THE DIAPHANOUS-RELATED FORMINS: DYNAMIC REGULATORS OF SMOOTH MUSCLE CELL-SPECIFIC GENE TRANSCRIPTION

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ABSTRACT

DEAN PATRICK STAUS: The Diaphanous-Related Formins, Dynamic Regulators of Smooth Muscle Cell-Specific Gene Transcription
(Under the direction of Christopher P. Mack, PhD)

We and others have previously shown that RhoA-dependent actin polymerization stimulates SMC-specific gene transcription by promoting the nuclear accumulation of the myocardin-related transcription factors (MRTF)-A and -B. Very little is known about the downstream RhoA effectors that mediate this response, and the goal of the studies described herein was to define the role of the diaphanous-related formins (DRFs) in regulating smooth muscle cell (SMC) differentiation. The DRFs mDia1 and mDia2 are highly expressed in cultured SMCs and in tissues containing a high smooth muscle component. Activation of mDia1 or mDia2 by RhoA stimulated actin polymerization, MRTF nuclear accumulation, and SMC-specific gene transcription. Interestingly, we found that phosphorylation of the Diaphanous Autoregulatory Domain (DAD) by Rho-kinase also stimulated mDia2 activity and SM-marker gene expression. Knockdown of mDia1/2 using siRNA significantly attenuated expression of numerous SM-marker genes in primary aortic SMCs. While we originally attributed these findings to the regulation of cytoplasmic actin dynamics by the DRFs, recent evidence linking nuclear globular (G)-actin to MRTF nuclear export led us to investigate a possible role for the DRFs in the nucleus. We found that mDia2, but not mDia1 or FHOD1, accumulated in the nucleus following treatment with leptomycin, an inhibitor of Crm-1 dependent nuclear export. Deletion and mutation analyses identified nuclear

localization sequences (NLS) in the core formin homology 2 (FH2) domain and extreme N-terminus, and a leucine-rich nuclear export sequence (NES) was identified in the C-terminus of mDia2. Importantly, mDia2 variants that were excluded from the nucleus did not stimulate SMC-specific gene transcription and MRTF-B nuclear accumulation as well as wild-type mDia2. Taken together, these data support a model in which mDia2 activity in the nucleus and cytoplasm depletes cellular G-actin pools resulting in MRTF nuclear accumulation and activation of SMC-specific gene transcription.

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LIST OF ABBREVIATIONS

DAD: *Diaphanous Autoregulatory Domain*

DID: Diaphanous Inhibitory Domain

DRF: Diaphanous-Related Formin

FH: Formin Homology

G-actin: Globular actin

GBD: GTPase Binding Domain

MHC: Myosin Heavy Chain

MRTF: Myocardin-Related Transcription Factor

ROCK: Rho kinase

S1P: Sphingosine 1-Phosphate

SM: Smooth Muscle

SMC: Smooth Muscle Cell

SRF: Serum Response Factor

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Vascular Development

The vascular system encompasses a complex network of capillaries, arteries and veins to efficiently deliver oxygen and nutrients to all bodily tissue. Since oxygen diffusion is limited to approximately 100-200 μM, vasculature formation begins very early in development [1]. Vasculogenesis is the earliest process in blood vessel formation and is characterized by the differentiation of vascular endothelial cells from mesodermal angioblasts (figure 1.1A) [2] . These cells form primitive hollow tubes that give rise to the vascular plexus, which serves as a scaffold for future circulation [3]. Shortly thereafter, new capillaries sprout from preexisting vessels in a process called angiogenesis [4]. Since this vasculature remains immature and poorly functional, additional vascular remodeling is required to meet the high metabolic needs of the embryo.

An increase in blood flow and pressure within these primitive vascular networks likely triggers the recruitment of pericytes and smooth muscle cells (SMCs) by the endothelium. These supportive cells enable the vasculature to withstand increases in hemodynamic forces that would otherwise induce vessel rupture or regression. Platelet-derived growth factor B (PDGF-B) is produced by the endothelium during this process, and acts as a potent stimulator of SMC migration and proliferation. Indeed, vessels in PDGF-B-deficient mice exhibit failed vascular recruitment of pericytes and SMCs, and these animals

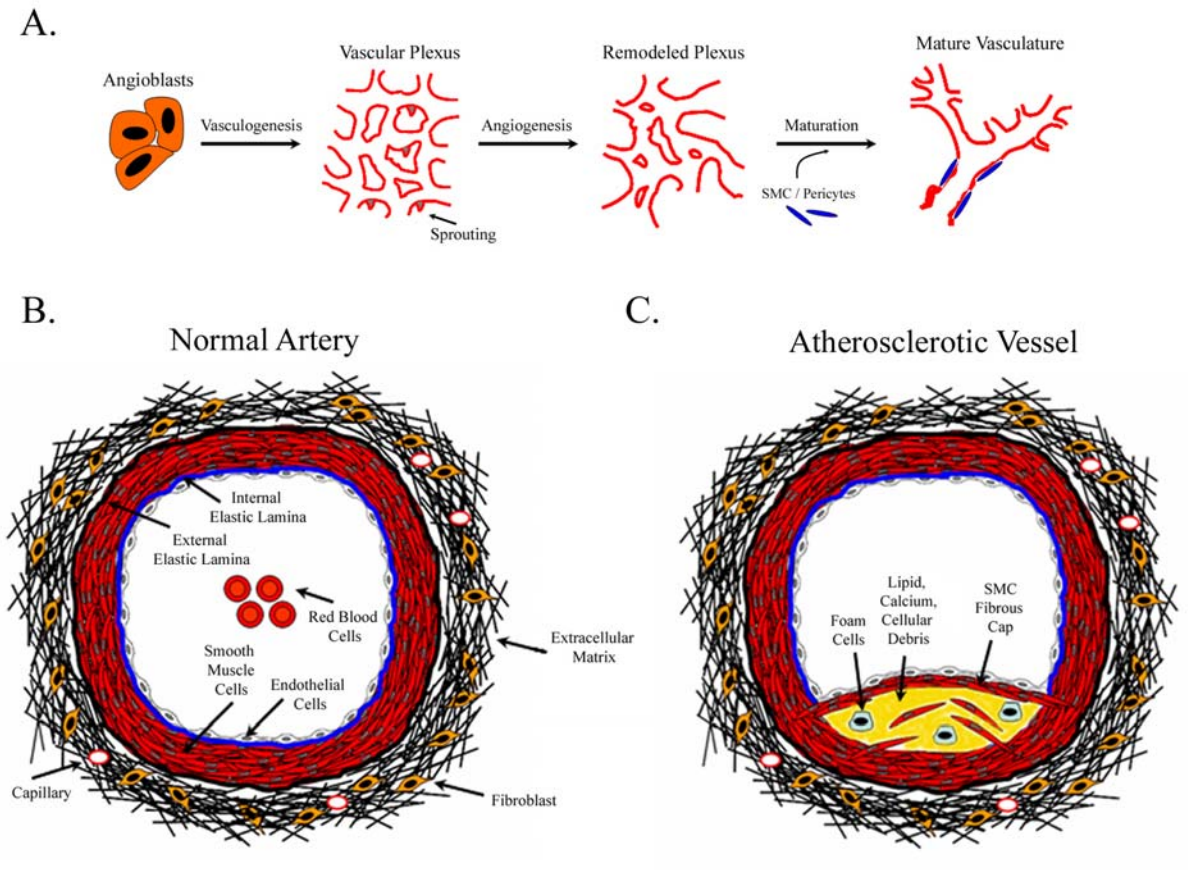

Figure 1.1. Vascular Development and the role of SMCs in atherosclerosis. A) Mesodermal angioblasts give rise to the vascular plexus through the process of vasculogenesis. Angiogenesis leads to vascular remodeling and further vessel maturation requires the recruitment of pericytes and SMCs. **B)** Normal artery consisting of three main layers; intima, media and adventitia. The intima is composed of a single layer of endothelial cells and is separated from the media by the internal elastic lamina. SMCs are the main component of the media and are critical for maintaining blood flow and vessel tone. The external elastic lamina is the boundary between the media and adventitia. This adventitia is primarily derived from extracellular matrix and fibroblasts. **C)** SMCs play atherogenic roles by migrating and proliferating into the vessel lumen. They further aid in plaque development by enhancing the deposition of lipids (as foam cells), calcium, extracellular matrix and inflammatory cytokines in the intima. SMC can also have a protective role in atherosclerosis by contributing to the fibrous cap, which may help to stabilize the lesion by preventing rupture and subsequent thrombosis.

die in early gestation due to multiple capillary microaneurysms [5, 6]. In addition to providing physical support, the medial SMC layer will also facilitate vascular contractility to dynamically regulate blood flow and pressure (figure 1.1B). The adventitial layer, which mainly consists of fibroblasts and extracellular matrix, is recruited to provide additional metabolic and structural support for larger vessels.

It is worthy of note that SMCs can differentiate from multiple distinct progenitor populations during development. While SMCs have traditionally been regarded as mesenchymal in origin, genetic fate mapping has identified at least eight different origins of vascular SMCs [7]. These regions include: the neural crest, which gives rise to SMCs of the pharyngeal arch and ascending aorta; the secondary heart field, which gives rise to SMCs of the pulmonary trunk and base of the aorta; and the proepicardium, which gives rise to coronary artery SMCs. Other identified SMC origins include somites, stem cells, mesoangioblasts, splanchnic mesoderm and mesothelium (see [8] for a comprehensive review).

Once integrated into the vasculature, these different SMC populations, although morphologically indistinguishable, can exhibit distinct characteristics. For example, they display spatial separation; while SMCs derived from separate progenitors can occupy the same vessel, they rarely intermix within vessels. Additionally, they can respond differentially to extracellular agonists [9]. These distinctions indicate that variance between SMC origins has clear functional consequences in the developed organism. Indeed, SMC progenitors were recently discovered within the walls of adult arteries, suggesting that differentiation of SMCs may not be exclusive to the developing embryo [10]. Identifying the signaling mechanisms and environmental cues that regulate differentiation of distinct SMC

progenitors will be essential to determine the contribution of each subtype to the vascular network.

Smooth Muscle Cells in Vascular Disease

Differentiated SMCs proliferate at a very low rate, are mainly non-migratory, and are characterized by the expression of multiple contractile-associated proteins, including SM22, SM α-actin, SM myosin heavy chain (MHC), vinculin, calponin, smoothelin and aortic carboxypeptidase-like protein (ACLP) [11]. Unlike skeletal and cardiac muscle, smooth muscle is not terminally differentiated; rather, SMCs exhibit a specialized plasticity that allows them to revert to a more proliferative and synthetic state. This unique process of phenotypic switching has been closely associated with the vascular diseases atherosclerosis and restenosis.

Atherosclerosis is the number one cause of illness and death in the U.S. as determined by the National Heart, Lung, and Blood Institute. Although the etiology of atherosclerosis is not completely understood, diets high in cholesterol and fat, cigarette smoking, high blood pressure and diabetes have been closely linked to its development and progression [12]. During atherosclerosis, a plaque develops within the intimal layer of the vessel as a result of interactions between many cells, including endothelial cells, SMCs, lymphocytes and monocytes [13]. As this inflammatory lesion progresses in size and severity, it can occlude the arterial lumen, resulting in myocardial infarction or stroke. Early atherosclerotic lesions are often described as “fatty streaks”, due to an accumulation of lipid-rich macrophages and lymphocytes within the vessel wall [14]. Late-stage atherosclerotic lesions are characterized by an abundance of necrotic debris, SMCs, and extracellular matrix

proteins (figure 1.1C). Interestingly, many studies have shown that SMCs within atherosclerotic lesions, which have invaded the arterial intima in response to various extracellular cues, are phenotypically distinct from normal medial SMCs [15-17]. These phenotypically switched intimal SMCs contribute to the progression of the lesion by 1) depositing extracellular matrix within the neointima; and 2) endocytosing lipid deposits to form foam cells [18, 19]. However, intimal SMCs can also serve a protective function by forming a fibrous cap over the plaque, which acts to stabilize the lesion by preventing rupture and subsequent thrombosis.

Atherosclerotic blockage is most commonly treated using balloon angioplasty and stent placement. In brief, a balloon attached to a catheter is placed over the plaque and inflated to increase lumen diameter. To prevent closure of the artery after catheter removal, a stent (wire mesh tube) is inserted. While this procedure temporarily relieves vessel occlusion, approximately 30-50% of the 400,000 angioplasties performed each year will undergo restenosis, or re-narrowing of the artery [20]. The mechanical injury induced by stent placement, especially to the endothelium, triggers SMC phenotypic modulation (dedifferentiation, migration and proliferation) leading to intimal hyperplasia and luminal narrowing [21]. While it is well established that SMCs play a critical role in atherosclerosis and restenosis, their contribution to other pathologies, such as cancer, hypertension and asthma, is less clear.

Extracellular Cues that Regulate SMC Growth and Differentiation

The differentiation state of SMCs is influenced by many environmental cues, including growth factors, contractile agonists, extracellular matrix proteins, endothelial cell

interactions, mechanical forces, and vascular injury (reviewed in [22]). PDGF is a growth factor produced by platelets, macrophages and endothelial cells that potently stimulates SMC migration and proliferation *in vitro* and *in vivo,* while it dramatically suppresses expression of multiple SM-specific marker genes [23, 24]. These cellular responses require PDGF-β receptors on the SMC surface, and antibody-mediated blockage of these receptors reduces intimal SMC invasion in an atherosclerotic mouse model [25]. Taken together, these data suggest that PDGF stimulates SMC growth and migration by affecting phenotypic modulation.

Conversely, other growth factors have been shown to promote SMC differentiation. For example, transforming growth factor (TGF)-β is a potent stimulator of SM-specific marker gene transcription [26-28]. Genetic deletion of TGF-β or its receptors results in early embryonic lethality, partially due to defects in vascular development [29-31]. Furthermore, TGF-β is required for the upregulation of SM marker genes in SMC precursors co-cultured with endothelial cells [32]. Interestingly, new agonists have been identified that are capable of inducing both SMC differentiation *and* proliferation, suggesting these processes are not mutually exclusive. Our laboratory previously showed that Sphingosine-1-Phosphate (S1P) can increase both SM α-actin expression and SMC proliferation by activating the RhoA GTPase and ERK MAP kinase, respectively [33]. Recent studies have shown that the differential regulation of SMC biology by S1P is accomplished through the activation of different S1P receptors. S1P2 receptor activation elicits SMC differentiation, whereas the S1P1/3 receptors facilitate SMC proliferation and phenotypic modulation *in vitro* and *in vivo* [34, 35].

Several other less extensively studied cues can also modulate SMC phenotype. Angiotensin II and arginine vasopressin are potent contractile agonists that increase expression of SM α-actin, myosin heavy chain (MHC), vimentin, and tropomyosin [36]. Extracellular matrix signaling can also regulate SMC differentiation. Culturing SMCs on fibronectin decreases SM α-actin levels, while SMCs plated on matrigel exhibit a more differentiated morphology than those grown on tissue culture plastic [37, 38]. Furthermore, a recent study by Orr *et al.* demonstrated that collagen I located within the arterial wall enhances SM α-actin and SM-MHC expression more potently than collagen I found within atherosclerotic lesions [39]. Finally, mechanical forces can also determine the phenotypic state of SMCs. It has previously been shown that blood flow commencement correlates with vascular SMC investment, and that exposure to stretch increases expression of SM-marker genes [40, 41]. This evidence suggests that arterial shear stress may play important roles in determining the phenotypic status of vascular SMCs.

CArG Boxes are Required for SMC-Specific Gene Transcription

Cellular differentiation is ultimately regulated at the level of gene transcription. Curiously, the DNA element responsible for effecting SMC-specific gene transcription was first identified in the ubiquitously expressed early growth gene, *c-fos* [42]. An enhancer was discovered within the *c-fos* promoter that was regulated by serum and growth factor stimulation. Further mapping studies identified a 23 base pair element (aggatgtccatattaggacatct) possessing dyad symmetry (underlined regions) that was critical for this response; thus, this region was later named the serum response element (SRE) [43].

Shortly after this discovery, an element was identified in the cardiac α-actin gene that resembled the SRE but lacked its dyad symmetry; this sequence was termed the CArG box ($CC(A/T)_6GG$) [44]. Mutations within the CArG boxes of cardiac α-actin were shown to attenuate its expression, demonstrating the functional importance of these elements [45]. Interestingly, additional promoter analysis studies have identified CArG boxes in nearly all SMC-specific genes [46]. *In vitro* studies demonstrated that these CArG boxes are essential for transcriptional activation of numerous SM-specific genes in cultured SMCs [11, 47, 48]. Furthermore, *in vivo* studies utilizing *lacZ* transgenes driven by different SM promoters demonstrated that mutation of CArG elements within SM-MHC, SM22α and SM α-actin results in dramatic promoter inactivation [49, 50]. These studies indicate that the integrity of CArG boxes within SMC-specific genes is essential to their expression. There are currently more than 60 characterized genes containing functional CArG boxes, all of which are either growth-related or from the skeletal, smooth, or cardiac muscle lineages [51].

Serum Response Factor

Serum response factor (SRF) was first purified from HeLa nuclear extracts by virtue of its interaction with the *c-fos* SRE [52, 53]. SRF is a 62-67 kDa protein that is a member of the MADS box family of transcription factors [54]. The MADS box is located on the N-terminus of SRF, and contains a basic DNA-binding domain and a region predicted to promote protein-protein interactions (figure 1.2A). This region also contains a dimerization domain (DD) that is required for the homodimerization of SRF on CArG boxes (figure 1.2B) [55]. The C-terminus of SRF contains the transactivation domain, which is indispensable for

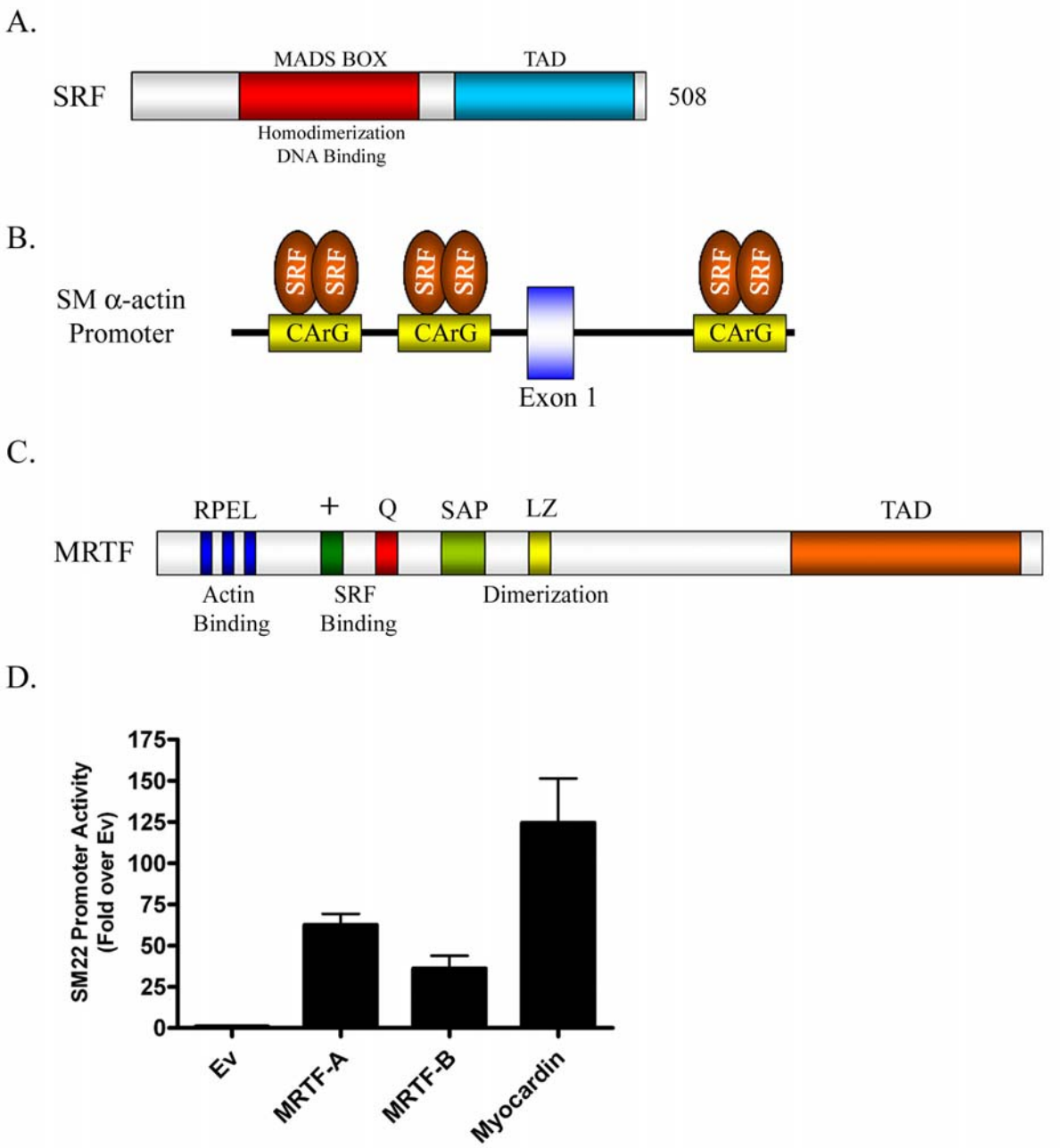

Figure 1.2. Regulation of SMC-specific gene transcription by serum response factor (SRF) and the myocardin related transcription factors (MRTF). A) Domain structure of SRF; transactivation domain (TAD) **B)** SRF (as a homodimer) regulates nearly all SMC-specific genes by binding to CArG elements located in their promoter or intronic regions. **C)** MRTF domain schematic.RPEL, amino acids (AA) RPXXXEL where X is any AA; +, basic region; Q, glutamine rich region; SAP (SAF-A/B, Acinus, PIAS) domain; LZ, leucine zipper-like domain. **D)** 10T1/2 cells were transfected with the SM22-luciferase reporter gene (125ng) in the presence of empty vector (EV) or flag-tagged myocardin family members (125ng). Data compiled from three independent experiments and represented as fold over EV.

its transcriptional activity, and many conserved phosphorylation sites that are extensively modified after serum stimulation [56].

There is strong evidence that SRF is essential to proper embryonic development. Mice with a non-functional SRF allele do not develop past gastrulation due to defects in mesoderm formation [57]. More specifically, several *in vivo* studies suggest that SRF plays an important role in cardiac development. Proepicardial cells derived from quail embryos, which normally give rise to coronary SMCs, exhibit attenuated differentiation in the presence of two different dominant negative SRF constructs [58]. In mice, cardiac-specific conditional mutagenesis or restricted inactivation of SRF results in severe cardiovascular defects and significant reduction of cardiac and SM-marker gene expression [59, 60]. Specific cardiovascular defects include: abnormal heart looping, chamber maturation, and Z-disk organization, failed SMC recruitment to the dorsal aorta, and aberrant cytoskeletal structure in vascular SMCs. In addition, skeletal muscle-specific deletion of SRF results in death during the perinatal period as a result of severe skeletal muscle hypoplasia [61]. Since SRF is ubiquitously expressed and regulates the expression of a number of muscle-specific, cytoskeletal, and early response growth genes, it is likely that additional mechanisms are important in facilitating cell type-specific SRF-dependent transcription [62-65].

To date, multiple SRF-binding partners have been identified, suggesting a model in which SRF-dependent transcription is regulated by interaction with other transcription factors and co-factors. Elk-1, SAP-1, and SAP-2/NET/ERP, the so-called ternary complex factors (TCFs), were the first identified cofactors that linked serum and growth factor stimulation to SRF-mediated activation of the *c-fos* SRE [66]. SRF also interacts directly with the transcription factors GATA-4 and Nkx2.5 to modulate cardiomyocyte

differentiation, while interaction with MyoD and myogenin promotes skeletal muscle-specific gene transcription [64, 67-69]. Finally, the cysteine-rich LIM-only proteins CRP1 and CRP2 are also capable of regulating SRF-dependent transcription, likely by organizing SRF in complex with other transcription factors or chromatin remodeling factors [70, 71].

Myocardin Family of Transcription Factors

The identification of the SRF cofactor myocardin greatly advanced our understanding of the molecular mechanisms governing SMC-specific gene transcription. Myocardin was originally identified in a bioinformatic screen for novel cardiac-restricted genes, but was later found to be highly expressed in adult and developing smooth muscle [72]. Several reports have utilized dominant negative myocardin variants or siRNA to demonstrate that myocardin is a potent stimulator of SMC-specific transcription [73-76]. The *in vivo* importance of myocardin is illustrated by the finding that germline deletion of myocardin is embryonic lethal and results in a complete lack of vascular SMCs [77]. Interestingly, cardiac development occurred normally, with no observable defects in heart looping or chamber formation.

While it is clear that myocardin is important to SMC differentiation, an accumulating amount of data question whether it is essential for SMC lineage determination in all SMC subtypes. For example, SM22 and SM α-actin are detected in SMC tissue as early as E9.5, even though myocardin expression is not observed until E12.5 [75, 78]. Furthermore, forced expression of myocardin in embryonic stem cells stimulates expression of many, but not all, SMC-specific genes [79]. Finally, myocardin -/- stem cells can still give rise to vascular SMCs *in vivo* [79, 80]. These data clearly indicate that factors other than myocardin can

regulate SMC differentiation during vascular development. Indeed, a bioinformatic screen for myocardin homologs led to the identification of the myocardin-related transcription factors A and B (MRTF-A, MRTF-B) [81].

The overall amino acid conservation between myocardin, MRTF-A, and MRTF-B is only 35%, but the N-terminal basic, glutamine (Q)-rich, and SAP domains share more than 60% amino acid identity (figure 1.2C). A critical seven-residue sequence called the B1 domain is located between the basic and Q-rich domains and is required for SRF binding [82]. The conserved SAP domain has not been well characterized *in vivo,* but other SAP domains have been shown to regulate nuclear organization and chromosomal dynamics [83]. The most distal N-terminal domains shared among the myocardin family members are a series of RPEL motifs that mediate actin monomer binding. C-terminally located are the leucine zipper (LZ) motif and transactivation domain (TAD), which mediate dimerization and transcriptional activation, respectively [84]. Unlike myocardin expression, which is cardiomyocyte- and SMC-specific, MRTF-A and MRTF-B are more widely expressed, but are particularly abundant in cultured aortic SMCs and SMC-rich tissues [33, 81, 85, 86].

We and others demonstrated that MRTF-A can potently stimulate transcription of SMC-specific genes (figure 1.2d) [33, 85-87]. Furthermore, a dominant negative of MRTF-A inhibits transcription of SM α-actin, SM22 and SM-MHC in primary aortic SMCs [86, 88]. Germline deletion of MRTF-A in mice results in reduced SRF-dependent gene transcription in mammary myoepithelial cells, providing additional evidence for an *in vivo* role for MRTF-A in regulating SMC-specific gene transcription [89, 90]. Given the widespread expression pattern of MRTF-A and the potency with which it activates SMC gene transcription, the restricted phenotype of these animals is rather surprising. However,

the close functional homology and similar expression pattern of MRTF-B suggests that compensation may mask additional *in vivo* roles of MRTF-A.

MRTF-B is the least studied of the myocardin family members. Along with the human homolog MKL-2, MRTF-B enhances promoter activity of several SM marker genes, but less potently than myocardin and MRTF-A (figure 1.2d) [86, 91]. Mice containing a loss-of-function mutation within the MRTF-B gene die at mid-gestation due to multiple cardiovascular abnormalities, including failed differentiation of neural crest-derived SMCs within the branchial arch [92, 93]. These data suggest that MRTF-B is important in the differentiation of at least some SMC subsets. Unfortunately, the redundancy observed amongst the myocardin family members, as well as the early lethality associated with the myocardin and MRTF-B knockouts, hampers dissection of lineage-specific roles for each protein. Nonetheless, further characterization of the molecular mechanisms that regulate the myocardin family members will be essential to better understanding their role in regulating SMC phenotype.

The Rho Family of GTPases

As discussed earlier, it is clear that a variety of extracellular cues are capable of stimulating SMC differentiation, and identifying the signaling pathways that sense and integrate these signals has been a major focus of our laboratory. Indeed, we and others have identified the small GTPase RhoA as an important regulator of SMC-specific gene expression. RhoA belongs to the family of Rho GTPases, which also includes Rac1 and Cdc42. These proteins act as molecular switches that activate downstream effector proteins to regulate cellular processes such as cytoskeletal remodeling, membrane trafficking and cell

adhesion (see [94, 95] for reviews). Activation of RhoA, Rac and Cdc42 induces the formation of dynamic actin structures, including stress fibers, lamellipodia or filopodia, respectively. While the Rho GTPases have traditionally been studied in the context of migration and adhesion, it has become clear they are also critical regulators of gene transcription [96-98].

