

MEF2 Activates a Genetic Program Promoting Chamber Dilation and Contractile Dysfunction in Calcineurin-Induced Heart Failure

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Background—Hypertrophic growth, a risk factor for mortality in heart disease, is driven by reprogramming of cardiac gene expression. Although the transcription factor myocyte enhancer factor-2 (MEF2) is a common end point for several hypertrophic pathways, its precise cardiac gene targets and function in cardiac remodeling remain to be elucidated.

Methods and Results—We report the existence of synergistic interactions between the nuclear factor of activated T cells and MEF2 transcription factors triggered by calcineurin signaling. To circumvent the embryonic lethality and mitochondrial deficiency associated with germ-line null mutations for MEF2C and MEF2A respectively, we used conditional transgenesis to express a dominant-negative form of MEF2 in the murine postnatal heart and combined this with magnetic resonance imaging to assess MEF2 transcriptional function in Ca^{2+} /calcineurin-induced cardiac remodeling. Surprisingly, end-diastolic and end-systolic ventricular dimensions and contractility were normalized in the presence of severely hypertrophied left ventricular walls on MEF2 inhibition in calcineurin transgenic mice. In line, we generated lines of transgenic mice expressing MEF2A in the heart, which displayed primarily chamber dilation. Microarray profiling indicated that MEF2 promotes a gene profile functioning primarily to or at the nucleus, cytoskeletal and microtubular networks, and mitochondria.

Conclusions—These findings assign a novel function to MEF2 transcription factors in the postnatal heart, where they activate a genetic program that minimally affects cardiac growth yet promotes chamber dilation, mechanical dysfunction, and dilated cardiomyopathy. (*Circulation*. 2006;114:298-308.)

Key Words: heart failure ■ hypertrophy ■ magnetic resonance imaging ■ signal transduction

The heart responds to stress signals by hypertrophic growth, which is accompanied by activation of select transcription factors and reprogramming of cardiac gene expression. Pathological hypertrophy frequently progresses to dilated cardiomyopathy, a chronic disorder of the heart muscle. Dilation of the ventricular chamber and the associated increase in stress on the ventricular wall are often the first irreversible steps toward heart failure,¹ but the genetic underpinnings of dilated cardiomyopathy remain largely elusive.

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Cytoplasmic members of the nuclear factor of activated T cells (NFATc1–c4) represent a family of Ca^{2+} /calcineurin-dependent transcription factors² that contribute to the development of cardiac hypertrophy.^{3,4} Because DNA binding by NFAT proteins is quite weak, NFAT family members prob-

ably rarely act alone but rather need transcriptional partners to elicit full gene activation. Ca^{2+} responsiveness of a number of muscle promoters/enhancers also has been mapped to another set of response elements recognized by the transcription factor myocyte-enhancer factor-2 (MEF2). In normal adult myocardium, MEF2 exhibits only basal activity, whereas cardiac stress signaling stimulates MEF2 transcriptional activity by causing the nuclear export of class II histone deacetylases (HDACs), which associate with MEF2 and suppress its activity.⁵ In line, mice lacking HDAC9 and/or HDAC5 are hypersensitive to cardiac stress signaling and display excessive cardiac growth.^{6,7} MEF2 also serves as a transcriptional platform for several prohypertrophic signaling cascades. Although calcineurin has been reported to promote MEF2 factors by direct dephosphorylation in neurons^{8–10} or by promoting ternary complex formation between NFAT and MEF2 in T lymphocytes,¹¹ the mechanism whereby MEF2

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transcription factors are activated downstream of cardiac calcineurin signaling awaits formal experimental demonstration.

Using a multidisciplinary approach, we analyzed the mechanisms whereby MEF2 is activated downstream of calcineurin signaling in heart muscle. We tested the consequences of inactivating the transcription factor MEF2 downstream of calcineurin activation, starting from the premise that MEF2 would be involved primarily in calcineurin-induced hypertrophic remodeling of the heart muscle. The coexistence of multiple MEF2 isoforms in the adult myocardium,¹² the embryonic lethal phenotype of a germ-line null mutation for MEF2C,¹³ and mitochondrial defects and lethal arrhythmias of a germ-line null mutation for MEF2A¹⁴ pose a formidable barrier to using null mutations as a way to test the function of MEF2-dependent transcription in hypertrophy. To circumvent this issue, we used a Cre recombinase activated “Flox-ON” approach to obtain heart-specific overexpression of a dominant-negative form of MEF2 (DNMEF2) in transgenic mice. Surprisingly, crossbreeding of Flox-DNMEF2 mice with calcineurin transgenic mice only modestly repressed cardiac hypertrophy but profoundly reduced chamber dilation and improved contractility. Gene chip analysis of MEF2 target genes downstream of calcineurin activation provided insights into the rescue mechanisms of heart failure. Finally, we generated lines of transgenic mice expressing MEF2A in the heart that primarily displayed dilated cardiomyopathy. Our combined results are consistent with a model wherein MEF2 controls cardiac gene expression by acting as a target for Ca²⁺-activated NFAT signaling that primarily promotes chamber dilation and loss of contractility in calcineurin-induced heart failure.

Methods

Mice

Details on the generation of transgenic mice expressing a DNMEF2 or wild-type MEF2A are given in the expanded Methods section in the online Data Supplement. Mice expressing Cre recombinase or an activated mutant of calcineurin under control of the 5.5-kb murine cardiac α -myosin heavy chain promoter¹⁵ (MHC-Cre and MHC-CnA transgenic mice, respectively) were described previously.^{3,16}

Live Cardiac Magnetic Resonance Imaging

Details on magnetic resonance imaging are given in the expanded Methods section (see online-only Data Supplement).

Histological Analysis

Hearts were arrested in diastole, perfusion fixed with 4% paraformaldehyde, and embedded in paraffin. Sections (6 μ m) were cut and stained with hematoxylin and eosin (H&E).

Recombinant Plasmids

All expression vectors used are provided in the expanded Methods section (see online-only Data Supplement).

Coimmunoprecipitation Assays

Details on coimmunoprecipitation assays are provided in the expanded Methods section (see online-only Data Supplement).

Chromatin Immunoprecipitation

Details on chromatin immunoprecipitation are given in the expanded Methods section (see online-only Data Supplement).

Recombinant Adenoviruses

C-terminal Myc-tagged MEF2, encompassing residues 1 to 117 and incorporating the R24L mutation¹⁷ and C-terminal FLAG-tagged full-length human MEF2A, were cloned into the pAdTrack-CMV viral shuttle vector¹⁸ to generate AdDNMEF2 and AdMEF2A. AdGFP was generated as described previously.¹⁸ An adenovirus expressing an activated mutant of calcineurin (AdCnA) was described earlier.¹⁹

Cell Culture, Transfections, and Luciferase Assays

Isolation and culture of neonatal rat ventricular cardiomyocytes and COS7 cells were performed as described before in detail.²⁰ Transient transfections were performed as described²⁰ with FuGENE 6 reagent (Roche, Basel, Switzerland) according to the manufacturer's recommendations.

