BIOCHEMICAL AND FUNCTIONAL ANALYSIS OF MEMBERS OF THE MYOCARDIN FAMILY DURING CARDIOVASCULAR DEVELOPMENT

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To

My Family

To
My Husband, Sang-Won Min

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The various stages of muscle development are characterized by distinct patterns of gene expression precisely controlled by combinatorial interaction between a large number of muscle-specific and ubiquitous transcription factors.

Myocardin is a cardiac and smooth muscle-specific transcriptional coactivator of serum response factor (SRF) that forms a ternary complex with SRF on DNA and provides its strong transcriptional activation domain (TAD) to SRF. SRF has been shown to stimulate expression of smooth and cardiac muscle genes in association with GATA transcription factors, which play important roles in cardiac and smooth muscle development. I show that

GATA transcription factors can either stimulate or suppress the transcriptional activity of myocardin, depending on the target gene. Modulation of myocardin activity by GATA4 is mediated by the physical interaction of myocardin with the DNA binding domain of GATA4 but does not require binding of GATA4 to DNA. The ability of GATA transcription factors to modulate myocardin activity provides a potential mechanism for fine tuning the expression of serum response factor target genes in a gene-specific manner.

Two Myocardin Related Transcription Factors, referred to as MRTF-A and B, are expressed in numerous embryonic and adult tissues, implying their potential to modulate SRF target genes in a wide range of tissues. To determine the functions of MRTF-B *in vivo*, I generated MRTF-B mutant mice by targeted inactivation of the *MRTF-B* gene. I show that mice homozygous for an *MRTF-B* loss-of-function mutation die during mid-gestation from a spectrum of cardiovascular defects. These abnormalities are accompanied by a failure in differentiation of smooth muscle cells within the branchial arch arteries, which are derived from the neural crest. The phenotype of MRTF-B mutant mice is distinct from that of mice lacking myocardin and MRTF-A, revealing unique roles for these SRF coactivators in the development of different subsets of smooth muscle cells *in vivo*.

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LIST OF PUBLICATIONS

1. **Oh JY**, Eun YM, Yoo SJ, Seol JH, Seong IS, Lee CS, Chung CH (1998). LonR9 carrying a single Glu614 to Lys mutation inhibits the ATP-dependent protease La (Lon) by forming mixed oligomeric complexes. *Biochem Biophys Res Commun.* 250:32-5.

2. Seong IS, **Oh JY**, Yoo SJ, Seol JH, Chung CH (1999). ATP-dependent degradation of SulA, a cell division inhibitor, by the HslVU protease in *Escherichia coli*. *FEBS Lett.* 456:211-4.

3. Seong IS, **Oh JY**, Lee JW, Tanaka K, Chung CH (2000). The HslU ATPase acts as a molecular chaperone in prevention of aggregation of SulA, an inhibitor of cell division in *Escherichia coli. FEBS Lett.* 477:224-9.

4. **Oh J***, Wang Z*, Wang DZ*, Lien CL, Xing W, Olson EN (2004). Target gene-specific modulation of myocardin activity by GATA transcription factors. *Mol Cell Biol.* 24:8519-28. (*contributed equally)

5. Cao D, Wang Z, Zhang CL, **Oh J**, Xing W, Li S, Richardson JA, Wang DZ, Olson EN (2005). Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. *Mol Cell Biol.* 25:364-76.

6. **Oh J**, Richardson JA, Olson EN (2005). Requirement of myocardin-related transcription factor-B for remodeling of branchial arch arteries and smooth muscle differentiation. *Proc Natl Acad Sci U S A.* 102:15122-7.

7. Creemers EE, Sutherland LB, **Oh J**, Barbosa AC, Olson EN (2006). Coactivation of MEF2 by the SAP domain proteins myocardin and MASTR. *Mol Cell.* 23:83-96.

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LIST OF ABBREVIATIONS

ANF	atrial natriuretic factor
bHLH	basic helix-loop-helix
cDNA	complementary DNA
Cf	C-terminal zinc finger
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
DN	dominant-negative
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione S-transferase
IP	immunoprecipitation
MADS box	MCM1, Agamous, Deficiens, and SRF box
MAP (kinase)	mitogen activated protein kinase
MRTF	myocardin-related transcription factor
MEF2	myocyte enhancer factor 2
MRF	muscle regulatory factor
Nf	N-terminal zinc finger
NLS	nuclear localization signal
PBS	phosphate-buffer saline

PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PMSF	Phenymethylsulfonyl fluoride
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SM	smooth muscle
SMC	smooth muscle cell
SM22	smooth muscle 22 kDa protein
SRF	serum response factor
TAD	transcription activation domain
TCF	ternary complex factor
Tg	transgenic
tk	thymidine kinase
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Chapter I

Introduction: Transcriptional Control of Muscle Development

Introduction

The three types of muscles in vertebrates are skeletal muscle, cardiac muscle and smooth muscle. These different muscles share common properties, mainly supporting the body, providing contractility and excitability, and express overlapping sets of muscle structural genes. However, each of them is unique in many respects, including the spectrum of muscle gene isoforms expressed, morphology, contractile properties and the ability to divide.

During vertebrate embryogenesis, skeletal, cardiac and smooth muscle cells arise from distinct mesodermal precursors in different regions of the embryo [1]. Skeletal muscle arises from the somites which form in a rostrocaudal progression by segmentation of the paraxial mesoderm lateral to neural tube. Somites initially appear as epithelial spheres, which subsequently compartmentalize to form dermamyotome and sclerotome. The sclerotome is comprised of mesenchymal cells that eventually give rise to ribs and vertebrae. Cells from dorsomedial region of the dermamyotome adjacent to neural tube later form the myotome, which gives rise to the vertebral and back muscles. Cells from the ventrolateral region of the dermamyotome migrate out to form limb muscle and body wall muscle [2].

Cardiac muscle is derived from cells in the anterior lateral plate mesoderm which are committed to the cardiogenic fate soon after gastrulation. After their specification, cardiac muscle cells converge along the ventral midline to form the primitive linear heart tube, which undergoes looping and chamber maturation to form the mature four chambered heart [3].

Unlike cardiac and skeletal muscles, which arise from distinct embryonic origins, smooth muscle arises from multiple, not very well defined areas throughout the embryo. For

example, portions of smooth muscle cells in the great vessels (systemic aorta, pulmonary arteries and carotid arteries) come from neural crest cells; the coronary artery smooth muscle cells arise from the proepicardial origin; and the majority of smooth muscle cells, including those of visceral organs, are derived from local mesenchymal cells [4, 5].

Recently, there has been dramatic progress toward understanding the molecular mechanism controlling skeletal muscle development, and to some extent, cardiac muscle development, whereas the transcriptional control of smooth muscle development has only begun to be elucidated.

Skeletal Muscle Development

Embryonic skeletal muscle development has become a paradigm for understanding the molecular basis of how cell lineages are established and how cells differentiate into specialized structures. During anterior-posterior axis formation, epithelial condensations of paraxial mesoderm give rise to somites in pairs on either side of the neural tube/notochord. Within several hours after epithelialization, cells of the ventral somite undergo an epithelial-mesenchymal transformation to produce sclerotome progenitors that form vertebrae or migrate ventrally to form ribs. Dorsal somite cells form the dermomyotome, a sheet of columnar cells that gives rise to progenitor lineages for all of the body musculature as well as to dermal progenitors [2, 7]. Medial dermomyotome cells at the dorsal medial lip (DML) produce epaxial muscle progenitors that migrate ventrolaterally to form the vertebral and back muscles (Fig. 1.1). Lateral dermomyotome cells at the ventral lateral lip (VLL) are the source of progenitor lineages for the ventral aspect of the myotome, as well as migratory

progenitors from interlimb somite that form hypaxial muscle at ventral sites (Fig. 1.1). Also, VLL progenitors from limb level somites migrate into limb buds to form limb muscles.

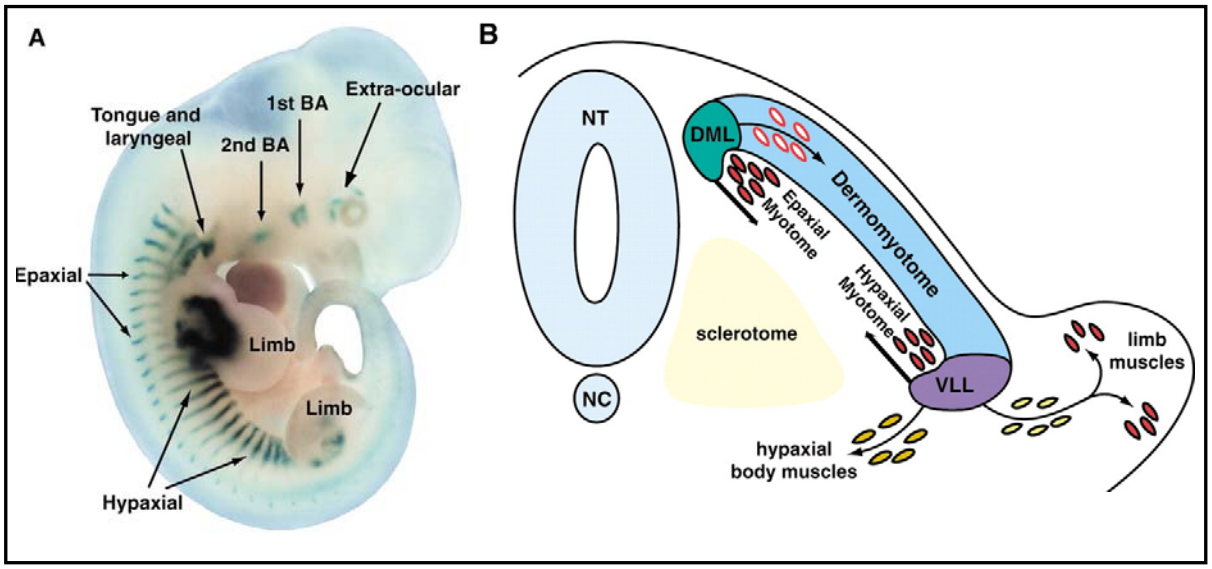

Figure 1.1. Mouse embryonic skeletal muscle development. (*A*) Myogenic progenitors in the mouse embryo at E11.5, as visualized through expression of the *MyoD* Core Enhancer/*LacZ* reporter. *MyoD* transgene expression is localized to trunk somite progenitors at the sites of epaxial, hypaxial, and limb muscle differentiation, and the head mesoderm progenitors, including the first and second branchial arches (BA), the tongue and larynx, and the extra-ocular muscles. (*B*) Somite origins of myogenic progenitors for epaxial, hypaxial, and limb muscles in mouse embryos. Muscle progenitors originate in the dorsal-medial and ventral-lateral lips of the dermomyotome. Cells of the dorsal-medial lip (DML) migrate ventrolaterally, differentiate, and form the myotomal muscles, which will give rise to epaxial deep back muscles. The ventral-lateral lip (VLL) provides progenitors that migrate ventrally to form the ventral body wall muscles (*orange*); that migrate dorsolaterally to form the hypaxial myotome; and that delaminate from the VLL and migrate to the dorsal and ventral muscle-forming regions of the limb (*yellow*) where they differentiate to form the limb musculature (*red*). NT, neural tube; NC, notochord (Adapted from Pownall et. al., 2002)

Skeletal muscle differentiation is accompanied by cell cycle arrest, fusion of individual myoblasts into multinucleate myotubes, and the transcriptional activation of muscle-specific genes. Paracrine factors instruct these myotome cells to become muscles. Wnts proteins (Wnt1 and Wnt3a) from the dorsal neural tube, in combination with low concentrations of Sonic hedgehog from the notochord and floor plate, induce the epaxial myotome, while Wnt proteins from the epidermis, in conjunction with BMP4 and FGF5 from the lateral plate mesoderm, are thought to induce the hypaxial myotome [6, 7]. The myotome cells produce myogenic bHLH transcription factors and become committed muscle cell precursors, myoblasts. These cells then align with each other and fuse to form the multinucleated myotubes characteristic of muscle tissue. Thus, the multinucleated myotube cells are the product of several myoblasts joining together and fusion of their cell membranes [8, 9]. The specification, proliferation and terminal differentiation of skeletal muscle cells is controlled by the combinatorial activities of several transcriptional factors. Identification and isolation of regulatory genes involved in controlling specific transcriptional events has led to greater understanding of the molecular mechanisms that control myogenesis in the embryo. In particular, the muscle regulatory factor (MRF) and myocyte enhancer factor 2 (MEF2) families have been implicated in establishing the myogenic lineage, as well as controlling terminal differentiation. The transcription factors that play important roles during myogenesis are discussed below.

Myogenic bHLH proteins The myogenic regulatory factors (MRFs) are part of a superfamily of basic helix-loop-helix (bHLH) transcription factors. The MRF subfamily, pivotal in skeletal muscle biology, consists of MyoD, myogenin, myf5 and MRF4, which are

expressed exclusively in skeletal muscle [9, 10]. The MRF proteins contain a conserved basic DNA-binding domain and a helix-loop-helix motif required for heterodimerization. These proteins form heterodimers with ubiquitously bHLH proteins known as E-proteins, to bind to and regulate expression from a consensus DNA sequence CANNTG (E-box), a specific DNA motif present in the control regions of most skeletal muscle genes. These myogenic proteins have the striking ability to activate the entire skeletal muscle differentiation program when introduced into a variety of non-muscle cells, including cells from all three germ layers. Thus, they are considered to be the "master regulators" of skeletal muscle differentiation. Their roles in muscle development have been confirmed by gene knockout studies. MyoD and myf5 play redundant roles in the specification and proliferation of myoblasts, whereas myogenin and MRF4 controls myoblast differentiation and myofiber maturation (Fig. 1.2) [1, 9].

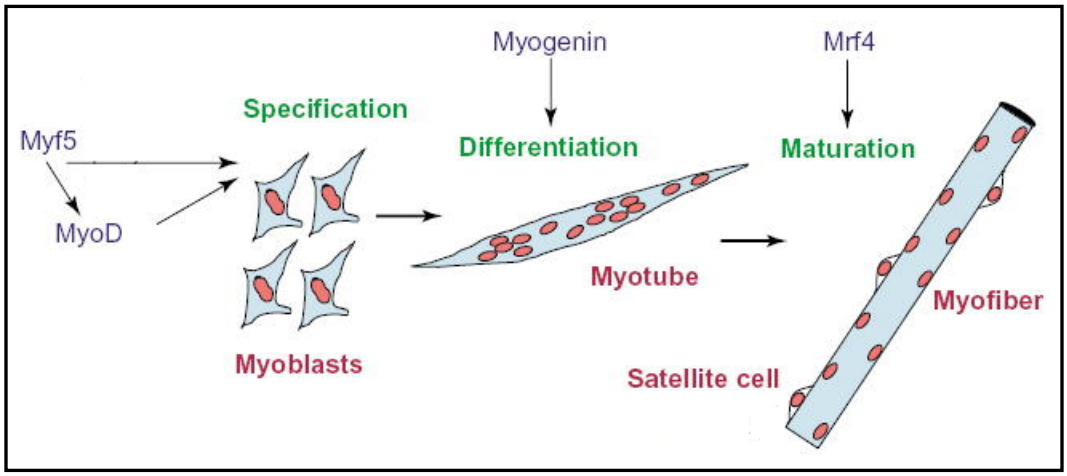

Figure 1.2. Hierarchical relationship of muscle regulatory factors. Myf5 can activate MyoD. Myf5 and MyoD act as specification genes, whereas myogenin is required for differentiation of specified myoblasts into myotubes, and MRF4 is involved in myofiber formation. (Adapted from Sartorelli et. al., 2005)

MEF2 proteins Another family of transcription factors that plays essential roles in skeletal muscle development is the MEF2 family of proteins, including MEF2 A-D in vertebrates, which are highly enriched in all three muscle lineages [11, 12]. MEF2 proteins belong to the MADS box family of transcription factors. MADS box is named after the first four proteins in which this domain is identified, including MCM1, which regulates mating type-specific gene expression in yeast; Agamous and Deficiens, which act as homeotic factors that control flower development; and Serum Response Factor (SRF), which controls serum-inducible and muscle-specific gene expression [13]. The MADS box of MEF2 proteins binds to a consensus DNA sequence YTA$(A/T)_4$TAR, termed the MEF2 site, which has been found in a variety of muscle-specific promoters, and in many cases to be essential for muscle-specific expression. The definitive proof of the requirement of MEF2 for muscle development comes from the inactivation of the only MEF2 gene, *D-mef2* in flies. In the mutant fly, the precursor cells for all three muscle lineages are specified and positioned normally, but failed to form normal differentiated muscles, suggesting an obligatory role of MEF2 in muscle differentiation [14].

Importantly, the myogenic bHLH proteins and MEF2 proteins physically interact and the interaction precisely correlates with their myogenic activity [15]. Furthermore, myogenic bHLH proteins upregulate their own expression and that of MEF2 factors. MEF2 proteins then feedback on myogenic bHLH factors and themselves to amplify and maintain their expression in committed muscle cell, thus establishing a mutually reinforcing regulatory circuit for skeletal myogenesis (Fig. 1.3) [11].

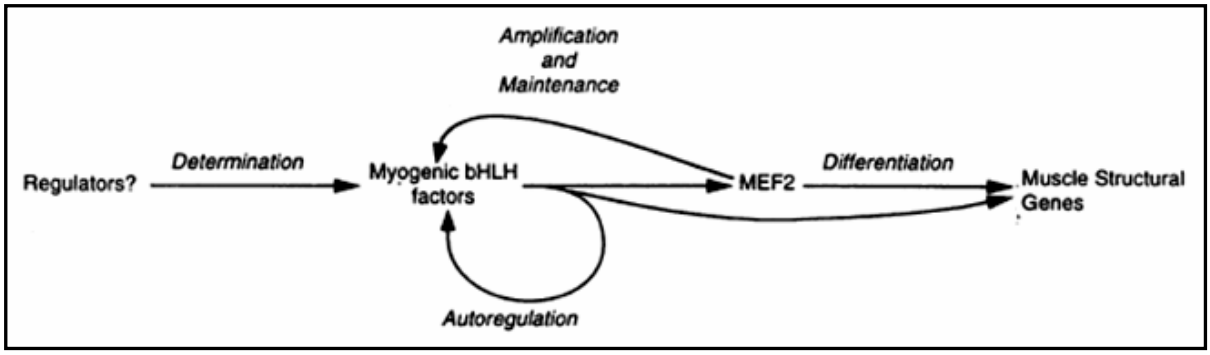

Figure1.3. Combinatorial control of muscle development by bHLH and MEF2. MEF2 and the myogenic bHLH proteins regulate the expression of each other. The first events in myogenesis are the determination of mesodermal progenitor cells to the myogenic lineage which express myogenic bHLH proteins. The myogenic bHLH proteins then directly activate a number of muscle-specific strutural genes as well as other transcription factors. One of these factors is MEF2, which can then feedback on the promoters of the myogenic bHLH genes to amplify and maintain their expression as well as to directly upregulate a number of skeletal muscle-specific structural genes. Together, these two factors amplify the expression of each other to augment muscle-specific gene expression. (Adapted from Molkentin et al., 1996)

Heart Formation and Cardiac Muscle Development

The circulatory system, consisting of a heart, blood cells and an intricate system of blood vessels, is the first functional unit in the developing embryo, and the heart is the first functional organ. Heart formation requires complex interactions among cells from multiple embryonic origins. Many of the genes within the pathways that control cardiac morphogenesis are conserved across vast phylogenetic distances, which has allowed cardiac development to be dissected in organisms ranging from flies to mammals. Cardiomyocytes originate in the anterior lateral mesoderm soon after gastrulation [16]. They are produced in response to protein factors, including bone morphogenetic proteins, which are secreted from adjacent endoderm (Fig. 1.4.*B*) [17]. Soon after their specification, cardiac muscle cells

converge along the ventral midline of the embryo to form a beating linear heart tube composed of distinct myocardial and endocardial layers separated by an extracellular matrix (Fig. 1.4.*A*). The linear heart tube then undergoes rightward looping, which is essential for proper orientation of the pulmonary (right) and systemic (left) ventricles, and for alignment of the heart chambers with the vasculature. Each cardiac chamber balloons out from the outer curvature of the looped heart tube in a segmental fashion. The four chambers then form and are separated by atrial and ventricular septa [3, 18].

In contrast to skeletal muscle, in which a single transcription factor, MyoD, is sufficient to activate the entire program of muscle differentiation, cardiac muscle differentiation is dependent on combinations of transcription factors [18, 19, 20].

Homeobox proteins The homeobox gene *tinman* is required for the formation of the primitive heart in the fruit fly. A mammalian ortholog of tinman, called Nkx2-5 or Csx, is expressed in cardiac muscle cells from the onset of embryonic heart formation until adulthood. Tinman in flies is necessary for specification of cardiac lineage and directly activates D-mef2 expression [21, 22]. But in contrast to tinman, Nkx2.5 in mice, although highly conserved and restricted to the cardiac lineage, is not necessary for cardiac specification, instead it is required for proper cardiac looping and left ventricle development [23, 24], suggesting that other homeobox genes may play redundant roles.

GATA factors Tinman and Nkx2.5 interact with zinc finger transcription factors of the GATA family to activate cardiac gene expression [25]. These two classes of transcription factors also regulate each other's expression through mutually reinforcing positive feedback loops [26]. The fly GATA gene *pannier* is required for cardiogenesis [27]. In mice, loss of

GATA-4 leads to bilateral heart tubes and a reduced number of cardiomyocytes [28]. The less severe phenotype in mice can be partly attributed to the expression of other GATA factors (GATA 5 and 6) in the mouse heart.

