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Calreticulin Signals Upstream of Calcineurin and MEF2C in Early Cardiac Development

by

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A thesis submitted to the faculty of Graduate Studies and Research

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ABSTRACT

Calreticulin is a Ca^{2+}-binding protein essential for heart development. At present, there is an incomplete understanding of: 1) genes of the fetal cardiac program; 2) transcription factors involved in activation of these genes; and, 3) Ca^{2+}-dependent signaling pathways involved in activation of these transcription factors. It is believed that a better understanding of Ca^{2+}-dependent signaling pathways will permit identification of novel drug targets to prevent, treat, and perhaps even cure many forms of cardiomyopathy. In this study, I investigated what cardiac transcription factors regulate cardiac expression of the calreticulin gene and identified myocyte enhancer factor 2C (MEF2C) as a strong activator. Examination of MEF2C cellular localization in calreticulin-deficient cells enabled identification of a novel signaling pathway in cardiomyocytes linking Ca^{2+} released by calreticulin to calcineurin activation and dephosphorylation of MEF2C. Calcineurin dephosphorylation of MEF2C carboxyl-terminus was necessary for MEF2C to translocate to the nucleus to impact target genes. The finding that MEF2C activates the calreticulin gene suggests the existence of a novel positive feedback mechanism in cells. In this feedback, MEF2C targets the gene of a Ca^{2+}-binding protein thereby ensuring an adequate supply of releasable Ca^{2+} be maintained within the cell for calcineurin activation and MEF2C nuclear translocation. Results of this investigation emphasize the importance of calreticulin in early stages of cardiac development and suggest a possible molecular explanation for embryonic lethality observed in calreticulin-deficient mice as well as insight as to how expression of activated-calcineurin in the heart rescues calreticulin-deficient phenotype.

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I would like to thank members of my supervisory committee, Dr. Luis Agellon and Dr. Michael Schultz. They were always helpful and I greatly benefited from their knowledge and input.

Monika Dabrowska, Sandi Aldred, Yue Dai, Irfan Ahsan, Dr. Anna Zuppini, and Dr. Lei Guo are current and former members of the Michalak Lab which I would like to thank. All made the work place a fun and positive environment. In particular, I would like to acknowledge Lei. She is a true friend and the best post-doc I have ever encountered.

The heart diagrams presented in Chapter One of this thesis were graciously drawn by my talented and beautiful fiancée, Kaari Chilibeck. She makes life extremely enjoyable and fun and I am so thankful to have her.

Also, I would like to thank my parents, Keith and Yola Lynch. They are fantastic parents and great friends. They have helped me so much and so many times. I am so very fortunate and so very grateful to have them.

Finally, I would like to acknowledge both the Alberta Heritage Foundation for Medical Research and the Heart and Stroke Foundation of Canada for financial support during my Ph.D. studies. I am honoured to have been a recipient of such precious awards.

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LIST OF ABBREVIATIONS

ABC method: Avidin-biotin complex method

ANF: Atrial natriuretic factor

AngII: Angiotension II

APMSF: 4-amidinophenylmethanesulfonyl fluoride hydrochloride

ATP: Adenosine triphosphate

BMP: Bone morphogenetic protein

BNP: B-type natriuretic peptide

bp: Base-pairs

BSA: Bovine serum albumin

CaM: Calmodulin

CaMK: Calmodulin-dependent protein kinase

CaMK*: Constitutively-active calmodulin-dependent protein kinase

CaN: Calcineurin

CaN*: Constitutively-active calcineurin

CBP: CREB-binding protein

cDNA: Complementary DNA

CHD: Congenital heart disease

ChIP: Chromation immunoprecipitation

CIAP: Calf intestinal alkaline phosphatase

CICR: Ca^{2+} induced Ca^{2+} release

CKII: Casein protein kinase II

CnA: Calcineurin A subunit

CnB: Calcineurin B subunit

COUP-TF: Chicken ovalbumin upstream promoter transcription factor

CP: Calreticulin promoter

CPF: Calreticulin promoter full-length

CREB: Cyclic adenosine (cAMP) monophosphate response element-binding protein

CRM1: Chromosome region maintenance protein 1

CRT: Calreticulin

CsA: Cyclosporin A

DAB: Diamino-benzidine tetrahydrochloride

DARP32: Dopamine and cyclic AMP (adenosine monophosphate) regulated phosphoprotein

dHAND: *Deciduum*, heart, autonomic nervous system and neural crest derivatives-expressed protein 2

DHPR: Dihydropyridine receptor

DMEM: Dulbecco's modified eagle medium

DNA: Deoxyribonucleic acid

dNTPs: Deoxyribonucleotide triphosphates

DTT: Dithiothreitol

ECL: Enhanced chemiluminescence

EDTA: Ethylene diamine tetra-acetic acid

eHAND: Extraembryonic tissues, heart, autonomic nervous system and neural crest derivatives-expressed protein 1

EGTA: Ethylene bis(oxyethylenenitrilo) tetra-acetic acid

EMSA: Electrophoretic mobility shift assay

ER: Endoplasmic reticulum

ES: Embryonic stem cell

ET-1: Endothelin-1

Evi-1: Ecotropic viral integration site-1

FBS: Fetal bovine serum

FKBP12: FK506-binding protein-12

FOG-2: Friend of GATA-2

GFP: Green fluorescent protein

GSK-3β: Glycogen synthase kinase-3β

HAT: Histone acetyl transferase

HDAC: Histone deacetylases

IGF-1: Insulin-like growth factor 1

$InsP_3$: Inositol-1,4,5-triphosphate

$InsP_3R$: Inositol-1,4,5-triphosphate receptor

Irx4: Iroquois homeobox protein 4

L-type Ca^{2+} channels: Long-lasting Ca^{2+} channels

MADS: MCM1, *Agamous*, *Deficiens*, and serum response factor

MAPK: Mitogen-activated protein kinase

MEF2: Myocyte-enhancer factor 2

MHC: Myosin heavy chain

MLC: Myosin light chain

mRNA: Messenger ribonucleic acid

NFAT: Nuclear factor in activated T-cells

NKE2: Nkx-2.5 response element-2

NLS: Nuclear localization signal

NMDA: N-methyl-D-aspartate

NP40: Nonidet P40

NRS: Nuclear retention signal

OCT compound: Optimal cutting temperature compound

ONPG: O-nitrophenyl-β-D-galactopyranoside

PBS: Phosphate buffered saline solution

PCR: Polymerase chain reaction

PKC: Protein kinase C

PMCA: Plasma membrane Ca^{2+}-transporting ATPases

Poly dI•dC: Poly(deoxyinosinic-deoxycytidylic)

Ptx2: Pituitary homeobox 2 transcription factor

RNA: Ribonucleic acid

RSRF: Related to serum response factor

RT-PCR: Reverse-transcriptase polymerase chain reaction

RyR: Ryanodine receptor

SAP domain: SAF-A/B, *Acinus*, PIAS domain

SD-PCR: Step-down PCR

SDS: Sodium dodecyl sulfate

SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase

SR: Sarcoplasmic reticulum

SRF: Serum response factor

TBX5: T-box 5 transcription factor

TNT: Transcription and translation

TD-PCR: Touch-down polymerase chain reaction

TLCK: Nα–p-Tosyl-L-lysine chloromethyl ketone hydrochloride

TPCK: N-p-Tosyl-phenylalanine chloromethyl ketone

wt: Wild-type

Y1H: Yeast-one hybrid

ZF: Zinc-finger

LIST OF OLIGONUCLEOTIDES UTILIZED IN THIS STUDY

OLIGOS FOR CALRETICULIN PROMOTER DELETION MUTANTS:

5' primer for CP8:

5'- ATTGAGCTCGTGTTAAAGCAGTG -3'

5' primer for CP7:

5'- ATTGAGCTCTTGACCTCAAGTG -3'

5' primer for CP6:

5'- ATTGAGCTCCTGCTTATCTCCC -3'

5' primer for CP5:

5'- ATTGAGCTCTAGAACTATCACC -3'

5' primer for CP4:

5'- AATGAGCTCTGTGGGAGACCACAAG -3'

3' primer for calreticulin promoter deletion mutants:

5'- CCTGAAGATCGTTTTAAAAAGCTTAAT -3'

FORWARD OLIGOS FOR EMSA EXPERIMENTS:

- Complementary reverse primers not shown.
- Potential MEF2C binding sites underlined.

Desmin-ME2C site (positive control; Naya *et al.*, 1999) forward primer

5' - TTCTCCT**CTATAAATAC**CAGCTCTG - 3'

Predicted calreticulin promoter MEF2C site #1 forward primer

5' – CATAGTGCGACC**AATAGAAATC**AGCCATCTGGG - 3'

Predicted calreticulin promoter MEF2C site #2 forward primer

5' - GAGAAAGAGAAAA**AAAAAAAATC**AAAAAACCCC - 3'

Predicted calreticulin promoter MEF2C site #3 forward primer

5' – GGAAGCACGC**CATTTTTGTA**AGGATTCCTTCTG - 3'

Predicted calreticulin promoter MEF2C site #4 forward primer

5' – AGCTTT**TAATTTTTATT**TTATTTGTATAGATGT - 3'

Positive control for MEF2C site #2 forward primer

5' - GAGAAAGAGAAAA**CTAAAAATAA**AAAAAACCCC - 3'

GL28-pXP1 ***(single-stranded oligo for EMSA reaction mixture)***

5'- CACTGCATTCTAGTTG -3'

OLIGOS FOR ChIP EXPERIMENTS:

5' primer for MEF2C cDNA cloning into pcDNA3.1/myc-His(-)A

5'- CCGCTCGAGCGGATGGGGAGAAAAAAGATTCAG -3'

3' primer for MEF2C cDNA cloning into pcDNA3.1/myc-His(-)A

5'- CGCGGATCCTGTTGCCCATCCTTCAGAG -3'

5' primer for GATA6 cDNA cloning into pcDNA3.1/myc-His(-)A

5'-
CCGCTCGAGCGGATGTACCAGACGCTCGCCGCTCTGTCCAGCCAGGGGCCCG
CCGCTTACGACGGCGC -3'

3' primer for GATA6 cDNA cloning into pcDNA3.1/myc-His(-)A

5'-
CGCGGATCCGGCCAGGGCCAGAGCACACCAAGAATCCTGTCGCACCGAGGA
TGTAACTTCGGC -3'

MEF2C ChIP forward primer

5'- CCCTTGCCTTCTGCCGTTTATTG -3'

MEF2C ChIP reverse primer

5'- CCCGTAGTTCCCTGCAGCCTGT -3'

GATA6/Evi-1 ChIP forward primer

5'- CAGGTACTGTTCTTCCTCCTCC -3'

GATA6/Evi-1 ChIP reverse primer

5'- GCTGTCCACGGTTCAAGAGC -3'

OLIGOS FOR CREATION OF GFP PLASMIDS:

5' primer for GFP-MEF2C

5'- CCGCTCGAGCAATGGGGAGAAAAAAGA -3'

3' primer GFP-MEF2C

5'- CCCAAGCTTTGATGTTGCCCATCC -3'

5' primer for GFP-MEF2C-NRS-NLS

5'- CCGCTCGAGCGCATATGGGCTCTGTAACTGGCTGGCAGCAG -3'

3' primer for GFP-MEF2C-NRS-NLS

5'- CGCGGATCCTCATGTTGCCCATCCTTCAGAG -3'

5' primer for GFP-MEF2C-NLS

5'-
CCGCTCGAGCGCATATGCACTCCCCCATTGGACTCACCAGACCTTCGCCGGA
CGAAAGGGAAAGTCCTTCAGTCAAGCGCATGCGACTCTCTGAAGGATGGGCA
ACATGAGGATCCGCG -3'

3' primer for GFP-MEF2C-NLS

5'-
CGCGGATCCTCATGTTGCCCATCCTTCAGAGAGTCGCATGCGCTTGACTGAAG
GACTTTCCCTTTCGTCCGGCGAAGGTCTGGTGAGTCCAATGGGGGAGTGCAT
ATGCGCTCGAGCGG -3'

5' primer for GFP-MEF2A

5'- CCGCTCGAGCGATGGGGCGGAAGAAAATACAAATCAC -3'

3' primer for GFP-MEF2A

5'- CCGGAATTCTTAGGTCACCCACGCGTCCATCCTCATTCGC -3'

5' primer for GFP-MyoD

5'- CCGAATTCGATGGAGCTTCTATCGCCGCC -3'

3' primer for GFP-MyoD

5'- CTAGTCTAGATCAAAGCACCTGATAAATCGC -3'

OLIGOS FOR EMBRYO GENOTYPING:

TO DETECT CALRETICULIN GENE:

Calreticulin gene forward primer (T8)

5'- CTCCAGGTCCCCGTAAAATTTGCC -3'

Calreticulin gene reverse primer (T19)

5'- AGGTCTAAACCAGTCAAAAGGACC -3'

TO DETECT NEOMYCIN CASSETTE DISRUPTION OF CALRETICULIN GENE:

Neomycin forward primer (Neo3'for)

5'- TCGTGCTTTACGGTATCGCCGCTCCCGATT -3'

Neomycin reverse primer (GL31)

5'- CGCGGATCCACCTCCCATGACAGCCATTTA -3'

LIST OF PRIMARY ANTIBODIES UTILIZED IN THIS STUDY

Anti-GFP: Goat polyclonal anti-green gluorscent protein antibody. Generous gift from Dr. Luc Berthiaume, University of Alberta.

Anti-HA: Anti-HA tag antibody. Mouse monoclonal anti-HA antibody. Roche Diagnostics, Cat. 1 867 423.

Anti-His: Anti-Histidine tag antibody. Mouse monoclonal anti-His antibody. Santa Cruz; Cat. SC-8036.

Anti-MEF2C: Polyclonal rabbit anti-MEF2C antibody. Cell Signaling Technology; Cat. 9792.

Ca^{2+}-DEPENDENT SIGNALING IN CARDIAC DEVELOPMENT AND PATHOLOGY

Versions of portions of this chapter have been published or submitted for publication:

1. Lynch, J., and Michalak, M. 2002. Ca^{2+}-dependent signaling pathways in the heart: potential drug targets for cardiac disease. *Curr. Drug Targets Cardiovasc. Haematol. Disord.* **2**:1-11.
2. Lynch, J and Michalak, M. 2003. Calreticulin is an upstream regulator of calcineurin. *Biochem. Biophys. Res. Commun.* **311**:1173-9.
3. Lynch, J., Guo, L., Gelebart, P., Chilibeck, K., Xu, J., Molkentin, J.D., Agellon, L.B., and Michalak, M. 2004. Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca^{2+}-dependent signaling pathway of early cardiac development. *Status: Submitted.*

Heart development

Formation of the heart in an embryo involves a precisely orchestrated series of molecular events. During heart development, even the most subtle of changes can have catastrophic consequences in the form of congenital heart disease (CHD). The physiological steps involved in heart development have been known for decades; however, little is really known about molecular aspects governing the development of this organ and genes controlling its developmental program. Because of its integrated structures and cell types, formation of an organ as complex as the heart involves highly regulated expression of numerous genes. Interestingly, it has been postulated that most of these genes are not cardiac specific (Srivastava and Olson, 2000).

After gastrulation, bone morphogenetic proteins (BMPs) are secreted from the endoderm (Schultheiss *et al.*, 1995) and these factors permit cardiomyocytes to originate in the anterior lateral mesoderm. BMPs activate expression of *Nkx2.5* gene, the earliest molecular marker of the cardiac lineage (Harvey, 1996). Nkx2.5 is a transcription factor that activates transcription of several genes, one of which is *Mef2* gene. *Mef2* gene encodes a transcription factor controlling myocyte differentiation (Gajewski *et al.*, 1998). In addition, Nkx2.5 interacts with zinc-finger transcription factors of the GATA family to activate cardiac gene expression (Durocher *et al.*, 1997). Nkx2.5 and GATA transcription factors regulate expression of one another through mutually reinforcing positive feedback loops (Schwartz and Olson, 1999). Although only a few transcription factors have been briefly described here as participating in heart development, the process is very complex and requires participation of many known (Frey and Olson, 2003; Srivastava and Olson, 2000) and likely several yet-to-be-identified transcription factors. Soon after their

specification, cardiac muscle cells converge along the ventral midline of the embryo to form a beating linear heart tube (Figure 1-1, *A*) composed of distinct myocardial and endocardial layers separated by an extracellular matrix (Srivastava and Olson, 2000). The linear heart tube is positioned along the anterior-posterior axis and the various regions and chambers of the looped and mature heart (Figure 1-1, *B*) are predestined (Srivastava and Olson, 2000). The molecular mechanisms governing cardiac looping (Figure 1-1, *C*) remain unknown, but identification of genes differentially expressed along the outer and inner curvatures of the looped heart tube suggest that intrinsic properties of the two surfaces permit this critical morphogenetic event. The direction of cardiac looping is influenced by an asymmetric axial signaling system that affects the position of other organs such as lungs, liver, and gut (Capdevila *et al.*, 2000). Interpretation of left–right signals is mediated in part by the transcription factor Ptx2, which is expressed along the left side of the early heart tube (Srivastava and Olson, 2000). Following cardiac looping, individual cardiac chambers become morphologically distinguishable (Figure 1-1, *C*). Interestingly each cardiac chamber differs in its morphological and contractile properties as well as patterns of gene expression (Srivastava and Olson, 2000). How the identity of each chamber is established remains a mystery, but it is believed to involve a combination of transcription factors (Figure 1-2). From the looped heart tube each cardiac chamber expands outward and neural crest cells populate the aortic arch arteries and aortic sac which together form segments of the mature aortic arch. Mesenchymal cells form cardiac valves from the conotruncal and atrioventricular valve segments (Srivastava and Olson, 2000). After separation of the aorta and pulmonary artery, the arteries rotate to achieve their final connections with the left and right ventricles,

FIGURE 1-1

Figure 1-1. Diagrams depicting stages of cardiac development. Morphologically related regions are colour coded. Cardiac precursor cells form a crescent (A) that has regions predestined to form segments of the linear heart tube (B). The heart tube undergoes looping (C) and each cardiac chamber balloons out to form the mature heart (D). Mesenchymal cells form the cardiac valves from the conotruncal (CT) and atrioventricular valve (AVV) segments. *Abbreviations*: A, atrium; Ao, aorta; AS, aortic sac; LA, left atrium; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RV, right ventricle; V, ventricle. Figure is modified from Srivastava and Olson (2000) and was graciously drawn by Kaari Chilibeck.

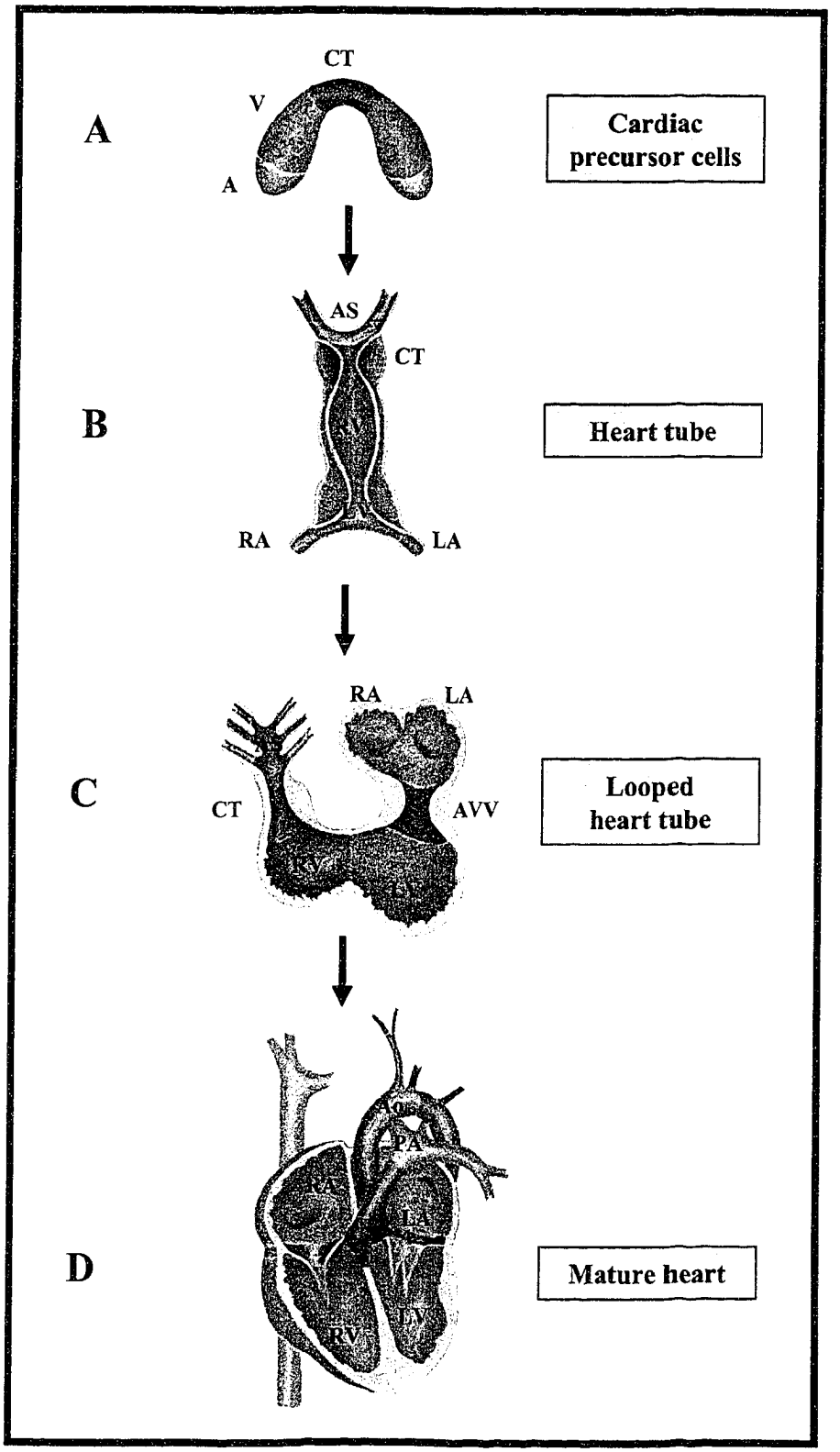
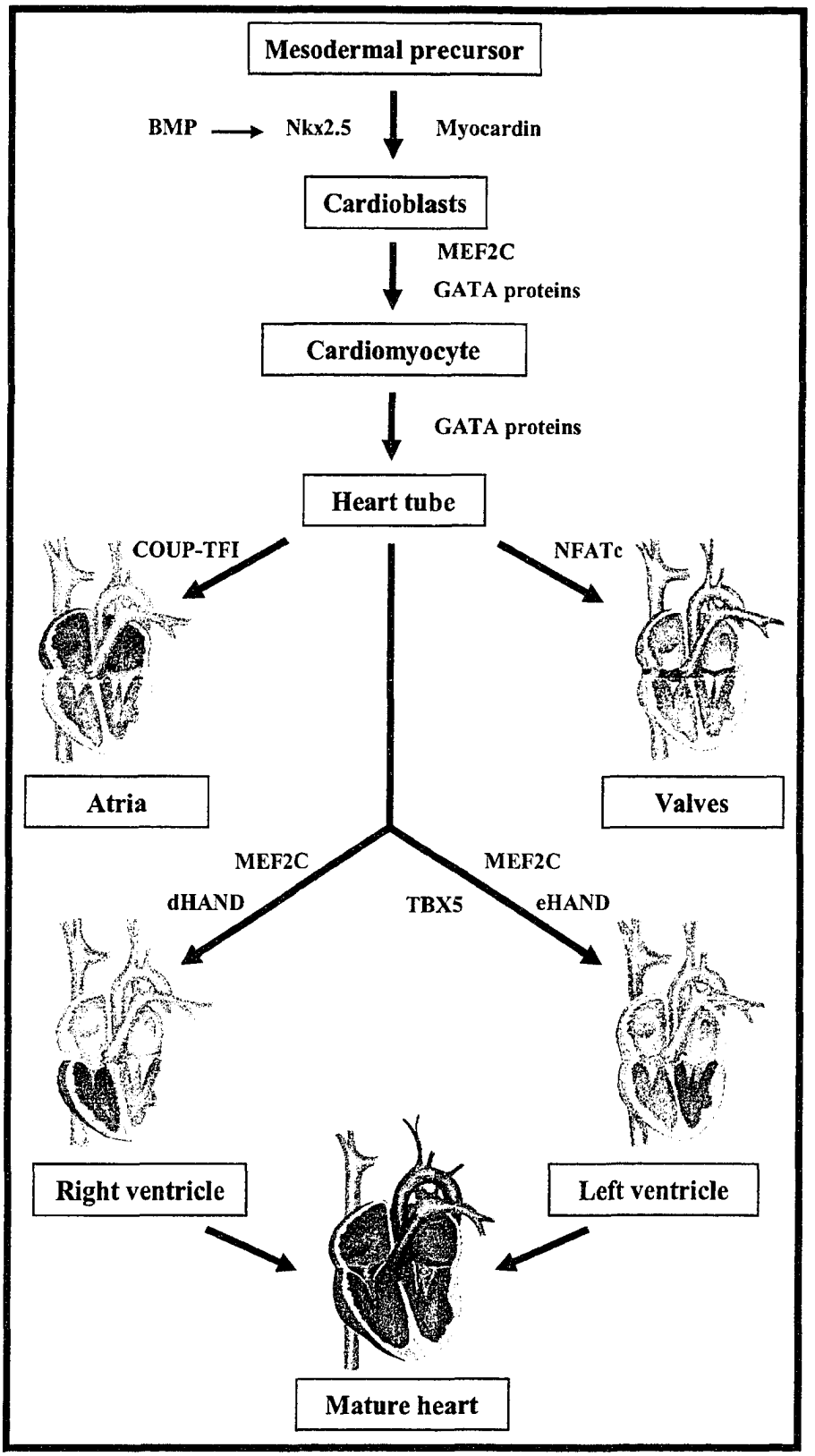


Figure 1-2. Diagram outlining steps of heart development. Early stages of heart development up to the linear heart tube stage are highly conserved among organisms. Distinct regulatory proteins and transcription factors (Nkx2.5, MEF2C, GATA, NFATc, COUP-TF1) known to serve particular roles during different stages of heart development are indicated. dHAND and eHAND transcription factors play an important role in right and left ventricle formation, respectively, while T-box 5 (TBX5) transcription factor plays a role in ventricular separation. *Abbreviations*: bone morphogenetic proteins (BMP); myocyte enhancer factor 2C (MEF2C); and, nuclear factor in activated T-cells c (NFATc). Figure is modified from Srivastava and Olson (2000) and was graciously drawn by Kaari Chilibeck.



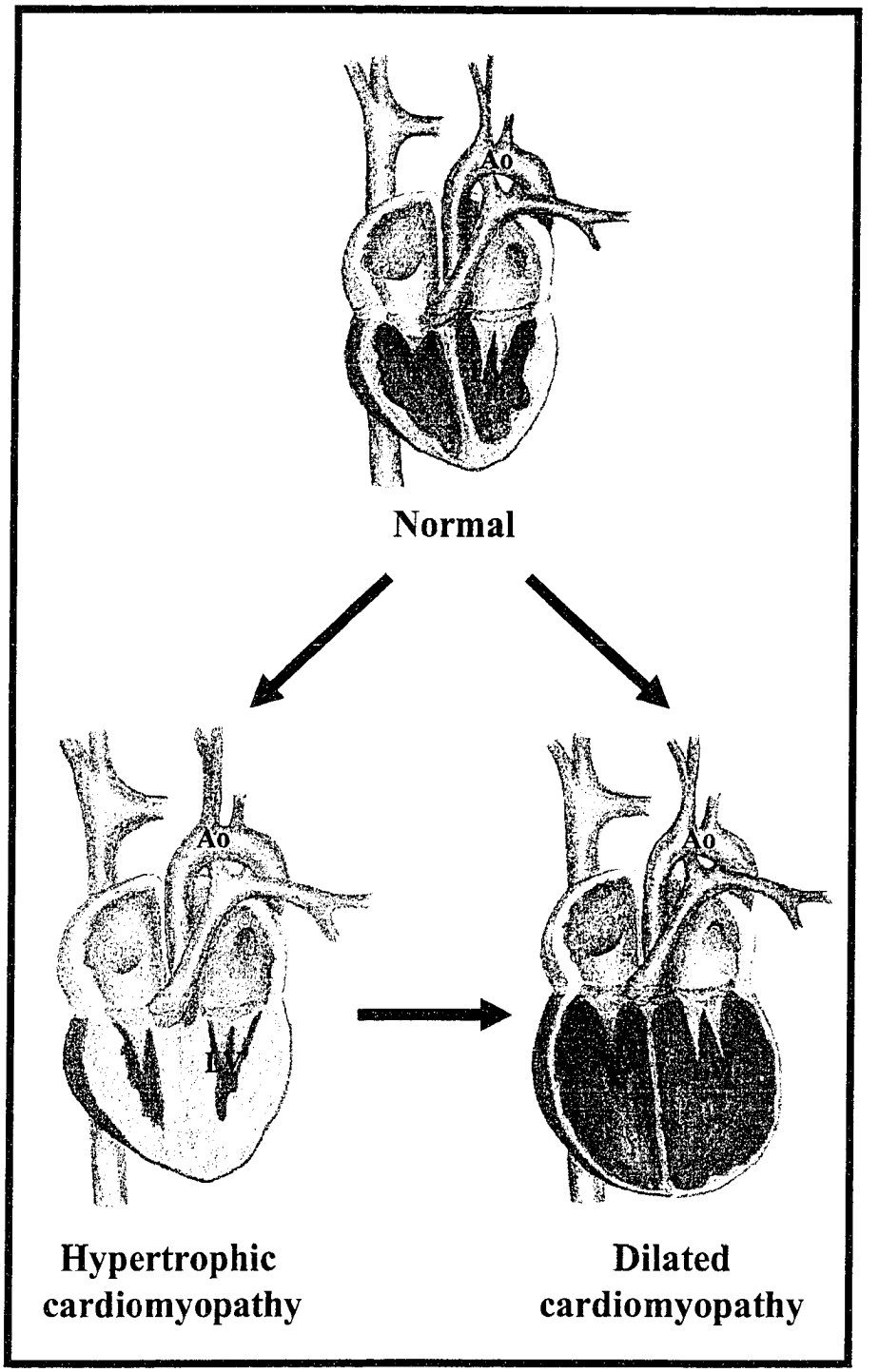
respectively. The final result is a mature, four-chambered heart (Figure 1-1, *D*).

Cardiac pathology

At present, the major cause of fatality in North America is heart disease (Hunter and Chien, 1999). As such, much research has been conducted so as to better understand this ailment. Heart-related mortality occurs due to CHD as well as a result of the two most common cardiac pathologies, cardiac hypertrophy and dilated cardiomyopathy (Figure 1-3). The condition of cardiac hypertrophy (Frey and Olson, 2003) arises when the body's need for oxygenated blood exceeds that supplied by the heart. The heart responds to this demand by increasing the size of the left ventricle thereby enhancing cardiac output. Cardiac hypertrophy is initially beneficial as it permits the heart to satisfy the demand of the body; however, prolonged stress eventually results in excessive hypertrophy. Well-documented characteristics of cardiac hypertrophy (Frey and Olson, 2003; Molkentin *et al.*, 1998) include: (1) an increase in cardiomyocyte size without cell division, (2) an overall enlargement and thickening of the heart muscle, (3) an increase in muscle mass, (4) an up-regulation of fetal cardiac genes, and (5) disorganization of sacromeres. Interestingly, excessive hypertrophy eventually causes the contractile ability of the heart to become weakened which results in heart failure and death.

A second very common type of cardiomyopathy is dilated cardiomyopathy. In dilated cardiomyopathy, the change experienced by the heart can be likened to a balloon being expanded (Figure 1-3). Dilated cardiomyopathy is characterized by: (1) enlargement of the heart with thinning of ventricular chamber walls, (2) a decrease in contractile elements, (3) microfilament disarray, and (4) interstitial fibrosis

Figure 1-3. Figure depicting two common cardiomyopathies. At the top is the picture depicting the relative size and ventricular wall thickness of a normal heart. The condition of cardiac hypertrophy is shown on the left while on the right is dilated cardiomyopathy. An arrow indicates that a hypertrophic heart can eventually worsen and become a dilated heart. The condition of dilated cardiomyopathy is severe and rapidly progresses to death. *Abbreviations*: A, atrium; Ao, aorta; LV, left ventricle. Figure was graciously drawn by Kaari Chilibeck.



(Chien, 1999). A condition of dilated cardiomyopathy rapidly progresses to death.

Acquired cardiovascular diseases such as cardiac hypertrophy or dilated cardiomyopathy can arise due to environmental factors (e.g., athlete's heart) and/or genetic susceptibility. It is likely that several intracellular signaling pathways are involved in initiation and progression of heart disease (Frey and Olson, 2003; Molkentin and Dorn, 2001; Srivastava and Olson, 2000) making studies on heart diseases equate to solving a very complex jigsaw puzzle (Chien, 1999). In this puzzle there are few obvious connecting pieces and many uncertain connections. The significance of these complicated webs of signaling pathways is only recently emerging with the help of molecular biology at the animal level. Findings from numerous laboratories over the past decade suggest that proteins involved in Ca^{2+} signaling cascades play a significant role in heart disease (Frey and Olson, 2003; Molkentin and Dorn, 2001; Molkentin *et al.*, 1998). Results within the past decade suggest that cardiomyopathies may arise as a result of: (1) disordered Ca^{2+} cycling (Frey *et al.*, 2000), (2) activation of fetal cardiac genes (Frey *et al.*, 2000; Frey and Olson, 2003; Molkentin *et al.*, 1998), and (3) compromised contractibility (Minamisawa *et al.*, 1999).

Research has shown that cardiac development and cardiac disease are very similar processes as both require transcriptional activation of many of the same genes (Frey and Olson, 2003; Srivastava and Olson, 2000). As such, a better understanding of cardiac development will provide valuable information which could potentially be utilized and applied in the design of novel drugs to treat and perhaps even cure some heart ailments. To better understand cardiac development, aspects of the fetal cardiac program and Ca^{2+}-dependent signaling pathways must be further deduced.

Ca^{2+} as a cytoplasmic signaling molecule

Ca^{2+} is a very important signal transduction element in cells (Berridge *et al.*, 1998; Berridge *et al.*, 2000; Clapham, 1995). Ca^{2+} regulates a variety of diverse cellular functions including contraction-relaxation, cell mobility, metabolism, protein synthesis and folding, gene expression, cell cycle progression, and activation of apoptosis (Berridge *et al.*, 2000; Ghosh and Greenberg, 1995; MacLennan, 2000; Putney, 2001). Thus, any alteration to intracellular Ca^{2+} signaling pathways or components of these pathways can have a profound physiological effect on cardiomyocytes (Chien, 1999; Chien, 2000; Kiriazis and Kranias, 2000). In cardiomyocytes, Ca^{2+} functions as an important secondary messenger for diverse chemical and physical signals including: (1) angiotensin II (AngII); (2) endothelin-1 (ET-1); (3) β-adrenergic agents, and (4) mechanical stretch. All of these signals can contribute to cardiac hypertrophy (Frey *et al.*, 2000). Thus, an understanding of the targets of Ca^{2+}, the functions of Ca^{2+}-binding proteins, and solving molecular aspects of Ca^{2+} signaling pathways will provide insight into the fetal cardiac program and help identify molecular differences between normal and diseased hearts.

External cellular concentration of Ca^{2+} is usually in the 1 to 2mM range (Berridge *et al.*, 2000; Clapham, 1995) and most membranes are impermeable to this ion. For most mammalian cells, the resting cytoplasmic Ca^{2+} concentration is low, approximately 100nM (Clapham, 1995). In order for cells to utilize Ca^{2+} as a universal signal, Ca^{2+} gradients must be maintained across the plasma membrane and within intracellular organelles. This is achieved by many membrane associated Ca^{2+} pumps and Ca^{2+} transport proteins. This includes plasma membrane Ca^{2+}-transporting ATPases (PMCA)

(Santiago-Garcia *et al.*, 1996; Shull, 2000), Na^+/Ca^{2+} exchanger (Philipson and Nicoll, 2000; Shigekawa and Iwamoto, 2001), sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Kimura *et al.*, 1998) and the mitochondrial uniporter (Bers, 2000). Of these membrane proteins, SERCA and the Na^+/Ca^{2+} exchanger transport the greatest amount of Ca^{2+} (Bers, 2000).

Heart contraction is necessary so that oxygenated blood can be delivered to various regions of the body. Heart contraction is mediated by a sequence of actions, beginning with a depolarization event in cardiomyocytes that induces conformation changes in long-lasting (L-type) Ca^{2+} channels (e.g., dihydropyridine receptor, DHPR) that permits these channels to open and Ca^{2+} to enter the cell. Ca^{2+} influx into the cytoplasm through L-type channels triggers ryanodine receptor (RyR) to release Ca^{2+} from the sarcoplasmic reticulum (SR) store in a mechanism known as "Ca^{2+} induced Ca^{2+} release" (CICR) (Berridge, 2002; Fabiato, 1985). RyR is negatively regulated by a high cytoplasmic Ca^{2+} concentration, so the frequency of opening is affected (Kiriazis and Kranias, 2000). Ca^{2+} released from the SR binds to the thin-filament protein troponin C (Katz, 1997) leading to muscle contraction (Bers, 2000). Following muscle contraction, some of the free Ca^{2+} (~30% depending on species) is extruded from the cardiomyocyte by the Na^+/Ca^{2+} exchanger while the majority of the remaining free Ca^{2+} is quickly pumped into the SR lumen by SERCA (Shigekawa and Iwamoto, 2001). Ca^{2+} pumped into the SR by SERCA is stored bound to Ca^{2+} buffers such as calsequestrin and the Ca^{2+} is readily available for release to participate in another contraction.

How Ca^{2+} impacts cellular function depends on the route of entry into the cell and pattern of modulation (Bading *et al.*, 1993; Chin and Means, 2000). The membrane

channels or intracellular receptors responsible for Ca^{2+} entry into the cytoplasm can have an effect on the Ca^{2+} signal (Ghosh and Greenberg, 1995). It is hard to image that an ubiquitous ion is capable of differential signaling, but duration, amplitude, and spatio-temporal patterning are all factors that determine what downstream targets will be activated by Ca^{2+} influx into the cytoplasm (Berridge *et al.*, 2000). Therefore, Ca^{2+} is capable of activating various signaling pathways due to versatility in its signaling and this is an area requiring further investigation.

SR/ER and Ca^{2+} signaling

The majority of Ca^{2+} stored within the cell is in the lumen of the SR and/or ER. Both the SR and ER store Ca^{2+} and relinquish their intracellular Ca^{2+} stores via Ca^{2+} release channels, RyR and inositol-1,4,5-triphosphate receptor ($InsP_3R$). The ER is an extensive membrane network throughout the cytoplasm (Clapham, 1995; Papp *et al.*, 2003) and the common paradigm is that SR is essentially specialized ER for muscle; however, it has been hypothesized that SR and ER are distinct functional organelles (Mesaeli *et al.*, 2001). Support for this hypothesis comes from studies on formation of SR during skeletal and cardiac muscle development which found that SR is not a continuation of existing ER membranes (Flucher and Franzini-Armstrong, 1996; Franzini-Armstrong and Jorgensen, 1994). Additional support for this hypothesis has come from investigations which found that cardiomyocytes contain an $InsP_3$-sensitive ER-like compartment functionally distinct from the SR (Jaconi *et al.*, 2000; Lipp *et al.*, 2000). Thus, it can be concluded that the majority of Ca^{2+} stored within the cell is in the lumen of the SR and/or ER.

The ER is an organelle capable of storing a substantial amount of Ca^{2+}. The total

concentration of Ca^{2+} in the ER is very high and estimated to be 1 to 3mM (Corbett and Michalak, 2000; Michalak *et al.*, 2002). Most of the ER Ca^{2+} is bound to chaperone proteins (Meldolesi and Pozzan, 1998), but a significant amount is unbound. The free Ca^{2+} concentration in the ER ranges from 60 to 400μM when stores are full, and from 1 to 50μM when ER stores are empty (Corbett and Michalak, 2000; Meldolesi and Pozzan, 1998). Hence, during agonist stimulation, free Ca^{2+} concentration in the ER can fluctuate substantially, from 400μM to 1μM. Ca^{2+}-binding chaperones that reside in the ER sense these changes in free Ca^{2+} and react by modulating Ca^{2+} homeostasis (Michalak *et al.*, 2002). Subsequently, changes in free Ca^{2+} concentration can impact ER functions including: 1) protein synthesis (Michalak *et al.*, 1999); 2) protein folding (Lodish *et al.*, 1992); 3) interaction of chaperones with substrates (Baksh *et al.*, 1995; Corbett *et al.*, 1999; Lodish *et al.*, 1992; Ware *et al.*, 1995); 4) cell adhesion (Johnson *et al.*, 2001; Michalak *et al.*, 1999); and 5) transcriptional activation/suppression of genes associated with stress or metabolic processes (Mesaeli *et al.*, 1999).

Our current understanding of components of Ca^{2+} signaling pathways indicate that downstream Ca^{2+}-dependent proteins cannot be activated without significant Ca^{2+} release from intracellular Ca^{2+} stores, the SR/ER compartments. Hence, Ca^{2+}-binding proteins of the SR/ER represent important components of intracellular Ca^{2+} signaling cascades. Although a very complex model depicting cardiac Ca^{2+}-depending signaling cascades is beginning to emerge (Frey and Olson, 2003), our understanding of all the proteins involved in these events is far from complete. At present, there is a limited understanding of: 1) genes of the fetal cardiac program; 2) transcription factors involved with activation of these genes; and, 3) Ca^{2+}-dependent signaling pathways that participate in activation of

these transcription factors. A better understanding of Ca^{2+}-dependent signaling pathways will permit identification of novel drug targets to prevent, treat, and perhaps even cure some forms of cardiomyopathy. The purpose of this study was to investigate Ca^{2+}-dependent signaling cascades essential for embryonic heart development with a major emphasis on the role played by an ER Ca^{2+}-binding protein, calreticulin.

Calreticulin

Calreticulin is an ER luminal Ca^{2+} binding chaperone highly conserved in mammals (Burns *et al.*, 1994; Eggleton and Michalak, 2003; Michalak *et al.*, 1992). In humans, the mature protein (46 kDa) consists of a 17-residue ER signal sequence and 400 amino acids (Johnson *et al.*, 2001; Michalak *et al.*, 1999). The protein possesses three distinctive regions, N-domain (amino acid residues 1 to 180), P-domain, (amino acid residues 181 to 280), and C-domain (amino acid residues 281 to 400). The three-dimensional structure of the calreticulin P-domain (amino acid residues 189 to 288) has been deduced by NMR (Ellgaard *et al.*, 2001a; Ellgaard *et al.*, 2001b) and appears to be a single hairpin fold. The N-domain is the most conserved region (Krause and Michalak, 1997) and is hypothesized to possess ATP and zinc-binding sites (Corbett *et al.*, 2000). In comparison, the P-domain, so named because it is proline-rich, has lectin-binding sites and the P-domain is important for the chaperone function of calreticulin. The P-domain contains three two amino acid sequence repeats of 17 (PXXIXDPDAXKPEDWDE; type 1 repeat) and 14 (GXWXPPXIXNPXYX; type 2 repeat) amino acids, respectively, in an "111222" arrangement (Krause and Michalak, 1997; Michalak *et al.*, 1999). The P-domain binds Ca^{2+} with low-capacity and high-affinity whereas the C-domain serves to bind Ca^{2+} with high-capacity, but low-affinity (Baksh and Michalak, 1991). The C-

domain of calreticulin is highly negatively-charged, binding 25mol of Ca^{2+} per mol of protein (Baksh and Michalak, 1991; Ellgaard *et al.*, 2001a). At its amino-terminus, calreticulin has a signal sequence and at the carboxyl-terminus it possess an ER KDEL retrieval sequence (Fliegel *et al.*, 1989a; Fliegel *et al.*, 1989b), so the cellular targeting of calreticulin is restricted to the ER lumen (Opas *et al.*, 1996). The unique domains of calreticulin and its near mM concentrations in the ER lumen (Tatu and Helenius, 1997) permit it to serve several cellular functions including: (1) maintaining Ca^{2+} homeostasis (Baksh and Michalak, 1991; Michalak *et al.*, 1998), and (2) as a molecular chaperone (Helenius *et al.*, 1997; Leach, 2002).

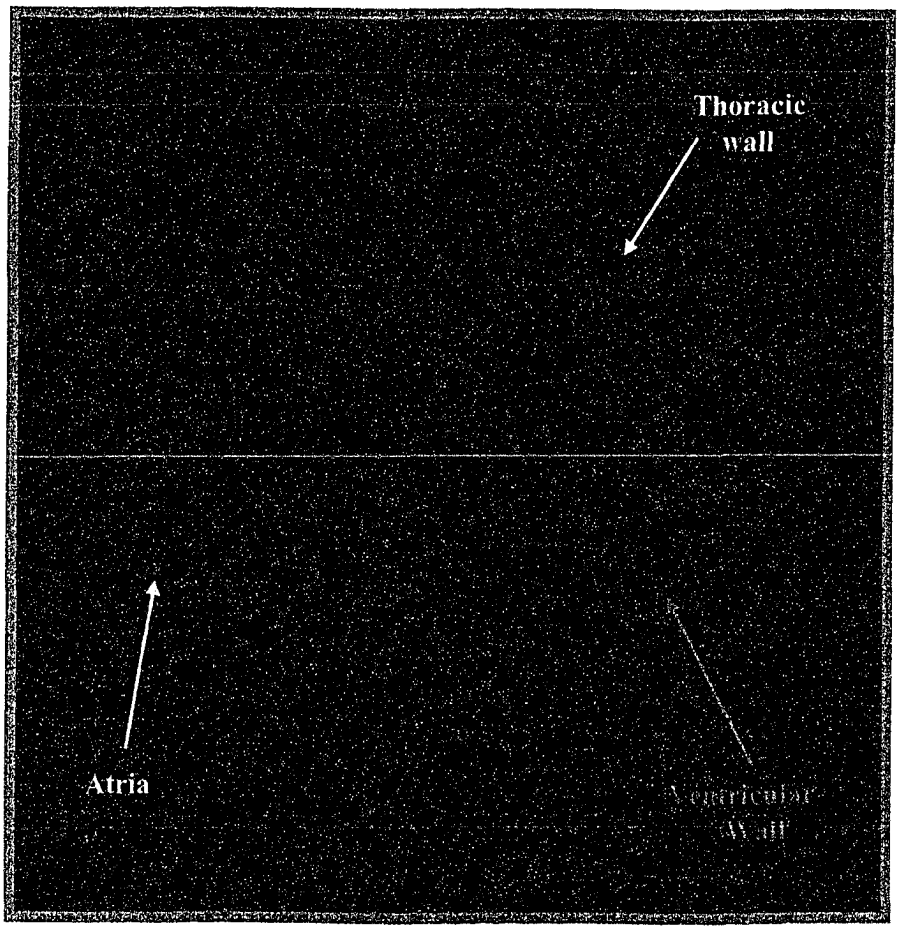
Elevated cytoplasmic Ca^{2+} concentration was achieved by emptying ER Ca^{2+} stores and resulted in enhanced expression of calreticulin (Waser *et al.*, 1997). This finding suggests that the cell may adapt to a decrease in ER Ca^{2+} or an elevation in cytoplasmic Ca^{2+} by increasing its concentration of Ca^{2+}-buffer proteins in the ER lumen. This likely occurs by having the enhanced Ca^{2+} concentration activate a component of the Ca^{2+} signaling pathway that terminates with activation of specific transcription factor(s). Hence, it is probable that feedback mechanisms exist in cells, so that transcription factors activated by Ca^{2+} target a variety of genes including genes which encode for Ca^{2+} buffer protein(s) or perhaps even proteins which influence nuclear import/export.

Mice with the calreticulin gene disrupted have been created (Mesaeli *et al.*, 1999) and the defect is embryonic lethal. Calreticulin null-mutant embryos die at embryonic day 14.5 (E14.5), where term is approximately 21 days. The calreticulin-deficient animal dies as a result of severe underdevelopment of the ventricular region of the heart (Mesaeli *et al.*, 1999). To understand expression of the calreticulin gene, transgenic mice with

green fluorescent protein (GFP) under control of 1722bp of calreticulin promoter were created (Mesaeli *et al.*, 1999). Calreticulin promoter driven GFP expression in the heart of these transgenic mice was observed from E9.0, reaching maximal expression at E14.5 (Figure 1-4). After E15.5, expression of GFP steadily declined and was not detectable in hearts of three week-old mice (Mesaeli *et al.*, 1999). Similar findings of calreticulin expression were observed through immunocytochemical analyses of fetal and neonatal rat hearts (Imanaka-Yoshida *et al.*, 1996). Therefore, calreticulin is highly expressed in the developing heart; however, expression of calreticulin gene is tightly regulated following birth and calreticulin protein is weakly expressed in the healthy, mature heart.

To determine what happens when calreticulin gene is not down-regulated following heart development, mice with cardiac over-expression of calreticulin were created. Interestingly, these animals died approximately 21-days after birth as a result of complete heart block, bradyarrhythima, and sudden cardiac death (Nakamura *et al.*, 2001a). L-type Ca^{2+} channels, which are activated and inactivated by a Ca^{2+} (Imredy and Yue, 1994), were observed at a decreased density in calreticulin over-expressor mice (Nakamura *et al.*, 2001a). Most importantly, these transgenic animals demonstrated how disruption of components of Ca^{2+} signaling pathways can lead to cardiac disease as well as confirmed that decreased expression of calreticulin after birth is essential to maintain normal cardiac function.

Figure 1-4. Calreticulin is highly expressed in the developing heart. This figure is a sagittal section though an E14.5 heart of transgenic mouse expressing GFP under control of calreticulin promoter. GFP expression can be seen to be very high throughout the atria and ventricular regions. Reproduced from *The Journal of Cell Biology*, 1999, **144**: 857-868 by copyright permission of The Rockefeller University Press.



Mesaeli *et al.*, 1999

It can be concluded that calreticulin is one protein essential for heart development. In the developing heart, calreticulin gene is highly expressed, but this protein is virtually absent from the healthy, mature heart. When calreticulin is over-expressed in the developed heart dilated cardiomyopathy arises and death occurs. Thus, expression of calreticulin gene must be tightly regulated. Therefore, the starting-point of this study was to determine which transcription factors of the fetal cardiac program regulated expression of calreticulin gene.

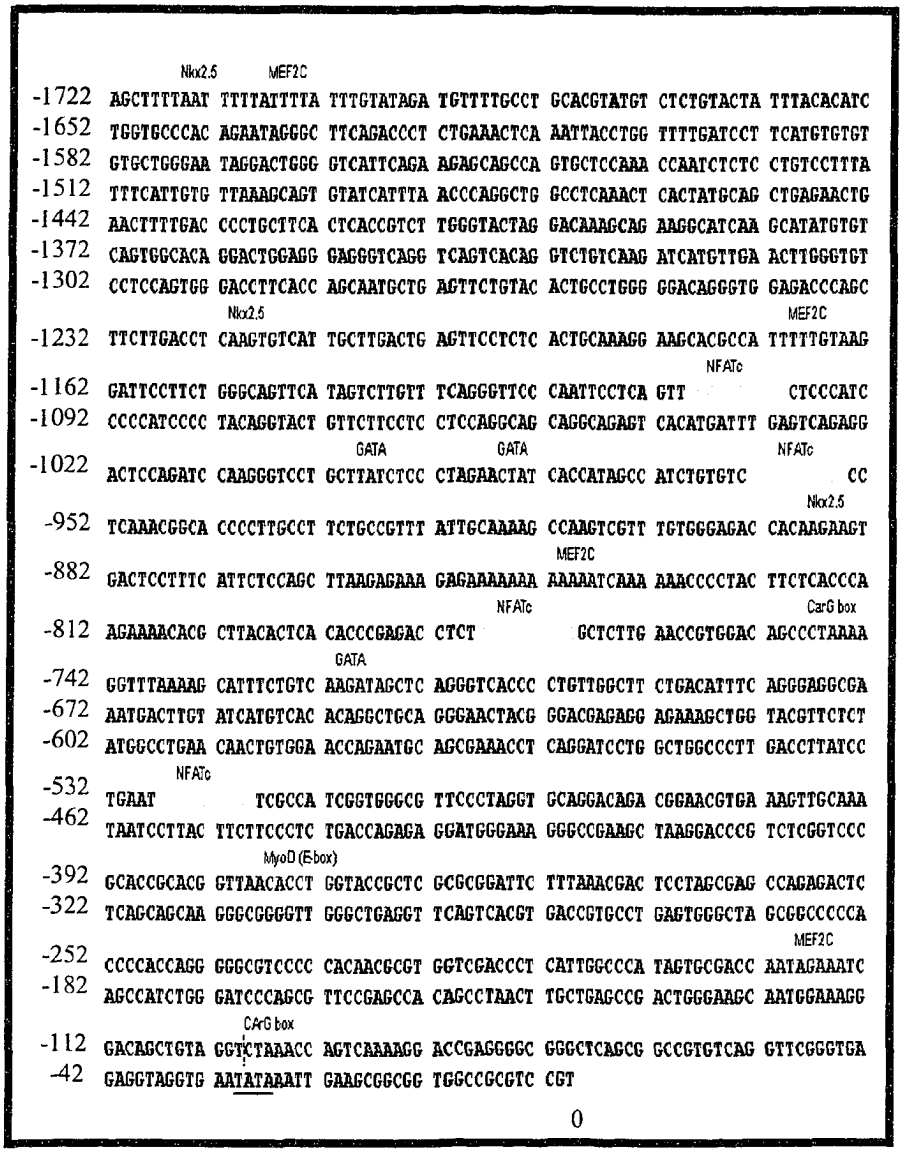
Potential transcriptional regulators of the calreticulin promoter

Previously, 1722bp of calreticulin promoter (Figure 1-5; GenBank Accession number: U38249) was isolated from a mouse liver genomic library and subcloned into a promoterless luciferase expression vector, pXP1 (de Wet *et al.*, 1987), producing plasmid pLC1, respectively (Waser *et al.*, 1997). This plasmid has since been renamed to CPF (Calreticulin Promoter Full-length). Examination of the sequence of CPF revealed several tentative binding sites (Figure 1-5) for several well documented transcription factors, many of which are expressed in the developing heart. Preliminary experiments with the CPF luciferase reporter gene plasmid were conducted to determine exactly which transcription factors regulated calreticulin promoter. Described below are the transcription factors investigated as potential regulators of calreticulin gene expression.

Nkx2.5

Nkx2.5 is the vertebrate homologue of the *Drosophila* gene *tinman* and is a homeobox transcription factor. The homeodomain consists of 60 amino acids folded into a stable helix-turn-helix motif. Nkx2.5 binds with high-affinity to the NKE2 (Nkx-2.5 response element-2) site, CTCAAGTGT (Durocher *et al.*, 1996). Nkx2.5 is a nuclear protein (Kasahara and Izumo, 1999) whose expression has been reported in developing pharyngeal arches, spleen, thyroid, tongue and heart (Komuro and Izumo, 1993; Buchberger *et al.*, 1996). In the heart, Nkx2.5 physically interacts with other transcription factors such as GATA4 (Durocher *et al.*, 1997; Lee *et al.*, 1998) to synergistically enhance gene expression (Molkentin, 2000b). The calreticulin promoter has three tentative Nkx2.5 binding sites (Figure 1-5), of which one site has been confirmed to bind this transcription factor (Guo *et al.*, 2001).

Figure 1-5. There are several tentative transcription factor binding sites within calreticulin promoter. The nucleotide sequence of the mouse calreticulin promoter (GenBank Accession: U38249). The nucleotides are numbered with the putative transcriptional initiation site of the mouse calreticulin gene at +1 (Smith and Koch, 1989) and nucleotides after ATG have been omitted. All tentative binding sites for all transcription factors studied in this investigation are indicated. TATA box is underlined.



Nkx2.5 is currently regarded as one of the earliest embryonic markers for cells that have acquired cardiogenic potential (Black and Olson, 1998), appearing in mice during embryonic day 7 (Searcy *et al.*, 1998). Nkx2.5 is essential for cardiac development and disruption of the *Nkx2.5* gene in mice is embryonic lethal before E9 as a result of severe heart defects (Lyons *et al.*, 1995). In these null-mutant embryos, cardiac development is arrested at the linear heart tube stage and the heart tube does not undergo looping (Lyons *et al.*, 1995; Schott *et al.*, 1998). At present, only five gene promoters [ANF (atrial natriuretic factor), MEF2C, GATA6, myocardin, calreticulin] have been identified as targets of Nkx2.5 (Durocher *et al.*, 1997; Guo *et al.*, 2001; Molkentin *et al.*, 2000a; Skerjanc *et al.*, 1998; Small and Krieg, 2003; Ueyama *et al.*, 2003). Research has also found that positive feedback mechanisms exist between Nkx2.5 and GATA (Searcy *et al.*, 1998) factors as well as exists between Nkx2.5 and myocardin (Ueyama *et al.*, 2003). It is probable that feedback mechanisms serve to enhance expression of the transcription factors. Expression of Nkx2.5 in hearts of mice continues throughout development into adult life, but the function associated with continued expression remains unknown (Komuro and Izumo, 1993; Searcy *et al.*, 1998).

Myocardin

Promoters for many muscle genes have a nucleotide sequence known as a CArG box ($CC(A/T)_6GG$) and it serves as the binding site for serum response factor (SRF) (Shore and Sharrocks, 1995). CArG boxes have been identified in promoters of genes expressed in skeletal, smooth, and cardiac muscle and this includes normal and diseased hearts (Parlakian *et al.*, 2004). However, because SRF is required for mesoderm formation (Arsenian, 1998), researchers were unable to determine the role of SRF in

heart formation. Recently, development of Cre-loxP technology has permitted an embryo to be created that lacks SRF only in the heart. Disruption of SRF gene was found to be lethal at E10.5 due to cardiac defects such as abnormally thin myocardium, dilated cardiac chambers, poor trabeculation, and a disorganized interventricular septum (Parlakian *et al.*, 2004). Examination of E9.5 SRF-deficient hearts showed that there was a marked reduction in expression of essential regulators of heart development, including Nkx2.5, GATA4, myocardin, and c-fos (Parlakian *et al.*, 2004). Thus, SRF is a global regulator of multiple developmental genes and is essential for cardiac differentiation and maturation.

SRF belongs to the MADS box family of transcription factors (Miano, 2003). The name MADS is derived from the first four member identified MCM1 (a yeast factor involved in mating selection), *Agamous*, *Deficiens* (homeotic factors that control leaf identity in plants), and serum response factor) to possess this conserved deoxyribonucleic acid (DNA)-binding domain (Black and Olson, 1998). The MADS box has conserved residues for DNA-binding (Black and Olson, 1998) and is important as it mediates homodimerization as well as recruits a variety of transcriptional cofactors that influence DNA binding affinity, transcriptional activity, and target gene specificity (Treisman, 1994). An attribute of MADS domain proteins is their ability to activate different sets of genes through association with other transcription factors. For example, SRF has been shown to cooperate with GATA4 (Belaguli *et al.*, 2000; Morin *et al.*, 2001) as well as Nkx2.5 (Chen and Schwartz, 1996), but SRF does not form a stable ternary complex with either of these factors on DNA (Wang *et al.*, 2001). However, SRF forms associations with accessory factors and these factors permit SRF to regulate transcription of numerous

muscle and growth factor-inducible genes. As SRF is not muscle specific, it has been postulated to activate muscle genes by recruiting accessory factors such as myocardin (Wang *et al.*, 2001).

Myocardin consists of 807 amino acids and belongs to the SAP (SAF-A/B, *Acinus*, PIAS) domain family of nuclear proteins. The SAP domain is a 35 amino acid putative DNA-binding motif with two predicted α-helices separated by a region of glycines (Wang *et al.*, 2001). The SAP domain of myocardin is common in a variety of proteins that influence transcription (Aravind and Koonin, 2000). Other notable features of myocardin include a basic region, an extended α-helix that resembles a leucine zipper, and a stretch of glutamine residues (Wang *et al.*, 2001).

Myocardin is localized in nuclei of smooth and cardiac muscle cells (Wang *et al.*, 2001). Although present in cardiac tissue at all times, cardiac expression of myocardin is higher in the neonatal heart than the adult heart (Torrado *et al.*, 2003). As development progresses, myocardin is found throughout the developing atrial and ventricular chambers of the heart until birth (Wang *et al.*, 2001). Northern blot analysis of adult mouse tissues revealed multiple myocardin transcripts specifically in heart (Wang *et al.*, 2001). Immunohistochemical analysis of mouse embryos indicated that myocardin was present in the cardiac crescent during embryonic day 7 (Wang *et al.*, 2001), very soon after expression of Nkx2.5, the earliest known marker for cardiogenic specification (Lints *et al.*, 1993) To determine if Nkx2.5 appeared in the developing heart before myocardin, subtractive hybridization using RNA isolated from wild-type and E8.5 Nkx2.5-null hearts was completed and mouse complementary DNA (cDNA) encoding myocardin isolated (Ueyama *et al.*, 2003). Expression of myocardin was significantly down-regulated in

Nkx2.5-null mouse hearts (Ueyama *et al.*, 2003). Reporter gene analysis studies indicated that Nkx2.5 activates the myocardin promoter (Ueyama *et al.*, 2003). Thus, in heart development Nkx2.5 appears before myocardin and the myocardin promoter is a target of Nkx2.5.

Myocardin appears very early in heart development and is a very potent activator of cardiac muscle promoters (Wang *et al.*, 2001). Myocardin acts preferentially through multiple CArG boxes (Wang *et al.*, 2001) and is known to strongly activate promoters for SM22 (a calponin-like protein that associates with cytoskeletal actin filaments), ANF, myosin light chain-2v (MLC-2v), α-myosin heavy chain (α-MHC), as well as serves as an enhancer for the *nkx2.5* gene (Wang *et al.*, 2001). The calreticulin promoter (Figure 1-5) lacks an ideal CArG box.