Western Blot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting were performed as described in detail.²⁰ Antibodies included monoclonal anti-green fluorescent protein (GFP) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif, 1:500), monoclonal anti-VP16 (Santa Cruz Biotechnology, Inc, 1:500), polyclonal rabbit anti-MEF2A (Upstate Inc, Charlottesville, Va, 1:1000), and monoclonal anti-FLAG (Sigma, St. Louis, Mo, 1:5000), followed by corresponding horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark, 1:5000) and enhanced chemiluminescence detection.

Immunocytochemistry

Neonatal rat cardiomyocyte isolation and preparation for immunofluorescence were performed as described previously in detail.²⁰

Agilent Gene Expression Profiling and Data Analysis

RNA extraction, cRNA production, and labeling and hybridization on Agilent 22k mouse microarray slides (Agilent Technologies, Palo Alto, Calif) are described in detail in the expanded Methods section (see online-only Data Supplement). Gene classifications were assigned on the basis of publicly available software and Web sites.

Real-Time Polymerase Chain Reaction

RT-RNA material from cardiac tissue from mice was analyzed by real-time polymerase chain reaction (PCR) with the BioRad iCycler (Bio-Rad, Hercules, Calif) using SYBR Green buffer for labeling and detection. Primer sequences are available on request.

Statistical Analysis

The results are presented as mean \pm SEM. Statistical analyses were performed with SPSS and InStat 3.0 software (GraphPad Software, Inc, San Diego, Calif) 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.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Calcineurin-Induced MEF2 Activation Is Mediated by NFAT

MEF2 transcription factors are activated downstream of calcineurin activity in the heart, but the precise mechanism whereby this class of transcription factors are activated remains to be determined. In T lymphocytes, NFATc2 has been shown to interact directly with MEF2D in a synergistic transcriptional complex to activate the Nur77 gene,¹¹ but the functional significance of NFATc2 in the heart, unlike

NFATc3,²¹ has not been addressed so far. To test whether an MEF2/NFAT complex is functional in cardiac muscle, we ectopically expressed Gal4-MEF2C, NFATc3, and an active form of calcineurin (Δ CnA) in cultured cardiomyocytes, which gave a 15-fold increase in Gal4-dependent reporter gene activity, whereas addition of p300 further enhanced this level to 25-fold activation (Figure 1A). These findings were confirmed by cotransfecting FLAG-MEF2C, NFATc3, and Δ CnA using a reporter with luciferase under control of multimerized MEF2 binding sites (Figure 1B). Collectively, these data suggest that calcineurin activates MEF2 transcriptional activity by promoting ternary complex formation between NFAT, MEF2, and CBP/p300 chromatin remodeling enzymes.

Recently, interacting domains on MEF2D and NFATc2 were mapped to the MADS-box DNA binding in MEF2D and the C-terminal transactivation domain (C-TAD) of NFATc2.¹¹ To test whether these domains were conserved in other MEF2 and NFAT isoforms, various truncation fragments of MEF2C were fused to Gal4. Coexpression of FLAG-NFATc3 and Δ CnA in the presence of the pG5-Luc reporter demonstrated efficient activation only in the presence of full-length MEF2C and deletion fragments harboring the MADS box (Data Supplement Figure IA). Next, FLAG-NFATc3, Δ CnA, and the Gal4-MEF2C deletion constructs were coexpressed in COS7 cells and immunoprecipitated with an anti-NFATc3 antibody. The presence of Gal4-MEF2C deletion mutants was detected by immunoblotting against Gal4. Among the NFATc3-interacting clones detected were full-length MEF2C (Figure 1C, lane 3) and only mutants that harbored the MADS-box domain (Figure 1C, lanes 4, 6, and 7, and Figure 1D).

To map the domain on NFATc3 that interacts with MEF2, 4 Gal4-NFATc3 fusions were constructed, harboring full-length NFATc3, the N-terminal regulatory domain, the Rel homology domain, or the C-TAD. Cotransfection of Gal4-NFATc3 mutants and FLAG-MEF2C demonstrated efficient transactivation when either full-length NFATc3 or the C-TAD of NFATc3 was linked to Gal4 (Data Supplement Figure IB). Next, FLAG-MEF2C, with distinct Gal4-NFAT mutants, was coimmunoprecipitated with a pan-MEF2 antibody. Among the interacting proteins detected were full-length NFATc3 (Figure 1E, lane 3) and the C-TAD of NFATc3 coupled to Gal4 (Figure 1E, lane 6, and Figure 1F). Collectively, these findings indicate that specific interactions exist between the MADS box of MEF2C and the C-TAD of NFATc3. To verify whether an NFAT-MEF2 interaction exists *in vivo*, MEF2 was immunoprecipitated from heart lysate obtained from wild-type and calcineurin transgenic mice. Immunoblotting for NFATc3 readily indicated that endogenous NFAT and MEF2 interacted only in calcineurin transgenic mouse hearts (Figure 1G).

Inhibition of MEF2 Reduces Calcineurin-Induced Cardiomyocyte Hypertrophy

To begin to assess the requirement of MEF2 downstream of calcineurin-mediated gene expression, we created a DNMEF2 harboring the first 117 amino acid residues from MEF2C with a C-terminal myc-epitope tag under control of

the CMV promoter. Additionally, we replaced the arginine residue at position 24 to leucine (R24L), which creates a non-DNA binding mutant form of MEF2 (Data Supplement Figure IIA).¹⁷ The effectiveness of the DNMEF2 construct was confirmed in cotransfection assays with wild-type MEF2C or MEF2A with increasing amounts of the DNMEF2 construct using 3 \times MEF2-Luc reporter as a readout, which dose dependently abrogated MEF2-dependent transcription activity (Figure 2A and 2B).

To investigate the requirement of MEF2 in calcineurin-mediated cardiomyocyte hypertrophy, we generated bicistronic adenoviral vectors expressing either the DNMEF2 construct or MEF2A and GFP under separate CMV promoters (AdDNMEF2 and AdMEF2A; see Data Supplement Figure IIA) or only GFP (AdGFP; Figure 2C and 2D). To monitor the change in cell size or sarcomere organization, cardiomyocytes preinfected with the control adenovirus AdGFP or AdDNMEF2 were stained for sarcomeric α -actinin (Figure 2E). After 24 hours, cells were either left untreated (Figure 2E, left) or infected with AdCnA for 24 hours (Figure 2E, right). AdCnA resulted in a >2-fold increase in cell surface area (3478 ± 140 versus $1601 \pm 101 \mu\text{m}^2$; $P < 0.01$ versus AdGFP; Figure 2F). AdDNMEF2 infection modestly abrogated the prohypertrophic effects of AdCnA ($2272 \pm 76 \mu\text{m}^2$; $P < 0.05$ versus AdCnA, $P < 0.05$ versus AdGFP). The data demonstrate that MEF2 is partially involved in aspects of hypertrophic remodeling of cardiomyocytes after calcineurin activation.

Conversely, cardiomyocytes infected with AdMEF2A demonstrated mild elongation (Figure 2G). Of note, the cells did not display the typical features of hypertrophied cells, including sarcomere assembly, vigorous beating, or ANF positivity (Figure 2G and data not shown), and did not reveal a significant increase in average cell surface area ($1924 \pm 87 \mu\text{m}^2$) compared with AdGFP-infected cultures ($1823 \pm 83 \mu\text{m}^2$; Figure 2H). We next determined the ratio of the length of the major and minor axes of cells in our experimental groups as a measure of cellular elongation. AdGFP-infected cardiomyocytes had a ratio of 2.54 ± 0.13 , whereas AdMEF2A-infected cells displayed a 2-fold increase in the ratio of major to minor axis (5.15 ± 0.43 ; $P < 0.05$) compared with AdGFP-infected cells (Figure 2I). Combined, these data suggest that MEF2 transcriptional activity in cultured cardiomyocytes does not provoke a classic hypertrophic response but rather promotes elongation of cardiomyocytes.