Rho GTPases cycle between a GDP- and GTP-bound state, but can only interact with downstream effectors when bound to GTP (see [98] for a comprehensive review). Although they have some intrinsic GTP hydrolase activity, it is tightly controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs activate GTPases by promoting exchange of GDP for GTP binding, while GAPs inactivate by enhancing GTP hydrolysis. GTP binding induces a conformational change in the conserved switch I and II regions, subsequently exposing the effector loop domain that interacts with downstream target proteins. Sequence variation in the effector loop determines the specific effectors bound by different GTPases. Rho proteins are further regulated by GDP-dissociation inhibitors (GDIs), which inhibit GTPase activity by preventing nucleotide exchange [99].

Our specific interest in RhoA as a determinant of SMC differentiation arose from studies by the Treisman laboratory linking RhoA signaling to SRF-dependent transcription. These studies demonstrated that activation of RhoA by serum, lysophosphatidic acid (LPA), or G-protein coupled receptor agonists induced transcription of a modified *c-fos* promoter lacking the TCF binding Ets domain [100-102]. Interestingly, increases in promoter activity were dependent on SRF and RhoA-mediated actin polymerization, but were independent of the TCF family of transcription factors. Since these studies were conducted with artificial *c-*

fos promoters in immortalized cells, the physiological importance of this signaling cascade in regulating SRF-dependent transcription remained unclear.

Our laboratory subsequently demonstrated that RhoA signaling is an important modulator of SMC-specific gene expression [103]. Inhibition of RhoA activity by dominant negative RhoA (N19) or RhoA-specific toxin C3 transferase significantly decreased transcription of numerous SM marker genes in primary rat aortic SMCs. Furthermore, a constitutively active variant of RhoA (L63) potently increased SMC-specific gene transcription, but only modestly activated the *c-fos* promoter. This increase in promoter activity was inhibited by the actin depolymerizing agent, latrunculin B (LB), further supporting a role for actin polymerization in SRF-dependent transcription. RhoA signaling has now been implicated in the regulation of SMC-specific transcription induced by various extracellular stimuli, including TGF-β, thrombin, Ca2+ currents, and mechanical force [104-108]. In addition, *in vivo* studies demonstrated that RhoA activity is required for differentiation of isolated proepicardial cells into SMCs [109]. Since activation or inhibition of RhoA has little effect on early growth gene expression, activation of RhoA-dependent signaling pathways may regulate expression of SMC-specific genes without effecting transcription of other SRF-dependent genes.

Actin Dynamics Regulate Cellular Localization of the MRTFs

We have shown that stimulation of SRF-dependent SMC gene transcription by RhoA requires actin polymerization, in excellent agreement with other studies [62, 103, 110]. However, a RhoA target that interacted with SRF had not been described, making it difficult to determine the precise mechanisms by which RhoA modulates SMC-specific transcription.

Recently, an elegant study by Miralles *et al.* shed light on this question [88]. The authors found MRTF-A to be predominantly cytoplasmic in serum-starved NIH3T3 cells. Upon serum treatment, however, MRTF-A rapidly localized to the nucleus, accumulated in an SRF-DNA complex, and potently stimulated transcription of a modified *c-fos* promoter. Nuclear localization was blocked using C3 transferase or latrunculin B, implicating RhoA and actin polymerization in the nuclear accumulation of MRTF-A. MRTF-A was subsequently shown to be required for RhoA signaling to SRF, as inhibition of MRTF-A by a dominant negative or RNAi blocked RhoA- and serum-mediated activation of SRF-dependent transcription [87]. To determine the importance of this pathway in regulating SMC phenotype, we conducted similar studies in SMCs and found that RhoA-mediated activation of SMC-specific gene transcription also required MRTF-A [33, 86]. Taken together, these data suggest that MRTF-A links RhoA-actin signaling to SRF.

While this evidence clearly indicated that nuclear accumulation of MRTF-A is enhanced by actin polymerization, the molecular mechanisms regulating this process remained elusive. These were elucidated by the finding that destruction of MRTF-A's RPEL motifs results in nuclear sequestration of MRTF-A under serum starved conditions [88]. The RPEL motifs were subsequently identified as globular (G)-actin binding interfaces, and RhoA-dependent increases in actin polymerization reduces the actin-MRTF complex, leading to nuclear accumulation of MRTF-A (figure 1.3). These and other studies suggest that under serum-starved conditions, when cellular G-actin levels are high, binding of actin to the RPEL motifs prevents nuclear localization of MRTF-A by masking a nuclear localization signal [88, 111]. However, a functional nuclear localization sequence for MRTF-A has yet to be identified, suggesting an alternative mechanism may determine

MRTF-A distribution. Sequence divergence within the RPEL motifs is likely responsible for the differing localization patterns of the myocardin family members [112]. For example, G-actin has a lower affinity for the RPEL motifs of myocardin than for those of MRTF-A, accounting for the constitutively nuclear localization of myocardin. In addition, we have shown that cytoplasmic retention of MRTF-B is greater than MRTF-A in the presence and absence of serum, suggesting that MRTF-B may possess the highest G-actin binding affinity of the three myocardin family members [113]. Taken together, the current model of SMC differentiation suggests that activation of RhoA by a variety of extracellular cues leads to actin polymerization and subsequent reductions in cytoplasmic G-actin pools. This event promotes localization of MRTF-A/B to the nucleus resulting in SRF binding and activation of SMC-specific gene transcription (figure 1.3)

Signaling Downstream of RhoA

While considerable evidence has demonstrated RhoA to be an important regulator of SMC differentiation, the elucidation of downstream signaling pathways that modulate this process has received little attention. In the GTP-bound state, RhoA interacts with a wide variety of effectors, including Rho kinase (ROCK), diaphanous proteins 1 and 2 (Dia1 and Dia2), protein kinase N (PKN), citron kinase, rhophilin, and rhotekin. Since the effect of RhoA on SMC-specific gene transcription is at least partially dependent on actin remodeling, studies of Rho-mediated differentiation have focused primarily on ROCK, given its ability to stimulate actin polymerization, stress fiber formation, and cell contractility (especially in SMCs) [114, 115]. ROCK promotes actin polymerization by activating LIM-kinase, which subsequently inhibits cofilin-mediated F-actin disassembly [116]. Interestingly, we and

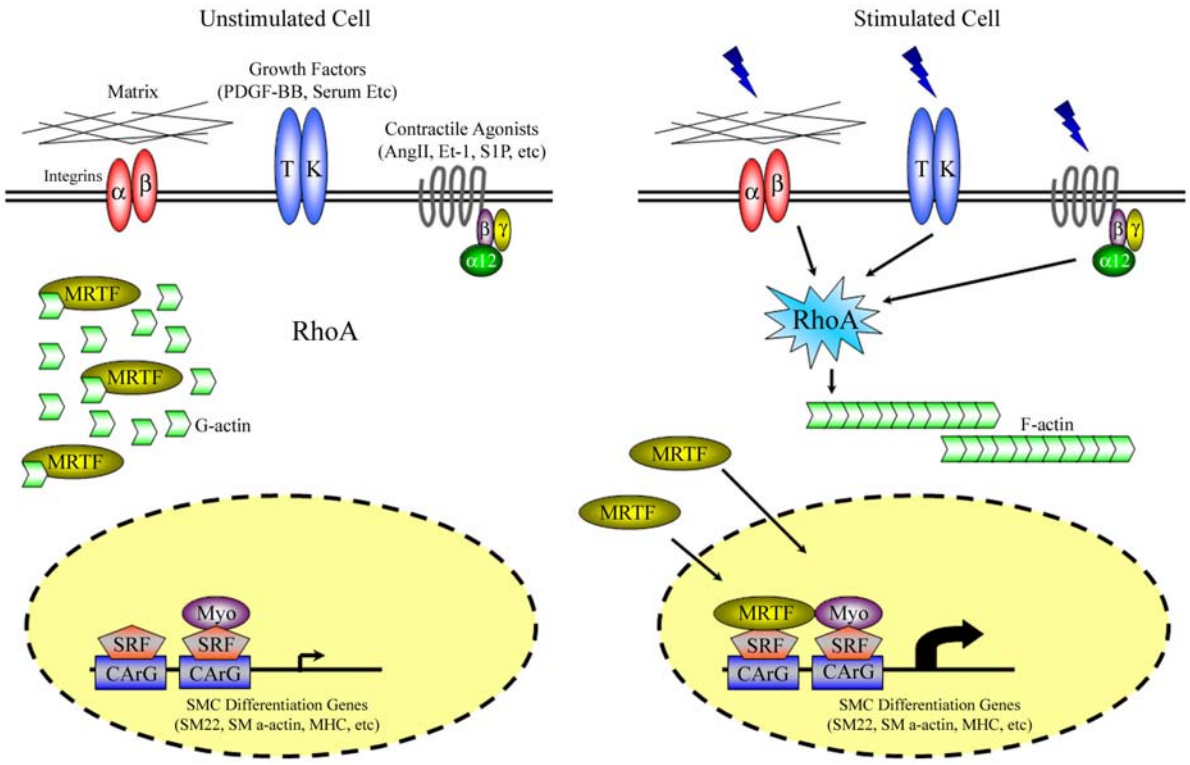

Figure 1.3. Schematic of signaling cascades that regulate SMC-specific gene transcription. In an unstimulated (serum starved) cell, elevated cytoplasmic G-actin levels sequester MRTF in the cytoplasm. The lack of MRTF nuclear localization results in low levels of SMC-specific gene transcription. Activation of RhoA by a variety of extracellular stimuli (blue lighting bolts), stimulates actin polymerization and subsequent reductions in cellular G-actin pools. This leads to nuclear localization of MRTF, where it binds SRF and enhances transcription of SM-marker genes.

others have demonstrated that the ROCK inhibitor Y-27632 only partially inhibits SM-specific gene expression, suggesting that additional RhoA effectors are involved in SMC differentiation [33, 117].

Two other RhoA effectors known to stimulate actin polymerization are Dia1 and Dia2 [118-120]. These proteins belong to the subfamily of diaphanous-related formins (DRFs), which also includes Dia3 and the Formin Homology Domain Protein (FHOD1) (see [121-123] for reviews). The DRFs are identified by two highly conserved formin homology (FH) domains, a GTPase binding domain (GBD) that interacts with Rho family GTPases, and a diaphanous autoregulatory domain (DAD) (figure 1.4A). The dimerization domain (DD) and coiled-coil region both mediate dimerization of the N-terminal region of the DRFs, although the physiological importance of this dimerization is still unclear. The molecular mechanisms that control DRF activity have been fairly well described. In the inactive state, the DRFs are inhibited by an intramolecular interaction between the C-terminal DAD and the N-terminal diaphanous inhibitory domain (DID) (figure 1.4B) [124, 125]. High affinity binding of GTP-RhoA to the GBD disrupts the DAD-DID interaction, thereby exposing the catalytically active FH1/FH2 region to stimulate actin polymerization. Interestingly, several studies have identified Dia1 and Dia2 as binding partners for multiple GTPases. For example, Cdc42 and Rif bind Dia2 to induce filopodia formation, while RhoB-Dia2 signaling governs endosomal trafficking [126-128]. The association of DRFs with multiple GTPases likely explains their ability to regulate a diverse group of actin-dependent phenomena, including membrane ruffling, cell migration, cytokinesis, mitochondrial distribution, cell polarity, stress fiber formation, and cell adhesion. A more in depth

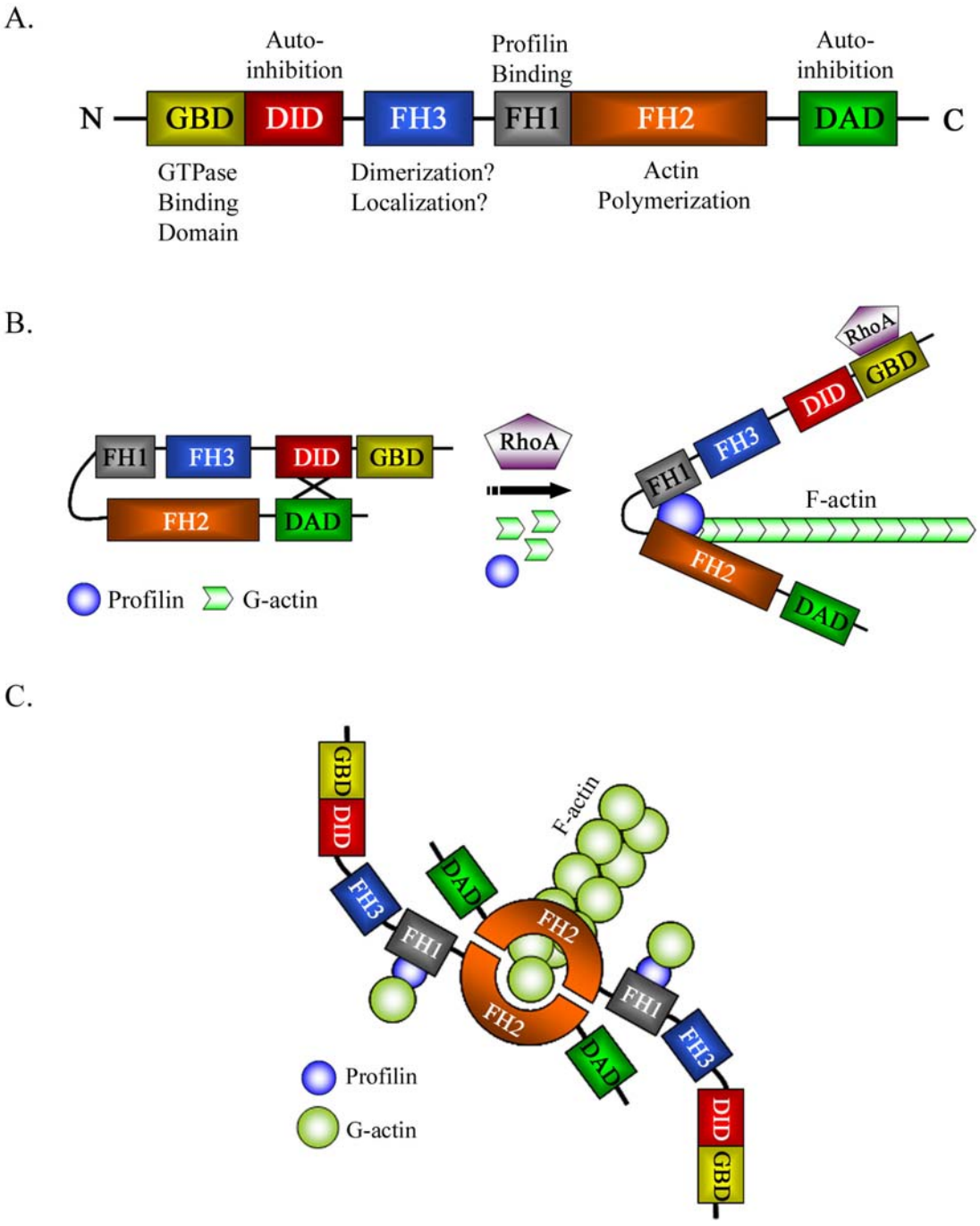

Figure 1.4. Regulation of actin dynamics by the diaphanous-related formins (DRF), mDia1 and mDia2 A) Domain structure of the DRFs. GBD, GTPase Binding Domain; DID, Diaphanous Inhibitory Domain; FH, Formin Homology; DAD, Diaphanous Autoregulatory Domain. **B)** mDia1/2 are autoinhibited by an interaction between the DID and DAD, which sterically hinders the catalytic function of the FH2. Binding of RhoA (or other GTPases) alleviates autoinhibition resulting in stimulation of actin polymerization by the FH2 domain. The binding of profilin to the FH1 domain enhances actin filament formation. **C)** Current schematic models suggest that the FH2 dimerizes to form a flexible "donut", which binds actin monomers and allows the formin to "stair step" along the growing filament.

discussion on the role of the DRFs in these processes is beyond the scope of this dissertation, but the reader is directed to [123] for an excellent review.

Further characterization of the DRFs will require identifying the molecular mechanisms by which formins nucleate actin. Early *in vivo* studies of the formin Bni1 determined that the ~400 residue FH2 domain was indispensable for actin cable assembly in yeast [129, 130]. Subsequent studies found that the FH2 directly nucleated actin, and that polymerization occurred at the barbed ends [131]. Furthermore, an interaction between the proline-rich FH1 domain and the actin-binding protein profilin accelerated actin filament elongation [132]. Unlike other actin nucleators, formins remain continually associated with the filaments during elongation, a property that is characteristic of all formins described to date [133-135]. Current schematic models suggest that the FH2 dimerizes to form a flexible "donut", which binds actin monomers and allows the formin to "stair step" along the growing filament (figure 1.4C) [136]. Indeed, mutation of conserved tryptophan residues within the FH2 required for dimerization inhibits formin-mediated actin polymerization [137]. The processive nature of formins facilitates rapid actin polymerization. As a formin adds actin monomers to the barbed end of the filament, it simultaneously inhibits the binding of capping proteins that would otherwise block filament extension. Although less extensively studied, formins also regulate microtubule-dependent processes. Several studies indicate that Dia1 and Dia2 associate with stable microtubules and these associations may play critical roles in cytokinesis and cell migration [127, 138-140].

Regulation of MRTFs by Nuclear Actin

Evidence linking nuclear actin to the regulation of gene transcription was first reported in 1984 by two separate groups [141, 142]. However, these studies were initially rebuffed on suspicion of experimental contamination by abundant cytoplasmic actin. Skepticism grew when reagents used to visualize actin filaments in the cytoplasm, such as phalloidin, did not identify similar structures within the nucleus. More than 20 years later, it is now widely accepted that actin exists in the nucleus and is involved in regulating gene transcription by interacting with ATP-dependent chromosomal remodeling factors, heterogeneous nuclear particles of Ribouclear protein (RNP), and all three RNA polymerases (see [143] for a review). Whether actin is monomeric or polymeric in these complexes remains unknown, primarily because tools to identify polymerized nuclear actin are lacking. However, nuclear actin filaments were identified in the nuclear pore complexes of amphibian oocytes, and immunochemical studies have had some success in identifying other nuclear actin structures [144, 145]. Interestingly, a recent study by McDonald *et al.* identified a dynamic pool of polymeric GFP-β-actin within the nucleus by using fluorescence recovery after photobleaching (FRAP) [146].

The cytoplasmic binding of G-actin was originally believed to sequester MRTF-A in the cytoplasm, but recent observations suggest that nuclear translocation of the MRTFs is mechanistically more complicated than originally thought. For example, a mutant G-actin has been described that can stimulate MRTF-A nuclear translocation independently of its effects on actin treadmilling, indicating that G-actin may be more directly involved in MRTF-A transport [147]. Indeed, Vartiainen *et al.* recently demonstrated that G-actin binding is required for nuclear export of MRTF-A and that export, and not import, is the

major limiting step in regulating MRTF-A nuclear accumulation [148]. Although an interaction with actin did not prevent the association of MRTF-A with SRF-target genes, its activity was inhibited. These data suggest that nuclear actin dynamics may be an important determinant in regulating MRTF-A subcellular localization. Taken together, these lines of evidence indicate that MRTF-A nuclear localization and SRF-dependent transcription are controlled by communication between nuclear and cytoplasmic actin pools. Knowledge of RhoA-dependent signaling pathways that regulate nuclear actin dynamics is currently lacking, and will likely be a prosperous area for future research.

Objective of this dissertation research

Further elucidation of the signaling pathways that govern SMC-specific gene transcription will be essential to our understanding of SMC differentiation and its contribution to diseases such as atherosclerosis, and restenosis. Although it is clear that RhoA plays a critical role in regulating SM-marker gene expression, the downstream signaling cascades that mediate this regulation remain poorly understood. The objectives of this dissertation were to (1) determine whether the RhoA effectors Dia1 and Dia2 are important determinants of SMC phenotype, (2) identify the mechanism(s) that control the activity or activation of Dia1/2, and (3) characterize a potential role of Dia1/2 in regulating subcellular localization of the MRTFs by controlling nuclear actin dynamics.

CHAPTER 2

Diaphanous 1 and 2 Regulate Smooth Muscle Cell Differentiation by Activating the Myocardin-Related Transcription Factors

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ABSTRACT

We have previously shown that smooth muscle cell (SMC) differentiation marker gene expression is regulated by the small GTPase, RhoA. The objective of the present study was to determine the contributions of the RhoA effectors, mouse diaphanous 1 and 2 (mDia1 and mDia2), to this regulatory mechanism. mDia1 and mDia2 are expressed highly in aortic SMC and in a number of SMC-containing organs including bladder, lung, and esophagus. Activation of mDia1/2 signaling by RhoA strongly stimulated SMC-specific promoter activity in multiple cell-types including primary aortic SMC, and stimulated endogenous SM α-actin expression in 10T1/2 cells. Expression of a dominant negative mDia1 variant that

inhibits both mDia1 and mDia2 significantly decreased SMC-specific transcription in SMC. The effects of mDia1 and mDia2 required the presence of SRF and the activity of the myocardin transcription factors and were dependent upon changes in actin polymerization. Importantly, stimulation of mDia1/2 signaling synergistically enhanced the activities of the myocardin-related transcription factors, MRTF-A and MRTF-B, and this effect was due to increased nuclear localization of these factors. These results indicate that RhoA-dependent signaling through mDia1/2 and the MRTFs is important for SMC-specific gene expression in SMC.

INTRODUCTION

A large number of environmental cues including growth factors, contractile agonists, matrix components, and cell-cell interactions have been shown to regulate smooth muscle cell (SMC) differentiation (see [46] for review). However, the signaling mechanisms by which these factors regulate SMC-specific transcription are poorly understood. Expression of most SMC differentiation markers, including SM α-actin, SM myosin heavy chain (SM MHC), SM22, calponin, and telokin requires serum response factor (SRF) binding to one or more conserved CArG ($CC(A/T_6)GG$) cis elements present within their promoters [49, 149-153]. However, because SRF is a ubiquitously expressed transcription factor that also regulates the early response growth genes, c-fos and egr-1 (see [154] for review), it is clear that additional factors are required to differentially regulate these gene programs.

The identification of the myocardin family of SRF co-factors by Wang et al. was an important advance in our understanding of the transcription mechanisms that regulate SMC-specific transcription (see [155] and [84] for reviews). The founding member of this family,

myocardin, strongly enhances SMC differentiation marker gene expression through direct interactions with SRF. Myocardin is expressed specifically in heart and SMC and its genetic deletion leads to embryonic lethality due to failure of SMC differentiation/investment of the developing aorta. The Myocardin-Related Transcription Factors, MRTF-A/MKL-1 and MRTF-B/MKL-2, are also expressed in some SMC sub-types including the aorta, and like myocardin, can strongly activate SMC differentiation marker gene expression [33, 81, 85, 87, 91]. Because the MRTFs are thought to be expressed more widely, their involvement in regulating cell-type-specific gene expression is less clear.

We have previously shown that the small GTPase, RhoA, regulates SM differentiation marker gene expression, and we hypothesize that RhoA acts as an integrator of many of the environmental cues that regulate this process [103]. For example, SMC-specific transcription in primary SMC cultures was stimulated by expression of constitutively active of RhoA (L63), and completely abolished by the RhoA inhibitor, C3 transferase. Neither of these interventions had dramatic effects on c-fos expression, suggesting that this pathway may be important for differentially regulating SRF-dependent growth and differentiation. A number of laboratories including our own have demonstrated that RhoA's downstream effects on the actin cytoskeleton were critical for activation of SRF-dependent transcription [33, 62, 103, 110, 156]. The RhoA effector, Rho-kinase (ROCK), has been the most thoroughly studied in this regard because it has major effects on actin polymerization, stress fiber formation, and contractility (see [94] for review). Interestingly, the ROK inhibitor, Y-27632, only partially inhibits SMC differentiation marker gene expression, strongly suggesting that other RhoA effectors are important [103, 104].

Two other RhoA effectors that have been shown to stimulate actin polymerization are the diaphanous formins 1 and 2 (mDia1 and mDia2) [118-120]. These proteins belong to the subfamily of diaphanous-related formins (DRFs) that also includes mDia3 and FHOD1. Previous studies have demonstrated that the DRFs act as potent actin and microtubule polymerizing factors that regulate a number of processes including cell migration and division (see [121]and [122] for reviews). The DRFs are identified by three highly conserved formin homology (FH) domains, a conserved GTPase binding domain (GBD) that interacts with Rho family GTPases, and a Diaphanous Auto-regulatory Domain (DAD). The molecular mechanisms that control DRF/Dia activity have been fairly well described. In the inactive state, the DRFs are inhibited by an interaction between the DAD and the N-terminal Diaphanous Inhibitory Domain (DID) (See figure 2.1a). High affinity binding of activated RhoA (or another GTPase) to the GBD disrupts the DAD-DID interaction to expose the catalytically active FH1/FH2 region. The precise mechanisms by which mDia1/2 stimulate actin polymerization are not completely understood. However, mDia1/2 seem to promote polymerization from actin filament barbed ends in cooperation with the actin binding protein, profilin [133, 157, 158].

The goal of these studies was to determine the contributions of mDia1 and mDia2 to the regulation of SMC-specific transcription. Our results demonstrate that both of these RhoA effectors are highly expressed in SMC, that they strongly activate SMC-specific transcription, and that their effects are mediated by the myocardin family of SRF co-factors.

METHODS

Plasmids and Reagents

mDia1 and mDia2 cDNAs were generous gifts from Shuh Narumiya (Kyoto University, Japan). All mDia cDNAs were subcloned into flag pcDNA3.1 and/or pEGFP-C3 (Clontech) and include, full length mDia1, full length mDia2, ΔGBDmDia1 (AA 238-1255), ΔGBDmDia2 (AA 257-1171), mDia2DAD (AA1030-1171), mDia1FH1FH2Δ1 (AA567-1182 minus the 20AA from 750-770), EGFP-ΔGBDmDia1, and EGFP-ΔGBDmDia2. Myocardin, MRTF-A, and MRTF-B cDNAs were generous gifts of Da-Zhi Wang (University of North Carolina, Chapel Hill, NC). All myocardin cDNAs were subcloned into flag pcDNA3.1 and pEGFP-C3. The dominant negative MRTF-A was generated by PCR and consists of AA 1-630.

Cell Culture, Transfections, and Reporter Assays

SMCs from rat thoracic aorta were isolated and cultured as previously described [33]. 10T1/2 and A7r5 SMC were obtained from ATCC. SRF -/- embryonic stem cells were a generous gift from Alfred Nordheim (Tubingen University, Germany) and have been previously described [159]. For transfections, cells were cultured in 24 or 48 well plates, maintained in 10% serum, and transfected 24 h after plating at 70-80% confluency using the transfection reagent, TransIT-LT1 (Mirus, Madison, WI), as per protocol. The SM22, SM α-actin, and c-fos promoter luciferase reporter constructs have been previously described [33]. The SM α-actin promoter constructs containing CArG mutations were a generous gift of Gary Owens (University of Virginia) and have been previously described [74]. When transfecting expression constructs (mDia1/2, MRTFs, etc.) the total amount of expression vector in each well was equalized by addition of empty vector. For experiments involving

pharmacological inhibitors, cells were serum starved for 6 h and then treated with Y-27632 (10μM) (Calbiochem, San Diego, CA) or Latrunculin B (0.5μM) (Calbiochem, San Diego, CA) for 24 h before luciferase measurements.

RT-PCR and Western Blots

Tissues were obtained from adult C57/Bl6 mice and confluent dishes of primary rat aortic SMC, A7r5 SMC, and 10T1/2 cells. For Western blots, tissue and cells were lysed in radio immunoprecipitation assay (RIPA) buffer plus protease inhibitors. Protein concentrations were determined using the BCA assay (Pierce). Protein lysates (40ug) from each tissue and cell type were run on an 10% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with anti-mDia1, anti-mDia2 (generous gifts of Henry Higgs, Dartmouth Medical School, Hanover, NH), anti-SM α-actin (Sigma), anti-tubulin (Sigma), or anti-flag (Sigma) antibodies. In some experiments cells were serum starved for 24 h and then treated with TGF-β (1ng/ml) for 24 h before lysate preparation.

