CAMTA: A SIGNAL-RESPONSIVE TRANSCRIPTION FACTOR THAT PROMOTES CARDIAC GROWTH BY OPPOSING CLASS II HISTONE DEACETYLASES

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To

My Wife Yan, My Son Yuqian, and My Family

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CAMTA: A SIGNAL-RESPONSIVE TRANSCRIPTION FACTOR THAT PROMOTES CARDIAC GROWTH BY OPPOSING CLASS II HISTONE DEACETYLASES

by

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CAMTA: A SIGNAL-RESPONSIVE TRANSCRIPTION FACTOR THAT PROMOTES CARDIAC GROWTH BY OPPOSING CLASS II HISTONE DEACETYLASES

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Cardiac growth is finely regulated by transcriptional circuits. In an effort to discover new regulators of cardiac growth, I performed a eukaryotic expression screen for activators of the *atrial natriuretic factor* (*ANF*) gene, a cardiac-specific marker of hypertrophic signaling and embryonic development. I discovered that a family of transcription factors, called CAMTAs, regulate the *ANF* promoter. CAMTA proteins were first discovered in plants, however, little was known of the mechanism of their action and biological function and virtually nothing was known about mammalian CAMTA proteins, CAMTA1 and CAMTA2.

CAMTA1 and CAMTA2 are enriched in embryonic and adult hearts, skeletal muscle at the embryonic stage, and brain. To define the mechanism whereby CAMTA2 activates the *ANF* promoter, I used a series of promoter deletion mutants to map the cis-regulatory sequences that confer responsiveness to CAMTA2. I found that CAMTA activates the *ANF* gene, at least in part, by associating with Nkx2-5, a cardiac transcription factor. CAMTA

proteins also activate promoters of *myogenin* and *β myosin heavy chain* via direct DNA binding. Therefore, CAMTAs activate target genes through diverse mechanisms.

Over-expression of CAMTA2 in vitro and in vivo promotes cardiac growth. Based on the ability of CAMTA2 to induce hypertrophy, I tested whether signaling molecules implicated in cardiac hypertrophy might enhance the activity of CAMTA2. I discovered that 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 the CAMTA2 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 pro-hypertrophic actions of CAMTA. CAMTA proteins are also required for embryonic heart development, as demonstrated by heart defects in mice with low dosage of CAMTA1.

These findings reveal a transcriptional regulatory mechanism that modulates cardiac growth and gene expression by linking cardiac growth signals to the cardiac genome.

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LIST OF ABBREVIATIONS

ANF	atrial natriuretic factor
bHLH	basic helix-loop-helix
cDNA	complementary DNA
CAMTA	calmodulin binding transcription activator
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EnR	Engrailed suppressor domain
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione S-transferase
IP	immunoprecipitation
MADS box	MCM1, Agamous, Deficiens, and SRF box
MAP (kinase)	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2
NLS	nuclear localization signal
NES	nuclear export signal
PBS	phosphate-buffer saline
PCR	polymerase chain reaction
PMSF	Phenymethylsulfonyl fluoride

RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
TAD	transcription activation domain
TBP	TATA binding protein
TCF	ternary complex factor
Tg	transgenic
tk	thymidine kinase
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Chapter I

Introduction: Transcriptional Regulation of Cardiac Development and Disease

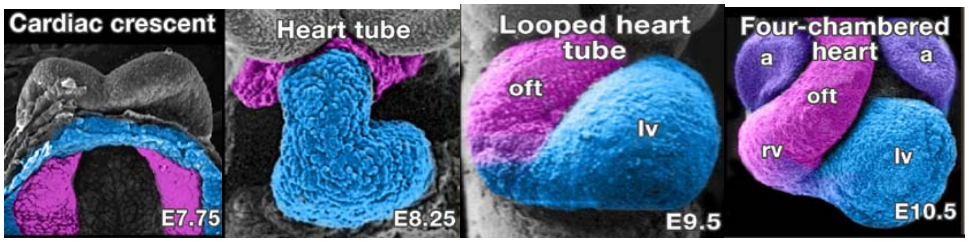
The heart is the first organ to form during the embryogenesis, and all events in the life of organism are dependent on the normal function of the heart. Congenital heart disease (CHD) is the most common human birth defect, occurring in about 1% of the world population (Hoffman and Kaplan, 2002). CHD is responsible for more deaths in the first year of a human life than any other birth defect. In the industrialized world, heart disease is the number one killer of adult men and women. Heart disease causing insufficient cardiac function affects an estimated five million Americans (Thom et al., 2006). Research in the last several decades has revealed that complex molecular signaling pathways and transcriptional and translational networks regulate heart formation and heart disease progression. Deciphering the mechanisms that control cardiomyocyte-fate specification, cardiomyocyte differentiation and cardiogenesis not only builds knowledge in the cardiac field, but also provides the possibility of developing new therapies for human disease.

Developmental Growth and Integration of the Embryonic Heart

Heart formation is initiated in vertebrate embryos from a population of cells in the region of the anterior lateral plate mesoderm, known as the cardiac crescent. The cardiac crescent appears at approximately embryonic (E) day 7.5 in the mouse embryo, corresponding roughly to about week two of human gestation (Olson, 2001). By E8.0, or 3 weeks in the human, cells in the cardiac crescent coalesce along the ventral midline to form

a primitive linear heart tube. The heart tube is composed of an interior layer of endocardial cells and an exterior layer of myocardial cells, separated by extracellular matrix for reciprocal signaling between the two layers. The linear heart tube undergoes rightward looping and chamber formation (Figure 1.1).

A



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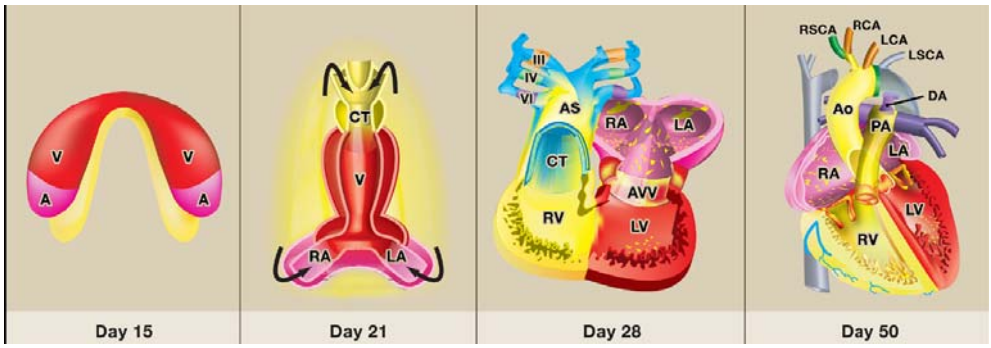

primary heart field secondary heart field

Figure 1.1. Mammalian heart development. (A) mouse embryonic heart development, and **(B)** a schematic of human heart development. Cardiac progenitor cells form two heart fields. The primary heart field cells form a crescent shape in the anterior plate of the embryo with the secondary heart field cells medial and anterior to the primary heart field. Cardiac progenitor cells in the primary heart field migrate, proliferate and differentiate into the atria and the left ventricle of the heart. However, cells in the secondary heart field contribute to the outflow tract, the right ventricle and the atria of the heart. The linear heart tube undergoes rightward looping to form the mature heart with four chambers: right and left atria, and ventricles. Swellings of the endocardium known as cardiac cushions, give rise to the valves. RV/rv, right ventricle; LV/lv, left ventricle; RA, right atrium; LA, left atrium. A/a, atrium; oft, outflow tract; CT, conotruncus; AS, aortic sac; AVV, atrioventricular valves; PA, pulmonary artery; Ao, aorta; DA, ductus arteriosus; LSCA, left subclavian artery; SACA; right subclavian artery; LCA, left carotid artery; RCA, right carotid artery. (Adapted from Garry and Olson, 2006; Srivastava, 2006)

Transcriptional Regulation of Cardiac Precursors

Heart development follows the establishment of cardiac-specific cell populations. Cardiac identity is regulated by signaling pathways and a set of transcription factors/activators (Figure 1.2). Signaling pathways have positive and negative effects on the establishment of cardiac cell fate (Olson and Schneider, 2003). Bone morphogenetic proteins (BMPs), a subfamily of the transforming growth factors (TGFs), promote cardiogenesis in vertebrate embryos through activation of Smad and the kinases TAK1 and P38. Expression of Oct-3/4, one of the earliest transcription factors expressed in the embryo, is activated by TGFβ signaling through Smad2/4. SiRNA-mediated inhibition of Oct-3/4 blunted TGFβ-induced upregulation of cardiac genes encoding Nkx2.5, and Mef2c. Similarly, injection of Oct-3/4 small interfering RNA (siRNA) in the inner cell mass of blastocysts impairs cardiogenesis in early embryos (Zeineddine et al., 2006). Therefore, Oct proteins play a pivotal role in cardiac commitment of embryonic stem (ES) cells (Figure 1.2). Wnt proteins signal through their seven-transmembrane frizzled receptors to promote cardiogenesis. Wnt signaling activates the kinases PKC and JNK or represses cardiogenesis through the activation of expression of target genes of T cell factor (TCF) proteins. However, the mechanism whereby these signaling molecules induce cardiac cell fate still remains unclear.

The Primary and Secondary Heart Field

Recent studies indicate that two distinct mesodermal heart fields, the primary heart field and the secondary heart field, are derived from a common progenitor and contribute to the formation of the heart in a temporally and spatially specific manner (Figure 1.1). The primary heart field known as the cardiac crescent is composed of the earliest population of cardiac progenitors. Cells from the primary heart field migrate medially and form the linear heart tube, which ultimately contributes to the left ventricle and atria (Buckingham et al., 2005). The secondary heart field is derived from the pharyngeal mesoderm located medial to the cardiac crescent. The secondary heart field has been shown to contribute to the outflow tract and right ventricle using lineage tracing experiments (Cai et al., 2003; Laugwitz et al., 2005). The primary and secondary heart fields can be distinguished by the differential expression of specific transcription factors and other molecules. The bHLH transcription factor HAND1 (heart and neural crest derivatives expressed transcript 1) and T-box transcription factor Tbx5 are markers of the primary heart field, whereas the LIM homeodomain transcription factor Isl1, forkhead box H1 Foxh1, and fibroblast growth factor 8/10 (Fgh8 and Fgf10) mark the secondary heart field. Some transcriptional regulators are expressed in both heart fields, for example, a homeodomain transcription factor Nkx2-5, bHLH transcription factor HAND2, T-box transcription factor Tbx1, Tbx20, and a MADS (MCM1, Agamous, Deficiens, Serum response factor box) transcription factor, MEF2C (Buckingham et al., 2005). Deciphering how these cardiac-restricted transcription factors/activators transmit signaling cues from adjacent endoderm

to cardiac progenitor cells to control their downstream target genes may provide possible therapies for congenital heart disease, myocardial infarction, and heart failure. For example, understanding the mechanism of commitment of cardiomyocytes may make it possible to generate functional cardiomyocytes from bone marrow stem cells of a patient with injured heart. Transplantation of these cardiomyocytes into the patient may restore cardiac function.

Labeling Cardiac Progenitor cells by Nkx2-5 and Isl1

The homeodomain transcription factor Nkx2-5 is one of the earliest markers of cell populations in the primary and secondary heart fields. Disruption of tinman, the Nkx2-5 ortholog in Drosophila leads to a complete loss of cardiac cells and visceral muscle cells (Bodmer, 1993), whereas, deletion of Nkx2-5 in mice results in abnormal heart looping, single and hypoplastic ventricle with embryonic lethality at E9.5 (Lyons et al., 1995; Tanaka et al., 1999). Moreover, overexpression of Nkx2-5 in *Xenopus* and Zebrafish embryos can expand the heart-formation area (Chen and Fishman 1996; Cleaver et al., 1996). In the fly, tinman determines cardiac cell fate, but it appears more complex in mammals. The relationship between Nkx2-5 and the specification of cardiac progenitor cells has been illuminated by recent studies. The majority of Nkx2-5 expressing cells isolated from developing embryos (E9.5) are able to differentiate into cardiomyocytes and cardiac conduction system cells (Wu et al., 2006).

Isl1 marks the secondary heart field, but its expression is decreased during cardiac differentiation. Interestingly, Isl1 marks niches of undifferentiated cardiac progenitor cells in the postnatal heart (Laugwitz et al., 2005). Hearts of mice lacking Isl1 completely lack the outflow tract, right ventricle, and part of atria (Cai et al., 2003).

Nkx2-5 may combine with other transcription factors, such as Isl1 to specify cardiac cell fate in mammals. This concept is supported by the recent observation that the transcriptional signature of $Isl1^{+}/Nkx2\text{-}5^{+}/Flk1^{+}$ (fetal liver kinase 1) defines embryonic stem cell-derived cardiovascular progenitors giving rise to cardiac muscle, smooth muscle and endothelial cells in vitro (Moretti et al., 2006).

Summary and Questions

Expression of a set of transcription factors is regulated by inductive signals from different germ layers (Figure 1.2). Although Nkx2-5, Isl1, and other transcription factors determine the fate of cardiac progenitors, unanswered questions remain. How is the expression of a set of transcription factors temporally and spatially regulated by inductive signals? It is reasonable to imagine that many more upstream transcriptional networks regulate Nkx2-5, Isl1 and other key factors. Disruption of genes encoding Nkx2-5, Tbx5, Isl1, MEF2C, HAND1 and HAND2 in mice does not prevent specification of cardiomyocytes. The lack of the phenotype of early cardiac cell fate in mutant mice lacking these factors is probably due to redundant mechanisms for regulation of the initial step of cardiac specification. Another reason is that transcription factors involved in

cardiac specification are still not identified. In addition, physical interactions among these transcriptional regulators may be a mechanism to regulate the specification and differentiation of cardiomyocytes. For instance, Nkx2-5 associates with Tbx5, members of the GATA family, and with serum response factor, a MADS box transcription factor, to activate cardiac genes (Hiroi et al., 2001; Chen and Schwartz, 1996; Bruneau et al., 2001; Sepulveda et al., 2002).

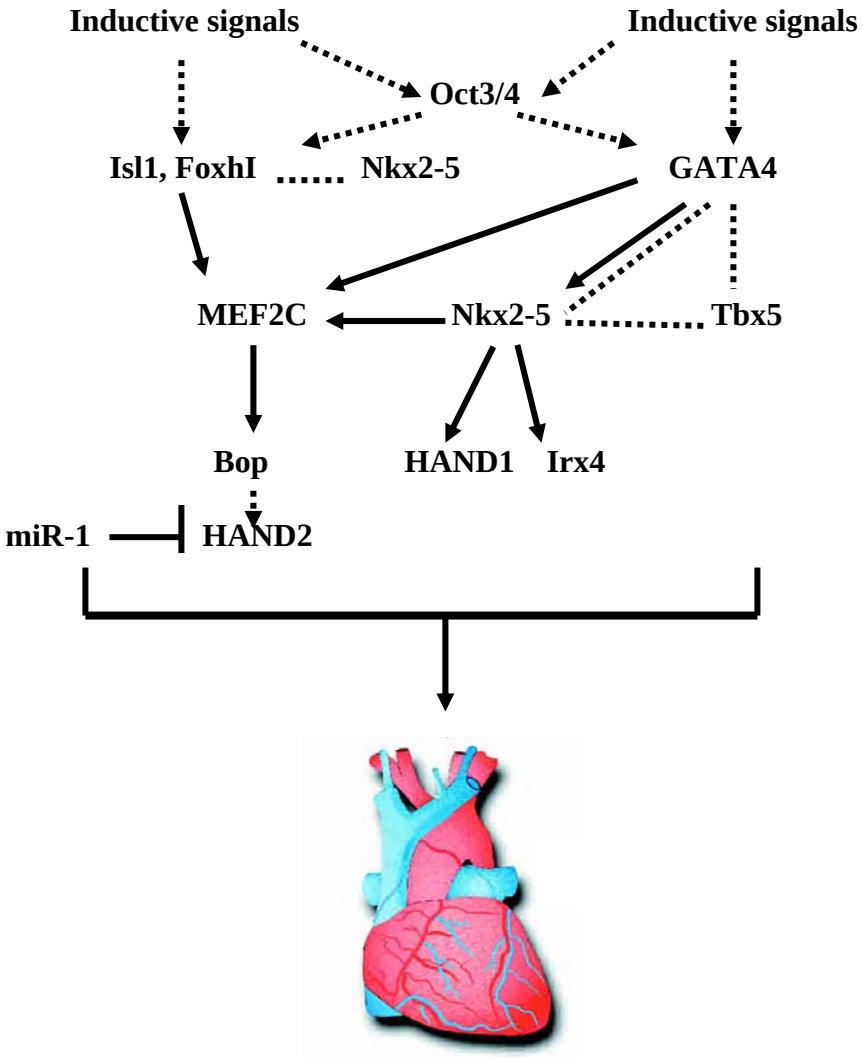

Figure 1.2. Transcriptional networks involved in cardiac specification, differentiation, and morphogenesis. Inductive signals activate a set of genes, encoding transcription factors in the primary and secondary heart field. Products of these genes program the core transcription factors: MEF2C-Nkx2-5-Tbx5-GATA4. These regulators activate their target genes encoding molecules involved in cardiac growth, patterning, and morphogenesis. Positive regulations are indicated by arrowheads, and negative influences by bar. Solid arrows indicate direct transcriptional connections; dotted arrows indicate connections not yet shown to be direct. Dashed lines indicate physical interaction between factors. (Adapted from Olson, 2006)

Transcriptional Regulation of Cardiomyocyte Differentiation

Embryonic cardiomyocytes undergo differentiation with assembly of contractile machinery, sarcomeres, when they are dividing. Differentiation of cardiac cells begins in the presomite stage prior to formation of the beating heart tube. Myocardial differentiation is initiated by a set of transcription factors/activators including Nkx2-5, members of MEF2 family, members of GATA family, HAND1, and HAND2, which contribute to the activation of many genes encoding components of the cardiac contractile machinery (Figure 1.2). Post-transcriptional control of cardiac structural genes has also been shown by microRNAs. For example, a cardiac specific microRNA, miR-208 was shown to regulate the expression of βMHC in response to cardiac stress (Van Rooij and Olson, unpublished data). In addition, miR-1 was shown to promote cardiac gene expression by targeting a transcriptional repressor, histone deacetylase 4 (HDAC4) (Zhao et al., 2005; Chen et al., 2006).

MEF2 proteins are important factors in regulating cardiomyocyte differentiation. MEF2 members control cardiomyocyte differentiation by switching on expression of cardiac structural genes. There are four members, MEF2A, MEF2B, MEF2C and MEF2D in mammals, but only one MEF2 in the fly. Cardiac cells do not differentiation in flies with a loss-of-function mutation of D-MEF2; however tinman, a homolog of mammalian Nkx2-5 is still expressed in the mutant heart (Lilly et al., 1995), which indicates that MEF2 is a key transcriptional regulator controlling differentiation of cardiomyoblasts into mature cardiomyocytes in the fly. Targeted deletion of MEF2C in mice causes embryonic lethality

with a cardiac looping defect. These embryos show severe ventricular hypoplasia with reduced expression of a set of cardiac specific genes such as cardiac α-actin, ANF and myosin light chain 1A (MLC1A) (Lin et al., 1997). Cardiomyocytes are still formed in these MEF2C mutant mice, even though some cardiac structural genes are not correctly expressed. Interestingly, MEF2B is up-regulated in these embryos. It's highly possible that members of the MEF2 family play overlapping functions in controlling cardiomyocyte differentiation during cardiac development.

To probe the function of MEF2 in myocardial differentiation, a dominant-negative MEF2 mutant (MEF2C/EnR) was driven by an Nkx2-5 enhancer and expressed in cardiac lineage cells (Karamboulas et al., 2006). P19 cells can be induced by DMSO into beating cardiomyocytes. Nkx2-5 is weakly expressed in P19 cells and is up-regulated once P19 cells are treated with DMSO. The early enhancement of cardiomyoblast markers, such as Nkx2-5, and MEF2C, is observed in P19 (Nkx2-5-MEF2C/EnR), a stable P19 cell line, treated with DMSO. However, the subsequent differentiation of cardiomyoblasts into cardiomyocytes is inhibited and the expression of cardiac structural proteins such as myosin heavy chain and cardiac α-actin was blocked (Karamboulas et al., 2006). Injection of Nkx 2-5-MEF2C/EnR into one-cell zygotes causes two types of phenotypes, total loss of heart formation and thin-walled myocardium with cardiac insufficiency (Karamboulas et al., 2006). The above observation indicates that MEF2 plays a critical role in cardiomyocyte differentiation. Total deletion all MEF2 family members in vivo may

answer the question if MEF2 is the exclusive transcription factor controlling cardiomyocyte differentiation.