MEF2 proteins MEF2 proteins have been found to interact with Nkx2.5 and GATA factors to synergistically activate expression of a variety of cardiac genes [12]. Mutation of the only MEF2 gene in flies, *D-mef2*, led to differentiation defects in all three muscle lineages. This is partly recapitulated by the deletion of the *MEF2C* gene in mice, which develop hypoplasia of the right and left ventricles and vascular defects, leading to early embryonic lethality [29]. Deletion of the *MEF2A* gene in mice leads to a mitochondrial deficiency and cardiac sudden death [30].

HAND proteins During mouse heart development , the related bHLH transcription factors dHAND/HAND2 and eHAND/HAND1 are expressed predominantly in the right and left ventricle, respectively [31]. Deletion of *dHAND/HAND2* in the heart results in hypoplasia of the right ventricle [32]. *eHAND/HAND1* has also been implicated in left ventricle development, although early placenta defects precluded a detailed analysis of its role in the heart [33, 34]. This is corroborated by a conditional knockout of *eHAND/HAND1* in the heart [35]. The role of HAND proteins in heart development is further confirmed in zebrafish. Loss of the only *HAND* gene in zebrafish abolishes ventricle development [36].

More and more transcription factors have now been implicated in the network of regulating cardiac gene expression. It is increasingly evident that the combination of different factors determines the specificity and intensity of gene expression. However, so far no single

factor or a combination of factors is able to activate the entire cardiac gene program in non-muscle cells.

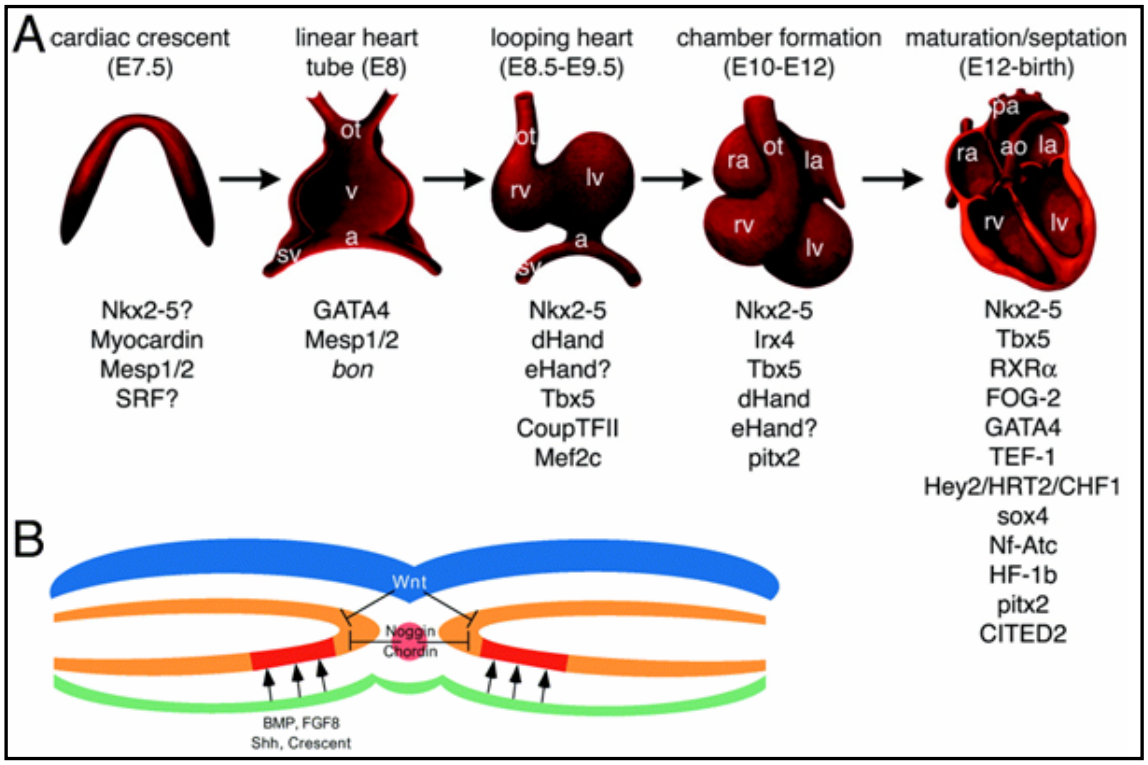

Figure 1.4. Schematic of mouse heart development. *(A)* Five major stages of heart development are shown: 1) cardiac crescent formation at embryonic day (E) 7.5; 2) formation of the linear heart tube at E8; 3) looping and the initiation of chamber morphogenesis at E8.5 to E9.5; 4) chamber formation; and 5) chamber maturation and septation and valve formation. The transcription factors involved or suspected of involvement in these processes are listed below each stage. ao, aorta; a, atrium; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle; ot, outflow tract; sv, sinus venosa; and pa, pulmonary artery. (Adapted from Bruneau, 2002) *(B)* Spatial relationship between cardiac inducing factors, which are mainly secreted by pharyngeal endoderm (green) and inhibitory factors such as canonical Wnt signals that are secreted by ectodermal tissue (blue) and Noggin and Chordin that are expressed in the notochord (pink). Cardiogenic mesoderm (red) is only formed in splanchnic mesoderm, which remains in close contact to pharyngeal endoderm. (Adapted from Brand, 2003)

Blood Vessel Formation and Smooth Muscle Development

Although the heart is the first functional organ of the body, it does not even begin to pump until the vascular system of the embryo has established its first circulatory loops of blood vessels. Blood vessels are constructed by two processes, vasculogenesis and angiogenesis. During vasculogenesis, blood vessels are created de novo from the lateral plate mesoderm. The splanchnic mesoderm cells are specified to become hemangioblasts, common precursors of both the blood cells and the blood vessels. These cells condense into aggregations called blood islands. The inner cells of these blood islands become hematopoietic stem cells, while the outer layer cells become angioblasts, the progenitor cells of the blood vessels. The angioblasts then multiply and differentiate into endothelial cells, which form the lining of the blood vessels. Finally the endothelial cells form tubes and connect to form the primary capillary plexus, a network of capillaries. This process of primary vascular network formation occurs both within the embryo and in extra-embryonic tissue, the yolk sac [37-39].

After the phase of vasculogenesis, angiogenesis begins. New vessels form by sprouting from the capillaries or splitting an existing vessel. The capillaries also fuse with each other to form wider vessels, the arteries and veins. Vessel maturation requires the subsequent recruitment of surrounding mesenchymal cells and their differentiation into vascular smooth muscle cells. This process has been shown to involve the interaction of endothelial cells with mesenchymal cells and the release of specific growth factors such as platelet-derived growth factor [40, 41].

Therefore, endothelial differentiation is an early event followed by the formation of primitive tubes. The subsequent recruitment and differentiation of vascular smooth muscle cells is a later event leading to the formation of stable blood vessels. Growth factors including platelet-derived growth factor, βFGF, VEGF, angiopoietin-1, and transforming growth factor-ß (TGF-ß) are key mediators of these events promoting proliferation, differentiation and migration of these cells. A series of transcription factors are either key regulators of the expression of either the growth factors or their receptors, or mediators of the cellular responses to these growth factors, as summarized in Fig. 1.5 [42].

Smooth muscle cells have a complex origin depending on their location, such as splanchnic mesoderm, epicardial cells and neural crest cells. One family of transcription factors that is crucial for smooth muscle development, in general, is the MEF2 transcription factor family. For example, MEF2C has recently been shown to be important in vascular development and in smooth muscle cell differentiation. Targeted disruption of MEF2C leads to abnormalities in smooth muscle cell differentiation and the inability of endothelial cells to form into vascular structures [43, 44]. However, the breakthrough in revealing the mechanism of smooth muscle differentiation is identification of the *cis*-elements that control smooth muscle-specific gene expression. Using a transgenic mouse approach, virtually every smooth muscle specific gene analyzed to date contains two or more CArG boxes in its control region, and mutation of these CArG elements abolishes smooth muscle specific gene expression [45]. The CArG box sequence $CC(A/T)_6GG$ is the binding site for Serum Response Factor (SRF), which is also a MADS box transcription factor.

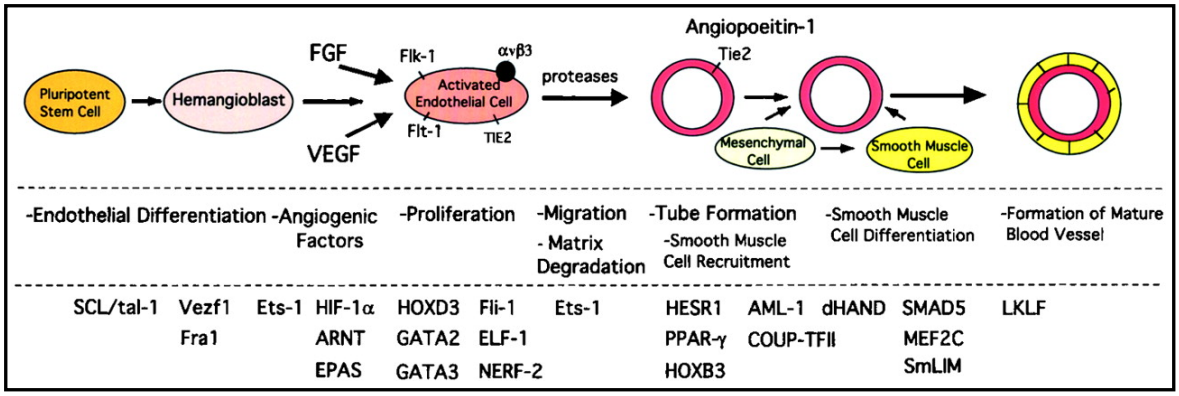

Figure 1.5. Role of transcription factors during different stages of vascular development. (Adapted from Oettgen, 2001)

Serum Response Factor and its Role during Muscle Development

SRF was first identified for its ability to bind CArG box and confer serum inducibility to the growth-responsive gene *c-fos* [46]. Later it was found to regulate other immediate early genes, such as *fosB, junB* and *egr-1*, and numerous muscle-specific genes, such as *α-myosin heavy chain, cardiac and skeletal α-actin*, etc. [47]. SRF, a member of the MADS box family of transcription factors, contains a DNA binding domain, a transactivation domain and several phosphorylation sites. Overexpression of SRF in the heart causes hypertrophic cardiomyopathy in transgenic mice, and inhibition of SRF by microinjection of SRF antibody or expression of antisense RNA suppresses muscle gene expression and blocks differentiation of myoblasts to myotubes [48, 49]. The requisite role of SRF in smooth muscle (SM) gene expression has been demonstrated by the ability of dominant negative SRF mutants to prevent smooth muscle cell (SMC) differentiation [50]. However, *SRF* null mice die at early gastrulation and fail to form mesoderm, prohibiting a detailed analysis of its role in the development of each muscle lineage [51]. Several groups recently have generated conditional *SRF* null alleles allowing for temporal and spatial specificity of gene deletion in

the mouse [52-54]. Cardiac-specific ablation of *SRF* results in embryonic lethality from cardiac defects with disrupted cardiac sarcomerogenesis [54], and deletion of the gene in smooth muscle results in embryonic lethality from a deficiency of differentiated smooth muscle cells [53]. Mice lacking skeletal muscle expression of SRF died during the first few days after birth with a severe skeletal muscle myopathy characterized by a deficiency in muscle growth [73]. These findings reveal an essential role for SRF in the development of each muscle lineage.

How ubiquitously expressed SRF achieves muscle-specific gene activation, how SRF coordinates the activation of both growth genes and muscle genes, which normally belong to exclusive gene programs, and how SRF potentiates gene transcription with its weak transcription domain, are still unanswered questions. One of the explanations for these questions lies at the ability of SRF to interact with different cofactors to regulate different sets of genes. For example, SRF interacts with members of ternary complex factor (TCF) family of the Ets domain transcription factors to activate *c-fos* gene expression upon growth factor stimulation [55]. On the other hand, it cooperates with the cardiac-restricted transcription factors GATA4 and Nkx2.5 to activate cardiac specific gene expression [56, 57]. No smooth muscle or skeletal muscle specific SRF cofactors had been identified until the myocardin family was revealed as potent coactivators of SRF, specifically acting on muscle-specific SRF target genes.

Myocardin Family of SRF Cofactors

The fact that SRF, which is expressed ubiquitously, is required for the expression of muscle genes suggests that muscle-specific SRF cofactors contribute to the muscle-specificity of SRF target genes. Myocardin, the founding member of a family of extraordinarily powerful myogenic SRF coactivators, was discovered in a bioinformatics screen for novel cardiac-restricted genes by Da-zhi Wang, a former postdoctoral fellow in Dr. Eric Olson's lab [58]. Myocardin stimulates SRF activity by forming a ternary complex with SRF on DNA and providing its strong transcription activation domain (TAD) to SRF, which by itself is a very weak activator of transcription. Myocardin preferentially activates promoters containing two or more CArG boxes, an observation consistent with the known cooperativity of CArG boxes associated with muscle genes. Two additional members of the myocardin family, referred to as MRTF-A (MAL, MKL1, BSAC) and MRTF-B (MKL2) have also been identified in mammals [59-62], whereas a single member of myocardin family, DMRTF, exists in Drosophila [63].

Consistent with its discovery as a novel cardiac-restricted gene, myocardin expression is largely restricted to the cardiovascular system. In mouse embryos, the onset of myocardin expression in the cardiac crescent at E7.75 coincides with that of Nkx2-5, the earliest known marker of the cardiac lineage [58]. Thereafter, myocardin is expressed throughout the heart, as well as in a subset of vascular and visceral SMCs within the cardiovascular system and internal organs, but not in skeletal muscle. MRTF-A and -B are expressed widely during embryogenesis and in adulthood [59], suggesting that those factors might be involved in other biological processes in addition to muscle cell differentiation.

Myocardin family members share homology in multiple functional domains and belong to the SAP (SAF-A/B, Acinus, PIAS) family of proteins, which play diverse roles in chromatin remodeling, transcriptional control, and fragmentation of DNA during apoptosis [64]. The 35-amino acid SAP domain has been predicted to adopt a helix-linker-helix structure with the potential to bind to DNA. Interestingly, deletion of the SAP domain of myocardin abolishes the ability to activate the atrial natriuretic factor (ANF) gene, a marker of the cardiac stress response, without affecting the activation of the *SM22* gene [58]. This finding suggests that the SAP domain of myocardin may discriminate between SRF target genes, possibly by differential association with additional factors. Myocardin and MRTFs contain a conserved N-terminal domain composed of RPEL repeats that have been implicated in Rho-dependent nuclear import of MRTF-A [65]. Deletion of the N-terminal domain of myocardin or MRTFs enhances the transcriptional activity of the proteins. The basic domain of myocardin family proteins has been shown to mediate nuclear localization, as well as SRF interaction [3], and a leucine zipper-like domain mediates homo- or heterodimerization among myocardin family members [65, 66]. Dimerization may provide a mechanism for interlinking myocardin or MRTF/SRF complexes bound at different CArG boxes within the control regions of SM genes. Myocardin is a highly potent transcriptional activator and the transactivation domain resides at the C-terminus of the protein. Myocardin and SRF form a ternary complex with DNA fragments containing a CArG box, and the interaction of myocardin and SRF is also detectable by co-immunoprecipitation assay [58]. Myocardin requires at least two CArG boxes within the responsive promoter region for optimal

transcriptional activity. The functional domains of myocardin family members and the mechanism of activation of gene transcription are summarized in Fig. 1.6 [67].

Expression of a dominant-negative myocardin mutant in *Xenopus* embryos blocks heart formation, suggesting that myocardin cooperates with SRF to activate cardiac gene expression [58]. Conversely, ectopic expression of myocardin in *Xenopus* embryos results in activation of cardiac gene expression throughout the embryo [68]. Interestingly, when overexpressed in ES cells and nonmuscle cells *in vitro*, myocardin activates smooth but not cardiac muscle gene expression [66, 69–71]. MRTF-A and MRTF-B also can activate smooth muscle gene expression in transfected fibroblasts, which seems paradoxical given that MRTFs are expressed in a wide range of nonmuscle cell types [66]. MRTF-A also has been implicated in skeletal muscle differentiation *in vitro*. Si-RNA for MRTF-A, as well as a dominant negative form of MRTF-A have been shown to block differentiation of the C2C12 skeletal muscle cell line *in vitro* and prevent activation of SRF-dependent muscle genes [72], suggesting that these factors are required for SRF-dependent activation of skeletal muscle genes. Similarly, transgenic expression of dominant-negative MRTF-A in skeletal muscle *in vivo* results in abnormally thin muscle fibers with significant fibrosis, similar to the myopathy observed in mice lacking *SRF* in skeletal muscle [73].

A homozygous null mutation of myocardin in mice results in embryonic lethality at embryonic day 10.5 (E10.5) accompanied by a loss of vascular smooth muscle gene expression, but no apparent decrease in cardiac gene expression [74]. Mice lacking *MRTF-A* are viable and display an intriguing defect specific to lactating females in which mammary myoepithelial cells, which are similar to smooth muscle cells and required for milk ejection,

fail to differentiate and undergo apoptosis during lactation. As a result, female *MRTF-A* mutant mice fail to nurture their offspring [75].

Both *in vivo* and *in vitro* studies on myocardin and MRTFs strongly suggest their involvement in the development of all three muscle lineages. From the highly restricted expression pattern of myocardin and the broad but differential expression patterns of MRTF-A and B, they are likely to perform unique roles in a subset of tissues, as well as redundant roles where their expression profile overlaps. This thesis mainly focuses on the functional roles of members of the myocardin familiy in cardiovascular development, specifically:

(1) To examine the functional interaction between myocardin and GATA *in vitro*.

(2) To analyze the functions of myocardin related transcription factors *in vivo*.

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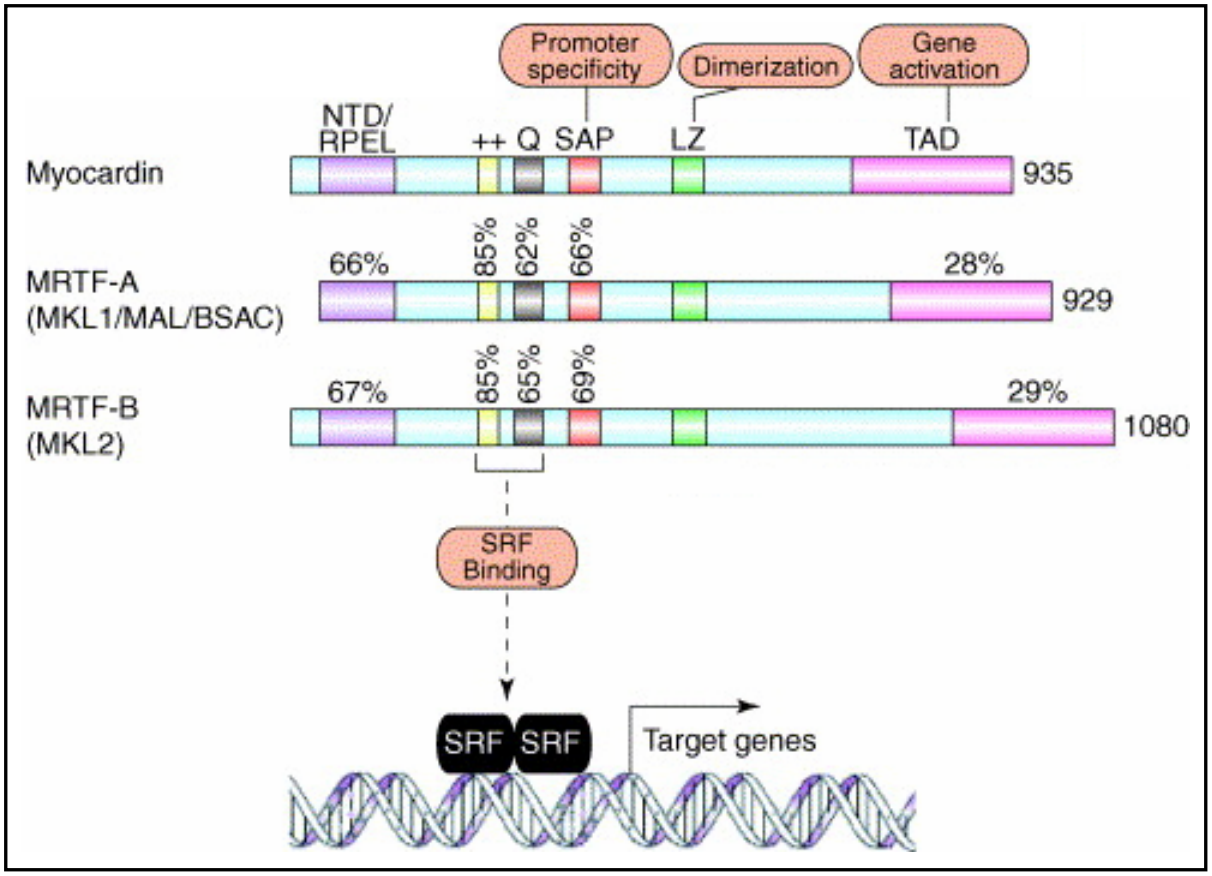

