Nuclear Factor of Activated T cells (NFAT):

Key regulator of cardiac hypertrophy and skeletal muscle adaptation

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Nucleaire Factor van Geactiveerde T cellen (NFAT):
Voornaamste regulator van cardiale hypertrofie en skelet spier adaptatie
(met een samenvatting in het Nederlands)

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A fact is a simple statement that everyone believes.
It is innocent, unless found guilty.
A hypothesis is a novel suggestion that no one wants to believe.
It is guilty, until found effective.

Edward Teller

Ter herinnering aan mijn oom
Aan mijn ouders

Contents

PREFACE

Despite significant progress in the prevention and treatment of cardiovascular diseases, heart failure is still a leading cause of morbidity and mortality in industrial countries. Sustained cardiac hypertrophy, which is defined as an increase in heart size resulting from an increase in cardiomyocyte cell volume, has been recognized as the single most important risk factor for heart failure development. Cardiac hypertrophy can be initiated by a wide array of (neuro/humoral) growth factors in response to increased workload, injury, or intrinsic defects in contractile performance. To understand the molecular determinants of the hypertrophic response and to achieve future rational drug design to treat heart failure, investigation currently focuses on identifying and characterizing intracellular signal transduction pathways in the heart.
The experiments presented in this thesis focus on a signaling pathway which plays a role in the hypertrophic transcriptional response of the myocyte. This signaling route employs the Ca^{2+}-calmodulin-dependent phosphatase calcineurin and its immediate downstream transcriptional effector Nuclear Factor of Activated T-cells (NFAT), and further focuses on the immediate downstream NFAT target genes in cardiac muscle.

In **chapter 1** we review the involvement of NFAT in cardiac hypertrophic remodeling in response to increased ventricular wall tension or neurohumoral stimulation, cardiac decompensation and heart failure. In this chapter, we describe the fundamental properties of NFAT transcription factors and discuss the principal studies implicating NFAT signaling so far as vital molecular motors behind the cardiac hypertrophy response. NFAT signaling is modulated by additional input from diverse signaling pathways, which affect NFAT kinases and (nuclear) partner proteins. We focus on the identity of these molecular inputs on the nuclear–cytoplasmic distribution and concurrent transcriptional activity of NFAT. Finally, an evaluation of the involvement of NFAT signaling in cardiac homeostasis or maladaptation is provided, as well as the rationale for future therapeutic approaches based upon NFAT antagonism.

In **chapter 2** the function of the different NFAT (splice)isoforms in vivo were addressed. One vital finding of this study is the relative high abundance of the NFATc2 isoform in cardiac muscle. It also shows that mRNAs for *nfatc4* and *nfatc1* are relatively low abundant in the heart. Indeed, it has been previously shown that *nfatc4*-null mice harboring a cardiac-specific calcineurin transgene did not display a compromise of cardiac hypertrophy and heart failure. In contrast, transcripts for *nfatc3* and *nfatc2* are relatively most abundant in the heart, with the latter still present at several folds more than those for *nfatc3*. A prior study showed that *nfatc3*-null mice are also partially deficient in their ability to undergo cardiac hypertrophy. 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 data presented in this chapter reinforces the paradigm that NFAT signaling is genetically restricted to pathological cardiac growth and maladaptive in nature. It shows 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 calcineurin/NFAT signaling is not activated after exercise because the cardiac growth response in response to voluntary wheel running remained unaffected in *nfatc2*-null mice. These data are fully in line with earlier findings in a transgenic mouse model harboring an NFAT-sensitive luciferase reporter gene, which was selectively activated by pathological hypertrophic remodeling and not by forced swimming exercise as a model to provoke physiological hypertrophy. Combined, these data demonstrate that NFAT transcriptional activity is a required genetic pathway that is selectively activated in pathological hypertrophy and ensuing heart failure. The data in this study suggest 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.

Since the calcineurin-NFAT pathway plays a very important role in cardiac hypertrophy, it could be very possible that it also plays an important role in later steps of pathological cardiac remodeling, which involve dilation of the left ventricle, the end stage form of heart failure. In **chapter 3**, we examined the involvement of NFATc2 in cardiac remodeling of *csrp3*-deficient mice. Disruption of the gene for *csrp3* leads to impaired cytoskeletal organization in cardiomyocytes leading to a form of pure dilated cardiomyopathy and severe heart failure.
Crossbreeding *csrp3*-null mice with *nfatc2* knockout mice (*csrp3/nfatc2* DKO) resulted in a remarkable rescue of the dilated cardiac phenotype and normalized cardiac function in double KOs. These data suggest that calcineurin/NFAT signaling may be more prominently and selectively involved in later stages of heart failure development (when dilation ensues), since we only obtained partial rescue of pressure overload-induced cardiac hypertrophy and dysfunction by *nfatc2* gene deletion. We can conclude that pathological cardiac remodeling in *csrp3* deficient mice is dependent of NFATc2.

Calcineurin is a required component of cardiac hypertrophy and dependent upon its transcriptional effector, Nuclear Factor of Activated T cells (NFAT), but limited information is available on the nature and number of NFAT target genes. In **chapter 4** we describe a study where we constructed murine ventricular clones allowing inducible expression of activated forms of calcineurin or NFAT using a binary, doxycyclin-dependent system. Two double-stable calcineurin and NFAT clones were selected to control for potential cell based variations and subjected to whole genome Agilent arrays. Among the 27 earliest target genes, the zinc finger-containing transcription factor Gata4 was identified as an early calcineurin/NFAT target gene. Electromobility shift and chromatin immunoprecipitation assays demonstrated the existence of an evolutionary conserved NFAT consensus-bindingsite

in a gata4 enhancer. Gata4 transcripts were increased in calcineurin Tg mice and pressure overloaded hearts. In line, elevated Gata4 protein levels were evident in calcineurin Tg mice as well as in human heart failure biopsies. Finally, siRNA-mediated knockdown of Gata4 abrogated calcineurin-mediated cardiomyocyte hypertrophy in primary cardiomyocyte cultures, indicating the functional requirement of Gata4 downstream of cardiac calcineurin/NFAT signaling. Given that NFAT and pre-existing Gata4 proteins also function as transcriptional partners, the present data reveal a novel feedforward mechanism where NFAT signaling provokes expression of the pro-hypertrophic Gata4 transcription factor, allowing amplification of calcineurin/NFAT signaling in the ventricular myocyte.

Calcineurin/NFAT signaling is also involved in multiple aspects of skeletal muscle development and disease. The myogenic basic helix-loop-helix (bHLH) transcription factors, MyoD, myogenin, Myf5, and MRF4 specify the myogenic lineage. In chapter five, we show that calcineurin/NFAT signaling is required for primary myogenesis by transcriptional cooperation with the bHLH transcription factor MyoD. Calcineurin/NFAT signaling is required for myogenin expression in differentiating myoblasts, where the myogenic regulatory factor MyoD selectively recruits NFATc3 to the myogenin promoter. In **chapter 5** we used gelshift and chromatin immunoprecipitation assays to identify three evolutionary conserved NFAT binding sites in the myogenin promoter, which were occupied by NFATc3 upon skeletal muscle differentiation. The transcriptional integration between NFATc3 and MyoD is crucial for primary myogenesis in vivo, since myogenin expression is absent in *myod:nfatc3* double null embryos, while myogenin expression is unaffected in embryos with null mutations for either factor alone. Thus, the combined findings provide a novel transcriptional paradigm for the first steps of myogenesis, where a calcineurin/NFATc3 pathway regulates myogenin induction in cooperation with MyoD.

Chapter 6 reviews our findings and extrapolates the results described in this thesis to the current knowledge on hypertrophic signaling and transcriptional remodeling to give direction to future research.

CHAPTER 1

NFAT transcription factors in cardiac (mal)adaptation

Paula A. da Costa Martins; Meriem Bourajjaj and Leon J. De Windt

Summary

In response to increased ventricular wall tension or neurohumoral stimuli, the myocardium undergoes hypertrophic remodeling, which promotes decompensation and heart failure. Central regulators implicated in the hypertrophic response are the members of the transcription factor family of Nuclear Factor of Activated T-cells (NFAT). In this review, we describe the fundamental properties of NFAT transcription factors and discuss the principal studies implicating NFAT signaling as a vital molecular pathway for the cardiac hypertrophy response. NFAT signaling is modulated by additional input from diverse signaling pathways, which affect NFAT kinases and (nuclear) partner proteins. We focus on the identity of these molecular inputs on the nuclear–cytoplasmic distribution and concurrent transcriptional activity of NFAT. Finally, an evaluation of the involvement of NFAT signaling in cardiac homeostasis or maladaptation is provided, as well as the rationale for future therapeutic approaches based upon NFAT antagonism.

Fundamental properties of NFAT transcription factors

The nuclear factor of activated T cells (NFAT), functionally related to the Rel/NFkB family of transcriptional activators (1, 2), is a family of transcription factors composed of five proteins. These include NFATc1 (NFATc, NFAT2), NFATc2 (NFAT1, NFATp), NFATc3 (NFATx, NFAT4), NFATc4 (NFAT3), and NFAT5 (TonEBP: tonicity element binding protein or OREBP: osmotic response element binding protein), the only NFAT related protein represented in the *Drosophila* genome (3), a transcription factor crucial for cellular responses to hypertonic stress (4). We focus here on the remaining four NFAT proteins (NFATc1–c4; see Table 1), referring to them collectively as NFAT, given that the role of NFAT5 in the cardiovascular system remains unexplored.

NFAT activation is initiated by dephosphorylation of the NFAT regulatory domain, a conserved ~300-amino acid region located N-terminal to the DNA-binding domain (Fig. 1), which is encoded in a single exon in four NFAT proteins from all vertebrate species (2). The domain is heavily phosphorylated in resting cells, with the phosphorylated serine residues distributed among four classes of conserved serine-rich sequence motifs (SRR domains, SP repeats, and KTS motifs; Fig. 1) (5, 6). Calcineurin dephosphorylates three of the four types of motifs, thus triggering NFAT nuclear accumulation and increasing the affinity of NFAT for its target sites in DNA (6) (7) (8).

Efficient activation of NFAT proteins requires a docking interaction between NFAT and calcineurin (9) (10). The major docking site for calcineurin is located at the N terminus of the NFAT regulatory domain, and has the consensus sequence PxIxIT (SPRIEIT in NFATc2; Fig. 1). The individual NFAT proteins possess characteristic PxIxIT sequences with a low affinity for calcineurin (Kd = 10–30 μM) needed to maintain sensitivity to environmental signals and to prevent constitutive activation of NFAT. Substitution of the SPRIEIT sequence of NFAT1 with HPVIVIT, a higher-affinity version obtained by peptide selection, increased the basal calcineurin sensitivity of the protein and resulted in partial nuclear localization (9). The surface of NFAT–calcineurin interaction is more extensive than the PxIxIT motif, however, because a second interacting sequence has been identified in NFAT2 and in NFAT4 (11). This sequence (calcineurin-binding sequence B in Fig. 1) is moderately conserved in the NFAT proteins and resembles a highly conserved sequence in the calcineurin inhibitors DSCR1/MCIP1 (12) (13, 14). Structural and cell-biological studies suggest that NFAT and DSCR1/MCIP1 use this region for calcineurin binding and inhibition, and compete for calcineurin binding in cells (14, 15).

Recent structural data emphasize the remarkable versatility of NFAT binding to DNA. Transcriptional activity of NFATs is either activating or deactivating depending on their binding partners, which include AP-1 (composed of Fos and Jun proteins), MEF2, GATA proteins and histone deacetylases (HDACs). The role of NFAT as a transcriptional activator, by coupling with different transcriptional regulators in the cardiovascular system, is slowly being elucidated (16) (17) (18). These studies suggest diverse mechanisms by which NFAT can function as an activator or a deactivator or regulate distinct cardiac phenotypes (16), depending on its binding partners in the nucleus where NFAT binds to specific loci in NFAT-regulated genes.

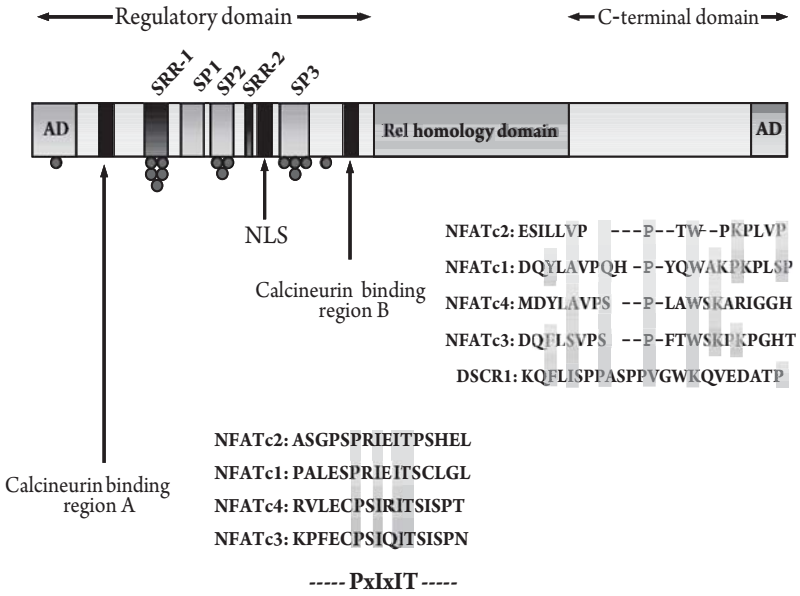

Figure 1. Schematic overview of the functional domains in NFAT: sites of phosphorylation and interaction with calcineurin. (Top) Overall structure of NFAT proteins, based upon on murine NFATc2. Regulatory-domain phosphorylations in conserved sequence motifs are shown as circles below the motif. Red circles indicate phosphate groups that are removed by calcineurin. The two regions involved in contacting calcineurin are indicated. Calcineurin binding Region A is the PxIxIT sequence found in all NFAT proteins, and region B has been defined in NFAT2 and in NFAT4. (AD) Activation domains; (NLS) nuclear localization signal in the regulatory domain. Sequences of the calcineurin-binding region A and calcineurin binding region A of NFAT2 aligned with the corresponding sequences of other NFAT proteins and the similar sequence in DSCR1.

Evidence for calcineurin/NFAT signaling in cardiac (mal)adaptation

Gain- and loss-of-function studies in genetically altered mice and in cultured cardiomyocytes have demonstrated the necessity and sufficiency of calcineurin, the direct upstream activator of NFAT (Fig. 2), to regulate pathologic cardiac hypertrophy (19) (20) (21). Calcineurin was originally implicated as a hypertrophic signaling factor based on its overexpression in the hearts of transgenic mice (17), which demonstrate a profound hypertrophic response (a tripling in heart size) that rapidly progressed to fulminant heart failure (22). In vitro, infection of cultured cardiomyocytes with an adenovirus expressing an activated form of calcineurin similarly induced a hypertrophic response, supporting the sufficiency of calcineurin as a hypertrophic mediator in two different model systems (17, 22).

The requirement of calcineurin activation in response to pathophysiologic stress *in vivo* is now broadly accepted. Given that calcineurin is considered to be the main target for the immunesuppressants cyclosporine and FK506 (tacrolimus), investigators first employed these pharmacological agents to directly assess the cause-and-effect relationship between calcineurin activation and the initiation and propagation of cardiac hypertrophy in a wide variety of rodent models for pathological hypertrophy (19) (20) (21). Although these agents proved to be instrumental in assessing calcineurin/NFAT involvement in initial studies (23), a number of negative reports quickly succeeded, which concluded no regulatory role for calcineurin in aortic-banded mice or rats (24, 25) (26), or proved to be detrimental for disease progression in MyHC 403 mutant mice (27). These contradictory results spurred a great deal of successive pharmacologic studies and, more importantly, genetic assessments into the causal linkage between calcineurin/NFAT signaling in cardiac hypertrophy. Nearly all of these subsequent studies have supported the initial hypothesis that this signaling paradigm is both sufficient and required for the hypertrophy response *in vivo* (19, 20) (21).

More indirect evidence derives from studies employing genetic defects in the direct upstream activator of NFAT signaling, calcineurin. Calcineurin Aβ null mice display a slight but significant reduction in heart weight in the unstimulated state, suggesting that calcineurin/NFAT signaling might also underly early maturational heart size at baseline (28). Moreover, these null mice have a clearly failed cardiac hypertrophy response to pressure overload and catecholamine infusion. Overexpression of MCIP1 (myocyte enriched calcineurin interacting protein-1), a small product of the *DSCR1* gene (Down's syndrome critical region 1), can function as an effective protein inhibitor of calcineurin (see below). Mice overexpressing the MCIP1 gene product in postnatal heart tissue nullify the cardiomyopathic

phenotype of mice harboring a cardiac specific transgene of calcineurin, and are resistant to cardiac hypertrophy in response to β-adrenergic stimulation or exercise training (29). In addition, cardiac overexpression of MCIP1 provided protection against postinfarction-induced pathological LV remodeling (30).

Finally, other calcineurin inhibitors are Cain (for calcineurin inhibitory protein), a noncompetitive inhibitor of calcineurin/NFAT signaling (31), and AKAP79 (for A-kinase anchoring protein), a scaffold protein, which besides calcineurin binds protein kinase A and protein kinase C and is thought to anchor multiple classes of signaling modules in the vicinity of substrates to facilitate their proper and timed activation (32). The calcineurin binding domains of either Cain (Δcain) and AKAP79 (ΔAKAP79) were overexpressed in postnatal mouse hearts, resulting in reduced calcineurin activity and resistance to pressure overload- and agonist-induced cardiac hypertrophy. Moreover, viral gene transfer of the ΔCain peptide into the adult rat myocardium yielded resistance to calcineurin activation and pressure overload hypertrophy (33). These reports utilizing such diverse genetic strategies to inhibit calcineurin activity (null mutation in the calcineurin Aβ gene, ΔCain, ΔAKAP79, MCIP1) have made it hard to dispute that calcineurin/NFAT signaling is a required component of hypertrophic signaling following *in vivo*.

Fewer studies have directly focused on NFAT itself. Two studies employed ectopic expression of a dominant negative NFAT factor or the high affinity peptide HPVIVIT in cultured cardiomyocytes by viral gene transfer. Transduced cardiomyocytes demonstrated an impaired ability to cytoplasm-nuclear translocation of endogenous NFAT isoforms and were resilient to calcineurin or agonist-induced cardiomyocyte hypertrophy in vitro (34, 35). Even less information is available in terms of *in vivo* genetic studies to deduce the function of individual NFAT genes using NFAT gene-targeted mice. While targeted disruption of NFATc4 did not diminish the magnitude of calcineurin transgene-dependent hypertrophy or pressure overload hypertrophy, NFATc3 null mice showed a significant and long-standing reduction in calcineurin-induced hypertrophy and in response to pressure overload and chronic endocrine stimulation (36). Although these results suggest that the NFATc3 isoform may be a mediator of cardiac hypertrophy and validates the original hypothesis that calcineurin/NFAT signaling provokes myocyte hypertrophy, it remains to be established what contributions other NFAT isoforms might have in cardiac disease.

Positive modulation of NFAT activation

Ca^{2+} entry leading to Ca^{2+}/calmodulin signaling positively modulates calcineurin and results in activation of NFAT transcription factors. Given the mechanism of activation of NFAT (dephosphorylation of critical residues in the regulatory domain; Fig.1), when Ca^{2+} entry is prevented or calcineurin activity is inhibited, NFAT is rephosphorylated by NFAT kinases, rapidly leaves the nucleus (t1/2 ≈ 15 min), and NFAT dependent gene expression is terminated (37, 38), (39). As a result of this absolute dependence on Ca^{2+}/calcineurin signaling, NFAT has a remarkable ability to sense dynamic changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_I$) and frequencies of Ca^{2+} oscillations in cells (40-42).

Nevertheless, despite the large and convincing body of literature supporting the involvement of calcineurin in cadiac hypertrophy, the actual source of Ca^{2+} that activates calcineurin/NFAT signaling in heart muscle (or many other parallel Ca^{2+}/calmodulin signaling modules for that matter) is incompletely understood. This query is further complicated by the fundamental uncertainty how the cardiac muscle can distinguish between changes in Ca^{2+} that result in calmodulin activation versus the vast fluctuations in Ca^{2+} that provoke each cycle of contraction and relaxation during excitation-contraction coupling.

Studies using the L-type Ca^{2+} blockers verapamil and nicadipine suggest a plausible mechanism that involves this Ca^{2+} entry current ($I_{Ca,L}$) in calcineurin activation following agonist stimulation with endotheline-1, angiotensin II, phenylephrine or leukemia inhibitory factor (43, 44). More recently, inositol 1,4,5-trisphosphate receptors (InsP3Rs) in ventricular myocytes, which are mainly localted in the nuclear envelope, where implicated in providing the Ca^{2+} source to activate another Ca^{2+}/calmodulin signaling cascade, CaMKII, and activation of the hypertrophic gene program. The group of Bers (45) demonstrated that ET-1, which activates plasmalemmal G protein-coupled receptors and InsP3 production, elicits local nuclear envelope Ca^{2+} release via InsP3R. This localized Ca^{2+} release in turn activated nuclear CaMKII, which triggered derepression of gene transcription via histone deacytelases (HDACs). The global Ca^{2+} transients that cause contraction at each heartbeat could not activate this Ca^{2+}-dependent pathway, suggesting one mechanism for how excitation-contraction coupling is physically and functionally insulated from excitation-transcription coupling (45).

A more recently uncovered and fundamentally distinct mechanism of NFAT activation involves the activation of calpain-dependent proteolytic pathways in heart failure, leading to proteolysis of the autoinhibitory domain of the catalytic calcineurin subunit. This proteolytic event renders calcineurin constitutively

nuclear and active, even after removal of the hypertrophic stimulus (46). Inhibition of proteolytic cleavage of calcineurin by a noninhibitory control peptide prevents the development of myocardial hypertrophy. These findings suggest that calcineurin not only activates NFAT by direct dephosphorylating NFAT, enabling its nuclear import, but its presence in the nucleus is also important for full NFAT transcriptional activity (46, 47).

ERK1/2 (extracellular signal-regulated kinase), a branch of the mitogen-activated protein kinase (MAPK) superfamily, signaling was associated with increased concentrations of NFATc4 in the nucleus and transcriptional activity in cardiac myocytes (48). More recently, the group of Molkentin (18) uncovered that MEK1-ERK1/2 signaling in heart muscle stimulates NFAT transcriptional activity by two distinct mechanisms. First, ERK1/2 signaling induces cardiac AP-1 activity, which functions as an essential NFAT-interacting transcriptional partner. As a second mechanism, MEK1/ERK1/2 and calcineurin-NFAT proteins form a complex in cardiac myocytes, resulting in direct phosphorylation of NFATc3 within its C terminus and augmenting its DNA binding activity. These findings established the calcineurin/NFAT and MEK1/ERK1/2 signaling paradigms as a co-dependent signaling module in cardiomyocytes and suggest that a productive hypertrophic response requires transcriptional cross-talk between both pathways in the heart (18).

Modulators of NFAT nuclear export

Several studies have evidenced the ability of select members of the mitogen-activated protein kinase (MAPK) branches to directly antagonize calcineurin/NFAT signaling. JNK1/2 and p38, two branches of the MAPK superfamily, directly phosphorylate the N-terminal regulatory domain of specific NFAT transcription factors, including NFATc1, NFATc2, NFATc3, resulting in nuclear export and suppression of NFAT transcriptional activity (7) (49) (50-52). In line, several studies evidenced that activation of either JNK or p38 MAPK do not correlate with cardiac hypertrophy (53, 54), but rather antagonize the *in vivo* growth response through negative crosstalk with NFAT activation (55).

NFAT dephosphorylation by calcineurin is also countered by other distinct NFAT kinases, among them casein kinase 1 (CK1) (56), protein kinase A (PKA) (57) and glycogen synthase kinase 3β (GSK3β) (5). The involvement of GSK3β as a NFAT kinase has been established in heart muscle, where it phosphorylates conserved serines in the N-terminal regulatory region of NFAT proteins, thereby promoting their nuclear export and contributing to its antihypertrophic effects

(58, 59). This ability of GSK3β to oppose calcineurin signaling fits with the demonstration that GSK3 is a potent negative regulator of cardiac hypertrophy (60). GSK3β regulation of NFAT signaling is likely more complicated, given that Hilioti and coworkers (61)demonstrated that kinases from the GSK3 family were able to phosphorylate the yeast homologue of MCIP1 converting it from an inhibitor to a stimulator of calcineurin activity (61).

More recently, DYRKs (dual-specific tyrosine-phosphorylation regulated kinases) have been identified as novel regulators of NFAT from a genome wide RNA interference (RNAi) screen in *Drosophila*. DYRK1 and DYRK2 directly phosphorylate the conserved serine-proline repeat 3 (SP-3) motif of the NFAT regulatory domain. DYRK, GSK3 and CK1 target distinctive motifs of the NFAT1 regulatory domain but DYRK-mediated phosphorylation of the SP-3 motifs primes for further phosphorylation of the distinct SRR-1 and SP-2 motifs by CK1 and GSK3, respectively, thus facilitaing complete phosphorylation and deactivation of NFAT1 (62). Whether DYRKs also function as NFAT kinases in the heart muscle and antagonize cardiac hypertrophy remains to be elucidated.

One very distinct mechanism for NFAT antagonism involves the modulatory calcineurin-interacting protein (MCIP), or Down Syndrome Critical Region 1 (DSCR1) and DSCR1-like proteins calcipressins, which is part of a gene family that includes *mcip1*, *mcip2*, and *mcip3* (63). In invertrebrates species, the MCIP homologue, RCN1 CBP1, has functional and phenotypic characteristics of a calcineurin activator (13, 61). Consistent with this observation, *mcip1* and *mcip2* null mice were recently shown to impair the cardiac hypertrophic response to pressure overload, suggesting that it may also function as a calcineurin facilitator *in vivo* (15). When overexpressed, however, MCIPs are able to effectively block calcineurin/NFAT activity both *in vitro* and *in vivo* by directly binding to the active site on calcineurin (29).

Signaling via cGMP and cGMP-dependent protein kinase type I (PKG I), has been recognized as a negative regulator of cardiac myocyte (CM) hypertrophy by targeting the calcineurin-NFAT signaling pathway. PKG I suppresses single L-type Ca^{2+}-channel open probability, $[Ca^{2+}]_i$ transient amplitude, and L-type Ca^{2+}-channel current-induced NFAT activation, indicating that PKG I targets Ca^{2+}-dependent steps upstream of calcineurin (64). Taken together, it seems that several protein kinases have fairly overlapping functions in the heart where they counterregulate NFAT activity, and as such play an antagonistic role in the orquestration of the cardiac growth response.