The role of GATA proteins in cardiomyocyte differentiation is uncertain. Stable expression of GATA-4 antisense RNA blocks DMSO-induced differentiation of P19 cells into beating cardiomyocytes and expression cardiac contractile components cTnc, α-MHC, and β-MHC (Grepin et al., 1995). However, $GATA4^{-/-}$ ES cells are able to differentiate into contracting cardiomyocytes (Narita et al., 1997). GATA-6 is expressed in presumptive cardiac mesoderm before gastrulation. Xenopus and zebrafish embryos, injected with antisense morpholino oligonucleotides designed specifically to knock-down translation of GATA-6 protein, are severely compromised in heart development with greatly reduced expression levels of contractile protein genes (Peterkin et al., 2003). GATA transcription factor family members may play a redundant function in differentiation of cardiomyocytes in vivo.

Transcriptional Regulation of Cardiac Morphogenesis

Soon after their specification, cardiac progenitor cells merge along the ventral midline to form the linear heart tube, which undergoes rightward looping, growth and specification of chambers to generate the multi-chambered heart (Figure 1.1). Many cell types, cardiomyocytes, endothelial cells, epithelial cells, smooth muscle cells, are involved in this complex and integrated process. As the heart tube loops to the right, the anteroposterior alignment at the ventricular level gives rise to a right/left asymmetry, and

the anteroposterior alignment of atria and left ventricle is converted into dorsoventral asymmetry. The ventral surface of the heart tube rotates to be the outer curvature of the looped heart, and the dorsal surface forms the inner curvature. The myocardium at the ventral level of the tube has high proliferation activity (Thompson et al., 1990). Therefore, proliferation activity and/or cell size change leads to ballooning out of the chambers from the primary heart tube. The four chambered heart is formed following this event (Christoffels et al., 2000).

Two cardiac lineages are well defined using lineage tracing experiments (Buckingham et al., 2005). The first lineage within the primary heart field contributes to the left ventricle, while the second lineage, corresponding to cells within the secondary heart field contributes to outflow tract and right ventricular myocardium. Atrial myocardium is formed by contributions of both types of progenitor cells (Figure 1.1).

The program of genes during cardiac morphogenesis is regulated by several transcriptional cascades (Figure 1.2).

Regulation of Morphogenesis by Transcriptional Cascades in the Primary Heart Field

Nkx2-5 is a key transcription factor marking the primary and secondary heart fields. Mice lacking Nkx2-5 show a normal linear heart tube, but heart-looping in the mutant is perturbed. HAND1 is a target of Nkx2-5 in the primary heart field (Yamagishi et al., 2001) (Figure 1.2). The expression of HAND1 is restricted to the primary heart field and later to the atria and left ventricle of the heart tube. Analysis of chimeras of HAND1 mutant and

wild type cells indicates that cells lacking HAND1 fail to contribute to the left ventricle (Riley et al., 1998). Cardiac specific deletion of HAND1 results in hypoplasia in the left ventricle (McFadden et al., 2005). In HAND1 mutant mice, cells within the primary heart field likely fail to expand into the left ventricle. A significant elevation of cardiomyocyte differentiation was observed in cultured embryonic stem cell-derived cardiomyocytes lacking HAND1. However, upregulation of HAND1 in HAND1-expression cells results in an extension of the heart tube and extraneous looping due to elevated proliferation of cardioblasts (Risebro et al., 2006). It's likely that HAND1 controls the balance of proliferation and differentiation of cardiomycytes during morphogenesis of the developing heart. In addition to HAND1, Nkx2-5 also targets an Iroquois family of transcription factors Irx4 which is ventricle-restricted (Bao et al., 1999; Bruneau et al., 2000; Bruneau et al., 2001b).

Tbx5 is an interaction partner of Nkx2-5 in the primary heart field (Figure 1.2). Nkx2-5 and Tbx5 synergistically activate genes encoding ANF and connexin 40 (Bruneau et al., 2001a). Humans with Holt-Oram syndrome, caused by mutations in the TBX5 gene have an atrial septal defect, ventricular septal defect, electrical conduction defect, as well as limb abnormalities (Mori and Bruneau, 2004). Tbx5 mutant mice show severe defects in the atria-inflow region and left ventricle hypoplasia. However, the outflow tract and right ventricle, which develop from the secondary heart field, are able to grow (Bruneau et al., 2001a). The expression of Tbx5 is excluded from the right ventricle during cardiogenesis. Ubiquitous misexpression of Tbx5 in the ventricular myocardium results in

a single ventricle with repression of the right ventricle factor HAND2, whereas, the left ventricle markers HAND1 and ANF are induced in the entire ventricle. Misexpression of Tbx5 in the right ventricle leads to right ventricular expansion. Both HAND1 and ANF are induced in the right ventricle, but HAND2 is repressed (Takeuchi et al., 2003). These results indicate that Tbx5 plays a role in left-ventricle specification and negative regulation of right-ventricle formation during morphogenesis.

Regulation of Morphogenesis by Transcriptional Cascades in the Secondary Heart Field

Nkx2-5 activates MEF2C expression in the secondary heart field associated with the forkhead box H1 transcription factor Foxh1. Foxh1 mutant embryos have similar phenotypes to MEF2C mutant embryos: no right ventricle and a truncated outflow tract (Von Both et al., 2004). LacZ transgenes driven by a MEF2C regulatory region show that early expression of MEF2C is medial to the cardiac crescent (Dodou et al., 2004). Later, the expression of MEF2C is restricted to the outflow tract, and the right ventricle, which develop from cardiac progenitor cells in the secondary heart field (Dodou et al., 2004; Verzi et al., 2005). The expression of *lacZ* in the secondary heart field is dependent on GATA and Isl1 sites (Dodou et al., 2004). These observations indicate that the function of MEF2C in the secondary heart field is regulated by its upstream transcription factors including Isl1, Foxh1, Nkx2-5, and GATA (Figure 1.2). MEF2C mutant embryos die at E9.5 with a reduced outflow tract and no right ventricle. The linear heart tube of a MEF2C

mutant embryo is normally formed, but the tube does not undergo looping morphogenesis. The future right ventricle is not developed (Lin et al., 1997). Isl1 is an upstream transcription factor of MEF2C. Isl1 mutant embryos have only one atrium and ventricle compartment, and no outflow tract (Cai et al., 2003). Isl1 mutant mice have more severe second heart-field defects than MEF2C mutant embryos, which supports the notion that MEF2C is a downstream target of Isl1. The expression of Bop, a putative methyl transferase, is dependent on MEF2C (Phan et al., 2005). Bop is expressed in cardiac precursors in the secondary heart field and myocardium. Mutation of Bop causes interference with maturation of ventricular myocytes and the right ventricle which is similar to the phenotype of MEF2C mutant mice. Bop regulates expression of HAND2, a transcriptional regulator essential for development of the right ventricle (Gottlieb et al., 2002). Deletion of HAND2 in mice causes right-ventricular hypoplasia (Srivastava et al., 1995, 1997). Therefore, regulation of HAND2 expression by MEF2 may be through Bop (Figure 1.2).

Transcriptional Cascades in Morphogenesis Are Patterned within the Heart Tube

How are chamber specific genes activated during cardiac morphogenesis so as to be inactive in the primary myocardium? The above observations show that a linear heart tube can form in mice lacking certain transcription factors, but these mutant mice lose the ability to generate chamber myocardium. Ballooning out of the chamber myocardium is associated with chamber specific genes programmed by existing transcriptional cascades

during cardiac morphogenesis. For example, a set of genes such as ANF, HAND1, and MLC2v that are up-regulated in the ballooning chamber myocardium are down-regulated in Nkx2-5 knockout mice (Lyons et al., 1995; Tanaka et al., 1999). Therefore, transcriptional cascades regulating chamber specific genes are already patterned within the linear heart tube. One more example of patterning is based on a mouse model lacking Pitx2, a homeobox transcription factor, in the secondary heart field. Pitx2 is asymmetrically expressed along the left-right axis in the heart tube. Pitx2 activates gene expression at the final stage of a complex network regulating left-right asymmetry. Deletion of all Pitx2 isoforms in the secondary heart field results in defects of the outflow tract (Ai et al., 2006). This finding indicates that the secondary cardiac progenitor field is asymmetrically patterned. Further identification of novel transcription factors that are restricted to certain segments of the heart tube and chamber myocardium is needed to understand how chamber specific genes are activated during heart looping and chamber formation.

Cardiac Growth in the Adult Heart

Congenital heart disease is the most common form of human birth defects. When survivors of cardiac malformations go into their third and fourth decades of life, cardiac abnormalities become apparent, including abnormal electrophysiology in conduction system, and diminished cardiac contractile capacity. Even normal adult hearts are

susceptible to abnormalities in response to neurohormonal signals and various forms of hemodynamic stress, including hypertension, pressure overload, myocardial infarction, and ischemia. Cardiac hypertrophy is thought to be an adaptive mechanism to normalize ventricular wall stress for sustaining cardiac output. However, prolonged hypertrophy can result in diastolic and systolic heart failure and cardiac sudden death from cardiac arrhythmias. Apoptosis may be involved in this process. Under internal or external stress condition, the adult heart may undergo dilated cardiomyopathy (Figure 1.3).

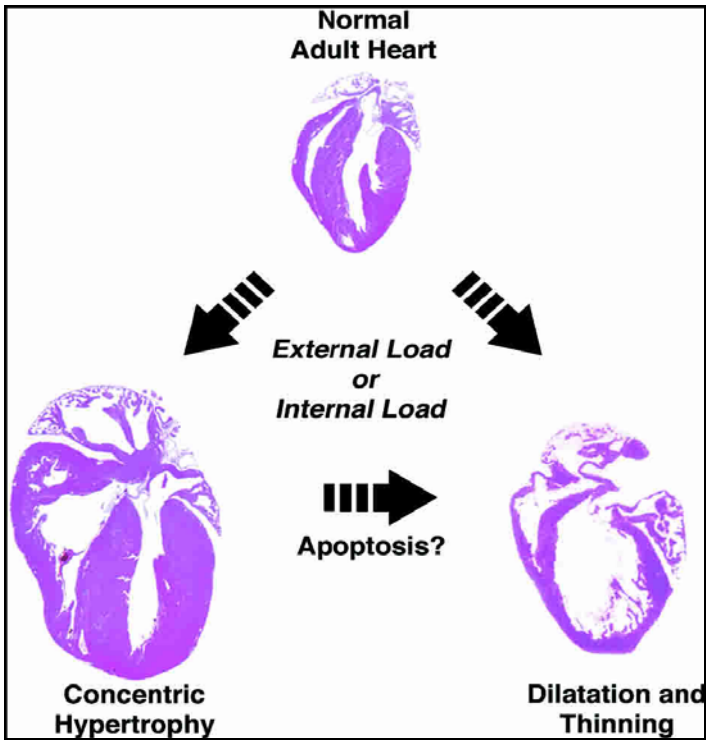

Figure 1.3. Abnormal growth of the adult heart. The adult heart undergoes growth through hypertrophy progressing to dilated myopathy or dilation in response to external and internal stresses. (Adapted from Olson and Schneider, 2003)

Transcriptional Regulation of Cardiac Hypertrophy

Cardiac growth in the developing heart is mainly through proliferation or hyperplasia of cardiomyocytes. However, cardiac growth in the adult heart occurs through hypertrophy, enlargement of cardiac muscle cells because most cardiomyocytes have loss the capacity to divide after birth. There are two types of cardiac hypertrophy during heart growth. One is named physiological hypertrophy, which is able to increase cardiac output to meet increased metabolic demands. Physiological hypertrophy is observed during normal growth of the postnatal heart or in athletes. The other type of cardiac hypertrophy is pathological hypertrophy, abnormal cardiac growth in response to injury and stress signals. Molecular pathways in physiological and pathological hypertrophy are distinct. Reprogramming of fetal genes such as atrial natriuretic factor (ANF), β-myosin heavy chain (MHC) and α-skeletal actin, and down-regulation of adult cardiac genes-α-MHC and α-cardiac actin, correlate with damage of the cardiac function in pathological hypertrophy (Molkentin et al., 1998; Lowes et al., 2002). Hypertrophic growth is also accompanied by an increase of protein synthesis, enhanced sarcomeric organization, and induction of immediate-early genes such as c-fos and c-myc (Sadoshima and Izumo, 1997).

Heart failure affects more than 4.5 million people in the United States, with approximately half a million new cases being diagnosed each year, with a mortality rate of nearly 50%. Deciphering the molecular mechanism of how stress signals are transmitted to

the cardiac genome to reprogram cardiac fetal genes should facilitate the discovery of new targets of drugs to cure cardiac hypertrophy and heart failure.

Calcineurin and NFAT Pathways

Many neurohormonal factors, including angiotensin II (AngII), phenylephrine (PE), and endothelin-1 (ET-1) are able to induce cardiac hypertrophy through activation of G-protein coupled receptors (GPCRs) (Arimoto et al., 2006). Agonists and other stress signals, such as overload and mechanical stress, induce cardiac hypertrophy and also share the ability to enhance intracellular Ca^{2+} concentration. Calcineurin is activated by Ca^{2+}. Calcineurin is a serine/threonine phosphatase existing as a heterodimer, composed of a calmodulin-binding catalytic A subunit and a Ca^{2+} -binding regulatory B subunit. Inhibition of calcineurin with cyclosporine A or FK-506, or by overexpression of the calcineurin inhibitory domains of Cain/Cabin-1 and myocyte-enriched calcineurin-interacting protein-1 (MCIP1) specifically in the heart attenuates cardiac hypertrophy (Rothermel et al., 2001; De Windt et al., 2001). Activated calcineurin dephosphates NFATs and precedes nuclear import of the dephosphorylated transcription factors (Rao et al., 1997; Graef et al., 2001). Nuclear NFATs and GATA transcription factors synergistically activate cardiac fetal genes by physical interaction with each other (Molkentin et al., 1998). Overexpression of active calcineurin and a nuclear form of NFAT3 in the heart induces cardiac hypertrophy and dilated cardiomyopathy, which provides a mouse model for heart failure (Molkentin et al., 1998) (Figure 1.4). Recently, a

positive feedback loop for the homeostasis of cellular Ca^{2+} was suggested when it was shown that nuclear NFATs enhance the expression of Transient receptor potential (TRP) proteins controlling Ca^{2+} entry into cells in response to a variety of signals (Kuwahara et al., 2006).

Class II HDACs and MEF2 Pathways

MEF2 exhibits basal activity in the adult heart, but hypertrophic signals stimulate the transcriptional activity of MEF2 (Passier et al., 2000). Association of MEF2 with class II histone deacetylases (HDACs) represses transcriptional activity of MEF2 (Figure 1.3) (Lu et al., 2000). Class II HDACs: HDAC4, HDAC5, HDAC7, and HDAC9, can be phosphorylated by protein kinase D (PKD), CaM kinase II and IV, which are activated by diverse stress signals (Vega et al., 2004; Backs et al., 2006; McKinsey et al., 2000). Identification of other HDAC kinases may further elucidate this critical signaling pathway in response to stress. Phosphorylation of class II HDACs provides docking sites for 14-3-3 chaperone proteins. Binding of class II HDACs to 14-3-3 proteins results in dissociation of HDACs from MEF2 or other transcription factors and nuclear export of the repressors (Figure 1.4). Nuclear export of HDAC5 is mediated by the exportin pathway in response to pathological hypertrophy signals (Harrison et al., 2004). Consistent with the notion of class II HDACs as negative regulators of pathological cardiac growth and remodeling, mice lacking HDAC5 or HDAC9 develop massively enlarged heart in response to pathological stress (Zhang et al., 2002; Chang et al., 2004). However, whether MEF2 is the

only target of class II HDACs remains unclear. Finding other transcription factors involved in cardiac hypertrophy will be helpful to further understand the relevant signaling pathways and to reveal novel mechanisms involved in pathological cardiac growth and remodeling.

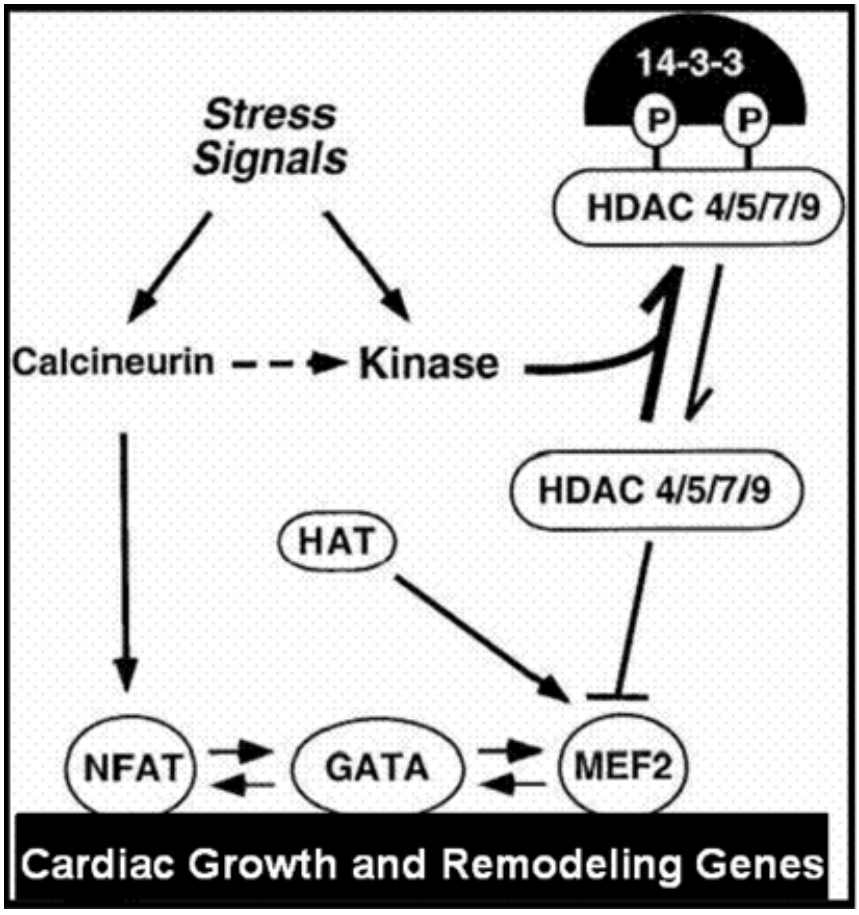

Figure 1.4. Signaling pathways and transcriptional regulation involved in cardiac hypertrophy. MEF2 and other potential transcription factors are repressed by association with class II HDACs. Activation of PKD, CaMK and other HDAC kinases by neurohormonal signals or overload leads to phosphorylation of class II HDACs. Phosphorylated HDACs are able to bind to 14-3-3 proteins. The complex of HDAC and 14-3-3 is exported to cytoplasm. The nuclear export releases MEF2 and other transcription factors from repression and promotes expression of cardiac growth and remodeling genes. Activation of calcineurin, a Ca^{2+}, and calmodulin-dependent phosphatase, by stress signals leads to dephosphorylation of nuclear factors of activated T cells (NFATs). The dephosphorylated NFAT transcription factors translocate to the nucleus and activate hypertrophic genes with other transcription factors, such as GATA. (Adapted from Zhang et al., 2002)

Nkx2-5 and GATA in Cardiac Hypertrophy

Some transcription factors involved in building the embryonic heart also serve as targets of signaling pathways during cardiac hypertrophy. GATA4 is critical to regulate embryonic heart development. Mice lacking GATA4 in the adult heart develop rapid decompensation and heart failure in response to pressure overload (Oka et al., 2006).

Numerous lines of evidence have implicated Nkx2-5 in the control of cardiac growth. Nkx2-5 expression is up-regulated during hypertrophy (Thompson et al., 1998; Saadane et al., 1999). Over-expression 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). A potential regulatory mechanism through which growth signals might impinge on Nkx2-5 is proposed in my dissertation.

Other Pathways in Cardiac Growth and Remodeling

An additional mechanism to control pathological growth in the adult heart at the transcriptional level is through hyperphosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Sano et al., 2002). Phosphorylation of the CTD by cyclin-dependent kinases-9 (Cdk9) is required for transcription elongation initiated by RNA polymerase II.

Cdk9 is activated by several hypertrophic stress signals such as, calcineurin, endothelin-1, signaling by G-protein Gq, and mechanical load. Activation of Cdk9 by hypertrophic signals causes cardiomyocyte enlargement, and defective mitochondrial function resulting in susceptibility to apoptotic cardiomyopathy (Sano et al., 2004).

In addition to the key roles of transcriptional pathways, some micro RNAs (miRNA) may play potential role in cardiac hypertrophy and remodeling via controlling the step of mRNA translation. More than 12 microRNAs are up- or down-regulated in hypertrophic and remodeling hearts in mice. Similar expression patterns are observed in failing human hearts (Van Rooij et al., 2006). These findings reveal a novel regulatory mechanism in mammalian hearts in response to pathological stress.