Figure 1.6. Schematic diagrams of myocardin/MRTFs and activation of SRF target genes by myocardin family. Structural domains of myocardin and MRTFs are shown. The percent identity between different domains is shown. Myocardin contains an NTD/RPEL domain. A related domain in MAL has been shown to regulate nuclear import. A segment of the protein including the basic and Q-rich domains mediates interaction of myocardin with SRF homodimers. The SAP domain is required for activation of a subset of SRF target genes, suggesting it confers promoter specificity. The leucine zipper mediates homodimerization or heterodimerization of myocardin with MRTFs. The transcription activation domain at the C terminus is required for gene activation. ++, basic region; Q, glutamine-rich region. (Adapted from Wang and Olson, 2003)

References

1. **Olson, E.N., M. Perry, and R.A. Schulz, *Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors.* Dev Biol, 1995. 172(1): p. 2-14.**
2. **Krenn, V., et al., *On the origin of cells determined to form skeletal muscle in avian embryos.* Anat Embryol (Berl), 1988. 179(1): p. 49-54.**
3. **Olson, E.N. and D. Srivastava, *Molecular pathways controlling heart development.* Science, 1996. 272(5262): p. 671-6.**
4. **Gittenberger-de Groot, A.C., et al., *Smooth muscle cell origin and its relation to heterogeneity in development and disease.* Arterioscler Thromb Vasc Biol, 1999. 19(7): p. 1589-94.**
5. **Li, L., et al., *Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells.* J Cell Biol, 1996. 132(5): p. 849-59.**
6. **Cossu, G., et al., *Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm.* Development, 1996. 122(2): p. 429-37.**
7. **Pownall, M.E., M.K. Gustafsson, and C.P. Emerson, Jr., *Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos.* Annu Rev Cell Dev Biol, 2002. 18: p. 747-83.**
8. **Konigsberg, I.R., *Clonal analysis of myogenesis.* Science, 1963. 140: p. 1273-84.**
9. **Sartorelli, V. and Caretti G., *Mechanisms underlying the transcriptional regulation of skeletal myogenesis.* Curr Opin Genet Dev, 2005. 15: p. 528–535.**
10. **Olson, E.N., *Signal transduction pathways that regulate skeletal muscle gene expression.* Mol Endocrinol, 1993. 7(11): p. 1369-78.**
11. **Molkentin, J.D. and E.N. Olson, *Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors.* Proc Natl Acad Sci U S A, 1996. 93(18): p. 9366-73.**
12. **Black, B.L. and E.N. Olson, *Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins.* Annu Rev Cell Dev Biol, 1998. 14: p. 167-96.**
13. **Pollock, R. and R. Treisman, *Human SRF-related proteins: DNA-binding properties and potential regulatory targets.* Genes Dev, 1991. 5(12A): p. 2327-41.**
14. **Lilly, B., et al., *Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila.* Science, 1995. 267(5198): p. 688-93.**
15. **Kaushal, S., et al., *Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD.* Science, 1994. 266(5188): p. 1236-40.**
16. **Redkar, A., M. Montgomery, and J. Litvin, *Fate map of early avian cardiac progenitor cells.* Development, 2001. 128(12): p. 2269-79.**
17. **Brand, B., *Heart development: molecular insights into cardiac specification and early morphogenesis.* Dev Biol, 2003. 258: p. 1-19.**
18. **Bruneau, B.G., *Transcriptional Regulation of Vertebrate Cardiac Morphogenesis,* Circ Res, 2002. 90: p.509-519.**

19. **Olson, E.N., *A decade of discoveries in cardiac biology.* Nat Med, 2004. 10(5): p. 467-74.**
20. **Lin, Q., D. Srivastava, and E.N. Olson, *A transcriptional pathway for cardiac development.* Cold Spring Harb Symp Quant Biol, 1997. 62: p. 405-11.**
21. **Bodmer, R., *The gene tinman is required for specification of the heart and visceral muscles in Drosophila.* Development, 1993. 118(3): p. 719-29.**
22. **Gajewski, K., et al., *D-mef2 is a target for Tinman activation during Drosophila heart development.* Embo J, 1997. 16(3): p. 515-22.**
23. **Lyons, I., et al., *Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5.* Genes Dev, 1995. 9(13): p. 1654-66.**
24. **Yamagishi, H., et al., *The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation.* Dev Biol, 2001. 239(2): p. 190-203.**
25. **Durocher, D., et al., *The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors.* Embo J, 1997. 16(18): p. 5687-96.**
26. **Schwartz, R.J. and E.N. Olson, *Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription.* Development, 1999. 126(19): p. 4187-92.**
27. **Gajewski, K., et al., *Pannier is a transcriptional target and partner of Tinman during Drosophila cardiogenesis.* Dev Biol, 2001. 233(2): p. 425-36.**
28. **Kuo, C.T., et al., *GATA4 transcription factor is required for ventral morphogenesis and heart tube formation.* Genes Dev, 1997. 11(8): p. 1048-60.**
29. **Lin, Q., et al., *Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C.* Science, 1997. 276(5317): p. 1404-7.**
30. **Naya, F.J., et al., *Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor.* Nat Med, 2002. 8(11): p. 1303-9.**
31. **Srivastava, D., P. Cserjesi, and E.N. Olson, *A subclass of bHLH proteins required for cardiac morphogenesis.* Science, 1995. 270(5244): p. 1995-9.**
32. **Srivastava, D. and E.N. Olson, *Knowing in your heart what's right.* Trends in Cell Biol., 1997. 7: p. 447-453.**
33. **Firulli, A.B., et al., *Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1.* Nat Genet, 1998. 18(3): p. 266-70.**
34. **Riley, P., L. Anson-Cartwright, and J.C. Cross, *The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis.* Nat Genet, 1998. 18(3): p. 271-5.**
35. **McFadden, D.G., et al., *The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner.* Development, 2005. 132(1): p. 189-201.**
36. **Yelon, D., et al., *The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development.* Development, 2000. 127(12): p. 2573-82.**
37. **Risau, W. and I. Flamme, *Vasculogenesis.* Annu Rev Cell Dev Biol, 1995. 11: p. 73-91.**

38. **Risau, W., *Differentiation of endothelium.* Faseb J, 1995. 9(10): p. 926-33.**
39. **Hanahan, D.J., *The continuing biochemical challenge of PAF and closely related lipid mediators.* Adv Exp Med Biol, 1996. 416: p. 1-3.**
40. **Hirschi, K.K., S.A. Rohovsky, and P.A. D'Amore, *PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate.* J Cell Biol, 1998. 141(3): p. 805-14.**
41. **Hirschi, K.K., et al., *Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact.* Circ Res, 1999. 84(3): p. 298-305.**
42. **Oettgen, P., *Transcriptional regulation of vascular development.* Circ Res, 2001. 89(5): p. 380-8.**
43. **Lin, Q., et al., *Requirement of the MADS-box transcription factor MEF2C for vascular development.* Development, 1998. 125(22): p. 4565-74.**
44. **Yang, X., et al., *Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5.* Development, 1999. 126(8): p. 1571-80.**
45. **Owens, G.K., et al., *Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease.* Physiol Rev, 2004. 84: p.767-801.**
46. **Treisman, R., *Identification and purification of a polypeptide that binds to the c-fos serum response element.* Embo J, 1987. 6(9): p. 2711-7.**
47. **Chai, J. and A.S. Tarnawski, *Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing.* J Physiol Pharmacol, 2002. 53(2): p. 147-57.**
48. **Gauthier-Rouviere, C., et al., *Expression and activity of serum response factor is required for expression of the muscle-determining factor MyoD in both dividing and differentiating mouse C2C12 myoblasts.* Mol Biol Cell, 1996. 7(5): p. 719-29.**
49. **Soulez, M., et al., *Growth and differentiation of C2 myogenic cells are dependent on serum response factor.* Mol Cell Biol, 1996. 16(11): p. 6065-74.**
50. **Landerholm, T.E., et al., *A role for serum response factor in coronary smooth muscle differentiation from proepicardial cells.* Development, 1999. 126(10): p. 2053-62.**
51. **Arsenian, S., et al., *Serum response factor is essential for mesoderm formation during mouse embryogenesis.* Embo J, 1998. 17(21): p. 6289-99.**
52. **Wiebel, F.F., et al., *Generation of mice carrying conditional knockout alleles for the transcription factor SRF.* Genesis, 2002. 32(2): p. 124-6.**
53. **Miano, J.M., et al., *Restricted inactivation of serum response factor to the cardiovascular system.* Proc Natl Acad Sci U S A, 2004. 101(49): p. 17132-7.**
54. **Parlakian, A., et al., *Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality.* Mol Cell Biol, 2004. 24(12): p. 5281-9.**
55. **Treisman, R., *Ternary complex factors: growth factor regulated transcriptional activators.* Curr Opin Genet Dev, 1994. 4(1): p. 96-101.**

56. **Chen, C.Y. and R.J. Schwartz, *Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription.* Mol Cell Biol, 1996. 16(11): p. 6372-84.**

57. **Belaguli, N.S., et al., *Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators.* Mol Cell Biol, 2000. 20(20): p. 7550-8.**

58. **Wang, D., et al., *Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor.* Cell, 2001. 105(7): p. 851-62.**

59. **Wang, D.Z. and S. Li, et al., *Potentiation of serum response factor activity by a family of myocardin-related transcription factors.* Proc Natl Acad Sci USA, 2002. 99: p.14855-14860.**

60. **Ma, Z., et al., *Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia.* Nat Genet, 2001. 28: p.220-221.**

61. **Mercher, T., et al., *Involvement of a human gene related to the Drosophila spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia.* Proc Natl Acad Sci USA, 2001. 98: p.5776-5779.**

62. **Sasazuki, T., et al., *Identification of a novel transcriptional activator, BSAC, by a functional cloning to inhibit tumor necrosis factor-induced cell death.* J Biol Chem, 2002. 277: p.28853-28860.**

63. **Han, Z., et al., *A myocardinrelated transcription factor regulates activity of serum response factor in Drosophila.* Proc Natl Acad Sci USA, 2004. 101: p.12567–12572.**

64. **Aravind, L. and E.V. Koonin, *SAP - a putative DNA-binding motif involved in chromosomal organization.* Trends Biochem Sci, 2000. 25(3): p. 112-4.**

65. **Miralles, F., et al., *Actin dynamics control SRF activity by regulation of its coactivator MAL.* Cell, 2003. 113: p.329-342.**

66. **Wang, Z., et al., Myocardin is a master regulator of smooth muscle gene expression. Proc Natl Acad Sci USA, 2003. 100: p.7129–7134.**

67. **Wang, D.Z. and E.N. Olson, *Control of smooth muscle development by the myocardin family of transcriptional coactivators.* Curr Opin Genet Dev, 2004. 14(5): p. 558-66.**

68. **Small, E.M., et al., *Myocardin is sufficient and necessary for cardiac gene expression in Xenopus. Development,* 2005. 132: p.987–997.**

69. **Chen, J., et al., *Myocardin: a component of a molecular switch for smooth muscle differentiation.* J Mol Cell Cardiol, 2002. 34: p.1345-1356.**

70. **Du, K.L., et al., *Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation.* Mol Cell Biol, 2003. 23: p.2425-2437.**

71. **Yoshida, T., et al., *Myocardin Is a Key Regulator of CArG-Dependent Transcription of Multiple Smooth Muscle Marker Genes.* Circ Res, 2003. 92: p.856-864.**

72. **Selvaraj, A. and R. Prywes, *Megakaryoblastic leukemia-1/2, a transcriptional co-activator of serum response factor, is required for skeletal myogenic differentiation.* J Biol Chem, 2003. 278: p.41977-41987.**

73. **Li, S., et al.,** ***Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice.*** **Proc Natl Acad Sci USA, 2005. 102: p.1082-1087.**

74. **Li, S., et al.,** ***The serum response factor coactivator myocardin is required for vascular smooth muscle development.*** **Proc Natl Acad Sci USA, 2003. 2003. 100: p.9366-9370.**

75. **Li, S., et al.,** ***Requirement of a myocardin-related transcription factor for development of mammary myoepithelial cells.*** **Mol Cell Biol, 2006. 26: p.5797-808.**

Chapter II

Target Gene-Specific Modulation of Myocardin Activity by GATA Transcription Factors

Introduction

Serum response factor (SRF) is a widely expressed transcription factor belonging to the MADS (MCM1, Agamous, Deficiens, SRF) box family of proteins [28, 34]. SRF binds as a homodimer to a DNA consensus sequence known as a CArG box ($CC[A/T]_6GG$), which is found in the control regions of numerous growth factor-regulated and muscle-specific genes [30]. The spectrum of SRF target genes expressed by a cell is dependent on association of SRF with a wide variety of positive and negative cofactors, many of which are signal responsive and tissue restricted.

Myocardin is a powerful SRF coactivator expressed specifically in cardiac and smooth muscle cells [37, 38]. Myocardin belongs to the SAP (scaffold-attachment factor A/B, Acinus, PIAS) domain family of nuclear proteins, which bind A/T-rich genomic regions known as scaffold or matrix attachment regions and have been implicated in chromatin remodeling [1]. Although myocardin lacks sequence-specific DNA binding activity, it forms a stable DNA-protein complex with SRF, resulting in activation of SRF target genes in muscle cells [37]. Myocardin is necessary [19] and sufficient [8, 39, 40] to activate smooth muscle gene expression in nonmuscle cells and uses SRF as an obligate partner in this process [38, 39]. Expression of a dominant negative myocardin mutant in *Xenopus* embryos is also sufficient to extinguish cardiac gene expression [37], suggesting an essential role for myocardin or other members of the myocardin family in cardiogenesis. Two myocardin-related transcription factors (MRTFs), referred to as MRTF-A [21, 22, 32, 38] and MRTF-B [38], also interact with SRF and stimulate transcription through the CArG box, but these

factors are not muscle restricted and are likely to modulate SRF activity in response to growth factor signaling [24].

SRF has also been shown to stimulate expression of smooth and cardiac muscle genes in association with a variety of homeodomain proteins [6, 9], LIM domain proteins [4], and GATA transcription factors [2, 26, 33]. The six GATA factors share homology in two zinc finger domains that mediate DNA binding and cofactor interactions [5, 23]. GATA4, -5, and 6 are expressed predominantly in cardiac and smooth muscle cell lineages, where they play diverse roles in differentiation, morphogenesis, and growth [15]. GATA 4 is required for proper embryonic folding and heart tube formation [13, 25]. GATA 4 and 5 have also been implicated in cardiac gene expression [14, 31], and GATA 6 is essential for mesoderm formation during gastrulation [12, 27].

Because myocardin and GATA factors both interact with SRF and participate in cardiac and smooth muscle gene expression, we investigated whether they might modulate each other's activities. Here, I show that GATA4 augments the activity of myocardin on some genes, such as the cardiac homeobox gene *Nkx2.5*, whereas it interferes with the activity of myocardin on other genes, such as the atrial natriuretic factor (*ANF*) gene. Modulation of myocardin activity by GATA4 is mediated by the direct physical interaction between the factors and is dependent on SRF DNA binding sites (CArG boxes) but not on GATA4 DNA binding. Modulation of myocardin activity by GATA4 provides a mechanism for fine tuning the expression of SRF target genes in a promoter-specific manner.

Materials and Methods

Cell Culture and Transfection Assays

COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.). Cells were grown to 60 to 70% confluence in six-well dishes at 37^{o} in the presence of 95% O_2 and 5% CO_2 and transfected using FuGENE 6 (Roche Applied Science) with 100 ng of reporter plasmid and 100 ng of each activator plasmid, unless otherwise indicated. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. 40 h after transient transfection, cells were harvested for measurement of luciferase activity using a Luciferase Reporter Assay System (Promega). pCMV-lacZ was included as an internal control for variations in transfection efficiency.

All myocardin expression vectors were cloned in pcDNA3.1 (Invitrogen), which was driven by the CMV promoter [37, 38]. A CMV-driven human SRF eukaryotic expression vector was used [6]. Myocardin and GATA4 deletion mutants were generated through PCR-based mutagenesis with the QuikChange kit from Stratagene. GATA4 point mutants in the DNA binding domain were generously provided by Bruce Markham and have been described [7]. All mutations were confirmed by DNA sequencing. The SM22-luciferase reporter contained the 1,343-bp promoter [17]. The 4xSM22-CArG-luciferase reporter has a luciferase reporter linked to the E1b basal promoter and four tandem copies of CArG-near from the *SM22* promoter [3]. The ANF-luciferase reporter contains the 638-bp promoter [35]. GATA-less ANF promoter harbors mutations in the two GATA sites from CTGATAAC to

CTCTAGAC and from AAGATAAC to AAAGCTAC (GATA site is underlined). The NK-tk-luciferase reporter was constructed by linking 5' upstream sequences from –9432 to –8923 of the mouse *Nkx2.5* gene [20] to a luciferase reporter with a thymidine kinase promoter. The NK-CArG-mutant-luciferase reporter has mutations in the CArG box from CCTTTTAAGG to AAGCTTAAGG.

Cardiomyocyte Culture and Construction of Adenoviruses

Cardiomyocyte cultures were prepared by dissociation of 1-day-old neonatal rat hearts and were plated differentially to remove fibroblasts. Cells were plated in 4:1 Dulbecco's modified Eagle's medium (DMEM):199 medium with 10% horse serum and 5% fetal calf serum at a density of 2×10^4 cells/cm^2. Eighteen hours after plating, cells were changed into serum-free media and infected with adenovirus at a multiplicity of infection (m.o.i.) of 100. The myocardin adenoviral expression construct (Ad-myocardin) contained a cDNA encoding amino acids 129–935 of mouse myocardin.

GST Protein Binding Assays

The plasmid encoding a glutathione *S*-transferase (GST) fusion protein was transformed into *Escherichia coli* BL21-Codon Plus cells (Stratagene). The cells were grown at 37°C in 2XYT medium to an optical density of 1.0. Isopropylthiogalactopyranoside (IPTG) (50 μM) was then added to the culture to induce protein expression. After shaking at room temperature for 4 to 6 h, the cells were harvested by spinning at 2,000g for 10 minutes at 4 ^{o}C. The cells were lysed in 10 ml cold PBS containing 0.5 mM EDTA, 0.5 mg/ml Lysozyme

(Sigma), 1 mM PMSF and protease inhibitor cocktail (Roche). After spinning at 10,000 rpm at 4 ^{o}C for 30 minutes, the lysate was incubated with 1 ml of 50% slurry glutathione beads (Amersham). After rotation at 4 ^{o}C for 90 minutes, the mixture was then washed with cold PBS containing 0.5% Triton X-100 for 3 times. Proteins translated in vitro were labeled with [^{35}S] methionine with a TNT T7 reticulocyte lysate system (Promega). For GST-protein binding assays, glutathione beads conjugated with 1 μg of GST fusion protein or GST protein alone were incubated with 10 μl of TNT product at 4°C for 2 h in 500 μl GST binding buffer (20 mM Tris, pH 7.3, 150 mM NaCl, 0.5% NP-40, protease inhibitor cocktail from Roche, and 1 mM PMSF). The beads were washed three times with GST binding buffer. Fifty microliters of sodium dodecyl sulfate (SDS) loading buffer was then added to the beads. After boiling, 20 μl was loaded onto an SDS-polyacrylamide gel electrophoresis (PAGE) gel to analyze proteins associated with glutathione beads.

Reverse Transcription-PCR

Total RNA was isolated with Trizol reagent (Invitrogen). After treatment with DNase I, 1 μg of RNA was used as a template for reverse transcription with random hexamer primers that spanned introns in the genes. Reverse transcription-PCRs were performed under conditions of linearity with respect to input RNA. The sequences of primers used are: Nkx2.5: 5'-ACTTGAACACCGTGCAGAGTCC-3', 5'-TCCTAGTGTGGAATCCGTCGA -3'; GAPDH: 5'-GCAGTGGCAAAGTGGAGATTG3-'; 5'-TTTGGCTCCACCCTTCAAG TG-3'.

Gel Mobility Shift Assays

SRF and myocardin were translated in vitro with a TNT T7-coupled reticulocyte lysate system (Promega), and gel mobility shift assays were performed with double-stranded probes as described [3]. The sequence of the top strand of the *Nkx2.5* CArG box probe was GCCCCCCCAAGTTTAAATGCT*CCTTTAAGGG*CTTGAGTGTCTGCAGC (CArG box is in italics).

Results

Synergistic Activation of the *Nkx2.5* Enhancer by Myocardin and GATA4

A dominant negative myocardin mutant suppresses *Nkx2.5* expression in *Xenopus* embryos and in the P19 embryonal carcinoma cell line [36, 37]. To test whether myocardin was able to stimulate the expression of *Nkx2.5* in cardiac myocytes, primary rat neonatal cardiac myocytes were infected with an adenovirus encoding myocardin (Ad-myocardin). Indeed, the myocardin-expressing virus up-regulated *Nkx2.5* expression by fivefold compared to a control virus expressing *lacZ* (Ad-lacZ) (Fig. 2.1.*A*).

Based on the above findings, I examined the early cardiac enhancer of the mouse *Nkx2.5* gene for CArG boxes that might confer responsiveness to myocardin. The enhancer, which is located between bp –9432 and –8923 upstream of the *Nkx2.5* gene, contains a single CArG box [20]. In gel mobility shift assays, SRF bound avidly to this sequence and gave rise to a ternary complex in the presence of myocardin (Fig. 2.1.*B*).

Transfection of COS cells with a myocardin expression vector and a luciferase reporter linked to the *Nkx2.5* enhancer and the viral E1b promoter (NK-E1b-luc) showed that myocardin was able to transactivate the enhancer (Fig. 2.1.*C*). Similar results were obtained when the enhancer was combined with the thymidine kinase (tk) basal promoter (NK-tk-luc), whereas an enhancer with a mutation in the CArG box (NK-CArGmut-tk-luc) was refractory to the activity of myocardin (Fig. 2.1.*C*). Myocardin also activated the *Nkx2.5* enhancer in transfected HeLa and 10T1/2 cells (data not shown).