Inhibition of MEF2 Reduces Biventricular Dilation and Cardiac Dysfunction but Minimally Affects Calcineurin-Induced Cardiac Hypertrophy

To more accurately assess the role of MEF2 transcription factors downstream of cardiac calcineurin signaling, we designed an approach to inhibit MEF2 transcriptional activity in the adult cardiac muscle *in vivo*. To this end, we generated lines of Flox-DNMEF2 transgenic mice that express DNMEF2 on activation of Cre recombinase and crossed them with MHC-Cre, a transgenic line expressing Cre recombinase in postnatal cardiac muscle¹⁶ (Figure 3A). Three Flox-DNMEF2 founder lines were obtained (lines 29, 32, and 40); lines 29 and 32 expressed the myc-tagged DNMEF2 protein

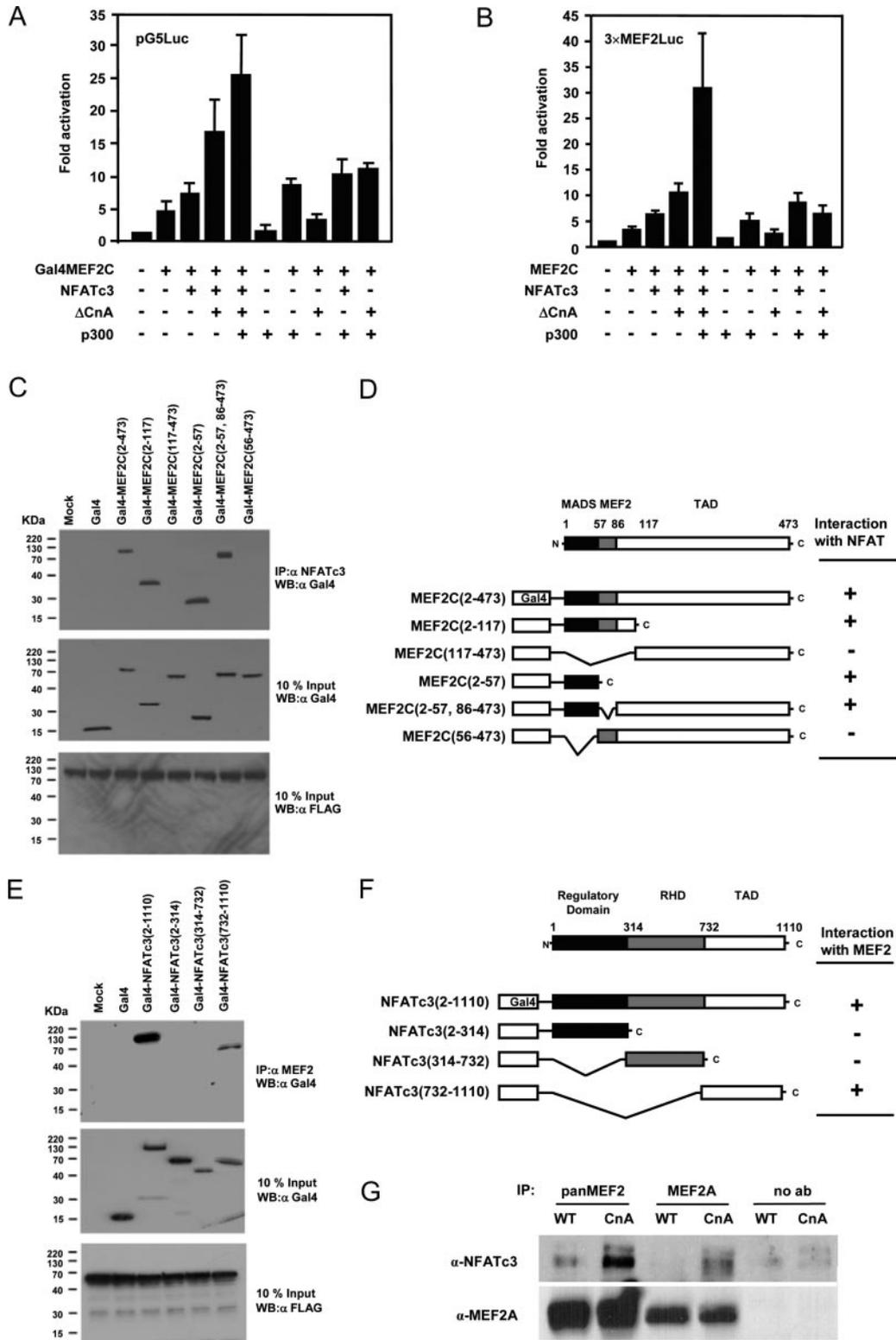


Figure 1. NFAT activates MEF2 transcriptional activity by direct protein-protein interaction. A, COS7 cells were transiently transfected with a Gal4 luciferase reporter construct and a Gal4-MEF2C fusion protein either in or not in the presence of NFATc3, ΔCnA, and p300. B, Transfections using a multimerized MEF2 luciferase reporter showing activation of MEF2 transcriptional activity by NFATc3, ΔCnA, and p300. C, Protein extracts from COS7 cells transfected with empty vectors (Gal4), FLAG-NFATc3, and/or Gal4-MEF2C deletion constructs were immunoprecipitated with an anti-NFATc3 antibody and subjected to Western blotting using an anti-Gal4 antibody. D, Schematic overview of the Gal4-MEF2C deletion constructs and their ability to bind NFATc3. E, Protein extracts from COS7 cells transfected with empty vectors (Gal4), Gal4-NFATc3 deletion constructs, and/or FLAG-MEF2C were immunoprecipitated with an anti-MEF2 antibody and subjected to Western blotting with an anti-Gal4 antibody. F, Schematic overview of the Gal4-NFATc3 deletion constructs and their ability to bind MEF2C. G, Protein extracts from wild-type and calcineurin transgenic mouse hearts were immunoprecipitated with anti-MEF2 antibodies and subjected to Western blotting using an anti-NFATc3 antibody.