Transcriptional Regulation of Arrhythmias

The electrical impulse is generated in the sinoatrial node (SAN). The generated impulse is propagated through the rest of the cardiac conduction system composed of the atrioventricular node (AVN), His-bundle branches and Purkinje fibers. Abnormally conducted signals cause alterations of the normal heart's beat referred to arrhythmia. Cardiac arrhythmia accounts for mortality of about 500,000 and medical cost of more than $2 billion in 2001 in the United States (Thom et al., 2006). Many arrhythmias are believed to be congenital such as long-QT syndrome caused by mutations in the sodium-channel gene SCN5A and Wolff-Parkinson-White syndrome which can occur in people with mutations in the gene PRKAG2 encoding an AMP-activated protein kinase (Sidhu and

Roberts, 2003). However many more arrhythmias are acquired and associated with cardiac hypertrophy, heart failure, cardiac ischemia, and myocardial infarction. This part focuses on congenital arrhythmia regulated by transcriptional pathways. Recent studies show that transcriptional pathways play roles in cardiac arrhythmia through controlling the development of the conduction system or gradient expression of ion channel genes.

Controlling the Development of the Conduction System by Transcription Factors

Mutations in transcription factors Nkx2-5, and HF-1b are attributed to cardiac arrhythmia due to the loss of the conduction system lineage cells. Human mutations in Nkx2-5 lead to congenital heart disease and conduction defects (Benson et al., 1999). Ventricular knockout of Nkx2-5 in mice leads to a hypoplastic atrioventricular (AV) node around birth and then selective dropout of these conduction cells. Mutant mice display secondary and third AV block at a young age. At older stages, the AV block progresses to a complete heart block (Pashmforoush et al., 2004). HF-1b is a cardiac zinc-finger transcription factor belonging to the Sp-1 family. Mice lacking HF-1b display sudden cardiac death and ventricular tachycardia and a high incidence of AV block. Continuous electrocardiographic recordings clearly documented cardiac arrhythmogenesis as the cause of death. HF-1b knockout mice show defects in the formation of ventricular Purkinje fibers (Nguyen-Tran et al., 2000). These mouse models indicate that development of components of the cardiac conduction system is finely regulated by distinct transcriptional pathways. Mis-regulation of the transcriptional pathways leads to developmental defects of

conduction-system components which contribute to cardiac arrhythmia and sudden death at old age.

Regulation of the Ion Channel Expression by Transcription Factors

Repolarization initiates cardiac relaxation after depolarization and contraction. In all mammals, ventricular repolarization proceeds in a synchronized wave advancing from the base of the heart to its apex and from epicardial to endocardial myocardium, which is believed to ensure efficient pump function and maintains an arrhythmia-free heart. Regional differences in action potential waveforms and frequency dependences reflect the differential expression of voltage-gated K^+ channels. This heterogeneity impacts the normal dispersion of ventricular repolarization. For example, the outward current, $I_{to,f}$ is seen in the highest density in epicardial myocytes, whereas its expression is much lower in endocardial myocytes (Nerbonne and Guo, 2002). $I_{to,f}$ is assembled as a heterotetramer of the pore-forming α subunits, $K_V4.2$ and $K_V4.3$, in association with accessory (β) subunits such as KChIP2 or frequenin/NCS-1. Altered heterogeneity of $I_{to,f}$ is linked to a diseased myocardium in humans (Antzelevitch, 2004). Mice lacking KChIP2 are susceptible to induction of arrhythmia due to a complete loss of heterogeneity of repolarization (Kuo et al., 2001). Repolarization patterning is accomplished largely via transcription factors expressed in specific compartments of the developing heart. The Iroquois homeobox (*Irx*) genes encode a conserved family of transcription factors. Irx5 largely distributes through the interventricular septum and endomyocardium (Costantini et al., 2005). The myocardial

K_v4.2 potassium-channel is expressed in an epicardial-to-endocardial gradient, whereas Irx5 is in a gradient from endocardium to epicardium. Disruption of Irx5 by gene targeting disturbs the gradient of K_v4.2. Irx5 mutant mice are susceptible to induced cardiac arrhythmia (Costantini et al., 2005). In this case, the expression of the gene encoding K_v4.2 is repressed by the transcription factor Irx5 via recruitment of the cardiac repressor mBop (Costantini et al., 2005). Correct maintenance of the repolarization gradient by transcriptional pathways is required to reduce the risk of cardiac arrhythmia.

Summary

The heart is the earliest organ to function during development of mammals. Transcriptional circuits are involved in the early development and maintenance of normal cardiac function to meet metabolic needs. Transcriptional pathways play critical roles in controlling specification and differentiation of cardiomyocytes, cardiac morphogenesis and heart disease. In spite of dramatic progress on the complex transcriptional circuits, many gaps in the networks still remain to be filled. In skeletal muscle, the transcription factor MyoD is sufficient to initiate differentiation of myocytes, however, no known transcription factor is able to do the same thing in cardiac muscle. Several key cardiac transcription factors are well identified including Nkx2-5, MEF2 family, GATA family, and Tbx5, but our knowledge of the transcription circuits remains limited. How cardiac progenitor cells are specified and how cardiomyocytes and other type of cells are integrated into chambered hearts represent major fronts. Transcriptional pathways in embryonic heart are redeployed in cardiac hypertrophy and heart failure. Pathways transmitting signals to transcription factors NFATs and MEF2 are discovered, but many genes that are up-regulated during the progression of cardiac hypertrophy and failure are not targets of these transcription factors, which indicates that unknown factors remain to be identified. Transcriptional pathways regulating the development of the conduction system and the expression patterns of ion channel genes may be potential targets of drugs to cure cardiac arrhythmia. This dissertation is mainly focused on discovery of novel components of

transcriptional networks controlling cardiac development, growth and remodeling. My specific aims are as follows:

1) **To discover new components in transcriptional networks regulating cardiac growth and remodeling.**
2) **To characterize the molecular mechanism of activating gene expression by a family of Calmodulin Binding Transcription Activators (CAMTAs).**
3) **To dissect the biological function of CAMTAs during cardiac growth and remodeling *in vitro* and *in vivo*.**

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Chapter II

The Calmodulin Binding Transcription Activator Stimulates Cardiac Growth by Opposing Class II Histone Deacetylases

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 the 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, it's shown that class II histone deacetylases (HDACs) act as signal-responsive co-repressors 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., 2002). Activation of 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, I 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). I showed that CAMTAs were 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. I showed that CAMTA proteins activated muscle specific genes encoding myogenin and β-Myosin heavy chain by direct DNA binding, and endogenous CAMTA proteins associated with these promoters in native chromatin. Through gain- and loss-of-function approaches in vivo and in vitro, I showed 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. My findings suggest that CAMTA proteins are not only required for maximum hypertrophic growth in the adult heart in response to stress, but also for cardiac development during embryogenesis. These findings have uncovered a role for mammalian CAMTA proteins as signal-responsive transcription factor of cardiac growth and targets for the anti-hypertrophic actions of class II HDACs.

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 h 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 transcrips 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 h and subsequently cultured in serum-free medium for 48 h to examine cellular hypertrophy or for 120 h to count myocytes.

Chromatin Immunoprecipitation (ChIP) 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 ChIP assays were performed. Cardiomyocytes (~5×10^6) were infected with recombinant adenovirus expressing Flag-CAMTA2 at a MOI of 50 and the ChIP assay was performed. C2C12 mouse myoblasts (American Type Culture Collection, Manssasw, VA) were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM Lglutamine, and penicillin-streptomycin. Cells were grown to 70% confluence and induced to differentiate by replacing the growth medium with the differentiation medium (DMEM with 2% horse serum). Endogenous

CAMTA1-chromatin complex was pulled down using a CAMTA1 specific antibody, then ChIP assay was performed using the ChIP assay kit (Upstate Biotech). Briefly, cells were crosslinked with 1% formaldehyde at 37^0C for 10 minutes. After washing with cold PBS twice, cells were scraped in 500 ul of PBS and centrifuged at 5000 g for 5 minutes at 4^0C. The pellets were then resuspended in 300 ul of SDS Lysis Buffer (1% SDS, 10 mM EDTA, 5 0 mM Tris-HCl, pH 8.1, and protease inhibitors). The lysate was sonicated six times for 10 seconds each, to shear the DNA to between 200 bp to 1000 bp in length. After centrifugation at 14,000 rpm for 15 minutes, the supernatant was diluted 10 times with ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL, pH 8.1, 167 mM NaCl, and protease inhibitors). 1 ml of the diluted lysate was then cleared with 10 ug salmon sperm DNA and 20 ul Protein A-agrose beads (Santa Cruz) at 4^0C for 2 hrs. After a brief centrifugation to pellet the beads, the supernatant was incubated with 2 ug of specific antibodies over night at 4^0C. The next day, 10 ug salmon sperm DNA and 20 ul Protein Aagarose beads were added to the supernatant and incubated for 2 hrs. The beads were then washed sequentially with Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8., 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), and LiCl Wash Buffer (0.25 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCL, pH 8.1) for 5 minutes each. The beads were then washed with TE twice, and the complex of protein-DNA was eluted twice with 250 ul of fresh Elution Buffer (1% SDS, 0.1 M NaHCO3). 20 ul of 5M NaCl was added to each elute and incubated at 65^0C

for 4 hrs to reverse crosslink. DNA fragments were extracted with 500 ul of phenol/chloroform, and then precipitated in 700 ul of isopropanol including 1 ug glycogen. Sequences of PCR primers used for ANF promoter are:

CAAGATAACCAAGGACTCTTTTCTGC, and CCGTCACTGTCTCCCAGCTGC.

Sequences of primers for amplifying *myogenin* promoter and coding region are:

P1: GGTAGGAGTAATTGAAAGGAGCAGATGAGAC

P2: CCAACTGCTGGGTGCCATTTAAACC

P3: CTTCCCAGACGAAACCATGCCCAAC

P4: GAGGGACATTAACAAGGGGGCTCTCTG

Sequences of primers for amplifying the *β-MHC* promoter are:

CAATGACGCCCACTTCCAATTGTTTCCTAGC, and

CCTGAGGGCAGCAAGTAAAGAAACAGC.

Electromobility Shift Assay

GST and MBP fusion proteins were expressed in bacterial cells. Probes were labeled by [αP^{32}]-dCTP using Klenow enzyme (New England Biolabs). Purified proteins and 100,000 cpm of hot probe were incubated in 20 ul of binding buffer (10 mM Hepes pH7.9, 50 mM KCl, 25 mM NaCl, 1mM EDTA pH8.0, 10% glycerol, 1 ug Poly dI/C) at room temperature for 20 minutes. The protein-DNA complex was supershifted by incubation with 1 ug anti-Flag antibody. The complex of DNA-protein was resolved using 4% native PAGE gel at 4^0C.

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). Briefly, 1 ug of GST alone or GST fused proteins were incubated with 25 ul of in vitro translated CAMTA proteins in binding buffer including 20 mM Tris-Cl (pH 7.4), 200 mM NaCl, 0.5% NP-40 at 4^0C for 1 hr. Beads-proteins complex was washed three times with GST binding buffer at 4^0C.

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'-TCACACCTTTGAAGTGGGGGCCTCTTGAGGCAAAT-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 ug of poly dI/dC (Sigma), and 10 ul of streptavidin beads (Amersham Biosciences) in 200 ul of DNA binding buffer (10 mM Hepes pH7.6, 1 mM EDTA pH8.0, 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 ul 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 ug of RNA as a template with random hexamer primers to generate cDNA. Sequences of RT-PCR and real time PCR primers were listed in Table 2.1. 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 FLAG-agarose beads (Sigma) at 4°C for 1.5 h. 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 germ-line transmission of the mutant allele.

Generation of gene trap CAMTA1$^{GT/GT}$ mice

A gene trap ES cell line that interrupts the CAMTA1 gene was obtained from Baygenomics. This ES cell line was used for blastocyst injection. The resulting chimeric mice were bred to C57BL/6 to obtain germ-line transmission of the mutant allele.

Thoracic Aorta 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 8-16 weeks 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 was performed by standard techniques. Tissues were fixed in 10% phosphate-buffered formalin at 4°C. Samples were then embedded in paraffin, sectioned at 5 um, and stained with hematoxylin and eosin or Masson Trichrome. These protocols were executed by members of the laboratory of Dr. James Richardson.

Immunohistochemistry was performed by standard techniques. Briefly, cells were fixed with cold methanol at -20^{0}C for 10 minutes. Samples were dried in air for 5 minutes. After 3 washes with PBS, 5 minutes each, cells were then permeated with blocking buffer (0.1% Triton X-100 and 10% horse serum in PBS) for 30 minutes and incubated with primary antibody, which is diluted at 1:200 with the blocking buffer, for 60 minutes at room temperature. After 3 washes with PBS, 5 minutes each, cells were incubated with secondary antibody diluted at 1:400 with blocking buffer for 30 minutes. After 3 washes again with PBS, the coverslips were mounted with mounting medium containing DAPI from Vectashield.

Immunostaining of frozen tissue sections for β-galactosidase expression was performed as described at the website of the Wellcome Trust Sanger Institute (www.sanger.ac.uk)**.** Briefly, frozen 5-μm tissue sections on pap-penned slides were completely dry for 10 minutes. Sections were fixed in PBS with 2% formaldehyde and 0.2% glutaraldehyde for 5 minutes. After wash twice, 5 minutes each, Sections were incubated in X-gal solution (5 mM potassium ferricyanide, crystalline; 5 mM potassium ferrocyanide, trihydrate; 2 mM magnesium choloride, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside) in humidity chamber at 37°C overnight. After wash 3 times, 5 minutes each, sections were mounted with mounting medium containing DAPI.

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.

Table 2.1. Sequences of RT-PCR and real time PCR primers

CAMTA1:

CCAGACATGGTAGACAGCCCGGTTGTC;

CATCTGCTCGAGGGTGGTGCTGGGACTC

CAMTA1 P1: CCCAAAGTGTATTCTGCGGAACTAGC

CAMTA1 P2: GGTCTGTACGTAGACAGCCCGGTTGTC

CAMTA1 P3: CAGGCCTCCCGGATCCACTGTGC

CAMTA1 P4 (located in pGT0Lxf**):** AGTATCGGCCTCAGGAAGATCG

CAMTA1 P5: ATTGCTGCAGGTCCATTTGATACCATGG

CAMTA1 P6: CATCTGCTCGAGGGTGGTGCTGGGACTC

CAMTA1 P7: CCTTGGCCTTTTTCAATTCTTTCACTCC

CAMTA2 (CAMTA2 P1 and P2 were used to detect CAMTA2 transcripts in hearts)**:**

CAMTA2 P1: CCCCCTGGAACCTTCACCATGAATAC;

CAMTA2 P2: CTTACGATTGTAGAGGATAATGGAGC

CAMTA2 P3:

CAGTCTTTCGTCATTCCCAGACCTCATGG

CAMTA2 P4:

GTGACAACCAGTCGAGCTGTGTACTAGG

ANF:

CTGAGAGACGGCAGTGCTTCTAGG; TGCAGAGTGGGAGAGGCAAGAC

BNP:

AGGTGCTGTCCCAGATGATTCTGT; CTTGTGCCCAAAGCAGCTTGAGAT

β-MHC:

TGCAAAGGCTCCAGGTCTGAGGGC; GCCAACACCAACCTGTCCAAGTTC

GAPDH:

GCAGTGGCAAAGTGGAGATTG; TTTGGCTCCACCCTTCAAGTG

Results

Discovery of CAMTA in an expression screen for regulators of the *ANF* promoter

I 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 (Figure 2.1), which is cardiac-specific and responsive to a variety of signaling pathways involved in cardiac growth and remodeling (Sprenkle et al., 1995; Tamsah 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, 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 (Figure 2.2A), but their functions have not been investigated.

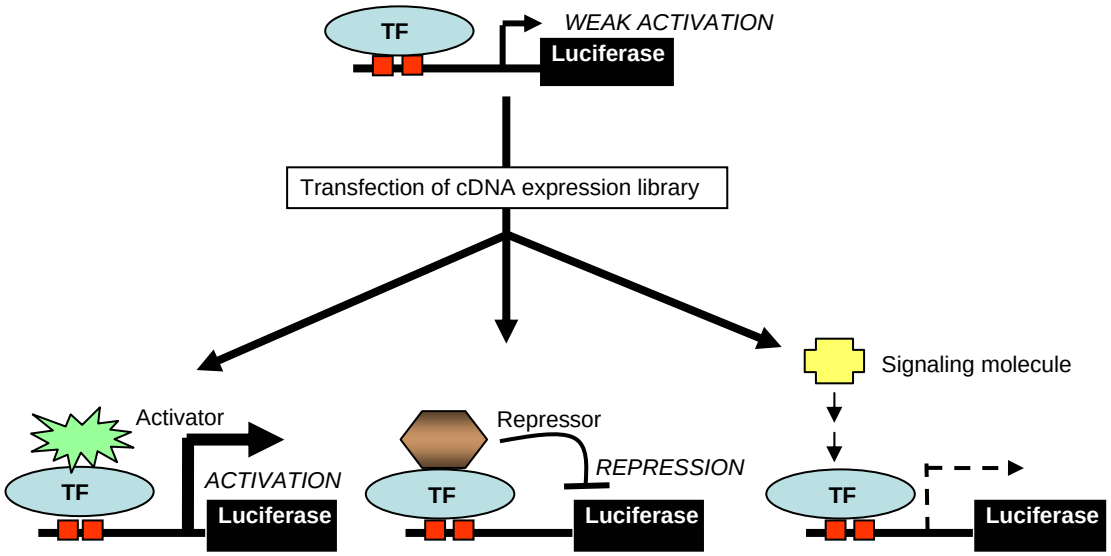

Figure 2.1. A eukaryotic expression screening strategy for discovering regulators of an interesting promoter. Luciferase cDNA is fused to the interesting promoter. A cDNA library and the reporter plasmid are cotransfected into cultured eukaryotic cell lines. Transcription factors or activators, repressors and signaling molecules are identified based on the expression level of the luciferase reporter. TF, transcription factor.

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 2.2A). 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 on other tissues (Figure 2.2B). 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 2.2B). During embryogenesis, CAMTA2 transcripts were detected only at a background level in the heart followed by pronounced up-regulation after birth (Figure 2.2C). Isolated cardiomyocytes showed an enrichment of CAMTA2 expression compared to the whole heart. However, CAMTA1 is highly expressed in embryonic heart of mice.

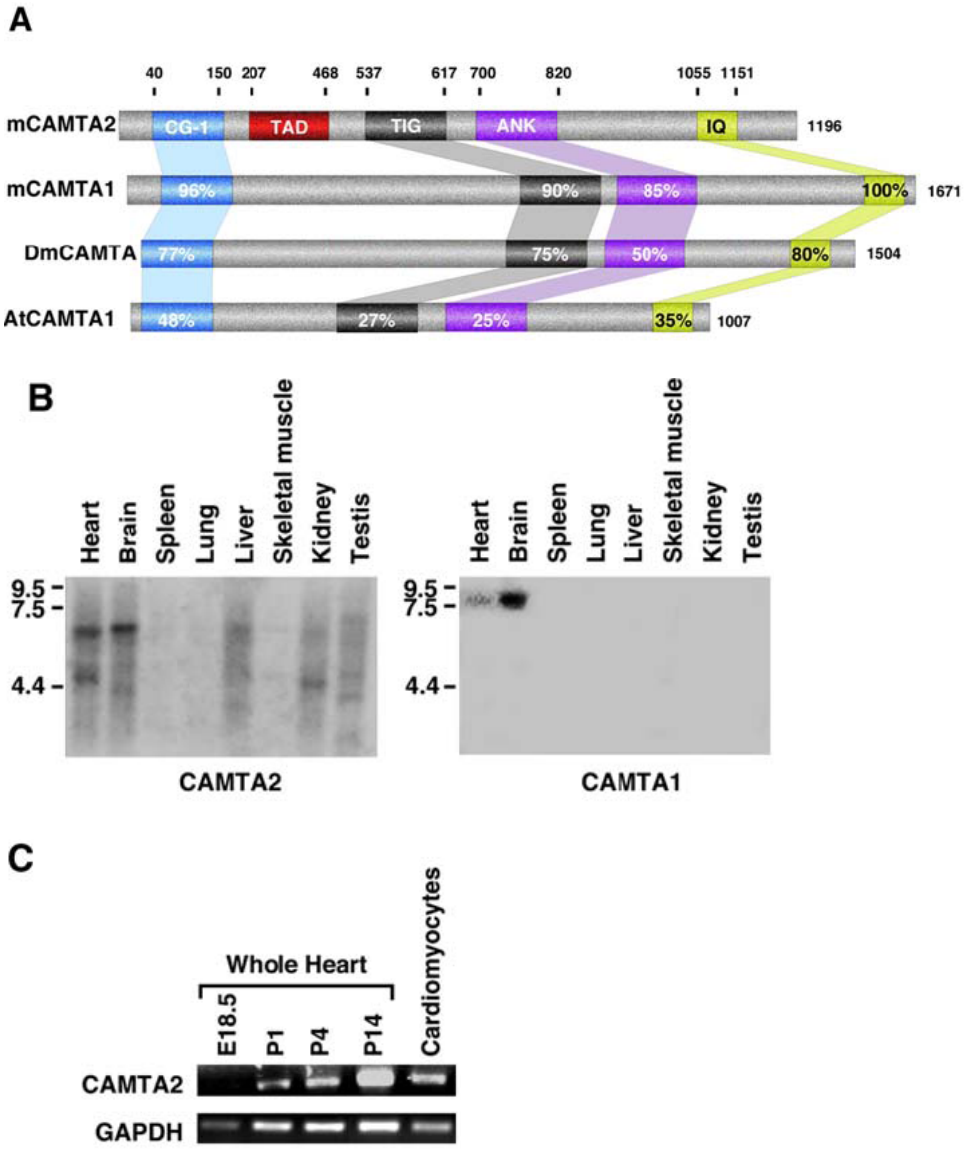

Figure 2.2. Identification of mammalian CAMTAs. **(A)** Schematic diagram of CAMTA proteins from mouse (m), Drosophila melanogaster (Dm) and Arabidopsis thaliana (At) CAMTA proteins. Amino acid identities within each domain are shown. **(B)** Detection of CAMTA transcripts by Northern blot analysis of adult mouse tissues. **(C)** Detection of transcripts for CAMTA2 and GAPDH (as a control) by semi-quantitative RT-PCR in hearts from rats at E18.5 and postnatal days (P) 1, 4 and 14.