Immunohistochemistry

For immunohistochemical visualization of mDia1/2, the MRTFs, or SM α-actin, cells fixed in 3.7% paraformaldehyde were permeabilized in 0.5% Triton X-100 for 3 min, blocked in 20% goat serum/3% BSA for 2 h, then exposed to antibody for 2 h at a dilution of 1:500. Texas Red or FITC-conjugated secondary antibodies were used for visualization at 1:1000. In some studies Texas Red-conjugated phalloidin (1:400; Molecular Probes) was used to detect actin stress fibers, and DAPI (1:10,000) was used to detect nuclei.

RESULTS

mDia 1/2 signaling regulates SMC-specific transcription - To test whether the RhoA effectors, mDia1 and mDia2, contribute to the regulation of SMC-specific transcription we co-transfected mDia1 and mDia2 along with SM22 or SM α-actin promoter/luciferase constructs into multi-potential 10T1/2 mouse cells. We and others have shown that these cells up-regulate the expression of many SMC-specific genes upon treatment with TGF-β or sphingosine 1-phosphate (S1P) making them very useful for studying the regulation of SMC-specific transcription [32, 33]. Figure 2.1b demonstrates that expression of full length mDia1 or mDia2 induced a modest (2-3 fold) increase in SM22 and SM α-actin promoter activity. However, co-expression of constitutively active L63RhoA with mDia1 or mDia2 strongly an synergistically activated SM22 and SM α-actin promoter activity to approximately 25 fold, suggesting that basal RhoA activity is a limiting factor for mDia activation. Previous studies have shown that N-terminal truncations that remove the GBD/DID inhibitory domain of mDia1 and mDia2 (ΔGBD) resulted in constitutive activation of these proteins. Remarkably, ΔGBDmDia1 and ΔGBDmDia2 activated SM22 and SM actin by 30-40 fold, indicating that these signaling molecules are powerful activators of SMC-specific transcription. The increased efficacy of mDia1 versus mDia2 was most likely due to increased expression of the mDia1 constructs (data not shown). ΔGBDmDia1 or ΔGBDmDia2 had comparatively little effect on c-fos promoter activity. Both ΔGBDmDia1 or ΔGBDmDia2 also activated the endogenous expression of SM α-actin as shown by the Western blot or immunohistochemistry (figure 2.1c). Given that transfection efficiency in the

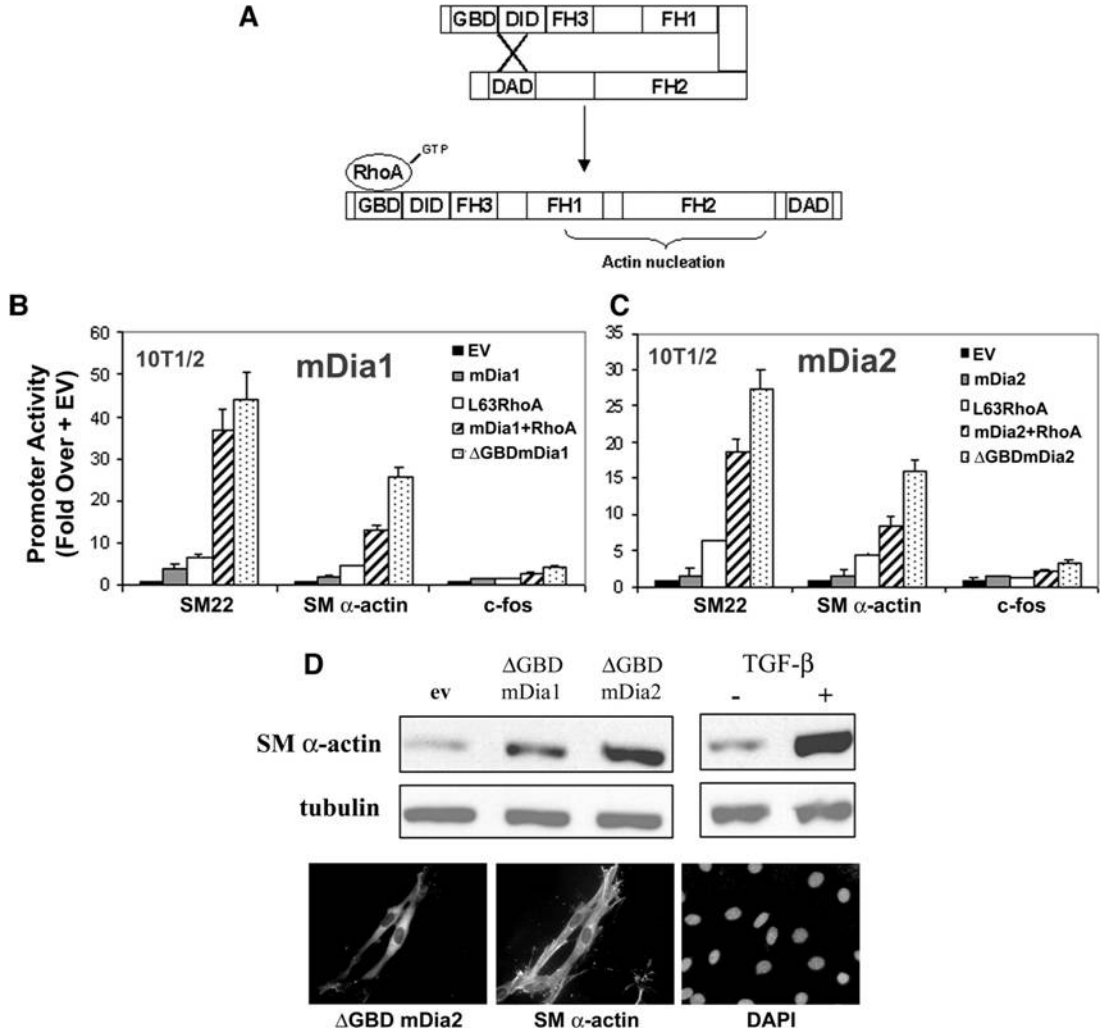
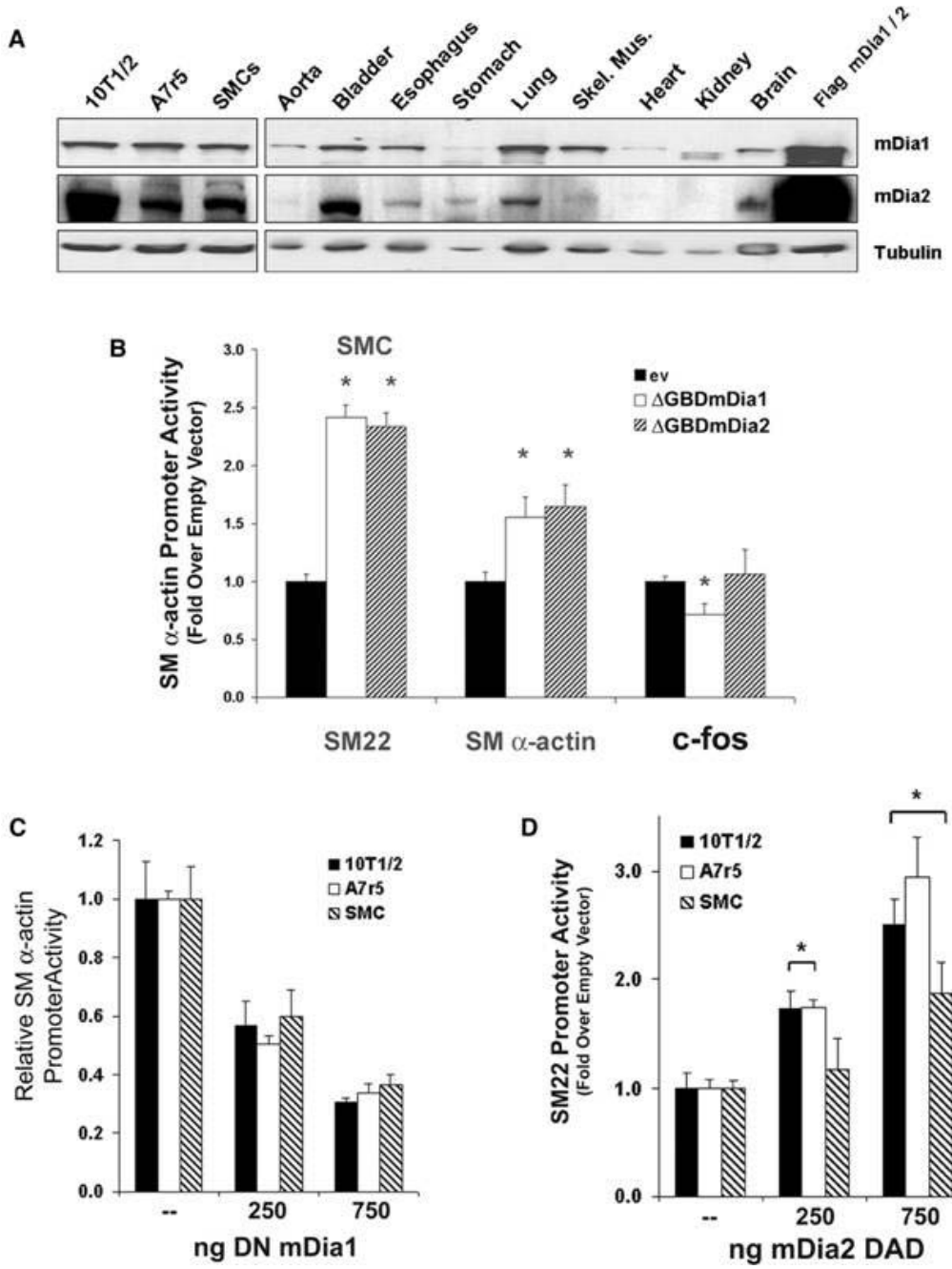


Figure 2.1. RhoA-dependent activation of mDia1 or mDia2 increased SMC-specific promoter activity. A, Schematic of mDia activation by RhoA. The inhibitory DID–DAD interaction is relieved by binding of activated RhoA. FH indicates formin homology domain; GBD, GTPase binding domain; DID, diaphanous inhibitory domain; DAD, diaphanous autoregulatory domain. B, 10T1/2 cells were cotransfected with SM22, SM α-actin, or c-fos luciferase reporter constructs along with Wt mDia1, constitutively active L63RhoA, Wt mDia1 plus L63RhoA, or ΔGBDmDia1. The total amount of expression vector in each well was equalized by addition of empty vector (EV). Luciferase activity was measured 48 hours after transfection. C, The same experiments were performed with mDia2. D, Endogenous SM α-actin expression was detected by Western blot and immunohistochemistry in cells transfected with ΔGBDmDia2. SM α-actin expression in TGF-ß–treated cells is shown as a control.

10T1/2 cell line was typically around 25%, this increase was as strong as or stronger than that seen upon treatment of cells with TGF-β.

Dia1 and Dia2 are highly expressed in SMC - To further investigate the role of mDia1 and mDia2, we analyzed their expression in tissues and cell lines. The Western blot shown in Figure 2.2a demonstrates that mDia1 and mDia2 are very highly expressed in primary rat aortic SMC, A7r5 rat SMC, and 10T1/2 cells as well as in numerous mouse tissues that contain a high SMC component, such as aorta, bladder, and lung. Given that mDia1 and mDia2 could strongly activate SMC-specific transcription and that these proteins were highly expressed in SMC it is likely that they play important roles in regulating SMC differentiation.

Endogenous mDia1/2 signaling regulates SMC-specific transcription in SMC - We next tested whether mDia signaling was important for regulating SMC-specific promoter activity in SMC. Expression of ΔGBDmDia1 or ΔGBDmDia2 in SMC significantly up-regulated SM22 and SM α-actin promoter activity (figure 2.2b). However, these effects were much less dramatic than those observed in 10T1/2 cells most likely due to the relatively high levels of SMC-specific transcriptional activity already exhibited by primary SMC. To determine whether endogenous mDia1/2 signaling was required for SMC-specific promoter activity we utilized a dominant negative form of mDia1 (FH1FH2Δ1) originally described by Copeland *et al.* that has been shown to inhibit the function of both of these proteins [160]. This strategy also allowed us to avoid known redundancy between mDia1 and mDia2 signaling.



Results

Figure 2.2. Endgenous mDia1 and mDia2 signaling was important for SMC-specific transcription. A, Expression of mDia1 and mDia2 in mouse tissues and cell lines was measured by Western blot. B, Rat aortic SMCs were cotransfected with SM22, SM α-actin, or c-fos luciferase reporter constructs along with ΔGBDmDia1 or ΔGBDmDia2. *P<0.05 vs empty vector. C, SMC, A7r5 SMC, and 10T1/2 cells were transfected with SM α-actin luciferase and increasing concentrations of DN mDia1. D, SMC, A7r5 SMC, and 10T1/2 cells were transfected with SM22 reporter and increasing concentrations of an mDia2 DAD peptide (AA 1030-1171). *P<0.05 vs empty vector.

shown in figure 2.2c demonstrate that FH1FH2Δ1mDia1 almost completely inhibited the effects of ΔGBDmDia1 or ΔGBDmDia2 on SM22 promoter activity supporting its effects as a dominant negative. Importantly, FH1FH2Δ1mDia1 strongly inhibited SMC-specific promoter activity in a dose-dependent manner in primary rat aortic SMC, the A7r5 rat SMC line, and in 10T1/2 cells (figure 2.2c, right panel). Results from previous studies have shown that expression of DAD peptides can activate endogenous Dia signaling by interfering with the intra-molecular repression that is mediated by the DID-DAD interaction [125]. Expression of a Dia2 DAD peptide (AA 1030-1171) in all three cell types resulted in a dose-dependent increase in SM22 promoter activity (figures 2.2d). Taken together, these results suggest that endogeous mDia signaling is very important for regulating SMC-specific promoter activity in SMC and other SMC-like cell lines.

The effects of mDia1/2 require SRF binding to CArG elements - It is clear that RhoA-dependent regulation of SMC-specific transcription involves activation of SRF. To test whether the effects of mDia1 and mDia2 were also dependent upon SRF, we performed similar experiments in an SRF knockout embryonic stem cell line. ΔGBDmDia2 did not significantly increase SM α-actin promoter activity in SRF -/- ES cells (figure 2.3a). Re-expression of SRF strongly activated the SM α-actin promoter (~50 fold) and rescued the effects of ΔGBDmDia2 on SM α-actin promoter activity strongly supporting the involvement of SRF in this response. We have previously shown that three CArG elements were required for SM α-actin promoter activity in vivo [49], and it has been suggested that the presence of multiple CArG elements may at least partially explain myocardin's gene-specific effects [161]. Thus, we used a series of SM α-actin promoter constructs that

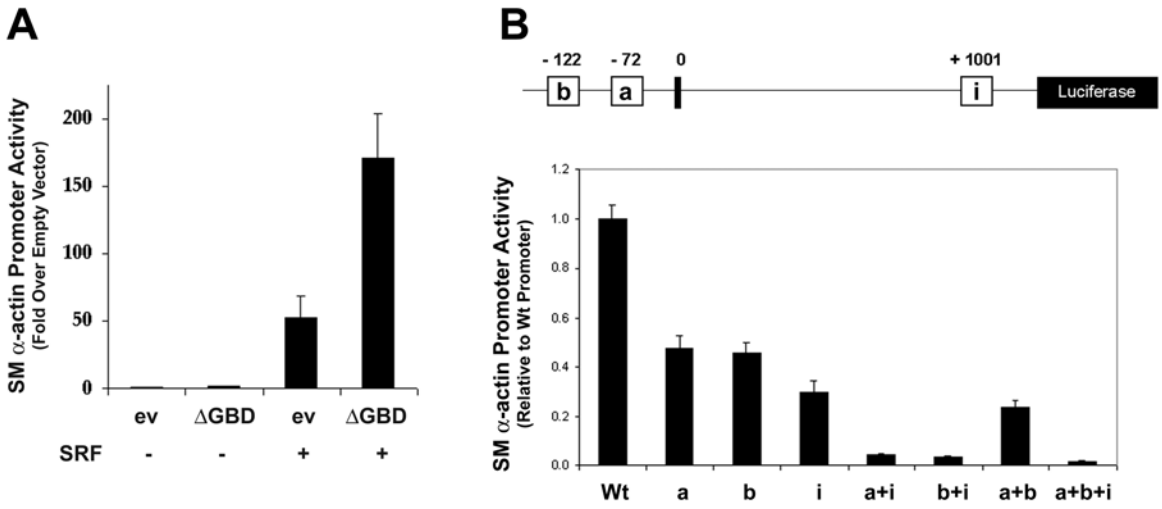


Figure 2.3. The effects of ΔGBDmDia2 required SRF and specific CArG elements. **A)** SRF -/- embryonic stem cells were transfected with ΔGBDmDia2 in the presence or absence of flag-SRF. **B)** 10T1/2 cells were co-transfected with ΔGBDmDia2 and SM α-actin reporters containing single or combinatorial CArG mutations (a, b, and i). *Inset*: SM α-actin promoter showing the three CArG elements that are required for its expression in vivo

contained CArG mutations to determine whether the effects of mDia were also dependent upon this mechanism. Mutation of all three CArG elements, two within the proximal promoter (a & b) and one within the first intron (i) (see figure 2.3b), completely inhibited the effects of ΔGBDmDia2 on SM α-actin promoter while single mutations to any one CArG reduced ΔGBDmDia2-induced activity by about 50%. Interestingly, the intronic CArG, by itself, could mediate a significant portion of the effects of ΔGBDmDia2, and any combination of mutations that included the intronic CArG resulted in complete loss of promoter activity.

The myocardin family of transcription factors are important for the effects of mDia1/2 - To test whether the myocardin transcription factors were critical for the effects of mDia1 and mDia2, we used a dominant negative form of MRTF-A lacking the C-terminal transactivation domain that inhibits all three myocardin family members. As shown in figure 2.4a, this dominant negative dose-dependently inhibited the effects of ΔGBDmDia1 and ΔGBDmDia2 on SM22 promoter activity. In addition, ΔGBDmDia1 (data not shown) and ΔGBDmDia2 (figure 2.4b) enhanced the abilities of the myocardin factors to transactivate the SM22 promoter activity. Interestingly, diaphanous signaling had a relatively greater effect on the activities of the MRTFs than on myocardin. For example, even though all three myocardin transcription factors were expressed at similar levels, myocardin, MRTF-A, and MRTF-B activated SM22 promoter activity by 110, 60, and 30 fold, respectively, a result in relatively good agreement with previous studies [81]. However, in the presence of ΔGBDmDia2 all three myocardin factors stimulated SMC-specific promoter activity to a

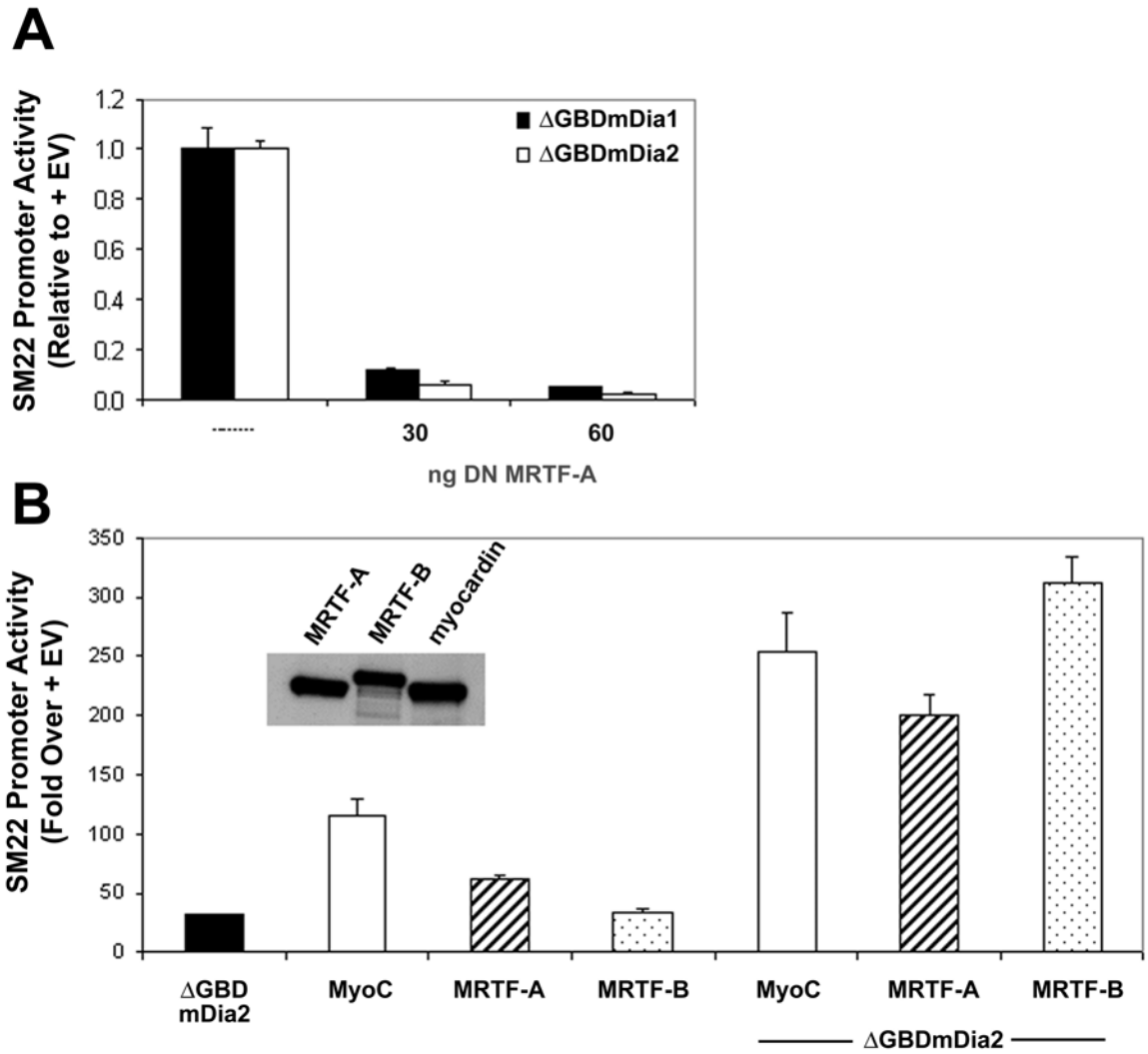


Figure 2.4. Dia1 and Dia2 signal through the myocardin family. A) 10T1/2 cells were co-transfected with ΔGBDmDia1 or ΔGBDmDia2 and increasing concentrations of DN MRTF-A. **B)** Cells were co-transfected with submaximal concentrations of myocardin, MRTF-A, or MRTF-B in the presence or absence of ΔGBDmDia2. Inset – myocardin (MyoC), MRTF-A, and MRTF-B expression levels as measured by Western Blot.

similar extent. Taken together, these data strongly suggest that mDia1 and mDia2 signal through the myocardin transcription factors to activate SMC-specific transcription.

The effects of Dia1/2 are mediated by actin polymerization – Since we had previously shown that RhoA-mediated stimulation of actin polymerization was important for SMC-specific transcription [103], we wanted to test whether the effects of ΔGBDmDia1 and ΔGBDmDia2 were also mediated by changes in actin dynamics. Thus, we examined F-actin in over 200 ΔGBDmDia2 expressing cells using Texas Red-conjugated phalloidin. The representative micrographs shown in figure 2.5a demonstrate that ΔGBDmDia2 significantly enhanced actin polymerization in 10T1/2 (top panels). This effect was not as easily observed in SMC due to high basal levels of actin polymerization in primary SMC cultures even under serum-starved conditions. However, expression of dominant negative FH1FH2Δ1mDia1 in SMC significantly inhibited actin polymerization, indicating that mDia signaling was important for maintaining actin polymerization in this cell-type (figure 2.5a, bottom panels). As shown in figure 2.5b, treatment of SMC and 10T1/2 cells with the actin depolymerizing drug latrunculin B (LB) or co-expression of G-actin nearly completely inhibited the effects of ΔGBDmDia2 on SM22 reporter activity, suggesting that changes in G-actin pools were important. Treatment of cells with the ROK inhibitor, Y-27632, did not affect ΔGBDmDia2-induced promoter activity. Identical results were obtained with ΔGBDmDia1 (data not shown).

mDia1/2 signaling induces MRTF nuclear localization - The data presented so far indicate that mDia1 and mDia2 strongly up-regulate SMC-specific transcription by stimulating actin

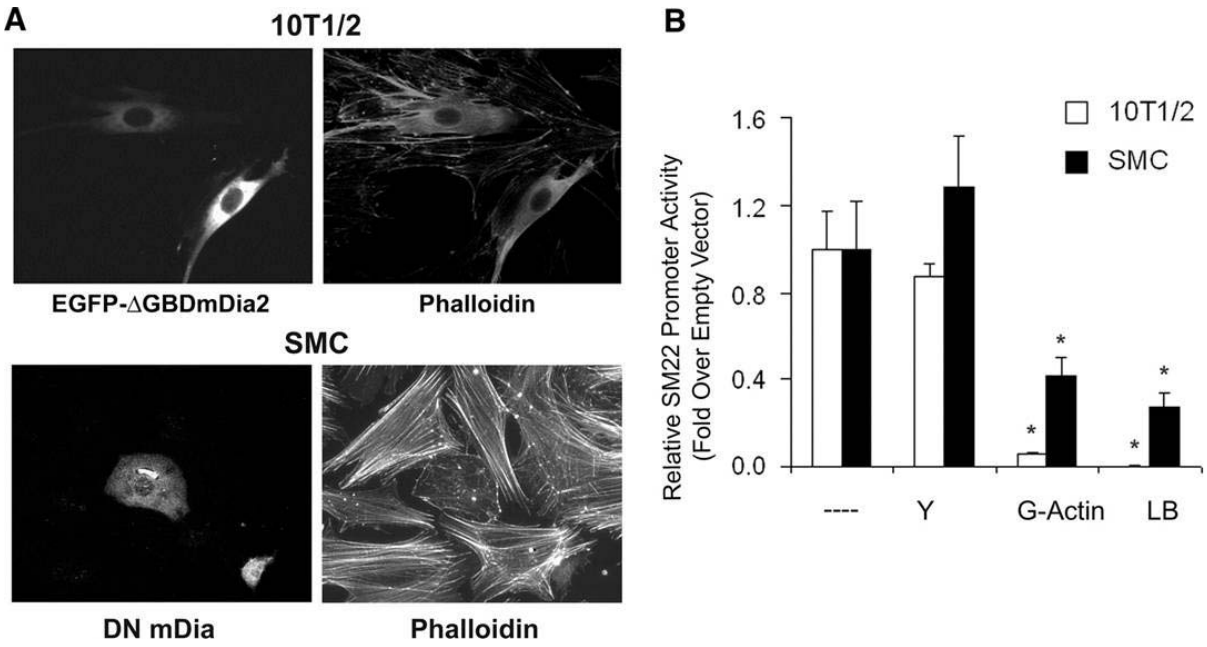


Figure 2.5. The effects of mDia1 and mDia2 were mediated by actin dynamics. A, 10T1/2 cells were transfected with EGFP-ΔGBDmDia2 (top panels) or Flag-DN mDia1 (bottom panels) and serum starved for 24 hours. F-actin was visualized using Texas Red phalloidin. B, 10T1/2 cells and SMCs were transfected with ΔGBDmDia2 and then treated with 0.5 μmol/L latrunculin B (LB) or 10 μmol/L Y-27632 for 24 hours before measuring luciferase activity. In separate experiments, cells were cotransfected with ΔGBDmDia2 and YFP-G-actin. Values are expressed relative to untreated cells transfected with ΔGBDmDia2 alone. **P*<0.05 vs untreated/empty vector.

polymerization and myocardin family transcription factor activity. Recent studies by Miralles et al indicated that the effects of RhoA on SRF-dependent transcription were mediated by changes in MRTF-A localization [88]. These authors demonstrated that MRTF-A was excluded from the nucleus in serum starved cells, through an interaction that involved G-actin binding to conserved RPEL domains present in MRTF-A's N-terminus. Previous results from our lab suggested that stimulation of MRTF-A localization by the strong RhoA activator, sphingosine 1-phosphate (S1P), was important for S1P-induced up-regulation of SMC differentiation marker gene expression [33]. We also demonstrated that MRTF-A and especially MRTF-B localized to the cytoplasm in a large percentage of serum starved SMC and 10T1/2 cells [86]. To test whether the effects of ΔGBDmDia1 and ΔGBDmDia2 were mediated by changes in MRTF nuclear localization, we co-transfected flag tagged-versions of MRTF-A and MRTF-B along with GFP-ΔGBDmDia2 into primary SMC and 10T1/2 cells. Results shown in figure 2.6 demonstrate that MRTF-B was localized almost exclusively to the cytoplasm in serum-starved SMC and 10T1/2 (left panel), and co-expression of ΔGBDmDia2 in either cell type resulted in MRTF-B nuclear localization in nearly all cells (right panel). Expression of ΔGBDmDia2 also increased the percentage of cells containing MRTF-A in the nucleus to nearly 100% (data not shown). When coupled with our previous results, these data strongly indicate that RhoA-dependent activation of mDia1 and mDia2 stimulates SMC differentiation marker gene expression by promoting nuclear localization of MRTF-A and MRTF-B.