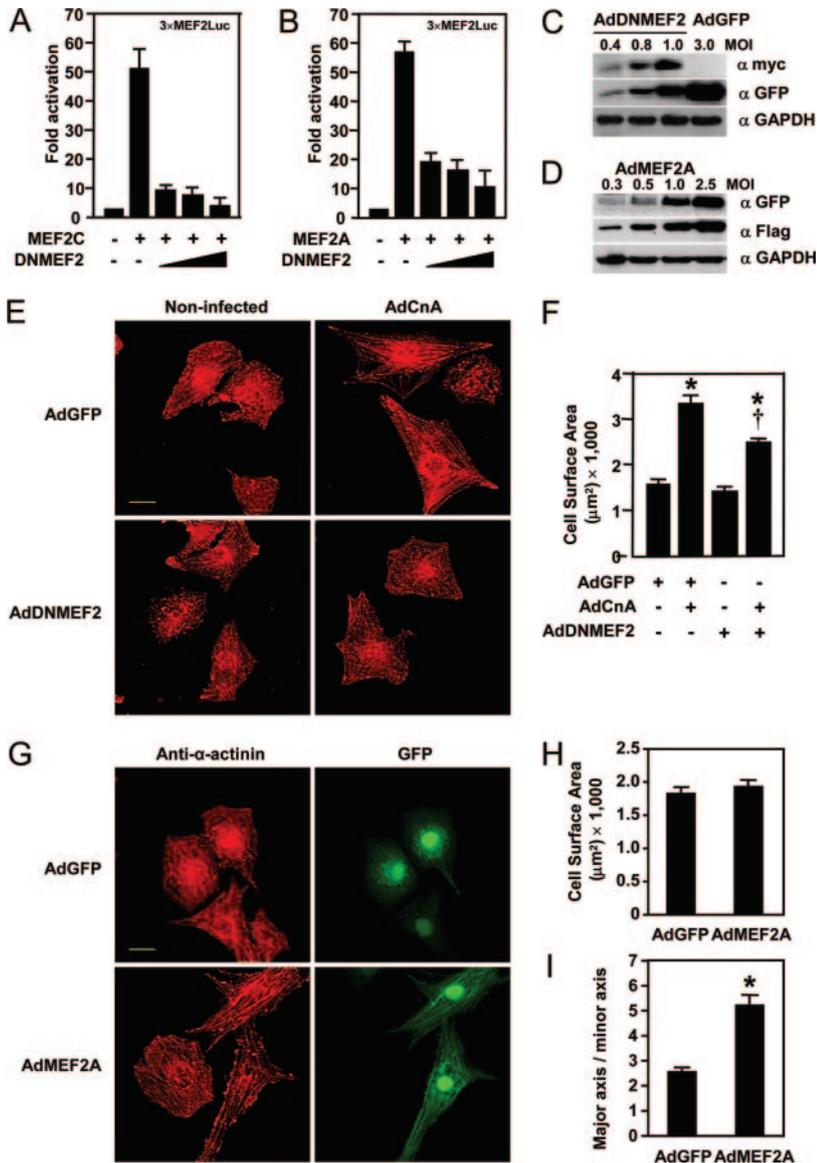


Figure 2. Inhibition of MEF2 modestly attenuates calcineurin-induced cardiomyocyte hypertrophy. A, B, COS7 cells were transiently transfected with a multimerized MEF2 reporter in the presence of either MEF2A (A) or MEF2C (B) constructs and increasing amounts of DNMEF2 construct. C, D, Western blot analysis with anti-Myc, anti-FLAG, or anti-GFP antibody on COS7 cell lysates infected with AdDNMEF2, AdMEF2A, or AdGFP. E, Representative images of immunostained cardiomyocytes infected with the indicated adenoviruses. F, Quantification of cell surface areas of cardiomyocytes preinfected with AdGFP or AdDNMEF2 and subsequently left uninfected or infected with AdCnA. Data represent mean ± SEM of 3 independent experiments. G, Representative images of immunostained cardiomyocytes infected with the indicated adenoviruses. H, Quantification of cell surface areas of cardiomyocytes infected with AdGFP or AdMEF2A. I, Quantitative examination of ratios of cell length to cell width of cardiomyocytes (≥100 per experiment) infected with AdGFP or AdMEF2A. **P*<0.05, AdCnA infected vs noninfected cells, †*P*<0.05, AdCnA-infected cells preinfected with AdDNMEF2 vs AdCnA-infected cells preinfected with AdGFP control virus.

only on activation by Cre recombinase (Figure 3A, bottom). We continued with Flox-DNMEF2 transgenic line 32, which displayed the highest amount of transgenic product in the heart.

Heart size and function of MHC-Cre/Flox-DNMEF2 mice were indistinguishable from nontransgenic and MHC-Cre control mice (Figure 3B and Data Supplement Movies). In line with previous findings,^{3,19} MHC-Cre/MHC-CnA mice displayed a 3-fold increase in heart size with severe biventricular dilation. Surprisingly, however, once MHC-Cre/MHC-CnA mice were crossbred with Flox-DNMEF2 mice to generate calcineurin transgenic mice expressing DNMEF2 only in heart muscle cells, gross cardiac morphology analysis indicated reduced biventricular dilation with little effect on heart weight (Figure 3B). Indeed, ratios of heart weight to body weight obtained from all experimental groups indicated only a minimal reduction in heart weight between MHC-Cre/MHC-CnA mice and MHC-Cre/MHC-CnA/Flox-DNMEF2 littermates (heart weight to body weight: MHC-Cre, 5.84 ± 0.32 mg/g; MHC-Cre/Flox-DNMEF2, 5.64 ± 0.33 mg/g; MHC-Cre/MHC-CnA, 15.10 ± 0.56 mg/g; MHC-Cre/

MHC-CnA/Flox-DNMEF2, 12.86 ± 0.74 mg/g; *P*<0.05 versus MHC-Cre; *P*<0.05 versus MHC-Cre/MHC-CnA; Figure 3C).

Next, in vivo cardiac morphology and function were analyzed in live mice with magnetic resonance imaging. Cardiac mass, end-diastolic and end-systolic dimensions, and contractility were comparable between MHC-Cre and MHC-Cre/Flox-DNMEF2 transgenic mice (Figure 3D and Data Supplement Movies I and II). In contrast, 8-week-old MHC-Cre/MHC-CnA mice displayed severely increased wall thickness of all ventricular walls, increased end-diastolic and end-systolic intraventricular dimensions, and substantial loss of contractility (Figure 3D and Data Supplement Movie III). Remarkably, MHC-Cre/MHC-CnA/Flox-DNMEF2 littermates displayed biventricular end-diastolic and end-systolic biventricular dimensions and contractility that were comparable to control MHC-Cre mice in the presence of persistently hypertrophied septal and left ventricular (LV) free wall thicknesses (Figure 3D and Data Supplement Movie IV).

