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The Transcriptional Coactivator CAMTA2 Stimulates Cardiac Growth by Opposing Class II Histone Deacetylases

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SUMMARY

Postnatal cardiac myocytes respond to diverse signals by hypertrophic growth and activation of a fetal gene program. In an effort to discover regulators of cardiac hypertrophy, we performed a eukaryotic expression screen for activators of the *atrial natriuretic factor* (*ANF*) gene, a cardiac-specific marker of hypertrophic signaling. We discovered that a family of transcriptional coactivators, called CAMTAs, promotes cardiomyocyte hypertrophy and activates the *ANF* gene, at least in part, by associating with the cardiac homeodomain protein Nkx2-5. The transcriptional activity of CAMTAs is governed by association with class II histone deacetylases (HDACs), which negatively regulate cardiac growth. Mice homozygous for a mutation in a *CAMTA* gene are defective in cardiac growth in response to pressure overload and neurohumoral signaling, whereas mice lacking HDAC5, a class II HDAC, are sensitized to the prohypertrophic actions of CAMTA. These findings reveal a transcriptional regulatory mechanism that modulates cardiac growth and gene expression by linking hypertrophic signals to the cardiac genome.

INTRODUCTION

Mammalian cardiac muscle cells respond to mechanical load and various extracellular stimuli by hypertrophic growth, characterized by an increase in cell size and protein synthesis, enhanced assembly of contractile units, and reactivation of a fetal cardiac gene program (Seidman and Seidman, 2001; Olson and Schneider, 2003). While cardiac hypertrophy can have initial salutary effects on cardiac function, when prolonged it is a major predictor of heart failure and sudden death.

A variety of stress-responsive signaling pathways promote cardiac hypertrophy, but the mechanisms that link these pathways to the cardiac genome are only beginning to be unveiled. Recently, we showed that class II histone deacetylases (HDACs) act as signal-responsive corepressors of the fetal cardiac gene program and cardiac growth (Zhang et al., 2002; Chang et al., 2004). The class II HDACs, HDAC5 and HDAC9, associate with the MEF2 transcription factor and repress its activity (McKinsey et al., 2000a, 2000b, 2002). Atypical protein kinase C (PKC) isoforms and the downstream effector kinase protein kinase D (PKD) regulate cardiac growth by promoting the phosphorylation of class II HDACs, which triggers their export from the nucleus and consequent activation of MEF2 target genes (Vega et al., 2004). Consistent with the proposed roles of class II HDACs as negative regulators of pathological cardiac growth, knockout mice lacking HDAC5 or HDAC9 develop massively enlarged hearts in response to stress (Zhang et al., 2002; Chang et al., 2004). Whether class II HDACs act solely through MEF2 to modulate cardiac growth or whether they have additional transcriptional targets remains an important question.

In an effort to discover regulators of cardiac gene expression and growth, we devised a eukaryotic expression screen for cDNAs encoding activators of the *atrial natriuretic factor* (*ANF*) promoter, a cardiac-specific marker of hypertrophy and pathological remodeling of the adult heart. This screen revealed a family of activators of the *ANF* promoter, called calmodulin binding transcription activators (CAMTAs), which are conserved from plants to humans (Bouche et al., 2002). We show that CAMTAs are recruited to the *ANF* promoter, at least in part, by association with the cardiac homeodomain protein Nkx2-5 and function as inducers of cardiac growth. Through gain- and loss-of-function approaches in vivo and in vitro, we show that class II HDACs restrain the activity of CAMTA proteins. Nuclear export of class II HDACs in response to PKC/PKD signaling releases CAMTAs from HDAC-dependent

repression with consequent expression of genes involved in cardiac growth. These findings uncover a role for mammalian CAMTA proteins as signal-responsive transcriptional coactivators of cardiac growth and targets for the antihypertrophic actions of class II HDACs.

RESULTS

Discovery of CAMTA2 in an Expression Screen for Regulators of the *ANF* Promoter

We performed a cDNA expression screen by expressing pools of clones from various cDNA expression libraries in COS cells and assaying for activation of a luciferase reporter controlled by the *ANF* promoter, which is cardiac specific and responsive to a variety of signaling pathways involved in cardiac growth and remodeling (Sprenkle et al., 1995; Temsah and Nemer, 2005). A total of 2000 cDNA pools, each containing ~100 individual cDNA clones, was screened, yielding ~20 positive pools. Individual cDNA clones capable of activating *ANF-luciferase* were identified by sib-selection. One cDNA, which strongly activated the *ANF-luciferase* reporter, encoded CAMTA2 (Figure 1A), a member of the CAMTA family of transcription factors discovered in plants as stress-responsive regulators of gene expression that respond to calcium/calmodulin (Yang and Poovaiah, 2002; Bouche et al., 2002). Two *CAMTA* genes (*CAMTA1* and *CAMTA2*) are predicted to exist in mice and humans, but their functions have not been investigated.

The CAMTA2 cDNA isolated in the expression screen encoded the full-length 1196-amino acid CAMTA2 protein. Members of the CAMTA family share homology in multiple domains (Figure 1A). A conserved domain of ~110 amino acids, referred to as the CG-1 domain, is located near the N termini of CAMTA proteins. This domain, which was first identified in a sequence-specific DNA binding protein from parsley, has been shown to bind to the DNA sequence CGCG (da Costa e Silva, 1994). Near the center of CAMTA proteins is a conserved TIG domain, which has been implicated in establishing nonspecific DNA contacts in other transcription factors such as the Rel proteins NFAT and NF-kB. This domain is followed by ankyrin repeats, which participate in protein-protein interactions, and a series of IQ motifs, which bind calmodulin (Bahler and Rhoads, 2002). CAMTA2 shares high homology with CAMTA1 and with CAMTA proteins from fruit flies and plants in each of the above domains, whereas the intervening regions of the proteins are less conserved.

Northern blot analysis revealed a predominant *CAMTA2* transcript of ~6 kb in adult mouse heart and brain, as well as minor species in other tissues (Figure 1B). The human *CAMTA2* transcript was detected specifically in heart, skeletal muscle, and brain (data not shown). Mouse *CAMTA1* transcripts were also detected in brain and heart (Figure 1B). During embryogenesis, *CAMTA2* transcripts were detected only at a background level in the heart (data not shown) followed by pronounced upregulation after birth (Figure 1C). Isolated cardiomyocytes showed an enrichment of CAMTA2 expression compared to the whole heart.

Transcriptional Activation by CAMTA Is Mediated by Nkx2-5

We used a series of *ANF* promoter mutants to map the *cis*-regulatory sequences that conferred responsiveness to CAMTA2. Deletion mutations from −624 bp to −97 bp relative to the transcription initiation site did not impair activation of the promoter by CAMTA2 in transfected COS cells (Figure 1D). However, deletion to −74 bp resulted in a precipitous decline in responsiveness to CAMTA2. The latter construct retained residual responsiveness to CAMTA2, which we attribute to the existence of cryptic CAMTA2 response elements in the reporter plasmid. CAMTA1 activated the *ANF* promoter even more strongly than CAMTA2 (Figure 1E).

The CAMTA-responsive region of the *ANF* promoter between −97 and −74 contains binding sites for the cardiac homeodomain protein Nkx2-5 and the T box factor Tbx5, both of which have been shown to be important for *ANF* transcription (Durocher et al., 1996, 1997; Hiroi et al., 2001; Sepulveda et al., 1998; Bruneau et al., 2001). The specific DNA sequence responsible for transcriptional activation by CAMTA2 was further delineated by point mutations in this region. Mutations in the Nkx2-5-response element (NKE) abrogated responsiveness to CAMTA2, whereas mutations in the Tbx binding element (TBE) had no effect on expression (Figure 1D), suggesting that CAMTA2 required the NKE to maximally stimulate the *ANF* promoter. Consistent with this conclusion, the *connexin-40* promoter, which contains two NKEs (Bruneau et al., 2001), was activated ~20-fold by CAMTA2, as was a reporter containing a single NKE linked to a basal promoter (Figure 1E).

Gel mobility shift assays with GST-CAMTA2 fusion protein and the CAMTA2-responsive region of the *ANF* promoter showed no evidence of CAMTA2 DNA binding (data not shown), suggesting that CAMTA2 might activate *ANF* expression via an effect on the expression or transcriptional activity of another factor that bound the NKE. Indeed, CAMTA2 enhanced the ability of Nkx2-5 to activate the *ANF* promoter by ~8-fold (Figure 1F). Because COS cells used for the original expression screen do not express Nkx2-5, we surmise that NK-type homeodomain proteins (or other factors) expressed by these cells satisfy the apparent requirement of CAMTA2 for the NKE in the *ANF* promoter.