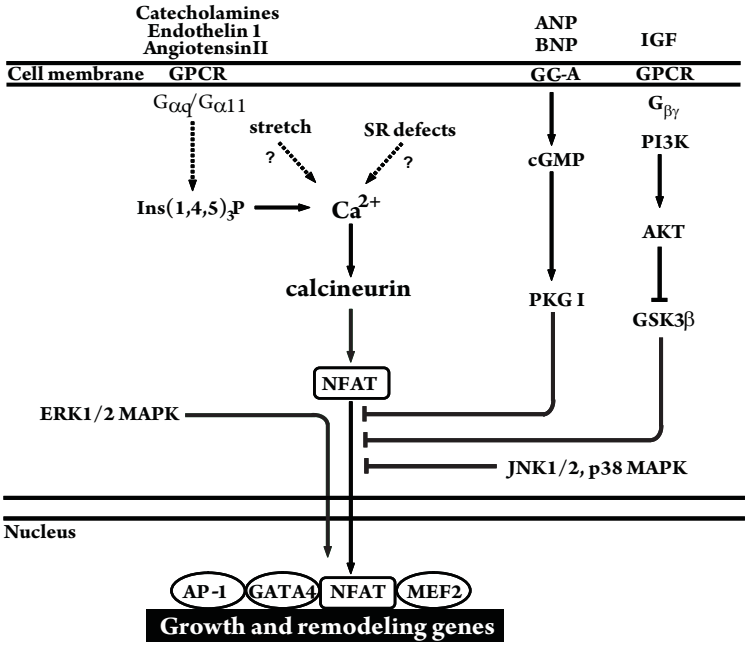

Figure 2. Schematic representation of intracellular signaling cascades that modulate NFAT transcriptional activity. Signaling that initiates at the cell membrane by various agonists is shown at the top, intermediate signaling enzymes (kinases, phosphatases) is shown in the middle, and below the nucleus is depicted harboring various transcriptional signaling events, including activated NFAT, which activates gene transcription in cooperation with other transcription factors as GATA4 and MEF2. Green arrows indicate signaling events that directly activate NFAT; red lines indicate kinases that rephosphorylate NFAT and antagonize NFAST transcriptional activity. ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; IGF-1, insulin-like growth factor-1; GPCR, G-protein-coupled receptors; GC-A, guanyl cyclase-A; Ins(1,4,5)P3, inositol-1,4,5-triphosphate; SR, sarcoplasmatic reticulum; cGMP, cyclic GMP; PKG I, cGMP-dependent protein kinase I; GSK3b, glycogen synthase kinase-3b; ERK1/2, extracellular signaling regulated kinase; JNK1/2, c-Jun N-terminal kinase; MEF2, myocyte enhancer factor-2.

NFAT transcriptional activity in cardiac (mal)adaptation

One final vexing question relates to whether calcineurin/NFAT signaling is exclusively restricted to pathological forms of cardiac hypertrophy, or whether it is also implicated in physiological and/or developmental myocardial growth. Hypertrophy as a consequence of overload is still considered "compensatory" on the premise that it facilitates ejection performance by normalizing systolic wall stress (65). Recent experimental results, however, call into question the necessity of normalization of wall stress that results from hypertrophic growth of the heart (66). Another study fueled the controversy by providing evidence that inhibition of calcineurin/NFAT signaling abrogated postinfarction-induced pathological LV remodeling, and thus challenging the adaptive value of post-MI hypertrophy of the remote myocardium (30). These findings raise the prospect of therapeutically modulating hypertrophic growth of the myocardium to afford clinical benefit without provoking hemodynamic compromise, provided that primarely pathological signaling paradigms would be targeted.

The premise that calcineurin/NFAT signaling is restricted to pathological forms of hypertrophy stems from the phenotypic characterizations of calcineurin transgenic mice, which quickly transitions to heart failure following an initial massive increase in cardiac mass (16, 20, 22). Another convincing study failed to observe cardiac NFAT transcriptional activity following swimming exercise or combined IGF-1/GH injections (approaches that mimic physiological cardiac growth) using transgenic NFAT reporter mice (67). Finally, MCIP1 transgenic mice display improved contractility following myocardial infarction (30) or pressure overload (68). Studies suggestive of an additional role for calcineurin/NFAT signaling in developmental/physiological forms are inferred from the use of the calcineurin inhibitors cyclosporine A and FK506, and the reduced basal heart size in calcineurin Aβ-gene targeted mice, although NFATc2, -c3 and –c4 gene targeted mice have normal heart size (36) and our unpublished observations). The overall experimental data suggest promising outcomes from future inhibitory approaches to NFAT signaling in the adult heart.

Although the role for NFAT signaling circuit as a mediator of pathological growth has been largely established, its current therapeutic potential is wrought with complications, given that the existing small molecule inhibitor repertoire cyclosporine A and FK506 (which indirectly target calcineurin) are associated with toxicity and lack of tissue-specificity. Future experimental efforts will likely uncover new and more specific NFAT inhibitory compounds (69), the testing of which should not be confined to caged rodents with short life spans, but extended to large

animal models. The recent discoveries demonstrating that the "compensatory" role of cardiac hypertrophy is not universally valid will facilitate the search for therapies targeting hypertrophy (70), with heart failure patients likely being the primary beneficiaries.

Table 1. The NFAT transcription factor family.

Name	Synonyms	Regulation	Chromosomal localization * (human/mouse)	Phenotype knockout mice	Cardiovascular phenotype
NFATc1	NFAT2, NFATc	Ca^{2+}/calcineurin	18q23/18 54.0 cM	Embryonic lethal due to deficient cardiac valve generation, reduced B and T cell proliferation response, impaired Th2 response with decreased IL-4 production	Embryo: Expression restricted to endocardium, essential for normal atrioventricular and pulmonary valve and septal morphogenesis. Adult: Role in adult cardiac homeostasis unknown.
NFATc2	NFAT1, NFATp	Ca^{2+}/calcineurin	20q13.2-q13.3/195.5cM	Enhanced B and T cell response. Th2 bias with increased Th2 cytokines. NFATc2/NFATc1 double KO has impaired effector T cell function, but hyperactive B cell function. NFATc2/NFATc3 double KO display lymphoproliferative disorder, allergic blefaritis, intestinal pneumonitis.	Role in embryonic and adult cardiac homeostasis unexplored
NFATc3	NFAT4, NFATx	Ca^{2+}/calcineurin	16q22.2/8 51.0cM	Viable with reduced fertility, defects in axon outgrowth, reduced vascularization in yolk sac.	Embryo: Reduced vascularization of yolk sac; Adult: Implicated in induction of brain natriuretic peptide induction in synergy with GATA4; no role in adult cardiac hypertrophy response
NFATc4	NFAT3	Ca^{2+}/calcineurin	14q11.2/14 C1 cM	Viable and fertile with defects in axon outgrowth. NFATc3/NFATc4 double KO is embryonic lethal (E11) with generalized defects in vessel assembly into neural tube and somites.	Embryo: normal cardiovascular development. When combined with NFATc3 null, double KO mice display severe vascularization defects. Adult: defective ability to mount a cardiac hypertrophy response to pressure overload or agonists.
NFAT5	TonEBP	Osmotic stress, integrin activation	16q22.1/8 52.0cM	Renal atrophy and lack of tonicity - responsive gene expression	Role in embryonic and adult cardiac homeostasis unexplored

cM: centimorgan
KO: knockout.
* Information from http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi

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CHAPTER 2

NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure

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ABSTRACT

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 of other *nfat* genes in the unstimulated heart by several folds. 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 myocardium 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 pathological cardiac remodeling and heart failure.

INTRODUCTION

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 chronically 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 of the myocardium 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) 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

LV 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, 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 - alpha-myosinheavychain-calcineurin (αMHC-CnA) transgenic mice, described previously (5) and generously provided by Eric N. Olson, were crossbred with mice harboring a *nfatc2* null mutation (17), generously provided by Laurie Glimcher.

Aortic Banding - Transverse aortic banding (TAC) 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).

Transthoracic echocardiography - Cardiac remodeling and function was serially assessed at two, four, six and eight 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 and immunofluorescence microscopy - Hearts were arrested in diastole and perfusion fixed with 4% paraformaldehyde and embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E), Sirius red, or FITC-labeled wheat germ agglutinin (WGA-FITC). 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).

Immunohistochemistry - 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 mg 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 BioRad iCycler (Biorad) and SYBR Green was used as described in detail previously (20).

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 ± SEM. Statistical analyses were performed using INSTAT 3.0 software (GraphPad Software) and consisted of ANOVA, followed by Tukey's posttest when group differences were detected at the 5% significance level or the Student *t* test when 2 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, since 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, skeletal 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 wildtype 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 in NFATc1.1, NFATc3.1 and NFATc3.2, and a relative decrease in NFATc4 compared to unstimulated hearts (Fig. 1b). Collectively, these data indicate that *nfatc2*

transcripts outnumber those from other *nfat* genes in the unstimulated heart by several folds, and that mild auto amplification loops involving *nfatc1* and *nfatc3* exist following calcineurin signaling.

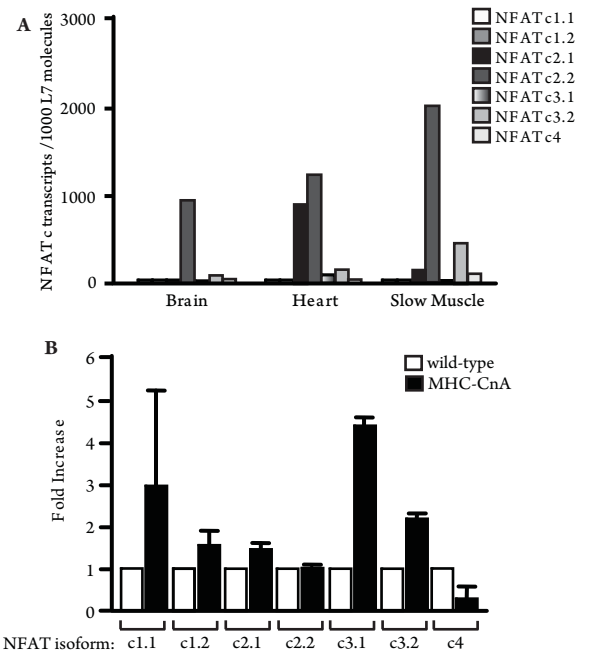


Figure 1. NFAT splice-isoform distribution in excitable tissues. (A) Quantitative assessment of transcript abundance of different NFAT (splice)-isoforms in murine brain, heart and skeletal muscle, normalized to the relative abundance of transcripts for 60S ribosomal protein L7. (B) Detection of NFAT autoregulation in hearts with calcineurin activation. The data demonstrate that upon calcineurin/NFAT activation, *nfatc1.1*, *nfatc3.1*, *nfatc3.2* splice transcripts are relatively enriched compared to baseline, and *nfatc4* transcripts decrease (n=3 per group).

NFATc2-deficiency compromises pathophysiologic 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 crossbred *nfatc2*-null mice with MHC-CnA mice. At three 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. 2a,c). Remarkably, MHC-CnA mice harboring a null mutation for the *nfatc2* 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 to MHC-CnA/*nfatc2*+/+ mice.

Histopathological analysis from H&E-, 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/*nfatc2*+/+ hearts, whereas MHC-CnA/*nfatc2-/-* 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. *Nfatc2*+/+ and *nfatc2*-null mice had similar myofiber cross-sectional areas, while MHC-CnA/*nfatc2*+/+ mice had significantly increased individual myofibril size (Fig. 2d). In contrast, a 47 % reduction was observed in MHC-CnA mice lacking *nfatc2.* These data confirm that loss of *nfatc2* attenuates calcineurin-induced cardiac hypertrophy.

To examine the impact of *nfatc2* ablation on calcineurin-induced hemodynamic dysfunction, all cohorts were subjected to serial 2-D and M-mode echocardiography at 4 weeks of age. Representative images of M-mode recordings are displayed in Fig. 2e. An increase in left ventricular internal diameter (LVID) and a proportional decrease in systolic contractility (FS) were evident in the MHC-CnA/*nfatc2*+/+ mice, whereas these parameters were clearly improved in MHC-CnA/*nfatc2*-/- animals (Fig. 2f,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 signaling in the heart (12). Transcripts for *rcan1.4* were substantially upregulated in MHC-CnA/*nfatc2*+/+ mice, and reduced to 50% in MHC-CnA/*nfatc2-/-* 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* (beta-myosin heavy chain) were substantially repressed upon *nfatc2* deletion (Fig. 2i). In conclusion, these results indicate that loss of *nfatc2* led to a significant reduction of all major calcineurin-induced structural alterations in the myocardium.

Nfatc2-deficient mice display modest splenomegaly, hyperproliferation of T-and B cells and dysregulated IL-4 production (17,26). To exclude the possibility that the observed cardiac phenotype was indirectly related to the relative immunodeficiency due to loss of NFATc2, we analyzed histological sections of hearts from the experimental groups for macrophages (MAC3) and leukocytes (CD45). Cardiac sections of *nfatc2*-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 *nfatc2* 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 *nfatc2*-null mice compared to wildtype hearts, either or not harboring the MHC-CnA transgene. These results indicate that *nfatc2* deficiency produces a fundamental deficit in the ability of calcineurin to execute a full myocyte hypertrophy response.

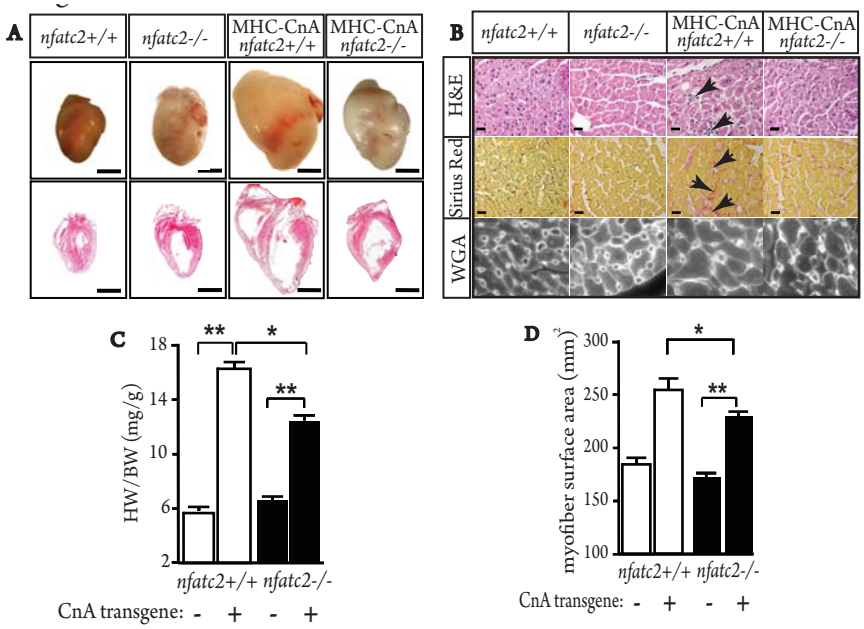


Figure 2. Gravimetrical, histological, functional and molecular analysis of calcineurin-transgenic mice crossed into a nfatc2-null background. (A) Representative gross morphology and H&E-stained four-chamber view of hearts dissected from 3 week-old mice of indicated genotypes, demonstrating a profound 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 panel b. (bar 0.2 mm). H&E-stained images reveal remarkable myocyte hypertrophy, myofiber disarray, and cellular infiltrates (arrowheads) in MHC-CnA/*nfatc2*+/+ mice, while 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 to *nfatc2*+/+ and *nfatc2*-/- mice and myofiber size of the MHC-CnA/*nfatc2*-/-mice was visibly smaller. (C) Heart weight/body weight ratios of 3 week-old *nfatc2*+/+, *nfatc2*-/-, MHC-CnA/*nfatc2*+/+ and MHC-CnA/*nfatc2*-/- mice (n=5 per group). (D) Quantification of myofiber cross-sectional area from indicated genotypes shows significant attenuation of myocyte hypertrophy in MHC-CnA/nfatc2-/- mice compared to MHC-CnA/nfatc2+/+ mice (n=3 per group, with 100 fibers counted per animal).

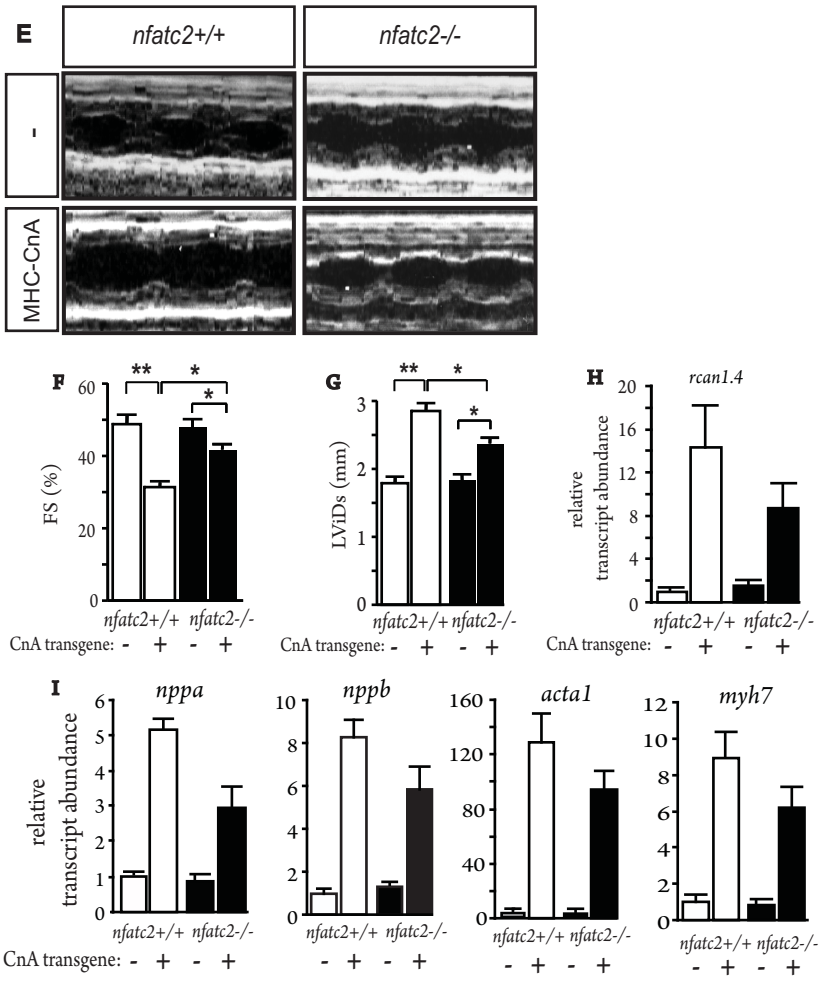


Figure 2 (continued). (E) Representative M-mode images of *nfatc2+/+*, *nfatc2-/-*, MHC-CnA/*nfatc2+/+* and MHC-CnA/*nfatc2-/-* mice at 4 weeks of age, indicating dilation and loss of contractile behavior in MHC-CnA/*nfatc2+/+* mice, which was substantially attenuated in MHC-CnA/*nfatc2* null mice. (F, G) 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 to *nfatc2+/+* mice (n=4-6 per group). (H, 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). * indicates $P < 0.05$, ** indicates $P < 0.01$.

NFATc2-deficiency compromises pathophysiologic cardiac hypertrophy
To determine whether NFATc2 also regulates hypertrophy in response to more physiologic stimuli, continuous angiotensin II infusion was performed. Vehicle treated *nfatc2*+/+ and *nfatc2*-/- mice displayed similar HW/BW ratios (4.1 ± 0.2 and 4.3 ± 0.1 mg/g, respectively). In response to angiotensin II, *nfatc2*-null mice still developed some degree of hypertrophy, although this was significantly blunted compared to the response displayed by *nfatc2*+/+ mice (Fig. 3a,b).

Next, transverse aortic banding (TAC) was performed, a surgical technique where the aorta was partially constricted for one week to mimic chronic hypertensive disease in human. To validate that the surgical procedure produced equal pressure gradients in all experimental groups, transcarotid pressures were measured invasively (Fig. 3c). Sham operated *nfatc2*+/+ and *nfatc2*-/- mice displayed similar HW/BW ratios (4.7 ± 0.1 and 5.3 ± 0.1 mg/g, respectively). In response to TAC, *nfatc2*-null mice still developed some degree of hypertrophy, although this was significantly blunted compared to the response displayed by *nfatc2*+/+ 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.

H&E- and Sirius red-stained cardiac histological sections did not show any signs of histopathology in *nfatc2*+/+ and *nfatc2*-/- 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 *nfatc2*+/+ hearts, whereas *nfatc2*-/- mice displayed these abnormalities in a much milder form in response to TAC (Fig. 3e,f). Myofibril cross-sectional areas were quantified from WGA stained histological sections. *Nfatc2*+/+ and *nfatc2*-/- sham operated mice had similar myofiber cross-sectional areas (232 ± 7 and 256 ± 4 mm^2, respectively), while pressure-overloaded *nfatc2*-/- mice had significantly decreased individual myofibril size compared to *nfatc2*+/+ mice after TAC surgery (434 ± 10 and 588 ± 14 mm^2, respectively). These data confirm that loss of *nfatc2* reduced pressure overload-induced myofibril hypertrophy (Fig. 3f).

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* (beta-myosin heavy chain) were substantially repressed upon pressure overload in *nfatc2*-null mice compared to wild-type controls (Fig. 3g). Collectively, these results demonstrate a clear defect in the structural and molecular program of pathological cardiac hypertrophy in the absence of *nfatc2*.

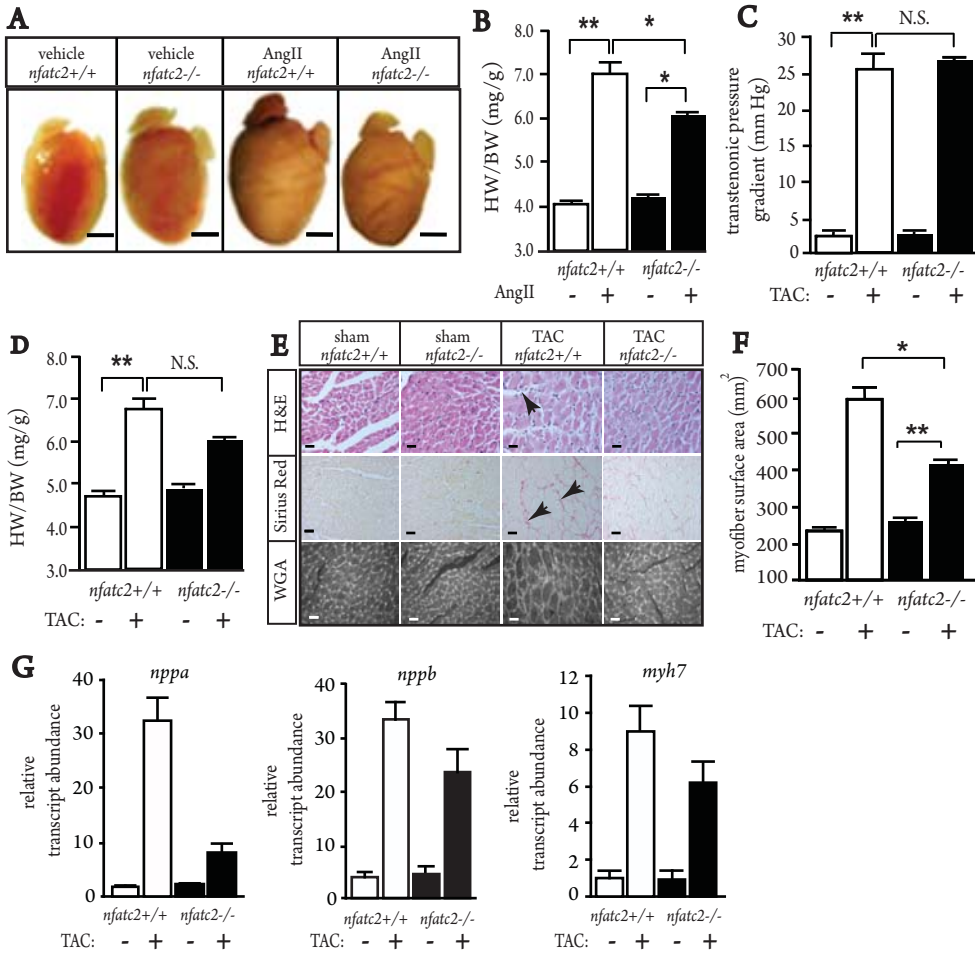


Figure 3. Nfatc2 ablation attenuates agonist- and pressure overload induced cardiac hypertrophy. (**A**) Representative gross morphology of hearts dissected from mice of indicated genotypes continuously infused with angiotensin-II or vehicle, demonstrating a profound rescue of cardiac enlargement by *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 to wild type hearts after two weeks of vehicle or angiotensin-II infusion. (**C**) Pressure gradients across the proximal and distal transverse aorta were measured invasively to validate the TAC procedure. (**D**) Heart weight to body weight (HW/BW) ratios of indicated genotypes subjected to sham or TAC surgery show a decreased hypertrophic response for *nfatc2-/-* hearts compared to wild type hearts after one week of TAC. (**E**) 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 myofibercross-sectional areas in *nfatc2+/+* mice subjected to TAC compared to sham-operated genotypes, while 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). * indicates $P < 0.05$, ** indicates $P < 0.01$.

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 eight weeks. To ensure equal loading conditions of all experimental groups, pressure gradients were measured non-invasively (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; N.S.; Fig. 4b,c). In contrast, substantial cardiac enlargement was evident in *nfatc2*+/+ mice at 8 weeks after TAC surgery, while *nfatc2-/-* mice had visibly smaller hearts (Fig. 4b,c). This was further reflected by HW/BW ratios (6.2 ± 0.3 and 5.8 ± 0.2 mg/g, respectively; $P < 0.05$; Fig. 4c), indicating that *nfatc2-/-* mice displayed a sustained reduction in cardiac hypertrophy over longer periods of pressure overload. H&E 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 to pressure-overloaded *nfatc2*+/+ hearts (Fig. 4g). Sirius red staining of hearts demonstrated a profound reduction in fibrosis in pressure-overloaded *nfatc2-/-* hearts compared to 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 2-D 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 Figure 4d. Four weeks after TAC, an increase in left ventricular internal diameter (LVID) (Fig. 4d, e) and a proportional decrease in systolic contractility (FS) were evident in the *nfatc2*+/+ mice subjected to pressure overload (Fig. 4d, 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 LV dilation and heart failure (Fig. 4e and Table 1). *Nfatc2*-deficient mice displayed a significant reduction of these geometrical and functional deteriorations (Fig. 4d, e, 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.

