THE REGULATION OF MYOCARDIN FACTOR DEPENDENT TRANSCRIPTION IN VASCULAR SMOOTH MUSCLE

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ABSTRACT

Jeremiah Stephen Hinson: The Regulation of Myocardin Factor Dependent Transcription in Vascular Smooth Muscle

(Under the direction of Christopher P. Mack, PhD)

This work explores the role of the myocardin family of serum response factor (SRF) co-factors in smooth muscle cell (SMC) differentiation, and identifies novel modes of regulating their activities. Extensive evidence indicates that SRF regulates muscle-specific gene expression by binding to conserved promoter regions known as CArG boxes. Myocardin and the myocardin-related transcription factors MRTF-A and MRTF-B are all capable of potently transactivating SRF-dependent gene expression. The sub-cellular localization of MRTFs is regulated by RhoA signaling, an established determinant of SMC marker gene activity. Studies included here demonstrate that the MRTFs are expressed in primary and cultured SMC and in multiple organs with a large SMC component, and that they are capable of upregulating SMC-specific gene activity in multipotential 10T1/2 cells. We demonstrate that the myocardin factors have dramatically different localization patterns and that the stimulation of SMC-specific transcription by certain RhoA-dependent agonists is likely mediated by increased nuclear translocation of the MRTFs. Gel shift assays were used to show that myocardin factor activity correlates well with SRF/CArG ternary complex formation, and that MRTF-SRF interactions are partially dependent upon CArG sequence. In a yeast-two-hybrid screen for novel SRF binding partners in aortic SMC, we identified four

and a half LIM domain protein 2 (FHL2). We showed that FHL2 also interacted with all three myocardin factors and enhanced myocardin and MRTF-A-dependent transactivation of the SM α-actin, SM22, and cardiac ANF promoters by increasing the half-lives of these proteins. Treatment of cells with the proteasome inhibitors MG132 and lactacystin strongly upregulated myocardin factor protein levels and resulted in a substantial increase in ubiquitin immunoreactivity in MRTF-A immunoprecipitants. Importantly, these data are the first to indicate that the myocardin factors are regulated by proteasome-mediated degradation, and that SRF-dependent gene activity can be upregulated via inhibition of their degradation. We have further shown that the muscle-specific ring finger protein MuRF3 physically interacts with the myocardin factors and inhibits transactivation of the SM α-actin and SM22 promoters. MuRF3 also inhibited myocardin factor protein expression, and may act as an E3 ubiquitin ligase for this very important family of SRF co-factors.

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things that he had probably just read on Wikipedia. Matt has also been a worthy adversary, in general, these past six years. Dean Staus has been really tall, and has loved science with a passion few find for any single thing in life. I am glad to have had a part in recruiting and mentoring him. Alicia Blaker has a frugal culinary genius that is rivaled by few, and a kindness and positive spirit that are rivaled by none. Lisa Galante was [and is] my first friend in Chapel Hill, and it was from her that I learned Johnny Cash's rendition of Sweet Home Alabama. Laura DiMichele, Rebecca Sayers, and a sturdy legal pad have all worked without end to make sure that we as a lab stayed on track, and that we always had enough food and paper products at every event. For these things we are all very thankful. Jason Doherty has always been hungry, an attribute which has made him a faithful lunch partner. Lee Mangiante has been a selfless helper and a constant supplier of mixed CDs, and without Liisa Sundberg, I would have had no protocols at all. Many others in the lab, including Marisa DeBurkarte, Xeenat Hakim, and Ward Jarvis have all been invaluable sources of friendship and support. Thank you all.

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LIST OF ABBREVIATIONS

Ab	antibody
AG	agamous
ANF	atrial natriuretic factor
ATP	adenosine 5' triphosphate
BCA	bicinchoninic acid
BMP	bone morphogenic protein
cAMP	cyclic adenosine monophosphate
CArG	C(A/T6)G
cDNA	complimentary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CRP	cysteine rich protein
DEFA	deficiens
DMRTF	drosophila myocardin-related transcription factor
DN	dominant negative
DNA	deoxyribonucleic acid
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E1	enzyme 1
E2	enzyme 2
E3	enzyme 3
EC	endothelial cell
EDG-1	endothelial differentiation gene 1
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGR-1	early growth response factor 1
ES	embryonic stem
FAK	focal adhesion kinase
FHL1	four and a half LIM domains protein 1
FHL2	four and a half LIM domains protein 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA binding protein 4
GATA6	GATA binding protein 6
GDP	guanosine diphosphate
GPCR	G-coupled protein receptor
GST	glutathione S-transferase
GTP	guanosine triphosphate

HECT	homologous to the E6-AP carboxyl terminus
ICAM-1	intercellular adhesion molecule 1
KCl	potassium chloride
KLF2	kruppel-like factor 2
LIM	Lin11, Isl-1, Mec-3
LPA	lysophosphatidic acid
LZ	leucine zipper
MADS	mdm1, agamous, deficiens, SRF
MAPK	mitogen-activated protein kinase
mDia	mouse diaphanous
MDM1	murine double minute 1
MEF-2	myocyte enahncing factor 2
MKL1	megakaryoblastic leukemia (translocation) 1
MKL2	megakaryoblastic leukemia (translocation) 2
mRNA	messenger ribonucleic acid
MRTF-A	myocardin-related transcription factor A
MRTF-B	myocardin-related transcription factor B
NaCl	sodium chloride
NF-kB	nuclear factor kappa B
NIH	National Institutes of Health
NKE	Nkx2.5 responsive element
Nkx2.5	NK2 transcription factor related, locus 5
NLS	nuclear localization signal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
RING	really interesting new gene
RIPA	radioactive immunoprecipitation assay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
S1P	sphingosine-1-phosphate
SAP	SAF-A/B, acinus and PIAS
SDS	sodium dodecyl sulfate
SEM	standard error of mean
siRNA	small interfering RNA
SM a actin	smooth muscle a actin
SM MHC	smooth muscle myosin heavy chain
SM22	smooth muscle 22 a

SMAD	small mothers against decapentaplegic
SMC	smooth muscle cells
SRE	serum response element
SRF	serum response factor
STARS	striated muscle activator of RhoA signaling
TAD	transcriptional activation domain
TBE	tris borate EDTA
TGF	transforming growth factor
Ub	ubiquitin
UPS	ubiquitin proteasome system
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

BACKGROUND AND SIGNIFICANCE

VASCULOGENESIS AND ANGIOGENESIS

A functioning circulatory system is required very early in mammalian development, when diffusion from surrounding uterine tissues becomes inadequate to supply all cells of the growing embryo with appropriate levels of oxygen. Mice unable to establish such a circulatory system die by embryonic day 10, and numerous human diseases involve alterations in circulatory system function and oxygen delivery (143). Blood vessel formation during embryogenesis is accomplished *de novo* via vasculogenesis, a process that involves the recruitment of progenitor cells from the mesoderm to previously avascular areas within the embryo. These angiogenic precursor cells differentiate into endothelial cells and coalesce to form a rudimentary tube network, referred to as the primary vascular plexus (130, 131). The vascular plexus supplies the very early embryo with adequate amounts of blood, but increasing oxygen demands stimulate increased vascularization via the process of angiogenesis, defined by the budding or sprouting of new endothelial capillary branches from pre-existing blood vessels (54).

Further vascular development requires the transformation of newly formed endothelial tubes into multi-layered arteries and veins. This process is highly dependent upon the investment of endothelial tubes with a medial layer of smooth muscle cells (SMC), which will provide the vasculature with a capacity for contraction, and throughout the life of the organism function in the regulation of blood pressure, blood distribution, and vessel tone

diameter. Larger vessels also become invested with an outer layer comprised of fibroblasts and extracellular matrix that provides further structural support and cushioning for the vasculature. As demonstrated in figure 1.1A, the result of these developmental processes is a mature blood vessel consisting of three layers: the intima (endothelial cells and basement membrane), media (SMC and their extracellular matrix), and adventitia (fibroblasts and their exracellular matrix) (143).

THE VASCULAR SMOOTH MUSCLE CELL: ORIGINS AND FUNCTION

At least three distinct embryological origins of vascular SMC have been experimentally identified in chick and mouse (see (61) for review). The cardiac outflow tract and pharyngeal arch arteries contain SMC derived from cardiac neural crest cells(75), while the media of the nearby coronary arteries is comprised of SMC that arise from mesothelial progenitors within the proepicardial organ (102). The remainder of the vasculature is populated with SMC that are derived largely from lateral and/or splanchnic mesoderm (65). Several recent studies have suggested that at least some SMC may also be derived directly from endothelial progenitor cells, through transdifferentiation of mature endothelial cells, or from persisting stem cell populations in adult organisms (61).

Mature SMC are highly specialized cells that normally proliferate at very low rates and whose primary function is contractile. SMC differentiation is measured by the expression of a unique set of marker genes that includes the contractile and contractile-associated smooth muscle myosin heavy chain (SM MHC), smooth muscle α-actin (SM α-actin), smooth muscle 22α (SM22), telokin, and calponin (see (122) for review). Unlike skeletal and cardiac myocytes, SMC do not undergo terminal differentiation. Instead, SMC retain a

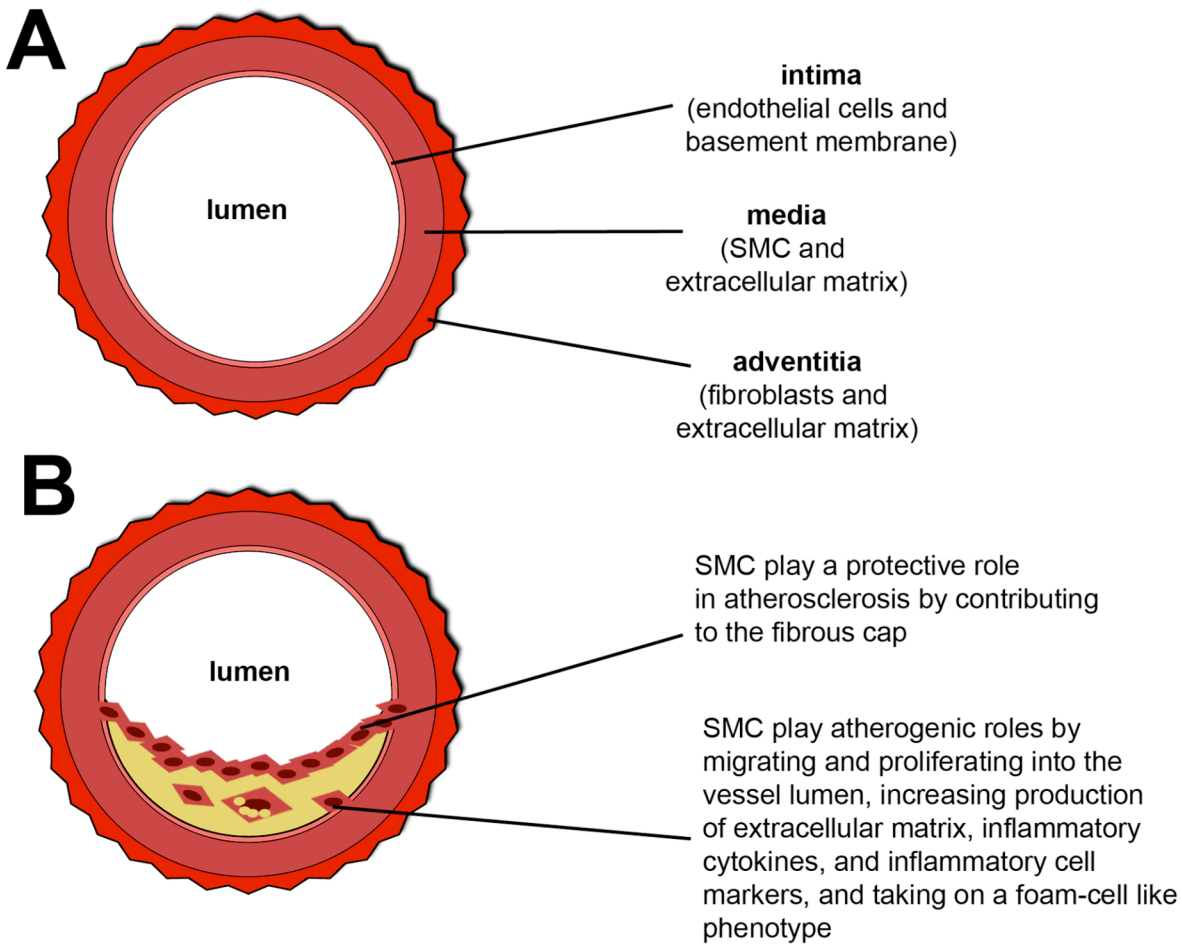

Figure 1.1. Vascular structure and the role of SMC in atherosclerosis. A) Large vessels consist of three primary layers as shown: the intima, media, and adventitia **B)** SMC play both atherogenic and protective roles in the pathogenesis of atherosclerosis.

plasticity that allows them to undergo significant and reversible changes in phenotype in response to environmental cues, even as fully mature cells. These changes occur through a process known as phenotypic switching (or phenotypic modulation), and are accompanied by often dramatic shifts in gene expression toward a profile resembling that of more proliferative and synthetic progenitor cell types. Phenotypic switching of SMC is very important for vascular morphogenesis and injury repair. Increases in proliferative, migratory, and synthetic capacities of SMC allow their growth into areas of new vascularization during development, and in response to certain types of vascular insults, facilitates similar roles in injury response (14), (122).

SMOOTH MUSCLE CELLS IN CARDIOVASCULAR DISEASE

While it is clear that a capacity for phenotypic switching is critical for proper function of SMC, in response to abnormal external cues, it can also lead to their involvement in multiple human diseases. Phenotypic modulation of SMC has been implicated in the pathogenesis of such diseases as atherosclerosis, restenosis, hypertension, and cancer. Atherosclerosis is the most common disease associated with SMC, and it appears that SMC play both pathologic and protective roles in its progression, as shown in figure 1.1B and discussed below (14).

SMC are one of the many cell-types implicated in the initiation of atherosclerosis that also includes endothelial cells, lymphocytes, monocytes, and macrophages (90). Phenotypically modulated SMC contribute to atherogenesis partly through increased production of proteases (44), inflammatory cytokines, and inflammatory cell markers (134, 136). The production of these factors results in recruitment and activation of leukocytes,

endothelial cell dysfunction, and increased proliferation of SMC themselves (34). Increased SMC growth and proliferation contribute to plaque expansion through increased intimal migration and increased production of extracellular matrix (169). SMC also directly promote lipid deposition within plaque. Not only are SMC capable of lipid uptake and foam cell transformation themselves (135), in concert with endothelial cells they also interact directly with recently recruited monocytes and facilitate their differentiation into macrophages (154), the major precursors of foam cells and source of lipid in the plaque. This interaction is facilitated in part via cell surface receptors (ie. VCAM-1 and ICAM-1) that are not expressed in SMC except during atherosclerosis (10, 37). SMC simultaneously play a protective role in atherosclerosis by contributing to formation of the plaque's fibrous cap. This cap is important for plaque stabilization, and helps to prevent of rupture and thrombosis (44). The protective role of SMC in atherosclerosis is accomplished via many of the same mechanisms that facilitate their pathological role, including increases in proliferation and intimal migration, and increased production of extracellular matrix.

Blockage of vessels due to atherosclerosis is commonly treated using balloon angioplasty and stent placement. While these procedures successfully clear atherosclerotic plaques, up to 50% of patients present with recurrence of symptoms due to post-angioplasty restenosis (150). Restenosis is characterized by intimal hyperplasia that leads to further luminal narrowing and vessel occlusion. This is caused in large part by aberrant growth and proliferation of medial SMC following mechanically-induced denuding of the endothelium (108). The development and experimental implementation of many elegant animal models has yielded a great deal of information regarding the pathogenesis of both atherosclerosis and

restenosis (2, 186), yet many of the molecular mechanisms governing initiation of SMC involvement remain to be determined.

Systemic hypertension, a complex disorder involving many factors, is characterized by increases in SMC contractility and vascular tone, as well as increases in SMC growth and synthesis of matrix materials (42, 111). These alterations in SMC function result in increased peripheral resistance, and elevated blood pressures. Similar changes in SMC function outside the vasculature are also believed to lead to their involvement in the progression of such diseases as athsma, obstructive bladder disease, and numerous reproductive disorders (122).

Additionally, phenotypic switching of SMC plays a significant role in the progression of many cancer types. Tumor growth is dependent upon increased oxygen supply via neovascularization, and larger tumors require larger vessels. Oftentimes, moderate to large diameter vessels formed via tumor-associated angiogenesis are underinvested with SMC or invested with poorly differentiated SMC, leading to leaky vasculature and increased opportunity for metastasis (107). The mechanisms responsible for defective SMC recruitment and differentiation observed in tumor-associated angiogenesis are poorly understood, and a greater understanding of the mechanisms governing SMC differentiation in development will undoubtedly aid in their dissection.

ENVIRONMENTAL CONTROLS OF SMOOTH MUSCLE CELL GROWTH AND DIFFERENTIATION

It is well established that SMC growth and differentiation are regulated by a complex array of local environmental cues including growth factors, contractile agonists, cell-cell and cell-matrix interactions, inflammatory stimuli, reactive oxygen species, and mechanical stresses (see (122) for review). However, the signal transduction pathways by which these

cues are transmitted to the nucleus, and the mode by which these cues promote either growth or differentiation, have not been well elucidated. An extensive review of this field is beyond the scope of this dissertation, but several studies are worth noting.

The D'Amore laboratory has demonstrated that co-culture of endothelial cells (EC) with 10T1/2 smooth muscle precursor cells leads to an increase in SMC differentiation marker gene expression in a manner that is dependent upon cell-cell contact, and that is mediated at least in part by transforming growth factor (TGF)-β signaling, a factor that has been shown to up-regulate SMC marker gene expression by many groups (33, 62, 122). Vascular endothelial growth factor (VEGF) has been shown by the same group to promote migration of SMC in culture (49), and several studies have strongly implicated platelet-derived growth factor (PDGF)-BB in the down-regulation of SMC differentiation marker genes and promotion of SMC growth and proliferation (7, 63, 83). An increasing number of agents appear to be capable of promoting both growth and differentiation in SMC. For example, we have recently identified the lipid agonist shingosine-1-phosphate (S1P) as a stimulator of early response gene and differentiation marker gene expression in SMC. We demonstrated that S1P activates multiple signaling pathways in SMC, including those of MAPK and RhoA, and that the end result of S1P-initiated signaling may depend largely upon competition between transcription factors in the nucleus (88).

Many of the findings above have also been supported by work in animal models. The importance of S1P signaling for SMC function was first illustrated *in vivo* by global knockout of Edg-1, a G-protein coupled receptor for S1P. Mice homozygous for this deletion died in utero due to defective SMC recruitment and function in established vasculature (87). Importantly, a similar effect was seen upon endothelial cell-specific deletion of this receptor,

indicating that Edg-1 activity in endothelial cells is important to SMC function (1). A further illustration of cross-talk between the endothelium and SMC was seen with the knockout of the Krupple-like transcription factor KLF2. In this model, KLF2 was expressed in endothelial cells but not SMC, yet its deletion was embryonic lethal due to in part to defective SMC maturation (71). Further analysis of KLF2-/- embryos revealed defective SMC migration in the developing vasculature (185). A similar phenotype is seen upon genetic deletion of PDGF-BB or its ligand (76, 85). Interestingly, PDGF-BB has been suggested to exert its effects on SMC function via KLF2 (185). Furthermore, genetic deletion of any one of a number of TGF-β signaling components, including TGFβ1 and two different TGF-β receptors results in embryonic lethality due in at least in part to defective vascular development and/or defective SMC differentiation (32, 119, 121). There are many other external cues regulate SMC function, and there is still much to be learned regarding SMC signal transduction pathways. However, it is clear that the small GTPase RhoA and the myocardin family of transcription factors (both discussed below) play important roles in coordinating many of these signals.

THE CArG BOX: AN ESSENTIAL DNA ELEMENT FOR GROWTH AND DIFFERENTIATION

In 1985, Treisman *et al* discovered a region within the promoter of the early growth response gene c-fos that was highly sensitive to serum stimulation and displayed all the characteristics of a classic enhancer element (166). Further studies of this enhancer region revealed that it contained a critical protein-binding sequence, which was later termed the serum response element (SRE) (46, 128, 164). The c-fos SRE was initially described as a 23-bp stretch of DNA with the sequence **aggatgtcc**atatta**ggacatct**, displaying repeats of inverted

dyad symmetry (regions of inverted dyad symmetry indicated by bolded type) (164). Almost simultaneously, a region of DNA with high sequence homology to the SRE core was discovered in the promoter of the cardiac α-actin gene. This region lacked the dyad symmetry of the SRE, and was described as a 10-bp stretch with the sequence $CC(A/T_6)GG$ (103). The cardiac α-actin sequence was later termed the "CArG box", and similar CArG boxes were soon discovered in the promoters of multiple muscle-restricted genes, including skeletal α-actin, alpha myosin heavy chain, myosin light chain, and cardiac troponin T (99, 103). Over 60 genes have since been identified which contain an SRE or CArG box variant sequence within their promoters, with a very high percentage of these being growth or muscle-restricted genes (see (99) for review).

Nearly every SMC differentiation marker gene described to date contains at least one CArG box in the vicinity of its promoter (122). Several SMC genes, including SM MHC and SM α-actin, contain multiple CArG elements, each of which appear to be required for proper expression of these genes *in vivo* (91, 96). Mack *et al* illustrated this paradigm using multiple deletion variants of a Lac Z transgene driven by the SM α-actin promoter. Mutation of any one of the three SM α-actin CArG boxes resulted in a loss of expression in smooth muscle-containing tissues (Fig 1.2). While it is still not entirely clear how multiple CArG elements function together within a single gene, it has been suggested that the multi-protein complex required for transcription of SMC differentiation marker genes may rely on transcription factor binding at each CArG, which would in turn facilitate further interactions between components of the transcriptional machinery (93). This continues to be an important area of study in the field.

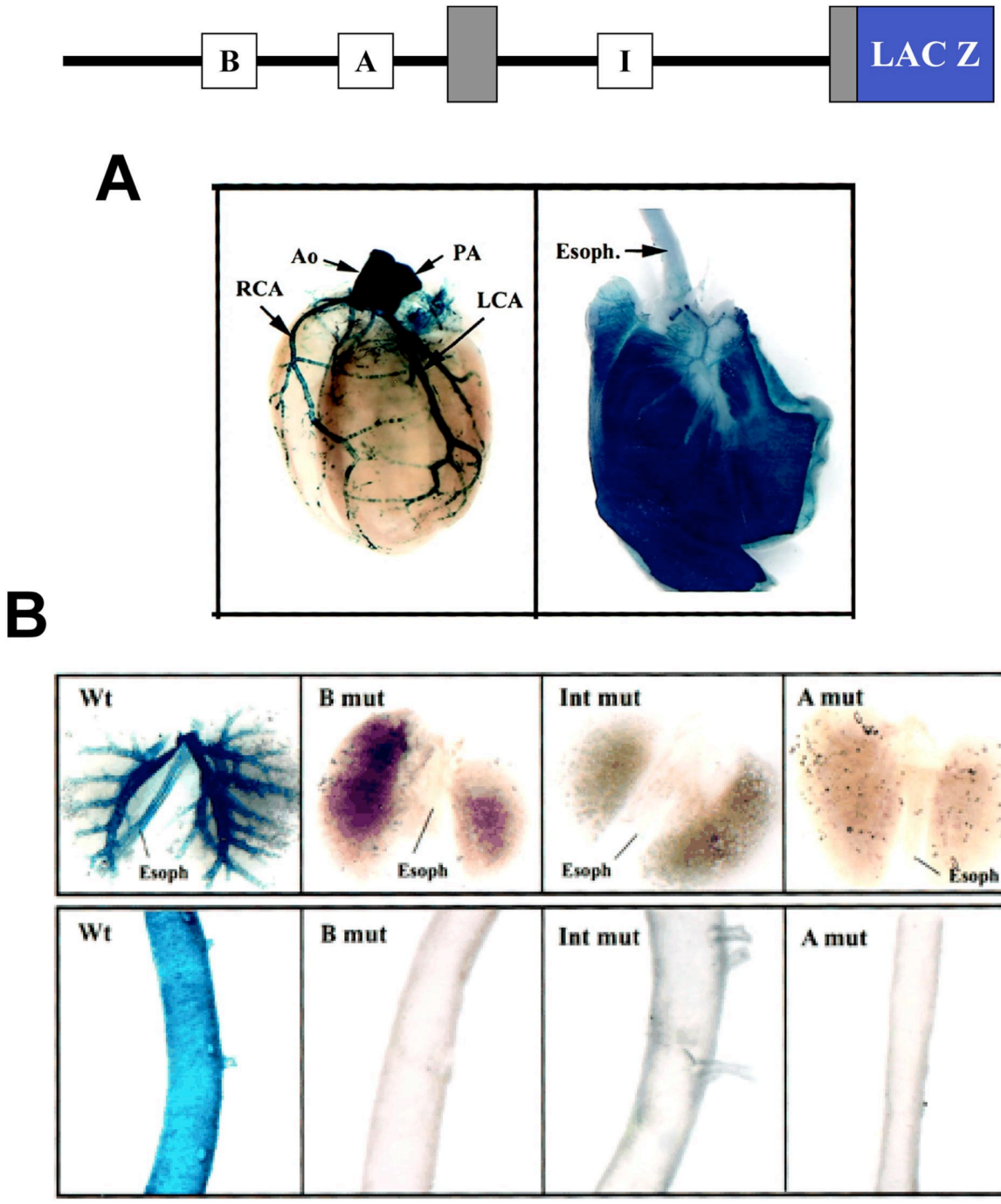

Figure 1.2. Expression of the SM α-actin gene is regulated by multiple CArG boxes in vivo. **A)** The SM α-actin promoter region from -2650 to +2750 (shown above encompassing the three CArGs, A, B and Intronic (I), as well as exon 1 and part of exon 2) drove SMC-specific expression of Lac Z in nearly all SMC tissues. **B)** Mutation to any one of the highly conserved CArG boxes abolished transgene expression in most SMC tissues. Lung and aorta are shown. (Images adapted from Mack, C. P. et al. Circ Res 1999;84:852-861)

REGULATION OF SMOOTH MUSCLE CELL DIFFERENTIATION BY SRF

Serum response factor (SRF) was originally purified and cloned based upon its interaction with the c-fos SRE, and was named based upon that interaction (116, 163). The central CArG box within the SRE was determined to be the sequence required for SRF binding, and later studies showed that the bases flanking the CArG box mediated interactions between the SRE and additional transcription factors known as the ternary complex factors (TCFs) (see (165) for review). The importance of SRF-cofactor interactions will be discussed later in this chapter.

SRF has been extensively studied since its discovery (see (99) for review). The 64 kDa transcription factor contains 508 amino acids and belongs to the MADS (MDM1, AG, DEFA, and SRF) family of proteins. These proteins are characterized by the presence of a conserved 56 amino acid region referred to as the MADS box. This region is comprised of an amino-terminal DNA binding domain, a dimerization domain, and a domain capable of mediating interactions with accessory factors (148). As shown in figure 1.3A, the MADS box of SRF occupies amino acids 142-223 and is flanked by an amino terminal nuclear localization signal (aa 95-100) and a carboxy-terminal transactivation domain (265-508)(45, 99, 105). SRF binds to the CArG box of target genes as a homodimer, where it interacts with various accessory factors, and activates gene expression via its carboxy-terminus.

SRF activity has been shown to be critical for processes central to development in multiple species. Genetic deletion of SRF in the mouse leads to lack of mesodermal formation and a halt in development at gastrulation (4). Similar deletion of SRF in other organisms including Dictyostelium, *Caenorhabditis elegans*, and *Drosophila melanogaster*, as well as in mouse embryonic stem cells have revealed that SRF is essential to the processes

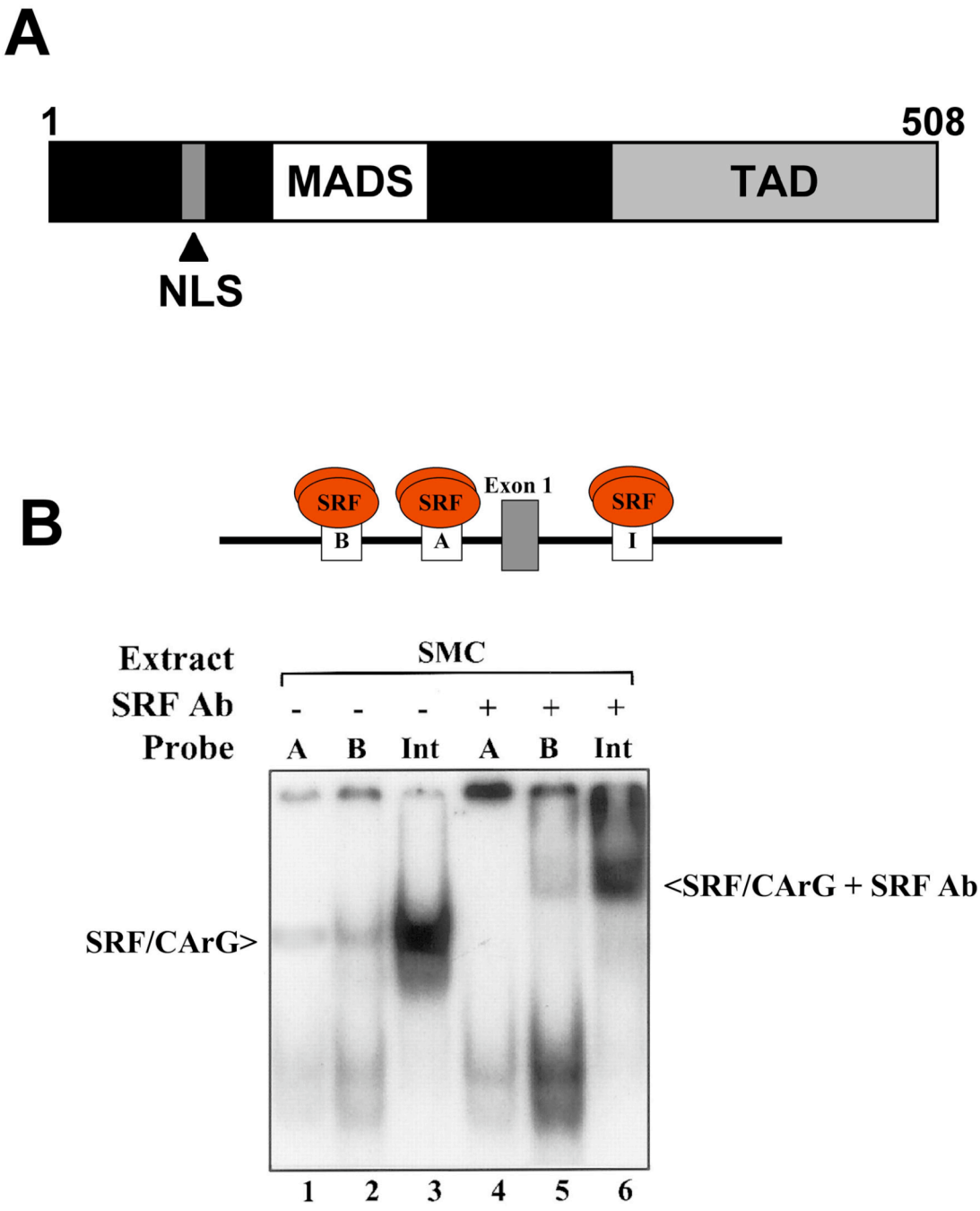

Figure 1.3. Serum Response Factor Binds to CArG elements. A) Serum response factor contains a single nuclear localization signal (NLS) at aa 95-100, a MADS domain at aa 142-223, and a carboxy-terminal transactivation domain (TAD) at aa 265-508. **B)** SRF interacts as a homodimer with the CArG elements present in the promoters of nearly all SMC genes. SRF binding to the SM α-actin B, A and Intronic (I) CArG boxes is demonstrated schematically above, and by electrophoretic mobility shift assays below. An SRF antibody was added to lanes 4-6 that super-shifted the SRF binding complex. (Image adapted from Mack, C. P. et al. Circ Res 1999;84:852-861)

of cell migration and adhesion, cytoskeletal structure formation, and animal movement (38, 43, 50, 141). The early lethality of SRF null mice has made traditional SRF lack of function studies in more differentiated cell types difficult. Recently, Miano *et al* partly overcame this issue by using Cre-lox technology to generate a line of mice in which SRF is selectively deleted in the cardiovascular system (101). Mutant mice in this study survived past gastrulation but died later in development due to incomplete development of the heart and vasculature. Ultrastructural examination of cardiomyocytes and SMC in these mice confirmed previous reports that SRF plays a vital role in cytoskeletal structure and contractile function.