Myocardin is a relatively weak activator through single CArG boxes [37]. I therefore tested whether its transcriptional potency might be enhanced in the presence of GATA4,

which binds two essential GATA sites in the *Nkx2.5* enhancer [20]. As shown in Fig. 2.1.*D*, GATA4 alone was a very weak activator of the *Nkx2.5* enhancer, but it synergized with myocardin to activate the enhancer. The synergy between GATA4 and myocardin was abolished by mutation of the CArG box (NK-CArG mut-tk-luc; Fig. 2.1.*D*). Surprisingly, however, the synergy was not affected when the two GATA sites were mutated (NK-GATA-mut-tk-luc; Fig. 2.1.*D*). I conclude that myocardin transactivates the *Nkx2.5* enhancer by associating with SRF on the CArG box in this enhancer and that GATA4 can potentiate the effect of myocardin on the enhancer through a mechanism independent of GATA4 DNA binding.

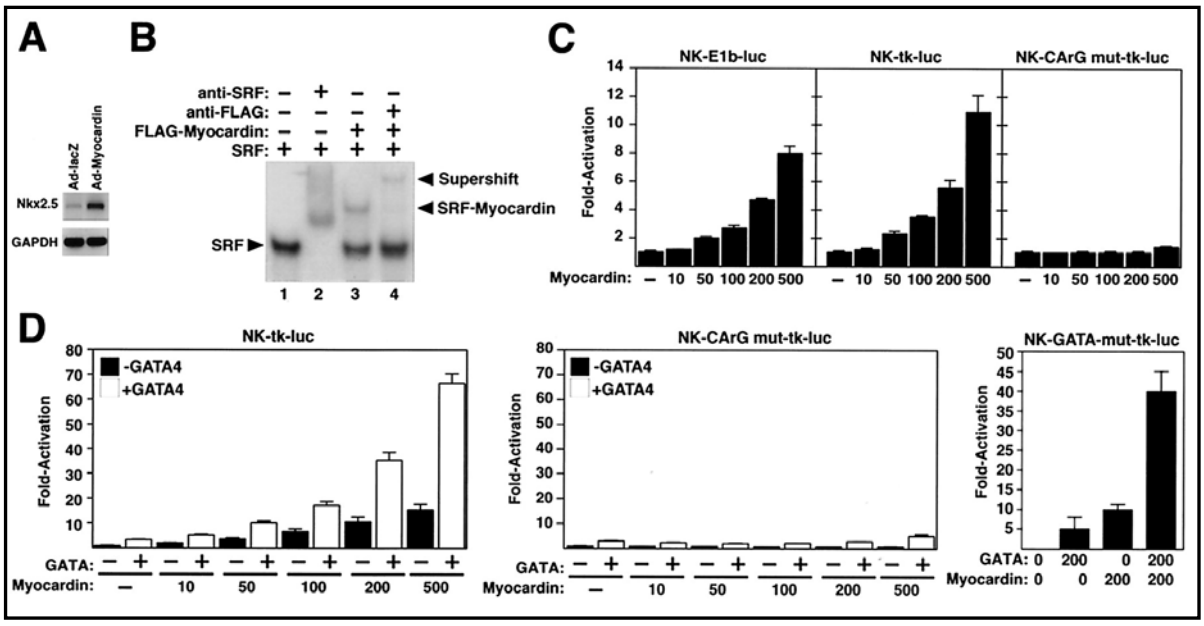

Figure 2.1. Myocardin and GATA4 synergistically activate the *Nkx2.5* enhancer. *(A)* Primary neonatal cardiomyocytes were infected with adenoviruses encoding myocardin or *lacZ* (as a negative control). Four days later, expression of Nkx2.5 transcripts was measured by semiquantitative reverse transcription-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were detected as a loading control. *(B)* Gel mobility shift assays were performed with a radiolabeled oligonucleotide corresponding to the CArG box in the *Nkx2.5* enhancer and in vitro-translated SRF and myocardin proteins, as indicated. Myocardin formed a stable ternary complex with SRF on this sequence (lane 3). Anti-SRF antibody supershifted the SRF complex (lane 2), and anti-Flag antibody supershifted the ternary complex formed by SRF and Flag-myocardin (lane 4). *(C)* COS cells were transiently transfected with the luciferase reporter plasmids shown above each panel and the indicated amounts (in nanograms) of myocardin expression vector. Luciferase activity was assayed and is expressed as fold activation above the level of expression of the reporter gene alone. *(D)* COS cells were transiently transfected with the luciferase reporter plasmids shown above each panel and the indicated amounts (in nanograms) of myocardin expression vector with and without a GATA4 expression vector (100 ng), and luciferase activity was determined as in panel *C*.

Repression of Myocardin Activity by GATA4

I next tested whether myocardin and GATA4 could cooperate to activate the *ANF* promoter, which contains a pair of binding sites for both factors [10, 16, 26]. Myocardin activates this promoter much more effectively than the *Nkx2.5* enhancer, at least in part because it contains two CArG boxes (Fig. 2.2.A) [37]. Unexpectedly, GATA4 potently repressed myocardin-dependent activation of the *ANF* promoter, such that as little as 10 ng of GATA4 expression plasmid resulted in a near-complete inhibition of ANF-luciferase expression (Fig. 2.2.*A*).

To determine whether the GATA sites in the promoter were required for repression, I examined the effects of myocardin and GATA4 on a mutant *ANF* promoter in which the two GATA sites were mutated. The mutant promoter (GATA-less ANF-luc) was activated by myocardin and repressed by GATA4 as effectively as the wild-type promoter (Fig. 2.2.*A*). These results suggested that the repressive effect of GATA4 did not require direct binding of GATA4 to DNA.

To further investigate the potential modulation of myocardin activity by GATA4, I tested the effect of GATA4 on the *SM22* promoter, which is extremely sensitive to myocardin and contains two essential CArG boxes but no GATA sites [8, 11]. GATA4 potently suppressed the ability of myocardin to activate the *SM22* promoter (Fig. 2.2.*B*), supporting the notion that DNA binding by GATA4 was not involved in the repressive mechanism.

GATA4 Enhances Myocardin Activity on a Multimerized CArG Box

To determine whether the CArG box was sufficient to confer responsiveness of myocardin to GATA4, we tested a reporter gene containing the *tk* promoter linked to four tandem copies of the promoter-proximal *SM22* CArG box (4xSM22 CArG-luc). As shown in Fig. 2.2.*C*, this reporter was activated by myocardin but, in contrast to the behavior of the *ANF* and *SM22* promoters, activation by myocardin was dramatically augmented by GATA4. In the absence of myocardin, GATA4 had no effect on the reporter, demonstrating that the stimulation of transcription by GATA4 required myocardin. Myocardin did not affect the expression of a reporter containing six tandem copies of the GATA binding site without a CArG box (data not shown), further suggesting that the positive and negative influences of GATA4 on myocardin were dependent on the presence of SRF binding sites and were independent of GATA4 DNA binding. Together, the above results demonstrated that GATA4 was able to discriminate between myocardin target genes and modulate myocardin activity, either positively or negatively, in a promoter context-dependent manner.

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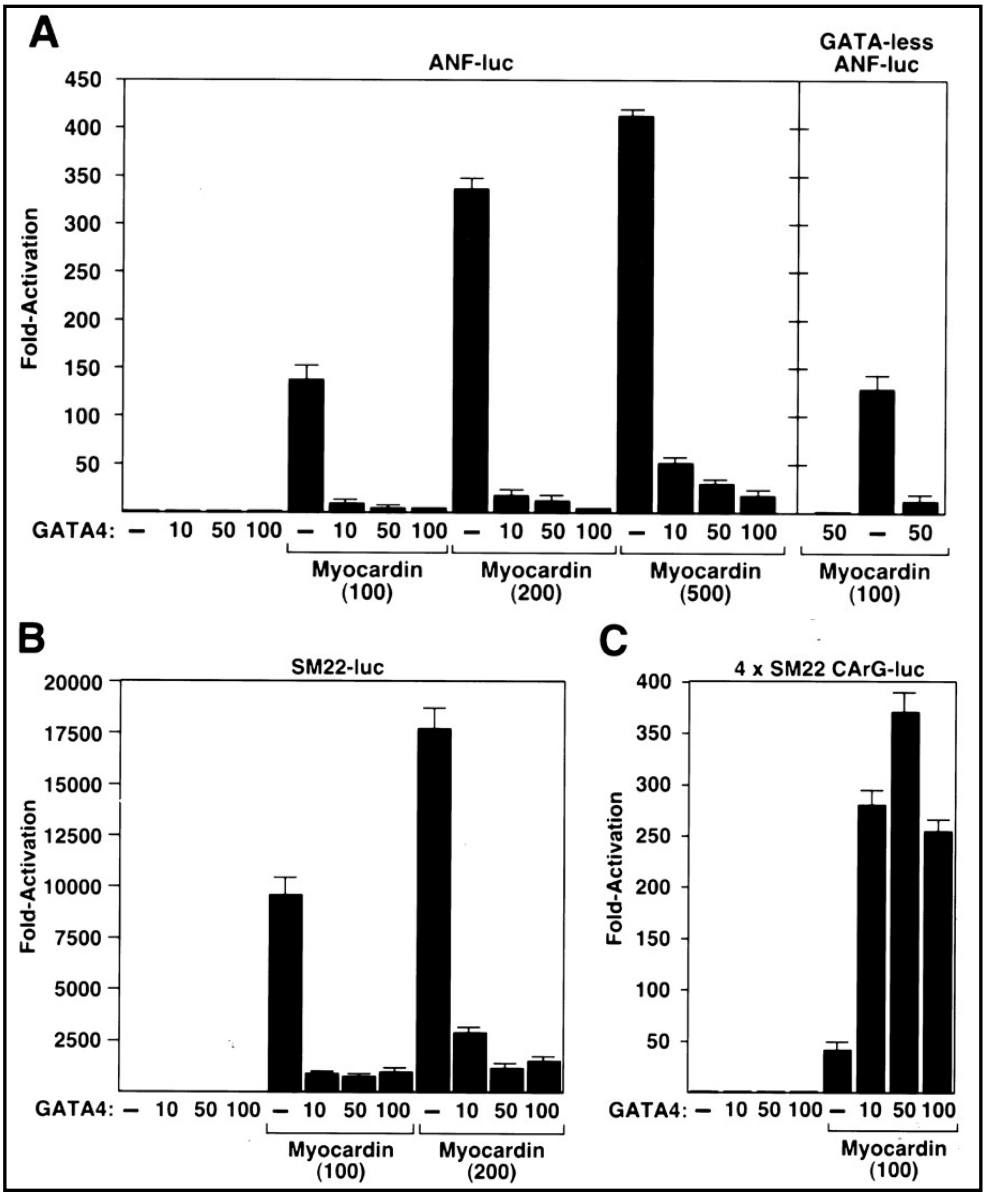


Figure 2.2. Differential effects of GATA4 on myocardin-dependent transcription. COS cells were transiently transfected with luciferase reporter plasmids controlled by *(A)* the *ANF* promoter or a mutant promoter lacking the two GATA4 binding sites, *(B)* the *SM22* promoter, or *(C)* four copies of the *SM22* CArG box and the indicated amounts (in nanograms) of myocardin and GATA4 expression vectors. Luciferase activity was assayed and is expressed as fold activation above the level of expression of the reporter gene alone.

Mapping the GATA4-Responsive Region of Myocardin

To determine whether stimulation and suppression of myocardin activity by GATA4 were dependent on the same domain of the myocardin protein, I examined the effects of GATA4 on a series of myocardin deletion mutants (Fig. 2.3 *A*-*C*). The transcriptional activity of a myocardin deletion mutant lacking the conserved N-terminal domain (ΔNTD) was stimulated by about an order of magnitude in the presence of GATA4 when assayed with the reporter controlled by the multimerized SM22 CArG box (4xSM22-CArG-luc; Fig. 2.3.*B*). Deletion mutants in which residues from amino acid 513 to the C terminus, including the transcription activation domain (TAD), were deleted and replaced with VP16 (mutants 129-513-VP16 and 129-713-VP16) were also stimulated by GATA4 (Fig. 2.3.A and B and data not shown). Deletion mutants that were stimulated by GATA4 on the multimerized SM22 CArG box reporter retained the ability to be repressed by GATA4 on the *SM22* and *ANF* promoters (Fig. 2.3 *A*-*C* and data not shown). These results suggested that the positive and negative effects of GATA4 were dependent on the same region of myocardin.

To determine whether the effects of GATA4 were mediated by myocardin or SRF (or another protein), I tested whether GATA4 affected the transcriptional activity of myocardin fused to the GAL4 DNA binding domain (Fig. 2.3.D and E). GATA4 stimulated activity of a GAL4-myocardin fusion protein extending from amino acid 129 to the C terminus (GAL4-129-935). The TAD of myocardin, located between amino acid 713 and the C terminus, shows a greater than 10-fold increase in activity when it is isolated from the remainder of the protein [37]. The TAD fused to the GAL4 DNA binding domain (GAL4-670-935) showed only a marginal response to GATA4. In contrast, residues 129 to 713, which are inactive

alone (GAL4-129-713), showed about a sevenfold increase in transcriptional activity in the presence of GATA4. The finding that GATA4 was able to stimulate transcription via a portion of myocardin lacking its own transcription activation domain suggested that myocardin acted as a bridge between GATA4 and the transcriptional machinery.

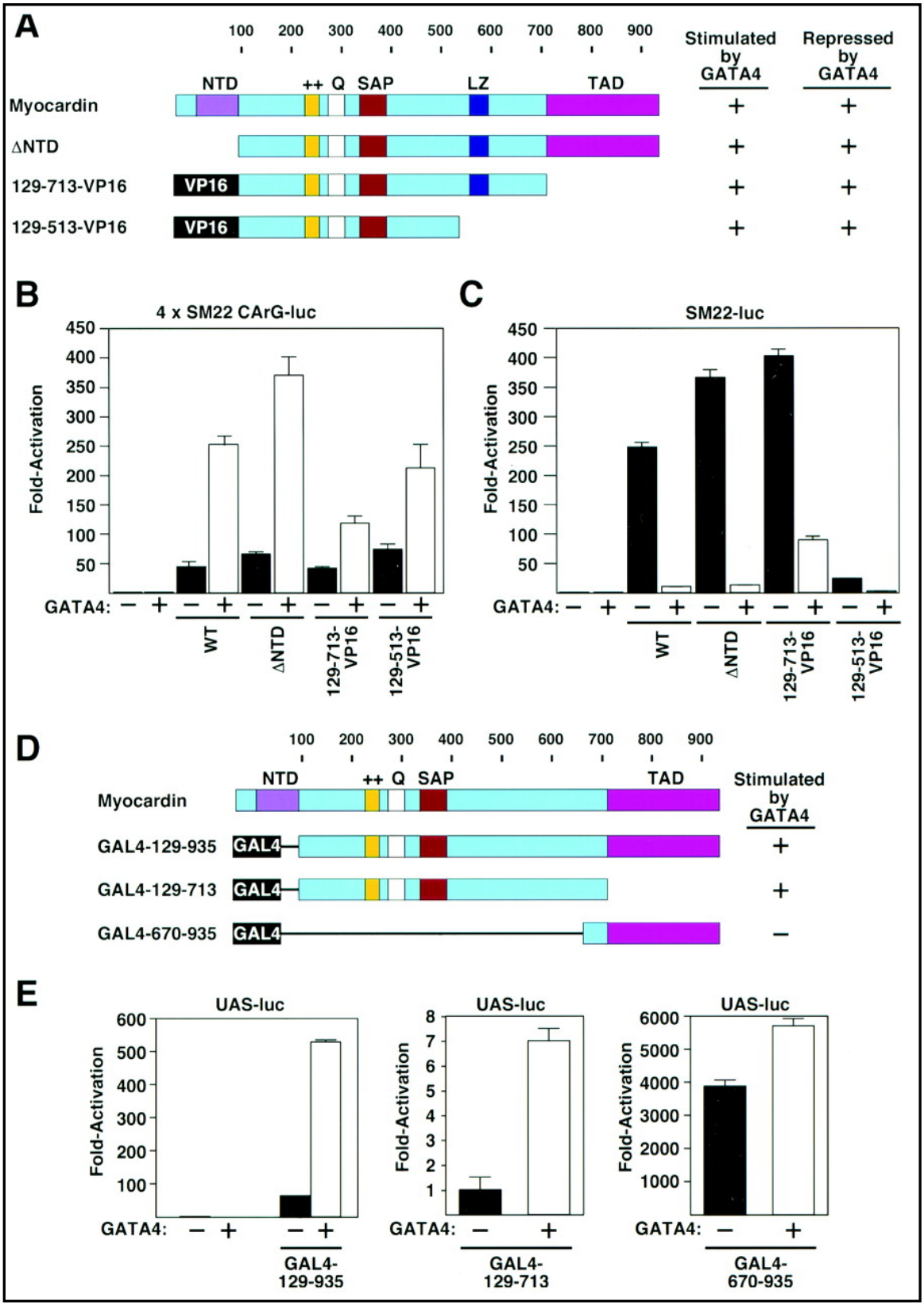

A
100 200 300 400 500 600 700 800 900
NTD ++ Q SAP LZ TAD
Stimulated by GATA4
Repressed by GATA4
Myocardin + +
ΔNTD + +
129-713-VP16 + +
129-513-VP16 + +
B
4 x SM22 CArG-luc
Fold-Activation
GATA4: – + – + – + – + – +
WT
ΔNTD
129-713-VP16
129-513-VP16
C
SM22-luc
Fold-Activation
GATA4: – + – + – + – + – +
WT
ΔNTD
129-713-VP16
129-513-VP16
D
100 200 300 400 500 600 700 800 900
NTD ++ Q SAP TAD
Stimulated by GATA4
Myocardin
GAL4-129-935 +
GAL4-129-713 +
GAL4-670-935 –
E
UAS-luc
Fold-Activation
GATA4: – + – +
GAL4-129-935
UAS-luc
Fold-Activation
GATA4: – +
GAL4-129-713
UAS-luc
Fold-Activation
GATA4: – +
GAL4-670-935

Figure 2.3. Mapping the region of myocardin that responds to GATA. *(A)* Schematic diagrams of myocardin mutants. The potential of each protein to be stimulated by GATA4 on the 4xSM22-CArG promoter or repressed by GATA4 on the *SM22* promoter is indicated. *(B and C)* COS cells were transiently transfected with the luciferase reporter plasmids shown above each panel and expression vectors encoding GATA4 (100 ng) and myocardin or myocardin mutants (100 ng) shown in panel A, as indicated. Luciferase activity was assayed and is expressed as fold activation above the level of expression of the reporter gene alone. *(D)* Schematic diagrams of GAL4-myocardin fusion proteins. The potential of each protein to be stimulated by GATA4 is indicated. *(E)* COS cells were transiently transfected with an upstream activation sequence-luciferase reporter plasmid and expression vectors encoding GAL4-myocardin fusion proteins (100 ng) shown in panel D with and without a GATA4 expression vector (100 ng), as indicated. Luciferase activity was assayed and is expressed as fold activation above the level of expression of the reporter gene alone.

Mapping the GATA4 Binding Region of Myocardin.

To determine whether the modulation of myocardin activity by GATA4 required direct physical interaction between the proteins, I performed GST pulldown assays with GST-GATA4 fusion protein and myocardin deletion mutants translated in vitro. As shown in Fig. 2.4, GATA4 interacted efficiently with myocardin. Amino-terminal deletion mutants up to residue 348 retained the ability to interact strongly with GATA4. A deletion mutant lacking residues 1 to 438 (mutant 439-935) was also able to interact with GATA4, albeit less strongly than mutants containing additional N-terminal sequences. The TAD of myocardin, contained within residues 713 to 935, did not interact with GATA4.

Deletions from the C terminus showed that residues 129 to 513 retained strong GATA4 binding activity. A smaller deletion mutant lacking additional C-terminal sequences (mutant 129-438) also bound GATA4, but its binding activity was reduced. Further deletions from the C-terminal end to residue 348 or 298 (mutants 129-348 and 129-298, respectively) abolished binding.