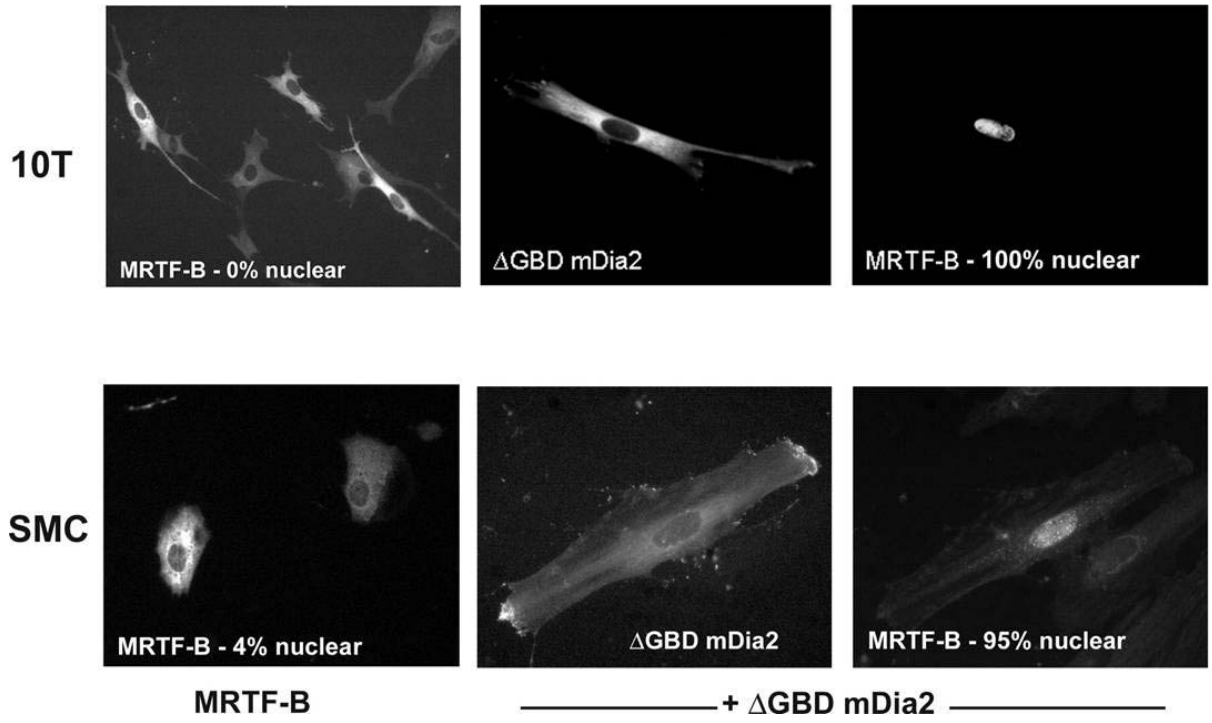


Figure 2.6. mDia activation promoted nuclear localization of MRTF-A and MRTF-B. A, 10T1/2 cells and SMCs were transfected with Flag-MRTF-B±EGFP-ΔGBDmDia2. Cells were serum starved for 24 hours, fixed, and visualized. Note that MRTF-B localized almost exclusively to the cytoplasm in control cells (left panels) and to the nucleus in cells expressing constitutively active mDia2 (right panels).

DISCUSSION

The molecular signaling mechanisms that regulate SMC differentiation are poorly understood. We have previously shown that RhoA regulates SMC-specific transcription by stimulating actin polymerization [33, 103], but the RhoA effectors involved have not been completely described. Results from the present study indicate that mDia1 and mDia2 are important regulators of SMC differentiation marker gene expression. First, both of these RhoA effectors were highly expressed in SMC. Second, RhoA-mediated activation of Dia1/2 strongly stimulated SMC-specific transcription in SMC and SMC-like cell lines. Third, a dominant negative version of mDia1 that inhibits endogenous mDia1 and mDia2 signaling strongly inhibited SMC-specific promoter activity in SMC and SMC-like cell lines. Finally, the effects of mDia1/2 were dependent upon SRF and were likely due to nuclear translocation of the SRF cofactors, MRTF-A and MRTF-B.

Our analysis of mDia expression is in relatively good agreement with a previous study of mDia1 expression by Northern blotting [162]. While neither mDia1 nor mDia2 is expressed specifically in SMC, relatively strong expression in isolated SMC and many SMC-containing tissues suggests that these RhoA effectors play an important role in regulating SMC function. Given their abilities to stimulate SMC differentiation marker gene expression, it will be crucial to further characterize mDia1 and mDia2 expression (and perhaps activity) during all stages of development, using methods that provide better cell-type-specific resolution. It is also important to emphasize that lack of SMC-specificity of mDia1 or mDia2 does not necessarily preclude their involvement in regulating SMC differentiation. Cell-type-specific signaling can also result from differences in proteins

downstream of the more general signaling pathways and we believe that the myocardin transcription factors may serve this function in SMC (see below).

It is clear that actin polymerization mediated by the catalytically active FH1/FH2 domain is inhibited by an intra-molecular interaction between the DAD auto-regulatory domain and the DID inhibitory domain. Extensive structure-function analyses, including two recent X-ray crystallography studies, indicate that the GBD and DID domains overlap slightly and that GTP-RhoA binding to the GBD displaces DAD from the DID binding pocket to activate the mDia proteins [124, 125, 127, 163, 164]. In the present studies, we used several different strategies to activate mDia signaling, including co-expression of constitutively active RhoA, deletion of the mDia GBD/DID domain, and expression of a competing DAD peptide. All three of these interventions significantly up-regulated SMC-specific transcription in 10T1/2 cells and rat aortic SMC. In 10T1/2 cells, expression of ΔGBDmDia1 or ΔGBDmDia2 transactivated the SM22 promoter by 45 and 30 fold, respectively, levels of transactivation usually only seen upon over-expression of the myocardin transcription factors. Expression of ΔGBDmDia1, ΔGBDmDia2 or DAD peptide in SMC also led to significant increases in SMC-specific transcription, but these effects were more modest. A similar difference in relative activities is observed upon over expression of the myocardin factors in SMC and is most likely due to the relatively high levels of SMC-specific transcriptional activity already exhibited by primary SMC. Our data would suggest that strong mDia expression (and activity) probably contributes to this high basal activity. Nevertheless, when taken together with the inhibitory effects of DN mDia1 in SMC and the high level of mDia1 and mDia2 expression in SMC, these data provide strong support for the involvement of RhoA-dependent mDia signaling in SMC differentiation.

Our results also demonstrated that mDia1 and mDia2 had very little effect on the activity of the c-fos promoter, further supporting the idea that RhoA signaling differentially regulates SRF-dependent SMC differentiation vs SRF-dependent SMC growth. The selectivity and/or promiscuity of individual DRFs for various Rho family GTPases may also be interesting in regard to gene-specific regulation by SRF. For example, mDia1 and mDia2 have been shown to interact with RhoA, RhoB, and RhoC, but not with Rac1 [118, 162], while FHOD1 interacts with Rac1, but not RhoA [165, 166]. Interestingly, mDia2 has also been shown to bind Cdc42 and a newly described GTPase, Rif, and these interactions are thought to play a role in filopodial extension [126, 127]. Expression of constitutively active Cdc42 did increase mDia2-dependent activation of the SMC-specific promoters, but these effects were not as great as those observed with L63RhoA (Staus and Mack, unpublished observation). Since the small GTPases are regulated by different signaling inputs, reside in different cellular compartments, and regulate the formation of slightly different actin structures, it is interesting to speculate that the DRFs may relay specific information to the nucleus that could lead to different levels of SRF-dependent gene expression or to differential expression of subsets of SRF-dependent genes.

The inhibitory effects of latrunculin B and over-expression of G-actin clearly implicate actin dynamics in mDia-induced regulation of SMC-specific transcription. RhoA also stimulates actin polymerization through ROK/LIMK/cofilin and it is thought that these pathways interact functionally to regulate SRF activity [62, 119]. In the present study, Y-27632 did not affect transactivation by ΔGBDmDia1, suggesting that ROK activity was not required for this response. When coupled with the strong effects of constitutively active mDia1 and mDia2 on SMC-specific promoter activity, these data suggest that RhoA

signaling to mDia1 and mDia2 may be more important for regulating SMC differentiation than RhoA signaling to ROK. Another RhoA effector, PKN, has recently been shown to be important for the induction of SMC-specific promoter activity by TGF-β, but whether changes in actin dynamics were involved was not addressed [167]. The present studies also demonstrated that mDia2 expression was up-regulated upon TGF-β treatment, suggesting additional cross-talk between these two pathways.

A growing body of evidence indicates that the effects of actin dynamics on SRF-dependent transcription are mediated by nuclear localization of the MRTFs. G-actin binding to the N-terminal RPEL domains found in MRTF-A and MRTF-B has been shown to be important for sequestering the MRTFs in the cytoplasm, and reduction of G-actin pools by RhoA-dependent signaling is important for relieving this inhibitory signal [33, 88, 147]. Results from the present study provide further support for this model. Importantly, expression of constitutively active mDia1 or mDia2 stimulated the nuclear localization of both MRTF-A and MRTF-B and synergistically activated MRTF-A- and MRTF-B-dependent SMC-specific transcription. Interestingly, in the presence of constitutively active mDia1 or mDia2, the activities of MRTF-A and MRTF-B were equal to that of myocardin, suggesting that mDia1/2 activity may be a limiting factor for their activities. Somewhat surprisingly, constitutively active mDia1 and mDia2 also increased myocardin-dependent transactivation even though myocardin was constitutively nuclear. This most likely reflects cooperative effects with endogenously expressed MRTFs, but it is possible that mDia signaling activates myocardin by a separate mechanism. Kuwahara *et al.* have recently identified another protein called STARS (STriated muscle Activator of Rho Signaling) that stimulates SRF-dependent transcription by regulating MRTF nuclear localization [168, 169].

Whether mDia1/2 activity is important for differentiation of other muscle cell types is an interesting question for future studies.

It is clear that MRTF-A and MRTF-B, like myocardin, can upregulate a number of CArG-containing muscle-specific genes. While the MRTFs are expressed in some SMC subsets including the aorta, these SRF co-factors are thought to be expressed more widely, which complicates their contribution to cell-type-specific gene expression [33, 81, 85, 93]. Several recent studies, however, indicate that the MRTFs are important for regulating SMC differentiation. Two separate groups have shown that genetic disruption of MRTF-B leads to a lethal embryonic defect in pharyngeal arch remodeling and that this phenotype is accompanied by a failure of SMC differentiation in the cardiac neural crest cells that populate the cardiac outflow tract [92, 93]. In addition, Pipes *et al.* used a chimeric mouse model to demonstrate that myocardin -/- cells could populate the developing aorta suggesting that the MRTFs probably regulate SMC differentiation under certain circumstances [80].

Interestingly, several groups have reported somewhat contrasting results on the regulation of MRTF-A localization by signaling pathways, suggesting that this mechanism may be more complicated than originally described and that cell-type-specific differences or local environmental cues may be important parameters for regulating this process [85, 91]. Given the observation that MRTF expression does not necessarily correlate with MRTF transcriptional activity, it is possible that differential RhoA/mDia signaling could play an important role in the control of cell-type-specific gene expression by the MRTFs. Since SMC never terminally differentiate, this mechanism could be particularly important for modulating SMC phenotype in response to environmental signals.

In summary, our results indicate that mDia1 and mDia2 are very important regulators of SMC-specific gene expression. These RhoA effectors strongly activate the MRTFs by increasing actin polymerization, which induces their nuclear localization. The precise roles that mDia1 and mDia2 play in the regulation of SMC differentiation during vascular development or in the regulation of SMC phenotype during the progression of cardiovascular disease will be an important area for future studies.

CHAPTER 3

Rho-Kinase-Dependent Phosphorylation of the Diaphanous Autoregulatory Domain Enhances mDia2 Activity

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ABSTRACT

The RhoA effector, mDia2, regulates SRF-dependent smooth muscle cell (SMC)-specific transcription by stimulating actin polymerization and myocardin-related transcription factor (MRTF) nuclear localization. While it is clear that RhoA activates mDia2 by disrupting the interaction between the diaphanous autoregulatory domain (DAD) and the diaphanous inhibitory domain (DID), it is currently unknown whether additional signaling pathways regulate mDia2 activity. In the present study we demonstrate that mDia2 is phosphorylated by Rho-kinase at two conserved residues (T1061 and S1070) just C-terminal to the DAD basic region that stabilizes the DID-DAD interaction. Phosphomimetic mutations (S/T to E) resulted in enhanced mDia2 activity in 10T1/2 cells as measured by SRF-dependent

transcription, actin polymerization, and MRTF nuclear localization and activity. Although the T1061E/S1070E double mutation was not sufficient to fully activate mDia2, it resulted in stronger association with and activation by L63RhoA. An mDia2 DAD peptide containing the double E mutation showed dramatically decreased binding to the DID, and when overexpressed in 10T1/2 cells, failed to activate endogenous mDia as well as a wild-type peptide. Based upon structural and sequence information, we also identified an acidic region in the mDia2 DID that when disrupted resulted in significant mDia2 activation Taken together our results indicate that phosphorylation of the mDia2 DAD by Rho-kinase enhances mDia2 function by destabilizing the DID-DAD interaction and sensitizing mDia2 to activation by RhoA. These results will have important implications for a number of cell functions that involve actin polymerization as well as for the control of SMC phenotype and contractility.

INTRODUCTION

Extensive studies indicate that RhoA signaling regulates gene expression by activating the MADS box transcription factor, known as serum response factor (SRF) (see [170] for review). We and others have shown that this pathway is important for the expression of many muscle differentiation marker genes that contain SRF binding CArG elements within their promoters [58, 103, 171]. Additional studies have shown that the effects of RhoA on SRF-dependent gene expression are secondary to its effects on actin polymerization and are mediated by nuclear translocation of the SRF co-factors Myocardin-Related Transcription Factor-A (MRTF-A) and MRTF-B [33, 88, 113, 169]. Given the importance of the MRTFs in regulating SMC differentiation marker gene expression in vivo

[89, 90, 92, 93], it is likely that this pathway is an important mechanism by which extracellular signals regulate SMC phenotype.

When activated by GTP binding, RhoA interacts with several effector molecules that regulate actin polymerization. Rho-kinase has been the most thoroughly studied in this regard and inhibits the disassembly of actin polymers through LIM-kinase-dependent inhibition of cofilin [116]. Rho-kinase also inhibits myosin phosphatase to stimulate actin-myosin-based contraction, which in turn, promotes actin fiber bundling and stress fiber formation [114, 115]. The RhoA effectors, mDia1 and mDia2, also strongly stimulate actin polymerization and SRF-dependent transcription [162, 164], and recent studies from our lab demonstrate that these formin proteins are highly expressed in SMC and are powerful activators of SMC-specific transcription [172].

mDia1 and mDia2, along with mDia3 and FHOD1, are members of a subfamily of diaphanous-related formins (DRFs) that act as potent actin polymerizing factors (see [121] and [122] for reviews). The DRFs are identified by three highly conserved formin homology (FH) domains, an N-terminal GTPase binding domain (GBD), an adjacent Diaphanous Inhibitory Domain (DID), and a C-terminal Diaphanous Auto-regulatory Domain (DAD) (see figure 3.1a). The molecular mechanisms that control DRF activity have been fairly well described. In the inactive state, the catalytic FH1/FH2 domain is inhibited by a DRF conformation that is maintained by an interaction between the N-terminal DID and C-terminal DAD domains [124, 160]. Crystal structure analysis of this interaction in mDia1 revealed that the core DAD sequence forms an amphipathic helix that binds to a highly conserved hydrophobic pocket formed by the DID [173]. Recent studies on mDia2 by Waller *et al*. and on FHOD1 by Schonichen *et al.* suggest that a conserved basic region C-

terminal to the core DAD domain may also be important for DID-DAD binding [125, 174]. Since the GBD and DID binding pockets slightly overlap, high affinity binding of an activated GTPase to the GBD disrupts the DAD-DID interaction to expose the catalytically active FH1/FH2 region [173, 175].

While there is some promiscuity in the activation of the DRFs by the small GTPases, mDia1 and mDia2 are activated most strongly by RhoA while mDia3 and FHOD1 are activated by Cdc42 and Rac1, respectively (see [176] for review). Interestingly, mDia2 has also been shown to bind Cdc42 and the Rho GTPase, Rif, which may target mDia2 and actin polymerization to the tips of filopodia. [165, 166, 177]. mDia1 and mDia2 promote actin polymerization from actin filament barbed ends in physical cooperation with the actin binding protein profilin [133, 157, 158], but the precise mechanisms involved are not completely understood.

Given that mDia1 and mDia2 have such a dramatic effect on SMC-specific transcription, we have become interested in the molecular mechanisms that regulate their activities. Since most studies have focused on DRF activation by the small GTPases, it is unclear whether post-translational modification plays a significant role. Wang *et al.* demonstrated that the FHOD1 DAD was phosphorylated by PKG at S1131, but the functional consequences of this modification were not examined [178]. The mDia2 DAD contains a very similar site at T1061, and based upon its proximity to the basic domain, we hypothesized that T1061 phosphorylation would stimulate mDia2 activity by disrupting the contributions of the basic region to the DID-DAD interaction.

MATERIALS AND METHODS

Plasmids and Reagents – Full length mDia2 was a generous gift of Shuh Narumiya (Kyoto University, Japan). Constitutively active Rho-kinase (pcMV-Myc-ROCKΔ3) and GST-L63 RhoA were kind gifts from Keith Burridge (University of North Carolina). Wild-type ROCK (pCAG-Myc-ROCK) and cDNA for MRTF-B were gifts from Channing Der and Da-Zhi Wang (University of North Carolina), respectively. GST fusion proteins and flag-tagged expression plasmids for mDia2 were generated by subcloning into the pcDNA3.1 (Clontech), pGEX-4T1 (Amersham Biosciences), or pEGFP (Clontech) vectors. The mDia2 DID and DAD fragments were generated by PCR and comprise amino acids 1-533 and 1030-1171, respectively. The S1070 and T1061 mutations to alanine (A) or glutamic acid (E) were made by the Quikchange Site-Directed Mutagenesis Kit (Stratagene).

Transient Transfections and Reporter Gene Assays – The maintenance and transfection of multipotential 10T1/2 cells were performed as previously described [172]. In brief, cells were maintained in 48 well plates in 10% serum and were transfected 24 h after plating at 70-80% confluency using the transfection reagent TransIT-LT1 (Mirus, Madison, WI), as per protocol. Cells were harvested 24 h after transfection for luciferase assay, which used the luciferase assay kit from Promega (Madison, WI). The SM22 (from –450 to +88) and SM α-actin promoters (from -2560 to +2784) used in this study have been previously described [103, 149].

GST-fusion Protein Expression – The expression of the DID, DAD and L63 RhoA GST fusion proteins were induced in BL-21 bacteria by 18 h incubation at room temperature with 100 μM isopropyl-D-thiogalactopyranoside. Following bacterial lysis, GST-fusion proteins were purified using glutathione-Sepharose beads (Sigma).

In Vitro and In Vivo Kinase Assays – In vitro Rho-kinase assays were performed as previously described [179]. In brief, constitutively active Rho-kinase (ROCKΔ3) was immunoprecipitated from Cos-7 cells and then incubated for 20 min at 30 °C in 25 µl of kinase buffer containing 200 µM ATP, 10 µCi of [γ-^{32}P]ATP and 2 µg GST-fusion protein. The Rho-kinase inhibitor Y-27632 (30µM) (Calbiochem) was added to some reactions. For *in vivo* [^{32}P] phosphate labeling, Cos-7 cells were transfected with either Wt or phosphorylation mutants of flag-tagged mDia2 in the presence or absence of Myc-ROCKΔ3. Cells were pre-incubated with phosphate-free medium 3 h and then labeled with 1 mCi/ml ^{32}P orthophosphate for 4 h. mDia2 was then immunoprecipitated using M2 Flag antibody conjugated to agarose beads (Sigma) and analyzed by SDS-PAGE and autoradiography.

Western Blotting - 10T1/2 cells were grown to confluency, lysed in RIPA buffer, and cleared by centrifugation (4^{o}C for 30 min at 14,000 rpm) and protein concentrations were determined by BCA assay (Pierce). Fifty µg of total protein was run on a 10% SDS polyacrylamide gel, transferred to nitrocellulose and detected with M2 anti-flag antibody (Sigma).

GST Pull-Downs – Cos-7 cells were transfected and scraped into 1% NP40 Buffer (0.15 M NaCl, 10 mM $NaPO_4$ (pH 7.2), 1% NP40, and 0.5% (DAD and L63 pull-downs) or 2% (DID pull-downs) Triton X-100) 24 h post-transfection. After lysis, 250 µg of total protein was incubated with 10 µg of GST-DID or 20 µg of GST-L63 RhoA / GST-mDia2 DAD fusion beads at 4°C for 3 h. Complexes were pelleted by centrifugation and washed 2X in lysis buffer and 1X in cold Tris-buffered saline. Samples were subsequently boiled in SDS-PAGE buffer, electrophoresed by SDS-PAGE, and probed with M2 anti-flag antibody.

Immunoflourescence -10T1/2 cells were plated and transfected in 8-well chamber slides, maintained in 10% serum overnight. Cells transfected with Myc-ROCK or GFP-MRTF-B

were serum-starved for 16 h or 8 h, respectively. Cells were fixed in 3.7% paraformaldehyde/PBS for 20 min and permeabilized in 0.5% Triton X-100/PBS for 3-4 min. Cells were then incubated with M2 anti-flag (1:500) (Sigma) or anti-Myc (1:500) (Cell Signaling) in 20% goat serum / 3% BSA in PBS for 2 h. Texas Red, FITC (Jackson ImmunoResearch), or Cy5 (Molecular Probes) conjugated secondary antibodies were used at 1:1000 while Texas Red Phalloidin (Molecular Probes) and DAPI (Molecular Probes) were used at 1:500 and 90nM, respectively.

RESULTS

The mDia2 DAD domain is phosphorylated by Rho-kinase

Based upon the previous demonstration that the FHOD1 DAD was phosphorylated by PKG [178], we originally tested whether two highly conserved amino acids, T1061 and S1070, just C-terminal to the mDia2 DAD basic region were phosphorylated by this enzyme (see figure 3.1a). However, we failed to detect a significant increase in mDia2 phosphorylation upon activation of PKG in Cos-7 cells even though the PKG substrate, VASP, was strongly phosphorylated in these experiments (data not shown). T1061 and S1070 also conform to the consensus Rho-kinase phosphorylation site (R/KXS/T). When coupled with the fact that Rho-kinase phosphorylates LIM-kinase at a threonine residue just C-terminal to a highly conserved basic domain [179], we tested whether the mDia2 DAD was phosphorylated by Rho-kinase. We first performed in vitro kinase assays with constitutively active Rho-kinase immunoprecipitated from Cos-7 cells. As shown in figure 3.1b, Rho-kinase strongly phosphorylated GST-DAD (but not GST alone) and mutation of

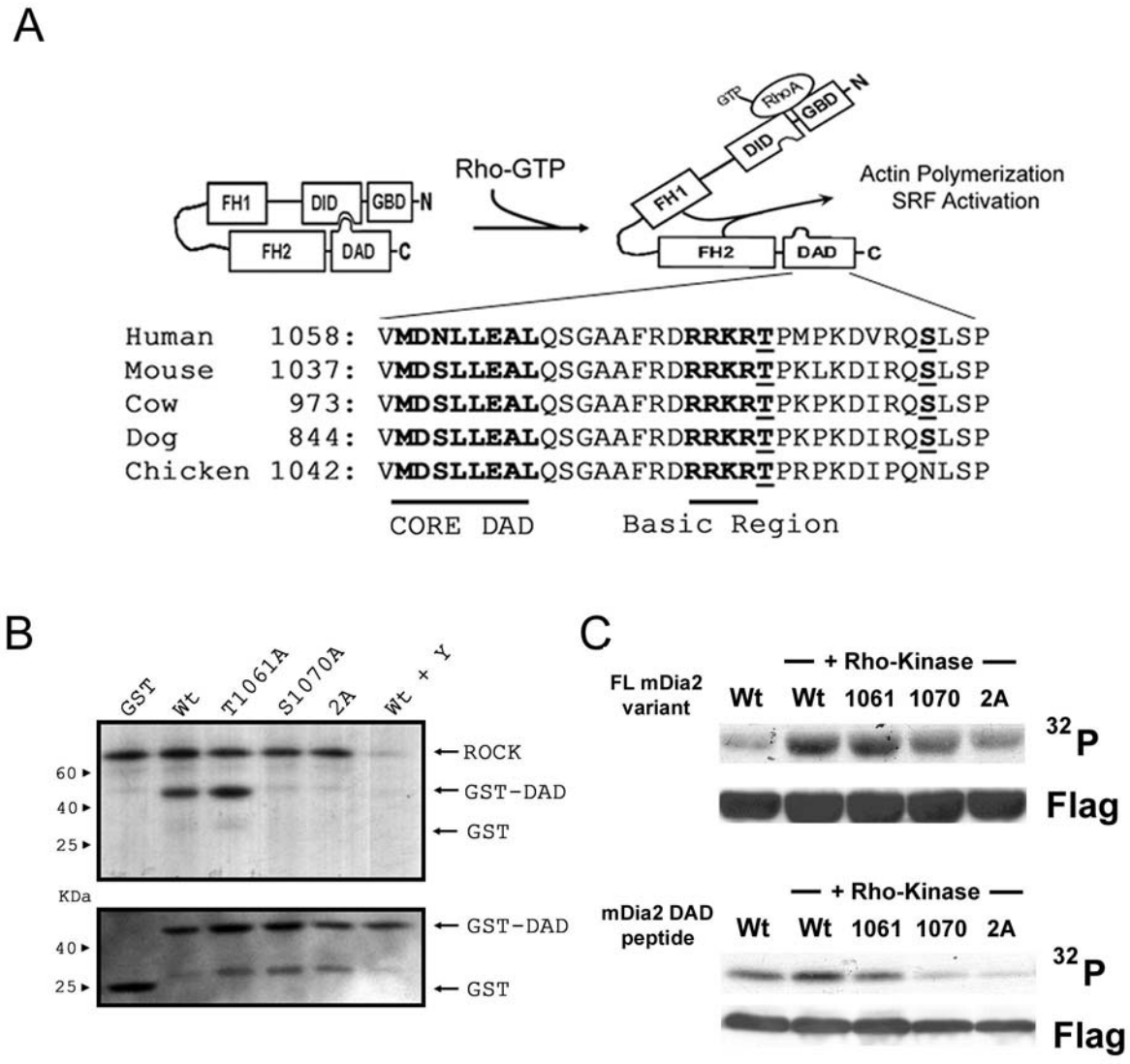

Figure 3.1. Phosphorylation of mDia2 by Rho-kinase. A) Schematic of mDia2 activation by RhoA and sequence conservation in the DAD domain. **B)** Rho-kinase (ROCK) *in vitro* kinase assay. mDia2 DAD GST fusion proteins (Wt and the indicated phosphorylation mutations) were ^{32}P labeled for 15 minutes by constitutively active ROCK that was immunoprecipitated from Cos lysates. Samples were separated on an SDS PAGE gel, transferred to nitrocellulose, and exposed to film. The Ponceau-stained blot in the lower panel demonstrates equal GST-fusion protein loading. **C)** Cos-7 cells were transfected with Wt or phophorylation deficient variants of full-length (top panel) or DAD (bottom panel) mDia2 along with constitutively active Rho-kinase (ROCKΔ3). Following ^{32}P orthophosphate labeling for 4 h, mDia2 was then immunoprecipitated and analyzed by autoradiography.