Soon after the initial discovery that SRF interacted with the SRE CArG, an independent report indicated that it also interacted with the cardiac α-actin CArG, albeit with a lower affinity than seen with the SRE in tandem experiments (9). These studies were the first to suggest that SRF may be responsible for positively regulating the expression of both growth genes and muscle differentiation genes, a surprising finding considering that these gene expression programs are somewhat opposing. We and others have demonstrated that SRF also regulates nearly all of the known SMC differentiation marker genes, indicating that it is a key regulator of SMC differentiation. As is true for growth genes and skeletal and cardiac muscle genes, SRF binds to the multiple CArG boxes that are found in the promoters of nearly all of the SMC differentiation marker genes to regulate their expression (Fig 1.3B) (55, 79, 91, 93, 95, 100).

While recent studies have shown that differentiation of skeletal and cardiac muscle is controlled by the expression of master regulatory transcription factors that work in concert with SRF such as MyoD, Nkx2.5, and Gata-4 (120, 127, 160), a transcription factor that

specifies SMC lineage or completely explains SMC-specific transcription has not yet been described. SRF is expressed highly in all three muscle cell-types during development (28), but as described above, is a ubiquitously expressed protein that also regulates the expression of a variety of other CArG-containing genes including the growth genes, c-fos and egr-1, as well as the expression of several skeletal- and cardiac-specific genes (11, 21, 152, 165, 173). While the molecular mechanisms by which SRF regulates these disparate gene programs are still not completely known, it is clear that mechanisms in addition to the presence and activity of SRF must exist that regulate SMC-specific transcription.

REGULATION OF SRF FUNCTION VIA COFACTOR INTERACTIONS

A large number of studies have established the paradigm that SRF function is regulated to a great extent by interaction with other transcription factors or co-factors. The first SRF co-factors identified, the ternary complex factors (TCFs), Elk-1, Sap-1, SAP-2/NET/ERP, regulate early response gene expression. These transcription factors are activated by growth factor signaling and only bind SRF after they have been phosphorylated by MAP-kinase (30, 56, 97). Based upon the demonstration of a SMC-selective SRF complex in gel shift analyses, our laboratory was one of the first to suggest that SMC-specific transcription was regulated by a similar mechanism (93). Chang *et al* later demonstrated that SRF associates with the SMC-selective cysteine- rich LIM-only proteins, CRP1 and CRP2, to enhance SMC differentiation marker gene expression (20). Since the CRPs do not contain transcription activation domains, it is likely that these proteins act as adapter molecules to facilitate interactions between SRF and other transcription factors including the GATA proteins. Indeed, SRF has been shown to interact with GATA-4 and

Nkx2.5 to regulate cardiac-specific gene expression and MyoD and myogenin to regulate skeletal muscle-specific gene expression (21, 22, 48, 145). Interestingly, forced expression of CRP2 has recently been shown to activate SRF-dependent SMC gene activity in adult mouse cardiomyocytes via an interaction with Brg-1, a component of the SWI/SNF chromatin remodeling complex. Brg-1 was also shown to interact with GATA-4, strengthening the idea that the CRPs may function to facilitate interactions between various components of the transcriptional complex (18).

Philippar *et al* have implicated an additional LIM-only protein, four and a half LIM domain protein 2 (FHL2), as a binding partner of SRF that also modulates its activation of SMC differentiation marker genes. FHL2 is unable to bind to DNA directly, but was shown to be present at the promoters of SMC differentiation marker genes (but not of growth genes) following activation of RhoA signaling (discussed below). The authors of this study reported that FHL2 functioned as an inhibitor of SRF-dependent SMC gene activation by FHL2, and that this was accomplished via inhibition of higher order complex formation between SRF and another cofactor (124). Prior to the publication of this srudy, we independently detected an interaction between FHL2 and SRF in human aorta using a genetic screen approach. We have subsequently found FHL2 to be both a positive and negative regulator of SMC differentiation marker gene expression, depending upon its interactions with additional SRF cofactors. The details of these studies will be discussed at length in a later chapter.

ACTIVATION OF SRF-DEPENDENT GENE EXPRESSION BY THE SMALL GTPASE RHOA

As discussed previously, SMC growth and differentiation are controlled by a diverse array of extrinsic factors, but the cell signaling mechanisms by which these factors initiate

SRF-dependent transcription of SMC differentiation marker genes is not well understood. Our laboratory group and others have recently demonstrated that the regulation of SRF-dependent transcription, including that of SMC differentiation marker genes, is accomplished at least in part by signaling through the small GTPase RhoA.

The binding of GTP regulates small GTPase activity, and the Rho family of small GTPases cycle continuously between active GTP-bound and inactive GDP bound forms (6). RhoA is activated by multiple serum components, many of which signal through G-protein coupled receptors (GPCRs). Agonists shown to activate RhoA in SMC include platelet-derived growth factor, angiotensin II, lipophosphatidic acid (LPA), and S1P (88, 112). The Treisman laboratory was the first to show that activation of RhoA could lead to an up-regulation of SRF-dependent genes. These studies focused on the *c-fos* promoter, and also showed that RhoA was capable of activating the expression of early response genes in a TCF-independent fashion (57). Additional studies have shown that the effects of RhoA on SRF-dependent transcription require RhoA-mediated actin polymerization and possibly reductions in G-actin pools (152). RhoA signaling has also been shown to be important in the regulation of multiple SRF-dependent skeletal muscle differentiation marker genes (159, 181) and cardiac genes during hypertrophy (3). Interestingly, a link between SMC-specific gene expression was made by Mack *et al* who showed that SMC differentiation marker gene expression is upregulated in response to RhoA activation, and that this upregulation is dependent upon RhoA-mediated actin polymerization (92). In addition, it has been reported that RhoA-mediated actin reorganization is also required for the differentiation of coronary SMC from proepicardial cells (89). Collectively, these studies suggest that RhoA signaling is an important point of convergence for the multiple external cues that promote SMC

differentiation. However, until recently, no factors had been identified which linked cytoplasmic activation of RhoA with up-regulation of SMC marker genes in the nucleus.

COACTIVATION OF SRF BY MYOCARDIN

An important breakthrough in the study of the molecular mechanisms that regulate SMC differentiation came in 2001 with the discovery of the myocardin family of SRF co-factors by Wang et. al (175). This family includes myocardin as well as two Myocardin-Related Transcription Factors, MRTF-A/MKL-1/Mal and MRTF-B/Mkl-2 (176). Myocardin was identified through a bioinformatics-based screen of cardiac-specific expression sequence tags, and results from Northern analysis initially showed that myocardin was specifically expressed in the myocardium of adult mice. *In situ* analysis demonstrated that myocardin was expressed very early during cardiac development, but interestingly, was also detected in a subset of developing smooth muscle that included the aortic arch, pulmonary outflow tract, lung, esophagus, and gut. Later studies confirmed and extended myocardin's expression pattern to the heart and most developing and adult SMC compartments including dorsal aorta, bladder, stomach, intestines, and uterus (36, 176).

Myocardin localization to the nucleus suggested that it played a role in transcriptional regulation, and Wang *et al* went on to show that myocardin powerfully transactivated a number of cardiac and SMC-specific genes in Cos7 cells including ANF, Nkx2.5, and SM22 (175, 176). Myocardin has no consensus DNA binding domain, but these authors presented several lines of evidence strongly indicating that myocardin was recruited to CArG-containing genes by a direct physical interaction with SRF. First, CArG mutations that prevented SRF binding also inhibited myocardin's ability to transactivate the SM22

promoter. Second, myocardin and SRF were shown to physically interact by coimmunoprecipitation and gel shift studies. Finally, myocardin was unable to transactivate the SM22 promoter in SRF -/- ES cells, an ability that could be rescued by re-expression of SRF.

Subsequent studies demonstrated that myocardin was very important for regulating SMC-specific gene expression. Chen *et al* demonstrated that myocardin was expressed robustly in rat aortic media and that this expression was attenuated upon culture of rat aortic SMC (23). Interestingly, loss of myocardin expression correlated well with loss of SM MHC and SM-calponin expression that is frequently seen in cultured SMC, and over-expression of myocardin was sufficient to restore calponin promoter activity in these cells. It was eventually shown that myocardin could strongly transactivate most, if not all, of the CArG-containing SMC differentiation marker gene promoters including SM MHC, SM22, calponin, and SM α-actin in a variety of cell types including SMC (36, 179, 189). Importantly, forced expression of myocardin was sufficient to activate the endogenous expression of SMC differentiation marker genes in a number of non-SMC cell-types including mouse ES cells, 10T1/2 cells, NIH 3T3 fibroblasts, L6 myoblasts, and cardiac fibroblasts indicating that myocardin was sufficient to activate the SMC gene program (23, 36, 179, 189).

Studies *in vitro* and *in vivo* have indicated that myocardin is required for SMC-specific gene expression and SMC differentiation. Several groups used dominant negative myocardin variants (lacking the transactivation domain) and siRNAs to inhibit SMC differentiation marker gene expression in primary rat aortic SMC, and A7r5, Pac1 and A10 SMC lines (36, 179, 189). Importantly, Li and associates confirmed a requirement for myocardin in SMC differentiation by introducing a deletion in the mouse genome at a locus

that encoded for 3 myocardin domains that were shown to be essential for its activity (Basic, Q-rich, and SAP, see below) (82). Mouse embryos homozygous for this deletion died at E10.5 due to hemorrhage. Examination of myocardin -/- embryos at E9.5 revealed normal heart development but severe defects in vascular development, including a reduction of vessels in the yolk sac and underdevelopment of the dorsal aorta. Immunohistochemical staining for PECAM indicated that endothelial cell differentiation and initial formation of the dorsal aorta were unaffected, but staining for SM α-actin, a marker normally expressed in the aorta at this time-point, revealed a complete lack of SM α-actin positive cells. These results indicated that while myocardin may be a redundant factor in the regulation of cardiac development, it is critically important for SMC differentiation, and further, that SRF is insufficient to confer SMC lineage in the absence of myocardin. An additional study by Parmacek and associates, which utilized Cre-lox technology to ablate expression of myocardin specifically in neural crest-derived SMC, further confirmed that myocardin is required for expression of SRF-dependent SMC differentiation marker genes. Mice deficient for SRF expression in neural crest-derived SMC displayed a lack of contractile SMC gene expression in this cell subset, and died prior to postnatal day 3 due to lack of ductus arteriosis closure (64).

While the importance of myocardin in the regulation of SMC differentiation has clearly been established, several studies suggest that myocardin does not entirely explain SMC lineage determination. For example, myocardin is not expressed in all developing SMC and detection of several SMC differentiation markers preceded detectable myocardin mRNA expression in the dorsal aorta (36). While these results obviously depended upon the sensitivity of *in situ* and immunohistochemical analyses for proteins that differ widely in

their expression levels, these observations may indicate that mechanisms independent of myocardin expression might specify SMC lineage in the vasculature. It has also recently been reported that forced expression of myocardin in primary SMC, ES cells, or 10T1/2 cells was incapable of inducing expression of the few SMC marker genes that do not contain CArG elements, including smoothelin-B, ACLP, and FRNK and inappropriately activated skeletal- and cardiac-specific gene expression (188), further indicating that mechanisms in addition to myocardin may be required for SMC differentiation.

MYOCARDIN DOMAIN STRUCTURE

Deletion and domain mapping studies have identified a number of regions in myocardin that are important for its function (see Figure 1.4) (175). Myocardin contains a basic region that is important for nuclear localization, and along with the Q-rich domain, mediates its binding to the MADS box domain of SRF. The very strong trans-activation domain (TAD) found at the C-terminus seems to function as a general TAD in that it can be replaced by the TAD from VP16 without significant changes in protein function or specificity. Myocardin is a member of the SAP (SAF-A/B, Acinus, PIAS) family and this domain has been linked to chromatin remodeling and nuclear matrix binding. Although the precise function of the SAP domain remains unclear, its deletion inhibited myocardin's ability to transactivate the ANF promoter without affecting its ability to activate the SM22 promoter indicating that it may be important for gene-specific regulation (175). The N-terminal region has an overall inhibitory effect on transcriptional activity as well as on MRTF-A's ability to associate with SRF in gel shift assays (104). It also contains several RPEL motifs that have been implicated in the regulation of MRTF-A nuclear translocation

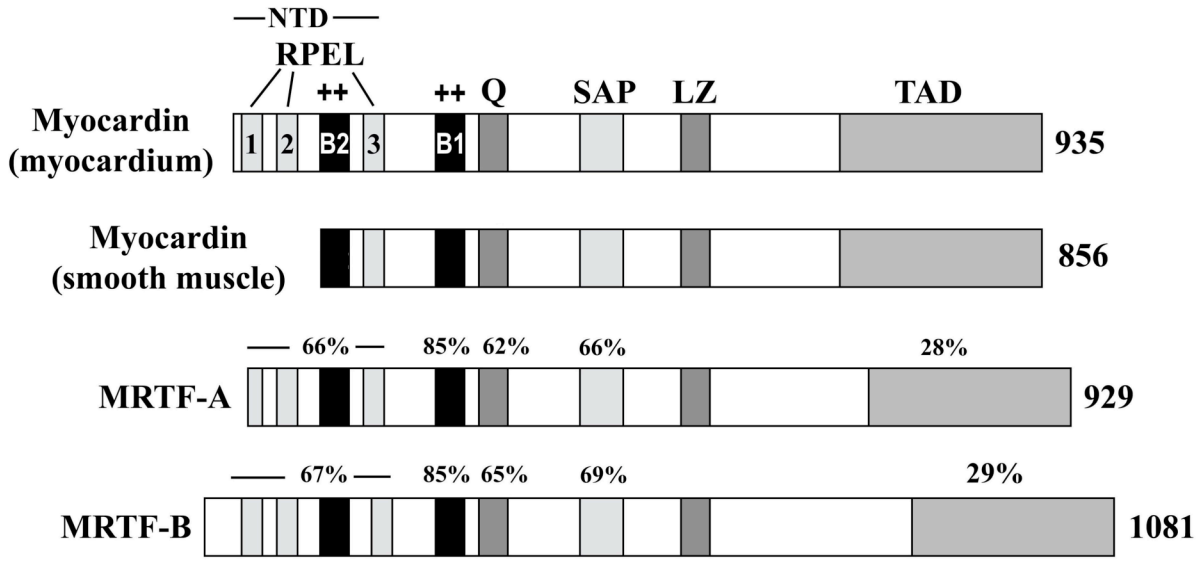

Figure 1.4. Myocardin family domain structure and homology. NTD, N-terminal Domain; ++, basic region; Q, glutamine rich region; SAP (SAF-A/B, Acinus, PIAS) domain; LZ, leucine zipper-like domain; TAD, transactivation domain. Percent identity between the MRTFs and myocardin are shown.

by RhoA (see below) (104). Finally, myocardin contains a leucine zipper region that mediates homo-dimerization and possibly hetero-dimerization with MRTFs (36, 104, 179).

Interestingly, Creemers *et al* have recently discovered an alternative splice variant of myocardin that is expressed specifically in smooth muscle (27). This variant contains an additional 44bp exon that harbors an early termination codon after amino acid 40 of myocardin. Re-initiation of translation from a second start site gives rise to an 856aa protein product (as opposed to the full-length 935aa version) that includes all components necessary for transactivation, but lacks 79aa of the N-terminal region (see Figure 1.4). The authors of this study demonstrated that both the smooth muscle (856aa) and cardiac (935aa) variants of myocardin were capable of transactivating SRF/CArG-dependent transcription, but that the 935aa cardiac variant alone was also capable of transactivating promoters driven by the SRF-related MADS box protein MEF-2. As MEF-2 is required for the expression of multiple cardiac marker genes (84, 114), the existence and expression patterns of these two versions of myocardin indicate that they may play an important role in cardiac versus smooth muscle cell fate determination.

MYOCARDIN RELATED TRANSCRIPTION FACTORS, MRTF-A AND MRTF-B

The myocardin-related transcription factors, MRTF-A/MAL/MKL-1 and MRTF-B/MKL-2 are also involved in the regulation of SRF-dependent transcription. All three myocardin family members have very similar domain structures with greater than 60% homology in their N-terminal, basic, Q-rich, and SAP domains and approximately 35% homology overall (176) (Fig 1.4). Unlike myocardin whose expression is specific to cardiac and SMC, the MRTFs are expressed more widely. Northern analysis has shown that MRTF-

A message is expressed in nearly all adult tissues, with strongest expression reported in heart, aorta, bladder, skeletal muscle, spleen, and brain (35, 176). In agreement with its widespread *in vivo* expression, MRTF-A is also expressed in multiple cell lines, including primary rat aortic SMC, the A7R5, A10, and PAC-1 SMC lines, mouse embryonic stem cells, 10T1/2 mutipotential cells, C2C12 skeletal myoblasts, and NIH 3T3 fibroblasts (35, 88). MRTF-B expression in adult mice is somewhat more restricted, but based on Northern analysis, it is expressed in heart, brain, testis, liver, and lung (176). In a separate study in humans, Selvaraj *et al* detected high levels of MRTF-B/MKL-2 message in skeletal muscle and lower levels in brain, heart, placenta, liver, kidney, and pancreas. These authors also detected MRTF-B and in mouse C2C12 skeletal myoblasts (142).

Similar to myocardin, MRTF-A transactivates many muscle-specific SRF-dependent genes, and several lines of evidence suggest that MRTF-A regulates SMC-specific gene expression perhaps in concert with myocardin. First, while initial results indicated that MRTF-A was not detected in the developing aorta, several groups have shown that MRTF-A message and protein are expressed highly in aortic SMC, a number of SMC cell lines, and in other tissues with large SMC components suggesting that it may play a role in regulating transcription in SMC (35, 88). Second, several laboratories including our own have shown that MRTF-A up-regulates most of the SMC-specific differentiation marker gene promoters (including SM MHC) to a level equal to or greater than that of myocardin (16, 35, 88, 142). Most of these studies have been performed in cell-types that do not express myocardin indicating that MRTF-A is sufficient for this response. Third, we and others have used dominant negative MRTF-A variants that lack the transactivation domain to inhibit SMC-specific promoter activity (16, 35, 88, 142). A potential weakness of this approach is that

these variants show some specificity toward MRTF-A, but they also strongly affect myocardin-dependent transcription (35), making it difficult to interpret experiments in SMC that express both transcription factors. Finally, the importance of the LZ motif for both myocardin and MRTF-A activity strongly suggests that dimerization of these transcription factors is important for their function. MRTF-A and myocardin have been shown to interact by yeast 2 hybrid as well as co-immunoprecipitation (35). Thus, the expression of both of these transcription factors in at least some SMC compartments indicates that it is likely that they interact to regulate SMC-specific transcription.

The third myocardin family member, MRTF-B/MKL2, has also been implicated in CArG-dependent transcription (16, 176). In the original report by Wang *et al* MRTF-B had a much smaller effect on SM22 or ANF promoter activity than did myocardin and MRTF-A (176). However, its human homologue, MKL-2, was shown to strongly activate the SM22, ANF, and SM α-actin genes in HeLa cells (142). The role of both MRTFs in SMC-specific transcription is further examined in this dissertation research.

MYOCARDIN FACTOR HOMO- AND HETERO-DIMERIZATION

One interesting aspect of the myocardin factors is the potential homo- and hetero-dimerization that can occur between the members of this transcription factor family. As stated previously, all three myocardin family members contain a highly conserved coiled-coil leucine zipper motif that has been shown to mediate direct interactions between these proteins. Disruption of this domain in myocardin, MRTF-A, or MRTF-B drastically reduced transactivation by all of these transcription factors (35, 142, 179) indicating that homo- and/or hetero-dimerization is important for their activity.

Based on gel mobility shift assays Wang *et al* suggested that myocardin binds SRF as a monomer (179). Since myocardin preferentially activates promoters with multiple CArG elements (175), these authors proposed a model in which dimerization between myocardin bound to neighboring CArG elements leads to exposure of an otherwise cryptic TAD. This could explain both myocardin's preference for promoters with multiple CArG elements and the requirement for multiple CArGs in many of the SMC differentiation marker genes.

The demonstration of heterodimeric interactions between myocardin family members may make this model somewhat more complicated in cell-types such as aorta that express more than one of these transcription factors (35, 88). In addition, evidence suggests that MRTF-A, in contrast to myocardin, preferentially binds SRF as a dimer (104), raising the possibility that MRTF-A and myocardin could simultaneously bind the same CArG element under certain conditions. Clearly, more information is needed concerning the requirements for formation of the CArG/SRF/myocardin factor ternary complex as well as on the potential interactions between myocardin family members.

REGULATING SMC DIFFERENTIATION VERSUS SMC GROWTH

Given SRF's ubiquitous expression pattern and its promiscuous involvement in a number of gene regulatory programs, including the immediate early response, it has been difficult to explain how SRF regulates cell-type-specific gene expression. An important observation in this regard is that, in general, myocardin and MRTF-A transactivate gene promoters that contain multiple CArG elements much more strongly than those regulated by a single CArG resulting in preferential activation of the SMC-specific promoters over the early response growth genes (175). In addition, dominant negative forms of MRTF-A had

little effect on the expression of c-fos, providing further support for differential effects of the myocardin family on SRF-dependent differentiation and SRF-dependent growth (16). As previously discussed, LZ domain-mediated interactions between myocardin molecules bound to separate CArG elements may at least partially explain myocardin's selective effects on SMC differentiation marker gene expression.

Another important observation made by Wang *et al* was that competition for SRF binding between myocardin and the TCFs may be involved in regulating SRF-dependent differentiation versus growth (178). Using gel shift and co-immunoprecipitations, these authors showed that myocardin and Elk-1 competed for binding to the same SRF domain within the MADS box, and that activation of MAP-kinase signaling by PBGF-BB could increase Elk-1-SRF association at the expense of myocardin-SRF association. They also found that mutation of a potential Elk-1 binding site in the SM22 promoter resulted in continued transgene expression in the heart after the wild-type transgene had been down-regulated.

Additionally, there is evidence indicating that myocardin factors may preferentially bind to certain CArG elements over others. Chromatin immunoprecipitation assays showed that MRTF-A interacted with SRF bound to the CArG boxes within the vinculin and SRF promoters but not with SRF bound to the *c-fos* or *egr-1* promoters (104). A better understanding of interactions between the different myocardin family members and specific SRF-CArG complexes will be important for delineating the role of the myocardin family in both cell-type-specific and gene-specific transcriptional regulation.

REGULATION OF MYOCARDIN

Very little is known about the regulation of myocardin expression and/or activity. One of the first parameters shown to affect myocardin-dependent transactivation was SRF expression levels, with excess SRF leading to attenuation of myocardin activity (175). All three muscle cell types express high levels of SRF which may partially explain why myocardin trans-activates the SMC-specific genes relatively weakly in SMC (10-20 fold) compared non SMC-types (>200 fold) (23, 176, 189). The precise mechanism for this inhibition is unknown, but these data suggest that SRF and myocardin can associate in transcriptionally inactive complexes, possibly in the absence of SRF-DNA binding. Interestingly, myocardin has also been shown to physically bind to GATA-4, an interaction mediated by the SAP domain (118). Depending upon the CArG-dependent promoter studied, this interaction either stimulated or inhibited promoter activity providing further evidence that complex interactions between myocardin, SRF, and other transcription factors is probably important for regulating muscle-specific transcription.

Several studies have shown that myocardin expression can be altered by extrinsic factors that are known to have effects on SMC differentiation. Yoshida *et al* demonstrated that treatment of primary rat aortic SMC with angiotensin II increased myocardin expression in a dose-dependent manner and that this increase correlated with increased expression of SM α-actin (187). Wamhoff *et al* from the same group also showed that membrane depolarization with KCl led to an increase in myocardin expression (174). The observed increase in this model was mediated by intracellular calcium and RhoA signaling in that it could be inhibited by the calcium channel blocker, nifedipine, or the Rho-kinase inhibitor, Y-27632. The molecular mechanisms that control myocardin expression have been examined by Ueyama *et al* (170). These authors identified myocardin in a subtractive hybridization

screen for gene targets of Nkx2.5 and went on to demonstrate that Nkx2.5 could transactivate myocardin expression by binding to an Nkx2.5 responsive element (NKE) within the myocardin promoter. Further examination of the myocardin promoter will certainly be important for identifying the mechanisms by which myocardin gene expression is regulated. Moreover, it will be equally important to determine whether myocardin activity (as opposed to myocardin expression) is modulated by agonist-induced changes in post-translational modification.

REGULATION OF MRTF ACTIVITY

Miralles *et al* were the first to show that MRTF-A's ability to stimulate transcription was regulated by RhoA-dependent signaling (104). These authors demonstrated in NIH3T3 cells that MRTF-A resides nearly exclusively in the cytoplasm in serum-starved cells, and that MRTF-A translocated to the nucleus upon stimulation by serum or other interventions that increased RhoA activity or actin polymerization (Fig 1.5). The RPEL domains in MRTF-A's N-terminus (see figure 1.1 for domain map) were shown to interact directly with G-actin (126), and deletion or mutation of RPEL domains 2 and 3 resulted in MRTF-A variants that were constitutively nuclear (104). Taken together these results indicated that in un-stimulated cells, MRTF-A is retained in the cytoplasm through an interaction with G-actin and that RhoA-induced depletion of the G-actin pool frees MRTF-A to enter the nucleus where it can stimulate SRF-dependent transcription. The C-terminal and Q-rich regions were also implicated in MRTF-A localization (104). These domains were not important for RhoA mediated nuclear import but possibly other aspects of MRTF-A localization such as nuclear

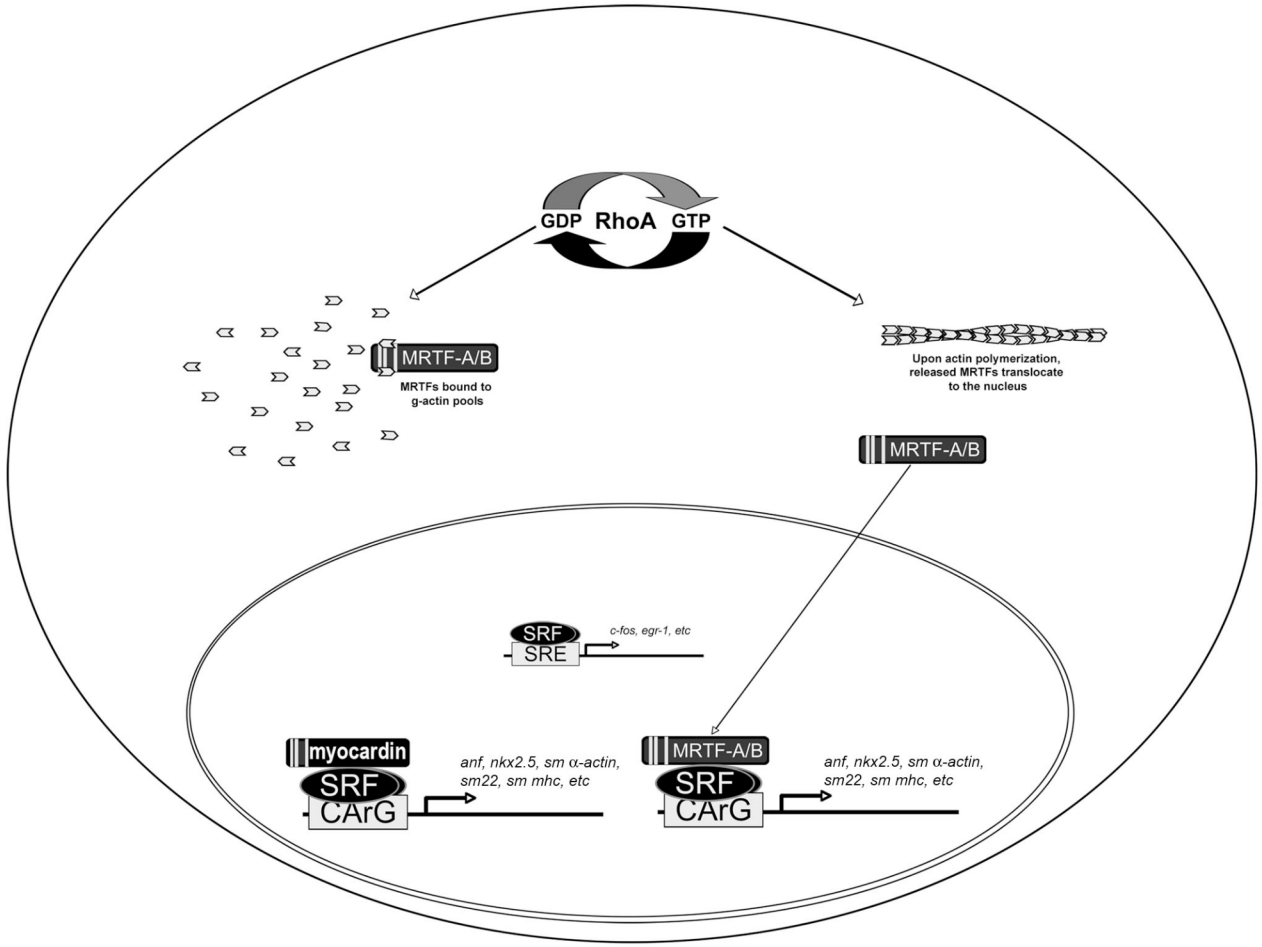

Figure 1.5. Regulation of SRF-dependent gene transcription by myocardin factors. Myocardin, MRTF-A, and MRTF-B have all been shown to bind SRF directly, and to potently up-regulate SRF/CARG-dependent transcription of cardiac and SMC-specific genes. The myocardin factors do not activate SRF-dependent transcription of the early response genes c-fos and egr-1. Myocardin localizes constitutively to the nucleus, while sub-cellular localization of the MRTFs is regulated by RhoA signaling. MRTF-A and MRTF-B are thought to be sequestered in the cytoplasm via interactions between their N-terminal RREL domains and G-actin. Upon activation of the RhoA pathway, MRTFs translocate to the nucleus in a manner dependent on RhoA-mediated actin polymerization.

retention or nuclear export. Interestingly, myocardin has a slight mismatch in RPEL domain 2 which may explain why myocardin is constitutively nuclear.