Mice lacking myocardin die by E10.5 because of the absence of vascular smooth muscle cells (Li *et al.*, 2003). Somewhat unexpected for the researchers, cardiac development was observed to be normal in the homozygous embryos (Li *et al.*, 2003). The reason for this observation may be that myocardin plays a minor role in early heart development (i.e. prior to E10.5) and/or embryonic death occurred before the absence of myocardin in the heart became an issue. Also, it is possible that other transcription factors compensate for loss of myocardin and sustain cardiac expression of myocardin specific target genes. For example, a SM22-lacZ transgene (having 1343bp of SM22 promoter linked to lacZ) was introduced into the myocardin null background by breeding and examination of hearts from these mice indicated that SM22-lacZ expression was removed from the dorsal aortae, but not from the heart or somites (Li *et al.*, 2003). Thus, other transcription factors likely activate SM22 expression in the heart and myocardin is

required specifically for vascular smooth muscle gene expression. Considering the phenotype of myocardin mutant mice as well as findings from studies that demonstrated that myocardin is essential for activation of smooth muscle genes (Chen *et al.*, 2002; Wang *et al.*, 2003), it would seem that the primary function of myocardin is for smooth muscle development. However, since myocardin is found in the heart very early in development and its targets include promoters for some cardiac genes, its potential role in the fetal cardiac program can not be disregarded.

Expression of myocardin mRNA in human hearts that are failing due to dilated cardiomyopathy has been examined by both Northern blot hybridization and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (Torrado *et al.*, 2003). Expression of myocardin was significantly higher in the failing human myocardium as compared to non-failing heart samples (Torrado *et al.*, 2003). Thus, myocardin expression in the mature heart could activate myocardin target genes resulting in initiation of the fetal cardiac program.

MEF2

Myocyte-enhancer factor 2 (MEF2) is a MADS-box family member containing a 57 amino acid MADS domain at its extreme amino-terminus. Because of the MADS domain, MEF2 proteins were originally referred to as RSRF (related to serum response factor) proteins (Pollock and Treisman, 1991). Adjacent to the MADS box is a 29 amino acid extension called the MEF2 domain. The MEF2 domain is necessary for high-affinity DNA binding and dimerization (Morin *et al.*, 2000).

Members of the MEF2 family of transcription factors bind A/T-rich DNA nucleotide sequences (Gossett *et al.*, 1989) and the consensus DNA binding site for

MEF2C is (C/T)TA(A/T)$_4$TA(G/A) (Andres *et al.*, 1995; Fickett, 1996; Pollock and Treisman, 1991). MEF2s can bind DNA as homo- or heterodimers (Andres *et al.*, 1995; Fickett, 1996; Naya *et al.*, 1999; Pollock and Treisman, 1991), but do not interact with other MADS family members. As the MEF2 domain itself can homo- or heterodimerize, MEF2s have enormous potential for different heterodimeric associations (Black and Olson, 1998).

Vertebrates express four MEF2 genes (*mef2a-d*) located on different chromosomes and the RNA can be alternatively sliced (Black and Olson, 1998). The four MEF2 isoforms share approximately 50% homology overall and the conserved MADS box and MEF2 domain are approximately 95% similar (Black and Olson, 1998; Theiben, 1996). Thus, MEF2s are divergent in their carboxyl-terminal transactivation domain. In the carboxyl-termini of MEF2s, there is relatively little amino acid sequence homology except for four Ser/Thr-rich regions (Black and Olson, 1998) and a nuclear localization signal (NLS) at the extreme carboxyl-termini of MEF2A, MEF2C, and MEF2D (Black and Olson, 1998).

All four MEF2 protein isoforms are highly enriched in brain, muscle, and heart (Black and Olson, 1998; Naya *et al.*, 1999) with the greatest expression being in muscle. In muscle, MEF2 proteins are essential for muscle-specific gene expression and differentiation of all three muscle types (Black and Olson, 1998; Morin *et al.*, 2000). MEF2 transcription factors regulate cardiac structural genes (e.g., myosin, actin) as well as genes involved with growth, stress-responsiveness, and apoptosis (Frey *et al.*, 2000).

In the mouse embryo heart, MEF2C appears after the arrival of Nkx2.5 and this likely occurs as MEF2C promoter is targeted by Nkx2.5 (Skerjanc *et al.*, 1998). MEF2C

is the first of the MEF2 family genes expressed, with transcripts appearing in mesodermal precursors that give rise to the heart (Black and Olson, 1998) at E7.5 (Edmondson *et al.*, 1994; Molkentin *et al.*, 1996b). At E9.5, MEF2C expression is throughout heart atria and ventricles and MEF2C is highly expressed until E14.5 (Naya *et al.*, 1999). After E14.5, there is a decline in *mef2c* gene expression and in adult mice, MEF2C is absent from every tissue except skeletal muscle (Naya *et al.*, 1999). MEF2B is co-expressed along with MEF2C in the precardiogenic mesoderm at E8.0 while MEF2A and MEF2D are expressed approximately 12 hours later (Edmondson *et al.*, 1994; Molkentin *et al.*, 1996).

Targeted disruption of *mef2C* gene is embryonic lethal at E9.5 due to right ventricular dysplasia (Lin *et al.*, 1997). In MEF2C-deficient embryos the heart tube does not undergo looping and the future right ventricle does not form (Lin *et al.*, 1997). Expression of cardiac muscle genes such as cardiac α-actin and dHAND (*Deciduum*, heart, autonomic nervous system and neural crest derivatives-expressed protein 2) were analyzed in hearts of MEF2C mutant embryos by RT-PCR analysis and mRNA transcripts for these genes were not detectable (Lin *et al.*, 1997). It is believed that the absence of the right ventricle in MEF2C null-mutant mice correlated with reduced expression of dHAND gene, which encodes a basic helix-loop-helix transcription factor required for cardiac morphogenesis (Lin *et al.*, 1997). Therefore, MEF2C is an essential regulator of cardiac myogenesis and ventricular development.

MEF2C is a very intriguing target for treatment of cardiomyopathies as MEF2C is a common endpoint for several diverse hypertrophic signaling pathways (Frey and Olson, 2003). As the endpoint, MEF2C factors regulate cell proliferation for a variety of growth factor-related signaling pathways (Black and Olson, 1998). MEF2s function under

positive and negative controls (Black and Olson, 1998) and protein-protein interactions play a critical role in determining target genes of MEF2 proteins (Black and Olson, 1998).

Protein-protein interactions of MEF2C include interactions with other transcription factors. The expression patterns of many early cardiac transcription factors overlap (Molkentin *et al.*, 2000a; Srivastava and Olson, 2000) and Nkx2.5, MEF2s, and GATAs are all present in precardiac mesoderm. Consequently, it is believed that these transcription factors regulate cardiogenesis in a synergistic and coordinated manner. Synergism between transcription factors significantly increases the number of potential target genes that a particular transcription factor can influence. That is to say that the absence of a transcription factor-binding site in a promoter is not necessarily an indication that a gene is not influenced by that factor. For example, the ANF promoter has GATA and Nkx2.5 sites, but no MEF2 site (Morin *et al.*, 2000). However, MEF2 proteins can activate the ANF gene as GATA4 recruits MEF2 proteins to the ANF promoter through protein-protein interactions (Morin *et al.*, 2000). MEF2/GATA4 synergy involves physical interaction between the MEF2 DNA-binding domain and the carboxyl zinc finger of GATA4 and requires the activation domains of both proteins (Morin *et al.*, 2001). It is believed that MEF2 further enhances GATA activity through recruitment of GATA cofactors and/or stabilization of co-activators. Co-activators include p300/CBP (CREB-binding protein) as both MEF2 and GATA have been shown to independently interact with these proteins (Morin *et al.*, 2001). Alternatively, a direct MEF2/GATA interaction may displace or overcome a co-repressor of one or both factors. For example, MEF2 interaction with GATA4 or GATA6 in the heart may displace FOG-

2 (Friend of GATA-2) that is a cofactor that represses GATA4 activity. Fetal cardiac genes identified to have promoters synergistically activated by MEF2 and GATA are ANF, BNP (B-type natriuretic peptide), α-MHC, MLC-2v, eHAND (Extraembryonic tissues, heart, autonomic nervous system and neural crest derivatives-expressed protein 1), dHAND, and α-cardiac actin (Morin *et al.*, 2000). Therefore, it can be concluded that protein-protein interactions between transcription factors are an important attribute and significantly increase the number of promoters targeted by MEF2 proteins. Examination of the calreticulin promoter indicated four tentative MEF2C binding sites.

GATA

The helix-loop-helix zinc-finger GATA family of transcription factors has six members, named GATA1 through GATA6. GATA1, 2, 3 are expressed in hemotopoietic cells while GATA4, 5, 6 are restricted to the heart and gut. GATA family members have two transcriptional activation domains within the amino-terminus and possess a NLS adjacent to the carboxyl-terminal zinc finger (amino acid residues 251-324) (Molkentin, 2000b). As such, GATA transcription factors are localized to the nucleus. It has been postulated that transcriptional activity of some GATA factors such as GATA4 may be regulated by protein kinases (Molkentin, 2000b); however, currently there is no published evidence to support this hypothesis.

The zinc finger motifs allow GATA transcription factors to bind to the A/TGATA/G consensus sequence (Koutsourakis *et al.*, 1999). In the heart, GATA activates numerous promoters such as α-MHC, cardiac troponin-C, ANF, BNP, cardiac troponin-1, Na^{+}/Ca^{2+} exchanger, cardiac restricted ankyrin repeat protein, A1 adenosine receptor, m2 muscarnic receptor, and MLC-2v (Molkentin, 2000b). GATA4, 5, 6 are

important regulators of gene expression in multiple endoderm- and mesoderm-derived tissues and it is likely that GATA4, 5, 6 regulate tissue-specific gene expression in different cell types via synergistic interactions with other semi-restricted transcription factors or cofactors (Molkentin, 2000b).

GATA4 and 6 are potential regulators of cardiac development and hypertrophy as they appear early in development in the precardiac mesoderm along with Nkx2.5 and MEF2 proteins (Liang *et al.*, 2001). Immunoprecipitation experiments have demonstrated that Nkx2.5 and GATA4 directly interact (Durocher *et al.*, 1997) and this interaction permits these transcription factors to synergistically activate target genes (Durocher *et al.*, 1997; Lee *et al.*, 1998; Molkentin, 2000b; Molkentin *et al.*, 2000a). GATA4 interaction with the carboxyl-terminal homeodomain of Nkx2.5 unmasks the Nkx2.5 activation domain (Lee *et al.*, 1998). The GATA/Nkx2.5 synergism is specific for GATA4 and GATA5, but not GATA6. In addition to Nkx2.5, GATA factors have a synergistic interaction with NFATc4 (Molkentin *et al.*, 1998). Thus, in the developing heart numerous transcription factors are co-expressed and GATA4 can physically interact with Nkx2.5, NFATc4, and MEF2 proteins (Molkentin, 2000) to fully activate gene expression. Synergistic interactions between GATA family members and other transcription factors suggest that the number of GATA gene targets in the developing heart is numerous.

GATA4 is highly expressed in the precardiogenic splanchnic mesoderm at the posterior edge of the anterior intestinal portal, which is the region of the embryo that undergoes ventral fusion (Molkentin *et al.*, 1997). GATA4 is expressed in the cardiac splanchnic mesoderm as early as E7 (Arceci *et al.*, 1993; Heikinheimo *et al.*, 1994;

Kelley *et al.*, 1993) and is expressed in the endocardium and myocardium of the folding heart tube. GATA4 expression is observed in the heart throughout development and adult life (Koutsourakis *et al.*, 1999). In GATA4-deficient embryos, the primitive heart tubes and foregut developed partially outside the yolk sac. As such, GATA4 null-mutants die early in embryonic development (E7 to E9.5) as a result of failure of the two bilaterally symmetric promyocardial primordia to fuse at the ventral midline; hence, in absence of GATA4 two separate heart tubes with differentiated cardiomyocytes develop (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). Thus, GATA4 transcription factor is essential for heart development.

As GATA4, 5 and 6 have overlapping patterns of expression the question arises as to the role of the other GATAs in the heart. Interestingly, GATA5-deficient mice were found to be viable with the only defect being genitourinary abnormalities observed in females (Molkentin *et al.*, 2000b). This finding strongly suggests that in most tissues other GATA factors have functions overlapping those of GATA5. In comparison, examination of GATA6 found that this isoform is essential during early embryogenesis. RNase protection analysis indicated that GATA6 mRNA is abundantly expressed in the heart (Narita *et al.*, 1996). Mouse embryos with the GATA6 gene disrupted by insertion of the β-galactosidase gene have been created and gene disruption was found to be lethal at E5.5 due to an indirect effect on the epiblast resulting from a defect in an extraembryonic tissue (Koutsourakis *et al.*, 1999). Examination of GATA6/lacZ heterozygous embryos showed that GATA6 expression was first evident at the blastocyst stage (E3.5) in the inner cell mass (Koutsourakis *et al.*, 1999). A transverse section through the heart of GATA6/lacZ heterozygous embryos showed that GATA6 expression

was in the myocardium and pericardium, but absent from the endocardium (Koutsourakis *et al.*, 1999). GATA6/lacZ was later observed to be expressed in both mesoderm and endoderm that formed the heart and gut (Koutsourakis *et al.*, 1999). Thus, GATA6 is essential for early embryonic development and is expressed early in heart development.

Of the six members of the GATA family of transcription factors, it can be concluded that only GATA4 and GATA6 are essential for heart development. When either GATA4 or GATA6 are over-expressed in the heart, cardiac hypertrophy occurs (Liang *et al.*, 2001). Within the calreticulin promoter there are three tentative sites for GATA factors. As such, both GATA4 and GATA6 were of interest in this investigation.

NFAT

Originally identified in T-cells, nuclear factor in activated T-cells (NFAT) has recently been shown to be important for activation of the fetal cardiac program in both normal and diseased hearts. At present there are five members in the NFAT family of transcription factors, named NFATc1 to NFATc4 and NFAT5 (Rao *et al.*, 1997), many of which can be alternatively spliced (Rao *et al.*, 1997). These transcription factors have distinct temporally and spatially regulated expression patterns (Rao *et al.*, 1997) and contribute to development of several organ systems including the immune system, vasculature, and heart (Graef *et al.*, 2001b; Horsley and Pavlath, 2002).

NFAT proteins have a highly conserved regulatory domain at their amino-terminus that mediates nuclear translocation and a Rel homology domain near their carboxyl-terminus that mediates DNA-binding (Molkentin *et al.*, 1998). The unusual structure of the Rel-like DNA binding domain (Wolfe *et al.*, 1997) means that binding to the consensus site T/A GGAAA N A/T/C N (Rao *et al.*, 1997) by NFATc proteins is

weak; thus, NFATc proteins require a partner to strongly bind DNA (Crabtree, 2001; Molkentin *et al.*, 1998). In the heart, NFATc proteins are essential for formation of heart valves and activate gene targets via interactions with GATA transcription factors (Crabtree, 2001; Srivastava and Olson, 2000). It has been documented that the fetal cardiac gene BNP is synergistically activated by GATA4 and NFATc4 despite absence of a NFAT promoter binding site (Molkentin *et al.*, 1998). Also, NFAT has been reported to synergistically interact with other factors such as SRF and MEF2 proteins to impact gene expression (Chin *et al.*, 1998; Delling *et al.*, 2000). Thus, NFAT is essential for heart development and/or synergism of NFATc proteins with other transcription factors is necessary for normal cardiac development.

NFATc1 to NFATc4 are localized in the cytoplasm (Rao *et al.*, 1997) and their activation is calcineurin-dependent. An elevation in cytoplasmic Ca^{2+} activates the phosphatase calcineurin which binds directly to a conserved motif, PxIxIT, in the amino-terminus of NFATc proteins (Aramburu *et al.*, 1998; Aramburu *et al.*, 1999; Northrop *et al.*, 1994; Wesselborg *et al.*, 1996). Calcineurin dephosphorylates serines within the amino terminal serine and proline (SP) repeats (SPxxSPxxSPxxxxx) (Ho *et al.*, 1995; Masuda *et al.*, 1995) as well as within the serine-rich region of NFATc family members (Beals *et al.*, 1997a; Beals *et al.*, 1997b). Dephosphorylation of NFATc induces a conformational change that exposes the NFATc NLS thereby permitting these proteins to translocate to the nucleus to impact target genes (Molkentin *et al.*, 1998; Rao *et al.*, 1997).

The NFAT family of transcription factors contribute to development of several organ systems. The cardiovascular system is dependent on NFAT activity for

development and for adult adaptation to cardiac stress (Graef *et al.*, 2001b; Molkentin, 2000a). NFATc1-deficiency results in cardiac valve and septal defects while over-expression of NFATc4 results in cardiac hypertrophy (Graef *et al.*, 2001b; Molkentin, 2000a). During cardiac hypertrophy, NFATc4 over-expression increases expression of ANF, BNF, SERCA2, α-MHC and β-MHC (Molkentin *et al.*, 1998). In comparison to NFATc1 and NFATc4, NFATc2 is known to regulate growth of skeletal muscle while NFATc3 is essential for creation of primary myofibers (Crabtree and Olson, 2002).

Mouse models have provided great insight into the role of NFATc proteins in the heart. Mice with disruption of NFATc3 were not born at the expected Mendelian ratio, but those surviving were viable and lacked any physical abnormalities (Oukka *et al.*, 1998). Examination of these mice later indicated that they had a thymic defect suggesting a problem with T-cell production (Oukka *et al.*, 1998). Mice with NFATc4 gene disrupted are viable without detectable phenotypic abnormalities (Wilkins *et al.*, 2002). Thus, it is likely that loss of NFATc4 can be compensated for by another cardiac-expressed NFATc isoform (Wilkins *et al.*, 2002). *In situ* hybridization showed that expression patterns of NFATc3 and NFATc4 significantly overlap (Graef *et al.*, 2001a). To get a better understanding of the role of NFATc proteins in the heart, a mouse with disruption of both NFATc3 and NFATc4 was created. NFATc3/c4 null-deletion was found to be embryonic lethal at E11 due to cardiovascular defects resulting from abnormal vascular patterning (Bushdid *et al.*, 2003; Graef *et al.*, 2001a). Therefore, investigation of NFATc in mouse models confirmed importance of this factor in cardiac development.

Evi-1

Ecotropic viral integration site-1 (Evi-1) is a gene that has been associated with both murine and human acute myeloid leukemia and is considered a protooncogene (Nucifora, 1997). Mouse and human Evi-1 cDNAs are 91% homologous in nucleotide sequence (Morishita *et al.*, 1990) suggesting that the Evi-1 protein has been very important during evolution. Evi-1 is a nuclear protein (Matsugi *et al.*, 1990) possessing ten Cys_2His_2 type zinc finger motifs found with two zinc finger domains, designated ZF1 and ZF2, as well as a carboxyl-terminal acidic domain (Morishita *et al.*, 1988; Perkins *et al.*, 1991). It has been postulated that the acidic domain functions as an activation domain for gene transcription (Struhl, 1987), but there is no supporting evidence for this function. In contrast to the other regions of Evi-1, much is known about the zinc finger (ZF) domains. ZF1 possesses seven of the ten zinc finger repeats and is situated near the amino terminus whereas ZF2 contains three of the zinc finger repeats and is positioned near the carboxyl-terminus (Morishita *et al.*, 1988; Nucifora, 1997). Both zinc finger domains of Evi-1 have their own distinct DNA binding sites (Bartholomew *et al.*, 1997). ZF1 recognizes and binds a consensus site of 15 nucleotides (GA(C/T)AAGA(T/C)AAGATAA) (Delwel *et al.*, 1993) while ZF2 binds to the consensus sequence GAAGATGAG (Funabiki *et al.*, 1994). Of the two sites, ZF2 site is the one that is essential for transcriptional activation (Morishita *et al.*, 1995). Therefore, when considering that Evi-1 is highly conserved in organisms, it is likely that it serves a very important role in regulating gene expression.

In addition to activation properties, Evi-1 has also reported to have transcriptional repressor ability (Bartholomew *et al.*, 1997). Since GATA transcription factor binding

sites are within the Evi-1 binding site, it has been observed that Evi-1 can repress GATA-dependent transactivation (Kreider *et al.*, 1993). Another study demonstrated that a genomic promoter with several GATA repeats was strongly repressed by Evi-1 (Nucifora, 1997). Therefore Evi-1 can act as a transcriptional repressor and it has been postulated that this attribute has important implications for the mechanism of action of Evi-1 protein both in development and in the progression of some myeloid leukemias (Bartholomew *et al.*, 1997). Although calreticulin promoter lacks an ideal Evi-1 consensus binding site, the promoter does possess several tentative GATA binding sites. Thus, Evi-1 could potentially regulate calreticulin expression via binding to the GATA site.

In situ hybridization analysis of Evi-1 expression in mouse adult and embryonic tissues suggested that Evi-1 plays an important regulatory role during mouse embryogenesis. Evi-1 expression was found to be confined to a few distinct organs, most of which were mesoderm-derived (Perkins *et al.*, 1991). Examination of E12.5 mouse hearts showed that Evi-1 expression was very high in the truncal (more distally located) and conal (more proximally located) ridges (Perkins *et al.*, 1991); thus, Evi-1 is highly expressed in regions that develop into heart septa and valves.

Disruption of the Evi-1 gene in mice rendered results that correlated with the *in situ* hybridization analysis. Disruption of Evi-1 gene was found to be lethal at E10.5 (Hoyt *et al.*, 1997). In homozygous embryos, the peripheral nervous system failed to develop and the embryos exhibited widespread hypocellularity and hemorrhaging as well as heart defects (Hoyt *et al.*, 1997). These data suggest that Evi-1 has important roles in general cell proliferation, vascularization, and heart development.

COUP-TFI

Chicken ovalbumin upstream promoter transcription factor (COUP-TF) is a transcription factor that can repress transcription of many genes (Smirnov *et al.*, 2000). There are three isoforms named COUP-TFI to COUP-TFIII (Avram *et al.*, 2000), and they bind a DNA consensus site TGACC C/T (Cooney *et al.*, 1992; Scott *et al.*, 1996) as homodimers or heterodimers (Smirnov *et al.*, 2000). Both isoforms belong to a group of orphan nuclear receptors and are highly conserved among species, from *Drosophila* to human. COUP-TFs play an essential role in nervous system development (Qiu *et al.*, 1997) and gene disruption is lethal shortly after birth as neonates starve and dehydrate. Examination of COUP-TFI deficient mice indicates serious abnormalities with neurological development (Qiu *et al.*, 1997).

Nkx2.5 is of great importance to cardiac development, and it is interesting that COUP-TFI DNA binding site is very similar to that of Nkx2.5. The COUP-TFI consensus DNA binding site is a palindrome of the Nkx2.5 binding site (Guo *et al.*, 2001). It is interesting that an essential transcriptional activator like Nkx2.5 has such a similar DNA binding site as a strong transcriptional repressor. The mechanism by which COUP is able to repress transcription has yet to be determined, but it is believed that its carboxyl-terminus associates with histone deacetylase (Smirnov *et al.*, 2000). Western blot analysis showed that COUP-TFI was expressed in both embryonic and postnatal hearts (Guo *et al.*, 2001). The level of Nkx2.5 decreases during development while the relative level of COUP-TFI does not change significantly during cardiogenesis (Guo *et al.*, 2001). Perhaps competitive binding of COUP-TFI to Nkx2.5 sites is the reason why Nkx2.5 can remain in a healthy adult heart, but not activate the fetal cardiac genes. Future

investigation is necessary to determine if some forms of heart disease arise as a result of decreased COUP-TFI expression.

MyoD1

MyoD1 is a transcription factor that converts fibroblasts to stable myoblasts (Davis *et al.*, 1987; Hopwood *et al.*, 1989). MyoD1 specifically interacts with a single consensus core nucleotide sequence, CANNTG, known as an E-box (French *et al.*, 1991; Sartorelli *et al.*, 1990). The E-box is frequently found in promoters of muscle-specific genes and is documented to bind members of the basic helix-loop-helix family of transcription factors (Skerjanc and McBurney, 1994). The calreticulin promoter has an E-box (Figure 1-5) situated at -372 to -377 (CACCTG).

MyoD1 is skeletal muscle-specific and is not expressed in the embryonic heart (Evans *et al.*, 1993; Hopwood *et al.*, 1989). In skeletal muscle, MyoD1 synergistically enhances activation of target genes by direct interaction with other transcription factors such as MEF2 family members (Black and Olson, 1998). Since MEF2C was a transcription factor of interest in my investigation, I wanted to know if such a synergistic interaction occurred on the calreticulin promoter. Subsequently, testing of MyoD1 was included in the experiments.

Therefore, a total of nine transcription factors, eight of which are expressed in the developing heart, were tested in this study to determine if any of these factors influenced expression of a calreticulin promoter-controlled reporter gene. Experimental design and results follow.

Calreticulin and Ca^{2+} signaling

The role of calreticulin as an ER Ca^{2+}-binding protein is well documented (Baksh and Michalak, 1991; Bastianutto *et al.*, 1995; Camacho and Lechleiter, 1995; John *et al.*, 1998; Mery *et al.*, 1996; Mesaeli *et al.*, 1999; Michalak *et al.*, 1999; Nakamura *et al.*, 2001b; Xu *et al.*, 2000). In calreticulin-deficient fibroblasts, ER Ca^{2+} storage capacity is reduced (Nakamura *et al.*, 2001b) while in calreticulin over-expressing fibroblasts there is an increased amount of Ca^{2+} in intracellular stores (Bastianutto *et al.*, 1995; Mery *et al.*, 1996). It is likely that changes in ER Ca^{2+} storage capacity and changes in Ca^{2+} binding to calreticulin affect chaperone function and impact the quality control of ER protein folding. Thus, in calreticulin-deficient fibroblasts, there is impaired $InsP_3$-dependent Ca^{2+} release owning to inefficient binding of a ligand (e.g., bradykinin) to the plasma membrane receptor (Nakamura *et al.*, 2001b). Thus, an alteration in ER Ca^{2+} concentration disrupts normal ER dynamics thereby impacting Ca^{2+} homeostasis and protein folding, such that these two functions are interdependent (Berridge, 2002; Michalak *et al.*, 2002).

Calreticulin-deficient fibroblasts have impaired agonist-induced Ca^{2+} release and exhibit compromised NFATc4 nuclear localization (Mesaeli *et al.*, 1999). Nuclear translocation of NFATc is dependent on dephosphorylation of its NLS by calcineurin. The observation that NFATc4 cannot translocate to the nucleus in the absence of calreticulin suggests that calcineurin requires Ca^{2+} from the ER for activation and this Ca^{2+} comes from calreticulin. Subsequently, the inability of a cell to release Ca^{2+} from the ER means impaired Ca^{2+}-dependent signaling cascades.

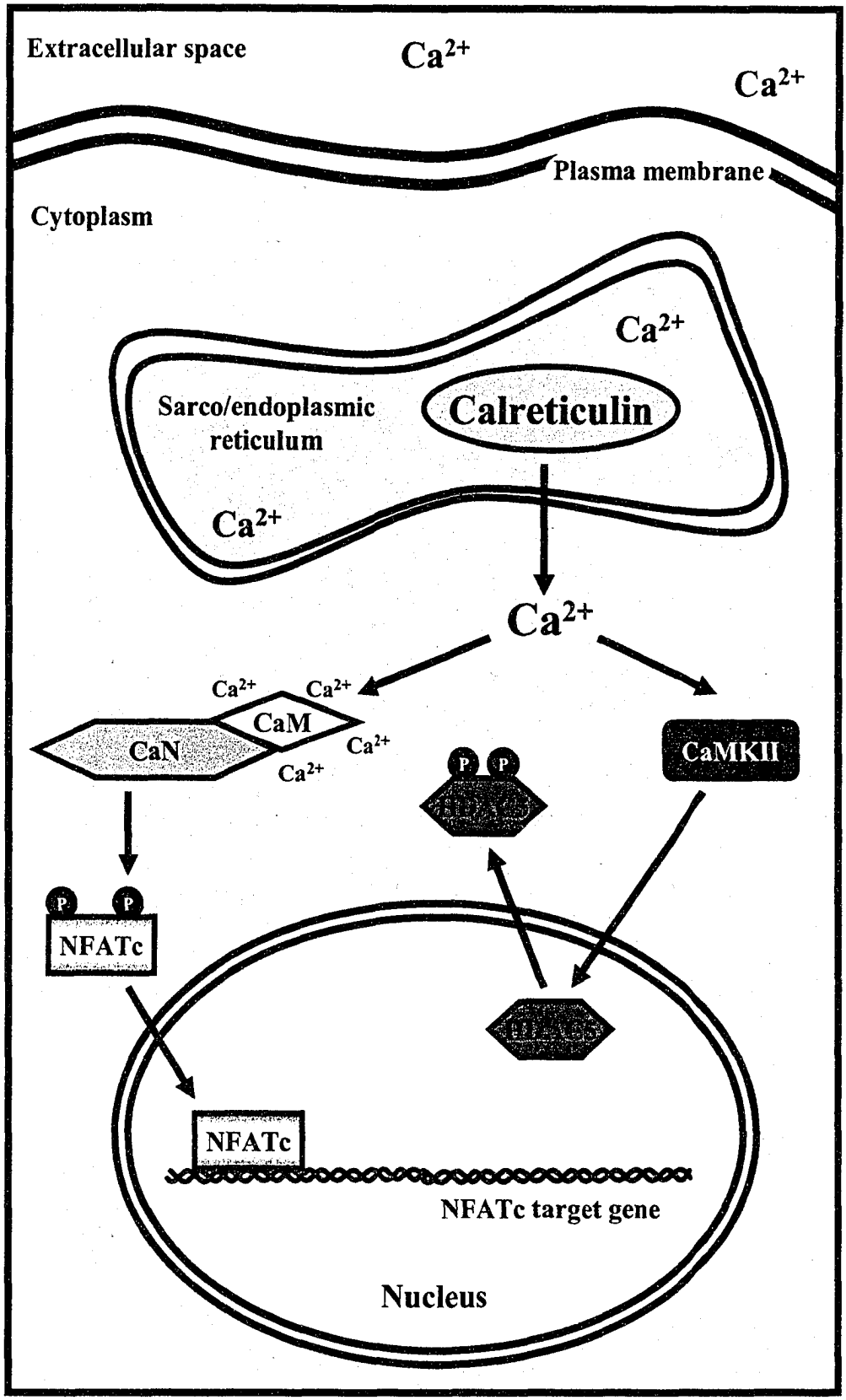
Our current understanding of components of Ca^{2+} signaling pathways indicate that

downstream Ca^{2+}-dependent proteins cannot be activated without significant Ca^{2+} release from intracellular Ca^{2+} stores, the SR and ER compartments. Hence, Ca^{2+}-binding proteins of the SR and ER represent essential components of intracellular Ca^{2+} signaling cascades. MEF2C is a transcription factor dependent on an upstream Ca^{2+} signaling event for activation. In order to investigate MEF2C function in calreticulin-deficient cells, it is important to understand how Ca^{2+} released by calreticulin in the ER could impact this transcription factor.

Calmodulin

Calmodulin (CaM) is one of the most intensely studied members of the E-F hand family of Ca^{2+} sensors (Figure 1-6). CaM is a small cytoplasmic Ca^{2+}-binding protein that is expressed in all eukaryotes (Chin and Means, 2000), but not prokaryotes. CaM is found throughout the cell and in the nucleus (Chin and Means, 2000). The protein consists of 148 amino acids with globular amino- and carboxyl-termini linked by an α-helix. Each globular end (E-F hand) of the protein binds two Ca^{2+} ions. In the absence of Ca^{2+}, CaM has an elongated "dumb-bell" structure, but when Ca^{2+} is present the helical region condenses and CaM assumes a globular conformation. These conformational changes enhance affinity of CaM for CaM-binding proteins. Most CaM targets are enzymes (Chin and Means, 2000) that rely on the Ca^{2+}/CaM complex for activation. Therefore, the main function of CaM is to convert Ca^{2+} signals into cellular function. CaM is essential for cell growth, proliferation, and movement (Chin and Means, 2000). CaM has several targets and, at present, it is unknown how CaM selectively activates downstream signaling pathways (Frey *et al.*, 2000).

Figure 1-6. Cartoon figure showing Ca^{2+}-dependent signalling in a hypothetical cell. A signal at the plasma membrane causes ligand binding to an SR/ER receptor and this encourages Ca^{2+} release from the SR/ER. This Ca^{2+} enters the cytoplasm via the $InsP_3R$ (RyR in SR) and binds to CaM. Ca^{2+} binding to CaM induces a conformation change in this protein that enables to interact with downstream proteins such as CaN or CaMK. CaN has many downstream targets of which the most well-documented are NFATc proteins. CaMK acts to phosphorylate transcriptional repressor HDAC proteins and this phosphorylation encourages nuclear export of HDACs. *Abbreviations*: CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; CaN, calcineurin; HDAC5, histone deacetylase 5; and NFATc, nuclear factor in activated T-cells c.



Relevant to cardiac physiology, is an observation that CaM interacts directly with a region of histone deacetylases (HDACs) that associate with and suppress activity of MEF2 transcription factors (Youn *et al.*, 2000). MEF2 transcription factors are essential for heart development (Lin *et al.*, 1997) and the CaM/HDAC interaction may be indicative of a feedback loop. CaM over-expression in the mouse heart results in severe cardiac hypertrophy (Frey *et al.*, 2000; Gruver *et al.*, 1993). In hearts of these animals, CaM-dependent protein kinase II (CaMKII) activity was observed to be higher (Frey *et al.*, 2000). CaMKII is a downstream target of Ca^{2+}/CaM complex and is less sensitive to Ca^{2+} than other downstream targets of CaM. It is unknown if activity of other downstream proteins were measured in these animals, or if CaMKII was the only Ca^{2+}/CaM complex target activated.

Calmodulin-dependent protein kinase

Well-documented targets of the Ca^{2+}/CaM complex are CaMKs. Four genes code for four isoforms of CaM-dependent protein kinase, named CaMKI to CaMKIV. CaMKI is widely expressed (Frey *et al.*, 2000), CaMKII is the main isoform in the heart, CaMKIII is ubiquitously expressed (Ryazanov *et al.*, 1997) and selectively activated in proliferating cells (Parmer *et al.*, 1999) while CaMKIV is expressed mainly in brain and testis (Frey *et al.*, 2000). As CaMKII is the main cardiac isoform, it is of particular interest in this investigation.

All CaMKs are dependent on Ca^{2+}/CaM complex for activation. CaMKs are at least one order of magnitude less sensitive to Ca^{2+} than calcineurin (Lisman, 1994) and can be activated by a transient high amplitude Ca^{2+} spike (Frey *et al.*, 2000). The Ca^{2+}/CaM complex activates CaMKII (Chin and Means, 2000) by binding two protein

kinases and promoting kinase autophosphorylation (Means, 2000). It is not known if CaMK activation is initiated by ligand binding to the plasma membrane or by mechanical activation (e.g., stretch); however, it is probable that CaMK can be activated by both methods. Evidence of ligand activation has come from studies in which myocytes were treated with hormones in the absence or presence of a CaMK inhibitor. CaMK is activated by insulin-like growth factor (IGF-1) (Muscat and Dressel, 2000) while KN-62, a synthetic inhibitor of CaMK (Tokumitsu *et al.*, 1990), prevented endothelin-1 (ET-1) induced hypertrophy (Irons *et al.*, 1992; Zhu *et al.*, 2000). At present, findings have demonstrated that CaMK is a downstream target of both ET-1 and IGF-1.

CaMK interaction with membrane components of the SR enhances muscle contractility. For example, CaMK phosphorylates RyR to modulate Ca^{2+} release from the SR (Simmerman *et al.*, 1986) while it may also phosphorylate SERCA to affect Ca^{2+} uptake into the SR (Xu and Narayanan, 2000). Furthermore, CaMK phosphorylates Thr^{17} on phospholamban disrupting phospholamban interaction with SERCA and promoting Ca^{2+} uptake into the SR (Simmerman *et al.*, 1986). It is possible that interaction of CaMK with SR membrane proteins represents a feedback mechanism. Hence, in heart disease it is likely that CaMK phosphorylation of SR proteins is altered (Frey *et al.*, 2000), but this has yet to be thoroughly investigated.

Indirect downstream targets of CaMK are members of the MEF2 family of transcription factors. CaMK disrupts MEF2/HDAC4 and MEF2/HDAC5 complexes to promote nuclear export of HDAC4 and HDAC5 (Corcoran and Means, 2001; Lu *et al.*, 2000; McKinsey *et al.*, 2000; McKinsey *et al.*, 2001b; Miska *et al.*, 1999; Muscat and Dressel, 2000) (Figure 1-6). In addition, CaMKII has other mechanisms by which to

further enhance MEF2 transcriptional activity. CaMKII can phosphorylate Ser^{133} and Ser^{142} of CREB (cyclic AMP response element binding protein) (Sheng *et al.*, 1991) which could result in p300 interaction with MEF2 transcription factors (Eckner *et al.*, 1996; Sartorelli *et al.*, 1997). p300 is a very important regulator of gene expression (Janknecht and Hunter, 1996) as it has histone acetyl transferase (HAT) activity (Ogryzko *et al.*, 1996) and functions to disrupt repressive (i.e. condensed) chromatin structures. Hence, p300 interaction with MEF2 could enhance transcription of MEF2 target genes (e.g., genes for muscle growth). Thus, MEF2 proteins are indirectly activated by CaMK.

Mice over-expressing CaMKIV in the heart have been created and these animals develop cardiac hypertrophy (Passier *et al.*, 2000). CaMKIV is not the major CaMK isoform in the heart, but likely mimics the function of CaMKII (Frey *et al.*, 2000). In humans, an increase in CaMK activity has been observed in patients with dilated cardiomyopathy (Kirchhefer *et al.*, 1999). Thus, it can be concluded from transgenic and clinical studies that activated CaMK plays an important role in cardiomyopathies.

Calcineurin

Calcineurin (CaN), also called protein phosphatase 2B, is a highly conserved, Ca^{2+}/CaM-dependent serine/threonine phosphatase (Klee, 1998; Rusnak and Mertz, 2000). To date, it is the only serine/threonine protein phosphatase directly regulated by Ca^{2+}/CaM (Klee *et al.*, 1979; Klee *et al.*, 1988). Although CaN is ubiquitously expressed (Klee, 1998), it is found in brain and muscle at approximately 10-fold higher concentrations than in any other cell type (Olson and Williams, 2000b). As skeletal and cardiac muscle are very Ca^{2+}-dependent tissues, it is not surprising that a Ca^{2+}/CaM-

dependent serine/threonine protein phosphatase is able to regulate many cellular events in these tissues.

CaN cellular localization can vary depending on cell type. In the brain, 50 to 70% of CaN is membrane bound or associated with cytoskeletal elements (Shibasaki *et al.*, 2002). CaN binding to membranes is myristoyl independent and is mediated by anionic phospholipids, diacylglycerol, and the presence of CaM (Kennedy *et al.*, 1996). Although CaN has been found in the nucleus (Moriya *et al.*, 1995; Pujol *et al.*, 1993; Shibasaki *et al.*, 1996), it is generally considered to be predominantly a cytoplasmic protein (Rusnak and Mertz, 2000) (Figure 1-6).

CaN is a heterodimer consisting of two subunits, A and B. The two subunits are tightly bound, only dissociating under denaturing conditions (Klee *et al.*, 1988). Subunit A is the CaM-binding catalytic subunit while subunit B is the Ca^{2+}-binding regulatory subunit (Olson and Williams, 2000a; Rusnak and Mertz, 2000; Stemmer and Klee, 1994). In the presence of less than 10^{-7} M Ca^{2+}, subunit A binds subunit B (Klee *et al.*, 1988). If alone, subunit A exhibits only very low enzymatic activity, but when coupled with the B subunit the phosphatase has high activity (Milan *et al.*, 1994; Perrino *et al.*, 1992).

CaN subunit A (CnA) is encoded by three genes, α, β, and γ. In humans, the α, β, and γ genes are located on chromosomes 4, 10, and 8, respectively (Wang *et al.*, 1996). The α and β gene products can be alternatively spliced (Guerini and Klee, 1989) further adding to their complexity. Depending on the isoform CnA is 57- to 59-kDa in mammals and can be as much as 20% larger in lower eukaryotic species (Rusnak and Mertz, 2000). CnAα and β exist in an overlapping expression pattern in multiple tissues while CnAγ has a testis-restricted distribution (Chang *et al.*, 1994). It is not surprising that all three

CnA isoforms share substantial homology in the domain encoding the catalytic site (Bueno *et al.*, 2002a; Cohen *et al.*, 1996; Goto *et al.*, 1986; Klee *et al.*, 1988; Kuno *et al.*, 1992). One major difference between the isoforms is variable amino- and carboxyl-terminal regions, whose functions are largely unknown (Guerini and Klee, 1989; Klee, 1998).

Two mouse lines with CnA gene disruptions have been created. Mutant mice lacking CnAα do not significantly differ from wild-type littermates. CnAα-deficient mice display only minor defects in T-cell response (Zhang *et al.*, 1996) and altered synaptic plasticity in the central nervous system (Kayyali *et al.*, 1997; Zhuo *et al.*, 1999). The second CnA gene disrupted was CnAβ. CnAβ-deficient mice were viable, and fertile; however, these mice displayed a 80% decrease in cardiac CaN activity and a 12% reduction in basal heart size (Bueno *et al.*, 2002b). Additionally, CnAβ-deficient mice showed impaired hypertrophic response to pressure overload or angiotensin II infusion (Bueno *et al.*, 2002b). The fact that the CnA gene disruptions were not lethal suggests that CnAα and CnAβ isoforms may compensate for one another.

The mature CnA subunit can be subdivided into 5 domains: 1) amino-terminal region (amino acids 15-24), which interacts with the CaN B subunit and may impact CaN catalytic activity (Kissinger *et al.*, 1995); 2) catalytic domain, a 350 amino acid core which binds an Fe^{3+} and Zn^{2+} ion and has sequence homology to other protein phosphatases; 3) CaN B-binding domain (amino acids 350-370); 4) CaM-binding domain (amino acids 390-414); and 5) an autoinhibitory (AI) domain (amino acids 469-486) that blocks access to the catalytic site. The three-dimensional X-ray crystal structure of CaN has been solved to a resolution of 2.1Å (Griffith *et al.*, 1995; Kissinger *et al.*, 1995).

Structurally, the catalytic domain is comprised of a sandwich-like segment of six β-sheets covered by three α–helices and three β-sheets which is layered by five β–sheets covered by a predominantly helical structure (Klee, 1998). Bound to residues at the two faces of the β sandwich are the Fe^{3+} and Zn^{2+} ions of the active site. Interestingly, the CaN B-binding domain, CaM-binding domain, and AI domain are all α–helices. As expected, the AI α–helix runs perpendicular to the CaN catalytic site and binds to the substrate-binding cleft of the enzyme thereby blocking substrate access to the active site (Kissinger *et al.*, 1995). CnA amino-terminal residues are responsible for interaction with carboxyl-terminal residues of CnB and these resides account for the high-affinity of CnB for CnA ($K_d < 10^{-13}$ M) (Anglister *et al.*, 1995; Watanabe *et al.*, 1995).

Much of the information regarding the active site of CaN has come from comprehensive sequence alignments of serine/threonine protein phosphatases (Barton *et al.*, 1994; Guerini *et al.*, 1992; Koonin, 1994; Lohse *et al.*, 1995; Zhuo *et al.*, 1993). These alignments have permitted identification of a consensus "phosphoesterase motif" (Koonin, 1994; Zhuo *et al.*, 1993). Site-directed mutagenesis of active site resides (Mertz *et al.*, 1997; Zhuo *et al.*, 1993) has assisted in identification of essential CaN active site residues. X-ray diffraction studies indicate that CaN has a dinuclear metal core separated by 3.14Å (Kissinger *et al.*, 1995). In addition to metal ions, there are several conserved residues within the CaN active site (Rusnak and Mertz, 2000). Although the role attributed to some active site residues is still tentative, a mechanism for CaN dephosphorylation has been proposed (Rusnak and Mertz, 2000).

In mammals, CnB is approximately 19-kDa and is encoded by two genes, each having alternatively spliced isoforms (Ueki *et al.*, 1992). The tissue expression pattern of

the two genes differs with CnB1 being ubiquitously expressed while CnB2 is expressed only in the testis (Chang *et al.*, 1994; Mukai *et al.*, 1991; Ueki *et al.*, 1992). The mature CnB protein consists of 170 amino acids and possesses four Ca^{2+}-binding EF-hand motifs (Aitken *et al.*, 1984). As such, CnB has structural similarity to members of the EF-hand family of Ca^{2+}-binding proteins (Guerini *et al.*, 1989; Klee, 1998) sharing homology with CaM (35% identity) and troponin (29% identity) (Rusnak and Mertz, 2000). NMR analysis has indicated that the structure of CnB is similar to CaM, with two adjacent Ca^{2+}-binding loops linked by a flexible α–helix (Anglister *et al.*, 1994; Kakalis *et al.*, 1995). CnB binds four Ca^{2+}, one with high-affinity ($k_d < 10^{-7}$ M) and three with mM affinity (Kakalis *et al.*, 1995).

At its amino-terminus, CnB has a myristoyl group. The mature CnB protein has a glycine at position 2 that is acylated with myristic acid (Aitken *et al.*, 1982). Although this attribute is highly conserved from yeast to mammals, indicative of a critical physiological role (Cyert, 1993; Klee, 1998), the myristoyl group is not essential for CaN function. Mutation of this glycine to an alanine demonstrated that myristolation is not required for membrane association and myristoylated and non-myristoylated CaN heterodimers showed equivalent enzymatic activity (Zhu *et al.*, 1995). The only difference identified between the myristoylated and non-myristoylated CaN heterodimers was that the myristoylated protein exhibited greater thermal stability (Kennedy *et al.*, 1996). X-ray structural investigation of myristoylated and non-myristoylated CnB indicated that the non-myristoylated amino-terminus is disordered whereas in the myristoylated form the myristoyl group is anchored via multiple hydrophobic contacts and this may enhance CnB stability (Kennedy *et al.*, 1996; Kissinger *et al.*, 1995). At

present, the role of CnB myristoylation is debatable; however, it may not only enhance protein stability, but it can be suggested that it may also facilitate interaction of CaN with substrates near membranes (Shibasaki *et al.*, 2002).

The mechanism of CnB activation has yet to be elucidated (Shibasaki *et al.*, 2002). What is known is that Ca^{2+} binding to CnB plays a role in activation of CaN phosphatase activity (Stemmer and Klee, 1994). It is hypothesized that Ca^{2+} binding to CnB plays a structural role rather than a regulatory role (Stemmer and Klee, 1994) and the interaction between CnA and CnB is necessary for allosterically activating CnA (Milan *et al.*, 1994). Future investigation is necessary to fully deduce the mechanism of CaN activation.

In addition to the post-translational modification of myristoylation, CaN itself can be phosphorylated. CaN can be phosphorylated by protein kinase C (PKC) (Hashimoto and Soderling, 1989; Tung, 1986), casein kinase I (Singh and Wang, 1987), casein kinase II (CKII) (Hashimoto *et al.*, 1988; Hashimoto and Soderling, 1989; Martensen *et al.*, 1989) and CaMKII (Hashimoto and Soderling, 1989). Interestingly, the kinetics of the phosphorylated and dephosphorylated forms are similar (Rusnak and Mertz, 2000). Thus, the significance of CaN phosphorylation as a means of regulating CaN is an area requiring further investigation.

Ca^{2+} and CaN activation

CaN is a Ca^{2+}-dependent phosphatase tightly regulated by intracellular Ca^{2+} concentration. There is some discrepancy in the identity of the Ca^{2+} source that activates CaN, but depending on the cell type, it is possible that RyR/$InsP_3$R, and L-type Ca^{2+} channels are all involved (Cameron *et al.*, 1995; Koide *et al.*, 1997). During a signaling

event, Ca^{2+} released by the SR/ER enters the cytoplasm and binds to CaM thereby activating this cytoplasmic Ca^{2+} buffer (Crabtree, 1999; Rao *et al.*, 1997). Activated CaM binds CaN at the CaM-binding domain with high-affinity (Guerini, 1997) and this binding induces a reversible conformational change in CaN. This structural modification causes the AI domain to dissociate from the catalytic groove thereby unblocking the CaN catalytic domain and activating the phosphatase (Kincaid and Vaughan, 1986; Sugiura *et al.*, 1998). The Ca^{2+}/CaM dependence of this enzyme allows CaN to respond to narrow Ca^{2+} thresholds following stimulation (Dolmetsch *et al.*, 1997; Shibasaki *et al.*, 2002). Interesting, CaN is activated by sustained Ca^{2+} release from the ER and Ca^{2+} influx from the plasma membrane (Aramburu *et al.*, 2000; Crabtree, 2001), but is insensitive to Ca^{2+} transients such as those necessary for muscle contraction (Dolmetsch *et al.*, 1997).

CaN substrates

Contrary to other protein phosphatases, CaN has a relatively narrow substrate specificity (Guerini, 1997; Klee, 1998). CaN is able to bind some substrates due to a conserved calcineurin-binding motif, PxIxIT (Chow *et al.*, 2000; Kim *et al.*, 2003). Substrate specificity of CaN is not only due to a specific sequence, but rather is determined by structural features (Blumenthal *et al.*, 1985; Donella-Deana *et al.*, 1994). Substrates of CaN include: 1) two inhibitors of phosphatase 1, DARP32 (dopamine and cyclic AMP regulated phosphoprotein) and inhibitor-1; 2) two membrane receptors, $InsP_3R$ (Cameron *et al.*, 1995) and N-methyl-D-aspartate (NMDA) synaptic receptor (Lieberman and Mody, 1994; Tong *et al.*, 1995); 3) Bad, a pro-apoptotic protein of the Bcl-2 family (Wang *et al.*, 1999); 4) nitric oxide synthase (Dawson *et al.*, 1993; Herskovits *et al.*, 1993); 5) substrates co-localized to the cytoskeleton, tau (Garver *et al.*,

1999; Goto *et al.*, 1985; Kayyali *et al.*, 1997), microtubule-associated protein 2 (Goto *et al.*, 1985; Montoro *et al.*, 1993), tubulin (Goto *et al.*, 1985), dystrophin (Earnest *et al.*, 1996; Michalak *et al.*, 1996; Walsh *et al.*, 1995), and dynamin (a GTPase involved with endocytosis) (Earnest *et al.*, 1996; Herskovits *et al.*, 1993; Liu *et al.*, 1994); 6) heat shock protein 25 (Gaestel *et al.*, 1992); and, 7) transcription factors, Elk-1 (Gaestel *et al.*, 1992; Sugimoto *et al.*, 1997), NFATc (Barford, 1996; Molkentin *et al.*, 1998), and MEF2A (Wu *et al.*, 2000). It has been suggested that tight binding of substrates by CaN may assist dephosphorylation of substrates whose intracellular concentration is very low (Jain *et al.*, 1993; Wesselborg *et al.*, 1996). Although several substrates of CaN have been identified, it is possible that there are others yet to be discovered.

Adaptor proteins permit CaN to interact with a larger number of proteins. For example, association of CaN with FK506-binding protein-12 (FKBP12) protein allows CaN to regulate $InsP_3R$ (Cameron *et al.*, 1995). $InsP_3R$ is found within the ER membrane and is a channel that permits Ca^{2+} efflux from the ER in response to Ca^{2+} or $InsP_3$ binding. FKBP targeting of CaN to this ER receptor may induce a structural change to the receptor via dephosphorylation (Brillantes *et al.*, 1994; Cameron *et al.*, 1997) thereby inducing a structural change that could ensure rapid Ca^{2+} release from the ER.

As CaN is a ubiquitous protein, it is highly probable that it is involved in many different Ca^{2+}-dependent signaling pathways in multiple organ systems. To date, several important cellular functions of CaN have been documented (Rusnak and Mertz, 2000). One very important function of CaN is to dephosphorylate NFATc transcription factors. Dephosphorylation of a serine-rich region of the amino-terminal of NFAT unmasks two NLSs that permit NFAT to translocate to the nucleus and modify gene expression

(Flanagan *et al.*, 1991; Molkentin *et al.*, 1998; Okamura *et al.*, 2000; Rusnak and Mertz, 2000). Interestingly, Ca^{2+} binding to CaN encourages an association between CaN and NFAT resulting in the co-localization of both to the nucleus (Shibasaki *et al.*, 1996). The significance of this co-localization is uncertain, but may be to maintain the signaling event and prevent NFAT nuclear export (Shibasaki *et al.*, 1996). By any means, CaN translocation of NFAT is necessary for immune responses in B- and T-cells (Rao *et al.*, 1997) as well as is essential for cardiac development and pathology (Akazawa and Komuro, 2003; Mesaeli *et al.*, 1999; Molkentin et al., 1998; Olson and Molkentin, 1999). When intracellular Ca^{2+} concentration is less than 150nM, glycogen synthase kinase-3β (GSK-3β) will phosphorylate nuclear NFAT resulting in NFAT being exported from the nucleus in a chromosome region maintenance protein 1- (CRM1)-dependent manner (Fukuda *et al.*, 1997; Gorlich, 1997) with a t½ ~12 minutes (Shibasaki *et al.*, 1996). In addition to influencing NFAT, CaN in skeletal muscle influences activity of MEF2 family of transcription factors by an unknown mechanism (Akazawa and Komuro, 2003; Blaeser *et al.*, 2000; Frey *et al.*, 2000; Mao and Wiedmann, 1999; Wu *et al.*, 2000; Youn *et al.*, 1999).

CaN in the heart

Although the physiological function of CaN dephosphorylating NFAT was originally elucidated in T-cells, the same basic pathway applies in other cell types including cardiomyocytes (Frey and Olson, 2003; Molkentin *et al.*, 1998; Sugden, 1999; Wilkins and Molkentin, 2002). Mice with NFATc1 gene disruption die *in utero* due to an inability to develop normal heart valves and septa (de la Pompa *et al.*, 1998; Ranger *et al.*, 1998). In another study, mice with cardiac over-expression of a constitutively-active

form of NFAT were found to develop severe cardiac hypertrophy (Molkentin *et al.*, 1998). Therefore, in the heart, NFAT is essential for cardiac development and participates in the occurrence of cardiac hypertrophy (de la Pompa *et al.*, 1998; Lim and Molkentin, 1999; Lim and Molkentin, 2000; Molkentin *et al.*, 1998).

Even though both CnA and CnB subunits are essential for catalytic activity, the CnA subunit can be activated constitutively by deletion of the carboxyl-terminal regulatory region (O'Keefe *et al.*, 1992). Deletion of both the AI domain and a carboxyl-terminal portion of the CaM-binding domain within CnA renders a constitutively-active form of CnA (CaN*), which is Ca^{2+} and CaM independent (Hubbard and Klee, 1989; O'Keefe *et al.*, 1992). Transgenic mice with CaN* under control of the α-MHC promoter will over-express constitutively-active CaN only in the heart. It was found that these α-MHC-CaN* mice developed severe cardiac hypertrophy and died approximately 70 days after birth as a result of heart failure (Molkentin *et al.*, 1998; Olson and Molkentin, 1999). The reason for development of hypertrophy was attributed to NFATc activation of genes associated with the fetal cardiac program (Molkentin *et al.*, 1998). When CaN* mice were treated with an immunosuppressant drug, cyclosporin A (CsA), to target CaN and CaN*, cardiac hypertrophy was essentially eliminated (Molkentin *et al.*, 1998). As such, it was implied that inhibitors of CaN may one day be utilized in a clinical setting to treat cardiac hypertrophy (Olson and Molkentin, 1999; Sussman *et al.*, 1998).

In the clinic, elevated levels of activated CaN were observed in hearts from patients in a compensatory hypertrophic phase and to a lesser extent in patients with overtly failing hearts (Haq *et al.*, 2001). In another investigation, CaN activity was found to be several fold higher in cardiac extracts from heart failure patients (Lim and

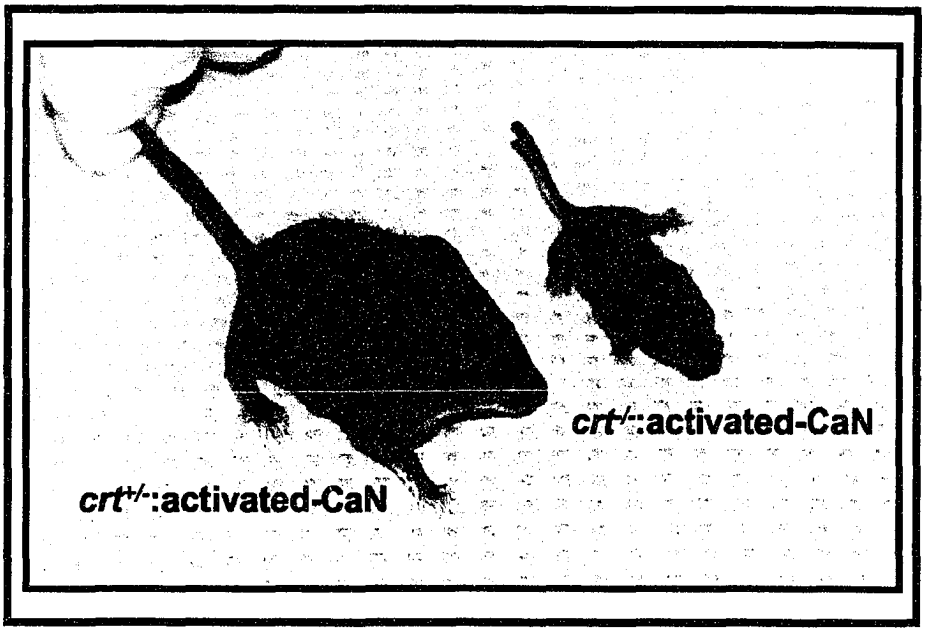
Molkentin, 1999). Clinical observations pertaining to CaN and heart disease as well as findings pertaining to CaN in animal models all suggest the importance of CaN to development of cardiac hypertrophy. Therefore, identification of CaN inhibitors could potentially be utilized to avoid, treat, or perhaps even cure some cases of cardiac hypertrophy.

Expression of CaN* in hearts of *crt*$^{-/-}$ mice

Calreticulin-deficient cells have impaired $InsP_3$-induced Ca^{2+} release (Nakamura *et al.*, 2001b) and compromised NFATc nuclear translocation (Mesaeli *et al.*, 1999), implying that CaN activity is impaired in absence of calreticulin. Calreticulin-deficiency is embryonic lethal in mice due to severe cardiac defects and, at present, no animal completely deficient for CaN exists. Subsequently, without a CaN-deficient animal model it is difficult to fully understand the relationship between the ER, calreticulin, Ca^{2+}, and CaN. Recently, this challenge was overcome by breeding calreticulin heterozygote mice with α–MHC-CaN* mice. This breeding was an attempt to link ER Ca^{2+} to activation of CaN. Interestingly, such breeding rendered a viable calreticulin-deficient mouse (Guo *et al.*, 2002) that had CaN* only in the heart (Figure 1-7). The fact that this animal was born indicated that calreticulin is a key upstream regulator of CaN in the Ca^{2+}-signaling cascade.

Although expression of CaN* in the heart reversed embryonic lethality because of calreticulin-deficiency, the surviving mice exhibited postnatal pathology and died 7 to 35 days after birth. In comparison to littermates, the rescue mice exhibited massive growth retardation and had severe metabolic problems as evidenced by blood cholesterol and sugar concentrations (Guo *et al.*, 2002). Blood analysis indicated hypoglycemia as

Figure 1-7. Cardiac expression of calcineurin rescues calreticulin-deficient phenotype. A photograph of calreticulin-deficient mouse rescued with the activated calcineurin (*crt* $^{-/-}$: *activated CaN*) and its sibling (*crt*$^{+/-}$: *activated CaN*) at 4 weeks of age. Reproduced from *The Journal of Biological Chemistry*, 2002, **277**: 50776-50779 with copyright permission.



Guo *et al.*, 2002

early as the first week of postnatal life and the "milky" appearance of the serum was indicative of abnormal lipid metabolism. Biochemical analysis of serum revealed significantly high levels of cholesterol (over 4-fold) and triacylglycerols (over 9-fold) in the rescue mouse relative to a heterozygote littermate. The underlying cause for the metabolic aberrations is not yet understood, but suggests the importance of the ER and calreticulin for normal energy homeostasis following birth.

MEF2C and Ca^{2+}

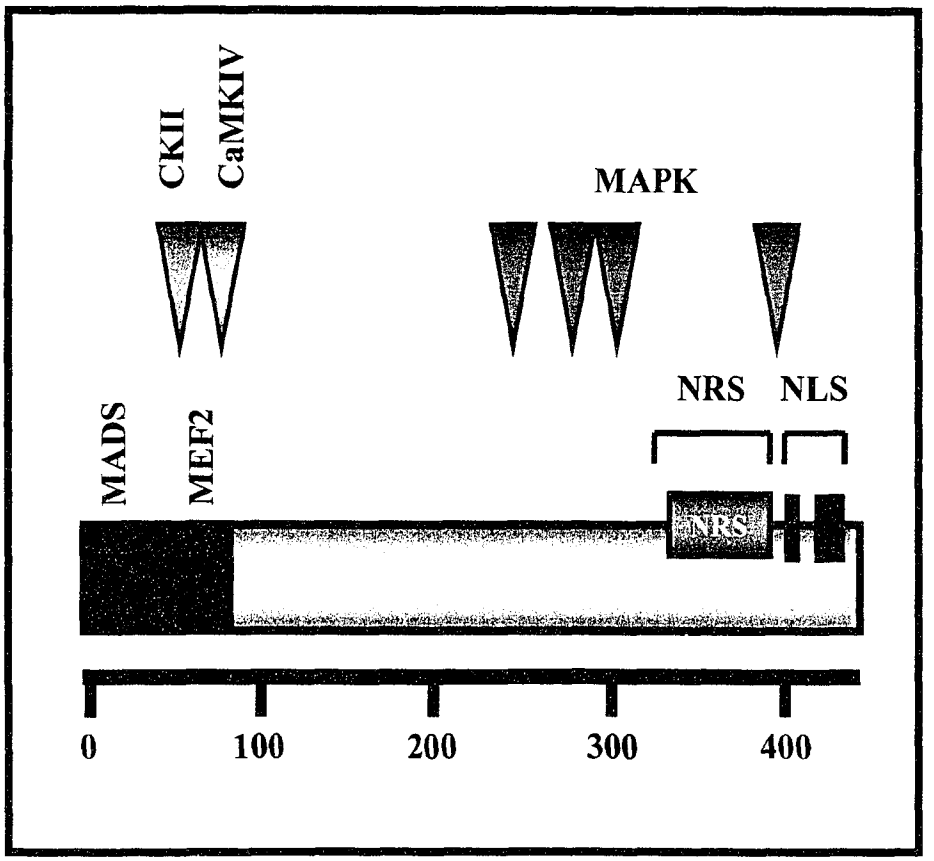
Contractions initiated in heart tubes of MEF2C homozygous embryos were observed to be slower and less rhythmic than that of wild-type embryos. At E9, the heart rate of wild-type and *MEF2C* heterozygous embryos was recorded as 58 ± 3 beats per minute, whereas for the mutants it was 26 ± 4 beats per minute (Lin *et al.*, 1997). In wild-type embryos, the heart tube was noted to exhibit a peristaltic beat that become sequential, with strong atrial contraction preceding ventricular contraction (Lin *et al.*, 1997). However, in MEF2C homozygotes, the atrial chamber exhibited weak contractions and the hypoplastic ventricular chamber appeared to vibrate only in response to the atrial contractions (Lin *et al.*, 1997). Therefore, the poor contractile properties of the heart suggest that Ca^{2+} homeostasis and/or Ca^{2+} handling proteins are compromised in absence of MEF2C.