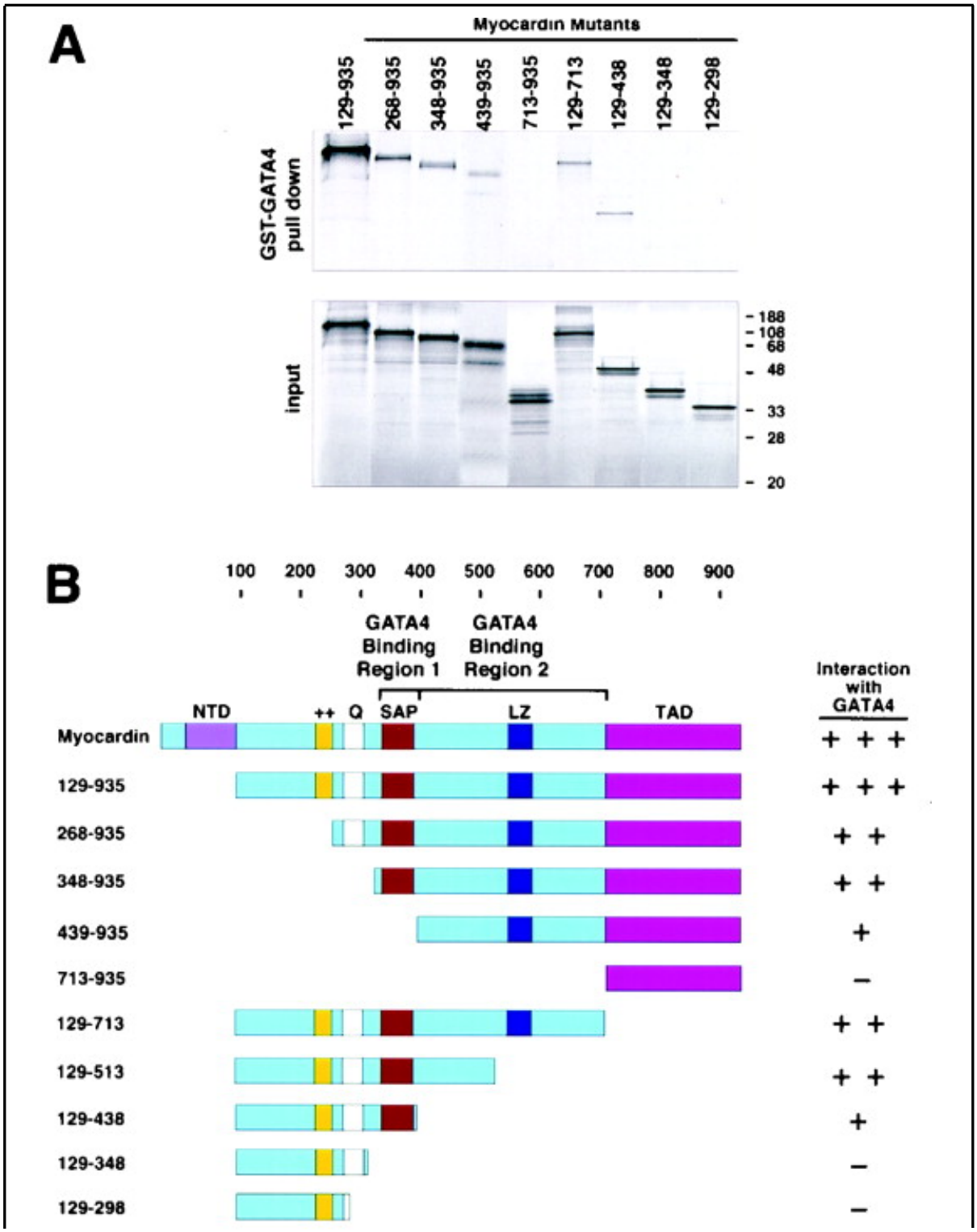
The above deletions suggested that myocardin contained two GATA4-binding regions, one between residues 129 and 438 (region 1) and another between residues 439 and 713 (region 2) (Fig. 2.4.*B*). Since the former region encompasses the SRF-binding domain and SAP domains, which are critical to the functions of myocardin, I created a series of smaller deletions within this region in order to further pinpoint this GATA4-binding domain. As shown in Fig. 2.4.*C* and *D*, deletion of the SAP domain (amino acids 380 to 414) severely impaired but did not eliminate GATA4-binding activity (mutant 129-513ΔSAP). Similarly, deletion of residues 326 to 377 reduced GATA4 activity, and a deletion combining both

regions (mutant Δ326 to 438) abolished GATA4 binding. I conclude that the GATA4-binding region 1 of myocardin maps to an extended sequence from 348 to 438 that encompasses the SAP domain. This GATA4-binding region of myocardin defined by GST pulldown assays correlated with the region of myocardin required for stimulation and suppression of myocardin activity by GATA4.

Mapping the Myocardin-Interacting Region of GATA4.

The region of GATA4 that interacted with myocardin was mapped by coimmunoprecipitation and GST pulldown assays with a GST-myocardin fusion protein containing residues 129 to 510 and a series of GATA4 deletion mutants translated in vitro (Fig. 2.5.*A* and *B*, and data not shown). Deletion of residues from amino acid 332 to the C terminus (mutant 1-332) did not affect binding of GATA4 to myocardin. Residues 1 to 177 of GATA4, which encompass the two TADs, did not interact with myocardin, whereas a strong interaction was observed with residues 177 to 332 and 190 to 332, which encompass the two zinc fingers and the nuclear localization sequence (NLS). Deletion of the N-terminal zinc finger (Nf) did not affect binding to myocardin (mutant 239 to 332), whereas deletion of the C-terminal zinc finger (Cf) or the NLS abolished myocardin binding (mutants 1-332ΔCf and 190-302, respectively). GATA5 and GATA6, which share extensive homology with GATA4, also interacted with myocardin (Fig. 2.5.*B*).

The ability of GATA4 deletion mutants to interact with myocardin correlated precisely with the ability to synergize with myocardin to activate the *Nkx2.5* enhancer or the


A
Myocardin Mutants
129-935
268-935
348-935
439-935
713-935
129-713
129-438
129-348
129-298
GST-GATA4 pull down
input
188
108
68
48
33
28
20
B
100
200
300
400
500
600
700
800
900
GATA4 Binding Region 1
GATA4 Binding Region 2
NTD
++
Q
SAP
LZ
TAD
Interaction with GATA4
Myocardin
129-935
268-935
348-935
439-935
713-935
129-713
129-513
129-438
129-348
129-298

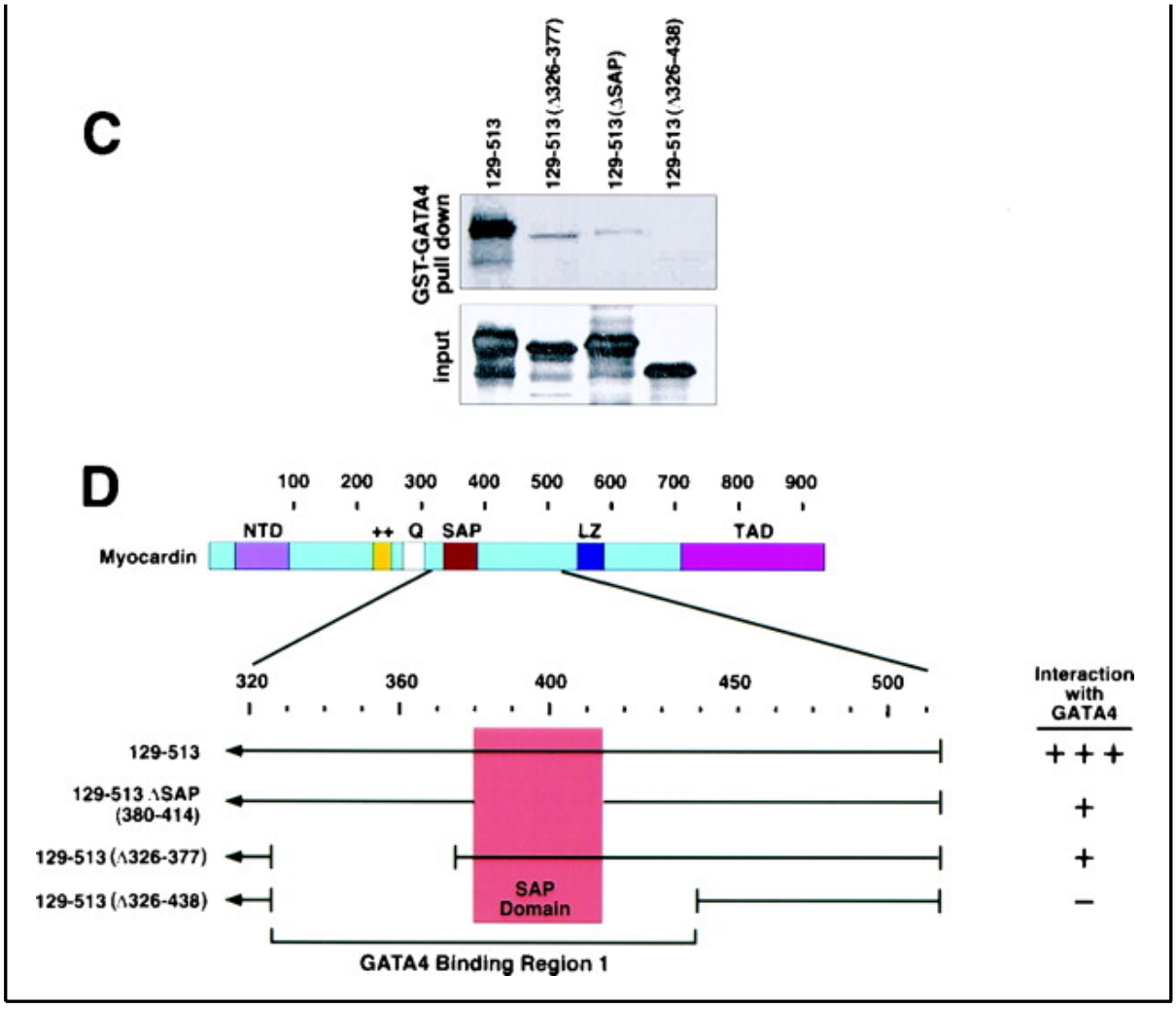


Figure 2.4. Mapping the region of myocardin that interacts with GATA4. *(A)* A GST-GATA4 fusion protein encompassing amino acids 177 to 332 was incubated with [^{35}S]methionine-labeled myocardin proteins translated in vitro. Myocardin proteins associated with GST-GATA4 are shown at the top, and 10% of the input proteins are shown at the bottom. *(B)* Summary of the GST-GATA4 pulldown and coimmunoprecipitation assays (data not shown). *(C)* GST pulldown assays were performed with the indicated myocardin mutants and GST-GATA4 as described for panel A. *(D)* Summary of the GST-GATA4 pulldown assays in panel C.

multimerized *SM22* CArG box (Fig. 2.5.*A* and *C*). Thus, only the Cf and NLS of GATA4 appeared to be required for myocardin binding and stimulation of myocardin activity. In contrast, binding to myocardin and repression of myocardin activity on the *SM22*-luciferase reporter could be uncoupled in certain deletion mutants. For example, repression required the amino-terminal TADs of GATA4 and the myocardin-binding region; deletion mutants containing only one domain or the other were unable to repress myocardin activity (e.g., mutants 1-177 and 177-332). In fact, the only GATA4 deletion mutant able to repress myocardin activity was mutant 1-332, which lacks the extreme C-terminal residues. GATA5 and GATA6 also activated and repressed myocardin activity as effectively as GATA4.

To further pinpoint the residues in the Cf of GATA4 that are critical for myocardin binding, we tested a series of point mutants for their abilities to bind the GST-myocardin fusion protein. Mutations that disrupted myocardin binding were also defective in stimulation of the multimerized *SM22* CArG box and repression of the *SM22* promoter with myocardin (Fig. 2.6 *A*-*C*). The two mutants (WRR and C1) that were defective in myocardin binding also failed to interact with SRF (data not shown). Thus, I cannot distinguish whether GATA4 modulates myocardin activity on SRF-dependent promoters by associating with myocardin directly or with SRF or both. However, the ability of GATA4 to enhance the activity of a GAL4-myocardin fusion protein, which is SRF independent, suggests that GATA4 can act through a direct effect on myocardin.

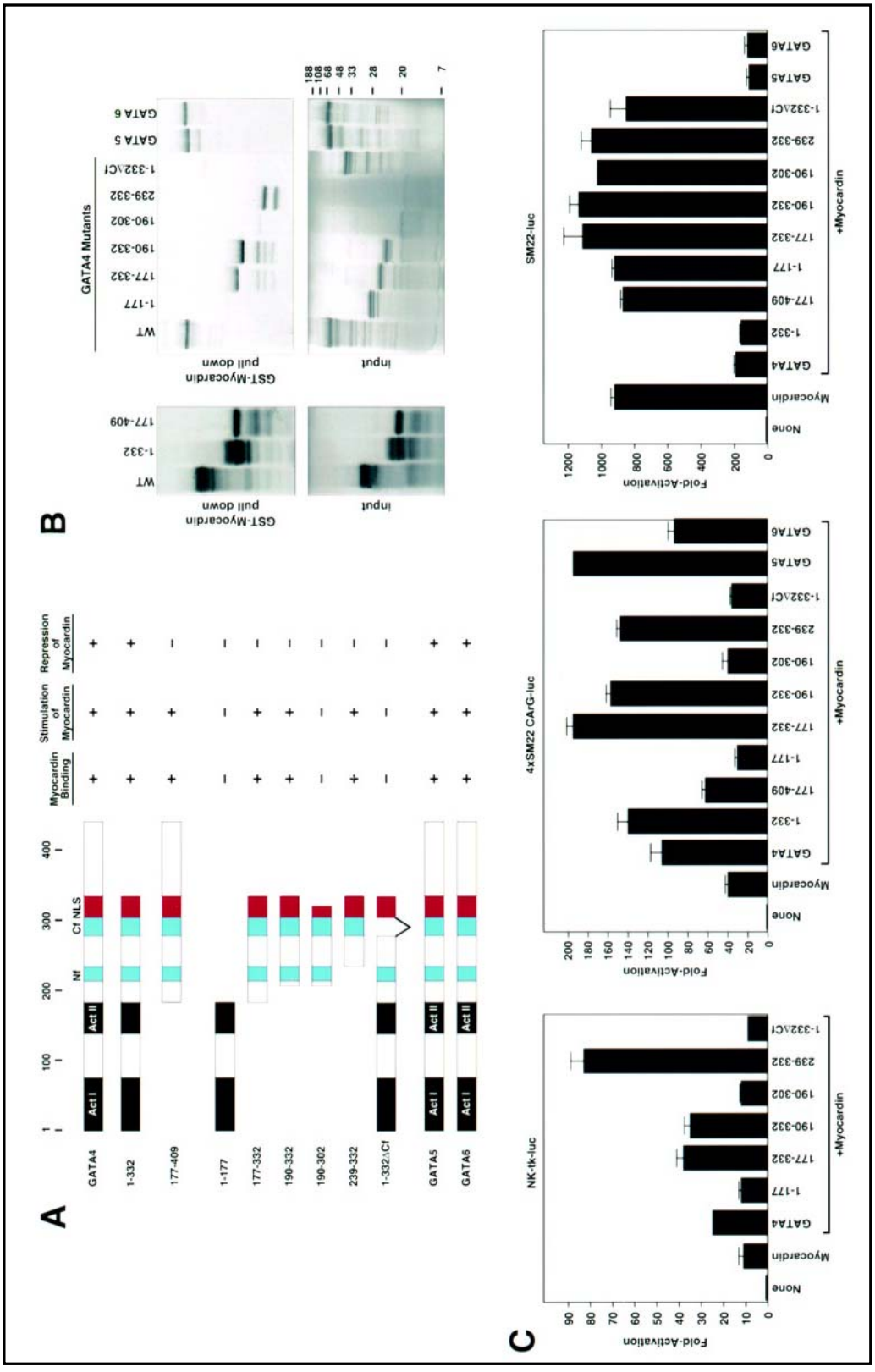

A
GATA4
1-332
177-409
1-177
177-332
190-332
190-302
239-332
1-332ΔCf
GATA5
GATA6
Act I
Act II
Nf
Cf
NLS
Myocardin Binding
Stimulation of Myocardin
Repression of Myocardin
B
GST-Myocardin pull down
Input
WT
1-332
177-409
GATA4 Mutants
1-177
177-332
190-332
190-302
239-332
1-332ΔCf
GATA 5
GATA 6
188
168
68
48
33
28
20
7
C
NK-tk-luc
4xSM22 CArG-luc
SM22-luc
Fold-Activation
None
Myocardin
+Myocardin
GATA4
1-332
177-409
1-177
177-332
190-332
190-302
239-332
1-332ΔCf
GATA5
GATA6

Figure 2.5. Mapping the region of GATA4 that interacts with myocardin. *(A)* Schematic diagrams of GATA4 mutants and their activities. *(B)* A GST-myocardin fusion protein encompassing amino acids 129 to 510 was incubated with [^{35}S]methionine-labeled GATA proteins translated *in vitro*. GATA4 proteins associated with GST-myocardin are shown at the top, and 10% of the input proteins are shown at the bottom. *(C)* COS cells were transiently transfected with the luciferase reporter plasmids shown above each panel and expression vectors encoding myocardin (100 ng) and GATA4 or GATA4 mutants (100 ng) shown in panel A, as indicated. Luciferase activity was assayed and is expressed as fold activation above the level of expression of the reporter alone.

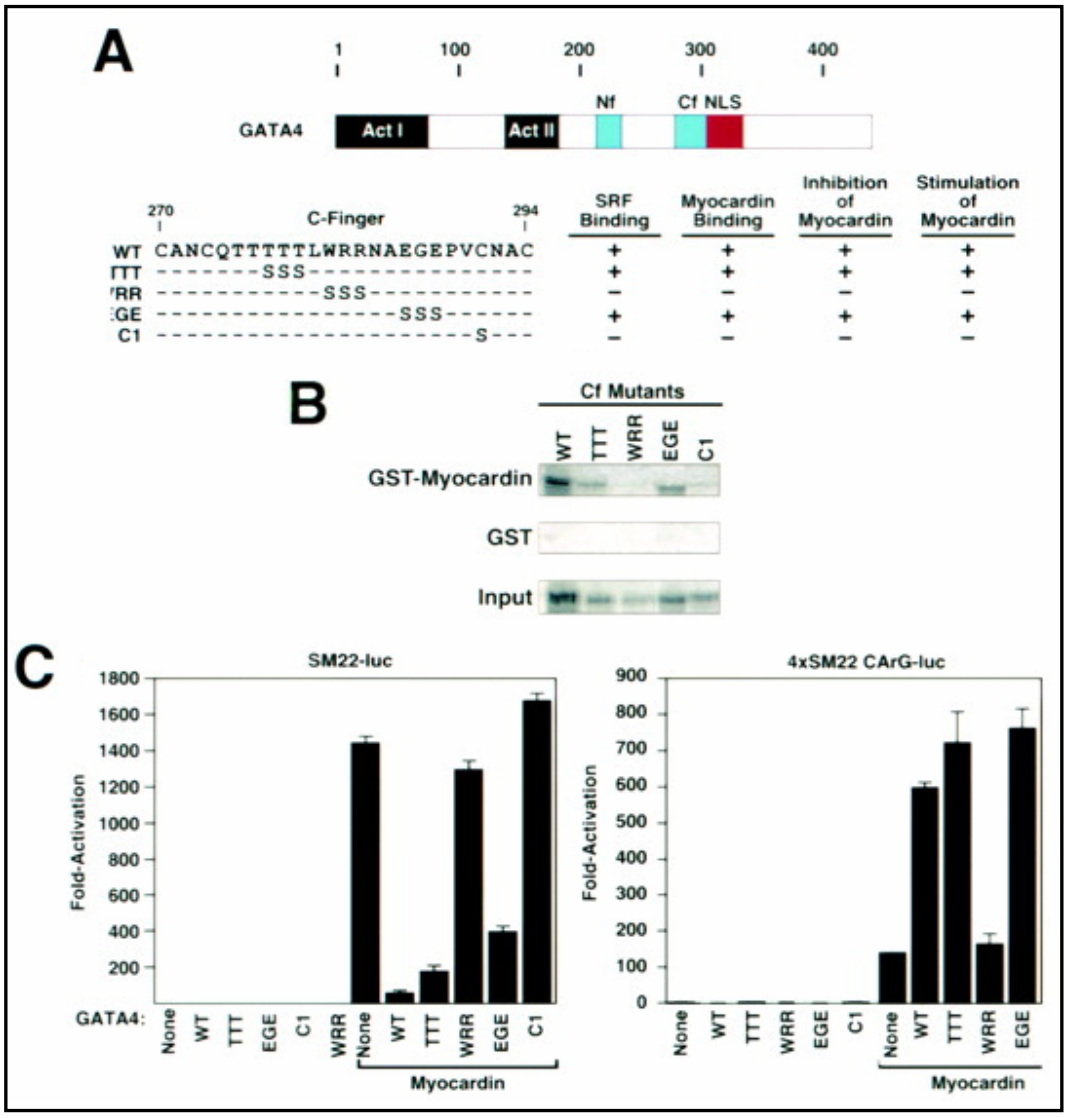


Figure 2.6. Identifying the residues in the Cf of GATA4 that are critical for myocardin binding. *(A)* Point mutants in Cf of GATA4 and their activities. *(B)* A GST-myocardin fusion protein encompassing amino acids 129 to 510 was incubated with [^{35}S]methionine-labeled GATA proteins translated in vitro. GATA4 proteins associated with GST-myocardin or GST are shown at the top and middle, respectively, and 10% of the input proteins are shown at the bottom. *(C)* COS cells were transiently transfected with the luciferase reporter plasmids shown above each panel and expression vectors encoding myocardin (100 ng) and GATA4 or GATA4 mutants (100 ng) shown in panel A, as indicated. Luciferase activity was assayed and is expressed as fold activation above the level of expression of the reporter alone.

Discussion

The major finding of this study is that GATA4 can stimulate or suppress myocardin activity in a target gene-specific manner. Modulation of myocardin activity by GATA4 is mediated, at least in part, by the direct interaction of GATA4 with myocardin and does not require binding of GATA4 to DNA. Paradoxically, stimulation of myocardin activity by GATA4 requires only the DNA binding domain, but not the TAD of GATA4, whereas repression of myocardin activity by GATA4 requires the DNA binding domain and the TAD (shown in Fig. 2.7). These findings reveal an unusual complexity to the mechanism(s) involved in myocardin- and GATA-dependent gene regulation with the potential to provide gene-specific transcriptional control and fine tuning of the expression of SRF target genes.