When taken together with our observations that RhoA was an important determinant of SMC-specific gene expression and that MRTF-A protein is expressed in rat aortic SMC cultures (88), we and others hypothesized that MRTFA may serve as an important link between the extrinsic cues that regulate SMC function and the transcriptional machinery that ultimately determines SMC phenotype (35). Indeed, Cen *et al* reported that DN MRTF-A could inhibit the effects of constitutively active RhoAV14 on SRF-dependent promoter activity in HeLa cells (16). Similarly, our laboratory demonstrated that dominant negative N19RhoA could inhibit MRTF-A transactivation and that DN variants of MRTF-A could inhibit S1P-induced activation of SMC-specific gene expression, an effect that is dependent upon activation of RhoA (88). Other studies from our laboratory have further demonstrated that the actin remodeling-dependent effects of RhoA on MRTF-A activity are mediated at least in part by the formin proteins mDia1 and mDia2, and that inhibition of these actin-polymerizing proteins also inhibits MRTF-A activity (155).

Several studies from other laboratories have suggested that the regulation of MRTF-A localization may be more complicated than originally described. Du *et al* reported that over-expressed MKL-1 was constitutively nuclear in rat aortic SMC (35). Selvaraj *et al* also failed to observe any increase in nuclear localization of MKL-1 upon serum stimulation of NIH3T3 or HeLa cells (142). Although differences in MRTF-A detection (i.e endogenous vs epitope, tagged vs EGFP fusion), species differences, or slight differences in culture conditions could explain these discrepancies, there are probably cell-type or species-specific differences in the regulation of MRTF-A localization. Furthermore, the differences seen in these model

systems may reflect inherent differences in RhoA signaling in these cell-types. Many different environmental factors affect RhoA signaling (see (51) for a review), making it likely that multiple environmental cues are important for regulating MRTF-A localization, and it is possible that specific cues may have different roles in different cell types.

Less has been reported regarding the regulation of MRTF-B activity, but it appears that MRTF-B is also regulated by RhoA signaling. Olson and associates first demonstrated this, showing that both MRTF-A and MRTF-B nuclear localization were increased upon serum stimulation (a known activator of RhoA). These authors demonstrated that over-expression of the protein striated muscle activator of Rho signaling (STARS) led to increases in MRTF/SRF/CArG-dependent gene expression. STARS was shown to promote release of MRTFs from actin pools in a manner dependent upon its actin-binding domain (72).

Important differences in the regulation of MRTF-A and MRTF-B localization by RhoA have also been noted. Zhoa *et al* used an *in vitro* force-induced model of RhoA activation to study the role of Rho-kinase in the regulation of SM α-actin gene expression in myofibroblasts. These authors observed in fibroblasts that following activation of the RhoA pathway by force, MRTF-A translocated to the nucleus while MRTF-B did not (192). A potentially important difference between MRTF-A and MRTF-B is the presence of a 71 AA N-terminal region in MRTF-B that does not seem to be present in MRTF-A (although the precise translational start site of MRTF-A is not completely clear) (Fig 1.4). In addition, the MRTF-B message contains a long 3'UT that is not found in myocardin or MRTF-A, and this region contains several binding elements that may regulate MRTF-B translation.

Recent studies have identified a single MRTF (DMRTF) in drosophila that regulates tracheal branching and cell migration (52, 151). Interestingly, nuclear accumulation of

DMRTF is stimulated by increased cell tension, an environmental parameter that is known to increase RhoA activity (151). These findings suggest that not only is the MRTF-SRF interaction highly conserved throughout evolution, but that the regulation of MRTF-A translocation by RhoA may be as well.

The precise mechanisms that regulate MRTF nuclear/cytoplasmic translocation are somewhat unclear. In the simplest model proposed by Treisman and colleagues, G-actin binding to the RPEL domain could mask a nuclear localization signal that is exposed when G-actin pools are reduced following RhoA stimulation. However, the two basic domains (B1 and B2) that are required for RhoA mediated nuclear translocation do not seem to function as nuclear localization sequences in that they do not promote nuclear localization when linked to a heterologous protein (104). Furthermore, Posern *et al* has described a mutant actin that can stimulate MRTF-A nuclear translocation independent of its effects on actin treadmilling suggesting a more direct mechanism by which actin may stimulate MRTF-A translocation (126). Interestingly, in NIH3T3 and C2C12 cells, MRTF-A's C-terminus is S/T phosphorylated upon serum treatment (104). While this phosphorylation does not seem to be involved in RhoA-dependent nuclear import, it may be important for the C-terminus' role in mediating nuclear retention or export or may have other effects on MRTF-A function.

Over-expression of MRTFs, even in cells that already express these proteins endogenously, has been shown to strongly activate SRF-dependent gene expression (16, 35, 88, 142). It follows that regulation of myocardin factor expression, either at the mRNA or protein level, may be an important mode of regulating their activities. However, no studies to date have been published identifying mechanisms that regulate MRTF expression. Two separate studies from our laboratory, detailed here in later chapters, have each examined this

topic in detail. In the first we demonstrate that many of the mechanisms known to stimulate myocardin factor activity do not affect their expression at the mRNA level, and in the second we provide the first lines of evidence implicating the proteasome as an important determinant of myocardin factor protein expression and activity.

THE UBIQUITIN-PROTEASOME SYSTEM

The ubiquitin-proteasome system (UPS) is the major regulator of non-lysosomal protein degradation within the cell. Efficient degradation of misfolded and malfunctioning proteins is critical for proper cellular function. The proteasome has been directly implicated in a wide array of processes and pathways including cellular metabolism, immune surveillance, apoptosis, signal transduction, gene transcription, proliferation, and differentiation (29). Despite UPS involvement in such varied cellular activities, UPS-mediated protein degradation is highly specific and tightly regulated. Improper proteasomal function has been implicated in the pathogenesis of multiple human diseases, including cancer, atherosclerosis and congestive heart failure (98, 123, 171). A brief description of this system is included below.

Protein ubiquitination occurs in a three-step process, each relying on a specific class of enzyme (E1-E3) (Fig 1.6A). First, an E1 ubiquitin activating enzyme binds free ubiquitin in an ATP-dependent manner. The ubiquitin molecule is then transferred to an E2 ubiquitin conjugating enzyme in a manner independent of ATP. In the third and final step of the process, an E3 ubiquitin ligase recognizes the substrate to be ubiquitinated and transfers the ubiquitin molecule from E2 to the substrate (162). In this manner, it is the E3 ligase that confers specificity in ubiquitination. Accordingly, there are a great many more E3 ligases

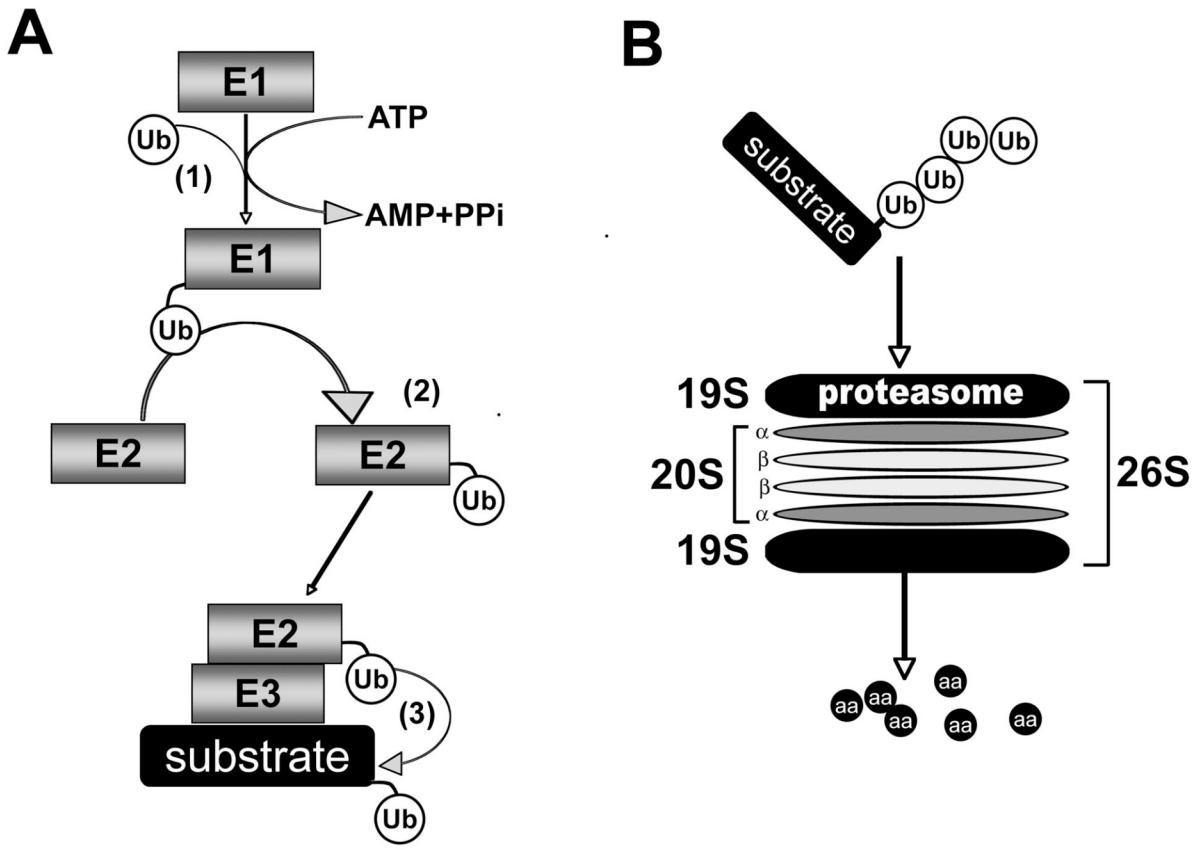

Figure 1.6. The Ubiquitin-Proteasome System. A) Substrate proteins are ubiquitinated via a multi-step process involving 3 classes of enzymes. First, free ubiquitin is bound by ubiquitin activating enzymes (E1) in an ATP-dependent manner. Second, ubiquitin is transferred directly to ubiquitin conjugating enzymes (E2). Finally, substrates are bound by ubiquitin ligases, which also interact with E2 and facilitate transfer of ubiquitin to substrate. **B)** Poly-ubiquitinated proteins are degraded via the 26S proteasome. This multi-protein complex is comprised of a core 20S subunit and two 19S subunits. The barrel-shaped 20S core is comprised of four ring structures, the outer α rings and inner proteolytic β rings.

than there are E2 conjugating enzymes, and there are very few E1 ubiquitin activating enzymes.

E3 ubiquitin ligases belong to one of two groups, divided by their mode of substrate ubiquitination. The first of these groups consists of the RING (really interesting new gene) domain-containing proteins and the structurally similar U-box proteins. These E3 ligases bind to ubiquitin carrying E2 ubiquitin conjugating enzymes and to their substrates simultaneously, and facilitate shape changes that lead to transfer of ubiquitin to the substrate. The second group is the HECT (homologous to E6-associated protein C terminus) domain proteins. These E3 ligases bind ubiquitin carrying E2 ubiquitin conjugating enzymes, and transfer the ubiquitin molecule to their own HECT domain. The ubiquitin molecule is transferred to the substrate in a second step. Regardless of the type of E3 enzyme used, the result is the formation of a covalent bond between a lysine residue of the ubiquitin molecule and a lysine residue of the substrate, usually located at its N-terminus. Poly-ubiquitin chains are often formed next, and are accomplished by E4 enzymes that catalyze the formation of similar covalent bonds between individual ubiquitin molecules (171).

Mono-ubiquitination of substrates has been demonstrated to result in various effects, including alterations in protein activity and localization, while poly-ubiquitination most often leads to ATP-dependent degradation via the 26S proteasome (Fig 1.6B)(113). The 26S proteasome contains three main subunits. Two outer 19S subunits allow for recognition of poly-ubiquitinated substrates, and sit on either side of a central barrel-shaped 20S subunit. The 19S subunits also harbor ATP activity at their bases that is used to unfold and de-ubiquitinate proteins as they enter the central tunnel of the proteasome. The 20S subunit is

comprised of 4 rings (2 outer α and 2 inner β rings), and as proteins pass through this barrel shaped structure, they are degraded by the proteolytic β-ring core (171).

OBJECTIVES OF THIS DISSERTATION RESEARCH

A better understanding of SMC differentiation and phenotypic modulation depends upon further description of the mechanisms that regulate SMC-specific transcription. The central role of SRF and myocardin factors in this regulatory pathway makes them excellent targets for investigation. The overall objectives of this dissertation research were to (1) determine the extent to which myocardin factors MRTF-A and MRTF-B contribute to SMC-specific gene expression and to identify molecular mechanisms that regulate MRTF activity, (2) identify novel members of the transcriptional complex present at the promoters of SMC differentiation marker genes using a yeast-2-hybrid screen approach, and to define the role of identified factors in the regulation of SRF-dependent SMC gene transcription, and (3) characterize the role of the E3 ubiquitin ligase muscle specific ring finger 3 in the regulation of myocardin factor activity and protein stability.

SMOOTH MUSCLE CELL SPECIFIC TRANSCRIPTION IS REGULATED BY NUCLEAR LOCALIZATION OF THE MYOCARDIN-RELATED TRANSCRIPTION FACTORS

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ABSTRACT

Based upon our previous studies on RhoA signaling in smooth muscle cells (SMC), we hypothesized that RhoA-mediated nuclear trans-localization of the Myocardin-Related Transcription Factors (MRTFs) was important for regulating SMC phenotype. MRTF-A protein and MRTF-B message were detected in aortic SMC and in many adult mouse organs that contain a large SMC component. Both MRTFs up-regulated SMC-specific promoter activity as well as endogenous SM22α expression in multipotential 10T1/2 cells, although to a lesser extent than myocardin. We used EGFP fusion proteins to demonstrate that the

myocardin factors have dramatically different localization patterns and that the stimulation of SMC-specific transcription by certain RhoA-dependent agonists was likely mediated by increased nuclear translocation of the MRTFs. Importantly, a dominant negative form of MRTF-A (ΔB1/B2) that traps endogenous MRTFs in the cytoplasm inhibited the SM α-actin, SM22α, and SM MHC promoters in SMC and attenuated the effects of S1P and TGF-β on SMC-specific transcription. Our data confirmed the importance of the N-terminal RPEL domains for regulating MRTF localization, but our analysis of MRTF-A/myocardin chimeras and myocardin RPEL2 mutations indicated that the myocardin B1/B2 region can over-ride this signal. Gel shift assays demonstrated that myocardin factor activity correlated well with ternary complex formation at the SM α-actin CArGs, and that MRTF-SRF interactions were partially dependent upon CArG sequence. Taken together our results indicate that the MRTFs regulate SMC-specific gene expression in at least some SMC sub-types, and that regulation of MRTF nuclear localization may be important for the effects of selected agonists on SMC phenotype.

INTRODUCTION

Vascular smooth muscle cell (SMC) differentiation is a very important process during vasculogenesis and angiogenesis, and it is recognized that alterations in SMC phenotype play a role in the progression of several prominent cardiovascular disease states including atherosclerosis, hypertension, and restenosis (122, 138). The identification of the transcription factors involved in this process has been complicated by the lack of terminal differentiation in this cell-type and the fact that SMC derive from multiple locations including local mesoderm, cardiac neural crest, the proepicardial organ, and possibly

circulating stem cells (see (122) for review). A completely SMC-specific transcription factor has yet to be described, and evidence suggests that SMC differentiation may be regulated by interactions between multiple transcription factors with overlapping expression patterns (15, 19, 48). We and others have shown that the SMC differentiation marker genes are regulated by serum response factor (SRF), a MADS Box transcription factor that binds to conserved CArG boxes found in nearly all of the SMC marker gene promoters (55, 79, 91, 93, 95, 100). Since SRF is ubiquitously expressed and regulates a variety of other muscle-specific genes as well as early response genes, c-fos and egr-1 (11, 22, 152, 168, 173), it is clear that additional mechanisms are involved.

An important breakthrough in the study of the molecular mechanisms that regulate SMC differentiation was the discovery of the myocardin family of SRF co-factors by Wang *et al* (175). The founding member of this family, myocardin, is selectively expressed in the heart and SMC and very powerfully transactivates SMC differentiation marker gene expression by physically interacting with SRF. Importantly, genetic deletion of myocardin resulted in embryonic lethality at E10.5 due, at least in part, to failure of SMC differentiation in the mesodermal cells surrounding the descending aorta (82). In more recent studies, Pipes *et al* used a chimeric mouse model to demonstrate that myocardin -/- cells could populate the developing aorta suggesting that myocardin-independent mechanisms are likely to be important for SMC differentiation (125).

Two Myocardin-Related Transcription Factors, MRTF-A/MKL-1 and MRTF-B/MKL-2, have been identified that have similar transcriptional activity to myocardin. These factors are thought to be expressed more widely, and their role in regulating cell-type-specific gene regulation is much less clear. MKL-1 message is expressed strongly in a

variety of human tissues that have a large SMC component including the aorta and bladder, and we and others have detected MRTF-A/MKL-1 protein in multiple SMC lines, including primary rat aortic SMC, A7R5, A10, and PAC-1 as well as mouse embryonic stem cells and multipotential 10T1/2 cells (35, 88). Over-expression of MRTF-A/MKL-1 strongly up-regulated several muscle-specific promoters in a variety of cell-types and importantly was sufficient to activate endogenous expression of SMC differentiation marker genes in ES cells. MRTF-B was originally shown to have little effect on CArG-dependent transcription (176). However, its human homologue, MKL-2, strongly activated the ANF, SM22α, and SM α-actin promoters in HeLa cells (142). Since these proteins share greater than 80% homology, the reason for this discrepancy is currently unknown. Two separate groups have recently shown that genetic disruption of MRTF-B led to a lethal defect in pharyngeal arch remodeling and that this phenotype was accompanied by a failure of SMC differentiation of the cardiac neural crest cells that populate the cardiac outflow tract (78, 117). Interestingly, both Li *et al* and Sun *et al* have shown that MRTF-A knockout females have a nursing defect that is accompanied by a loss of SMC differentiation marker gene expression that normally occurs in the myoepithelial layer of the mammary gland during lactation (81, 156).

Identification of the mechanisms that regulate the myocardin transcription factors will be very important for our understanding of their role in cell-type-specific gene regulation. Miralles *et al* were the first to demonstrate that the activity of MRTF-A was regulated by RhoA-dependent signaling (104). These authors demonstrated in NIH3T3 cells that MRTF-A resided nearly exclusively in the cytoplasm in serum-starved cells, that MRTF-A translocated to the nucleus upon serum stimulation, and that this process was regulated by RhoA-dependent changes in actin polymerization. The RPEL domains in MRTF-A's N-terminus

were shown to bind to G-actin (126), and this interaction was shown to be important for retaining MRTF-A in the cytoplasm. A recent study indicated that MRTF-B localization may be regulated by the same mechanism (72). Interestingly, myocardin is constitutively nuclear, and it has been suggested that lack of conservation in myocardin's second RPEL domain may inhibit cytoplasmic retention (104). Several groups have reported somewhat contrasting results on MRTF-A localization (35, 142). For example, Du *et al* reported that in primary rat aortic SMC, MRTF-A/MKL-1 was constitutively nuclear in serum starved cells or in the presence of dominant negative RhoA. In addition, Selvarej *et al* did not observe nuclear translocation of MKL-1 upon serum stimulation of NIH 3T3 and HeLa cells (142). The reason for these discrepancies is unknown.

We have previously shown that RhoA is an important determinant of SMC differentiation marker gene expression and that MRTF-A was required for the up-regulation of SMC-specific transcription observed upon treatment of aortic SMC with the strong RhoA agonist, sphingosine 1-phosphate (S1P) (88, 92). The goals of the present study were to determine the contributions of the MRTFs to SMC-specific gene regulation, to test whether regulation of MRTF nuclear localization is an important signaling mechanism for controlling SMC-specific transcription and to further characterize the differences between the three myocardin transcription factors.

MATERIALS AND METHODS

Plasmids and Proteins - Myocardin, MRTF-A and MRTF-B plasmids were a generous gift of Da-Zhi Wang (University of North Carolina, Chapel Hill, NC). MRTF deletions lacking the N-terminal RPEL domains were created by PCR. The dominant

negative ΔB1/B2 MRTF-A was a generous gift of Richard Treisman (Cancer UK, London) and has been described previously (104). MRTF-A/myocardin chimeras that fused N-terminal fragments of MRTF-A to C-terminal fragments of myocardin were made by PCR. An exogenous XhoI restriction site (that codes LE) was inserted at the MRTF-A/myocardin junction to facilitate cloning, and junction sites were placed in regions of low homology. More details on the MRTF-A/myocardin chimeras can be found in figure 2.3 and are available upon request. The myocardin double mutation S72P, S76E that restored RPEL2 was made using the QuikChange method (Stratagene). All myocardin factors were subcloned into a flag-tagged pcDNA3.1 and/or an EGFP expression vectors.

Cell Culture, Transient Transfections, and Reporter Assays – SMCs from rat thoracic aorta were isolated, cultured, and transfected as previously described (91, 147). In short, cells were maintained in 24 well plates in 10% serum and were transfected 24 h after plating at 70-80% confluency using the transfection reagent, Superfect (Qiagen), as per protocol. The SM22α promoter (from –450 to +88), SM α-actin promoters (from -2560 to +2784), SM MHC promoter (from –4200 to + 11600), and c-fos promoter (from -356 to +109) used in this study have been previously described (80, 93, 94). In some experiments myocardin, MRTF-A, MRTF-B, or variants thereof were co-transfected along with the promoter-luciferase constructs.

Prior to agonist treatments, SMC were placed in serum free media for 24 h while 10T1/2 cells were placed in 0.2% charcoal treated serum (to remove serum lipids). Cells were treated with S1P (1uM), 10% serum, TGF-β (1ng/ml), PDGF-BB (20ng/ml), and luciferase assays were performed after 24 h. In some experiments the Rho-kinase inhibitor, Y-27632 (10 uM), was added 15 minutes prior to addition of agonist. The S1P used in these

experiments was obtained from Matreya and was maintained in 4 mg/ml fatty-acid free BSA which was used as a vehicle control. Relative promoter activities are expressed as the means +/- standard error computed from a set of at least three separate transfection experiments. We did not co-transfect a viral promoter/Lac Z construct as a control for transfection efficiency since we have previously shown that such constructs exhibit unknown and variable squelching effects on the SM-specific promoters presumably due to competition for common transcription factors (147). Moreover, we have found that inclusion of such controls are unnecessary in that variations in transfection efficiency between independent experimental samples is routinely very small (<10%)(147).

Analysis of MRTF expression - Adult C57/Black6 mice were sacrificed, and blood was removed by perfusing phosphate-buffered saline through the vasculature via a puncture of the left ventricle. Tissues were excised and homogenized in RIPA buffer plus inhibitors by sonication. Lysates were clarified by centrifugation and protein concentrations were determined using BCA protein assay (Pierce). 150ug total protein from each tissue lysate was run on an 8% SDS polyacrylamide gel, and subsequently transferred to nitrocellulose. MRTF-A was detected using MRTF-A antiserum generously provided by Richard Treisman (Cancer Research UK, London). *In vitro* translated MRTF-A was prepared using Promega's TNT T7 Coupled Reticulocyte Lysate System and was run along side the lysates as a positive control. Semi-quantitative PCR was used to measure MRTF-B expression. In brief, RNA was prepared from cell and tissues as above using Trizol Reagent (Invitrogen), and quantified by Ribogreen Assay (Molecular Probes). cDNA was generated with the iScript cDNA synthesis kit (Biorad) using 1ug of RNA per manufacturer's protocol. The following exon spanning primers were used for amplification reactions: MRTF-B, 5'-atgaggaagccatcaagcag-

3’ and 5’-atctgctgactgtgcaca-3’; GAPDH, 5’-atgggtgtgaaccacgaagaa -3’ and 5’-ggcatggactgtggtcatga-3’.

Visualization of myocardin factor localization – The myocardin factors were subcloned into the pEGFP-C3 vector (Clontech). SMC and 10T1/2 were transfected with fusion protein plasmids as described above, maintained in 10% serum overnight, and then held in serum free media for 24 h. After addition of agonist, myocardin factor localization was monitored in real time on an inverted fluorescent microscope, and images were taken at 5 min intervals for 80 min using a Spot digital camera. Micrographs were converted into time-lapse movies using Isee imaging software and QuickTime. To quantify localization in the entire cell population, cells were fixed after 80 minutes of treatment in 4% paraformaldehyde, and localization was scored into 3 separate categories: nuclear, diffuse, and cytoplasmic.

Gel shift analyses - SRF and flag-tagged myocardin factors were translated *in vitro* using the Promega T7 TnT kit. Binding reactions contained 1 uL SRF, 2 uL of myocardin factor, 20,000 cpms of a P^{32}-labeled oligonucleotide probe containing CArGA, CArGB or the intronic CArG from the rat SM α-actin promoter, 0.20 ug dIdC in binding buffer (10 mM Tris, pH 7.5, 50mM NaCl, 100 mM KCl, 1mM DTT, 1mM EDTA, 5% glycerol). Reactions were incubated for 30 min before loading on non-denaturing 4% polyacrylamide gel that was pre-run at 170V for 1hr. Electrophoresis was performed at 170V in 0.25X TBE (45 mM Tris Borate, 1 mM EDTA). Gels were dried and exposed to film for 24-72 h at -80°C. For supershift studies, 1 µl of M2 Flag antibody (Sigma) was added after 20 min of incubation.

RESULTS

MRTF-A and MRTF-B were expressed in aortic SMC and many SM-containing tissues. Previous studies have demonstrated that the MRTFs are expressed more widely than myocardin (176). However, MRTF-A message expression is extremely high in the human aorta and fairly high in bladder, stomach, intestine, and in many SMC or SMC-like cell lines including primary rat aortic SMC, A10, PAC-1, and 10T1/2 (35) suggesting that it may have an important role in SMC. To get a better idea of MRTF-A protein expression in adult mouse SMC-containing tissues, we performed Western analysis. Results shown in Figure 2.1 demonstrate that MRTF-A levels were high in aorta, bladder, lung, and uterus and in rat aortic and A7r5 SMC cultures. The size of immunoreactive bands varied slightly between tissues indicating that MRTF-A may be post-translationally modified or processed in a cell-type-specific manner. Due to the lack of a suitable MRTF-B antibody, we used semi-quantitative RT PCR to measure MRTF-B expression in adult mouse SMC-containing tissues. Figure 2.1b demonstrates that MRTF-B message was more evenly distributed, but was relatively high in aorta, lung, stomach, liver, and rat aortic SMC cultures.

The MRTFs regulated SMC differentiation marker gene expression. Myocardin has been shown to regulate SMC differentiation(36, 82, 189), but the role played by the MRTFs in this process is less clear. To directly compare activation of SMC-specific gene expression by all three myocardin factors, we expressed them in multi-potential 10T1/2 cells. This cell line is a very useful and consistent model for studying the regulation of SMC-specific transcription because many endogenous SMC-specific differentiation marker genes including SM α-actin, SM22α, and calponin can be induced by agonists such as TGF-β or sphingosine-1 phosphate (62, 88). As shown in figure 2.2, all three of the myocardin factors transactivated the SM α-actin and SM22α promoters and stimulated the endogenous

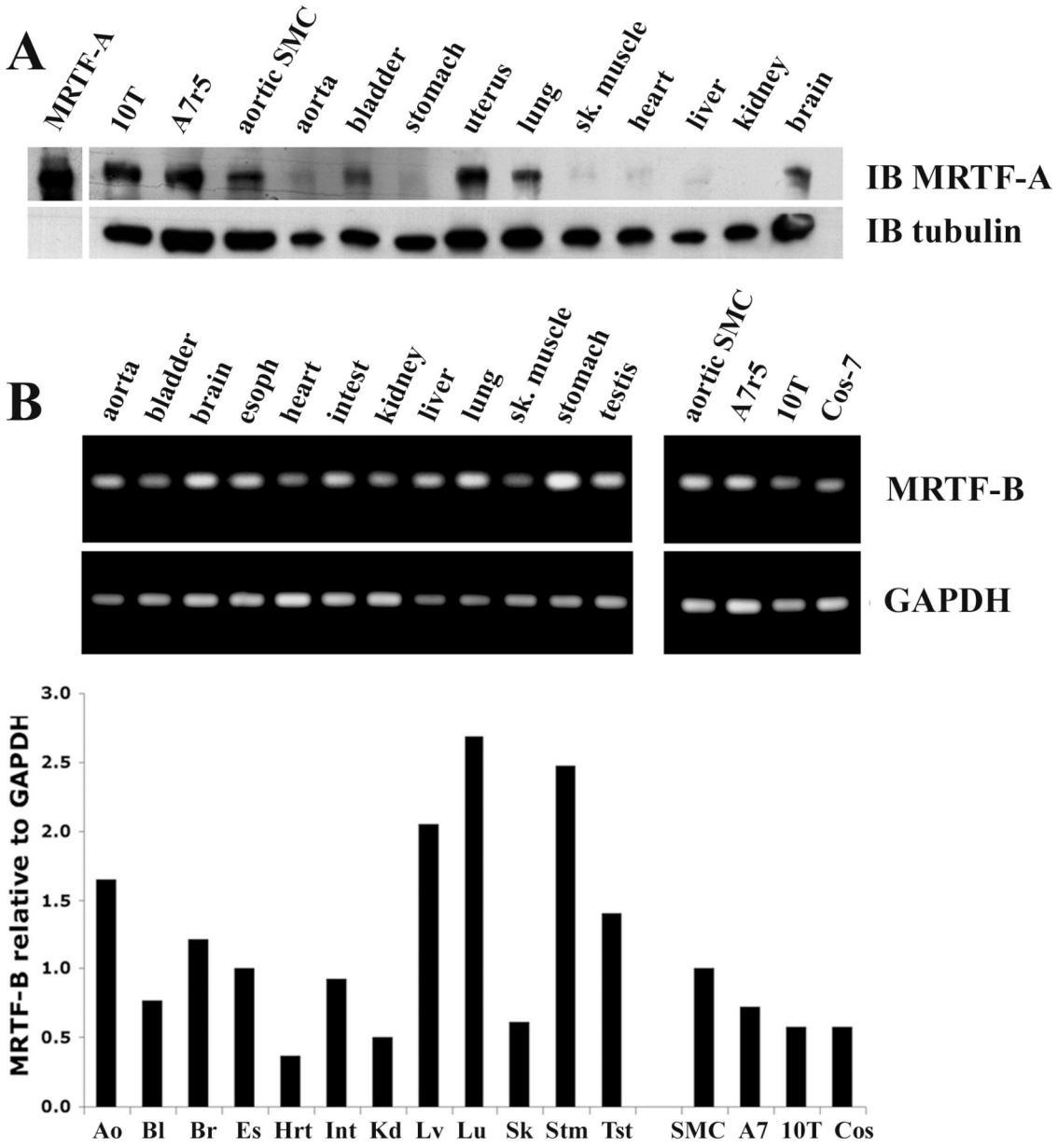

Figure 2.1. MRTF expression in SMC and SMC tissues. A) RIPA lysates were prepared from the indicated mouse tissues and cultured cell lines, separated on a polyacrylamide gel, transferred to nitrocellulose, and probed with anti-MRTF-A antiserum. B) RNA was prepared from the indicated cell lines and mouse tissues using the Trizol Reagent. Following first strand cDNA synthesis, MRTF-B and GAPDH primers were used in amplification reactions. PCR products were run on a 1% agarose gel and visualized with ethidium. Bottom panel shows MRTF-B expression normalized to GAPDH as quantified by ImageJ software.