HDACs repress MEF2 activity (McKinsey *et al.*, 2001b) and CaMKs relieve this repression. CaMKs activate MEF2C by phosphorylating Ser^{259} and Ser^{498} of class II HDACs (HDAC4 or HDAC5) resulting in HDAC release from MEF2C (Lu *et al.*, 2000; McKinsey *et al.*, 2000). HDAC4 and -5 each possess a signal-responsive nuclear export sequence (NES) at their extreme carboxyl-termini (McKinsey *et al.*, 2001b). As such,

evidence suggests that the chaperone protein 14-3-3 associates with phosphorylated Ser^{259} and Ser^{498} thereby causing nuclear export of HDACs (Ellis *et al.*, 2003; Frey *et al.*, 2000; McKinsey *et al.*, 2000; McKinsey *et al.*, 2001b). Nuclear export of HDACs is not essential for activation of MEF2s, but was found to help sustain expression of target genes (McKinsey *et al.*, 2000). In the cytoplasm, CaM may associate with the MEF2-binding domain of HDAC (Frey *et al.*, 2000), perhaps representing a Ca^{2+}-feedback mechanism. Further studies to determine the significance of the CaM interaction with HDACs are necessary.

MEF2 proteins possess numerous sites of potential phosphorylation. CKII, protein kinase C (PKC), calmodulin-dependent protein kinase IV (CaMKIV), and MAP-dependent protein kinase (MAPK) can all phosphorylate MEF2 (Black and Olson, 1998) (Figure 1-8). p38 (MAPK) is a terminal component of the Ras signaling pathway and a p38 target is MEF2C (Han *et al.*, 1997). p38 phosphorylates several sites in the carboxyl-terminal transactivation domain of MEF2; however, MEF2 bound by HDACs prevents phosphorylation by p38. Thus, CaMK must be activated before MEF2 phosphorylation by p38 is possible (Frey *et al.*, 2000). p38 phosphorylation of MEF2 serves to enhance MEF2 transcriptional activity. Although some aspects of MEF2C phosphorylation are known, it is an area that requires further investigation.

Figure 1-8. Schematic diagram of MEF2C with identified sites of phosphorylation indicated. Only a few sites of MEF2C phosphorylation have been currently identified by *in vitro* studies and they are indicated in the figure along with respective phosphorylating kinases. MADS and MEF2 domains are indicated as are the relative locations of the NRS and bipartite NLS. *Abbreviations*: CKII: casein kinase II; CaMKIV: calmodulin-dependent protein kinase IV; MAPK: mitogen-activated protein kinase; NRS: nuclear retention signal; and NLS: nuclear localization signal.



Objectives

The objective of this study was to determine what cardiac expressed transcription factors activate the calreticulin promoter and determine if any of these factors were regulated by CaN. In cardiomyocytes, Ca^{2+} serves several roles ranging from a secondary messenger involved with gene expression to a controller of excitation-contraction coupling (Molkentin *et al.*, 1998; Passier *et al.*, 2000). As such, Ca^{2+} is an essential component in both heart development and in healthy and diseased hearts. Disruption of calreticulin gene, an ER Ca^{2+}-binding protein, is embryonic lethal in mice as a result of improperly developed heart ventricles (Mesaeli *et al.*, 1999). Intracellular Ca^{2+} modulates phosphatase-mediated cellular signaling and cardiac expression of constitutively-active CaN, a Ca^{2+}/calmodulin-dependent phosphatase, will rescue the calreticulin-deficient phenotype (Guo *et al.*, 2002). CaN is known to influence activation of several transcription factors including NFATc4 (Molkentin *et al.*, 1998) and MEF2A (Wu *et al.*, 2000). An important Ca^{2+}-dependent cardiac transcription factor essential for heart development and associated with hypertrophy is MEF2C (Lin *et al.*, 1997; Passier *et al.*, 2000). Disruption of the MEF2C gene in mice is embryonic lethal early in development due to severe malformation of heart ventricles (Lin *et al.*, 1997). Hence, an aim of this investigation was to provide a molecular explanation as to why the calreticulin-deficient embryo dies during development while cardiac over-expression of constitutively-activate CaN rescues the *crt*$^{-/-}$ phenotype.

Rationale and hypothesis

The ER participates in Ca^{2+}-dependent signaling cascades by releasing its stored Ca^{2+} which serves to activate cytoplasmic proteins that impact transcriptional activation/suppression of genes associated with growth, stress, or other cellular processes (Berridge, 2002). A change in the dynamics of the ER, such as absence of a molecular chaperone or a change in Ca^{2+} homeostasis, can have a substantial influence on cellular signaling cascades. An ER lumen protein that functions as both a molecular chaperone and a Ca^{2+} buffer is calreticulin. Calreticulin is highly expressed in the embryonic heart and is essential for heart development. Examination of calreticulin-deficient fibroblasts indicated that basal ER Ca^{2+} concentration is lower than wild-type fibroblasts and $InsP_3R$ Ca^{2+} release is compromised when calreticulin is absent. Thus, one hypothesis is that calreticulin-deficient cells have compromised intracellular Ca^{2+} signaling cascades. To date, the only Ca^{2+}/CaM-dependent serine/threonine phosphatase identified is CaN. CaN is an ubiquitously expressed cytoplasmic protein whose activation is dependent on Ca^{2+} released from the ER via $InsP_3R$ (Mesaeli *et al.*, 1999). Cardiac over-expression of CaN* results in cardiac hypertrophy (Molkentin *et al.*, 1998) and breeding heterozygote calreticulin mice with mice that expressed CaN* in the heart rescued calreticulin-deficient embryos (Guo *et al.*, 2002). Survival of the rescue mouse suggests that calreticulin is an upstream regulator of CaN; however, it also raises the question of why the calreticulin-deficient rescue mouse survives. Does it survive solely due to NFATc translocation or are other effectors of CaN responsible? Thus, identification of transcription factors dependent on CaN activation is necessary to suggest an explanation as to why the calreticulin-deficient rescue mouse is viable.

CaN has been reported to target MEFA in skeletal muscle (Wu *et al.*, 2000). MEF2A is the MEF2 isoform most similar to MEF2C (Black and Olson, 1998) and MEF2C is essential for development of heart ventricles (Lin *et al.*, 1997). Therefore, my hypothesis is that calreticulin-dependent Ca^{2+} released from the ER is essential for activation of CaN and this phosphatase acts on MEF2C to increase expression of MEF2C target genes.

Specific aims

The phosphatase CaN and the transcription factor MEF2C are essential for cardiac development, normal post-natal cardiomyocyte growth, and both proteins can contribute to disease hypertrophy. Does activated CaN target MEF2C in the heart to rescue the calreticulin-deficient phenotype? To answer this question and further deduce signaling pathways involved in cardiac development and pathology, it is necessary to determine if and how CaN influences MEF2C transcriptional activity in cardiomyocytes by performing the following experiments:

1) Utilize reporter genes to determine if MEF2C and CaN* independently and/or synergistically activate the calreticulin promoter.

2) Demonstrate interaction of MEF2C with the calreticulin promoter.

3) Demonstrate that CaN activation is compromised in the absence of calreticulin.

3) Determine if CaN* influences activation of MEF2C.

4) Demonstrate direct targeting of MEF2C by CaN*.

For this investigation, established biochemical and molecular biological techniques were to be utilized.

Significance of proposed research

Cardiac hypertrophy is a major illness contributing to hundreds-of-thousands of deaths annually. Subsequently, it is essential that cardiac hypertrophy be understood at a molecular level so that novel targets to prevent, treat, or perhaps even cure the disease be identified. Researchers have found that cardiac disease and cardiac development are very similar processes as both utilize Ca^{2+}-dependent signaling pathways and both require expression of many similar genes. Consequently, the purpose of this study was to expand current understanding of cardiac Ca^{2+}-dependent signaling pathways by providing a molecular explanation as to why calreticulin-deficient embryos die *in utero* and how cardiac expression of CaN* rescues calreticulin-deficient mice. It is hypothesized that the Ca^{2+}-dependent phosphatase, CaN, regulates activity of a transcription factor essential for cardiac development and muscle growth, MEF2C. If the hypothesis is correct and CaN regulates MEF2C activity, then targeting ER Ca^{2+} release, CaN, and/or MEF2C with drugs or endogenous inhibitors could represent an effective treatment for cardiac hypertrophy. From a pharmacological perspective, MEF2C represents a promising drug target as it is an endpoint upon which multiple hypertrophic signaling cascades converge (Black *et al.*, 1998; Kolodziejczyk, 1999). Thus, this research will provide major insight into molecular mechanisms underlying control of fetal cardiac genes responsible for cardiac pathology seen in clinics throughout the world.

Luciferase and β-galactosidase assays

1722bp of the mouse calreticulin promoter (Waser *et al.*, 1997) was inserted into a promoterless luciferase reporter vector (de Wet *et al.*, 1987), pXP1 (ATCC), to create CPF plasmid. cDNAs encoding transcription factors were generous gifts supplied by other investigators as follows: Nkx2.5 from Dr. Issei Komuro (University of Tokyo Graduate School of Medicine, Tokyo, Japan); MEF2C and myocardin from Dr. Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, Texas, USA); GATA4 and GATA6 from Dr. Mona Nemer (Institut de Recherches Cliques de Montreal, Montreal, Quebec, Canada); NFATc4 from Dr. T. Hoey (Tularik Inc., San Francisco, California, USA); Evi-1 from Dr. Archibald S. Perkins (Yale School of Medicine, New Haven, Connecticut, USA); COUP-TFI from Dr. Larry Fliegel (University of Alberta, Edmonton, Alberta, Canada); and MyoD1 from Dr. Michel Puceat (Centre de Recherches de Biochime Macromoléculaire, Montpellier, France).

For luciferase reporter gene activity analysis, NIH3T3 mouse fibroblasts were transiently transfected with plasmid DNA by the Ca^{2+}-phosphate transfection method (Ausubel *et al.*, 1989). Transfections methods such as Lipofectamine (Invitrogen, Cat. 18324-012), Effectene (Qiagen, Cat. 301425), and FuGENE 6 (Roche, Cat 1 814 443) were all tested to determine transfection efficiencies. In all instances, much higher efficiencies were observed with the commercial products (FuGENE 6 > Effectene > Lipofectamine), but because similar trends relative to controls were observed, I opted to transfect with the very economical Ca^{2+}-phosphate method. Between 5 and 10μg total DNA (with typically 2μg of DNA plasmid containing cDNA encoding each transcription

factor) was transfected into cells (~50% confluent; approximately 6 x 10^6 cells) grown on 10cm plates. For controls, empty vector was used to normalize DNA amounts (Guo *et al.*, 2001; Liang *et al.*, 2001). 48 hours after transfection, cells were washed three times with PBS [137mM NaCl; 2.7mM KCl; 8.1mM Na_2HPO_4; 1.5mM KH_2PO_4; pH 7.4], lysed with 100μL of a Nonidet P40 (NP40) lysis buffer [100mM Tris, pH 7.8; 0.5% NP40; 50mM dithiothreitol (DTT)] for 20 minutes on ice, harvested, and centrifuged at 7500rpm for 5 minutes at 4^0C. Supernatant was collected and 10μL utilized for each luciferase measurement [combined with 100μL luciferase buffer (20mM Tricine; 1.1mM $MgCO_3$; 2.7mM $MgSO_4$; 0.1mM EDTA (Ethylene diamine tetra-acetic acid); 33mM DTT; 270μM coenzyme A; 470μM luciferin (Sigma, Cat. L-6882); 530μM ATP)] and 20μL utilized for each β-gal measurement [combined with 80μL H_2O and 13nM O-nitrophenyl-β-D-galactopyranoside (ONPG)]. Data for luciferase and β-galactosidase activity assays were collected as described previously (Guo *et al.*, 2001; Waser *et al.*, 1997) using Lumat LB9501 and Molecular Devices microplate reader machines, respectively.

Cells and Cultures

NIH3T3 mouse fibroblasts, wild-type embryonic fibroblasts, and calreticulin-deficient embryonic fibroblasts were maintained in Dulbecco's modified eagle medium (DMEM; Sigma; Cat. D5796) containing 10% fetal bovine serum (FBS; Sigma, Cat. F-1051) and 1% penicillin-streptomycin (Invitrogen; Cat. 15140-122) at a pH of 7.8. Cells were grown at 37^0C in an 5% CO_2, 95% air incubator. In some instances it was desirable to elevate cytoplasmic Ca^{2+} concentration, so cells were treated with 200nM bradykinin (Nakamura *et al.*, 2001b). Cells after more than 25 passages were not utilized.

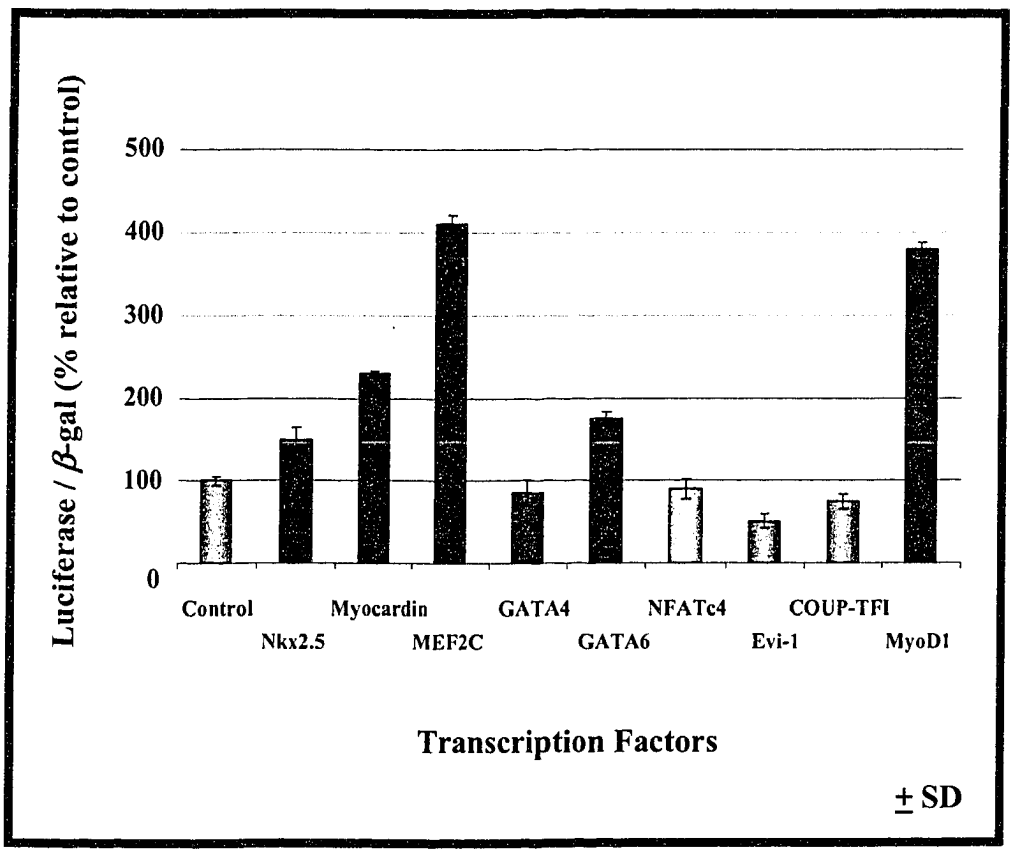
RESULTS

Analysis of transcriptional activation of the calreticulin promoter

Reporter gene analysis suggested that numerous factors bind to the calreticulin promoter to influence reporter gene activity (Figure 1-9). The transcription factors that induced the greatest amount of reporter gene activity in order of most active were: MEF2C, MyoD1, myocardin, GATA6, and Nkx2.5. Both GATA4 and NFATc4 were unable to activate reporter gene activity even when co-transfected with other factors and/or pre-treated with bradykinin to elevate cytoplasmic Ca^{2+} concentrations. Interestingly, two repressors of calreticulin promoter were identified, COUP-TFI and Evi-1, with the latter being the more potent.

When Nkx2.5 was expressed with the calreticulin promoter-controlled luciferase reporter gene, activity of the reporter gene was increased by approximately 50% (Figure 1-9). Investigation of Nkx2.5 influence on calreticulin promoter has been published (Guo *et al.*, 2001) and my contribution to this work was determining the influence Nkx2.5 had on reporter gene activity in both NIH3T3 fibroblasts as well as neonatal rat cardiomyocytes (Guo *et al.*, 2001). Interestingly, when COUP-TFI expression vector was co-transfected with plasmid containing cDNA encoding Nkx2.5 into fibroblasts, there was a dose-dependent repression of reporter gene activity (Guo *et al.*, 2001). When considering that the expression of Nkx2.5 decreases during development and the relative amount of COUP-TFI does not change significantly (Guo *et al.*, 2001) it is reasonable to hypothesize that in the developing heart expression of calreticulin is at least in part down-regulated by COUP-TFI binding to the Nkx2.5 sites within the calreticulin promoter.

Figure 1-9. MEF2C activated calreticulin promoter-controlled luciferase reporter gene. Activity of calreticulin promoter-controlled luciferase reporter gene in NIH3T3 cells co-transfected with plasmid encoding various transcription factors. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.



Myocardin activated reporter gene activity approximately 2-fold (Figure 1-9). At the time of this investigation little was known about the consensus DNA binding site for myocardin and few research tools (e.g., antibodies) were available. Thus, although myocardin activates the calreticulin promoter-controlled luciferase reporter gene further investigation of this transcription factor was not performed.

MEF2C was the transcription factor that activated calreticulin promoter-controlled reporter gene to the greatest extent, consistently inducing a 3- to 4-fold enhancement in reporter gene activity (Figure 1-9). MEF2C binds A/T rich DNA sequences and within the calreticulin promoter there were four tentative binding sites (Figure 1-5).

Of the nine transcription factors initially studied, only GATA4 and NFAT4c did not to impact calreticulin promoter-controlled reporter gene activity (Figure 1-9). Both of these factors were tested several times and under several different conditions. Different conditions included: 1) co-transfection of cDNA encoding these two transcription factors; 2) co-transfection with cDNA encoding other transcription factors; 3) co-transfection with cDNA encoding constitutively-active calcineurin plasmid to encourage NFATc4 nuclear accumulation; and 4) drug treatment to enhance cytoplasmic Ca^{2+} to activate endogenous calcineurin and permit NFATc4 nuclear translocation. Despite the effort, neither GATA4 nor NFATc4 activated calreticulin promoter luciferase reporter gene.

Within the calreticulin promoter there are three tentative binding sites for GATA factors (Figure 1-5). A yeast-one hybrid (Y1H) experiment which utilized an E11 mouse cardiac library (Guo *et al.*, 2001) and a probe which had the double GATA site (-981bp to -1000bp) from calreticulin promoter found that both GATA6 (Identity: 276bp/283bp)

and Evi-1 (Identity 362bp/372bp) strongly bound to this region of the calreticulin promoter (Dr. Lei Guo, unpublished data). Activation of calreticulin promoter driven luciferase reporter gene by GATA6 was observed to be approximately 80%, while Evi-1 inhibited reporter gene activity by 50% (Figure 1-9). Both GATA6 and Evi-1 are expressed in the developing heart and the probable DNA binding site was known from the Y1H, so further investigation of these factors was tempting.

Protein-protein interactions enable many transcription factors to regulate gene expression and these interactions are an important aspect of the fetal cardiac program. Many reports have found a synergistic relationship between Nkx2.5 and GATA transcription factors for many fetal cardiac gene promoters (Durocher *et al.*, 1997). For this reason, a GATA6 investigation had to include experiments involving Nkx2.5 and COUP-TFI.

MyoD1 was found to activate calreticulin promoter-controlled reporter gene activity approximately 3-fold (Figure 1-9). This observation was not surprising as the calreticulin promoter possesses one ideal DNA consensus binding site for MyoD1 (Figure 1-5). Reporter gene results for MyoD1 served as a positive control and were an indication as to the level of reporter activity that could be expected from a transcriptional activator binding to an ideal consensus site within the calreticulin promoter. However, MyoD1 is a transcription factor expressed exclusively in skeletal muscle and is absent from the heart. Since the primary objective of this investigation was to determine which transcription factors regulated cardiac expression of calreticulin, further investigation of MyoD1 was not beneficial.

DISCUSSION

Nkx2.5 binds to and activates calreticulin promoter-controlled luciferase reporter gene. Binding of calreticulin promoter by Nkx2.5 was not completely unexpected as the promoter possessed three tentative binding sites for this transcription factor. As Nkx2.5 is the earliest embryonic marker for cells committed to become cardiomyocytes, calreticulin gene regulation by Nkx2.5 suggested that calreticulin may play an important role in early cardiogenesis. A previous investigation involving transgenic mice with GFP under control of calreticulin promoter found that calreticulin was highly expressed throughout atria and ventricles of E9 hearts (Mesaeli *et al.*, 1999). As Nkx2.5 is highly expressed in cardiomyocytes at E7.5, it is probable that GFP expression would have been observed in these embryos had it been examined prior to E9. Regardless, the observation that Nkx2.5 activates the calreticulin promoter and this activation is later repressed by COUP-TFI binding to the Nkx2.5 site (Guo *et al.*, 2001) suggests importance of calreticulin in early heart development, and provides clues as how the calreticulin gene is regulated in embryonic and adult hearts.

Myocardin is expressed in the embryonic heart at almost the same time as Nkx2.5. Discovery of myocardin is relatively recent (Wang *et al.*, 2001) and since being first reported, many researchers have suggested that the role of myocardin may not be greatest in the heart, but rather for expression of smooth muscle genes (Chen *et al.*, 2002; Li *et al.*, 2003; Wang *et al.*, 2003). The finding that myocardin activated the calreticulin promoter-controlled luciferase reporter gene was unexpected as calreticulin promoter lacks an ideal DNA binding site for this transcription factor. As such, future investigation

will be necessary to precisely determine where myocardin binds to the calreticulin promoter as well as the significance of myocardin transcriptional activation.

MEF2C was the transcription factor that most strongly activated calreticulin promoter-controlled luciferase reporter gene. MEF2C binds A/T-rich DNA sequences and inspection of calreticulin promoter suggested four tentative MEF2C binding sites. Interestingly, cardiac expression of MEF2C overlaps with that of calreticulin. Mice with MEF2C gene disrupted die at E9 as a result of malformed heart ventricles. In comparison, calreticulin-deficient embryos die at E14.5 due to a similar defect. Hence, MEF2C influence on calreticulin promoter-controlled luciferase reporter gene was a finding that required further investigation.

GATA4 and NFATc4 did not influence activity of the calreticulin promoter-controlled luciferase reporter gene. The calreticulin promoter possesses three tentative GATA4/6 consensus binding sites that, interestingly, are in close proximity to four putative NFAT DNA binding sites (Figure 1-5). As neither GATA4 nor NFATc4 influenced calreticulin promoter-controlled luciferase reporter gene, it was decided that GATA4 and NFATc4 would not be further studied in this investigation.

GATA6 activated calreticulin promoter-controlled luciferase reporter gene. The observation that a cardiac expressed GATA family member activated the reporter gene was not surprising as calreticulin promoter has three tentative GATA binding sites. Y1H data suggested identity of the GATA6 binding site within calreticulin promoter as well as a potential binding site for a repressor of GATA6 activity, Evi-1. Thus, it was decided that GATA6 and Evi-1 influence on calreticulin promoter would be further studied.

Experiments with luciferase reporter genes and transcription factors were a source of discovery. These experiments provided clues as to which transcription factors potentially regulated calreticulin promoter during cardiac development and data suggested that several cardiac transcription factors could influence calreticulin promoter. Of nine factors originally considered for investigation, seven were observed to impact calreticulin promoter-controlled luciferase reporter gene activity. Of the seven, five factors (Nkx2.5, myocardin, MEF2C, GATA6, and MyoD1) were deemed to be transcriptional activators while two were found to be transcriptional repressors (Evi-1, COUP-TFI). Given that so many factors potentially regulated calreticulin gene expression, a decision had to be made as to which factors were to be focused upon. MEF2C was the transcription factor that activated the reporter gene to the greatest extent, consistently inducing a 3- to 4-fold enhancement in reporter gene activity. As MEF2C is known to be highly expressed in both developing and diseased hearts, has a similar expression pattern as calreticulin, and has been suggested to have transcriptional activity influenced by Ca^{2+} it was decided that this factor would be included in this investigation. In addition, luciferase assay findings pertaining to GATA6 and Evi-1 were complemented nicely by Y1H data. Therefore, the project to determine which cardiac transcription factors regulated expression of calreticulin gene was to become more focused and so further investigation was to involve only three factors: MEF2C, GATA6, and Evi-1.

As will be seen in following chapters, the MEF2C aspect of the promoter investigation would become the primary thesis project. As a result, data for GATA6 and Evi-1 has been presented in Supplementary Chapter 1.

MEF2C BINDS TO THE CALRETICULIN PROMOTER

A version of portions of this chapter has been submitted for publication:

1. Lynch, J., Guo, L., Gelebart, P., Chilibeck, K., Xu, J., Molkentin, J.D., Agellon, L.B., and Michalak, M. 2004. Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca^{2+}-dependent signaling pathway of early cardiac development. *Status: Submitted.*

Data from calreticulin promoter-controlled luciferase reporter gene experiments showed that MEF2C strongly activated reporter gene activity. MEF2C binds A/T-rich DNA nucleotide sequences (Gossett *et al.*, 1989) and the consensus DNA binding site for MEF2C is (C/T)TA(A/T)$_4$TA(G/A) (Andres *et al.*, 1995; Fickett, 1996; Pollock and Treisman, 1991). Within the calreticulin promoter there is no ideal MEF2C DNA binding site, but there were several A/T-rich sequences similar to the consensus site. Thus, the next steps in this investigation involved determining if MEF2C: 1) synergistically interacted with any other transcription factors to activate a calreticulin promoter-controlled luciferase reporter gene; 2) influenced reporter gene activity in a dose-dependent manner; and, 3) directly interacted with the calreticulin promoter to activate gene expression. Determining synergism between transcription factors and constructing a dose-response curve required performing more experiments with the calreticulin promoter-controlled luciferase reporter gene. However, precisely determining where a transcription factor binds to a site within a promoter is a laborious task. Current methods utilized to establish DNA binding sites include electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP).

EMSA is a technique utilized to demonstrate protein-DNA interaction. In EMSA, a probe possessing a DNA sequence of interest is radiolabelled and incubated with a protein of interest. Samples are analyzed on a native polyacrylamide gel and the radioactivity visualized. DNA to which the protein binds will move more slowly in the gel and mobility is relative to the sample with no protein. EMSA for MEF2C has been previously described (McDermott *et al.*, 1993; Molkentin *et al.*, 1996a; Ornatsky and

McDermott, 1996). Advantages of the EMSA technique are that it is technically easy and can be applied to reveal multiple complexes. A disadvantage of the method is that false positives can occur in this non-physiological "pure probe – pure ligand" setting. To correct for weakness of this technique, supporting evidence of protein-DNA interaction can be collected from the following experiments: 1) supershift (antibody against the ligand will supershift the band on a polyacrylamide gel); 2) competition EMSA; and 3) ChIP. Despite its limitations, EMSA remains a powerful method by which to observe protein-DNA interaction.

ChIP is a procedure (Lee and Kraus, 2001; Orlando, 2000) utilized to detect *in vivo* protein-DNA interaction. The ChIP assay involves a step of *in vivo* formaldehyde cross-linking of whole cells to stabilize protein-protein and protein-DNA interactions. DNA is later sheared by sonication and antibodies specific for the protein of interest are utilized for immunoprecipitation of the protein-DNA complexes. With ChIP it is possible to isolate a transcription factor bound to DNA and later purify the DNA for PCR (polymerase chain reaction) analysis. Therefore, ChIP provides biologically significant evidence of protein binding to a specific region of DNA.

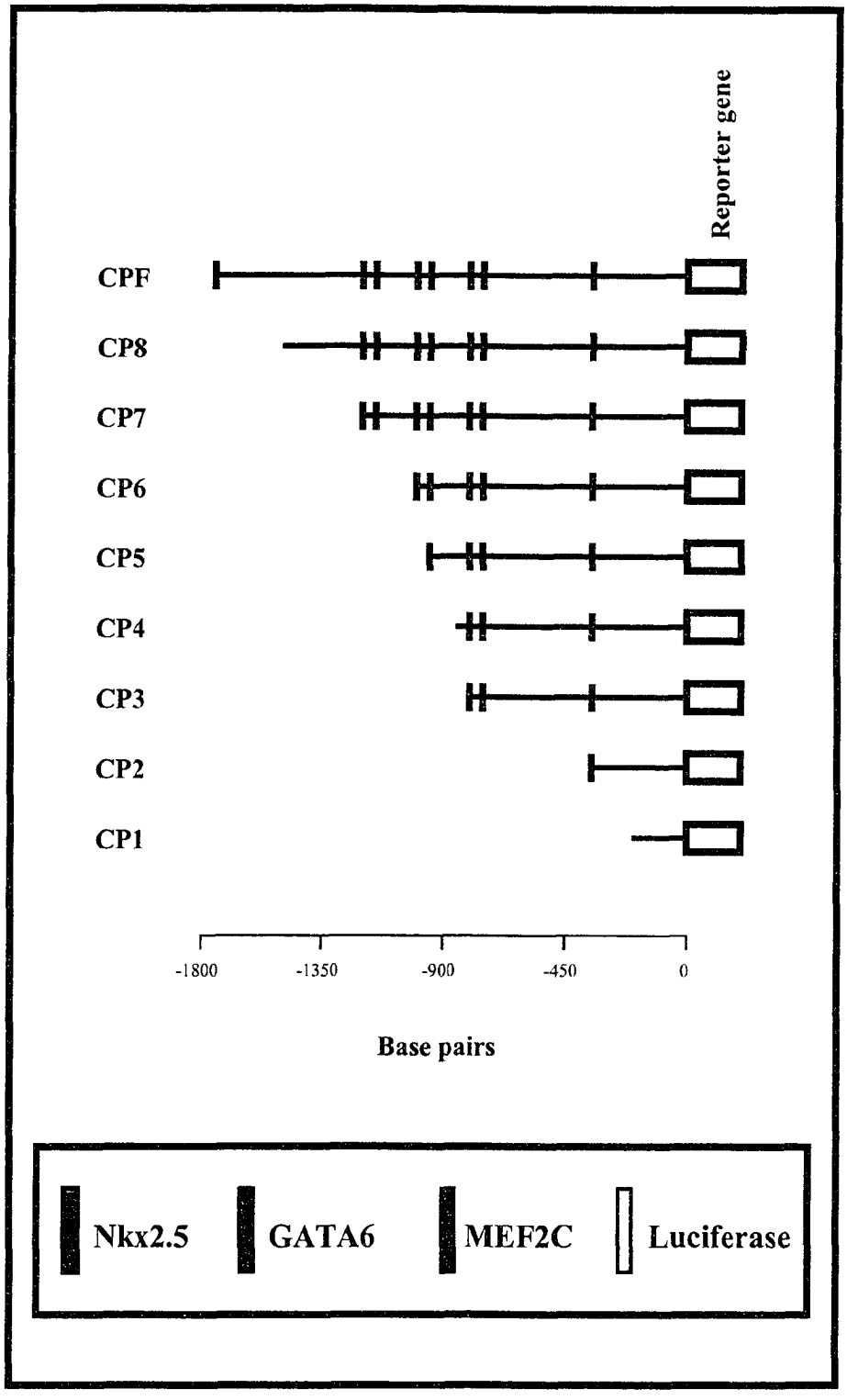
In this investigation various methods were utilized to provide evidence of MEF2C binding to the calreticulin promoter. First, several luciferase reporter plasmids with deletion mutants of the calreticulin promoter were constructed and utilized to determine the region of the promoter bound by MEF2C. Second, EMSA and competition EMSA were completed to show that MEF2C specifically bound to the calreticulin promoter *in vitro*. Finally, a ChIP experiment provided *in vivo* evidence supporting the EMSA results.

It was believed that by utilizing several different techniques the precise MEF2C site within the calreticulin promoter would be correctly identified.

Luciferase and β-galactosidase assays

Calreticulin promoter deletion mutants were created to assist in determining the potential MEF2C binding site within calreticulin promoter. Three deletion mutants of the calreticulin promoter had previously been made (Waser *et al.*, 1997). For this investigation extra calreticulin promoter deletion mutant plasmids were necessary and so five additional deletion mutants of the calreticulin promoter were constructed by PCR method. For each deletion mutant a 5' Sstl site and a 3' HindIII site was introduced. PCR was performed in a GeneAmp PCR System 9700 in 50μL total reaction mixture consisting of: Taq buffer [20mM Tris-HCl (pH 8.8), 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 5μg BSA (bovine serum albumin)]; 0.3mM deoxyribonucleotide triphosphates (dNTPs; Invitrogen; Cat. 55085); 200ng forward primer; 200ng reverse primer; 2 units Vent Polymerase (NEB; Cat. M0254S). PCR products were purified with Qiaquick PCR purification kit (Qiagen; Cat. 28106) according to manufacturer instructions. PCR products were digested with restriction enzymes as recommended by the manufacturer and separated by 1% low-melting-point agarose gel (Invitrogen; Cat. 15517-014). DNA was purified from the gel by MinElute gel extraction kit (Qiagen; Cat. 28606) as recommended by the manufacturer. In total, eight deletion mutants of the calreticulin promoter were cloned into pXP1 with promoter sizes ranging from 172 to 1510bp. Deletion mutants were named from smallest to largest, CP1 to CP8 (Figure 2-1). For all cloning: 1) synthetic oligos were purchased from Sigma, 2) plasmid DNA was purified by QIAGEN plasmid maxi kit (Cat. 12165) according to manufacturer instructions, 3) restriction enzymes were from Gibco, 4) ligation was with

Figure 2-1. Schematic diagrams of calreticulin promoter and promoter deletion mutants. The schematics indicate location of Nkx2.5 (red), MEF2C (green), GATA6 (blue) DNA binding sites as well as luciferase reporter gene (yellow). Vectors contained the following DNA fragments of the calreticulin promoter: CPF: 1722bp; CP8: 1510bp; CP7: 1234bp; CP6: 1009bp; CP5: 992bp; CP4: 907bp; CP3: 862bp; CP2: 371bp; CP1: 172bp.



T4 ligase from NEB (Cat. M0202L), and 5) nucleotide sequencing of all plasmids was performed by the DNA Core Facility at the University of Alberta.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA for MEF2C had previously been described (Kolodziejczyk, 1999) and the protocol for MEF2C EMSA described in this study was generously provided by Dr. John McDermott (York University, Toronto, Ontario, Canada). For EMSA, MEF2C protein was required and so both MEF2C and luciferase proteins were synthesized using a coupled transcription and translation (TNT) reticulocyte system (Promega, Cat. L4610) according to manufacturer instructions. Luciferase protein was synthesized as a positive control to ensure that TNT protein synthesis was completed successfully. Following protein synthesis, it was necessary to confirm that the TNT method was successful. As such, 5μL of synthesized luciferase and MEF2C protein samples were loaded onto a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The proteins were visualized by the Transcend Colourmetric Translation Detection System (Promega; Cat. L5070) using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega; Cat. S3841) according to manufacture instructions.

For the EMSA experiment, two 33-mer probes were synthesized. Probe 1 was representative of the predicted calreticulin promoter MEF2C site (5'-GAGAAAGAGAAAA**AAAAAAAATC**AAAAAACCCC-3'), while probe 2 was a positive control, (5'-GAGAAAGAGAAAA**CTAAAAATAA**AAAAAACCCC-3') with an ideal MEF2C consensus site flanked by bases from the tentative MEF2C site within the calreticulin promoter. Probes were annealed to their complementary deoxyoligonucleotides and annealed oligonucleotides (5pmol) were labeled with 2.5μL

[γ^{32}P]ATP (10μci/μL; Amersham Pharmacia Biotech.) using 1μL T4 polynucleotide kinase (Gibco; Cat. 18004-010) for 15 minutes at 37^{0}C. Quick spin G-25 sepheadex columns (Boehringer Mannheim; Cat. 1 273 949) were utilized to purify radiolabelled DNA. MEF2C protein was pre-incubated on ice for 20 minutes in binding buffer [final concentrations: 20mM HEPES (pH 7.8), 50mM KCl, 1mM DTT, 1mM EDTA, 1.25% glycerol] in presence of 1μg poly(deoxyinosinic-deoxycytidylic) (poly dI•dC; Sigma; Cat. P-4929) and 50ng single-stranded oligo, GL28-pXP1 (Guo *et al.*, 2001). Following pre-incubation, purified radiolabelled probe was added and incubation continued for 20 minutes at room temperature. Loading buffer (20% Ficoll 400; 0.1M EDTA, pH 8; 1% sodium dodecyl sulfate (SDS); 0.25% bromophenol blue) was added to each sample (Voytas, 1998) and samples were loaded onto a native 7.5% acrylamide gel. Gels were run in TBE (Tris, Boric acid, EDTA, pH 8.0) buffer at 220 Volts. Dried gels were exposed to a phosphoimager plate and the plate was scanned using a Typhoon 8600 Variable Mode Imager.

Chromatin Immunoprecipitation (ChIP)

cDNA encoding mouse MEF2C (432 amino acids; GenBank Accession: NM_025282) was amplified by PCR and 5' XhoI and 3' BamHI sites incorporated so that it could be ligated into pcDNA3.1/myc-His(-)A (Invitrogen) cloning vector. FuGENE 6 was utilized to transiently transfect wild-type mouse embryonic fibroblasts with MEF2C-myc-His according to manufacturer instructions. 48 hours after transfection, cells were fixed in 1% formaldehyde (Sigma; Cat. F-1635) at room temperature for 20 minutes. Cells were harvested and lysed with Extract-N-Amp for tissue (Sigma; Cat. XNAT2) according to manufacturer instructions. A Branson 450 Sonifier was utilized to shear the

chromatin to a final average size of 850bp. Extracts were pre-incubated with a small volume of Protein A sepharose beads (Amersham Biosciences; Cat. 17-0780-01) to pre-clear the extracts. Pre-cleared extracts were incubated at 4^0C for 3 hours with mouse monoclonal anti-His antibody (Santa Cruz; Cat. SC-8036) at a dilution of 150:1. Following this incubation, the extracts were added to a tube with 30μL bead volume of Protein A sepharose beads (Amersham Biosciences; Cat. 17-0780-01). Immunoprecipitates were washed, eluted, and incubated at 65^0C to reverse the cross-links. DNA was purified and analyzed by Touch-down PCR (TD-PCR). TD-PCR was performed in an automated Biometra thermocycler in 50μL total reaction mixture consisting of: 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 0.5mM dNTP mix, 2 units of Taq DNA polymerase (Invitrogen; Cat. 10342-020) and 1ng/μL of the forward (5'-CCCTTGCCTTCTGCCGTTTATTG-3') and reverse primers (5'-CCCGTAGTTCCCTGCAGCCTGT-3'). PCR samples were loaded along with 1kb+ DNA ladder (Invitrogen; Cat. 10787-018) onto a 1% agarose gel. The gel was visualized with the Gel Doc 1000 (Bio-Rad Laboratories, Hercules, CA) coupled to Molecular Analyst software version 2.1.2.

RESULTS

Co-transfection of MEF2C with other transcription factors suggested that MEF2C interacted with and activated calreticulin promoter (Figure 2-2). In this experiment MEF2C plasmid DNA was co-transfected with Nkx2.5, myocardin, GATA6, as well as NFATc4. In all instances, activation of the calreticulin promoter was not observed to be greatly increased or decreased by presence of the other factors. Therefore, MEF2C likely bound to the calreticulin promoter alone.

Deletion mutants of the calreticulin promoter were necessary to provide insight as to the location of MEF2C binding site within the calreticulin promoter. In total, eight deletion mutants of the calreticulin promoter were created and co-transfected into NIH3T3 cells with MEF2C plasmid. In presence of MEF2C, CPF activity was increased approximately 3-fold. Deletion mutants CP3 to CP8 had activity comparable to CPF whereas CP1 and CP2 had activity similar to the control (Figure 2-3). The difference between CP3 and CP2 is one tentative MEF2C binding site (Figure 2-3). Thus, analysis of calreticulin promoter deletion mutants suggested that a probable MEF2C binding site was within CP3 (862bp).

EMSA provides *in vitro* evidence of protein binding to a specific DNA sequence and this experiment was necessary to provide evidence of MEF2C directly binding to a site within the calreticulin promoter. For this experiment, a probe containing the tentative MEF2C binding site as suggested by the promoter deletion mutant experiment was tested. The positive control was MEF2C protein binding to a probe with an ideal MEF2C consensus DNA binding site (probe 2) whereas the negative control was

Figure 2-2. The presence of other transcription factors does not enhance MEF2C activation of the calreticulin promoter-controlled luciferase reporter gene. cDNA of the calreticulin promoter with luciferase reporter gene was co-transfected with cDNA for MEF2C and other transcription factors into NIH3T3 cells. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Data are mean representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.

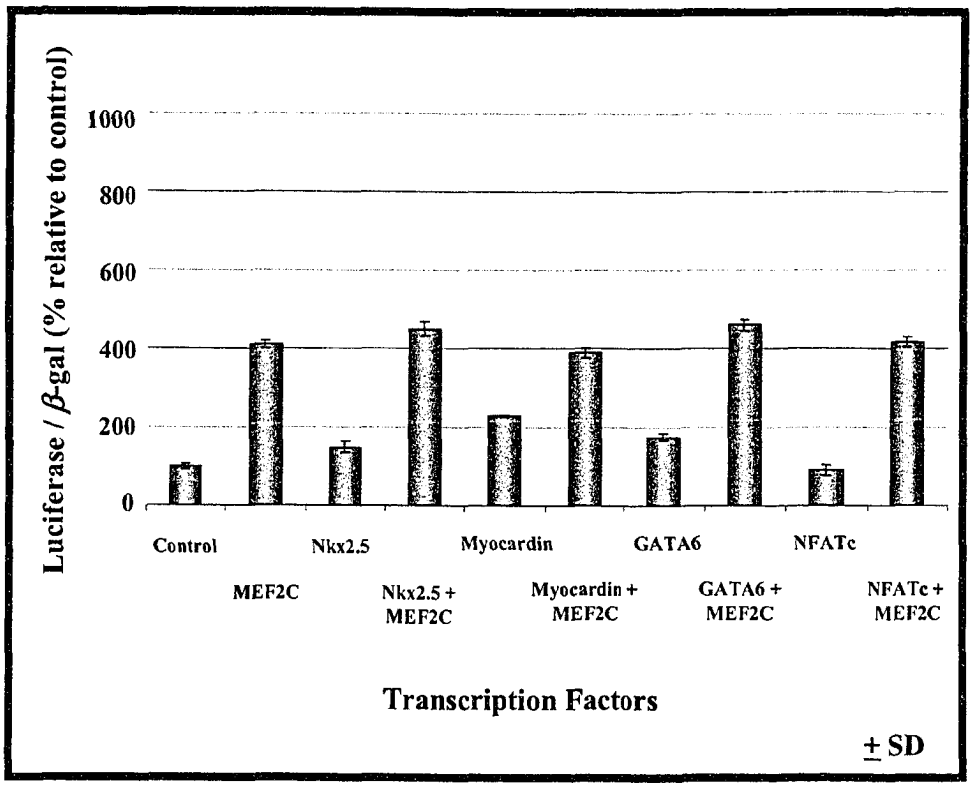
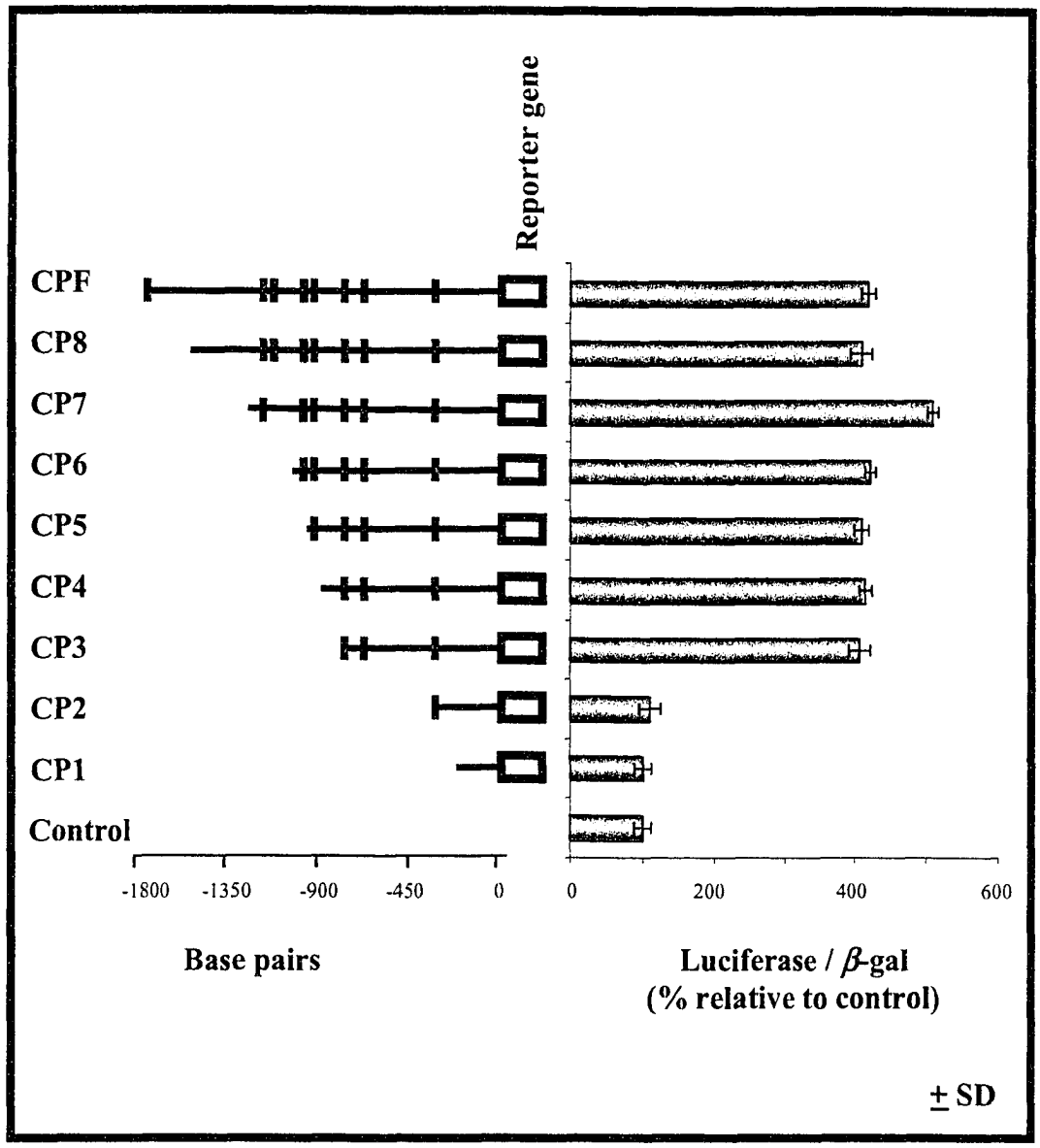


Figure 2-3. Schematic diagrams of calreticulin promoter and promoter deletion mutants as well as reporter gene activity in response to MEF2C. The schematics indicate location of tentative Nkx2.5 (red), MEF2C (green), and GATA6 (blue) DNA binding sites. Vectors contained the following DNA fragments of the calreticulin promoter: CPF: 1722bp; CP8: 1568bp; CP7: 1292bp; CP6: 1068bp; CP5: 1053bp; CP4: 965bp; CP3: 928bp; CP2: 438bp; CP1: 212bp. The calreticulin promoter deletion mutants were co-transfected with cDNA encoding MEF2C and the luciferase reporter gene activity measured. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each deletion mutant. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.



luciferase protein interaction with the identical probe. Results showed that probe 2 did not bind to the luciferase protein, but did bind strongly to the MEF2 protein (Figure 2-4, *lanes 1 and 2*, respectively). The probe with the tentative MEF2C site (probe 1) was observed to bind MEF2C protein (Figure 2-4, *lane 3*), but the intensity of the binding was slightly less than that for the positive control. Thus, results from EMSA clearly indicated that MEF2C bound to a nucleotide sequence found at position -836 to -845 within the calreticulin promoter.

In a competition EMSA excess unlabelled competitor probe identical to the labeled probe should abolish the observed protein-DNA complexes if the DNA contains a high-affinity site for the protein. In this experiment, non-labeled competing probe ranging from 2 to 20 times the amount of labeled probe was included in the reaction mixture. The results indicate that when competing probe was present, the interaction of the labeled probe with MEF2C protein was disrupted (Figure 2-5, *lanes 3 to 7*). This result suggested that MEF2C association with its calreticulin promoter binding site was relatively weak. When the competition EMSA was completed using the positive control probe which had the ideal MEF2C binding site, MEF2C was able to bind the probe in the presence of 5 times the amount of unlabeled probe (Appendix Figure 1). In summary, the results from EMSA and competition EMSA indicated that MEF2C bound specifically to position -836 to -845 within calreticulin promoter.

ChIP is a technique by which to demonstrate *in vivo* interaction between a transcription factor and DNA. With ChIP it was possible to isolate MEF2C bound to DNA and later utilize this DNA for PCR analysis. In this investigation, mouse embryonic fibroblasts were first transfected with MEF2C-myc-His plasmid DNA and later harvested

Figure 2-4. EMSA shows *in vitro* binding of MEF2C to probe that includes the MEF2C site within calreticulin promoter. *Lane 1*: Luciferase protein with probe for ideal MEF2C consensus binding site (negative control); *Lane 2*: MEF2C with probe for ideal MEF2C consensus binding site (positive control); and *Lane 3*: MEF2C with probe for predicted MEF2C binding site within calreticulin promoter.

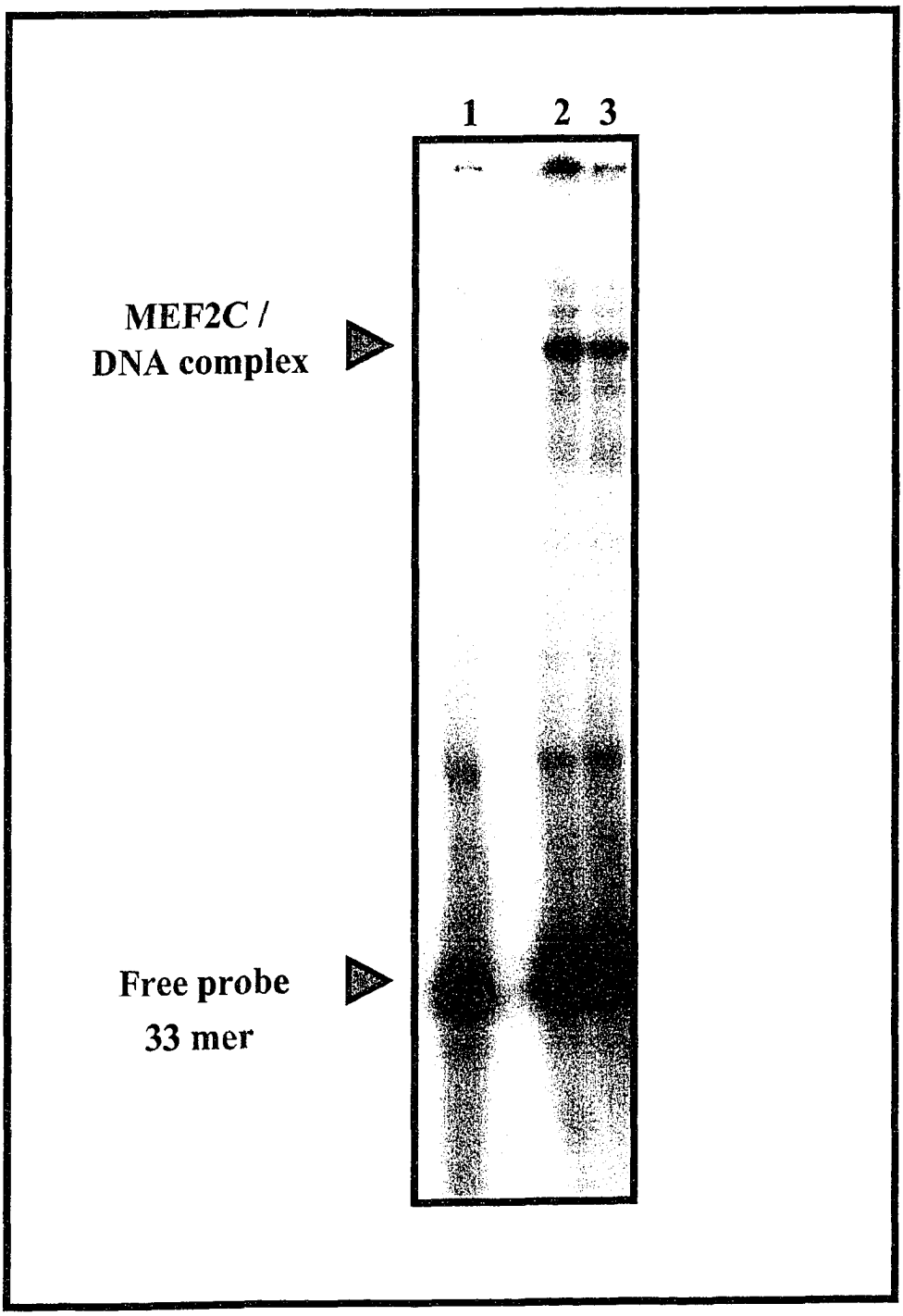
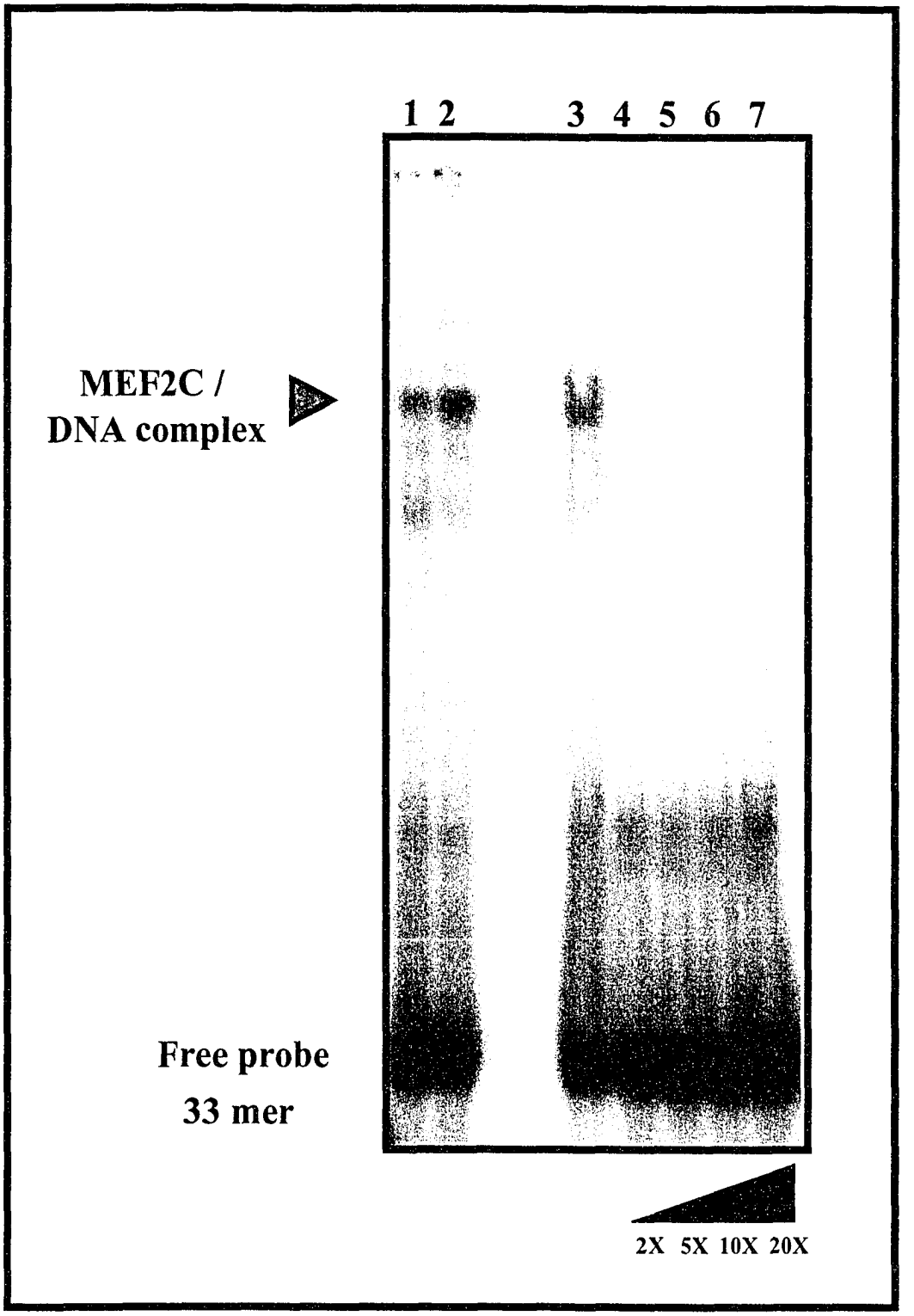


Figure 2-5. Competition EMSA of non-labeled probed competing for binding to MEF2C site within calreticulin promoter. *Lane 1*: MEF2C with probe for predicted MEF2C binding site within calreticulin promoter; *Lane 2*: MEF2C with probe for ideal MEF2C consensus binding site (positive control); *Lane 3*: MEF2C with probe for predicted MEF2C binding site within calreticulin promoter; and *Lanes 4 to 7*: MEF2C with labeled and unlabeled probe for predicted MEF2C binding site within calreticulin promoter.



and fixed with formaldehyde.

When the DNA was analyzed, a PCR product was present for the plasmid positive control, but not for the negative control (Figure 2-6, *lanes 2 and 3*, respectively). PCR analysis of ChIP-isolated DNA produced a PCR product of identical size to the positive calreticulin promoter control control (Figure 2-6, *lane 4*). Thus, ChIP provided *in vivo* evidence of MEF2C binding to calreticulin promoter. When the ChIP experiment was performed using isolated neonatal rat cardiomyocytes, the findings support that observed in the fibroblasts (Appendix Figure 2). Therefore, ChIP findings supported the EMSA results and confirmed that MEF2C bound calreticulin promoter at position -836 to -845 (Figure 2-7).

Figure 2-6. ChIP demonstrates *in vivo* binding of MEF2C to calreticulin promoter. PCR products using ChIP isolated DNA as template were loaded onto an agarose gel. *Lane 1*: 1kb+ DNA ladder; *Lane 2*: Calreticulin promoter plasmid (positive control); *Lane 3*: ChIP analysis with no MEF2C antibody utilized (negative control); and, *Lane 4*: ChIP sample in which anti-His antibody was utilized. PCR product size was 310bp.