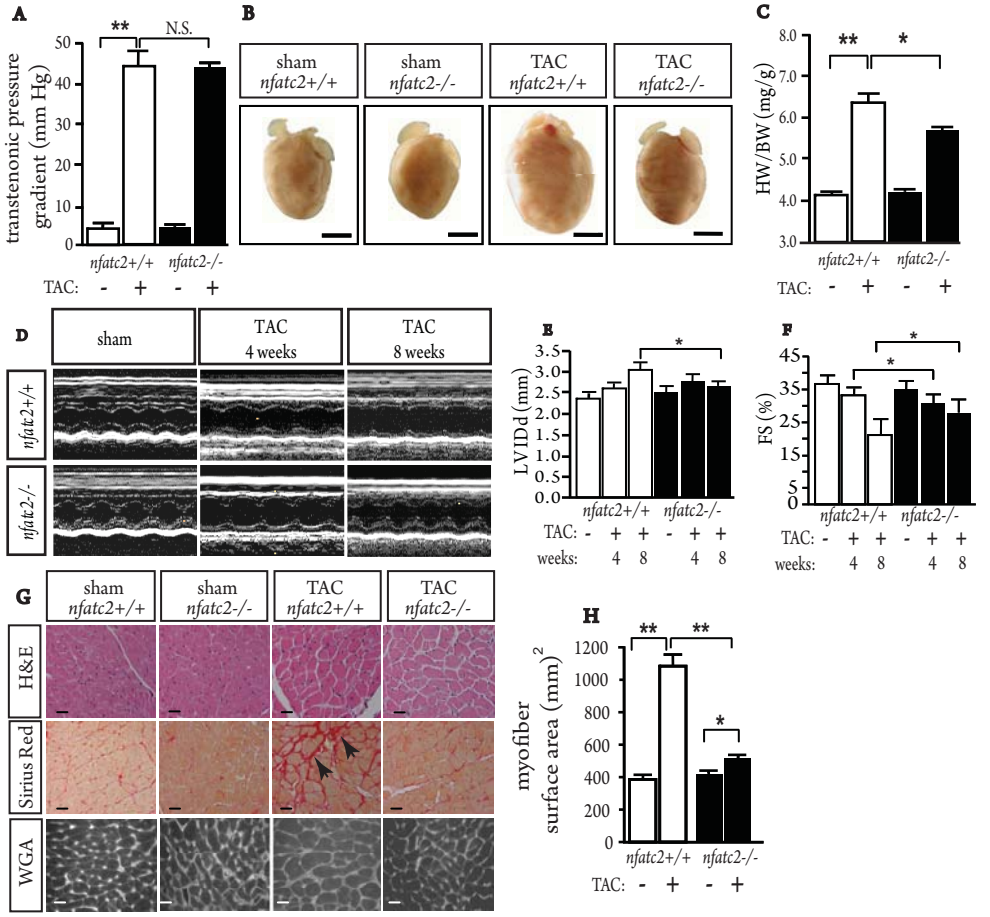


Figure 4. *Nfatc2*-deficiency prevents pressure overload induced heart failure. (A) Pressure gradients across the proximal and distal transverse aorta were measured noninvasively to validate the TAC procedure. (B) Representative gross morphology of hearts disseced from mice of indicated genotypes subjected 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 to wild type hearts after 8 weeks of TAC. (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, 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 to *nfatc2+/+* mice (n=6-10 per group). (G) 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 myofibercross-sectional areas in *nfatc2+/+* mice subjected to TAC compared to sham-operated genotypes, while 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). * indicates $P < 0.05$, ** indicates $P < 0.01$.

NFATc2 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, since latter form of cardiac growth does not provoke hemodynamic demise nor 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. 5b,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.

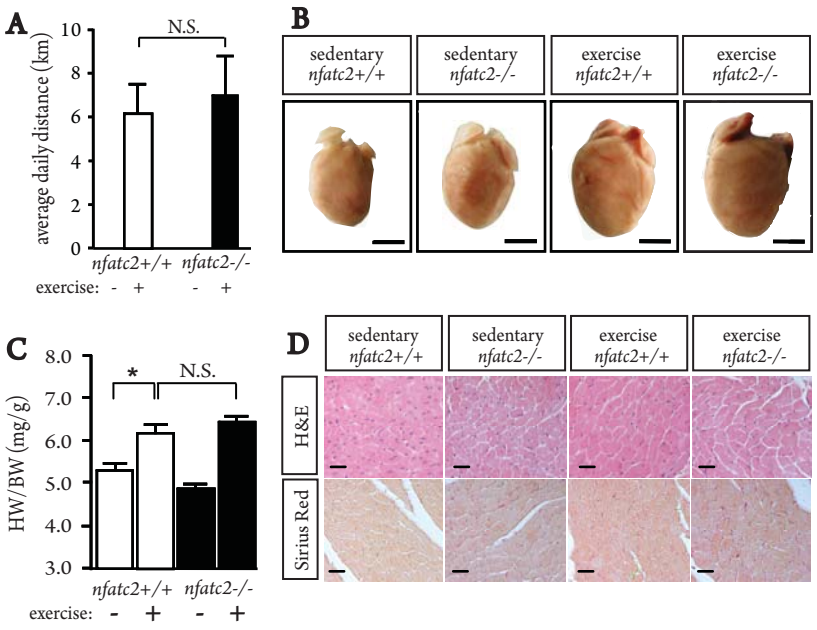


Figure 5. *Nfatc2* deficiency does not affect physiological hypertrophy. (a) Average daily distance that mice ran voluntarily. (b) Representative gross morphology from sedentary and exercised mice, indicating that exercised *nfatc2+/+* and *nfatc2-/-* develop equal cardiac enlargement. (c) Heart weight to body weight (HW/BW) ratios of indicated genotypes either sedentary or exercised (n=8 per group). (d) Representative H&E- and Sirius staining of heart sections of indicated genotypes indicates no histopathological alterations following exercise.

DISCUSSION

Functional hierarchy among cardiac NFAT isoforms in cardiac pathology - One unanticipated finding of the present 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, since 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 folds 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 the present 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 to 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 several folds, and that mild auto-

amplification loops involving *nfatc1* and *nfatc3* exist following calcineurin activation.

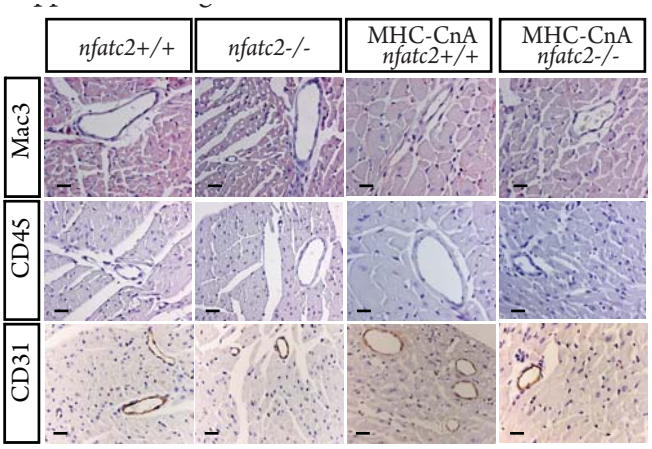
NFAT signaling is genetically restricted to pathological cardiac growth and maladaptive in nature - Classical conceptualization has it that left ventricular hypertrophy would start as an adaptive, beneficial response in order to normalize wall stress to either altered mechanical loading conditions (e.g. resulting from valvular disease or chronic hypertension) or decreased performance due to 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 the present study we noted a pronounced decrease in β-MHC gene expression in *nfatc2*-null mice compared to 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 manuscript 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 calcineurin/NFAT signaling is not activated after exercise because the cardiac growth response in response to voluntary wheel running remained unaffected in *nfatc2*-null mice. 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).

Combined, these data demonstrate that NFAT transcriptional activity is a required genetic pathway and selectively activated in pathological hypertrophy and ensuing heart failure. The data in this study suggest 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.



Supplemental Figure. Absence of aberrant macrophage/leukocyte infiltration or angiogenesis in nfatc2 null hearts. The relative amount of Mac-3 positive macrophages, CD45 positive leukocytes or capillary density (CD31-positive structures) does not differ in the four genotypes studied.

ACKNOWLEDGEMENTS

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CHAPTER 3

NFATc2 inactivation protects Cysteine rich protein 3 (CSRP3) deficient mice from dilated cardiomyopathy

Meriem Bourajjaj, Jenny M. Meerding, Hamid el Azzouzi, Marcel G. Nederhoff, Cees J. van Echteld and Leon J. De Windt

ABSTRACT

Cysteine rich protein 3 (CSRP3), also known as Muscle LIM Protein (MLP), knockout mice have been shown to exhibit many features of human dilated cardiomyopathy. Although disruption of the cytoskeletal architecture, due to absence of functional CSRP3, likely results in impaired force transmission by individual cardiomyocytes, the signaling mechanism that links this cytoskeletal defect with the onset of cardiac dilation and heart failure is unclear.

We have recently demonstrated that *nfatc2*-null mice displayed abrogation of calcineurin-provoked cardiac growth and a clear protection against the geometrical, functional, and molecular deterioration of the myocardium following hemodynamic loading. These findings established a predominant role for *nfatc2* in the execution of cardiac remodeling and heart failure downstream of calcineurin. The collective findings suggest that mice deficient for *nfatc2* may also display improvements of late phase heart failure downstream of seemingly calcineurin-independent mechanisms (Chapter 2). To investigate the significance of *nfatc2* deletion in the severe heart failure phenotype of *csrp3* null mice, we crossbred *csrp3* knockout mice with *nfatc2* null mice. Histological and hemodynamic analyses revealed dramatic improvements in cardiac morphology and function in *csrp3/nfatc2* double knockout mice.

In conclusion, deletion of the *nfatc2* gene prevents maladaptive remodeling and cardiac dysfunction of *csrp3* deficient hearts, and infers a role for calcineurin/NFAT signaling in Z-disk related stretch sensing.

INTRODUCTION

Dilated cardiomyopathy (DCM) is characterized by wall thinning and ventricular chamber dilation, accompanied by severe contractile dysfunction. A growing number of mutations in either cytoskeletal or sarcomeric genes has been described to account for the development of human DCM (1, 2). One of the genes linked to DCM is cysteine rich protein 3 (CSRP3), also known as Muscle LIM Protein (MLP) (3, 4). CSRP3 is a muscle specific member of the LIM-only class of the LIM domain protein family that possesses two tandem LIM domains. CSRP3 has been originally identified in striated muscle, where it promotes myogenesis and regulates myogenic differentiation (5, 6). Mice homozygous deficient for *csrp3* display features of DCM and develop heart failure (7). Furthermore, a decrease in CSRP3 protein level has been observed in human heart failure (8). Finally, CSRP3 interacts and colocalizes with telethonin (T-cap), a titin interacting protein, and a human *csrp3* mutation (W4R) associated with DCM results in a marked defect in T-cap interaction/localization, suggesting that a Z-disc CSRP3/T-cap complex is a key component of the cardiomyocyte stretch sensor machinery, and that defects in this complex underlie human DCM (3).

In striated muscle, CSRP3 is localized at the Z-disc, where it directly binds structural proteins, such as T-cap, α-actinin, βI-spectrin and N-RAP, further implying a role in the existence of an intracellular stretch sensor apparatus (3, 9-11). Impaired cytoskeletal force transmission, due to myofibrillar disorganization caused by disruption of this scaffold complex, could give a straightforward explanation for the cardiac phenotype observed in *csrp3* knockout mice. It has been further demonstrated that the cardiomyopathic phenotype of these mice is related to calcium cycling defects, as complete phenotypic rescue occurs by crossbreeding *csrp3* null mice with either phospholamban deficient mice or β-adrenergic receptor kinase-1 inhibitor overexpressing mice (12-14). As calcium is a very potent secondary messenger, a decrease in CSRP3 protein expression could, apart from the direct structural effects on the contractile apparatus, give rise to alterations in intracellular signaling. So far, the identity of signaling pathways activated by reduction in CSRP3 protein and its ramifications on the cardiac remodeling process have remained elusive.

NFATc2 is a well-known downstream effector of calcium signaling and other pro-hypertrophic pathways in the cardiomyocyte. Recently, we have demonstrated 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. These findings imply predominant roles for *nfatc2,* in the execution of cardiac remodeling and heart failure downstream of calcineurin. To investigate the significance of *nfatc2* deletion in the pathological remodeling of *csrp3*-deficient hearts, we crossbred *csrp3*-knockout mice with *nfatc2* null mice to generate *csrp3/nfatc2* double knockout (DKO) mice. Histological and hemodynamic analyses revealed dramatic improvements in cardiac morphology and function in *csrp3/nfatc2* double knockout mice, suggesting a role for calcineurin/NFAT signaling in Z-disk related stretch sensing.

MATERIALS AND METHODS

Mice. Mice harboring a *nfatc2*-null mutation were generously provided by Laurie Glimcher (Harvard) (15). *Crsp3*-deficient mice have been described elsewhere (7) and were generously provided by Howard Rockman (Duke University). Studies were performed in animals of 6 weeks of age.

Live cardiac magnetic resonance imaging. Cardiac and respiratory triggered cine MR images were acquired on a 9.4 T scanner (Bruker Biospin GmbH, Rheinstetten, Germany) using a birdcage RF coil. A gradient echo pulse sequence was used to acquire data with TR=9.8 ms, TE=1.9 ms, a matrix of 256 x 256, a field of view of 3.0 x 3.0 cm, a slice thickness of 1 mm, flip angle 18º, and four signal averages. The number of phases was 11-13 depending on the heart rate. Seven to eight shortaxis slices were needed to image the entire left ventricle. Images were processed with dedicated imaging software (CAAS-MRV, Pie Medical Imaging BV, Maastricht, the Netherlands).

Immunolabeling and immunofluorescence microscopy - Hearts were arrested in diastole and perfusion fixed with 4% paraformaldehyde and embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) or Sirius red. Sections were visualized using a Nikon Eclipse E600 microscope.

Statistical analysis. The results are presented as mean ± SEM. Statistical analyses were performed with InStat 3.0 (GraphPad Software, Inc, San Diego, CA). The analyses consisted of ANOVA, followed by Tukey's posttest when group differences were detected at the 5% significance level. Statistical significance was accepted at a p value <0.05.

RESULTS

Deletion of nfatc2 prevents cardiac dilation in csrp3 knockout mice

To investigate whether *nfatc2* gene activation is involved in the genesis of chamber dilation of the heart in dilated cardiomyopathy (DCM), we intercrossed *csrp3* knockout mice with *nfatc2* null mice.

Gross morphology of hearts obtained from 6-week-old mice demonstrated morphological difference between *csrp3* null and *csrp3/nfatc2* DKO mice (Fig. 1A). In line with earlier observations, hearts from *csrp3* null mice displayed thinning of cardiac walls and biventricular dilation (7). These morphological changes were rescued in *csrp3/nfatc2* DKO mice, where the wall remained thicker, shown by H&E-stained four chamber view of hearts (Fig. 2A). Sirius red staining indicated massive interstitial and perivascular fibrosis in both hearts of *csrp3* null and *csrp3/nfatc2* DKO mice (Fig 2B).

Furthermore, measurements of heart-weight-to-body-weight (HW/BW) ratios indicated a similar increase in cardiac mass for *csrp3* null mice (7.5±1.2 mg/g) and *csrp3/nfatc2* DKO mice (6.2 ±0.9 mg/g) (Fig. 1B). These data demonstrate that deletion of *nfatc2* prevents cardiac enlargement secondary to *csrp3* gene deletion.

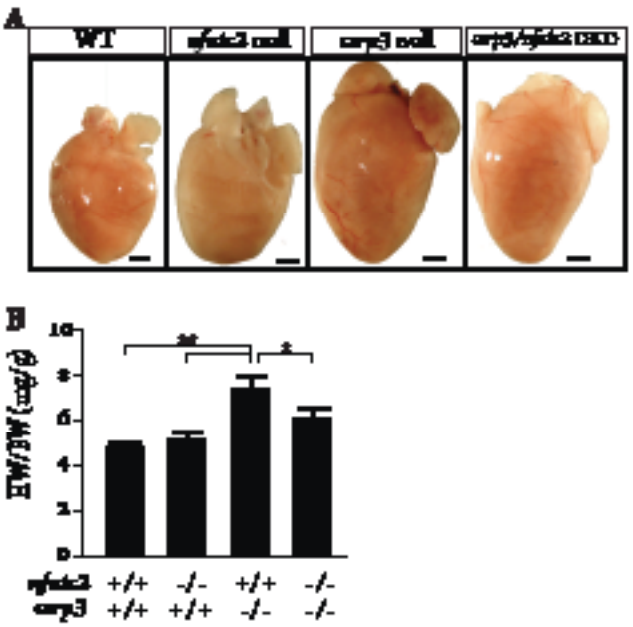

Figure 1. Deletion of *nfatc2* attenuates cardiac enlargement in *csrp3*-null mice. (**A**). Representative gross morphology of hearts dissected from 6 week-old mice of indicated genotypes, demonstrating a visible rescue of cardiac enlargement by *nfatc2* deletion in *csrp3*-null mice (bar 5 mm). (**B**). Ratios of heart-weight-to-body-weight (HW/BW) of the indicated groups show a decrease in cardiac mass for *csrp3/nfatc2*-DKO mice hearts at 6 weeks of age compared to hearts of *csrp3*-null mice. *Indicates $p<0.05$ versus corresponding *csrp3*-null group.

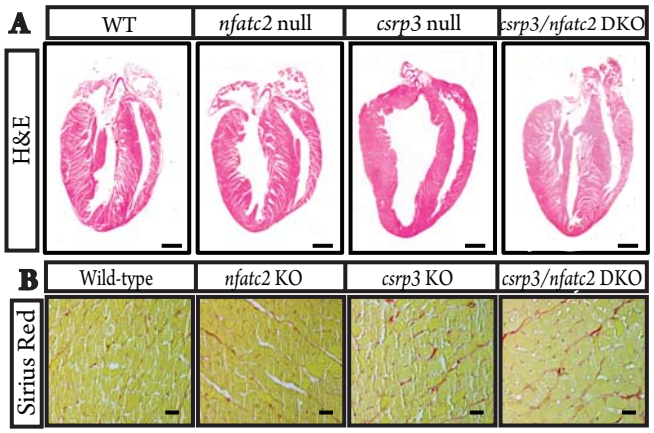

Figure 2. Histological characteristics of hearts from *csrp3*-null mice crossed into a *nfatc2*-null background.
(**A**). H&E stained cardiac sections demonstrate ventricular wall thinning and chamber dilation in *csrp3*-null hearts, which is not present in *csrp3/nfatc2*-DKO mice. (**B**). Representative histological images of hearts from mice with genotypes indicated under panel b. (bar 2 mm). Sirius red staining indicates massive interstitial and perivascular fibrosis in hearts of *csrp3*-null mice, which is not attenuated in *csrp3/nfatc2*-DKO mice.

Deletion of nfatc2 preserves cardiac function in csrp3 knockout mice

Next, in vivo cardiac morphology and function were analyzed in live mice using magnetic resonance imaging. Cardiac mass, end-diastolic and end-systolic dimensions and contractility were comparable between WT and *nfatc2* null mice (Figure 3A). 8-week-old *csrp3* null mice displayed increased wall thickness of all ventricular walls, increased end-diastolic and end-systolic intraventricular dimensions, and increased contractility compared to WT and *nfatc2* null mice (Figure3A). Remarkably, *csrp3/nfatc2* DKO mice displayed end-diastolic and end-systolic biventricular dimensions and contractility that were comparable to WT and *nfatc2* null mice (Figure 3A).

Comparison of the images at maximal diastole and systole (Figure 3A) indicated the markedly reduced function in the *csrp3* null mice compared with control genotypes, WT- and *nfatc2* null mice. *csrp3/nfatc2* DKO mice displayed markedly improved ejection fraction (Figure 3B), a slight reduction in LV mass (Figure 3C), and a striking reduction in biventricular dilation (end-diastolic and end-systolic volume; Figure 3D). LV mass determined at end systole by nuclear magnetic resonance (NMR) imaging amounted to 58.5±12.0, 57.7±4.2, 72.9±6.5

and 65.5±19.8 mg in WT, *nfatc2* null, *csrp3* null mice, and *csrp3/nfatc2* DKO mice, respectively. End-diastolic volume amounted to 36.9±2.8 35.9±2.0, 42.2±5.5 and 51.6±7.2 µL in WT-, *nfatc2* null-, *csrp3* null-, and *csrp3/nfatc2* DKO mice, respectively. Likewise, ejection fraction was normalized when *nfatc2* was deleted in vivo 59.0±11.8%, 66.6± 4.0%, 21.9±6.1% and 65.0±6.3% in WT-, *nfatc2* null-, *csrp3* null-, and *csrp3/nfatc2* DKO mice, respectively. Wall thickness and wall thickening was significantly increased in *csrp3/nfatc2* DKO mice compared to *csrp3* null mice (Figure 3E, F).

Conclusively, *nfatc2* deletion in *csrp3* knockout mice prevents the development of dilated cardiomyopathy in this mouse model. Together, our data suggest that cardiomyopathic remodeling in MLP knockout mice is dependent on NFAT-calcineurin pathway.

DISCUSSION

Here, we examined the involvement of NFAT signaling in remodeling of *csrp3* deficient hearts. CSRP3 is also known as Muscle LIM Protein (MLP). Disruption of the gene encoding *csrp3* leads to impaired cytoskeletal organization in cardiomyocytes associated with dilated cardiomyopathy and heart failure (7). CSRP3 is a muscle specific member of the LIM-only class of the LIM domain protein family that possesses two tandem LIM domains. CSRP3 has been originally identified in striated muscle, where it promotes myogenesis and regulates myogenic differentiation (5, 6). Furthermore, a decrease in CSRP3 protein level has been observed in human heart failure (8). Finally, CSRP3 interacts and colocalizes with telethonin (T-cap), a titin interacting protein, and a human *csrp3* mutation (W4R) associated with DCM results in a marked defect in T-cap interaction/localization, suggesting that a Z-disc MLP/T-cap complex is a key component of the cardiomyocyte stretch sensor machinery, and that defects in this complex underlie human DCM (3).

Although the mechanistical link between disturbances of the cytoskeleton in individual cardiomyocytes and the progression to dilated cardiomyopathy is unclear, several studies have reported the involvement of impaired intracellular calcium homeostasis (12, 14, 16). Altered intracellular calcium levels/fluctuations could provide an explanation for the involvement of *nfatc2*, since NFAT is a well-known downstream effector of calcium signaling (17, 18).

In line of the presumption that calcium/calmodulin signaling may be

activated by alterations of Z-disk architecture, calsarcin-1, another Z-disc protein, has been found to interact with calcineurin and mice lacking calsarcin-1 protein are sensitized to calcineurin signaling and display an accelerated progression to cardiomyopathy (19). A decrease in Ca^{2+} uptake is a central feature of human and animal heart failure (20, 21) and an increase in the relative ratio of PLB to SERCA2a is an important determinant of SR dysfunction in heart failure (22, 23). In line, Minamisawa et al. showed that *csrp3/plb* DKO mice display a rescue of the entire spectrum of the *csrp3*-deficient heart failure phenotype at the structural, functional, and molecular level. The rationale being that in *plb* KO hearts, the inhibitory effect of PLB on the SERCA2 SR pump is removed, resulting in normalization of SR calcium uptake. At the same time, the resulting increase in SR calcium content in *csrp3/plb* DKO mice results in maintenance of normal calcium release, thereby leading to normalization of normal contractility and relaxation (12) .

Our results are in contrast to two separate studies. In one study, activation of NFAT signaling pathway was reduced in *csrp3+/-* mice after MI, as shown by a blunted transcriptional activation of NFAT in cardiomyocytes isolated from *csrp3+/-*/NFAT-luciferase reporter gene compound transgenic mice. Calcineurin was found to be co-localized with CSRP3 at the Z-disk in WT mice but was displaced from the Z-disk in *csrp3+/-* mice, indicating that CSRP3 is essential for calcineurin anchorage to the Z-disk. In vitro assays in cardiomyocytes with down-regulated CSRP3 confirmed that CSRP3 is required for stress-induced calcineurin–NFAT activation. This study by Heineke *et al.* implies a link between the stress sensor CSRP3 and the calcineurin–NFAT pathway at the sarcomeric Z-disk in cardiomyocytes and indicates that reduced MLP–calcineurin signaling predisposes to adverse remodeling after MI (24). In a second and more recent study, PICOT (protein kinase [PK]C–interacting cousin of thioredoxin) activity was found to constitute a negative feedback loop for cardiac hypertrophy (25). Jeong *et al.* demonstrated that PICOT overexpression by adenoviral overexpression impaired calcineurin signaling in vitro. Further, using a transgenic model engineered to overexpress a PICOT mutant, they were able to demonstrate that the induction of brain natriuretic peptide (*nppb*) and the exon 4 splice isoform of *rcan1*, a direct NFAT target gene, was blunted in response to pressure loading (26). Remarkably, no whole heart morphological or physiological parameters were presented from this mouse model at baseline or after stress.

At this moment it is not clear why our findings are in disparate to the previous studies. First, our study employs an unequivocal approach by creating double mutant mice for both *csrp3* and *nfatc2,* yielding a very clear phenotype and rescue

of many aspect of dilated cardiomyopathy. In contrast, it should be noted that in the study by Heineke *et al.* (24) heterozygous knockout mice for *csrp3* were employed. Heterozygous mutant *csrp3* mice (*csrp3* +/-) do not show impaired Z-disc structure, nor any other apparent phenotype, so that the choice of this particular genotype to study this signaling phenomenon is remarkable. The other major finding of Heineke *et al.*, suggesting co-localization between CSRP3 and calcineurin, is intriguing and fully in line with our findings.