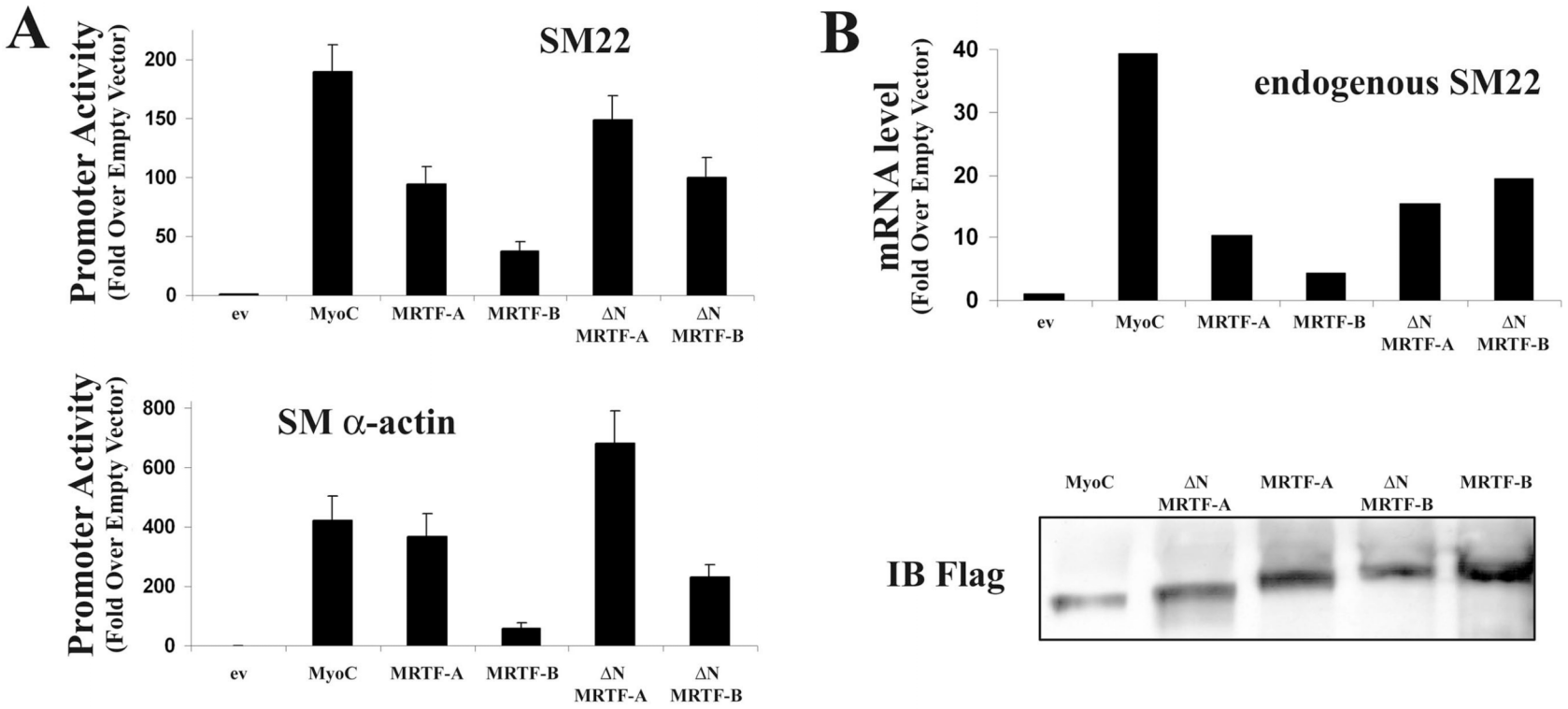

Figure 2.2. The MRTFs activated SMC-specific gene transcription. A) Luciferase constructs driven by the SM α-actin and SM22α promoters were transfected into 10T1/2 cells along with myocardin, or full length or ΔN versions of MRTF A and MRTF-B. Cells were maintained in 10% serum and assayed for luciferase activity after 48 h. Empty expression vector was transfected as a control (ev) **B)** The effects of the myocardin factors on endogenous SM22α message were measured using quantitative PCR (TaqMan).

expression of SM22α. However, transactivation by myocardin was significantly greater than that induced by MRTF-B and in most instances greater than that induced by MRTF-A even though these transcription factors were expressed at similar levels. Our data also support previous studies that demonstrated that the N-terminal actin-binding region of both MRTF-A and MRTF-B had inhibitory effects on their activities.

MRTF activity was regulated by nuclear localization. MRTF-A nuclear localization has been shown to be regulated by RhoA signaling (88, 104) and we hypothesize that changes in MRTF nuclear localization may help explain the effects of certain environmental cues on SMC-specific transcription. To begin to test this, we constructed EGFP fusion proteins so that we could monitor myocardin factor localization in real time. Fusion protein expression was confirmed by Western blot using an anti-EGFP antibody and results from co-transfection experiments demonstrated that these fusion proteins significantly activated the SM α-actin promoter suggesting that the EGFP moiety did not dramatically disrupt myocardin protein function (data not shown). Initially, 10T1/2 cells were serum starved for 24 h, fixed, and scored for localization into 3 separate categories: nuclear, diffuse, and cytoplasmic. Myocardin localization was constitutively nuclear even after prolonged serum deprivation while MRTF-B localization was nearly completely cytoplasmic under the same conditions (figure 2.3). Interestingly, MRTF-A could localize to the nucleus or cytoplasm but was often found in a more diffuse pattern. In addition, neighboring cells with dramatically different MRTF-A localization patterns were frequently observed. Since it has been shown that RhoA activity is down regulated in confluent SMC and other cell-types (115, 191), we also tested whether cell density could affect MRTF-A localization. As shown in figure 2.3B, the percentage of cells containing MRTF-A exclusively in the nucleus was

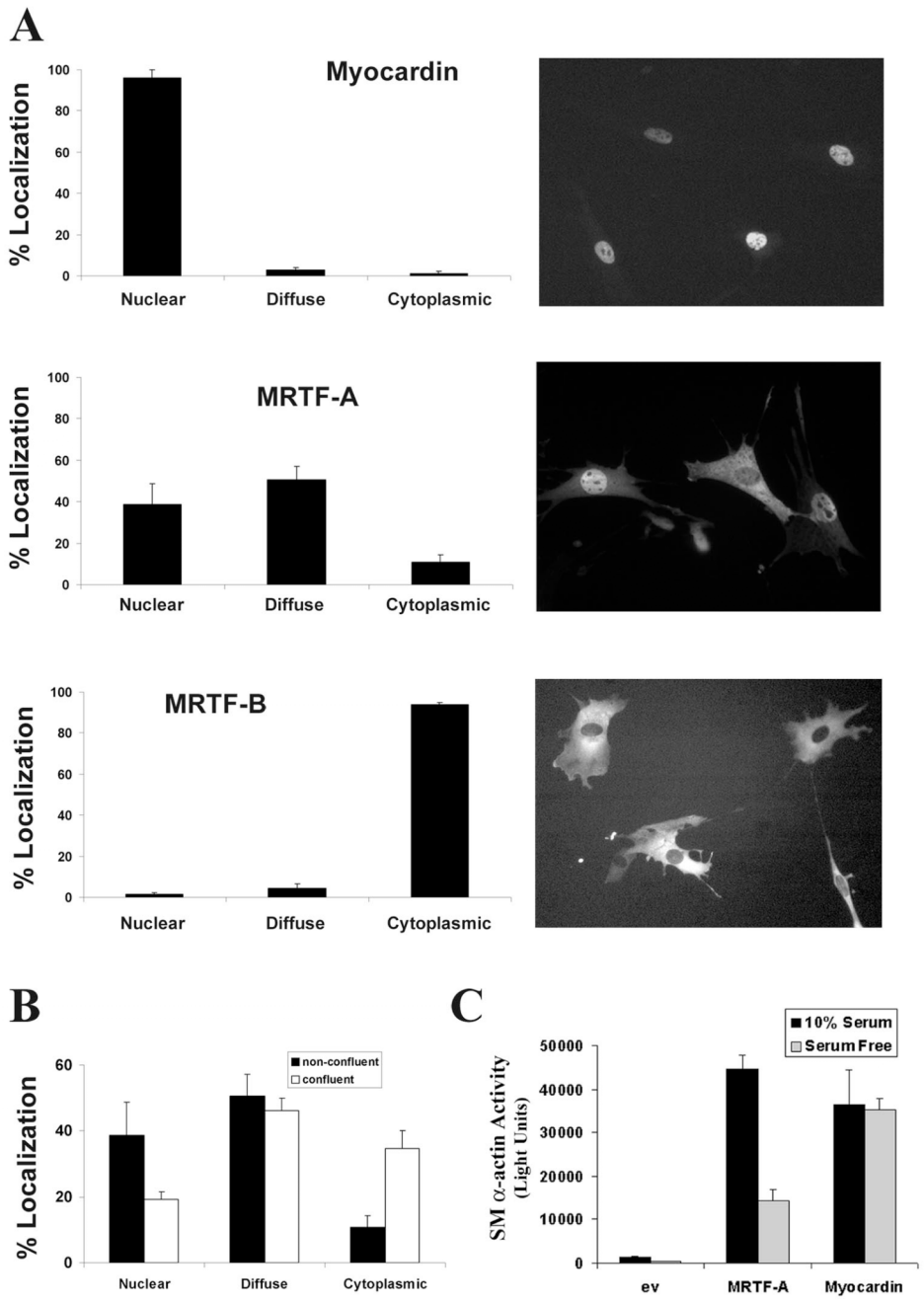

Figure 2.3. The myocardin transcription factors were differentially regulated by nuclear localization. A) All three myocardin factors were expressed as EGFP fusion proteins in 10T1/2 cells. Cells were serum-starved for 24h and then fixed in 4% paraformaldehyde. Cells were scored into three separate localization categories, nuclear, diffuse and cytoplasmic. The data represent the averages +/- SEM of three separate transfections. On the right are representative micrographs depicting typical localization patterns for each myocardin factor. **B)** EGFP-MRTF-A localization was scored in serum-starved cells that were confluent or sub-confluent (50-70%). **C)** SM α-actin promoter activity was measured in cells expressing myocardin or MRTF-A under serum containing or serum-free conditions.

significantly inhibited in confluent cells versus cells that were 50-70% confluent. Because these localization patterns correlated fairly well with the relative activities of the myocardin factors shown (figure 2.2), we tested whether serum starvation had differential effects on myocardin and MRTF-A activity. Results shown in figure 2.3C demonstrate that transactivation by myocardin was unaffected by serum withdrawal. In contrast, MRTF-A activity was reduced by approximately 70%, a result in excellent agreement with previous studies (104).

MRTF localization was regulated by specific agonists. We also monitored the localization of MRTF-A and MRTF-B in real time following treatment with several different agonists that have been shown to have variable effects on SMC differentiation marker gene expression including 10% serum, S1P, TGF-β, and PDGF-BB. In cells that contained MRTF-A in the cytoplasm, stimulation of cells with 10% serum or S1P (both strong activators of RhoA signaling) caused MRTF-A to translocate to the nucleus within a time span of about 45-60 min (Fig 2.4A). To better quantify the effects of these agonists, we fixed cells after 80 minutes and scored for localization. Results shown in figure 2.4B demonstrate that serum or S1P treatment significantly increased the number of cells that exhibited nuclear localization of the MRTFs and significantly decreased the number of cells exhibiting cytoplasmic or diffuse localization. Pre-treatment of cells with the Rho-kinase inhibitor, Y-27632, completely inhibited the effects of S1P on nuclear translocalization of MRTF-A. Somewhat surprisingly, Y-27632 did not seem to cause MRTF-A that was already nuclear (~ 20% of cells) to translocate to the cytopjlasm during the time frame of these studies suggesting that nuclear retention and/or export are regulated by a separate mechanism. Treatment of cells with TGF-β slightly increased MRTF-A nuclear localization but had no

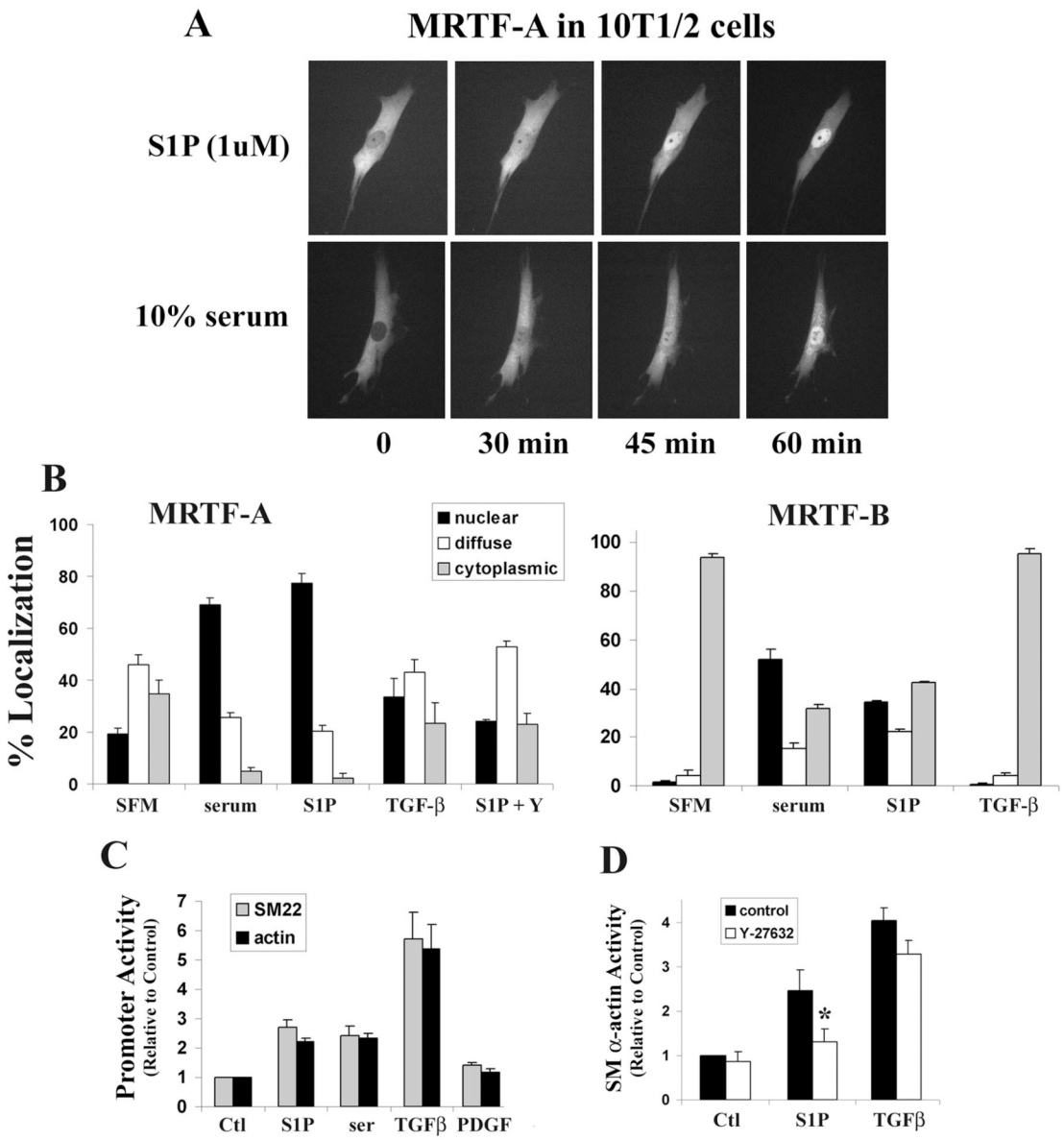

Figure 2.4. MRTF nuclear localization was regulated by specific agonists. EGFP-MRTF-A and EGFP-MRTF-B expressing 10T1/2 cells were placed in serum free media for 24h and then treated with S1P (1uM), 10% serum, TGF-β (1ng/ml), or PDGF-BB (20ng/ml). In some experiments the Rho-kinase inhibitor, Y-27632 (Y) was added 15 minutes prior to agonist addition. MRTF localization was monitored in real time using an inverted fluorescent microscope. Digital images were taken every 5 minutes for the next 80 minute, and time lapse movies were created using Isee imaging software and QuickTime (see supplemental data). **A)** Representative images from EGFP-MRTF-A expressing cells treated with 10% serum and S1P at the indicated time-points. **B)** MRTF-A and MRTF-B localization was scored in fixed cells after 80 min of treatment. **C)** SM22α and SM α-actin promoter activity was measured in cells following treatment for 24 h. Data are expressed relative to luciferase activity in untreated cells. **D)** Effects of Y-27632 on S1P and TGF-β-induced SM α-actin promoter activity. * $p<0.05$ compared to S1P only.

effect on MRTF-B. PDGF-BB had no effect on localization of either of the MRTFs. Parallel transfection experiments demonstrated that the effects of S1P, serum, and PDGF-BB on MRTF localization correlated well with their abilities to stimulate SM α-actin promoter activity in this model system (figure 2.4C). In contrast, although TGF-β had only minor effects on MRTF-A nuclear translocation at 80 min, it strongly stimulated SM α-actin promoter activity. Analysis of MRTF localization in TGF-β treated cells at 3h, 6h, and 24h demonstrated no further changes in MRTF localization.

As shown in figure 2.5, MRTF nuclear localization in SMC was slightly different. Under serum starved conditions, a significantly greater proportion of SMC contained MRTFs in the nucleus or in the diffuse pattern instead of exclusively in the cytoplasm. Also, S1P- and serum-induced nuclear translocation occurred much more quickly (~10 min vs ~60 min) (figure 2.5A).

MRTF nuclear translocation was required for SMC-specific transcription. Results presented so far indicate that MRTF-A and MRTF-B are expressed in aortic SMC, can activate SMC-specific transcription when over-expressed, and show increased nuclear localization upon stimulation with S1P or serum. To test whether nuclear localization is important for SMC differentiation marker gene expression in SMC, we used a dominant negative generated by Miralles *et al* that contains deletions to two N-terminal basic domains that were shown to be required for nuclear localization of MRTF-A (ΔB1/B2) (104). These authors showed that ΔB1/B2 MRTF-A trapped endogenous MRTF-A in the cytoplasm through dimerization, thus inhibiting its activity as a transcription factor (104). Indeed, ΔB1/B2 MRTF-A acted as a dominant negative in our model system attenuating, in a dose-dependent manner, the increase in SM α-actin activity mediated by over expression of Wt

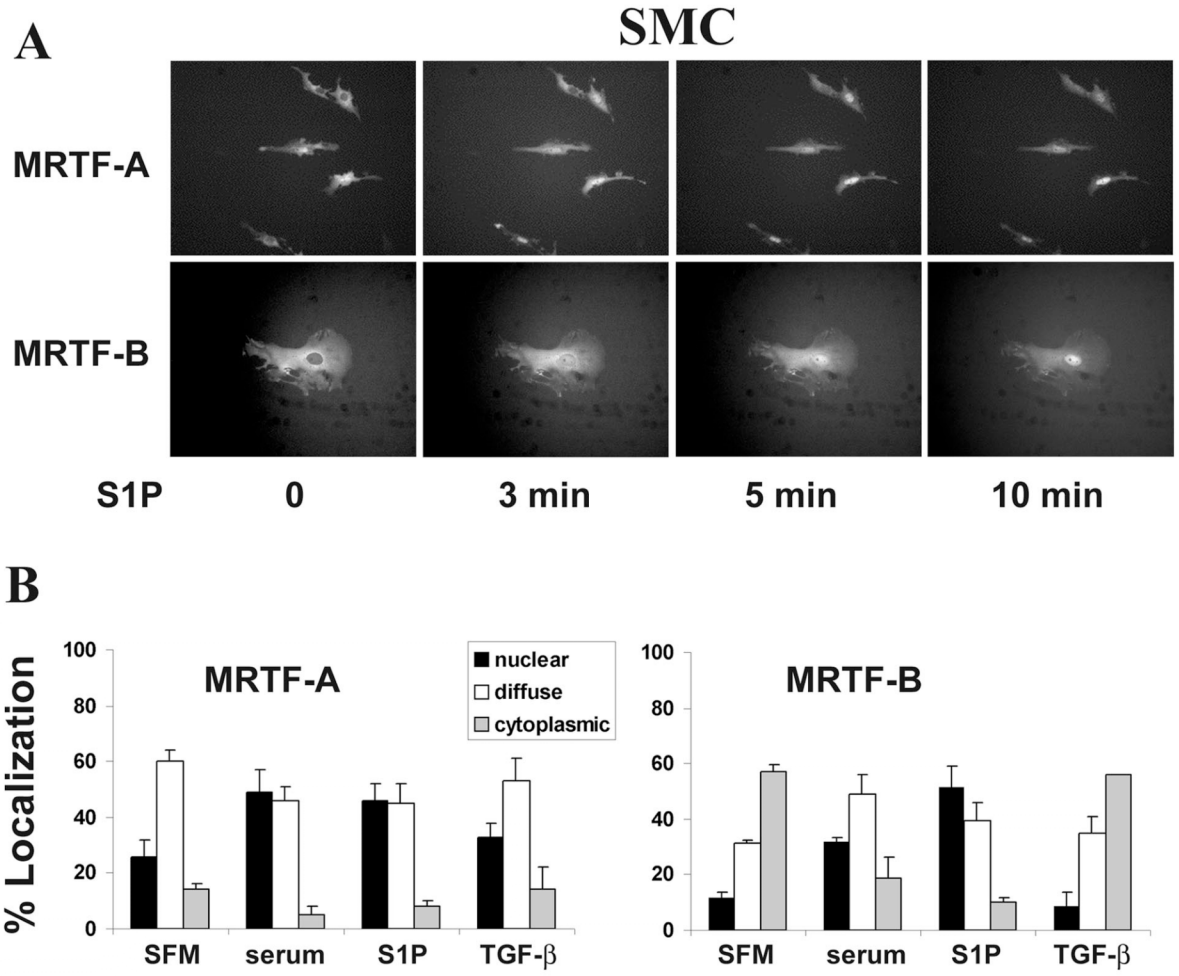

Figure 2.5. MRTF localization was regulated in SMC. EGFP-MRTF A and EGFP-MRTF-B localization was examined in treated SMC as in figure 4. **A)** Representative images of MRTF-A and MRTF-B localization at the indicated time-points in SMC treated with S1P. **B)** Scoring of MRTF localization in treated SMC.

MRTF-A (figure 2.6A). Importantly, ΔB1/B2 MRTF-A inhibited the activity of the SM22α, SM α-actin, and SM MHC promoters in SMC indicating that MRTF activity is important for regulating SMC differentiation marker gene expression (figure 2.6B). ΔB1/B2 MRTF-A also inhibited the up-regulation of SM22α and SM α-actin promoter activity in 10T1/2 cells treated with sphingosine-1-phosphate (S1P) or TGF-β (figure 2.6C,D). Interestingly, ΔB1/B2 significantly augmented activation of the c-fos promoter by S1P and TGF-β in this model (figure 2.6E). Taken together, these data indicate that nuclear translocation of the MRTFs regulates SMC-specific transcription and may serve as an important link between the extrinsic cues that regulate SMC function and the transcriptional machinery that ultimately determines SMC phenotype.

Myocardin factor nuclear localization was regulated by N-terminal-dependent and N-terminal-independent mechanisms. Results shown in Figure 2.2 demonstrate that the N-terminal actin binding domains of MRTF-A and MRTF-B inhibit their activity. Studies have shown that the N-terminal MRTF RPEL motifs bind non-polymerized G-actin to trap the MRTFs in the cytoplasm and that RhoA-dependent actin polymerization reduces the G-actin pool to release this inhibitory mechanism (126). Interestingly, although the removal of the N-terminal RPEL domains increased MRTF activity, the ΔN MRTFs (especially ΔN MRTF-B) were still less active than myocardin suggesting that additional regulatory mechanisms may affect their nuclear localization or activity. Further supporting this idea was our finding that ΔN MRTF-B did not localize exclusively to the nucleus under serum free conditions and was found in a diffuse pattern in about 50% of the cells examined.

To further identify regions in the myocardin factors that govern their nuclear localization we made a series of chimeric molecules that replaced N-terminal portions of

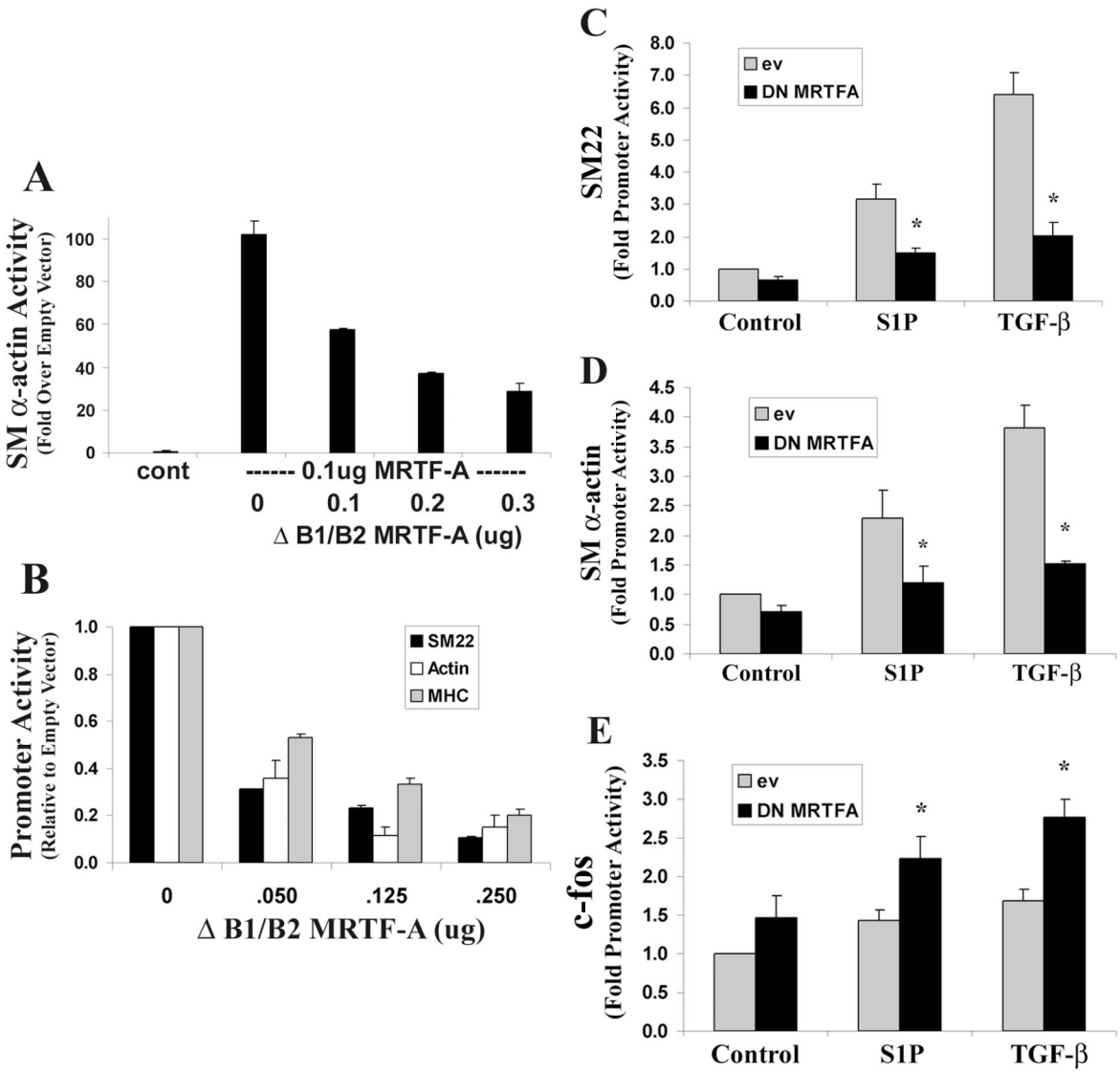

Figure 2.6. The MRTFs were required for SMC-specific promoter activity in SMC. A) Multi-potential 10T1/2 cells were co-transfected with SM α-actin/luciferase, 0.1 ug MRTF-A, and increasing concentrations of dominant negative ΔB1/B2 MRTF-A. **B)** SMC were transfected with SM22α, SM α-actin, and SM MHC luciferase reporter constructs along with increasing concentrations of ΔB1/B2 MRTF-A. At right, 10T1/2 cells were transfected with SM22α **(C)**, SM α-actin **(D)** and c-fos **(E)** promoter/luciferase constructs along with dominant negative MRTF-A. Cells were serum starved for 48 h, and then treated with either S1P (1uM) or TGF-β (1ng/ml). Luciferase assays were performed following 24 h of agonist treatment. Note that ΔB1/B2 MRTF-A inhibited the SMC-specific promoters, but increased the effects of S1P and TGF-β on c-fos. * $P<0.05$ compared to treated + empty vector (ev).

myocardin with those of MRTF-A. The myocardin used for these experiments was the 935 amino acid form that contains the entire N-terminus. Although the myocardin N-terminal region is highly homologous to that of the MRTFs, this form of myocardin is still constitutively nuclear. The chimeric constructs that were generated are depicted in Figure 2.7A along with the percentage of cells that showed nuclear localization for each chimera. Interestingly, MRTF-A sequences up to the second RPEL domain did not significantly affect localization of myocardin while replacement with an MRTF-A sequence that also included the second basic (B2) and third RPEL domains decreased nuclear localization to 57%. Another large decrease in nuclear localization (to 33%) was seen upon inclusion of an MRTF-A sequence that contained the region containing basic domain 1 (B1). Further inclusion of C-terminal MRTF-A sequences gradually reduced chimera localization to that of MRTF-A (20%).

It has been suggested that variations in myocardin that disrupt the actin-binding RPEL motif in RPEL domain 2 may be responsible for the constitutive nuclear localization of full-length myocardin. To directly test this hypothesis, we mutated the divergent sequences in RPEL2 back to a consensus RPEL domain and monitored nuclear localization and transcriptional activity of the mutated protein (see figure 2.7B). This myocardin RPEL2 mutant (MC-R2) localized to the nucleus in 100% of the cells examined and had transcriptional activity that was identical to that of Wt myocardin (data not shown).