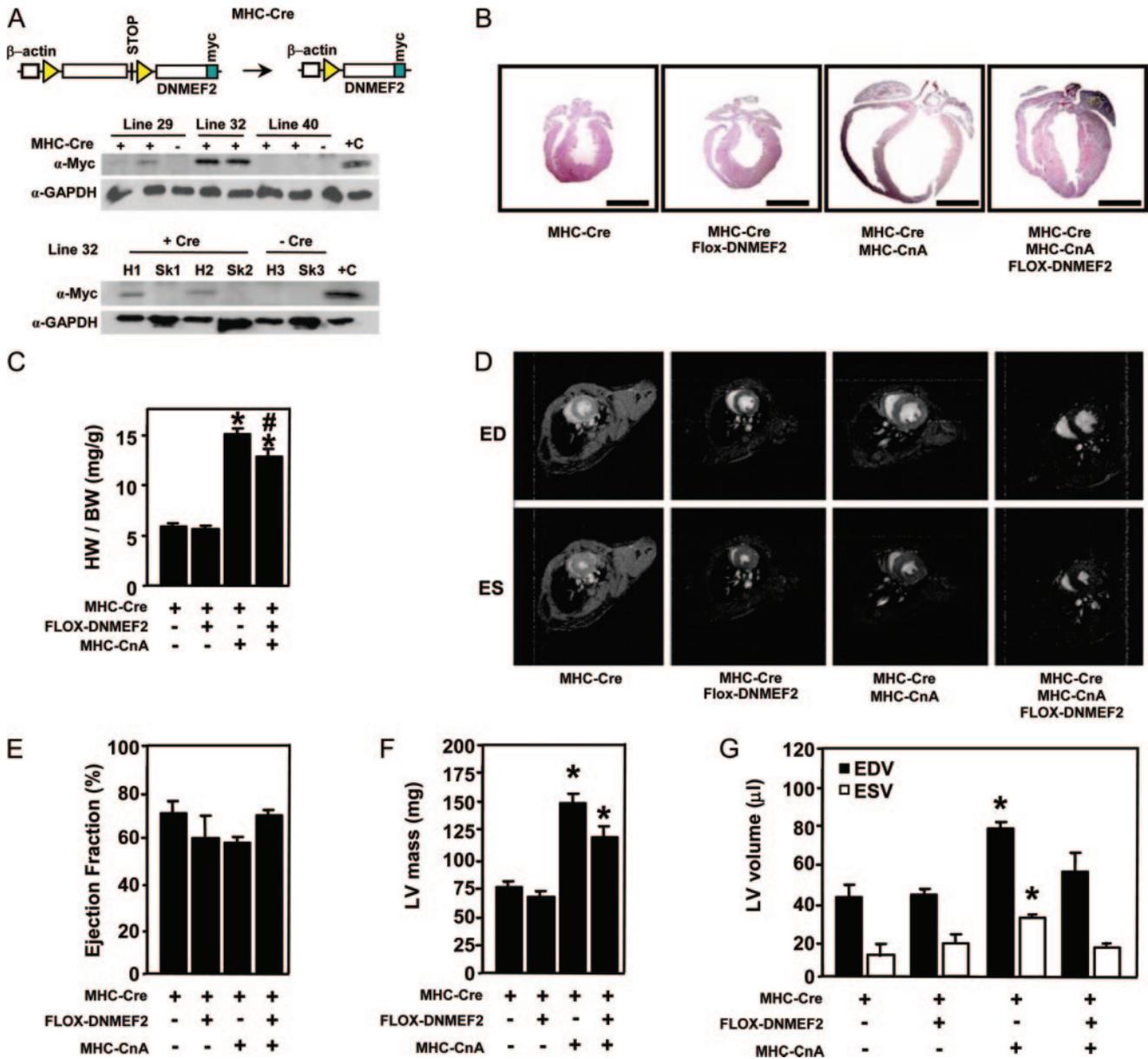


Figure 3. Inhibition of MEF2 reduces calcineurin-induced cardiac dysfunction. A, Schematic representation of Flox-DNMEF2 transgenic construct (top panel) before and after Cre induced recombination and Western blot analysis on heart lysate (top blot) and heart (H) and skeletal muscle (Sk) lysate (bottom blot) using an anti-myc antibody to detect DNMEF2 expression. B, H&E-stained coronal sections of the 4 experimental genotypes indicating a massive hypertrophic and dilated cardiomyopathy in 8-week-old MHC-Cre/MHC-CnA transgenic mice and rescue of chamber dilation in MHC-Cre/MHC-CnA/Flox-DNMEF2 transgenic mice. C, Ratios of heart weight to body weight (HW/BW) of the indicated genotypes (n=12 to 17 per group). D, 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 thickened walls of MHC-Cre/MHC-CnA hearts and less dilation but similar wall thickness of MHC-Cre/MHC-CnA/Flox-DNMEF2 transgenic mice. E, Ejection fraction determined from NMR imaging in live mice with the indicated genotypes (n=3 per group). F, LV mass determined by nuclear magnetic resonance imaging in live mice with the indicated genotypes (n=3 per group). G, Maximal LV volumes as a measure of chamber dilation calculated at maximal diastole (solid bars) or maximal systole (open bars) determined by nuclear magnetic resonance imaging in live mice or the indicated genotypes. **P*<0.05 vs MHC-Cre control mice. #*P*<0.05 vs MHC-Cre/MHC-CnA.

Comparison of the images at maximal diastole and systole (Figure 3D) indicated the markedly reduced function in the MHC-Cre/MHC-CnA mice compared with control genotypes and MHC-Cre/MHC-CnA/Flox-DNMEF2 mice, which displayed markedly improved ejection fraction (Figure 3E), a slight reduction in LV mass (Figure 3F), and a striking reduction in biventricular dilation (end-diastolic and end-systolic volume; Figure 3G). LV mass determined at end diastole by nuclear

magnetic resonance (NMR) imaging amounted to 75.2±4.2, 67.3±2.5, 146.6±8.1, and 118.6±9.9 mg in MHC-Cre, MHC-Cre/Flox-DNMEF2, MHC-Cre/MHC-CnA, and MHC-Cre/MHC-CnA/Flox-DNMEF2 mice, respectively. End-diastolic volume amounted to 43.3±6.8, 44.5±2.5, 78.4±2.6, and 56.2±9.2 μL in MHC-Cre, MHC-Cre/Flox-DNMEF2, MHC-Cre/MHC-CnA, and MHC-Cre/MHC-CnA/Flox-DNMEF2 mice, respectively. Likewise, ejection fraction was normalized