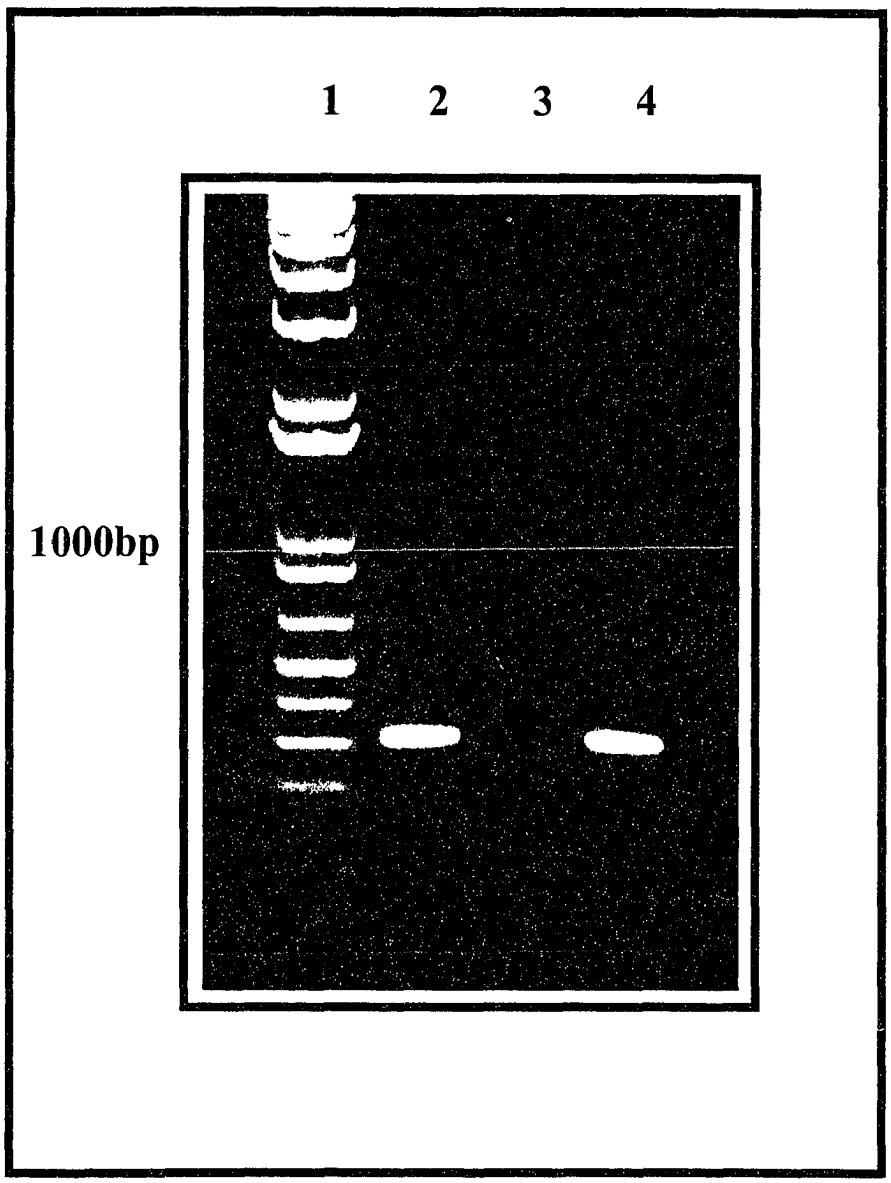
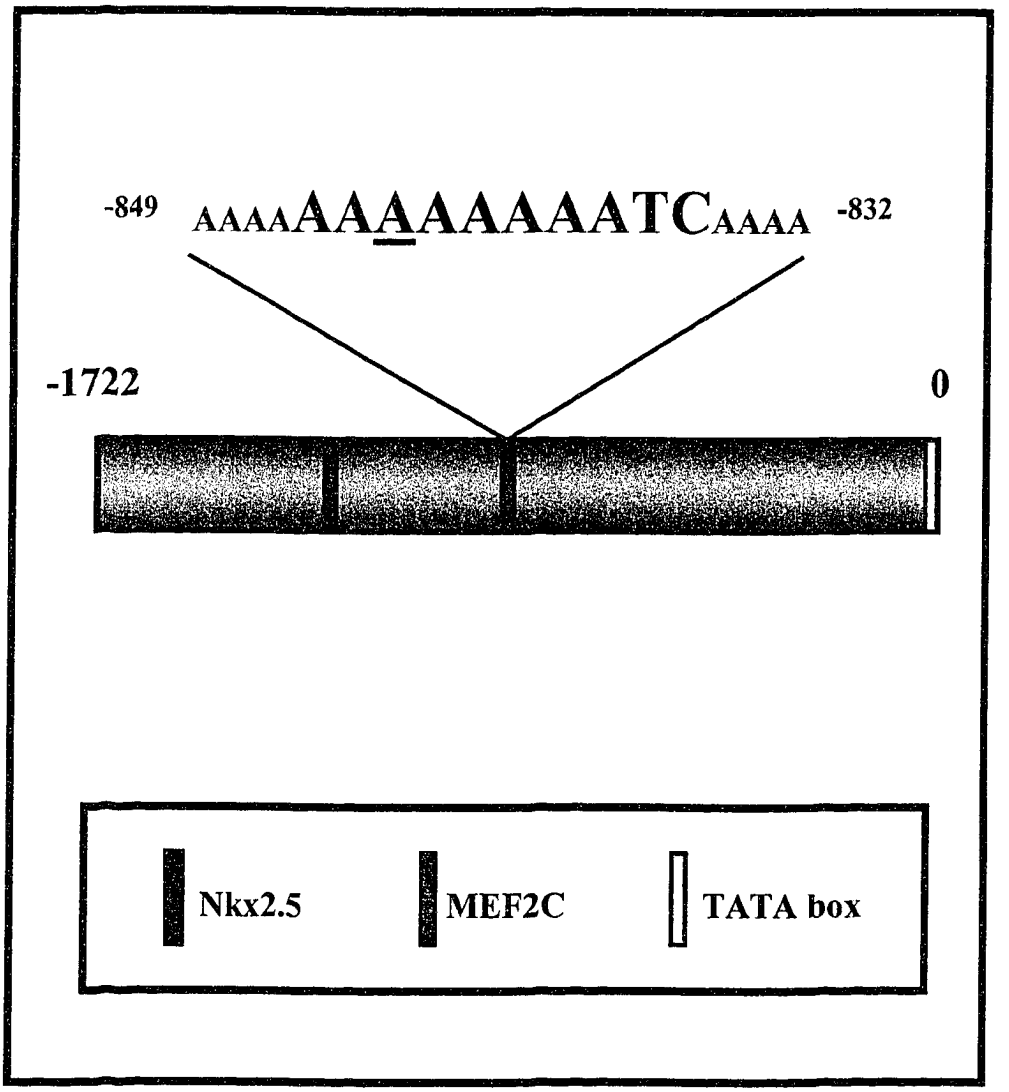


Figure 2-7. Schematic figure of the calreticulin promoter indicating the position of identified MEF2C DNA binding site. The MEF2C site in the calreticulin promoter is located from -836 to -845. The binding site is a reversal of the ideal MEF2C binding site suggested by the literature with exception of one T to A base substitution (*underlined*). Locations of the identified Nkx2.5 DNA binding site (red), MEF2C DNA binding site (green), and TATA box (yellow) are indicated.



DISCUSSION

Co-transfection experiments suggested that MEF2C independently activated the calreticulin promoter. The paradigm for MEF2 proteins is that they weakly bind DNA and rely on interactions with other transcription factors, such as MyoD, for DNA binding (Black and Olson, 1998). This stereotype is starting to change and recently it was reported that MEF2 proteins independently activated skeletal muscle genes (Wu *et al.*, 2000). Thus, the observation that MEF2C independently activated the calreticulin promoter is not without precedent.

The observation that MEF2C activated the calreticulin promoter-controlled luciferase reporter gene was unexpected as initial inspection of the calreticulin promoter suggested that the promoter did not possess an ideal MEF2 DNA consensus binding site. MEF2 transcription factors bind A/T-rich sequences and minor mutations to the sequence can still permit MEF2C binding (Molkentin *et al.*, 1996a). With that considered, a second examination of the calreticulin promoter was completed and enabled four tentative MEF2C sites to be identified (Figure 1-5). Probes for all four tentative MEF2C sites and a desmin-MEF2C (Naya *et al.*, 1999) positive control were made and utilized for EMSA. Of the four tentative sites, MEF2C protein bound only to one and the location of this site identified by EMSA correlated with that suggested by the calreticulin promoter-controlled deletion mutant reporter gene experiments (Figure 2-3). The MEF2C site identified within the calreticulin promoter (AAAAAAAATC) is a reversal of the MEF2C consensus site (C/T)TA(A/T)$_4$TA(G/A) (Andres *et al.*, 1995; Fickett, 1996; Pollock and Treisman, 1991) with the exception of one T to A base substitution (underlined). Perhaps this base change is the reason why the intensity of the band captured by the

phosphoimager appears less than that for the ideal consensus site used as a positive control (Figure 2-4). The binding of MEF2C to the calreticulin site is likely of moderate strength and can be disrupted by presence of unlabelled probe (Figure 2-5). The ChIP experiment not only confirmed the *in vitro* EMSA findings as to the identity of the MEF2C site, but also provided *in vivo* evidence of MEF2C binding to the calreticulin promoter (Figure 2-6). Thus, the MEF2C binding site within the calreticulin promoter was identified as being at position -836 to -845. When the MEF2C binding site within the calreticulin promoter was mutated (Appendix Figure 3), MEF2C could not activate reporter gene activity (Appendix Figure 4) thereby confirming identification of this site.

In this chapter, MEF2C alone activated calreticulin promoter-controlled luciferase reporter gene. Several different techniques to observe protein-DNA interaction were utilized to demonstrate MEF2C interaction with the calreticulin promoter. These results clearly indicated that MEF2C interacted with calreticulin promoter *in vitro* and *in vivo*. These results enabled a precise identification of the MEF2C site within calreticulin promoter to be determined at position -836 to -845. Therefore, at this stage of the investigation it can be concluded that MEF2C directly binds calreticulin promoter to activate gene expression. Determining the biological significance of MEF2C activation of calreticulin promoter was a future direction.

MEF2C NUCLEAR LOCALIZATION IS Ca^{2+}- AND CALCINEURIN-DEPENDENT

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2. Lynch, J and Michalak, M. 2003. Calreticulin is an upstream regulator of calcineurin. *Biochem. Biophys. Res. Commun.* **311**:1173-9.
3. Lynch, J., Guo, L., Gelebart, P., Chilibeck, K., Xu, J., Molkentin, J.D., Agellon, L.B., and Michalak, M. 2004. Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca^{2+}-dependent signaling pathway of early cardiac development. *Status: Submitted.*

INTRODUCTION

Examination of calreticulin-deficient fibroblasts indicated that basal ER Ca^{2+} concentration is lower than wild-type fibroblasts and $InsP_3R$ Ca^{2+} release is compromised when calreticulin is absent (Mesaeli *et al.*, 1999; Nakamura *et al.*, 2001b). Thus, it is believed that calreticulin-deficient cells have compromised intracellular Ca^{2+} signaling cascades (Mesaeli *et al.*, 1999; Nakamura *et al.*, 2001b). To date, the only Ca^{2+}/CaM-dependent serine/threonine phosphatase identified is CaN. CaN is a ubiquitously expressed cytoplasmic protein that is dependent on the ER releasing Ca^{2+} via $InsP_3R$ for its activation (Mesaeli *et al.*, 1999). Cardiac over-expression of CaN* results in severe cardiac hypertrophy (Molkentin *et al.*, 1998). A recent investigation to deduce cardiac Ca^{2+} signaling pathways involved breeding heterozygote calreticulin mice with mice that expressed a constitutively-active form of CaN in the heart (Guo *et al.*, 2002). Interestingly, cardiac CaN* rescued the calreticulin-deficient phenotype (Guo *et al.*, 2002). Survival of this mouse suggests that calreticulin is an upstream regulator of CaN. However, it also raises the question of why the calreticulin-deficient mouse survives. What are the molecular evens affected by CaN? CaN is a Ca^{2+}-dependent protein and affects transcriptional pathways. Therefore, identification of other transcription factors downstream of CaN was necessary to deduce the mechanism of why and how the calreticulin-deficient rescue mouse survives. In this chapter calreticulin-deficient cells were studied to determine significance of Ca^{2+} dependence of MEF2C. I wanted to determine if there was a relationship between Ca^{2+} released by calreticulin and MEF2C activity.

MATERIALS & METHODS

Plasmids

cDNAs for transcription factors and constitutively-active enzymes were generous gifts supplied by other investigators as follows: Green fluorescent protein-HDAC5 (GFP-HDAC5), constitutively-active CaN (CaN*), and constitutively-active CaMKI (CaMKI*) from Dr. Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, Texas, USA), MEF2A and MEFD from Dr. John McDermott (York University, Toronto, Ontario, Canada), and constitutively-active CaMKII (CaMKII*) from Dr. Howard Schulman (Stanford University, Stanford, California, USA).

Isolation and culturing of cardiomyocytes

Cardiomyocyte primary cultures were obtained from day 12 mouse embryos. Hearts collected from embryos were incubated at 37^0C in 200μL F-12K nutrient mixture (Gibco; Cat. 21127-022) with 0.1% type II collagenase (358U/mg; Worthington Biochemical Corporation, Lakewood, New Jersey, USA) and every eight minutes the hearts were removed from incubation and minced using pipette tips. After 30 minutes of incubation cardiomyocytes were plated in 6-well plates (Falcon; Cat. 353046) onto 0.1% gelatin-coated (Bio-Rad; Cat. 170-6537) 25mm coverslips (Fisher Scientific; Cat. 12-545-107). Cardiomyocytes were maintained in F-12K nutrient mixture supplemented with 20% FBS, 1% MEM non-essential amino acid solution (Invitrogen; Cat. 11140-050), 1% L-glutamine (Invitrogen; Cat. 25030081), 0.008% $CaCl_2$, and 1% penicillin-streptomycin at a pH of 7.45.

Embryo tail clippings were incubated at 65^0C overnight in 500μL buffer (10mM Tris-HCl, pH 8; 150mM NaCl; 10mM EDTA; 0.1% SDS) containing 10μg proteinase K

(Gibco, Cat. 25530-015). Following incubation, genomic DNA was purified by phenol/chloroform precipitation method and genomic DNA was utilized for PCR genotyping of the embryos. One set of PCR primers (T8 and T19) enabled detection of calreticulin gene while another set (Neo3'for and GL31) permitted detection of the neomycin insert.

DNA Arrays

Cardiomyocytes were isolated from E12 wild-type ($crt^{+/+}$) and calreticulin-deficient ($crt^{-/-}$) embryos. Some calreticulin-deficient cells were treated with 1μM ionomycin (Sigma; Cat. I-0634) and 250μM $CaCl_2$ (Sigma; Cat. 4901) for 4.5 hours prior to harvesting. 48 hours after transfection, nuclear proteins were isolated by a nuclear extraction kit (Panomics, Inc.; Cat. AY2002) as described by the manufacturer. Nuclear extracts were incubated in a probe mix supplied by the manufacturer that contained biotin-labeled oligonucleotides. Protein/DNA complexes were permitted to form and these complexes were loaded into wells of a 1% low-melting-point agarose gel. Complexes were removed from the gel and the DNA purified from the complexes. DNA was hybridized to TranSignal™ cAMP/calcium protein DNA arrays (Panomics, Inc.; Cat. MA1500) according to manufacturer instructions. DNA binding was visualized using biotin antibody followed by enhanced chemiluminescence (ECL; SuperSignal West Dura Extended Duration Substrate; Pierce, Rockford, IL; Cat. 34075) development reaction.

DNA arrarys were later stripped according to manufacturer instructions and treated with nuclear extracts isolated from wild-type and calreticulin-deficient mouse embryonic fibroblasts which had been transiently transfected with plasmid DNA containing cDNA encoding MEF2C. FuGENE 6 was utilized for the transfections

according to manufacturer instructions. DNA arrarys for fibroblasts were similar to that for cardiomyocytes, so only the cardiomyocyte data is shown.

RT-PCR experiment

TRIZOL reagent (Gibco, Cat. 15596) was utilized to isolate total RNA from E12 $crt^{+/+}$ and $crt^{-/-}$ embryo hearts according to manufacturer instructions. 500ng samples of isolated RNA were incubated for 5 minutes at 65^0C with oligo (dT) 12-18 primer (Invitrogen; Part Y01212), and 10mM dNTPs mix. Following this incubation, 5X first strand buffer (Invitrogen; Part Y00146), 0.1M DTT (Invitrogen; Part Y00147), RNASE OUT ribonuclease inhibitor (Invitrogen; Cat. 10777-019), and SuperScript II RNase H reverse transcriptase (Invitrogen; Cat. 18064-014) were added to the reaction tube and incubated at 42^0C for 50 minutes. Enzyme was inactivated by heating at 70^0C for 15 minutes. RNase H was then added to the reaction tube and a final incubation at 37^0C for 20 minutes followed. cDNA produced from the RNA was analyzed by step-down PCR (SD-PCR). SD-PCR was performed in an GeneAmp PCR system 9700 (PE Applied Biosystems) in 50μL total reaction mixture consisting of: PCR Buffer [20mM Tris-HCl (pH 8.4), 50mM KCl], 1.5mM $MgCl_2$, 1μL of 0.1mM dNTP mix, 2 units of Taq DNA polymerase (Invitrogen; Cat. 10342-020) and 200ng of forward and reverse primers. For myosin light chain 2v (MLC-2v; GenBank Accession: NM_010861) forward and reverse primers were 5'-CCCAGATCCAGGAGTTCAAGGAAGC-3' and 5'-GGTCAGCATCTCCCGGACATAGTC-3', respectively. For eHAND (GenBank Accession: S79216) the forward primer was 5'-CCGCCGCACCCCATGCTCCACG-3' and the reverse primer was 5'-GCTTGTGCATCCTTGGCCAGC-3'. For iroquois homeobox protein 4 (Irx4; GenBank Accession: AF124732) the forward and reverse

primers utilized to investigate this factor were 5'- GGCAGCTCGCAGGGCTATGG-3' and 5'-GGCGTCTTCAGCTCACATTCTG-3', respectively. The final factor investigated was GATA6 (GenBank Accession: AF179425). GATA6 is not a MEF2C gene target and was selected to serve as a control. GATA6 was studied using forward 5'-GAGTGGAGGTGGCGGCAGCCTG-3' and reverse 5'-GAGCCACTGCTGTTACCGGAGC-3' primers. PCR products were loaded along with 1kb+ DNA ladder (Invitrogen; Cat. 10787-018) onto a 1% agarose gel. The gel was visualized with the Gel Doc 1000 (Bio-Rad Laboratories, Hercules, CA) coupled to Molecular Analyst software version 2.1.2.

Luciferase and β-galactosidase assays

Luciferase and β-gal assays were conducted as described previously. An addition to the protocol involved treatment of cells with 1μM ionomycin and 250μM $CaCl_2$ 18 hours before cells were harvested.

In comparison to NIH3T3 fibroblasts, wild-type and calreticulin-deficient embryonic fibroblasts have very low transfection efficiencies. As such, for transfection of the embryonic fibroblasts FUGENE 6 transfection reagent was utilized.

Immunohistochemistry

Wild-type and calreticulin-deficient embryonic and mouse fibroblasts were grown on 25mm coverslips in 6 well plates. MEF2C was transfected into the cells using FuGENE 6 according to manufacturer protocol. Approximately 30 hours after transfection, cells were fixed with 2% formaldehyde (Sigma; Cat. P-6148) at pH 7.15 and later permeabilized with 0.2% Triton X-100 (Bio-Rad; Cat. 161-040) in accordance with

previously established protocol (Spector *et al.*, 1998). The primary antibody utilized was polyclonal rabbit anti-MEF2C (Cell Signaling Technology; Cat. 9792) at a dilution of 1:70 while the secondary antibody was Texas Red dye-conjugated affinipure donkey anti-rabbit (Jackson ImmunoResearch, Inc.; Cat. 711-075-152) at a dilution of 1:70. Coverslips were fixed to slides with Cytoseal 60 mounting medium (Richard-Allan Scientific, Kalamazoo, Michigan, USA). Slides were examined with a Zeiss Axiovert S100 fluorescent microscope using an excitation wavelength of 595nm and an emission wavelength of 615nm. Images were captured with a Microview Camera (Princeton Instruments, Inc.; Model: RTE/CCD-1317K2) and pseudo coloured in Northern Eclipse 5.0 software.

GFP plasmids

The cloning vector utilized to create GFP fusion proteins was pEGFP-C1 (Clontech; GenBank Accession: U55763). Method for construction of GFP-NFATc4 has been described previously and involved insertion of NFATc4 fragment into the BamHI site (Guo *et al.*, 2002). GFP plasmids were transfected into wild-type and calreticulin-deficient embryonic fibroblasts and cardiomyocytes using FuGENE 6 transfection reagent. All GFP images were of live cells grown on 25mm coverslips. GFP images were captured with a Zeiss Axiovert S100 fluorescent microscope using an excitation wavelength of 490nm and emission wavelength of 520nm. All pictures were pseudo-coloured using Northern Eclipse 5.0 software (Empix Imaging Inc., Mississauga, Ontario, Canada).

RESULTS

MEF2C localization and function was impaired in absence of calreticulin

The Panomic DNA array had DNA consensus binding sites for several Ca^{2+}-dependent transcription factors, three of which were initially studied in this investigation: NFAT, MEF2, and GATA. Nuclear expression of these three transcription factors was observed to be strong in wild-type mouse cardiomyocytes (Figure 3-1). In comparison, calreticulin-deficient embryonic fibroblasts had GATA present in the nuclear extracts, but both NFAT and MEF2 were absent (Figure 3-1). Interestingly, when calreticulin-deficient cells were treated with Ca^{2+} ionophore, nuclear accumulation of both NFAT and MEF2 in these cells was equivalent to that observed for wild-type cells (Figure 3-1). Therefore, in the absence of calreticulin both NFAT and MEF2 are essentially absent from the nucleus, but became nuclear when there was a high concentration of cytoplasmic Ca^{2+}.

The results from DNA array analysis suggested that MEF2C was absent from the nucleus of calreticulin-deficient cells. As MEF2C is a transcription factor, then an inability to localize to the nucleus means that MEF2C function might be compromised. Thus, if MEF2C function was compromised in calreticulin-deficient cells then there should be reduced expression of MEF2C gene targets. To examine expression of MEF2C target genes, RT-PCR was conducted on mRNA isolated from E12 cardiomyocytes. RT-PCR of wild-type cardiomyocytes for MLC-2v, eHAND, Irx4, and GATA6 all rendered a PCR product (Figure 3-2). In comparison, RT-PCR of calreticulin-deficient cardiomyocytes produced a PCR product for GATA6, but not for any targets of MEF2C (Figure 3-2). Thus, RT-PCR results suggested that MEF2C function was compromised in

Figure 3-1. Panomic TranSignal™ Protein/DNA array analysis of nuclear fractions isolated from wild-type and calreticulin-deficient cells. Each square contains 4 spots representing an individual transcription factor. Genes on the array are spotted in duplicate with the bottom of the two spots representing a 1:10 dilution of the top spots. *Top panel*, wild-type (wt) cells; *middle panel*, calreticulin-deficient (*crt*$^{-/-}$) cells; and *bottom panel*, calreticulin-deficient cells treated with Ca^{2+} ionophore.

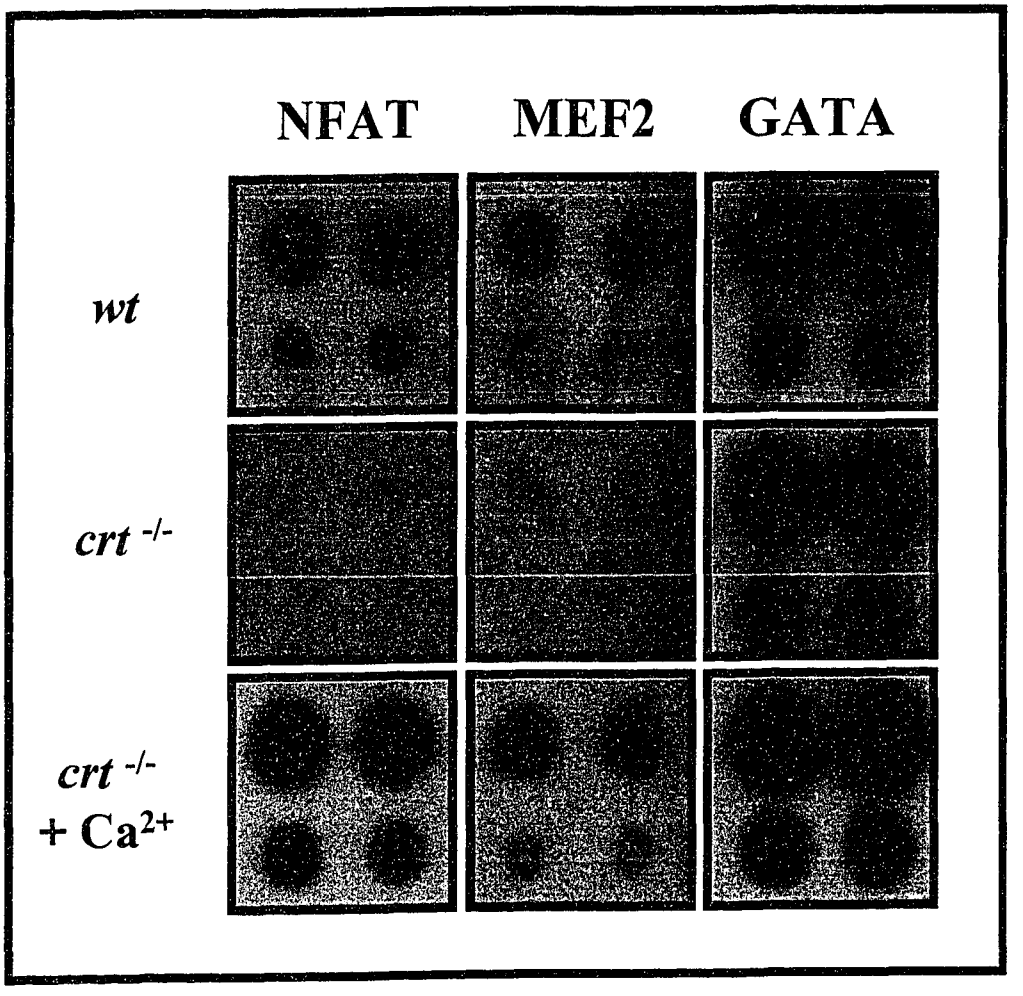
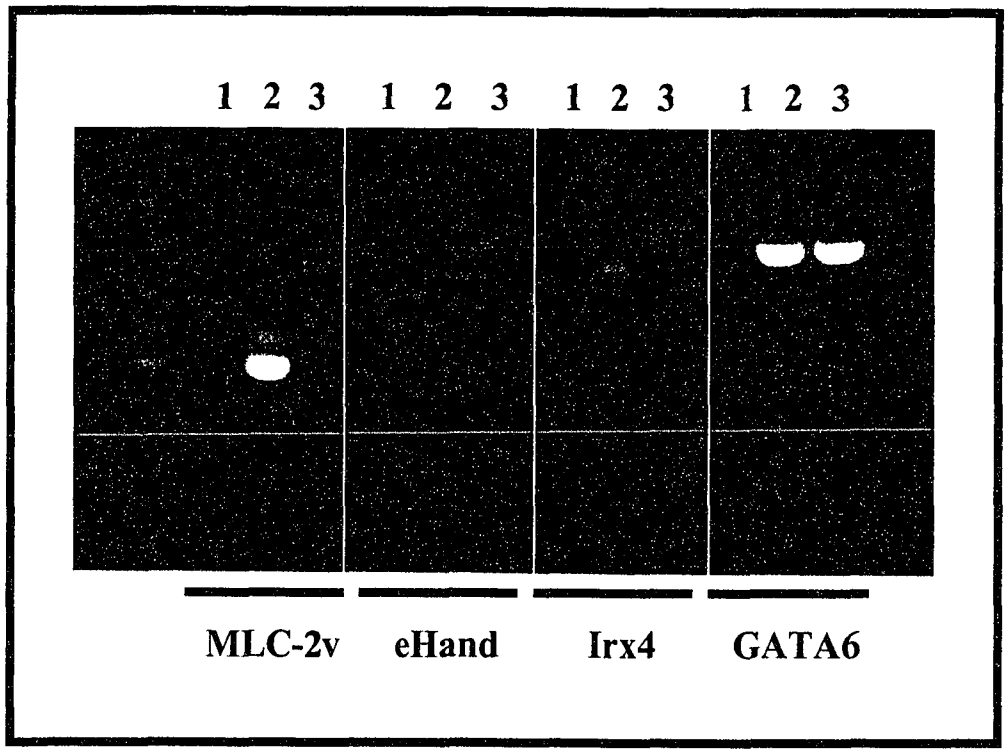


Figure 3-2. RT-PCR of MEF2C target gene products. Expression of MEF2C-activated genes is repressed in absence of calreticulin. RNA isolated from E12 wild-type and calreticulin-deficient embryonic mouse hearts was utilized to produce DNA for PCR analysis of MEF2C target genes. *Lane 1*: control, no RNA; *Lane 2:* amplification of RNA isolated from wild-type cardiomyocytes; and *Lane 3*, amplification of RNA isolated from calreticulin-deficient cardiomyocytes. PCR product sizes for MLC-2v, eHand, Irx4, and GATA6 are 301bp, 415 bp, 604bp, and 641bp, respectively.



the absence of calreticulin.

MEF2C influence on luciferase reporter gene activity was enhanced by Ca^{2+} or CaN*

DNA array analysis suggested that Ca^{2+} treatment of calreticulin-deficient cells permitted MEF2C nuclear localization while RT-PCR results suggested that MEF2C function was compromised in the absence of calreticulin. To determine if Ca^{2+} influenced the function of MEF2C, calreticulin promoter-controlled luciferase reporter gene experiments were completed, but in these experiments cells were treated with Ca^{2+}or co-transfected with constitutively-active forms of Ca^{2+}-dependent enzymes, CaN* or CaMKII*. The results indicate that MEF2C in the presence of Ca^{2+} or CaN* resulted in a doubling of reporter gene activity (Figure 3-3). CaMKII* was found not to enhance MEF2C activity beyond that observed for MEF2C alone (Figure 3-3). Surprisingly, co-transfection of MEF2C with CaN* and CaMKII* did not render reporter gene activity beyond that observed for co-transfection of MEF2C and CaN* (data not shown). Transfection experiments with NIH3T3 fibroblasts suggested that Ca^{2+} treatment or co-transfection with CaN* significantly enhanced MEF2C ability to activate the reporter gene.

To determine if MEF2C function was compromised in the absence of calreticulin, luciferase reporter gene experiments conducted in NIH3T3 cells were repeated in wild-type and calreticulin-deficient embryonic mouse fibroblasts. MEF2C activated the calreticulin promoter-controlled luciferase reporter gene over 2-fold in wild-type cells (Figure 3-4); however, in cells lacking calreticulin MEF2C was unable to activate the reporter gene (Figure 3-4). Interestingly, treatment of wild-type and calreticulin-deficient

Figure 3-3. MEF2C and CaN* synergistically activate the calreticulin promoter reporter gene. The activity of calreticulin promoter-controlled luciferase reporter gene in NIH3T3 cells indicated that reporter gene activity was strongly influenced by presence of both MEF2C and CaN*. CaMK* had no influence on MEF2C activation of the reporter gene. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each plasmid utilized. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.

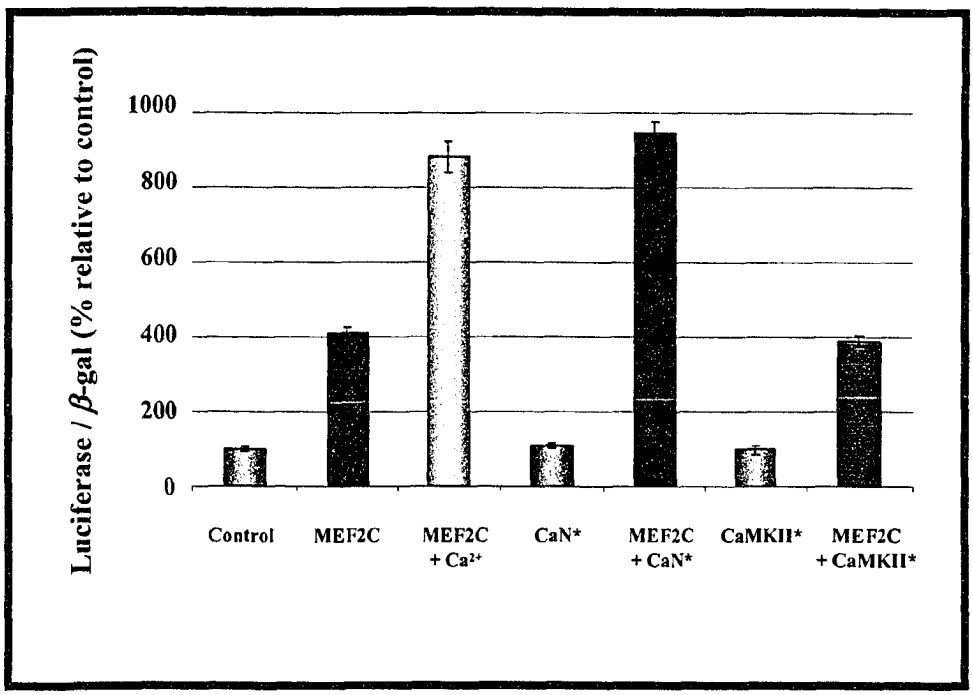
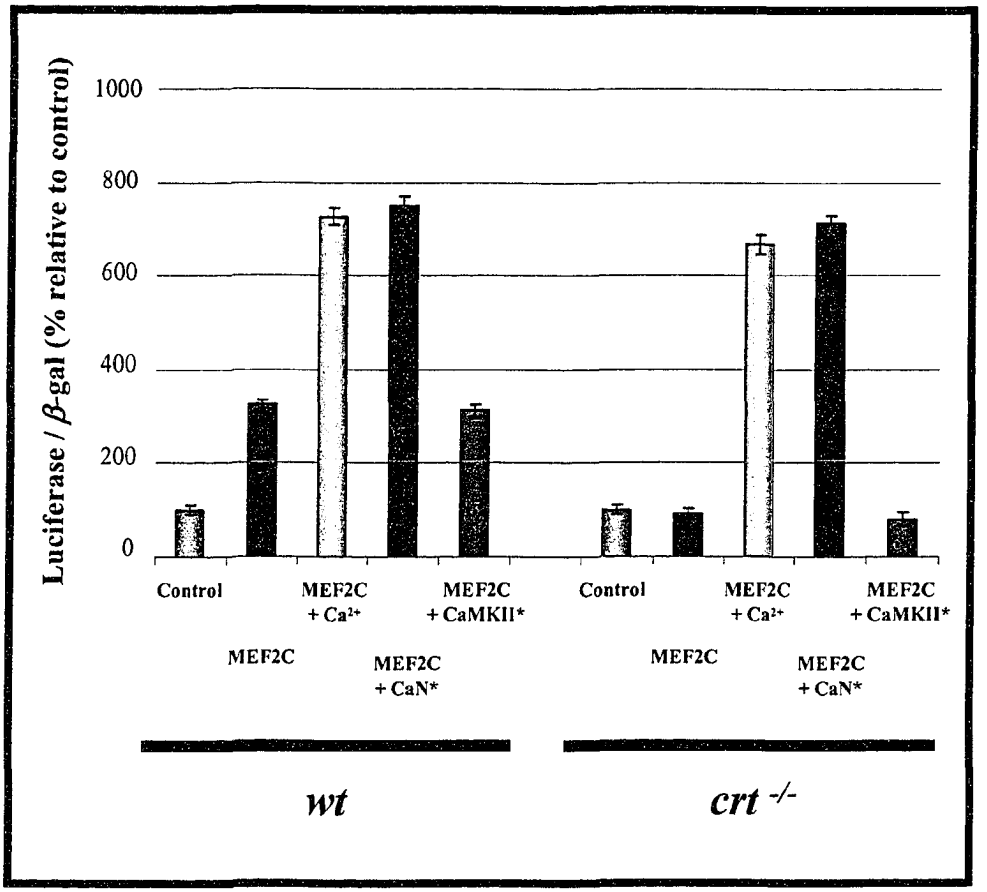


Figure 3-4. Activity of calreticulin promoter-controlled luciferase reporter gene in mouse embryonic fibroblasts is strongly influenced by Ca^{2+} and CaN*. In calreticulin-deficient cells MEF2C did not activate the reporter gene unless the cells were treated with Ca^{2+} or co-transfected with CaN*. CaMK* had no influence on MEF2C activation of the reporter gene. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each plasmid utilized. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.



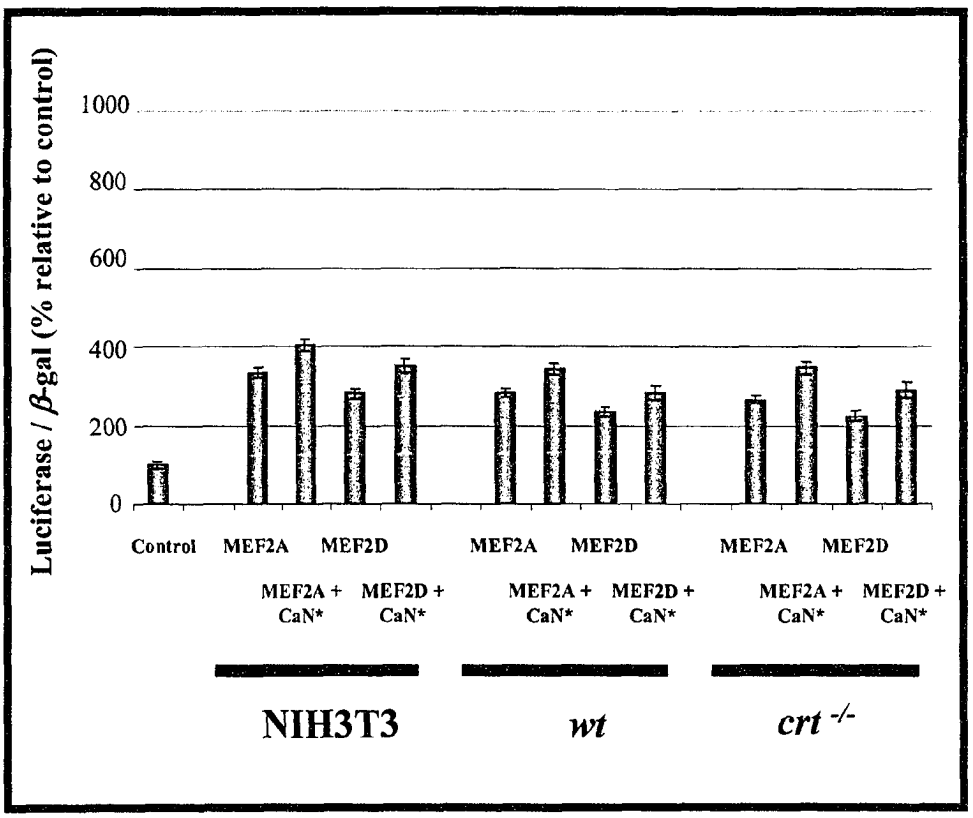
cells with Ca^{2+} ionophore or presence of CaN* render reporter gene activity that was greatly enhanced and similar between the two embryonic cell types (Figure 3-4). The presence of CaMKII* had no influence on MEF2C activation of the reporter gene. Thus, it can be concluded that in calreticulin-deficient cells MEF2C function was compromised, but was restored by an increase in cytoplasmic Ca^{2+} or the presence of activated CaN. Similar results were observed when this experiment was conducted using a 6X MEF2C minimal promoter-controlled luciferase reporter plasmid (Appendix Figure 5).

There are four members of the MEF2 family of proteins and all bind a similar DNA consensus site. To determine if other MEF2 isoforms influenced calreticulin promoter-controlled luciferase reporter gene activity, NIH3T3 cells as well as wild-type and calreticulin-deficient embryonic fibroblasts were transfected with plasmid DNA for MEF2A or MEF2D. MEF2A and MEF2D activated reporter gene activity 2- to 3-fold in all cell types, even cells lacking calreticulin (Figure 3-5). Co-transfection of MEF2A and MEF2D with CaN* resulted in a slight increase in reporter gene activity, but not as large an increase as observed for MEF2C and CaN*. Thus, it can be concluded that ability of MEF2A and MEF2D to activate the reporter gene was not influenced by presence or absence of calreticulin. Therefore, it would seem that the compromised function observed for MEF2C in calreticulin-deficient cells was specific for the MEF2C isoform.

Ca^{2+} and CaN restore nuclear localization of MEF2C in calreticulin-deficient cells

To understand why MEF2C function was compromised in calreticulin-deficient cells, the localization of MEF2C was examined in embryonic mouse fibroblasts. MEF2C cellular localization was determined by immunostaining with a MEF2C primary antibody and a Texas-red conjugated secondary antibody. In wild-type cells MEF2C was localized

Figure 3-5. Calreticulin promoter-controlled luciferase reporter gene was activated by MEF2A and MEF2D, but co-transfection of CaN* with these MEF2 isoforms did not greatly influence reporter gene activity. NIH3T3, wild-type embryonic fibroblasts, and calreticulin-deficient embryonic fibroblasts were utilized to determine reporter gene activation by MEF2A or MEF2D in absence or presence of CaN*. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.



to the nucleus (Figure 3-6, *a*). This localization was unaffected by the addition of a Ca^{2+} ionophore and unaffected by the expression of CaN*, CaMKII*, or CaMKI* (Figure 3-6, *b, c, d, e*). In comparison, the nuclear import of MEF2C in calreticulin-deficient fibroblasts was impaired and the majority of the transcription factor was found in the cytoplasm (Figure 3-6, *f*). Furthermore, MEF2C was found in the nucleus of calreticulin-deficient cells following cell treatment with Ca^{2+} ionophore or presence of CaN* (Figure 3-6, *g, h*). As observed in wild-type cells, CaMK* had no influence on MEF2C localization (Figure 3-6, *i, j*). Examination of calreticulin-deficient fibroblasts that had been stably transfected with calreticulin indicated that MEF2C was localized in the nucleus and the cells had MEF2C localization identical to that observed in wild-type cells (Figures 3-6, *k* to *o*). When this experiment was conducted in mouse fibroblasts (Figure 3-7), similar results to the embryonic fibroblasts were observed. Therefore, observations suggest that translocation of MEF2C into the nucleus is calreticulin-dependent, but in absence of calreticulin can be corrected by increased concentration of cytoplasmic Ca^{2+} or presence of activated CaN.

Confirmation of activity of constitutively-active enzymes

To be certain that constitutively-active CaN and CaMKII were functioning within cells, embryonic mouse fibroblasts were co-transfected with these plasmids as well as GFP-NFATc4 or GFP-HDAC5. GFP-NFATc4 was found to be localized in the cytoplasm of wild-type and calreticulin-deficient cells and translocated to the nucleus in presence of CaN* (Figure 3-8). In comparison, GFP-HDAC5 was initially nuclear and became localized in the cytoplasm in presence of CaMKII*, but not as a result of CaN* (Figure 3-10). When these experiments were conducted in mouse fibroblasts (Figures 3-9

Figure 3-6. Immunolocalization of MEF2C in wild-type, heterozygote, and calreticulin-deficient mouse embryonic fibroblasts indicates that nuclear localization of MEF2C was impaired in the absence of calreticulin. *Panels a, f, and k*, localization of MEF2C in wild-type (wt), calreticulin-deficient ($crt^{-/-}$) cells and $crt^{-/-}$cells expressing recombinant calreticulin ($crt^{-/-}$ + CRT), respectively. *Panels b, g, and l,* cells were treated with Ca^{2+} ionophore while *panels c, h, and m*, are cells transfected with CaN* expression vector. *Panels d, i, n* and *panels e, j, o* are results for cells transfected with CaMKI* and CaMKII* expression vectors, respectively. Scale bar = 25μm.

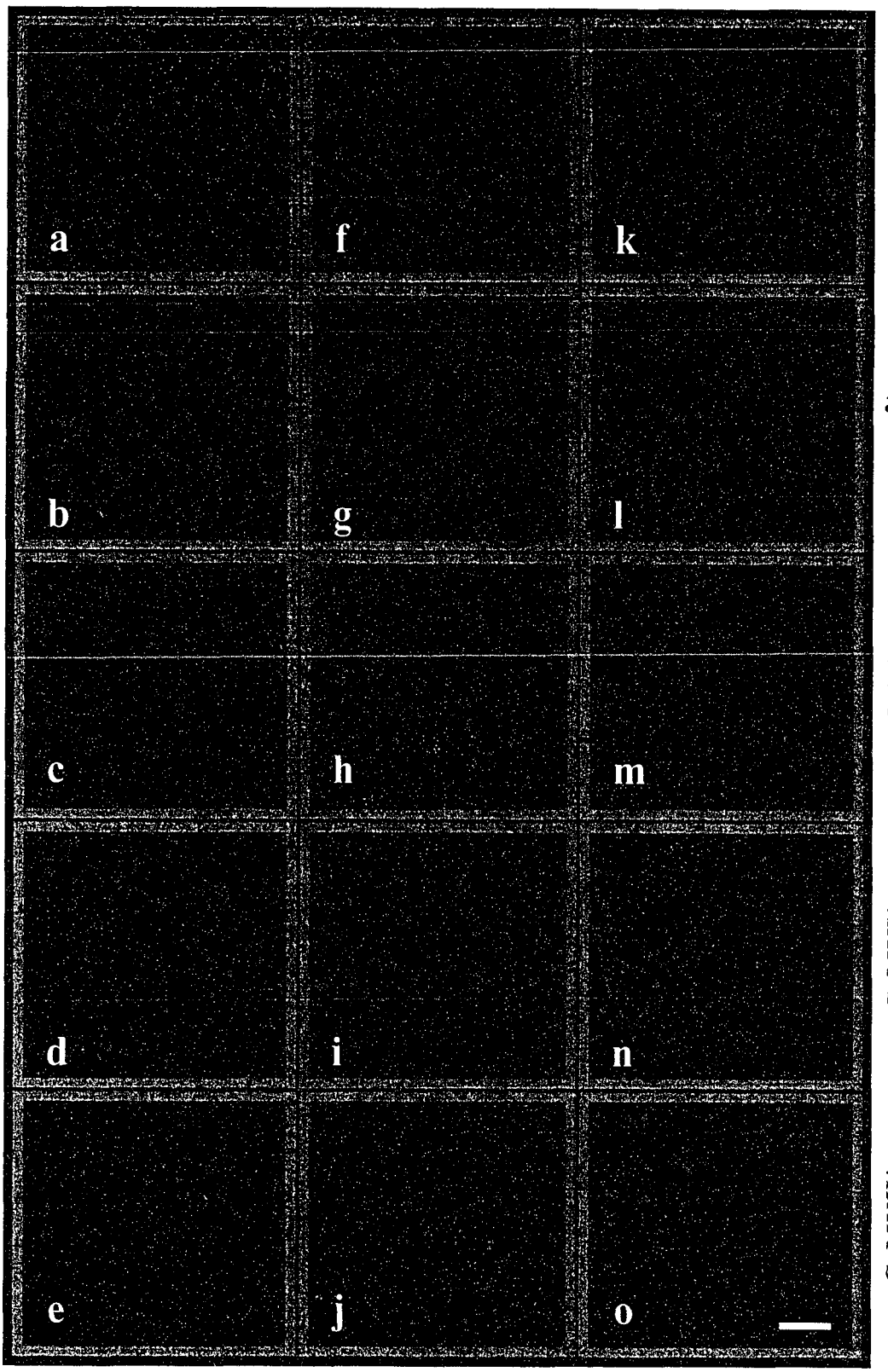


Figure 3-7. Immunolocalization of MEF2C in wild-type, heterozygote, and calreticulin-deficient mouse fibroblasts indicates that nuclear localization of MEF2C was impaired in the absence of calreticulin. *Panels a, f, and k*, localization of MEF2C in wild-type (wt), heterozygote ($crt^{+/-}$), and calreticulin-deficient ($crt^{-/-}$) cells, respectively. *Panels b, g, and l*, cells were treated with Ca^{2+} ionophore while *panels c, h, and m*, are cells transfected with CaN* expression vector. *Panels d, i, n* and *panels e, j, o* are results for cells transfected with CaMKI* and CaMKII* expression vectors, respectively. Scale bar = 25μm.

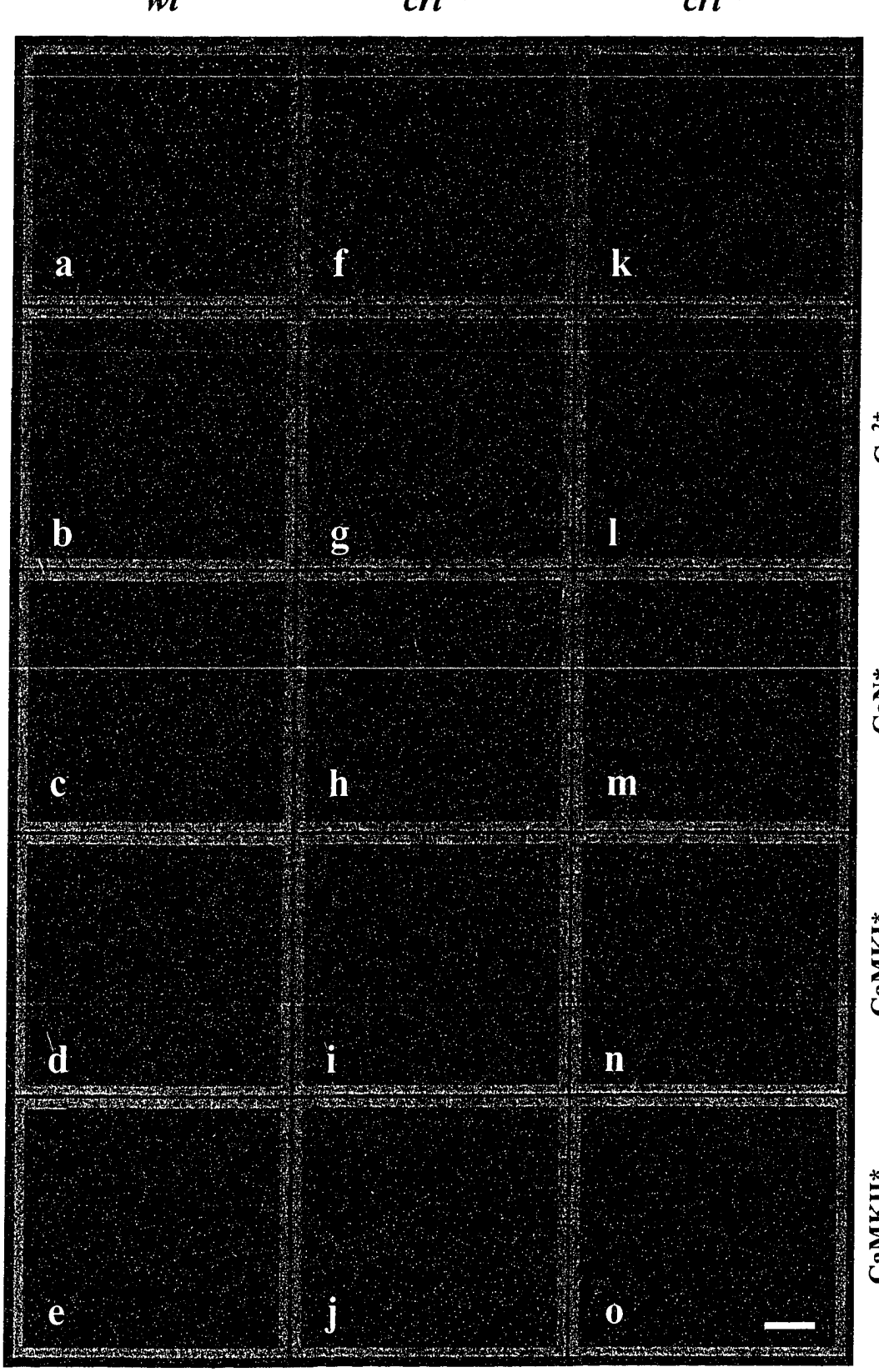


Figure 3-8. Localization of GFP-NFATc4 in wild-type and calreticulin-deficient mouse embryonic fibroblasts. GFP-NFATc4 was cytoplasmic in wild-type (*panel a*) and calreticulin-deficient (*panel c*) embryonic fibroblasts. The presence of CaN* enabled GFP-NFATc4 to translocate to the nucleus (*panels b and d*). Scale bar = 25μm.

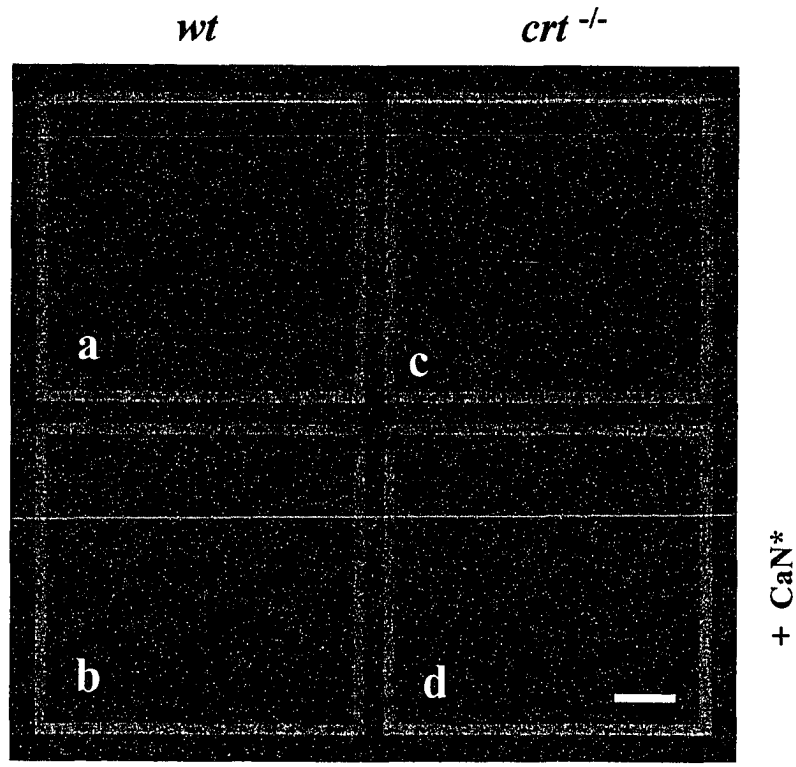


Figure 3-9. Localization of GFP-NFATc4 in wild-type and calreticulin-deficient mouse fibroblasts. GFP-NFATc4 was cytoplasmic in wild-type (*panel a*) and calreticulin-deficient (*panel c*) embryonic fibroblasts. Presence of CaN* enabled GFP-NFATc4 to translocate to the nucleus (*panels b and d*). Scale bar = 25μm.

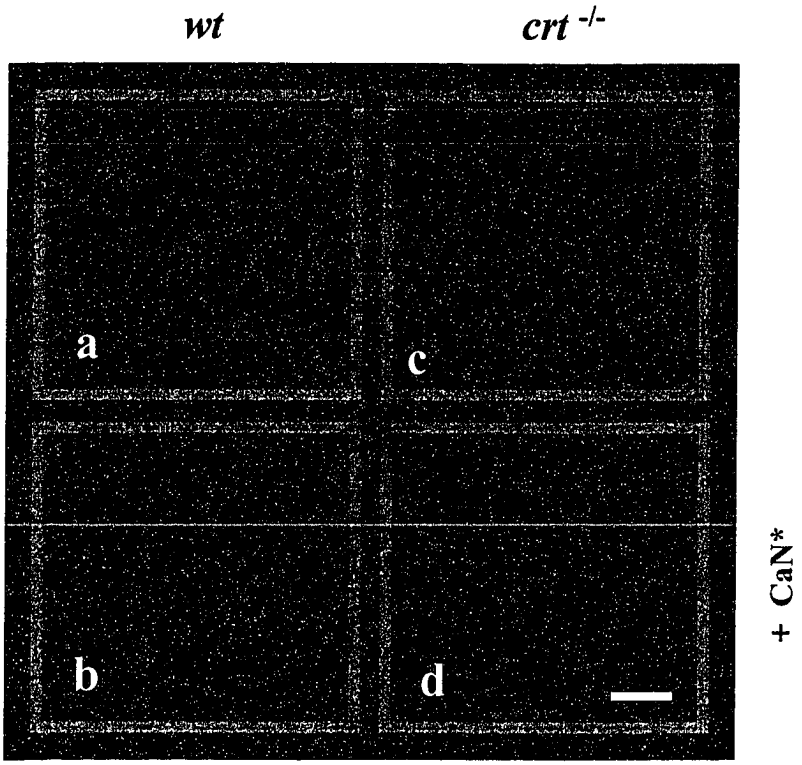
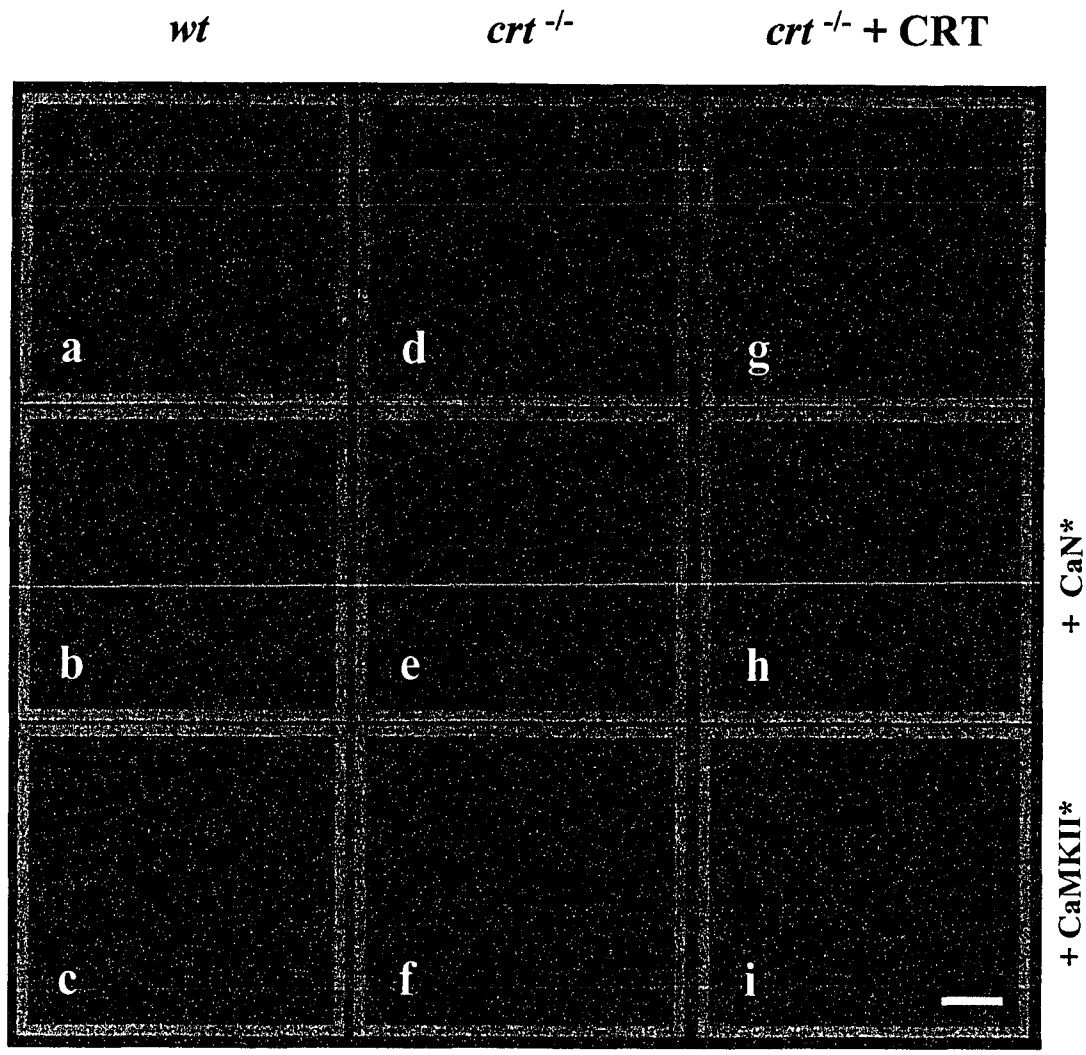
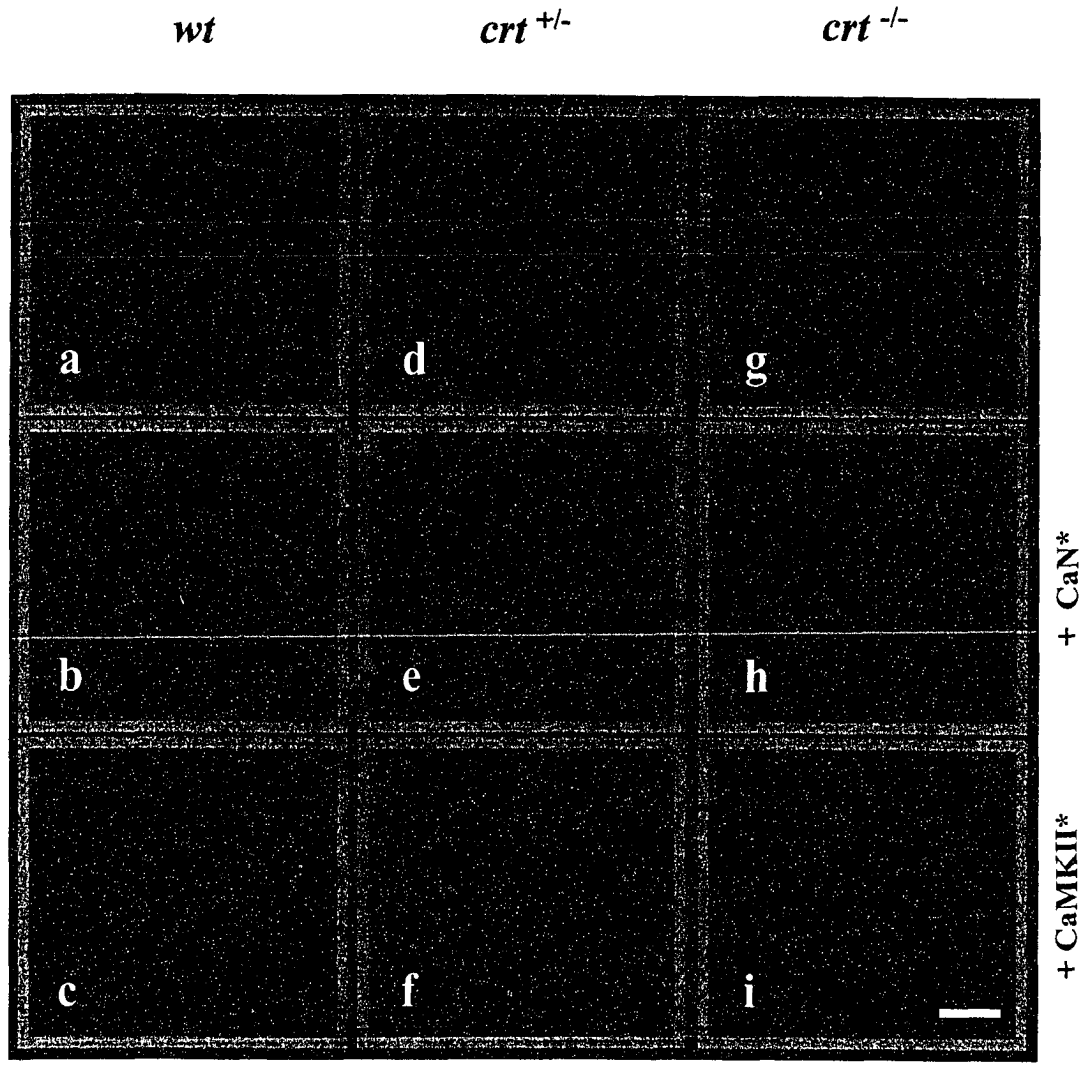


Figure 3-10. Localization of GFP-HDAC5 in in wild-type and calreticulin-deficient mouse embryonic fibroblasts. GFP-HDAC5 was found to be nuclear in wt (*panel a*), $crt^{-/-}$ (*panel d*), and $crt^{-/-}$ + CRT (*panel g*) embryonic fibroblasts. In all cells, the presence of CaMKII* (*panels c, f, i*) caused GFP-HDAC5 to be exported to the cytoplasm whereas the presence of CaN* did not influence localization of GFP-HDAC5 (*panels b, e, h*). CaMKI* was studied only in wt and $crt^{-/-}$ embryonic fibroblasts (data not shown) and results were identical to CaMKII*. Scale bar = 25μm.



and Figures 3-11), similar results to the embryonic fibroblasts were observed. Thus, both embryonic and mouse fibroblasts exhibited similar localization attributes. Therefore, localization and nuclear translocation of GFP-NFATc4 and GFP-HDAC5 was found to be normal in wild-type and calreticulin-deficient cells and CaN* and CaMKII* were functional.