Association of CAMTA2 with Nkx2-5 on the *ANF* Promoter

Chromatin immunoprecipitation (ChIP) assays with primary neonatal rat cardiomyocytes showed that CAMTA2 associated with the NKE in the *ANF* promoter in native chromatin (Figure 2A). In transfected COS cells, CAMTA2 was also detected on an exogenous *ANF* promoter, and its association with the promoter was enhanced when coexpressed with Nkx2-5 (Figure 2A), supporting the

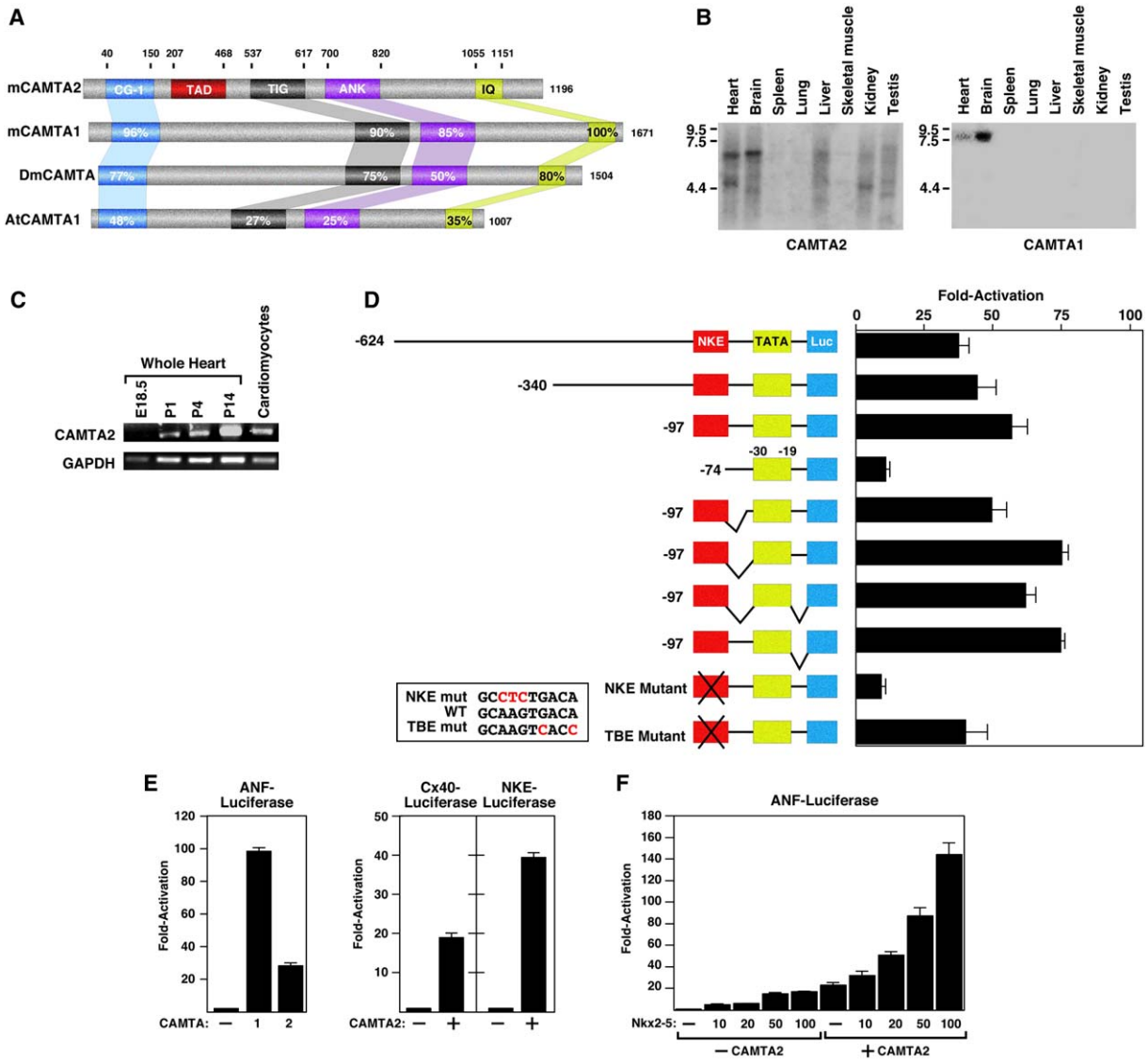

Figure 1. Identification of CAMTA2 as an Activator of the *ANF* Promoter

(A) Schematic diagram of CAMTA proteins from mouse (m), *Drosophila melanogaster* (Dm), and *Arabidopsis thaliana* (At). Amino acid identities within each domain are shown.

(B) Detection of *CAMTA* transcripts by Northern blot analysis of adult mouse tissues.

(C) Detection of transcripts for *CAMTA2* and *GAPDH* (as a control) by semiquantitative RT-PCR in hearts from rats at E18.5 and postnatal days (P) 1, 4, and 14.

(D) COS cells were transfected with a CAMTA2 expression plasmid and the indicated ANF-luciferase reporters. Values are expressed as the fold-increase in luciferase expression (± SD) in the presence compared to the absence of CAMTA2. (mutations in the NKE and TBE sites are shown in red.)

(E) COS cells were transfected with a CAMTA2 expression plasmid and the indicated luciferase reporters. Values are expressed as the fold-increase in luciferase expression (± SD) in the presence compared to the absence of CAMTA. The left panel compares the activities of CAMTA1 and CAMTA2.

(F) COS cells were transfected with ANF-luciferase, the indicated amounts of Nkx2-5 expression plasmid (ng) alone (left side), and 100 ng of CAMTA2 (right side). Values are expressed as the fold-increase in luciferase expression (± SD) with Nkx2-5 and/or CAMTA2 expression plasmids compared to the reporter alone.

conclusion that an endogenous protein in COS cells recruits CAMTA2 to the *ANF* promoter, allowing its detection in the expression screen.

As an independent test of the ability of CAMTA2 to associate with Nkx2-5 on the NKE DNA sequence, we incubated ^{35}S-labeled CAMTA2 protein with a biotinylated NKE binding site. As shown in Figure 2B, ^{35}S-labeled CAMTA2 protein associated with the NKE in the presence of GST-Nkx2-5, but not in the presence of GST alone.

CAMTA2 was distributed in the nucleus and cytoplasm (Figure 2Ca). In the presence of leptomycin B, an inhibitor of nuclear export, CAMTA2 became localized exclusively

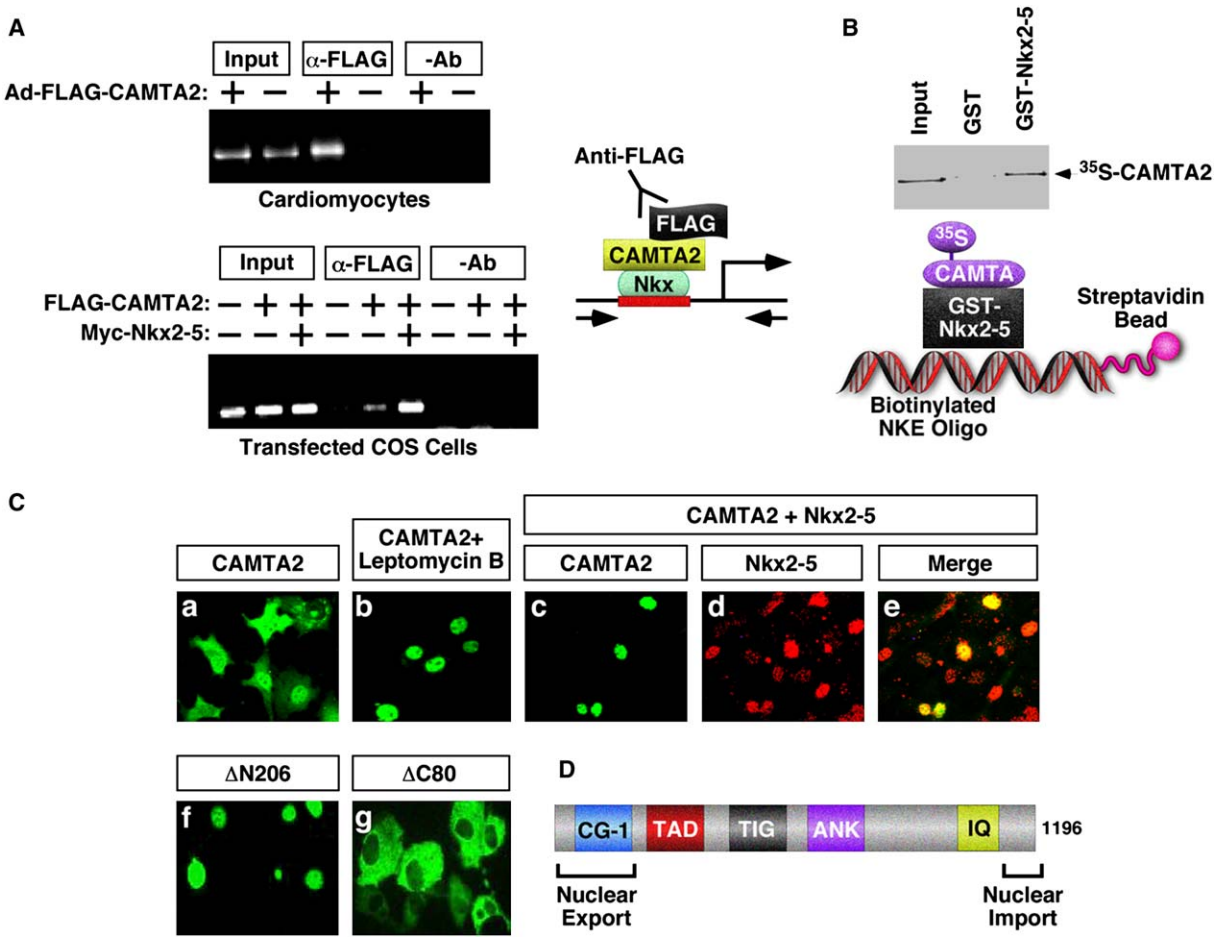

Figure 2. Interaction of CAMTA and Nkx2-5

(A) Primary neonatal cardiomyocytes (upper panel) were infected with adenovirus encoding FLAG-CAMTA2, ChIP was performed with anti-FLAG antibody or without antibody, and PCR was performed with primers flanking the NKE in the *ANF* promoter, as indicated. In the lower panel, COS cells were transfected with expression vectors encoding FLAG-CAMTA2 or Myc-Nkx2-5, as indicated, and ChIP was performed with the indicated antibodies. Input DNA was detected using primers for amplification of the NKE site on the *ANF* promoter. A schematic of the *ANF* promoter is shown to the right.