Differential SRF binding also regulates myocardin factor activity. Our results suggest that differential nuclear localization explains, at least in part, the differences in myocardin factor activity observed in these studies. However, once in the nucleus, myocardin factor activity may also be regulated by differential binding to SRF. To analyze myocardin

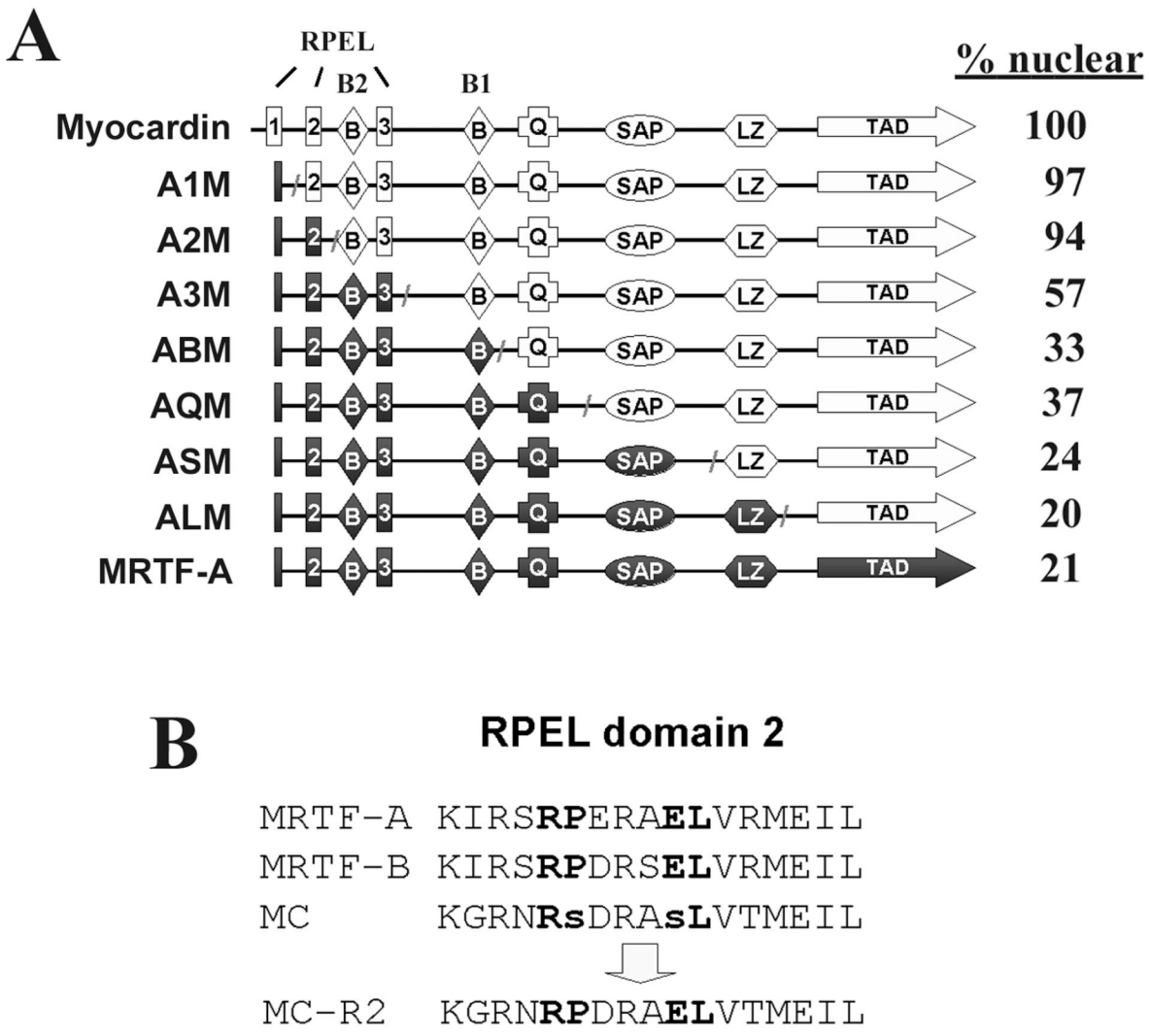

Figure 2.7. Myocardin factor localization was determined by regions in addition to those contained in the N-terminus. A) Flag-tagged MRTF-A/myocardin chimeras were created by PCR and expressed in 10T1/2 cells. Following serum starvation for 24h, cells were fixed and scored for chimera localization using immunohistochemical detection of the Flag epitope. Myocardin sequence is depicted in white while MRTF sequence is depicted in gray. The percentage of cells showing nuclear localization is shown on the right. **B)** Conservation in RPEL domain 2 between myocardin family members and the double mutation to myocardin (S72P, S76E) that restored the RPEL motif (MC-R2).

factor binding to SRF we performed electromobility shift assays. We also wanted to test whether variations in SRF binding contributed to interactions with specific myocardin factors. Thus, we used the three SM α-actin CArG elements (A, B, and intronic) that have dramatically different abilities to bind SRF due to G/C substitutions in their A/T rich regions. It is important to note that we used the ΔN versions of the MRTFs in this assay because actin binding to the full length MRTFs interferes with ternary complex formation in gel shift assays (104, 142)and data not shown). Results shown in figure 2.8 demonstrate that SRF binds to all three CArGs with varying affinity (intronic>B>>A). All three myocardin factors formed higher order complexes on the intronic CArG and could be super-shifted with anti-flag antibody (lanes 13-15). However, even though these SRF co-factors were present at equal amounts (see inset), myocardin binding to SRF was stronger than ΔN MRTF-A and much stronger than ΔN-MRTF-B. There were also differences in complex formation that were CArG-specific. For example, the intensity of the MRTF-A containing ternary complex (relative to SRF binding alone) was much less for the weaker CArGs than for the strong intronic CArG, a difference that was not apparent with myocardin (compare lane 7 to 11 and 6 to 10).

DISCUSSION

Extensive evidence indicates that myocardin is an important regulator of SMC differentiation. While the role played by the MRTFs is less clear, several lines of evidence from the present study support their involvement in regulating SMC phenotype. First, both MRTFs are expressed strongly in isolated SMC and in a variety of adult mouse tissues that contain a significant SMC component. While these studies obviously lack sufficient

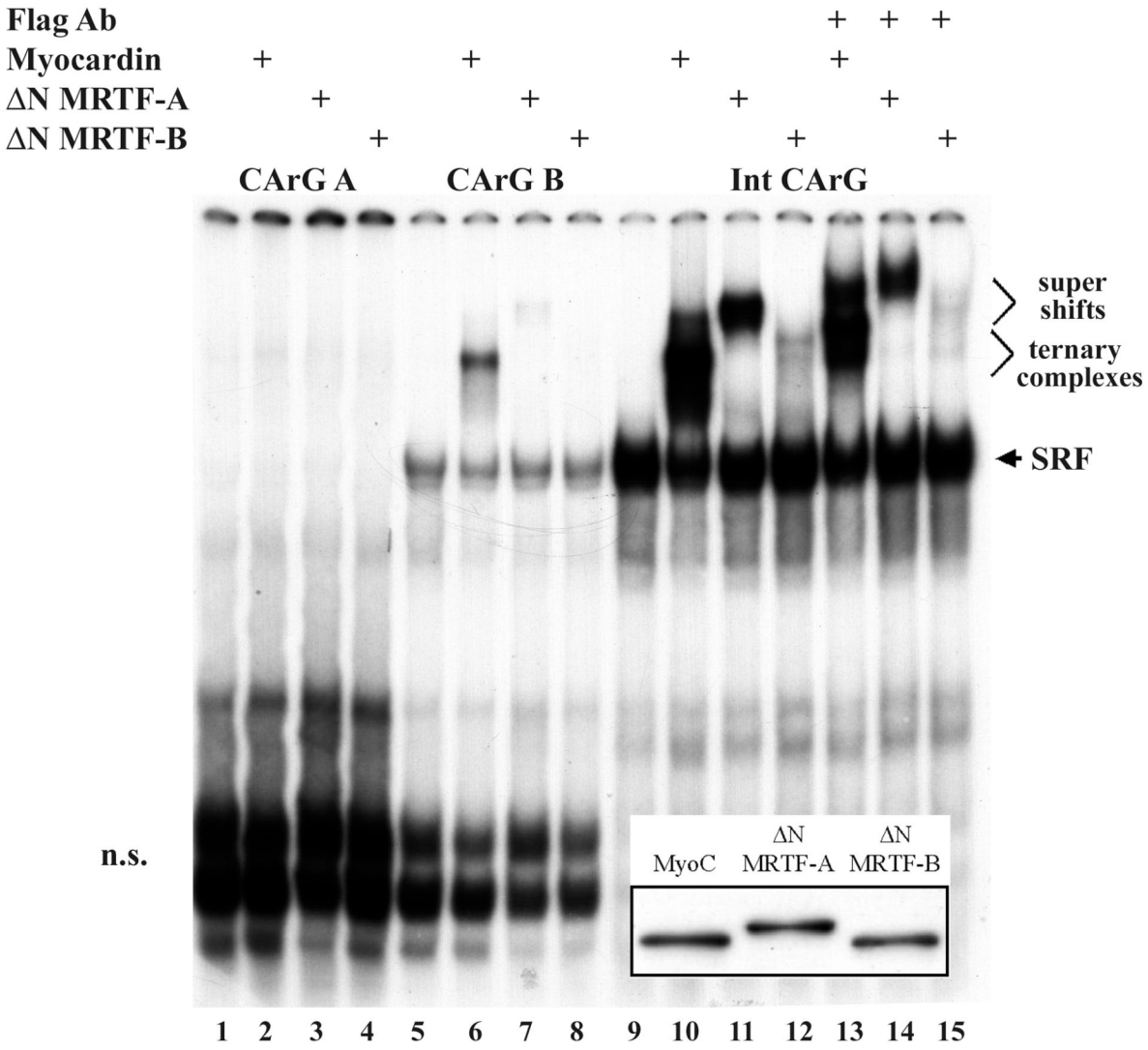

Figure 2.8. SRF binding by the MRTFs was relatively weak and dependent upon CArG sequence. Gel shift analysis was performed by incubating SRF, flag-tagged myocardin, MRTF-A, or MRTF-B (all in vitro translated) with radiolabeled CArGA, CArGB, or intronic CArG shift probes. After incubation for 30 min, samples were loaded on a 4 % nondenaturing polyacrylamide gel and electrophoresed at 170V for 4 hrs. For supershift analysis (lanes 13-15), flag antibody was added 10 min before gel loading. The SRF, ternary complex, and supershift bands are labeled at right. Note that all three myocardin factors were present at equal amounts (see inset) and that shorter gel runs show equal amounts of free probe for each CArG element (data not shown). n.s. - non-specific band.

resolution to determine whether the SMC within these organs express MRTF-A or MRTF-B, our results support a more in depth examination of MRTF expression by in situ or immunohistochemistry. Second, both MRTFs up-regulated SMC-specific promoter activity as well as endogenous SMC differentiation marker gene expression in 10T1/2 cells. 10T1/2 cells do not express myocardin (189) indicating that either of the MRTFs was sufficient for this response. Third, a dominant negative form of MRTF-A (ΔB1/B2) significantly attenuated SMC-specific transcription in SMC. We and others have previously used dominant negative myocardin family variants that lack the transactivation domain to inhibit SMC-specific promoter activity. Importantly, however, these variants inhibit all three of the myocardin factors making it difficult to interpret experiments in SMC that express multiple members of this family (35). In the present study we used ΔB1/B2 MRTF-A to trap the endogenous MRTFs in the cytoplasm without affecting myocardin which is constitutively nuclear (175). Taken together, these results indicate that the MRTFs regulate SMC-specific transcription in SMC, perhaps in concert with myocardin. The observations that myocardin and MRTF-A can associate directly though conserved leucine zipper motifs and that these motifs are required for full activity of these transcription factors supports this idea (35, 179).

The phenotypes of the myocardin family member knockouts indicate that each member has essential non-redundant functions in the regulation of SMC differentiation marker gene expression (78, 81, 82, 117, 156). The early lethality associated with the myocardin and MRTF-B knockouts as well as the potential redundancy between these very similar transcription factors has made it difficult to determine whether the MRTFs are important for regulating SMC differentiation in the SMC sub-types where they are expressed. It is also unclear whether MRTFs are required for other aspects of SMC function that do not

directly involve specification such as the changes in gene expression that are known to occur during environmental stresses such as hypertension and atherosclerosis. Based upon the mammary myoepithelial defect observed in MRTF-A knock-out mice, the MRTFs may be responsible for the up-regulation of SMC marker gene expression that is observed in many SMC-like cells (i.e. myofibroblasts or mesangial cells) following injury (68, 177).

Given the importance of the myocardin family for regulating SMC-specific transcription, the identification of the signaling mechanisms that regulate the expression and/or activities of these transcription factors will be very important to our understanding of the regulation of SMC phenotype. In our studies, agonist-induced up-regulation of SMC-specific transcription did not correlate with increased expression of any of the myocardin factors ((88) and data not shown). Instead, our data indicated that RhoA-dependent regulation of MRTF nuclear localization was important and involved cytoplasmic retention of the MRTFs by a mechanism involving G-actin binding to the MRTF N-terminus. Results obtained with the MRTF-A/myocardin chimeras and the myocardin RPEL2 domain mutant suggest that other regions are also important including basic region 1 (B1) and perhaps B2. These regions have already been implicated in nuclear import, and it is likely that a delicate balance exists between these nuclear localization and cytoplasmic retention signals.

Since a number of extrinsic cues that regulate SMC differentiation also regulate RhoA activity, these studies indicate that regulation of MRTF nuclear localization may be an important mechanism by which environmental factors regulate SMC phenotype. The observation that ΔB1/B2 MRTF-A inhibited the induction of the SM22α and SM α-actin promoters by S1P and serum supports a role for MRTF nuclear translocation in this response, as does the direct correlation between the effects of S1P, serum, and PDGF-BB on promoter

activity and nuclear translocation of the MRTFs. Interestingly, ΔB1/B2 MRTF-A actually increased c-fos promoter activity in S1P and TGF-β treated cells providing additional evidence that the myocardin factors differentially regulate SRF-dependent growth and SRF-dependent differentiation. Wang *et al* have shown that myocardin and the ternary complex factors compete for SRF binding and the positive effects of ΔB1/B2 MRTF-A on the c-fos promoter would be consistent with this model (178).

TGF-β was the strongest activator of SMC-specific transcription in these studies and has been shown to activate RhoA in some cell culture model systems (5, 24). The inhibitory effect of ΔB1/B2 MRTF-A on this response indicated that a basal level MRTF activity is required for TGF-β-induced up-regulation of SMC-specific promoter activity. It is also possible that our inability to detect partial changes in MRTF localization following TGF-β treatment could explain these results. However, the observations that TGF-β had only minor effects on MRTF localization and that Y-27632 had little to no effect on TGF-β-induced SM α-actin promoter activity suggest that non RhoA-dependent mechanisms are more important. TGF-β's effects on SMC-specific transcription are thought to be mediated by activation of the SMADs (SMADs 2 and 3 in particular) (129, 146, 149). Our data would indicate while both pathways are essential for SMC differentiation marker gene expression, they probably act, at least to some extent, in parallel. Interestingly, Chen *et al* demonstrated in Monc-1 cells that Y-27632 inhibited TGF-β-induced SMC-specific gene expression and that dominant negative RhoA inhibited SMAD 2 and SMAD 3 nuclear localization (24). This same study showed that inhibition of RhoA in 10T1/2 cells (by DN-RhoA and C3 exotoxin) yielded more modest results suggesting that there are probably cell-type-specific differences in the importance of RhoA on TGF-β signaling. Two recent studies have shown that

myocardin interacts directly with SMAD3 and SMAD1 to regulate transcription in smooth and cardiac muscle, respectively (13, 149). Therefore, it will be very important to further identify the mechanisms that integrate the RhoA and TGF-β signaling pathways.

Because many environmental cues are known to affect RhoA activity (see (51) for a review), the regulation of MRTF localization is probably complicated by many cell-type-specific and microenvironmental differences. Our observations that basal MRTF nuclear localization was higher in SMC than in 10T1/2 cells and that nuclear translocation occurred more quickly in SMC in response to treatment supports this idea. While our data suggest that cell confluency (a signal that inhibits RhoA signaling) attenuated MRTF nuclear localization, others have shown that application of cell tension (a signal that stimulates RhoA) promoted nuclear localization of drosophila MRTF (52, 151). The later findings suggest that not only is the MRTF-SRF interaction highly conserved throughout evolution, but that the regulation of MRTF translocation by RhoA may be as well. It is also possible that the contrasting results on MRTF localization that have been reported (35, 142) may reflect inherent differences in RhoA signaling between cell-types.

Our gel shift assays demonstrated that the transcriptional activities of the myocardin factors are also regulated by their ability to interact with SRF. Even after removal of the inhibitory the N-terminal region, MRTF-SRF complexes were still much weaker than those formed with myocardin. As with nuclear localization, these differences correlated well with the relative activities of the myocardin factors suggesting that this parameter is also an important determinant of myocardin factor activity. Another interesting finding of our studies was that, relative to SRF binding, MRTF-A formed a more robust ternary complex at the intronic CArG than at CArGs A or B which was not the case for myocardin. Little is known

about the mechanisms by which the myocardin factors discriminate between SRF bound to the large number of CArG-dependent gene promoters, but these data suggest that variations in CArG sequence may be an important determinant. MRTF-A has been shown to preferentially interact with SRF at the vinculin and SRF promoters but not with SRF at the c-fos or egr-1 promoters (104). While this difference may be due to known competition for SRF binding between the Elk-1 and the myocardin factors, slight differences in SRF conformation (or perhaps DNA bending) due to variations in CArG sequence may also play a role. In support of this, Zaromytidou *et al* have recently shown that MRTF-A co-immunoprecipitated with SRF more strongly in the presence of a consensus CArG oligonucleotide than in the presence of a CArG that contained substitutions to the AT rich region (190). It is clear that a better understanding of interactions between the different myocardin family members and specific SRF-CArG complexes will be important for delineating the role of the myocardin family in both cell-type-specific and gene-specific transcriptional regulation.

In summary, results from the present study indicate that the MRTFs play an important role in the regulation of SMC differentiation marker gene expression in at least some SMC sub-types. Unlike myocardin, MRTF nuclear localization, and hence MRTF transcriptional activity, is regulated by agonists that activate RhoA, and we feel that this signaling pathway may be an important mechanism by which a variety of environmental cues regulate SMC-specific transcription. Since SMC are known to maintain a significant level of plasticity even in adult animals, perhaps signaling mechanisms such as these have evolved as a mechanism by which SMC can quickly and reversibly alter their phenotype in response to environmental cues.

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REGULATION OF MYOCARDIN FACTOR PROTEIN STABILITY BY THE LIM-ONLY PROTEIN, FHL2

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ABSTRACT

Extensive evidence indicates that serum response factor (SRF) regulates muscle-specific gene expression and that the myocardin family SRF co-factors are critical for smooth muscle cell (SMC) differentiation. In a yeast-two-hybrid screen for novel SRF binding partners expressed in aortic SMC, we identified Four and a Half LIM domain protein 2 (FHL2) and confirmed this interaction by GST pull-down and co-immunoprecipitation assays. FHL2 also interacted with all three myocardin factors and enhanced myocardin and MRTF-A-dependent transactivation of the SM α-actin, SM22, and cardiac ANF promoters in 10T1/2 cells. Expression of FHL2 increased myocardin and MRTF-A protein levels, and importantly, this effect was due to an increase in protein stability not due to an increase in myocardin factor mRNA expression. Treatment of cells with the proteasome inhibitors, MG132 and lactacystin, strongly up-regulated endogenous MRTF-A protein levels and resulted in a substantial increase in ubiquitin immunoreactivity in MRTF-A immunoprecipitants.

Interestingly, expression of FHL2 attenuated the effects of RhoA and MRTF-B on promoter activity perhaps through decreased MRTF-B nuclear localization or decreased SRF-CArG binding. Taken together, these data indicate that the myocardin factors are regulated by proteasome-mediated degradation and that FHL2 regulates SRF-dependent transcription by multiple mechanisms including stabilization of myocardin and MRTF-A.

INTRODUCTION

The MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor, serum response factor (SRF), regulates cell-type-specific gene expression in cardiac, skeletal, and smooth muscle by binding to CArG elements present in most of the muscle differentiation marker gene promoters. Although SRF is highly expressed in all three muscle cell-types, it is a ubiquitously expressed factor that also regulates several early response growth genes including c-fos and egr-1 (11, 21, 152, 167, 173). Extensive evidence indicates that SRF activity is regulated mainly by its physical interaction with additional general and cell-type-specific transcription factors. The first SRF co-factors identified were the ternary complex factors (Elk-1, Sap-1, SAP-2/NET/ERP) that bind to SRF as well as to the Ets domain adjacent to the c-fos CArG following their phosphorylation by MAP kinase (see (12) for review). SRF has also been shown to interact with cell-type specific factors such as MyoD and GATA-4 to regulate skeletal and cardiac muscle-specific gene expression, respectively (120, 144).

The discovery of myocardin was a major advance in our understanding of the mechanisms that regulate SMC differentiation. This SRF co-factor is specifically expressed in cardiac and smooth muscle, powerfully stimulates SRF-dependent transcription in a

variety of cell-types, and is critical for SMC differentiation *in vivo* (23, 82, 175). Two Myocardin-Related Transcription Factors, MRTF-A and MRTF-B were also described that have similar activities to that of myocardin (176). Although the MRTFs are expressed more widely, recent results in knock-out mice indicate that MRTF-B is required for SMC differentiation of cardiac neural crest cells while MRTF-A is required for SMC differentiation marker gene expression that normally occurs in the myoepithelial layer of the mammary gland during lactation (78, 81, 117, 156). Interestingly, the myocardin factors are differentially regulated by sub-cellular localization, and our lab and others have shown that RhoA-dependent nuclear translocalization of the MRTFs is an important mechanism by which some extrinsic factors stimulate SMC-specific gene expression (35, 58, 88, 104).

To identify additional factors involved in the regulation of SRF-dependent SMC-specific transcription, we conducted a yeast-two-hybrid screen of a human aortic library using an amino terminal version of SRF (aa 1-201) as bait. Three of the clones identified coded for Four and a Half LIM domain-containing protein 2 (FHL2), a LIM-only protein that has been shown to be selectively expressed in the heart and SMC during development (26) (67) (158) and that functions as a transcriptional co-activator or co-repressor for a variety of transcription factors including the androgen receptor, cAMP-responsive element binding protein, AP-1, FOXO1, E4F1, and β-catenin (see (67) for review). Since FHL2 does not bind DNA directly, these effects are thought to be mediated by FHL2's ability to facilitate protein-protein interactions through its multiple LIM domains.

In addition to its expression pattern during development, several features of FHL2 function led us to examine its role in regulating SMC- and cardiac-specific transcription. First, Müller *et al* demonstrated that, like the MRTFs, FHL2 nuclear localization and

transactivation were dependent upon RhoA signaling (110). Second, Philippar *et al* used a genetic screen in SRF -/- ES cells to identify FHL2 as an SRF target gene whose up-regulation correlated with increased SMC-specific gene expression in an ES cell model of SMC differentiation (124). These authors demonstrated that FHL2 interacted physically with SRF and that over-expression of FHL2 inhibited RhoA-dependent activation of SM22. Finally, Chang *et al* demonstrated that the SMC-specific LIM only proteins, CRP1 and CRP2, stimulated SMC-specific transcription by facilitating SRF's interaction with GATA factors (18, 19).

Our results confirm that FHL2 interacts with SRF, but we also demonstrate that FHL2 binds directly to all three myocardin factors and has differential effects on their abilities to regulate cardiac and SMC-specific transcription. Importantly, FHL2 increased myocardin and MRTF-A transactivation and this effect may be due to protection of these factors from proteasomal degradation.

MATERIALS AND METHODS

Yeast 2-hybrid screen and plasmid construction - FHL2 was identified in a Matchmaker yeast two hybrid screen (Clontech) using the SRF N-terminus (aa 1-201) as bait. Full length FHL2 was subcloned into flag-pcDNA3 and PGEX4T1 vectors. Expression constructs for SRF, L63RhoA, Myocardin, MRTF-A, MRTF-B, and myocardin factor derivatives have been previously described (58, 88). The GST fusions, FHL2 0-2 and FHL2 3/4, were generous gifts from Roland Schüle (University of Freiburg, Freiburg, Germany) and have been previously described (109).

Cell Culture, Transient Transfections, and Reporter Assays – The rat aortic SMC and 10T1/2 cultures and the SMC-specific promoter assays used have been previously described (88). In brief, cells were maintained in 10% serum media and transfected 24 h after plating at 70-80% confluency using LT-1 transfection reagent (Mirus) per protocol. Luciferase activity measurements were made 24 h post transfection. Luciferase activity was measured in relative light units (RLUs) and expressed as fold activity over empty vector. For harvesting of protein, cells were plated in 15cm dishes and lysed in RIPA + 0.5% triton.

GST fusion pull-downs and co-immunoprecipitations - GST pull-down assays and co-immunoprecipitations were performed as previously described (157). In brief, GST fusion proteins were purified from bacterial lysates using glutathione sepharose (Amersham Biosciences). Interacting proteins were *in vitro* translated and 35S labeled using the Promega TnT kit. Interacting complexes were pelleted by centrifugation and washed 2X in NETN solution and 1X in cold Tris-buffered saline. For co-immunoprecipitations flag-FHL2 was expressed in 10T1/2 cells and immunoprecipitations were performed using anti-flag or anti-SRF Abs.

Semi-quantitative PCR - RNA was prepared from cell and tissue lysates using Trizol Reagent (Invitrogen) and was quantified by Ribogreen Assay (Molecular Probes). cDNA was generated from 1ug of RNA using in the iScript cDNA synthesis kit (Biorad). Exon spanning primers were used to amplify FHL2, myocardin, MRTF-A, MRTF-B and GAPDH (sequences available upon request).

Electrophoretic Mobility Shift Assays - FHL2, SRF and myocardin factors were *in vitro* translated using the Promega TnT kit. Binding reactions contained 5ul of total TnT lysate, a 32P-labeled SM α-actin Intronic CArG probe (20,000 cpm), and 0.25 μg dI·dC in

binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol). Reactions were incubated for 30 m before loading on a nondenaturing 5% polyacrylamide gel.

Sub-cellular Localization Studies – 10T 1/2 cells were transfected with EGFP-MRTF-B +/- FHL2. After serum starvation, cells were fixed in 4% paraformaldehyde and EGFP-MRTF-B localization was then scored into 3 categories: nuclear, cytoplasmic, and diffuse.

FHL2 knockdown - The following siRNAs were ordered from Invitrogen: Control (NTC) - ugguuuacaugucgacuaa, FHL2 - gcaaggacuuguccuacaa. siRNAs were transfected into primary rat aortic SMC using dharmafect reagent 1 (Dharmacon) per manufacturer's protocol. FHL2 protein expression in SMC RIPA lysates was measured by Western Blot using an anti-FHL2 Ab (Santa Cruz). For promoter-luciferase assays, transfections were performed 24 hrs after oligo introduction.

Detection of ubiquitinated MRTF-A – Myocardin factor expressing or non-expressing 10T1/2 cells were treated for 12 h with MG132 (20uM), lactacystin (10uM), or DMSO vehicle. RIPA lysates were run on an SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-flag, anti-tubulin, or anti-MRTF-A Abs. MRTF-A was immunoprecipitated from MG132-treated cells using a monoclonal hamster anti-MRTF-A Ab coupled to protein G beads (Sigma). Western Blots were performed on immunoprecipitants using the P4G7 anti-ubiquitin antibody (Covance).

Determination of MRTF-A half-life – 10T1/2 cells +/- FHL2 were treated with cycloheximide (50ug/ml) or vehicle. Endogenous MRTF-A expression was measured by Western Blot at 0, 0.5, 3, and 6 h. Similar experiments were done in Cos cells expressing flag-MRTF-A and in 10T1/2 cells treated with MG132.

RESULTS

FHL2 interacted with SRF. To identify novel members of the transcriptional complex required for expression of SMC differentiation marker genes, we screened a human aortic yeast-two-hybrid library using the N-terminal third of SRF (aa 1-201) as bait. This SRF fragment included the inhibitory N-terminal region, nuclear localization signal, and MADS box motifs αI (aa 153-179), βI (aa 182-188), and βII (aa 194-198) (see figure 2.1A). Three of the fifty interacting clones coded for the LIM-only protein FHL2, and a series of secondary yeast screens were performed to eliminate the possibility that the FHL2-Gal4 activation domain fusion construct activated yeast reporter genes on its own. Using co-immunoprecipitation assays and GST fusion pull-downs we observed a consistent, but relatively weak interaction between FHL2 and SRF (Figs 3.1b and 3.1c). These data confirm the observation by Philippar *et al* that FHL2 interacts with SRF (124).

FHL2 was strongly expressed in SMC and SMC-containing tissues. FHL2 belongs to a subclass of LIM-only proteins that is characterized by four full LIM domains and one half LIM domain at the amino termini separated by short linker peptides. To date, six members of this family (FHL1-5 and ACT) have been identified that have varying expression patterns (26, 41). FHL2 was originally thought to be specifically expressed in the myocardium, but examination of mice containing LacZ knocked into the endogenous FHL2

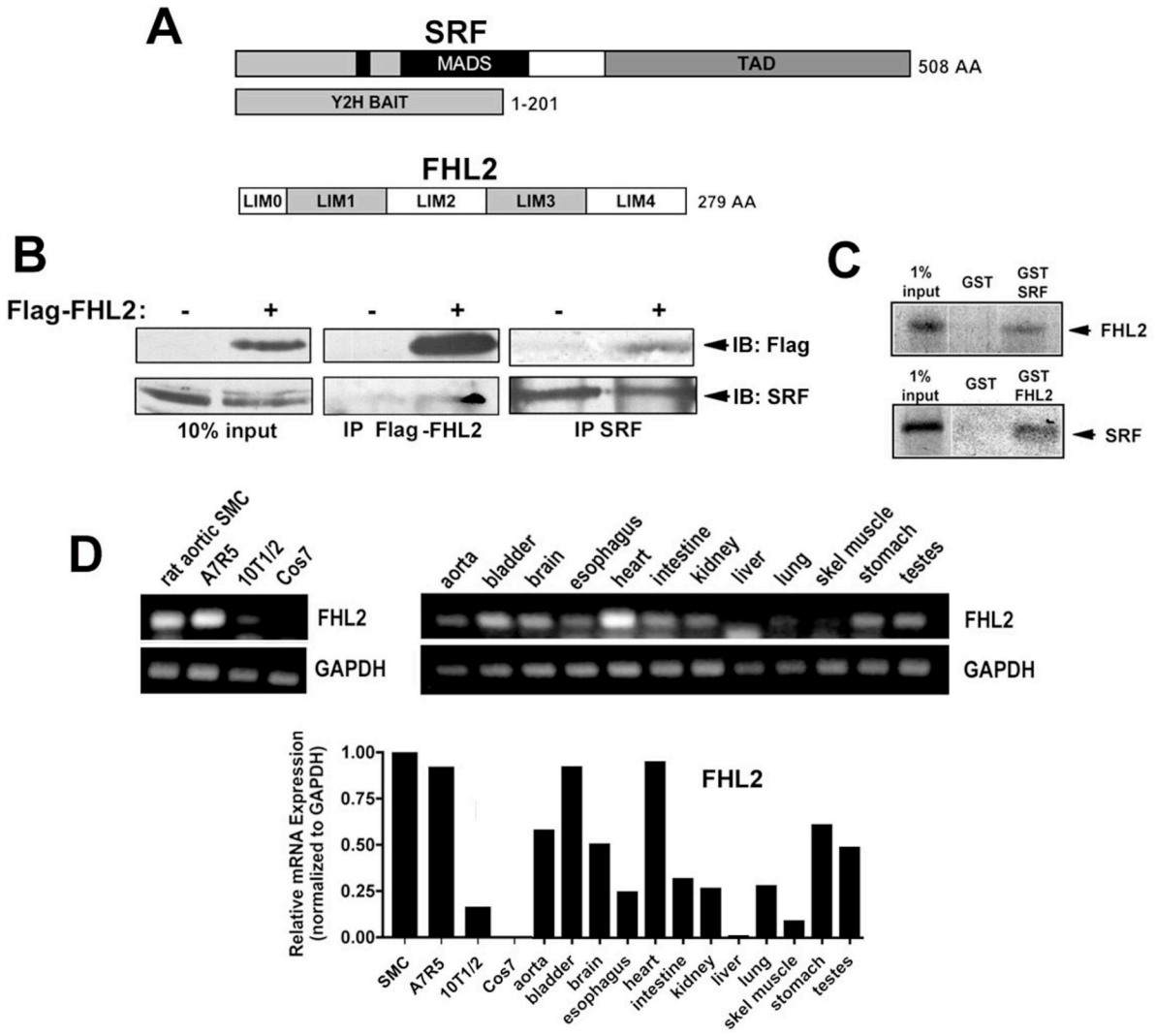

Figure 3.1. **FHL2 interacted with SRF and was strongly expressed in cardiac and SMC. A)** Schematics of the SRF bait used in the two-hybrid screen and FHL2. **B)** SRF and flag-FHL2 were immunoprecipitated from 10T1/2 lysates. Immunoprecipitants were washed, subjected to SDS-PAGE, and immunoblotted with anti-flag and anti SRF antibodies. **C)** Pull-down assays were performed using the indicated GST fusions and *in vitro* translated, ^{35}S-labeled proteins. **D)** Semi-quantitative RT-PCRs for FHL2 were performed on mRNA isolated from the indicated cultured cells and mouse tissues. Band intensities were quantified using ImageJ software and are expressed relative to GAPDH.

locus revealed high expression of FHL2 in the developing vasculature (26). To specifically examine FHL2 expression in adult smooth muscle, we used semi-quantitative RT-PCR (Fig 3.1d). As expected, FHL2 message was highest in the heart, but significant levels were also detected in tissues that contain a large SMC component including aorta, bladder, esophagus, and stomach. In addition, FHL2 expression was very high in primary rat aortic and A7r5 SMC, weak in multipotential 10T1/2 cells, and absent in cos-7 cells. FHL1, which has also been shown to be expressed in the developing heart and outflow tract, was expressed in a similar pattern (data not shown).