Figure 3-11. Localization of GFP-HDAC5 in wild-type and calreticulin-deficient mouse fibroblasts. GFP-HDAC5 was found to be nuclear in wt (*panel a*), $crt^{+/-}$ (*panel d*), and $crt^{-/-}$ (*panel g*) mouse fibroblasts. In all cells, the presence of CaMKII* (*panels c, f, i*) caused GFP-HDAC5 to be exported to the cytoplasm whereas presence of CaN* did not influence localization of GFP-HDAC5 (*panels b, e, h*). Scale bar = 25μm.



DISCUSSION

Calreticulin-deficient embryonic fibroblasts have impaired $InsP_3$-induced ER Ca^{2+} release (Nakamura *et al.*, 2001b) and impaired NFATc nuclear localization (Mesaeli *et al.*, 1999). In addition, calreticulin-deficient ES-derived cardiomyocytes have been reported to have normal expression of MEF2C, but exhibited compromised MEF2C nuclear import (Li *et al.*, 2002a). Subsequently, we wanted to confirm these observations and test if the problem of nuclear localization of these transcription factors could be corrected by addition of Ca^{2+}. For this experiment, nuclear proteins were isolated from wild-type and calreticulin-deficient cells and applied to DNA arrays. The results indicated that both NFAT and MEF2C were virtually absent from nuclear extracts of calreticulin-deficient cells, but localization of GATA transcription factors was not affected. A DNA array hybridized with a nuclear extract from calreticulin-deficient cells treated with Ca^{2+} ionophore prior to harvesting appeared similar to a DNA array hybridized with wild-type nuclear extracts. Thus, NFAT and MEF2C localization problems in calreticulin-deficient cells likely occurred as a result of calreticulin-deficient cells having impaired Ca^{2+} release from cellular stores. Therefore, DNA array analysis showed that calreticulin-deficient cells have comprised localization of NFAT and MEF2C transcription factors and this nuclear localization problem could be overcome by increasing cytoplasmic Ca^{2+} concentration.

If MEF2C nuclear localization is compromised in absence of calreticulin then calreticulin-deficient cells should have diminished expression of genes activated by this transcription factor. To determine if expression of MEF2C target genes differed between wild-type and calreticulin-deficient cells, RT-PCR analysis of known MEF2C gene

targets (Morin *et al.*, 2000) was completed using mRNA isolated from E12 wild-type and calreticulin-deficient hearts. Results from RT-PCR indicated reduced expression of known MEF2C target genes (MLC-2v, eHAND, Irx4) in $crt^{-/-}$ embryonic hearts. Expression of a GATA6, a non-MEF2C target, was observed to be similar in both wild-type and calreticulin-deficient hearts and served as a control for this experiment. Hence, RT-PCR data indicated that expression of MEF2C target genes was diminished in calreticulin-deficient cells and adds support to earlier observations that MEF2C localization is compromised in absence of calreticulin. A complete lack of expression of all MEF2C target genes would render a lethal phenotype for calreticulin-deficient embryos very similar to that of *mef2c* gene disrupted mice (Lin *et al.*, 1997). As this is not the case, it is probable that there was some expression of MEF2C genes in calreticulin-deficient cells. As such, quantitative PCR would be necessary to precisely determine expression of MEF2C target genes. In conclusion, RT-PCR results suggest that in absence of calreticulin MEF2C function is compromised.

MEF2 isoforms bind the same DNA consensus sequence and so all MEF2 isoforms should be able to activate the calreticulin promoter. As expected, all MEF2 isoforms activated the calreticulin promoter-controlled reporter gene with MEF2C being the isoform that induced the greatest activity. When co-transfected with CaN*, MEF2C exhibited a synergistic increase in reporter gene activity while MEF2A and MEF2D only induced a slight enhancement in reporter gene activity. CaN* had previously been reported to strengthen MEF2 binding to DNA (Mao and Wiedmann, 1999) and this many be responsible for the modest increase in reporter gene activity observed when MEF2 isoforms are co-transfected with CaN*; however, it does not explain the substantial

increase in reporter gene activity observed when MEF2C was co-transfected with CaN*.

Problems with MEF2C nuclear localization appeared to be corrected with increased cytoplasmic Ca^{2+} and so it was necessary to determine which Ca^{2+}-dependent cytoplasmic enzymes influenced MEF2C localization and/or activity. The results from co-transfection of luciferase reporter gene into NIH3T3 fibroblasts suggested that MEF2C activity could be synergistically enhanced following Ca^{2+} treatment or the presence of CaN*, but was not influenced by the presence of CaMKI* or CaMKII*. Synergism among MEF2C, CaN*, and CaMKIV* had previously been reported (Wu *et al.*, 2000) and all MEF2 isoforms have been observed to have synergistic activity with CaN* (Wu *et al.*, 2000) in C2C12 myoblasts (i.e. skeletal muscle). However, in this study no change in reporter gene activity was observed in the presence of CaMKI* or CaMKII*. This observation does not fully agree with the earlier investigation and discrepancies may have occurred because of: 1) differences in cell type utilized, 2) differences between reporter gene promoters, and/or 3) differences between CaMK* isoforms. Recently, other researchers have reported no synergism between CaN* and CaMK* (Schaeffer *et al.*, 2004) which is in agreement with findings in this investigation. In conclusion, the results of this study indicated that MEF2C influence on calreticulin promoter reporter gene was greatly enhanced following an increase in cytoplasmic Ca^{2+} or the presence of CaN*.

When luciferase reporter gene experiments were completed using wild-type and calreticulin-deficient embryonic fibroblasts, it was observed that in the absence of calreticulin MEF2C was unable to activate the calreticulin promoter reporter gene. Interestingly, calreticulin-deficient cells treated with Ca^{2+} or co-transfected with CaN*

exhibited reporter gene activity equivalent to that observed for wild-type cells. Hence, these results indicated that ability of MEF2C to activate the calreticulin promoter was compromised in absence of calreticulin and the problem was corrected after an increase in cytoplasmic Ca^{2+} or the presence of CaN*. Therefore, data from both reporter gene experiments and from DNA arrays suggested that it was necessary to examine MEF2C cellular localization in calreticulin-deficient cells.

Immunostaining of MEF2C indicated that ability of this transcription factor to localize in the nucleus was compromised in cells lacking calreticulin. Similar immunostaining results were obtained for both mouse embryonic and mouse fibroblasts and the observations were in agreement with those observed for MEF2C localization in ES cell-derived cardiomyocytes (Li *et al.*, 2002a). Treatment of cells to elevate cytoplasmic Ca^{2+} or co-transfection of cells with CaN* corrected the problem MEF2C had with nuclear localization. CaMKs had no influence on MEF2C localization which was an expected observation as CaMKs interact with HDACs rather than directly with MEF2C (McKinsey *et al.*, 2000; McKinsey *et al.*, 2001b). Since CaN is a Ca^{2+}-dependent phosphatase found in the cytoplasm, it is probable that endogenous CaN was activated by Ca^{2+} ionophore treatment. Hence, immunostaining experiments indicated that MEF2C nuclear localization was compromised in absence of calreticulin, but was corrected by presence of activated CaN.

To confirm that constitutively-active enzymes were functioning within cells, embryonic mouse fibroblasts were co-transfected with these plasmids as well as GFP-NFATc4 or GFP-HDAC5. GFP-NFATc4 was found to be localized in the cytoplasm of wild-type and calreticulin-deficient cells and only translocated to the nucleus in the

presence of CaN*. In comparison, GFP-HDAC5 was initially nuclear and became localized in the cytoplasm in the presence of CaMKII*, but not as a result of CaN* (Figure 3-10). The findings for CaMKs were in agreement with those previously observed (McKinsey *et al.*, 2001b). As identical localization results were obtained for both mouse embryonic and mouse fibroblasts, it was decided that because these cell types exhibited such strong similarities further investigation would only involve the embryonic fibroblasts. Therefore, in wild-type and calreticulin-deficient cells GFP-NFATc4 and GFP-HDAC5 localization and nuclear translocation were found to be normal and both CaN* and CaMKII* were functional.

It can be concluded that MEF2C function was compromised in calreticulin-deficient cells. DNA array data showed that MEF2C was absent from calreticulin-deficient cell nuclear extracts, but was present in the extracts following Ca^{2+} ionophore treatment. MEF2C possesses both a NLS and a nuclear retention signal (NRS) and because it is a transcription factor it is imperative that it be localized in the nucleus to impact target genes. The observation that MEF2C was originally absent from the nucleus of cells lacking calreticulin suggested that its function may be compromised in cells lacking calreticulin. The belief that MEF2C function was impaired in absence of calreticulin was investigated by RT-PCR and results suggested that expression of MEF2C target genes was decreased in calreticulin-deficient cardiomyocytes relative to wild-type. Experiments with calreticulin promoter-controlled luciferase reporter gene confirmed that MEF2C function was compromised in the absence of calreticulin as well as demonstrated that Ca^{2+} ionophore treatment or the presence of CaN* could correct the problem. Thus, in the absence of calreticulin MEF2C was unable to function efficiently.

DNA array data had indicated that Ca^{2+} ionophore treatment permitted MEF2C to translocate to the nucleus. To further scrutinize this observation, immunostaining of MEF2C in wild-type and calreticulin-deficient fibroblasts was conducted. The results from both embryonic and mouse fibroblasts indicated that MEF2C was nuclear in wild-type cells, but predominantly cytoplasmic in calreticulin-deficient cells. Interestingly, co-transfection of calreticulin-deficient cells with CaMK* did not induce MEF2C nuclear localization, but nuclear localization occurred following Ca^{2+} ionophore treatment or co-transfection with CaN*. Hence, in calreticulin-deficient cells MEF2C nuclear localization was compromised, but was made possible following an increase in cytoplasmic Ca^{2+} concentration or the presence of activated CaN. Further studies were necessary to determine if this localization problem was specific for MEF2C transcription factor or if localization of other proteins was compromised in the absence of calreticulin.

GFP-MEF2C NUCLEAR LOCALIZATION IS Ca^{2+} AND CALCINEURIN-DEPENDENT

Versions of portions of this chapter have been published or submitted for publication:

1. Lynch, J and Michalak, M. 2003. Calreticulin is an upstream regulator of calcineurin. *Biochem. Biophys. Res. Commun.* **311**:1173-9.
2. Lynch, J., Guo, L., Gelebart, P., Chilibeck, K., Xu, J., Molkentin, J.D., Agellon, L.B., and Michalak, M. 2004. Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca^{2+}-dependent signaling pathway of early cardiac development. *Status: Submitted.*

INTRODUCTION

In the previous chapter immunostaining of fixed cells indicated that MEF2C localization was compromised in the absence of calreticulin, but was corrected by increased cytoplasmic Ca^{2+} or the presence of activated CaN. Further evidence was desired to support these observations and so other methods for localization of MEF2C were utilized. At present, there are several techniques available by which proteins can be localized within a cell. In this chapter, methods were utilized that enabled localization of MEF2C within: 1) living cells; 2) cardiomyocytes; and, 3) cardiac tissue. In addition, experiments were conducted which permitted localization of MEF2C to be examined in cells treated with inhibitors of CaN or CaMK.

Green fluorescent protein

Green fluorescent protein (GFP) is a small protein (27kDa) first isolated from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). GFP has stimulated a great deal of excitement among molecular, developmental, and cell biologists as the DNA sequence for GFP can be manipulated to create gene fusions between GFP and any protein of interest (Chalfie *et al.*, 1994). A major advantage of the GFP tag is that a protein of interest can be localized within living cells and organisms from the moment of its expression. Therefore, GFP fusion proteins represent one method by which to determine protein localization.

Immunohistochemistry

Immunohistochemistry is a technique that enables localization of antigens in tissue sections by the use of labeled antibodies. The technique was originally developed

over 50 years ago (Coons, 1951) and continues to be improved upon. At present, the avidin-biotin complex (ABC) method is the most sensitive immunohistochemical technique available. Avidin is a large glycoprotein that is labeled with peroxidase and has a very high-affinity for biotin while biotin is a low molecular weight vitamin that can be conjugated to a variety of biological molecules such as antibodies. The ABC technique involves three major steps: 1) addition of unlabeled primary antibody; 2) addition of biotinylated secondary antibody; and, 3) addition of a complex of avidin-biotin peroxidase. The peroxidase is then developed by addition of diamino-benzidine tetrahydrochloride (DAB) substrate to produce an intensely coloured (purple to black) end product. A counterstain such as hematoxylin (blue colour) is used to identify nuclei. Thus, immunostaining is a powerful method that permits *in situ* localization of protein.

CaN Inhibitor

CsA is an important immunosuppressive agent used in organ transplantation and treatment of diverse immune disorders. CsA suppresses the immune response by binding to and inhibiting CaN function (Liu *et al.*, 1991). CsA was originally extracted from *Tolypocladium inflatum Gams* and is a neutral, lipophilic, very hydrophobic, cyclic polypeptide of 11 amino acids (Bueno *et al.*, 2002a). The drug, administered orally or intravenously, distributes rapidly between blood cells and plasma eventually accumulating in erythrocytes (50 to 60%), in leukocytes (10 to 20%), and the remainder bound to plasma lipoproteins (Bueno *et al.*, 2002a). In a cell, CsA binds to an ubiquitous, cytoplasmic protein, cyclophilin, to form a complex that is capable of high-affinity association with CaN. Complex association with CaN inhibits phosphatase activity as well as prevents substrate interaction. The actual tissue distribution of CsA is dependent

on cyclophilin and lipid content (Bueno *et al.*, 2002a). CsA is metabolized in the liver by P-450IIIA enzymes to render more than 30 metabolites (Bueno *et al.*, 2002a). It is estimated that the half-life of CsA is six hours (Faulds *et al.*, 1993).

In some instances, CsA can protect against the onset of cardiac hypertrophy (Molkentin *et al.*, 1998). In the case of aortic banding to induce hypertrophy by pressure-overload, one study showed the protectory effect of CsA (Sussman *et al.*, 1998) whereas several other investigations have failed to confirm this observation (Ding *et al.*, 1999; Luo *et al.*, 1998; Zhang *et al.*, 1999). In another study, CsA was found to protect against pressure-overload induced hypertrophy after 7 days, but not after 21 days (Molkentin, 1998). It is possible that discrepancies occurred due to dose of immunosuppressant drug and/or mode of administration. Additionally, it is possible that the differences occurred because hypertrophy is a complex process involving multiple signaling pathways. Hence, immunosuppressive drugs to suppress CaN may delay, but not prevent the hypertrophic response (Rusnak and Mertz, 2000).

CsA has severe side effects. Human patients maintained on CsA for the purpose of immunosuppression can develop hypertension (Lipkin *et al.*, 1993; Lo and Alloway, 2002) and cardiac hypertrophy (Bueno *et al.*, 2002a; Chang *et al.*, 1998). At therapeutic doses, CsA was found to induce sustained SR Ca^{2+} leakage from adult rabbit and human cardiomyocytes (Janssen *et al.*, 2000). Long-term CsA treatment disrupts Ca^{2+} homeostasis and alters contractile properties of cardiomyocytes via modifications to SERCA and L-type Ca^{2+} channels (Mijares *et al.*, 1997). In addition to influencing Ca^{2+} homeostasis, CsA has CaN-independent effects in multiple organs, including neural tissue, smooth muscle, and kidney (Bueno *et al.*, 2002a; Lo and Alloway, 2002). *In vivo*,

CsA prevents muscle regeneration in response to damage (Abbott *et al.*, 1998). In the clinic, transplant patients treated with CsA exhibit severe skeletal muscle weakness (Goy *et al.*, 1989). Additionally, metabolic toxicities (Lo and Alloway, 2002) and weight loss have been associated with CsA treatment (Frey and Olson, 2003). It is worth noting that the doses of CsA required to prevent cardiac hypertrophy in experimental animals is approximately 10-fold higher than that needed to achieve immunosuppression in humans. This phenomenon may be attributed to high cardiac CaN content, different rates of drug absorption, or perhaps due to the higher metabolic rate of the mice (Bueno *et al.*, 2002a). When considering the large number of side-effects associated with CaN inhibitor treatment, there is a great need to correctly determine the dosage and mode of drug administration (Bueno *et al.*, 2002a).

To date, a vast number of studies have attempted to determine if CsA could be utilized to treat cardiac hypertrophy (Bueno *et al.*, 2002a). In all instances, the investigations were hampered to some degree by intra- and extra-cardiac side effects associated with the drug. Since CsA has targets independent of CaN (Kaibori *et al.*, 1999; Lipkin *et al.*, 1993), the extra-cardiac side effects virtually exclude CsA usage as a potential treatment for patients with cardiac hypertrophy.

CaMK inhibitors

Two well known inhibitors of CaMK are KN-62 and KN-93. KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-l-tyrosyl]-4-piperazine) is a synthetic inhibitor of CaMK (Tokumitsu *et al.*, 1990) and acts on CaMKII in a dose-dependent manner (Minami *et al.*, 1994). KN-62 inhibits DNA synthesis and slows progression through S phase (Minami *et al.*, 1994; Williams *et al.*, 1996). In comparison, KN-93 (Sumi *et al.*,

1991) is a methoxybenzenesulfonamide (2-[N-(2-Hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) that acts by antagonizing CaM binding and reversibly arrests rodent and human cells in G1 phase of the cell cycle (Morris *et al.*, 1998; Rasmussen and Rasmussen, 1995; Tombes *et al.*, 1995). Studies have found that cultured cardiomyocytes treated with KN-62 (Okazaki *et al.*, 1994) or KN-93 (Vittone *et al.*, 2002) have a dose-dependent decrease in rate of beating due to decreased phosphorylation of phospholamban and depressed SERCA activity. Thus, CaMK inhibitors impact Ca^{2+} cycling and may also influence sinoatrial node pacemaker activity (Anderson *et al.*, 1998; Maier *et al.*, 2003; Vinogradova *et al.*, 2000). Pressure-overloaded rat hearts in a heart perfusion model treated with KN-62 showed that CaMKII could be inhibited by KN-62 *in situ* (Saito *et al.*, 2003). Although KN-62 and KN-93 inhibit CaMK activity, they also inhibit the cell cycle and encourage apoptosis; thus, these drugs are unlikely to have clinical applications.

GFP plasmids

The cloning vector utilized to create GFP fusion proteins was pEGFP-C1 (Clontech; GenBank Accession: U55763). All cDNA inserts were in frame with the GFP cloning vector. For GFP-MEF2C, full-length MEF2C was amplified by PCR and 5' XhoI site and 3' HindIII sites introduced. To create GFP-MEF2A, full-length MEF2A was subcloned into pEGFP-C1 following PCR amplification and introduction of 5' XhoI and 3' EcoRI sites. To create GFP-MyoD, cDNA for MyoD was amplified by PCR and 5' EcoRI and 3' XbaI sites introduced. Creation of GFP-GATA6-NLS involved restriction enzyme cutting of the NLS region of GATA6 with SstI and EcoRI. GFP plasmids were transfected into wild-type and calreticulin-deficient embryonic fibroblasts and cardiomyocytes using FuGENE 6 transfection reagent. All GFP images were of live cells grown on 25mm coverslips. GFP images were captured with a Zeiss Axiovert S100 fluorescent microscope using an excitation wavelength of 490nm and emission wavelength of 520nm. All pictures were pseudo-coloured using Northern Eclipse 5.0 software (Empix Imaging Inc., Mississauga, Ontario, Canada).

Drug treatment of fibroblasts

Fibroblasts were transfected with GFP-MEF2C and were treated with drugs 60 minutes after addition of DNA. CsA (1mM stock in 3.8% ethanol; Sigma; Cat. C-3662) was added to media to produce a final CsA concentration of 0.1μM. KN-62 (40μM stock in DMSO; Sigma Cat. I-2142) was added to media to render a final concentration of 1μM (Zhu *et al.*, 2000). Controls were treated with the appropriate drug vehicle.

Histological Analysis

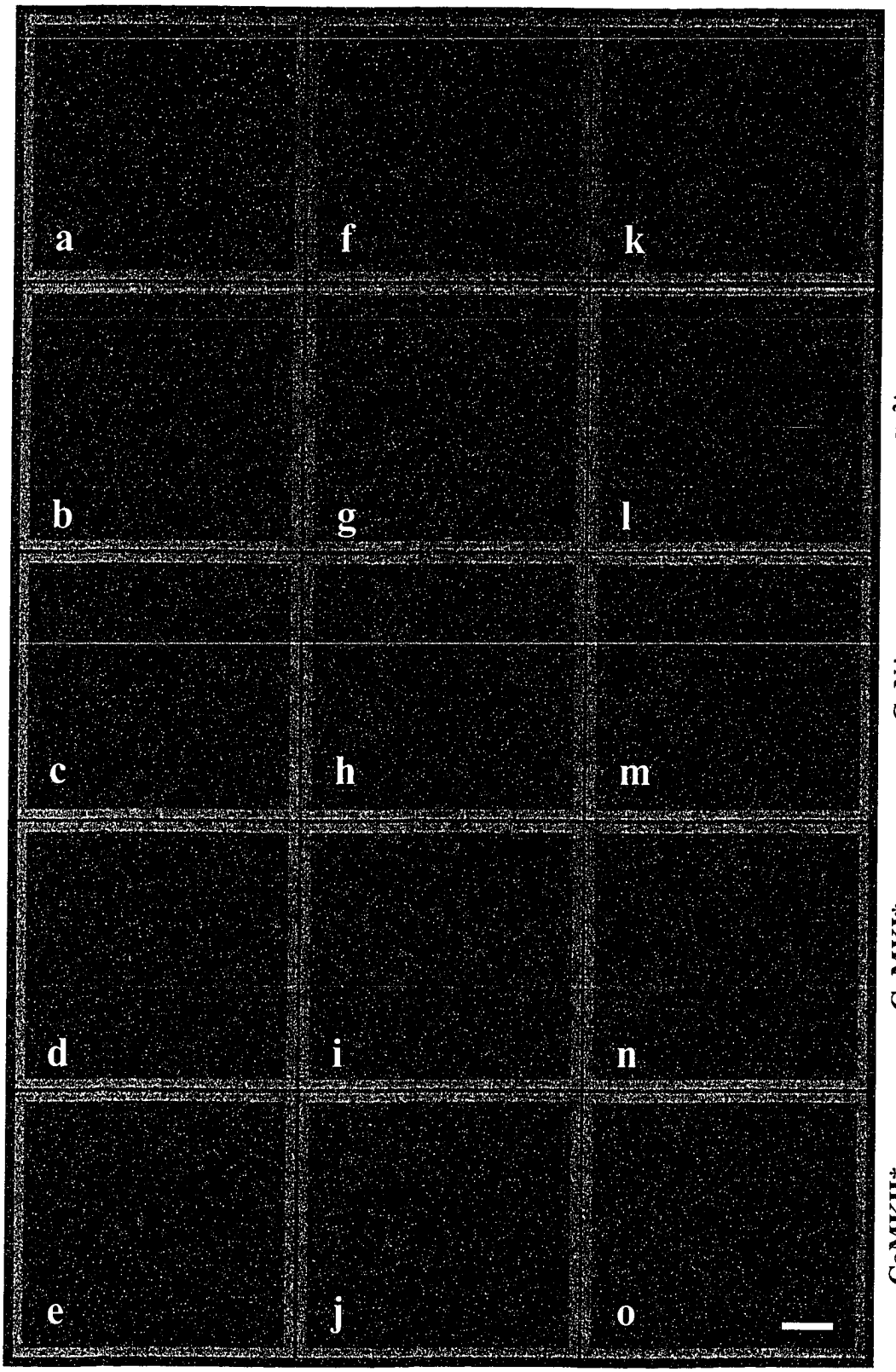
E12 mouse embryos were dissected out of the uterus and fixed in 4% paraformaldehyde for 8 hours. The embryos were washed with PBS for 30 minutes, placed in 50% PBS/50% Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, California, USA; Cat. 4583) for 16 hours at room temperature, and then incubated in 100% OCT for 24 hours at 4^0C. The embryos were frozen in 2-methylbutane (Fisher Scientific; Cat. 03551-4) cooled in liquid nitrogen. Embryos were stored at -80^0C until time of sectioning. A cryostat (Ames/Miles Tissue Tek II Cryostat) was utilized to produce 4μm sagittal sections of embryos and these sections were placed on poly-L-lysine (concentration = 500μg/mL; Sigma; Cat. P-1399) coated slides. Following methanol/acetone fixation, embryo sections were incubated in 3% H_2O_2 to block endogenous peroxidase. Embryo sections were stained using Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, California, USA; Cat. PK-6200) and Vector DAB substrate kit (Cat. SK-4100). Sections were counterstained with hematoxylin (Cat. H-3404) and mounted in Vectamount Permanent Mounting Medium (Cat. H-5000). The application of Vector Laboratory products was in accordance with manufacturer instructions. The primary antibody utilized was polyclonal MEF2C (1:10 dilution). Embryo sections were examined using a Zeiss Axioskop 2 mot plus microscope fitted with a 100X objective. Images were captured with a Nikon Coolpix 995 digital camera.

Specificity of compromised MEF2C nuclear localization

A GFP-MEF2C plasmid was created and transfected into wild-type and calreticulin-deficient mouse embryonic fibroblasts. As expected, GFP-MEF2C was localized only in the nucleus of wild-type cells (Figure 4-1, *a*), and this localization did not change following Ca^{2+} treatment or the presence of CaN*, CaMKI*, or CaMKII* (Figure 4-1, *b to e*). However, for calreticulin-deficient cells GFP-MEF2C was not found to be predominantly cytoplasmic as suggested by the immunostaining data, but rather was found to be distributed throughout the cell, in both cytoplasm and nucleus (Figure 4-1, *f*). Immunoblot analysis of cytoplasmic and nuclear extracts indicated that over 90% of the GFP-MEF2C was localized in the nucleus of wild-type cells while in calreticulin-deficient cells only 15 to 38% of the GFP-MEF2C was nuclear (Appendix Figure 6). Results from the investigation of GFP-MEF2C localization suggested that MEF2C localization was not completely inhibited in cells devoid of calreticulin, but rather indicated that in absence of calreticulin MEF2C localization was impaired. As with the immunostained cells, MEF2C accumulated in the nucleus of calreticulin-deficient cells following Ca^{2+} treatment or presence of CaN* (Figure 4-1, *g, h*), but was not influenced by CaMKI* or CaMKII* (Figure 4-1, *i, j*). Also, as with the immunostaining experiment, calreticulin-deficient cells stably transfected with calreticulin were found to always have nuclear localization of MEF2C (Figure 4-1, *k, o*). Therefore, examination of GFP-MEF2C supported immunostaining data and suggested that MEF2C localization was not entirely inhibited in absence of calreticulin, but rather was substantially impaired.

To better understand if compromised MEF2C nuclear translocation was specific

Figure 4-1. Nuclear localization of GFP-MEF2C was compromised in the absence of calreticulin. Localization of GFP-MEF2C in mouse embryonic fibroblasts. Wild-type (*panels a to e*) and calreticulin-deficient cells stably transfected with calreticulin (*panel k to o*) had nuclear localization of GFP-MEF2C. Calreticulin-deficient cells had GFP-MEF2C distributed throughout the cell, in both cytoplasm and nucleus (*panel f*). In the absence of calreticulin, GFP-MEF2C localized to the nucleus only after cells were treated with Ca^{2+} (*panel g*) or co-transfected with CaN* (*panel h*). Scale bar = 25μm.



for MEF2C, cDNAs for several other transcription factors were subcloned into a GFP vector and localization was observed in wild-type and calreticulin-deficient embryonic fibroblasts. GFP-MEF2A localization was identical in both cells types (Figure 4-2, *a, f*) independent of Ca^{2+} treatment (Figure 4-2, *b, g*) or co-transfection with CaN* (Figure 4-2, *c, h*) or CaMK* (Figure 4-2, *d, e, i, j*). GFP-MyoD (Figure 4-3, *a, b*) and GFP-GATA6-NLS (Figure 4-3, *c, d*) were also observed to be nuclear in both wild-type and calreticulin-deficient cells. Thus, in wild-type and calreticulin-deficient fibroblasts the nuclear localization of GFP-NFATc4, GFP-HDAC5, GFP-MEF2A, GFP-MyoD and GFP-GATA6-NLS were similar. Hence, it is probable that the compromised nuclear localization of MEF2C observed in calreticulin-deficient cells was specific for this transcription factor.

Role of CaN and CaMK inhibitors

CsA treatment impaired nuclear localization of GFP-MEF2C in wild-type cells (Figure 4-4, *a, b, c, d*) and GFP-MEF2C localization was found to be similar to that observed for calreticulin-deficient fibroblasts (Figure 4-4, *e, f, g, h*). In comparison, KN-62 treatment of embryonic fibroblasts had no influence on GFP-MEF2C localization (Figure 4-5) beyond that observed for GFP-MEF2C alone (Figure 4-1). Therefore, CsA treatment impaired GFP-MEF2C nuclear localization whereas KN-62 treatment had no influence on localization.

MEF2C localization was impaired in cardiomyocytes

Since MEF2C is a component of the fetal cardiac program, it was desirable to determine if the localization data collected for GFP-MEF2C in fibroblasts was what

Figure 4-2. Localization of another nuclear protein (GFP-MEF2A) was not compromised in the absence of calreticulin. GFP-MEF2A was localized to the nucleus of wild-type (*panels a to e*) and calreticulin-deficient (*panels e to g*) embryonic fibroblasts. Scale bar = 25μm.

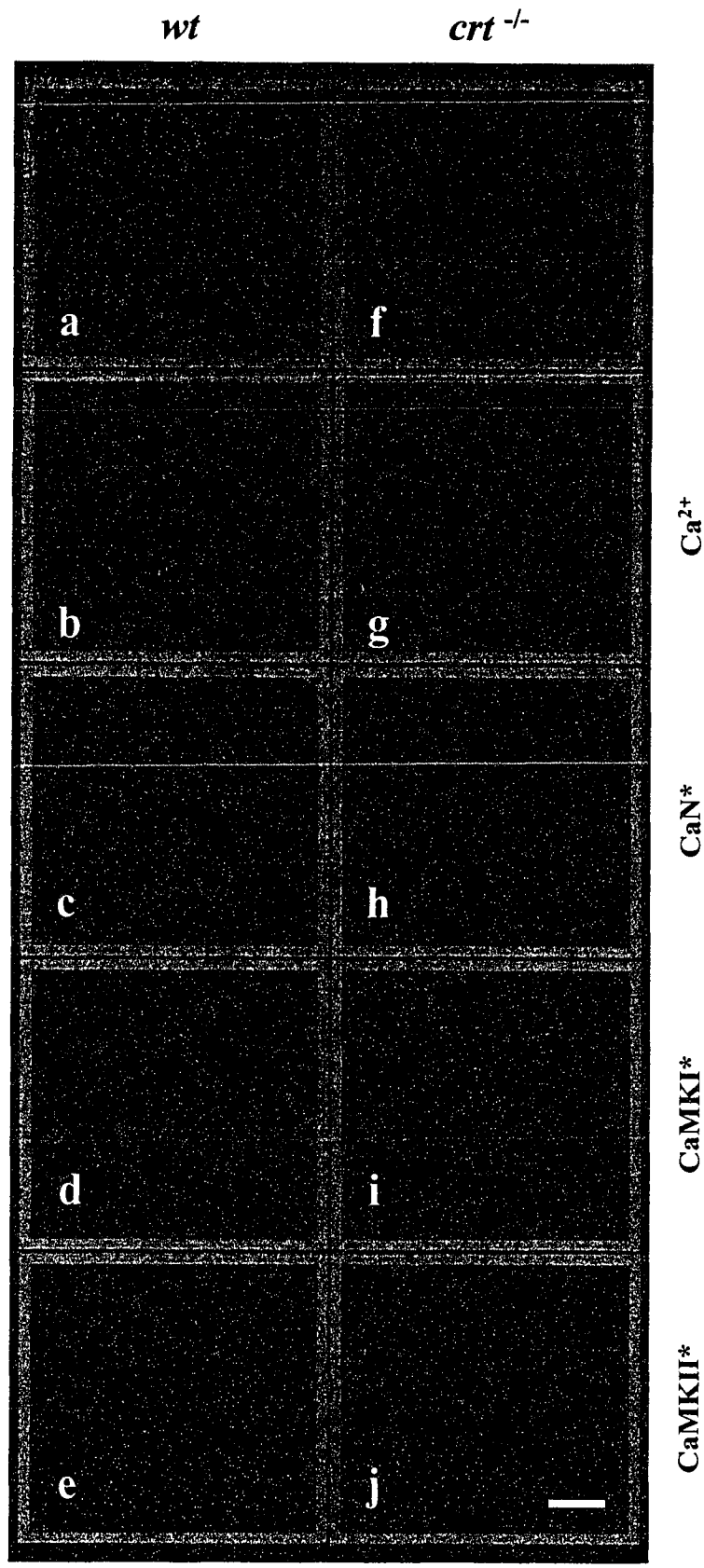


Figure 4-3. Localization of other nuclear proteins (GFP-MyoD1 and GFP-GATA6-NLS) was not compromised in the absence of calreticulin. GFP-MyoD1 was localized to the nucleus of wild-type (*panel a*) and calreticulin-deficient (*panel b*) embryonic fibroblasts. Localization of GFP-GATA6-NLS was also observed to be nuclear in wild-type (*panel c*) and calreticulin-deficient (*panel d*) embryonic fibroblasts. Scale bar = 25μm.

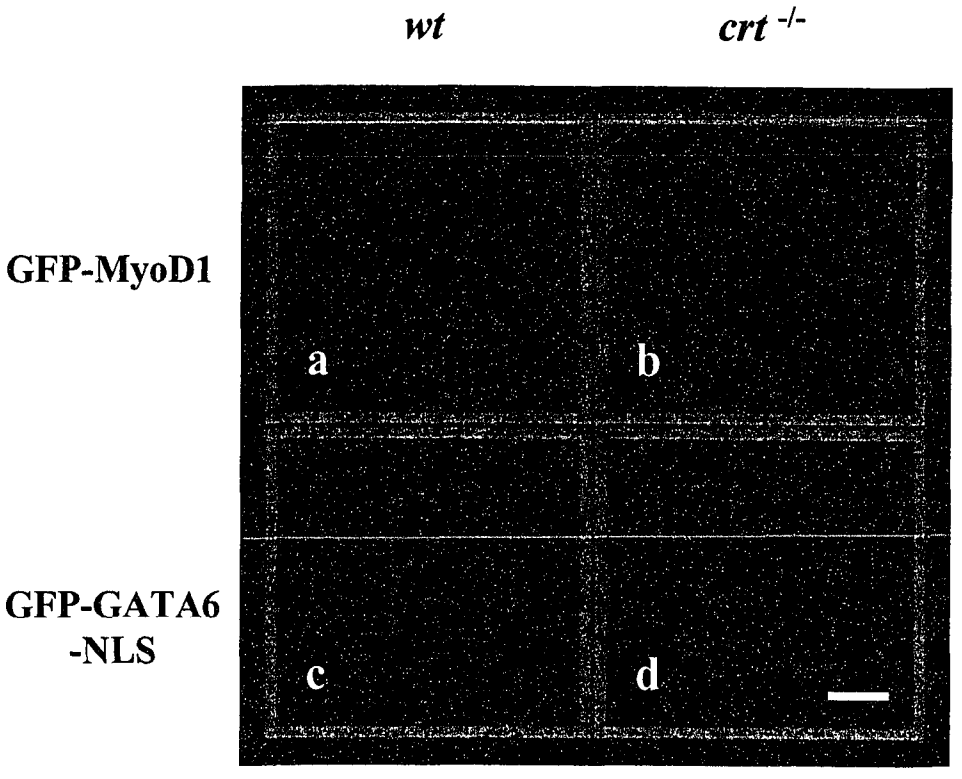


Figure 4-4. Treatment of cells with a CaN inhibitor impaired nuclear localization of GFP-MEF2C. Localization of GFP-MEF2C in wild-type (*panels a to d*) and calreticulin-deficient (*panels e to h*) embryonic fibroblasts was observed to be throughout the cell, in both cytoplasm and nucleus, following CsA treatment (CaN inhibitor). Scale bar = 25μm.

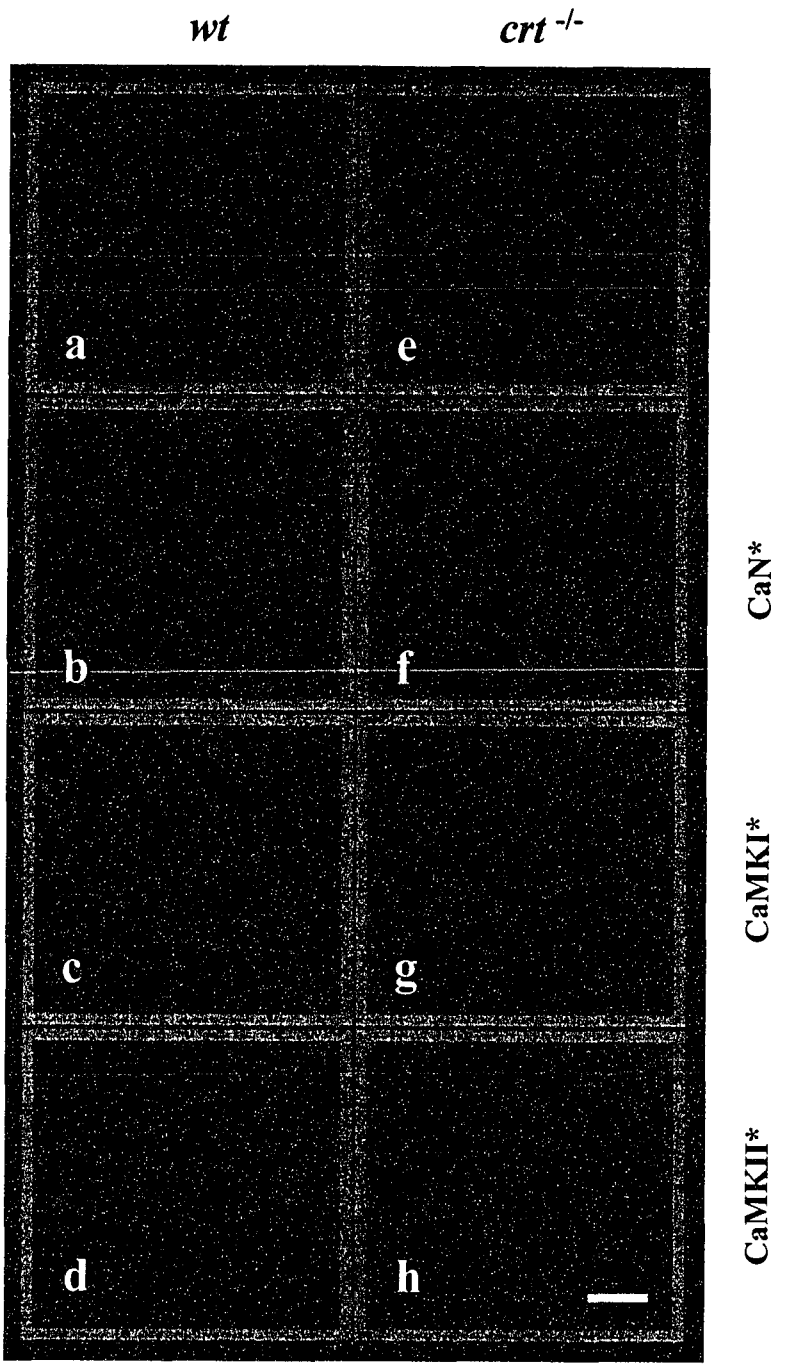
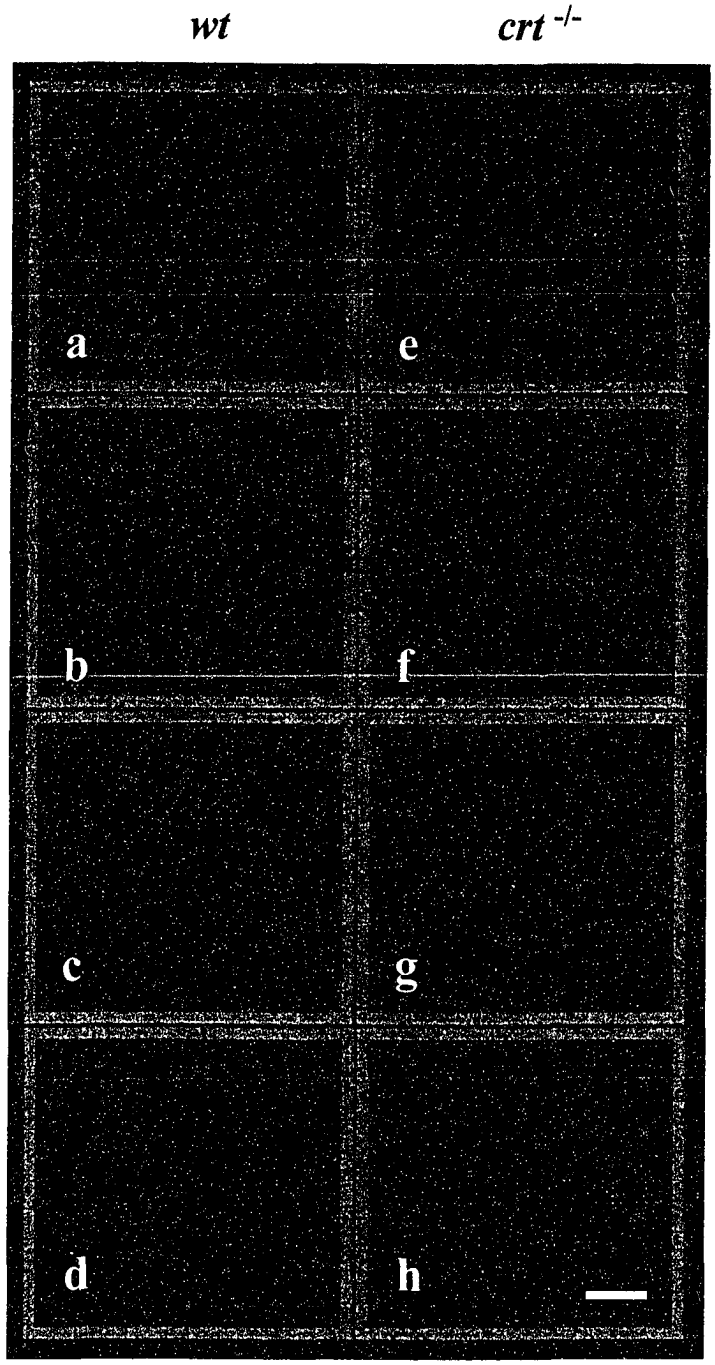


Figure 4-5. Treatment of cells with a CaMK inhibitor had no influence on GFP-MEF2C localization. KN-62 (CaMK inhibitor) treatment of wild-type (*panels a to d*) and calreticulin-deficient (*panels e to h*) embryonic fibroblasts had no influence of GFP-MEF2C localization beyond that observed for no KN-62 treatment (Figure 4-1). Scale bar = 25μm.



would be observed in cardiomyocytes. For this experiment, cardiomyocytes isolated from E12 wild-type and calreticulin-deficient embryos were transfected with GFP-MEF2C in the presence or absence of CaN*. In wild-type cardiomyocytes, GFP-MEF2C was found only in the nucleus whereas in calreticulin-deficient cells it was distributed throughout the cell, in both cytoplasm and nucleus (Figure 4-6). Similar to what was observed in fibroblasts, the presence of CaN* permitted GFP-MEF2C to completely translocate to the nucleus in the absence of calreticulin (Figure 4-6). Therefore, the results for GFP-MEF2C in cardiomyocytes supported observations in fibroblasts.

In vitro and *in vivo* data suggested that MEF2C nuclear localization is impaired in calreticulin-deficient cells. To determine if this was the case *in situ*, E12 mouse embryos were sectioned for histological examination. Wild-type embryo hearts were found to have very dark MEF2C nuclear staining (Figure 4-7, *c*). In comparison, hearts from calreticulin-deficient embryos were found to have MEF2C in the cytoplasm (Figure 4-7, *d*). The results from this experiment were in agreement with *in vitro* and *in vivo* observations and confirmed that MEF2C nuclear localization was disrupted in absence of calreticulin.

Figure 4-6. GFP-MEF2C localization in cardiomyocytes was compromised in the absence of calreticulin. Cardiomyocytes isolated from wild-type (*panels a, c*) and calreticulin-deficient (*panels b, d*) E12 embryos were cultured and transfected with expression vectors for GFP-MEF2C and CaN*. GFP-MEF2C was exclusively nuclear in wild-type cells (*panels a, c*), but not in calreticulin-deficient cells (panel c). Expression of activated-CaN promoted nuclear translocation of GFP-MEF2C in the absence of calreticulin (*panel d*). Scale bar = 50μm.

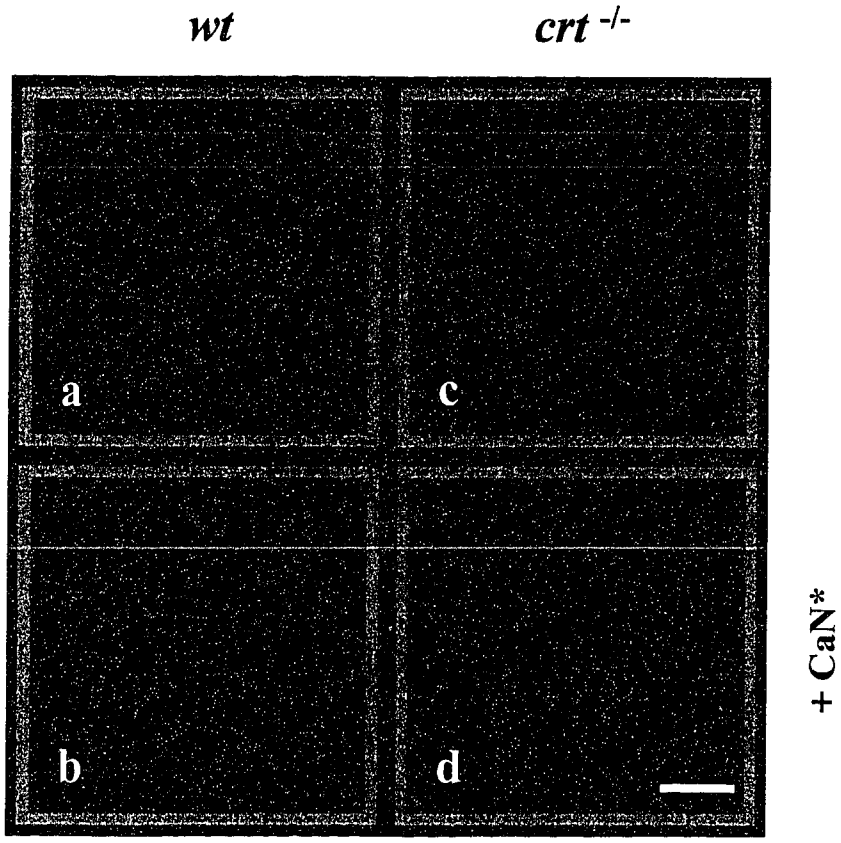
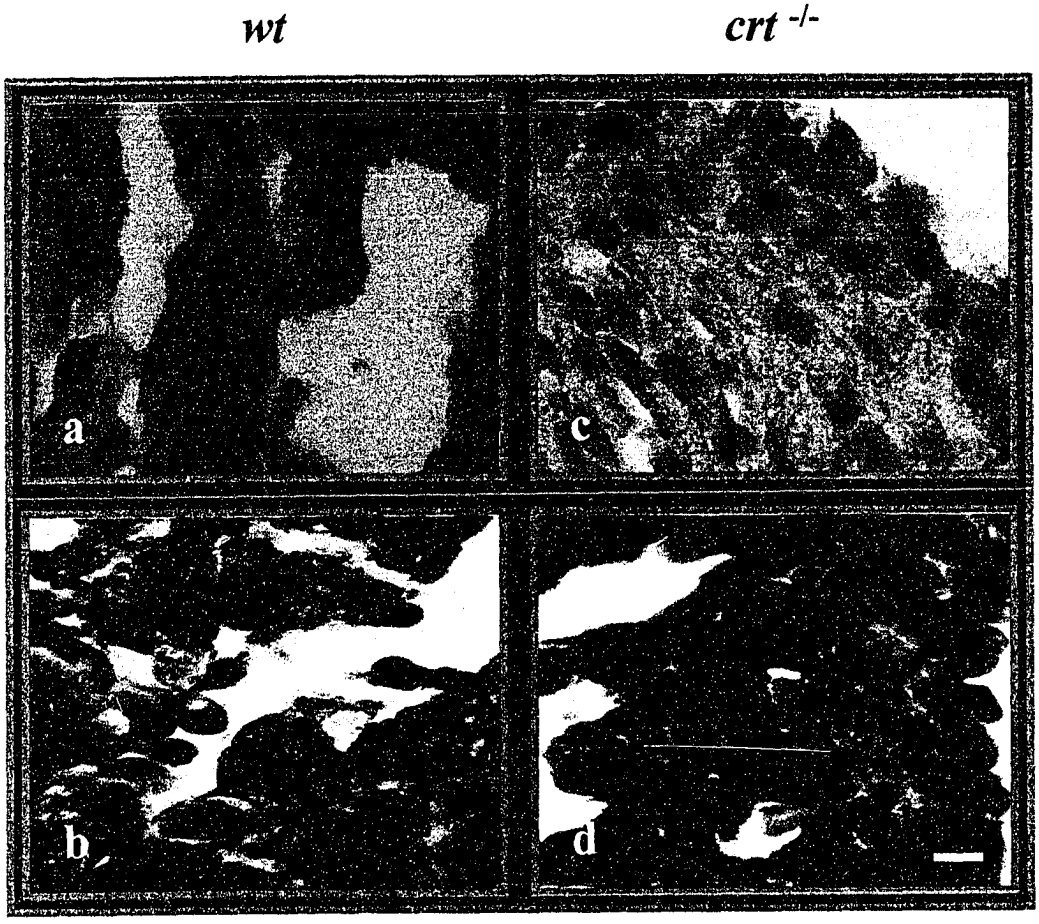


Figure 4-7. MEF2C localization in cardiomyocytes was compromised in absence of calreticulin. *In situ* localization of MEF2C in sagittal sections of E12 heart ventricle walls. Black colour is the primary staining and represents location of MEF2C while blue staining is due to hematoxylin and represents location of nuclei. Wild-type sections had very dark nuclei staining owning to a combination of the black and blue staining (*panel b*). In comparison, nuclei in calreticulin-deficient fibroblasts were blue in colour and most of the black colour (MEF2C) was observed in the cytoplasm (*panel d*). Controls were sections that were only stained with hematoxylin (*panels a, c*). Scale bar = 50μm.



DISCUSSION

Localization of GFP-MEF2C was compromised in calreticulin-deficient cells. For additional evidence to support the MEF2C immunostaining data, a GFP-MEF2C plasmid was created and localization examined in mouse embryonic fibroblasts. As expected, GFP-MEF2C was localized only in the nucleus of wild-type cells (Figure 4-1, *a*), and this localization did not change following Ca^{2+} treatment or the presence of CaN*, CaMKI* or CaMKII* (Figure 4-1, *b, c, d, e*). However, for calreticulin-deficient cells GFP-MEF2C was found to be distributed throughout the cell, in both cytoplasm and nucleus (Figure 4-1, *f*). The results from GFP-MEF2C localization suggested that MEF2C localization was not completely inhibited in cells devoid of calreticulin, but rather indicated that in absence of calreticulin MEF2C localization was impaired. As with the immunostained cells, MEF2C accumulated in the nucleus of calreticulin-deficient cells following Ca^{2+} treatment or presence of CaN* (Figure 4-1, *g, h*), but was not influence by CaMK* (Figure 4-1, *i, j*). As with MEF2C immunostaining experiment, calreticulin-deficient cells stably transfected with calreticulin were found to always have nuclear localization of MEF2C (Figure 4-1, *k, l, m, n, o*). Therefore, examination of GFP-MEF2C supported immunostaining data and suggested that MEF2C localization was not entirely inhibited in absence of calreticulin, but rather was significantly impaired.

The slight difference between Texas-red MEF2C immunostaining and GFP-MEF2C observations may have occurred as a result of several factors. The pEGFP-C1 vector in which MEF2C was cloned has a more robust CMV promoter than the MEF2C plasmid utilized for immunostaining. As a result, more molecules of GFP-MEF2C may have been in cells transfected with this plasmid. Also, there is an issue of signal

sensitivity. In addition, GFP-MEF2C may have been in the cytoplasm over the nucleus. Finally, minor differences may have occurred because one method required cell fixation with formaldehyde (which could have resulted in some masking of antigen binding sites) while the other method viewed localization in live cells. Regardless of minor discrepancies between Texas red immunostaining and GFP localization, it can be concluded that obvious differences existed between wild-type and calreticulin-deficient cells and in the latter cell type MEF2C localization was impaired.

Recently, it was reported that glucocorticoid receptor (Holaska *et al.*, 2001) and p53 (Mesaeli and Phillipson, 2004) exhibit impaired nuclear translocation in the absence of calreticulin. As such, it was necessary to determine if the problem observed for MEF2C localization was specific for this transcription factor or if the problem was due to some physical abnormality with the cells resulting from absence of calreticulin. Subsequently, localization of other transcription factors in calreticulin-deficient fibroblasts was studied. The GFP-MEF2A results were particularly interesting as MEF2A and MEF2C have the same DNA binding specificity (Black and Olson, 1998) as well as a similar NLS (Yu, 1996). In this investigation, GFP-MEF2A was observed to localize to the nucleus in the absence of calreticulin. Examination of several other GFP-linked transcription factors indicated that cellular location of all was normal. Thus, any problem a transcription factor has with nuclear translocation in absence of calreticulin appears to be specific for that transcription factor and is not owing to problems with the nuclear pore. Therefore, compromised MEF2C nuclear translocation in calreticulin-deficient cells likely occurs because these cells have impaired Ca^{2+} release from cellular stores, which

results in diminished activity of CaN and an inability of CaN to post-translationally modify MEF2C thereby permitting MEF2C to translocate to the nucleus.

For further evidence to suggest CaN was directly influencing MEF2C, wild-type and calreticulin-deficient cells were transfected with cDNA encoding GFP-MEF2C and treated with CsA or KN-62. The results indicated that the CaN inhibitory drug, CsA, impaired GFP-MEF2C nuclear translocation whereas the CaMKII* inhibitory drug, KN-62, had no influence on GFP-MEF2C localization. In both cell types, GFP-MEF2C was observed to be throughout the cell, in both the cytoplasm and nucleus and so it is probable that treatment of cells with CsA likely inhibited both endogenous CaN as well as co-transfected CaN*. Hence, CsA treatment caused impaired GFP-MEF2C nuclear translocation and this result suggests that MEF2C is dependent on activated CaN for nuclear localization.

At this point, all localization studies had been conducted in fibroblasts, so it was necessary to determine if MEF2C localization was compromised in calreticulin-deficient hearts. As such, MEF2C localization in wild-type and calreticulin-deficient cardiomyocytes were studied. Localization of GFP-MEF2C in cardiomyocytes was observed to be impaired in the absence of calreticulin, but was corrected by the presence of CaN*. Thus, the cardiomyocyte data was in complete agreement with that observed for mouse embryonic fibroblasts. Histological analysis of hearts from E12 embryos provided *in situ* evidence to support *in vivo* observations regarding MEF2C localization. Therefore, it can be concluded that nuclear localization of MEF2C was compromised in the absence of calreticulin.

In conclusion, when localization of GFP-MEF2C in fibroblasts and cardiomyocytes was examined, differences between wild-type and calreticulin-deficient cells were obvious. Localization findings for GFP-MEF2C differed somewhat from Texas red immunostaining data and this was likely due to several factors. Regardless, GFP-MEF2C findings suggested that MEF2C nuclear localization was not completely inhibited in absence of calreticulin, but rather was compromised. Interestingly, treatment of cells to elevate cytoplasmic Ca^{2+} or co-transfection of cells with CaN* corrected the problem MEF2C had with nuclear localization. Since CaN is a Ca^{2+}-dependent phosphatase found in the cytoplasm, it is probable that endogenous CaN is activated by Ca^{2+} treatment. Hence, CaN* was responsible for MEF2C nuclear translocation. Determining if CaN was directly targeting MEF2C was the next experiment.

MEF2C CARBOXYL-TERMINUS IS DEPHOSPHORYLATED BY CALCINEURIN

Versions of portions of this chapter have been published or submitted for publication:

1. Lynch, J and Michalak, M. 2003. Calreticulin is an upstream regulator of calcineurin. *Biochem. Biophys. Res. Commun.* **311**:1173-9.
2. Lynch, J., Guo, L., Gelebart, P., Chilibeck, K., Xu, J., Molkentin, J.D., Agellon, L.B., and Michalak, M. 2004. Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca^{2+}-dependent signaling pathway of early cardiac development. *Status: Submitted.*

INTRODUCTION

MEF2C is a transcription factor that should be localized in the nucleus of any cell as it possesses both a NRS and a NLS in its carboxyl-terminus (Black and Olson, 1998; Borghi *et al.*, 2001). The MEF2C NLS is rather unique as it is believed to be bipartite (Borghi *et al.*, 2001). Sequence comparisons of MEF2 isoform carboxyl-termini indicated that MEF2A and MEF2C possessed the most similar NLS; however, these two isoforms differ substantially in the regions flanking the NLS (Borghi *et al.*, 2001). In particular, the MEF2C carboxyl-terminal around the NLS is much more serine- and threonine-rich than that of MEF2A. CaN is a serine/threonine phosphatase and this may be the reason as to why this investigation has found that CaN* influences GFP-MEF2C localization (Figure 4-1), but does not impact GFP-MEF2A localization (Figure 4-2). Previously, it was reported that deletion mutants lacking carboxyl-terminal portions of MEF2C have impaired nuclear localization (Borghi *et al.*, 2001). Hence, it was hypothesized that CaN* was targeting a region within the carboxyl-terminal of MEF2C and this region was responsible for MEF2C nuclear translocation.

There are several methods by which to demonstrate if a protein is being phosphorylated and/or dephosphorylated. Difficulty arises if the specific kinase involved in the phosphorylation reaction is unknown. In such an instance, it is best to perform an *in vitro* experiment using purified protein and phosphorylate this protein with a mixture of kinases such as that extracted from the heart (Moor and Fliegel, 1999; Moor *et al.*, 2001; Wang *et al.*, 1997). Preliminary experiments found that GST-MEF2C protein aggregates and MEF2C stable cell lines cannot be established (J. Lynch, unpublished data). As such, it was necessary to use a different approach to demonstrate CaN targeting

of MEF2C. Recently an *in vitro* experiment involving skeletal muscle cell extracts treated with CsA, Ca^{2+} ionophore, and/or calf intestinal alkaline phosphatase (CIAP) was published and in this report MEF2A was observed to be hyperphosphorylated in presence of CsA and hypophosphorylated in presence of Ca^{2+} ionophore or CIAP (Wu *et al.*, 2000). Therefore, there are several methods to demonstrate protein dephosphorylation and it was necessary to perform such an experiment to determine if CaN was directly dephosphorylating MEF2C.

GFP plasmids

The GFP plasmid utilized was pEGFP-C1. This plasmid was selected as GFP would be in frame with the amino-terminal portion of MEF2C and the GFP was less likely to potentially interfere with the MEF2C NLS. To create a plasmid that would have GFP fused in frame to only the NRS-NLS region of MEF2C, the carboxyl-terminal portion of MEF2C was amplified by PCR and 5' XhoI and 3' BamHI sites incorporated. To create a GFP plasmid with GFP linked only to the MEF2C NLS, long oligos were synthesized, annealed, and cloned into 5' XhoI and 3' BamHI sites. Wild-type and calreticulin-deficient fibroblasts and cardiomyocytes were transfected with GFP expression plasmids using FuGENE 6 transfection reagent. All GFP images were of live cells grown on 25mm coverslips. GFP images were captured with a Zeiss Axiovert S100 fluorescent microscope using an excitation wavelength of 490nm and emission wavelength of 520nm. All pictures were pseudo-coloured using Northern Eclipse 5.0 software (Empix Imaging Inc., Mississauga, Ontario, Canada).

Dephosphorylation of MEF2C

For dephosphorylation investigation, the GFP plasmid containing cDNA encoding the carboxyl-terminal NLS (Figure 5-1) region of MEF2C was transiently transfected into NIH3T3 fibroblasts. Cellular proteins were isolated using a buffer containing 50mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA (ethylene bis(oxyethylenenitrilo) tetra-acetic acid), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and a mixture [0.1 mg/mL aprotinin, Sigma Cat. A-1153; 0.05 mg/mL phosphoramidone, Sigma Cat.

Figure 5-1. Protein sequence of mouse MEF2C. This figure shows the 432 amino acids of MEF2C. Shown in bold is the 99 amino acid carboxyl-region which possesses the MEF2C NRS and NLS. Amino acids corresponding to the NRS are underlined while the amino acids corresponding to the bipartite NLS are shown in green.