(B) A biotinylated DNA probe encompassing the NKE from the *ANF* promoter was incubated with GST or GST-Nkx2-5 and ^{35}S-methionine-labeled CAMTA2 protein translated in vitro. Proteins were then captured by binding to streptavidin beads and analyzed by SDS-PAGE.

(C) The subcellular distribution of FLAG-CAMTA2 in transfected COS cells was detected by immunofluorescence. CAMTA2 is distributed in the nucleus and cytoplasm (panel a). In the presence of leptomycin B, CAMTA2 becomes localized to the nucleus (panel b). Similarly, when coexpressed with Nkx2-5, CAMTA2 colocalized with Nkx2-5 in the nucleus (panels c–e). Deletion of the N-terminal 206 residues (ΔN206) or C-terminal 80 residues (ΔC80) resulted in nuclear or cytoplasmic localization, respectively (panels f and g).

(D) The positions of nuclear export and import sequences in CAMTA2 are shown.

to the nucleus (Figure 2Cb), suggesting that the protein cycles between the cytoplasm and the nucleus. When coexpressed with Nkx2-5, which is exclusively nuclear, all CAMTA2 protein became localized to the nucleus, consistent with a possible interaction between the proteins (Figures 2Cc–2Ce).

Functional Domains of CAMTA2

The transcriptional activity of CAMTA2 was assayed by fusing portions of the protein to the DNA binding domain of GAL4. As shown in Figure 3A, CAMTA2 fused to the GAL4 DNA binding domain activated a GAL4-dependent luciferase reporter in transfected COS cells; a region between amino acids 285 and 468 acted as a transcription activation domain (TAD). This region was approximately two orders of magnitude more effective in activating transcription than the full-length protein, suggesting that other regions may suppress its transcriptional activity, as discussed later.

To further define the mechanism of action of CAMTA2, we generated a series of deletion mutants and assayed their subcellular distribution and ability to activate the *ANF* promoter (Figure 3B). Deletion of the CG-1 domain (mutant ΔN206) completely abolished the ability of CAMTA2 to activate the *ANF* promoter, despite the presence of the TAD in this mutant. In contrast to the wild-type

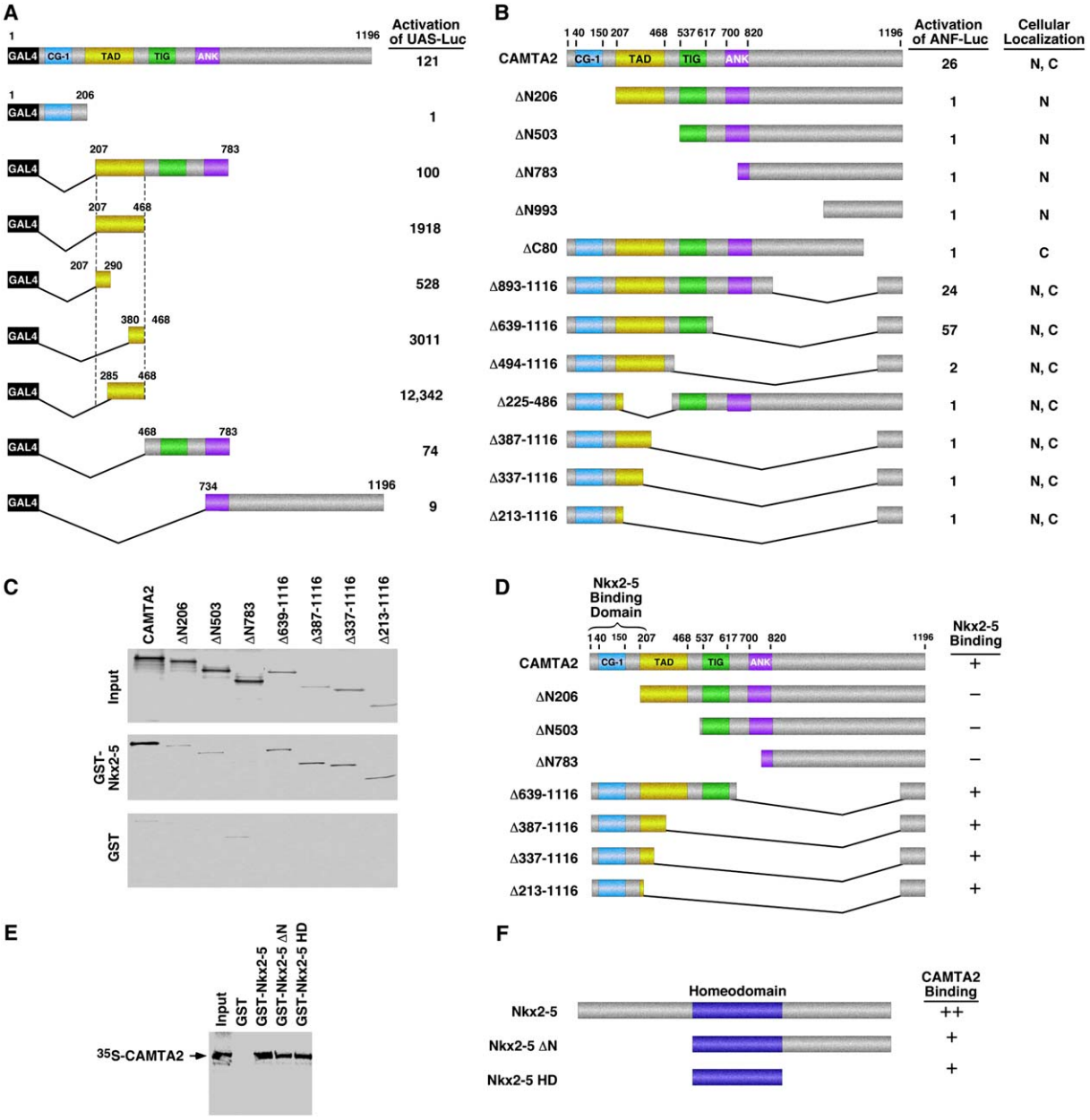

Figure 3. Interaction of CAMTA2 and Nkx2-5

(A) Portions of CAMTA2 were fused to the GAL4 DNA binding domain and assayed for activity with a UAS-luciferase reporter. Values are expressed as the fold-increase in luciferase expression in the presence of each GAL4-CAMTA2 mutant protein compared to the reporter alone.

(B) Deletion mutants of CAMTA2 were tested for their ability to activate ANF-luciferase in transfected COS cells. Values are expressed as the fold-increase in luciferase expression in the presence of each CAMTA2 deletion mutant compared to the reporter alone. The presence of the mutant protein in the nucleus (N) or cytoplasm C, as detected by immunostaining, is shown.

(C) GST-Nkx2-5 was incubated with ^{35}S-methionine-labeled CAMTA2 deletion mutants translated in vitro. Input CAMTA2 proteins are shown in the top panel. CAMTA2 proteins bound to GST-Nkx2-5 are shown in the middle panel. The lack of binding of CAMTA2 proteins to GST is shown in the bottom panel.

(D) Summary of binding data for CAMTA deletion mutants.

(E) GST alone or GST fused to portions of Nkx2-5 was incubated with ^{35}S-methionine-labeled CAMTA2 translated in vitro, as indicated.

(F) Summary of binding data for Nkx2-5 deletion mutants.