Interaction of GATA4 with Myocardin

My results demonstrate that GATA4 can interact with two regions of myocardin. GATA4-binding region 1 of myocardin maps to residues 326 to 438. This region encompasses the SAP domain (residues 380 to 414), but deletions to either side of this domain impair GATA4 binding, indicating that binding requires an extended sequence of myocardin such that deletions throughout this region perturb this protein-protein interaction. GATA4 can also interact with residues 439 to 713 independently of binding region 1. I have not further defined this region, which includes the TAD, since replacing it with VP16 does not diminish the responsiveness of myocardin to GATA4.

What mechanism(s) might account for the opposite effects of GATA factors on myocardin activity? Because GATA4 can stimulate myocardin activity on some regulatory

regions (e.g., the *Nkx2.5* enhancer) and inhibit activity on others (e.g., the *ANF* promoter), it is likely that the differential effects of GATA4 are dependent on other factors that bind myocardin target genes. Stimulation of myocardin activity by GATA4 was observed with an artificial reporter containing multimers of the SRF binding site, which suggests to me that this type of stimulatory activity may reflect a simpler mechanism and that repression may involve more complex interactions between GATA4 and other nuclear factors. The *ANF* and *SM22* promoters were both repressed by GATA4 in the presence of myocardin, suggesting that they are regulated by the same mechanism. The *ANF* promoter contains two GATA binding sites, neither of which is required for repression by GATA4, and the *SM22* promoter contains no GATA sites. Inspection of these promoters has not revealed any obvious binding sites for common factors that might mediate the repressive influence of GATA4.

Understanding the mechanisms involved in GATA-dependent modulation of myocardin activity is complicated by the fact that both myocardin and GATA4 interact with SRF [2, 26, 37]. This raises the possibility that some of the observed effects of GATA4 may reflect competition between myocardin and GATA4 for interaction with SRF or recruitment of both factors by SRF, independent of their interaction with each other. The finding that GATA4 can stimulate the activity of a GAL4-myocardin fusion protein that activates transcription independently of SRF suggests that the stimulatory effects of GATA4 do not require its direct association with SRF and are likely to be mediated by direct physical association with myocardin. This conclusion is supported by the precise correlation between the ability of GATA4 mutants to interact with myocardin and to stimulate myocardin activity.

Although GATA4 interacts with myocardin and enhances myocardin activity, myocardin cannot stimulate transcription through GATA binding sites. The inability of myocardin to activate transcription by tethering to GATA factors on DNA may be explained by the fact that myocardin interacts with the same residues in the C-terminal zinc finger of GATA4 that mediate GATA4 DNA binding, which could preclude the formation of a stable GATA-myocardin ternary complex on DNA. In contrast, GATA4 interacts with domains of myocardin that are not required for association with SRF. Therefore, GATA4 can interact with myocardin without perturbing myocardin's ability to interact with SRF.

It is intriguing that stimulation of myocardin activity requires only the Cf and NLS of GATA4, whereas suppression of myocardin activity requires these domains in addition to the N-terminal transcription activation domain. The ability to separate the stimulatory and suppressive effects of GATA4 by deletion of the TAD suggests that these occur through distinct mechanisms. I can envision at least three mechanisms that might account for the ability of GATA4 to stimulate myocardin activity. GATA4 could induce a conformational change in myocardin that augments its transcriptional activity, possibly by unmasking the TAD or stabilizing its interaction with the transcriptional machinery. GATA4 could recruit a coactivator to the myocardin-SRF complex, or it could displace an inhibitory protein from the complex.

With respect to the mechanism for GATA4-mediated suppression of myocardin activity, the requirement for the TAD in this process suggests that the presence of GATA4 may cause "squelching" [29] such that this domain competes with an activation domain of another factor, which could be myocardin itself, SRF, or another factor required for

activation of the set of genes that are suppressed by GATA4. Another mechanism may involve competition between myocardin and GATA4 for SRF interaction. It is not obvious, however, how the TAD of GATA4 would contribute to such a repressive mechanism.

My finding that GATA4 can suppress the activity of myocardin on SRF target genes is somewhat surprising in light of numerous previous studies demonstrating that GATA4 can stimulate SRF-dependent transcription [2, 26, 33]. The stimulatory effect of GATA4 on SRF is mediated by the direct interaction of the SRF MADS box with the Cf of GATA4 [2, 26, 33], the same region that I showed to mediate interaction of GATA4 with myocardin. This seeming discrepancy from previous studies may be explained, at least in part, by the fact that SRF and GATA4 are relatively weak activators that stimulate transcription by about an order of magnitude under optimal conditions. In contrast, myocardin stimulates expression of target genes with two or more SRF binding sites by several orders of magnitude. Thus, a modest stimulatory effect of GATA4 on SRF activity would be overcome by the dramatic repressive effect of GATA4 on myocardin activity.

Modulation of Myocardin Activity through the Stoichiometry of its Partners

I have previously shown that the transcriptional activity of myocardin is exquisitely sensitive to the level of SRF, such that relatively minor increases in SRF expression above an optimal level result in pronounced suppression of myocardin activity analogous to the effects of GATA4 seen in this study [37]. These findings illustrate the importance of precise control over the stoichiometry of these transcriptional activators. Given the importance of myocardin and GATA factors in smooth and cardiac muscle development, it will be especially

interesting to explore whether perturbations in the relative levels of expression of the genes encoding these factors in vivo influences development of the cardiovascular system. The importance of GATA factors and the myocardin-related transcription factor MRTF-A in hematopoietic development also suggests that the types of regulatory interactions described in this study will be of importance in that system as well.

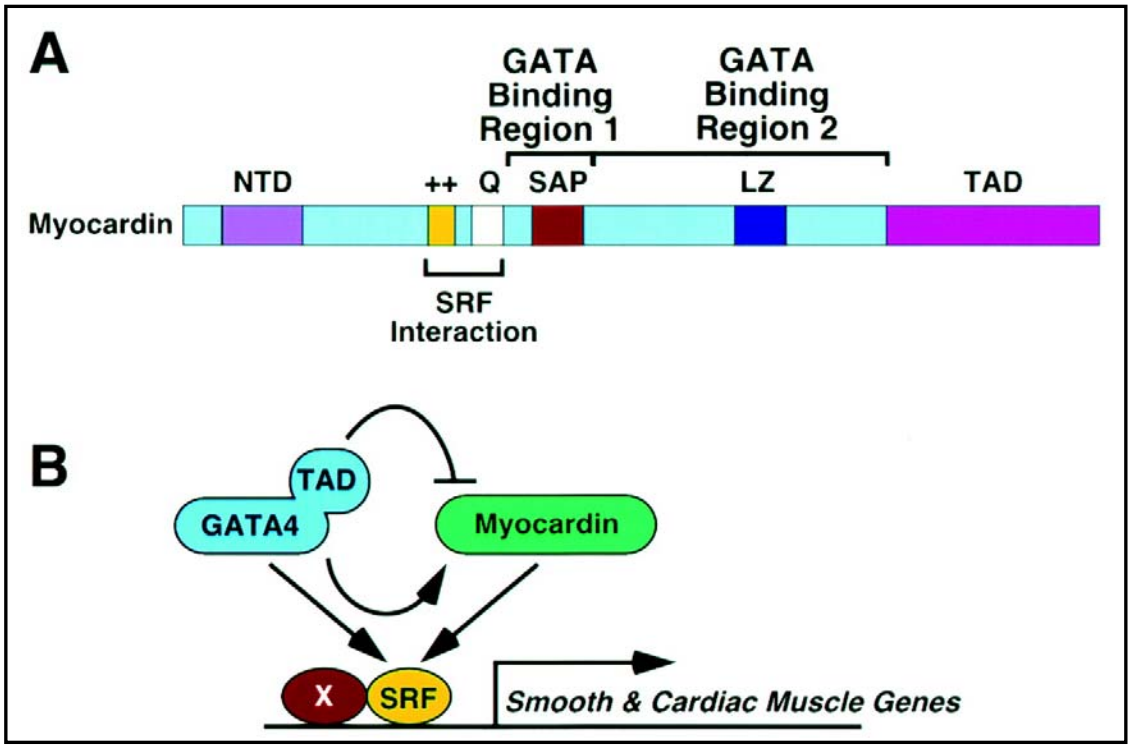


Figure 2.7. Schematic diagram of myocardin and a model. *(A)* The domains of myocardin are shown. *(B)* The regions of GATA4 that associate with myocardin and mediate activation and repression are shown.

References

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

2. **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.

3. **Chang, P. S., Li, L., McAnally, J. and Olson, E. N.** 2001. Muscle specificity encoded by specific serum response factor-binding sites. *J. Biol. Chem.* **276:**17206-17212.

4. **Chang, D. F., Belaguli, N. S., Iyer, D., Roberts, W. B., Wu, S. P., Dong, X. R., Marx, J. G., Moore, M. S., Beckerle, M. C., Majesky, M. W. and Schwartz, R. J.** 2003. Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent smooth muscle differentiation cofactors. *Dev. Cell.* **4:**107-118.

5. **Charron, F. and Nemer, M.** 1999. GATA transcription factors and cardiac development. *Semin. Cell Dev. Biol.* **10:**85-91.

6. **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.

7. **Dai, Y. S. and Markham, B. E.** 2001. p300 Functions as a coactivator of transcription factor GATA-4. *J. Biol. Chem.* **276:**37178-37185.

8. **Du, K. L., Ip, H. S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M. M., Owens, G. K. and Parmacek, M. S**. 2003. Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Mol. Cell. Biol.* **23:**2425-2437.

9. **Grueneberg, D. A., Natesan, S., Alexandre, C. and Gilman, M. Z.** 1992. Human and Drosophila homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science* **257:**1089-1095.

10. **Hines, W. A., Thorburn, J. and Thorburn, A.** 1999. A low-affinity serum response element allows other transcription factors to activate inducible gene expression in cardiac myocytes. *Mol. Cell. Biol.* **19:**1841-1852.

11. **Kim, S., Ip, H. S., Lu, M. M., Clendenin, C. and Parmacek, M. S.** 1997. A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. *Mol. Cell. Biol.* **17:**2266-2278.

12. **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.

13. **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.

14. **Latinkic, B. V., Kotecha, S. and Mohun, T. J.** 2003. Induction of cardiomyocytes by GATA4 in Xenopus ectodermal explants. *Development* **130:**3865-3876.

15. **Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. and Evans, T.** 1994. GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269:**23177-23184.

16. **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.

17. **Li, L., Miano, J. M., Mercer, B. and Olson, E. N.**1996. Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J. Cell Biol.* **132:**849-859.

18. **Li, L., Liu, Z., Mercer, B., Overbeek, P. and Olson, E. N.** 1997. Evidence for serum response factor-mediated regulatory networks governing SM22alpha transcription in smooth, skeletal, and cardiac muscle cells. *Dev. Biol.* **187:**311-321.

19. **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. USA* **100:**9366-9370.

20. **Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A. and Olson, E. N.** 1999. Control of early cardiac-specific transcription of an Nkx2-5 by a GATA-dependent enhancer. *Development* **126:**75-84.

21. **Ma, Z., S. W. Morris, V. Valentine, M. Li, J. A. Herbrick, X. Cui, D. Bouman, Y. Li, P. K. Mehta, D. Nizetic, Y. Kaneko, G. C. Chan, L. C. Chan, J. Squire, S. W. Scherer, and J. K. Hitzler.** 2001. Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. *Nat. Genet.* **28:**220-221.

22. **Mercher, T., Coniat, M. B., Monni, R., Mauchauffe, M., Khac, F. N., Gressin, L., Mugneret, F., Leblanc, T., Dastugue, N., Berger, R. and Bernard, O. A.** 2001.

Involvement of a human gene related to the Drosophila spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia. *Proc. Natl. Acad. Sci. USA* **98:**5776-5779.

23. **Merika, M., and Orkin, S. H.** 1993. DNA-binding specificity of GATA family transcription factors. *Mol. Cell. Biol.* **13:**3999-4010.

24. **Miralles, F., Posern, G., Zaromytidou, A. I. and Treisman, R.** 2003. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113:**329-342.

25. **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.

26. **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.

27. **Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S.** 1998. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12:**3579-3590.

28. **Norman, C., Runswick, M., Pollock, R. and Treisman, R.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. *Cell* **55:**989-1003.

29. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. *Nature* **335:**683-689.

30. **Reecy, J., Belaguli, N. S. and Schwartz, R. J.** 1998. SRF/homeobox protein interactions, p. 273-290. *In* R. Harvey and N. Rosenthal (ed.), Heart development. Academic Press, San Diego, Calif.

31. **Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D. Y.** 1999. Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13:**2983-2995.

32. **Sasazuki, T., Sawada, T., Sakon, S., Kitamura, T., Kishi, T., Okazaki, T., Katano, M., Tanaka, M., Watanabe, M., Yagita, H., Okumura, K. and Nakano, H.** 2002. Identification of a novel transcriptional activator, BSAC, by a functional cloning to inhibit tumor necrosis factor-induced cell death. *J. Biol. Chem.* **277:**28853-28860.

33. **Sepulveda, J. L., Vlahopoulos, S., Iyer, D., Belaguli, N. and Schwartz, R. J.** 2002. Combinatorial expression of GATA4, Nkx2-5, and serum response factor directs early cardiac gene activity. *J. Biol. Chem.* **277:**25775-25782.

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

35. **Sprenkle, A.B., Murray, S.F. and Glembotski, C.C.** 1995. Involvement of multiple Cis-elements in α-adrenergic agonist-inducible ANF transcription. Roles for SREs and an Sp-1-like element. *Circ. Res.* **77**:1060–1069.

36. **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.

37. **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.

38. **Wang, D.-Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Nordheim, A. and Olson, E. N.** 2002. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc. Natl. Acad. Sci. USA* **99:**14855-14860.

39. **Wang, Z., Wang, D.-Z., Teg Pipes, G. C. and Olson, E. N.** 2003. Myocardin is a master regulator of smooth muscle gene expression. *Proc. Natl. Acad. Sci. USA* **100:**7129-7134.

40. **Yoshida, T., Sinha, S., Dandre, F., Wamhoff, B. R., Hoofnagle, M. H., Kremer, B. E., Wang, D.-Z., Olson, E. N. and Owens, G. K.** 2003. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ. Res.* **92:**856-864.

Chapter III

Requirement of Myocardin Related Transcription Factor-B for Remodeling of Branchial Arch Arteries and Smooth Muscle Differentiation

Introduction

Myocardin and the myocardin-related transcription factors (MRTFs) A and B comprise a family of coactivators that provides transcriptional activity to serum response factor (SRF) [refs. 1 and 2; reviewed in refs. 3 and 4]. SRF binds a DNA sequence known as a CArG box and serves as a docking site for myocardin and MRTFs [1, 2]. CArG boxes are required for expression of numerous smooth, cardiac, and skeletal muscle genes [5, 6]. Tissue-specific deletion of the *SRF* gene in these muscle cell types results in severe abnormalities in muscle cell differentiation [7–10].

Members of the myocardin family share homology in a basic and glutamine-rich region that interacts with SRF [1, 2]. An adjacent (Scaffold attachment factor, Acinus, PIAS) domain is involved in target gene specificity, such that its deletion prevents activation of a subset of SRF target genes [1]. A coiled-coil domain resembling a leucine zipper mediates dimerization of myocardin and MRTFs [11–13] and has been proposed to allow cooperativity between CArG boxes [11]. The C-terminal regions of myocardin and MRTFs function as transcription activation domains [1, 2].

Myocardin and the MRTFs enhance the transcriptional activity of SRF by forming a ternary complex with SRF on DNA and providing their powerful transcription activation domains [1, 2]. MRTF-A and -B also convey stimulatory signals from the Rho GTPase and the actin cytoskeleton to SRF via their regulated translocation into the nucleus [12, 14–16].

Myocardin expression is restricted to cardiac and smooth muscle cells [1], whereas MRTF-A and MRTF-B are expressed in a broad range of cell types [2, 17]. Consistent with the postulated role of myocardin as a regulator of smooth muscle gene expression, myocardin

knockout mice die by embryonic day (E) 10.5 with an absence of differentiated smooth muscle cells (SMCs) [18]. Although a dominant negative myocardin mutant is sufficient to prevent cardiac gene expression in *Xenopus* embryos [1], myocardin null mouse embryos do not show cardiac defects [18]. Mice homozygous for a *lacZ* enhancer trap allele of *MRTF-B* display perinatal lethality [19], which has been attributed to abnormalities in vascular development [20]. However, this *lacZ* insertion has the potential to generate a dominant negative mutant of MRTF-B that would be predicted to perturb the function of other myocardin family members. Moreover, a fraction of transcripts generated from this allele encode the wild-type protein, thereby preventing determination of a true loss-of-function phenotype [19].

To unequivocally determine the function of MRTF-B *in vivo*, I created an *MRTF-B* mutant allele by homologous recombination in embryonic stem cells, which I used to generate *MRTF-B* null mice. These mice die between E13.5 and E14.5 and display severe defects in the anatomy of branchial arch arteries and cardiac outflow tract, accompanied by a defect in smooth muscle differentiation. I conclude that MRTF-B plays an essential early role in development of a specific subset of vascular SMCs.

Materials and Methods

Transfection Assays

Transfection assays were described in detail in Chapter II. COS cells were transiently transfected with 100 ng of pcDNA expression plasmids encoding wild-type or mutant forms of myocardin, MRTF-A and MRTF-B, and an SM22-luciferase reporter. Forty hours later, cell extracts were prepared, and luciferase activity was determined and normalized to the β-galatosidase acitivity.

Generation of MRTF-B Mutant Mice

I created an *MRTF-B* targeting vector so as to delete exon 8 of the gene and introduce a *lacZ* and *neomycin-resistance* gene by using a pN-Z-TK2 vector, which contains a nuclear *LacZ* (*nLacZ*) cassette and a *neomycin-resistance* gene under the control of the RNA polymerase II promoter and two herpes simplex virus *thymidine kinase* (*TK*) gene cassettes (a generous gift of R. Palmiter, University of Washington, Seattle). The targeting vector was electroporated into 129 SvEv-derived ES cells, and selection was performed with G-418 and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil, respectively. Four hundred ES cell clones were isolated and analyzed for homologous recombination by Southern blotting with both 5' and 3' probes. Three clones with a targeted *MRTF-B* gene were used for blastocyst injection, and the resulting chimeric male mice were bred to C57BL/6 females to obtain germ-line transmission of the mutant allele. Genotyping was performed by PCR with genomic DNA prepared from tail biopsies or from embryo yolk sacs using the following primers: fwd, 5'-ATCAGGAACAGACATTTCCTACACTGTC-3'; wt-rvs, 5'-GGCATAGT

TGGAGTCCATCTGTGGCTCG-3'; mut-rvs, 5'-CATCGTAACCGTGCATCTGCCAGTTT GAG-3'

RT-PCR

Total RNA was purified from the hearts of E10.5 embryos with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, total RNA was used as a template for reverse transcriptase and random hexamer primers. RT-PCR primers used are as follows: myocardin: 5'-GCCAACGACAGTGACGACGAACA-3, 5'-CGTGAAGCTC AGCTGCAGAC-3'; MRTF-A: 5'- CACTGTGACCAATAAGAGTGC-3', 5'-GCCTGTG GAGCATCAATG-3'; MRTF-B: 2F, 5'-GTGCTCCAGCTGAGGCTGCAACAAAGG-3; 5R, 5'-GTGTGGCCCTGAAGAGATGGC-3'; 7F, 5'-CCTCAGTGTCCCCAGCAGTTC-3'; 8F, 5'-GCCATCCCAAGAATCCAAACGACG-3'; 8R, 5'-GCATAGTTGGAGTCCATCT GTG-3'; 9R, 5'-GTCATCCAAGCTGGAGGGCAGC-3'; 10F, 5'-GCAGAGCAGCAGAA CGTTGTCTCGC-3'; 10R, GCTGCCTCTGATACTGAAGAACTG-3'; lacZR, 5'-CATCGT AAGCATCTGCCAGTTTGAG-3'; GAPDH: 5'-GCAGTGGCAAAGTGGAGATTG-3'; 5'-TTTGGCTCCACCCTTCAAGTG-3'.

India Ink Injection

Embryos were collected at E11.5, and India ink was injected intracardially by using a finely drawn glass pipette. The embryos were then immediately fixed in 4% paraformaldehyde overnight, dehydrated, and cleared in benzyl alcohol: benzyl benzoate.

Histology and Immunohistochemistry

Histology and LacZ staining were performed as described in ref. 21. Briefly, embryos used for histology were fixed in 4% paraformaldehyde, sectioned, and processed for hematoxylin and eosin (H&E) staining. For LacZ staining, the embryos were dissected out free of yolk sac and amnion. After 2 washes in cold PBS, they were fixed with cold PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde. Embryos younger than E9.5 were fixed for 30 minutes; E10.5-E13.5 embryos were fixed for 1-1.5 hrs. After fixation, the embryos were washed with cold PBS for 3 times, 10 minutes each. Then they were stained in the dark overnight at room temperature with staining solution. The staining solution contains: 4 mM Ferrocyanide, 4 mM Ferricyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal in PBS. The X-gal stock is 40 mg/ml in Dimethylformamide. After staining, the embryos were washed with PBS for 3 times and post-fixed with the same fix solution overnight at 4 ^{o}C.

For immunohistochemistry, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol to PBS, and permeabilized in 0.3% Triton X-100 in PBS. Nonspecific binding was blocked by 1.5% normal horse serum in PBS and monoclonal α-smooth muscle (SM) actin antibody [clone 1A4, Sigma] was applied at a 1:200 dilution in 0.1% BSA in PBS overnight at 4°C. Sections were washed in PBS and fluorescein-conjugated secondary antibody (Vector Laboratories) were applied at a 1:200 dilution in 1% normal horse serum for 1 hr.