Transcriptional activation on ANF promoter by CAMTA is mediated by Nkx2-5

I 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 2.3A). However, deletion to –74 bp resulted in a precipitous decline in responsiveness to CAMTA2. The latter construct retained residual responsiveness to CAMTA2, which I attribute to the existence of cryptic CAMTA2 response elements in the reporter plasmid. CAMTA1 activated the *ANF* promoter even more strongly than CAMTA2 (Figure 2.3B).

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 2.3A), 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 2.3B).

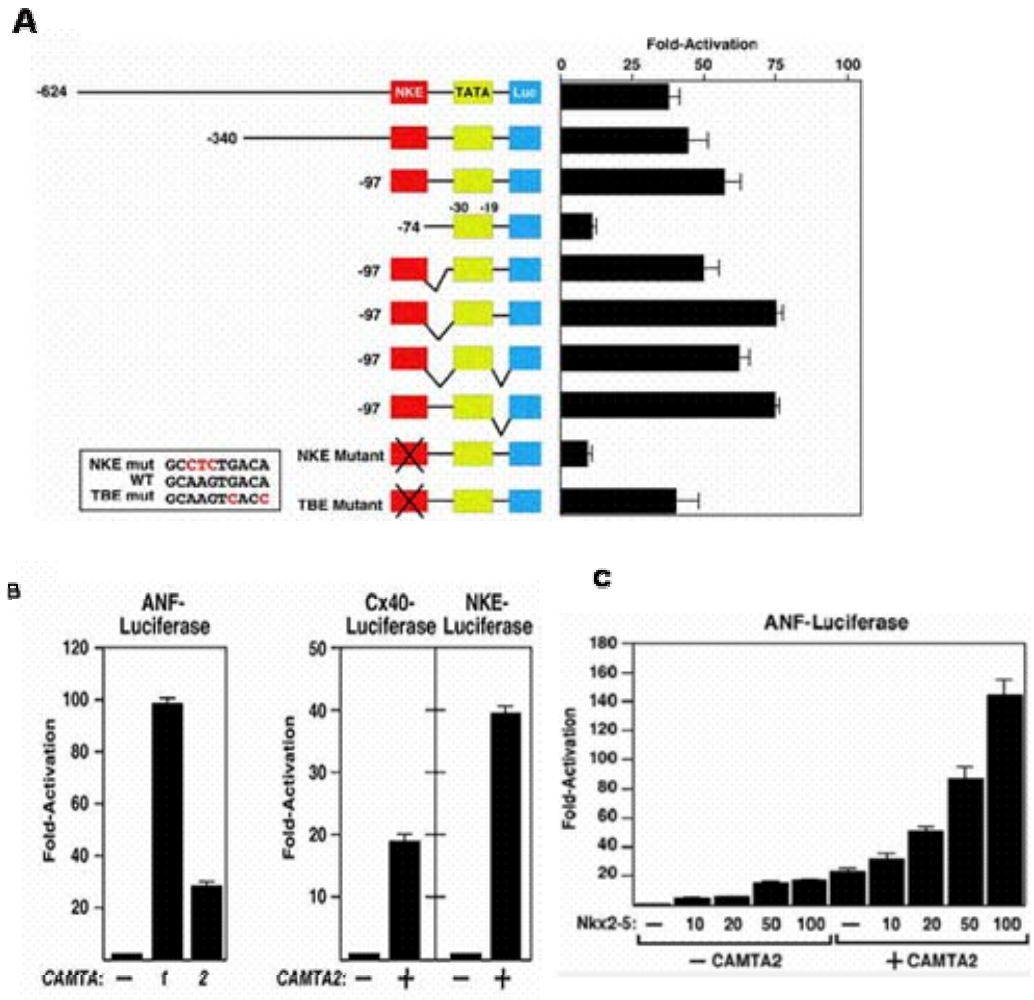

Figure 2.3. Activation of *ANF* promoter by CAMTA via a NK site. **(A)** COS cells were transfected with a CAMTA2 expression plasmid and the indicated ANF-luciferase reporters. Values are expressed as the fold-increase in luciferase expression (± S.D.) in the presence compared to the absence of CAMTA2. (mutations in the NKE and TBE sites are shown in red). **(B)** COS cells were transfected with a CAMTA2 expression plasmid and the indicated luciferase reporters. Values are expressed as the fold-increase in luciferase expression (± S.D.) in the presence compared to the absence of CAMTA. The left panel compares the activities of CAMTA1 and CAMTA2. **(C)** 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 (± S.D.) with Nkx2-5 and/or CAMTA2 expression plasmids compared to the reporter alone.

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 ~6-fold (Figure 2.3C). 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 2.4A). In transfected COS cells, CAMTA2 was also detected on an exogenous *ANF* promoter, and its association with the promoter was enhanced when co-expressed with Nkx2-5 (Figure 2.4A), supporting the 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 2.4B, ^{35}S-labeled CAMTA2 protein associated with the NKE in the presence of GST-Nkx2-5, but not in the presence of GST alone.

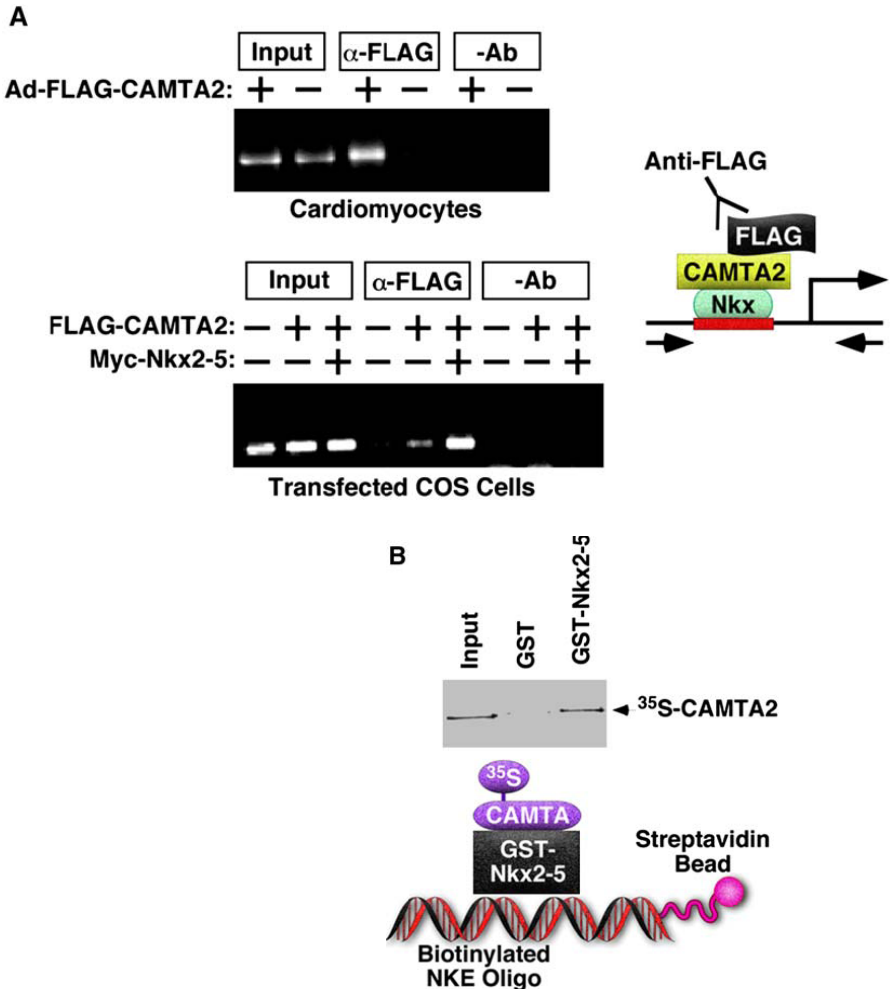

Figure 2.4. Interaction of CAMTA2 and Nkx2-5. (A) Primary neonatal cardiomyocytes (upper panel) were infected with adenovirus encoding FLAG-CAMTA2, ChIP was performed with anti-FLAG antibody or without antibody and PCR was performed with primers flanking the NKE in the *ANF* promoter, as indicated. In 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.

CAMTA2 was distributed in the nucleus and cytoplasm (Figure 2.5Aa). In the presence of leptomycin B, an inhibitor of nuclear export, CAMTA2 became localized exclusively to the nucleus (Figure 2.5Ab), suggesting that the protein cycles between the cytoplasm and the nucleus. When co-expressed with Nkx2-5, which is exclusively nuclear, all CAMTA2 protein became localized to the nucleus, consistent with a possible interaction between the proteins (Figure 2.5Ac-e).

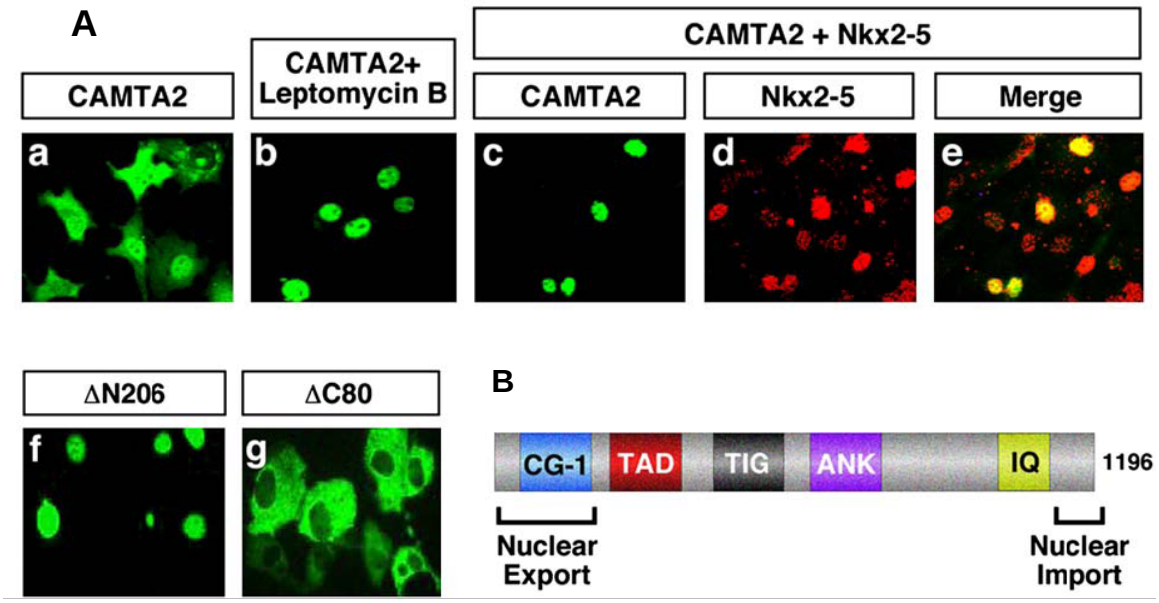

Figure 2.5. Cellular distribution of CAMTA2. (A) The subcellular distribution of FLAG-CAMTA2 in transfected COS cells was detected by immunofluorescence. CAMTA2 is distributed in the nucleus and cytoplasm (panel a). In the presence of leptomycin B, CAMTA2 becomes localized to the nucleus (panel b). Similarly, when co-expressed 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). **(B)** The positions of nuclear export and import sequences in CAMTA2 are shown.

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 2.6A, CAMTA2 fused to the GAL4 DNA binding domain activated a GAL4-dependent luciferase reporter in transfected COS cells. These findings showed that 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, I generated a series of deletion mutants and assayed their subcellular distribution and ability to activate the *ANF* promoter (Figure 2.6B). 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 protein, the ΔN206 mutant was localized exclusively to the nucleus (Figure 2.5Af), suggesting that the CG-1 domain contains a nuclear export sequence (NES) (Figure 2.5B). 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 2.5Ag), indicative of a nuclear localization sequence (NLS) in this region (Figure 2.5B). As expected from its cytoplasmic localization, mutant ΔC80 was unable to activate the *ANF* promoter (Figure 2.6B). Because the NLS was contained in the C-terminal 80 residues of CAMTA2, I 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. I 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.

I 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 (Figure 2.7A and B). 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 (Figure 2.7C and D).

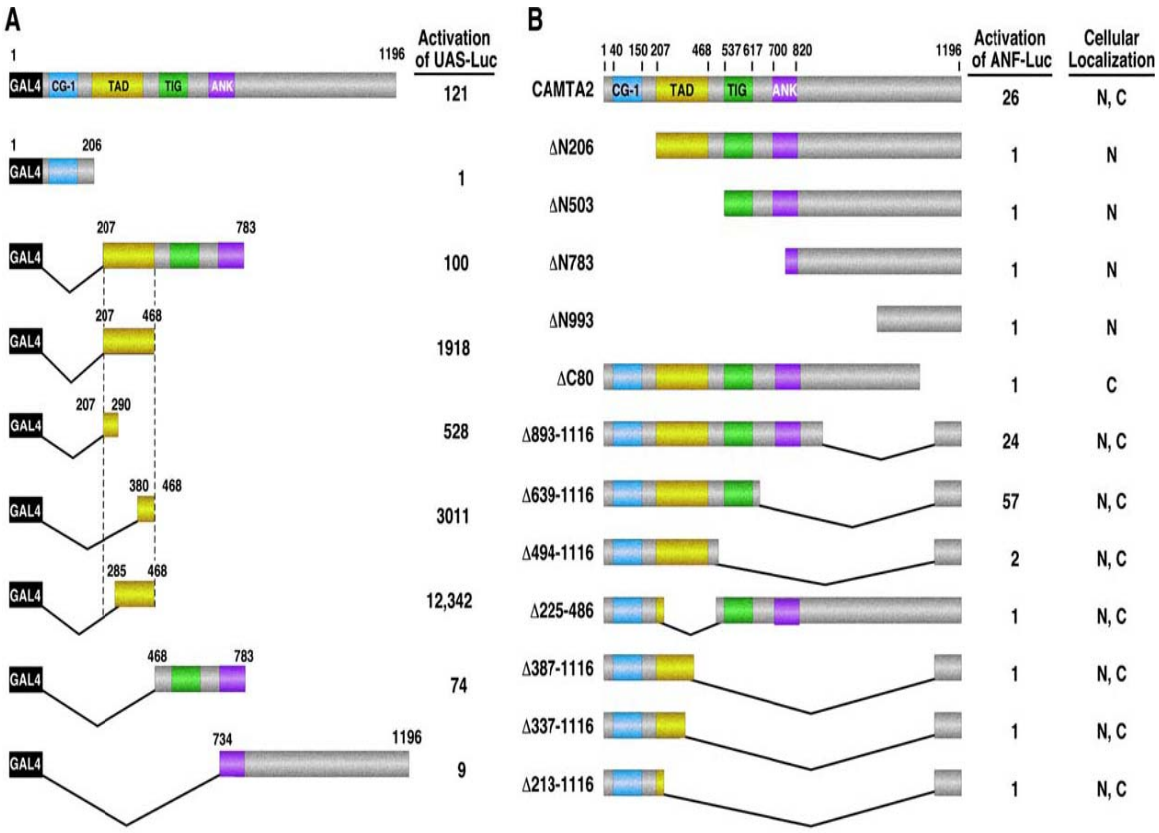

Figure 2.6. Functional domains of CAMTA2. (A) Portions of CAMTA2 were fused to the GAL4 DNA binding domain and assayed for activity with a UAS-luciferase reporter. Values are expressed as the fold-increase in luciferase expression in the presence of each GAL4-CAMTA2 mutant protein compared to the reporter 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.

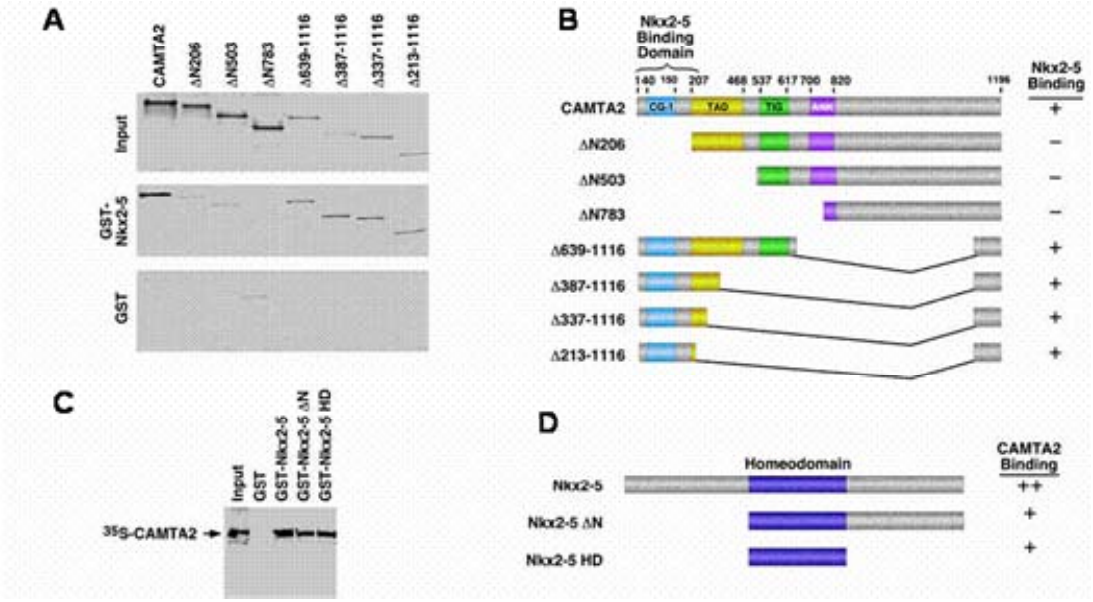

Figure 2.7. Interaction of CAMTA2 and Nkx2-5. (A) GST-Nkx2-5 was incubated with ^{35}S-methionine-labeled CAMTA2 deletion mutants translated in vitro. Input CAMTA2 proteins are shown in the top panel. CAMTA2 proteins bound to GST-Nkx2-5 are shown in the middle panel. The lack of binding of CAMTA2 proteins to GST is shown in the bottom panel. **(B)** Summary of binding data for CAMTA deletion mutants. **(C)** GST alone or GST-fused to portions of Nkx2-5 was incubated with ^{35}S-methionine-labeled CAMTA2 translated in vitro, as indicated. **(D)** Summary of binding data for Nkx2-5 deletion mutants.

CAMTA activates *β-MHC* and *myogenin* promoters by direct DNA binding

I have shown that CAMTA activation of *ANF* promoter is mediated by Nkx2-5. it has been shown that plant and fly CAMTA proteins are capable of binding to DNA (Mitsuda, et al., 2003; Han, et al., 2006). I hypothesized that mammalian CAMTA

proteins bind DNA and could activate target genes through direct DNA binding. To test this hypothesis, I examined many luciferase reporters to look for candidate promoters which were activated by CAMTA in COS cells using transfection assay. Both *myogenin* and *β-MHC* promoters were activated by CAMTA in vitro (Figure 2.8A and Figure 2.10A). CAMTA1 has much higher transcriptional activity on these two promoters than CAMTA2. The *β-MHC* gene is highly activated in the embryonic heart. In the adult heart, the *β-MHC* gene is transcribed at a low level. However, the *β-MHC* gene is up-regulated in response to hypertrophic stress in the adult heart. The *β-MHC* promoter was activated several hundred-fold by CAMTA1 (Figure 2.8A). The similar DNA motif, CTGGGTGC, was found in both promoters. Nucleotide substitution of 8 base pairs, CTGGGTGC, between -77 and -70 relative to transcriptional initiation site reduced the transcriptional activity of CAMTA on the *β-MHC* promoter by ~80%. The substitution of 8 base pairs totally abolished the activity of CAMTA1, using low concentration of a CAMTA1 expression plasmid in transfection assays (Figure 2.8A), indicating that the binding motif of CAMTA remains unclear. To examine if CAMTA proteins can directly bind to this region, I performed a gel mobility shift with a DNA probe located between -90 and -46 of the *β-MHC* gene with purified CAMTA2 recombinant fusion protein, MBP-FLAG-CAMTA2 (1-250 amino acids). The gel mobility shift assay showed that the first 250 amino acids of CAMTA binds to the β-MHC promoter (Figure 2.8B). FLAG-CAMTA-DNA complex was supershifted in the presence of anti-FLAG antibody (Figure 2.8B).