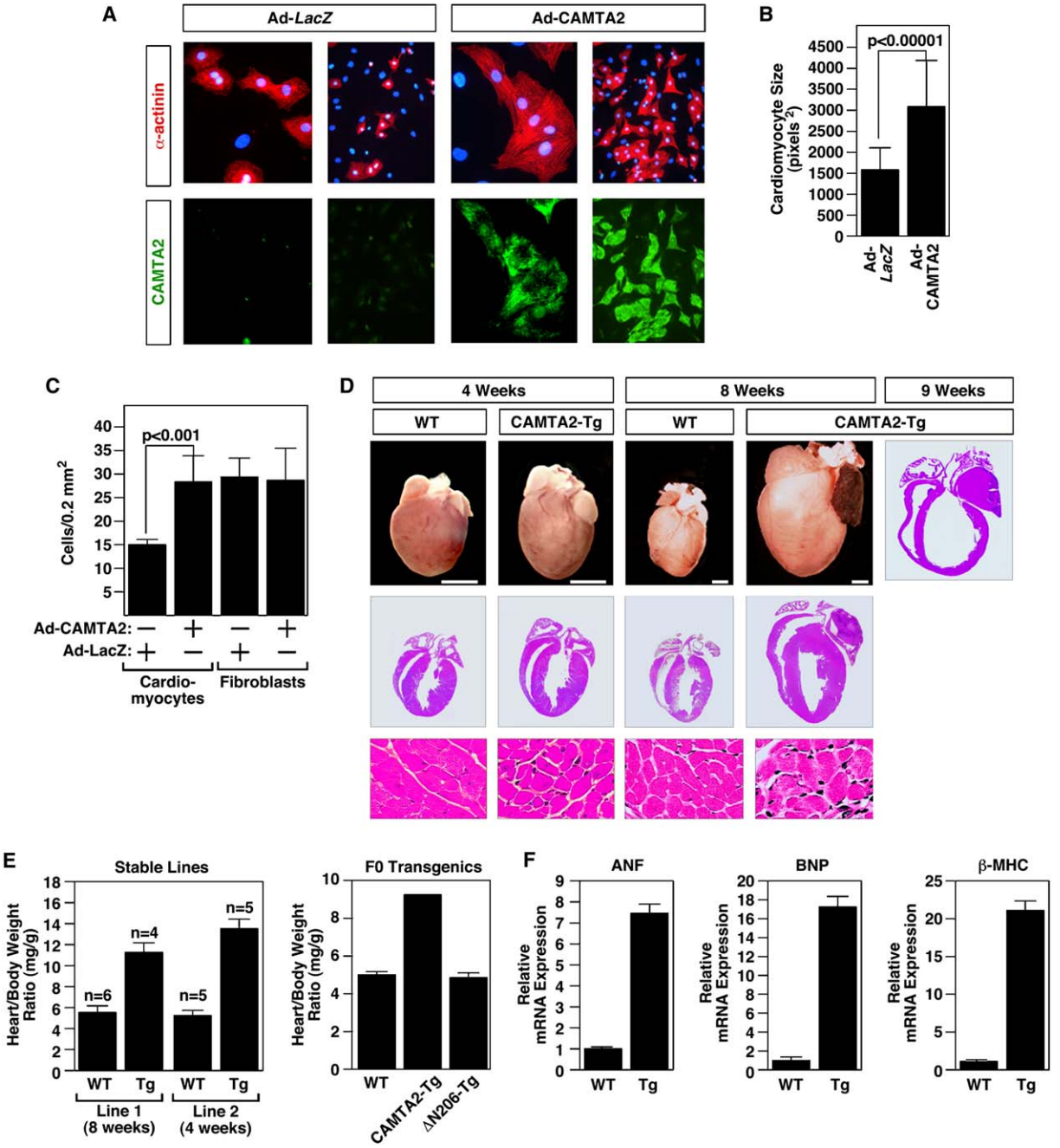

Figure 4. Induction of Cardiac Growth by CAMTA

(A) Primary neonatal rat cardiomyocytes were infected with adenoviruses encoding FLAG-CAMTA2 (right panels) or lacZ (left panels) as a control. Cells were stained with anti-α-actinin antibody (red) to mark cardiomyocytes, DAPI (blue), and anti-FLAG (green). Ad-CAMTA2 induces profound hypertrophy and sarcomere assembly. Left panels of each set are 10× and right panels are 40× magnification.

(B) Cell size (± SD) in (A) was determined as described in Experimental Procedures.

(C) Numbers of cardiomyocytes and fibroblasts (± SD) in cultures from (A) were determined by counting 20 fields. The adenovirus selectively infects cardiomyocytes, but not fibroblasts. Hence, the number of fibroblasts remains constant, but the number of cardiomyocytes increases in the presence of Ad-CAMTA2.

(D) Transgenic mice were generated bearing an α-MHC-CAMTA2 transgene. Hearts from wild-type and transgenic mice (line 1) at 4, 8, and 9 weeks of age are shown at the top. Histological sections are shown in the middle panel and high magnification views of ventricular cardiomyocytes are shown at the bottom. At 9 weeks of age, hypertrophy progresses to dilated cardiomyopathy in α-MHC-CAMTA2 transgenic mice. Bars = 2 mm.

protein, the ΔN206 mutant was localized exclusively to the nucleus (Figure 2Cf), suggesting that the CG-1 domain contains a nuclear export sequence (NES) (Figure 2D). Mutant proteins with larger N-terminal deletions were also transcriptionally inactive and localized to the nucleus. Deletion of the C-terminal residues 1116–1196 (mutant ΔC80) resulted in complete exclusion of CAMTA2 from the nucleus (Figure 2Cg), indicative of a nuclear localization sequence (NLS) in this region (Figure 2D). As expected from its cytoplasmic localization, mutant ΔC80 was unable to activate the *ANF* promoter (Figure 3B). Because the NLS was contained in the C-terminal 80 residues of CAMTA2, we generated internal deletion mutants that retained this domain. Deletion of the ankyrin-repeat region (mutant Δ639–1116) enhanced transcriptional activity, suggesting that this region suppresses activity of the TAD. Deletion mutants that removed the TIG domain resulted in a total loss of transcriptional activity. We conclude that transcriptional activity of CAMTA2 requires the combined activities of the CG-1, TAD, and TIG domains together with the NLS at the C terminus.

We performed GST pull-down experiments using a GST-Nkx2-5 fusion protein and mutants of CAMTA2 translated in vitro to map the Nkx2-5 binding domain of CAMTA2. An interaction of the full-length CAMTA2 protein with GST-Nkx2-5 was readily detectable in this assay (Figures 3C and 3D). Deletion of the CG-1 motif resulted in nearly a complete loss in binding to Nkx2-5. Further deletion of the TIG domain (mutant ΔN783) abolished the residual Nkx2-5 binding activity. Deletion mutations from the C terminus showed that residues 1–207, which encompass the CG-1 motif, were sufficient to interact with Nkx2-5. GST pull-down assays showed that the homeodomain of Nkx2-5 was sufficient for association with CAMTA2 (Figures 3E and 3F).

CAMTA2 Induces Cardiac Hypertrophy In Vivo and In Vitro

To further investigate the potential function of CAMTA2 as a regulator of cardiac gene expression, we elevated CAMTA2 expression in primary neonatal rat cardiomyocytes by adenoviral delivery. Ad-CAMTA2 infected cells displayed a phenotype of hypertrophy and sarcomere assembly, compared with control cultures infected with Ad-lacZ (Figures 4A and 4B). Cultures infected with Ad-CAMTA2 also contained a greater number of cardiomyocytes than control cultures, suggesting that CAMTA2 enhanced myocyte proliferation and/or survival (Figures 4A and 4C).

Next, we generated transgenic mice that overexpressed CAMTA2 in the heart under control of the α-*MHC* promoter. Two independent stable lines of transgenic mice and three transgenic founders were viable, but their hearts were grossly enlarged and showed extensive myocyte hypertrophy (Figures 4D and 4E). In transgenic line 1, which expressed exogenous CAMTA2 at a level approximately 6-fold higher than endogenous CAMTA2, cardiac hypertrophy worsened between 4 and 8 weeks of age. By 9 weeks of age, hypertrophy progressed to dilated cardiomyopathy and heart failure; and all transgenic animals died by 12 weeks of age. Transgenic line 2 expressed CAMTA2 at a level 11.4-fold above normal and displayed more pronounced hypertrophy (Figure 4E) and high susceptibility to sudden death. Consistent with the ability of CAMTA2 to activate the *ANF* promoter, *ANF* transcripts were elevated in the hearts of α-MHC-CAMTA2 transgenic mice, as were *b-type natriuretic peptide (BNP)* and β-*MHC* transcripts, which are markers of hypertrophy (Figure 4F).

Four transgenic founders expressing the ΔN206 CAMTA2 mutant, which failed to associate with Nkx2-5, showed no evidence of cardiac hypertrophy, even though the transgene was expressed at levels ranging from 10- to 58-fold higher than endogenous CAMTA2 (Figure 4E). These findings support the conclusion that the CG-1 domain of CAMTA2 is required to promote cardiac growth.

PKC and PKD Signaling Stimulate CAMTA2 Activity

We investigated whether the activity of CAMTA2 might be enhanced by signaling molecules implicated in cardiac hypertrophy, including activated calcineurin, activated MAP kinase MKK6, PKC, and PKD. The transcriptional activity of CAMTA2 was unaffected by calcineurin or MKK6. However, as shown in Figure 5A, activated PKCε, an atypical PKC isoform, and PKD, which act in a hypertrophic signaling cascade (Vega et al., 2004), stimulated the transcriptional activity of CAMTA2. Wild-type PKCε also enhanced CAMTA2 activity, albeit to a lesser extent than the constitutively active enzyme, while a dominant-negative form of PKCε suppressed CAMTA activity (Figure 5A).