Results

Potential Dominant Negative Function of an MRTF-B lacZ Insertion Allele

My goal was to analyze the function of *MRTF-B in vivo* by creating *MRTF-B* null mice. We were aware of a mutation created by random insertion of a β-*geo* cassette between exons 10 and 11 of the *MRTF-B* gene [20]. This *lacZ* insertion allele would generate an MRTF-B protein truncated at amino acid 730 (Fig. 3.1.*A*). Based on prior studies, I anticipated that this truncated protein would act as a dominant negative mutant [1, 2, 13, 16]. Before embarking on the creation of a new *MRTF-B* mutation, I examined the function of this mutant protein in transfected COS cells. The truncated MRTF-B protein (1–730) failed to activate an SRF-dependent reporter alone (data not shown); however, as anticipated, it interfered with the transcriptional activity of the wild-type myocardin and MRTF-A proteins when comparable amounts of each plasmid were transfected (Fig.3.1.*B*). The dominant negative activity of the MRTF-B 1–730 mutant was weaker than that of a previously described myocardin mutant protein (129–715) [1], presumably because the N-terminal domain of MRTF-B causes partial sequestration in the cytoplasm [2, 16]. The 1–730 mutant protein, like wild-type MRTF-B, showed nuclear localization in ≈15% of transfected COS cells. Fusion of an SV40 nuclear localization sequence to the amino terminus of this truncated protein to more closely reflect the nuclear localization of the endogenous MRTF-B protein in SMCs enhanced its dominant negative activity (Fig. 3.1.*B*).

Based on these findings, I was concerned that the potential dominant negative activity of the *lacZ* insertion allele might cloud the interpretation of the potential role of MRTF-B *in*

vivo. In addition, it has been reported that a minor fraction of wild-type transcripts are generated from this allele by splicing around the *lacZ* insertion [20]. Thus, the *lacZ* insertion allele does not function as a true null.

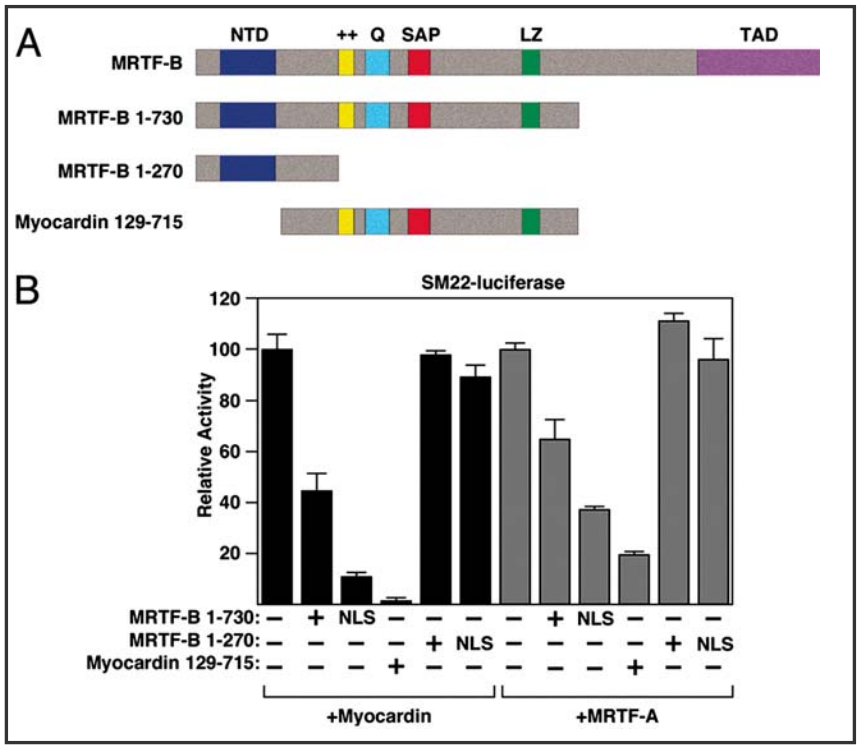


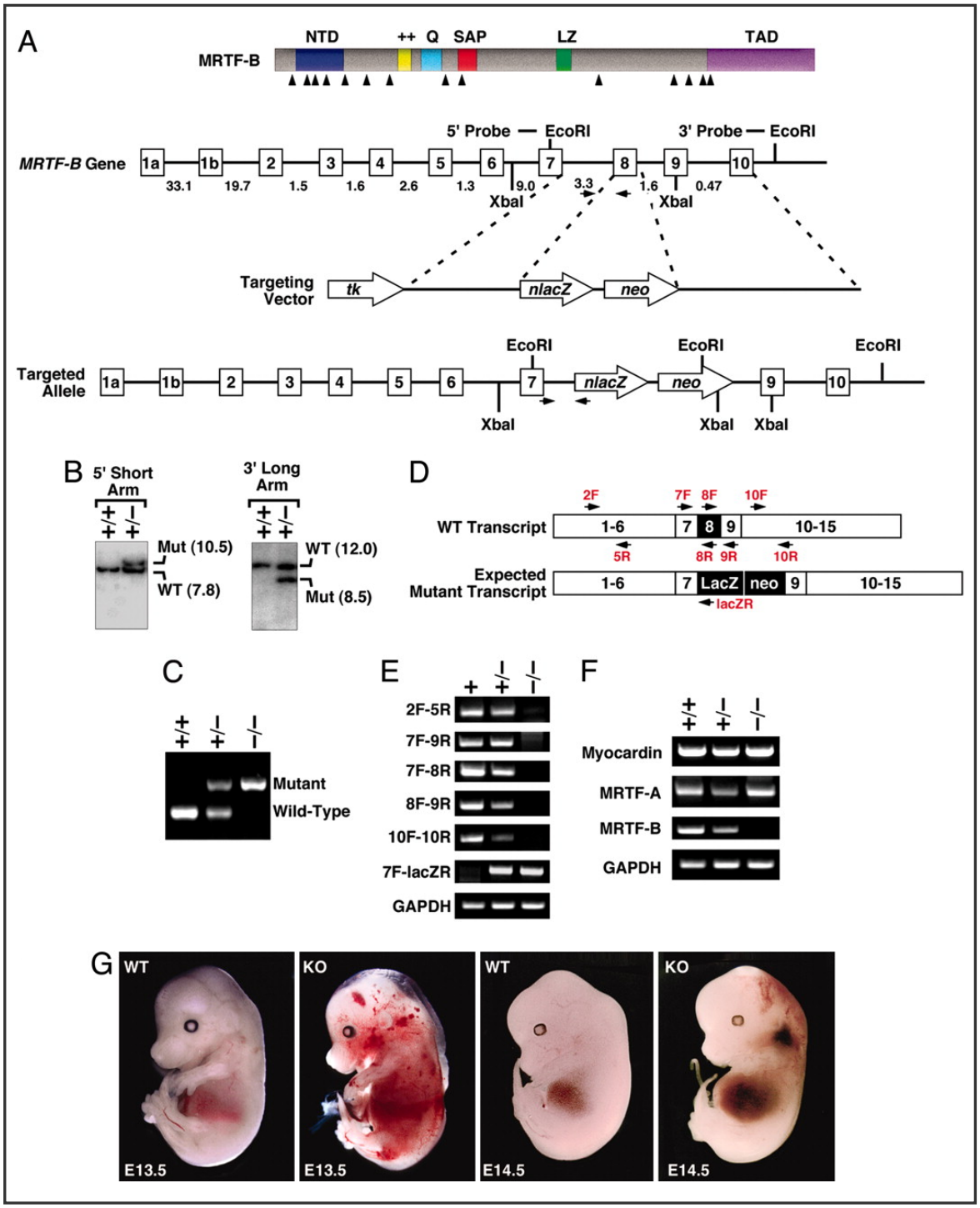
Figure 3.1. Transcriptional activities of MRTF-B mutant proteins. (*A*) The structures of wild-type and mutant MRTF-B and myocardin proteins are shown. The *lacZ* gene trap line (20) is predicted to generate a truncated MRTF-B protein containing residues 1–730. The targeted mutation we introduced into the *MRTF-B* gene is predicted to generate a truncated MRTF-B protein containing residues 1–270. A dominant negative mutant of myocardin is also shown. NTD, N-terminal domain; ++, basic domain; SAP, Scaffold attachment factor. Q, glutamine-rich domain; LZ, leucine zipper; TAD, transcription activation domain. (*B*) Wild type and mutant proteins were transfected into COS cells and assayed for their ability to activate an SM22-luciferase reporter. Truncated MRTF-B proteins containing the SV40 nuclear localization sequence (NLS) at the amino terminus were also tested. The MRTF-B 1–730 mutant functions as a dominant negative, whereas the 1–270 mutant lacks inhibitory activity.

Generation of MRTF-B Knockout Mice.

To create a loss-of-function allele of the mouse *MRTF-B* gene, we introduced a *lacZ* cassette after exon 7 and deleted exon 8 of the gene by homologous recombination in ES cells (Fig. 3.2 *A*-*C*). This mutation results in a truncated protein containing only the first 270 amino acids of MRTF-B and lacking the SRF-binding, dimerization, and transcription activation domains. In contrast to the dominant negative activity of the predicted product of the *lacZ* insertion allele, the 270-aa mutant protein is functionally inert (Fig. 3.1.*B*).

Germ-line transmission of the mutant *MRTF-B* allele was achieved from three independent ES cell clones. Intercrosses of *MRTF-B*$^{+/-}$ mice in the isogenic C57BL/6 background or in mixed backgrounds failed to produce any *MRTF-B* null offspring among >100 offspring analyzed. Contrary to findings observed with the *MRTF-B* mutant mice generated by random insertion of a β-*geo* cassette [20], we conclude that our homozygous *MRTF-B* mutation results in embryonic lethality with complete penetrance.

The gene-targeting event was confirmed by RT-PCR analysis of mRNA from the hearts of E10.5 embryos by using primers representing exon sequences within and surrounding the deleted region of the gene (Fig. 3.2.*D*). These assays showed that exon 7 was spliced to the *lacZ* allele as predicted (primers 7F and lacZR in Fig. 3.2.*D* and *E*). Sequencing of this PCR product confirmed that the mutant allele generated a protein in which amino acid 270 of MRTF-B was fused inframe to lacZ (data not shown). Portions of the *MRTF-B* transcript 3' of the *lacZ* insertion site could not be detected in homozygous mutant embryos. There was no change in expression of *myocardin* or *MRTF-A* in mutant hearts (Fig. 3.2.*F*), indicating that these genes were not up-regulated to compensate for the lack of MRTF-B.


A
MRTF-B
NTD
++
Q
SAP
LZ
TAD
MRTF-B Gene
5' Probe
EcoRI
3' Probe
EcoRI
1a
1b
2
3
4
5
6
7
8
9
10
33.1
19.7
1.5
1.6
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1.3
9.0
3.3
1.6
0.47
XbaI
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Targeting Vector
tk
nlacZ
neo
Targeted Allele
EcoRI
EcoRI
EcoRI
XbaI
XbaI
XbaI
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5' Short Arm
3' Long Arm
+/+
+/−
Mut (10.5)
WT (7.8)
WT (12.0)
Mut (8.5)
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10-15
2F
7F
8F
10F
5R
8R
9R
10R
Expected Mutant Transcript
LacZ
neo
lacZR
C
Mutant
Wild-Type
E
2F-5R
7F-9R
7F-8R
8F-9R
10F-10R
7F-lacZR
GAPDH
F
Myocardin
MRTF-A
MRTF-B
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E13.5
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E14.5

Figure 3.2. Generation and analysis of *MRTF-B* knockout mice. (*A*) Gene targeting strategy. The mouse MRTF-B protein is schematized at the top. The targeting vector contained a 3.2-kb 5' arm and a 4.7-kb 3' arm and replaced a 0.5-kb region of the gene with a lacZ-neo cassette. Intron junctions within the coding region are shown by arrowheads beneath the schematized protein. Exons 1–10 are shown in boxes, and sizes of introns are indicated. Positions of 5' and 3' probes used for Southern analysis in *B* are indicated. Positions of PCR primers used for genotyping are shown by horizontal arrows. (*B*) Southern analysis. Genomic DNA from ES cell clones was isolated and analyzed by Southern blot with 5' and 3' probes after digestion with XbaI and EcoRI, respectively. (*C*) PCR analysis. Genomic DNA from E11.5 embryos was analyzed by PCR with primers shown in *A*. (*D*) Positions of primers used for RT-PCR. A schematic of the exons of the *MRTF-B* gene and positions of primers used for RT-PCR is shown. The expected mutation would contain a lacZ-neo cassette between exons 7 and 9 and would delete exon 8. (*E*) RT-PCR was performed by using RNA isolated from hearts of E10.5 embryos, and the primers shown in *D*. Genotypes of embryos are shown at the top. The truncated transcript generated from the mutant allele (2F-5R) was expressed at a much lower level than the WT MRTF-B transcript. (*F*) RT-PCR was performed by using RNA isolated from hearts of E10.5 embryos with primers specific for myocardin, MRTF-A, and MRTF-B. Transcripts for GAPDH were detected as a control for RNA loading and integrity. (*G*) Appearance of wild type and *MRTF-B* mutant embryos at E13.5 and E14.5.

Early Embryonic Lethality of MRTF-B$^{-/-}$ Mice.

Genotyping of litters from timed pregnancies revealed no homozygous mutants after E14.5 (Table 3.1). At E14.5, I observed only 2 homozygous mutants of 35 embryos analyzed, a number substantially lower than expected from Mendelian inheritance. Homozygous mutant embryos at E13.5 and E14.5 showed pericardial edema and widespread hemorrhaging, suggesting that vascular abnormalities contributed to embryonic demise (Fig. 3.2.*G* and data not shown). At E12.5 and earlier, homozygous mutants appeared outwardly normal and were present approximately at Mendelian ratios. I conclude that the targeted mutation in the *MRTF-B* gene results in complete lethality between E13.5 and 14.5.

Table 3.1. Genotypes of offspring from MRTF-B$^{+/-}$ intercrosses

Genotype	+/+	+/−	−/−	Total
P10	33	61	0	94
E15.5–16.5	17	36	0	53
E14.5	14	19	2 (6)	35
E13.5	17	30	12 (20)	59
E12.5	14	41	14 (20)	69
E9.5≈11.5	24	50	25 (25)	99

The number of offspring of each genotype from intercrosses of *MRTF-B*$^{+/-}$ is shown. Numbers in parentheses indicate % of total offspring at each age.

Expression of lacZ from the Targeted MRTF-B Allele.

The *lacZ* reporter integrated into the targeted *MRTF-B* allele was expressed in a pattern corresponding to that of the wild-type gene. At E8.5, strong expression of lacZ was observed along the ventral neural tube and in two zones in the developing hindbrain, previously reported to correspond to rhombomeres 3 and 5 (Fig. 3.3.*A*) [20]. At E9.5, lacZ expression was also detected in the otic vesicle, heart, dorsal aorta, and branchial arch arteries 1, 2, and 3 (Fig. 3.3 *B* and *C*). At E10.5, lacZ continued to be expressed in the above structures, as well as the first branchial arch and the third and fourth branchial arch arteries of homozygous mutant embryos (Fig. 3.3 *D*–*F*).

Cardiovascular Abnormalities in MRTF-B Mutant Embryos.

Because of the expression of *MRTF-B* in the developing cardiovascular system and the mid-gestation lethality with obvious cardiovascular demise, I examined the cardiovascular system of mutant embryos by injecting India ink into the beating hearts of E11.5 embryos. In contrast to wild-type embryos, in which the third, fourth, and sixth branchial arch arteries were clearly intact bilaterally, in the mutant embryo both sixth branchial arch arteries were hypoplastic and narrow, whereas the third and fourth branchial arch arteries were normal (Fig. 3.4).

Histological sections through the branchial arch arteries at E10.5 and E11.5 consistently confirmed that the sixth branchial arch arteries of mutant embryos were abnormally narrow and prematurely regressed compared with those of wild type.

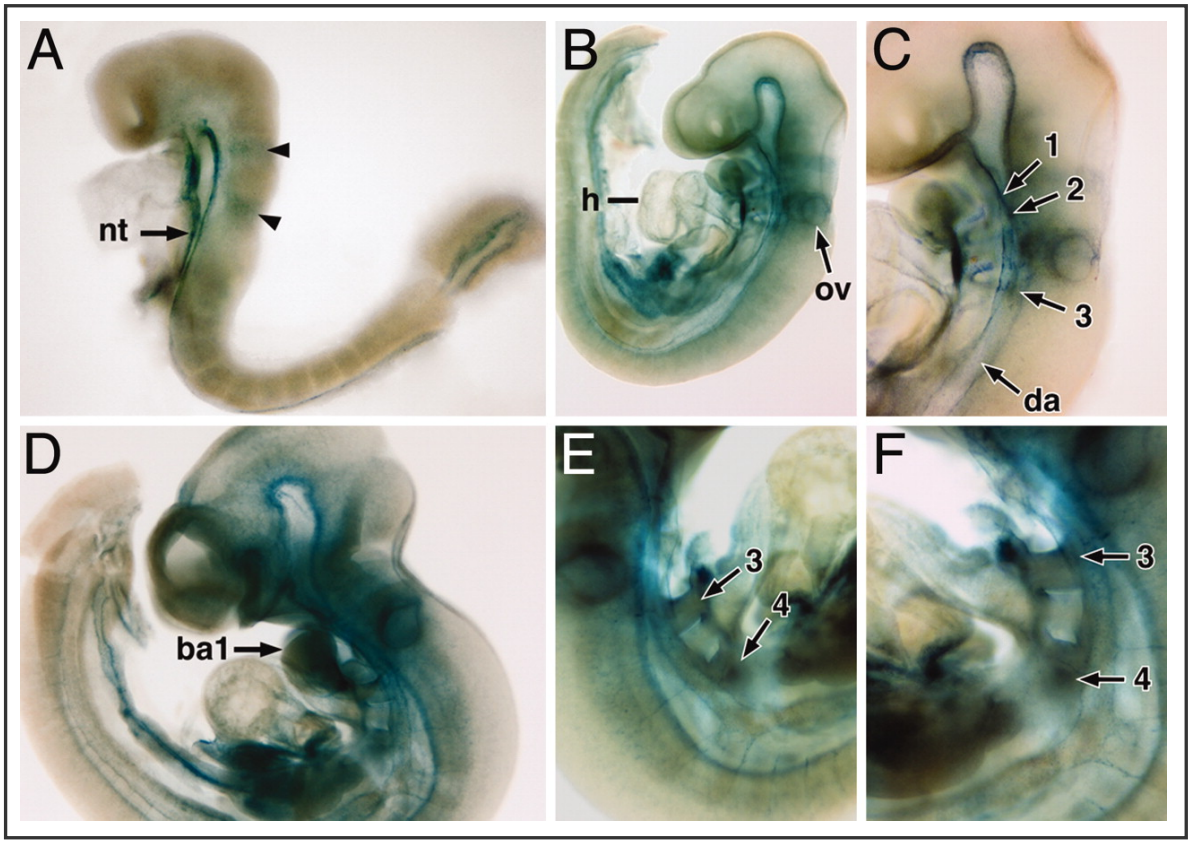

Figure 3.3. LacZ staining of *MRTF-B* mutant embryos. Embryos were stained for lacZ expression. At E8.5, staining was apparent in the ventral neural tube and two distinct stripes (arrowheads) in the developing hindbrain (*A*). LacZ staining is observed in the otic vesicle, heart, dorsal aorta, and branchial arch arteries 1, 2, and 3 at E9.5 (*B* and *C*). At E10.5, lacZ expression becomes more widespread, with high level of expression in the first branchial arch (*D*). LacZ expression is also detected in the third and fourth arch arteries of MRTF-B mutant embryos as shown in the right and left lateral view (*E* and *F*). Branchial arch arteries are numbered. ba1, first branchial arch; nt, neural tube; ov, otic vesicle, h, heart; da, dorsal aorta.