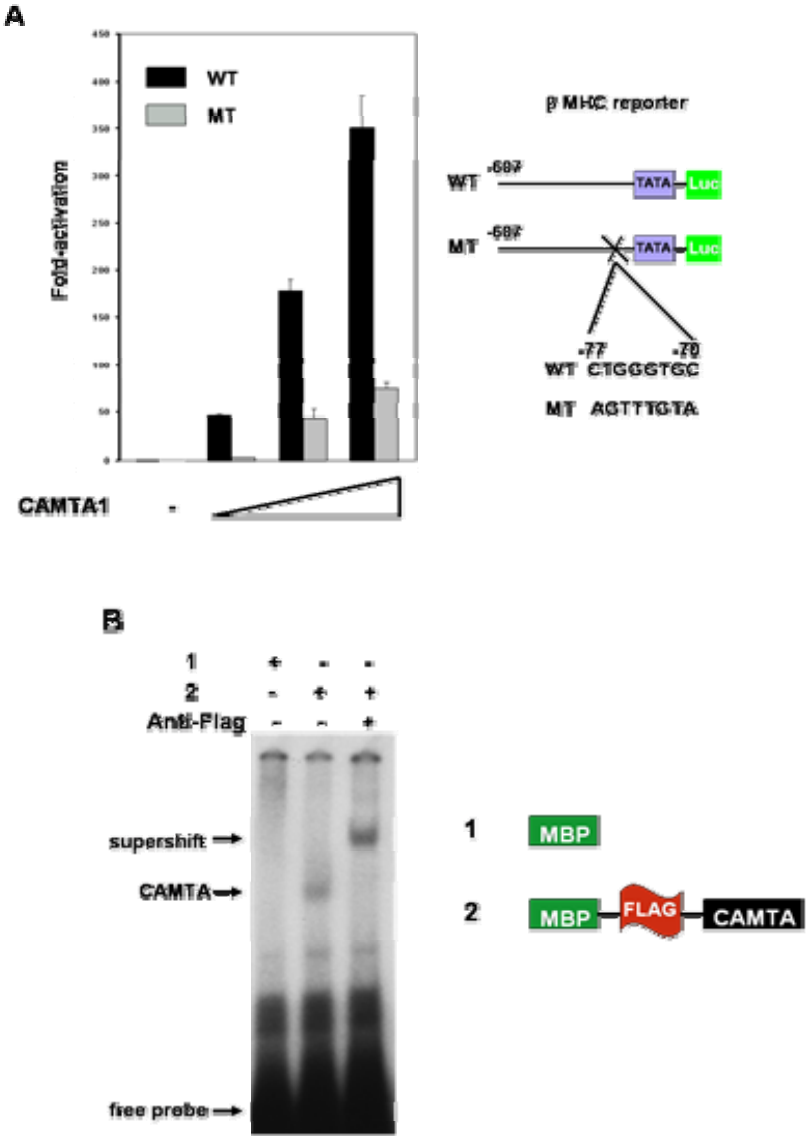

Figure 2.8. Activation of the *β-MHC* promoter by CAMTA via DNA binding. (A) COS cells were transfected with a CAMTA1 expression plasmid and the indicated β-MHC-luciferase reporters. Values are expressed as the fold-increase in luciferase expression (± S.D.) in the presence compared to the absence of CAMTA1. The diagram in the right panel shows wildtype and mutant reporters used in the assay. **(B)** Gel mobility shift assay was performed with MBP-FLAG-CAMTA2 fusion protein purified from *E.coli* and the labeled β-MHC probe located between -90 and -46 of the gene. The complex of FLAG-CAMTA2-DNA was supershifted in presence of an anti-FLAG antibody.

The basic-helix-loop-helix (bHLH) transcription factor myogenin is a key regulator during skeletal muscle differentiation. Mammalian CAMTA1 and CAMTA2 are highly expressed in differentiating skeletal muscle cells, such as C2C12 myotubes (Figure 2.9). C2C12 myoblasts faithfully mimic skeletal muscle differentiation in vitro. C2C12 myoblasts can be induced to differentiate into myotubes by a differentiating medium containing low serum concentration. CAMTA1, CAMTA2, and myogenin were up-regulated during induction of myoblasts to myotubes (Figure 2.9).

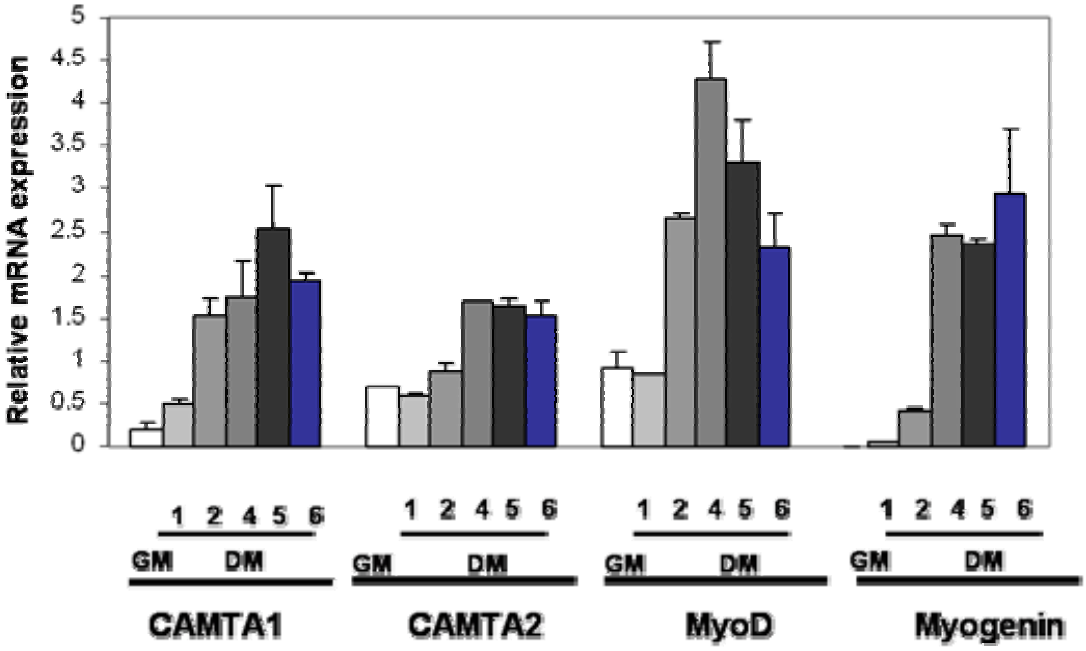

Figure 2.9. Up-regulation of CAMTA1, CAMTA2, and myogenin in C2C12 myotubes. Transcripts of CAMTA1, CAMTA2, myogenin, and MyoD (± S.D.) were detected in C2C12 myoblasts, and differentiating myotubes by real time PCR . MyoD was used to be a positive control. GM, growth medium; DM, differentiating medium.

The *myogenin* promoter was activated ~60-fold by CAMTA1 (Figure 2.10A). I used a series of *myogenin* promoter mutants to map the cis-regulatory sequences that conferred responsiveness to CAMTA1. Deletion mutations from –1110 bp to –146 bp relative to the transcription initiation site did not impair activation of the promoter by CAMTA1 in transfected COS cells. There are two (CTGGGTGC) motifs within the region between -146 to +1. To define if CAMTA proteins directly bind to the region between -146 to +1 of the *myogenin* gene, I performed gel mobility shift assay with purified MBP-CAMTA2 (1-250) fusion protein. A complex of FLAG-CAMTA2-DNA was formed in the assay. Anti-FLAG antibody was able to supershift the complex (Figure 2.10B). CAMTA binds to DNA via the first ~250 hundred amino acids.

Gel mobility shift assay with *myogenin* and *β-MHC* promoters suggested that CAMTA was capable of binding directly to DNA.

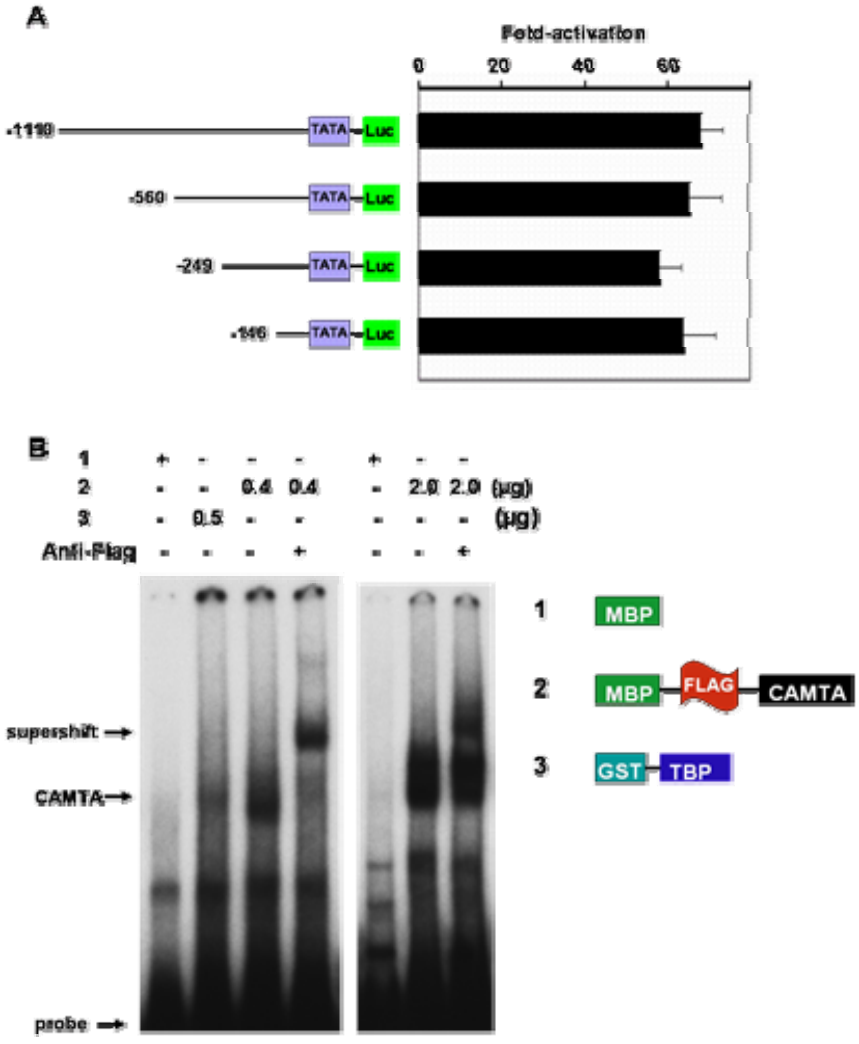

Figure 2.10. Activation of the *myogenin* promoter by CAMTA via DNA binding. (A) COS cells were transfected with a CAMTA1 expression plasmid and the indicated myogenin-luciferase reporters. Values are expressed as the fold-increase in luciferase expression (± S.D.) in the presence compared to the absence of CAMTA1. **(B)** Gel mobility shift assay was performed with MBP-FLAG-CAMTA2 fusion protein purified from *E.coli* and the myogenin probe labeled by P^{32}. The probe is located from -146 to +1 of the *myogenin* gene. The complex of FLAG-CAMTA2-DNA was supershifted in presence of an anti-FLAG antibody. The diagram in the right panel shows structures of protein used in the assay. GST-TBP was used to be as a positive control. TBP, TATA binding protein.

Association of CAMTA1 with *β-MHC* and *myogenin* promoters in native chromatin

To validate in vivo binding of CAMTA 1 to the *β-MHC* and *myogenin* promoters, I performed chromatin immunoprecipitation (ChIP) assays. A polyclonal antibody was generated in rabbits to recognize CAMTA1 (Figure 2.11A). The antibody recognizes the C-terminus of the CAMTA1 protein which is not conserved with CAMTA2. I performed ChIP assays with this antibody and showed that endogenous CAMTA occupied the *β-MHC* promoter in native chromatin in primary neonatal rat cardiomyocytes (Figure 2.11B) and CAMTA occupied the native *myogenin* promoter in C2C12 myotubes (Figure 2.11C). These findings suggest that CAMTA activates target genes in striated muscle, cardiac and skeletal muscle.

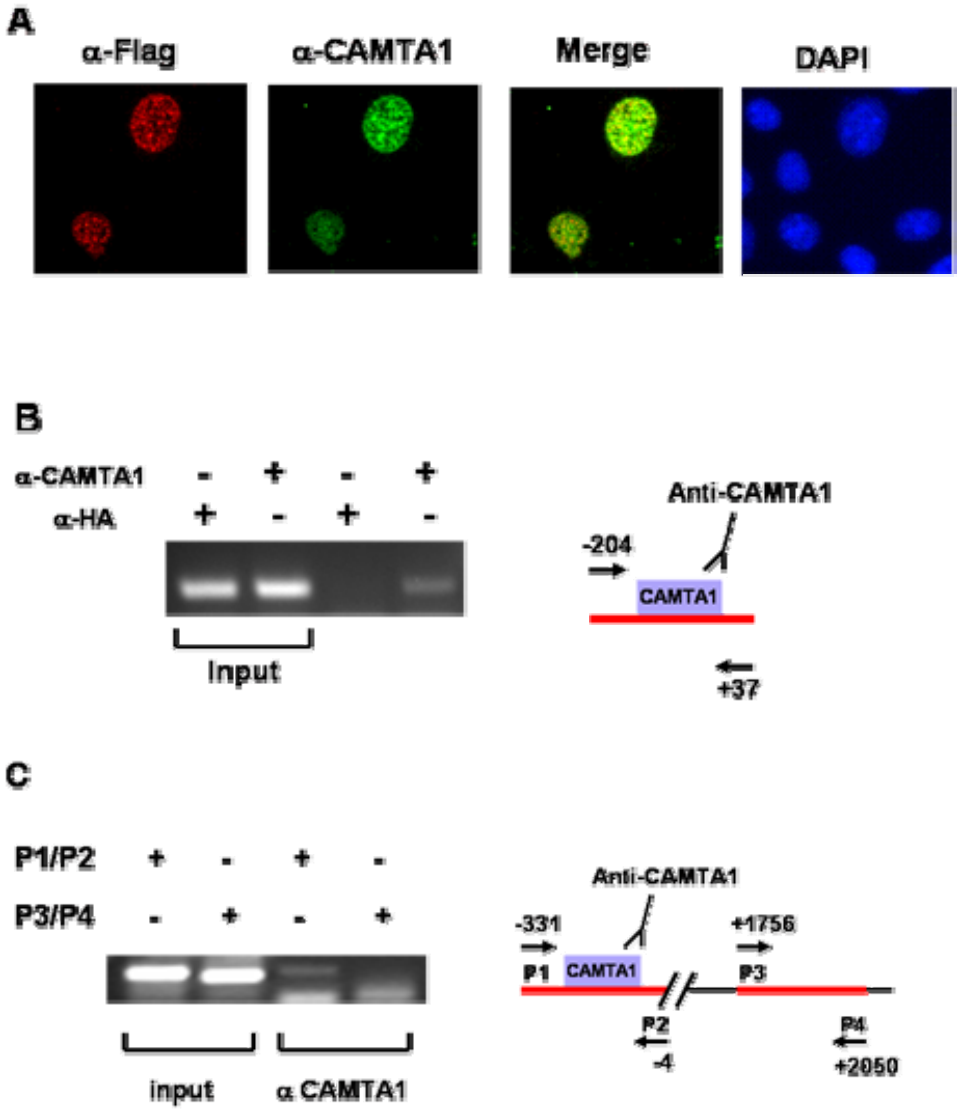

Figure 2.11. Association of CAMTA with *myogenin and β-MHC* promoters in vivo. (A) COS cells were transfected with a Flag-CAMTA1 expression plasmid. The Flag-CAMTA1 protein was detected with anti-flag antibody (red) and anti-CAMTA1 antibody (green). DAPI (blue) was used to mark nuclei. **(B)** ChIP was performed with primary neonatal rat cardiomyocytes and anti-CAMTA1 antibody, and PCR was performed with primers flanking a putative CAMTA1 binding region on the *β-MHC* promoter as indicated in the right panel. **(C)** ChIP was performed with C2C12 myotubes. The complex of CAMTA1-chromatin was pulled down with anti-CAMTA1 antibody, and PCR was performed with primers flanking a putative CAMTA binding region and coding area of *myogenin* gene as indicated.

CAMTA2 induces cardiac hypertrophy in vivo and in vitro

To further investigate the potential function of CAMTA2 as a regulator of cardiac gene expression, I overexpressed CAMTA2 in primary neonatal rat cardiomyocytes by adenoviral delivery. Ad-CAMTA2 infected cells displayed a phenotype of hypertrophy and increased sarcomere assembly, compared with control cultures infected with Ad-lacZ (Figure 2.12A and B). Cultures infected with Ad-CAMTA2 also contained a greater number of cardiomyocytes than control cultures, suggesting that CAMTA2 enhanced myocyte proliferation and/or survival (Figure 2.12A and C).

Transgenic mice that over-expressed CAMTA2 in the heart under control of the α-MHC promoter were generated. Two independent stable lines of transgenic mice and three transgenic founders were viable, but their hearts were grossly enlarged and showed extensive myocyte hypertrophy (Figure 2.13A and B). In transgenic mouse 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 2.13B) 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 2.14A).

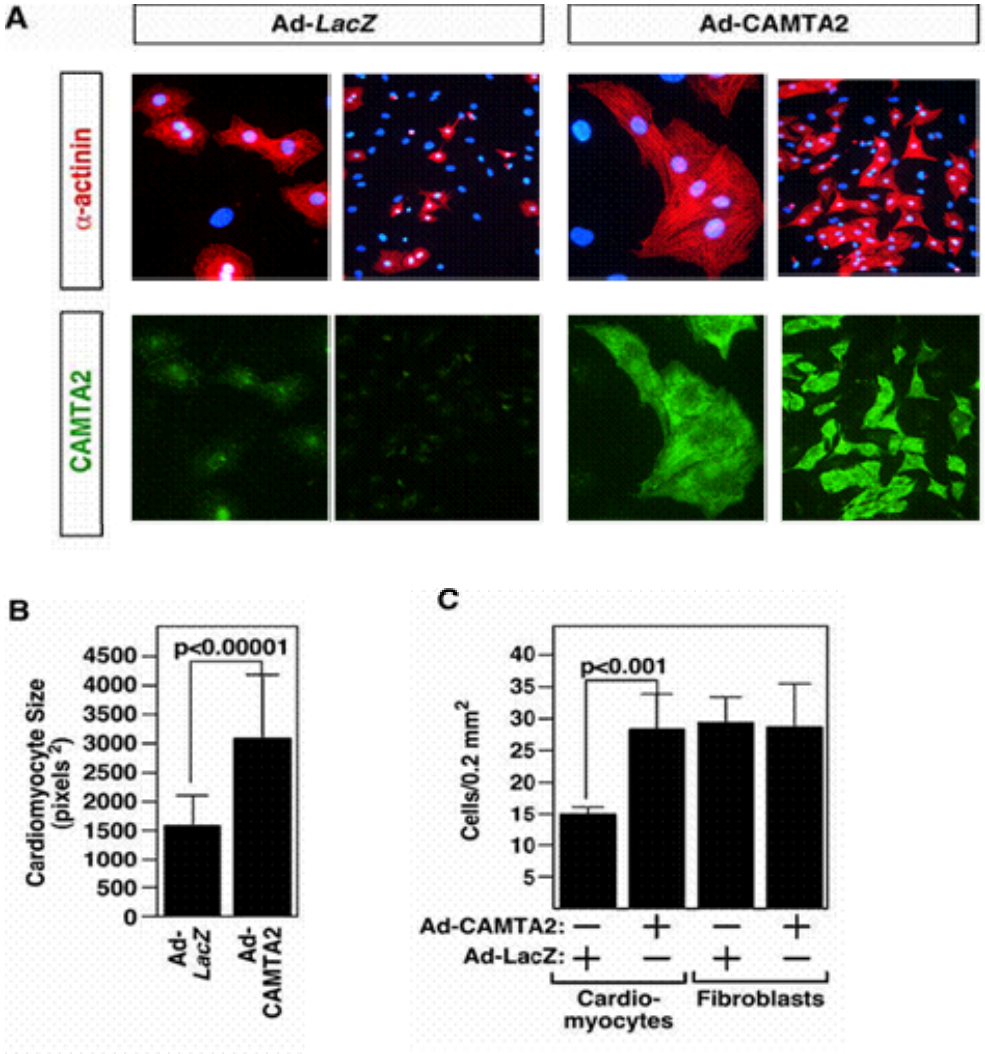

Figure 2.12. Induction of cardiac growth by CAMTA2 in vitro. (A) Primary neonatal rat cardiomyocytes were infected with adenoviruses encoding FLAG-CAMTA2 (right panels) or lacZ (left panels) as a control. Cells were stained with anti-α-actinin antibody (red) to mark cardiomyocytes, DAPI (blue) and anti-FLAG (green). Ad-CAMTA2 induces profound hypertrophy and sarcomere assembly. Left panels of each set are 10X and right panels are 40X magnification. **(B)** Cell size (± S.D.) in Panel A was determined as described in Experimental Procedures. **(C)** Numbers of cardiomyocytes and fibroblasts (± S.D.) in cultures from Panel C 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.