Association of CAMTA2 with Class II HDACs

Signaling by atypical PKCs can induce *ANF* expression, at least in part, by stimulating the phosphorylation of class II HDACs, which results in their translocation from the nucleus to the cytoplasm with consequent derepression of fetal cardiac genes (Vega et al., 2004). To determine whether CAMTA2 might be a target for the repressive effects of class II HDACs on hypertrophic signaling, we tested whether HDAC5, a class II HDAC, could interfere with the ability of CAMTA2 to activate the *ANF* promoter. Indeed, HDAC5 blocked activation of the *ANF* promoter

(E) Heart weight/body weight ratios (± SD) of wild-type and stable lines α-MHC-CAMTA2 transgenic mice at 4 and 8 weeks of age are shown in the left panel. Heart weight/body weight ratios of wild-type and F0 transgenic mice harboring α-MHC-CAMTA2 and α-MHC-ΔN206 transgenic mice (n = 4) at 4 weeks of age are shown in the right panel.

(F) Transcripts representing hypertrophic gene markers (± SD) were detected in hearts of wild-type and α-MHC-CAMTA2 transgenic mice (line 1) (n = 2) at 8 weeks of age by real-time PCR.

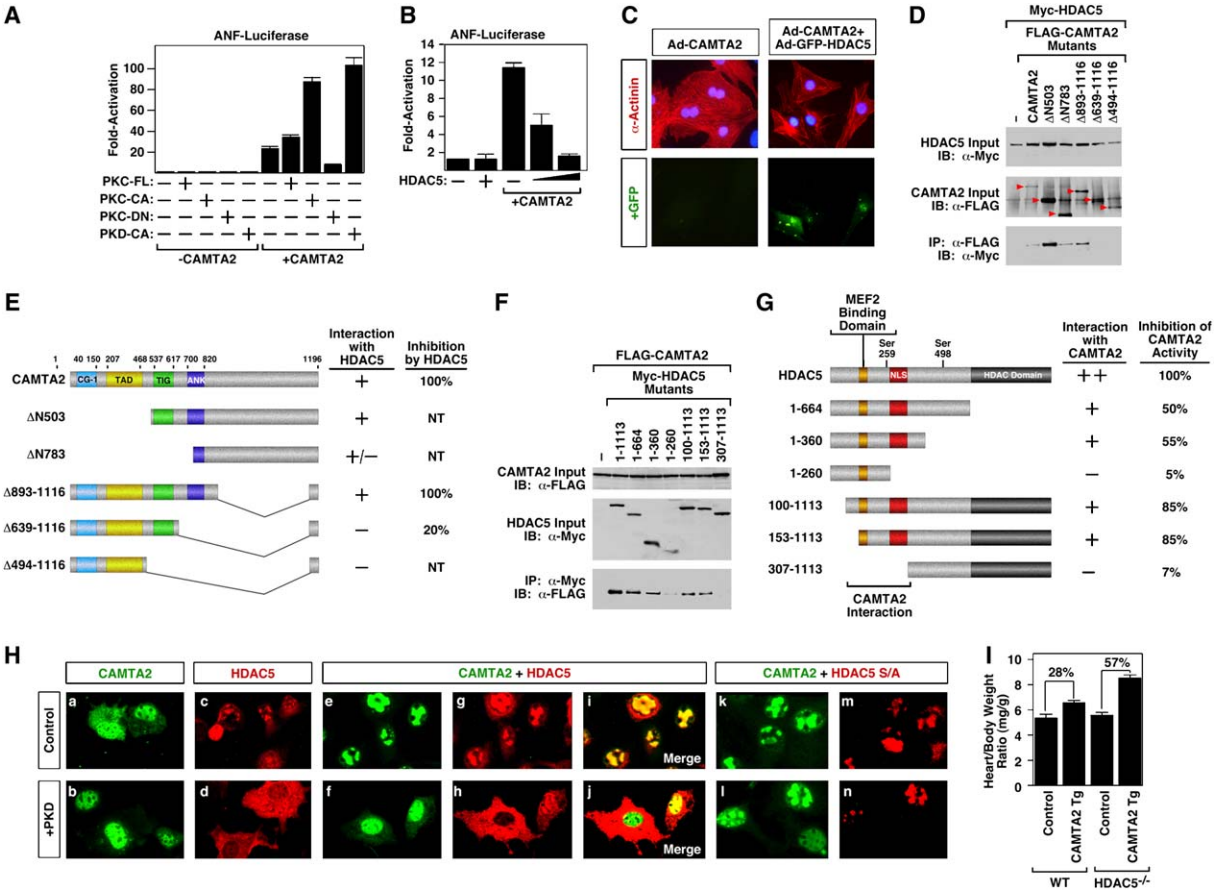

Figure 5. Signal-Dependent Regulation of CAMTA2 and Its Association with Class II HDACs

(A) COS cells were transfected with expression plasmids encoding full-length (FL), constitutively active (CA), or dominant-negative (DN) PKCε or PKD, and CAMTA2 (100 ng each), as indicated, along with the ANF-luciferase reporter (250 ng). Values are expressed as the fold-increase in luciferase expression (± SD) compared to the reporter alone.

(B) COS cells were transfected with expression plasmids encoding CAMTA2 (100 ng) or HDAC5 (5 and 25 ng), as indicated, and the ANF-luciferase reporter (150 ng). Values are expressed as the fold-increase in luciferase expression (± SD) compared to the reporter alone.

(C) Primary neonatal rat cardiomyocytes were infected with adenoviruses encoding FLAG-CAMTA2 (all panels) and GFP-HDAC5 (right panels). Cells were stained with anti-α-actinin antibody (red) to mark cardiomyocytes, DAPI (blue) to mark nuclei, and GFP (green) to detect HDAC5. Ad-HDAC5 prevents hypertrophy in response to CAMTA2. Magnification = 40×.

(D) COS cells were transfected with expression plasmids encoding Myc-HDAC5 and FLAG-CAMTA2 proteins (500 ng each). Input HDAC5 and CAMTA2 proteins detected by immunoblot (IB) are shown in the top and middle panels, respectively. HDAC5 proteins coimmunoprecipitated (IP) with CAMTA2 are shown in the bottom panel. Red arrowheads point to CAMTA2 proteins. The full-length protein is expressed at a lower level than the deletion mutants.

(E) The ability of each CAMTA2 deletion mutant to associate with HDAC5 in (D) is shown. The extent to which each protein is inhibited by HDAC5 is indicated in the right column. Maximum repression by full-length HDAC5 is set at 100%. NT, not tested because these mutants are inactive.

(F) COS cells were transfected with expression plasmids encoding FLAG-CAMTA2 and Myc-HDAC5 proteins (500 ng each). Input CAMTA2 and HDAC5 proteins detected by immunoblot (IB) are shown in the top and middle panels, respectively. CAMTA2 proteins coimmunoprecipitated (IP) with HDAC5 are shown in the bottom panel.

(G) The ability of each HDAC5 deletion mutant to associate with CAMTA2 in (F) is shown. The relative effectiveness of each protein to inhibit CAMTA2 activity is indicated in the right column.

(H) COS cells were transfected with expression plasmids encoding CAMTA2 (300 ng) or HDAC5 (100 ng) either separately or together and a PKD expression plasmid (600 ng). CAMTA2 (green) and HDAC5 (red) proteins were detected by immunostaining. HDAC5 S/A (panels k–n) contains serine to alanine mutations at positions 259 and 498 and is refractory to nuclear export by PKD.

(I) Enhanced cardiac hypertrophy of α-MHC-CAMTA2 transgenic (line 1)/HDAC5 mutant mice. Heart weight/body weight measurements (± SD) of mice of the indicated genotypes were determined at 4 weeks of age. HDAC5$^{-/-}$ mice show twice the amount of hypertrophy as wild-type transgenics.

and prevented hypertrophy in response to CAMTA2 (Figures 5B and 5C). Similar repression was observed with HDAC4 (data not shown).

In coimmunoprecipitation assays, CAMTA2 interacted avidly with HDAC5, and deletion mutants identified the ankyrin-repeat domain of CAMTA2 (Figures 5D and 5E)

and the N-terminal regulatory region of HDAC5 (residues 153–360) as the interacting domains (Figures 5F and 5G). Consistent with the possibility that HDAC5 represses CAMTA2 through a direct interaction, the CAMTA2 deletion mutant Δ639–1116 lacking the HDAC5 interaction domain displayed higher activity than the full-length CAMTA2 protein (Figure 3B). Deletion mutants of HDAC5 lacking the HDAC domain (mutants 1–664 and 1–360) but retaining the CAMTA2 binding domain also repressed the transcriptional activity of CAMTA2 (Figure 5G), in agreement with prior studies demonstrating that the HDAC domains of class II HDACs are not required for repression (Zhang et al., 2002).