MGRKKIQITRIMDERNRQVTFTKRKFG
LMKKAYELSVLCDCEIALIIFNSTNKLF
QYASTDMDKVLLKYTEYNEPHESRTN
SDIVEALNKKENKGSESPDPDSSYALT
PRTEEKYKKINEEFDNMIKSHKIPAVPP
PSFEMPVTIPVSSHNSLVYSNPVSTLG
NPNLLPLAHPSLQRNSMSPGVTHRPP
SAGNTGGLMGGDLTSGAGTSAGNGY
GNPRNSPGLLVSPGNLNKNIQAKSPP
PMNLGMNNRKPDLRVLIPPGSKNTMP
SVNQRINNSQSAQSLATPVVSVATPTL
PGQGMGGYPSAISTTYGTEYSLSSAD
LSSLSGFNTASALH**LGSVTGWQQQHL**
HNMPPSALSQLGDRTTTPSRYPQHTT
RHEAGRSPVDSLSSCSSSYDGSDRE
DHRNEFHSPIGLTRPSPDERESPSVK
RMRLSEGWAT

R-9382; 0.1 mg/mL TLCK hydrochloride (Nα–p-Tosyl-L-lysine chloromethyl ketone hydrochloride), Sigma Cat. T-7254; 0.2 mg/mL TPCK (N-p-Tosyl-phenylalanine chloromethyl ketone), Sigma Cat. T-4376; 0.1 mg/mL APMSF (4-amidinophenylmethanesulfonyl fluoride hydrochloride) Sigma, Cat. A-6664; 0.1 mg/mL E-64, Sigma, Cat. E-3132; 0.05 mg/mL Leupeptin, Sigma Cat. L-2884; 0.02 mg/mL Pepstatin A, Sigma Cat. P-4265] of protease inhibitors (Milner *et al.*, 1991). Centricon concentrators (Amicon, Inc., Beverly, MA; Cat. 4202) were utilized to concentrate the cellular extracts into PBS containing 0.5% NP40. Aliquots of each protein extract were incubated with CIAP (NEB; Cat. M0290S) for 6 hours at 37^0C. Proteins were then loaded onto a 12.5% SDS-PAGE gel separated at room temperature at 230 volts for 2.25 hours. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California, USA; Cat. 162-0115) and immunoblotted with polyclonal goat anti-GFP (1:5000 dilution; generous gift from Dr. Luc Berthiaume, University of Alberta). Antibody binding was detected with peroxidase-conjugated rabbit anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, Inc.; Cat. 305-035-003) at a dilution of 1:10000 followed by ECL development reaction.

RESULTS

Dephosphorylation of MEF2C carboxyl-terminal by CaN* enabled MEF2C nuclear localization

Two deletion mutants of MEF2C were subcloned into a GFP vector and localization of these deletion mutants in wild-type and calreticulin-deficient embryonic fibroblasts was examined. The first deletion mutant (99 amino acids) had the carboxyl-terminal portion of MEF2C encompassing both the NRS and NLS while the second deletion mutant was much smaller (30 amino acids) and possessed only the NLS region (Figure 5-2). In wild-type cells, GFP-MEF2C-NRS-NLS fusion protein was found to be only in the nucleus regardless of Ca^{2+} treatment or co-transfection with other plasmids (Figure 5-3, *a, b, c, d, e*). In comparison, in calreticulin-deficient cells the GFP-MEF2C-NRS-NLS fusion protein was observed to be in both the nucleus and cytoplasm (Figure 5-3, *f, g, h, i, j*) and only in the nucleus following addition of Ca^{2+} or CaN* (Figure 5-3, *g, h*). Identical results were obtained when the second deletion mutant, GFP-MEF2C-NLS fusion protein, was visualized in wild-type (Figure 5-4, *a, b, c, d, e*) and calreticulin-deficient fibroblasts (Figure 5-4, *f, g, h, i, j*). Therefore, experiments using MEF2C deletion mutants suggested that at the extreme carboxyl-terminal of MEF2C there is a region of 30 amino acids essential and sufficient for MEF2C nuclear localization and this region is sensitive to presence of Ca^{2+} or CaN*.

In all calreticulin-deficient cells, impaired MEF2C nuclear localization was observed and it could only be overcome by Ca^{2+} treatment or presence of CaN*. Subsequently, I hypothesized that CaN* was essential for post-translational modification of the carboxyl-terminal of MEF2C and this event was necessary for MEF2C nuclear

Figure 5-2. Schematic diagrams of full-length MEF2C and GFP-MEF2C deletion mutants. In the MEF2C diagram, the blue box represents the MADS domain (57 amino acids) while the red box represents the MEF2 domain (29 amino acids). The locations of the NRS and NLS are indicated. The MEF2C-NRS-NLS deletion mutant has the carboxyl-terminal 99 amino acid residues of MEF2C (residues 333 to 432). In comparison, MEF2C-NLS possesses the carboxyl-terminal 30 amino acid residues of MEF2C (residues 402 to 432).

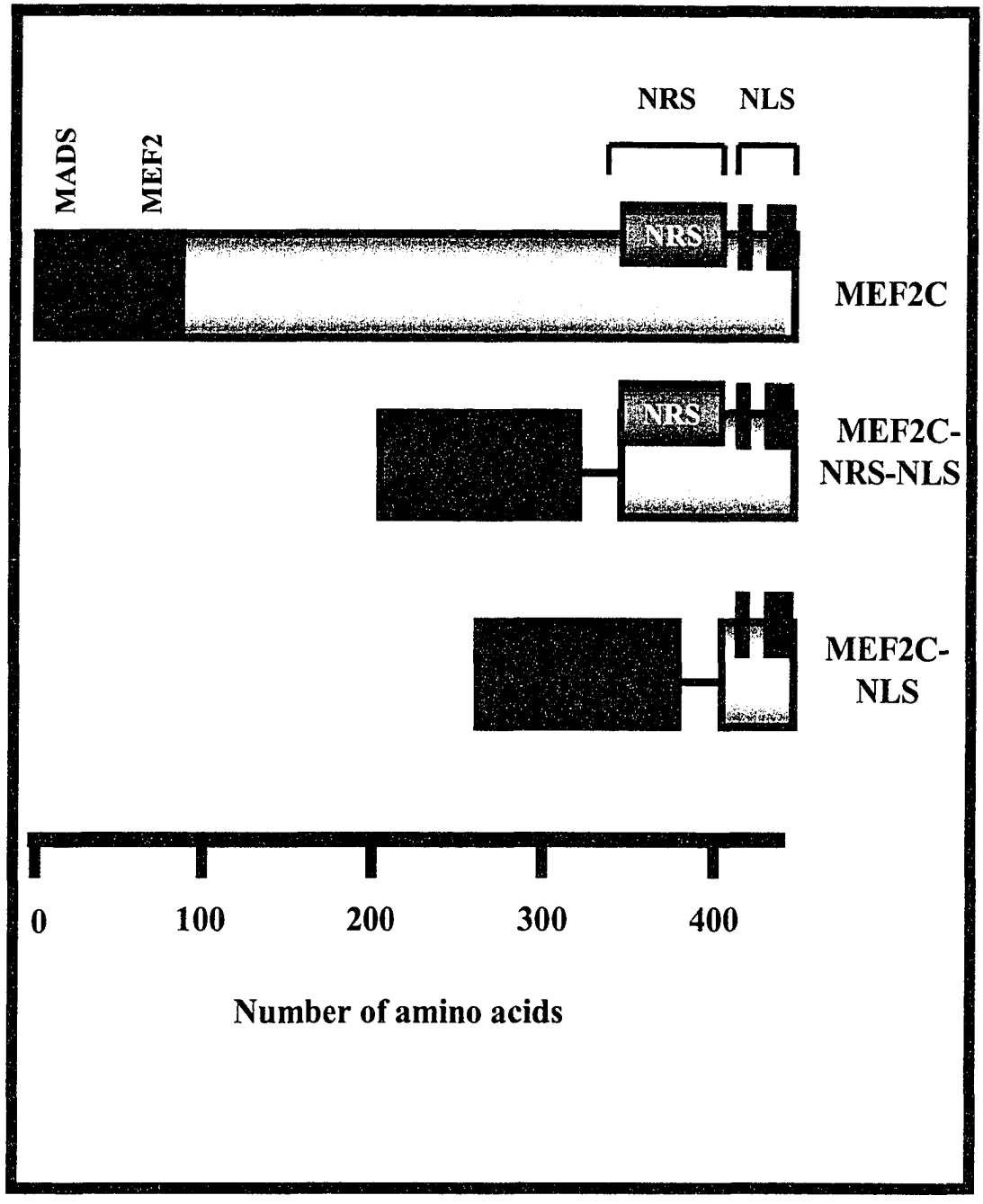


Figure 5-3. Localization of GFP-MEF2C-NRS-NLS fusion protein in mouse embryonic fibroblasts. Wild-type (*panels a to e*) cells had nuclear localization of GFP-MEF2C-NRS-NLS fusion protein while calreticulin-deficient cells had GFP-MEF2C-NRS-NLS fusion protein in both cytoplasm and nucleus (panels *f, i, j*). When calreticulin-deficient cells were treated with Ca^{2+} (*panel g*) or co-transfected with CaN* (*panel h*) then GFP-MEF2C-NRS-NLS fusion protein was in the nucleus. CaMK* had no influence on fusion protein localization (*panels d, e, i, j*). Scale bar = 25μm.

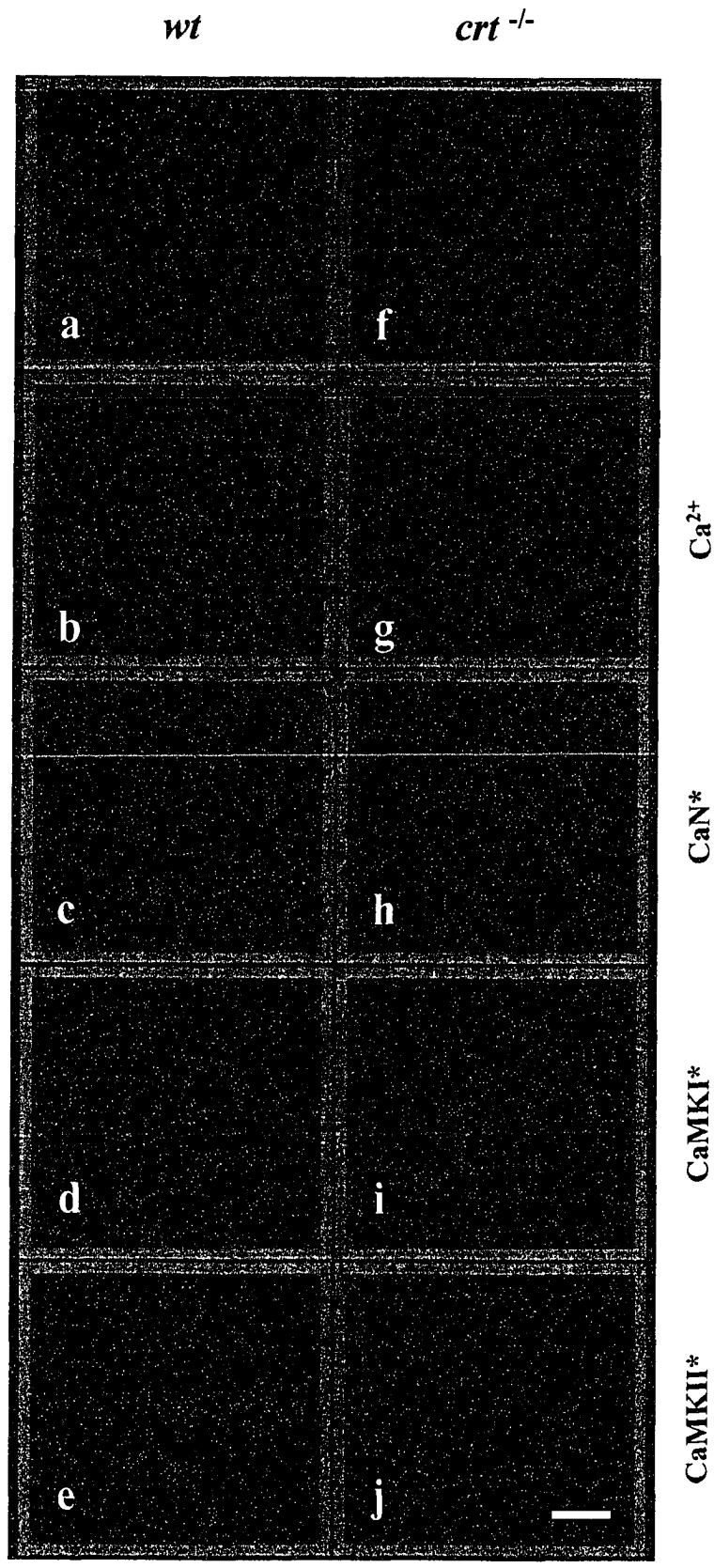
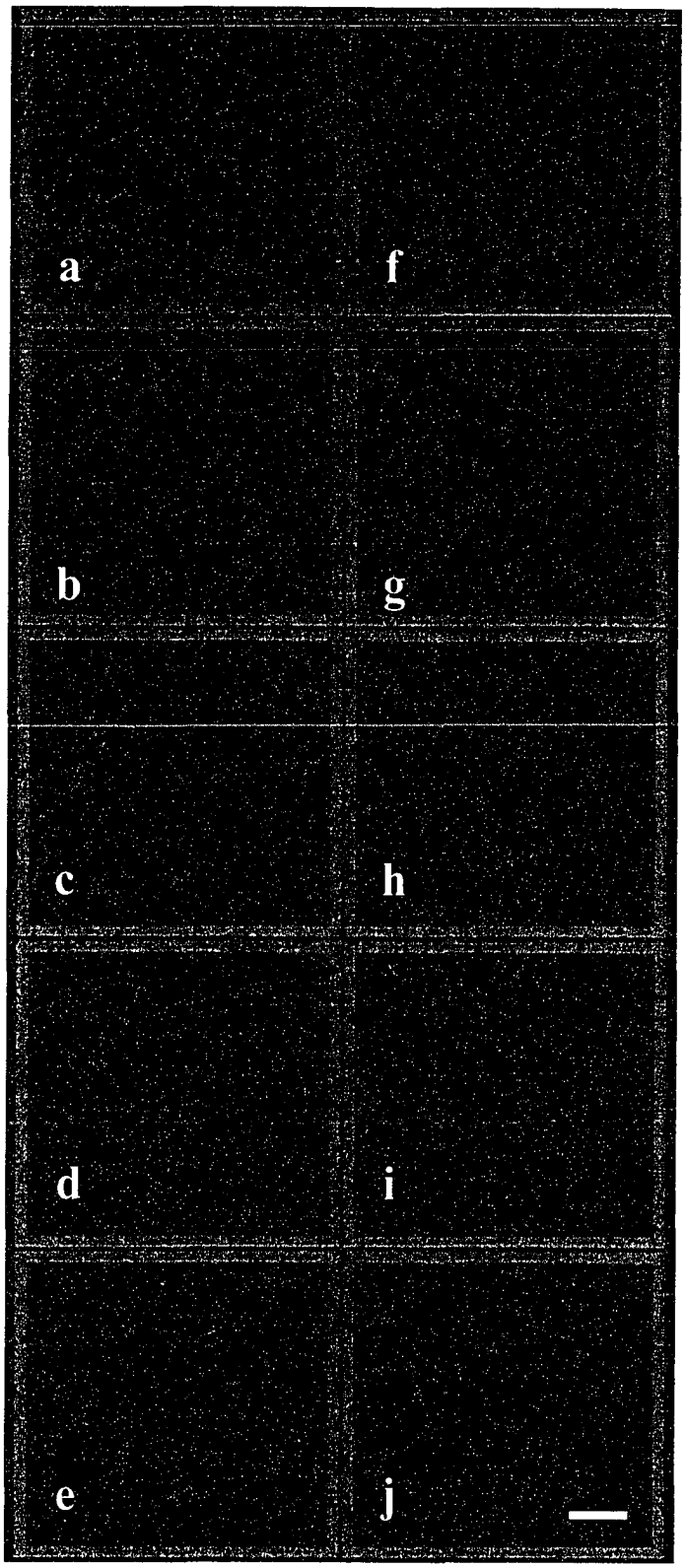
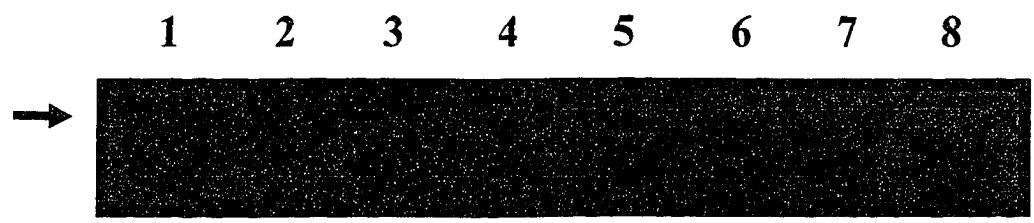


Figure 5-4. Localization of GFP-MEF2C-NLS fusion protein in mouse embryonic fibroblasts. Wild-type (*panels a to e*) cells had nuclear localization of GFP-MEF2C-NLS fusion protein while calreticulin-deficient cells had GFP-MEF2C-NLS fusion protein in both cytoplasm and nucleus (panels *f, i, j*). Calreticulin-deficient cells treated with Ca^{2+} (*panels g*) or co-transfected with CaN* (*panel h*) had GFP-MEF2C-NLS fusion protein in the nucleus. CaMK* had no influence on fusion protein localization (*panels d, e, i, j*). Scale bar = 25μm.



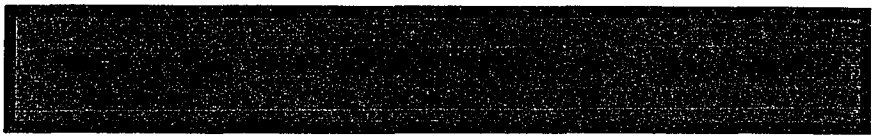
localization. To test this hypothesis, it was necessary to determine if CaN* directly dephosphorylated the carboxyl-terminal of MEF2C. For this experiment, DNA encoding GFP-MEF2C-NLS expression plasmid was transiently transfected into calreticulin-deficient fibroblasts and cells were treated with CsA or Ca^{2+} ionophore or co-transfected with CaN*. Immunoblots indicated that cells exposed to the CaN-inhibiting drug CsA had a slower migrating band (Figure 5-5, *lane 2*) while cells exposed to Ca^{2+} ionophore or CaN* had a more rapidly migrating form of GFP-MEF2C-NLS (Figure 5-5, *lanes 3 and 4*). The identity of this faster migrating band as a hypophosphorylated form of GFP-MEF2C-NLS was confirmed by incubation of the protein extracts with CIAP (Figure 5-5, *lanes 5 to 8*). Fibroblasts transfected with only pEGFP-C1 plasmid served as a negative control (Figure 5-6).

Figure 5-5. GFP-MEF2C carboxyl-terminal was directly dephosphorylated by CaN*. Calreticulin-deficient fibroblasts were transfected with an expression vector for GFP-MEF2C-NLS, or with vectors for GFP-MEF2C-NLS and CaN* (*Lanes 4 and 8*) followed by SDS-PAGE and Western blot analysis with anti-GFP antibodies. The upper and lower protein bands correspond to hyperphosphorylated GFP-MEF2C-NLS (*arrow*) and hypophosphorylated GFP-MEF2C-NLS, respectively. *Lane 1*, control cell extract; *Lane* 2, extract of cells treated with 0.25 μM cyclosporin (*CsA*); *Lane 3*, cells treated with Ca^{2+} ionophore; *Lane 4*, cells expressing activated-CaN; and *Lanes 5-8*, protein extracts were treated with alkaline phosphatase (*CIAP*). Three independent experiments rendered similar findings.



	1	2	3	4	5	6	7	8
CsA	-	+	-	-	-	+	-	-
Ca^{2+}	-	-	+	-	-	-	+	-
CaN*	-	-	-	+	-	-	-	+
CIAP	-	-	-	-	+	+	+	+

Figure 5-6. GFP was not targeted by CaN*. Calreticulin-deficient fibroblasts were transfected with GFP expression vector or GFP and CaN* expression vectors (*Lanes 4 and 8*) followed by SDS-PAGE and Western blot analysis with anti-GFP antibodies. *Lane 1*, control cell extract; *Lane* 2, cell extract treated with 0.25 μM cyclosporin (*CsA*); *Lane 3*, cells treated with Ca^{2+} ionophore; *Lane 4*, cells expressing CaN*; and *Lanes 5-8*, protein extracts were treated alkaline phosphatase (*CIAP*). Electrophoretic mobility of GFP was not influenced by CsA, increased Ca^{2+} concentration, CaN* or CIAP thus suggesting that GFP was not a target of calcineurin phosphatase activity.



	1	2	3	4	5	6	7	8
CsA	-	+	-	-	-	+	-	-
Ca^{2+}	-	-	+	-	-	-	+	-
CaN*	-	-	-	+	-	-	-	+
CIAP	-	-	-	-	+	+	+	+

DISCUSSION

Of the MEF2 isoforms, MEF2A and MEF2C are most similar (Black *et al.*, 1998) and in skeletal muscle, CaN dephosphorylates MEF2A in a dose-dependent manner to augment MEF2A transactivational domain (Wu *et al.*, 2001). Dephosphorylation of MEF2A was found to have reduced DNA binding ability whereas a hypophosphorylated from had enhanced DNA binding and maximum MEF2A transactivation capability (Mao and Wiedmann, 1999; Wu *et al.*, 2001). CaN hypophosphorylation of MEF2A has also been observed in cultured primary cerebellar granule neurons (Mao and Wiedmann, 1999). Therefore, as MEF2 isoforms have several potential phosphorylation sites, it is probable that CaN functions to dephosphorylate MEF2 proteins so as to enhance MEF2 transcriptional activity.

Data from localization of MEF2C deletion mutants enabled a hypothesis to be formed that suggested that CaN was targeting the extreme carboxyl-terminal of MEF2C near the MEF2C NLS. A dephosphorylation experiment confirmed that in presence of CaN*, MEF2C carboxyl-terminal was hypophosphorylated whereas in the presence of the CaN inhibitor, CsA, MEF2C carboxyl-terminal was hyperphosphorylated. Subsequently, it can be concluded that MEF2C is a direct target for CaN and a region of 30 amino acids in the MEF2C carboxyl-terminal must be dephosphorylated in order to unmask the MEF2C NLS and permit nuclear translocation of this transcription factor. Since the carboxyl-terminal of MEF2C is extremely serine- and threonine-rich, further investigation will be necessary to identify the kinase(s) responsible for phosphorylating MEF2C as well as determine the MEF2C residue(s) that are dephosphorylated by CaN.

Although disruption of calreticulin gene is not as severe as disruption of *mef2c*

gene, it is interesting that both null-mutants: 1) are much smaller than wild-type embryos; 2) die *in utero* as a result of severe malformation of heart ventricles; and, 3) have cardiomyocytes with severe conduction problems (Li *et al.*, 2002; Lin *et al.*, 1997). In this investigation, it was discovered that MEF2C localization was compromised in the absence of calreticulin rather than completely inhibited; subsequently, it is not surprising that the MEF2C-deficient phenotype is much more severe than the calreticulin-deficient phenotype. Interestingly, calreticulin-deficient embryos are rescued when CaN* is over-expressed in the heart (Guo *et al.*, 2002). It is possible that the rescue occurred because CaN* activated NFATc family members; however, from this investigation it can be seen that CaN* also targets MEF2C and MEF2C likely contributes to survival of the calreticulin-rescue mouse.

In summary, the data presented in this chapter are the first to demonstrate a direct link between CaN and MEF2C in the heart. Examination of MEF2C cellular localization in calreticulin-deficient cells has enabled identification of a novel signaling pathway in cardiomyocytes that links Ca^{2+} released by calreticulin to CaN activation and dephosphorylation of MEF2C. Dephosphorylation of the MEF2C NLS region by CaN is essential for MEF2C to translocate to the nucleus and impact target genes. The finding that MEF2C binds to the calreticulin promoter and potentially activates the calreticulin gene suggests existence of a novel positive feedback mechanism in cells where MEF2C targets the gene of a Ca^{2+}-binding protein so as to ensure a sufficient supply of releasable Ca^{2+} be maintained within the cell for CaN activation and MEF2C translocation. Support for this feedback mechanism comes from experiments with: 1) mice that have cardiac over-expression of MEF2C as these transgenic animals show an increase in calreticulin

expression in the heart (Appendix Figure 7); and, 2) HEK293 cells inducible for calreticulin as findings using these cells suggest that when there is more calreticulin present in a cell then the ability of MEF2C to activate a 6X MEF2C luciferase reporter gene is enhanced (Appendix Figure 8). Therefore, the results from this investigation add to the current understanding of cellular pathways responsible for cardiac development and provide a molecular explanation as to why the calreticulin-deficient embryos die and why the calreticulin rescue mice survive. The significance of the findings as well as future directions for this research will be discussed in the next chapter.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Calreticulin is a ubiquitously expressed protein found within the lumen of the SR/ER. Several cellular functions have been attributed to calreticulin and include functioning as: 1) a molecular chaperone; 2) a Ca^{2+} buffer helping to maintain both intracellular Ca^{2+} stores and Ca^{2+} homeostasis; and, 3) a component of intracellular Ca^{2+} signaling cascades. Calreticulin is known to be highly expressed early in the heart and gene disruption in mice is lethal at E14.5 as a result of severe underdevelopment of the heart. Interestingly, breeding calreticulin heterozygote mice with mice that have cardiac over-expression of the serine/threonine phosphatase CaN produces a viable calreticulin-deficient animal. Thus, calcineurin is essential for heart development and activation of calcineurin is dependent on calreticulin-dependent Ca^{2+} released from the ER. A molecular explanation as to why the calreticulin-deficient embryos die and why the rescue mice survive was unknown. Therefore, the purpose of this investigation was to determine a molecular explanation by examining the calreticulin promoter. It was hypothesized that Ca^{2+} released from calreticulin in the ER is essential for activation of CaN and this phosphatase acts on MEF2C to increase expression of MEF2C target genes and rescue calreticulin-deficient phenotype.

In this study, cardiac transcription factors that regulated expression of the calreticulin gene were investigated and MEF2C was identified as a strong activator. MEF2C was found to bind *in vitro* and *in vivo* to a site within the calreticulin promoter. The ability of MEF2C to activate the calreticulin promoter-controlled luciferase reporter gene was synergistically enhanced following treatment of cells with Ca^{2+} ionophore or presence of CaN*. A novel observation of this investigation was that MEF2C was unable to activate the reporter gene in calreticulin-deficient cells, but could following Ca^{2+}

ionophore treatment or presence of CaN*.

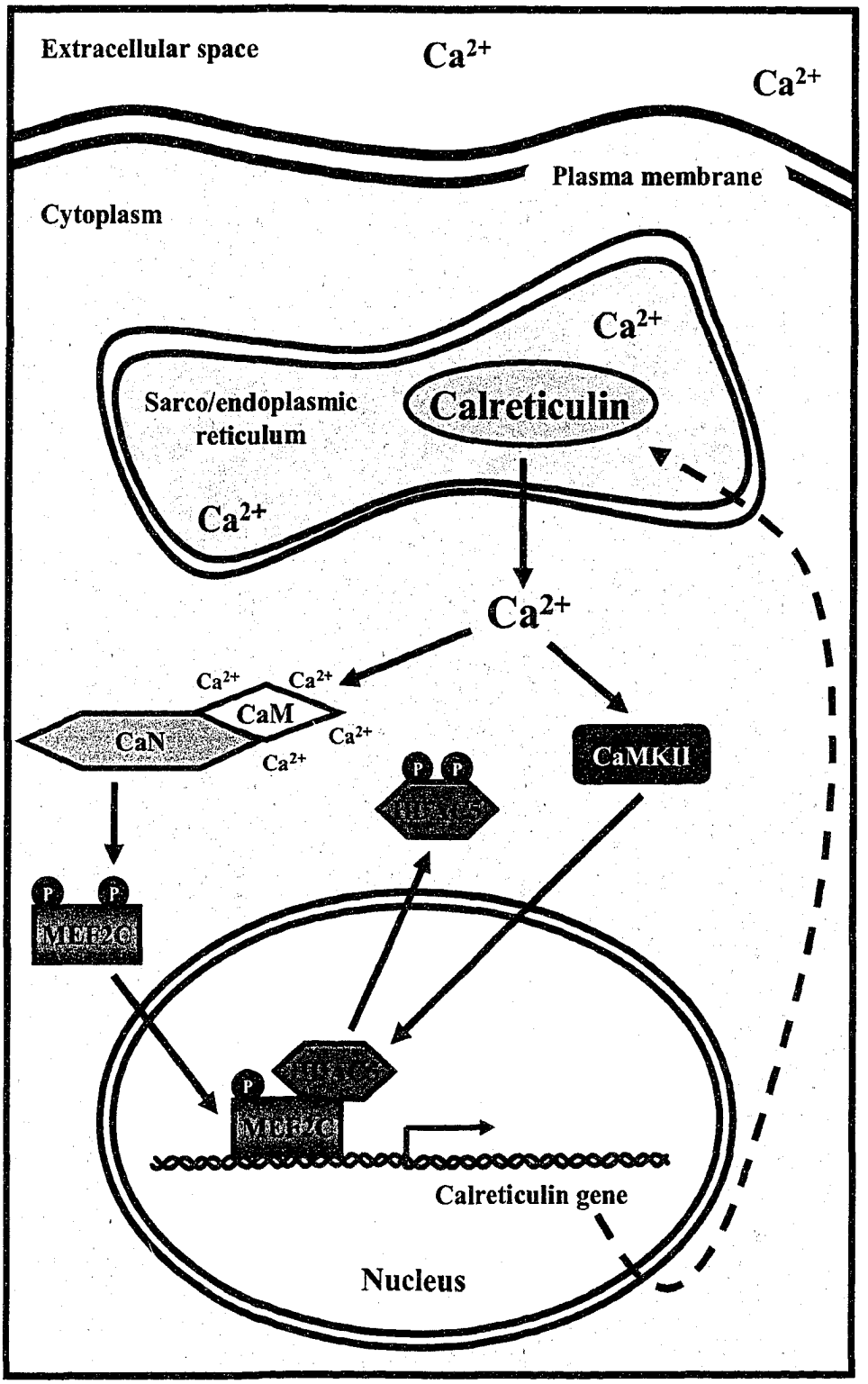
When nuclear extracts isolated from calreticulin-deficient cardiomyocytes were applied to DNA arrays it was discovered that MEF2C was present in the nuclear extracts only after the cells had been treated with Ca^{2+} ionophore. Thus, it appeared that MEF2C nuclear translocation was compromised in the absence of calreticulin and suggested that in calreticulin null-mutant cells the function of this transcription factor may also be jeopardized. To investigate MEF2C function, RT-PCR was conducted and the results indicated that MEF2C target genes were under-expressed in calreticulin-deficient hearts relative to wild-type hearts. Therefore, in the absence of calreticulin MEF2C did not function properly unless cytoplasmic Ca^{2+} concentration was increased or CaN* was present.

MEF2C was localized within fibroblasts and cardiomyocytes to determine if MEF2C function was impaired because of a problem with nuclear localization in the absence of calreticulin. Localization studies showed that MEF2C could not localize efficiently to the nucleus in absence of calreticulin, but could following Ca^{2+} ionophore treatment or presence of CaN*. Figure 6-1 illustrates the interactions among calreticulin, Ca^{2+}, calcineurin, and MEF2C which have been demonstrated in the present study. Changes in cytosolic Ca^{2+} concentration affect numerous signal transduction pathways and influence a wide range of cell and organ functions, including cardiogenesis (Bootman *et al.*, 2001; Webb and Miller, 2003). To activate Ca^{2+}-dependent signaling pathways, Ca^{2+} is released from the ER as well as comes from the extracellular space thereby causing an increase in cytoplasmic Ca^{2+} concentration which can impact Ca^{2+}-dependent processes (Berridge *et al.*, 2003). During cardiac development an important target protein

is calcineurin (Figure 6-1) which, when activated by a sustained increase in intracellular Ca^{2+} concentration, dephosphorylates MEF2C carboxyl-terminus promoting its translocation from the cytoplasm into the nucleus (Figure 6-1). Among its many effects, Ca^{2+} released from the ER also activates CaMKs. These kinases are known to influence MEF2C function in the heart, likely via phosphorylation of HDACs (McKinsey *et al.*, 2001a; McKinsey *et al.*, 2001b). In the absence of calreticulin, agonist-dependent Ca^{2+} release from the ER is inhibited (Mesaeli *et al.*, 1999; Nakamura *et al.*, 2001b) and, as a result, calcineurin is not fully activated and MEF2C is not translocated into the nucleus efficiently in calreticulin-deficient cells (Li *et al.*, 2002b). Therefore, calreticulin is an upstream regulator of CaN and MEF2C in cardiac Ca^{2+}-dependent signaling cascades (Figure 6-1). As the calreticulin promoter is targeted by MEF2C, it is probable that a positive feedback mechanism exists in cells (Figure 6-1) where MEF2C targets the gene of an upstream Ca^{2+} buffer protein to ensure an adequate supply of Ca^{2+} remaisn in the intracellular stores for CaN and MEF2C activation. Mutually reinforcing feedback loops are common in cardiomyocytes (Srivastava and Olson, 2000).

The hypothesis of a link between CaN and MEF2C has been proposed previously (Passier *et al.*, 2000), but this is the first study to have evidence establishing this Ca^{2+}-dependent signaling pathway component. The finding that calreticulin is an upstream regulator of CaN and MEF2C in early cardiac development provides a molecular explanation as to why calreticulin-deficiency is lethal and how activated CaN rescues the calreticulin-deficient phenotype. Thus, the hypothesis of this investigation has been supported and the objectives fulfilled.

Figure 6-1. Model of a hypothetical cell depicting the calcineurin-dependent transcriptional pathway essential for MEF2C nuclear translocation and cardiac development. In this model, Ca^{2+} released from calreticulin enters the cytoplasm and activates calmodulin (CaM). Ca^{2+}-bound CaM binds to and activates CaN. Activated CaN functions to dephosphorylate the carboxyl-terminal of MEF2C and this post-translational modification allows MEF2C to translocate to the nucleus to impact transcription of target genes. Since the calreticulin promoter is a target for MEF2C, a positive feedback mechanism exists. The role of CaMKII* in this model is to phosphorylate HDACs thereby facilitating their nuclear export and preventing HDAC repression of MEF2C activity. *Abbreviations*: CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; CaN, calcineurin; HDAC5, histone deacetylase 5; and MEF2C, myocyte enhancer factor 2C.



Future directions

MEF2C was found to activate the calreticulin promoter-controlled luciferase reporter gene. Numerous luciferase experiments confirmed this observation and the MEF2C site was identified by EMSA and confirmed by ChIP. However, it could have been interesting to mutate the identified MEF2C site within the calreticulin promoter and perform the EMSA or ChIP experiments. It can be hypothesized that mutation of the MEF2C site would likely have resulted in MEF2C being unable to bind to site within the probe or promoter. Although this experiment was not necessary, it could have been a nice addition.

In calreticulin-deficient fibroblasts MEF2C was unable to activate the calreticulin promoter-controlled luciferase reporter gene unless the cells were treated with Ca^{2+} ionophore or co-transfected with CaN*. Results of this investigation suggested that activation of CaN was compromised in absence of calreticulin. As such, a reporter gene (e.g., 9X NFATc consensus binding sites upstream of a minimal promoter) could have been utilized to show that CaN was inactive (Bueno *et al.*, 2004; Wilkins *et al.*, 2004) in calreticulin-deficient cells. Therefore, additional experiments with reporter genes could have been beneficial.

MEF2C nuclear localization was compromised in absence of calreticulin, but nuclear localization of another MEF2 isoform, MEF2A, was not. To further demonstrate that MEF2C carboxyl-terminal was responsible for the impaired nuclear localization, it would have been interesting to create MEF2A and MEF2C chimera proteins. In this experiment, the carboxyl-terminal of one could have been substituted for the other and cloned into a GFP plasmid thereby permitting cellular localization to be observed.

The model that I have proposed (Figure 6-1) suggests that a feedback mechanism exists within cells where MEF2C increases expression of calreticulin (Appendix Figure 7). Although calreticulin promoter-controlled luciferase reporter gene experiments demonstrated that MEF2C targets the calreticulin promoter, it would have been interesting to have transfected MEF2C plasmid into fibroblasts and: 1) performed a western blot for calreticulin to see if calreticulin protein concentration increased; as well as 2) performed Fura-2 Ca^{2+} measurements of these cells to determine if ER Ca^{2+} content was enhanced. Results from these experiments could have provided support for the positive feedback aspect of the model. In addition, experiments using hearts isolated from 13-day-old mice over-expressing calreticulin (Nakamura *et al.*, 2001a) suggests that when there is more calreticulin there is more MEF2C (Appendix Figure 9). The reason for this observation requires further investigation.

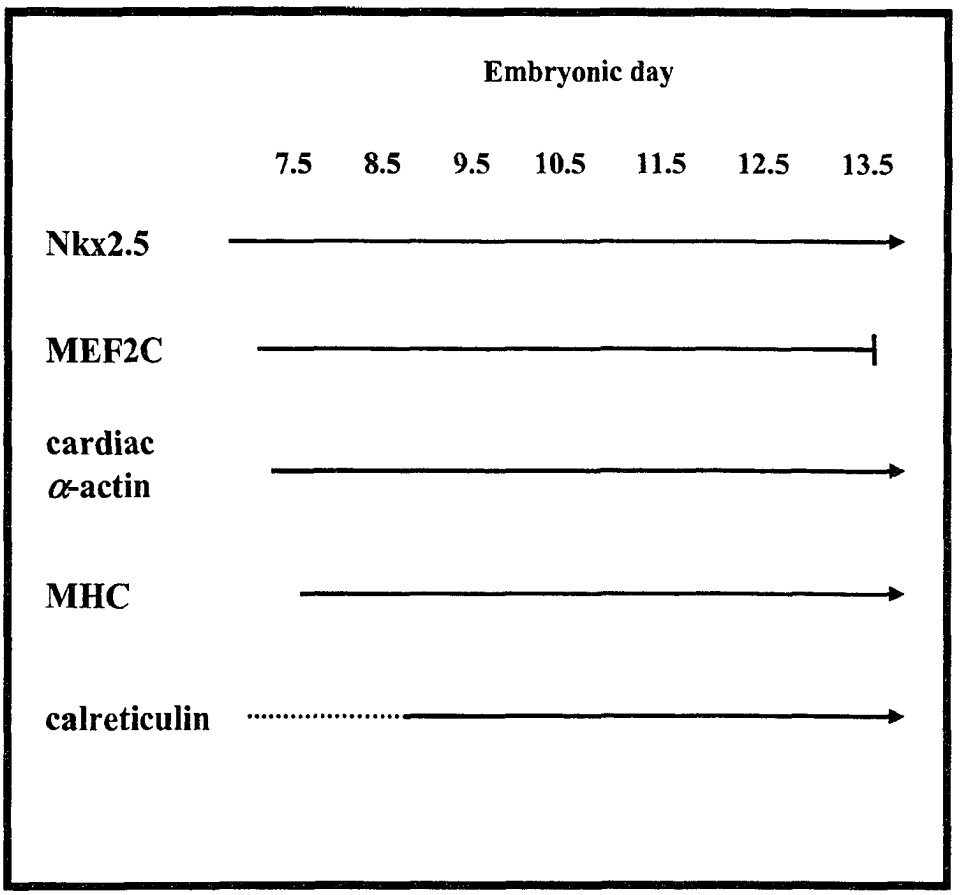
The novel pathway proposed in this thesis (Figure 6-1) creates new scientific questions and in so doing identifies areas requiring further research. As such, future investigation will be necessary to determine what proteins participate in post-translational modification of MEF2C. In addition, further studies are necessary to precisely determine a timeline as to when calreticulin is expressed in the developing heart as well as determine if expression of proteins other than CaN can rescue the calreticulin-deficient phenotype.

This investigation found that MEF2C is dephosphorylated by activated CaN. It would be interesting to utilized mass spectrometry to specifically identify the phosphorylation sites in the carboxyl-terminal of MEF2C as well as identify the residues dephosphorylated by CaN. Phosphorylation sites have been mapped for MEF2A (Cox *et*

al., 2003) and although undertaking such a project is an enormous task the technology is available. Once the sites of phosphorylation have been identified, sites could be mutated and incorporated into GFP fusion proteins to examine cellular localization. An additional future project that would be more difficult than mapping the phosphorylation sites would be to identify the kinase(s) responsible for phosphorylation of MEF2C carboxyl-terminal. Therefore, two large future directions for MEF2C research involve identifying kinases responsible for phosphorylating MEF2C and residues dephosphorylated by CaN.

If a feedback mechanism exists between MEF2C and calreticulin then a question that arises is: Which of these proteins appears first in the heart during development? To answer this question the cardiac expression of these factors as well as others must be considered (Figure 6-2). MEF2C has been reported to be in the heart at E7.5 (Edmondson *et al.*, 1994; Molkentin *et al.*, 1996b) while mice with calreticulin promoter controlling expression of GFP had GFP expressed in the heart at E9 (Mesaeli *et al.*, 1999). The calreticulin promoter is targeted by Nkx2.5, myocardin, and MEF2C which means that calreticulin should be expressed extremely early during heart development. Thus, it is unfortunate that calreticulin promoter-controlled GFP expression was not examined earlier than E9 because it is probable that it would have been observed. Therefore, one future study could involve re-establishing the calreticulin promoter-controlled GFP reporter mouse line and precisely determining when calreticulin is expressed during early heart development. In addition another future investigation could involve establishing a new calreticulin promoter-GFP transgenic mouse line which has the MEF2C site (-836 to -845) in the calreticulin promoter mutated and examine cardiac GFP expression in these animals.

Figure 6-2. Timeline of genes expressed in the developing heart. Nkx2.5 (Searcy *et al.*, 1998), MEF2C (Edmondson *et al.*, 1994), cardiac α-actin (Sassoon *et al.*, 1988), MHC (Lyons *et al.*, 1990), and calreticulin (Mesaeli *et al.*, 1999). Cardiac expression of calreticulin was investigated from E9.0 to 3-weeks-old; hence, calreticulin expression in the heart may be earlier than E9.0, but this has not been investigated, so is indicated by a dashed line.



Fibroblast studies have shown that removal of calreticulin from a cell causes a decrease in ER Ca^{2+} concentration. If calreticulin-deficiency lethality occurs as a result of decreased ER Ca^{2+} content then perhaps substituting another ER Ca^{2+} buffer such as calsequestrin for calreticulin may rescue the calreticulin-deficient phenotype. Preliminary experiments could be conducted in fibroblasts and ER Ca^{2+} concentration measured and/or MEF2C localization examined. If fibroblasts results are favorable then calsequestrin could potentially be used to create a calreticulin-deficient rescue mouse.

In this chapter a few future directions for this research have been suggested and no doubt numerous other possibilities exist. Now that this novel signaling pathway linking calreticulin to CaN and MEF2C has been established, it will be interesting to see what direction the science will take.

CONCLUSIONS

Heart disease and heart development are closely linked processes as both utilize similar molecular aspects. Thus, it is believed that an understanding of Ca^{2+}-dependent signaling cascades involved in one process likely occurs in the other. This study focused on the relationship between calreticulin, CaN, and MEF2C in cardiac development. The purpose of this investigation was to: 1) determine which cardiac transcription factors regulate calreticulin gene; and 2) provide a molecular explanation as to why calreticulin-deficiency is lethal and why cardiac expression of calcineurin rescues the calreticulin-deficient phenotype. The major findings of this investigation were:

1. MEF2C is a transcription factor that activates the calreticulin promoter.

2. MEF2C does not localize efficiently to the nucleus in theabsence of calreticulin and this can be corrected by enhancement of cytoplasmic Ca^{2+} or presence of activated CaN.

3. CaN was found to dephosphorylate MEF2C and this post-translational modification enabled MEF2C to localize in the nucleus.

4. This study provides a molecular explanation as to as to why calreticulin-deficiency is embryonic lethal.

5. This investigation also provides a molecular explanation why expression of activated CaN in the heart rescues calreticulin-deficient embryos.

The results of this study add to the current understanding of cellular pathways responsible for cardiac development. Since cardiac development and cardiac disease

utilize similar signaling cascades and require expression of similar genes, the results of this investigation suggest that disruption of function of Ca^{2+}-storage proteins or proteins that permit Ca^{2+} release from the SR/ER, are potential targets for pharmacological intervention to disrupt CaN, NFATc, and MEF2C function in an attempt to treat or prevent cardiomyopathies. By deducing Ca^{2+}-dependent signaling pathways involved in heart development and heart disease it is hoped that heart ailments may one day be prevented, treated, or perhaps even cured.

REFERENCES

REFERENCES

Abbott, K. L., Friday, B. B., Thaloor, D., Murphy, T. J., and Pavlath, G. K. (1998). Activation and cellular localization of the cyclosporine A-sensitive transcription factor NF-AT in skeletal muscle cells. Mol Biol Cell *9*, 2905-2916.

Aitken, A., Cohen, P., Santikarn, S., Williams, D. H., Calder, A. G., Smith, A., and Klee, C. B. (1982). Identification of the NH2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett *150*, 314-318.

Aitken, A., Klee, C. B., and Cohen, P. (1984). The structure of the B subunit of calcineurin. Eur J Biochem *139*, 663-671.

Akazawa, H., and Komuro, I. (2003). Roles of cardiac transcription factors in cardiac hypertrophy. Circ Res *92*, 1079-1088.

Anderson, M. E., Braun, A. P., Wu, Y., Lu, T., Schulman, H., and Sung, R. J. (1998). KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. J Pharmacol Exp Ther *287*, 996-1006.

Andres, V., Cervera, M., and Mahdavi, V. (1995). Determination of the consensus binding site for MEF2 expressed in muscle and brain reveals tissue-specific sequence constraints. J Biol Chem *270*, 23246-23249.

Anglister, J., Grzesiek, S., Wang, A. C., Ren, H., Klee, C. B., and Bax, A. (1994). 1H, 13C, 15N nuclear magnetic resonance backbone assignments and secondary structure of human calcineurin B. Biochemistry *33*, 3540-3547.

Anglister, J., Ren, H., Klee, C. B., and Bax, A. (1995). NMR identification of calcineurin B residues affected by binding of a calcineurin A peptide. FEBS Lett *375*, 108-112.

Aramburu, J., Garcia-Cozar, F., Raghavan, A., Okamura, H., Rao, A., and Hogan, P. G. (1998). Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol Cell *1*, 627-637.

Aramburu, J., Rao, A., and Klee, C. B. (2000). Calcineurin: from structure to function. Curr Top Cell Regul *36*, 237-295.

Aramburu, J., Yaffe, M. B., Lopez-Rodriguez, C., Cantley, L. C., Hogan, P. G., and Rao, A. (1999). Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science *285*, 2129-2133.

Aravind, L., and Koonin, E. V. (2000). SAP - a putative DNA-binding motif involved in chromosomal organization. Trends Biochem Sci *25*, 112-114.

Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H., and Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol *13*, 2235-2246.

Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998). Serum response factor is essential for mesoderm formation during mouse embryogenesis. EMBO J *17*, 6289-6299.

Avram, D., Fields, A., Pretty On Top, K., Nevrivy, D. J., Ishmael, J. E., and Leid, M. (2000). Isolation of a novel family of C(2)H(2) zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors. J Biol Chem *275*, 10315-10322.

Bading, H., Ginty, D. D., and Greenberg, M. E. (1993). Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science *260*, 181-186.

Baksh, S., Burns, K., Andrin, C., and Michalak, M. (1995). Interaction of calreticulin with protein disulfide isomerase. J Biol Chem *270*, 31338-31344.

Baksh, S., and Michalak, M. (1991). Expression of calreticulin in Escherichia coli and identification of its Ca2+ binding domains. J Biol Chem *266*, 21458-21465.

Barford, D. (1996). Molecular mechanisms of the protein serine/threonine phosphatases. Trends Biochem Sci *21*, 407-412.

Bartholomew, C., Kilbey, A., Clark, A. M., and Walker, M. (1997). The Evi-1 proto-oncogene encodes a transcriptional repressor activity associated with transformation. Oncogene *14*, 569-577.

Barton, G. J., Cohen, P. T., and Barford, D. (1994). Conservation analysis and structure prediction of the protein serine/threonine phosphatases. Sequence similarity with diadenosine tetraphosphatase from Escherichia coli suggests homology to the protein phosphatases. Eur J Biochem *220*, 225-237.

Bastianutto, C., Clementi, E., Codazzi, F., Podini, P., De Giorgi, F., Rizzuto, R., Meldolesi, J., and Pozzan, T. (1995). Overexpression of calreticulin increases the Ca2+ capacity of rapidly exchanging Ca2+ stores and reveals aspects of their lumenal microenvironment and function. J Cell Biol *130*, 847-855.

Beals, C. R., Clipstone, N. A., Ho, S. N., and Crabtree, G. R. (1997a). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev *11*, 824-834.

Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997b). Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science *275*, 1930-1934.

Belaguli, N. S., Sepulveda, J. L., Nigam, V., Charron, F., Nemer, M., and Schwartz, R. J. (2000). Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators. Mol Cell Biol *20*, 7550-7558.

Berridge, M. J. (2002). The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium 32*, 235-249.

Berridge, M. J., Bootman, M. D., and Lipp, P. (1998). Calcium--a life and death signal. Nature *395*, 645-648.

Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nature Rev Mol Cell Biol *4*, 517-529.

Berridge, M. J., Lipp, P., and Bootman, M. D. (2000). The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol *1*, 11-21.

Bers, D. M. (2000). Calcium fluxes involved in control of cardiac myocyte contraction. Circ Res *87*, 275-281.

Black, B. L., Molkentin, J. D., and Olson, E. N. (1998). Multiple roles for the MyoD basic region in transmission of transcriptional activation signals and interaction with MEF2. Mol Cell Biol *18*, 69-77.

Black, B. L., and Olson, E. N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol *14*, 167-196.

Blaeser, F., Ho, N., Prywes, R., and Chatila, T. A. (2000). Ca(2+)-dependent gene expression mediated by MEF2 transcription factors. J Biol Chem *275*, 197-209.

Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., and Krebs, E. G. (1985). Identification of the calmodulin-binding domain of skeletal muscle myosin light chain kinase. Proc Natl Acad Sci U S A *82*, 3187-3191.

Bootman, M. D., Collins, T. J., Peppiatt, C. M., Prothero, L. S., MacKenzie, L., De Smet, P., Travers, M., Tovey, S. C., Seo, J. T., Berridge, M. J., *et al.* (2001). Calcium signalling--an overview. Semin Cell Dev Biol *12*, 3-10.

Borghi, S., Molinari, S., Razzini, G., Parise, F., Battini, R., and Ferrari, S. (2001). The nuclear localization domain of the MEF2 family of transcription factors shows member-specific features and mediates the nuclear import of histone deacetylase 4. J Cell Sci *114*, 4477-4483.

Brillantes, A. B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks, A. R. (1994). Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. Cell *77*, 513-523.

Bueno, O. F., Lips, D. J., Kaiser, R. A., Wilkins, B. J., Dai, Y. S., Glascock, B. J., Klevitsky, R., Hewett, T. E., Kimball, T. R., Aronow, B. J., *et al.* (2004). Calcineurin Abeta gene targeting predisposes the myocardium to acute ischemia-induced apoptosis and dysfunction. Circ Res *94*, 91-99.

Bueno, O. F., van Rooij, E., Molkentin, J. D., Doevendans, P. A., and De Windt, L. J. (2002a). Calcineurin and hypertrophic heart disease: novel insights and remaining questions. Cardiovasc Res *53*, 806-821.

Bueno, O. F., Wilkins, B. J., Tymitz, K. M., Glascock, B. J., Kimball, T. F., Lorenz, J. N., and Molkentin, J. D. (2002b). Impaired cardiac hypertrophic response in Calcineurin Abeta -deficient mice. Proc Natl Acad Sci U S A *99*, 4586-4591.

Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C., and Michalak, M. (1994). Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature *367*, 476-480.

Bushdid, P. B., Osinska, H., Waclaw, R. R., Molkentin, J. D., and Yutzey, K. E. (2003). NFATc3 and NFATc4 are required for cardiac development and mitochondrial function. Circ Res *92*, 1305-1313.

Camacho, P., and Lechleiter, J. D. (1995). Calreticulin inhibits repetitive intracellular Ca2+ waves. Cell *82*, 765-771.

Cameron, A. M., Nucifora, F. C., Jr., Fung, E. T., Livingston, D. J., Aldape, R. A., Ross, C. A., and Snyder, S. H. (1997). FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine-proline (1400-1401) and anchors calcineurin to this FK506-like domain. J Biol Chem *272*, 27582-27588.

Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995). Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca2+ flux. Cell *83*, 463-472.

Capdevila, J., Vogan, K. J., Tabin, C. J., and Izpisua Belmonte, J. C. (2000). Mechanisms of left-right determination in vertebrates. Cell *101*, 9-21.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802-805.

Chang, C. D., Mukai, H., Kuno, T., and Tanaka, C. (1994). cDNA cloning of an alternatively spliced isoform of the regulatory subunit of Ca2+/calmodulin-dependent protein phosphatase (calcineurin B alpha 2). Biochim Biophys Acta *1217*, 174-180.

Chang, R. K., Alzona, M., Alejos, J., Jue, K., and McDiarmid, S. V. (1998). Marked left ventricular hypertrophy in children on tacrolimus (FK506) after orthotopic liver transplantation. Am J Cardiol *81*, 1277-1280.

Chen, C. Y., and Schwartz, R. J. (1996). Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. Mol Cell Biol *16*, 6372-6384.

Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002). Myocardin: a component of a molecular switch for smooth muscle differentiation. J Mol Cell Cardiol *34*, 1345-1356.

Chien, K. R. (1999). Stress pathways and heart failure. Cell *98*, 555-558.

Chien, K. R. (2000). Meeting Koch's postulates for calcium signaling in cardiac hypertrophy. J Clin Invest *105*, 1339-1342.

Chin, D., and Means, A. R. (2000). Calmodulin: a prototypical calcium sensor. Trends Cell Biol *10*, 322-328.

Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R., and Williams, R. S. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev *12*, 2499-2509.

Chow, C. W., Dong, C., Flavell, R. A., and Davis, R. J. (2000). c-Jun NH(2)-terminal kinase inhibits targeting of the protein phosphatase calcineurin to NFATc1. Mol Cell Biol *20*, 5227-5234.

Clapham, D. E. (1995). Calcium signaling. Cell *80*, 259-268.

Cohen, P. T., Chen, M. X., and Armstrong, C. G. (1996). Novel protein phosphatases that may participate in cell signaling. Adv Pharmacol *36*, 67-89.

Cooney, A. J., Tsai, S. Y., O'Malley, B. W., and Tsai, M. J. (1992). Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. Mol Cell Biol *12*, 4153-4163.

Coons, A. H. (1951). Fluorescent antibodies as histochemical tools. Fed Proc *10*, 558-559.

Corbett, E. F., Michalak, K. M., Oikawa, K., Johnson, S., Campbell, I. D., Eggleton, P., Kay, C., and Michalak, M. (2000). The conformation of calreticulin is influenced by the endoplasmic reticulum luminal environment. J Biol Chem *275*, 27177-27185.

Corbett, E. F., and Michalak, M. (2000). Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem Sci *25*, 307-311.

Corbett, E. F., Oikawa, K., Francois, P., Tessier, D. C., Kay, C., Bergeron, J. J., Thomas, D. Y., Krause, K. H., and Michalak, M. (1999). Ca2+ regulation of interactions between endoplasmic reticulum chaperones. J Biol Chem *274*, 6203-6211.

Corcoran, E. E., and Means, A. R. (2001). Defining Ca2+/calmodulin-dependent protein kinase cascades in transcriptional regulation. J Biol Chem *276*, 2975-2978.

Cox, D. M., Du, M., Marback, M., Yang, E. C., Chan, J., Siu, K. W., and McDermott, J. C. (2003). Phosphorylation motifs regulating the stability and function of myocyte enhancer factor 2A. J Biol Chem *278*, 15297-15303.

Crabtree, G. R. (1999). Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. Cell *96*, 611-614.

Crabtree, G. R. (2001). Calcium, calcineurin, and the control of transcription. J Biol Chem *276*, 2313-2316.

Crabtree, G. R., and Olson, E. N. (2002). NFAT signaling: choreographing the social lives of cells. Cell *109 Suppl*, S67-79.

Cyert, M. S. (1993). The function of Ca2+/calmodulin-regulated phosphatase in yeast. Adv Protein Phosphatases *7*, 429-443.

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987-1000.

Dawson, T. M., Steiner, J. P., Dawson, V. L., Dinerman, J. L., Uhl, G. R., and Snyder, S. H. (1993). Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proc Natl Acad Sci U S A *90*, 9808-9812.

de la Pompa, J. L., Timmerman, L. A., Takimoto, H., Yoshida, H., Elia, A. J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B. L., Crabtree, G.R., and Mak, T. (1998). Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature *392*, 182-186.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol *7*, 725-737.

Delling, U., Tureckova, J., Lim, H. W., De Windt, L. J., Rotwein, P., and Molkentin, J. D. (2000). A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. Mol Cell Biol *20*, 6600-6611.

Delwel, R., Funabiki, T., Kreider, B. L., Morishita, K., and Ihle, J. N. (1993). Four of the seven zinc fingers of the Evi-1 myeloid-transforming gene are required for sequence-specific binding to GA(C/T)AAGA(T/C)AAGATAA. Mol Cell Biol *13*, 4291-4300.

Ding, B., Price, R. L., Borg, T. K., Weinberg, E. O., Halloran, P. F., and Lorell, B. H. (1999). Pressure overload induces severe hypertrophy in mice treated with cyclosporine, an inhibitor of calcineurin. Circ Res *84*, 729-734.

Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997). Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature *386*, 855-858.

Donella-Deana, A., Krinks, M. H., Ruzzene, M., Klee, C., and Pinna, L. A. (1994). Dephosphorylation of phosphopeptides by calcineurin (protein phosphatase 2B). Eur J Biochem *219*, 109-117.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. Embo J *16*, 5687-5696.

Earnest, S., Khokhlatchev, A., Albanesi, J. P., and Barylko, B. (1996). Phosphorylation of dynamin by ERK2 inhibits the dynamin-microtubule interaction. FEBS Lett *396*, 62-66.

Eckner, R., Yao, T. P., Oldread, E., and Livingston, D. M. (1996). Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. Genes Dev *10*, 2478-2490.

Edmondson, D. G., Lyons, G. E., Martin, J. F., and Olson, E. N. (1994). Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. Development *120*, 1251-1263.

Eggleton, P., and Michalak, M. (2003). Introduction to calreticulin, 2nd edn (Austin, Texas, USA, R.G. Landes Company, Biomedical Publishers).

Ellgaard, L., Riek, R., Braun, D., Herrmann, T., Helenius, A., and Wuthrich, K. (2001a). Three-dimensional structure topology of the calreticulin P-domain based on NMR assignment. FEBS Lett *488*, 69-73.

Ellgaard, L., Riek, R., Herrmann, T., Guntert, P., Braun, D., Helenius, A., and Wuthrich, K. (2001b). NMR structure of the calreticulin P-domain. Proc Natl Acad Sci U S A *98*, 3133-3138.

Ellis, J. J., Valencia, T. G., Zeng, H., Roberts, L. D., Deaton, R. A., and Grant, S. R. (2003). CaM kinase IIdeltaC phosphorylation of 14-3-3beta in vascular smooth muscle cells: activation of class II HDAC repression. Mol Cell Biochem *242*, 153-161.

Evans, S. M., Walsh, B. A., Newton, C. B., Thorburn, J. S., Gardner, P. D., and van Bilsen, M. (1993). Potential role of helix-loop-helix proteins in cardiac gene expression. Circ Res *73*, 569-578.

Fabiato, A. (1985). Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. J Gen Physiol *85*, 247-289.

Faulds, D., Goa, K. L., and Benfield, P. (1993). Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. Drugs *45*, 953-1040.

Fickett, J. W. (1996). Quantitative discrimination of MEF2 sites. Mol Cell Biol *16*, 437-441.

Flanagan, W. M., Corthesy, B., Bram, R. J., and Crabtree, G. R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature *352*, 803-807.

Fliegel, L., Burns, K., MacLennan, D. H., Reithmeier, R. A., and Michalak, M. (1989a). Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J Biol Chem *264*, 21522-21528.

Fliegel, L., Burns, K., Opas, M., and Michalak, M. (1989b). The high-affinity calcium binding protein of sarcoplasmic reticulum. Tissue distribution, and homology with calregulin. Biochim Biophys Acta *982*, 1-8.

Flucher, B. E., and Franzini-Armstrong, C. (1996). Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. Proc Natl Acad Sci U S A *93*, 8101-8106.

Franzini-Armstrong, C., and Jorgensen, A. O. (1994). Structure and development of E-C coupling units in skeletal muscle. Annu Rev Physiol *56*, 509-534.

French, B. A., Chow, K. L., Olson, E. N., and Schwartz, R. J. (1991). Heterodimers of myogenic helix-loop-helix regulatory factors and E12 bind a complex element governing myogenic induction of the avian cardiac alpha-actin promoter. Mol Cell Biol *11*, 2439-2450.

Frey, N., McKinsey, T. A., and Olson, E. N. (2000). Decoding calcium signals involved in cardiac growth and function. Nat Med *6*, 1221-1227.

Frey, N., and Olson, E. N. (2003). Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol *65*, 45-79.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308-311.

Funabiki, T., Kreider, B. L., and Ihle, J. N. (1994). The carboxyl domain of zinc fingers of the Evi-1 myeloid transforming gene binds a consensus sequence of GAAGATGAG. Oncogene *9*, 1575-1581.

Gaestel, M., Benndorf, R., Hayess, K., Priemer, E., and Engel, K. (1992). Dephosphorylation of the small heat shock protein hsp25 by calcium/calmodulin-dependent (type 2B) protein phosphatase. J Biol Chem *267*, 21607-21611.

Gajewski, K., Kim, Y., Choi, C. Y., and Schulz, R. A. (1998). Combinatorial control of Drosophila mef2 gene expression in cardiac and somatic muscle cell lineages. Dev Genes Evol *208*, 382-392.

Garver, T. D., Kincaid, R. L., Conn, R. A., and Billingsley, M. L. (1999). Reduction of calcineurin activity in brain by antisense oligonucleotides leads to persistent phosphorylation of tau protein at Thr181 and Thr231. Mol Pharmacol *55*, 632-641.

Ghosh, A., and Greenberg, M. E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science *268*, 239-247.

Gorlich, D. (1997). Nuclear protein import. Curr Opin Cell Biol *9*, 412-419.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol Cell Biol *9*, 5022-5033.