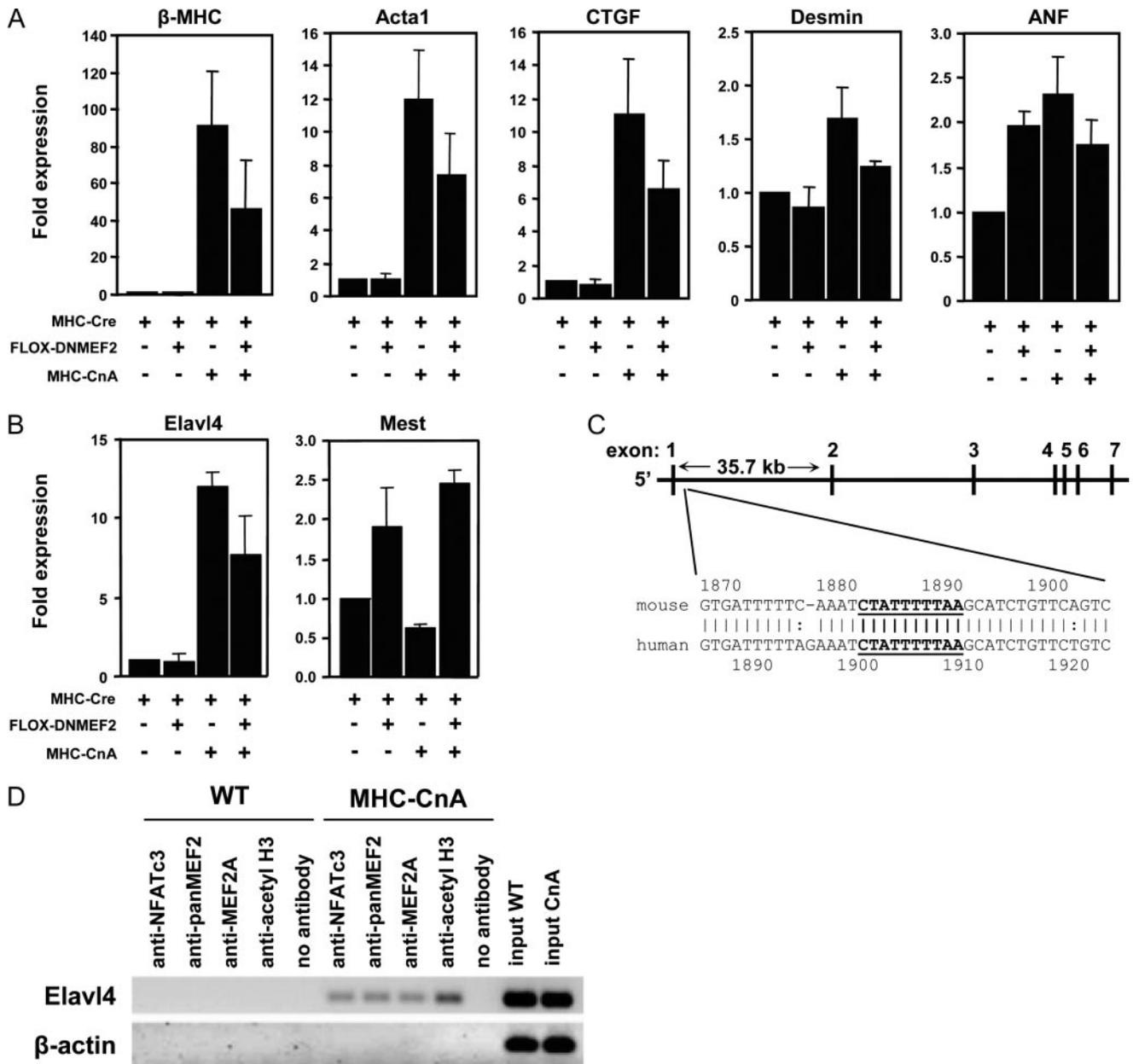


Figure 4. Calcineurin activates MEF2-dependent genes. Verification of transcript levels for β -MHC, Acta1, CTGF, desmin, ANF (A), *elavl4*, and *mest* (B) by real-time PCR detection in which transcript levels observed in MHC-Cre control mice were set at 1. C, Schematic of the intragenic region of *elavl4* showing the conserved MEF2 binding site. D, Chromatin immunoprecipitation demonstrates that NFATc3 associates with MEF2 to form a transcriptionally active complex on AT-rich motif in the *elavl4* gene in calcineurin transgenic hearts.

when MEF2 was inhibited in vivo ($69 \pm 7\%$, $57 \pm 9\%$, $58 \pm 2\%$, and $70 \pm 1\%$ in MHC-Cre, MHC-Cre/Flox-DNMEF2, MHC-Cre/MHC-CnA, and MHC-Cre/MHC-CnA/Flox-DNMEF2 mice, respectively). Taken together, the data indicate that inhibition of MEF2 transcriptional activity downstream of calcineurin signaling rescues biventricular dilation and contractility with a minimal effect on the massive hypertrophy response provoked by transgenic calcineurin activation.

Gene Expression Profiling of Calcineurin Transgenic Mice With or Without Inhibition of MEF2

We also analyzed hypertrophic marker gene expression, which demonstrated increased levels in MHC-Cre/MHC-

CnA transgenic mice and a modestly reduced expression in the presence of the Flox-DNMEF2 transgene (Figure 4A). To provide more insights into the mechanisms underlying the phenotypic rescue evoked by inhibition of MEF2 transcriptional activity downstream of calcineurin, we analyzed the relative gene expression profiles from the indicated experimental groups with Agilent mouse chips (Agilent technologies, Palo Alto, Calif) containing 22 000 genes. One hundred sixty-one gene transcripts (0.7% of all genes) displayed opposite expression between MHC-Cre/MHC-CnA and MHC-Cre/MHC-CnA/Flox-DNMEF2 mice with a fold change in expression ≥ 1.5 (with $P < 0.01$) without a change in abundance on Cre-mediated activation of Flox-DNMEF2 (the Table). Among these, 108 transcripts were upregulated in

Expression Profiling of Calcineurin Transgenic Hearts With or Without MEF2 Transcriptional Activity

Growth factors, cytokines, receptors (22): *Acvrinp1, Akap13, Apln, Arhgap24, Camkk1, Cav1, Dll1, Dok4, Dusp18, Ephb1, Gpr24, Gtbbp4, Map3k7, Olfr566, Pcp2, Pde7a, Plk3, Rassf2, Rgs3, Shank3, Tbc1d4, Wif1*

Cytoskeletal architecture, microtubular system, cell adhesion (20): *Ablim1, Cald1, Dnahc8, Elavl4, Kif1b, Krt1-15, Mtap7, Mtss1, Myo10, Neb, Ofd2, Slmap, A930004K21Rik, Amotl1, Cldn5, Emilin1, Itlna, Mcam, Mest, Mmp15*

Transcriptional regulation (14): *Ash11, Bcl6b, Cebpz, Cited2, Dnajc3, Flil1, L3mbtl3, Pou4f1, Ppp1r12b, Rcor2, Six4, Tlx2, Zfpm2, 2310076014Rik*

Proteolysis, ubiquitin complex (10): *Casp7, NE, Nedd4, Npepps, Prp19, Ube2q, Uhrf1, Usp48, 1700095N21Rik, 6430527G18Rik*

Energy metabolism, electron transport (10): *Aass, Bpgm, Dbt, Fmo2, Grp58, Lip1, Lpgat1, Pfkp, D2Bwg1356e, 5330438E18Rik*

Ion channels (5): *Cacna1s, Clic5, Kcnd2, Slc24a3, Slc28a2*

Cell cycle (4): *Ccng, Mcm6, Rif1, Tlm*

Ribosome biogenesis (4): *Hnrpa3, Rpl14, Rps26, Rrs1*

Intracellular protein transport (3): *Rab1b, Rab12, Rab21*

One hundred sixty-one transcripts were detected in calcineurin transgenic hearts that were regulated in an opposite manner in the presence of the Flox-DNMEF2 transgene, of which relevant transcripts with a 1.5-fold change in signal intensity are shown. Others include 27 ESTs and 10 transcripts without gene ontology description.

MHC-Cre/MHC-CnA double-transgenic mice and oppositely regulated on inhibition of MEF2 transcriptional activity in the setting of cardiac calcineurin signaling. Conversely, 53 transcripts showed decreased expression when calcineurin was activated in the myocardium, with the decrease in expression attenuated when crossbred with Flox-DNMEF2 mice (the Table and Data Supplement Table I). Gene ontology classifications revealed an overrepresentation of gene transcripts in 3 subclasses: signal transducers, cell-matrix adhesion and cytoskeletal architecture, and energy metabolism (Table). On MEF2 inhibition, calcineurin transgenic hearts differentially expressed transcripts for multiple signal transducers (*apln, camkk1, map3k7, pde7a*), transcriptional repressors (*cited2, dnajc3, rcor2*), cytoskeletal remodeling components (*neb, cldn5, emilin1*), and ubiquitin-related proteases (*npepp2, usp48*). Opposite expression of *elavl4* and *mest* by microarray profiling was confirmed by real-time reverse transcriptase-PCR (RT-PCR) (Figure 4B). Combined, these data indicate that MEF2 activity downstream of calcineurin signaling activates subsets of genes localized primarily to or functioning at the nucleus, the cytoskeletal and microtubular networks, and mitochondria.