Whereas CAMTA2 was distributed in the nucleus and cytoplasm (Figure 5Ha), when coexpressed with HDAC5, it became colocalized with HDAC5 in the nucleus (Figures 5He, 5Hg, and 5Hi). In the presence of activated PKD, HDAC5 translocates from the nucleus to the cytoplasm (Figures 5Hd, 5Hh, and 5Hj). Under these conditions, CAMTA2 remained nuclear (Figures 5Hf, 5Hh, and 5Hj). Moreover, the cytoplasmic pool of CAMTA2 appeared to enter the nucleus in the presence of PKD, even in the absence of HDAC5 (Figure 5Hb). CAMTA2 also colocalized in the nucleus with a mutant form of HDAC5 in which the signal-responsive serines in the N-terminal regulatory region were changed to alanines (HDAC5-S/A) (Figures 5Hk and 5Hl). In contrast to wild-type HDAC5, this mutant remains nuclear in the presence of activated PKD (Figures 5Hm and 5Hn). Thus, hypertrophic signaling leads to dissociation of CAMTA2 from HDAC5 as a consequence of HDAC5 phosphorylation. The retention of CAMTA2 in the nucleus, concomitant with the export of HDAC5 to the cytoplasm, provides a mechanism for signal-dependent activation of CAMTA2-responsive genes.

Antagonism between HDAC5 and CAMTA2 In Vivo

To test whether HDAC5 antagonized the growth-stimulatory influence of CAMTA2 on the heart in vivo, we interbred α-MHC-CAMTA2 transgenic mice (line 1) with mice harboring a loss-of-function mutation in *HDAC5*. Mice lacking HDAC5 do not display abnormalities in cardiac size or function at 1 month of age but are hypersensitive to stress signaling through the PKD pathway (Chang et al., 2004). As shown in Figure 5I, the cardiac growth response to CAMTA2 overexpression was dramatically enhanced in *HDAC5* null mice, providing genetic evidence for the opposing roles of CAMTA2 and HDAC5 in the control of cardiac growth in vivo.

CAMTA2 Knockout Mice Display Diminished Hypertrophy in Response to Multiple Stimuli

We generated a loss-of-function mutation in the mouse *CAMTA2* gene by homologous recombination to investigate the function of CAMTA2 in vivo. The targeting strategy resulted in the deletion of amino acids 5–554, encoded by exons 3–10 of the gene, and insertion of a lacZ reporter gene in-frame with amino acid 4 (Figures 6A and 6B). Mice homozygous for the *CAMTA2* null mutation were viable and fertile and did not display obvious cardiac defects. The absence of *CAMTA2* transcripts in mutant mice was confirmed by RT-PCR (Figure 6C). The lacZ gene inserted into the *CAMTA2* locus was expressed in cardiomyocytes, as detected by immunostaining (Figure 6D).

Although *CAMTA2* mutant mice show no overt cardiac phenotype, when these mice were subjected to a variety of hypertrophic stresses, their ability to mount a hypertrophic response was severely compromised (Figures 6E–6J). In response to thoracic aortic banding (TAB), which promotes hypertrophy by pressure overload, *CAMTA2* mutant mice showed only a 22% increase in cardiac mass compared to a 60% increase in wild-type littermates ($p < 0.006$) (Figures 6E and 6F). The induction of fetal cardiac genes, including *ANF*, was similarly diminished in *CAMTA2* mutant mice following TAB (Figure 6G). Cardiac hypertrophy was also suppressed in *CAMTA2* mutant mice following chronic infusion with angiotensin II (Figures 6H and 6I) and the adrenergic agonist isoproterenol (Figure 6J). In addition, angiotensin II infusion resulted in ventricular fibrosis, which was not observed in *CAMTA2* mutant mice (Figure 6I). We conclude that CAMTA2 is not only sufficient to induce cardiac hypertrophy but is necessary for a maximal hypertrophic response to diverse stimuli in vivo.

DISCUSSION

Diverse types of signals induce the heart to undergo hypertrophic growth, which is accompanied by transcriptional reprogramming of cardiac gene expression. Using the *ANF* promoter as a sensitive marker of hypertrophic signaling in a eukaryotic expression screen, we discovered CAMTA2 as a powerful activator of cardiac growth and gene expression and counterregulator of the growth-inhibitory activity of class II HDACs.

The CAMTA Family of Transcriptional Coactivators

CAMTA genes have been identified on the basis of nucleotide sequence homology in a wide range of eukaryotes including several plant species, nematodes, fruit flies, and mammals (Bouche et al., 2002), but their functions have not been examined in any organism. Through mutational analysis, we identified multiple evolutionarily conserved functional domains of CAMTA2 (Figure 7A). The CG-1 domain is required for association of CAMTA2 with Nkx2-5 and for transcriptional activation of Nkx2-5-dependent promoters, as well as for induction of cardiac growth. The TIG domain is essential for stimulation of the *ANF* promoter but is separable from the TAD, which may reflect a role in stabilizing the interaction with Nkx2-5. The ankyrin-repeat region of CAMTA2 associates with class II HDACs and negatively modulates the activity of the TAD. The structural determinants of this interaction may be similar to those that mediate association of class II HDACs with other ankyrin-repeat-containing transcriptional activators (McKinsey et al., 2006). The IQ motifs near the C terminus of CAMTA2 can be deleted without a loss in transcriptional

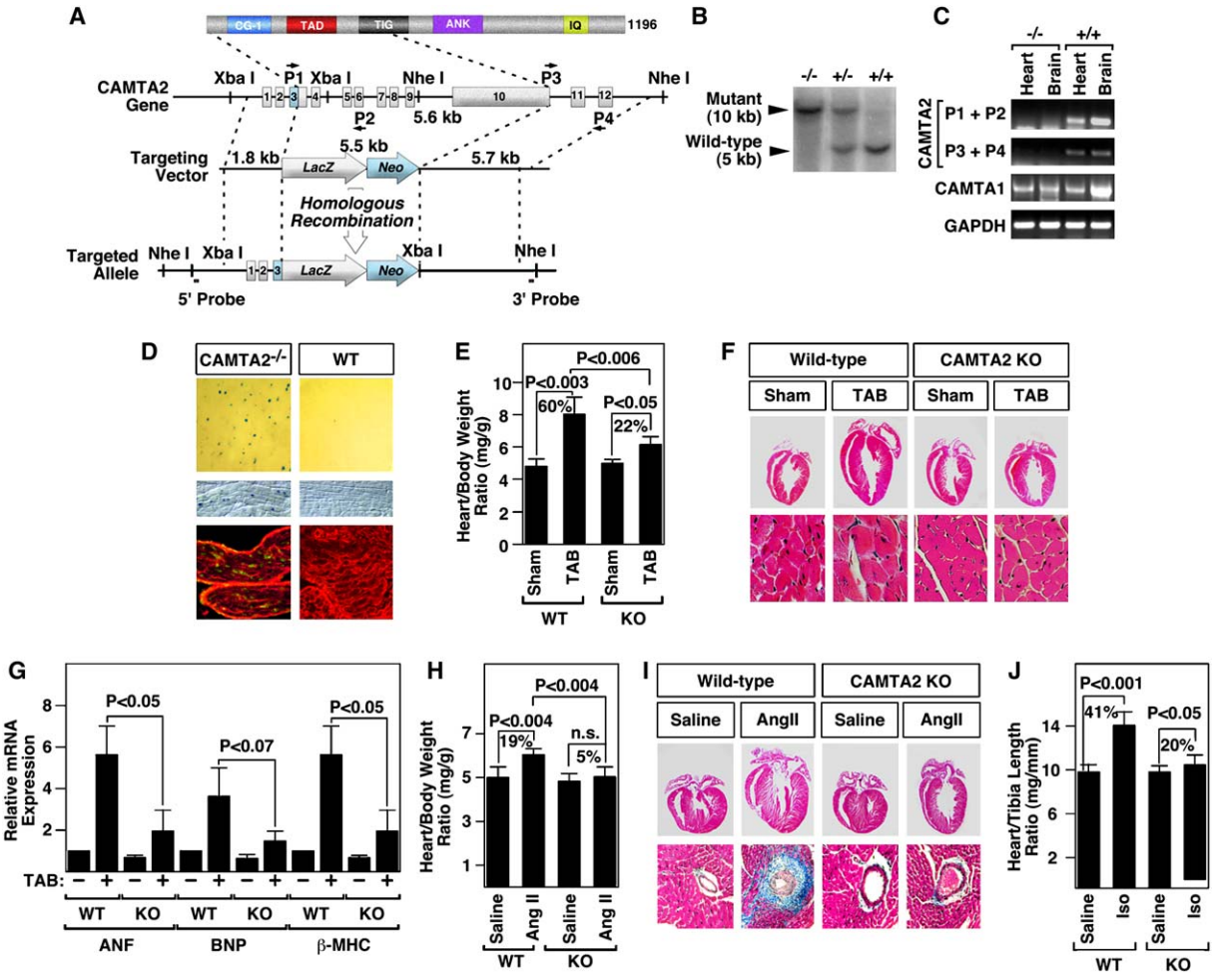

Figure 6. *CAMTA2* Knockout Mice Are Compromised in Their Ability to Mount a Hypertrophic Response

(A) The structure of the mouse *CAMTA2* gene is shown. The targeting strategy deleted exons 2–10, removing the CG-1, TAD, and part of the TIG domain. Positions of primers for PCR and probes for Southern blot are shown.