Goto, S., Matsukado, Y., Mihara, Y., Inoue, N., and Miyamoto, E. (1986). Calcineurin in human brain and its relation to extrapyramidal system. Immunohistochemical study on postmortem human brains. Acta Neuropathol (Berl) *72*, 150-156.

Goto, S., Yamamoto, H., Fukunaga, K., Iwasa, T., Matsukado, Y., and Miyamoto, E. (1985). Dephosphorylation of microtubule-associated protein 2, tau factor, and tubulin by calcineurin. J Neurochem *45*, 276-283.

Goy, J. J., Stauffer, J. C., Deruaz, J. P., Gillard, D., Kaufmann, U., Kuntzer, T., and Kappenberger, L. (1989). Myopathy as possible side-effect of cyclosporin. Lancet *1*, 1446-1447.

Graef, I. A., Chen, F., Chen, L., Kuo, A., and Crabtree, G. R. (2001a). Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell *105*, 863-875.

Graef, I. A., Chen, F., and Crabtree, G. R. (2001b). NFAT signaling in vertebrate development. Curr Opin Genet Dev *11*, 505-512.

Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995). X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. Cell *82*, 507-522.

Gruver, C. L., DeMayo, F., Goldstein, M. A., and Means, A. R. (1993). Targeted developmental overexpression of calmodulin induces proliferative and hypertrophic growth of cardiomyocytes in transgenic mice. Endocrinology *133*, 376-388.

Guerini, D. (1997). Calcineurin: not just a simple protein phosphatase. Biochem Biophys Res Commun *235*, 271-275.

Guerini, D., and Klee, C. B. (1989). Cloning of human calcineurin A: evidence for two isozymes and identification of a polyproline structural domain. Proc Natl Acad Sci U S A *86*, 9183-9187.

Guerini, D., Krinks, M. H., Sikela, J. M., Hahn, W. E., and Klee, C. B. (1989). Isolation and sequence of a cDNA clone for human calcineurin B, the Ca2+-binding subunit of the Ca2+/calmodulin-stimulated protein phosphatase. DNA *8*, 675-682.

Guerini, D., Montell, C., and Klee, C. B. (1992). Molecular cloning and characterization of the genes encoding the two subunits of Drosophila melanogaster calcineurin. J Biol Chem *267*, 22542-22549.

Guo, L., Lynch, J., Nakamura, K., Fliegel, L., Kasahara, H., Izumo, S., Komuro, I., Agellon, L. B., and Michalak, M. (2001). COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene during cardiac development. J Biol Chem *276*, 2797-2801.

Guo, L., Nakamura, K., Lynch, J., Opas, M., Olson, E. N., Agellon, L. B., and Michalak, M. (2002). Cardiac-specific expression of calcineurin reverses embryonic lethality in calreticulin-deficient mouse. J Biol Chem *277*, 50776-50779.

Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997). Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. Nature *386*, 296-299.

Haq, S., Choukroun, G., Lim, H., Tymitz, K. M., del Monte, F., Gwathmey, J., Grazette, L., Michael, A., Hajjar, R., Force, T., and Molkentin, J. D. (2001). Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. Circulation *103*, 670-677.

Harvey, R. P. (1996). NK-2 homeobox genes and heart development. Dev Biol *178*, 203-216.

Hashimoto, Y., King, M. M., and Soderling, T. R. (1988). Regulatory interactions of calmodulin-binding proteins: phosphorylation of calcineurin by autophosphorylated Ca2+/calmodulin-dependent protein kinase II. Proc Natl Acad Sci U S A *85*, 7001-7005.

Hashimoto, Y., and Soderling, T. R. (1989). Regulation of calcineurin by phosphorylation. Identification of the regulatory site phosphorylated by Ca2+/calmodulin-dependent protein kinase II and protein kinase C. J Biol Chem *264*, 16524-16529.

Heikinheimo, M., Scandrett, J. M., and Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. Dev Biol *164*, 361-373.

Helenius, A., Trombetta, E., Hebert, D., and Simons, J. (1997). Calnexin, calreticulin and the folding of glycoproteins. Trends in Cell Biology *7*, 193-200.

Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993). Effects of mutant rat dynamin on endocytosis. J Cell Biol *122*, 565-578.

Ho, S. N., Thomas, D. J., Timmerman, L. A., Li, X., Francke, U., and Crabtree, G. R. (1995). NFATc3, a lymphoid-specific NFATc family member that is calcium-regulated and exhibits distinct DNA binding specificity. J Biol Chem *270*, 19898-19907.

Holaska, J. M., Black, B. E., Love, D. C., Hanover, J. A., Leszyk, J., and Paschal, B. M. (2001). Calreticulin Is a receptor for nuclear export. J Cell Biol *152*, 127-140.

Hopwood, N. D., Pluck, A., and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. Embo J *8*, 3409-3417.

Horsley, V., and Pavlath, G. K. (2002). NFAT: ubiquitous regulator of cell differentiation and adaptation. J Cell Biol *156*, 771-774.

Hoyt, P. R., Bartholomew, C., Davis, A. J., Yutzey, K., Gamer, L. W., Potter, S. S., Ihle, J. N., and Mucenski, M. L. (1997). The Evi1 proto-oncogene is required at midgestation for neural, heart, and paraxial mesenchyme development. Mech Dev *65*, 55-70.

Hubbard, M. J., and Klee, C. B. (1989). Functional domain structure of calcineurin A: mapping by limited proteolysis. Biochemistry *28*, 1868-1874.

Hunter, J. J., and Chien, K. R. (1999). Signaling pathways for cardiac hypertrophy and failure. N Engl J Med *341*, 1276-1283.

Imanaka-Yoshida, K., Amitani, A., Ioshii, S. O., Koyabu, S., Yamakado, T., and Yoshida, T. (1996). Alterations of expression and distribution of the Ca(2+)-storing proteins in endo/sarcoplasmic reticulum during differentiation of rat cardiomyocytes. J Mol Cell Cardiol *28*, 553-562.

Imredy, J. P., and Yue, D. T. (1994). Mechanism of Ca(2+)-sensitive inactivation of L-type Ca2+ channels. Neuron *12*, 1301-1318.

Irons, C. E., Sei, C. A., Hidaka, H., and Glembotski, C. C. (1992). Protein kinase C and calmodulin kinase are required for endothelin-stimulated atrial natriuretic factor secretion from primary atrial myocytes. J Biol Chem *267*, 5211-5216.

Jaconi, M., Bony, C., Richards, S. M., Terzic, A., Arnaudeau, S., Vassort, G., and Puceat, M. (2000). Inositol 1,4,5-trisphosphate directs Ca(2+) flow between mitochondria and the Endoplasmic/Sarcoplasmic reticulum: a role in regulating cardiac autonomic Ca(2+) spiking. Mol Biol Cell *11*, 1845-1858.

Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature *365*, 352-355.

Janknecht, R., and Hunter, T. (1996). Versatile molecular glue. Transcriptional control. Curr Biol *6*, 951-954.

Janssen, P. M., Zeitz, O., Keweloh, B., Siegel, U., Maier, L. S., Barckhausen, P., Pieske, B., Prestle, J., Lehnart, S. E., and Hasenfuss, G. (2000). Influence of cyclosporine A on contractile function, calcium handling, and energetics in isolated human and rabbit myocardium. Cardiovasc Res *47*, 99-107.

John, L. M., Lechleiter, J. D., and Camacho, P. (1998). Differential modulation of SERCA2 isoforms by calreticulin. J Cell Biol *142*, 963-973.

Johnson, S., Michalak, M., Opas, M., and Eggleton, P. (2001). The ins and outs of calreticulin: from the ER lumen to the extracellular space. Trends Cell Biol *11*, 122-129.

Kaibori, M., Okumura, T., Ito, S., Oda, M., Inoue, T., and Kamiyama, Y. (1999). Inhibition of iNOS induction by FK506, but not by cyclosporine, in rat hepatocytes. Transplant Proc *31*, 804-805.

Kakalis, L. T., Kennedy, M., Sikkink, R., Rusnak, F., and Armitage, I. M. (1995). Characterization of the calcium-binding sites of calcineurin B. FEBS Lett *362*, 55-58.

Kasahara, H., and Izumo, S. (1999). Identification of the in vivo casein kinase II phosphorylation site within the homeodomain of the cardiac tisue-specifying homeobox gene product Csx/Nkx2.5. Mol Cell Biol *19*, 526-536.

Katz, A. M. (1997). Molecular biology of calcium channels in the cardiovascular system. Am J Cardiol *80*, 17I-22I.

Kayyali, U. S., Zhang, W., Yee, A. G., Seidman, J. G., and Potter, H. (1997). Cytoskeletal changes in the brains of mice lacking calcineurin A alpha. J Neurochem *68*, 1668-1678.

Kelley, C., Blumberg, H., Zon, L. I., and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. Development *118*, 817-827.

Kennedy, M. T., Brockman, H., and Rusnak, F. (1996). Contributions of myristoylation to calcineurin structure/function. J Biol Chem *271*, 26517-26521.

Kim, S. J., Ding, W., Albrecht, B., Green, P. L., and Lairmore, M. D. (2003). A conserved calcineurin-binding motif in human T lymphotropic virus type 1 p12I functions to modulate nuclear factor of activated T cell activation. J Biol Chem *278*, 15550-15557.

Kimura, Y., Asahi, M., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1998). Phospholamban domain Ib mutations influence functional interactions with the Ca2+-ATPase isoform of cardiac sarcoplasmic reticulum. J Biol Chem *273*, 14238-14241.

Kincaid, R. L., and Vaughan, M. (1986). Direct comparison of Ca2+ requirements for calmodulin interaction with and activation of protein phosphatase. Proc Natl Acad Sci U S A *83*, 1193-1197.

Kirchhefer, U., Schmitz, W., Scholz, H., and Neumann, J. (1999). Activity of cAMP-dependent protein kinase and Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human hearts. Cardiovasc Res *42*, 254-261.

Kiriazis, H., and Kranias, E. G. (2000). Genetically engineered models with alterations in cardiac membrane calcium-handling proteins. Annu Rev Physiol *62*, 321-351.

Kissinger, C. R., Parge, H. E., Knighton, D. R., Lewis, C. T., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, R. E., Moomaw, E. W., Gastinel, L., Habuka, N., Chen, X., Maldonado, F., Barker, J., Bacquet, R., and Villafranca, J.E. (1995). Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. Nature *378*, 641-644.

Klee, C. B., Crouch, T. H., and Krinks, M. H. (1979). Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc Natl Acad Sci U S A *76*, 6270-6273.

Klee, C. B., Draetta, G. F., and Hubbard, M. J. (1988). Calcineurin. Adv Enzymol Relat Areas Mol Biol *61*, 149-200.

Klee, C. B., Ren, H., and Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. Journal of Biological Chemistry *273*, 13367-13370.

Koide, M., Obata, K., Iio, A., Iida, M., Harayama, H., Yokota, M., and Tuan, R. S. (1997). Function of FK506 binding protein (FKBP) in chick embryonic cardiac development. Heart Vessels *Suppl 12*, 7-9.

Kolodziejczyk, S. M., et al., (1999). MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. Curr Biol *9*, 1203-1206.

Komuro, I., and Izumo, S. (1993). Csx: a murine homeobox-containing gene specifically expressed in the developing heart. Proc Natl Acad Sci U S A *90*, 8145-8149.

Koonin, E. V. (1994). Conserved sequence pattern in a wide variety of phosphoesterases. Protein Sci *3*, 356-358.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., and Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. Development *126*, 723-732.

Krause, K. H., and Michalak, M. (1997). Calreticulin. Cell *88*, 439-443.

Kreider, B. L., Orkin, S. H., and Ihle, J. N. (1993). Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. Proc Natl Acad Sci U S A *90*, 6454-6458.

Kuno, T., Mukai, H., Ito, A., Chang, C. D., Kishima, K., Saito, N., and Tanaka, C. (1992). Distinct cellular expression of calcineurin A alpha and A beta in rat brain. J Neurochem *58*, 1643-1651.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev *11*, 1048-1060.

Leach, M., Cohen-Doyle, M., Thomas, D., and Williams, D. (2002). Localization of the lectin, ERp57 binding, polypeptide binding sites of calnexin and calreticulin. J Biol Chem *277*, 29686-29697.

Lee, K. C. L., and Kraus, W. L. (2001). Nuclear receptors, coactivators and chromatin: new approaches, new insights. Trends in Endocrinology and Metabolism *12*, 191-197.

Lee, Y., Shioi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E., and Izumo, S. (1998). The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. Mol Cell Biol *18*, 3120-3129.

Li, J., Puceat, M., Perez-Terzic, C., Mery, A., Nakamura, K., Michalak, M., Krause, K. H., and Jaconi, M. E. (2002). Calreticulin reveals a critical Ca(2+) checkpoint in cardiac myofibrillogenesis. J Cell Biol *158*, 103-113.

Li, S., Wang, D. Z., Wang, Z., Richardson, J. A., and Olson, E. N. (2003). The serum response factor coactivator myocardin is required for vascular smooth muscle development. Proc Natl Acad Sci U S A *100*, 9366-9370.

Liang, Q., De Windt, L. J., Witt, S. A., Kimball, T. R., Markham, B. E., and Molkentin, J. D. (2001). The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. J Biol Chem *276*, 30245-30253.

Lieberman, D. N., and Mody, I. (1994). Regulation of NMDA channel function by endogenous Ca(2+)-dependent phosphatase. Nature *369*, 235-239.

Lim, H. W., and Molkentin, J. D. (1999). Calcineurin and human heart failure. Nat Med *5*, 246-247.

Lim, H. W., and Molkentin, J. D. (2000). Reply to revisiting calcineurin and human heart failure. Nat Med *6*, 3.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science *276*, 1404-1407.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development *119*, 419-431.

Lipkin, G. W., Tucker, B., Giles, M., and Raine, A. E. (1993). Ambulatory blood pressure and left ventricular mass in cyclosporin- and non-cyclosporin-treated renal transplant recipients. J Hypertens *11*, 439-442.

Lipp, P., Laine, M., Tovey, S. C., Burrell, K. M., Berridge, M. J., Li, W., and Bootman, M. D. (2000). Functional InsP3 receptors that may modulate excitation-contraction coupling in the heart. Curr Biol *10*, 939-942.

Lisman, J. (1994). The CaM kinase II hypothesis for the storage of synaptic memory. Trends Neurosci *17*, 406-412.

Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807-815.

Liu, J. P., Sim, A. T., and Robinson, P. J. (1994). Calcineurin inhibition of dynamin I GTPase activity coupled to nerve terminal depolarization. Science *265*, 970-973.

Lo, A., and Alloway, R. R. (2002). Strategies to reduce toxicities and improve outcomes in renal transplant recipients. Pharmacotherapy *22*, 316-328.

Lodish, H. F., Kong, N., and Wikstrom, L. (1992). Calcium is required for folding of newly made subunits of the asialoglycoprotein receptor within the endoplasmic reticulum. J Biol Chem *267*, 12753-12760.

Lohse, D. L., Denu, J. M., and Dixon, J. E. (1995). Insights derived from the structures of the Ser/Thr phosphatases calcineurin and protein phosphatase 1. Structure *3*, 987-990.

Lu, J., McKinsey, T. A., Nicol, R. L., and Olson, E. N. (2000). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proc Natl Acad Sci U S A *97*, 4070-4075.

Luo, Z., Shyu, K. G., Gualberto, A., and Walsh, K. (1998). Calcineurin inhibitors and cardiac hypertrophy. Nat Med *4*, 1092-1093.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev *9*, 1654-1666.

Lyons, G. E., Schiaffino, S., Sassoon, P., Barton, P., and Buckingham, M. (1990). Developmental regulation of myosin gene expression in mouse cardiac muscle. J Cell Biol *111*, 2427-36.

MacLennan, D. H. (2000). Ca2+ signalling and muscle disease. Eur J Biochem *267*, 5291-5297.

Maier, L. S., Zhang, T., Chen, L., DeSantiago, J., Brown, J. H., and Bers, D. M. (2003). Transgenic CaMKIIdeltaC overexpression uniquely alters cardiac myocyte Ca2+ handling: reduced SR Ca2+ load and activated SR Ca2+ release. Circ Res *92*, 904-911.

Mao, Z., and Wiedmann, M. (1999). Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. J Biol Chem *274*, 31102-31107.

Martensen, T. M., Martin, B. M., and Kincaid, R. L. (1989). Identification of the site on calcineurin phosphorylated by Ca2+/CaM-dependent kinase II: modification of the CaM-binding domain. Biochemistry *28*, 9243-9247.

Masuda, E. S., Naito, Y., Tokumitsu, H., Campbell, D., Saito, F., Hannum, C., Arai, K., and Arai, N. (1995). NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. Mol Cell Biol *15*, 2697-2706.

Matsugi, T., Morishita, K., and Ihle, J. N. (1990). Identification, nuclear localization, and DNA-binding activity of the zinc finger protein encoded by the Evi-1 myeloid transforming gene. Mol Cell Biol *10*, 1259-1264.

McDermott, J. C., Cardoso, M. C., Yu, Y. T., Andres, V., Leifer, D., Krainc, D., Lipton, S. A., and Nadal-Ginard, B. (1993). hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. Mol Cell Biol *13*, 2564-2577.

McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2000). Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proc Natl Acad Sci U S A *97*, 14400-14405.

McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001a). Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev *11*, 497-504.

McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001b). Identification of a signal-responsive nuclear export sequence in class II histone deacetylases. Mol Cell Biol *21*, 6312-6321.

Means, A. R. (2000). Regulatory cascades involving calmodulin-dependent protein kinases. Mol Endocrinol *14*, 4-13.

Meldolesi, J., and Pozzan, T. (1998). The endoplasmic reticulum Ca2+ store: a view from the lumen. Trends Biochem Sci *23*, 10-14.

Mertz, P., Yu, L., Sikkink, R., and Rusnak, F. (1997). Kinetic and spectroscopic analyses of mutants of a conserved histidine in the metallophosphatases calcineurin and lambda protein phosphatase. J Biol Chem *272*, 21296-21302.

Mery, L., Mesaeli, N., Michalak, M., Opas, M., Lew, D. P., and Krause, K. H. (1996). Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx. J Biol Chem *271*, 9332-9339.

Mesaeli, N., Nakamura, K., Opas, M., and Michalak, M. (2001). Endoplasmic reticulum in the heart, a forgotten organelle? Mol Cell Biochem *225*, 1-6.

Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K. H., Opas, M., MacLennan, D. H., and Michalak, M. (1999). Calreticulin is essential for cardiac development. J Cell Biol *144*, 857-868.

Mesaeli, N., and Phillipson, C. (2004). Impaired p53 expression, function and nuclear localization in calreticulin deficient cells. Mol Biol Cell *15*, 1862-1870.

Miano, J. M. (2003). Serum response factor: toggling between disparate programs of gene expression. J Mol Cell Cardiol *35*, 577-593.

Michalak, M., Corbett, E. F., Mesaeli, N., Nakamura, K., and Opas, M. (1999). Calreticulin: one protein, one gene, many functions. Biochem J *344 Pt 2*, 281-292.

Michalak, M., Fu, S. Y., Milner, R. E., Busaan, J. L., and Hance, J. E. (1996). Phosphorylation of the carboxyl-terminal region of dystrophin. Biochem Cell Biol *74*, 431-437.

Michalak, M., Mariani, P., and Opas, M. (1998). Calreticulin, a multifunctional Ca2+ binding chaperone of the endoplasmic reticulum. Biochem Cell Biol *76*, 779-785.

Michalak, M., Milner, R. E., Burns, K., and Opas, M. (1992). Calreticulin. Biochem J *285 (Pt 3)*, 681-692.

Michalak, M., Robert Parker, J. M., and Opas, M. (2002). Ca(2+) signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium *32*, 269-278.

Mijares, A., Malecot, C. O., Peineau, N., and Argibay, J. A. (1997). In vivo and in vitro inhibition of the L-type calcium current in isolated guinea-pig cardiomyocytes by the immunosuppressive agent cyclosporin A. J Mol Cell Cardiol *29*, 2067-2076.

Milan, D., Griffith, J., Su, M., Price, E. R., and McKeon, F. (1994). The latch region of calcineurin B is involved in both immunosuppressant-immunophilin complex docking and phosphatase activation. Cell *79*, 437-447.

Milner, R. E., Baksh, S., Shemanko, C., Carpenter, M. R., Smillie, L., Vance, J. E., Opas, M., and Michalak, M. (1991). Calreticulin, and not calsequestrin, is the major calcium binding protein of smooth muscle sarcoplasmic reticulum and liver endoplasmic reticulum. J Biol Chem *266*, 7155-7165.

Minami, H., Inoue, S., and Hidaka, H. (1994). The effect of KN-62, Ca2+/calmodulin dependent protein kinase II inhibitor on cell cycle. Biochem Biophys Res Commun *199*, 241-248.

Minamisawa, S., Hoshijima, M., Chu, G., Ward, C. A., Frank, K., Gu, Y., Martone, M. E., Wang, Y., Ross, J., Jr., Kranias, E. G., W.R. Giles, and Chien, K.R. (1999). Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell *99*, 313-322.

Miska, E. A., Karlsson, C., Langley, E., Nielsen, S. J., Pines, J., and Kouzarides, T. (1999). HDAC4 deacetylase associates with and represses the MEF2 transcription factor. Embo J *18*, 5099-5107.

Molkentin, J. D. (1998). Calcineurin inhibition and cardiac hypertrophy. Science *282*, 1007a.

Molkentin, J. D. (2000a). Calcineurin and beyond: cardiac hypertrophic signaling. Circ Res *87*, 731-738.

Molkentin, J. D. (2000b). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J Biol Chem *275*, 38949-38952.

Molkentin, J. D., Antos, C., Mercer, B., Taigen, T., Miano, J. M., and Olson, E. N. (2000a). Direct activation of a GATA6 cardiac enhancer by Nkx2.5: evidence for a reinforcing regulatory network of Nkx2.5 and GATA transcription factors in the developing heart. Dev Biol *217*, 301-309.

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1996a). Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. Mol Cell Biol *16*, 2627-2636.

Molkentin, J. D., and Dorn, I. G., 2nd (2001). Cytoplasmic signaling pathways that regulate cardiac hypertrophy. Annu Rev Physiol *63*, 391-426.

Molkentin, J. D., Li, L., and Olson, E. N. (1996b). Phosphorylation of the MADS-Box transcription factor MEF2C enhances its DNA binding activity. J Biol Chem *271*, 17199-17204.

Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev *11*, 1061-1072.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell *93*, 215-228.

Molkentin, J. D., Tymitz, K. M., Richardson, J. A., and Olson, E. N. (2000b). Abnormalities of the genitourinary tract in female mice lacking GATA5. Mol Cell Biol *20*, 5256-5260.

Montoro, R. J., Diaz-Nido, J., Avila, J., and Lopez-Barneo, J. (1993). N-methyl-D-aspartate stimulates the dephosphorylation of the microtubule-associated protein 2 and potentiates excitatory synaptic pathways in the rat hippocampus. Neuroscience *54*, 859-871.

Moor, A. N., and Fliegel, L. (1999). Protein kinase-mediated regulation of the Na(+)/H(+) exchanger in the rat myocardium by mitogen-activated protein kinase-dependent pathways. J Biol Chem *274*, 22985-22992.

Moor, A. N., Gan, X. T., Karmazyn, M., and Fliegel, L. (2001). Activation of Na+/H+ exchanger-directed protein kinases in the ischemic and ischemic-reperfused rat myocardium. J Biol Chem *276*, 16113-16122.

Morin, S., Charron, F., Robitaille, L., and Nemer, M. (2000). GATA-dependent recruitment of MEF2 proteins to target promoters. Embo J *19*, 2046-2055.

Morin, S., Paradis, P., Aries, A., and Nemer, M. (2001). Serum response factor-GATA ternary complex required for nuclear signaling by a G-protein-coupled receptor. Mol Cell Biol *21*, 1036-1044.

Morishita, K., Parganas, E., Douglass, E. C., and Ihle, J. N. (1990). Unique expression of the human Evi-1 gene in an endometrial carcinoma cell line: sequence of cDNAs and structure of alternatively spliced transcripts. Oncogene *5*, 963-971.

Morishita, K., Parker, D. S., Mucenski, M. L., Jenkins, N. A., Copeland, N. G., and Ihle, J. N. (1988). Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. Cell *54*, 831-840.

Morishita, K., Suzukawa, K., Taki, T., Ihle, J. N., and Yokota, J. (1995). EVI-1 zinc finger protein works as a transcriptional activator via binding to a consensus sequence of GACAAGATAAGATAAN1-28 CTCATCTTC. Oncogene *10*, 1961-1967.

Moriya, M., Fujinaga, K., Yazawa, M., and Katagiri, C. (1995). Immunohistochemical localization of the calcium/calmodulin-dependent protein phosphatase, calcineurin, in the mouse testis: its unique accumulation in spermatid nuclei. Cell Tissue Res *281*, 273-281.

Morris, T., DeLorenzo, R., and Tombes, R. (1998). CaMK-II inhibition reduces cyclin D1 levels and enhances the association of p27kip1 with Cdk2 to cause G1 arrest in NIH 3T3 cells. Experimental Cell Research *240*, 218-227.

Mukai, H., Chang, C. D., Tanaka, H., Ito, A., Kuno, T., and Tanaka, C. (1991). cDNA cloning of a novel testis-specific calcineurin B-like protein. Biochem Biophys Res Commun *179*, 1325-1330.

Muscat, G. E., and Dressel, U. (2000). Not a minute to waste. Nat Med *6*, 1216-1217.

Nakamura, K., Robertson, M., Liu, G., Dickie, P., Guo, J. Q., Duff, H. J., Opas, M., Kavanagh, K., and Michalak, M. (2001a). Complete heart block and sudden death in mice overexpressing calreticulin. J Clin Invest *107*, 1245-1253.

Nakamura, K., Zuppini, A., Arnaudeau, S., Lynch, J., Ahsan, I., Krause, R., Papp, S., De Smedt, H., Parys, J. B., Muller-Esterl, W., Lew, D., Krause, K., Demaurex, N., Opas, M., and Michalak, M. (2001b). Functional specialization of calreticulin domains. J Cell Biol *154*, 961-972.

Narita, N., Heikinheimo, M., Bielinska, M., White, R. A., and Wilson, D. B. (1996). The gene for transcription factor GATA-6 resides on mouse chromosome 18 and is expressed in myocardium and vascular smooth muscle. Genomics *36*, 345-348.

Naya, F. J., Wu, C., Richardson, J. A., Overbeek, P., and Olson, E. N. (1999). Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. Development *126*, 2045-2052.

Northrop, J. P., Ho, S. N., Chen, L., Thomas, D. J., Timmerman, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994). NF-AT components define a family of transcription factors targeted in T-cell activation. Nature *369*, 497-502.

Nucifora, G. (1997). The EVI1 gene in myeloid leukemia. Leukemia *11*, 2022-2031.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J. P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P. G., and Rao, A. (2000). Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol Cell *6*, 539-550.

Okazaki, K., Ishikawa, T., Inui, M., Tada, M., Goshima, K., Okamoto, T., and Hidaka, H. (1994). KN-62, a specific Ca++/calmodulin-dependent protein kinase inhibitor, reversibly depresses the rate of beating of cultured fetal mouse cardiac myocytes. J Pharmacol Exp Ther *270*, 1319-1324.

O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., and O'Neill, E. A. (1992). FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature *357*, 692-694.

Olson, E. N., and Molkentin, J. D. (1999). Prevention of cardiac hypertrophy by calcineurin inhibition: hope or hype? Circ Res *84*, 623-632.

Olson, E. N., and Williams, R. S. (2000a). Calcineurin signaling and muscle remodeling. Cell *101*, 689-692.

Olson, E. N., and Williams, R. S. (2000b). Remodeling muscles with calcineurin. Bioessays *22*, 510-519.

Opas, M., Szewczenko-Pawlikowski, M., Jass, G. K., Mesaeli, N., and Michalak, M. (1996). Calreticulin modulates cell adhesiveness via regulation of vinculin expression. J Cell Biol *135*, 1913-1923.

Orlando, V. (2000). Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci *25*, 99-104.

Ornatsky, O. I., and McDermott, J. C. (1996). MEF2 protein expression, DNA binding specificity and complex composition, and transcriptional activity in muscle and non-muscle cells. J Biol Chem *271*, 24927-24933.

Oukka, M., Ho, I. C., de la Brousse, F. C., Hoey, T., Grusby, M. J., and Glimcher, L. H. (1998). The transcription factor NFAT4 is involved in the generation and survival of T cells. Immunity *9*, 295-304.

Papp, S., Dziak, E., Michalak, M., and Opas, M. (2003). Is all of the endoplasmic reticulum created equal? The effects of the heterogeneous distribution of endoplasmic reticulum Ca2+-handling proteins. J Cell Biol *160*, 475-479.

Parlakian, A., Tuil, D., Hamard, G., Tavernier, G., Hentzen, D., Concordet, J. P., Paulin, D., Li, Z., and Daegelen, D. (2004). Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality. Mol Cell Biol *24*, 5281-5289.

Parmer, T. G., Ward, M. D., Yurkow, E. J., Vyas, V. H., Kearney, T. J., and Hait, W. N. (1999). Activity and regulation by growth factors of calmodulin-dependent protein kinase III (elongation factor 2-kinase) in human breast cancer. Br J Cancer *79*, 59-64.

Passier, R., Zeng, H., Frey, N., Naya, F. J., Nicol, R. L., McKinsey, T. A., Overbeek, P., Richardson, J. A., Grant, S. R., and Olson, E. N. (2000). CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest *105*, 1395-1406.

Perkins, A. S., Mercer, J. A., Jenkins, N. A., and Copeland, N. G. (1991). Patterns of Evi-1 expression in embryonic and adult tissues suggest that Evi-1 plays an important regulatory role in mouse development. Development *111*, 479-487.

Perrino, B. A., Fong, Y. L., Brickey, D. A., Saitoh, Y., Ushio, Y., Fukunaga, K., Miyamoto, E., and Soderling, T. R. (1992). Characterization of the phosphatase activity of a baculovirus-expressed calcineurin A isoform. J Biol Chem *267*, 15965-15969.

Philipson, K. D., and Nicoll, D. A. (2000). Sodium-calcium exchange: a molecular perspective. Annu Rev Physiol *62*, 111-133.

Pollock, R., and Treisman, R. (1991). Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev *5*, 2327-2341.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992). Primary structure of the Aequorea victoria green-fluorescent protein. Gene *111*, 229-233.

Pujol, M. J., Bosser, R., Vendrell, M., Serratosa, J., and Bachs, O. (1993). Nuclear calmodulin-binding proteins in rat neurons. J Neurochem *60*, 1422-1428.

Putney, J. W., Jr. (2001). Cell biology. Channelling calcium. Nature *410*, 648-649.

Qiu, Y., Pereira, F. A., DeMayo, F. J., Lydon, J. P., Tsai, S. Y., and Tsai, M. J. (1997). Null mutation of mCOUP-TFI results in defects in morphogenesis of the glossopharyngeal ganglion, axonal projection, and arborization. Genes Dev *11*, 1925-1937.

Ranger, A. M., Grusby, M. J., Hodge, M. R., Gravallese, E. M., de la Brousse, F. C., Hoey, T., Mickanin, C., Baldwin, H. S., and Glimcher, L. H. (1998). The transcription factor NF-ATc is essential for cardiac valve formation. Nature *392*, 186-190.

Rao, A., Luo, C., and Hogan, P. G. (1997). Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol *15*, 707-747.

Rasmussen, G., and Rasmussen, C. (1995). Calmodulin-dependent protein kinase II is required for G1/S progression in HeLa cells. Biochem Cell Biol *73*, 201-207.

Rusnak, F., and Mertz, P. (2000). Calcineurin: form and function. Physiol Rev *80*, 1483-1521.

Ryazanov, A. G., Ward, M. D., Mendola, C. E., Pavur, K. S., Dorovkov, M. V., Wiedmann, M., Erdjument-Bromage, H., Tempst, P., Parmer, T. G., Prostko, C. R., Germino, F.J., and Hait, W. (1997). Identification of a new class of protein kinases represented by eukaryotic elongation factor-2 kinase. Proc Natl Acad Sci U S A *94*, 4884-4889.

Saito, T., Fukuzawa, J., Osaki, J., Sakuragi, H., Yao, N., Haneda, T., Fujino, T., Wakamiya, N., Kikuchi, K., and Hasebe, N. (2003). Roles of calcineurin and calcium/calmodulin-dependent protein kinase II in pressure overload-induced cardiac hypertrophy. J Mol Cell Cardiol *35*, 1153-1160.

Santiago-Garcia, J., Mas-Oliva, J., Saavedra, D., and Zarain-Herzberg, A. (1996). Analysis of mRNA expression and cloning of a novel plasma membrane Ca(2+)-ATPase splice variant in human heart. Mol Cell Biochem *155*, 173-182.

Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. (1997). Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. Mol Cell Biol *17*, 1010-1026.

Sartorelli, V., Webster, K. A., and Kedes, L. (1990). Muscle-specific expression of the cardiac alpha-actin gene requires MyoD1, CArG-box binding factor, and Sp1. Genes Dev *4*, 1811-1822.

Sassoon, D., Garner, I., and Buckingham, M. (1988). Transcripts of alpha-cardiac and alpha-skeletal actins are early markers for myogenesis in the mouse embryo. Development *104*, 155-64.

Schaeffer, P. J., Wende, A. R., Magee, C. J., Neilson, J. R., Leone, T. C., Chen, F., and Kelly, D. P. (2004). Calcineurin and calcium/calmodulin dependent protein kinase activate distinct metabolic gene regulatory programs in cardiac muscle. J Biol Chem *279*, 39593-39603.

Schott, J. J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E., and Seidman, J. G. (1998). Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science *281*, 108-111.

Schultheiss, T. M., Xydas, S., and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. Development *121*, 4203-4214.

Schwartz, R. J., and Olson, E. N. (1999). Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. Development *126*, 4187-4192.

Scott, D. K., Mitchell, J. A., and Granner, D. K. (1996). The orphan receptor COUP-TF binds to a third glucocorticoid accessory factor element within the phosphoenolpyruvate carboxykinase gene promoter. J Biol Chem *271*, 31909-31914.

Searcy, R. D., Vincent, E. B., Liberatore, C. M., and Yutzey, K. E. (1998). A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. Development *125*, 4461-4470.

Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427-1430.

Shibasaki, F., Hallin, U., and Uchino, H. (2002). Calcineurin as a multifunctional regulator. J Biochem (Tokyo) *131*, 1-15.

Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996). Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature *382*, 370-373.

Shigekawa, M., and Iwamoto, T. (2001). Cardiac Na(+)-Ca(2+) exchange: molecular and pharmacological aspects. Circ Res *88*, 864-876.

Shore, P., and Sharrocks, A. D. (1995). The MADS-box family of transcription factors. Eur J Biochem *229*, 1-13.

Shull, G. E. (2000). Gene knockout studies of Ca2+-transporting ATPases. Eur J Biochem *267*, 5284-5290.

Simmerman, H. K., Collins, J. H., Theibert, J. L., Wegener, A. D., and Jones, L. R. (1986). Sequence analysis of phospholamban. Identification of phosphorylation sites and two major structural domains. J Biol Chem *261*, 13333-13341.

Singh, T. J., and Wang, J. H. (1987). Phosphorylation of calcineurin by glycogen synthase (casein) kinase-1. Biochem Cell Biol *65*, 917-921.

Skerjanc, I. S., and McBurney, M. W. (1994). The E box is essential for activity of the cardiac actin promoter in skeletal but not in cardiac muscle. Dev Biol *163*, 125-132.

Skerjanc, I. S., Petropoulos, H., Ridgeway, A. G., and Wilton, S. (1998). Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. J Biol Chem *273*, 34904-34910.

Small, E. M., and Krieg, P. A. (2003). Transgenic analysis of the atrialnatriuretic factor (ANF) promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of ANF. Dev Biol *261*, 116-131.

Smith, M., and Koch, G. (1989). Multiple zones in the sequence of calreticulin (CRP55, calregulin, HACBP), a major calcium binding ER/SR protein. EMBO J. *8*, 3581-3586.

Smirnov, D. A., Hou, S., and Ricciardi, R. P. (2000). Association of histone deacetylase with COUP-TF in tumorigenic Ad12-transformed cells and its potential role in shut-off of MHC class I transcription. Virology *268*, 319-328.

Spector, D., Goldman, R. Leinwand, L. (1998). Cells: A Laboratory Manual. Volume 3: Subcellular Localization of Genes and Their Products. (New York: Cold Springs Harbour Laboratory Press).

Srivastava, D., and Olson, E. N. (2000). A genetic blueprint for cardiac development. Nature *407*, 221-226.

Stemmer, P. M., and Klee, C. B. (1994). Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. Biochemistry *33*, 6859-6866.

Struhl, K. (1987). Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell *49*, 295-297.

Sugden, P. H. (1999). Signaling in myocardial hypertrophy: life after calcineurin? Circ Res *84*, 633-646.

Sugimoto, T., Stewart, S., and Guan, K. L. (1997). The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. J Biol Chem *272*, 29415-29418.

Sugiura, R., Toda, T., Shuntoh, H., Yanagida, M., and Kuno, T. (1998). pmp1+, a suppressor of calcineurin deficiency, encodes a novel MAP kinase phosphatase in fission yeast. Embo J *17*, 140-148.

Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., and Hidaka, H. (1991). The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem Biophys Res Commun *181*, 968-975.

Sussman, M. A., Lim, H. W., Gude, N., Taigen, T., Olson, E. N., Robbins, J., Colbert, M. C., Gualberto, A., Wieczorek, D. F., and Molkentin, J. D. (1998). Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science *281*, 1690-1693.

Tatu, U., and Helenius, A. (1997). Interactions between newly synthesized glycoproteins, calnexin and a network of resident chaperones in the endoplasmic reticulum. J Cell Biol *136*, 555-565.

Theiben, G., Kim, J., Saedler, H. (1996). Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J Mol Evol *43*, 484-516.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M., and Hidaka, H. (1990). KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazi ne, a specific inhibitor of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem *265*, 4315-4320.

Tombes, R. M., Grant, S., Westin, E. H., and Krystal, G. (1995). G1 cell cycle arrest and apoptosis are induced in NIH 3T3 cells by KN-93, an inhibitor of CaMK-II (the multifunctional Ca2+/CaM kinase). Cell Growth Differ *6*, 1063-1070.

Tong, G., Shepherd, D., and Jahr, C. E. (1995). Synaptic desensitization of NMDA receptors by calcineurin. Science *267*, 1510-1512.

Torrado, M., Lopez, E., Centeno, A., Medrano, C., Castro-Beiras, A., and Mikhailov, A. T. (2003). Myocardin mRNA is augmented in the failing myocardium: expression profiling in the porcine model and human dilated cardiomyopathy. J Mol Med *81*, 566-577.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. Curr Opin Genet Dev *4*, 96-101.

Tung, H. Y. (1986). Phosphorylation of the calmodulin-dependent protein phosphatase by protein kinase C. Biochem Biophys Res Commun *138*, 783-788.

Ueki, K., Muramatsu, T., and Kincaid, R. L. (1992). Structure and expression of two isoforms of the murine calmodulin-dependent protein phosphatase regulatory subunit (calcineurin B). Biochem Biophys Res Commun *187*, 537-543.

Ueyama, T., Kasahara, H., Ishiwata, T., Nie, Q., and Izumo, S. (2003). Myocardin expression is regulated by Nkx2.5, and its function is required for cardiomyogenesis. Mol Cell Biol *23*, 9222-9232.

Vinogradova, T. M., Zhou, Y. Y., Bogdanov, K. Y., Yang, D., Kuschel, M., Cheng, H., and Xiao, R. P. (2000). Sinoatrial node pacemaker activity requires Ca(2+)/calmodulin-dependent protein kinase II activation. Circ Res *87*, 760-767.

Vittone, L., Mundina-Weilenmann, C., Said, M., Ferrero, P., and Mattiazzi, A. (2002). Time course and mechanisms of phosphorylation of phospholamban residues in ischemia-reperfused rat hearts. Dissociation of phospholamban phosphorylation pathways. J Mol Cell Cardiol *34*, 39-50.

Voytas, D. (1998). Resolution and recovery of large DNA fragments. In: Current Protocols in Molecular Biology, Volume 1, F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, K. Struhl, L. Alright, D. Coen, and A. Varki, eds. (New York: John Wiley and Sons Ltd.), pp. 2.5A.1-2.5A.9.

Walsh, M. P., Busaan, J. L., Fraser, E. D., Fu, S. Y., Pato, M. D., and Michalak, M. (1995). Characterization of the recombinant C-terminal domain of dystrophin: phosphorylation by calmodulin-dependent protein kinase II and dephosphorylation by type 2B protein phosphatase. Biochemistry *34*, 5561-5568.

Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001). Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. Cell *105*, 851-862.

Wang, H., Silva, N. L., Lucchesi, P. A., Haworth, R., Wang, K., Michalak, M., Pelech, S., and Fliegel, L. (1997). Phosphorylation and regulation of the Na+/H+ exchanger through mitogen-activated protein kinase. Biochemistry *36*, 9151-9158.

Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999). Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science *284*, 339-343.

Wang, M. G., Yi, H., Guerini, D., Klee, C. B., and McBride, O. W. (1996). Calcineurin A alpha (PPP3CA), calcineurin A beta (PPP3CB) and calcineurin B (PPP3R1) are located on human chromosomes 4, 10q21-->q22 and 2p16-->p15 respectively. Cytogenet Cell Genet *72*, 236-241.

Wang, Z., Wang, D. Z., Pipes, G. C., and Olson, E. N. (2003). Myocardin is a master regulator of smooth muscle gene expression. Proc Natl Acad Sci U S A *100*, 7129-7134.

Ware, F. E., Vassilakos, A., Peterson, P. A., Jackson, M. R., Lehrman, M. A., and Williams, D. B. (1995). The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. J Biol Chem *270*, 4697-4704.

Waser, M., Mesaeli, N., Spencer, C., and Michalak, M. (1997). Regulation of calreticulin gene expression by calcium. J Cell Biol *138*, 547-557.

Watanabe, Y., Perrino, B. A., Chang, B. H., and Soderling, T. R. (1995). Identification in the calcineurin A subunit of the domain that binds the regulatory B subunit. J Biol Chem *270*, 456-460.

Webb, S. E., and Miller, A. L. (2003). Calcium signalling during embryonic development. Nature Rev Mol Cell Biol *4*, 539-551.

Wesselborg, S., Fruman, D. A., Sagoo, J. K., Bierer, B. E., and Burakoff, S. J. (1996). Identification of a physical interaction between calcineurin and nuclear factor of activated T cells (NFATp). J Biol Chem *271*, 1274-1277.

Wilkins, B. J., Dai, Y. S., Bueno, O. F., Parsons, S. A., Xu, J., Plank, D. M., Jones, F., Kimball, T. R., and Molkentin, J. D. (2004). Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. Circ Res *94*, 110-118.

Wilkins, B. J., De Windt, L. J., Bueno, O. F., Braz, J. C., Glascock, B. J., Kimball, T. F., and Molkentin, J. D. (2002). Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol *22*, 7603-7613.

Wilkins, B. J., and Molkentin, J. D. (2002). Calcineurin and cardiac hypertrophy: where have we been? Where are we going? J Physiol *541*, 1-8.

Williams, C. L., Phelps, S. H., and Porter, R. A. (1996). Expression of Ca2+/calmodulin-dependent protein kinase types II and IV, and reduced DNA synthesis due to the Ca2+/calmodulin-dependent protein kinase inhibitor KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenyl piperazine) in small cell lung carcinoma. Biochem Pharmacol *51*, 707-715.

Wolfe, S. A., Zhou, P., Dotsch, V., Chen, L., You, A., Ho, S. N., Crabtree, G. R., Wagner, G., and Verdine, G. L. (1997). Unusual Rel-like architecture in the DNA-binding domain of the transcription factor NFATc. Nature *385*, 172-176.

Wu, H., Naya, F. J., McKinsey, T. A., Mercer, B., Shelton, J. M., Chin, E. R., Simard, A. R., Michel, R. N., Bassel-Duby, R., Olson, E. N., and Williams, R. S. (2000). MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. Embo J *19*, 1963-1973.

Wu, H., Rothermel, B., Kanatous, S., Rosenberg, P., Naya, F. J., Shelton, J. M., Hutcheson, K. A., DiMaio, J. M., Olson, E. N., Bassel-Duby, R., and Williams, R. S. (2001). Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. Embo J *20*, 6414-6423.

Xu, A., and Narayanan, N. (2000). Reversible inhibition of the calcium-pumping ATPase in native cardiac sarcoplasmic reticulum by a calmodulin-binding peptide. Evidence for calmodulin-dependent regulation of the V(max) of calcium transport. J Biol Chem *275*, 4407-4416.

Xu, W., Longo, F. J., Wintermantel, M. R., Jiang, X., Clark, R. A., and DeLisle, S. (2000). Calreticulin modulates capacitative Ca2+ influx by controlling the extent of inositol 1,4,5-trisphosphate-induced Ca2+ store depletion. J Biol Chem *275*, 36676-36682.

Youn, H. D., Grozinger, C. M., and Liu, J. O. (2000). Calcium regulates transcriptional repression of myocyte enhancer factor 2 by histone deacetylase 4. J Biol Chem *275*, 22563-22567.

Youn, H. D., Sun, L., Prywes, R., and Liu, J. O. (1999). Apoptosis of T cells mediated by Ca2+-induced release of the transcription factor MEF2. Science *286*, 790-793.

Yu, Y. T. (1996). Distinct domains of myocyte enhancer binding factor-2A determining nuclear localization and cell type-specific transcriptional activity. J Biol Chem *271*, 24675-24683.

Zhang, B. W., Zimmer, G., Chen, J., Ladd, D., Li, E., Alt, F. W., Wiederrecht, G., Cryan, J., O'Neill, E. A., Seidman, C. E., Abbas, A., and Seidman, J.G. (1996). T cell responses in calcineurin A alpha-deficient mice. J Exp Med *183*, 413-420.

Zhang, W., Kowal, R. C., Rusnak, F., Sikkink, R. A., Olson, E. N., and Victor, R. G. (1999). Failure of calcineurin inhibitors to prevent pressure-overload left ventricular hypertrophy in rats. Circ Res *84*, 722-728.

Zhu, D., Cardenas, M. E., and Heitman, J. (1995). Myristoylation of calcineurin B is not required for function or interaction with immunophilin-immunosuppressant complexes in the yeast Saccharomyces cerevisiae. J Biol Chem *270*, 24831-24838.

Zhu, W., Zou, Y., Shiojima, I., Kudoh, S., Aikawa, R., Hayashi, D., Mizukami, M., Toko, H., Shibasaki, F., Yazaki, Y., Nagai, R., and Komuro, I. (2000). Ca2+/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. J Biol Chem *275*, 15239-15245.

Zhuo, M., Zhang, W., Son, H., Mansuy, I., Sobel, R. A., Seidman, J., and Kandel, E. R. (1999). A selective role of calcineurin aalpha in synaptic depotentiation in hippocampus. Proc Natl Acad Sci U S A *96*, 4650-4655.

Zhuo, S., Clemens, J. C., Hakes, D. J., Barford, D., and Dixon, J. E. (1993). Expression, purification, crystallization, and biochemical characterization of a recombinant protein phosphatase. J Biol Chem *268*, 17754-17761.

SUPPLEMENTARY CHAPTER 1:
GATA6 activates the calreticulin promoter

INTRODUCTION

Calreticulin promoter-controlled luciferase reporter gene data indicated that GATA6 activated reporter gene activity. To further investigate GATA6 influence on the calreticulin-promoter it was necessary to determine if GATA6 activated the reporter gene: 1) in a dose-dependent manner; 2) by direct interaction with the calreticulin promoter; 3) in other cell types such as cardiomyocytes; and, 4) in a synergistic manner with Nkx2.5 as previously reported for GATA4. Additionally, the influence of repressors (Evi-1 and COUP-TF1) on the calreticulin promoter was to be considered in the GATA6 investigation.

Y1H results found that GATA6 bound to the calreticulin promoter (Dr. Lei Guo, unpublished data). Additional evidence was necessary to demonstrate the interaction of these factors with the calreticulin promoter and so ChIP assay was completed. EMSA for GATA6 has been previously described (Hautala *et al.*, 2001; Liang *et al.*, 2001; Weidenfeld *et al.*, 2002; Yaar *et al.*, 2002) while ChIP assay has been completed for GATA4 (Brown *et al.*, 2004), but not for GATA6. As Y1H was one piece of evidence showing protein-DNA interaction it was decided that the second piece would be ChIP as it provides evidence from an *in vivo* condition.

Nkx2.5 binds to a site (-1217 to -1223) within the calreticulin promoter and activates gene expression (Guo *et al.*, 2001). Synergistic interaction of Nkx2.5 with GATA on promoters of fetal cardiac genes (e.g., ANF, BNP) is well documented (Durocher *et al.*, 1997; Molkentin, 2000; Molkentin *et al.*, 1998). The Nkx2.5 binding

site on calreticulin promoter is in close proximity to the double GATA site (-981 to -1000). As such, these two transcription factors were to be studied.

Nkx2.5 binding to the calreticulin promoter is disrupted in a dose-dependent manner by COUP-TF1 as these two factors compete for the same DNA binding site (Guo *et al.*, 2001). If there is synergistic interaction between Nkx2.5 and GATA6 on the calreticulin promoter then the question arises as to whether this interaction can be disrupted by increasing amounts of COUP-TF1. As such, experiments involving Nkx2.5 and COUP-TF1 were included in the GATA6 investigation.

Y1H findings suggested that Evi-1 bound to the same site as GATA6 within the calreticulin promoter (Dr. Lei Guo, unpublished data). EMSA for Evi-1 has previously been completed (Takahashi and Licht, 2002) whereas ChIP assay for Evi-1 has not been reported. As Y1H was one piece of evidence showing Evi-1 interaction with the calreticulin promoter it was decided that additional evidence would be ChIP assay. From the Evi-1 luciferase experiments it was known that Evi-1 was a repressor of calreticulin promoter-controlled luciferase reporter gene (Figure 1-9). To determine if GATA6 and Evi-1 competed for the same DNA binding site, experiments with the calreticulin promoter controlled-luciferase reporter gene were necessary.

Isolation of cardiomyocytes from neonatal rats

Hearts were removed from 5-day-old neonatal rats and placed in 1mL of a solution containing: Hanks Buffered Salt Solution (HBSS), 20mM HEPES, and 1% penicillin/streptromycin. Heart atria were removed and the ventricles were minced with a scalpel. Ventricular pieces were transferred to an Erlenmeyer flask and 15mL of HBSS supplemented with 0.1% collagenase (358U/mg; Worthington Biochemical Corporation, Lakewood, New Jersey, USA) added. The flask was placed on a magnetic stirrer and gentle heat applied for approximately 20 minutes. After which time, liquid in the flask was decanted and put in a tube with 15mL of a solution containing HBSS, 20% FBS, and 1% penicillin/streptromycin. The 30mL of solution was placed on ice and the incubation procedure with the minced heart pieces repeated three more times. At the end, there were four tubes each containing cardiomyocytes in a volume of 30mL. Solutions were gently vortexed and the mixture was filtered through cell strainers (Falcon; Cat. 35 2350) into new tubes. Cells were centrifuged at room temperature at 2000rpm for 5 minutes and then re-suspended in 25mL a solution containing: DMEM F12, 29mM $NaHCO_3$, 1% BSA, 1% penicillin-streptomycin, 0.7mM $CaCl_2$, 0.1mM L-ascorbic acid, 1% MEM-nonessential amino acids (Sigma; Cat. A-4403), 0.1% Vitamin 100X (Gibco; Cat. 11120052), and final pH of 7.4. Three pre-plating steps for 20 minutes at 37^0C in a humidified atmosphere (5% CO_2, 95% air) were necessary to reduce the number of fibroblasts. Following pre-incubation, cells were plated onto primaria easy grip surface modified polystyrene 35mm primary culture plates (Falcon; Cat. 353801). This protocol was developed in the Fliegel lab (University of Alberta, Edmonton, Alberta, Canada) and

produces several confluent 35mm culture plates each having a monolayer of spontaneously beating cardiomyocytes.

Cardiomyocytes are difficult cells to transfect and efficiency is often quiet low. Effectene reagent was utilized according to manufacturer instructions to transfect cardiomyocytes with 10μg of either GATA6 or Nkx2.5 plasmid. 48 hours after transfection cells were harvested and luciferase and β-galactosidase measurements recorded.

Chromatin Immunoprecipitation (ChIP)

cDNA encoding GATA6 was amplified by PCR and 5' XhoI and 3' BamHI sites incorporated so that it could be ligated into pcDNA3.1/myc-His(-)A cloning vector. Evi-1 plasmid was a generous gift from Dr. Archibald S. Perkins (Yale School of Medicine, New Haven, Connecticut, USA) that had been constructed with a HA-tag.

For GATA6 and Evi-1 ChIP analysis, the same protocol as described for MEF2C in Chapter 2 was utilized. For this experiment GATA6 antibody was a monoclonal anti-His (Santa Cruz Biotechnology, Cat. SC-8036) while Evi-1 antibody was monoclonal anti-HA (Roche Diagnostics, Cat. 1 867 423). A change to the method previously described was that different forward (5'-CAGGTACTGTTCTTCCTCCTCC-3') and reverse (5'- GCTGTCCACGGTTCAAGAGC-3') PCR primers were necessary. The ChIP PCR product was 332bp.

RESULTS

GATA6 activated the calreticulin promoter controlled luciferase reporter gene in NIH3T3 fibroblasts (Figure 1-9). When GATA6 ability to activate the reporter gene was examined in neonatal rat cardiomyocytes (Figure S-1), similar results to that observed in NIH3T3 cells were observed.

Dose-response experiment provides an indication as to how strongly a transcription factor activates gene expression. In this particular instance, calreticulin-promoter controlled luciferase reporter gene was co-transfected with between 1.5μg and 4μg of GATA6 plasmid DNA. The results indicate that calreticulin promoter-controlled luciferase reporter gene activity increased proportionally to the amount of cDNA encoding GATA6 co-transfected (Figure S-2). Therefore, activation of calreticulin promoter by GATA6 is dose-dependent.

Deletion mutants of the calreticulin promoter were necessary to provide insight as to the location of the GATA6 binding site within calreticulin promoter. CPF as well as the eight deletion mutants were co-transfected into NIH3T3 cells with GATA6 plasmid. In presence of GATA6, CPF activity was increased by approximately 80% (Figure S-3). Deletion mutants CP5 to CP8 had activity comparable to CPF whereas activity for CP1 to CP4 mutants was greatly reduced (Figure S-3). The difference between CP4 and CP5 is one tentative GATA6 binding site (Figure S-3). Thus, it would seem that GATA6 was binding to a GATA site within the double GATA region of the promoter. However, from this experiment it is difficult to determine which of the two GATA sites was being bound.

ChIP assay demonstrates an *in vivo* interaction between a transcription factor and DNA. With ChIP it was possible to isolate GATA6 bound to DNA and later utilize this

Figure S-1. GATA6 activated calreticulin promoter-controlled luciferase reporter gene in cardiomyocytes. Neonatal rat cardiomyocytes were co-transfected with a reporter gene plasmid and a plasmid containing cDNA for GATA6. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Mean +/- standard deviation is indicated.

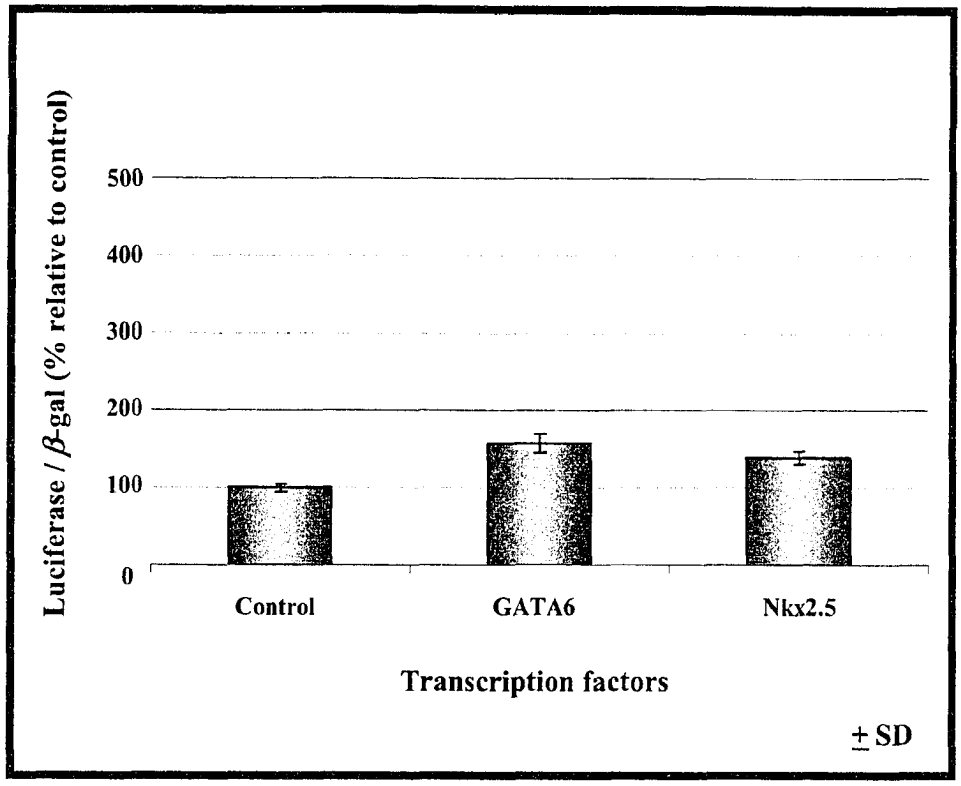


Figure S-2. GATA6 activation of the calreticulin promoter is dose-dependent. A constant amount (2μg) of CPF was transfected with increasing amounts of GATA6 plasmid. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Data is representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.

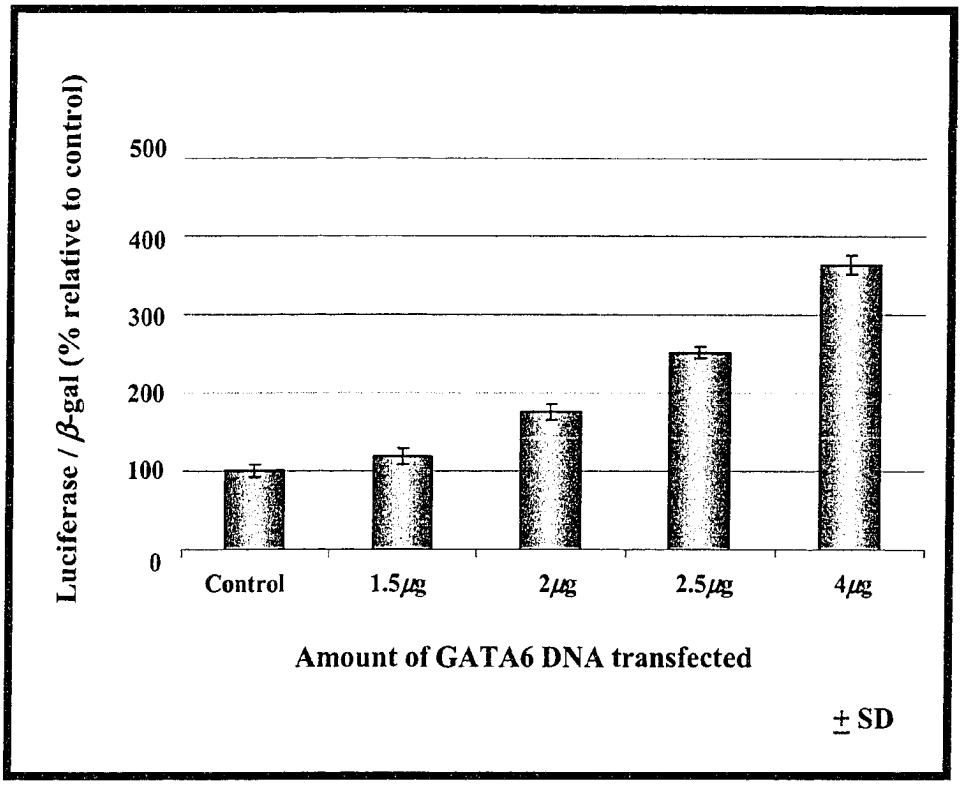
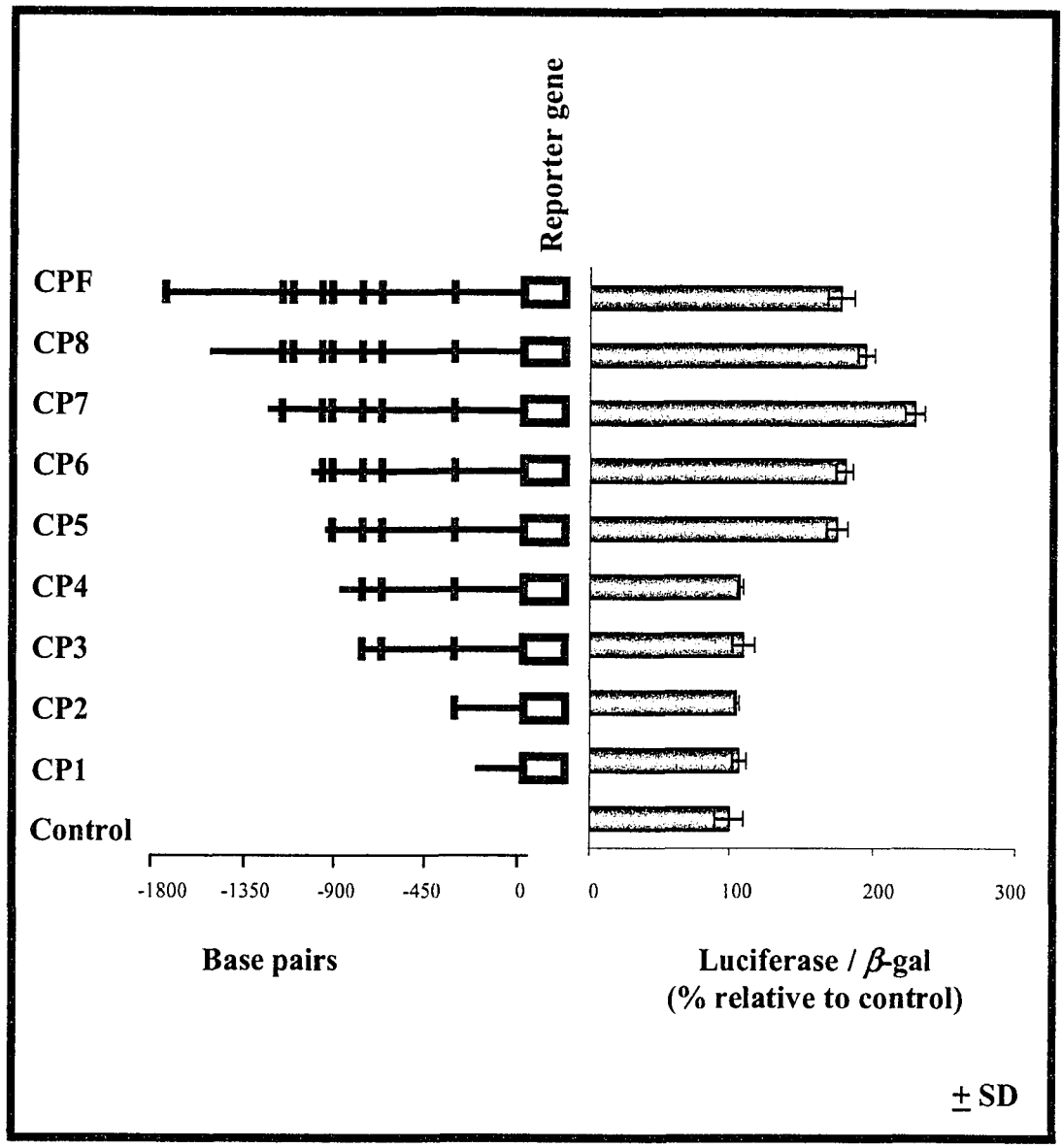


Figure S-3. Schematic diagrams of the calreticulin promoter and promoter deletion mutants as well as reporter gene activity in response to GATA6. The schematics indicate identified DNA binding site of Nkx2.5 (red), and tentative locations of MEF2C (green) and GATA6 (blue) DNA binding sites. Vectors contained the following DNA fragments of the calreticulin promoter: CPF: 1722bp; CP8: 151bp; CP7: 1234bp; CP6: 1009bp; CP5: 992bp; CP4: 907bp; CP3: 862bp; CP2: 371bp; CP1: 172bp. The calreticulin promoter deletion mutants were co-transfected with GATA6 and luciferase reporter gene activity measured. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each deletion mutant. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.