S1070 to alanine or addition of the Rho-kinase inhibitor, Y-27632, completely eliminated this phosphorylation. The upper band visible in these blots represents Rho-kinase auto-phosphorylation and serves as an excellent positive internal control in these experiments. To test for mDia2 phosphorylation in vivo, we co-transfected Wt and phosphorylation deficient variants of full-length mDia2 along with constitutively active Rho-kinase into Cos-7 cells. Phosphorylation of full-length mDia2 was significantly increased by the presence of Rho-kinase (figure 3.1c, top panel), and importantly, this increase was attenuated by T1061A and S1070A mutations. Although in vivo phosphorylation of mDia2 DAD peptides by Rho-kinase was not as strong (figure 3.1c, bottom panel), the T1061A and S1070A mutations nearly completely inhibited phosphorylation of the DAD peptide. These results suggest that Rho-kinase's ability to phosphorylate T1061 and S1070 may be facilitated by structural features of the full-length molecule. It is also possible that the DAD peptide exhibits higher baseline phosphorylation or that T1061 and S1070 are targeted by additional kinases in this context. During the course of our studies, Takeya *et al.* demonstrated that FHOD1 was phosphorylated by Rho-kinase at several conserved DAD residues including S1131, S1137, and T1141 [180], further supporting a role for Rho-kinase in DRF regulation.

Phosphorylation of T1061/S1070 enhances mDia2 activity

To examine the consequences of T1061/S1070 phosphorylation on mDia2 function, we tested a series of phosphomimetic (S/T to E) and inhibitory (S/T to A) mDia2 mutations on SRF-dependent, SMC-specific promoter activity in multi-potential 10T1/2 cells, a model in which SMC marker gene expression can be up-regulated by a variety of extrinsic cues. As shown in figure 3.2a, expression of Wt mDia2 increased SM22 promoter activity by 6 fold

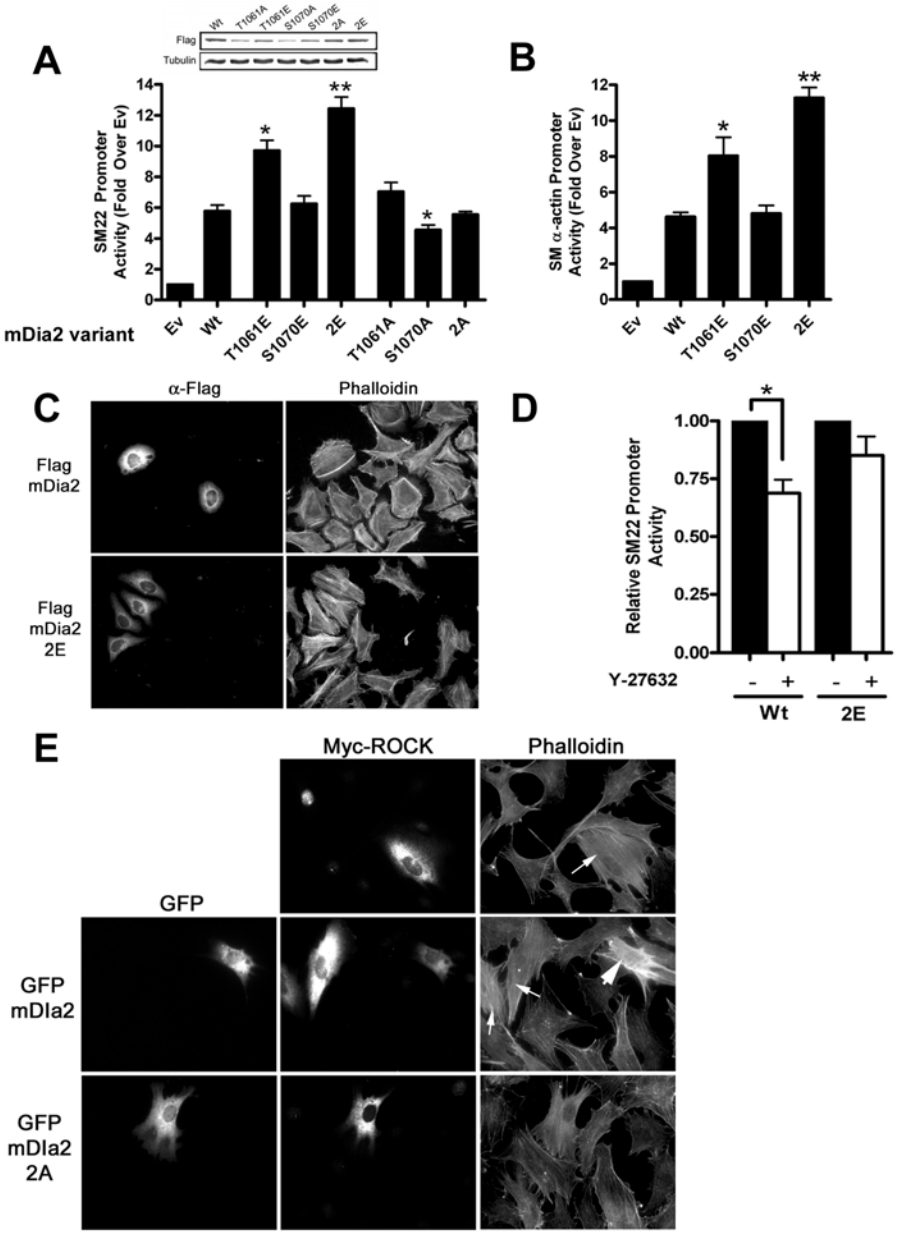

Figure 3.2. DAD phosphorylation by Rho-kinase enhances mDia2 activity. Full length mDia2 and the indicated mDia2 phosphorylation variants were transfected into 10T1/2 cells along with a luciferase reporter construct driven by the SM22 **(A)** or SM □-actin **(B)** promoters. Luciferase activity was measured in cell lysates 24 h post-transfection. Inset in A shows expression levels of the different mDia2 variants. * $p<0.05$ vs Wt; ** $p<0.05$ versus T1061E **C)** HeLa cells, used for their low basal levels of stress fibers, were transfected with flag-mDia2 Wt or 2E and actin polymerization visualized via phalloidin staining. **D)** 10T1/2 cells were co-transfected with SM22-luciferase and either the Wt or 2E mDia2 variant. 18 h post-transfection, cells were treated for 6 h with 10 □M Y-27632 and then luciferase assays were performed. Data are expressed relative to luciferase activity in untreated cells set to 1. * $p<0.05$ vs untreated. **E)** 10T1/2 cells were transfected with GFP, GFP-mDia2, or GFP-mDia2 2A plus or minus constitutively active myc-tagged Rho-kinase. Paraformaldehyde fixed cells were imaged for myc and GFP expression and for actin polymerization with phalloidin

and this effect was significantly increased by the T1061E mutation. Interestingly, while the S1070E mutation had no effect on its own, the mDia2 variant containing the T1061E/S1070E double mutation (2E) enhanced SM22 activity more strongly perhaps suggesting some cooperativity between the two sites. Although the S1070A mutation slightly but significantly decreased mDia2 activity, in general, the inhibitory alanine mutations had little effect suggesting that basal levels of T1061/S1070 phosphorylation in 10T1/2 cells are relatively low. The phosphomimetic mutations had nearly identical effects on the SRF-dependent, SM α-actin promoter (figure 3.2b).

We next tested the effects of DAD phosphorylation on mDia2's ability to stimulate actin polymerization. As shown in figure 3.2c, expression of full-length flag mDia2 in HeLa cells did not alter phalloidin staining. This is in excellent agreement with previous studies [125] and most likely results from the auto-inhibited nature of the full length molecule. In contrast, expression of the mDia2 2E variant led to a modest increase in phalloidin staining indicative of mDia2 activation. To further explore the relationship between Rho-kinase and mDia2, we treated mDia2 and mDia2-2E expressing cells with the Rho-kinase inhibitor Y-27632. As shown in figure 3.2d, Y-27632 reduced SM22 promoter activation by Wt mDia2, but had no effect on the 2E variant. Taken together these results indicate that phosphorylation of T1061 and S1070 by Rho-kinase enhances the effects of mDia2 on actin polymerization and SRF-dependent transcription.

To more closely examine the role of Rho-kinase in this process, we expressed the Wt and 2A mDia2 variants with constitutively active Rho-kinase. As shown in Figure 3.2e, expression of constitutively active Rho-kinase on its own modestly increased phalloidin staining (small arrow head) in 10T1/2 cells. Co-expression of Wt mDia2 significantly

enhanced this signal indicating that these molecules synergize to regulate actin polymerization (large arrows). Importantly, co-expression of Rho-kinase with the mDia2-2A variant did not result in increased phalloidin staining over that observed with Rho-kinase alone suggesting that phosphorylation of these residues is required for this synergy (bottom panel).

Based upon previous data from our lab and others, we hypothesized that the effects of the mDia2-2E variant on SMC-specific promoter activity were mediated by the effects of increased actin polymerization on MRTF nuclear localization. To test this more directly, we treated mDia2-2E-expressing cells with the actin polymerization inhibitor latrunculin B. As shown in Figure 3.3a, the presence of latrunculin B significantly inhibited the effects of mDia2-2E on SMC-specific promoter activity as previously observed with ΔGBDmDia2 (see chapter 2). In addition, expression of the mDia2-2E variant increased the activity and nuclear localization of MRTF-B (figs 3.3b and 3.3c). Since MRTF activity is directly regulated by changes in G-actin pools, these results provide further evidence that phosphorylation of the mDia2 DAD enhances actin polymerization.

It has been suggested that GTPase binding to the GBD is not sufficient to fully activate DRF-dependent actin polymerization and that an additional, as yet undescribed signal, may also be required [163]. The effects of mDia2 phosphorylation could be explained by increased catalytic activity or by increased sensitization of the phosphorylated form to activation by RhoA. To distinguish between these possibilities, we made similar mutations in the context of the constitutively active form of mDia2 that lacks the GBD domain. Neither mutation increased ΔGDBmDia2's ability to stimulate SM22 promoter activity suggesting that the positive effect of the 2E mutation was not due to increased catalytic activity (data not

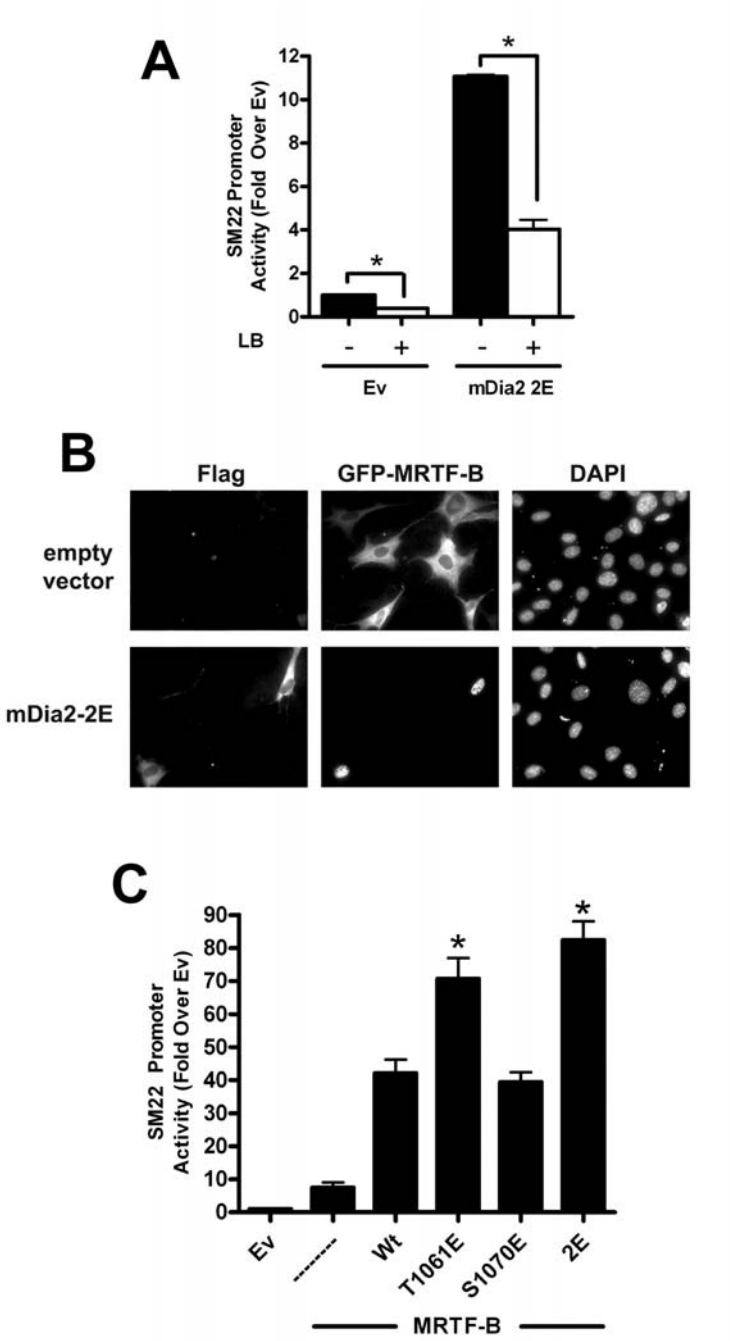

Figure 3.3. DAD phosphorylation enhances MRTF-B activation and nuclear localization. A) SM22-luciferase activity was measured in mDia2-2E expressing 10T1/2 cells in the presence or absence of the actin polymerization inhibitor, latrunculin B (LB). * $p<0.05$ vs untreated **B)** 10T1/2 cells were transfected with equal amounts of GFP-MRTF-B and flag-mDia2-2E and localization visualized after 18 h serum starvation (0.5% FBS). **C)** 10T1/2 cells were cotransfected with SM22 luciferase, MRTF-B, and the indicated mDia2 phosphorylation variant. Luciferase activity was measured at 24 h. * $p<0.05$ vs Wt

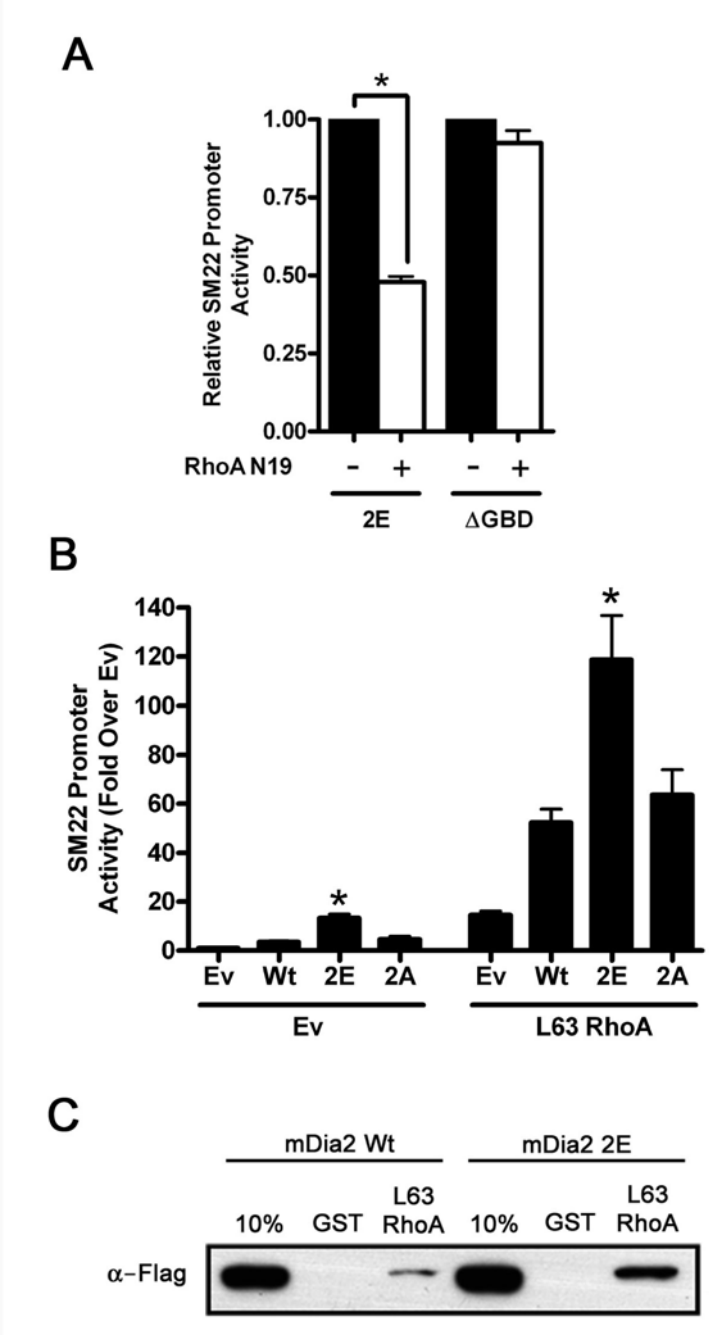

Figure 3.4. DAD phosphorylation sensitizes mDia2 to activation by RhoA. A) The 2E and GBD variants of mDia2 were transfected into 10T1/2 cells plus or minus dominant negative N19RhoA and SM22 luciferase activity was measured at 24 h. Data are expressed relative to luciferase activity measured in the absence of N19RhoA set to 1. * $p<0.05$ vs. minus N19RhoA **B)** The indicated mDia2 variant was transfected into 10T1/2 cells plus or minus constitutively active L63RhoA. * $p<0.05$ vs Wt **C)** A GST-L63RhoA fusion protein was used to pull-down flag-tagged Wt and 2E mDia2 DAD variants (AA 1030-1171) from Cos7 cell lysates.

shown). We also found that the transcriptional activity of the 2E variant, unlike that of ΔGBDmDia2, was inhibited by dominant negative N19RhoA (Fig 3.4a), suggesting that DAD phosphorylation does not render mDia2 constitutively active. Results shown in figure 3.4b demonstrate that the 2E variant was synergistically activated by L63RhoA, and importantly, this increase in RhoA-dependent activation was accompanied by a concomitant increase in mDia2 binding to RhoA as measured by GST pull down assays (Fig 3.4c). Taken together these data suggest that DAD phosphorylation regulates mDia2 activity by enhancing or sustaining mDia2 activation by RhoA.

T1061/S1070 phosphorylation inhibits DAD binding to the DID

We originally hypothesized that addition of negatively charged phosphate groups at T1061 and S1070 would activate mDia2 by interfering with the basic region's role in stabilizing the mDia2 DID-DAD interaction. To test this more directly we generated a GST-DID (AA 1-533) fusion protein and used it to pull down Wt, 2A, and 2E DAD (AA 1030-1162) variants from Cos-7 lysates. As shown in figure 3.5a, the Wt and 2A variants were efficiently precipitated in this assay, while the 2E variant was not. Another method used to study DID-DAD interactions is based upon the activation model shown in figure 3.1a and involves the stimulation of endogenous mDia activity by competitive inhibition of the DID-DAD interaction by exogenously expressed DAD peptides [125, 172]. In this assay the ability of a specific DAD peptide to stimulate endogenous mDia activity can be used as a rough measure of its ability to bind the DID. As expected, expression of Wt DAD in 10T1/2 cells activated endogenous mDia2 as measured by increased SM22 promoter activity (figure

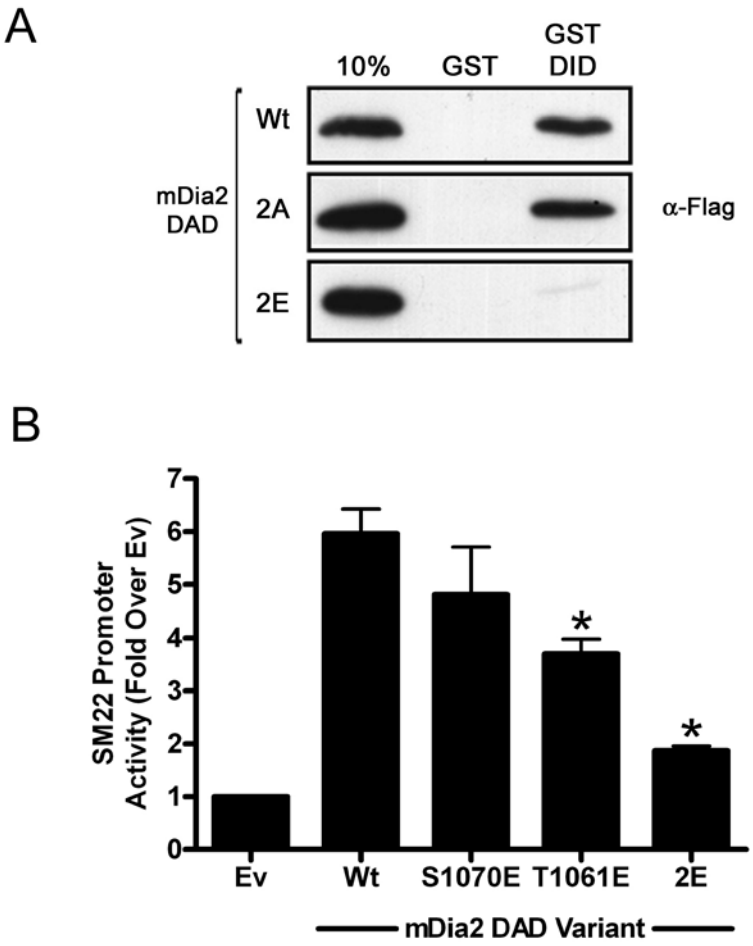

Figure 3.5. DAD phosphorylation weakens the DID-DAD interaction. A) A GST-mDia2 fusion protein (AA 1-533) containing the entire DID was used to pull-down the indicated flag-tagged mDia2 DAD variant from Cos7 cell lysates. **B)** 10T1/2 cells were transfected with the indicated flag-tagged mDia2 DAD variants peptides along with the SM22-luciferase. * $p<0.05$ vs. Wt

3.5b). Importantly, the T1061E and 2E variants had significantly less effect providing additional evidence that DAD phosphorylation decreases DID-DAD binding.

A conserved acidic region within the DID is important for the DID-DAD interaction.

Our results strongly support a role for the basic region in modifying mDia2 activation. However, the mechanisms by which this region alters DID-DAD binding is currently unclear because nothing is known about DID residues with which the DAD interacts with the DID. The DID binding pocket consists of 5 armadillo repeats with all five B helices making contact with the hydrophobic face of the DAD helix [173]. Although the basic domain did not show sufficient order to be placed into the DID-DAD crystal structure, it likely extends C-terminally from the core DAD sequence toward the α5B helix of the DID. Based upon sequence conservation (between species and other DRF family members, see figure 3.6a) and positioning within the α5B helix, we hypothesized that two acidic residues (E377 and D378) were important for the DID-DAD interaction. To test this directly, we generated a E377K/D378K double mutation within the context of an N-terminal mDia2 construct (AA 1-533) that contained the entire DID sequence. As shown in Figure 3.6b, this mutation dramatically inhibited the DID-DAD interaction as measured in pull down assays with a GST-DAD fusion protein. Importantly, in the context of full-length molecule, the E377K/D378K double mutation very strongly activated SM22 promoter activity providing additional evidence that this acidic region was important for maintaining mDia2 in the inactive state (figure 3.6c). To further test this hypothesis we used an assay recently described by Copeland *et al.* that measures the ability of the N-terminal half of mDia2 to inhibit the C-terminal half of mDia2 that contains the catalytically active FH1/FH2 domain

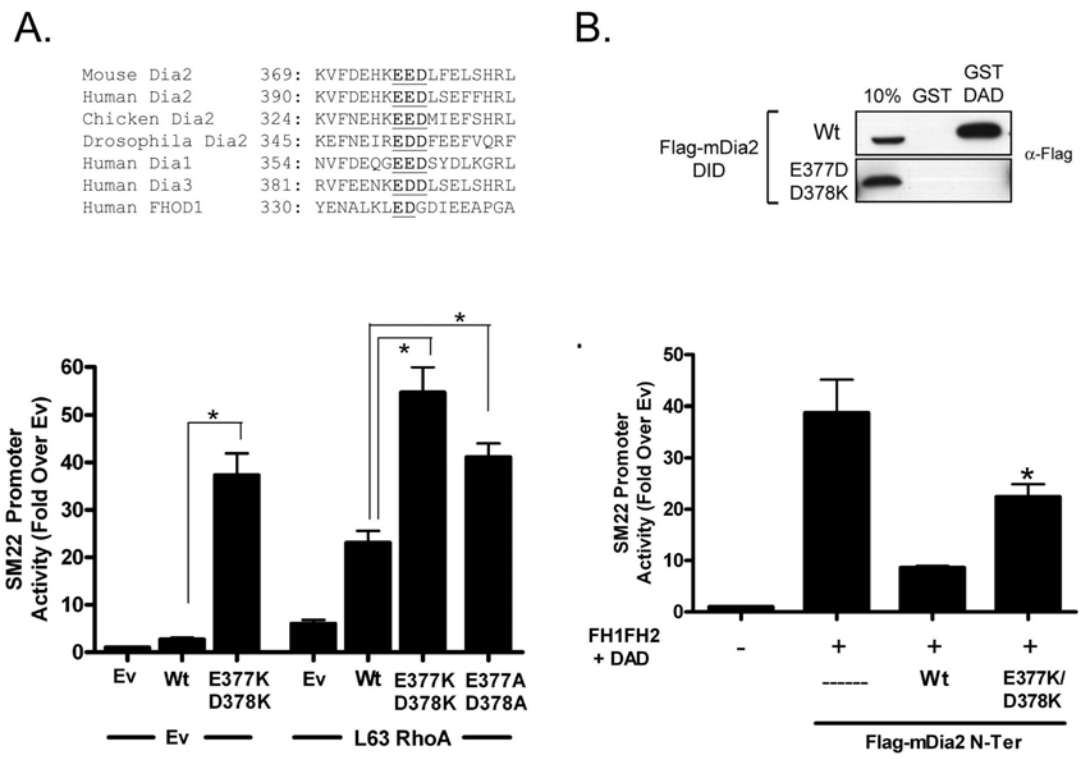

Figure 3.6. Identification of acidic DID residues that may interact with the DAD basic domain. **A)** Conservation of an acidic region in the DID α5B helix. **B)** A GST-DAD fusion protein was used to pull-down flag-tagged mDia2 N-terminal fragments (AA 1-533) containing Wt or E377K/D378K sequence from Cos7 cells. **C)** Full-length Wt or E377K/D378K mDia2 were transfected into 10T1/2 cells along with SM22 luciferase. L63RhoA was included in a second group of transfections. Luciferase activity was measured at 24 h. * $p<0.05$ vs Wt **D)** The catalytically active C-terminal half of mDia2 (flag-FH1FH2 + DAD) was transfected into cells in the presence or absence of the indicated flag tagged mDia2 N-terminal fragment. * $p<0.05$ vs Wt.

and DAD [181]. As expected, expression of the Wt N-terminal fragment attenuated the activation of the SM22 promoter by the C-terminal fragment while the N-terminal fragment containing the E377K/D378K double mutation was less effective (figure 3.6d).

DISCUSSION

It is clear that mDia2 activity is inhibited by the DID-DAD interaction and that RhoA binding displaces DAD from the DID binding pocket to expose the catalytic activity of the FH1/FH2 domain. Virtually nothing is known about additional signaling mechanisms that regulate mDia2 activity, and the goal of the current study was to examine the effects of DAD phosphorylation on this process. Our results demonstrate that mDia2 is phosphorylated at T1061 and S1070 by Rho-kinase. These modifications weaken the DID-DAD interaction sensitizing the phosphorylated form to activation by RhoA. To our knowledge this is the first demonstration of direct cross-talk between two RhoA effectors and our findings should have important implications for RhoA-dependent regulation of actin polymerization.