(B) Genomic DNA digested with Xba I from mice of the indicated genotypes was analyzed by Southern blot.

(C) RNA from heart and brain was analyzed by RT-PCR for the indicated transcripts. Primers for *CAMTA2* are shown in (A). Transcripts for GAPDH were detected as a control.

(D) Histological sections from the heart of a CAMTA2$^{-/-}$ mouse and wild-type littermate were stained for expression of lacZ (upper panels). Strong expression of lacZ was detected in cardiomyocyte nuclei. The middle panels show high-magnification views of cardiomyocytes photographed with DIC illumination with nuclear lacZ staining in the mutant. The lower panels were stained for lacZ (green) and α-actinin (red) by immunodetection. Rabbit anti-β-galactosidase (Ab cam) was used at a dilution of 1:3000.

(E) Wild-type and *CAMTA2* null mice were subjected to TAB or sham operation and heart weight/body weight ratios (± SD) were determined after 21 days.

(F) Histological sections of representative hearts from (E) are shown. Hypertrophy in response to TAB is inhibited in *CAMTA2* mutant mice.

(G) Transcripts for *ANF*, *BNP*, and β-*MHC* were detected by real-time PCR in hearts from wild-type (WT) and CAMTA2 null (KO) mice following TAB (+) or sham operation (−). Three animals in each group were tested. Values are expressed as the fold-increase in transcripts (± SD) compared to these markers in sham-operated wild-type mice.

(H) Wild-type and *CAMTA2* null mice were subjected to chronic infusion of saline or AngII and heart weight/body weight ratios (± SD) were determined after 14 days.

(I) Histological sections of representative hearts from (H) are shown. The lower panels are stained with Masson Trichrome to detect fibrosis.

(J) Wild-type and *CAMTA2* null mice were subjected to chronic infusion of saline or isoproterenol and heart weight/tibia length ratios (± SD) were determined after 7 days.

activity of CAMTA2. The IQ motifs in plant CAMTA proteins bind calmodulin (Bouche et al., 2002; Yang and Poovaiah, 2002). It will be interesting to further investigate the potential significance of this domain.

Regulation of Nkx2-5 Activity by CAMTA2

The following observations support the conclusion that CAMTA2 acts as a coactivator for Nkx2-5. (1) The Nkx2-5 binding site in the *ANF* promoter is required for maximal

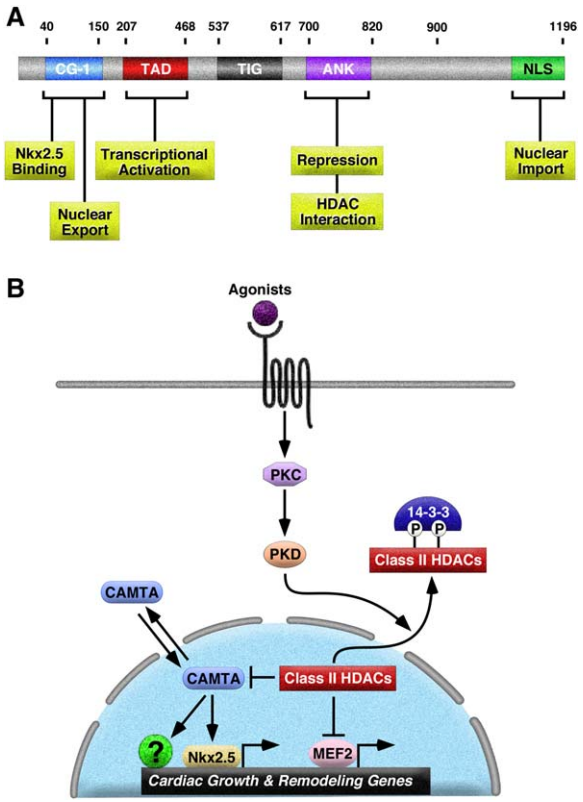

Figure 7. Domains of CAMTA2 and a Model of CAMTA2 Function in Hypertrophic Signaling

(A) The functional domains of CAMTA2 are shown.

(B) CAMTA2 cycles between the cytoplasm and the nucleus and stimulates the activity of Nkx2-5 and possibly other as yet unidentified transcription factors. CAMTA2 is repressed by association with class II HDACs. Activation of PKC/PKD signaling leads to phosphorylation of class II HDACs, which creates docking sites for 14-3-3 proteins and their nuclear export, releasing CAMTA2 from repression and promoting cardiac growth.

transcriptional activation by CAMTA2. (2) A single copy of the Nkx2-5 binding site is sufficient to confer CAMTA responsiveness to a basal promoter. (3) CAMTA2 synergizes with Nkx2-5 to activate NKE-dependent promoters. (4) Nkx2-5 interacts with CAMTA2. (5) CAMTA2 can be detected by chromatin immunoprecipitation on the Nkx2-5 binding region of the *ANF* promoter within native chromatin or on an exogenous plasmid template. (6) Mutations in CAMTA2 that disrupt interaction with Nkx2-5 abolish the ability of CAMTA2 to stimulate Nkx2-5 activity in vitro and ANF induction in vivo.

Overexpression of CAMTA2 appears to stimulate hypertrophy and proliferation of cardiomyocytes; whether these two responses are interrelated remains to be determined. Numerous lines of evidence have implicated Nkx2-5 in the control of cardiac growth, but the regulatory mechanisms through which growth signals might impinge on Nkx2-5 have not been defined. Nkx2-5 expression is upregulated during hypertrophy (Thompson et al., 1998; Saadane et al., 1999). Overexpression of Nkx2-5 results in cardiac hyperplasia in *Xenopus* and zebrafish embryos (Cleaver et al., 1996; Chen and Fishman, 1996) and hypertrophy and heart failure in transgenic mice (Kasahara et al., 2003). Conversely, expression of an Nkx2-5 dominant-negative mutant in *Xenopus* inhibits cardiac growth (Fu et al., 1998). Our results suggest that induction of cardiac hypertrophy by CAMTA2 is mediated, at least in part, by its association with Nkx2-5, although CAMTA2 may also have additional transcriptional targets. Nkx2-5 also associates with other transcription factors, including GATA4, Tbx5, and serum response factor, with consequent stimulation of Nkx2-5 activity (Chen and Schwartz, 1996; Durocher et al., 1996, 1997; Hiroi et al., 2001; Sepulveda et al., 1998; Small and Krieg, 2003).

Mutations in Nkx2-5 result in a spectrum of cardiac abnormalities in humans (Schott et al., 1998; Rosenthal and Harvey, 1999) and mice (Biben et al., 2000; Lyons et al., 1995; Tanaka et al., 1999), which have been attributed to dysregulation of cardiac growth and aberrant regulation of cell lineages contributing to the cardiac conduction system (Pashmforoush et al., 2004). CAMTA2 does not show appreciable expression in the heart until after birth, whereas CAMTA1 is strongly expressed in the embryonic heart (data not shown). Perhaps CAMTA1 modulates the developmental functions of Nkx2-5.

Signaling to CAMTA via Class II HDACs

PKC signaling is a powerful inducer of cardiac growth (Dorn and Force, 2005). We have shown that atypical PKCs activate PKD, which directly phosphorylates class II HDACs, resulting in their export from the nucleus to the cytoplasm and activation of fetal cardiac gene expression (Vega et al., 2004). Phosphorylation and nuclear export of class II HDACs are accompanied by the derepression of MEF2, a transcription factor implicated in fetal cardiac gene expression and myocardial growth (McKinsey et al., 2002). However, MEF2 does not regulate all of the genes that are induced during hypertrophy, suggesting the involvement of additional transcriptional regulators.

The results of this study identify CAMTA2 as an independent target of class II HDACs (Figure 7B). In the absence of hypertrophic signaling, the nuclear fraction of CAMTA2 can associate with HDAC5, resulting in repression of CAMTA2 transcriptional activity. Signaling by PKC and PKD can stimulate CAMTA activity by promoting the translocation of class II HDACs to the cytoplasm, relieving their repressive influence on CAMTA. Consistent with this model, a signal-resistant HDAC5 mutant lacking the phosphorylation sites required for nuclear export blocks CAMTA activity even in the face of PKC signaling (data not shown). Notably, HDAC5 associates with CAMTA2 and MEF2 through different regions of its N-terminal regulatory domain, potentially allowing it to independently repress both transcription factors in a signal-dependent manner.

Multiple G protein-coupled receptors drive cardiac growth by signaling through PKD to class II HDACs (Vega et al., 2004; Harrison et al., 2006). The finding that genetic

deletion of CAMTA2 desensitizes the heart to signaling by G protein-coupled receptor agonists, as well as pressure overload, whereas genetic deletion of HDAC5 sensitizes the heart to the growth-stimulatory activity of CAMTA2, suggests that CAMTA2 is a key downstream effector of hypertrophic signaling in vivo. The residual hypertrophic response of *CAMTA2* null mice is likely to reflect the involvement of parallel, partially redundant, signaling mechanisms possibly involving CAMTA1, as well as other effectors. It is noteworthy that the inability of *CAMTA2* null mice to mount a full hypertrophic response does not result in cardiac demise, indicating that hypertrophy is not a necessary response to cardiac stress.