With respect to the study of Jeong *et al.* (26), which analyzes PICOT function, it should be remarked that the suggested competition between PICOT and calcineurin for CSRP3 binding was very weak, questioning its physiological relevance. Further, it is as of yet unknown what exactly happens to PICOT levels in the diseased heart, which makes the choice of generating a transgenic model expressing a PICOT mutant unfortunate, since it may not reflect the correct physiological situation. Even more remarkable, very little details are known about the PICOT transgenic mouse apart from the transcript abundance of two fetal genes, so that at this stage it is impossible to draw any conclusions from this study. The other major finding of the study, suggesting direct binding between calcineurin and CSRP3 at the Z-disc, is again, of course, fully in line with our findings.

Histological and hemodynamic analyses revealed dramatic improvements in cardiac morphology and function in *csrp3/nfatc2* double knockout mice, suggesting a role for calcineurin/NFAT signaling in Z-disk related stretch sensing. The combined findings indicate that *csrp3*-deficiency, which is a model for disruption of the cytoskeletal architecture resulting in impaired force transmission by individual cardiomyocytes, mimicking human dilated cardiomyopathies, activates calcineurin/NFAT signaling to direct cardiac remodeling.

ACKNOWLEDGMENTS

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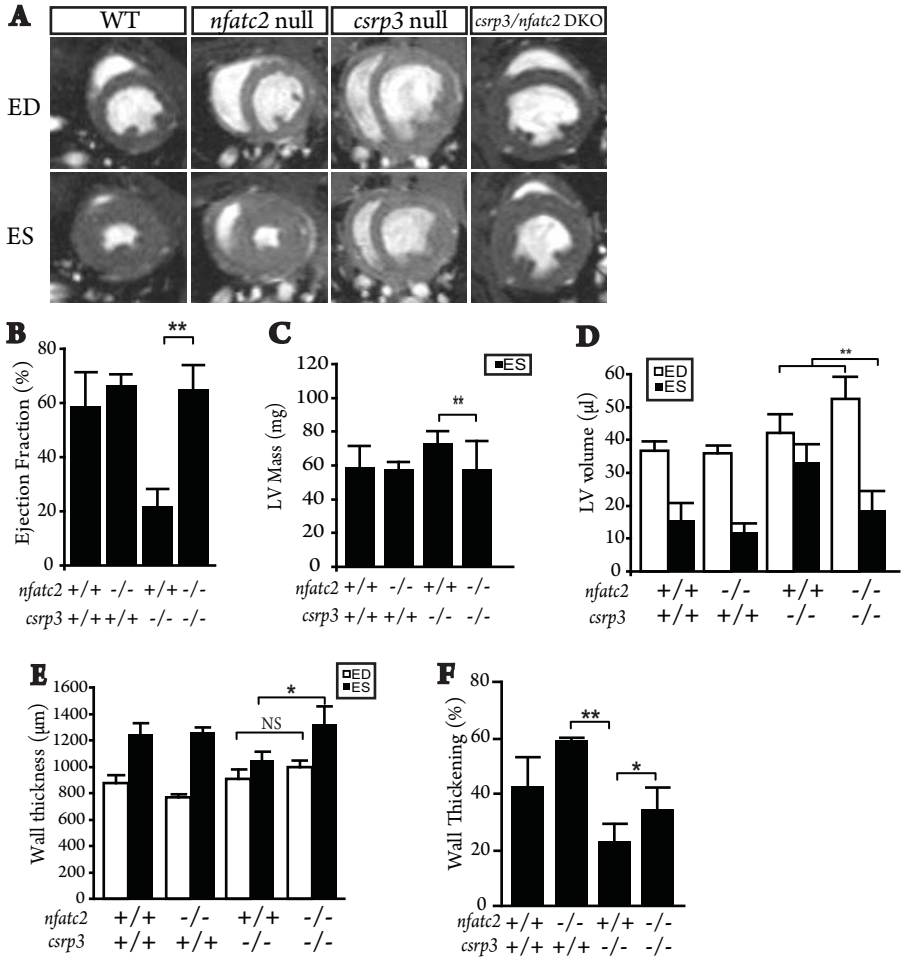

Figure 3. Deletion of nfatc2 reduces CSRP3-induced cardiac dysfunction.
(**A**). NMR images at mid thorax level of living mice of the indicated genotypes at maximal diastole (top panels) and maximal systole (bottom panels) indicating the severe chamber dilation and thinned walls of *csrp3*-null hearts and less dilation and less thinned walls of *csrp3/nfatc2*-DKO mice. (**B**). Ejection fraction determined from NMR imaging in live mice with the indicated genotypes (n=6 per group). (**C**). LV mass determined by NMR imaging in live mice with the indicated genotypes (n=6 per group). (**D**). Maximal LV volumes as a measure of chamber dilation calculated at maximal diastole (open bars) or maximal systole (solid bars) determined by NMR imaging in live mice with the indicated genotypes (n=6 per group). (**E**). Wall thickness as a measure of chamber dilation calculated at maximal diastole (open bars) or maximal systole (solid bars) determined by NMR imaging in live mice with the indicated genotypes (n=6 per group). (**F**). Wall thickening calculated at maximal diastole (open bars) or maximal systole (solid bars) determined by NMR imaging in live mice with the indicated genotypes (n=6 per group). * indicates $P < 0.05$, ** indicates $P < 0.01$.

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CHAPTER 4

Calcineurin/NFAT transcriptome profiling reveals Gata4 as an early target gene in a feedforward mechanism driving cardiomyocyte hypertrophy

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ABSTRACT

Calcineurin is a required component of cardiac hypertrophy and dependent upon its transcriptional effector, Nuclear Factor of Activated T cells (NFAT), but limited information is available on the nature and number of NFAT target genes. Here, we constructed murine ventricular clones allowing inducible expression of activated forms of calcineurin or NFAT using a binary, doxycyclin-dependent system. Two double-stable calcineurin and NFAT clones were selected to control for potential cell based variations and subjected to whole genome Agilent arrays. Among 27 target genes, the zinc finger-containing transcription factor Gata4 was identified as an early calcineurin/NFAT target gene. Electromobility shift and chromatin immunoprecipitation assays demonstrated the existence of an evolutionary conserved NFAT consensus-binding site in a *gata4* enhancer. *Gata4* transcripts were increased in calcineurin Tg mice and pressure overloaded hearts. In line, elevated Gata4 protein levels were evident in calcineurin Tg mice as well as in human heart failure biopsies. Finally, siRNA-mediated knockdown of Gata4 abrogated calcineurin-mediated cardiomyocyte hypertrophy in primary cardiomyocyte cultures, indicating the functional requirement of Gata4 downstream of cardiac calcineurin/NFAT signaling. Given that NFAT and pre-existing Gata4 proteins also function as transcriptional partners, the present data reveal a novel feedforward mechanism where NFAT signaling provokes expression of the pro-hypertrophic Gata4 transcription factor, allowing amplification of calcineurin/NFAT signaling in the ventricular myocyte.

INTRODUCTION

Heart failure is a leading cause of morbidity and mortality in industrial countries, affecting over 10 million Americans and Western Europeans, with a 5-year mortality approaching 50% despite current medical therapy (1). A primary event in the pathogenesis of heart failure is the development of pathological cardiac hypertrophy, characterized by increased cardiomyocyte size and altered gene expression, the latter being maladaptive and contributing to heart failure progression (2). One signaling pathway that links extracellular stimuli to hypertrophy employs the phosphatase calcineurin and its downstream transcriptional effector nuclear factor of activated T-cells (NFAT) (3). The Ca^{2+}-calmodulin-activated phosphatase calcineurin physically interacts with cytoplasmic NFATc members (NFATc1-c4), where it directly dephosphorylates multiple serine residues within the N-terminal regulatory domain of NFAT, resulting in the unmasking of two nuclear localization sequences required for nuclear import (4-7).

Calcineurin/NFAT signaling has been implicated as a signaling paradigm that is both sufficient and required to drive the cardiac hypertrophic growth response (8, 9). Notwithstanding its established requirement in heart failure development, limited information is available about the immediate downstream NFAT target genes in cardiac muscle. To address this caveat, we resorted to the use of a previously developed, ventricular muscle cell line, NkL-TAg (10). These cells were isolated from ventricular sarcomas from hearts of transgenic mice carrying a mouse Nkx2.5 promoter coupled to simian virus 40 large T-antigen (TAg) flanked by loxP sites. NkL-TAg cells actively proliferate without apparent senescence, while introduction of Cre recombinase by adenoviral delivery results in the elimination of TAg expression, permanent exit from the cell cycle and expression of cardiac markers (10).

The conditional expression of TAg allows stable integrations of NkL-TAg cells during their propagation phase, while its regulated growth termination permits reproducible genomics approaches to study cardiac signaling in a genome-wide fashion in the appropriate cellular context. Here, we have created double stable NkL-TAg clones harboring a stably integrated tetracyclin-dependent repressor and constitutively activated mutants of either calcineurin or NFAT under control of Tet-operator (TetO) sequences responsive to occupation by the TetR, allowing controlled activation of the calcineurin/NFAT pathway in a time- and dosage-dependent manner.

A genome-wide gene chip analysis uncovered the earliest calcineurin/

NFAT-regulated transcripts in cardiac muscle. Gene ontology classifications revealed an overrepresentation of genes in translational machinery/protein transport, transcription factors and signal transduction. Remarkably, the zinc finger containing transcription factor Gata4 was found to be a common target gene of calcineurin/NFAT signaling. In line, Gata4 transcripts and protein levels were elevated in cardiomyopathic hearts from calcineurin transgenic mice, pressure overloaded hearts and human failing hearts. Given that NFAT and pre-existing Gata4 proteins synergistically activate target genes as transcriptional partners, our findings suggest the existence of a novel feedforward mechanism where NFAT signaling provokes expression of the pro-hypertrophic Gata4 transcription factor, allowing amplification of calcineurin/NFAT signaling in the ventricular myocyte. The unique reproducibility of stably expressing clones with absence of senescence during the propagation phase and regulated growth termination, provides a valuable experimental resource allowing cardiovascular genomics studies in a genome-wide fashion to study stress signaling events in the correct cellular context.

EXPERIMENTAL PROCEDURES

Animals. *Nfatc2* null were generously provided by Laurie Glimcher (Harvard) and described previously (11). Transgenic mice expressing an activated mutant of calcineurin under control of the myosin heavy chain (*myh6*) promoter were generously provided by Eric Olson (Dallas) (10). All protocols were performed according to institutional guidelines and approved by local Animal Care and Use Committees.

Recombinant adenoviruses. AdGFP, a control virus expressing only GFP and the adenovirus expressing an activated mutant of calcineurin (AdCnA) were described and characterized previously (10, 12). AdCre, an adenovirus expressing Cre recombinase, was described previously (12).

Cell culture. Isolation and culture of neonatal rat ventricular cardiomyocytes was performed as described before in detail (13). Low passage COS7 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. NkL-TAg cells were cultured and mortalized by AdCre infection as described previously (10).

Generation of stable cardiac cell lines. NKL-TAg clones harboring stably integrated constructs were generated using the T-REX system (Invitrogen) with modifications. Briefly, 10.000 NkL-TAg cells were transfected using FUGENE 6 reagent (Roche) with 8 μg pCAgβTrs-hygro, a vector expressing the tetracyclin-dependent repressor (TR) under control of a β-actin promoter (generously provided by Hans Clevers, Hubrecht Institute) and stable clones selected with 250 μg/μl hygromycin. Select clones were transiently transfected using FUGENE 6 reagent with 0.2 μg pcDNA4/TO-luciferase (generously provided by Hans Clevers, Hubrecht Institute) to test their responsiveness to doxycyclin (Dox) using the Dual Luciferase assay system (Promega). Next, two different Tet-repressor clones (TR1 and TR4), showing high inducible luciferase activity and low background, were transfected with 8.5 μg pcDNA4/TO-FlagΔCnA or pcDNA4/TO-FlagΔNFAT using FUGENE 6 reagent (Roche) and cultured in the presence of 250 μg/μl hygromycin and 750 μg/μl zeocin to generate double stable cell lines. Zeocin/hygromycin resistant clones were transiently transfected with NFAT-sensitive luciferase reporters (pGL3-NFAT9merluc and pGL2-Intr3-DSCR1) (13) to test their DOX-inducible calcineurin or NFAT activation profile. Renilla was taken as an internal control.

Reporter constructs and expression vectors. Nine copies of an NFAT binding site from the IL-4 promoter (5'-TGGAAAATT-3') were positioned 5' to a minimal thymidine kinase promoter harboring the TATA box and inserted upstream of the luciferase reporter in pGL3 Basic (Promega) to create pGL3-NFAT9merluc. An N-terminal FLAG-tagged deletion construct with a constitutively active form of mouse calcineurin Aα (amino acids 1 to 398) was inserted into pcDNA4/TO (Invitrogen), a vector harboring Tet-operator (TetO) sequences responsive to occupation by the TetR, to create pcDNA4/TO-FlagΔCnA. Likewise, an N-terminal FLAG-tagged deletion of mouse NFATc3 (amino acids 291-1065) was used to create pcDNA4/TO-FlagΔNFAT. pCG-GATA-4, an expression vector with full-length rat GATA4 was generously provided by Antoon Moorman (Academic Medical Center, Amsterdam). pGL3-hMCIP1(Int3)Luc, an MCIP1-luciferase reporter, containing a 904-bp intragenic sequence encompassing the third intron of the human *rcan1* (*DSCR1*) gene was described previously (13).

Agilent gene expression profiling and data analysis. Two inducible CnA- and NFAT TR clones, designated TR1-CnA, TR4-CnA, TR1-NFAT and TR4-NFAT, were maintained in parallel cultures, mortalized by overnight AdCre infection, cultured for an additional 3 days in serum-free media, and cultured for 24 hrs in

the presence or absence of 1 μg/μl doxycyclin. Total RNA was extracted using TRIzol (Invitrogen), cleaned with Qiagen RNeasy Mini Kits (Qiagen), RNA quantity was measured with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Wilmington), and RNA quality was monitored using an Agilent 2100 bioanalyzer. Agilent 44k mouse whole genome microarray slides (Palo Alto) were used and a dye-swap experimental design applied. RNA samples (500 ng each) from three corresponding cultures were pooled, amplified and labeled with Cy5- and Cy3-CTP (Perkin Elmer) to produce labeled cRNA using Agilent low RNA input fluorescent linear amplification kits following the manufacturers protocol. Dye-incorporation ratio was determined with NanoDrop® ND-1000 UV-Vis Spectrophotometer. For hybridization, the guidelines for 44k format arrays with cRNA targets were strictly followed. Briefly, 750 ng of Cy3-labeled cRNA and 750 ng Cy5-labeled cRNA were mixed and incubated with an Agilent microarray slide for 17 hours using an Agilent in situ hybridization kit following SSC buffer washing. The washed slides were immediately dried, and scanned using Agilent DNA Microarray Scanner (G2565BA). Raw data were generated using Agilent's Feature Extraction software (FE v7.1). Gene classifications were assigned based upon publicly available software and websites, including FATIGO Data mining with Ontology (www.fatigo.org), Mouse Genome Informatics (MGS; www.informatics.jax.org), GenBank and Medline searces. The heatmap was generated using Cluster 3.0 and Java Treeview.

Real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). One μg RNA was used as template for Superscript reverse transcriptase II (Promega) using gene specific primer combinations (primer sequences available upon request). For real time RT-PCR, the BioRad iCycler (Biorad) and SYBR Green was used as described in detail previously (12).

Western blot analysis. Protein were isolated from cells or hearts by lysis in extraction buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and complete protease inhibitor cocktail (Roche). Total protein from centrifuged lysate was separated by electrophoresis on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Amersham). Antibodies used included rabbit polyclonal anti-GATA4 (Santa Cruz, 1:1000); mouse monoclonal anti-FLAG (Sigma F-3165, 1:5000) and mouse monoclonal GAPDH (Santa Cruz, 1:5000) in 5% non-fat dry milk, followed by corresponding horseradish peroxidase-conjugated secondary antibodies (DAKO, 1:5000) and processed for chemiluminescent detection as described by the manufacturer (ECL, Amersham).

Electrophoretic mobility shift assay. HPLC-purified, 5' FAM-labeled double-stranded oligonucleotides were used (sequences available upon request). For competition experiments a non-labeled oligonucleotide duplex containing the corresponding NFAT binding sequence was used. Forward and reverse FAM-labeled oligo (15 pmol) were incubated for 10 min at 95 C in a total volume of 50 µL annealing buffer (100mM NaCl, 50mM HEPES, pH 7.5), followed by slowly cooling down to room temperature. FAM-oligo duplex (1.5 pmol) and 2 µg of nuclear extract of NFATc3 transfected COS7 cells were incubated for 15 min at room temperature in a total volume of 15 µL binding buffer (final concentration 10% (vol/vol) glycerol, 50 mM KCl, 15 mM Tris pH 7.9, 0.2 mM EDTA, 1 mM MgCl2, 0.4 mM dithiothreitol, 33 µg/ml BSA, pH 7.9), subsequently kept on ice, and loaded on a 5% polyacrylamide (29:1), 2.5% glycerol, TAE (Tris-acetate EDTA, pH 8) gel. Tracking dye (15 µl) was loaded in a separate lane. 1xTAE, 1 mM MgCl2 was used as running buffer. For competition experiments nonlabeled oligo duplex was added in molar excess to the binding mix as indicated. The gel was run at 4°C in the dark at 150 V for 2.5 h. The fluorescent signal was immediately analyzed at 488 nm using a FluorImager 595 (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant software (ImageQuant 5.2, Amersham Biosciences).

Chromatin immunoprecipitation. Cells were treated with 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were washed with cold phosphate-buffered saline (PBS) and swelled on ice for 10 min in a solution containing 25 mM HEPES, pH 7.8, 1.5mM MgCl2, 10 mM KCl, 0.1%NP40, 1 mM dithiothreitol, and a protease inhibitor cocktail (Roche). Following Dounce homogenization (20 strokes), the nuclei were collected and resuspended in sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and protease inhibitors) and sonicated on ice to an average length of 200 to 1,000 bp. The samples were centrifuged at 20,000 g and precleared with protein G-Sepharose in the presence of 2 µg of sonicated DNA and 1 mg/ml BSA. Twenty-five A260 units of the precleared chromatin was immunoprecipitated with 5 µl of antibodies, and immune complexes were collected by adsorption to protein G-Sepharose. The beads were washed twice with sonication buffer, twice with sonication buffer containing 500 mM NaCl, twice with 20 mM Tris (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, and twice with Tris-EDTA buffer. The immunocomplexes were eluted with 50 mM Tris (pH 8.0), 1 mM EDTA, 1% SDS at 65°C for 10 min, adjusted to 200 mM NaCl, and

incubated at 65°C for 5 hrs to reverse the cross-links. After successive treatments with 10 μg /ml Rnase A and 20 μg /ml proteinase K, the samples were extracted with phenolchloroform and precipitated with ethanol. The immunoprecipitated DNA and input DNA was analyzed by qPCR using primers for the mouse Gata4 promoter and as control primers a region far upstream from the myogenin promoter was taken (sequences available upon request). The Gata4 promoter harbors three putative NFAT sites find by RVISTA software online. The primers were designed around three putative NFAT sites and designated as N1 (-6518 to -6527 relative to the start site of Gata4 exon1), N2 (-2785 to -2794) and N3 (-1273 to -1282).

Immunofluorescence and confocal microscopy. Cultured cardiomyocytes were fixed with PBS, 4% paraformaldehyde , 0.1% NP40, and processed for immunofluorescence as described (13) Nuclei were counterstained with TOPRO-3. Cells were washed PBS/0.1% NP40 and mounted with coverslips in vectashield (Vector laboratories) and analyzed by confocal microscopy using a Zeiss LSM 510 META instrument. Antibodies used included mouse anti α-actinin (Sigma, 1:500); Texas Red-conjugated goat polyclonal anti-mouse (Molecular Probes, 1:800); TOPRO-3 (Invitrogen, 1:100). Cell surface areas and cell length-width ratios were determined using SPOT-imaging software (Diagnostic Instruments).

Statistical analysis. The results are presented as mean values ± SEM. Statistical analyses were performed using InStat 3.0 software (GraphPad Software Inc., San Diego, CA) and consisted of ANOVA followed by Tukey's posttest when group differences were detected at the 5% significance level. Statistical significance was accepted at a P value < 0.05.

RESULTS

Inducible activation of calcineurin and NFAT in cardiac cell lines. To begin to reveal the immediate target genes underlying the phenotypic alterations evoked by calcineurin/NFAT transcriptional activity in cardiac muscle, we engineered a cellular system to inducibly activate either calcineurin or NFAT in NkL-TAg cells, a previously described ventricular muscle cell line (10). To this end, NkL-TAg cells were stably transfected with an expression vector harboring the Tet-repressor (TR) under control of a β-actin promoter using hygromycin as selectable marker (Fig. 1a). About 80 clones were obtained and tested for doxycyclin (Dox)-inducibility

by transient transfection of a luciferase reporter construct downstream of two Tet-operator (TetO) sites (Fig. 4b). We continued with 2 NkL-TAg-TR clones (designated TR1 and TR4), which showed at least a 100-fold increase of luciferase activity in the presence of Dox and background luciferase in the absence of Dox (Fig. 4b). Next, we stably transfected TR1 and TR4 clones with a construct harboring an activated form of either calcineurin or NFAT under transcriptional control of two Tet-operator (TetO) sequences responsive to occupation by the tetracycline-dependent repressor (TR) (Fig. 1a), using zeocin as a selectable marker. Up to 100 clones were obtained per group and tested for inducible calcineurin or NFAT transcriptional activity following Dox stimulation using two separate NFAT-luciferase reporters (Fig. 1d).

For both TR1 and TR4, 2 double-stable clones were selected which only expressed calcineurin or NFAT after Dox stimulation, as demonstrated by Western blotting using an antibody against FLAG (Fig. 1c) and by their ability to activate NFAT-reporter genes (Fig. 1d and data not shown). To control for cell-based variations, we selected two TR-CnA and two TR-NFAT clones (designated TR1-CnA and TR4-CnA for calcineurin, and TR1-NFAT and TR4-NFAT for NFAT). By RT-PCR (Fig. 1e), AdCre-mortalized and differentiated TR-CnA/NFAT clones expressed the cardiogenic transcription factors Nkx2.5 and gata4; cardiac ion channels were expressed regulating the calcium transient (*atp2a2* and *ryr2*) and the transient outward K+ current (*kncip2*). Sarcomeric components were also detected including α-myosin heavy chain (*myh6*), myosin light chain-1a (*myl4*), desmin and sarcomeric actin (*actc*) at levels comparable to parental differentiated NkL-TAg cells (Fig. 1e), demonstrating the maintenance of cardiac muscle identity of differentiated TR clones, even after double stable integration events.

Thus, given the proof-of-principle that our TR clones adopted a cardiac muscle fate upon sequential application of AdCre and differentiation in serum free medium, we next designed experiments to induce activation of either calcineurin or NFAT activity in cognate double stable clones by addition of doxycyclin or vehicle for 24 hrs and subjecting total RNA to genome-wide gene chips to identify differentially expressed genes regulated by this pathway (Fig. 1f).

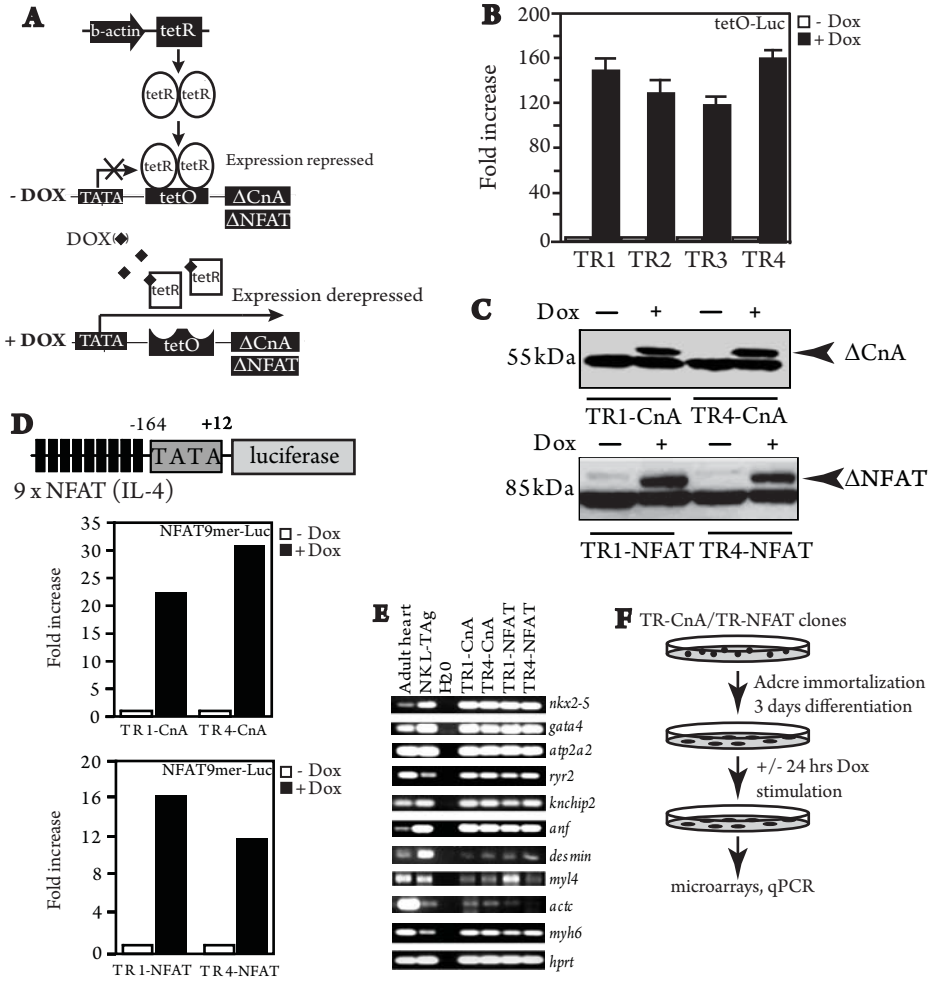

Figure 1. Generation of stable cardiac cell lines inducibly expressing calcineurin or NFAT. (A) Schematic representation of double-stable ventricular sarcoma harboring the conditional system encompassing constitutive expression of the tetracycline-sensitive repressor (TR) and activated mutants of calcineurin or NFAT under control of Tet operator (tetO) sequences, allowing derepressed expression after doxycyclin treatment. (**B**) Stable cell lines expressing the Tet repressor were tested for inducible expression using transient expression of a luciferase reporter downstream of Tet operators. (**C**) Western blot analysis using anti-Flag antibody on lysates of different double stable TR-calcineurin or NFAT clones, cultured in the absence of presence of doxycyclin (Dox) for 24 h. (**D**) Luciferase measurements on TR clones, transiently transfected with a NFAT-9mer reporter construct, indicates an increase in NFAT transcriptional activity after stimulation with Dox in all clones analyzed. (**E**) TR1-CnA, TR4-CnA, TR1-NFAT and TR4-NFAT clones were mortalized with AdCre, and differentiated for 3 days in serum free medium. Total RNA was analyzed RT-PCR for a series of cardiac genes and compared to RNA isolated from parental NkL-TAg cells and adult wild type heart. (**F**) Schematic representation of flow of experimental approach to mortalize TR-calcineurin or TR-NFAT clones with AdCre, differentiated for 3 days in serum free medium, and cultured for 24 hr in medium containing doxycyclin or not, and total RNA subjected to microarray profiling or real time RT-PCR.