The core DAD sequence, MDSLLEAL, is critically important for the DID-DAD interaction, and recent crystal structure analyses of mDia1 have demonstrated that this region forms an amphipathic helix that binds tightly to a hydrophobic pocket on the DID surface [173]. Mutation analyses by Wallar *et al*. have recently implicated the basic region (RRKR) N-terminal to the core domain in mDia2 regulation. These authors demonstrated that single glutamate substitutions at any one of these residues resulted in a significant reduction in DAD affinity for DID and that DAD peptides containing these mutations were incapable of activating endogenous mDia. Our demonstration that the T1061E/S1070E phosphomimetics enhanced mDia2 activity fits well with these results and supports a model in which the DID-

DAD interaction is regulated by phosphorylation. Importantly, during the completion of the current studies, Takeya *et al.* demonstrated that phosphorylation of the FHOD1 DAD inhibited its interaction with the putative N-terminal FHOD1 DID, providing further evidence for this model [180]. Moreover, our identification of acidic residues in the DID that potentially interact with the basic domain extends our understanding of the molecular interactions that regulate DID-DAD binding and mDia2 activity. It will certainly be important to further characterize this interaction by additional structural studies. Interestingly, basic sequences are found in many other DRFs (mDia1, mDia3, dDia, FHOD1, Bni1), but only mDia2 and FHOD1 have conserved consensus Rho-kinase phosphorylation sites near this domain, suggesting differential regulation of the DRFs by this mechanism.

Although difficult to determine at present, the timing of DAD phosphorylation during the mDia2 activation cycle could have important implications on the regulation of mDia2 activity. For example, since mDia2 activation is essentially determined by competitive binding of RhoA and DAD to the GBD/DID domain, a phosphorylation-mediated weakening of the DID-DAD interaction would favor RhoA binding and initial activation. Alternatively, DAD phosphorylation that occurred subsequent to RhoA binding could prevent the re-association of the DID-DAD complex leading to prolonged mDia2 activity. Qualitatively, full length mDia2 was more strongly phosphorylated by Rho-kinase than the DAD peptide, perhaps supporting the former mechanism. In addition, T1061 and T1070 could be targets for other kinases especially in the open conformation. It will be important to identify all of the kinases that can phosphorylate these residues and to determine whether activation state affects mDia2 phosphorylation by Rho-kinase. Likewise, the identification of phosphatases that could dephosphorylate these residues will also be important.

The precise physiologic significance of mDia2 activation by Rho-kinase is not completely clear. However, given that this mechanism occurs downstream of RhoA, we hypothesize that it allows for more dynamic temporal or spatial control of actin polymerization. This could be particularly relevant at the very leading edge of migrating cells where precise actin polymerization is required for cell extension. In addition, since mDia2 activity has been shown to be targeted to filopodia by Cdc42 and Rif [165, 166, 177], it will be important to test the effects of phosphorylation on mDia2 activation by these small GTPases. We also demonstrated that DAD phosphorylation increased mDia2 association with RhoA, which could have implications for the specificity of mDia2 activation by the different small GTPases with which it interacts. Finally, since both Rho-kinase and mDia2 activity are important for SMC-specific gene expression [172], this mechanism may play a role in the regulation of SMC phenotype. Rho-kinase is also a major regulator of SMC contractility and this mechanism could help ensure that levels of actin contractile fibers are sufficient to maintain correct SMC tone.

In summary, the results from the present study indicate that Rho-kinase regulates mDia2 activity by phosphorylating the DAD at conserved residues near the basic region. This phosphorylation weakens the intramolecular DID-DAD interaction and facilitates the activation of mDia2 by RhoA resulting in increased actin polymerization and SRF-dependent transcription. Given the importance of mDia2 in the regulation of a number of cellular processes (especially in SMC) and the fact that this mechanism may link two important RhoA effectors, it will be critical to further characterize this signaling mechanism and its physiologic consequences.

CHAPTER 4

Regulation of Smooth Muscle Specific-Gene Transcription and MRTF Subcellular Localization by Nuclear Shuttling of the RhoA Effector mDia2

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ABSTRACT

RhoA-mediated changes in the actin cytoskeleton can stimulate transcription of smooth muscle (SM)-specific genes by regulating the subcellular localization of the myocardin-related transcription factors (MRTFs) A and B. Herein, we show that silencing of the RhoA effectors mDia2 and mDia1, either in isolation or concurrently, significantly reduces expression of SM marker genes in primary mouse aortic smooth muscle cells (SMCs) and in multi-potential 10T1/2 cells treated with sphingosine-1-phosphate (S1P). While we originally attributed these results to an increase in cytoplasmic globular actin (G-actin), recent evidence linking nuclear G-actin to MRTF nuclear export led us to investigate a possible role for the diaphanous-related formins (DRF) in the nucleus. We found that

mDia2, but not mDia1 or FHOD1, accumulated in the nucleus following treatment with leptomycin, an inhibitor of Crm-1 dependent nuclear export. Deletion mapping and mutation analyses identified a nuclear localization sequence (NLS) in the core formin homology 2 (FH2) domain, and nuclear accumulation mediated by this NLS is likely inhibited by the autoinhibitory state of mDia2. Furthermore, an additional NLS and a leucine-rich nuclear export sequence (NES) were identified in the extreme N- and C-termini of mDia2, respectively. In comparison to wild-type mDia2, mutants of mDia2 excluded from the nucleus were significantly impaired in their ability to stimulate MRTF-B nuclear accumulation and SMC-specific gene transcription. Taken together, these data imply a novel role for nuclear mDia2 in the functional regulation and subcellular localization of the MRTFs.

INTRODUCTION

Serum response factor (SRF) regulates the expression of a number of muscle-specific, cytoskeletal, and early response growth genes by binding to conserved CArG ($CC(A/T)_6GG$) *cis* elements found within their promoters (see [170] for review). SRF's cell type- and gene-specific effects are mediated by direct interactions with additional cofactors, and extensive evidence indicates that the myocardin factors (myocardin and the Myocardin-Related Transcription Factors, MRTF-A/MKL-1 and MRTF-B/MKL-2) regulate SMC-specific transcription [72, 81, 182]. Indeed, genetic deletion of myocardin or MRTF-B in the mouse resulted in embryonic lethality due to defects in SMC differentiation in the dorsal aorta and brachial arches, respectively. Moreover, mice lacking MRTF-A fail to up-regulate SMC differentiation marker gene expression in myoepithelial cells during lactation [89, 90].

The precise contributions of each myocardin factor to SMC differentiation is certainly complicated by the high functional homology and overlapping expression patterns of the myocardin factors, the well-known plasticity of SMC, and the existence of multiple SMC lineages. However, it is clear that the identification of the molecular mechanisms that regulate the myocardin factors will be critical for our understanding of the control of SMC phenotype.

The Treisman lab was the first to demonstrate that MRTF-A activity was regulated by the small GTPase, RhoA. This group demonstrated that MRTF-A nuclear localization was inhibited by G-actin binding to the RPEL domains within the MRTF-A N-terminus and that this inhibitory mechanism was relieved by a RhoA-induced reduction in G-actin pools. Recent observations suggest that this mechanism is slightly more complicated. For example, a mutant G-actin has been described that can stimulate MRTF-A nuclear translocation independent of its effects on actin treadmilling, indicating that G-actin may be more directly involved in MRTF-A transport [147]. In addition, Vartiainen *et al.* recently demonstrated that the major factor regulating MRTF-A nuclear accumulation was Crm-1-dependent nuclear export and that binding of MRTF-A to G-actin present in the nucleus somehow facilitated this export mechanism. Furthermore, the association between MRTF-A and G-actin in the nucleus inhibited MRTF-A transcriptional activity without preventing its association with SRF-target genes. Taken together, these data suggest that nuclear G-actin is a critical determinant of MRTF-A nuclear accumulation and activity. Although actin's presence within the nucleus is now well-accepted, almost nothing is known about the regulation of nuclear actin levels or polymerization within this compartment.

We have previously shown that the RhoA effectors, mDia1 and mDia2, are highly expressed in SMCs and strongly activate SMC marker gene expression by promoting nuclear localization of the MRTFs [172]. mDia1 and mDia2 (along with mDia3 and FHOD1) belong to the subfamily of diaphanous-related formins (DRFs) that act as potent actin polymerizing factors (see [176] and [123] for reviews). The DRFs are identified by two highly conserved formin homology (FH) domains, a GTPase binding domain (GBD) that interacts with Rho family GTPases, and a Diaphanous Auto-regulatory Domain (DAD). The dimerization domain (DD) and coiled-coil region mediate N-terminal dimerization of the DRFs, although the physiological importance of this dimerization is still unclear. The molecular mechanisms that control DRF/mDia activity have been fairly well described. In their inactive state, the DRFs are inhibited by an intramolecular interaction between the C-terminal DAD and the N-terminal Diaphanous Inhibitory Domain (DID) [124, 125]. High affinity binding of activated RhoA to the GBD disrupts the DAD-DID interaction, thus exposing the catalytically active FH1FH2 region and stimulating actin polymerization

In the present study we demonstrate that mDia2 shuttles between the cytoplasm and nucleus by a CRM-1-dependent mechanism. We identify several mDia2 sequences that regulate its nuclear import and export and use mDia2 variants to show that the presence of mDia2 in the nucleus is important for its effects on MRTF activity and SMC-specific gene expression.

MATERIALS AND METHODS

Cell Culture -- SMCs were isolated from thoracic aortas of 8-week $FAK^{flox/flox}$ mice by enzymatic digestion as described previously [183]. 10T1/2 cells (ATCC) or low passage SMCs were maintained in normal Dulbecco's modified Eagle's medium or F12 plus 10% fetal bovine serum and 0.5% penicillin-streptomycin.

Plasmids – mDia2 constructs were generated by standard procedures and expressed in either N-terminal flag-tagged pcDNA3.1 or pEGFP (Clontech). The mDia2 K35A/R36A and L1064A/L1065A variants were generated using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). MRTF-A/B were obtained and cloned as described previously [184]. Flag-RhoA (L63) was received from Gary Owens (University of Virginia). GFP-FHOD1 and GFP-mDia1 were generous gifts from Michael Mendelsohn (Tufts University School of Medicine) and Shuh Narumiya (Kyoto University, Japan), respectively.

siRNA Knockdown - The following short interfering (si)RNAs were obtained from Invitrogen; control (GFP) 5'-GGUGCGCUCCUGGACGUAGCC-3' , mDia2 5'-GCAUGACAAGUUUGUGAUATT-3' and mDia1 5'-GGACCUCUAUUGCCCUCAATT-3'.10T1/2 or SMCs were harvested 48 or 96 h after siRNA transfection (Dharmafect; Dharmacon) for protein expression analysis. For S1P treatment, 10T1/2 were serum starved in 0.5% FBS for 12 h and then treated with S1P (10 μm) (Mateya) for 24 h. For luciferase-reporter assays, promoters were transfected 24 h prior to siRNA treatment and harvested 48 h thereafter.

Immunofluorescence – 10T1/2 cells were plated in 8- or 4-well chamber slides, maintained in 10% serum for 48 hours, fixed in 3.7% paraformaldehyde and permeabilized in 0.5% Triton X-100**.** Slides were incubated for 2-3 hours in M2 anti-flag antibody (1:500; Sigma) or anti-mDia2 (1:500; kind gift of Henry Higgs, Dartmouth). Texas Red- or FITC-

conjugated anti-mouse IgG (1:1000; Jackson ImmunoResearch) and DAPI (90 nM) were added for 1 h. Leptomycin B treatments (5 ng/mL; Sigma) were administered for 3 h.

Cell Fractionation – Cell fractionation was conducted as previously described [185]. In short, 10T1/2 cells were gently scraped into cytoplasmic lysis buffer, lysed on ice for 15 minutes and nuclei pelleted by centrifugation. Supernatant was saved as the cytoplasmic fraction. The nuclear pellet was washed three times and resuspended in cytoplasmic lysis buffer containing 0.5 M NaCl. 5% of the nuclear fraction volume and 2.5% of the cytoplasmic fraction volume were analyzed by SDS-PAGE. Antibodies to mDia1, mDia2 and FHOD1 were generous gifts of Henry Higgs (Dartmouth) and Michael Mendelsohn (Tufts University School of Medicine).

Transient Transfections and Reporter Gene Assays – 10T1/2 cells were seeded on 48-well plates and transfected with expression vectors at 70-80% confluency using TransIT-LT1 (Mirus), according to the manufacturer's protocol. Luciferase assays were conducted 24 hours after transfection using the Steady-Glo system (Promega). The SM22, SM α-actin, c-fos and TK promoters have been described elsewhere [33].

GST Fusion Pull-downs / Western Blots - GST pull-down assays were performed as previously described [186] . Cos7 or 10T1/2 cells expressing flag-tagged mDia2 variants were scraped in RIPA containing protease inhibitors. GST-fusion proteins (15 μg) were incubated with Cos7 lysates (500 μg total protein) for 3 hrs and interacting complexes were pelleted by centrifugation. Western blots were probed using anti-flag M2 antibody (1:1000; Sigma), Histone H3 (1:1000; Abcam), Vinculin (1:2000; Sigma) or Tubulin (1:5000; Sigma).

RESULTS

Knockdown of mDia1 and mDia2 inhibits SMC differentiation marker gene expression

We have previously implicated mDia-signaling in the regulation of SMC differentiation by overexpression of constitutively active or dominant variants of mDia1 and mDia2 [184]. To confirm and extend these studies, we examined the individual and combinatorial roles of mDia1 and mDia2 in regulating SMC differentiation marker gene expression in mouse aortic smooth muscle cells using siRNA. We consistently observed a 60% knock-down of mDia1 protein levels and even this relatively modest reduction inhibited SM22 and SM a-actin expression (figure 4.1a). The knockdown of mDia2 had less of an effect, but a compensatory function of mDia1 in mDia2 knockdown cells may have blunted this response. In support of this idea, the combinatorial knockdown of mDia1 and mDia2 inhibited SMC marker gene expression to a much greater extent, suggesting some redundancy in this pathway. We also demonstrated that the double knockdown strongly inhibited the SM22 and SM α-actin promoters but had little effect on the CArG-dependent c-fos promoter or a CArG-independent minimal thymidine kinase promoter (figure 4.1b).

We also tested whether mDia signaling was required for activation of SMC differentiation marker gene expression in multi-potential 10T1/2 cells treated with the RhoA-dependent agonist, sphingosine-1-phosphate (S1P). As shown in figure 4.1c, S1P strongly enhanced expression of SM α-actin, but knockdown of mDia1 and mDia2 significantly attenuated this response. Interestingly, we found that mDia1 and mDia2 protein levels were strongly increased following S1P treatment in this model, suggesting that both expression levels and activation state of mDia may be relevant to the regulation of SMC phenotype. The S1P-induced activation of SM22-luciferase reporter in 10T1/2 cells was significantly

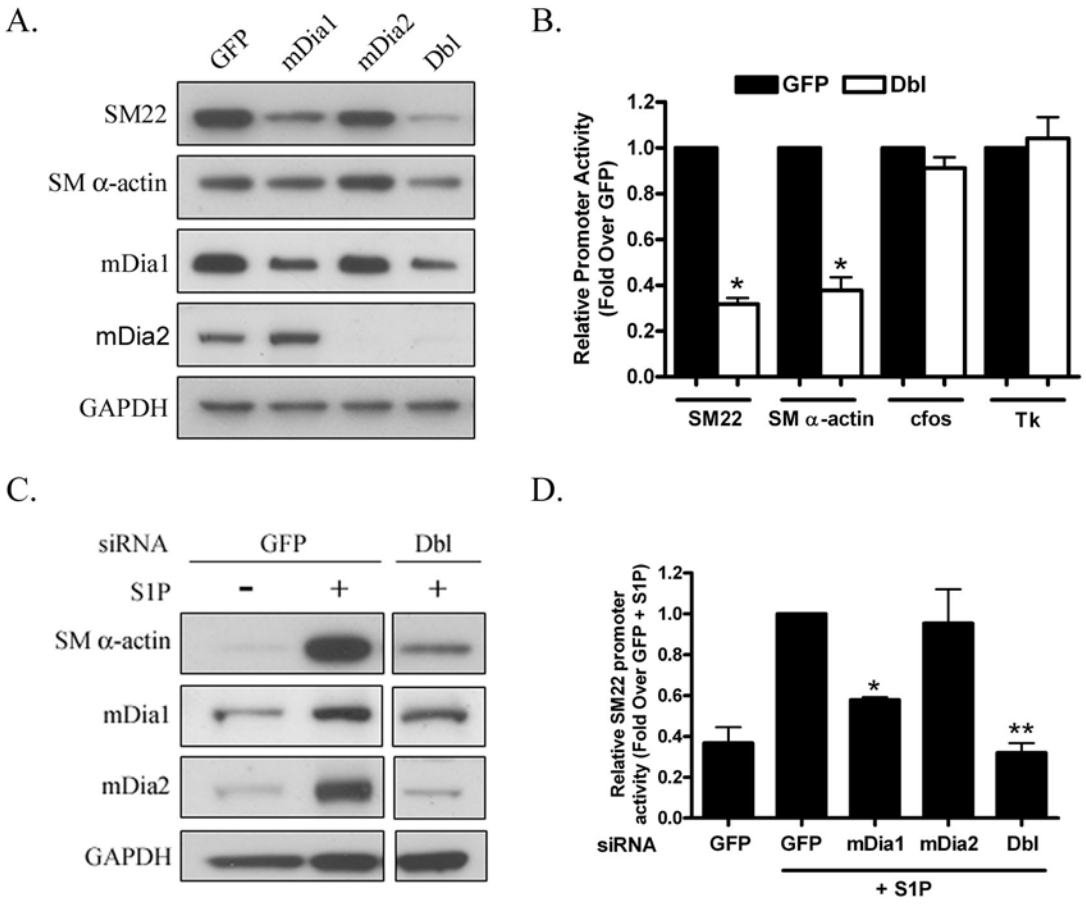

Figure 4.1. Knockdown of mDia1 and mDia2 inhibits SM marker gene expression. A) Primary mouse aortic SMCs were transfected with GFP, mDia1 and (Dbl) /or mDia2 siRNA and allowed to grow in serum containing 10% FBS for 96 h. Equal amounts of protein were separated by SDS-PAGE and probed with antibodies as indicated. GAPDH was used as loading control. **B)** SMCs were transfected with siRNA (as indicated) 24 h post transfection with 500ng of PGL3-SM22, SM α-actin, c-fos or TK. Luciferase activity measured 48 h later. **C)** 10T1/2 cells were transfected with the indicated siRNA. 24 h post transfection the cells were serum starved in 0.5% FBS for 18 h and stimulated with 10 μM S1P for 24 h. Protein expression determined as described in A. **D)** 10T1/2 cells were transfected with 500 ng of the PGL3-SM22 luciferase reporter and manipulated as discussed in C. Duration of S1P treatment was decreased to 8 h and the cells were subsequently measured for luciferase activity. (*$P \leq 0.05$).

attenuated by mDia1 knockdown, and as observed in SMC, this effect was enhanced by the combinatorial knockdown of mDia2 (figure 4.1d). Taken together, these data indicate that both mDia1 and mDia2 are critical for maintaining the differentiated SMC phenotype and for the differentiation of multi-potential 10T1/2 cells into SM (or SM-like) cells.

mDia2 shuttles through the nucleus

The effects of mDia1 and mDia2 on MRTF-dependent gene expression were originally attributed to a decrease in cytoplasmic G-actin levels that promoted MRTF-A nuclear translocation. It is clear that mDia1 and mDia2 can regulate actin polymerization in this compartment as well as at a variety of specific cytoplasmic structures (i.e. the leading edge, filopodia, and endosomes, etc). However, a recent report indicates that the rate limiting step in MRTF-A nuclear accumulation is nuclear export, and that the binding of MRTF-A to nuclear G-actin facilitates this process [148]. Based upon this important finding and several studies demonstrating that ROCK, profilin, and VASP can localize to the nucleus, we hypothesized that the regulation of nuclear actin dynamics could govern MRTF localization. Given the vital role of mDia signaling in this process, we further hypothesized that nuclear localization of one or more of these proteins was involved in this mechanism.

To begin to address the role of mDia1 and mDia2 in the nucleus, we visualized GFP-tagged fusions of mDia1, mDia2, and FHOD1 in multi-potential 10T1/2 cells. As shown in figure 4.2a, all three full-length DRFs were predominantly cytoplasmic, although a small amount of nuclear mDia2 was detected in some cells. Surprisingly, inhibition of Crm-1-dependent nuclear export with leptomycin B (LMB) induced nuclear accumulation of mDia2, but not mDia1 or FHOD1. These data indicate that mDia2 shuttles through the nucleus and that its

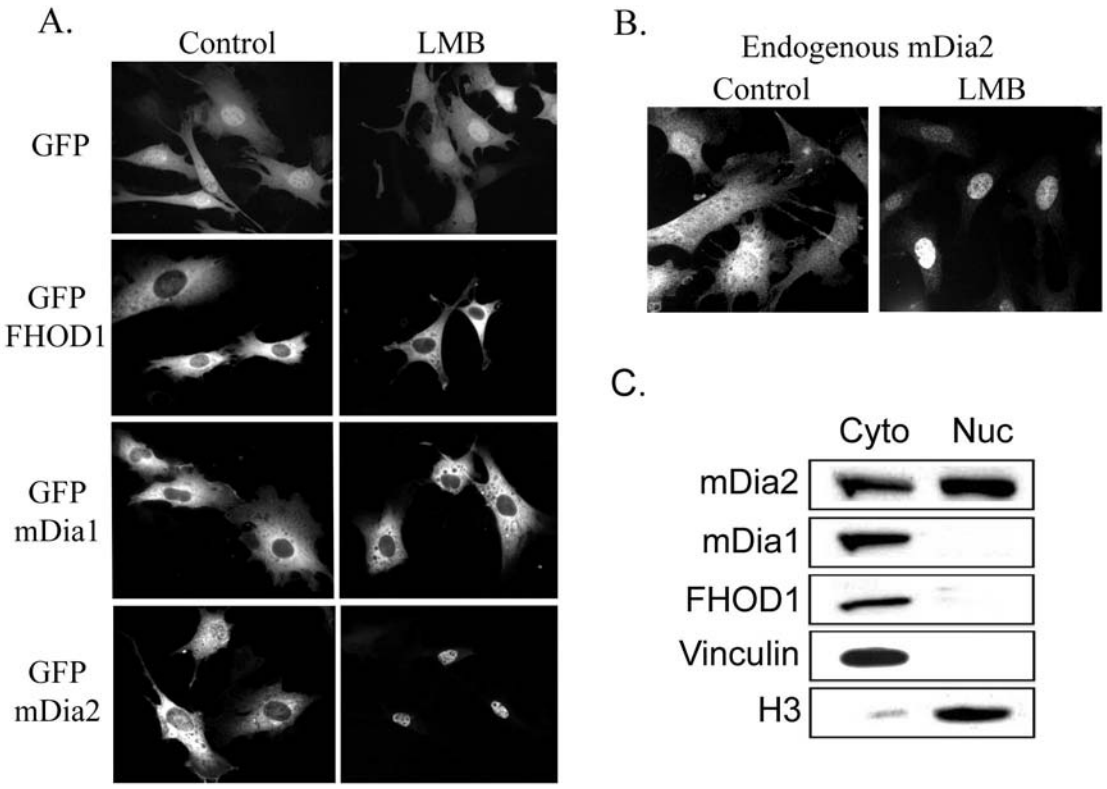

Figure 4.2: mDia2 shuttles between the nucleus and cytoplasm. A) 10T1/2 cells were transfected with GFP or GFP-tagged FHOD1, mDia1, or mDia2. After 48h in 10% serum, cells were treated with leptomycin B for 3 hours, fixed, and visualized. **B)** Leptomycin B (LMB)-treated 10T1/2 cells were fixed and stained with anti-mDia2. **C)** Nuclear and cytoplasmic fractions were prepared from 10T1/2 cells grown in 10%serum and probed with antibodies specific for mDia1, mDia2 and FHOD1. Anti-vinculin and anti-histone 3 were used as controls for cross contamination of the cytoplasmic and nuclear fractions, respectively

cytoplasmic localization is maintained, at least in part, by Crm-1-dependent nuclear export. It is important to note that we observed very little difference between Flag- and GFP-tagged proteins, suggesting that the presence of the GFP moiety had little effect on DRF localization. To examine localization of the endogenous proteins, we performed cell fractionation experiments and probed cytoplasmic and nuclear fractions with antibodies specific to mDia1, mDia2, and FHOD1. Even in the absence of leptomycin, a considerable amount of mDia2, but not mDia1 or FHOD1, was detected in the nuclear fraction (figure 4.2c). Further immunoflourescence analysis revealed that endogenous mDia2 localized almost exclusively to the nucleus in the presence of leptomycin (figure 4.2b). Although we did not detect mDia1 or FHOD1 in the nucleus following leptomycin treatment, it remains possible that cytoplasmic localization of these DRFs is maintained by a CRM-1-independent nuclear export mechanism. In fact, an mDia1 N-terminal deletion mutant was recently shown to be predominantly nuclear in NIH 3T3 cells [181], as was a similar caspase-3 cleavage fragment of FHOD1 in HeLa cells [187]. Thus, there is some evidence to suggest that these DRFs may enter the nucleus under some conditions. In addition, mDia1 has been shown to associate in a complex with exportin6, a recently identified nuclear envelope protein not inhibited by leptomycin that exports G-actin from the nucleus [188].

Identification of nuclear import and export sequences of mDia2

In order to test whether nuclear mDia2 played an important role in regulating MRTF activity, we needed to identify the import and export signals that regulate its localization. We first generated a series of GFP-mDia2 deletion constructs (see figure 4.3a) and expressed them in 10T1/2 cells. The FH2 domain of mDia2 localized predominantly to the nucleus, and

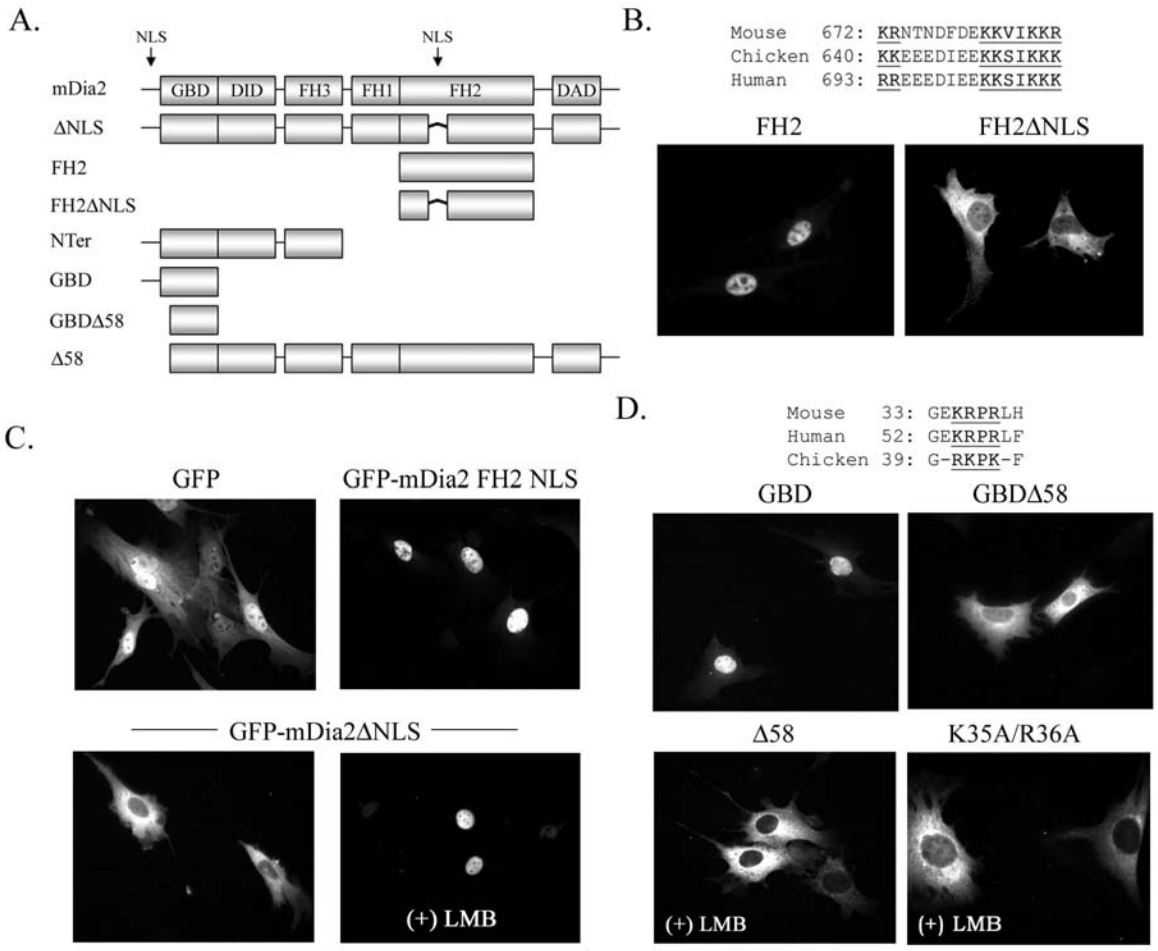

Figure 4.3: mDia2 contains two nuclear import signals. A) Schematic of mDia2 truncations used to identify nuclear import signals. **B-D)** 10T1/2 cells were transfected with GFP-tagged mDia2 variants (as indicated), maintained in 10% FBS for 48 hours, fixed and visualized. LMB (5ng/mL) treatments lasted 3 h. Note conservation of FH2 **(B)** and N-terminal **(D)** NLS sequences.

from residues 673-692 (figure 4.3b). Deletion of these amino acids (FH2ΔNLS) completely inhibited nuclear localization of the FH2. Furthermore, this putative NLS enhanced nuclear accumulation of GFP (figure 4.3c, upper panels), providing evidence that this region is sufficient to drive nuclear localization. Interestingly, deletion of this NLS from the full-length mDia2 molecule (ΔNLS) did not block nuclear accumulation in the presence of LMB (figure 4.3c), suggesting the presence of additional sequences outside the FH2 domain capable of stimulating nuclear import.