FHL2 interacted directly with all three myocardin family members. Based upon previous studies demonstrating that the CRP LIM only proteins facilitated the formation of an SRF/GATA6 complex and that FHL2's transcriptional activity, like that of MRTF-A and MRTF-B, was regulated by RhoA, we postulated that FHL2 may interact with the myocardin factors. As shown in figure 3.2a GST-FHL2 strongly precipitated *in vitro* translated myocardin, MRTF-A, and MRTF-B. Further mapping studies suggested that full length FHL2 was required for strong interactions with myocardin and MRTF-A, while an FHL2 fragment containing only the N-terminal 2 and 1/2 LIM domains (0-2) could mediate binding with MRTF-B (Fig 3.2b). In addition, endogenous MRTF-A co-immunoprecipitated with flag-FHL2 expressed in 10T1/2 cells, further suggesting that these proteins interact *in vivo* (Fig 3.2c).

We also mapped the domains of the myocardin factors that interacted with FHL2. As shown in figure 3.2d-f, FHL2 bound most strongly to myocardin, MRTF-A, and MRTF-B through an N-terminal fragment that contained the RPEL motifs, the two basic domains, and the Q-rich region. Interestingly, FHL2 bound fairly strongly to a myocardin fragment that

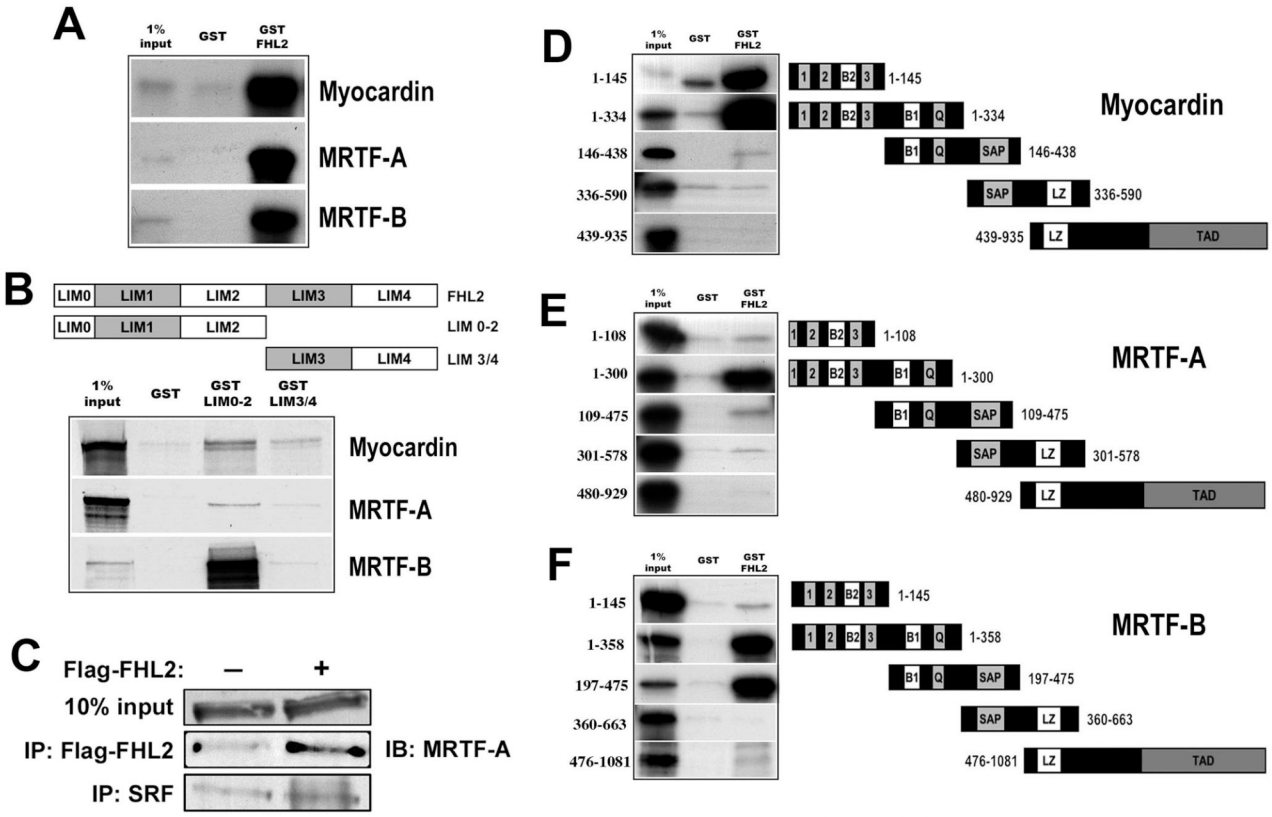

Figure 3.2. FHL2 interacted physically with the myocardin factors. **A)** GST or GST-FHL2 were incubated with in vitro translated, 35S labeled myocardin, MRTF-A, or MRTF-B. Complexes were pelleted, washed 3X, ran on an SDS page gel, and exposed to film. **B)** FHL2 truncations containing the first 21/2 LIM domains (0-2) or the last two LIM domains (3/4) were fused to GST and used to precipitate the myocardin factors in similar reactions. **C)** 10T1/2 cells were transfected with flag-FHL2 or empty expression vector. 48 h post-transfection RIPA lystates were immunoprecipitated with anti-flag or anti-SRF antibodies. Immunoprecipitants were then probed using a monoclonal anti-MRTF-A Ab. Note that expression of FHL2 had little effect on the interaction between SRF and MRTF-A. **D-F**) Several myocardin factor deletions were generated and used in pull down assays with GST and GST-FHL2.

contained only the RPEL motifs and basic domain 2 (Fig 3.2d) and to an MRTF-B fragment that contained only basic domain 1, the Q-rich region, and the SAP domain (Fig 3.2f). Taken together, these results suggest that the region near basic domain 1 is probably the most important for FHL2 binding. The slight differences in FHL2 binding to the myocardin factors were somewhat surprising and suggested that FHL2 may have differential effects on the myocardin factors.

FHL2 enhanced myocardin and MRTF-A transactivation. To test whether FHL2 had functional effects on myocardin factor activity we co-expressed FHL2 with each of the myocardin factors along with the SMα-actin and SM22 promoters in multipotential 10T1/2 cells. In these experiments we transfected sub-maximal levels of myocardin, MRTF-A, and MRTF-B that activated these promoters by approximately 60, 45 and 10 fold, respectively (data not shown). As shown in figure 3.3, co-expression of FHL2 dose-dependently increased myocardin's ability to stimulate SMC-specific promoter activity. In stark contrast, FHL2 inhibited transactivation by MRTF-B, and slightly stimulated transactivation by MRTF-A but only at high concentrations. Because FHL2 expression is the highest in the myocardium, we also examined the effects of FHL2 on the cardiac-specific atrial natriuretic factor (ANF) promoter. Similar to effects observed with the SMC-specific promoters, FHL2 increased transactivation of ANF by myocardin and MRTF-A. We initially hypothesized that FHL2 increased myocardin and MRTF-A activity by facilitating their interactions with SRF. However, expression of FHL2 had no effect on the association of SRF and MRTF-A as measured by co-immunoprecipitation of proteins from 10T1/2 lysates (Fig 3.2c) and did not increase ternary complex formation between SRF and the myocardin factors in gel shift assays (see Fig 3.6, below).

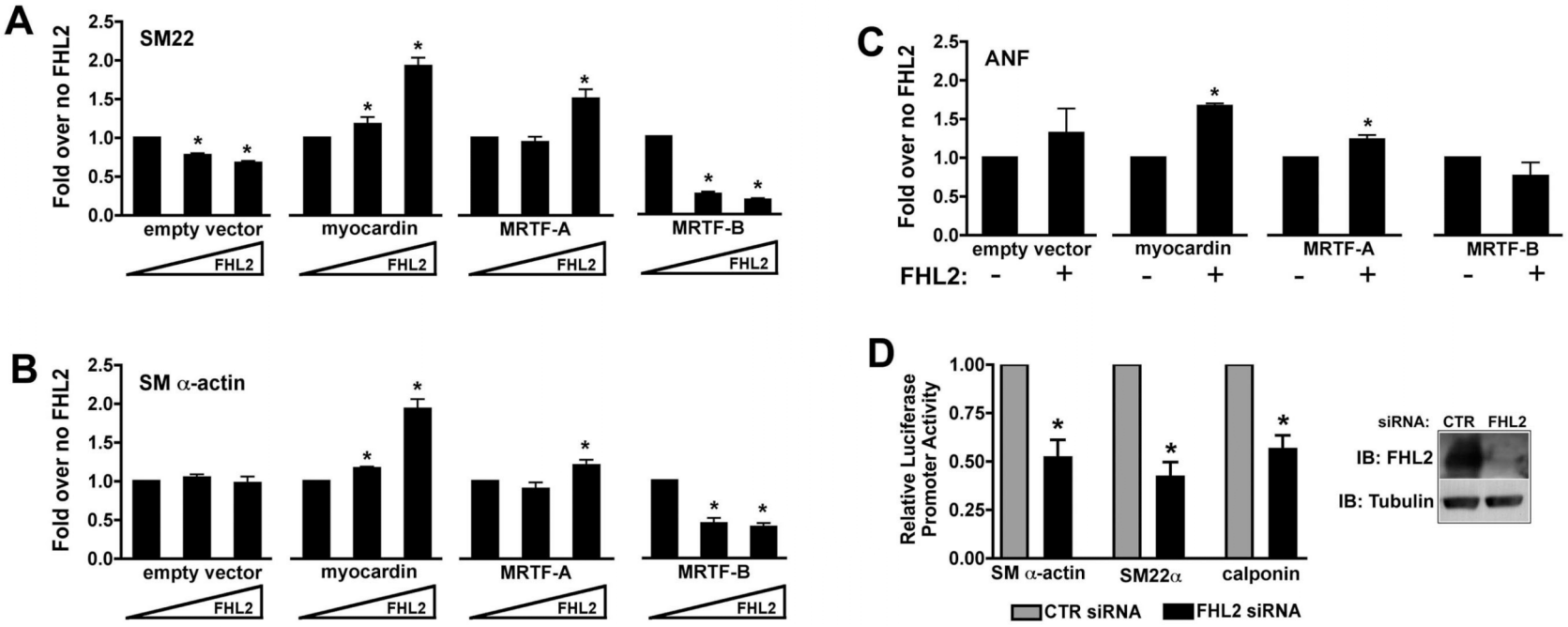

Figure 3.3. FHL2 differentially regulated myocardin factor transactivation. SM α-actin **(A),** SM22 **(B),** or atrial natriuretic factor **(C)** promoter-luciferase constructs were co-transfected into 10T1/2 cells along with one of the myocardin factors and increasing concentrations of FHL2. The total amount of expression plasmid in each transfection was equalized by addition of empty expression vector. Cells were maintained in 10% serum media and assayed for luciferase activity at 24h. Results are expressed relative to promoter activity in the presence of the indicated myocardin factor but without FHL co-expression. * $p \leq 0.05$ versus minus FHL2. **D)** Following siRNA-mediated knockdown of FHL2 expression in SMC (right panel), cells were transfected with the indicated luciferase reporter constructs, and luciferase activity was measured at 48h. * $p \leq 0.05$ versus control siRNA.

To further explore the role of FHL2 in SMC differentiation marker gene expression, we used siRNA to knock down FHL2 expression in primary rat aortic SMC. As shown in figure 3.3d, knockdown of FHL2 expression by 90% led to an approximately 50% decrease in the activities of the SM α-actin, SM22, and calponin promoters.

FHL2 protected myocardin and MRTF-A from proteasome-mediated degradation. A recent report demonstrated that FHL2 is a target for the ubiquitin E3 ligase, Murf3 (10). Given that other LIM domain containing proteins can inhibit proteasome-mediated degradation by interacting with E3 ligases or ubiquitin targets (60, 140), we tested whether myocardin factor protein levels were regulated by proteasomal degradation and whether the positive effects of FHL2 on myocardin factor activity were due to inhibition of this pathway. We have recently generated a highly specific monoclonal antibody for MRTF-A that we first used to determine whether endogenous MRTF-A protein levels were affected by FHL2 expression. Even with a transfection efficiency of only 40-50% (measured by parallel transfection of GFP), over-expression of FHL2 in 10T1/2 cells led to a significant increase (~3-fold) in MRTF-A protein as measured by Western Blot (Fig 3.4a). Importantly, over-expression of FHL2 did not affect myocardin factor mRNA levels (Fig 3.4b). Treatment of cells with the proteasome inhibitor MG132 resulted in a slightly larger increase in endogenous MRTF-A protein in both 10T1/2 cells and primary rat aortic SMC (Fig 3.4c).

We were also interested in the effects of FHL2 on myocardin and MRTF-B protein levels, but because we have had little success in measuring myocardin or MRTF-B protein levels with the available antibodies, we co-transfected FHL2 along with flag-tagged versions of all three myocardin factors into 10T1/2 cells. As shown in figure 3.4d, over-expression of FHL2 led to increased levels of exogenously expressed myocardin (compare lanes 1 and 2)

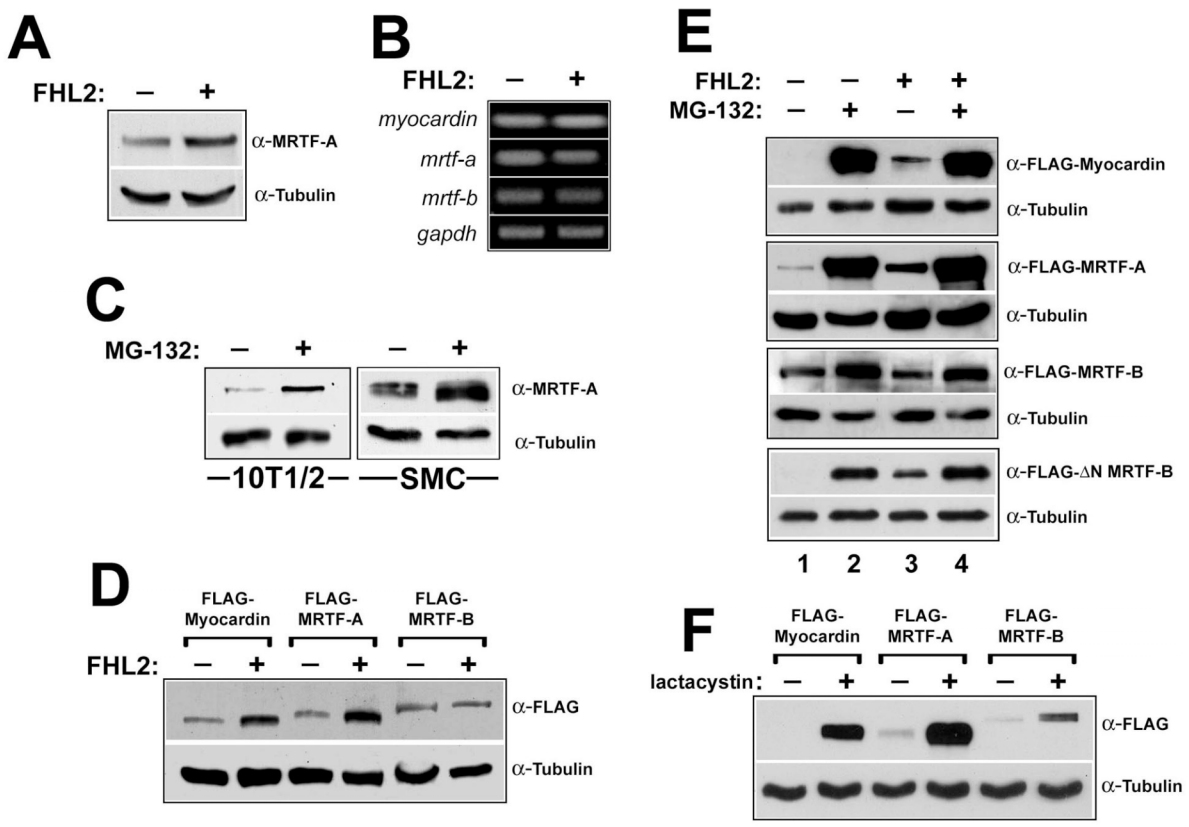

Figure 3.4. FHL2 inhibited proteasome-mediated degradation of myocardin and MRTF-A. **A)** 10T1/2 cells were transfected with FHL2 or empty expression vector, lysed after 24 h, and subjected to Western analysis using a monoclonal antibody to MRTF-A. **B)** RT-PCR for the each myocardin factor was performed on mRNA isolated from cells transfected with FHL2 or empty vector. C) MRTF-A expression was measured by Western Blot in 10T1/2 cells and SMC treated with the proteasome inhibitor MG-132 (20uM) or DMSO for 12 hrs. **D)** Exogenously expressed myocardin, MRTF-A, and MRTF-B were measured in 10T1/2 cells in the absence or presence of co-transfected FHL2. **E)** Flag-tagged myocardin factors were co-transfected with FHL2 or empty vector and treated with MG-132 (20um) or DMSO for 12 hrs. **F)** Exogenously expressed myocardin, MRTF-A, and MRTF-B were measured in 10T1/2 cells following treatment with 10uM lactacystin or DMSO vehicle for 12h hrs.

and MRTF-A (lanes 3 and 4), but had no effect on levels of MRTF-B (lanes 5 and 6). MG132 treatment led to more dramatic increase in myocardin and MRTF-A protein, and slightly increased MRTF-B levels (Fig. 3.4e, compare lanes 1 and 2). Interestingly, FHL2 expression had little if any effect on myocardin and MRTF-A levels in MG132-treated cells (compare lanes 2 and 4). We observed very similar changes in myocardin factor protein levels upon treatment of cells with lactacystin, a more specific inhibitor of the proteasome (Fig. 3.4f).

Interestingly, FHL2's ability to protect the myocardin factors from degradation correlated fairly well with myocardin factor nuclear localization. For example, myocardin which is constitutively nuclear, exhibited relatively lower expression levels under control conditions and was protected from degradation by FHL2. In contrast, MRTF-B, which is frequently cytoplasmic, was expressed at much higher levels but was not up-regulated by FHL2 expression. To test this more rigorously, we compared the effects of MG132 and FHL2 on ΔN MRTF-B, which lacks the N-terminal RPEL motifs that are required for localization of MRTF-B to the cytoplasm. ΔN MRTF-B levels were much lower than full-length MRTF-B levels under control conditions, and unlike full-length MRTF-B, ΔN MRTF-B protein levels were up-regulated by FHL2 co-expression and were strongly up-regulated by MG132 treatment (Fig 3.4e; bottom panel).

To directly measure the effects of FHL2 on MRTF-A protein stability we treated cells with the protein synthesis inhibitor, cycloheximide and measured MRTF-A protein by Western blot at 0.5, 3, and 6 h. As shown in figure 3.5, the half-life of over-expressed or endogenous MRTF-A was approximately 3 h and was significantly extended by the

expression of FHL2 (Figs 3.5a and 3.5b) or by proteasome inhibition (Fig 3.5c). Note that the protein half-life of FHL2 was substantially shorter (~1.5 h).

To determine whether the myocardin factors were targeted to the proteasome by ubiquitination, we immunoprecipitated MRTF-A from MG132-treated and control SMC and probed immunoprecipitates with an anti-ubiquitin antibody. As shown in figure 3.5d, ubiquitin immunoreactivity was only detected in MRTF-A (not control IgG) immunoprecipitants, and treatment of SMC with MG132 resulted in a substantial increase in this signal.

FHL2 did not increase myocardin factor association with SRF and may negatively regulate SRF-CArG binding. Philippar *et al* reported that FHL2 expression inhibited RhoA-dependent increases in SM22 and SM α-actin promoter activity in 293T cells and based upon gel shift analyses suggested that this effect was due to competitive inhibition of myocardin factor binding to SRF (20). We observed similar effects of FHL2 on RhoA-dependent stimulation of SMC-specific transcription in our 10T1/2 model (data not shown) and also demonstrated that FHL2 had negative effects on MRTF-B transactivation (Fig 3.3). However, our demonstration that FHL2 interacts directly with the myocardin factors and has differential effects on myocardin factor transactivation potentially complicates this model.

Based upon our observation that FHL2 binds slightly differently to each myocardin factor we used gel shift assays to test whether FHL2 had differential effects on myocardin factor binding to the SRF-CArG complex. Because the N-terminal RPEL domains of MRTF-A and MRTF-B inhibit their incorporation into the SRF-containing ternary complex, full-length and amino-terminally truncated variants (ΔN) of the MRTFs were used in these assays. Results shown in figure 3.6a demonstrate that only myocardin and the ΔN forms of

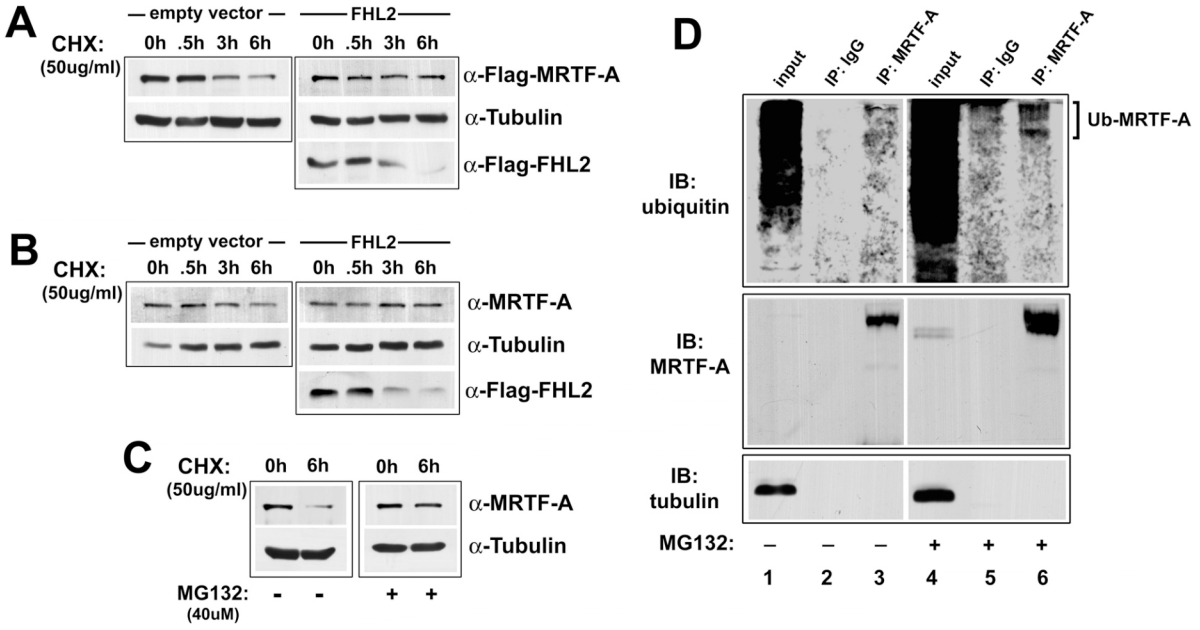

Figure 3.5. MRTF-A half-life was increased by overexpression of FHL2 or treatment with MG132. A) Cos7 cells were transfected with flag-MRTF-A +/- FHL2. Cells were treated with cycloheximide (CHX) and flag-MRTF-A and flag-FHL2 levels were measured by Western Blot at 0, 0.5, 3, or 6h. **B)** 10T1/2 cells +/- flag-FHL2 were treated with CHX as above and endogenous MRTF-A expression was measured by Western Blot at 0, 0.5, 3, or 6h. **C)** MRTF-A expression was measured in 10T1/2 cells treated simultaneously with MG132 and CHX. **D)** MRTF-A was immunoprecipitated from control and MG132-treated SMC lysates. Immunoprecipitants were probed with an antibody to ubiquitin (top panel) or MRTF-A (middle panel).

the MRTFs formed ternary complexes with SRF at the SM a-actin CArG B element. Addition of *in vitro* translated FHL2 to the binding reaction slightly decreased the intensity of all SRF containing complexes including those that did not contain a myocardin factor. This observation was particularly evident in the binding reactions that contained SRF only (far right). Taken together, although FHL2 did slightly decrease the levels of ternary complex, this was likely due to an overall reduction in SRF-CArG binding.

FHL2 modestly inhibited MRTF nuclear translocation. FHL2, like the MRTFs, has been shown to translocate from the cytoplasm to the nucleus in response to external stimuli that activate RhoA (110). Given that the effects of RhoA are strongly dependent upon nuclear translocation of the MRTFs, we hypothesized that the inhibitory effects of FHL2 could also be due to inhibition of MRTF-B nuclear translocation. To test this, we co-expressed flag-FHL2 along with EGFP-MRTF-B fusion proteins. MRTF-B localization under serum-free conditions was scored as nuclear, cytoplasmic, or diffuse (nuclear + cytoplasmic). As shown in figure 3.6b, expression of FHL2 slightly increased the percentage of cells exhibiting strictly cytoplasmic localization and decreased the percentage of cells exhibiting diffuse localization.

DISCUSSION

FHL2 has been shown to interact with a large number of transcription factors in a number of different model systems and has been shown to have both positive and negative effects on gene expression (see (67) for review). The goal of the present study was to further characterize the effects of FHL2 on SRF-dependent transcription. While our results confirm a previous report that FHL2 interacts directly with SRF, they also suggest that FHL2's role in

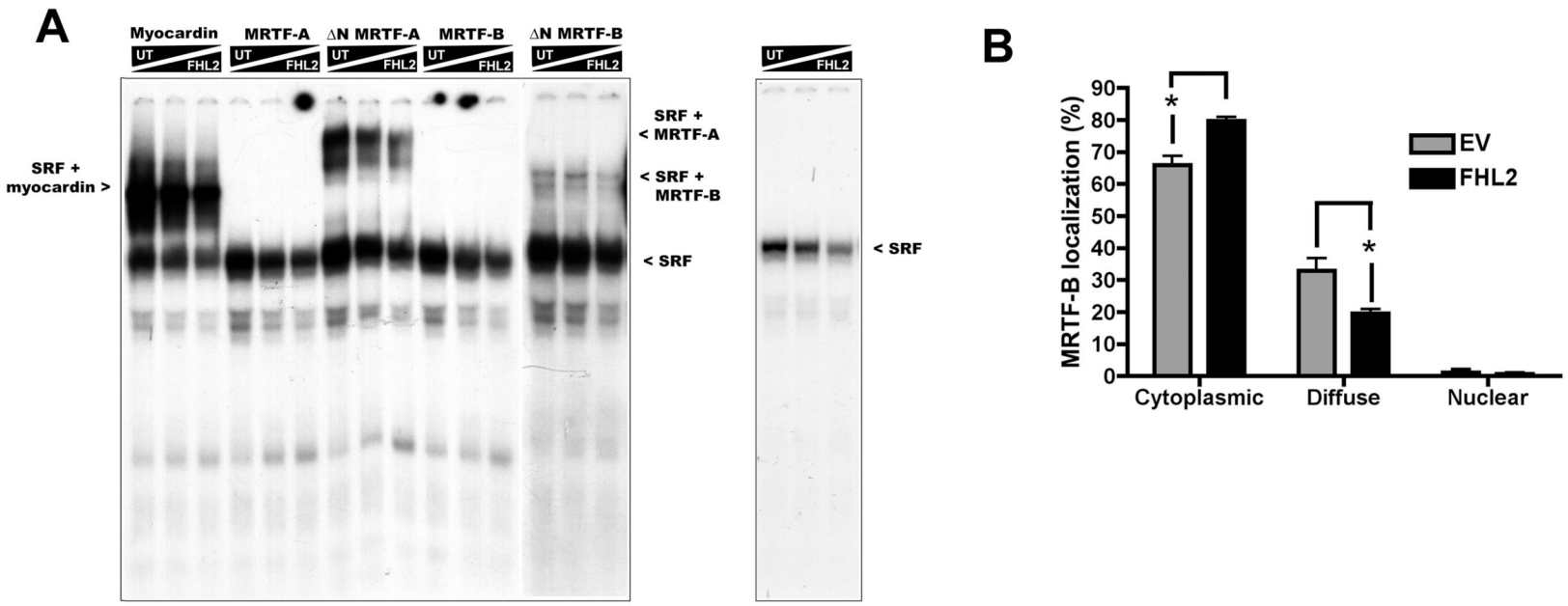

Figure 3.6. FHL2 modestly decreased SRF complex formation and nuclear translocation of MRTF-B. **A)** *In vitro* translated SRF and the indicated myocardin factors were incubated with radiolabeled SM α-actin Intronic CArG probe. Increasing concentrations of *in vitro* translated FHL2 (0, 2, or 4ul) were added to each binding reaction and the total amount of TnT lysate in each reaction was maintained by the addition of unprogrammed TnT lysate. After 30 min, binding reactions were run on a 5% non-denaturing gel, transferred to filter paper, and visualized by autoradiography. **B)** 10T1/2 cells were transfected with EGFP-MRTF-B +/- FHL2 and maintained in serum-free media for 24 h. Following fixation EGFP-MRTF-B localization was scored as nuclear cytoplasmic, or diffuse. Data represent averages ± SE of three separate transfections. * $p \leq 0.05$

the regulation of SRF-dependent transcription is complicated by additional interactions with the myocardin family of SRF co-factors. We demonstrate for the first time that expression of FHL2 stimulates myocardin-dependent transactivation of cardiac and SMC-specific promoter activity and that these effects are most likely due to protection of myocardin and MRTF-A from proteasomal degradation. Thus, based upon the known expression pattern of FHL2 during development, we propose that FHL2 promotes cardiac and SMC differentiation by preserving myocardin factor protein levels. In addition, since the FHL2 and SRF promoters are both regulated by SRF (124) (and probably the myocardin factors), FHL2 could be a key component of a feed-forward mechanism that maintains high levels of cardiac- and/or SMC-specific gene expression.

To our knowledge, these studies are the first to demonstrate that the myocardin factors are regulated by the proteasome and may provide a novel mechanism for the control of cardiac and SMC-specific transcription. Our finding that endogenous MRTF-A is ubiquitinated suggests that the myocardin factors may be direct targets for the proteasome, but it remains possible that myocardin factor stability is also regulated by another mechanism that is proteasome-sensitive. In addition, although the effects of FHL2 on MRTF-A protein half-life strongly suggest that FHL2 stabilizes myocardin factor protein, we cannot completely rule out an additional effect of FHL2 on the translation of myocardin factor message.