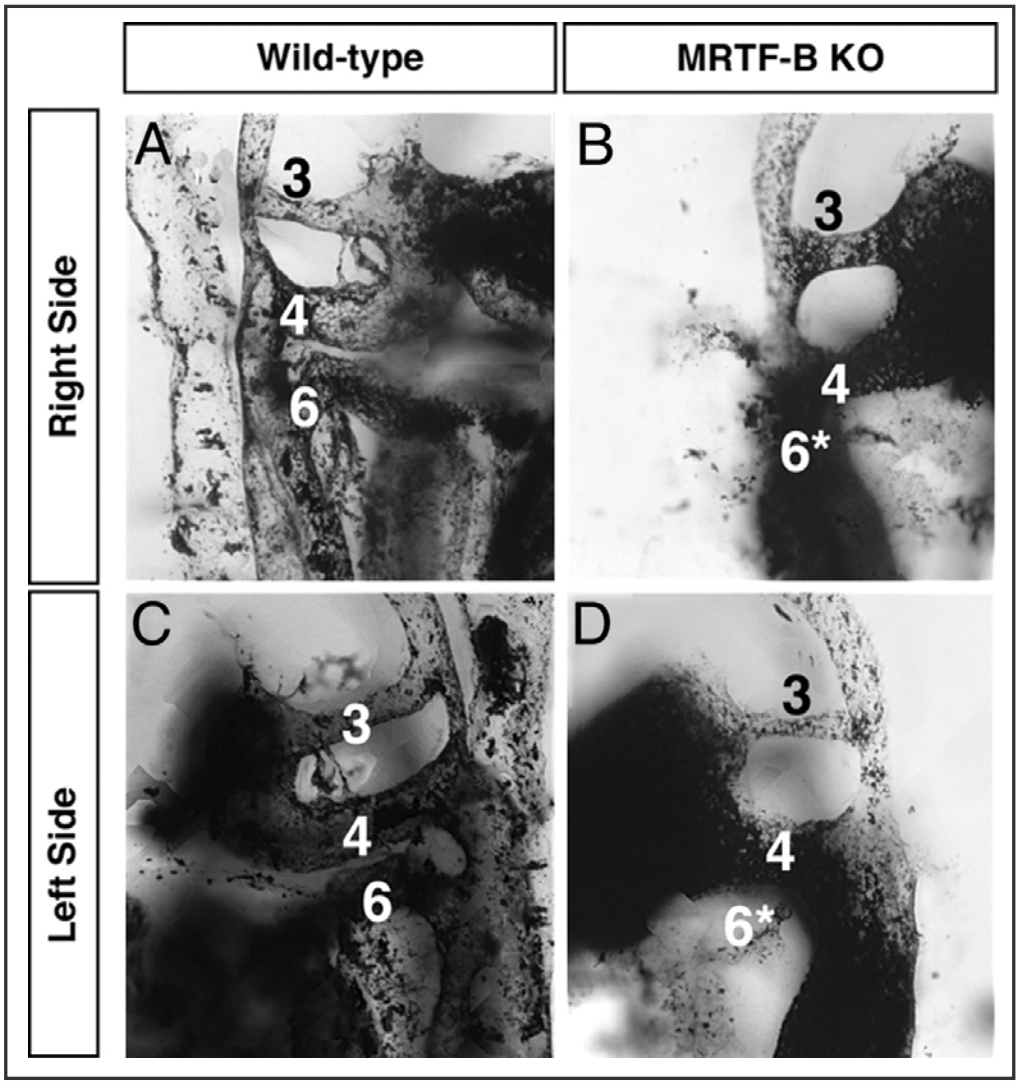

Figure 3.4. Visualization of vasculature by India ink injection. India ink was injected into the beating hearts of wild-type (*A* and *C*) and *MRTF-B*$^{-/-}$ embryos (*B* and *D*) at E11.5. The right (*A* and *B*) and left (*C* and *D*) branchial arch arteries for each embryo are shown in lateral view and numbered. Note that both the right and left sixth arch arteries of mutant embryo are hypoplastic and prematurely regressed (*****).

Furthermore, in serial histological sections, fusion between the fourth and sixth arteries was detected in E10.5 mutant embryos (Fig. 3.5.*A*).

Serial histological sections revealed numerous branchial arch artery defects in mutant embryos at E13.5. In several mutant embryos, the ductus arteriosus, normally derived from the left sixth arch artery, abnormally regressed and did not connect to the aortic arch (Fig. 3.6 *B* and *F*). The pulmonary trunk was hypoplastic, possibly secondary to the lack of the ductus, but did give rise to the pulmonary arteries that projected to the primitive lung buds. In other E13.5 mutant embryos, the ductus arteriosus developed on the right side and passed behind the trachea before joining the descending aorta, reflecting abnormal regression of the left sixth arch artery accompanied by abnormal persistence of the right sixth arch artery (Fig. 3.6 *C* and *G*). I also observed interruption of the aortic arch because of abnormal regression of the left fourth arch artery. In such embryos, the ductus arteriosus connected with the descending aorta distal to the defect (Fig. 3.6 *D* and *H*). Interestingly, this abnormality was associated with an absence of carotid arteries bilaterally due to abnormal patterning of the third arch arteries.

In addition to the abnormal development of the branchial arch arteries, *MRTF-B* mutant embryos displayed abnormalities in the origin of the great vessels at E12.5 and E13.5. Double outlet right ventricle was observed in all mutant embryos (data not shown). High ventricular septal defects and thin-walled myocardium were observed as well (Fig. 3.5.*E*).

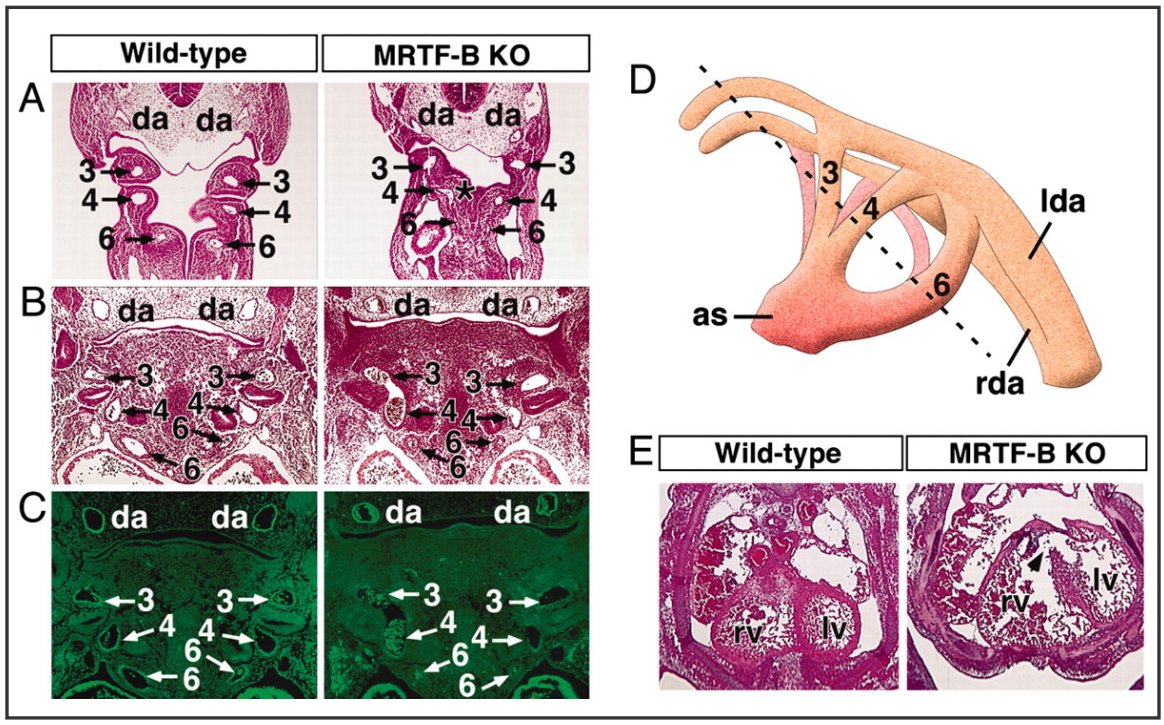

Figure 3.5. Branchial arch artery defects in *MRTF-B* mutant embryos. (*A*–*C*) Wild-type and MRTF-B mutant embryos at E10.5 (*A*) and E11.5 (*B* and *C*). Hematoxylin/eosin sections show hypoplastic sixth arch arteries in mutant embryos. Abnormal communication between the fourth and sixth arteries at E10.5 is indicated (*****). Histological sections stained for SM α-actin show smooth muscle differentiation within the wall of branchial arch arteries and dorsal aortae in the wild-type embryo. In contrast, SM α-actin expression is not detected in the branchial arch arteries of the mutant embryo, but expression is seen in the dorsal aorta and heart. Branchial arch arteries are numbered as 3, 4, and 6. da, dorsal aorta. (*D*) Schematic of branchial arch arteries. The plane of section in the embryos shown in *A*–*C* is indicated by a dashed line. as, aortic sac; lda, left dorsal aorta; rda, right dorsal aorta. (*E*) Transverse section through the heart shows incomplete ventricular septation (arrowhead) and thin myocardial wall in the mutant embryo at E13.5. lv, left ventricle; rv, right ventricle.

Lack of Differentiation of Branchial Arch Artery SMCs in MRTF-B Null Embryos.

Given the ability of myocardin and MRTFs to activate SMC gene expression *in vitro* [11, 17, 22–24], I anticipated that the vascular abnormalities of *MRTF-B* mutant embryos might be accompanied by defects in SMC differentiation. Indeed, immunostaining of histologic sections for SM-α-actin expression showed a clear reduction in smooth muscle differentiation within the walls of the third, fourth, and sixth branchial arch arteries of mutant embryos at E11.5 (Fig. 3.5.*C*). In contrast, SM-α-actin expression was unaffected in the dorsal aorta or heart of mutant embryos. Thus, the loss of *MRTF-B* appears to specifically disrupt SMC differentiation within the branchial arch arteries where morphological abnormalities are also observed.

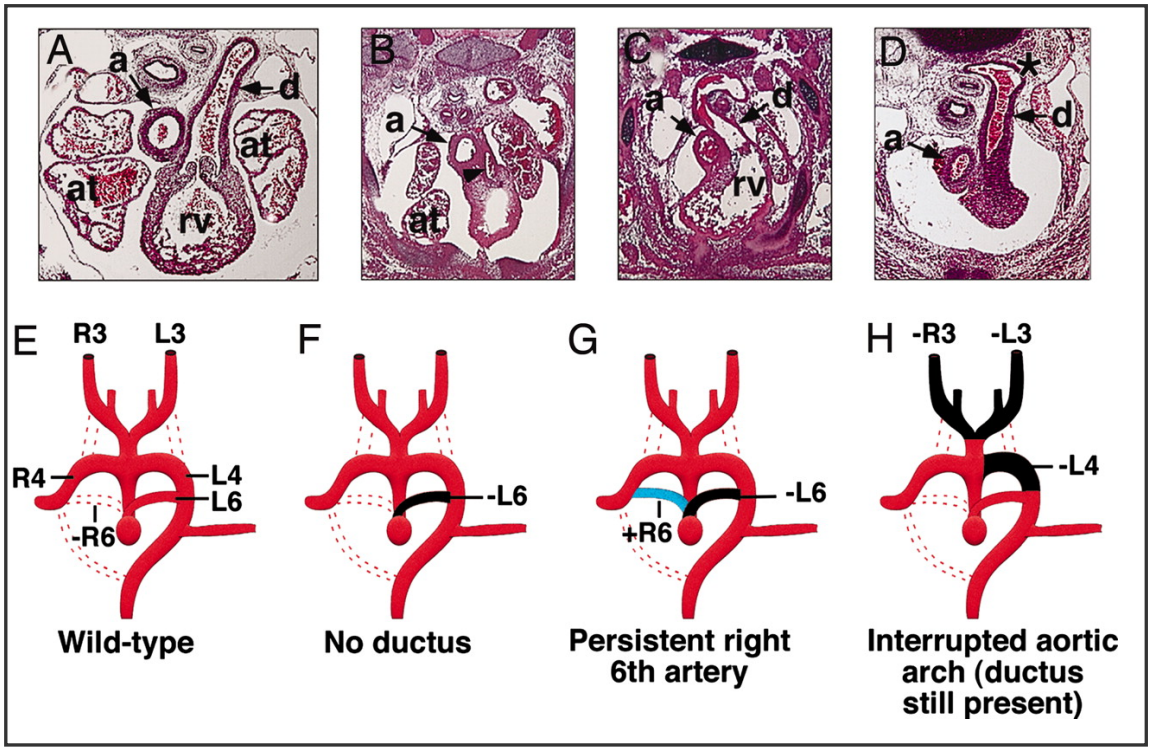

Figure 3.6. Branchial arch artery defects in *MRTF-B* mutant embryos. (*A–D*) Defects of the great vessels in E13.5 mutant embryos. In the transverse section of the wild-type embryo, the pulmonary trunk communicates with the descending aorta via the ductus arteriosus (*A*). Mutant embryos display hypoplastic pulmonary trunk (arrowhead) and no ductus arteriosus (*B*), persistent right-sided ductus arteriosus (*C*), and interrupted aortic arch (*D*). Note the abnormal shape and position of the descending aorta where the ductus arterious joins (*****). a, aorta; at, atrium; d, ductus arteriosus; rv, right rentricle. (*E–H*) Schematic diagrams of aortic arch defects seen in *A–D*. Dotted lines represent normal regression. Black areas depict abnormal regression, whereas blue areas indicate abnormal persistence. (*E*) Patterning of the branchial arch arteries in the wild-type embryo with normal regression of the right sixth artery (-R6). (*F*) Absence of the ductus arteriosus resulting from abnormal regression of the left sixth artery (-L6). (*G*) Persistent right-sided ductus caused by abnormal regression of the left sixth artery (-L6) and abnormal persistence of the right sixth artery (+R6). (*H*) Interrupted aortic arch and loss of both carotid arteries, due to abnormal regression of the left fourth (-L4) and both right and left third arteries (-R3, -L3). R3, R4, and R6, right arch arteries 3, 4, and 6, respectively. L3, L4, and L6, left aortic arch arteries, respectively.

Discussion

The phenotype of *MRTF-B* null mice demonstrates that MRTF-B is an obligate regulator of early cardiovascular development. The absence of MRTF-B results in lethal malformations of the branchial arch arteries and cardiac outflow tract, as well as cardiac anomalies, accompanied by a failure in differentiation of a specific subset of SMCs.

Cardiovascular Abnormalities in MRTF-B Mutant Embryos

The vascular structures affected by the *MRTF-B* null mutation are derived from the neural crest, which migrates into the branchial arches giving rise to vascular SMCs [25, 26]. Based on the lack of α-SM actin staining in the affected vascular structures of *MRTF-B* mutant embryos, I conclude that MRTF-B is required for activation of the SMC differentiation program in these cells, analogous to the function of myocardin in other SMCs [18]. Whether the block to differentiation of these cells is causally related to or independent of the profound remodeling defects in the branchial arch arteries is unclear.

In principle, the failure in differentiation of neural crest-derived SMCs could reflect an abnormality in neural crest cell migration, a block in differentiation of smooth muscle precursors after they reach their destination, or an increase in apoptosis of these cells. Expression of Plexin A, a marker of neural crest cells, showed no difference in wild-type and *MRTF-B* mutant embryos, nor was there an increase in apoptosis within the branchial arches of mutant embryos (data not shown). Thus, I conclude that MRTF-B acts specifically to control differentiation of SMCs in the branchial arch arteries.

Numerous mouse mutants display defects in patterning of the branchial arch arteries [26], but these defects do not result in lethality until birth. It is uncertain why the homozygous *MRTF-B* mutation results in embryonic lethality. This early lethality may result from the lack of the ductus arteriosus, which is necessary for fetal life after ventricular and outflow tract septation. Thin myocardium may also contribute to embryonic lethality. Alternatively, MRTF-B may have functions elsewhere in the embryo, such as the liver, that are required for embryonic viability. Tissue-specific deletion of *MRTF-B* will be required to resolve these issues.

Phenotypes Resulting from Different MRTF-B Mutant Alleles.

As this work was being completed, another report [20] described the phenotype resulting from a *lacZ* insertion allele of *MRTF-B*. In contrast to my results, suggesting that the truncated MRTF-B protein predicted to arise from that *lacZ* insertion allele behaves as a dominant negative mutant, that study concluded that the mutant protein failed to act as a dominant negative mutant. I cannot explain the discrepancy between the results of the two studies. However, it should be pointed out that the mutant protein produced by the *lacZ* insertion allele contains the dimerization and SRF-interaction domains and would therefore be expected to possess dominant negative activity, based on our prior analyses of myocardin and MRTF functions [1, 2]. The low level of the wild-type protein generated by that allele also further complicates the interpretation of phenotypes resulting from it [20]. Although both studies conclude that MRTF-B is required for differentiation of SMCs in the branchial arch arteries, the timing of lethality, as well as the severity and extent of cardiovascular defects,

are clearly different with the two mutations. It is difficult to know whether the different phenotypes resulting from the two mutant alleles reflect expression of the dominant negative protein or leaky expression of wild-type protein from the *lacZ* insertion allele or other variables.

Potential Involvement of Myocardin Family Members in Human Congenital Heart Disease

The requisite roles of myocardin and MRTF-B in vascular development raises the possibility that mutations or polymorphisms in these genes may contribute to outflow tract abnormalities in humans, which represent one of the most prevalent forms of congenital heart disease [25–27]. Although mice heterozygous for loss-of-function mutations in these genes do not display obvious phenotypes, it is not uncommon for heterozygous mutations in cardiovascular developmental control genes to cause congenital heart disease in humans [28]. Thus, myocardin and MRTFs warrant careful consideration as culprits in forms of congenital heart disease characterized by vascular abnormalities.

References

1. **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.

2. **Wang, D.-Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Nordheim, A. and Olson, E. N.** 2002. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc. Natl. Acad. Sci. USA* **99,** 14855–14860.

3. **Wang, D.-Z. and Olson, E. N.** 2004. Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Curr. Opin. Genet. Dev.* **14,** 558–566.

4. **Cen, B., Selvaraj, A. and Prywes, R.** 2004. Myocardin/MKL family of SRF coactivators: key regulators of immediate early and muscle specific gene expression. *J. Cell. Biochem.* **93,** 74–82.

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

6. **Owens, G. K., Kumar, M. S. and Wamhoff, B. R.** 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* **84,** 767–801.

7. **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.

8. **Miano, J. M., Ramanan, N., Georger, M. A., de Mesy Bentley, K. L., Emerson, R. L., Balza, R. O., Jr., Xiao, Q., Weiler, H., Ginty, D. D. and Misra, R. P.** 2004. Restricted inactivation of serum response factor to the cardiovascular system. *Proc. Natl. Acad. Sci. USA* **101,** 17132–17137.

9. **Niu, Z., Yu, W., Zhang, S. X., Barron, M., Belaguli, N. S., Schneider, M. D., Parmacek, M., Nordheim, A. and Schwartz, R. J.** 2005. *J. Biol. Chem.* **280,** 32531–32538.

10. **Li, S., Czubryt, M. P., McAnally, J., Bassel-Duby, R., Richardson, J. A., Wiebel, F. F., Nordheim, A. and Olson, E. N.** 2005. Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. *Proc. Natl. Acad. Sci. USA* **102,** 1082–1087.

11. **Wang, Z., Wang, D.-Z., Teg Pipes, G. C. and Olson, E. N.** 2003. Myocardin is a master regulator of smooth muscle gene expression. *Proc. Natl. Acad. Sci. USA* **100,** 7129–7134.

12. **Miralles, F., Posern, G., Zaromytidou, A. I. and Treisman, R.** 2003. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113,** 329–342.

13. **Selvaraj, A. and Prywes, R.** 2003. Megakaryoblastic leukemia-1/2, a transcriptional co-activator of serum response factor, is required for skeletal myogenic differentiation. *J. Biol. Chem.* **278,** 41977–41987.

14. **Cen, B., Selvaraj, A., Burgess, R. C., Hitzler, J. K., Ma, Z., Morris, S. W. and Prywes, R.** 2003. Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes. *Mol. Cell. Biol.* **23,** 6597–6608.

15. **Du, K. L., Chen, M., Li, J., Lepore, J. J., Mericko, P. and Parmacek, M. S.** 2004. Megakaryoblastic leukemia factor-1 transduces cytoskeletal signals and induces smooth muscle cell differentiation from undifferentiated embryonic stem cells. *J. Biol. Chem.* **279,** 17578–17586.

16. **Kuwahara, K., Barrientos, T., Pipes, G. C., Li, S. and Olson, E. N.** 2005. Muscle-specific signaling mechanism that links actin dynamics to serum response factor. *Mol. Cell. Biol.* **25,** 3173–3181.

17. **Du, K. L., Ip, H. S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M. M., Owens, G. K. and Parmacek, M. S**. Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. 2003. *Mol. Cell. Biol.* **23,** 2425–2437.

18. **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. USA* **100,** 9366–9370.

19. **Skarnes, W. C., Auerbach, B. A. and Joyner, A. L.** 1992. A gene trap approach in mouse embryonic stem cells: the lacZ reported is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice. *Genes Dev.* **6,** 903–918.

20. **Li, J., Zhu, X., Chen, M., Cheng, L., Zhou, D., Lu, M. M., Du, K., Epstein, J.A. and Parmacek, M.S.** 2005. Myocardin-related transcription factor B is required in cardiac neural crest for smooth muscle differentiation and cardiovascular development. *Proc. Natl. Acad. Sci. USA* **102,** 8916–8921.

21. **Li, L., Miano, J. M., Mercer, B. and Olson, E. N.** Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. 1996. *J. Cell Biol.* **132,** 849–859.

22. **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.

23. **Yoshida, T., Sinha, S., Dandre, F., Wamhoff, B. R., Hoofnagle, M. H., Kremer, B. E., Wang, D.-Z., Olson, E. N. and Owens, G. K.** 2003. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ. Res.* **92,** 856–864.

24. **Wang, Z., Wang, D.-Z., Hockemeyer, D., McAnally, J., Nordheim, A. and Olson, E. N.** 2004. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428,** 185–189.

25. **Creazzo, T. L., Godt, R. E., Leatherbury, L., Conway, S. J. and Kirby, M. L.** 1998. Role of cardiac neural crest cells in cardiovascular development. *Ann. Rev. Physiol.* **60,** 267–286.

26. **Stoller, J. Z. and Epstein, J. A.** 2005. Cardiac neural crest. *Sem. Cell Dev. Biol.*, **16,** 704-15

27. **Hoffman, J. I. and Kaplan, S.** 2002. The incidence of congenital heart disease. *J. Am. Coll. Cardiol.* **39,** 1890–1900.

28. **Srivastava, D. and Olson, E. N.** 2000. A genetic blueprint for cardiac development. *Nature* **404,** 221–226.

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