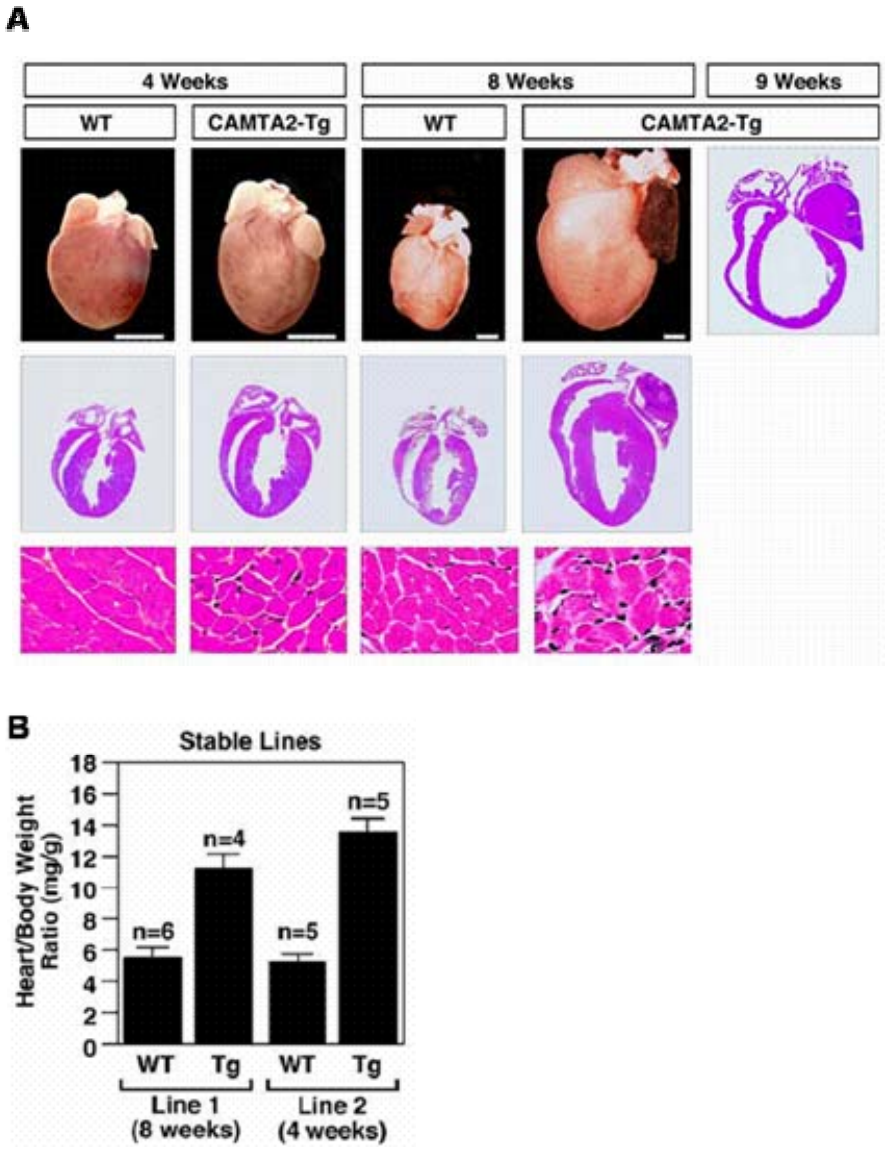

Figure 2.13 Induction of cardiac growth by CAMTA2 in vivo. (A) Transgenic mice were generated bearing an αMHC-CAMTA2 transgene. Hearts from wild type and transgenic mice (line 1) at 4, 8 and 9 weeks of age are shown at the top. Histological sections are shown in the middle panel and high magnification views of ventricular cardiomyocytes are shown at the bottom. At 9 weeks of age, hypertrophy progresses to dilated cardiomyopathy in αMHC-CAMTA2 transgenic mice. Bars = 2 mm. (B) Heart weight/body weight ratios (± S.D.) of wild type and stable lines αMHC-CAMTA2 transgenic mice at 4 and 8 weeks of age are shown.

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 2.14B). These findings support the conclusion that the CG-1 domain of CAMTA2 is required to promote cardiac growth.

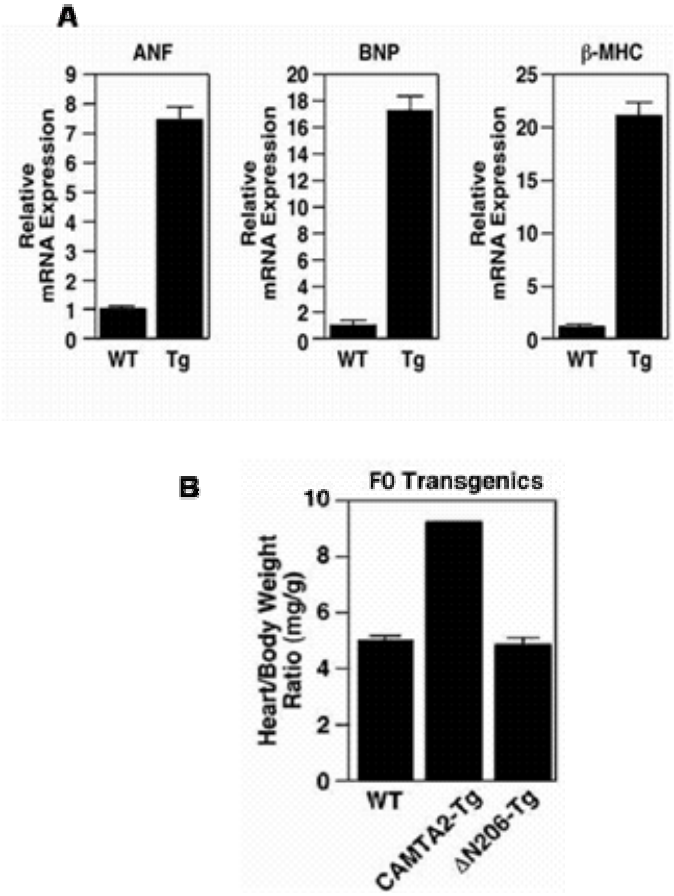

Figure 2.14. Induction of cardiac hypertrophy by CAMTA2 in vivo. (A) Transcripts representing hypertrophic gene markers (± S.D.) were detected in hearts of wild type and α-MHC-CAMTA2 transgenic mice (line 1) (n=2) at 8 weeks of age by real time PCR. **(B)** Heart weight/body weight ratios of wild type and F0 transgenic mice harboring αMHC-CAMTA2 and αMHC-ΔN206CAMTA2 transgenic mice (n=4) at 4 weeks of age.

PKC and PKD signaling stimulate CAMTA2 activity

I 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 2.15, 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 2.15).

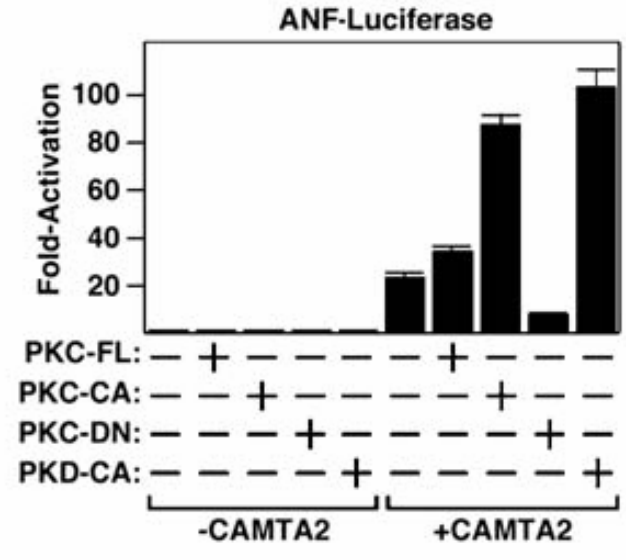

Figure 2.15. Regulation of CAMTA2 activity by PKC and PKD signaling. COS cells were transfected with a expression plasmids encoding full length (FL), constitutively active (CA) or dominant negative (DN) PKCε or PKD, and CAMTA2 (100 ng each), as indicated, along with the ANF-luciferase reporter (250 ng). Values are expressed as the fold-increase in luciferase expression (± S.D.) compared to the reporter alone.

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 de-repression 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, I 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 and prevented hypertrophy in response to CAMTA2 (Figure 2.16A and B). Similar repression was observed with HDAC4 (data not shown).

In co-immunoprecipitation assays, CAMTA2 interacted avidly with HDAC5, and deletion mutants identified the ankyrin-repeat domain of CAMTA2 (Figure 2.17A and B) and the N-terminal regulatory region of HDAC5 (residues 153-360) as the interacting domains (Figure 2.18C and D). 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 2.6B). 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 2.17D), in agreement with prior studies demonstrating that the HDAC domains of class II HDACs are not required for repression (Zhang et al., 2002).

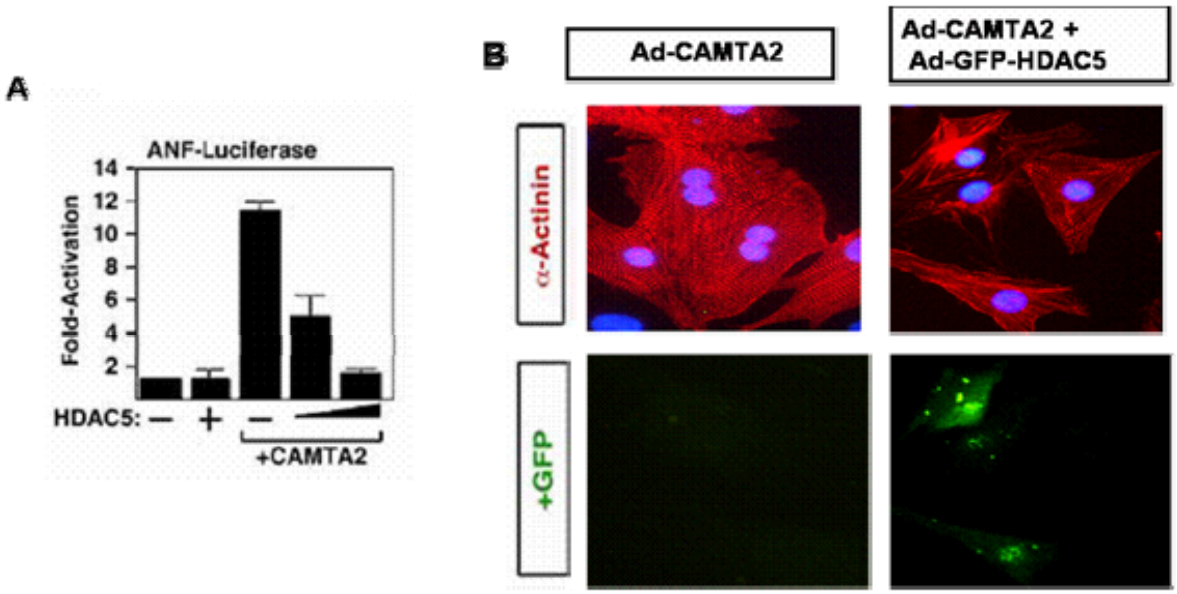

Figure 2.16. Interference with CAMTA2 activity by HDAC5. (A) COS cells were transfected with expression plasmids encoding CAMTA2 (100 ng) or HDAC5 (5 and 25 ng), as indicated, and the ANF-luciferase reporter (150 ng). Values are expressed as the fold-increase in luciferase expression (± S.D.) compared to the reporter alone. **(B)** 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 X.

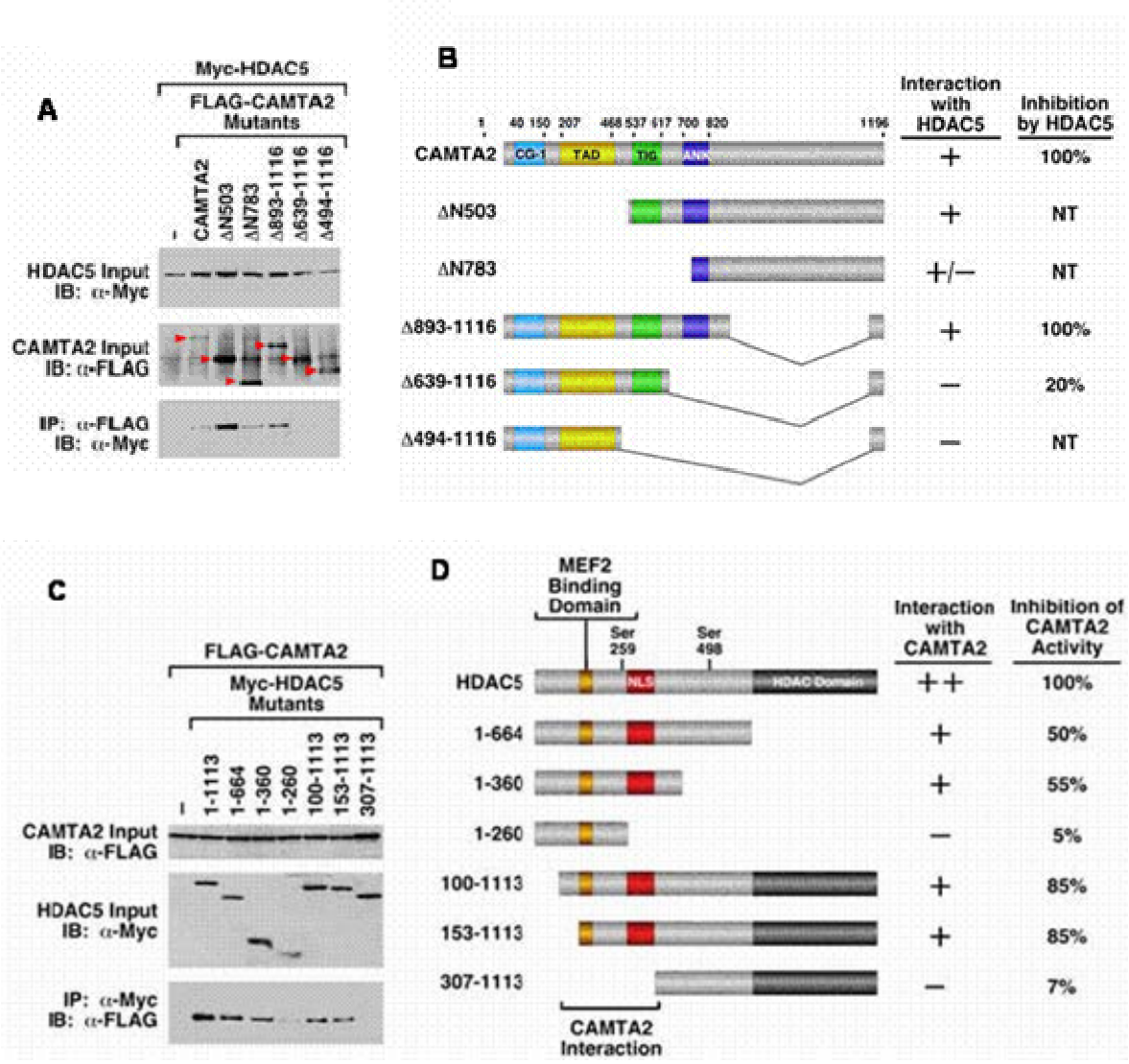

Figure 2.17. Interaction of CAMTA2 with HDAC5. (A) COS cells were transfected with expression plasmids encoding Myc-HDAC5 and FLAG-CAMTA2 proteins (500 ng each). Input HDAC5 and CAMTA2 proteins detected by immunoblot (IB) are shown in the top and middle panels, respectively. HDAC5 proteins co-immunoprecipitated (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. **(B)** The ability of each CAMTA2 deletion mutant to associate with HDAC5 in Panel A 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. **(C)** 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 co-immunoprecipitated (IP) with HDAC5 are shown in the bottom panel. **(D)** The ability of each HDAC5 deletion mutant to associate with CAMTA2 in Panel C is shown. The relative effectiveness of each protein to inhibit CAMTA2 activity is indicated in the right column.

Whereas CAMTA2 was distributed in the nucleus and cytoplasm (Figure 2.18a), when coexpressed with HDAC5, it became colocalized with HDAC5 in the nucleus (Figure 2.18e, g, i). In the presence of activated PKD, HDAC5 translocates from the nucleus to the cytoplasm (Figure 2.18d, h, j). Under these conditions, CAMTA2 remained nuclear (Figure 2.18f, h, j). Moreover, the cytoplasmic pool of CAMTA2 appeared to enter the nucleus in the presence of PKD, even in the absence of HDAC5 (Figure 2.18b). 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) (Figure 2.18k and l). In contrast to wild type HDAC5, this mutant remains nuclear in the presence of activated PKD (Figure 2.18m and n). 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.

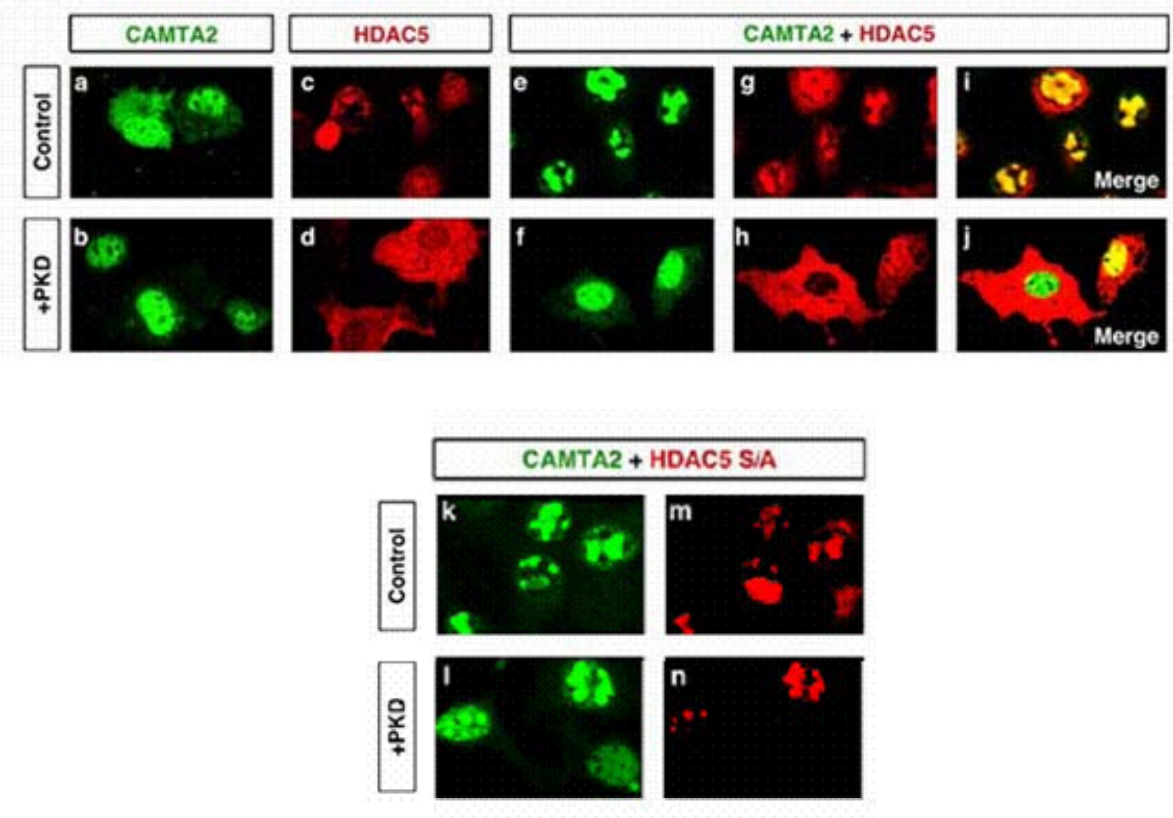

Figure 2.18. Signal-dependent regulation of CAMTA2 and its association with HDAC5. COS cells were transfected with expression plasmids encoding CAMTA2 (300 ng) or HDAC5 (100 ng) either separately or together with a PKD expression plasmid (600 ng). CAMTA2 (green) and HDAC5 (red) proteins were detected 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.

