypertrophic response and/or a delay in the progression from hypertrophy to heart failure (5–12). The data presented in this study extend this paradigm and demonstrate that NFAT transcriptional activity is activated in a sustained manner during pressure overload-induced cardiac remodeling and heart failure. Our results also provide genetic evidence that NFATC2 is not required for hypertrophic growth of the heart in response to exercise because heart weight remained unaffected in nfatc2-null mice following voluntary wheel running. These data are in line with earlier findings in a transgenic mouse model harboring an NFAT-sensitive luciferase reporter, which was selectively regulated by pathological hypertrophic remodeling and not by forced swimming exercise as a model to provoke physiological hypertrophy (34).

Because cardiac sections of wild-type and nfatc2 null mice did not show differences in infiltrating macrophages or leukocytes, nor displayed differences in capillary

**TABLE 1**

| Echo | \(n\) | Sham | TAC |
|------|------|------|------|
| DeltaP, mm Hg | 6 | 7 ± 2 | 50 ± 4* | 44 ± 3* |
| AWths, mm | 6 | 1.6 ± 0.1 | 1.6 ± 0.1 | 1.5 ± 0.1 |
| AWthd, mm | 7 | 1.2 ± 0.1 | 1.2 ± 0.1 | 1.1 ± 0.1 |
| LVIDs, mm | 6 | 2.6 ± 0.1 | 2.6 ± 0.1 | 2.7 ± 0.1 |
| LVIdm, mm | 6 | 3.6 ± 0.1 | 4.0 ± 0.1a | 3.7 ± 0.2b |
| PWths, mm | 6 | 1.1 ± 0.1 | 1.2 ± 0.1 | 1.3 ± 0.1 |
| PWthd, mm | 6 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 |
| FS (%) | 6 | 0.1 2.5 | 0.1 1.1 | 0.1 3.7 |

**Morphometry**

| HW (mg) | 6 | 127 ± 4 | 136 ± 8 | 193 ± 11a | 171 ± 7a,b *
| BW (g) | 6 | 32 ± 1 | 31 ± 1 | 31 ± 1 | 30 ± 1 |
| TL (mm) | 6 | 19 ± 1 | 18 ± 1 | 18 ± 1 | 18 ± 1 |
| HW/BW (mg/g) | 6 | 4.1 ± 0.2 | 4.4 ± 0.2 | 6.2 ± 0.3a | 5.8 ± 0.2ab |
| HW/TL (mg/mm) | 6 | 6.8 ± 0.2 | 7.6 ± 0.5 | 10.8 ± 0.7a | 9.5 ± 0.4ab |

* Values indicate \( p < 0.05 \) versus corresponding sham-operated group.

**FIGURE 5.** nfatc2 deficiency does not affect physiological hypertrophy. a, average daily distance that mice ran voluntarily. b, representative gross morphology from sedentary and exercised mice, indicating that exercised nfatc2+/+ and nfatc2−/− and cardiac remodeling. c, representative hematoxylin and eosin (H&E) and Sirius staining of heart sections of indicated genotypes indicates no histopathological alterations following exercise. N.S., not significant; *, \( p < 0.05 \).
density, we conclude that nfatc2 deficiency produces a fundamental deficit in the cardiac myocyte to execute a full hypertrophy response. Nevertheless, we cannot fully exclude the possibility that nonmyocyte-related effects secondary to systemic loss of nfatc2 may have influenced the cardiac phenotypes we observed. Combined, these data demonstrate that NFAT transcriptional activity is a required genetic pathway and selectively activated in pathological hypertrophy and ensuing heart failure. Furthermore, this study suggests that approaches targeting either NFATc2 activation or its immediate downstream target genes provide a suitable approach for future drug design to treat forms of pathological cardiac hypertrophy and heart failure.

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