Early cardiac muscle genes regulated by calcineurin and NFAT. Agilent mouse chips harboring 44,000 transcripts were used to identify the early gene expression pattern evoked by calcineurin/NFAT activation by 24 hr doxycyclin stimulation (Fig. 2a). 27 genes (0.06% of all genes) were differentially expressed in a consistent manner in the four TR clones with a fold change in expression ≥ 2 ($P < 0.01$) (Fig. 2a). Among these 27 genes, 20 showed an increase in expression, 7 genes decreased expression. Gene ontology classifications revealed an overrepresentation of genes in three specific subclasses: translational machinery/protein transport, transcription factors and signal transduction components (Fig. 2a). We picked 8 genes from the list to verify differential expression by real-time RT-PCR and validated whether their up- or downregulation was sustained in hearts from 3 week-old wildtype or calcineurin Tg mice (Fig. 2b, c).

The vast majority of genes identified, except for *gata4*, *alpha tropomyosin 1* (*tpm1*) and *regulator of calcineurin* (*rcan1* or *dscr1/mcip1*), have not been connected to cardiac pathogenesis so far. Unexpectedly, transcripts for the zinc-finger containing transcription factor GATA binding protein 4 (*gata4*) were found upregulated, while those for coiled-coil-like protein 1 (*cclp1* or *ppfibp2*), another transcription factor with unknown function, were consistently decreased, suggesting that calcineurin/NFAT signaling secondarily provokes distinct alterations in gene expression.

Another gene class differentially regulated by calcineurin/NFAT activity included genes involved in energy metabolism and mitochondrial energy production, such as *mitochondrial ribosomal protein L38* (*mrpl38*) and *procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3* (*plod3*), which contains oxidoreductase activity (Fig. 2a, b). This is in line with previous reports suggesting a direct or indirect role for calcineurin to control cardiac energy production, reactive oxygen species and apoptosis (8, 14).

Of interest to the specific cardiomyopathic alterations observed in calcineurin or NFAT transgenic mice, another interesting gene ontology class involves protein translation, protein transport and (de)ubiquitination processes. Obviously, calcineurin activity provokes a massive hypertrophic response, the latter of which requires increased protein synthesis. Exemplary are transcripts of genes including *NECAP endocytosis associated 2* (*necap2*), *alanyl-tRNA synthetase* (*aars*), *golgi apparatus protein 1* (*glg1*) and *deubiquitinating enzyme 1* (*dub1*) (Fig. 2a, b).

Finally, calcineurin/NFAT signaling invokes secondary signaling events, evidenced by upregulation of the small GTP-binding protein domain-containing protein *ADP-ribosylation factor 6* (*arf6*) and *G protein-coupled receptor kinase 5* (*gprk5*). In contrast, *protein phosphatase 1 (formerly 2C)-like* (*Ppm1l*) and *leucine*

zipper protein 1 (*luzp1*) were significantly downregulated, implicating each of them in pro-hypertrophic signaling (Fig. 2a, b, c). Combined, these data indicate that calcineurin/NFAT activates subsets of genes primarily localized to or functioning with respect to transcriptional processes, mitochondrial metabolism, signal transduction, and protein biogenesis.

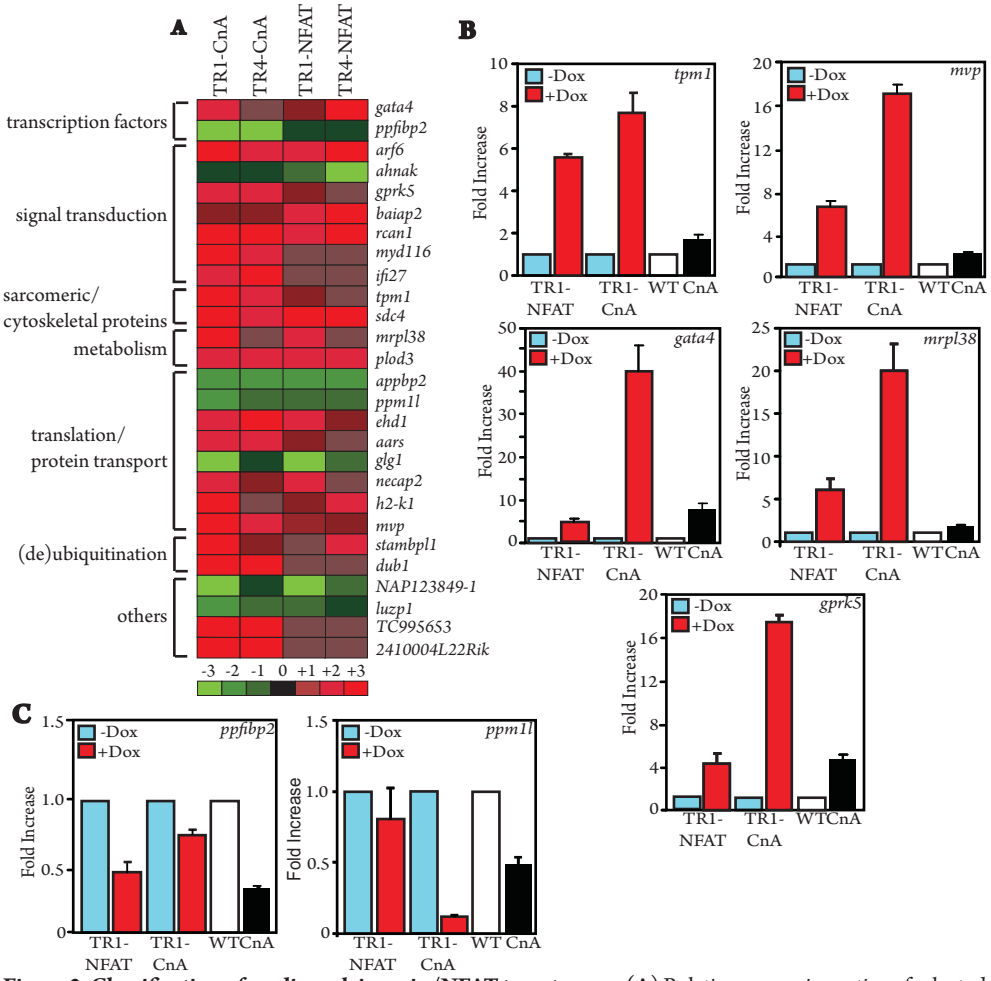

Figure 2. Classification of cardiac calcineurin/NFAT target genes. (**A**) Relative expression ratios of selected functional gene groups assessed from microarray screening. Cardiac RNA was collected from 3 separate TR clones (+/- Dox) and subjected to expression profiling using the Agilent 44k mouse whole genome microarray slides (Palo Alto) arrays. The color scale representation of gene expression levels is indicated, with black equal to 0 (no change), bright red equal to 3.0 (3.0-fold increased expression), and bright green equal to -3.0 (-3.0 decreased expression). (**B**) Real time RT-PCR validation of microarray results for mRNA levels of indicated genes that displayed increased expression in TR1-CnA, TR1-NFAT clones (+/- Dox). Adult wildtype and calcineurin transgenic hearts were taken along to analyze the corresponding gene expression values in myocardium. All values were corrected for L7 mRNA levels to control for loading variations. (**C**) Real time RT-PCR validation of microarray results for mRNA levels of indicated genes that displayed decreased expression.

NFAT directly activates the *gata4* promoter. In order to define the calcineurin/NFAT-responsiveness of Gata4 in cardiac muscle, we searched for enhancers that might regulate *in vivo* transcription of *gata4*. Comparison of genomic sequences across species using rVISTA revealed that a 3.0 kb genomic region immediately upstream of the first exon of gata4 was conserved between human and mouse, apart from discontinued more distal regions that also displayed high cross-species conservation (Fig. 3a). Within these regions we noted several *cis* elements conserved between human and mouse that represented potential binding sites for the essential (cardiac) transcription factors Mef2, Nkx2.5 and NFAT. Three potential NFAT consensus binding sites ([T/A]GGAAA or complementary) in the gata4 enhancers were nearly identical and conserved in human, mouse and rat, which we designated N1, N2 and N3 (Fig. 3b). Of interest, two highly conserved potential Mef2 sites were found in gata4 in close proximity to the NFAT enhancers N1 and N2, further lending support to the notion of a combinatorial MEF2/NFAT regulatory transcriptional pathway controlling gene expression in cardiac muscle cells as recently described by our group (12).

In order to confirm the binding of NFAT to the *gata4* enhancers *in vivo*, chromatin immunoprecipitation (ChIP) was carried out. To this end, TR1-CnA clones were mortalized, differentiated and either maintained in serum free media or cultured in the presence of doxycyclin fo 24 hr; the resultant nuclear fractions were immunoprecipitated using specific antibodies to NFATc3, and associated DNA was purified (Fig. 3c). Using specific primers to the *gata4* promoter flanking the N1, N2 or N3 sites, by real time PCR, only the PCR amplicon flanking N3 was observed to be significantly enriched in differentiated TR1-CnA clones upon activation of the calcineurin/NFAT pathway compared to differentiated TR1-CnA clones in the absence of doxycyclin (Fig. 3c). This association was specific for NFATc3 since enrichment of PCR products was not obtained when using beads alone, or when using primers to an unrelated promoter, such as myoglobin (data not shown). Of further evidence of the functionality of the N3 site, by electromobility shift assays, NFAT could specifically bind the most proximal N3 site in the *gata4* enhancer region (Fig. 2b, d). Taken together, these results indicate that calcineurin/NFAT signaling regulates the *gata4* gene by direct transcriptional activation, and unambiguously show the presence of endogenous NFAT proteins on the proximal *gata4* promoter *in vivo*, supporting the idea that *gata4* is a direct target gene of calcineurin/NFAT signaling.

Gata4 protein levels are increased in the failing postnatal myocardium. To determine whether calcineurin/NFAT-mediated *gata4* transcription also translates into increased Gata4 protein expression in the postnatal myocardium, we used western blot analysis to assay GATA4 abundance *in vivo*. Transgenic mice overexpressing murine calcineurin in myocardium (MHC-CnA) were mated with *nfatc2-/-* mice to obtain F2 mice with four different genotypes: wildtype, the CnA gain-of-function (MHC-CnA), the *nfatc2* loss-of-function (*nfatc2-/-*), and the combinatorial genotype (MHC-CnA / *nfatc2-/-*). At 3 weeks of age, *nfatc2*-null mice did not differ from wildtype controls; thus, NFATc2 is dispensable to achieving normal adult cardiac mass (data not shown). These findings are suggestive for the interpretation that calcineurin/NFAT signaling does not regulate embryonic Gata4 induction given that *nfatc2*-null mice achieve normal cardiac mass and function. By contrast, transgenic mice with activated calcineurin in the postnatal myocardium in an *nfatc2*-null background revealed a significant abrogation (>60%) of calcineurin-provoked cardiac growth (data not shown). At the age of 3 weeks, GATA4 protein abundance in myocardium was unchanged between wildtype and nfatc2-null mice (Fig. 3e). Postnatal calcineurin-mediated hypertrophy in MHC-CnA mice was accompanied by clear upregulation of Gata4 protein levels. By contrast, in hearts from mice with the combinatorial genotype (MHC-CnA / *nfatc2-/-*), Gata4 protein abundance was normalized to a level of wildtype mice (Fig. 3e).

To assess whether Gata4 is also upregulated in human heart failure, we determined Gata4 protein expression in left ventricular myocardium from three nonfailing and six failing hearts and found an approximate 5-fold increase of Gata4 in left ventricles from failing human hearts (Fig. 3f, g). In conclusion, from our complimentary approaches we surmise that postnatal myocardial hypertrophy and *gata4* gene induction critically relies upon calcineurin/NFAT signaling.

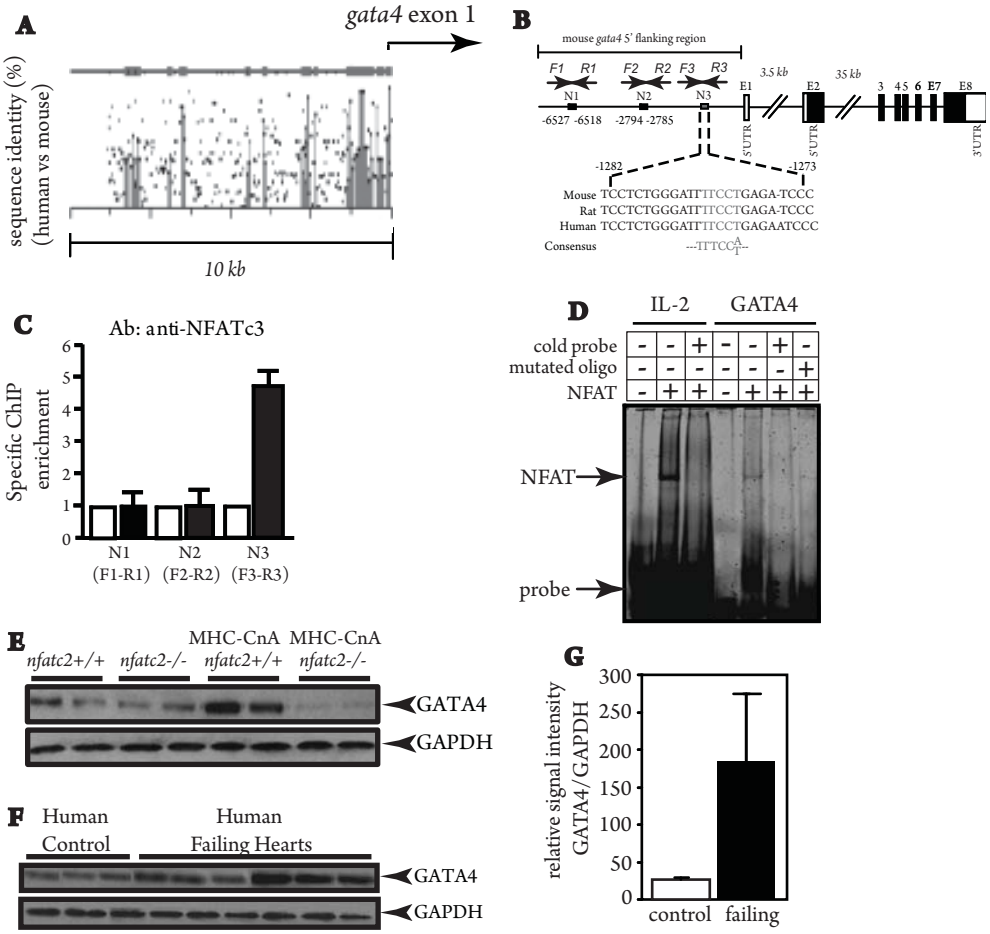

Figure 3. Presence of a functional NFAT site in the Gata4 enhancer regulates calcineurin-responsiveness in postnatal myocardium. (**A**) Comparison of the *gata4* genomic regions between mouse and human. Percentage conservation of a 5' 10.0 kb genomic region upstream of *gata4* first exon is shown. (**B**) Schematic presentation of a ± 7 kb 5' flanking region in mouse *gata4* and location of potential NFAT binding sites (N1, N2, and N3). Primers yielding ChIP amplicons are indicated. (**C**) Chromatin immunoprecipitation assays were performed on TR1-CnA (+/- Dox) and soluble chromatin was immunoprecipitated with a specific antibody for NFATc3. Bars represent fold enrichment of amplicons with indicated primer sets in (**B**), normalized to input controls obtained with primers spanning a non-coding genomic region 3' of the *gata4* gene. (**D**) Gel mobility shift assay was performed using fluorescent probes of NFAT consensus sequences (IL-2) or NFAT-like site N3 from the Gata4 promoter. (**E**) Western blot analysis of Gata4 protein abundance in 3 week-old hearts from indicated genotypes indicates that Gata4 is increased upon calcineurin stimulation *in vivo*, and this upregulation is prevented in an *nfatc2* null background. (**F**) Gata4 abundance in human heart failure biopsies. (**G**) Quantification of Gata4 protein expression in human failing heart compared to control hearts using GAPDH expression level as a loading control.

Inhibition of GATA4 induction reduces calcineurin-induced cardiomyocyte hypertrophy. To assess the functional requirement of Gata4 upregulation downstream of calcineurin/NFAT signaling in cardiac muscle, we resorted to short hairpin (siRNA) knockdown approaches of endogenous Gata4 in cultured cardiomyocytes. First, we tested the efficiency of a Gata4-specific siRNA in neonatal rat cardiomyocytes. By Western blotting, we confirmed that endogenous Gata4 was significantly downregulated in AdGFP or AdCnA-infected primary cardiomyoctes compared to cells infected with AdGFP or AdCnA-infected and transfected with a control siRNA (siRNA-scrambled) (Fig. 4a).

To monitor the change in cell size or sarcomere organization, cells were stained for sarcomeric α-actinin and counterstained with TO-PRO3 to visualize nuclei (Fig. 4b). First, cells were left untransfected, transfected with a non-specific siRNA or transfected with the siRNA specific for Gata4. After 24 h, cells were then left untreated (Fig. 4b, left panels), infected with AdGFP (Fig. 4b, middle panels) or infected with AdCnA for 24 h (Fig. 4b, right panels). Uninfected or AdGFP-infected cells left untransfected or transfected with either siRNA species, showed no signs of hypertrophy (885±68μm^2, 747±50 μm^2, 932±73 μm^2, AdGFP, AdGFP/control siRNA or AdGFP/Gata4-siRNA, respectively; N.S.) AdCnA resulted in a more than 2-fold increase in cell surface area (1703±134 μm^2 vs. 1601±101 μm^2, $p<0.01$ versus AdGFP; Fig. 4c). By contrast, Gata4-specific siRNA substantially abrogated the prohypertrophic effects of AdCnA (907±52 μm^2 vs 1542±122 μm^2, AdCnA/Gata4-siRNA and AdCnA/control siRNA; $p<0.05$; $p<0.05$ versus AdGFP; Fig. 4c). These data demonstrate that Gata4 is critically involved in all aspects of the initial hypertrophic response of cardiomyocytes following calcineurin/NFAT activation.

DISCUSSION

Re-activation of a "fetal" gene program is a conserved feature of the hypertrophic response and has been extensively studied as a means to identify physiological regulators of hypertrophy. Although the initial steps of the induction of embryonic or prohypertrophic genes are reversible, chronic changes in the cardiac transcriptome may trigger pathological changes in the myocardium that invoke irreversible cellular changes, dilation of the ventricular chamber and cardiac dysfunction, often the first irreversible steps towards heart failure (15). Accordingly, the transcription factors that connect biomechanical forces and the activation of stress pathways to morphological changes of the myocardium

are central to understanding the initiation and progression of heart failure. The calcineurin/NFAT transcriptional paradigm is both sufficient and required to drive the cardiac hypertrophic growth response (3, 8, 9). Notwithstanding its established requirement in provoking pathological gene expression preceding heart failure development, limited information is available about the immediate downstream calcineurin/NFAT target genes in cardiac muscle.

Using a multidisciplinary approach, we analyzed the consequences of activating the calcineurin/NFAT pathway in cardiac muscle, starting from the premise that the target genes of calcineurin and/or NFAT would provide novel insights into pathological hypertrophic remodeling of the heart muscle. Accordingly, we uncovered the earliest calcineurin/NFAT-regulated transcripts in cardiac muscle. Gene ontology classifications of these target genes revealed an overrepresentation of genes in translational machinery/protein transport, energy metabolism, signal transduction and transcription factors, allowing insight into the particulars of pathological cardiac remodeling. Specifically, calcineurin activity provokes a massive hypertrophic response, the latter of which requires increased protein synthesis, which are seemingly further fortified by secondary signaling events, thereby implicating such novel signaling components as the small GTP-binding protein domain-containing protein *ADP-ribosylation factor 6* (*arf6*), *G protein-coupled receptor kinase 5* (*gprk5*), *protein phosphatase 1-like* (*Ppm1l*) and *leucine zipper protein 1* (*luzp1*) in prohypertrophic signaling. Secondly, the genomic changes in mitochondrial energy metabolism are in line with previous reports suggesting a direct or indirect role for calcineurin to control cardiac energy production, reactive oxygen species and apoptosis, implicating a fundamental role for mitochondrial function and biogenesis in the pathology (14, 16-18).

Unexpectedly, Gata4 transcripts and protein levels were found to upregulated early after activation of calcineurin/NFAT stimulation and in a sustained manner in human heart failure biopsies. Moreover, genetic abrogation of Gata4 induction using a short hairpin knockdown approach revealed its functional requirement as a downstream NFAT target gene in provoking initial hypertrophic remodeling. Given that NFAT and pre-existing Gata4 proteins cooperatively activate target genes such as *brain natriuretic peptide, adenylosuccinate synthetase 1, endothelin-1, calcineurin A-beta* as transcriptional partners (3, 17, 19, 20), our findings suggest the existence of a novel transcriptional feedforward mechanism where NFAT signaling invokes expression of the pro-hypertrophic Gata4 transcription factor, allowing amplification of calcineurin/NFAT signaling in the ventricular myocyte (Fig. 5). Gata4 has been ascribed to function as a transcriptional integrator for various stress

signaling pathways in the postnatal myocardium. Forced viral overexpression of Gata4 provokes cardiomyocyte hypertrophy in culture, while dominant negative forms or antisense delivery efficiently counteracts Gata4-directed transcriptional responses and features of cardiomyocyte hypertrophy secondary to phenylephrine or endothelin-1 stimulation (21, 22). *In vivo*, Gata4 function is pleiotropic as it mediates stress-induced cardiac hypertrophy, survival of cardiac myocytes and postnatal myocardial angiogenesis (23-26).

Gata4 has also been ascribed to regulate the specification and differentiation of cardiac myocytes early in development through the direct transcriptional control of key cardiac structural and regulatory genes (27-29), alone or in concert with developmentally active transcription factors such as Tbx20, Nkx2.5, SRF, Hand2, and myocardin (30-35). We uncovered a novel gata4 enhancer near exon 1 that harbors a cluster of NFAT and MEF2 sites. Recently, a gata4 enhancer located more distal to the one described in this study was demonstrated to be responsive to Forkhead and GATA factors and required for gata4 transcription in the lateral mesoderm (36). The combinatorial observations suggest the existence of physically separated enhancers for gata4 developmental expression versus gata4 induction upon calcineurin/NFAT-mediated stress signaling. Our study remains inconclusive whether calcineurin/NFAT signaling is responsible for developmental gata4 regulation. Gene targeting studies in mice demonstrated that a combinatorial knockout for both NFATc3 and NFATc4 result in embryonic death due to defects in metabolic maturation of the myocardium at midgestation (37), while similar defects in heart development were observed in chicken embryos treated with cyclosporine A, the pharmacological calcineurin inhibitor (38). Moreover, *rcan1* gene expression is regulated by NFATc1 during valve maturation and coincides with abnormal development in Down syndrome (39). By contrast, our results indicate that mice harboring a null mutation for *nfatc2* achieve normal adult cardiac mass, suggesting a more specialized function for NFAT transcription factors as integrators of stress signaling in the postnatal myocardium. Future conclusive studies should elucidate whether the *gata4* enhancers are indeed developmentally and spatially separated.

Finally, our current approach used stably transfected NkL-TAg ventricular sarcoma clones harboring conditional expression of simian virus 40 large T-antigen (10), which allows stable integrations during their propagation phase, while its regulated growth termination permits reproducible genomics approaches to study cardiac signaling in a genome-wide fashion in the appropriate cellular context. In this study, we created double stable NkL-TAg clones harboring a stably integrated tetracyclin-dependent repressor and constitutively activated mutants

of either calcineurin or NFAT. This approach allowed us controlled activation of the calcineurin/NFAT pathway in a time- and dosage-dependent manner and elucidation of the earliest gene targets of this signaling pathway. Similar approaches can be envisioned to study other signaling components. The unique combination of reproducibility of stably expressing clones with absence of senescence during the propagation phase, provides a valuable resource to perform high throughput chemical compound or viral siRNA screens for the identification of novel inhibitory compounds or cellular modulators in a genome-wide fashion for myocardial stress signaling.

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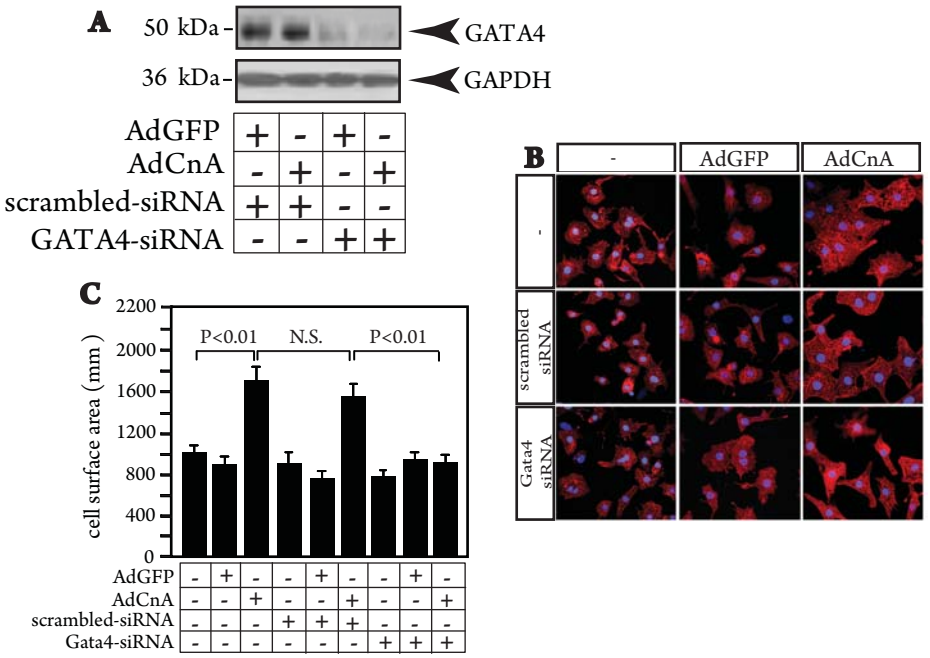

Figure 4. Inhibition of GATA4 induction reduces calcineurin-induced cardiomyocyte hypertrophy. (**a**) Western blot analysis of endogenous Gata4 levels in primary cardiomyocytes treated with scrambled-siRNAor Gata4-siRNA, indicating specific downregulation of Gata4 protein after treatment with a Gata4-specific Gata4-siRNA, indicating specific downregulation of Gata4 protein after treatment with a Gata4-specific siRNA. (**b**) Confocal microscopy of cultured neonatal rat cardiomyocytes treated with indicated siRNA species and/or infected with indicated adenoviruses. (**c**) Quantification of myofiber cross-sectional areas from indicated groups show significant attenuation of calcineurin-induced cardiomyocyte hypertrophy after treatment with Gata4-siRNA (n=50 cells per group).