Previous studies have shown that FHL2's effects on gene expression are affected by a number of parameters including cellular localization and interactions with a variety of general and cell-type-specific transcription factors. The positive and negative transcriptional effects of FHL2 observed in this study likely reflect a combination of these differences. For

example, although FHL2 may attenuate cardiac and SMC-specific promoter activity by inhibiting ternary complex formation or MRTF-B nuclear translocation, these effects may be counteracted by increased myocardin and MRTF-A protein levels. The failure of FHL2 to protect MRTF-B from degradation may help explain the negative effects of FHL2 on MRTF-B transactivation. It is also possible that the negative effects of FHL2 may be due to sequestration of the myocardin factors or SRF into inactive complexes.

It is currently unclear why FHL2 does not affect MRTF-B stability. Our results, especially the comparison between full-length and ΔN MRTF-B, demonstrated that myocardin factor protein levels and the ability of FHL2 to protect individual myocardin factors from degradation were affected by nuclear localization. These results suggest the interesting possibility that myocardin factor ubiquitination occurs mainly in the nucleus. It is also possible that small differences in FHL2 binding to each myocardin factor could affect interactions with the ubiquitination or proteasome machinery.

Other LIM-domain containing proteins have also been shown to inhibit ubiquitination. Sangadala *et al* demonstrated that LIM-mineralization protein-1 stimulated osteoblast differentiation in mesenchymal cells by an inhibitory interaction with the E3 ligase, Smurf1, and that this interaction prevented SMAD degradation (140). In addition, Hiratani *et al* demonstrated that the Xenopus LIM homeodomain protein, Xlim-1, prevented the degradation of its binding partner, Ldb1, by the E3 ligase XRnf12 (60). Importantly, during the preparation of this manuscript, Fielitz *et al* identified FHL2 as a substrate for the E3 ligase Murf3 (40), and a very important future goal is to determine whether the myocardin factors are potential targets of the MuRF proteins. Although we did not detect FHL2 in complex with the SRF myocardin factor complex by gel shift, flag-FHL2 was detected at the

CArG-containing regions of the SM α-actin and SM22 promoters *in vivo* (124). If FHL2 and the myocardin factors are targets of the same E3 ligase, FHL2 could shield the myocardin factors from degradation by competitively interfering with myocardin factor ubiquitination. This would lead to stabilization of an important component of the SMC-specific transcription initiation complex and increased SMC differentiation marker gene expression.

Deletion of FHL2 in the mouse did not result in an overt cardiac phenotype (25) , but it is unknown whether other FHL family members (most likely FHL1) can compensate for loss of FHL2 in this model. Importantly, FHL2 -/- mice were more susceptible to isoproterenol-induced the lack cardiac hypertrophy (70) suggesting that FHL2 does have a specific cardiac function. Although the effects of FHL2 deficiency on SMC function have not been directly examined, a recent study by Wixler *et al* indicates that FHL2 regulates SMC differentiation marker gene expression *in vivo* (184). These authors observed that FHL2 was activated in skin myofibroblasts following wounding and that FHL2 expression correlated with the activation of SM α-actin and SM22 expression that is known to occur in this model (59). Furthermore, they demonstrated that FHL2 -/- mice exhibited reduced SM α-actin expression and healing following wounding and that re-expression of FHL2 in FHL2 -/- MEFs and mesenchymal stem cells up-regulated SM α-actin promoter activity. These results support a closer examination of the role of FHL2 in the modulation of SMC phenotype following vessel injury.

In summary, FHL2 has multiple effects on SRF- and myocardin factor-dependent transcription. Our data suggest that that FHL2 protects myocardin and MRTF-A from proteasome mediated degradation, providing a novel mechanism for the control of myocardin factor activity. It will be very important to further characterize this regulatory mechanism in

cardiac and SMC and to test whether FHL2 (and perhaps other FHL family members) has similar effects on other transcription factors.

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REGULATION OF MYOCARDIN FACTOR PROTEIN EXPRESSION BY THE MUSCLE-SPECIFIC RING FINGER PROTEIN MURF3

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ABSTRACT

Members of the myocardin family of SRF cofactors play a central role in cardiac and smooth muscle cell-specific gene expression, yet little is known about their regulation. Here, we extend recent findings that these transcription factors are regulated by proteasomal degradation, and report that the muscle-specific ring finger protein MuRF3 likely acts as an E3 ubiquitin ligase for the myocardin factors. Over-expression of MuRF3 inhibited SM α-actin promoter activity in luciferase assays, and significantly reduced the capacity of myocardin factors for transactivating the SM α-actin or SM22 promoters. MuRF3 physically interacted with the myocardin factors and down-regulated their expression in culture. Examination of hearts and multiple smooth muscle rich organs from wild-type and MuRF3-/- mice revealed increased abundance of MRTF-A protein in the absence of MuRF3. Preliminary *in vivo* ubiquitination assays demonstrated that over-expression of MuRF3

increased the ubiquitinated fraction of MRTF-A, suggesting that the myocardin factors may be substrates for its ubiquitin ligase activity.

INTRODUCTION

Vascular smooth muscle cells (SMC) play a critical role in vasculogenesis and angiogenesis, and provide mature vessels with the capacity for contraction necessary for proper maintenance of blood pressure and distribution throughout the organism. SMC arise from multiple origins within the embryo including the proepicardial organ, the cardiac neural crest, local mesoderm, and potentially from circulating stem cells as well (see (122) for review). Unlike other muscle cell types, SMC do not undergo terminal differentiation, but retain a unique phenotypic plasticity that facilitates their involvement in vascular remodeling and injury repair. In response to aberrant signaling, this phenotypic plasticity also facilitates the involvement of SMC in the pathogenesis of multiple prominent cardiovascular disease states including hypertension, atherosclerosis, and post-angioplasty restenosis. A greater understanding of the mechanisms governing SMC differentiation will undoubtedly increase our knowledge of vascular development and our ability to treat cardiovascular disease. The elucidation of these mechanisms, however, has been complicated by many factors including the varied origins of SMC and their lifelong capacity for phenotypic modulation.

It is well accepted that the expression of nearly all SMC differentiation marker genes, including SM MHC, SM α-actin, and SM22, is driven by the binding of the transcription factor serum response factor (SRF) to a conserved region within the promoters of these genes known as CArG ($C(A/T)_6G$) boxes. SRF however, is ubiquitously expressed and is also involved in the CArG-dependent regulation of multiple other non-SMC genes including the

early response genes *c-fos* and *egr-1*, as well as many skeletal and cardiac muscle-specific genes. It follows therefore that other factors in addition to SRF are necessary for specifying SMC fate. An important advance in our understanding of the mechanisms governing SMC differentiation came with the discovery of the myocardin family of transcription factors (53, 175, 176) that includes myocardin and the myocardin-related transcription factors A and B (MRTF-A and MRTF-B). The myocardin factors have been shown to interact directly with SRF and to potently up-regulate SRF/CArG-dependent expression SMC differentiation marker genes. While the myocardin factors have been shown to be important in promoting the expression of SMC differentiation marker genes over growth genes, they have also been shown to up-regulate expression of cardiomyocyte differentiation marker genes in a similar manner. Thus, it remains an important goal to identify pathways that determine cardiac versus SMC fate, and to determine how the myocardin factors may be involved in these pathways.

Recent experiments performed in our laboratory have demonstrated that degradation of myocardin and MRTF-A could be inhibited via novel interactions with the LIM-only protein four and a half LIM domains 2 (FHL2) (manuscript under review). Addition of FHL2 increased myocardin- and MRTF-A-dependent transactivation of the SM α-actin and SM22 promoters. FHL2 did not affect myocardin factor mRNA levels, but significantly increased the protein half-lives of both myocardin and MRTF-A. Further experiments strongly implicated the ubiquitin-proteasome system (UPS) as a mediator of myocardin factor stability, and demonstrated that this may be an important mode of regulating their activities. Treatment of cells with the proteasome inhibitors MG132 and lactacystin resulted in

increased protein stability of all three myocardin family members, and ubiquitination of MRTF-A was established by co-immunoprecipitation in SMC.

The importance of the UPS in cardiovascular development and disease is still emerging. The UPS has long been recognized as a regulator of multiple cellular processes including cell cycle progression and control of transcription (86, 113). More recent reports have directly implicated the UPS as a major player in the progression of cardiac-specific disease, and as an important regulator of normal cardiac function (see (183) for review). Importantly, recent studies have also linked the UPS to multiple pathways in SMC, including RhoA signaling and SRF-dependent gene expression (133, 139).

Protein ubiquitination occurs by a three-step process, each relying on a specific class of enzyme (E1-E3). Substrate specificity is conferred during the final step of this process, in which an E3 ubiquitin ligase transfers the ubiquitin molecule directly to the substrate. Mono-ubiquitination of substrates has been demonstrated to result in various effects, including alterations in protein activity and localization, while poly-ubiquitination most often leads to degradation via the 26S proteasome (see (113) for review). To understand the signaling mechanisms that regulate proteasomal degradation of the myocardin factors, it will be important to identify the E3 ligases responsible for their ubiquitination.

Interestingly, Olson and colleagues have recently reported that FHL2 interacts with the E3 ubiquitin ligase muscle-specific RING finger 3 (MuRF3), and presented compelling data suggesting that FHL2 is a target of MuRF3-mediated proteasomal degradation (40). MuRF3 belongs to a subset of RING-finger E3 ubiquitin ligases that also includes MuRF1- and MuRF-2, all of which are believed to be expressed exclusively in striated muscle (17, 153). Accordingly, nearly all studies of the MuRF family proteins to date have focused on

their role in skeletal and cardiac muscle. MuRF1 has been shown to catalyze the ubiquitination and degradation of troponin I in cardiomyocytes, and to play a role in skeletal muscle atrophy and cardiac hypertrophy (8, 69, 182). MuRF2 also interacts with the sarcomeric apparatus, and has additionally been implicated as a mechanical stress-induced modulator of SRF activity in cardiomyocytes (74). MuRF3 has been shown to play an important role in microtubule assembly and is required for skeletal muscle differentiation and maintenance of ventricular integrity following myocardial infarction (40, 47, 153). Thus far, none of the MuRF family proteins have been shown to play a role in the modulation of SMC gene expression.

In light of our earlier findings that FHL2 protected myocardin and MRTF-A from proteasomal degradation, and the recent report indicating FHL2 is a binding partner of the E3 ubiquitin ligase MuRF3, we hypothesized that MuRF3 may also mediate the degradation of myocardin factors. Our results indicate that MuRF3 inhibits myocardin factor-mediated transactivation of SMC gene promoters and down-regulates myocardin factor expression *in vitro* and *in vivo*. Importantly, MuRF3 also physically interacted with the myocardin factors, and may act as an E3 ubiquitin ligase that facilitates their ubiquitination.

MATERIALS AND METHODS

Plasmids and Reagents – Flag-pcDNA3-Myocardin, MRTF-A, and MRTF-B, as well as the SMα-actin, SM22, and CRE luciferase promoter constructs have been previously described (58). Myc-pcDNA3.1-MuRF3 and rabbit anti-MuRF3 Ab were generous gifts of Monte Willis (University of North Carolina). Flag-ubiquitin was a generous gift of Liliana Ponguta and Elizabeth Wilson (University of North Carolina). Monoclonal anti-MRTF-A Ab

was generated by immunization of hamsters with MRTF-A peptide. All other Abs were commercially obtained including goat anti-MRTF-B (Santa Cruz) rabbit anti-FAK (Upstate), mouse anti-α-tubulin (Sigma), and mouse anti-ubiquitin (Covance).

Cell Culture, Transient Transfections, and Reporter Assays – 10T1/2 cultures and the SMC-specific promoter assays used have been previously described (88). In brief, cells were maintained in 10% serum media and transfected 24 h after plating at 70-80% confluency using LT-1 transfection reagent (Mirus) per protocol. Luciferase activity measurements were made 24 h post transfection. Luciferase activity was measured in relative light units (RLUs) and expressed as fold activity over empty vector. For harvesting of protein, cells were transfected in 10cm dishes using LT-1 reagent as above and lysed in RIPA + 0.5% triton.

GST fusion pull-downs and co-immunoprecipitations - GST pull-down assays and co-immunoprecipitations were performed as previously described (157). In brief, GST fusion proteins were purified from bacterial lysates using glutathione sepharose (Amersham Biosciences). Whole cell lysates were prepared from rat hearts as follows: organs were perfused with phosphate-buffered saline, excised from animals, homogenized, and lysed in RIPA+1% triton. For pull-downs, GST fusions were used to precipitate MuRF family proteins, and for co-immunoprecipitations endogenous MRTF-A was precipitated using hamster anti-MRTF-A Ab. Interacting complexes were pelleted by centrifugation and washed 3X in RIPA buffer and 1X in cold Tris-buffered saline.

Semi-quantitative PCR - RNA was prepared from cell and tissue lysates using Trizol Reagent (Invitrogen) and was quantified by optical density assay. cDNA was generated from 1ug of RNA using in the iScript cDNA synthesis kit (Biorad). Exon spanning primers were used to amplify MuRF3 and GAPDH (sequences available upon request).

In vivo ubiquitination assays –10T1/2 cells were transfected with myc-MuRF3 and flag-ubiquitin or flag empty vector and treated for 12 h with MG132 (10uM) (Sigma). Cells were lysed in RIPA buffer and immunoprecipitations were performed using M2 anti-flag (Sigma) or hamster anti-MRTF-A Abs coupled to protein G beads (Sigma). Precipitants were run on an SDS-PAGE gel, transferred to nitrocellulose, and probed with P4G7 anti-ubiquitin (Covance) or monoclonal anti-MRTF-A Abs.

RESULTS

MuRF3 was detected in heart and aorta and inhibited SM α-actin promoter activity. MuRF3 expression is thought to be limited to striated muscle, yet Spencer *et al* have reported that extended exposures of Northern analyses revealed low level MuRF3 mRNA expression in lung (an organ with a large SMC component), and no reports to date have specifically examined the expression of MuRF3 in smooth muscle (153). To further evaluate this question, we performed semi-quantitative RT-PCRs on RNA extracted from mouse hearts, primary cultures of rat aortic SMC, and multiple immortalized cell lines. As shown in figure 4.1A, MuRF3 mRNA expression was greatest in heart, as previously reported, but was also detectable at very low levels in primary cultures of SMC and in Cos-7 cells. MuRF3 mRNA was not detected in immortalized A7R5 SMC, or in 10T1/2 cells, which have been shown to differentiate into SMC upon treatment with TGF-β. We also examined MuRF3 protein levels in lysates derived from rat heart and aorta, and observed similar levels of MuRF3 protein in both organs (Fig 4.1B). The striking difference between MuRF3 mRNA levels in primary cultures of aortic SMC and MuRF3 protein levels in aortic lysate was surprising, and may reflect cell-type specific differences in post-transcriptional regulation of

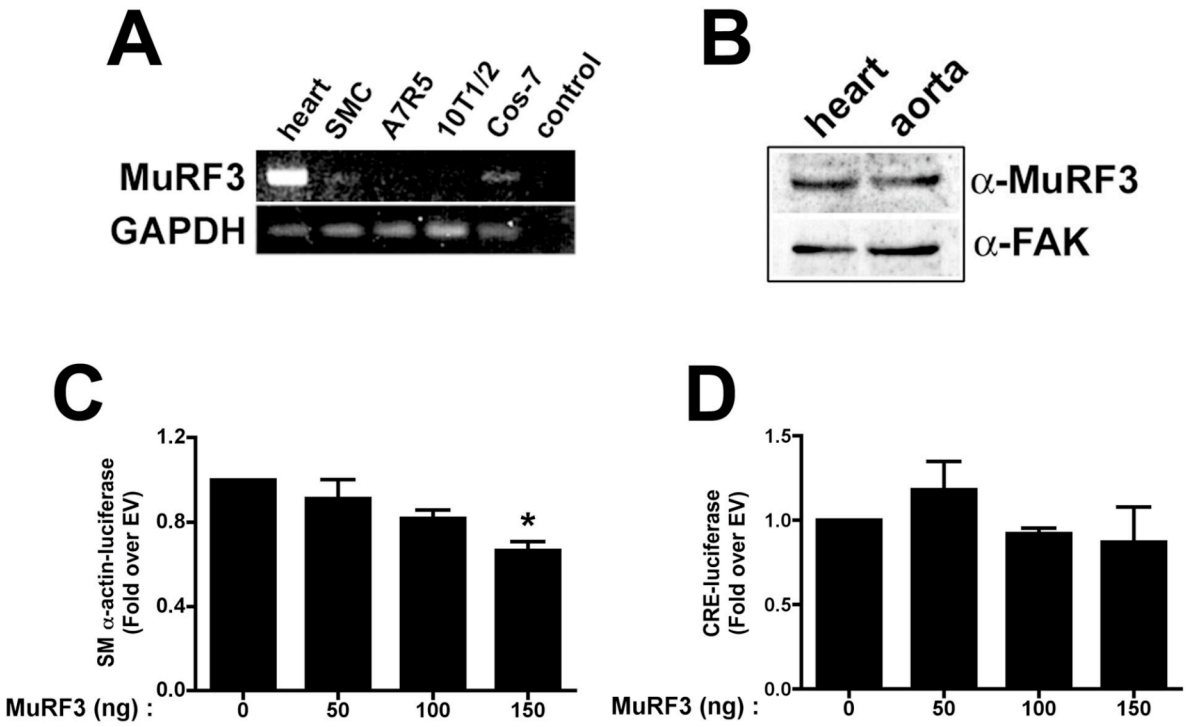

Figure 4.1. MuRF3 was detected in heart and aorta and specifically inhibited SM α-actin promoter activity. A) Semi-quantitative RT-PCR was used to examine MuRF3 transcript levels in heart and multiple cell lines. GAPDH was used as an internal control. **B)** MuRF3 protein was detected by Western analysis in lysates derived from rat heart and aorta. **C)** MuRF3 inhibited SM α-actin promoter activity in 10T1/2 cells at high concentrations. **D)** MuRF3 had no effect on activity of the minimal CRE promoter or TK promoter. Luciferase assays were performed 24h post transfection. Significance was determined by student's T-test (* $p \leq .05$).

MuRF3 message or rapid down-regulation of MuRF3 gene expression upon culture of SMC. At this time, we also cannot rule out nonspecific interaction of MuRF3 antibody with MuRF1 or MuRF2, as this antibody was generated before the existence of these two proteins was reported (17, 153).

MuRF3 inhibited transactivation of SMC marker genes by myocardin factors. To determine whether MuRF3 has any effect on SMC differentiation marker gene promoter activity, we first performed transcriptional reporter assays in which the SM α-actin or minimal CRE promoter was used to drive the luciferase reporter gene in the SMC precursor 10T1/2 cell line. Interestingly, co-transfection of increasing concentrations of MuRF3 with the SM α-actin promoter construct resulted in slightly decreased luciferase activity, while co-transfection with the CRE promoter construct resulted in no effect (Fig 4.1C,D).

We further explored the role of MuRF3 in SMC-specific transcription by testing its effects on myocardin factor transactivation. Co-transfection of increasing concentrations of MuRF3 with myocardin or MRTF-B decreased each transcription factor's ability to trans-activate either the SM α-actin or SM22 promoter in a dose-dependent manner (Fig 4.2A-B). A similar trend resulted upon co-transfection of increasing concentrations of MuRF3 with MRTF-A, although a statistically significant level of MRTF-A inhibition was not reached in these experiments (Fig 4.2A-B). As was observed before, addition of MuRF3 had no effect on CRE promoter activity, regardless of myocardin factor co-transfection (Fig4.2C).

MuRF3 down-regulated myocardin factor protein expression *in vitro* and *in vivo*. Because we have previously reported that the myocardin factors are regulated via proteasomal degradation, and MuRF3 is known to act as an E3 ubiquitin ligase, we tested whether MuRF3 expression had any effect on myocardin factor protein expression levels. We

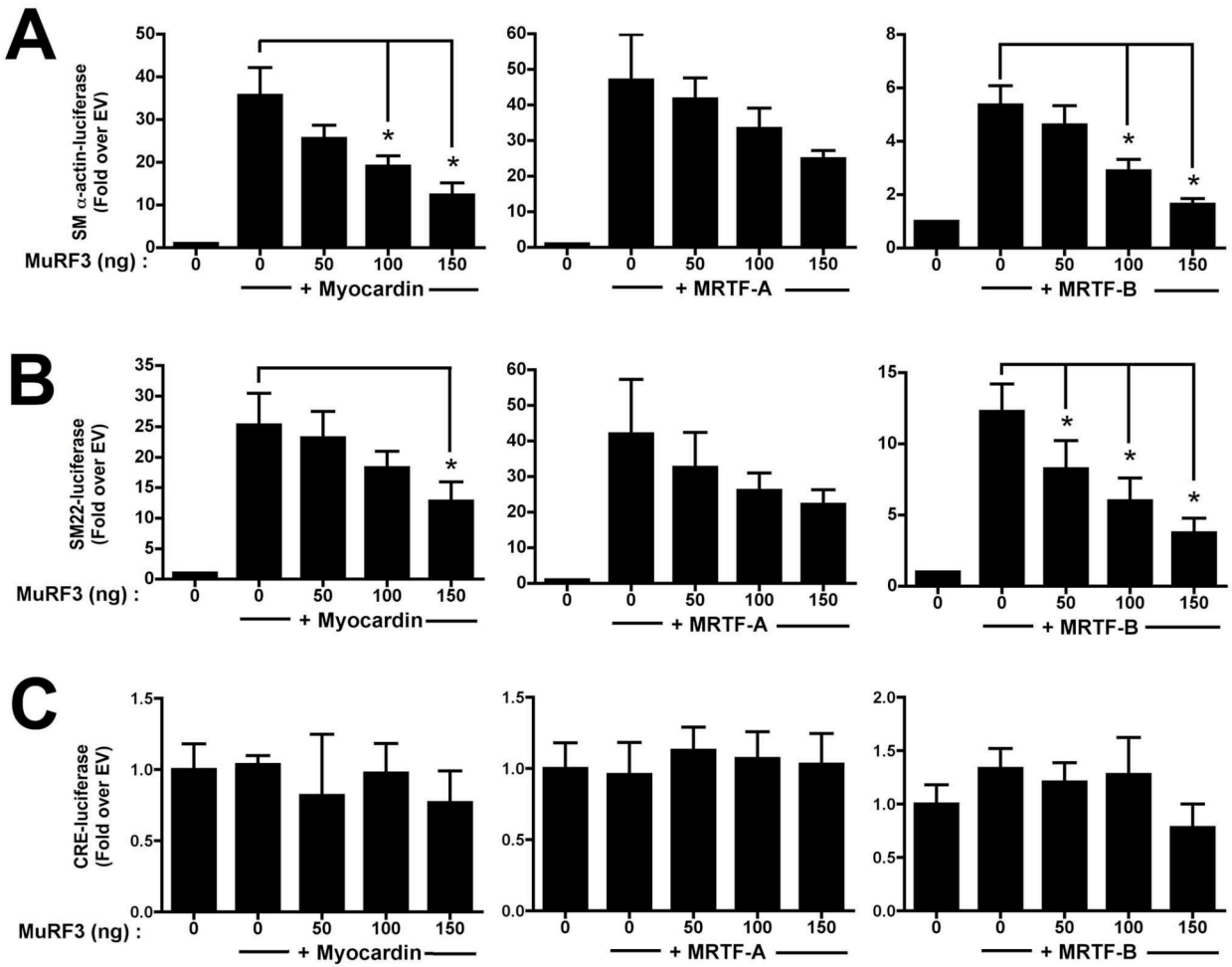

Figure 4.2. MuRF3 inhibited transactivation of the SM α-actin and SM22 promoters by the myocardin factors. Increasing concentrations of MuRF3 were co-transfected into 10T1/2 cells along with myocardin, MRTF-A, MRTF-B, and a luciferase reporter construct driven by the SM α-actin **(A)**, SM22 **(B)**, or minimal CRE **(C)** promoter. Luciferase assays were performed 24h post transfection. Significance was determined by Student's T-test (* $p \leq .05$).

did this first by co-transfecting each of the myocardin factors with either MuRF3 or empty vector, and measuring the expression of exogenous myocardin factor protein by Western analysis. Addition of MuRF3 strongly inhibited myocardin and MRTF-B expression, and led to the breakdown of exogenously expressed MRTF-A (Fig 4.3B). To further explore the effect of MuRF3 expression on MRTF-A protein expression, we transfected cells with either MuRF3 or empty vector, and measured the effects of MuRF3 expression on endogenous MRTF-A. Lack of suitable antibodies precluded examination of endogenous myocardin and MRTF-B protein. As shown in figure 4.3A, transfection of MuRF3 resulted in reduced protein levels of endogenous MRTF-A by approximately 50%. While this effect is less striking than that seen upon co-transfection of MuRF3 with myocardin and MRTF-B, these data may underestimate the potency of MRTF-A protein inhibition by MuRF3, as our transfection efficiency in this cell type is only 40-50%.

We next examined myocardin factor expression levels in the hearts and multiple smooth muscle-containing organs of wild-type and MuRF3 knockout mice. The mice used in these studies were obtained from the Willis laboratory, and genetic deletion of MuRF3 was accomplished using the same knock-in approach as has been previously published for MuRF1(8, 182). Deletion of MuRF3 in the mice tested by our laboratory was confirmed by PCR (data not shown). Due to lack of a suitable antibody against myocardin, our examination of myocardin factor protein levels in these tissues was limited to MRTF-A and MRTF-B. As predicted based on the over-expression studies described above, MRTF-A protein was up-regulated in the hearts, aorta, lung, and bladder of MuRF3-/- mice (Fig 4.3C). No differences in MRTF-B protein expression were detected between wild-type and MuRF3-/- mice using a sub-optimal commercially available antibody to MRTF-B (data not shown).

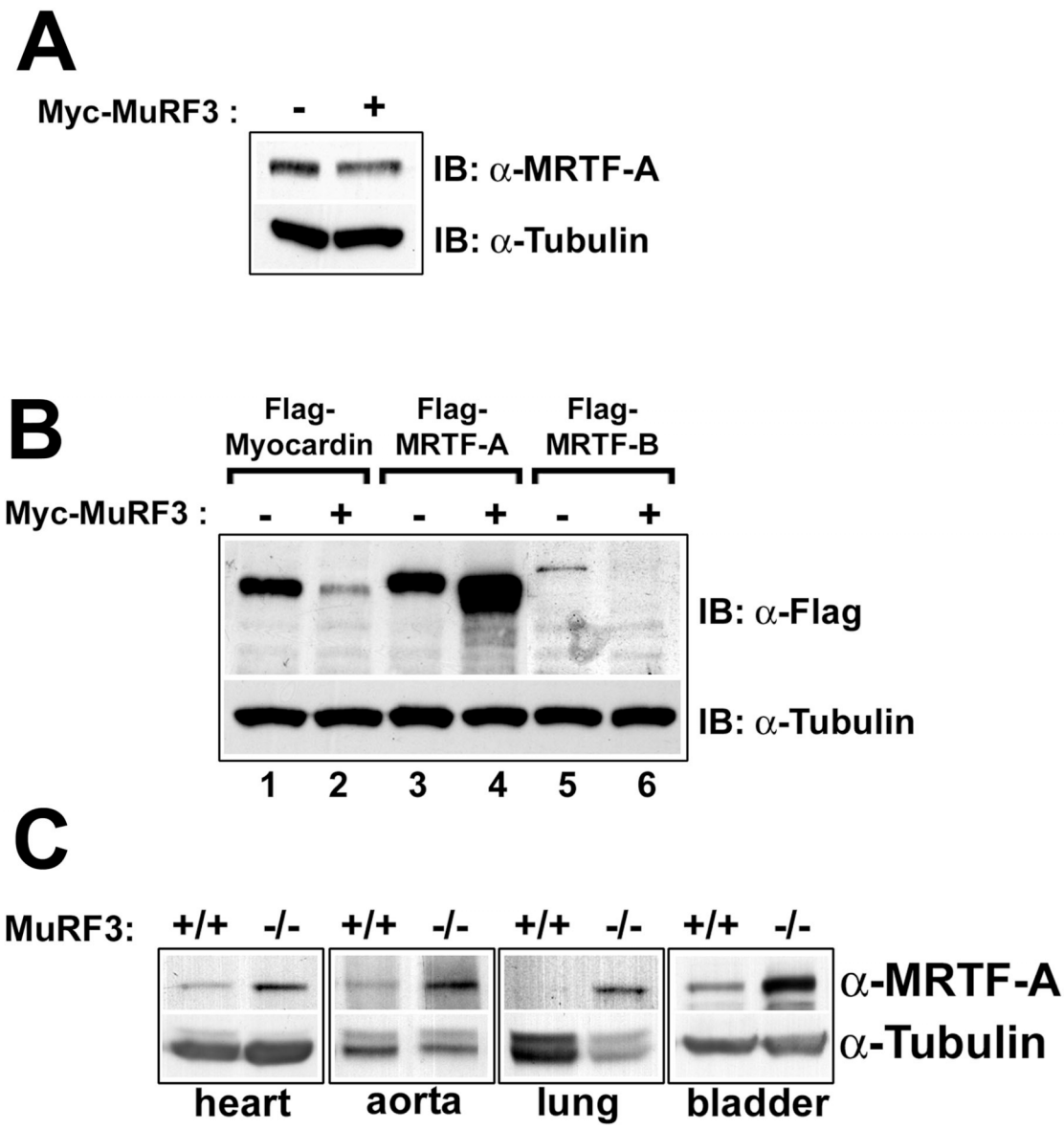

Figure 4.3. MuRF3 down-regulated myocardin factor protein expression in vitro and in vivo. A) 10T1/2 cells were transfected with Myc-MuRF3 or empty vector and maintained in serum-containing media for 48h. Cells were lysed in RIPA and subjected to Western analysis. Immunoblots were probed with antibodies against endogenous MRTF-A or tubulin (loading control) **B)** 10T1/2 cells were transfected with Myc-MuRF3 or empty vector along with flag-tagged variants of each myocardin factor. Following transfection cells were treated as above, and immunoblots were probed with antibodies against flag epitope or tublin. **C)** The indicated organs were obtained from MuRF3-/- and wild-type mice from the same genetic background, homogenized and lysed in RIPA, and subjected to Western analysis. Immunoblots were probed with antibodies against endogenous MRTF-A or tubulin.

MuRF3 physically interacted with the myocardin factors. Poly-ubiquitination of proteins is most often initiated by E3 ligases, which interact with both the E2 ubiquitin conjugating enzyme, and with the substrate protein to facilitate transfer of the ubiquitin molecule (172). To begin to explore the possibility that MuRF3 may act as an E3 ligase for the myocardin factors, we conducted experiments to detect an interaction between the proteins. Immunoprecipitation of endogenous MRTF-A from rat heart lysate resulted in co-immunoprecipitation of endogenous MuRF3, indicating that the two proteins do interact *in vivo* (Fig 4.4A). We next used GST fusion pull-down experiments to further substantiate an interaction between MuRF3 and MRTF-A and to investigate possible interactions between other MuRF family members and MRTF-A or MRTF-B. As shown in figure 4.4B, MRTF-A and MRTF-B were precipitated from rat heart lysate using GST-fusions of both MuRF1 and MuRF3, but not MuRF2. Lack of a suitable antibody against myocardin again limited our examination to MRTF-A and MRTF-B in these experiments.

MuRF3 may act as an E3 ubiquitin ligase for the myocardin factors. The data presented thus far have demonstrated that MuRF3 interacts with at least MRTF-A and MRTF-B (myocardin was not examined) and down-regulates the protein levels of all three myocardin factors (Figs 4.3-4.4). To further assess the ability of MuRF3 to act as an E3 ligase for myocardin factors, we performed *in vivo* ubiquitination assays of MRTF-A in the presence and absence of over-expressed MuRF3. We recently used a similar assay to demonstrate that MRTF-A is ubiquitinated *in vivo* (manuscript under review). These experiments have been only marginally successful (data not shown). We have also attempted to demonstrate MuRF3-mediated ubiquitination of the myocardin factors using *in vitro* ubiquitination assays, but have thus far been unsuccessful (data not shown).