To define a regulatory mechanism involving NFAT and MEF2 in the induction of *elavl4*, the mouse and human orthologs were analyzed and aligned for cross-species conservation. A fragment of between 1870 and 1900 bp relative to the transcriptional start site in the murine sequence displayed a remarkable conservation in an A/T-rich MEF2 binding site (¹⁸⁸³CTATTTTAA), which is completely homologous with the consensus MEF2 binding sequence (C/T)TA(T/A)₄TA(G/A) (Figure 4C). To determine whether a MEF2/NFAT complex binds to this A/T-rich motif in vivo, we performed ChIP assays using anti-MEF2, anti-NFATc3, and anti-acetylated histone H3 antibodies. No antibody immunoprecipitates (Figure 4D, lanes 5 and 10) were used as

negative controls; input chromatin served as a positive control (Figure 4D, lanes 11 and 12). When immunoprecipitated chromatin was subjected to PCR with primers spanning the A/T-rich motifs in *elavl4*, a specific 209-bp band was obtained only in MHC-CnA transgenic hearts, indicating that a complex consisting of MEF2/NFAT and histone acetyltransferase activity (indicative of p300 presence) occupied the MEF2 binding site. Collectively, these data support the notion that a complex consisting of MEF2, NFAT, and p300 accumulates on selected A/T-rich motifs in genes after calcineurin activity in vivo.

Generation and Characterization of MEF2A Transgenic Mice

To verify whether MEF2 transcriptional function in vivo is restricted primarily to promoting ventricular dilation and contractile loss, we designed a strategy to activate MEF2 transcriptional activity in the postnatal heart. We generated transgenic mice expressing the MEF2A isoform under control of the well-characterized 5.5-kb murine α -myosin heavy chain (*myh6*) promoter (Figure 5A). Seven independent founders were recovered; 6 produced progeny and established transgenic lines for further characterization. All viable MEF2A transgenic lines overexpressed human MEF2A in the myocardium, albeit at differing levels, varying between 1.5-fold and 4-fold more MEF2A protein at 12 weeks of age, when normalized to glyceraldehyde-3-phosphate dehydrogenase (Figure 5B and data not shown). One female transgenic founder died with an extreme form of peripartum cardiomyopathy and severely dilated ventricular chambers on necropsy (Figure 5C). Low-expressing MEF2A transgenic lines, characterized by a 1.5-fold to 2-fold increase in MEF2A protein, showed no lethality, no signs of cardiac hypertrophy, and no histological abnormalities of the myocardium on closer inspection (Figure 5D and data not shown). We suspect that the lack of a phenotype in low MEF2 expressors reflects the inability of a low level of MEF2 to overcome HDAC repression. One medium overexpressing MEF2A transgenic line, characterized by 2.5-fold more MEF2A protein, displayed a very mild increase in heart weight (corrected for body weight and tibial length), slightly thickened LV walls, and mild dilation of ventricular walls (Figure 5D and data not shown). Two high-expressor lines, with 4-fold more MEF2A expression, were characterized by substantial dilation of both right ventricular and LV chambers at 12 weeks of age and thinned ventricular walls (Figure 5D). Combined, these data indicate that cardiac MEF2A activation produces a dose-dependent, variable phenotype ranging from no apparent phenotype to slight hypertrophic/dilated remodeling to severe dilated cardiomyopathy characterized by ballooning of the ventricular chambers and thinning of ventricular walls.

Discussion

Here, we demonstrated that cardiac MEF2 transcription factors are activated in an NFAT-dependent fashion by calcineurin, a pivotal mediator of calcium-dependent cardiac growth, and that a Cre/LoxP-dependent approach to antagonize MEF2 transcriptional activity prevented chamber dilation and mechanical dysfunction, with minor effects on

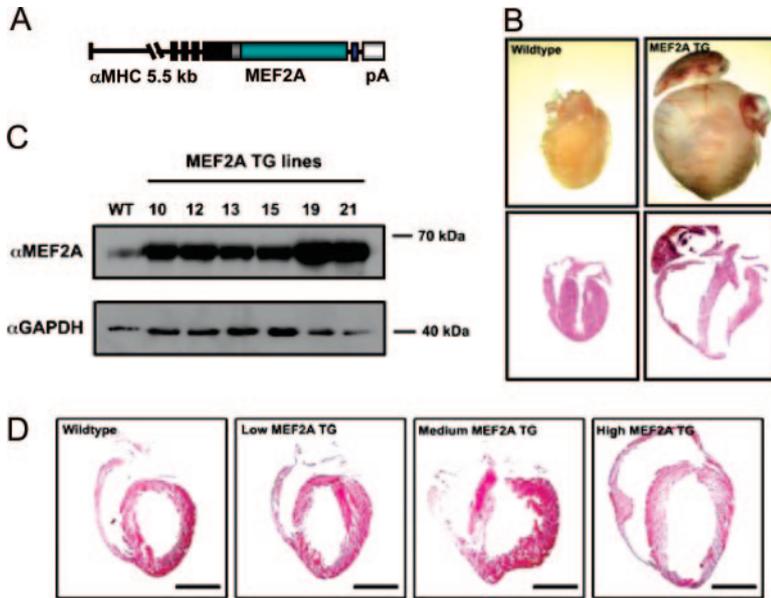


Figure 5. MEF2A transgenic mice display dilated cardiomyopathy. A, Schematic of transgenic construct consisting of 5.5-kb α -MHC promoter, FLAG-tagged human MEF2A, and human growth hormone polyadenylation signal. B, Western blot analysis on nuclear fractions of hearts from wild-type mice and different MEF2A transgenic lines indicates overexpression of MEF2A in cardiac muscle. C, Whole-heart image and H&E coronal section of wild-type and a transgenic founder that died suddenly from peripartum dilated cardiomyopathy. D, H&E-stained coronal sections of wild-type and low, medium, and high overexpressing MEF2A transgenic mice indicate that with medium and high MEF2A overexpression, mild and severe dilated cardiomyopathy, respectively, was observed.

cardiac growth. Chronic changes in the cardiac gene program may trigger pathological changes in the myocardium that incite irreversible cellular changes, with chamber dilation and the associated increase in stress on the ventricular wall representing the first irreversible steps toward heart failure. Accordingly, the transcription factors that connect biomechanical forces and the activation of stress pathways to a “fetal” gene program are central to understanding the initiation and progression of heart failure. Multiple signals and transcriptional events suffice to evoke cardiac hypertrophy in cultured myocyte systems and whole-animal gain-of-function models,²² but only a few examples exist in which gene deletion rescues the germane features of heart failure in mice, which are chamber dilation and mechanical dysfunction.^{23,24} Using genetic gain and loss-of-function approaches, we are the first, to the best of our knowledge, to prove the existence of separate genetic programs primary to cardiac growth versus chamber dilation and cardiac dysfunction within the venue of calcium-driven cardiac growth and failure (Figure 6).