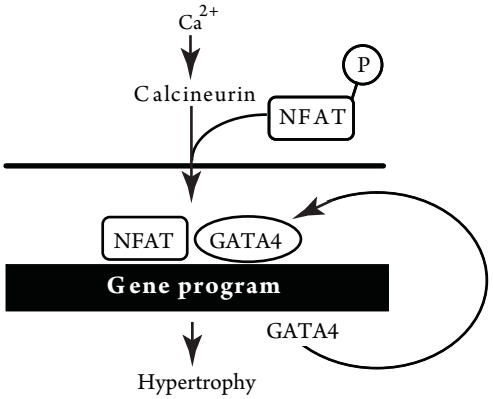

Figure 5. Following cardiac calcineurin activation, NFAT is able to recruit pre-existing Gata4 to cooperatively activate selected downstream target genes. One NFAT target gene is Gata4, allowing a transcriptional feedforward loop that reinforces calcineurin/NFAT mediated gene induction and myocyte hypertrophy.

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CHAPTER 5

Cooperative synergy between NFATc3 and MyoD regulates myogenin expression and myogenesis

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ABSTRACT

Calcineurin/NFAT signaling is involved in multiple aspects of skeletal muscle development and disease. The myogenic basic helix-loop-helix (bHLH) transcription factors, MyoD, myogenin, Myf5, and MRF4 specify the myogenic lineage. Here we show that calcineurin/NFAT signaling is required for primary myogenesis by transcriptional cooperation with the bHLH transcription factor MyoD. Calcineurin/NFAT signaling is required for myogenin expression in differentiating myoblasts, where the myogenic regulatory factor MyoD selectively recruits NFATc3 to the myogenin promoter. Using gelshift and chromatin immunoprecipitation assays, we identified three evolutionary conserved NFAT binding sites in the myogenin promoter, which were occupied by NFATc3 upon skeletal muscle differentiation. The transcriptional integration between NFATc3 and MyoD is crucial for primary myogenesis *in vivo*, since myogenin expression is absent in *myod:nfatc3* double null embryos, while myogenin expression is unaffected in embryos with null mutations for either factor alone. Thus, the combined findings provide a novel transcriptional paradigm for the first steps of myogenesis, where a calcineurin/NFATc3 pathway regulates myogenin induction in cooperation with MyoD.

INTRODUCTION

In vertebrates all trunk muscles originate from the dermomyotome, an epithelial sheet formed by the paraxial mesoderm that develops from the dorsal part of the epithelial somite and overlays the sclerotome. Vertebrate skeletal muscle develops through the fusion of a variable number of myoblasts, muscle precursors committed to the skeletal muscle lineage within the myotome, to form syncitial myofibers (1). Myf5 is the first myogenic regulatory protein expressed in the skeletal muscle lineage. In concert with Pax3, Myf5 activates a network of myogenic regulatory factors including MyoD, myogenin and MRF4 (Myf-6) in the muscle precursors to initiate and maintain the expression of muscle-specific genes (reviewed in (2)). Genetic studies indicate that both Myf-5 and MRF4 act upstream of MyoD to specify the myogenic lineage (3, 4), whereas myogenin has a crucial role in the terminal differentiation of committed muscle cells (5, 6). Protein motifs conserved in MyoD and Myf-5 are necessary to initiate the expression of a subset of genes critical for the myogenic program, including transcription of the *myogenin* gene (7, 8).

The second messenger calcium regulates many signaling pathways critical for skeletal muscle homeostasis. A number of studies demonstrate that the calcium/calmodulin-dependent protein phosphatase calcineurin plays a regulatory role in skeletal muscle adaptation and muscle regeneration by its ability to promote myotube differentiation (9, 10). Calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT) transcription factor family, allowing NFAT to translocate to the nucleus where it cooperates with other transcription factors to induce transcription of target genes. Five NFAT genes have been identified, NFATc1-c4 and NFAT5 (11). Forced calcineurin activity provokes nuclear translocation of NFATc3 and differentiation of myoblasts. These *in vitro* results are consistent with the muscle phenotype of *nfatc3* null mice, which display reduced muscle mass due to a decrease in the number of myofibers (10). Although the precise mechanisms by which this occurs remain unresolved, these findings suggest that NFATc3 may serve a specialized role in primary myogenesis.

In this study, we provide mechanistic insights how calcineurin/NFAT signaling regulates primary myogenesis. We show that calcineurin/NFAT signaling induces myogenin expression in differentiating C2C12 cells by transcriptional cooperation with the bHLH transcription factor MyoD. Our data demonstrate that the myogenic regulatory factor MyoD recruits NFATc3 to the myogenin promoter in differentiating myoblasts. We demonstrate that NFATc3 and MyoD both play a crucial role in somite differentiation since double null *myod*/*nfactc3* embryos express

very low amounts of myogenin transcripts, while myogenin expression in mice with single null mutations for either *nfatc3* or *myod* is unaffected. Thus, a calcineurin/NFATc3 pathway plays a crucial role in the first steps of myogenesis, by regulating myogenin induction in cooperation with MyoD.

EXPERIMENTAL PROCEDURES

Animals. *Myod*, *nfatc3* and *nfatc2* null mice were generously provided by Shahragim Tajbakhsh and Laurie Glimcher and were described previously (3, 12, 13). All protocols were performed according to institutional guidelines and approved by local Animal Care and Use Committees.

Processing of embryos and detection of transcripts. Embryos were processed for WM-ISH (14). Riboprobes for *myog* and *paraxis* were as described previously (15). For comparative WM-ISH experiments, age-matched and litter-matched embryos were used with independent probe sets and litters, and the ISH reactions were stopped at the same time.

Cell culture, transfections and adenoviruses. Cell culture of C2C12 and COS7 cells was described previously (17). Transfections were performed in 48-well plates using FuGENE 6 (Roche) and the dual luciferase system (Promega). AdGFP was generated as described previously (16). AdVIVIT was generated by using VIVIT-eGFP (17) and the pAdTrack-CMV system (18). AdNFAT9mer-luc was generated via ligation of NFAT9mer-Luc in vector pDC511 and FLP mediated recombination with pBGHfrt (Microbix).

Coimmunoprecipitation assays and western blot analysis. V5/His tagged MyoD or NFATc3 constructs were immunoprecipitated using Ni-NTA beads (Invitrogen), followed by western blotting procedures as described in detail previously (16) . Antibodies used included mouse monoclonal anti-GAPDH (Chemicon), rabbit polyclonal anti-myogenin (Santa Cruz), mouse monoclonal anti-V5 (Invitrogen), and mouse monoclonal anti-Gal4 (Santa Cruz). previously (11). The NFAT9mer-Luc plasmid harbors nine copies of a high-affinity NFAT binding site from the interleukin-4 enhancer inserted upstream of a minimal TATA box and pGL3. Detailed information about vectors and oligo sequences is available upon request. Reverse transcription and real-time PCR using the BioRad iCycler (Biorad) was described previously (11).

Cloning and real-time PCR. MyoD constructs were inserted in frame into pBind (Promega) and pCDNA3.1/V5/His (Invitrogen) vectors. Expression vectors for an activated mutant of CnA or deletion fragments for NFATc3 were described previously (16). The NFAT9mer-Luc plasmid harbors nine copies of a high-affinity NFAT binding site from the interleukin-4 enhancer inserted upstream of a minimal TATA box and pGL3. Detailed information about vectors and oligo sequences is available upon request. Reverse transcription and real-time PCR using the BioRad iCycler (Biorad) was described previously (16).

Chromatin immunoprecipitations. C2C12 cells cultured either in proliferating medium or in differentiation medium for 48 h and cross-linked with 2% formaldehyde for 20 min at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were washed with cold phosphate-buffered saline (PBS), collected and resuspended in sonication buffer containing 50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.5% SDS, and protease inhibitors and sonicated on ice to an average length of 200 to 1000 bp. Samples were centrifuged at 14000 rpm and precleared with protein G-Sepharose in the presence of 25 μg/mL of salmon sperm DNA and 1 mg of bovine serum albumin/ml. Precleared chromatin was immunoprecipitated with 5 μg of monoclonal anti-NFATc3 (sc-8405X, Santa Cruz) or polyclonal anti-MyoD (sc-760, Santa Cruz). The immune complexes were collected by adsorption to protein G-Sepharose. The beads were washed twice a non-labeled oligonucleotide duplex containing the corresponding NFAT binding sequence was used. Forward and reverse FAM-labeled oligo (15 pmol) were incubated for 10 min at 95 C in a total volume of 50 μL annealing buffer (100mM NaCl, 50mM HEPES, pH 7.5), followed by slowly cooling down to room temperature. FAM-oligo duplex (1.5 pmol) and 2 μg of nuclear extract of NFATc2 transfected COS7 cells were incubated for 15 min at room temperature in a total volume of 15 μL binding buffer (final concentration 10% (vol/vol) glycerol, 50 mM KCl, 15 mM Tris pH 7.9, 0.2 mM EDTA, 1 mM MgCl2, 0.4 mM dithiothreitol, 33 μg/ml BSA, pH 7.9), subsequently kept on ice, and loaded on a 5% polyacrylamide (29:1), 2.5% glycerol, TAE (Tris-acetate EDTA, pH 8) gel. Tracking dye (15 μl) was loaded in a separate lane. 1xTAE supplemented with 1 mM MgCl2 was used as running buffer. For competition experiments nonlabeled oligo duplex was added in molar excess to the binding mix as indicated. The gel was run at 4 C in the dark at 150 V for 2.5 h. The fluorescent signal was immediately analyzed at 488 nm using a FluorImager 595 (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant

with sonication buffer, twice with sonication buffer containing 500 mM NaCl, twice with 20 mM Tris (pH 8.0)-1 mM EDTA-250 mM LiCl-0.5% NP-40-0.5% sodium deoxycholate, and twice with Tris-EDTA buffer. The immunocomplexes were eluted with 50 mM Tris, pH 8.0-1 mM EDTA-1% SDS at 65°C for 10 min, adjusted to 200 mM NaCl, and incubated at 65°C for 5 h to reverse the cross-links. After successive treatments with 10 μg of Rnase A and 20 μg of proteinase K/ml, the samples were extracted with phenol-chloroform and precipitated with ethanol. One tenth of the immunoprecipitated DNA and input DNA (from extracts before immunoprecipitation) was analyzed using real-time PCR reactions on a MyIQ apparatus (Bio-Rad). Oligos used for the PCR amplifications were as follows: myogenin promoter amplicon 1, sense: 5'-AAGGAGAGGGAAGGGGAATC-3' and antisense: 5'-GCCAACGCCACAGAAACC-3'; myogenin promoter amplicon 2, sense: 5'-GATTTTCAAGACCCCTTCCC-3' and antisense: 5'-CCGTCGGCTGTAATTTGATTAG-3'; myogenin promoter amplicon 3, sense: 5'-TGATGTGGTAGTGGTAGGTC-3'; control region downstream myogenin, sense: 5'-TCCTGGATTACTGTCAAGC-3'.

Electromobility shift assay. The following HPLC-purified FAM-labeled double-stranded oligonucleotides were used: the NFAT site from the Interleukin-2 promoter (FAM-Il2: 5'-FAM-GGAGGAAAAACTGTTTCATACAGAAGGCGT-3'), the Myog-N1 (FAM-N1: 5'-TAATCCACTGGAAACGTCTTGA 3'), Myog-N2 (FAM-N2: 5'-TGTGGAGAAATGAAAACTAATC-3'), and Myog-N3 (FAM-N3: 5'-TGCTGAGCAGGAAAGAGAAGGC-3'). For competition experiments a non-labeled oligonucleotide duplex containing the corresponding NFAT binding sequence was used. Forward and reverse FAM-labeled oligo (15 pmol) were incubated for 10 min at 95 C in a total volume of 50 μL annealing buffer (100mM NaCl, 50mM HEPES, pH 7.5), followed by slowly cooling down to room temperature. FAM-oligo duplex (1.5 pmol) and 2 μg of nuclear extract of NFATc2 transfected COS7 cells were incubated for 15 min at room temperature in a total volume of 15 μL binding buffer (final concentration 10% (vol/vol) glycerol, 50 mM KCl, 15 mM Tris pH 7.9, 0.2 mM EDTA, 1 mM MgCl2, 0.4 mM dithiothreitol, 33 μg/ml BSA, pH 7.9), subsequently kept on ice, and loaded on a 5% polyacrylamide (29:1), 2.5% glycerol, TAE (Tris-acetate EDTA, pH 8) gel. Tracking dye (15 μl) was loaded in a separate lane. 1xTAE supplemented with 1 mM MgCl2 was used as running buffer. For competition experiments nonlabeled oligo duplex was added in molar excess to the binding mix as indicated. The gel was run at 4 C in the dark at 150 V for 2.5 h. The fluorescent signal was immediately analyzed at 488 nm using a FluorImager 595 (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant

software (ImageQuant 5.2, Amersham Biosciences).

Statistical Analysis. The results are presented as means ± SEM. Statistical analyses were performed using INSTAT 3.0 software (GraphPad, San Diego) and Student's t-test or ANOVA followed by Tukey's post-test when appropriate. Statistical significance was accepted at a P value < 0.05.

RESULTS

Calcineurin/NFAT signaling is required for myogenin expression.
Genetic studies indicate that MyoD and Myf-5 are necessary to specify the skeletal muscle lineage, whereas myogenin (Myog) has a critical role in the terminal differentiation of the specified muscle cells (5, 6). We first analyzed the timing of Myog induction in cultured C2C12 skeletal myoblasts upon differentiation to skeletal myotubes. Western blot analyses reveal a slight increase in Myog protein after 48h of differentiation, which is dramatically enhanced after 72 and 96 h of differentiation (Fig. 1a). Next, we induced myotube differentiation of myoblasts pre-infected with an adenovirus harboring 9 copies of the consensus NFAT-binding site from the IL-4 gene (9-mer) upstream of a minimal TATA box driving luciferase (AdNFAT-luc), to track activity of endogenous NFAT in skeletal myoblasts. In either proliferation medium (PM) or upon differentiation for 24 h, endogenous NFAT transcriptional activity is barely detectable (Fig. 1b). In contrast, NFAT activity is massively and transiently increased in C2C12 starting at 48h of differentiation (Fig. 1b), a time point that corresponds with the induction of Myog (Fig. 1a).

To determine whether NFAT transcriptional activity is required for the induction of Myog upon differentiation, myoblasts were infected with either a control adenovirus (AdGFP) or an adenovirus expressing a fusion between GFP and the high-affinity peptide VIVIT, which specifically inhibits calcineurin-mediated activation of NFAT (17). Cells were allowed to differentiate into myotubes, and Myog transcript and protein abundance were documented. AdGFP-infected myoblasts display a dramatic induction of Myog transcripts after 48 hr of differentiation (Fig. 1c), which mirrors the induction of Myog protein expression (Fig. 1a). In contrast, AdVIVIT-infected cells express substantially lower amounts of Myog transcripts at every time point analyzed (Fig. 1c). The inhibition of Myog induction by VIVIT-mediated inhibition of NFAT transcriptional activity was confirmed at the protein level (Fig. 1d). Conclusively, these findings demonstrate that NFAT activity is

induced upon differentiation of myoblasts to myotubes and that NFAT activity is required for Myog expression and skeletal muscle differentiation.

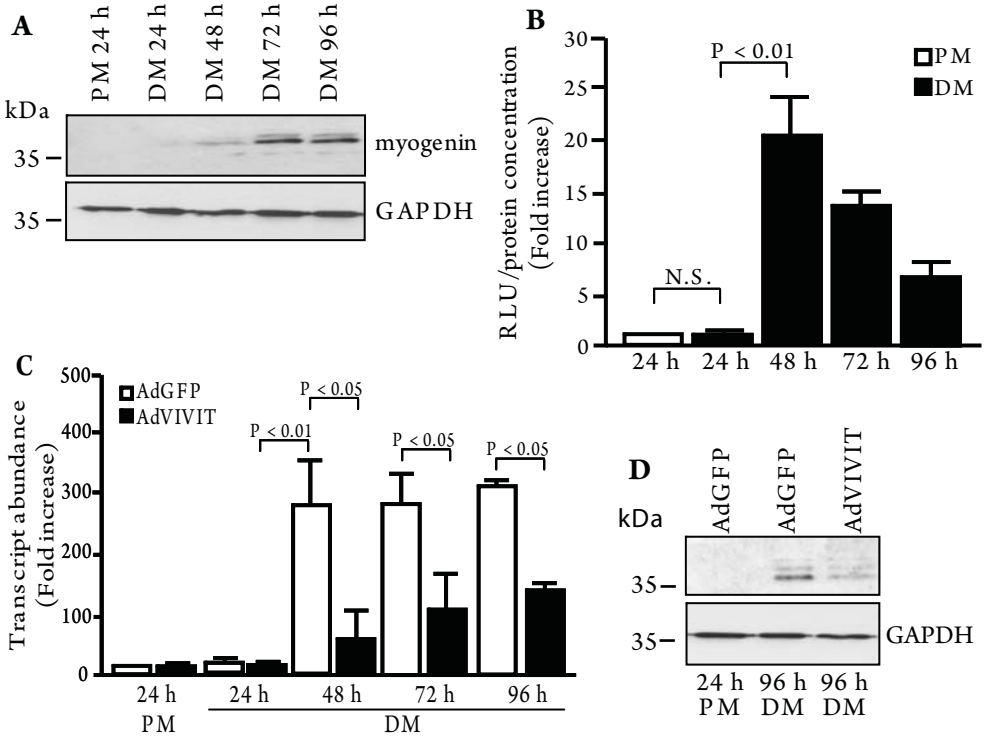

Figure 1. NFAT transcriptional activity is required for myogenin expression.
(A) C2C12 myoblasts were cultured in proliferation (PM) or differentiation medium (DM) and abalyzed for myogenin protein abundance using GAPDH as loading control. (B) C2C12 myoblasts were infected with AdNFAT9mer-luc, grown in PM or DM, and luciferase induction analyzed. Data is provided as mean ± SEM (n = 3) as fold increase of the ratio relative light units/ protein content in differentiation over proliferation. (C-D) C2C12 cells were infected with either control AdGFP or AdVIVIT adenoviruses and cultured in PM or DM for indicated time periods, and myogenin transcript (C) or protein abundance (D) analyzed by real-time PCR or western blotting, respectively. The data in (C) represents the mean ± SEM of two independent experiments.

NFATc3 directly activates the Myog promoter

In order to define the mechanisms behind the regulation of Myog by calcineurin/NFAT signaling, we searched for enhancers that might regulate *myog* transcription *in vivo*. Comparison of genomic sequences across species, using rVISTA, revealed several regions with high conservation between human, rat and mouse within a 5.0 kb 5' flanking genomic region of *myog* (Fig. 2a). Within the most proximal 0.6 kb part of murine *myog*, three potential NFAT consensus binding sites ([T/A] GGAAA) were found, which we designated N1, N2 and N3.

To determine whether endogenous calcineurin/NFAT signaling can activate the *myog* promoter, a DNA fragment extending from +73 to -522 bp relative to the transcription initiation site of the mouse *myog* gene was fused to a luciferase reporter (Myog-luc), transfected in myoblasts, and cells were allowed to differentiate to myotubes. Transcriptional activity of the Myog promoter was very low in myoblasts. Myog-luc was strongly activated after 48 hr of differentiation, mimicking the temporal activation profile of endogenous Myog transcripts (Fig. 1c), with a maximum at 96 hr (72±6 fold increase; Fig. 2c). In contrast, when Myog-luc was cotransfected with an expression vector expressing a fusion between GFP and the high-affinity peptide VIVIT, activation of the Myog promoter was significantly abrogated at each time point analyzed (Fig. 2c).

NFAT bound the labeled NFAT-like sequences from the *myog* promoter to a similar efficiency as the NFAT site at the *il2* promoter, and binding to the cognate sites was completely eliminated by the presence of unlabeled NFAT consensus sequences (Fig. 2d). Similarly, labeled oligonucleotides representing either of the *myog* NFAT-like sequences with the core NFAT site mutated were not able to bind NFATc3 (Fig. 2d).

In order to further confirm the binding of NFATc3 to the promoter of *myog*, chromatin immunoprecipitation (ChIP) was carried out. C2C12 myoblasts were either maintained in PM or were allowed to differentiate for 48 hr; the resultant nuclear fractions were immunoprecipitated using specific antibodies to NFATc3 or MyoD, and associated DNA was purified (Fig. 2e). Using specific primers to the *myog* promoter flanking the N1, N2 or N3 sites, by real time PCR, all three PCR amplicons were observed to be significantly enriched in differentiated C2C12 cells compared to undifferentiated myoblasts (Fig. 2e). This association was specific for NFATc3 since enrichment of PCR products was not obtained when using beads alone, or when using primers to an unrelated promoter, such as myoglobin (data not shown).

Conversely, ChIP analysis carried out with an antibody against MyoD

transcriptional activation, and unambiguously show the presence of endogenous MyoD and NFATc3 on the proximal *myog* promoter *in vivo*, supporting the idea that NFATc3 and the bHLH transcription factor MyoD may cooperate to activate *myog* transcription.

NFATc3 physically interacts with MyoD

Given that calcineurin/NFATc3 signaling is required for Myog induction and MyoD is also capable of directly activating Myog expression, we tested whether NFATc3 may physically associate with MyoD to cooperatively activate Myog expression. To map the MyoD binding site(s) on NFATc3, a panel of NFATc3 deletion mutants was used in coimmunoprecipitation assays (Fig. 3a). Next, a C-terminal His/V5 tagged form of MyoD and Gal4-NFATc3 deletion constructs were coexpressed in COS7 cells and MyoD immunoprecipitated with Ni-NTA beads, specific for the C-terminal His tag on MyoD. The presence of Gal4-NFATc3 deletion mutants was detected by immunoblotting against Gal4. Both the N-terminal regulatory domain of NFATc3 (residues 2-314) and the DNA binding Rel-homology domain (RHD) on NFATc3 (residues 314-732) interacted with MyoD (Fig. 3c, lane 4, 5 and 6). NFATc3(732-1110) did not interact with MyoD (Fig. 3c, lane 7). We conclude that two separate domains, located between residues 2-314 harboring the N-terminal regulatory domain, and residues 314-732 which includes the RHD, respectively, are sufficient for MyoD binding.

Next, to map the NFATc3 binding site on MyoD, coimmunoprecipitation assays were also performed by using extracts from COS7 cells overexpressing epitope-tagged derivatives of MyoD. To this end, a series of MyoD deletion mutants coupled to Gal4 (Fig. 3b) were coexpressed with a C-terminal His/V5 tagged full length NFATc3 and an activated mutant of CnA. NFATc3-His/V5 was immunoprecipitated with Ni-NTA beads and interacting MyoD mutants identified with an antibody against Gal4. Deletion of N-terminal sequences up to amino acid 101 had no effect of MyoD binding to NFATc3 (Fig. 3d, lanes 4 and 5). Likewise, deletion of carboxy-terminal sequences from amino acid 167 to 318 had no noticeable effect on binding of MyoD to NFATc3 (Fig. 3d, lanes 4, 6 and 9), indicating that interaction with NFATc3 centered on the bHLH region. Removal of residues between amino acids 125 and 166, the basic domain, led to a complete loss of MyoD binding (Fig. 3d, lanes 6, 7 and 8). In conclusion, these findings confirm that NFATc3 and MyoD physically interact, and that residues 125-166 on MyoD, the basic domain of this class II bHLH transcript factor, and two separate domains on NFATc3 are required for this interaction.

NFATc3 and MyoD cooperatively induce myogenin expression in somites

The myogenic regulatory factors MyoD, Myog, Myf-5 and MRF4 (Myf-6) regulate myogenesis in the developing embryo. Myog and MyoD are expressed in the myotome at E8.5 and E10.0 respectively (19). To analyze the implications of the uncovered transcriptional interaction between MyoD and NFATc3, we analyzed embryos deficient for *myod*, *nfatc3*, *nfatc2* or combinations thereof for Myog transcript expression at E10.5. To this end, we performed whole-mount *in situ* hybridization for Myog in wild-type (Fig. 4a), *myod* null (Fig. 4b), *nfatc2* null (Fig. 4c) and *nfatc3* null (Fig. 4d) mice. Mutant embryos for *myod*, *nfatc2* and *nfatc3* displayed strong Myog expression in developing somites to the same extent as in wild-type embryos (Fig.4a, b, c and d). Next, we analyzed *myod:nfatc3* and *myod:nfatc2* double null embryos. Remarkably, Myog transcripts were virtually absent in somites of *myod:nfatc3* double null embryos. In contrast, *myod:nfatc2* double null embryos displayed Myog transcript levels to the same extent as somites from wild-type embryos, demonstrating the specificity of the interaction between MyoD and the NFATc3 isoform during myogenesis (Fig. 4e, f). Paraxis, a member of the Twist subfamily of bHLH transcription factors, has been shown to regulate morphogenetic events during somitogenesis, including the transition of cells from mesenchyme to epithelium and maintaining anterior/posterior polarity (20). To exclude the possibility that the observed Myog phenotype derived from general somite dysmorphogenesis or premature embryonic death before E10.5, we performed whole-mount *in situ* hybridization for paraxis on *myod:nfatc2* and *myod:nfatc2* double null embryos (Fig. 4g, h). Paraxis expression in *myod:nfatc3* and *myod:nfatc2* double null embryos was unchanged, which relieves concerns about the specificity of the observed downregulation of Myog in *myod:nfatc3* double null mice (Fig. 5g, h). Collectively, these data confirm that Myog induction during primary myogenesis *in vivo* is established by cooperative interaction between MyoD and calcineurin/NFATc3 signaling.