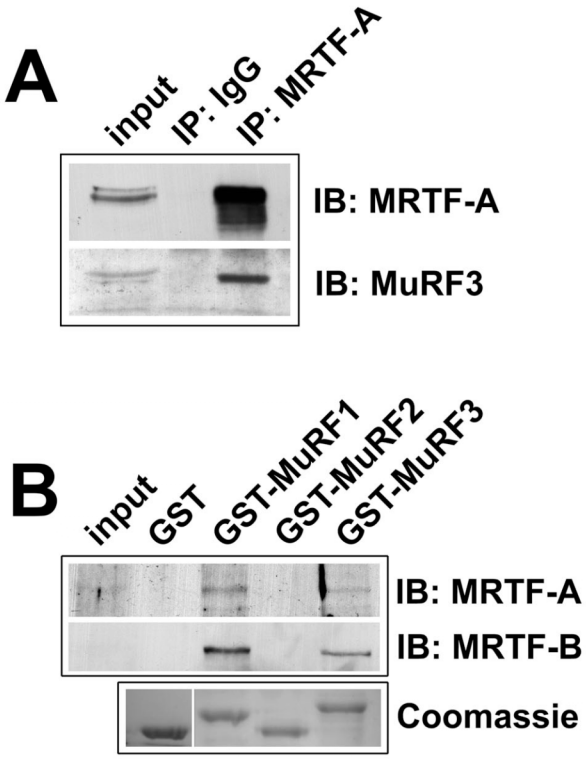

Figure 4.4. MuRF3 physically interacted with MRTF-A and MRTF-B. A) Immuno-precipitations were performed in RIPA lysates derived from rat heart using anti-MRTF-A or IgG control antibodies. Precipitants were subjected to SDS-PAGE, and immunoblots were probed by Western analysis using antibodies against endogenous MRTF-A or MuRF3. **B)** Pull-down assays were performed in RIPA lysates derived from rat hearts using GST alone and GST fusions of MuRF1, 2, and 3. Immunoblots were probed by Western analysis using antibodies against endogenous MRTF-A and MRTF-B. Coomassie staining was used to ensure equal addition of all GST fusion proteins to pull-down assays.

DISCUSSION

The goal of the present study was to explore a potential role for MuRF3 in the regulation of myocardin factor stability and SMC-specific transcription. The data presented here demonstrate for the first time that MuRF3 inhibits transactivation of the SM α-actin and SM22 gene promoters. We also show that MuRF3 down-regulates myocardin factor protein levels, physically interacts with MRTF-A and MRTF-B, and may act as an E3 ubiquitin ligase for this very important family of SRF co-factors. While MuRF3 was shown to down-regulate exogenous myocardin protein levels as well, an interaction between MuRF3 and endogenous myocardin was not investigated due to lack of suitable antibody. A present goal is to evaluate this possibility using ectopic expression studies. To our knowledge, the myocardin factors have not previously been identified as potential substrates for any other E3 ubiquitin ligase. These findings provide information critical to our understanding of the roles played by MuRF family proteins and the UPS in regulating SRF-dependent SMC-specific transcription.

Thus far, we have been unable to directly demonstrate ubiquitination of myocardin factors by MuRF3. We have attempted to do this using *in vitro* ubiquitination assays. Potential reasons for our lack of success in these experiments, as well as alternative future approaches, are discussed below.

In vitro ubiquitination assays require the addition of purified substrate protein. We have attempted to isolate myocardin factor protein from both yeast and insect cell systems. GST fusions of the myocardin factors were unstable in yeast, and insect cells infected with myocardin factor-encoding baculovirus grew at very low rates compared to uninfected cells. Ultimately, our attempts at isolation of full-length myocardin factor protein from cells have

been unsuccessful. This may be partially explained by the short half-lives of myocardin factors observed in mammalian cells (Fig 3.5). Rapid turnover of these proteins in yeast and/or insect cells might explain the degradation we have observed. In addition, Tang *et al* have recently shown that myocardin inhibits cellular proliferation by inhibiting NF-κB mediated cell-cycle progression (161). These findings may explain the lack of cellular growth observed in myocardin factor encoding baculovirus-infected insect cells.

We have had past success in generating recombinant myocardin factor protein using an *in vitro* transcription/translation coupled system (Promega). The amount of protein produced in this system is insufficient for visualizing the relatively small percentage of protein that undergoes ubiquitination in these assays by Western analysis, but radiolabeling of proteins with ^{35}S should overcome this problem. It may also be important to perform these experiments in the presence and absence of whole cell extracts (WCE) to determine if there are additional accessory factors (such as SRF, discussed below) that are required for the ubiquitination of MRTFs by MuRF3. WCE should be prepared from cells that do not express MuRF3, so that the presence of MuRF3 can be controlled for.

MuRF3 was originally identified in a yeast-2-hybrid screen for binding partners of SRF in adult mouse heart, yet subsequent attempts to demonstrate this interaction or co-localization of the two proteins were unsuccessful (153). The myocardin factors are known to interact with SRF and are expressed in the myocardium. Under certain conditions, MuRF3 may form complexes with SRF-bound myocardin factors, providing a possible explanation for detection of MuRF3 in the above screen.

The idea that MuRF3 may bind to myocardin factors in complex with SRF is further supported by a recent examination of the proteasome's role in regulating transcription *in vivo*

(106). In this study Molinari *et al* demonstrated that transcriptional activator protein levels did not correlate with mRNA levels, but were tightly regulated post-translationally by the ubiquitin-proteasome system. These authors further showed that protein turnover rate is directly correlated with potency of transcriptional activation domain and that mutations to DNA binding domains of transcriptional activators protected them from degradation, indicating that the proteasomal system is capable of both sensing and regulating transcriptional activity. The myocardin factors do not bind DNA directly, but exert transcriptional activation via interactions with DNA-bound SRF. It follows therefore, that proteasomal regulation of myocardin factor function in transcription would be dependent on SRF binding, and that interactions with the E3 ligase MuRF3 might be initiated while myocardin factors are still in complex with SRF (see figure 4.6 for model).

This model of proteasomal regulation of myocardin factor-dependent transcription also fits nicely with other recent findings. We have previously demonstrated that proteasome-mediated degradation of myocardin factors correlates well with nuclear localization, as does protection from degradation by FHL2 (see figure 3.4E in previous chapter). It is likely that myocardin factor degradation is associated with nuclear localization because ubiquitination of these factors occurs after initiation of transcriptional complex incorporation. An important future aim will be to test this experimentally.

While the mechanism by which FHL2 protects the myocardin factors from degradation is still unclear, the data presented here provide the basis for plausible hypotheses. FHL2 interacts directly with SRF and the myocardin factors (see figures 3.1 and 3.2 in previous chapter), and it is possible that FHL2-bound myocardin is resistant to ubiquitination by MuRF3. Alternatively, FHL2 also interacts with and is degraded by

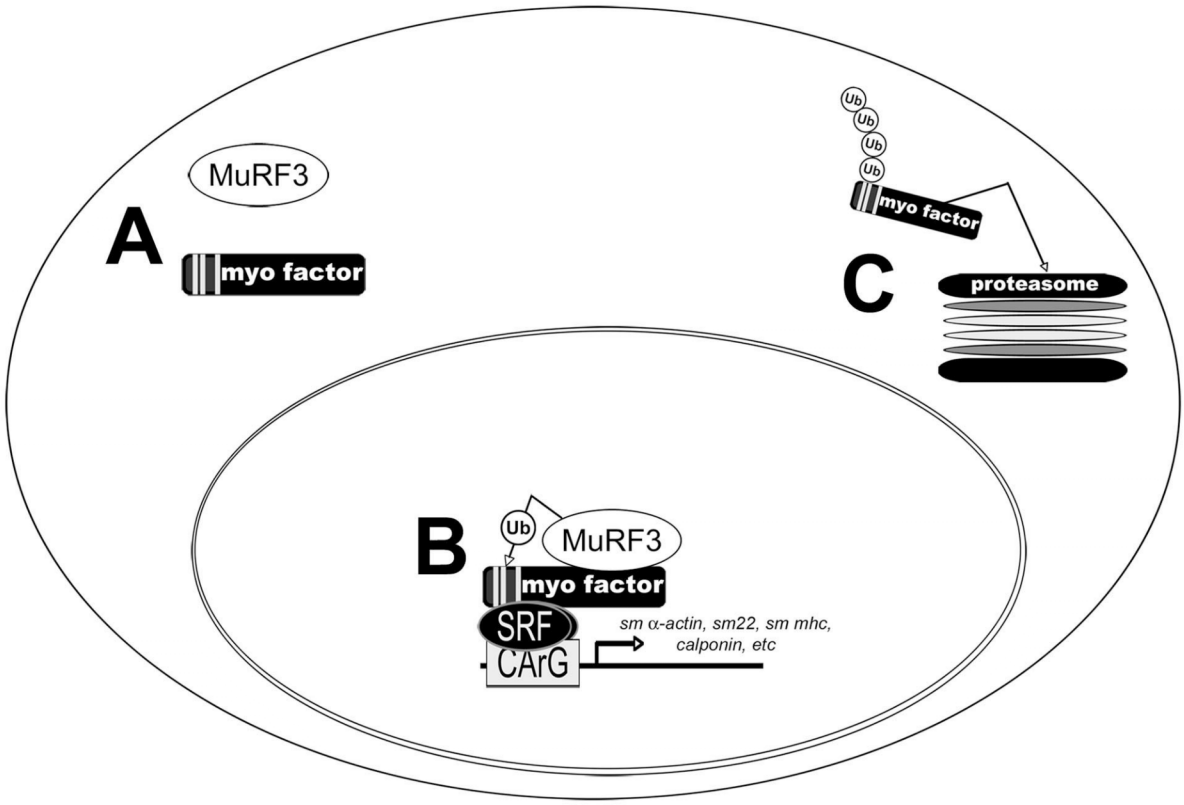

Figure 4.5. Hypothetical model of transcription-dependent MuRF3-mediated myocardin factor degradation. A) We have previously shown that cytoplasmic myocardin factors are less susceptible to degradation, therefore nuclear localization may be important for their interaction with the E3 ubiquitin ligase, MuRF3. **B)** Nuclear localization increases myocardin factor ubiquitination, and recent reports indicate that degradation of transcriptional co-activators is dependent on interaction with DNA binding complexes. In light of this, we propose that SRF binding of myocardin factors may be prerequisite for MuRF-3 mediated degradation. **C)** Once ubiquitinated, myocardin factors are sent to the 26S proteasome for degradation.

MuRF3 (40), and may prevent the ubiquitination of myocardin factors by competitive inhibition.

It remains possible however, that the myocardin factors are not ubiquitinated by MuRF3, and that MuRF3 exerts its effects on their protein expression by some as yet undefined mechanism. For instance, we have shown that FHL2 extends the half-life of myocardin and MRTF-A (Fig 3.4), and FHL2 is also a substrate for MuRF3 mediated ubiquitination (40). MuRF3 could therefore lead to down-regulation of myocardin and MRTF-A protein by promoting the degradation of FHL2. This model would not explain MuRF3-mediated inhibition of MTRF-B, as FHL2 did not promote its stability, but degradation of a similar protein by MuRF3 would have potentially similar effects. A logical first step in evaluating these possibilities will be to obtain more conclusive data regarding the ubiquitination of myocardin factors by MuRF3 using modified *in vitro* ubiquitination assays as described above.

Findings presented here demonstrate that MuRF3 regulates myocardin factor protein levels, and is capable of significant effects on SMC-specific transcription. This study is the first to demonstrate that MuRF3 protein is present in the vasculature, and the function of MuRF3 in vessels has not yet been examined. However, it is known that MuRF3-/- mice do not exhibit defective cardiac or SMC differentiation. Olson and colleagues have recently reported that while the hearts of MuRF3 null mice appear to develop properly, MuRF3 is important for protection of cardiomyocytes following myocardial infarction, and that mice lacking MuRF3 exhibit abnormal sarcomeric structure due at least in part to increased abundance of FHL2 and γ-filamin (40). It will be interesting to determine whether levels of myocardin factors are similarly dysregulated in this injury model, and whether MuRF3-/-

mice also exhibit altered function in SMC under pathologic stress. This question should be addressed using established models of induced hypertension and restenosis.

Additionally, we have shown that MuRF1 also interacts with the myocardin factors MRTF-A and MRTF-B in heart. It will be important to determine whether MuRF1 or MuRF2 play similar roles to MuRF3 in the regulation of myocardin factor degradation. It is very possible that loss of MuRF3 in mouse is compensated for by redundancy in function with other MuRF family proteins, and a greater understanding of the *in vivo* importance of these factors may require examination of mice in which these genes have been combinatorially deleted. Olson and colleagues have very recently reported that tandem deletion of MuRF1 and MuRF3 predisposes mice to striated muscle myopathies caused by increased accumulation of myosin heavy chain. This study reported no effects on myocardin factor stability or SMC function (39). To our knowledge, no other combinatorial deletions of the MuRF family proteins have been reported.

The data presented here further our knowledge of a recently described regulatory pathway in SMC-specific gene expression. While these studies are preliminary, they suggest the UPS may tightly regulate myocardin factor protein turnover via MuRF3. They also provide a basis for further exploration of UPS-mediated controls of SMC-specific transcription.

Acknowledgements:

We would like to thank Da-Zhi Wang (University of North Carolina, Chapel Hill, NC) for reagents.

PERSPECTIVES AND FUTURE DIRECTIONS

DISTINCT ROLES FOR MYOCARDIN FACTORS IN VASCULAR DEVELOPMENT

The studies included here have focused largely on the regulation and activities of the very important myocardin family transcription factors in tissue culture and *in vitro* model systems. Since the time that this dissertation research was begun, several studies have increased our understanding of individual myocardin factor function *in vivo*, and are important to a discussion of future studies in the field. As was described in the Background and Significance chapter of this dissertation, genetic deletion of myocardin results in embryonic lethality due to severe vascular defects caused by defective SMC differentiation, indicating that myocardin is requisite for smooth muscle development (82). Until recently, the *in vivo* role of the MRTFs was less clear.

Multiple laboratories have now shown that genetic deletion of MRTF-B in the mouse also leads to embryonic lethality caused by severe cardiovascular defects, including incomplete SMC differentiation. Mice homozygous for a loss of MRTF-B display defective cardiac outflow tract development. This region of the vasculature arises from progenitor cells within the cardiac neural crest, a region where MRTF-B is highly expressed. Loss of MRTF-B is accompanied by a lack of SMC differentiation marker gene expression in cardiac neural crest-derived cells, and specific restoration of MRTF-B expression in these cells is sufficient to rescue the MRTF-B -/- phenotype (78, 117). In a similar study performed by Wei *et al*,

these authors demonstrated that MRTF-B -/- mice also exhibit defective SMC gene expression in the liver sinusoids, vitilline veins, and yolk sac, extending our understanding of the *in vivo* role of MRTF-B (180).

The role of MRTF-A in regulating SMC differentiation *in vivo* is still somewhat unclear. It has been reported that MRTF-A knock out mice are viable, indicating that it is not required for SMC specification as is myocardin (177). Yet, two recent studies have shown that female mice deficient for MRTF-A are unable to nurse their offspring due to defective myoepithelial cell differentiation. Myoepithelial cells function similarly to SMC and provide the mammary gland with a contractile capacity necessary for milk ejection. MRTF-A -/- mice lack SRF-dependent contractile gene expression in these cells (81, 156). Whether other myocardin family members compensate for the loss of MRTF-A in SMC will not be known until mice are generated with multiple deletions of this family. It is also unclear whether MRTF-A is required for other aspects of SMC function such as changes in gene expression that are known to occur due to environmental stresses or whether MRTF-A is responsible for SMC marker gene expression in other non SMC-types like that observed in myofibroblasts during wound healing (31).

Deletion of each of the myocardin factors yields a distinct phenotype, indicating that each plays a unique role in cardiovascular development. This idea was further supported in a recent study by Pipes *et al*, in which these authors showed that although genetic deletion of myocardin is embryonic lethal, myocardin null mouse ES cells differentiated into SMC in culture, and when introduced into a chimeric mouse model gave rise to myocardin -/- SMC in the aorta(125). RT-PCR analyses revealed that MRTF-A was strongly expressed in myocardin -/- cells, and in wild-type cells, was expressed much earlier in development than

myocardin. Though myocardin null ES cells were able to differentiate into SMC and appeared to have normal contractile protein levels and function, many markers were dysregulated at the mRNA level, indicating myocardin as an important coordinator of gene transcription, a role of myocardin that was previously undemonstrated.

IMPORTANT AREAS FOR FUTURE INVESTIGATION

Expression Patterns of MRTFs in the Embryo and Adult Animal

Clearly, more information is needed concerning the specific role of each myocardin factor in cardiovascular development. A more rigorous examination of tissue and cell-type distribution of the myocardin factors throughout development and in adult animals will be an important aim to pursue, so that their contributions to tissue-specific transcription can be more precisely determined. MRTF-A and MRTF-B expression have thus far been examined primarily by Northern and Western blotting. Because of this, it has been difficult to determine precisely which cell-types express these SRF co-factors. In situ analyses of MRTF-A and MRTF-B will be of great benefit to our understanding of these factors, but histological examination of protein expression will also be important. This is especially true in light of our recent finding that the myocardin factor levels are highly regulated by the ubiquitin-proteasome system, a post-translational pathway.

The Role of Myocardin Factors in Cardiovascular Disease

An important area of study that has received little attention thus far is the role of the myocardin factors in vascular disease. It is well established that down-regulation of SMC differentiation marker genes plays an important role in the pathogenesis of multiple cardiovascular diseases including atherosclerosis and restenosis, and that myocardin factors

are normally responsible for activation of these genes. Recent studies have suggested that estrogen and BMP may exert athero-protective effects via activation of the myocardin factors (73, 77). It will be interesting to directly compare intracellular localization patterns of the MRTFs in healthy and diseased vessels, and to determine whether the expression of the myocardin factors is up- or down-regulated at either the mRNA or protein level. It would be worthwhile to evaluate this in vascular injury models as well.

Preferences of Myocardin Factors for SRF at Specific CARG elements

Another persisting question is whether the myocardin family members discriminate between SRF bound to different CArG elements found in the same or different promoters, and if so, how? Unpublished studies from our laboratory have revealed that mutations to specific CArG elements in the SM α-actin promoter differentially affect transactivation by myocardin and MRTF-A, indicating that MRTF-A and myocardin may interact with separate CArG elements within the same promoter, again supporting the idea that these proteins serve non-redundant roles in SMC-specific transcription. Detailed chromatin immunoprecipitation (ChIP) analyses of all three myocardin family members will be useful for identifying which CArG elements are important for the function of these SRF co-factors, and may aid in further determining whether the myocardin factors bind CArG elements as homo-or hetero-dimers.

Regulation of Myocardin Factors by the Ubiquitin-Proteasome System

Our recent discoveries that the myocardin factors are degraded by the proteasome, that MRTF-A and MRTF-B are stabilized by the LIM-only protein FHL2, and that MuRF3 may act as an E3 ubiqutin ligase for these factors constitute the establishment of a novel pathway for myocardin factor regulation. The description of this pathway has the potential

for far-reaching implications in the field. As mentioned above, little is known regarding the regulation of myocardin factor expression and activity in development and disease, and the importance of myocardin factor protein turnover to normal SMC and cardiomyocyte function has yet to be examined. It is interesting to postulate that UPS-mediated regulation of the myocardin factors may play a significant role in cardiac and smooth muscle lineage determination, as well as in normal vascular function. This is an area that warrants further investigation. It will also be interesting to determine whether the myocardin factors are differentially regulated by the UPS in cardiac and smooth muscle, and whether this has a bearing on the preference of myocardin factors for binding to SRF at promoters specific to each cell-type. Additionally, our demonstration of poly-ubiquitinated MRTF-A in SMC is the first indication that the myocardin factors are ubiquitinated at all. It will be important to determine whether these proteins are also subject to mono-ubiquitination or similar post-translational modifications, and if so, what effect these modifications have on myocardin factor function.

The Role of FHL2 and Related Proteins in Cardiovascular Development

FHL2 is expressed specifically in the heart and vasculature (26) (67) (158) and we have shown that it increases myocardin and MRTF-A activity by protecting them from proteasomal degradation. A logical conclusion based on these data would be that FHL2 plays a critical role in cardiovascular development. However, genetic deletion of FHL2 in the mouse had no observed affect on cardiac or smooth muscle differentiation or function (25). Further interpretation of these results requires additional studies. In particular, it will be important to determine whether other FHL family members function redundantly with FHL2 in the stabilization of myocardin factors. We observed similar expression patterns between

FHL1 and FHL2, and it is possible that FHL1 compensates for the loss of FHL2 *in vivo*. We have recently cloned FHL1 into mammalian expression vectors, and an important future goal is to evaluate the role of FHL1 in the stabilization of myocardin factors in culture. It will be important to evaluate this possibility *in vivo* by tandem deletion of these two genes.

CLINICAL IMPLICATIONS

It was recently estimated by the American Heart Association that over 80 million people in the United States suffer from one or more forms of cardiovascular disease including hypertension, coronary heart disease, stroke, and congestive heart failure. Cardiovascular disease is the number one cause of death in our country, and in 2004 alone, claimed nearly 870,000 lives. This number represents one third of all deaths that year, and nearly 20% of these occurred in individuals below the age of 65 (137). Phenotypic modulation of SMC plays a central role in the pathogenesis of nearly every major cardiovascular disease, and it follows that an increased understanding of the mechanisms governing SMC-specific gene expression will be useful in identifying potential drug targets.

This is especially true in the treatment and prevention of atherosclerosis and post-angioplasty restenosis. As discussed in the opening chapter of this dissertation, SMC that have undergone phenotypic switching contribute to these disease processes in many ways, including through increased proliferation and migration, and increased matrix and inflammatory cytokine production. The work presented in this dissertation has examined several proteins that may be useful drug targets for preventing phenotypic switching of SMC in at-risk patients. For example, the myocardin factors themselves are potent activators of contractile gene expression in SMC, and drugs that increase myocardin factor expression or

stability may reduce the role played by SMC in atherogenesis and restenosis. Drugs designed to increase expression of FHL2 may have a similar effect. Conversely, enzymes that down-regulate myocardin factor activity, including the muscle-specific ring fingers, present potential targets for repression. Finally, our demonstration that the ubiquitin-proteasome system plays a role in the regulation of this transcriptional pathway may have the greatest potential for future use in a clinical setting. Proteasome inhibitors have recently proven efficacious in the treatment of certain cancer-types (66, 132), and based on the data presented here, it is interesting to speculate that they may also have a use in the treatment of cardiovascular disease.

APPENDIX

INTRODUCTION

A major technical limitation to studying the myocardin factors has been a lack of suitable antibodies against these proteins. Accordingly, the majority of studies published to date examining myocardin factor expression patterns have used Northern analyses and *in situ* hybridizations. Likewise, functional studies of the myocardin factors have relied heavily on ectopic expression of epitope-tagged myocardin factor proteins. Because we and others have demonstrated that the myocardin factors are post-translationally regulated, it will be important to also closely examine their protein expression patterns, as these may be different from those of mRNA. Additionally, our understanding of myocardin factor function will be strengthened by further examination of endogenous protein behavior. Each of these pursuits requires that antibodies be generated against each member of this important family of SRF co-factors.

In the completion of this dissertation research, a highly specific monoclonal antibody was generated against MRTF-A that has been successfully used in Western analyses, immunoprecipitations, and indirect immunofluorescence studies. This antibody was generated in collaboration with the Immunology Core Facility at the University of North Carolina at Chapel Hill.

MATERIALS AND METHODS

Generation of Antibody - Multiple regions of MRTF-A were identified that are evolutionally conserved but are not shared with myocardin or MRTF-B. Two of these regions

were selected as templates for antigen production based on many factors including primary sequences, Kyte-Doolittle hyrophilicity plots, Jameson-Wolf antigenic indices, and Emini surface probability plots. Purified peptides with sequences corresponding to either aa 603-611 or aa 658-676 of murine MRTF-A were commercially obtained (Sigma-Genosys). Armenian hamsters were immunized with purified peptide, and hamster sera were screened for immunoreactivity with recombinant and endogenous MRTF-A protein by Western analysis. MRTF-A aintibody producing hamsters were sacrificed, and hybridomas were generated via fusion of B cells harvested from hamsters with P3x63-Ag8.653 murine myeloma cells. Hybridomas were grown in 96 well plates, and antibody-containing supernatants were evaluated by enzyme-linked immunosorbant assay for reactivity with MRTF-A peptide and by Western analysis for immunoreactivity with MRTF-A protein. Monoclonal antibody-producing cell lines were obtained by serial dilution of hybridomas, and MRTF-A antibody was purified using Protein G columns (Sigma). The monoclonal cell line chosen for final antibody purification was derived from mice immunized with MRTF-A peptide 603-611.

Plasmids and Proteins – In vitro translated myocardin, MRTF-A, and MRTF-B protein were generated using Promega's TNT T7 Reticulocyte Lysate System. pcDNA3.1-myocardin, MRTF-A, and MRTF-B were used as templates in these reactions, and were gereous gifts Da-Zhi Wang (University of North Carolina at Chapel Hill).

Immunoprecipitations and Western Analyses – Rat aortic SMC were lysed in RIPA + 0.5% triton X-100, and endogenous MRTF-A was precipitated from lysates using whole sera or purified MRTF-A antibody coupled to G protein beads (Sigma). Precipitants were washed 3 times in RIPA and 1X in tris-buffered saline. All samples were boiled and subjected to

SDS-PAGE, transferred to nitrocellulose and imunoblotted using whole sera or purified MRTF-A antibody at 1:1000. Peroxidase-conjugated Affinipure Goat Anti-Armenian Hamster IgG was used as secondary antibody at 1:10,000 (Jackson Immunoresearch).

Indirect Immunofluorescence – Sphingosine-1-phospahte (1uM) treated SMC were fixed using 3.7% paraformaldehyde and permeabilized with 0.4% triton X-100 in phosphate-buffered saline. Anti-MRTF-A antibody was added at 1:300, and MRTF-A localization was visualized by addition of FITC Anti-Hamster IgG at 1:1000 (Open Biosystems). Filamentous actin was visualized by addition of Texas Red phalloidin at 1:1000 (Invitrogen).

RESULTS

Purified hamster anti-MRTFA antibody detected *in vitro* transcribed and translated MRTF-A protein by Western analysis, and did not cross-react with highly homologous myocardin or MRTF-B (Fig 6.1B). Purified hamster anti-MRTF-A antibody also detected endogenous MRTF-A protein, as previously demonstrated (Fig 2.1). As can be seen in figure 6.1B, this antibody also efficiently immunoprecipitated endogenous MRTF-A from SMC lystate. Subcellular localization of endogenous MRTF-A was examined in S1P-treated SMC by indirect immunofluorescence using purified hamster anti-MRTF-A antibody and FITC Anti-Hamster IgG (Fig 6.1C).

DISCUSSION

The antibody generated here, hamster anti-MRTF-A, has been very useful in the completion of this dissertation research, and will be a valuable tool for future studies of MRTF-A function. Immunization of hamsters with peptides corresponding to the same

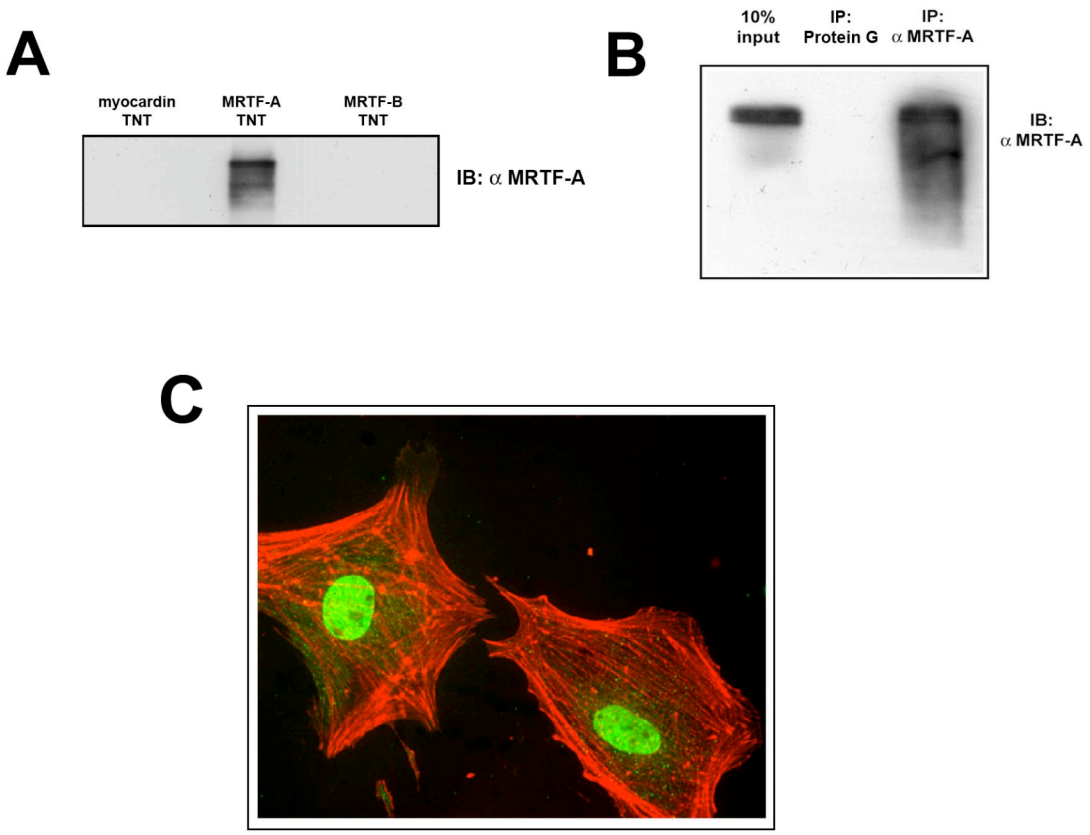

Figure 6.1. Monoclonal hamster anti-MRTF-A antibody. A) In vitro transcribed and translated myocardin, MRTF-A and MRTF-B were subjected to Western analysis and immunoblotted using purified hamster anti-MRTF-A at 1:1000. **B)** Endogenous MRTF-A was precipitated from rat aortic SMC using protein G beads alone or purified hamster anti-MRTF-A coupled to protein G beads. 10% input control and immuoprecipitates were subjected to SDS-PAGE and immunoblotted for MRTF-A using purified hamster anti MRTF-A at 1:1000. **C)** SMC were treated with 1uM S1P for 90m and fixed with 3.7% paraformaldehyde. MRTF-A localization (green) was visualized by addition of purified hamster anti-MRTF-A (1:300) and FITC anti-hamster (1:1000). Actin (red) was visualized using Texas Red phalloidin (1:1000).

regions of myocardin and MRTF-B may yield similarly efficacious antibodies for these proteins. This is a worthwhile pursuit, as its success would open the door to a wide array of studies that have this far been impossible. For example, ChIP analyses (as discussed in the previous chapter) performed in cells ectopically expressing epitope-tagged variants of the myocardin factors will certainly yield important data, but similar studies examining the behavior of endogenous myocardin factors would be much more useful. Likewise, examination of myocardin factor protein expression and localization patterns in development and disease requires that these antibodies be made available. In the meantime, we plan to begin detailed analyses of endogenous MRTF-A using the antibody described here.

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