The data in this study confirm the existence of a combinatorial MEF2/NFAT regulatory transcriptional pathway controlling gene expression in cardiac muscle cells. This observation is reminiscent of the cooperative interaction between MEF2 proteins and myogenic basic helix-loop-helix factors in skeletal muscles²⁵ and between MEF2 proteins and GATA factors in cardiac muscles,²⁶ and it suggests that MEF2 factors interact with a range of transcription factors to optimize gene expression. Calcineurin has been reported to activate the Nur77 promoter in T lymphocytes by promoting nuclear translocation of NFATc2, which in turn forms a ternary complex with MEF2 and CBP/p300.¹¹ Given the overlapping expression patterns of MEF2 and NFAT factors in smooth muscle, skeletal muscle, cardiac muscle, and the central nervous system, the NFAT/MEF2-dependent pathway described in this work suggests the existence of this molecular paradigm for Ca^{2+} /calmodulin signaling in excitable tissue.

Using a multidisciplinary approach, we analyzed MEF2 transcription factor function in adult cardiac muscle, starting from the premise that MEF2 would be involved primarily in the initial hypertrophic remodeling phase of the heart muscle.²⁷ Although transgenic overexpression of (truncated mutants of) transcription factors harbors the risk of unwanted transcriptional squelching, the consistency in opposite phenotypes we observed using our combinatorial dominant-negative versus gain-of-function models for MEF2 transcriptional activity justifies the overall conclusions drawn in this study. To our surprise, MEF2 promotes cardiac disease symptoms that are more compatible with later manifestations of cardiac disease that extends beyond and stems from initial pathological cardiac growth, namely chamber dilation, mechanical dysfunction, and end-stage heart failure. The seeming paradox—that cardiac growth and failure are not mutually exclusive yet cardiac growth initiates failure—could reflect the limitations of current knowledge on the specificity and

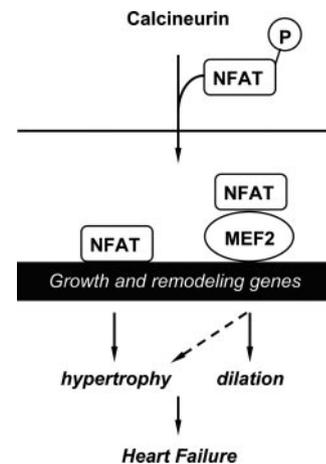


Figure 6. After cardiac calcineurin activation, MEF2 is able to recruit NFAT to activate selected downstream target genes that preferentially drive dilation, whereas NFAT alone or with other transcriptional partners provokes hypertrophy.

convergence of individual characterized stress pathways. Nevertheless, the mere existence of a genetic pathway specifically underlying ventricular dilation will be of fundamental clinical and therapeutic interest.

A large and convincing body of literature demonstrated that cardiac MEF2 activity is under control of HDAC regulation at the onset of cardiac hypertrophy. However, chromatin remodeling is a very general mode of transcription regulation,²⁸ and HDACs have been demonstrated to regulate other transcription factors that have been implicated in promoting cardiomyocyte growth, suggesting that the excessive cardiac growth response observed in HDAC9- or HDAC5-deficient mice²⁹ may not be limited to derepression of MEF2 activity alone. Indeed, MEF2 LacZ sensor mice injected with prohypertrophic agonists such as isoproterenol, angiotensin II, or thyroid hormone failed to display ventricular MEF2 activity despite an increase in ventricular mass.³⁰ Given these considerations and the findings with the loss-of-function and gain-of-function analyses for MEF2 in transgenic mice in the present study, it is reasonable to suggest that the function of MEF2 factors in the postnatal myocardium may be more restricted to molecular signals that promote dilated cardiomyopathy rather than the initial phases of hypertrophic remodeling. Nevertheless, genetic proof that MEF2 is dispensable for developmental or stress-induced postnatal cardiac growth but required for ventricular dilation and mechanical dysfunction suggests the logic of strategies to inhibit this class of transcriptional regulators in forms of heart disease involving calcineurin signaling.

The classification profiles of MEF2 target genes secondary to cardiac calcium stress signaling identified in the present study underscore the complexity of genetic events underlying chamber dilation and mechanical dysfunction yet also provide tangible entry points for mechanistic explanations of the findings presented here. First, the gene profiles identified in the present work correlate well with those observed in gene chip analysis of human end-stage idiopathic dilated cardiomyopathy³¹ and with the most characteristic pathological features of human end-stage dilated cardiomyopathy, which include a dynamic redistribution of cytoskeletal structures, extensive fibrosis, and extracellular alterations in mitochondria number and morphology.³² For example, the relative enrichment in transcripts of genes at the cardiomyocyte cytoskeletal network, including *nebulin*, *claudin*, *elastin microfibril interfaces 1 (emilin1)*, *intelectin a* and *microtubule-associated protein 7*, to mention a few, which couple adherens junctions and costameres to myofibrils and the extracellular matrix, underscores the germane role of the cytoskeletal architecture in force transmission. Transcripts for stress signaling components such as *Wnt inhibitory factor 1*, *EphB1*, *apelin*, and *transforming growth factor- β activated kinase 1 (map3k7 or tak1)* were differentially affected. Tak1 activation suffices to provoke ventricular dilation, mechanical dysfunction, and fulminant heart failure in mice.³³ Finally, ventricular dilation and MEF2 function concur with complex epigenetic effects, as suggested by altered transcript abundance for chromatin remodeling factors, including *cbp/p300-interacting transactivator*, *REST co-repressor 2* and *the DNAJ (Hsp40) homolog*.

Conclusions

The present study provides the first direct approach to studying the biological ramifications of the transcription factor MEF2 in the postnatal heart muscle. Our findings identify distinct gene profiles regulated by MEF2 activity, which suggest that MEF2 promotes multiple cellular changes, each of which may independently or in an interconnected fashion promote changes in the heart muscle that are characteristic of dilated cardiomyopathy.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Hypertension, aortic stenosis, and myocardial infarction lead to the development of a pathological form of cardiac hypertrophy that predisposes to the development of congestive heart failure and cardiac arrhythmias. One intracellular signaling pathway required for pathological cardiac hypertrophy uses the calcium-activated phosphatase calcineurin and the transcription factor nuclear factor of activated T cells. Several growth factors and hormones are known to activate calcineurin in heart tissue, including angiotensin II, endothelin-1, and β -adrenergic stimulation. In this work, the transcriptional mechanisms between nuclear factor of activated T cells and members of the myocyte enhancer factor-2 (MEF2) underlying calcineurin-induced cardiac hypertrophy were analyzed. Remarkably, mice with heart-restricted, impaired activation of MEF2 were able to undergo hypertrophic remodeling in response to calcineurin activation, but they were resistant to the development of later phases of pathological remodeling such as wall thinning, chamber dilation, and contractile dysfunction. Conversely, mice with transgenic activation of an MEF2 transcription factor developed dilated cardiomyopathy without prior cardiac hypertrophy. These results suggest that agents that specifically prevent MEF2 transcriptional activity or the interaction between nuclear factor of activated T cells and MEF2 transcription factors might avert the development of deleterious phases of pathological growth and therefore may have therapeutic utility in the treatment of congestive heart failure. One hope is that it will be possible to prevent chamber dilation and contractile dysfunction in pathological forms of cardiac hypertrophy.