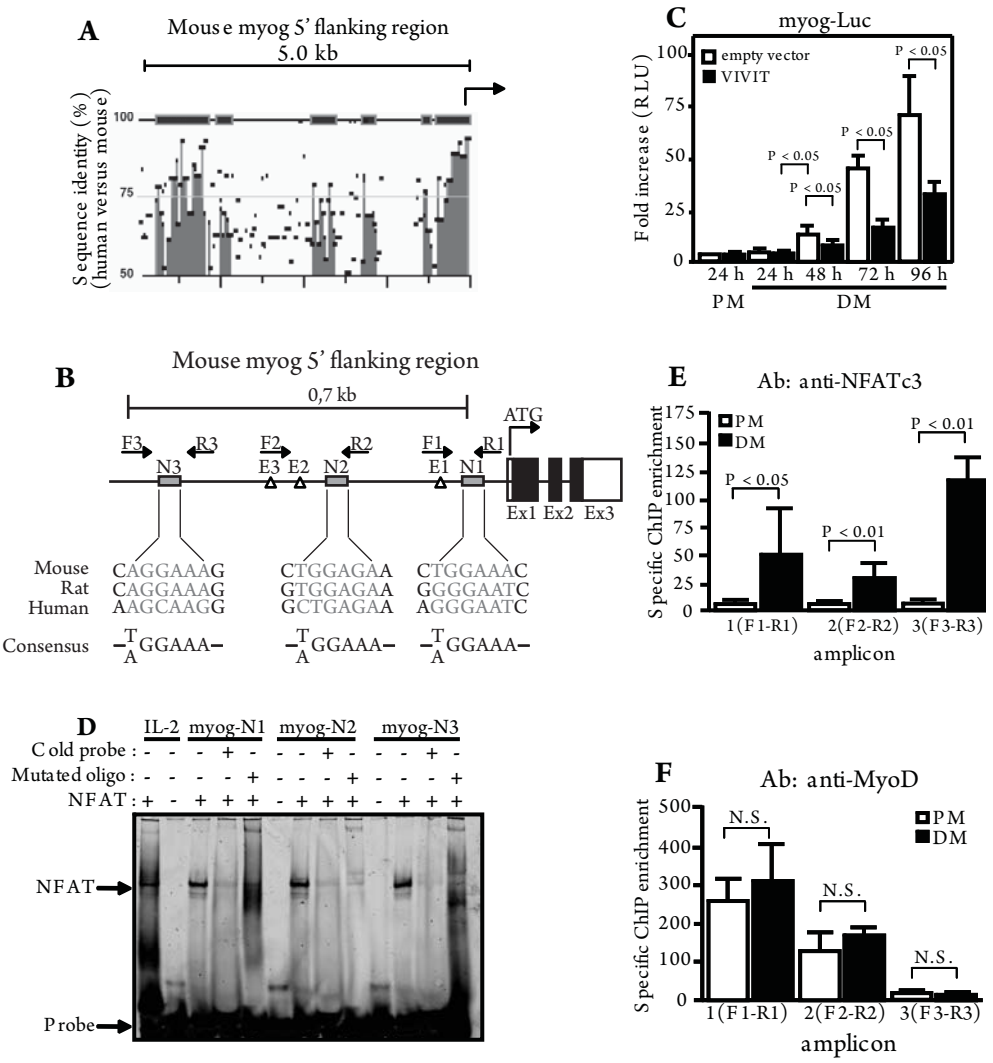

Figure 2. Presence of functional NFAT consensus binding sites in the myogenin promoter.
(A) Comparison of the *myog* genomic regions between mouse and human. Percentage conservation of a 5' 5.0 kb genomic region upstream myogenin first exon is shown. (B) Schematic presentation of 0.6 kb 5' flanking region in mouse myog and location of NFAT binding sites (N1, N2, N3) and E-boxes (E1, E2, E3). Primers yielding ChIP amplicons are indicated. (C) Transfection assay in C2C12 cells culture in PM or DM using a 0.6 kb myog-luc vector in the absence (empty vector) or presence of VIVIT-eGFP expression vector demonstrates the requirement of endogenous NFAT transcriptional activity for induction of the myog-luc vector. (D) Gel mobility shift assay was performed using fluorescent probes of NFAT consensus sequences (IL-2) or NFAT-like sites N1, N2 or N3 from the *myog* promoter. (E-F) Chromatin immunoprecipitation assays were performed on C2C12 cells grown in PM or DM with antibodies for NFATc3 (E) or MyoD (F). Bars represent fold enrichment of amplicons with indicated primer sets (B), normalized to input controls obtained with primers spanning a non-coding genomic region 3' of the *myog* gene.

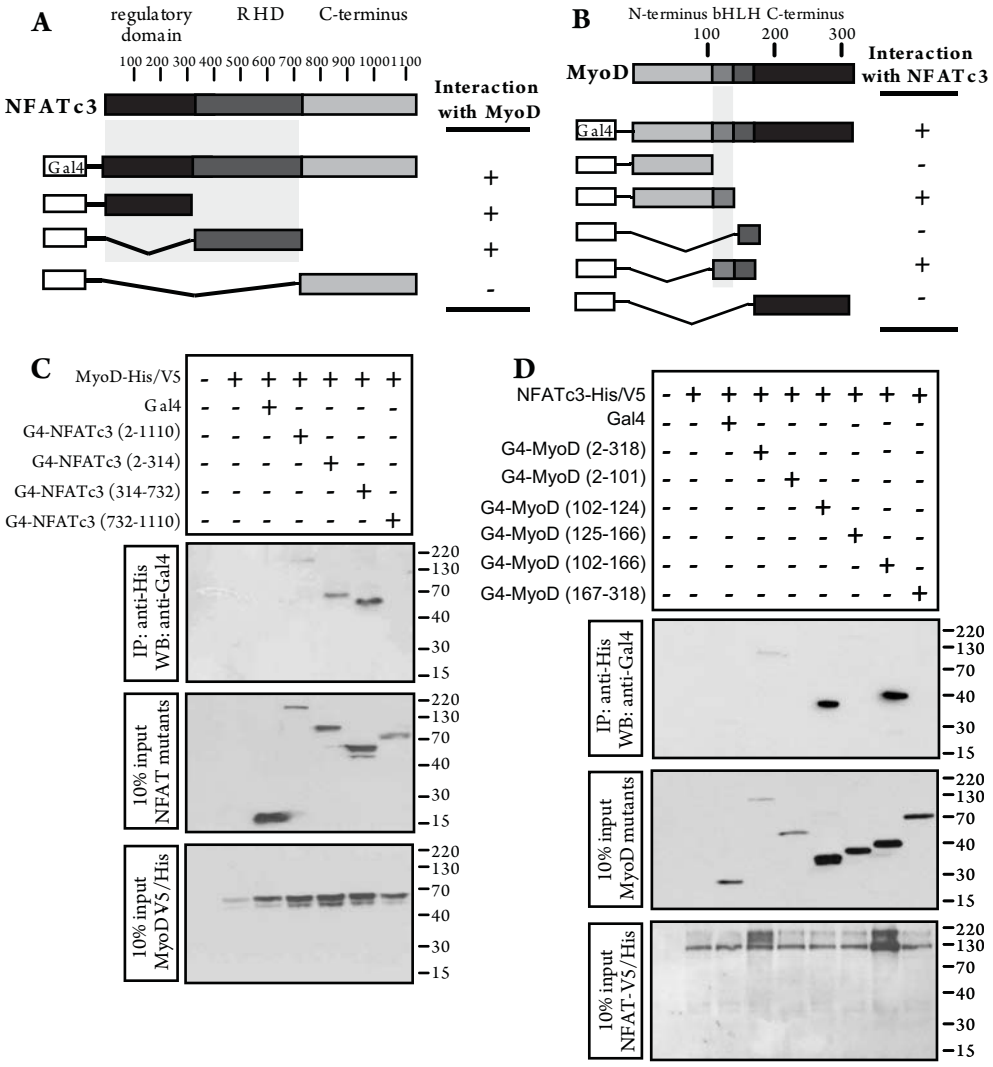

Figure 3. Mapping of NFATc3 and MyoD interaction.
(A-B) Schematic overview of Gal4-NFATc3 (A) and Gal4-MyoD (B) deletion constructs and their ability to bind MyoD or NFATc3, respectively. (C) Protein extracts from COS7 cells transfected with MyoD-V5/His, empty vector (Gal4), and/or Gal4-NFATc3 deletion constructs were immunoprecipitated with Ni-NTA beads and subjected to Western blotting using an anti-Gal4 antibody. (D) Protein extracts from COS7 cells transfected with NFATc3-V5/His, empty vector (Gal4), and/or Gal4-MyoD deletion constructs were immunoprecipitated with Ni-NTA beads and subjected to Western blotting using an anti-Gal4 antibody.

DISCUSSION

Calcineurin/NFAT signaling regulates skeletal muscle development, adaptation and regeneration

In the embryo, initial muscle development is controlled by the myogenic regulatory factors Myf-5, MyoD, myogenin and MRF4 (Myf-6) (2) in conjunction with the MEF2 MADS-box family of transcription factors (21). Notably, of the single-knockout mice for the myogenic regulatory factors, only *myog*-null mice exhibit severe skeletal muscle deficiencies, thereby demonstrating its unique stance in embryonic muscle differentiation and suggesting the absence of redundant or compensatory mechanisms to substitute its function *in vivo* (5, 6, 16).

Reminiscent to the myogenic factors, the calcineurin/NFAT pathway represents another pathway involved in skeletal muscle differentiation and muscle regeneration. Calcineurin activity affects myogenic differentiation of cultured myoblasts *in vitro* (22), while during pupal development in Drosophila, mutation of *canB2*, which encodes a regulatory subunit of calcineurin, provokes severe defects in the organization of indirect flight muscles (23). Calcineurin/NFAT also regulates postnatal skeletal muscle hypertrophy and fiber-type switching. Transgenic mice expressing an activated calcineurin mutant in skeletal muscle exhibit an increase in Type I fibers (24), while a constitutively active NFAT mutant stimulates the MyHC-slow promoter in adult fast muscles (25). Conversely, calcineurin deficient mice have a reduced oxidative slow muscle fiber-type profile (26), providing evidence that calcineurin/NFAT signaling acts as a nerve activity sensor and controls activity-dependent myosin switching in adult skeletal muscle. In response to injury, quiescent satellite cells become activated and migrate to the site of injury where they proliferate, differentiate, and fuse to form new myofibers. Calcineurin/NFAT signaling is required for muscle precursor cell differentiation and the regenerative capacity of postnatal skeletal muscle (27).

Rather than regulating muscle differentiation in parallel, here we demonstrate that MyoD and the calcineurin/NFATc3 pathway converge at the transcriptional level to initiate embryonic muscle differentiation by coactivating the *myog* gene with implications for proper differentiation of somite derivatives *in vivo*. While three NFAT isoforms are expressed in skeletal muscle, the combined findings from individual NFAT null mice now provides evidence that the individual NFAT isoforms have a unique role in skeletal muscle development. Indeed, the muscle phenotype of *nfatc2* null mice is distinct from *nfatc3* mutant mice. NFATc2 has been shown to participate in myofiber and myoblast fusion, leading to the growth

of multinucleated muscle tubes (9). Collectively, the combined observations point to isoform-specific and temporally distinct contributions of NFAT transcription factors to skeletal muscle development.

Calcineurin/NFAT-dependent, organ-specific responses by transcriptional synergy

The present study provides for the first time evidence of a combinatorial NFAT/MyoD transcriptional pathway controlling gene expression in myogenic cells. Our observations are reminiscent of the cooperative transcriptional integration between the basic helix-loop-helix (bHLH) transcription factor MyoD and members of the MEF2 family of transcription factors, which also plays an essential role in gene activation during muscle differentiation (21). Interestingly, MEF2 proteins also directly interact with NFAT transcription factors. In T-lymphocytes, NFATc2 interacts directly with MEF2D in a synergistic transcriptional complex to activate the *Nur77* gene (28), while a combinatorial MEF2/NFAT regulatory transcriptional pathway controls gene expression in cardiac muscle cells (16).

The HLH transcription factor family has been classified based upon tissue distribution, dimerization capabilities, and DNA-binding specificities (29). Class II HLH proteins, which include the myogenic bHLH proteins MyoD, Myf-5, myogenin and MRF4, show a tissue restricted pattern of expression and are required for vital developmental processes, including hematopoiesis, cardiogenesis, myogenesis and neurogenesis (29). In contrast to the restricted tissue distribution of class II HLH proteins, NFAT proteins are expressed ubiquitously, yet, individual or combinatorial loss of NFAT isoforms in mice reveals highly specific defects in cardiovascular, myogenic, neuronal or immune cell lineages (10, 12, 30-32). Consequently, one vexing question in NFAT biology relates to how the ubiquitously expressed NFAT factors can induce organ and cell type-selective responses despite their ubiquitous expression pattern. We now demonstrate that, during myogenesis, this specificity results from the transcriptional synergy between one single NFAT isoform and myogenic lineage restricted bHLH transcription factor MyoD. This involvement is specific to one NFAT isoform, NFATc3, since double null *myod:nfatc2* mice displayed no defect in myogenin expression at this stage of myogenesis. It is tempting to speculate that similar transcriptional cooperations may also exist between NFAT transcription factors and other members of the class II HLH transcriptional regulators to specify gene expression in distinct developmental processes and other organs. Future studies will be required to assess whether and to what extent the transcriptional cooperation between HLH proteins and the calcineurin/NFAT

pathway also impinges on postnatal skeletal muscle adaptation, other developmental processes and in disease.

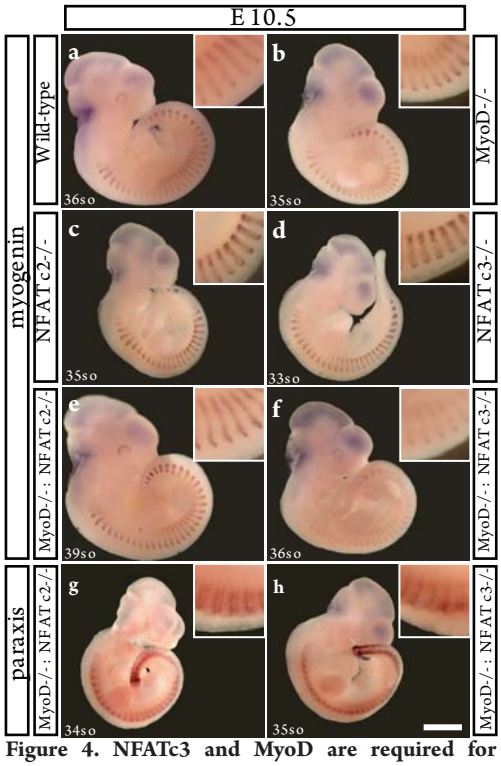

Figure 4. NFATc3 and MyoD are required for myogenin expression in myogenesis.
Myogenin whole mount *in situ* hybridization (WM-ISH) of wild-type (A), *myod* null (B), *nfatc2* null (C), *nfatc3* null (D), *myod:nfatc2* double null (E) and *myod:nfatc3* double null (F) E10.5 embryos. Paraxis WM-ISH in *myod:nfatc2* double null (G) and *myod:nfatc3* double null (H) E10.5 embryos.

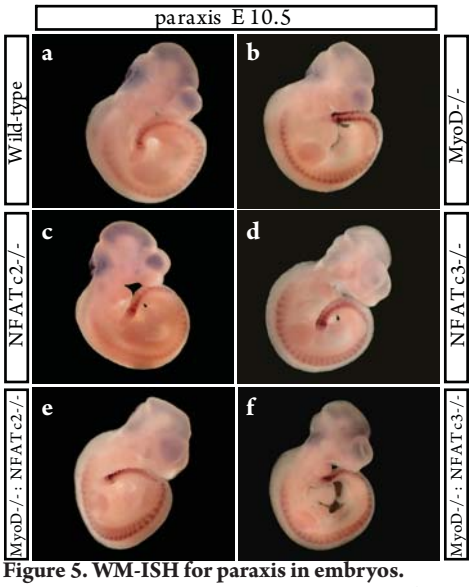

Figure 5. WM-ISH for paraxis in embryos.
Paraxis whole mount *in situ* hybridization (WM-ISH) of wild-type (A), *myod* null (B), *nfatc2* null (C), *nfatc3* null (D), *myod:nfatc2* double null (E) and *myod:nfatc3* double null (F) E10.5 embryos.

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CHAPTER 6

General Discussion

Meriem Bourajjaj

The calcineurin/NFAT pathway is selectively involved in pathological cardiac hypertrophy

The calcium-dependent phosphatase calcineurin was identified as a key mediator of cardiac hypertrophy (2). Cardiac specific overexpression of an activated form of calcineurin revealed to be sufficient to induce a robust hypertrophic response in transgenic mice and eventually result into a failing phenotype. Conversely, pharmacological inhibition of calcineurin using cyclosporine A or FK506, transgenic expression of a dominant negative form of calcineurin or specific protein inhibitors, or by the creation of a somatic knockout for the calcineurin Abeta isoform, largely protected the murine heart from the development of pathological hypertrophy, fibrosis, dysfunction and heart failure. These combined observations have raised considerable interest in this particular pathway. Notwithstanding the profound number of studies implicating the enzymatic part of the calcineurin/NFAT pathway in cardiac remodeling, remarkably little is known about its direct transcriptional effector molecule NFAT, nor the number or nature of its target genes. The studies presented in this thesis have been dedicated to elucidate the precise mode of action and physiological role of NFAT transcriptional activity in the hypertrophic growth process of the heart.

Mice and men share four distinct calcineurin-regulated NFAT genes encoded on four different chromosomes, designated *nfatc1*, *nfatc2*, *nfatc3* and *nfatc4*, which are expressed as multiple spliced transcripts in rodents and human (3-6). *Nfatc1, -c2, and -c3* are most highly expressed in immune cells and skeletal muscle, as well as weakly expressed in many other cell types, whereas *nfatc4* and *nfat5* are more evenly expressed throughout the body (3, 7). Targeted disruption of *nfatc1, -c2, and -c3* genes has identified critical roles for these factors in immune cell function and/or survival (3, 8-11). Disruption of the *nfatc1* gene resulted in embryonic lethality due to aberrant heart valve formation and cardiac insufficiency (12, 13). More recently, *nfatc2*- and *nfatc3*-null mice were shown to have defects in skeletal muscle fiber number or size (14, 15), while *nfatc2*-null mice were also shown to undergo aberrant chondrogenesis (16). Lastly, the combinatorial disruption of *nfatc3*/*nfatc4* in mice resulted in embryonic lethality due to vascular insufficiency, demonstrating a role for NFAT factors in developmental patterning (17). Collectively, NFAT factors are expressed in multiple cell types and at different developmental times, where they perform diverse functions.

In the initial description of calcineurin's involvement in cardiac hypertrophy, calcineurin was shown to dephosphorylate the transcription factor NFATc4 in cardiomyocytes, enabling it to translocate to the nucleus and activate cardiac

transcription (2). However, although transgenic mice expressing an activated mutant of NFATc4 in the heart displayed robust hypertrophy, targeted loss of *nfatc4* did not compromise the ability of the myocardium to undergo hypertrophic growth, which has largely excluded the absolute requirement of NFATc4 in the hypertrophic process. In contrast, *nfatc3*-null mice were demonstrated to be partially deficient in the their ability to undergo cardiac hypertrophy in response to calcineurin activation (18). Together, earlier observations established NFATc3 to be at least one critical downstream mediator of calcineurin-regulated hypertrophy in the heart and validated the original hypothesis that the calcineurin-regulated hypertrophic responses require NFAT effectors in vivo.

The high degree of homology for all calcineurin-sensitive NFAT isoforms and the observation the *nfatc3*-null mice were unable to completely block calcineurin- induced hypertrophy (18), led us to hypothesize that the partially protective phenotype of *nfatc3*-null mice might be a result of functional redundancy between NFAT isoforms. The existence of proteins for all four calcineurin-regulated NFAT isoforms in cardiomyocytes was previously demonstrated (18, 19). In Chapter 2, we analyzed the relative abundance of NFAT (splice) transcripts using real time PCR approaches, since most commercially available antibodies proved ineffective to quantify the relative abundance of the low levels of NFAT proteins in the adult heart (18) . In Chapter 2 we provide evidence that *nfatc2* mRNA levels are the most abundantly expressed in the heart among all NFAT isoforms. We show that mRNAs for *nfatc4* and *nfatc1* are scarcely expressed in the heart, which is fully in line with the observation that *nfatc4*-null mice cannot compromise the development of cardiac hypertrophy and heart failure following pleiotropic stimuli (18). In contrast, transcripts for *nfatc3* and *nfatc2* were relatively more abundantly present in the heart, with the latter still present at several folds more than those for *nfatc3*. Collectively, the data indicate that *nfatc2* transcripts outnumber those from other *nfat* genes in the heart by several folds.

In Chapter 2, we further demonstrated 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. Moreover, at 8 weeks after pressure overload, echocardiography indicated marked LV 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, reduced fibrosis and a diminished hypertrophic gene program. Intriguingly, *nfatc2*-null mice were not compromised in their ability to undergo athletic cardiac enlargement.

This observation is in line with a study by Wilkins et al., which demonstrated that voluntary wheel running and swimming exercise in mice resulted in significant cardiac hypertrophy enlargement without activation of calcineurin-NFAT signaling (20). Taken together, these findings indicate that the cardiac calcineurin-NFATc2 signaling axis is not activated after voluntary wheel running, given that cardiac enlargment was not affected in *nfatc2* null mice compared to wildtype mice.

NFAT transcriptional activation is required in the genesis of dilated cardiomyopathy (DCM)

In chapter 2 we demonstrated that *nfatc2*-null mice display abrogation of calcineurin-provoked cardiac growth and a clear protection against various aspects of pathological cardiac remodeling in response to hemodynamic loading and in a transgenic model of heart failure. In contrast, physiological cardiac enlargement in response to voluntary exercise training was not affected in *nfatc2*-null mice. Combined, these results revealed a major role for the NFATc2 transcription factor in pathological cardiac remodeling and heart failure.

To investigate the significance of the NFATc2 transcriptional response in pathological remodeling in a seemingly calcineurin unrelated murine model of severe heart failure, we crossbred mice deficient for Cysteine rich protein 3 (also known as Muscle LIM Protein or MLP) (*csrp3*-null mice) with *nfatc2*-null mice to create *csrp3/nfatc2* double knockout mice (DKO). Disruption of *csrp3* leads to impaired cytoskeletal organization in cardiomyocytes associated with dilated cardiomyopathy and heart failure (21). Csrp3 is a muscle specific member of the LIM-only class of the LIM domain protein family that possesses two tandem LIM domains. has been originally identified in striated muscle, where it promotes myogenesis and regulates myogenic differentiation (22, 23). Furthermore, a decrease in protein level has been observed in human heart failure (24). Finally, CSRP3 interacts with and colocalizes to telethonin (T-cap), a titin interacting protein. A human mutation (W4R) associated with DCM results in a marked defect in T-cap interaction/localization, suggesting that a Z-disc /T-cap complex is a key component of the cardiomyocyte stretch sensor machinery, and that defects in this complex underlie human DCM (25).

Although the mechanistical link between disturbances of the cytoskeleton in individual cardiomyocytes and the progression to dilated cardiomyopathy is unclear, several studies have reported the involvement of impaired intracellular calcium homeostasis (26-28). A resultant of altered intracellular calcium levels secondary to perturbations of Z-disk structure could be the activation of pro-hypertrophic

calcium/calmodulin-dependent signaling (18, 19). In line of the presumption that calcium/calmodulin signaling may be activated by alterations of Z-disk architecture, calsarcin-1, another Z-disc protein, has been found to interact with calcineurin and mice lacking calsarcin-1 protein are sensitized to calcineurin signaling and display an accelerated progression to cardiomyopathy (29). A decrease in Ca^{2+} uptake is a central feature of human and animal heart failure (30, 31) and an increase in the relative ratio of PLB to SERCA2a is an important determinant of SR dysfunction in heart failure (32, 33). (26).

Given the above described defects in SR calcium handeling, we reasoned that, secondary to *csrp3*-deficiency, calcium/calmodulin-activated calcineurin/NFAT signaling may be activated in the *csrp3*-deficient cardiac phenotype and drive the downstream remodeling processes. To test this premise, we crossbred *csrp3* knockout mice with *nfatc2* null mice and found that histological and hemodynamic parameters were dramatic improved in *csrp3/nfatc2* double knockout mice. In conclusion, deletion of the *nfatc2* gene prevented maladaptive remodeling and cardiac dysfunction of *csrp3* deficient hearts, and infers a role for calcineurin/NFAT signaling in Z-disk related stretch sensing.

Our results are in contrast to two separate studies. In one study, activation of NFAT signaling pathway was reduced in *csrp3+/-* mice after MI, as shown by a blunted transcriptional activation of NFAT in cardiomyocytes isolated from *csrp3+/-*/NFAT-luciferase reporter gene compound transgenic mice. Calcineurin was found to be co-localized with CSRP3 at the Z-disk in WT mice but was displaced from the Z-disk in *csrp3+/-* mice, indicating that CSRP3 is essential for calcineurin anchorage to the Z-disk. In vitro assays in cardiomyocytes with down-regulated CSRP3 confirmed that CSRP3 is required for stress-induced calcineurin–NFAT activation. This study by Heineke *et al.* implies a link between the stress sensor CSRP3 and the calcineurin–NFAT pathway at the sarcomeric Z-disk in cardiomyocytes and indicates that reduced MLP–calcineurin signaling predisposes to adverse remodeling after MI (34). In a second and more recent study, PICOT (protein kinase [PK]C–interacting cousin of thioredoxin) activity was found to constitute a negative feedback loop for cardiac hypertrophy (35). Jeong *et al.* demonstrated that PICOT overexpression by adenoviral overexpression impaired calcineurin signaling in vitro. Further, using a transgenic model engineered to overexpress a PICOT mutant, they were able to demonstrate that the induction of brain natriuretic peptide (*nppb*) and the exon 4 splice isoform of rcan1 was blunted in response to pressure loading (36). Remarkably, no whole heart morphological or physiological parameters were presented from this mouse model at baseline or

after stress.