Antagonism between HDAC5 and CAMTA2 in vivo

To test whether HDAC5 antagonizes the growth-stimulatory influence of CAMTA2 on the heart in vivo, I 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 2.19, the cardiac growth response to CAMTA2 over-expression 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.

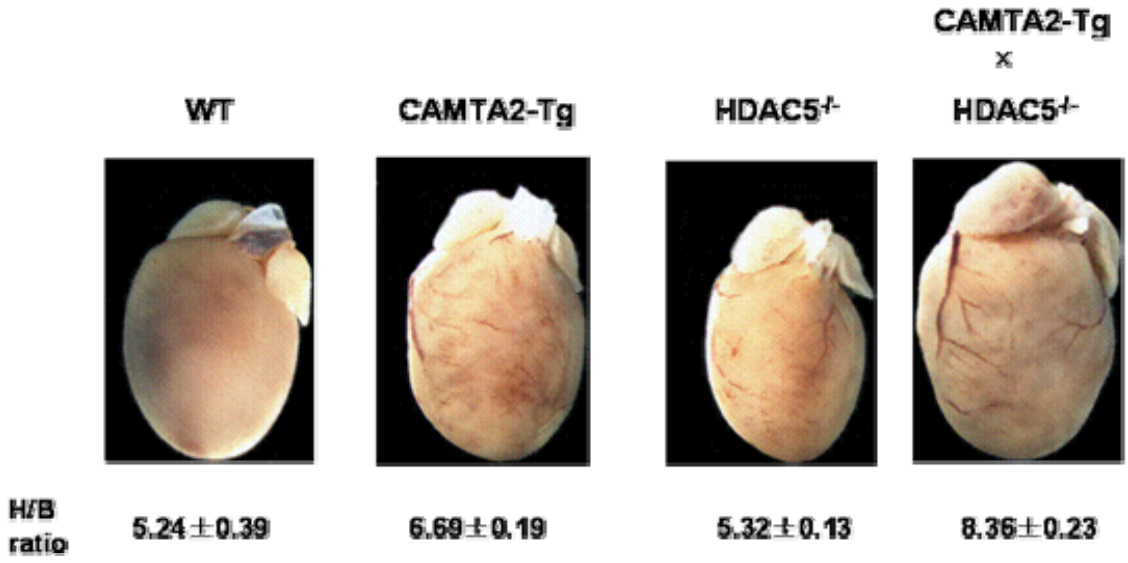

Figure 2.19. Antagonism between HDAC5 and CAMTA2 in vivo. Enhanced cardiac hypertrophy of αMHC-CAMTA2 transgenic (line 1)/HDAC5 mutant mice. Heart weight/body weight measurements (± S.D.) of mice of the indicated genotypes were determined at 4 weeks of age. $HDAC5^{-/-}$ mice show twice the amount of hypertrophy as wild type transgenics. H, heart weight in mg; B, body weight in g.

CAMTA2 knockout mice display diminished hypertrophy in response to multiple stimuli

I 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 (Figure 2.20A and B). 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 2.20C). The lacZ gene inserted into the *CAMTA2* locus was expressed in cardiomyocytes, as detected by immunostaining (Figure 2.20D).

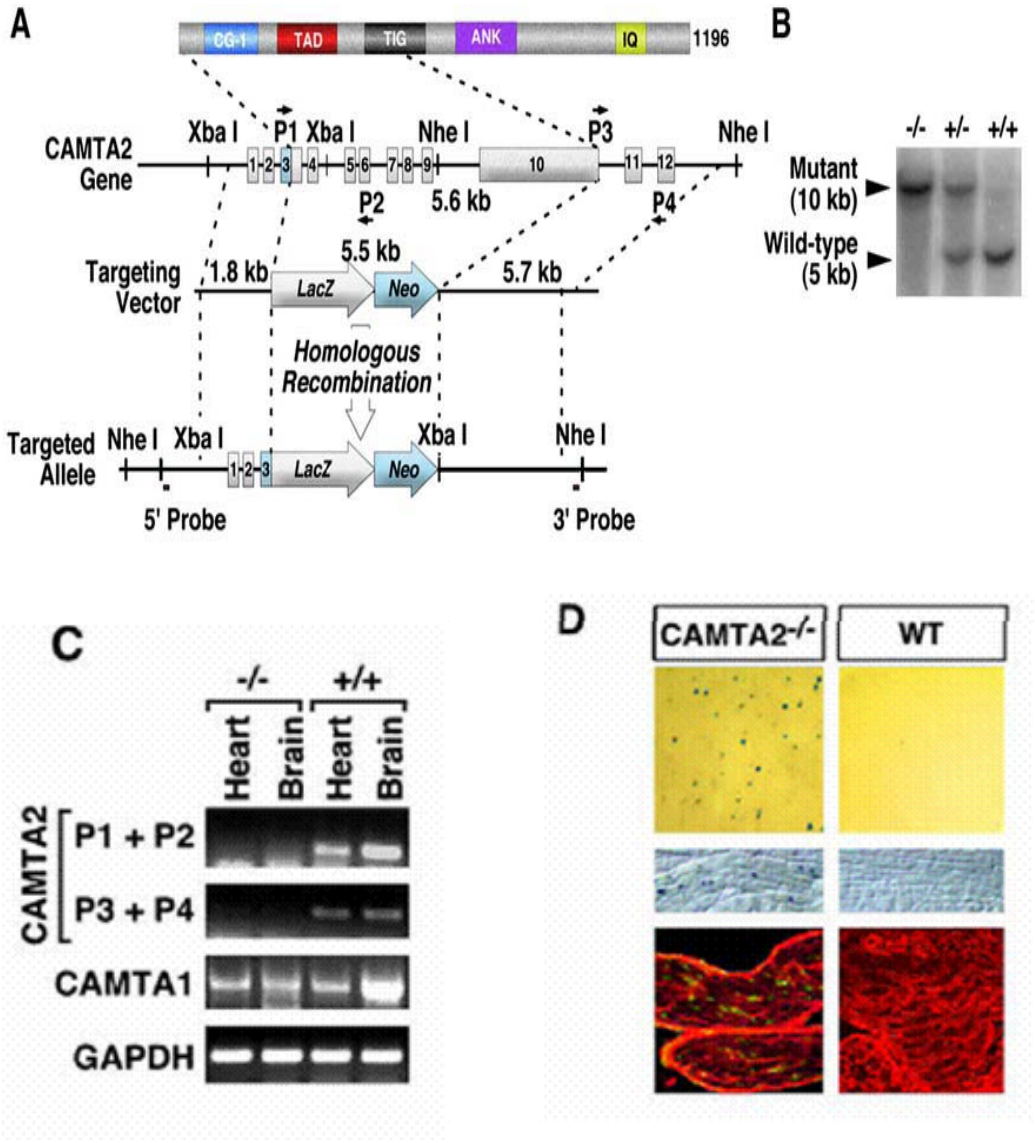

Figure 2.20. Mutation of CAMTA2 by gene targeting. (A) The structure of the mouse CAMTA2 gene is shown. The targeting strategy deleted exons 2-10, removing the CG-1, TAD and part of the TIG domain. Positions of primers for PCR and probes for Southern blot are shown. **(B)** Genomic DNA from mice of the 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 alpha-actinin (red) by immunodetection. Rabbit anti-beta-galactosidase (Ab cam) was used at a dilution of 1:3000.

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 (Figure 2.21-2.23). 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 ($p < 0.006$) (Figure 2.21A and B). The induction of fetal cardiac genes, including *ANF*, was similarly diminished in CAMTA2 mutant mice following TAB (Figure 2.21 C). Cardiac hypertrophy was also suppressed in CAMTA2 mutant mice following chronic infusion with angiotensin II (Ang II) (Figure 2.22A and B) and the adrenergic agonist isoproterenol (Figure 2.23A-C). Chronic infusion of isoproterenol leads to cardiac dilation and decreased cardiac function (Figure 2.23 B and C). But there was no significant change of cardiac dilation and function between isoproterenol-treated and untreated CAMTA2 knockout mice (Figure 2.23A-C). In addition, angiotensin II infusion resulted in ventricular fibrosis, which was not observed in CAMTA2 mutant mice (Figure 2.22B). I conclude that CAMTA2 is not only sufficient to induce cardiac hypertrophy, but is necessary for a maximal hypertrophic response to diverse stimuli in vivo.

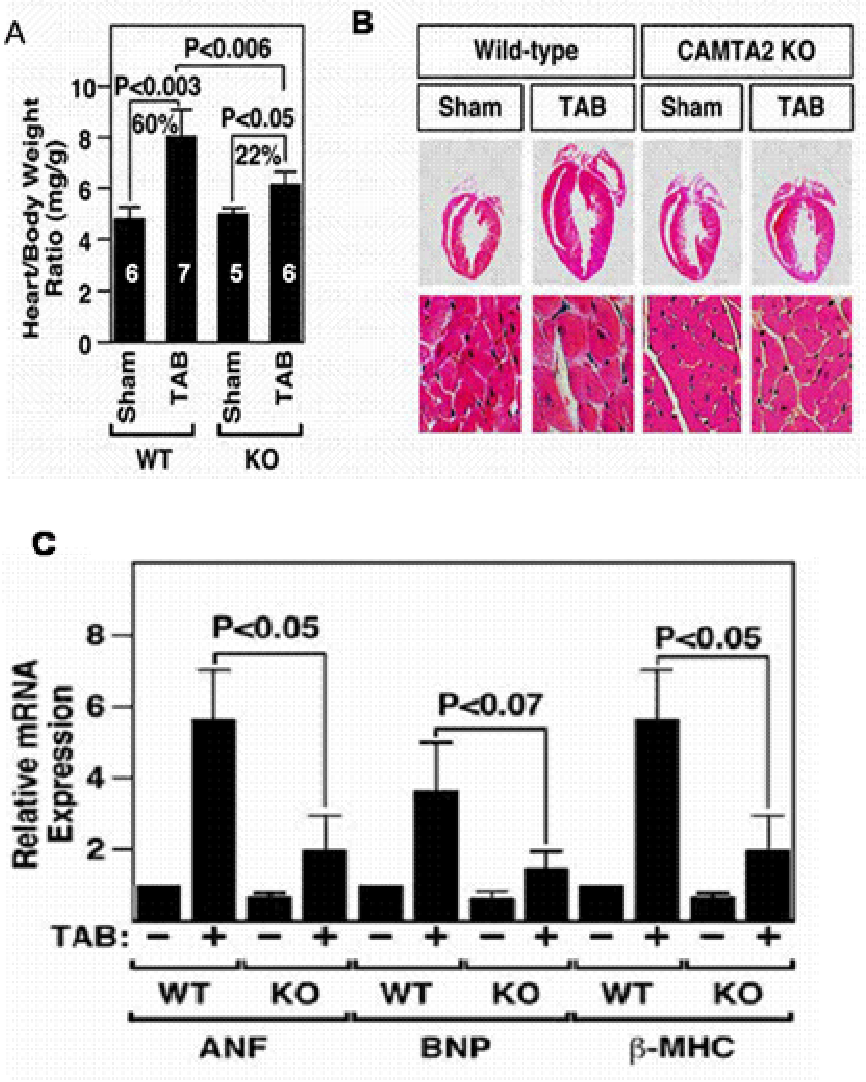

Figure 2.21. CAMTA2 knockout mice are compromised in their ability to mount a cardiac hypertrophic response to thoracic aortic banding (TAB). (A) Wild-type and CAMTA2 null mice were subjected to TAB or sham operation and heart weight/body weight ratios (± S.D.) were determined after 21 days. The number of mice per group was shown in the bar. **(B)** Histological sections of representative hearts from E are shown. Hypertrophy in response to TAB is inhibited in CAMTA2 mutant mice. **(C)** Transcripts for ANF, BNP and beta-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 (± S.D.) compared to these markers in sham-operated wild-type mice.

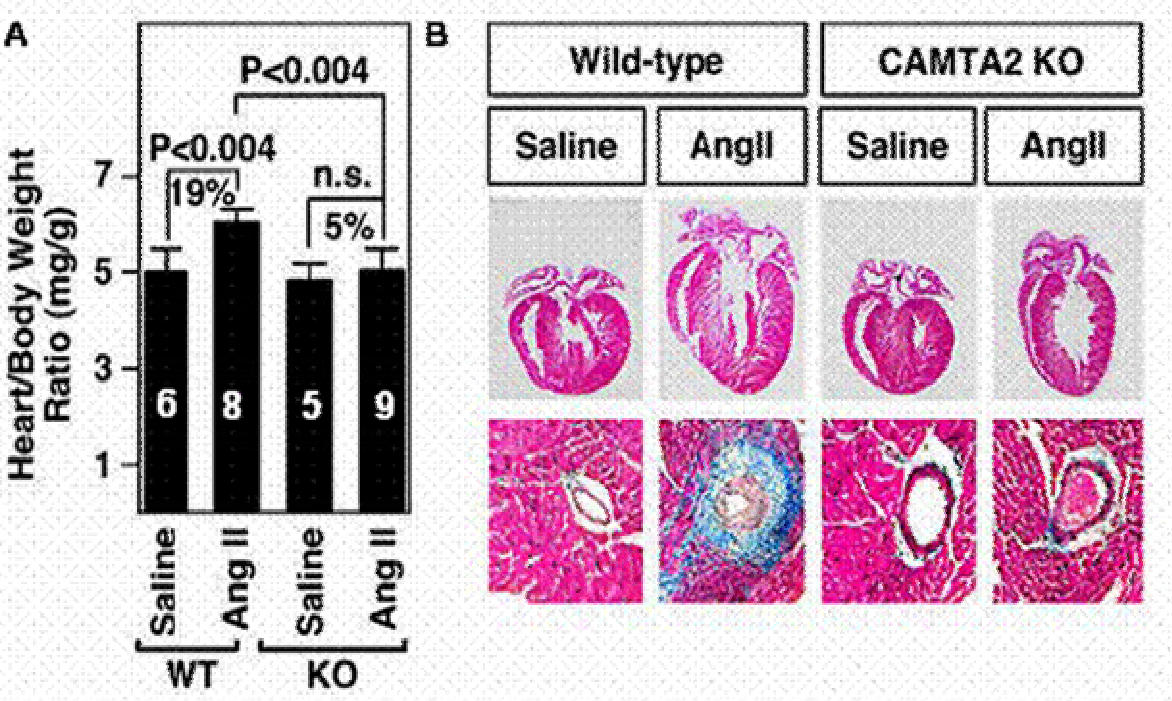

Figure 2.22. CAMTA2 knockout mice are compromised in their ability to mount a cardiac hypertrophic response to angiotensin II. (A) Wild type and CAMTA2 null mice were subjected to chronic infusion of saline or AngII and heart weight/body weight ratios (± S.D.) were determined after 14 days. The number of mice per group was shown in the bar. **(B)** Histological sections of representative hearts from H are shown. The lower panels are stained with Masson Trichrome to detect fibrosis.

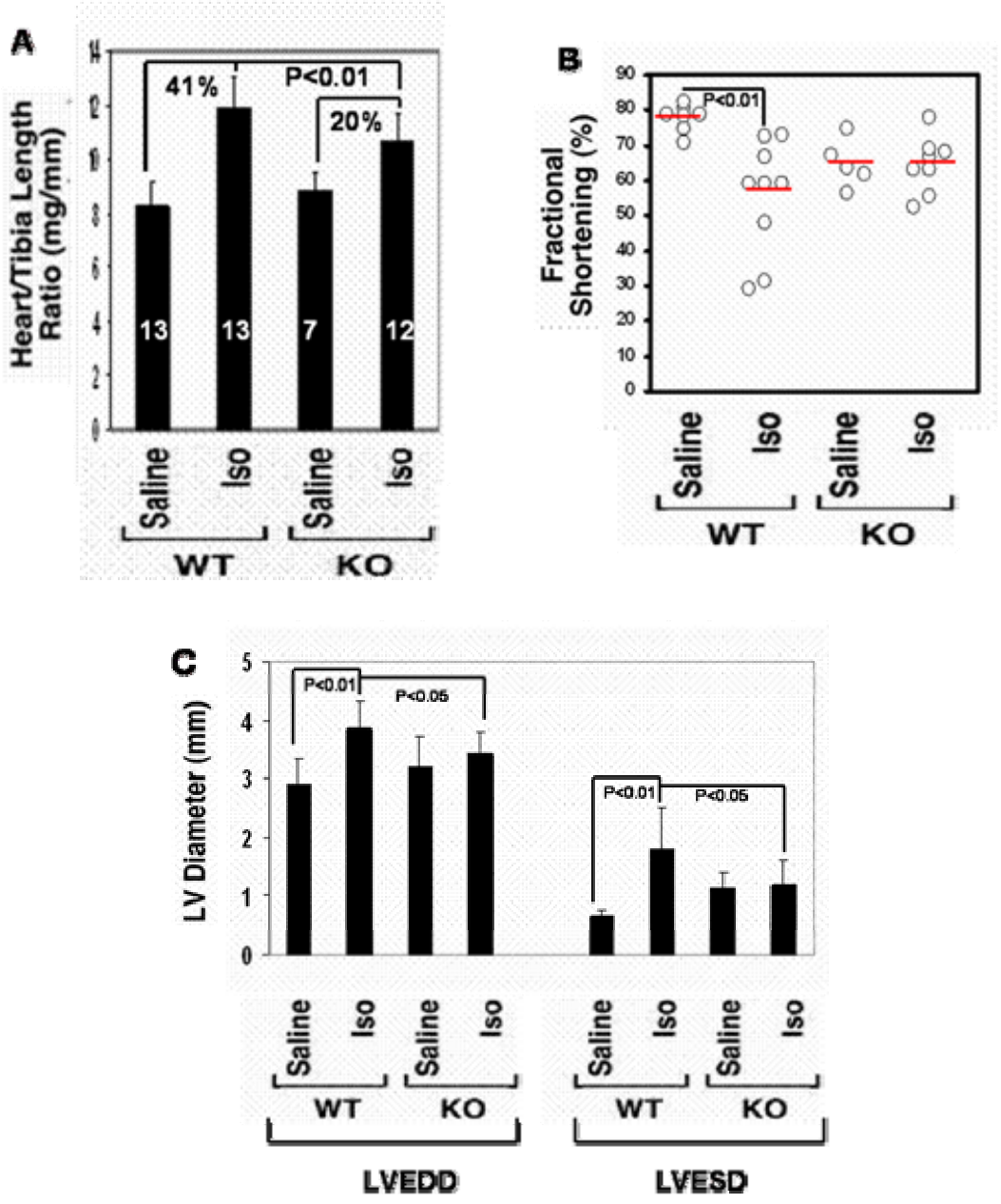

Figure 2.23. CAMTA2 knockout mice are compromised in their ability to mount a cardiac hypertrophic response to isoproterenol. (A) Wild-type and CAMTA2 null mice were subjected to chronic infusion of saline or isoproterenol and heart weight/tibia length ratios (± SD) were determined after 7 days. The number of mice per group was shown in the bar. **(B)** Echocardiographic measurement of fractional shortening. There was a significant decrease in

fractional shortening in wild type mice infused with isoproterenol (n=10) compared with untreated wild type mice (n=6). There was no significant difference between isoproterenol-treated (n=9) and untreated (n=5) CAMTA2 mutant mice. **(C)** Echocardiographic measurements of left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD). Wild type mice treated with isoproterenol show dilated left ventricle. However, chronic infusion of isoproterenol did not result in cardiac dilation of CAMTA2 knockout mice.

Cardiac defects in CAMTA1-deficient mice

CAMTA2 knockout mice are viable, but compromised in their ability to mount a maximal hypertrophic response to multiple stimuli. Reprograming of fetal genes is one characteristic of cardiac hypertrophy in the adult heart in response to stress (Olson and Schneider, 2003). No apparent phenotype in embryonic hearts of CAMTA2 knockout mice was observed. CAMTA1 is strongly expressed in embryonic heart (Figure 2.24C), suggesting that the loss of CAMTA2 in embryonic heart could be compensated by CAMTA1. To test this hypothesis, I obtained a gene trap ES cell line that interrupts the CAMTA1 gene. This ES cell line was used for blastocyst injection. The resulting chimeric mice were bred to C57BL/6 mice to obtain germ-line transmission of the mutant allele. These CAMTA1$^{GT(pGT0Lxf)}$ mice (referred to here as CAMTA1$^{GT/+}$) have an insertion in intron 4 (Figure 2.24A), which is predicted to result in a fusion between the CAMTA1 protein after amino acid residue 87 and the βgeo protein. Homozygous CAMTA1$^{GT/GT}$ mice from intercrosses of CAMTA1$^{GT/+}$ mice were born, but were underrepresented at P1 (9% of littermates, rather than 25% as expected) (Table 2.1). Insertion of vector pGT0Lxf leads to hypomorphic mutation of CAMTA1 (Figure 2.24B). I observed two major classes

of phenotypes in CAMTA1$^{GT/GT}$ mice: type 1 was quit severe and resulted in 16% of embryos dying during embryogenesis with heart defects, and type 2 was milder, resulting in live-born mice, of which all died before 6 weeks. This suggests that the dosage of CAMTA1 is critical. At E12.5, CAMTA1$^{GT/GT}$ mice of type 1 showed cardiac defects (Figure 2.25). The ventricular myocardium of hearts from these embryos at E12.5 was extremely thin and hypocellular, and less trabiculation was observed in mutant hearts (Figure 2.25). Mild ventricular septal defects (VSDs) were observed in some embryonic hearts of CAMTA1$^{GT/GT}$ mice. These findings suggest that CAMTA1 is required for cardiac morphogenesis and growth during embryogenesis.