DNA for PCR analysis. In this investigation, mouse embryonic fibroblasts were first transfected with a plasmid containing cDNA encoding His-tagged GATA6. When the DNA was analyzed, a PCR product was present for the plasmid positive control, but not for the negative control (Figure S-4, *lanes 2 and 3*, respectively). When ChIP DNA isolated from GATA6 transfected cells was utilized as the template for PCR a PCR product of identical size to the positive control was observed (Figure S-4, *lane 4*). Thus, ChIP provided *in vivo* evidence of GATA6 binding to calreticulin promoter. Therefore, ChIP findings supported the unpublished Y1H results and confirmed that GATA6 binds to calreticulin promoter at the region -981 to -1000 which included the double GATA site (Figure S-5).

The calreticulin promoter is synergistically activated by GATA6 and Nkx2.5. Individually GATA6 and Nkx2.5 activated reporter gene by approximately 80% and 50%, respectively; however, when GATA6 and Nkx2.5 were co-transfected the reporter activity was over 300%. Interestingly, the presence of COUP-TF1 repressed this synergism in a dose-dependent manner (Figure S-6).

Y1H indicated that GATA6 and Evi-1 bound to the same region of calreticulin promoter. Luciferase reporter gene experiments suggested that GATA6 was a transcriptional activator while Evi-1 was a transcriptional repressor. When equal amounts of GATA6 and Evi-1 plasmids were co-transfected into cells, there was a 34% reduction in GATA6 induced reporter activity (Figure S-7). Thus, it would seem that GATA6 and Evi-1 compete for binding to the same region of the calreticulin promoter. ChIP assay (Figure S-8) confirmed that Evi-1 bound to the same region (i.e. likely the double GATA site) of the calreticulin promoter as GATA6 (Figure S-5).

Figure S-4. ChIP demonstrated *in vivo* binding of GATA6 to calreticulin promoter. PCR products using ChIP isolated DNA as template were loaded onto an agarose gel. *Lane 1*: 1 kb+ DNA ladder; *Lane 2*: Calreticulin promoter plasmid (positive control); *Lane 3*: ChIP analysis with no His-antibody utilized (negative control); and, *Lane 4*: ChIP sample in which His-antibody was utilized. PCR product size was 332bp.

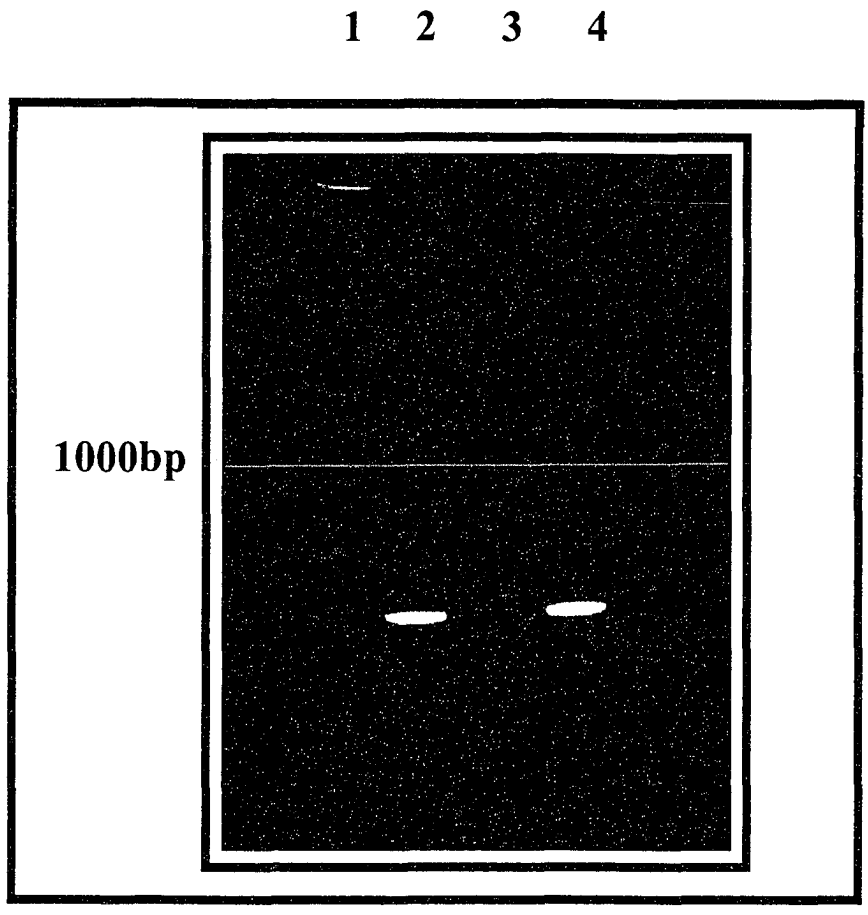


Figure S-5. Schematic figure of the calreticulin promoter indicating the position of identified GATA6 DNA binding region. GATA6 binding sites (*underlined text*) in the calreticulin promoter are located from -981 to -1000. Location of identified Nkx2.5 DNA binding site (red), MEF2C DNA binding site (green), and TATA box (yellow) are indicated.

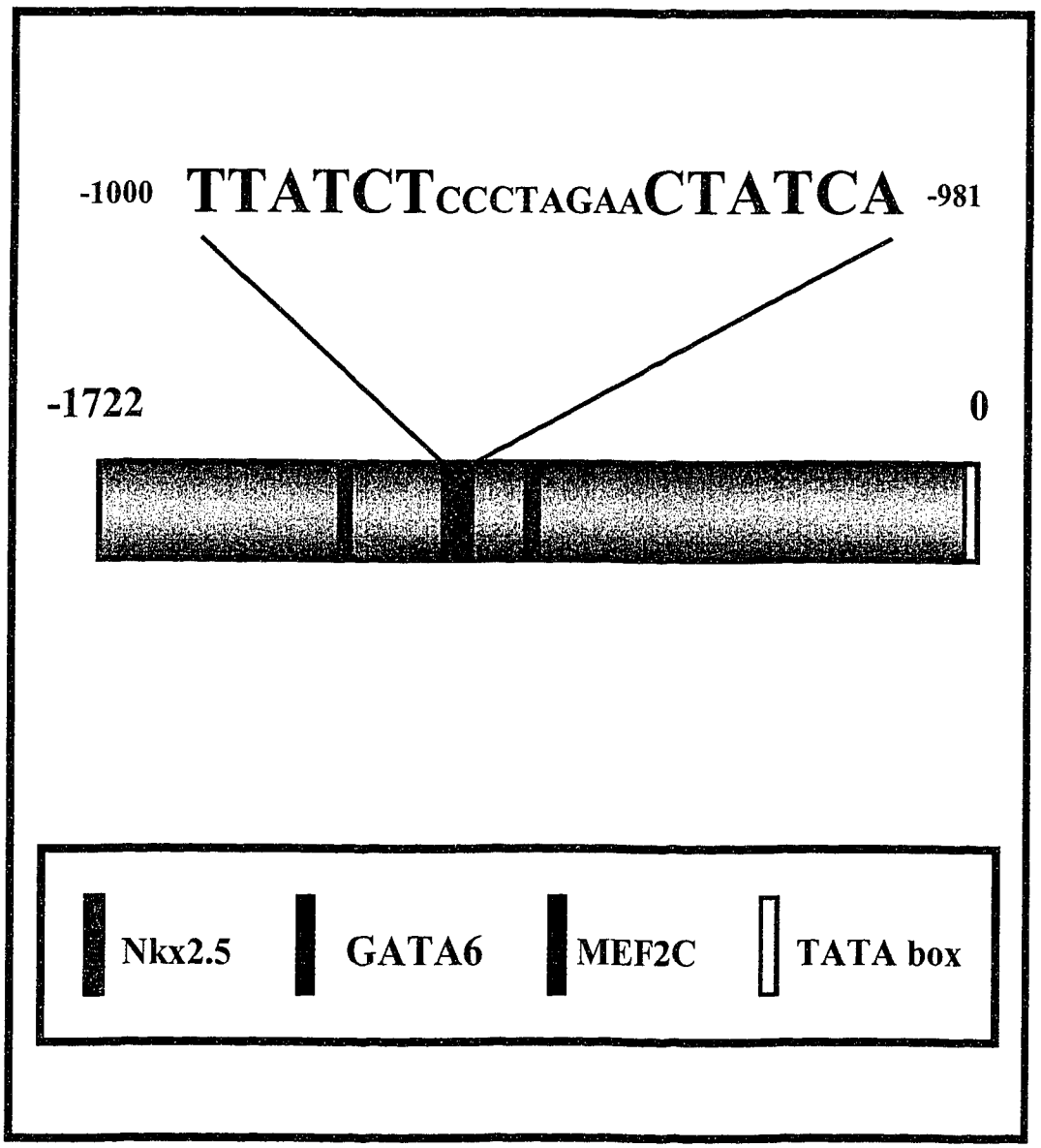


Figure S-6. GATA6 and Nkx2.5 synergistically activate the calreticulin promoter controlled luciferase reporter gene activity and COUP-TFI disrupted the synergism. CPF plasmid was co-transfected with cDNA encoding GATA6, Nkx2.5, COUP-TFI and luciferase reporter gene activity measured. GATA6 and Nkx2.5 synergistically activated reporter gene activity and presence of COUP-TFI diminished the synergism in a dose-dependent manner. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.

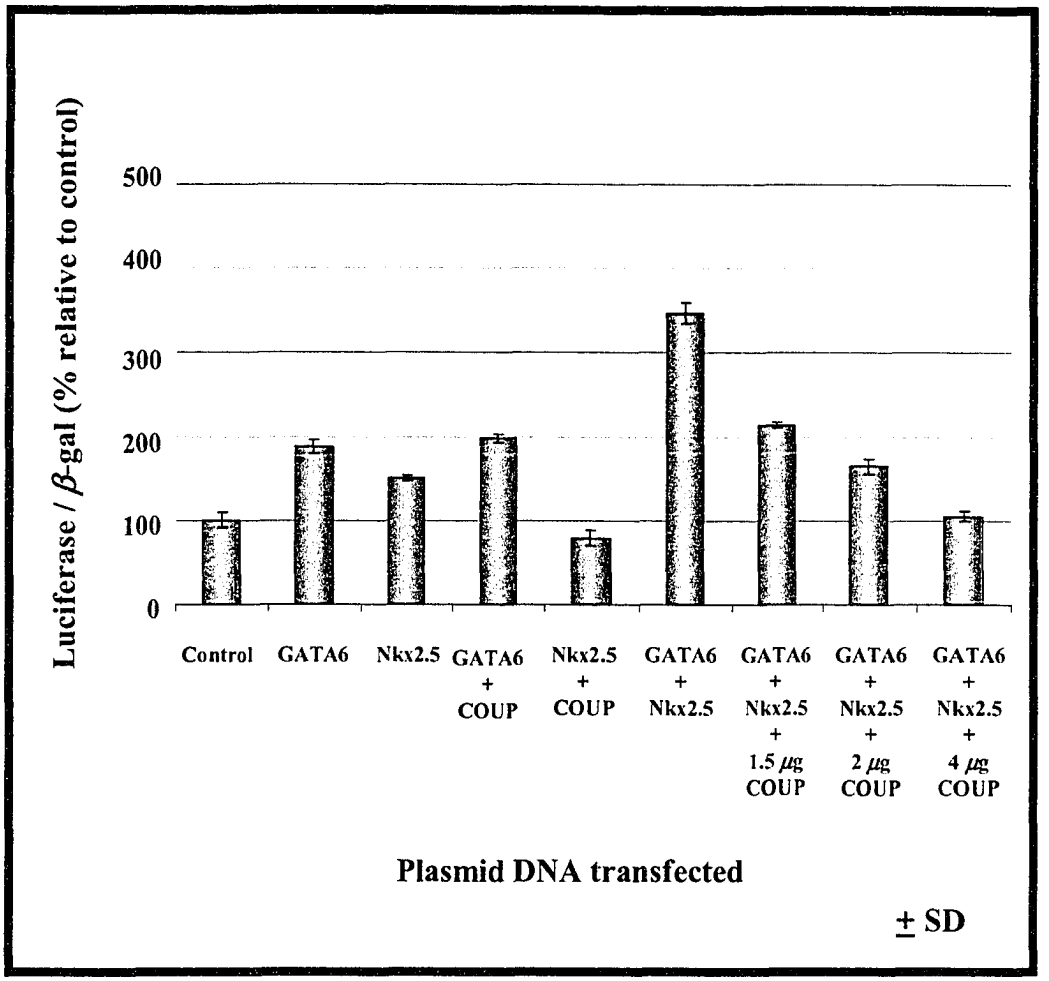


Figure S-7. Evi-1 repressed activity of the calreticulin promoter controlled reporter gene. CPF plasmid was co-transfected with cDNA for GATA6 and/or Evi-1 and reporter gene activity measured. Mean data are plotted relative to activity of the control. Although only a single control bar is present, it represents control data collected for each transcription factor. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.

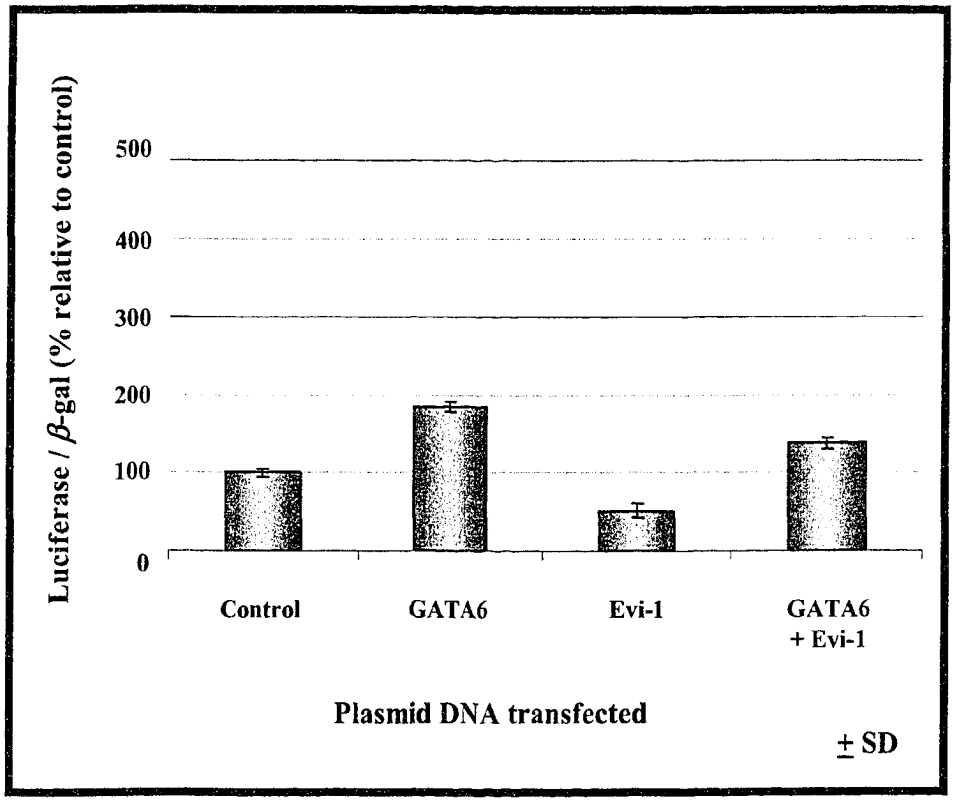
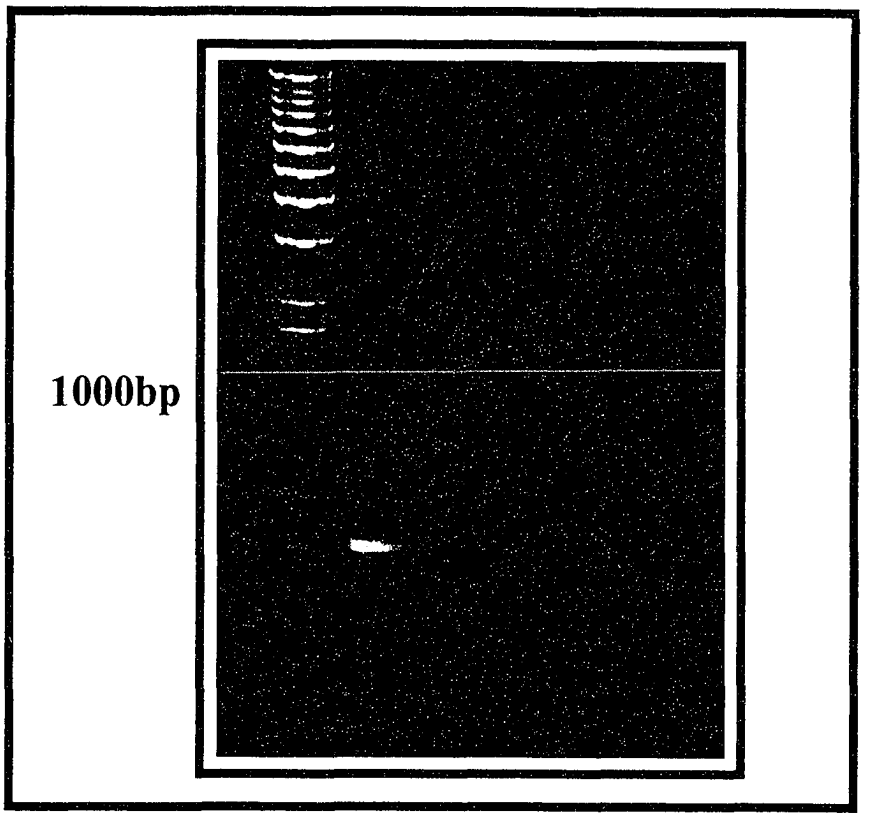


Figure S-8. ChIP demonstrated *in vivo* binding of Evi-1 to calreticulin promoter. PCR products using ChIP isolated DNA as template were loaded onto an agarose gel. *Lane 1*: 1 kb+ DNA ladder; *Lane 2*: Calreticulin promoter plasmid (positive control); *Lane 3*: ChIP analysis with no HA-antibody utilized (negative control); and, *Lane 4*: ChIP sample in which HA-antibody was utilized. PCR product size was 332bp.



DISCUSSION

The calreticulin promoter possesses three tentative GATA6 binding sites and GATA6 activated the calreticulin promoter-controlled luciferase reporter gene in fibroblasts and cardiomyocytes. Y1H data (Lei Guo, unpublished) and ChIP analysis indicated that GATA6 bound to the calreticulin promoter in the region of the double GATA6 site, -981 to -1000. Thus, it can be concluded that GATA6 binds to the calreticulin promoter and activates calreticulin gene.

Evi-1 was found to repress the calreticulin controlled-luciferase reporter gene in fibroblasts. The observation that Evi-1 was a transcriptional repressor has been previously reported (Bartholomew *et al.*, 1997; Kreider *et al.*, 1993). Y1H data (Lei Guo, unpublished) and ChIP analysis indicated that Evi-1 bound to a similar DNA nucleotide region of the calreticulin promoter as GATA6. As the DNA binding site for GATA factors is contained within the binding site for Evi-1 (Funabiki *et al.*, 1994), it is probable that Evi-1 represses GATA6 activation of the calreticulin promoter.

Several additional experiments are necessary to determine the significance of GATA6 and Evi-1 binding to the calreticulin promoter. In particular, it will be necessary to conduct reporter gene experiments using calreticulin promoter reporter gene in which the double GATA6 site within the calreticulin promoter has been mutated. In addition, EMSA for both factors is required to specifically identify both the GATA6 and Evi-1 DNA binding sites.

GATA4 has been reported to synergistically interact with Nkx2.5 (Durocher *et al.*, 1997), but no synergism has been reported to exist between GATA6 and Nkx2.5. As previously observed, COUP-TF1 is a repressor of calreticulin promoter (Figure 1-9) and

presence of COUP-TF1 disrupted GATA6/Nkx2.5 synergistic activation of the calreticulin promoter-controlled luciferase reporter gene. It is likely that COUP-TF1 disrupts this synergism by binding to the Nkx2.5 site within the calreticulin promoter (Guo *et al.*, 2001). Therefore, this study has evidence suggesting existence of a synergistic relationship between GATA6 and Nkx2.5, but additional experiments are necessary to specifically demonstrate this protein-protein interaction.

It can be concluded that GATA6 is an activator of the calreticulin promoter. Although the GATA6 investigation has produced some interesting findings, the study is not finished. Therefore, future investigations will be necessary to provide a complete understanding of the significance of GATA6 activation of the calreticulin promoter and how this may be of significance to heart development and heart disease.

REFERENCES

Bartholomew, C., Kilbey, A., Clark, A. M., and Walker, M. (1997). The Evi-1 proto-oncogene encodes a transcriptional repressor activity associated with transformation. Oncogene *14*, 569-577.

Brown, C. O., 3rd, Chi, X., Garcia-Gras, E., Shirai, M., Feng, X. H., and Schwartz, R. J. (2004). The cardiac determination factor, Nkx2-5, is activated by mutual cofactors GATA-4 and Smad1/4 via a novel upstream enhancer. J Biol Chem *279*, 10659-10669.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. Embo J *16*, 5687-5696.

Funabiki, T., Kreider, B. L., and Ihle, J. N. (1994). The carboxyl domain of zinc fingers of the Evi-1 myeloid transforming gene binds a consensus sequence of GAAGATGAG. Oncogene *9*, 1575-1581.

Guo, L., Lynch, J., Nakamura, K., Fliegel, L., Kasahara, H., Izumo, S., Komuro, I., Agellon, L. B., and Michalak, M. (2001). COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene during cardiac development. J Biol Chem *276*, 2797-2801.

Hautala, N., Tokola, H., Luodonpaa, M., Puhakka, J., Romppanen, H., Vuolteenaho, O., and Ruskoaho, H. (2001). Pressure overload increases GATA4 binding activity via endothelin-1. Circulation *103*, 730-735.

Kreider, B. L., Orkin, S. H., and Ihle, J. N. (1993). Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. Proc Natl Acad Sci U S A *90*, 6454-6458.

Liang, Q., De Windt, L. J., Witt, S. A., Kimball, T. R., Markham, B. E., and Molkentin, J. D. (2001). The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. J Biol Chem *276*, 30245-30253.

Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. J Biol Chem *275*, 38949-38952.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell *93*, 215-228.

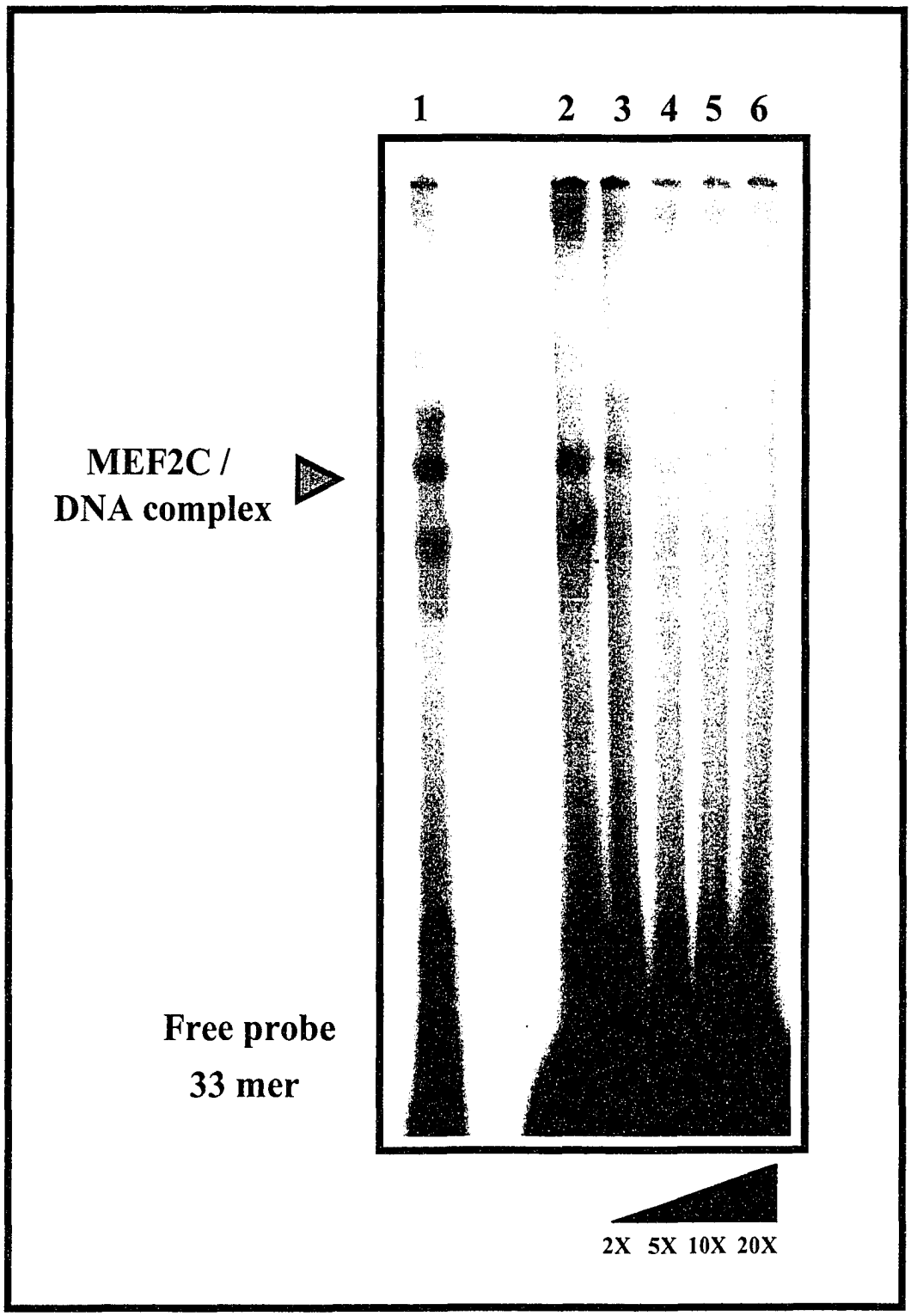
Takahashi, S., and Licht, J. D. (2002). The human promyelocytic leukemia zinc finger gene is regulated by the Evi-1 oncoprotein and a novel guanine-rich site binding protein. Leukemia *16*, 1755-1762.

Weidenfeld, J., Shu, W., Zhang, L., Millar, S. E., and Morrisey, E. E. (2002). The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. J Biol Chem *277*, 21061-21070.

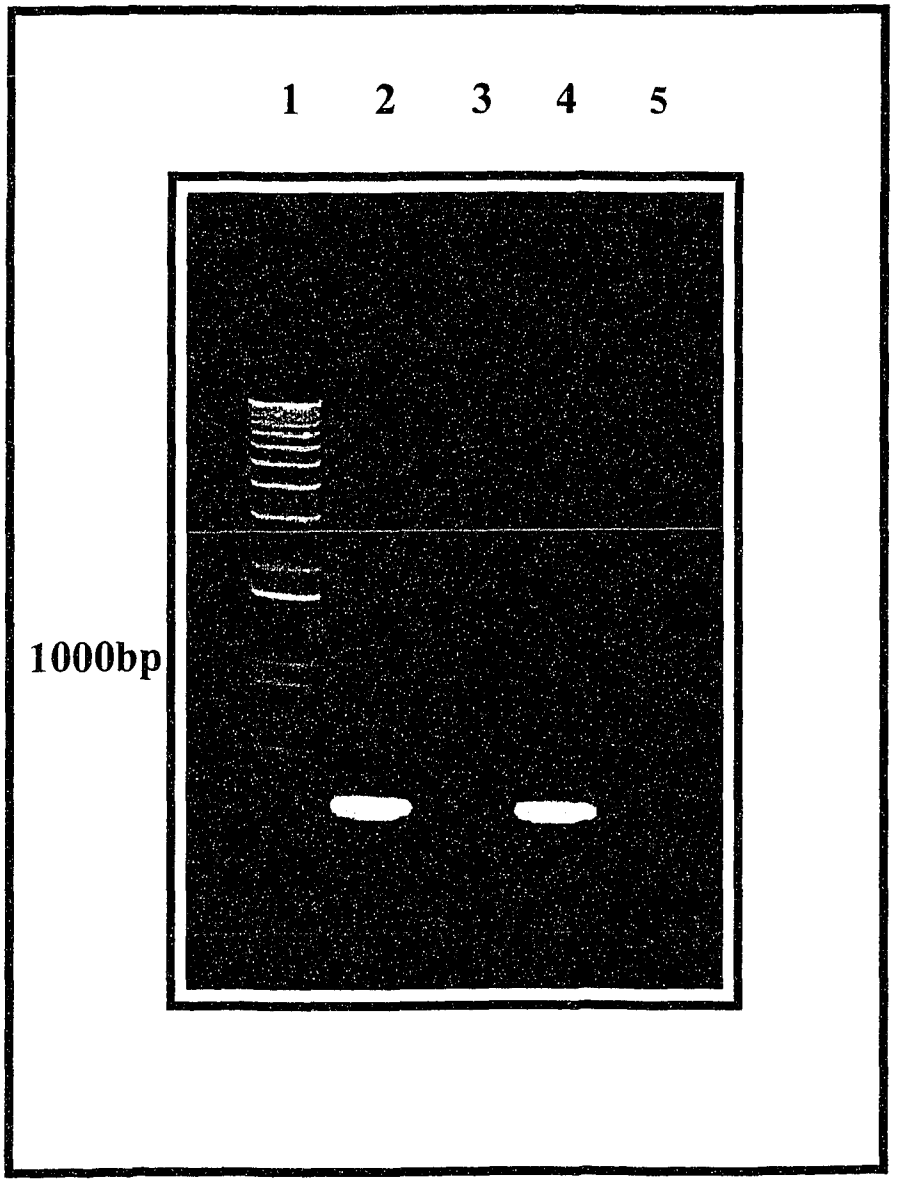
Yaar, R., Cataldo, L. M., Tzatsos, A., Francis, C. E., Zhao, Z., and Ravid, K. (2002). Regulation of the A3 adenosine receptor gene in vascular smooth muscle cells: role of a cAMP and GATA element. Mol Pharmacol *62*, 1167-1176.

APPENDIX

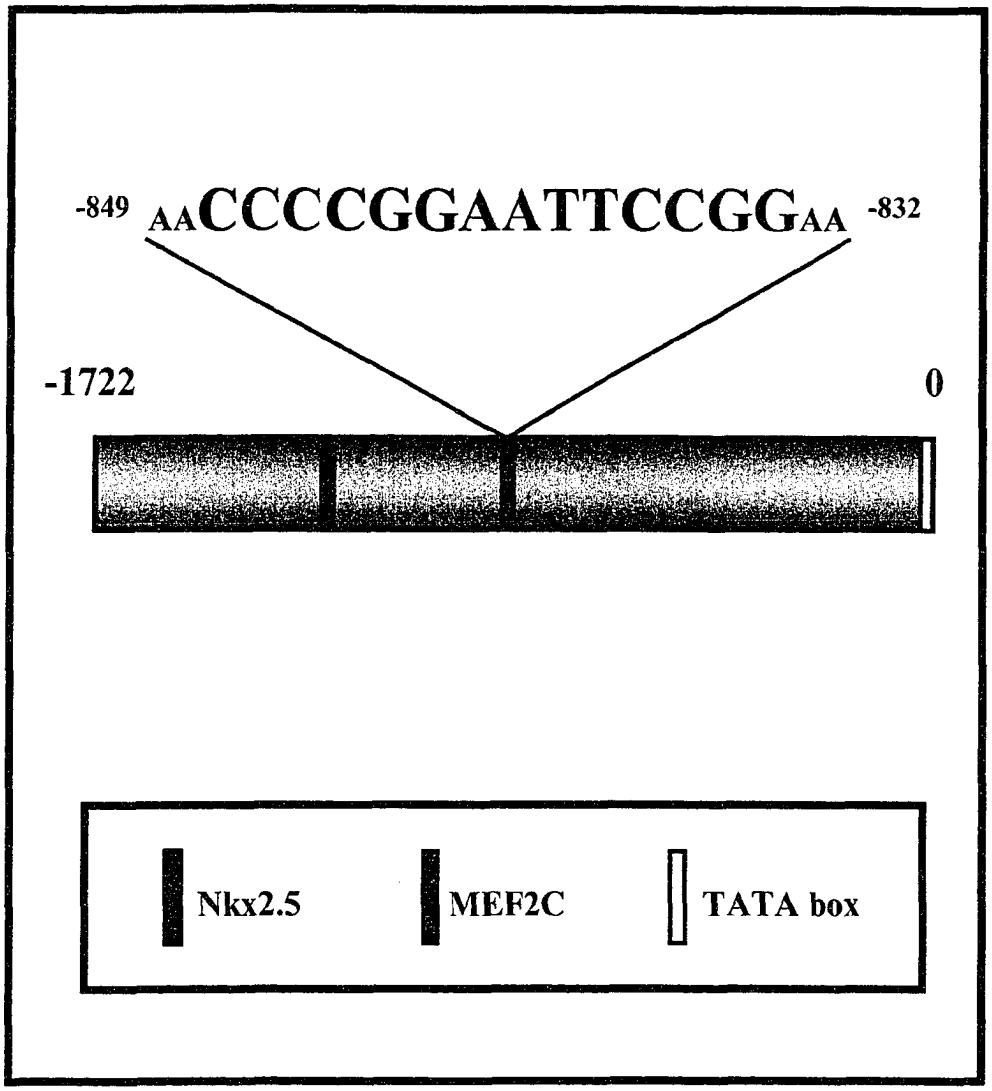
Appendix Figure 1. Competition EMSA of non-labeled probed competing for binding to ideal MEF2C site. *Lane 1*: MEF2C with probe for ideal MEF2C consensus binding site (positive control); *Lane 2*: MEF2C with probe for ideal MEF2C consensus binding site; and *Lanes 3 to 6*: MEF2C with labeled and unlabeled probe for predicted MEF2C binding site within calreticulin promoter.



Appendix Figure 2. ChIP assay for MEF2C conducted in 5-day-old neonatal rat cardiomyoctyes. *Lane 1*: 1 kbp+ DNA ladder, *Lane 2*: Positive plasmid control, *Lane 3*: Negative control, *Lane 4*: ChIP isolated DNA, *Lane 5*: Negative control (no antibody treatment). PCR product size was 310bp. For this experiment, hearts were removed from 5-day-old neonatal rats and cardiomyocytes isolated as described in Supplementary Chapter 1. Effectene reagent (Qiagen, Cat. 301425) was utilized according to manufacturer instructions to transfect cardiomyocytes with 10μg of cDNA encoding mouse MEF2C (432 amino acids; GenBank Accession: NM_025282) which had been cloned in pcDNA3.1/myc-His(-)A (Invitrogen) cloning vector. ChIP protocol for cardiomyocytes was completed as previously described for fibroblasts in Chapter 2.



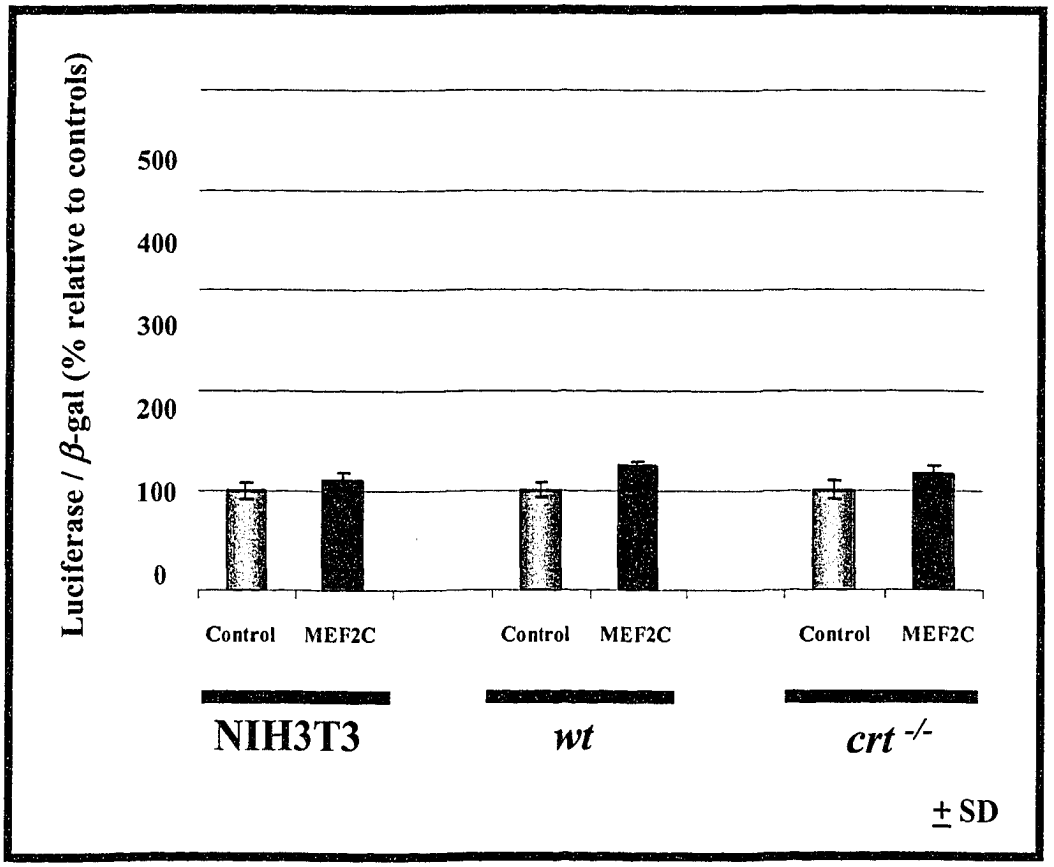
Appendix Figure 3. Schematic figure of the calreticulin promoter indicating the mutation made to the identified MEF2C DNA binding site. Locations of the identified Nkx2.5 DNA binding site (red), MEF2C DNA binding site (green), and TATA box (yellow) are indicated. To mutate the MEF2C DNA binding site within the calreticulin promoter (AAAAAAAATC at position -836 to -845) a block mutation was introduced by PCR method which enabled the MEF2C site and some addition bases to be changed (CCCCGGAATTCCGG; former MEF2C DNA binding site is underlined). For PCR a GeneAmp PCR System 9700 was utilized and two primer pairs were necessary (**5'-**TCCGAGCTCGTTAATTTTTATTTTATTTGTATAGA**-3'** and **5'-**CCGGAATTCCGGGGTTTCTCTTTCTCTTAAGCTGG**-3'** as well as **5'-**CCCCGGAATTCCGGAAACCCCTACTTCTCACCCAAGAAAACACGC**-3'** and **5'-**CCCAAGCTTGGGGCTCTGCGGTATTGACGGACGCGGCC**-3'**). Utilizing these primer sets permitted two PCR products to be produced. The first PCR product had a 5' Sst1 site and a 3' EcoR1 site while the second PCR product had a 5' EcoR1 site and a 3' HindIII site. The PCR products were cut with restriction enzymes (Gibco) and ligated into 5'Sst1 and 3' HindIII sites of pXP1 using T4 ligase (NEB; Cat. M0202L). Mutation of the MEF2C site was confirmed by nucleotide sequencing performed by the DNA Core Facility at the University of Alberta (Edmonton, Alberta).



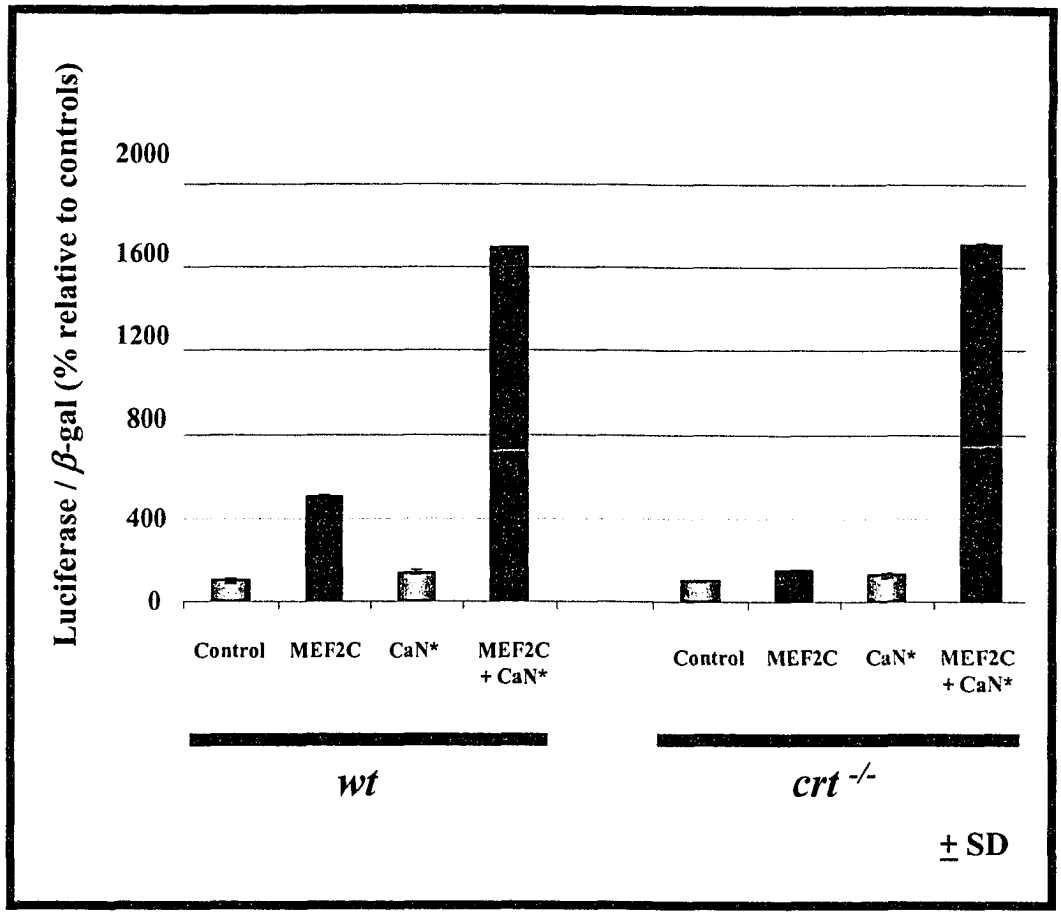
Appendix Figure 4. MEF2C activation of calreticulin promoter did not occur when MEF2C site within the promoter was mutated. NIH3T3, wild-type and calreticulin-deficient mouse embryonic fibroblasts were transfected with plasmid cDNA in an identical manner as described in Chapter 1. Data for luciferase and β-galactosidase activity assays were collected as described previously (Guo *et al.*, 2001; Waser *et al.*, 1997) using Lumat LB9501 and Molecular Devices microplate reader machines, respectively. In NIH3T3s as well as wild-type and calreticulin-deficient cells, MEF2C did not activate the reporter gene even when co-transfected with CaN*. Mean data are plotted relative to activity of the control. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated.

References:

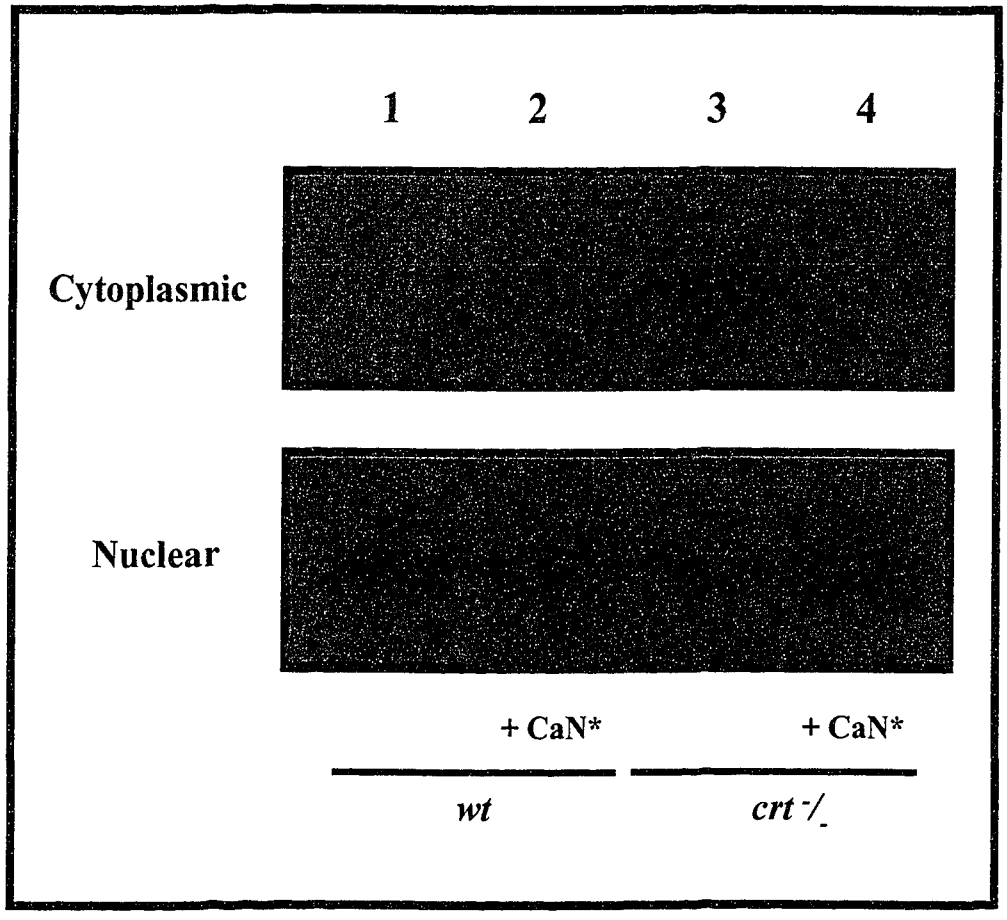
See Chapter 7.



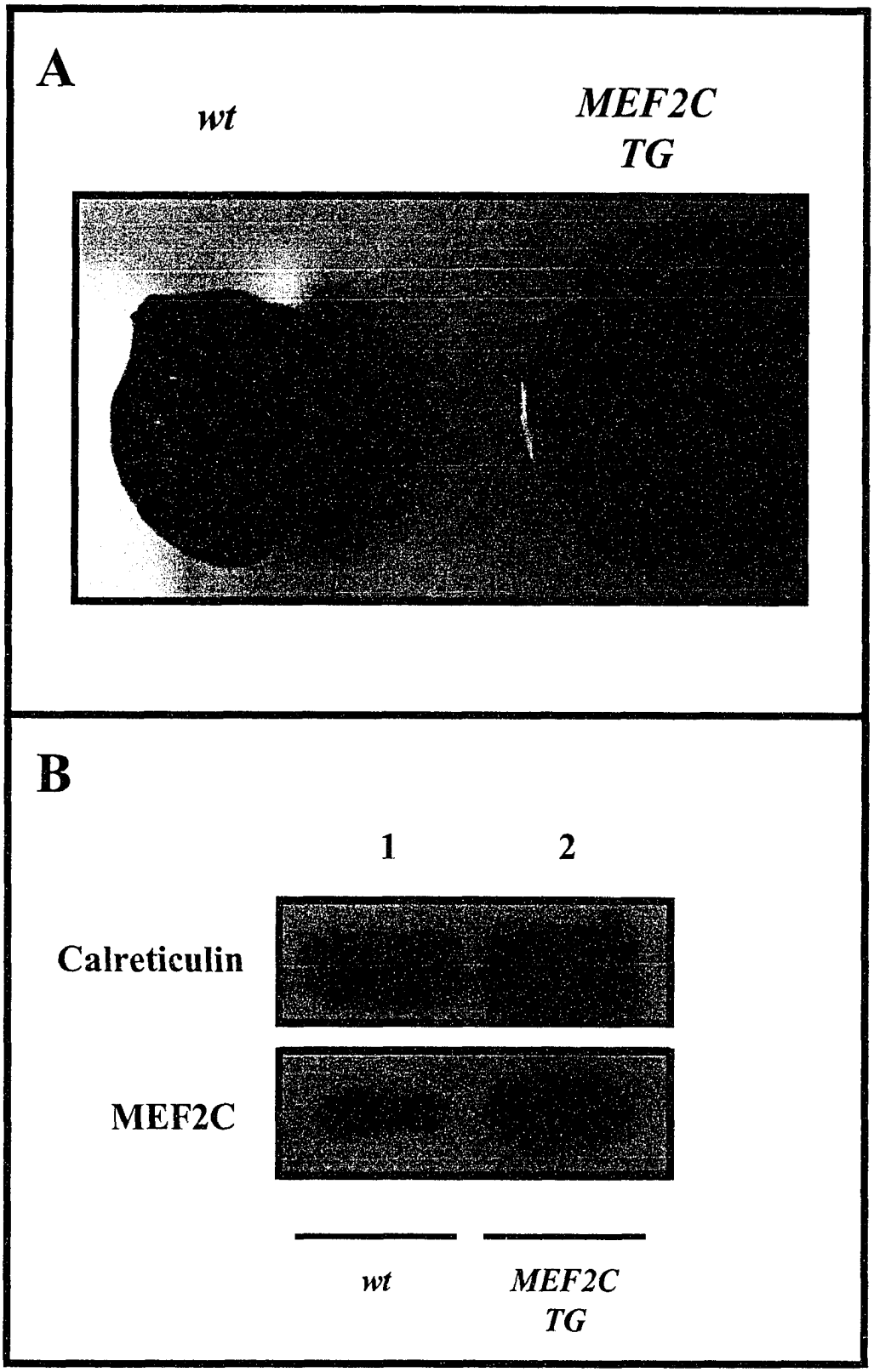
Appendix Figure 5. Activity of 6X MEF2C luciferase reporter gene in mouse embryonic fibroblasts is strongly influenced by the presence of CaN*. In wild-type mouse embryonic fibroblasts, MEF2C strongly activated the 6X MEF2C luciferase reporter gene and activation by MEF2C was synergistically enhanced by presence of CaN*. In comparison, in calreticulin-deficient cells MEF2C did not activate the 6X MEF2C reporter gene unless the cells were co-transfected with cDNA for CaN*. Mean data are plotted relative to activity of the control and respective control bars are present. Data are representative of a minimum of three independent experiments. Mean +/- standard deviation is indicated. The reporter plasmid had six ideal MEF2C binding sites upstream of a minimal promoter controlling a luciferase reporter gene. The 6X MEF2C reporter plasmid was a generous gift from Dr. Jeffrey Molkentin (Cincinnati's Children's Hospital, Cincinnati, Ohio, USA).



Appendix Figure 6. GFP-MEF2C immunoblot analysis indicated that GFP-MEF2C was predominantly nuclear in wild-type mouse embryonic fibroblasts and predominantly cytoplasmic in calreticulin-deficient mouse embryonic fibroblasts. In wild-type cells over 90% of GFP-MEF2C was found to be in nuclear extracts. In comparison, in calreticulin-deficient cells the majority of GFP-MEF2C was cytoplasmic and the amount of GFP-MEF2C in the nuclear extracts was much lower and varied from 15 to 38%. Wild-type and calreticulin-deficient mouse embryonic fibroblasts were transiently transfected with plasmid DNA containing cDNA encoding MEF2C using FuGENE 6 according to manufacturer instructions. 48 hours after transfection, cytoplasmic and nuclear proteins were isolated by a nuclear extraction kit (Panomics, Inc.; Cat. AY2002) as described by the manufacturer. Protein concentration of the extracts was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, California, USA) according to manufacture instructions. 30μg of each extract were loaded onto a 10% SDS-PAGE gel and separated at room temperature at 140 volts for 1 hour. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California, USA; Cat. 162-0115) and immunoblotted with polyclonal goat anti-GFP (1:5000 dilution) (generous gift from Dr. Luc Berthiaume, University of Alberta). Antibody binding was detected with peroxidase-conjugated rabbit anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, Inc.; Cat. 305-035-003) at a dilution of 1:10000 followed by ECL development reaction. Quantitative analysis was completed using a Kodak Digital Science Image Station 440CF with Kodak 1D v3.5.4 software (Kodak Scientific Imaging Systems, 4 Science Park, New Haven, Connecticut, USA).



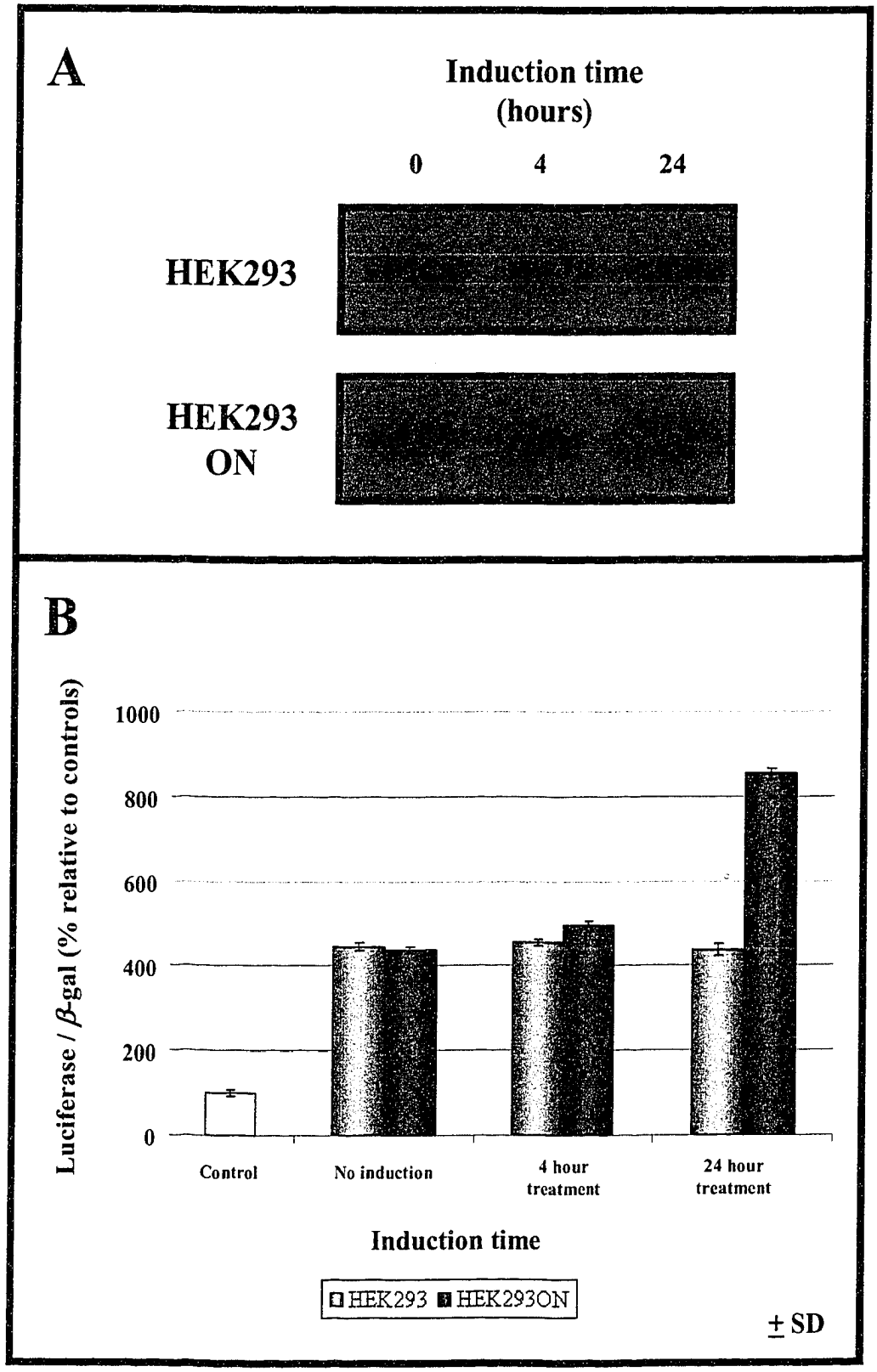
Appendix Figure 7. Two-month-old mice with cardiac over-expression of MEF2C have an increased concentration of calreticulin in the heart. *Panel A*: Gross morphology of two-month-old wild-type and MEF2C transgenic mouse hearts, and *Panel B*: Immunoblot with anti-calreticulin or anti-MEF2C antibodies indicated that the mice over-expressing MEF2C had approximately 53% more calreticulin and approximately 60% more MEF2C in the heart. Mouse MEF2C cDNA was cloned in the α-MHC promoter vector by blunt-end ligation. The vector was digested and a fragment containing the promoter and cDNA was purified and injected into newly fertilized oocytes for the standard generation of transgenic mice. The MEF2C transgenic line used in this study was created in the laboratory of Dr. Jeffery Molkentin and over-expresses approximately 50% more MEF2C compared with endogenous levels (unpublished results, Jian Xu and Jeffery D. Molkentin). These animals develop severe cardiac hypertrophy. For this investigation, hearts from two-month-old wild-type and MEF2C over-expresser male mice were isolated and immediately frozen in liquid nitrogen. Hearts were stored in 2mL eppendorf tubes at -80^0C until time of use. Hearts were homogenized in using a buffer described in Chapter 5 and protein concentration determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, California, USA) according to manufacture instructions. 30μg of each extract was mixed loaded onto a 10% SDS-PAGE gel and proteins were transferred to nitrocellulose membranes and immunoblotted. The primary antibody utilized was either polyclonal goat anti-calreticulin antibody or polyclonal rabbit anti-MEF2C antibody at a dilution of 1:500 and 1:1000, respectively. Following ECL development reaction, intensity of bands was determined.



Appendix Figure 8. HEK293 cells expressing more calreticulin have enhanced MEF2C activation of 6X MEF2C reporter gene. *Panel A*: Immunoblot of HEK293 and HEK293-ON (calreticulin over-expressing cells) shows an approximate 12% increase in calreticulin following 4 hours of induction and a 150% increase after 24 hours of induction, and *Panel B*: Ability of MEF2C to activate 6X MEF2C luciferase reporter gene increased following doxycycline induction of calreticulin. HEK293 cells inducible for full-length calreticulin have previously been described Arnaudeau *et al.*, 2002). These HEK293-ON cells as well as HEK293 cells were transiently transfected with cDNA encoding mouse MEF2C using FuGENE 6 reagent. 24 hours after transfection doxycycline (final concentration: 2 μg/mL) was added to some of the plates and 20 hours later doxycycline was added to additional plates of cells. 48 hours after the initial time of transfection, doxycycline treated and untreated HEK293 and HEK293-ON cells were harvested. Protein concentration was determined by Bio-Rad DC Protein Assay. 30μg of protein was loaded onto 10% SDS-PAGE gels and later transferred to nitrocellulose. Immunoblotting was performed using polyclonal rabbit anti-MEF2C antibody at a dilution of 1:1000 or polyclonal goat anti-calreticulin antibody at a dilution of 1:500. The secondary antibodies utilized for these imunoblots were diluted 1:10000 and were goat anti-rabbit horse radish peroxidase and rabbit anti-goat horse radish peroxidase (Jackson ImmunoResearch Laboratories, Inc.), respectively. ECL development reaction was completed and the intensity of bands determined by Kodak 1D v3.5.4 software.

Reference:

Arnaudeau, S., Frieden, M., Nakamura, K., Castelbou, C., Michalak, M., Demaurex, N. (2002). Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. J Biol Chem *277*, 46696-46705.



Appendix Figure 9. 13-day-old mice with cardiac over-expression of calreticulin have an increased concentration of MEF2C. *Panel A*: Gross morphology of 13-day-old wild-type and calreticulin transgenic mouse hearts, and *Panel B*: Immunoblot with anti-calreticulin or anti-MEF2C antibodies indicated that the mice over-expressing calreticulin had approximately 80% more calreticulin and approximately 50% more MEF2C in the heart. Calreticulin transgenic mice have previously been described (Nakamura *et al.*, 2001a). This experiment was completed in a similar manner as described for Appendix Figure 7.

Reference:

See Chapter 7.