Other Potential Functions of CAMTA Proteins

The association of transcription factors with coactivators (and corepressors) allows for signal-dependent regulation of gene expression and expands the regulatory potential of *cis*-acting DNA sequences as a consequence of combinatorial protein-protein interactions. While many, perhaps even most, cardiac transcription factors have been identified, the transcriptional coactivators that regulate cardiac growth or development are only beginning to be identified. CAMTA proteins join a growing list of transcriptional coactivators involved in the control of cardiac gene regulation during development and disease, including myocardin, EYA4 and TAZ (Wang et al., 2001; Schonberger et al., 2005; Murakami et al., 2005).

We speculate that CAMTA proteins may have transcriptional partners in addition to Nkx2-5 in cardiac myocytes, as well as other tissues (such as brain) where Nkx2-5 is not expressed. It is intriguing that CAMTA1 and 2 are expressed at the highest levels in heart and brain, which depend on calcium signaling for excitability and gene expression. Perhaps calcium-dependent HDAC signaling pathways modulate CAMTA activity in neurons. In the future, it will be interesting to identify other potential partners of CAMTA proteins, to determine the extent of redundancy and uniqueness of the functions of CAMTA1 and 2, and to investigate their potential involvement in human disease.

EXPERIMENTAL PROCEDURES

Expression cDNA Library and Expression Screen

The expression screen assay was performed using the *ANF638-luc* reporter (Sprenkle et al., 1995). cDNA expression libraries from human brain (Promega) and fetal heart (Invitrogen) were separated into pools containing ~50–100 clones each. Pooled plasmid DNA was purified using PerfectPrep Plasmid 96 Vac Direct Bind kit (Eppendorf). The initial CAMTA2 cDNA clone was isolated from the human brain library.

COS-1 cells plated in 24-well plates in DMEM with 10% FBS were transfected with 190 ng of pooled plasmid DNA from each expression library, 100 ng of *ANF638-luc* reporter (Sprenkle et al., 1995), and 10 ng of internal control *pCMV-LacZ* using FuGENE 6 (Roche). After 40 hr of transfection, the cells were harvested in 200 μl of passive lysis buffer (Promega), and cell lysates were used for luciferase and β-galactosidase assays. *E. coli* were transformed with positive plasmid pools and 12 colonies from each positive pool were picked and combined as a subpool. Plasmids were purified from 16 subpools and used to transfect COS-1 cells in the presence of *ANF638-luc* reporter and *pCMV-lacZ* as described above. Plasmid DNA from single colonies was prepared and sequenced.

Plasmids and Transfection Assays

Reporter plasmids containing regions of the rat *ANF* promoter were generated by PCR. Mutation of the NKE site in the rat *ANF* promoter was performed using the QuickChange kit (Stratagene). CAMTA1 and 2, FLAG- or c-*myc*-tagged cDNAs were ligated into pcDNA3.1 (Invitrogen). Transfections were performed using FuGENE 6 (Roche). Ten nanograms of a lacZ reporter controlled by the Rous sarcoma virus promoter and enhancer was included as an internal control in all transfection assays.

Isolation of Neonatal Rat Cardiomyocytes and Adenovirus Infection

Neonatal rat cardiac myocytes were isolated from 1- to 3-day-old Sprague-Dawley rats. Eighteen to thirty-six hours after plating, cardiomyocytes were infected with recombinant adenovirus for 2.5 hr and subsequently cultured in serum-free medium for 48 hr to examine cellular hypertrophy or for 120 hr to count myocytes.

Chromatin Immunoprecipitation Assay

COS-1 cells (~2×10^5) were transfected with an ANF reporter plasmid alone (200 ng) or with myc-Nkx2-5 (400 ng) and Flag-CAMTA2 (400 ng) expression plasmids. Twenty-four hours following transfection, chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate Biotech). Cardiomyocytes (~5×10^6) were infected with recombinant adenovirus expressing Flag-CAMTA2 at a MOI of 50 and the ChIP assay was performed.

Glutathione S-Transferase Pull-Down Assays

Mouse Nkx2-5 cDNA and a DNA fragment encoding the Nkx2-5 homeodomain were subcloned into the pGEX-KG vector (Amersham Biosciences). The plasmid containing the amino-terminal deletion of Nkx2-5 fused to GST and GST-Nkx2-5-ΔN were gifts from Dr. Issei Komuro (Hiroi et al., 2001). Pull-down assays were performed as previously described (Lu et al., 2000).

Detection of a Ternary Complex of CAMTA2, Nkx2-5, and NKE

The sequence of the biotinylated oligonucleotide corresponded to a high-affinity NKE site in the *ANF* promoter: 5′-TCACACCTT TGAAGTGGGGGCCTCTTGAGGCAAAT-3′. Ten microliters of in vitro [^{35}S]-labeled CAMTA2 protein, generated using the TNT Coupled Reticulocyte Lysate System (Promega), was incubated with 0.2 ug of GST or GST-Nkx2-5 fusion protein, 0.1 pM of biotinylated NKE, 10 μg of poly dI/dC (Sigma), and 10 μl of streptavidin beads (Amersham Biosciences) in 200 μl of DNA binding buffer (10 mM Hepes pH 7.6, 1 mM EDTA pH 8.0, 100 mM KCl, 1 mM DTT, 0.3 mg/ml BSA, and 5% glycerol) for 20 min at room temperature. The beads were washed three times using 500 μl of the above binding buffer, the bound proteins were resolved by SDS-PAGE, and [^{35}S]-CAMTA2 was detected by autoradiography.

RNA Analyses, Immunoprecipitation, and Western Blot Analysis

RNA analyses, immunoprecipitation, and Western blot analysis were performed as previously described (Lu et al., 2000). Total RNA was isolated from mouse hearts using TRIzol reagent (Invitrogen). RT-PCR was performed using 1 μg of RNA as a template with random hexamer primers to generate cDNA. Sequences of PCR primers are available upon request. Human or mouse multiple tissue Northern blots (Clontech) were hybridized with a ^{32}P-labeled probe containing CAMTA1 and CAMTA2 cDNA sequence.

Immunoprecipitations were performed by incubating 300 μl of lysate supernatant with 15 μl anti-FLAG-agarose beads (Sigma) at 4°C for 1.5 hr. The beads were washed three times with lysis buffer and boiled in SDS sample buffer. The immunoprecipitated proteins were resolved

by SDS-PAGE and analyzed by Western blot using rabbit anti-myc antibody (Santa Cruz) at a dilution of 1:1,000 and anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1:10000 with detection by Luminol Reagent (Santa Cruz).

Generation of Transgenic Mice

A cDNA encoding mouse FLAG-tagged CAMTA2 was cloned into an expression plasmid containing the α-*MHC* promoter and human GH (hGH) poly(A)$^+$ signal (Subramaniam et al., 1991), and transgenic mice were generated by standard techniques. Genotyping was performed by PCR using genomic DNA. Cardiac expression of CAMTA2 in transgenic mice was evaluated by real-time PCR or immunohistochemistry with anti-FLAG antibody (Sigma) to detect FLAG-tagged CAMTA2.

Generation of CAMTA2 Knockout Mice

The CAMTA2 targeting construct was built using the pN-Z-TK2 vector, which contains a nuclear LacZ (nLacZ) cassette and a neomycin-resistance gene (kindly provided by R. Palmiter). The 1.8 kb 5′ arm and 5.7 kb 3′ arm were amplified using PCR and confirmed by sequencing. The nLacZ and neomycin cassette were fused in-frame to exon 3 following the first 4 amino acids of CAMTA2, placing the LacZ reporter gene under the control of the endogenous *CAMTA2* promoter. The targeting construct was linearized and electroporated into 129 SvEv-derived ES cells. Using Southern blot analysis with 5′ and 3′ probes, two CAMTA2 targeted ES clones were identified and used for blastocyst injection. The resulting chimeric mice were bred to C57BL/6 to obtain germline transmission of the mutant allele.

Thoracic Aortic Banding, Infusion of Angiotensin II and Isoproterenol

Six-week-old mice underwent either a sham operation or were subjected to pressure overload induced by TAB as previously described (Hill et al., 2000). Cardiac hypertrophic agonists angiotensin II (3 mg/kg/d) (American Peptide) or saline were administered using miniosmotic pumps (model 2002, Alzet) subcutaneously implanted dorsally in 8-week-old male mice. Isoproterenol (Sigma) (8.7 mg/kg/d) or saline were administered to 16-week-old mice using osmotic minipumps (model 2001, Alzet). Mice were sacrificed 14 days after angiotensin II administration or 7 days following isoproterenol infusion. Cardiac hypertrophy was evaluated by measuring heart weight, body weight, and tibia length.

Histology and Immunohistochemistry

Histology and immunohistochemistry were performed by standard techniques. Immunostaining of frozen tissue sections for β-galactosidase expression was performed as described at the website of the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk). Cell size was measured using NIH Scion Image software.

Animal Care

All animal procedures were previously approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

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