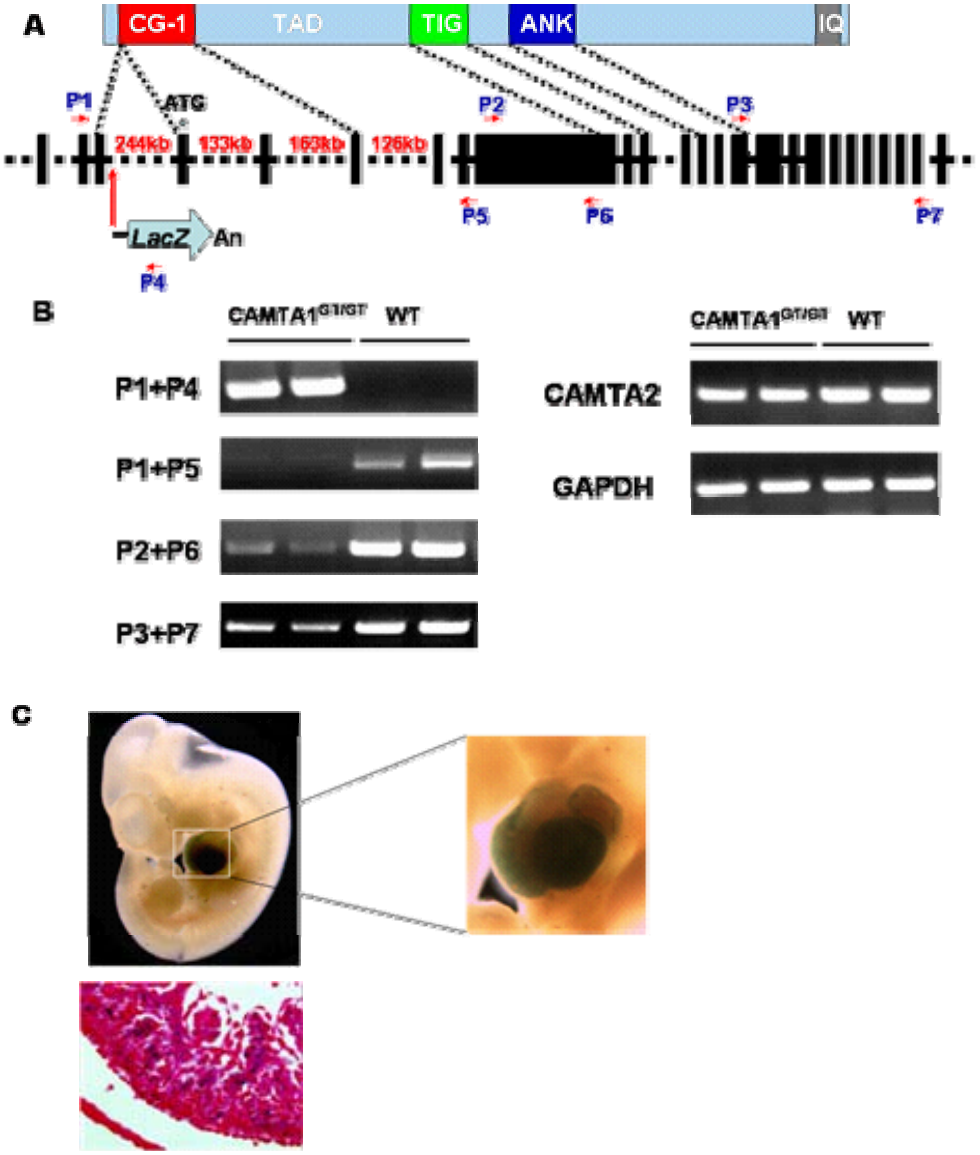

Figure 2.24. Interruption of the CAMTA1 gene in a gene trap mouse line. **(A)** CAMTA1 protein (top) and schematic of gene trap allele (bottom) were shown. **(B)** Detection of CAMTA1 and CAMTA2 transcripts in hearts of wild-type and CAMTA1$^{GT/GT}$ mice using RT-PCR. Positions of PCR primers were shown in panel (A). **(C)** Expression pattern of CAMTA1 in embryos at E11.5. Expressions of lacZ from gene trap allele. Box show region expanded in the right panel (top). Light eosin staining of histological sections of CAMTA1$^{GT/GT}$ embryos at E11.5 prestained for *lacZ* (bottom).

Table 2.2. Genotypes of offspring from intercrosses of CAMTA1GT$^{/+}$ mice at P1.

Genotype	observed (no.)	predicted (%)	observed (%)
+/+	35	25	29
+/-	73	50	61
-/-	11	25	9

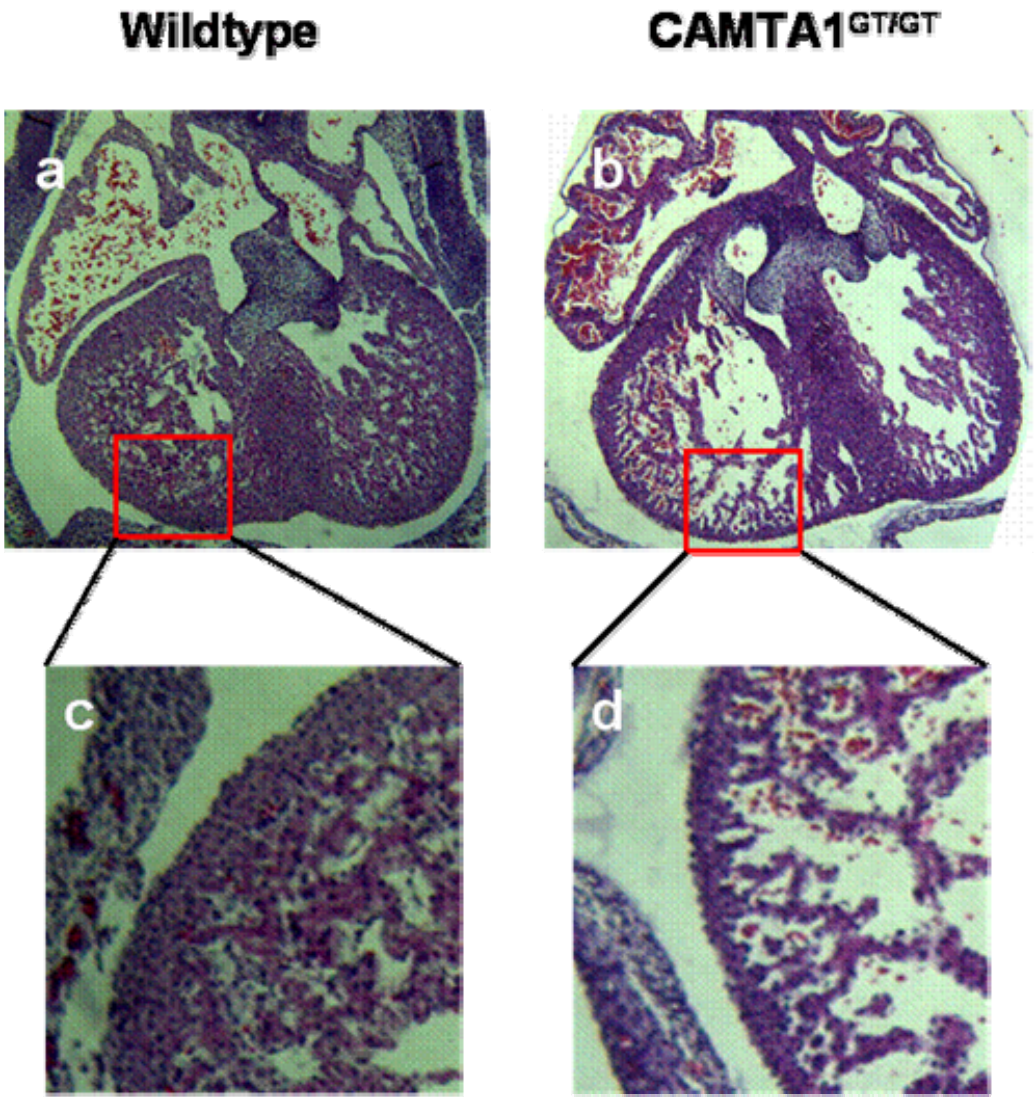

Figure 2.25. Abnormalities in cardiac development in CAMTA1 mutant mice. H and E sections of wild-type and CAMTA1 mutant embryos at E12.5. Panels (a) and (b) show whole hearts at E12.5. Boxes show regions of the heart expanded in panels (c) and (d). Note the thin-wall myocardium, less trabiculation, and smaller left ventricle in the mutant.

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, I discovered CAMTA2 as a powerful activator of cardiac growth and gene expression and counter-regulator of the growth inhibitory activity of class II HDACs.

Identification of regulators using a high-throughput expression screening strategy

One of my goals was to develop a method to identify molecules that regulate the expression of interesting genes. The features of this system should by high-throughput, rapid, low cost, and efficiency.

Understanding the function of transcriptional networks offers a forum to modulate genetic expression and provide new therapeutic biomedical research options. Identification of activators of known transcription factors or novel transcription factors is helpful to dissect the mechanism of the transcriptional networks in development and disease. The most commonly used genetic system to identify partners of a protein is the yeast two-hybrid system (Fields and Song, 1989). However, there are at least two disadvantages when the yeast two-hybrid system is used to identify cofactors of a transcription factor. First, since the readout of this system is mainly based on transcription, the transactivation domain of the target transcription factor must be deleted when this protein is fused to a

DNA binding domain. Therefore, if a protein interacts with this transcription factor in the transactivation domain, it cannot be identified using this system. Second, using a partial protein domain of this transcription factor may cause misfolding of the protein due to the absence of the transactivation domain and/or fusing the partial protein to a DNA binding domain. Recently, a recruitment system was developed to identify cofactors of a protein based on the fact that a Ras-GEF, hSos, is activated by its recruitment to the plasma membrane (Aronheim, 2000). This method also has some limitations in screening cofactors of transcription factors. First, an endogenous transcription factor functions in the nucleus, and this recruitment system localizes the transcription factor to the cytoplasm, perhaps altering the protein conformation or translational modifications of the transcription factor now localized to the cytoplasm instead of the nucleus. Second, fusing the cDNA libraries to Ras-GEF may result in misfolding of the cofactor proteins and abrogate protein-binding interactions.

I have developed a different method from the above two strategies. This method is based on small pool expression screening (Lusting et al., 1997), combined with a cell transfection assay. A small pool of cDNA constructs will be co-transfected with a reporter driven by a interesting promoter and/or selected transcription factor into cultured mammalian cells. Determination of positive cDNA pools is based on quantification of a reporter driven by the selected transcription factor. In this system, the target transcription factor and proteins encoded by a cDNA library are not modified, which should contribute to maintaining the authentic protein conformation and translational modifications. This

system also has the potential to identify not only physically interacting partners of the transcription factors, but also molecules that indirectly regulate transcription factor activity such as some components of a signaling pathway controlling the transcription factor and novel transcription factors (Figure 2.1).

This novel strategy described in this proposal offers a high-throughput, rapid, low cost, screen to identify regulators of transcription factors and novel transcription factors. Two months after I discovered CAMTAs using this strategy, a calcium-responsive transactivator (CREST) was identified using the same strategy and published in *Science* (Aizawa et al., 2004).

The CAMTA family of transcriptional factors

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). Mutations in the fly CAMTA, DmCAMTA, cause defects in photoresponse termination, and DmCAMTA activity is regulated by interaction with calmodulin (Han, et al., 2006).

Through mutational analysis, we identified multiple evolutionarily conserved functional domains of CAMTA2 (Fig. 2.26A). 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 first 250 amino acids of CAMTA2 is sufficient to bind to DNA. 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 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 in mammals.

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 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.

Over-expression 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 up-regulated during hypertrophy (Thompson et al., 1998; Saadane et al., 1999). Over-expression 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). My 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., 2002; Small et al., 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.

Low dosage of CAMTA1 results in abnormalities in embryonic hearts. It will be interesting to examine whether CAMTA1 modulates the developmental functions of Nkx2-5.

Activation of *myogenin* and *β-MHC* genes by CAMTA via DNA binding

Arabidopsis AtCAMTA and fly DmCAMTA were shown to bind to the CGCG box within promoters (Mitsuda, et al., 2003; Han, et al., 2006). There is only one CAMTA gene in the fly. A truncation mutation of DmCAMTA does not lead to lethality (Han et al., 2006). There are two CAMTAs in the mouse and human. Low dosage of CAMTA1 in mice leads to partial embryonic lethality, suggesting mammalian CAMTA proteins could be different in molecular mechanism and biological function. My discoveries indicate that mammalian CAMTA proteins activate target genes by at least two mechanisms. The first one is that CAMTA is recruited by other transcription factors to promoters. CAMTA modifies transcriptional activity of the transcription factor through physical interaction. For example, CAMTA activates the *ANF* gene, at least in part, by association with Nkx2-5. The second mechanism is that CAMTA behaves as a transcription factor which activates genes by directly binding to cis-elements within promoter. The following observations support this mechanism. 1) CAMTA activates the *myogenin* and *β-MHC* promoters in COS and Hela cells. 2) Gel mobility shift assay performed with purified recombinant CAMTA proteins suggests that CAMTA directly associates with DNA in vitro. 3) ChIP assay indicates that endogenous CAMTA1 associates with *myogenin* and *β-MHC*

promoters in native chromatin. It will be interesting to identify CAMTA binding elements in the future.

Signaling to CAMTA via class II HDACs

PKC signaling is a powerful inducer of cardiac growth (Dorn and Force, 2005). I 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 de-repression 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 2.26B). 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. 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 hypersensitive 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.

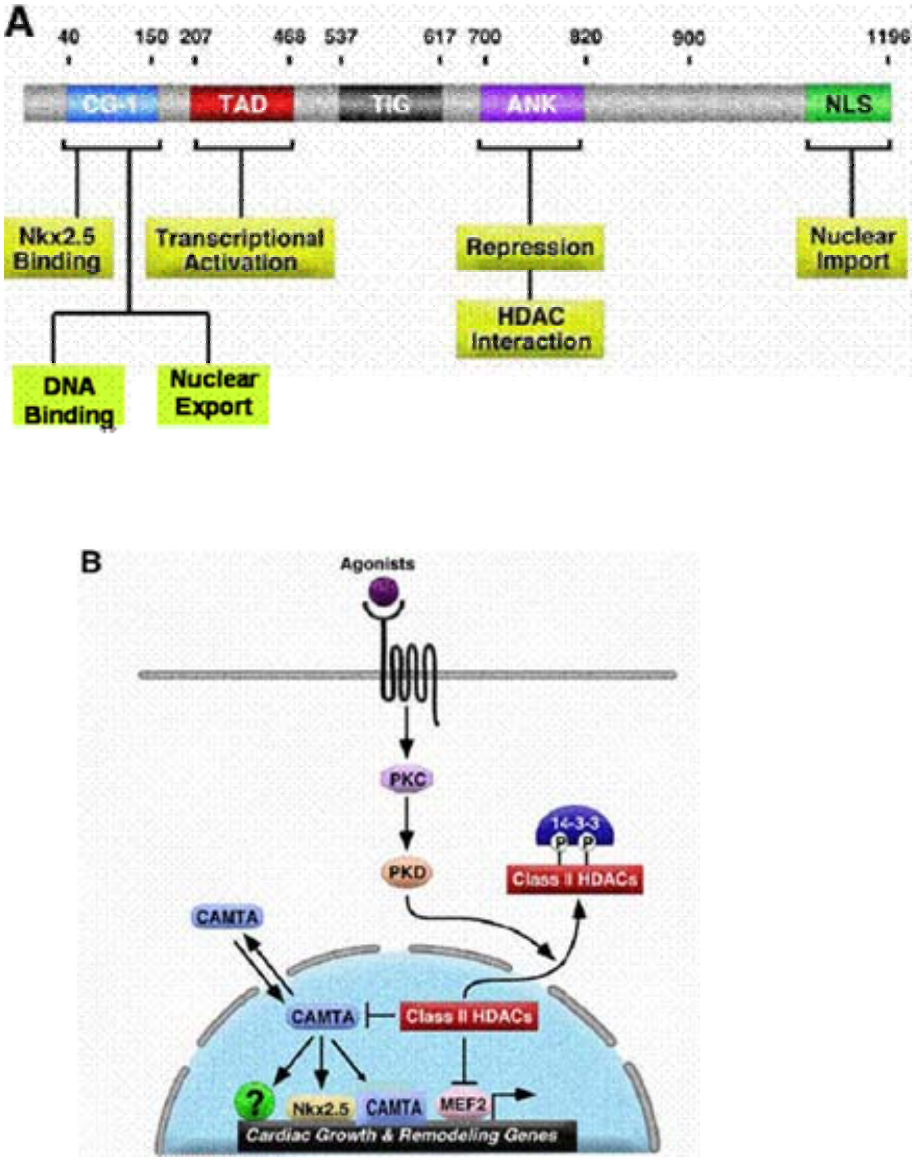

Figure 2.26. Domains of CAMTA2 and a model of CAMTA function in cardiac growth and remodeling signaling. (A) The functional domains of CAMTA2 are shown. **(B)** CAMTA cycles between the cytoplasm and the nucleus and stimulates the activity of Nkx2-5 and possible other as yet unidentified transcription factors. CAMTA also activates target genes by direct binding to DNA. CAMTA 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 CAMTA from repression and promoting cardiac growth.

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., 2002; Schonberger et al., 2005; Murakami et al., 2005).

I 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 mechanisms 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.

CAMTA1 is up-reguated in differentiating C2C12 myotubes. CAMTA1 associates with the *myogenin* promoter in vivo. Myogenin is a key transcription factor to control

skeletal muscle differentiation. Myogenin is up-regulated in differentiating muscle cells. Previous studies indicate that elements required for muscle specific expression of *myogenin*, and growth factor responsiveness are located within 184 nucleotides of the *myogenin* transcription initiation site (Cheng, et al., 1992; Edmondson, et al., 1992, Cheng, et al., 1993). My results showed that CAMTA1 associated with -146 to +1 relative to the myogenin transcription initiation site. In the future, it will be interesting to investigate if CAMTA controls skeletal muscle growth and/or fusion through regulating myogenin expression and/or other targets.

It has been shown that CAMTA1 could be a tumor suppressor of neuroblastoma (Katoh and Katoh, 2003; Henrich, et al., 2007). Neuroblastoma, a cancer of the sympathetic nervous system, and of undifferentiated neuroblasts, is the most common cancer in infancy and accounts for 9% of all childhood cancers. Reduced expression of CAMTA1 correlates with adverse outcome in neuroblastoma patients (Henrich, et al., 2006). It's interesting that association between neuroblastoma and congenital heart disease such as hypoplasia of the ventricular myocardium, and ventricular septum defect (VSD) was observed (Holzer and Franklin, 2002). My results show that CAMTA1 and CAMTA2 are involved in cardiac growth, especially, that CAMTA1 is involved in embryonic heart development. In the future, it will be interesting to investigate if CAMTA1 is the bridge between repression of neuroblastoma and regulation of cardiac development and growth.

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VITAE

Kunhua Song was born in Yunnan province, China, on January 16, 1972, the son of Shuifu Song and Feng Liu. After completing his work at the First Middle School of Yuxi, Yunnan, China in 1991, He entered Fudan University, China. During September of 1991 and July of 1992 he attended Dalian Academy of Military, China. He received the degree of Bachelor of Science with a major in biology from Fudan University in July, 1996. He entered Zhijiang University in September of 1996. He received the degree of Master in 1999. During the following one year he was employed as a research specialist at Tumor Hospital, Sun Yat-sen University of Medical Sciences. In August, 2000 he entered the Graduate School at University of Arkansas, Fayetteville, Arkansas. He was awarded the degree of Master of Science in May, 2002. In August 2002, he entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. He joined the laboratory of Dr. Eric Olson in May 2003. He graduated with a Ph.D. degree in March, 2007. He married Yan Hou in July 1999. His son, Alex Yuqian Song, was born in February 16, 2004.

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