Additional mapping studies revealed that an N-terminal 256 amino acids of mDia2 containing the GBD (GFP-GBD) localized to the nucleus (figure 4.3d). Further deletion analysis identified the presence of a NLS within the first 58 AA (GBDΔ58) and deletion of this region from full length mDia2 (Δ58) completely blocked nuclear accumulation in the presence of leptomycin (figure 4.3d, bottom left panel). To further define this N-terminal NLS, we made double alanine mutations to two conserved basic regions within this region in the context of full length mDia2. While a R18A/R19A mutation had no effect on mDia2 localization (data not shown), a K35A/R36A mutation completely blocked nuclear accumulation in the presence of leptomycin, clearly indicating that the nuclear import of full-length mDia2 is primarily controlled by this sequence. The contribution of the FH2 NLS to mDia2 localization will require further investigation, and at present, we cannot completely rule out a potential role for this sequence under other different circumstances.

The accumulation of mDia2 in the nucleus in the presence of leptomycin B strongly suggests that mDia2 contains a leucine-rich Crm-1-dependent nuclear export sequence (NES). To identify this sequence we analyzed the localization of another series of GFP-mDia2 deletions (figure 4.4a). Fusion of the DAD to either GFP alone (fig 4.4b) or GFP-

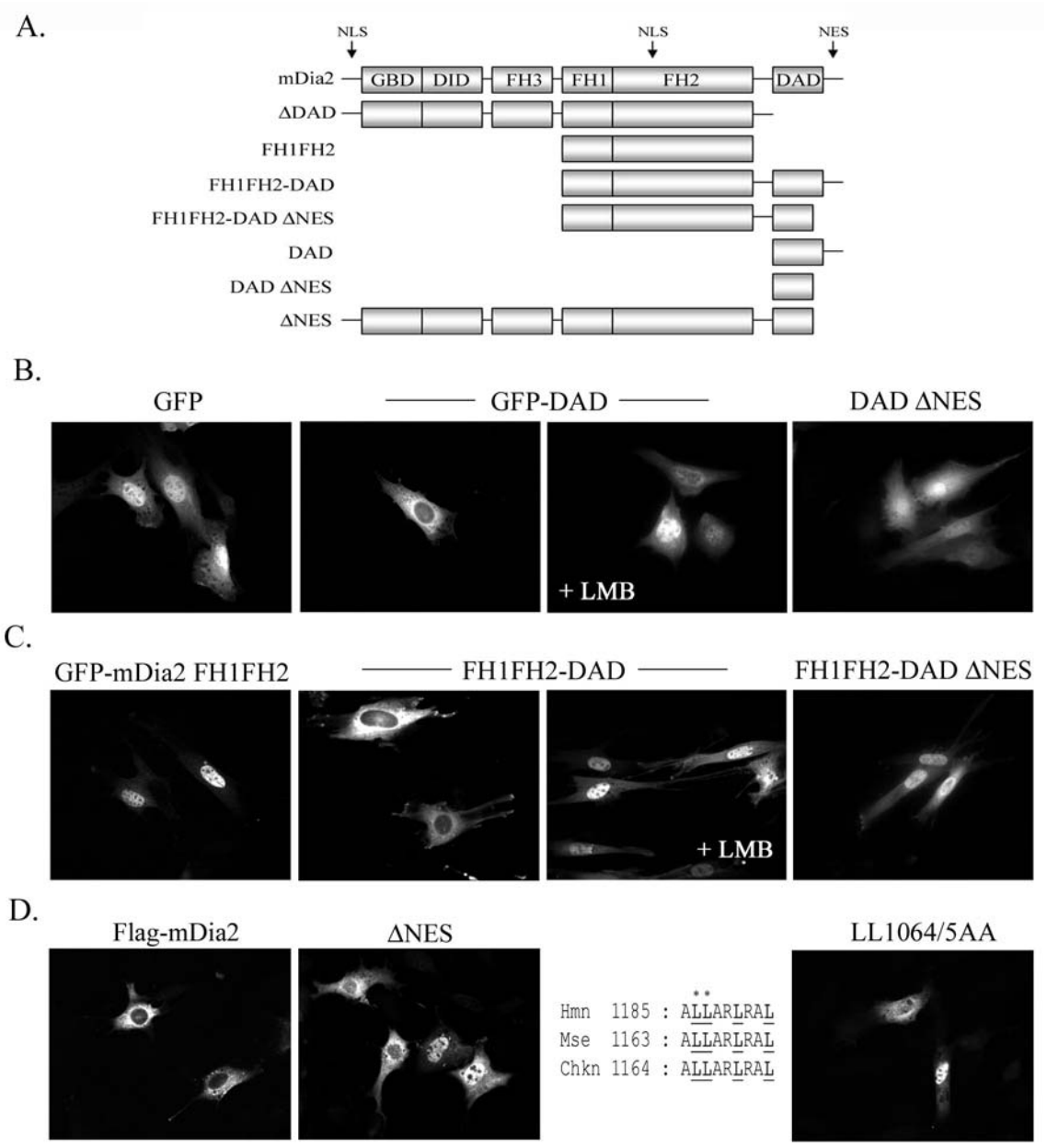

Figure 4.4: Identification of mDia2 nuclear export sequences. A) Schematic depicting the mDia2 deletions and mutations localized in these studies. **B-D)** GFP-tagged mDia2 variants (as indicated) were transfected into 10T1/2 cells and maintained in 10% FBS for 48 h. Cells were either treated with LMB for 3 hours or left untreated, fixed and then visualized. Note conservation of C-terminal NES **(D)**. * denotes specific amino acids that were mutated.

FH1FH2 (fig 4.4c) blocked nuclear localization of these constructs, and this effect was rescued by leptomycin B treatment, strongly suggesting that this region functions as a NES. We identified a leucine-rich region at the extreme C-terminus (AA 1064 to 1071), and deletion of this region (ΔNES) in the context of GFP-DAD or GFP-FH1FH2 resulted in nuclear localization in the absence of leptomycin B (Fig 4.4b and c, far right panels). Furthermore, both the ΔNES deletion and a double alanine mutation (L1044A/L1045A) promoted significant nuclear accumulation of full-length mDia2 (figure 4.4d). Taken together, these data suggest that mDia2 nuclear export is regulated, at least in part, by a Crm-1 dependent NES located within the extreme C-terminus.

Determining the molecular mechanisms that govern mDia2 nuclear shuttling

The identification of multiple NLS's in mDia2 suggests that its nuclear import may be more complex than originally thought. We and others have shown that the intramolecular interaction between the DAD and DID prevents actin polymerization by sterically inhibiting the FH2 domain. Since the FH2 NLS cannot drive full-length mDia2 nuclear accumulation following LMB treatment, perhaps the mDia2 auto-inhibited state blocks the function or availability of this NLS. To further test this possibility, we monitored flag-FH1FH2-DAD localization in 10T1/2 cells after LMB treatment in the presence of mDia2 N-terminal variants fused to GFP (see figure 4.5b for domain schematic). We found that FH1FH2-DAD nuclear accumulation following LMB treatment was inhibited in the presence of GFP-NTerΔ58, but not wild type NTer (figure 4.5a). This indicates that when bound to the N-terminus, FH1FH2-DAD nuclear import is mediated through the N-terminal NLS rather than the FH2 NLS. We also found that an A272D mutation to NTerΔ58 reversed its inhibitory

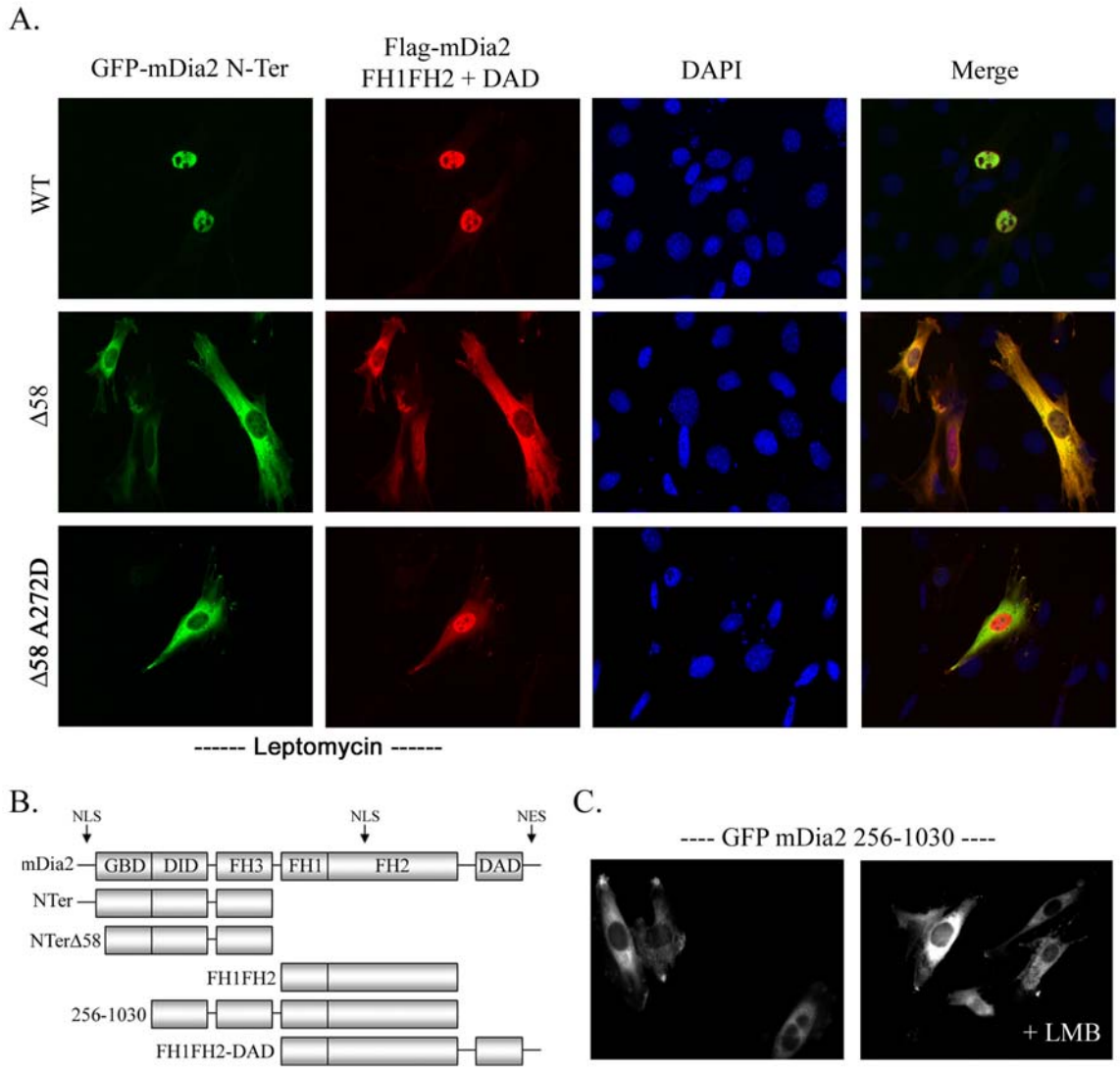

Figure 4.5: Autoinhibited state of mDia2 inhibits function of the FH2 NLS: A) 10T1/2 cells were co-transfected with flag-mDia2 FH1FH2-DAD and GFP-mDia2 NTer variants. All cells were treated with LMB for 3 h and nuclei were stained using DAPI (90 μM). **B)** Summary schematic of mDia2 truncations. **C)** Amino acids 256-533 were fused to GFP-mDia2 FH1FH2 (256-1030) and localization monitored before and after LMB treatment (3 h).

effect on FH1FH2-DAD nuclear localization. The A272D mutation, modeled after a similar mutation described in mDia1 [189], inhibits the DID-DAD interaction without affecting RhoA binding in mDia2 (data not shown). These data suggest that inhibition of FH1FH2-DAD nuclear accumulation by NTerΔ58 is dependent on the DID-DAD interaction. Taken together, these findings extend our data implicating a role for mDia2 autoinhibition in the regulation of the FH2 NLS. We also found that nuclear accumulation of FH1FH2 was completely abolished when directly fused to the FH3/DID domains (amino acids 256-533) and that this change in localization was not sensitive to LMB treatment (figure 4.5c). It is likely that this region inhibits FH1FH2 nuclear accumulation by regulating the function of the FH2 NLS and/or promoting cytoplasmic sequestration. However, it is also possible that this region may enhance nuclear export via a Crm-1 independent mechanism (see discussion). Additional studies are required to test these hypotheses.

Regulation of SMC-specific transcription by mDia2 nuclear localization

Our data so far indicates that mDia2 shuttles through the nucleus and is an important regulator of SMC phenotype. To determine if activation of SMC-specific gene transcription by mDia2 requires its nuclear localization, we compared the ability of wild type, Δ58 and K35A/R36A mDia2 to stimulate activity of the SM22 promoter in 10T1/2 cells. In the absence (figure 4.6a) or presence of constitutively active RhoA (figure 4.6b), the Δ58 and K35A/R36A mutants of mDia2 activated the SM22 promoter only half as robustly as wild type mDia2. Notably, these mutants were unimpaired in their ability to bind active RhoA, as shown by GST pull-down assays (figure 4.6c). These data support previous reports that the first 75 amino acids of mDia1 are dispensable for high affinity RhoA binding [163, 175] .

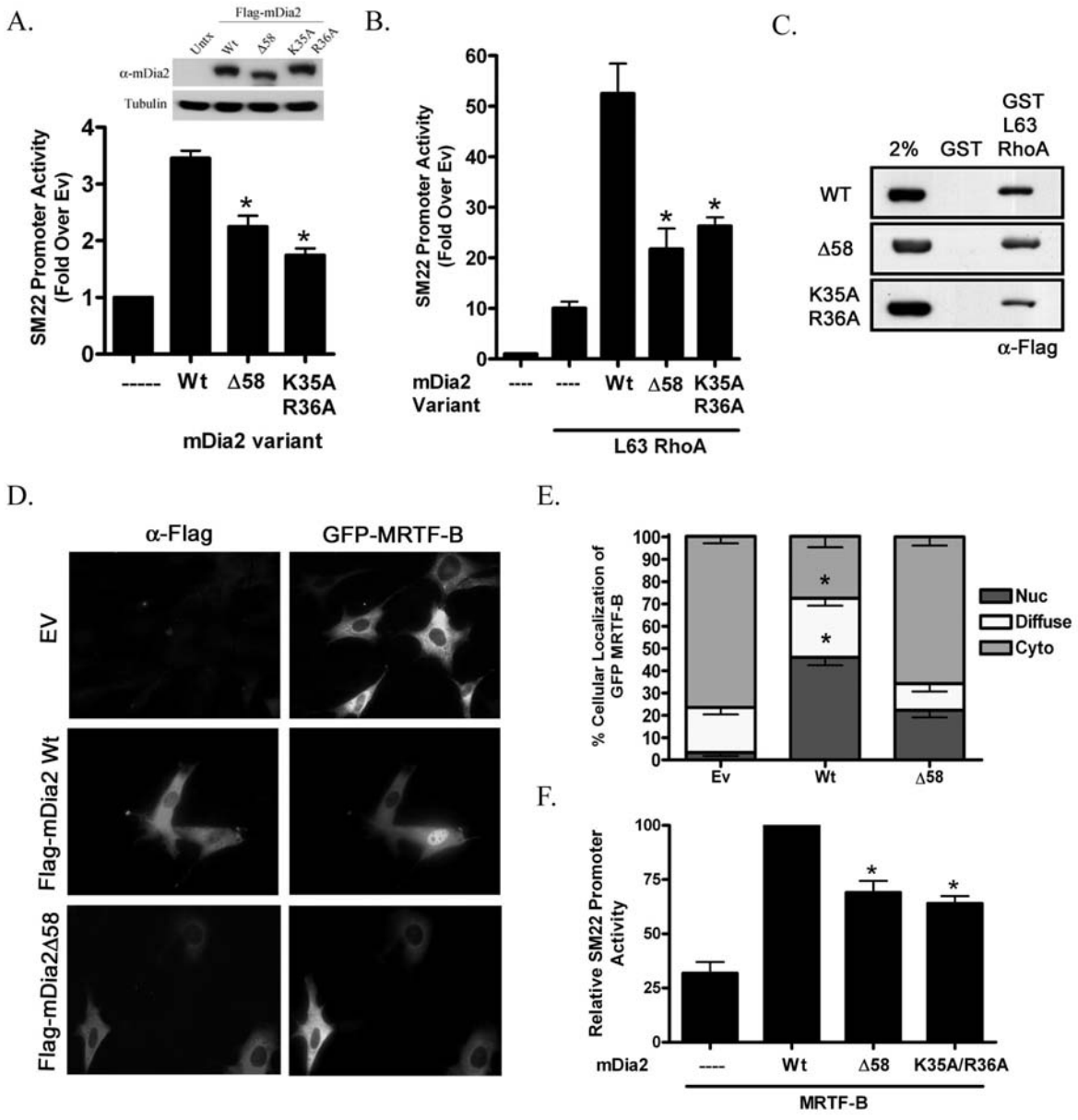

Figure 4.6: Deletion or mutation of N-terminal NLS inhibits mDia2-mediated regulation of SMC-specific gene transcription. 10T1/2 cells were transfected with 125 ng of individual flag-mDia2 variants and PGL3-SM22 in the absence **(A)** or presence **(B)** of flag-L63 RhoA (12.5ng) and luciferase activity measured 24 h later. **C)** GST-L63 RhoA pull-downs of flag-mDia2 variants expressed in Cos7 cells. **D)** 10T1/2 cells were transfected with equal amounts of GFP-MRTF-B and either Wt or Δ58 flag-mDia2. Cells were maintained in 10% FBS for 36 h, placed in 0.5% FBS for 18 h, and then fixed and visualized**. E)** Quantification of GFP-MRTF-B localization from at least 100 cells from three independent experiments. Nuc: Nucleus. Cyto: Cytoplasm **F)** 10T1/2 cells were transfected with PGL3-SM22 (125ng), flag-mDia2 variant (125ng) and flag-MRTFB (12.5ng) and luciferase activity recorded 24 h post-transfection (**P* ≦0.05).

Given recent evidence that nuclear export of the MRTFs is regulated by nuclear actin dynamics, we investigated whether inhibition of mDia2 nuclear import would affect localization or activity of MRTF-B. Indeed, mDia2 Δ58 was significantly less capable of stimulating nuclear accumulation of MRTF-B in comparison to wild type mDia2 (figure 4.6d-e). This variant and the K35A/R36A mutant were similarly deficient in stimulating transactivation of the SM22 promoter by MRTF-B (figure 4.6f). These findings suggest that mDia2 function in the nucleus is important in regulating SMC-specific gene transcription and subcellular localization of MRTF-B.

We also found that mDia2 FH1FH2 was predominantly nuclear and potently enhanced SM22 promoter activity and nuclear accumulation of MRTF-B (figure 4.7a, b), further supporting a nuclear role for mDia2. We were unable to measure the effects of FH1FH2ΔNLS on SM22 promoter activity and MRTF subcellular localization since mutation of the FH2 NLS had deleterious effects on the ability of FH1FH2 to stimulate actin polymerization (figure 4.7c). Therefore, we targeted the dominant negative mDia variant F1F2Δ1 to the nucleus (Nuc-F1F2Δ1) to determine the contribution of nuclear FH1FH2 signaling to SM marker gene transcription. For a description of how these dominant negative variants may function please see Chapter 2. We found that Nuc-F1F2Δ1 dose dependently inhibited FH1FH2-mediated stimulation of SM22 promoter activity to a comparable level to Wt F1F2Δ1 (figure 4.7d). Importantly, this inhibitory effect seems to be specific to the nucleus since we did not observe decreases in cytoplasmic actin polymerization from cell expressing Nuc-F1F2Δ1 (figure 4.7e). Taken together, these findings indicate that inhibiting the nuclear function of mDia2 attenuates MRTF-B nuclear accumulation and SM marker gene transcription.

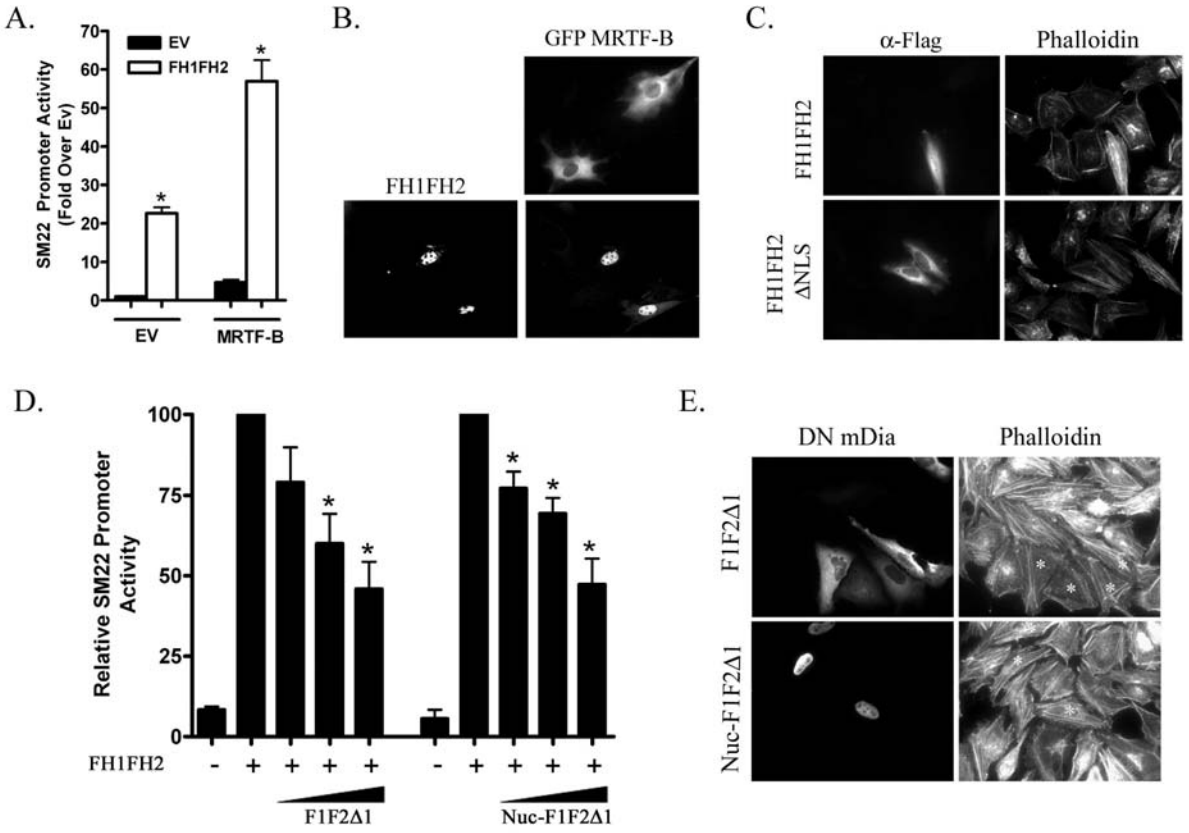

Figure 4.7: A nuclear targeted mDia dominant negative inhibited activation of the SM22 promoter by FH1FH2. A) 10T1/2 cells were transfected with PGL3-SM22 (125 ng) and FH1FH2 (112.5 ng) in the presence or absence of flag-MRTFB (12.5ng). Luciferase activity measured 24 h post transfection. **B)** GFP-MRTFB and flag-mDia2 FH1FH2 were transfected into 10T1/2 cells, maintained in 10% FBS for 36 h, serum starved in 0.5% FBS for 18 h, fixed, and visualized by fluorescence microscopy. **C)** GFP-mDia2 FH1FH2 Wt or ΔNLS were transfected into HeLa cells (chosen for their low basal levels of stress fibers), starved in 0.5% FBS for 12 h, fixed, and actin polymerization visualized with phalloidin (1:100). D) 10T1/2 cells were transfected with flag-mDia2 FH1FH2 (30 ng), PGL3-SM22 (125 ng) and increasing amounts of flag-mDia1 F1F2Δ1 or pCMV-Myc-Nuc-F1F2Δ1 (100-500ng). Luciferase measured 24 h later (**P* ≤0.05) **E)** HeLa cells were transfected with flag-F1F2Δ1 or Nuc-F1F2Δ1 and stained with flag or myc antibodies 48 h later, respectively. Changes in actin polymerization determined via phalloidin staining.

DISCUSSION

Herein we extend our previous studies implicating the DRFs in regulating SMC-specific gene transcription and MRTF subcellular localization. Double knockdown of mDia1 and mDia2 significantly inhibited expression of the SM marker genes SM22 and SM α-actin but had negligible effects on transcription of the c-fos and TK genes. mDia2, but not mDia1 or FHOD1, accumulated in the nucleus following LMB treatment and this nuclear shuttling is facilitated by an NLS and NES found in the distal N- and C-termini of mDia2, respectively. An additional NLS was found in the FH2 domain, but its function is likely blocked by the autoinhibitory state of mDia2. mDia2 variants that could not localize to the nucleus were significantly impaired in their ability to stimulate SM22 promoter activity and MRTF-B nuclear accumulation. Finally, activation of SM22 promoter activity by the FH1FH2 domain, which is predominantly nuclear, was dose dependently inhibited by a nuclear targeted mDia dominant negative. These data support a model in which mDia2-signaling in the nucleus reduces nuclear G-actin levels, prevents MRTF nuclear export, and subsequently stimulates SMC-specific gene transcription.

The DRFs mDia1 and mDia2 are important determinants of SMC phenotype

The diaphanous-related formins have been shown to regulate a diverse set of actin-dependent cellular events, including membrane ruffling, cell migration, cytokinesis, cell polarity, stress fiber formation, and cell adhesion (see [123] for review). Here we expand the role for DRFs to include regulation of SMC-specific gene transcription. Knockdown of mDia1 and mDia2 inhibited the expression of multiple SM marker genes in primary mouse aortic SMCs and 10T1/2 cells after treatment with S1P. Importantly, neither knockdown

impacted the activity of the c-fos or TK promoters, suggesting that mDia1 and mDia2 specifically regulate SMC gene transcription. We also found that the protein levels of mDia1 and mDia2 were elevated following S1P treatment, suggesting that their expression in addition to their activity may be an important determinant of SMC phenotype. It is intriguing to suggest that the expression of mDia1 and mDia2 may be at least in part regulated by SRF, but we cannot rule out changes in protein stability and additional studies will be need to decipher between these possibilities.

Germline deletion of mDia1in mice induced myeloproliferative defects and lymphopenia due to defective lymphocyte migration, but no gross vascular abnormalities were reported [190, 191]. A lack of a vascular phenotype may be attributed to a compensatory function of mDia2 in mDia1 knockout mice. In support of this, we find that knockdown of mDia1 enhances expression of endogenous mDia2 in both SMCs (figure 4.1a) and 10T1/2 cells (data not shown), which is in good agreement with other studies [127]. Closer examination of mDia1 knockout mice for defects in SMC phenotype, as well as generation of mDia2 and mDia1/2 knockout mice, may further elucidate our understating of formin function in SMC differentiation.