At this moment it is not clear why our findings are in disparate to the previous studies. First, our study employs an unequivocal approach by creating double mutant mice for both *csrp3* and *nfatc2,* yielding a very clear phenotype and rescue of many aspect of dilated cardiomyopathy. In contrast, Heineke *et al*. (34) employed heterozygous knockout mice for *csrp3*, which do not show impaired Z-disc structure, nor any other apparent phenotype, so that we question the choice of this particular mouse model to study this signaling phenomenon. The other major finding of Heineke *et al*., suggesting co-localization between CSRP3 and calcineurin, is intriguing and fully in line with our findings. With respect to the study of Jeong *et al*. (36), which analyzes PICOT function, it should be remarked that the suggested competition between PICOT and calcineurin for CSRP3 binding was very weak, questioning its physiological relevance. Further, it is as of yet unknown what exactly happens to PICOT levels in the diseased heart, which makes the choice of generating a transgenic model expressing a PICOT mutant, unfortunate since it may not reflect the correct physiological situation. Even more remarkable, very little details are known about the PICOT transgenic mouse apart from the transcript abundance of two fetal genes, so that at this stage it is impossible to draw any conclusions from this study. The other major finding of that study, suggesting direct binding between calcineurin and CSRP3 at the Z-disc, is again, of course, fully in line with our findings.

Our combined findings indicate that *csrp3*-deficiency, an excellent model for disruption of the cytoskeletal architecture in inherited forms of human dilated cardiomyopathies, is critically dependent upon calcineurin/NFAT signaling for the cardiac remodeling process.

Calcineurin/NFAT transcriptome profiling of target genes driving cardiomyocyte hypertrophy

Re-activation of a "fetal" gene program is a conserved feature of a pathological hypertrophic response and has been extensively studied as a means to identify physiological regulators of hypertrophy. Although the initial steps of the induction of embryonic or prohypertrophic genes are reversible, chronic changes in the cardiac transcriptome may trigger pathological changes in the myocardium that invoke irreversible cellular changes, dilation of the ventricular chamber and cardiac dysfunction, often the first irreversible steps towards heart failure (37). Accordingly, the transcription factors that connect biomechanical forces and the activation of stress pathways to morphological changes of the myocardium

are central to understanding the initiation and progression of heart failure. The calcineurin/NFAT transcriptional paradigm is both sufficient and required to drive the cardiac hypertrophic growth response (38-40). Notwithstanding its established requirement in provoking pathological gene expression preceding heart failure development, limited information is available about the immediate downstream calcineurin/NFAT target genes in cardiac muscle.

Using a multidisciplinary approach, we analyzed the consequences of activating the calcineurin/NFAT pathway in cardiac muscle, starting from the premise that the target genes of calcineurin and/or NFAT would provide novel insights into pathological hypertrophic remodeling of the heart muscle. Accordingly, in chapter 4 we performed studies to uncover the earliest calcineurin/NFAT-regulated transcripts in cardiac muscle. In this study, we created double stable NkL-TAg clones harboring a stably integrated tetracyclin-dependent repressor and constitutively activated mutants of either calcineurin or NFAT. This approach allowed us controlled activation of the calcineurin/NFAT pathway in a time- and dosage-dependent manner and elucidation of the earliest gene targets of this signaling pathway. Gene ontology classifications of these target genes revealed an overrepresentation of genes in translational machinery/protein transport, energy metabolism, signal transduction and transcription factors, allowing insight into the particulars of pathological cardiac remodeling. Unexpectedly, Gata4 transcripts and protein levels were found to upregulated early after activation of calcineurin/NFAT stimulation and in a sustained manner in human heart failure biopsies. The zinc finger-containing transcription factor GATA4 is highly expressed in cardiomyocytes throughout embryonic development, postnatal growth, and adulthood, during which it functions as a critical regulator of cardiac differentiation–specific gene expression (41). Genetic knockdown of Gata4 using specific short hairpins (shRNAs) revealed its functional requirement as a downstream NFAT target gene in provoking initial hypertrophic remodeling. Given that NFAT and pre-existing Gata4 proteins cooperatively activate target genes such as *brain natriuretic peptide, adenylosuccinate synthetase 1, endothelin-1, calcineurin A-beta* as transcriptional partners (40, 42-44), our findings suggest the existence of a novel transcriptional feedforward mechanism where *nfat* signaling invokes expression of the pro-hypertrophic *gata4* transcription factor, allowing amplification of calcineurin/NFAT signaling in the ventricular myocyte. Gata4 has previously been ascribed to function as a transcriptional integrator for various stress signaling pathways in the postnatal myocardium. Forced viral overexpression of *gata4* provokes cardiomyocyte hypertrophy in culture, while dominant negative forms or antisense delivery efficiently counteracts *gata4*-directed

transcriptional responses and features of cardiomyocyte hypertrophy secondary to phenylephrine or endothelin-1 stimulation (45, 46). *In vivo*, *gata4* function is pleiotropic as it mediates stress-induced cardiac hypertrophy, survival of cardiac myocytes and postnatal myocardial angiogenesis (47-50).

Gata4 has also been ascribed to regulate the specification and differentiation of cardiac myocytes early in development through the direct transcriptional control of key cardiac structural and regulatory genes (51-53), alone or in concert with developmentally active transcription factors such as Tbx20, Nkx2.5, SRF, Hand2, and myocardin (54-59). We uncovered a novel *gata4* enhancer near exon 1 that harbors a cluster of NFAT and MEF2 sites. Recently, a *gata4* enhancer located more distal to the one described in this study was demonstrated to be responsive to Forkhead and GATA factors and required for *gata4* transcription in the lateral mesoderm (60). The combinatorial observations suggest the existence of physically separated enhancers for *gata4* developmental expression versus *gata4* induction upon calcineurin/NFAT-mediated stress signaling. Our study remains inconclusive whether calcineurin/NFAT signaling is responsible for developmental *gata4* regulation. Gene targeting studies in mice demonstrated that a combinatorial knockout for both *nfatc3* and *nfatc4* result in embryonic death due to defects in metabolic maturation of the myocardium at midgestation (61), while similar defects in heart development were observed in chicken embryos treated with cyclosporine A, the pharmacological calcineurin inhibitor (62). Moreover, *regulator of calcineurin 1* (*rcan1*), gene expression is regulated by NFATc1 during valve maturation and coincides with abnormal development in Down syndrome (63). By contrast, our results indicate that mice harboring a null mutation for *nfatc2* achieve normal adult cardiac mass, suggesting a more specialized function for NFAT transcription factors as integrators of stress signaling in the postnatal myocardium. Future studies should elucidate whether the *gata4* enhancers are indeed developmentally and spatially separated.

In this study, we created stably expressing clones, with absence of senescence during the propagation phase and regulated growth termination, which control activation of the calcineurin/NFAT pathway in a time- and dosage-dependent manner and elucidation of the earliest gene targets of this signaling pathway. This provides a valuable experimental resource allowing cardiovascular genomics studies in a genome-wide fashion to study stress signaling events in the correct cellular context.

The calcineurin/NFAT pathway and MyoD are co-regulators of myogenin expression and myogenesis

The calcineurin/NFAT pathway is also involved in skeletal muscle differentiation and muscle regeneration. Calcineurin activity affects myogenic differentiation of cultured myoblasts in vitro (64), while during pupal development in Drosophila, mutation of CnAB2, which encodes a regulatory subunit of calcineurin, provokes severe defects in the organization of indirect flight muscles (65). Calcineurin/NFAT regulates postnatal skeletal muscle hypertrophy and fiber-type switching. Transgenic mice expressing an activated calcineurin mutant in skeletal muscle exhibit an increase in Type I fibers (66), while a constitutively active NFAT mutant stimulates the MyHC-slow promoter in adult fast muscles (67). Conversely, calcineurin-deficient mice have a reduced oxidative slow muscle fiber-type profile (68), providing evidence that calcineurin/NFAT signaling acts as a nerve activity sensor and controls activity-dependent myosin switching in adult skeletal muscle. In response to injury, quiescent satellite cells become activated and migrate to the site of injury where they proliferate, differentiate, and fuse to form new myofibers. Calcineurin/NFAT signaling is required for muscle precursor cell differentiation and the regenerative capacity of postnatal skeletal muscle.

Rather than regulating muscle differentiation in parallel, here we demonstrate that MyoD and the calcineurin/NFATc3 pathway converge at the transcriptional level to initiate embryonic muscle differentiation by coactivating the *myog* gene with implications for proper differentiation of somite derivatives in vivo. The HLH transcription factor family has been classified based upon tissue distribution, dimerization capabilities, and DNA-binding specificities (69). Class II HLH proteins, which include the myogenic bHLH proteins MyoD, Myf-5, myogenin and MRF4, show a tissue restricted pattern of expression and are required for vital developmental processes, including hematopoiesis, cardiogenesis, myogenesis and neurogenesis (69). In contrast to the restricted tissue distribution of class II HLH proteins, NFAT proteins are expressed ubiquitously, yet, individual or combinatorial loss of NFAT isoforms in mice reveals highly specific defects in cardiovascular, myogenic, neuronal or immune cell lineages (70, 71).

While three NFAT isoforms are expressed in skeletal muscle, the combined findings from individual *nfat* isoform knockout mice now provides evidence that the individual NFAT isoforms have a unique role in skeletal muscle development. Indeed, the muscle phenotype of *nfatc2* null mice is distinct from *nfatc3* mutant mice. NFATc2 has been shown to participate in myofiber and myoblast fusion, leading to the growth of multinucleated muscle tubes (66).

Consequently, one vexing question in NFAT biology relates to how the ubiquitously expressed NFAT factors can induce organ and cell type-selective responses despite their ubiquitous expression pattern. We now demonstrate that, during myogenesis, this specificity can result from the transcriptional synergy between one single NFAT isoform and myogenic lineage restricted bHLH transcription factors, in this case MyoD. This involvement is specific to one NFAT isoform, NFATc3, since *myod:nfatc2* double null mice displayed no defect in myogenin expression at this stage of myogenesis. It is tempting to speculate that similar transcriptional cooperations may also exist between NFAT transcription factors and other members of the class II HLH transcriptional regulators to specify gene expression in distinct developmental processes and other organs. Future studies will be required to assess whether and to what extent the transcriptional cooperation between HLH proteins and the calcineurin/NFAT pathway also impinges on postnatal skeletal muscle adaptation, other developmental processes and in disease.

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Samenvatting in het Nederlands en Arabisch

Hartfalen is een aandoening waarbij het hart niet meer in staat is om genoeg bloed te pompen om aan de behoefte van het lichaam te voldoen. Het hart reageert op stress signalen, zoals een hartinfarct of een chronisch verhoogde bloeddruk, door te groeien (hypertrofie). Hypertrofie is in eerste instantie een aanpassing van het hart om beter te kunnen samentrekken, maar door nog een onbekend mechanisme resulteert hypertrofie in een later stadium vaak in hartfalen, waarbij de hartwanden heel dun zijn en het hart slecht klopt. Er is op dit moment niet veel bekend over de signalen in de hartspiercel die hypertrofie en hartfalen veroorzaken. Een belangrijk eiwit dat hypertrofie veroorzakende signalen doorgeeft in spiercellen van het hart, is het door calcium geactiveerde calcineurine. Van calcineurine is bekend dat het de transcriptie factor Nucleaire Factor van Geactiveerde T cellen (NFAT) kan activeren. NFAT wordt gedefosforyleert en gaat zo de kern in van een hartspiercel. Samen met andere transcriptiefactoren kan NFAT bepaalde in het DNA gelegen genen activeren. In dit promotieonderzoek is de rol van NFAT en van NFAT gerelateerde genen tijdens de ontwikkeling van hypertrofie en hartfalen onderzocht. Ook is de rol van NFAT bekeken tijdens myogenese (spierontwikkeling).

Er bestaan vier *nfatc* genen, *nfatc1*, *nfatc2*, *nfatc3* en *nfatc4*. In hoofdstuk 2 laten we zien dat *nfatc2* de meest voorkomende isform in het hart is. *In vivo* studies gepubliceerd in het verleden, toonden aan dat een genetisch gemodificeerde muismodel, waarbij het gen voor *nfatc4* niet langer functioneel is (*nfatc4* knock-out muis) geen verandering bracht in cardiale hypertrofie en hartfalen veroorzaakt door calcineurine signalering. *Nfatc3* knock-out muizen zijn alleen gedeeltelijk deficiënt in cardiale hypertrofie en laten geen verbetering zien op het niveau van hypertrofe genetische markers expressie of cardiale dysfunctie in response op calcineurine activatie. In dit hoofdstuk leveren wij bewijs dat NFATc2 het meeste tot expressie komt in het hart vergeleken met overige NFATc isoformen. Wij hebben aangetoond dat *nfatc2*-deficiente muizen sterk verminderde cardiale hypertrofie vertonen. Echocardiografie laat zien dat dilatatie toeneemt in de wand van linker ventrikel en bovendien treedt er verlies op van de systolische functie in een wildtype muis na 8 weken aanhoudende hoge bloeddruk. *Nfatc2* knock-out muizen spelen een prominente rol in reductie in myofiber hypertrofie, preservatie van de geometrie en contractiliteit van het linker ventrikel, minder fibrose en laten een verminderde hypertrofische gen programma zien. Opmerkelijk was dat *nfatc2* knock-out muizen niet in staat waren atletische hart vergroting tegen te gaan. Alle bevindingen bij elkaar laten zien dat er een hoofdrol is voor NFATc2 onder de leiding van calcineurine signalering in pathologisch hart remodelering.

Aangezien NFATc2 een belangrijke rol speelt in de hypertrofie van het hart, was het belangrijk om in hoofdstuk 3 te kijken of het ook een rol speelt in dilatatie van het hart. Cysteïne rijk eiwit 3 (*Csrp3)* knock-out muizen hebben dezelfde karakteristieke eigenschappen als humane gedilateerde cardiomyopathie. Gedilateerde cardiomyopathie is een afwijking van de hartspier, waarbij één of beide kamers uitgezet zijn en een verminderde

functie vertonen. Om de significatie te onderzoeken van het *nfatc2* gen in pathologische remodelering van harten van *csrp3* knock-out muizen, hebben we *csrp3* knock-out muizen gekruist met *nfatc2* knock-out muizen. Histologische en and hemodynamische analyses lieten dramatische verbeteringen zien in cardiale morfologie en functie in *csrp3/nfatc2* dubbel knock-out muizen. In conclusie, deletie van het *nfatc2* gen voorkomt maladaptieve remodelering en cardiale disfunctioneren van *csrp3* deficiënte harten. Hieruit kunnen wij concluderen dat calcineurine/NFAT signalering ook erg belangrijke functie vervult in later stadia van hartfalen.

Calcineurin is een belangrijke component in hypertrofie van het hart. Zoals nu al duidelijk is het ook afhankelijk van zijn transcriptionele effector NFAT. Er is maar beperkte informatie beschikbaar over de aard en het aantal NFAT geactiveren genen. In hoofdstuk 4 hebben we gebruik gemaakt van gekweekte hartspiercellen waarin geactiveerd NFAT of calcineurine voorkomen. Twee dubbel-stabiele calcineurine klonen en twee NFAT klonen werden geselecteerd om potentiële cel gebaseerde variaties tegen te gaan. De cellen werden gebruikt om het gehele genoom te scannen door middel van microarray. Er werden 27 genen geïdentificeerd als vroeg calcineurin/NFAT gerelateerde genen, waaronder Gata4. Gata4 is een transcriptie factor dat een zinc finger bevat. Experimenten en bevindingen in dit hoofdstuk lieten zien dat Gata4, die bekend was als een transcriptionele partner van NFAT nu ook als NFAT gerelateerde gen voorkomt. Dit onthult een nieuwe versterkende mechanisme, waar NFAT signalering ervoor zorgt dat de prohypertrofe transcriptie factor Gata4 tot expressie komt, wat een amplificatie van calcineurine/NFAT signalering in de ventriculaire hartspiercel te weeg brengt.

Calcineurin/NFAT signalering is betrokken bij meerdere aspecten van skelet spier ontwikkeling en ziekte. Spier ontwikkeling wordt bepaald door de myogene Basic helix-loop-helix (bHLH) transcriptie factoren, waaronder MyoD, myogenine, Myf5 en MRF4. In hoofdstuk 5 laten we zien dat calcineurin/NFAT signalering nodig is voor myogenese door transcriptionele samenwerking tussen de bHLH transcriptie factor MyoD en de NFAT isoform NFATc3. Onze experimenten identificeerde drie evolutionaire geconserveerde NFAT binding plaatsen in de myogenine promoter, welke door NFATc3 werden bezet na skelet spier differentiatie. De transcriptionele integratie tussen NFATc3 and MyoD is cruciaal voor primaire myogenese *in vivo*, omdat myogenine expressie volledig afwezig was in *myod:nfatc3* dubbel knock-out embryo's, terwijl myogenine expressie niet aangetast is in embryo's met mutaties voor elke factor alleen. Al de bevindingen in hoofdstuk 5 geven een nieuw transcriptionele inzicht in de eerste stappen van myogenese, waar een calcineurin/NFATc3 route myogenine inductie reguleert in samenwerking met MyoD.

In hoofdstuk 6 bediscussiëren we de belangrijkste bevindingen van dit proefschrift en de toekomstige richting voor het calcineurine/NFAT signalering onderzoek. Onze data benadrukken de belangrijke bijdrage van de calcineurine/NFAT route in hypertrofie en falen van de hartspiercel, onthullen de identiteit van de belangrijke NFAT gerelateerde genen in de hartspier. Dit alles kan een mogelijke bijdrage leveren aan de ontwikkeling van toekomstige therapeutische medicijnen voor de behandeling van hart en spier ziekten.

تحدث #أزمات القلبية عندما يعجز القلب عن توزيع الدم بصفة منتظمة وعادية الى سائر أعضاء الجسم.
يتفعل القلب نتيجة #إشارات التوتر العصبي عند حدوث جلطة قلبية أو ارتفاع الضغط الدموي وذلك بامتلاء خلايا عضلة القلب.
يحدث هذا #الامتلاء في المرحلة #الأولى لكي يستطيع القلب أن يشتغل بطريقة عادية. لكن هذه الظاهرة و نتيجة عامل غير معروف تتسبب في #أزمات قلبية حيث يصبح غشاء القلب رقيق جدا و دقاته غير منتظمة.
حاليا #توجد معلومات كافية عن طبيعة الخلايا المكونة لعضلة القلب التي تؤدي الى أزمات قلبية أو #امتلاء.
المادة البروتينية المهمة في #إعطاء #إنذارات مسببة بامتلاء خلايا عضلة القلب هي Calcineurine وهي مادة يتم تفعيلها بواسطة الكالسيوم.
المعروف عن مادة Calcineurine أنها تقوم بتفعيل الجينات NFAT في خلايا T.
NFAT يقوم #بالتفاعل ويدخل في خلايا عضلة القلب، كما يلعب دورا هاما في تفعيل الجينات الموجودة في DNA.
في هذا البحث ندرس دور NFAT والجينات المتعلقة بها في حالات #الامتلاء و#الأزمات القلبية.
يوجد أربعة أنواع من الجينات NFAT :
$NFATc_1$, $NFATc_2$, $NFATc_3$, $NFATc_4$

في الفقرة 2 من البحث تبين بأن $NFATc_2$ هي الموجودة بكثرة في القلب.
في دراسات Vivo المنشورة في الماضي، في تجارب حول فأر لديه الجينات NFAT ولكن بدون وظيفة $NFATc_4$ knock-out تبين عدم حدوث أي تغيير في ما يتعلق بإنذارات Calcineurine المسببة لـ #امتلاء أو #الأزمات القلبية.
الفئران $NFATc_3$ knock-out تبين أن لديهم نقص في امتلاء خلايا عضلة القلب

في هذا البحث تبين أن $NFATc_2$ هو النوع الوحيد من الجينات الذي لديه مفعول في القلب، كما تبين أن الفئران $NFATc_2$ knock-out لم يحدث لديهم امتلاء خلايا القلب.
في الصورة القلبية تبين لنا توسعا في غشاء البطين #الأيسر كذلك يقل انبساط القلب عند الفئران الطافرة، يحدث هذا بعد استفاظهم لضغط دموي عالي لمدة 8 أسابيع.

الفئران NFATc2 knock-out يلعبون دورا في تقليل امتلاء خلايا القلب وحفظ حجم البطين #ايسر من التوسع، كما تبين لنا أيضا نقص في الجين المتعلق بامتلاء خلايا عضلة القلب. تبين لنا أيضا أن هذه الفئران لم تستطع تحمل تضخم حجم القلب.
كل هذه النتائج تبين أن لـ NFATc2 دور مهم في قيادة انذارات Calcineurine.
بما أن NFATc2 يلعب دورا مهما في امتلاء القلب كان من الضروري أن نبحث هل له دور في اتساع القلب.
الفئران التي تحتوي على البروتين Cysteine (Csrp3) لديها نفس صفات قلب انسان مصاب بأزمة قلبية.
لبحث انذارات NFATc2 في قلب الفئران Csrp3 null، جعلنا فئران Csrp3 knock-out تتزاوج مع فئران NFATc2 null.
التجارب بينت تحسينات في الشكل والوظيفة القلبية للفئران التي تحتوي على NFATc2 / Csrp3 knock-out.
استنتجنا أن انقسام NFATc2 يمنع حدوث أخطاء في الوظيفة القلبية للفئران التي ينقصها Csrp3.
من هذا نستطيع استنتاج أن #نذارات NFAT و Calcineurine وظيفة جد مهمة في #زمات القلبية.

Calcineurine جد مهمة في امتلاء القلب، كما تبين لنا أنها تعتمد في هذا على NFAT.
لدينا معلومات محدودة حول طبيعة وعدد الجينات NFAT.
في الفقرة 4 قمنا بتحليل خلايا عضلة القلب التي تحتوي على NFAT أو Calcineurine.
وجدنا 27 من الجينات في المرحلة #ولى لـ Calcineurine و NFAT، منها الجين Gata 4.
النتائج في هذه الفقرة أوضحت أن Gata 4، الذي تبين لنا في المراحل #ولى أنه جين مساعد لـ NFAT، تحول الى NFAT.
هذا يشير الى نظام مساند حيث أن انذارات NFAT التي تساعد في تكوين Gata 4 التي بدورها تساعد في اكثار انذارات NFAT/Calcineurine في خلايا عضلة بطين القلب.

انذارات Calcineurine / NFAT متعلقة بجوانب متعددة في تطوير عضلات الهيكل العضمي و#مراض.

تطور العضلات يتحدد بعامل Basic Helix-Loop-Helix (bHLH) من بينها MyoD، myogenin، Myf5 و MRF4.

في الفقرة 5 تبين أنه لحدوث هذا التطوير يجب أن يكون هناك تعاون استنساخي بين العامل bHLH و MyoD و الجين $NFATc_3$.
أبحاثنا تحدد ثلاث تطورات لأماكن ارتباط NFAT في myogenin التي تحولت الى $NFATc_3$ بعد التحول الى هيكل عضلي.
الاستنساخ بين $NFATc_3$ و MyoD يكون حاسما في التطور الابتدائي في vivo لعدم وجود myogenin في الجنين MyoD: $NFATc_3$.

كل النتائج في الفقرة 5 تعطي مفهوما جديدا في الاستنساخ خلال المراحل الأولى في التطور. حين يكون Calcineurine/$NFATc_3$ و myogenin في تعاون منتظم مع MyoD.

في الفقرة 6 نتحدث عن النتائج المهمة في هذا البحث ومستقبل أبحاث انذارات Calcineurine/NFAT.

استنتاجاتنا تبين مدى أهمية Calcineurine/NFAT في انشاء وأزمات خلايا عضلة القلب وتكشف عن هوية الجينات NFAT الضرورية في عضلة القلب.

هذا كله يمكن أن يساهم في اكتشاف أدوية لمعالجة أمراض القلب والعضلة.

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Like I said before: negative points from above.
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أسرتي في المغرب شكرا لكل الأوقات الجميلة
التي قضيتها معكم في العطلات الصيفية و
اللحظات الممتعة على شواطئ البحر.
أجل يا سناء, مريم و هدى لقد إنتهيت أخيرا.
أريد شكر جميع أفراد العائلة لإهتماماتكم ببحثي هذا.

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Als je vraagt wat echte vriendschap is
Zal iedereen wat anders zeggen
Het lijkt alsof iedereen er anders over denkt
Maar dan moet je ze vragen het uit te leggen

Je kent elkaar door en door
Je weet al wat de ander zeggen zal
Het is degene die je altijd zal vangen
Na weer zo'n eindeloze val

Het is iemand die je kan vertrouwen
Tegen alle anderen is die stil
Iemand die je altijd zal helpen
Als iets niet lukken wil

Iemand die je steun biedt
Of iemand die je raad geeft
Waar je bij uit kan huilen
Ook als die het zelf zwaar heeft

Diegene zal zoveel mogelijk doen
Om te zorgen dat je vrolijk bent
Iemand die zoveel voor je betekent
Omdat je die zo goed kent

Iemand die altijd voor je klaar staat
In voor en tegenspoed
In goede en in slechte tijden
Omdat die van je houdt, niet omdat het moet

Curriculum Vitae

Meriem Bourajjaj werd geboren op 28 augustus 1979 te Amsterdam. In 1998 behaalde zij het VWO (Atheneum) diploma aan het Amstel lyceum te Amsterdam, en ging zij Medische Biologie studeren aan de Vrije Universiteit te Amsterdam. In het kader van deze studie deed zij 2 onderzoeksstages. Haar eerste stage was bij de afdeling Radiotherapie, sectie Radiobiologie in het VUMC te Amsterdam, onder begeleiding van Dr. Peter Sminia. Haar tweede stage werd uitgevoerd in Crucell NV in Leiden, onder begeleiding van Dr. S. Verhaagh. In augustus 2002 behaalde zij het doctoraal examen voor Medische Biologie. In datzelfde jaar is zij begonnen als assistent in opleiding in het VUMC, afdeling Klinische Chemie onder begeleiding van Dr. Casper Schalkwijk. Na een jaar daar gewerkt te hebben besloot zij de moleculaire biologie weer in te gaan. Hierna is ze als onderzoeker in opleiding begonnen aan het Hubrecht Instituut te Utrecht, onder leiding van Dr. Leon de Windt in april 2004. De resultaten van dat onderzoek staan beschreven in dit proefschrift.

List of Publications

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Bourajjaj M, *Stehouwer CD, van Hinsbergh VW, Schalkwijk CG. Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus. Biochem Soc Trans. 2003; 31(6):1400-2.*

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