The human homologs of mDia1 and mDia2 are associated with the development of two different rare forms of non-syndromic genetic deafness, DFNA1 and DFN2, respectively [192, 193]. Genetic mapping and sequence analysis discovered that DFNAI patients contain a single nucleotide substitution in a splice donor of Dia1, which induces a frameshift mutation and C-terminal truncation [193]. The functional consequence of this mutation on Dia1 activity is still unclear, but similar truncations induced aberrant actin polymerization in Cos7 cells [162]. Electrophysiological data suggest deafness in DFNA1 patients may be due

in part to dysfunctional control of inner ear fluid homeostasis. These defects are commonly associated with functional abnormalities in the stria vascularis, which contains the majority of vasculature of the inner ear. Indeed, mice null for the $S1P_2$ receptor are deaf by one month of age due to altered homeostasis of the inner ear fluids, which is the result of defects in the vascular bed of the stria vascularis [194, 195]. Taken together, these findings suggest that vascular defects stemming from dysfunctional Dia signaling could contribute to deafness observed in DFNA1 and DFN2 patients.

Molecular mechanisms governing mDia2 Nuclear shuttling

The nucleoplasmic shuttling of macromolecules proceeds through nuclear pore complexes by either passive diffusion or facilitated transport. Passive diffusion is restricted to molecules smaller than 40 kDa; thus, nuclear transport of large proteins such as the DRFs are typically controlled by nuclear transport receptors of the importin β-superfamily. We demonstrated that shuttling of full-length mDia2 requires an N-terminal basic NLS and a C-terminal leucine-rich Crm-1 dependent NES. Indeed, during the final preparation of this manuscript, Miki *et al.* [196] also identified these regions as major regulatory elements of mDia2 nuclear localization in HeLa cells.

We identified an additional NLS within the FH2 domain but its role in regulating mDia2 nuclear import is still unclear. Given that the autoinhibited state of mDia2 can block FH2-mediated actin assembly, we hypothesized that this conformation may also mask the function of the FH2 NLS. Indeed, full-length constructs containing only this NLS failed to accumulate in the nucleus after LMB treatment. We found that although nuclear import of FH1FH2-DAD following LMB treatment was facilitated by the FH2 NLS, this localization

was completely inhibited by co-expression of N-terminal mDia2 fragments. Since these inhibitory effects required the DID-DAD interaction, these findings further support a role of mDia2 autoinhibition in regulating the FH2 NLS function. A cryptic NLS in the FH2 domain suggests that the activation state of mDia2 may differentially regulate its subcellular localization and may be a novel mechanism by which active mDia2 is imported into the nucleus. Additionally, since splice variants and cleavage products have recently been identified in other diaphanous family members, it is intriguing to consider that the FH2 NLS may be important in analogous variants of mDia2 [187, 197].

Nuclear accumulation of FH1FH2 was also completely blocked when fused to the N-terminal amino acids 256-533, but the mechanism underlying this inhibition is currently being investigated. We predict three possible mechanisms that could explain this inhibitory effect. (1) Residues 256-533 could inhibit nuclear import by directly binding and masking the region containing the FH2 NLS. Indeed, structure-function studies have proposed an interaction between the N-terminus and FH2 domain that is independent of the DID-DAD interaction [163]. (2) The N-terminal region could also alter FH1FH2 nuclear import by facilitating dimerization. Several conserved coiled-coil regions have been predicted in this region and similar residues in mDia1 was shown to be dimeric through the use of gel filtration chromatography [163]. Inhibition of nuclear import by dimerization has been reported for several other factors [198, 199]. (3) It is also possible that amino acids 256-533 inhibit nuclear accumulation of FH1FH2 through a Crm-1 independent export mechanism. Interestingly, mDia1 was identified as a substrate for exportin6 [188] and a variant of mDia1 consisting of the FH1,FH2 and DAD domains is predominantly nuclear [160], but we found that addition of the N-terminal 257-567 amino acids inhibited this localization (data not

shown). Since fusion of amino acids 256-533 to GFP or mDia2 GBD did not induce cytoplasmic localization (unpublished observation), nuclear export may require sequences within the FH1FH2 domain. Recent studies found that actin is required for nuclear export of MRTF [148]. Similarly, perhaps binding of nuclear actin to the FH2 is required for residues 256-533 to facilitate nuclear export of mDia2.

We have also identified a leucine-rich Crm-1 dependent export sequence within the C-terminus of mDia2. However, mutation or deletion of this NES induced nuclear accumulation to a lesser degree than LMB treatment, suggesting the presence of an additional NES. We and others have found LMB treatment induces nuclear accumulation of N-terminal fragments of mDia2, but no functional Crm-1 dependent export sequence has yet to be identified within this region [196]. It is possible that the DID within these N-terminal fragments binds to endogenous mDia2 and is subsequently carried to the nucleus upon inhibition of Crm-1. Recently, Alberts demonstrated that a DAD peptide containing just the core region, which contains several conserved leucines but lacks the NES discussed above, may also regulate mDia2 nuclear export [200]. These experiments showed that nuclear accumulation of GFP was blocked when fused to the DAD core, and that an L1044A mutation to the DAD restored nuclear localization. Since our NES mutations only partially inhibited nuclear export of mDia2, it is possible that an additional NES within the DAD core is sufficient to affect some nuclear export. Given that the DAD regulates both activation and nuclear export of mDia2, further studies will need to clarify if nuclear shuttling of mDia2 is influenced by its activation state.

Regulation of SMC-specific gene transcription by nuclear mDia2

Regulation of gene transcription by nuclear actin was first reported in the 1980s, but these studies were largely dismissed on the basis of possible experimental contamination from abundant cytoplasmic actin pools. More than 25 years later, it is well established that actin exists in the nucleus and regulates a plethora of nuclear proteins, including chromatin remodeling factors, RNP particles and all three RNA polymerases (reviewed in [201]). Interestingly, a recent study by Vartiainen *et al.* has implicated nuclear actin dynamics in the control of MRTF-A subcellular localization [148]. These authors demonstrate that nuclear G-actin stimulates Crm1-dependent nuclear export of MRTF-A and inhibits SRF-dependent gene transcription. Further, MRTF-A bound G-actin in both the cytoplasm and nucleus, but this interaction varied dramatically with changes in RhoA-mediated actin treadmilling. However, it is currently unknown whether RhoA directly regulates nuclear actin pools or if a change in nuclear actin dynamics simply reflects changes in cytoplasmic actin treadmilling. Herein, we show that the RhoA effector mDia2 shuttles to the nucleus and that inhibition of this localization reduces nuclear accumulation of MRTF-B and transcription of SMC-specific genes. To our knowledge, this is the first evidence identifying a nuclear role for a RhoA effector in the regulation of MRTF localization and SRF-dependent transcription. While we attribute these effects to changes in nuclear actin dynamics mediated by mDia2, we have been unable to confirm this premise due to the lack of a reliable label for nuclear actin. Only recently was McDonald *et al.* able to distinguish highly dynamic nuclear actin polymers, distinct from cytoplasmic actin filaments, by using fluorescence recovery after photobleaching (FRAP) of GFP-β-actin in HeLa cells [146]. These findings are in agreement with other immunofluorescence studies describing distinct immunological signatures of nuclear actin as detected by monoclonal antibodies [145, 202]. This may indicate that at

least a fraction of the nuclear actin pool has a unique conformation whose polymeric state may be unrecognizable by conventional actin stains such as phalloidin. Others have shown that latrunculin, which sequesters actin monomers, inhibited nuclear processes, such as export and transcription, providing functional data to support the presence of polymeric actin in the nucleus [146, 203]. Given abundant evidence that MRTF-A is a good sensor of actin monomer pools and that DRFs are dynamic regulators of actin polymerization, we highly favor a model in which mDia2 activity in the nucleus depletes nuclear G-actin, inhibits MRTF nuclear export, and activates SMC-specific gene transcription (figure 4.8). Based on our results, we cannot decipher whether activation of mDia2 by RhoA occurs prior or subsequent to nuclear import. In addition to its small size (~24 kDa), RhoA possesses a conserved canonical NLS in its C-terminus, and might thus utilize facilitated and/or passive diffusion to enter the nucleus [204]. The presence of Rho guanine nucleotide exchange factors in the nucleus further supports a nuclear function of RhoA-signaling [205-207]. Previously, interactions between Cdc42, RhoB, and mDia2 have been successfully detected using fluorescence resonance energy transfer (FRET) [127, 128] Similar experiments using probes for RhoA and mDia2 will be critical to determine whether these factors interact in the nucleus.

Conclusions

In this study, our findings suggest that nuclear shuttling of mDia2 plays an important role in the regulation of MRTF subcellular localization and SMC-specific gene transcription. We did not detect mDia1 or FHOD1 in the nucleus following leptomycin treatment and other studies suggest that cytoplasmic localization of these DRFs may be maintained by a CRM-1-

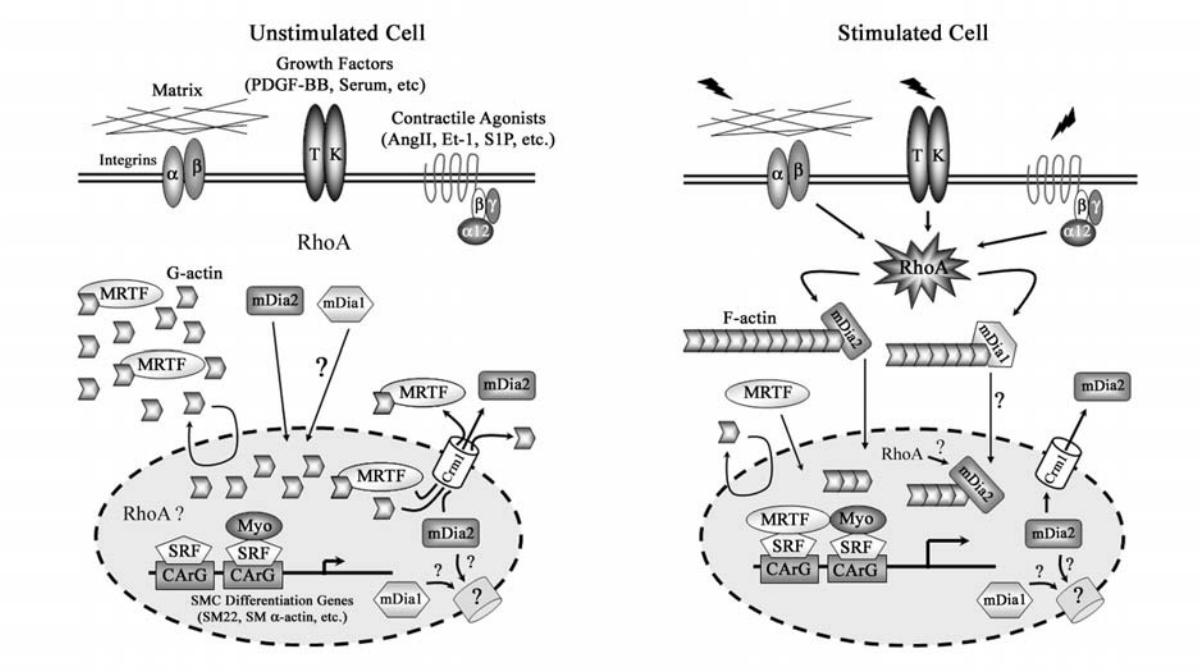

Figure 4.8: Current model of SMC differentiation by the DRFs. In unstimulated cells, a direct interaction with G-actin reduces MRTF nuclear accumulation by inhibiting nuclear import while simultaneously enhancing nuclear export, subsequently reducing transcription of SMC-specific genes. Herein we also demonstrate that although mDia2 is predominantly cytoplasmic, it shuttles from the cytoplasm to the nucleus and is exported in a Crm-1 dependent manner. A similar export mechanism has been identified for G-actin and MRTF, although the small size of actin likely allows it to also enter the nuclues by passive diffusion. Additional studies are required to determine if mDia1 and RhoA are present in the nucleus and whether they utilize similar or different export mechanisms. Following stimulation by a variety of extracellular stimuli, activation of RhoA leads to an increase in cytoplasmic actin treadmilling and nuclear import of MRTF, which is at least in part accomplished by activation of mDia1 and mDia2. Similarly, a reduction in nuclear G-actin pools (partially regulated by activation of mDia2) inhibits MRTF nuclear export, leading to an increase in MRTF nuclear accumulation and SMC marker gene expression.

independent nuclear export mechanism. We suspect that the DRFs at least in part regulate SM marker gene expression by regulating nuclear actin dynamics. In support of this, other *bona fide* actin nucleators, such as the Arp2/3 complex, localize to the nucleus and have been suggested to regulate nuclear actin polymerization [185, 208]. Since actin associates with nuclear lamins and can control the nuclear export of molecules, such as MRTF-A and HIV-1 mRNA, this suggests the regulation of nuclear actin dynamics by the DRFs (or other formins) may also modulate nuclear architecture and trafficking. With an abundance of new evidence implicating actin in a variety of nuclear processes, future studies to identify the roles of formins regulating the actin nucleoskeleton will be important

CONCLUSIONS, PERSPECTIVES, AND FUTURE DIRECTIONS

It is clear that the dynamic regulation of SMC phenotype plays an important role in vascular development and disease. A plethora of studies from our laboratory and others have made significant progress in identifying the signaling mechanisms that govern SMC-specific gene transcription. Given the inherent complexity of these signaling cascades, it is critical to incorporate the findings described within this dissertation into our current understanding of SMC differentiation.

Regulation of SMC-specific gene transcription: The role of SRF, MRTF and RhoA

Nearly all SMC-specific genes contain conserved promoter elements called CArG boxes that bind to the transcription factor SRF [46, 52]. Mutagenesis of SMC gene CArG elements or germline deletion of SRF in mice significantly inhibits SM marker gene expression *in vivo,* demonstrating the importance of these factors in SMC differentiation [49, 50, 59, 60]. Since SRF is ubiquitously expressed and controls the transcription of growth genes as well as skeletal muscle- and cardiomyocyte-specific genes, it is clear that additional factors are required to promote SMC-specific gene transcription [61-64]. Indeed, myocardin and the myocardin-related transcription factors (MRTF-A and MRTF–B) were identified as SRF cofactors that specifically enhanced transcription of SMC marker genes, and genetic disruption of any myocardin family member resulted in abnormal SMC differentiation [74, 77, 89, 92, 184].

The upstream signaling mechanisms that regulate SRF and the myocardin family were first elucidated by the Treisman laboratory, which showed that activation of the small GTPase RhoA could potently stimulate SRF-dependent transcription [100]. Interestingly, these effects were inhibited by latruculin, an actin monomer-sequestering drug, strongly implicating actin polymerization in the regulation of SMC-specific gene transcription. A pivotal study by Miralles *et al.* subsequently showed that subcellular localization of MRTF-A was similarly regulated by actin dynamics [88]. MRTF-A was predominantly cytoplasmic when G-actin levels were elevated by serum starvation, but translocated to the nucleus upon stimulation of actin polymerization. These authors further demonstrated that G-actin directly bound the N-terminus of MRTF-A, and that this interaction likely inhibited nuclear import. Taken together, these data supported a model in which activation of RhoA promotes actin polymerization, resulting in nuclear localization of MRTF-A and transcription of SMC-specific genes (see figure 1.3).

Adding the DRFs to the puzzle

Investigation of RhoA effectors that regulate SMC differentiation has mainly focused on Rho Kinase (ROCK), since it enhances actin polymerization, stress fiber formation, and contractility in SMCs [114, 115]. However, we and others have shown that treatment with Y-27632, a potent inhibitor of ROCK, only partially attenuates SMC-specific gene transcription [33, 117], suggesting that additional RhoA effectors are involved in this process. Chapter 1 of this dissertation demonstrated that the RhoA effectors mDia1 and mDia2 are highly expressed in SMCs, and potently stimulate SMC marker gene expression. Neither Diaphanous-Related Formin (DRF) activated transcription of the SRF-dependent

growth gene *cfos,* suggesting the transcriptional effect induced by mDia1/2 is unique to SMC genes. Activation of mDia2 was sufficient to induce MRTF-A/B nuclear accumulation, and this event was required for mDia2-mediated SMC-specific gene transcription. Furthermore, overexpression of a dominant negative variant that blocks mDia-mediated actin polymerization attenuated SM marker gene transcription in a dose-dependent fashion. These results are supported by the experiments described in chapter 3, which showed that silencing of mDia1/2 with siRNA significantly inhibits SM marker gene expression in primary aortic SMCs. When compiled, these data indicate that activation of mDia1 and mDia2 by RhoA can increase transcription of SMC-specific genes by stimulating nuclear accumulation of the MRTFs.

Initial studies, including our own, proposed that MRTF localization was primarily regulated via G-actin binding. According to this model, when the MRTFs were bound to G-actin, they were sequestered in the cytoplasm; when dissociated from G-actin, they were imported to the nucleus. However, a recent report by Vartiainen *et al.* argues against this paradigm, providing elegant evidence that nuclear export, and not import, is the major determinant of MRTF localization. Interestingly, direct binding of actin to MRTF-A within the nucleus stimulated Crm-1-mediated nuclear export of MRTF-A and inhibited SRF-dependent transcription. Given our evidence that mDia1 and mDia2 potently stimulate nuclear accumulation of MRTF-A, we chose to investigate a potential function of these DRFs in the nucleus. We found that mDia2, but not mDia1 or FHOD1, accumulated in the nucleus after leptomycin treatment, suggesting that mDia2 shuttles through the nucleus via a Crm-1 dependent export mechanism. Deletion mapping and mutation analysis revealed two nuclear localization signals (NLS) within mDia2: one in the FH2 domain, and a second in the

distal N-terminus. Furthermore, nuclear export of mDia2 was mediated by a leucine-rich region adjacent to the DAD. In comparison to wild-type mDia2, variants that were excluded from the nucleus were weak stimulators of SMC-specific gene transcription and MRTF-B nuclear accumulation. We also found that the nuclear-localized FH1FH2 domain of mDia2 strongly stimulated SM22 promoter activity, an effect that was dose-dependently inhibited by a nuclear-targeted dominant negative of mDia2. Taken together, these data support a model in which mDia2 activity in the nucleus and cytoplasm depletes cellular G-actin pools resulting in MRTF nuclear accumulation and activation of SMC-specific gene transcription (see figure 4.8 for schematic of current SMC differentiation model).

RhoA signaling in the nucleus

Although our data indicate that nuclear localization of mDia2 is important to its role in SMC-specific gene transcription, whether mDia2 is activated prior to or following nuclear import remains unknown. It is entirely plausible that RhoA could enter the nucleus passively or actively, since it possesses not only a low molecular weight (~24kDa), but also a canonical C-terminal NLS [204]. While most reports have described RhoA localization as predominantly cytoplasmic, cell fractionation studies have in fact identified a small nuclear RhoA pool [209]. Additionally, the Rho GEFs ECT2 and NET1 have also been detected in the nucleus, further supporting a nuclear function of RhoA [206, 207]. While we have focused on the role of RhoA in regulating nuclear mDia2 signaling, it is certainly possible that other factors may regulate nuclear mDia2 activity. RhoB has been reported to bind mDia2 [128] and to localize to the nucleus [210, 211], but additional studies are required to determine if RhoB (or RhoC) might play a role in mDia2-mediated transcription. In chapter

2, we demonstrate that ROCK activates mDia2 by phosphorylating the DAD. Given that ROCK has also been detected in the nucleus, one might hypothesize that ROCK regulates nuclear mDia2 activity [212]. Since it is clear that RhoA can regulate a plethora of cellular processes, a closer examination of nuclear RhoA signaling will be critical to better understand its myriad functions.

A role for the DRFs in nuclear actin dynamics

There is now overwhelming evidence implicating the DRFs in the regulation of actin polymerization (see [123] for review). Since we and others have shown that the DRFs, or variants of them, localize to the nucleus, it is intriguing to consider these factors as potentially important regulators of nuclear actin dynamics. In support of this, other *bona fide* actin nucleators, such as the Arp2/3 complex, localize to the nucleus and have been suggested to regulate nuclear actin polymerization [185, 208]. Additionally, the actin-binding protein profilin, which is required for robust DRF-mediated actin polymerization, has also been detected in the nucleus [188, 213]. Since traditional actin labeling tools such as phalloidin are unable to detect nuclear actin structures, methodological limitations currently pose an impediment to the study of nucleoskeletal remodeling. While non-physiological approaches (*e.g.,* latrunculin treatments and *in vitro* polymerization assays) have been used with some success, it is clear that the development of new tools to visualize and quantitatively measure nuclear actin polymerization *in situ* will be critical to the advancement of this field. Two groups have recently developed monoclonal antibodies that may be specific for nuclear actin [145, 202]. One antibody recognizes actin in its dimeric form, while the other binds to its nucleotide-binding cleft. However, these promising tools

are still in their infancy, and have only been tested in a limited number of cell types. We are currently evaluating the ability of these antibodies to recognize nuclear actin structures in SMCs and 10T1/2 cells.

The most convincing evidence of nuclear F-actin comes from a recent study by McDonald *et al*. [146]. By measuring fluorescence recovery after photobleaching (FRAP), the authors demonstrated that the recovery rate of GFP-β-actin in the nucleus depends on the equilibrium between nuclear G- and F-actin. Latrunculin B treatment, which inhibits actin polymerization, increased the GFP-actin recovery rate, while jasplakinolide, which promotes polymerization, decreased the recovery rate. These data suggest that monomeric actin diffuses into the photobleached nucleus at a faster rate than polymeric actin. Using this FRAP-based approach, we hope to directly assess the role of the DRFs in nuclear actin dynamics. Given its nuclear localization, we predict that the catalytically active FH1FH2 fragment will attenuate GFP-β-actin recovery by enhancing nuclear actin polymerization and impeding G-actin diffusion. Similar experiments will be conducted in cells transfected with various DRF-targeted siRNAs. FRET has also been used to quantitate the equilibrium of F- and G-actin in live cells [214]. In brief, when CFP- and YFP-actin are co-expressed in cells, polymerization of the labeled monomers facilitates energy transfer between the CFP and YFP fluorophors, resulting in a FRET signal. As yet, this technique has not been used to monitor nuclear actin, but could prove a useful tool for spatiotemporal analysis of nucleoskeletal remodeling in the context of overexpressed mDia2 variants.

Mechanisms regulating mDia2 autoinhibition

Given the important roles of mDia2 in SMC-specific gene transcription, we sought to further characterize the molecular mechanisms that govern its activity. The autohibitory state of mDia2 is mainly controlled by the binding of the core DAD motif (MDXLLXL) to residues within the DID. Binding of active RhoA to the GTPase binding domain (GBD) results in the dissociation of the DID from the DAD and exposure of the catalytically active FH1FH2 domain. Additional studies identified a basic region adjacent to the DAD core motif which is conserved throughout the DRFs, and mutation of these residues partially disrupts the DID-DAD interaction [125]. We found that this basic region bound to corresponding acidic residues within the DID, and mutation of these acidic residues partially activated mDia2 by weakening DID-DAD binding. Indeed, a similar acidic region was recently identified in mDia1 [215]. Interestingly, we identified two conserved ROCK consensus phosphorylation sites near the basic region, and hypothesized that addition of negatively charged phosphate groups could disrupt DID-DAD binding, thereby enhancing mDia2 activity. We found that ROCK phosphorylated the mDia2 DAD at T1061 and S1070 *in vitro* and *in vivo*. Phospho-mimetic mutation (mDia2-2E) of both residues decreased the DID-DAD interaction, thereby stimulating actin polymerization, MRTF activation, and SMC-specific gene transcription.

Detailed analysis of mDia activation has revealed that RhoA binding only partially alleviates the DID-DAD interaction, suggesting that additional signaling events are required for full activation [163]. In good agreement with this claim, we found that overexpression of constitutively active (L63) RhoA activated SMC-specific gene transcription to a lesser extent than constitutively active mDia2 (ΔGBD) [184]. Thus, we hypothesized that while RhoA binding may alleviate the core DAD-DID interaction, additional disruption of the basic

region via phosphorylation might be required to completely activate mDia2. In support of this, we found that L63 RhoA activated mDia2-2E much more robustly than wild-type mDia2. Taken together, these data suggest that phosphorylation of the DAD by ROCK may partially activate mDia2 by disrupting the electrostatic attraction between the basic and acidic motifs in the DAD and DID regions, respectively. In sum, our data supports a model in which full activation of mDia2 requires both RhoA binding and phosphorylation of the DAD.

The ability of mDia2 to regulate numerous cellular processes is likely achieved by its promiscuous binding of various GTPases, including Cdc42, Rif, and RhoA-C [118, 126, 127]. Although the presence of a unique threonine-serine-histidine (TSH) motif in the mDia2 DID has been shown to mediate binding of these GTPases [215], very little is known of how conformational changes in mDia2 can alter these interactions. For example, could phosphorylation-induced changes in the DID-DAD interaction trigger a conformational shift in the GBD that determines the binding affinity of specific GTPases? We found that RhoA bound more strongly to mDia2-2E than to wild-type mDia2, and it will be interesting to see if a similar increase in binding occurs with other GTPases. Currently, our model assumes that phosphorylation occurs when mDia2 is in the "closed" conformation, and activates the molecule by relieving DID-DAD binding. However, it remains possible that phosphorylation may occur when mDia2 is active. According to this model, phosphorylation of the DAD could prevent reassociation of the DID and the DAD, thus trapping the molecule in its "open" conformation and prolonging its activity. A closer examination of DAD phosphorylation in active and inactive mDia2 variants will be needed to evaluate the accuracy of these two models.

In addition to regulating SMC differentiation, RhoA also modulates vascular tone. Vascular SMC contraction is closely associated with myosin light chain (MLC) phosphorylation, which is regulated by RhoA-dependent activation of ROCK and subsequent inhibition of MLC phosphatase [216]. Protein kinase G (PKG) is one of the most well known physiological inducers of SMC relaxation, which is in part accomplished by inhibition of RhoA-mediated contractility [217, 218]. Interestingly, numerous studies have indicated that PKG positively regulates SMC differentiation, despite its inhibitory effect on RhoA-mediated cell contractility (see [217] review). This paradoxical evidence could be explained by direct phosphorylation of mDia2 by PKG. In this model, activation of PKG would inhibit the RhoA-ROCK-MLC pathway of contractility, but could simultaneously activate mDia2 to maintain the differentiated state of the SMC. Although we found that PKG can phosphorylate the mDia2 DAD *in vitro* (data not shown), additional *in vivo* studies will be essential to determine the biological consequence of this phosphorylation event. During the course of our studies, two separate reports identified FHOD1 as a substrate for both PKG and ROCK [178, 180]. Although the functional consequence of PKG phosphorylation was not investigated, phosphorylation by ROCK occurred in the DAD domain and significantly enhanced the activity of FHOD1. Taken together, our studies and others suggest that the activation state of the DRFs is regulated not only by GTPase binding, but also by multiple phosphorylation events.

Developmental and pathological roles of the DRFs **in vivo**

Although the DRFs have been fairly well described as dynamic regulators of the actin cytoskeleton, their *in vivo* function is poorly understood. The human homologs of

mDia1 and mDia2 (Dia1 and Dia3) are associated with the development of two different rare forms of non-syndromic genetic deafness, DFNA1 and DFN2 [192, 193]. Genetic mapping and sequence analysis discovered that a single nucleotide substitution in a splice donor induced a frameshift and C-terminal truncation of Dia1 in the DFNA1 patients [193]. A similar truncation in mDia1 has been shown to disrupt DID-DAD binding and induce aberrant stress fiber formation in Cos-7 cells [118]. This cytoskeletal perturbation has been proposed to be responsible for dysregulated function of stereocilia within the ear, but additional studies are needed to support this claim [219].

Alternatively, Dia might be important for the proper formation and function of the inner ear vasculature. Studies that have further characterized DFNA1 have identified that hydrops, or fluid imbalances of the ear, could be the etiology of deafness in these patients [220]. The presence of hydrops can be indicative of functional abnormalities in the *stria vascularis*, which encompasses the majority of the inner ear vessels and is important in maintaining fluid homeostasis [221]. Indeed, two separate studies have reported that mice lacking the $S1P_2$ receptor have defects in the vascular bed of the *stria vascularis* and are deaf by one month of age due to dysfunctional control of inner ear fluid homeostasis [194, 195]. Given our data strongly implicating the diaphanous proteins in SMC differentiation, it is intriguing to hypothesize that dysregulated Dia signaling could result in failed SMC maturation, leading to abnormal structure and function of the *stria vascularis,* and ultimately deafness. In chapter 3, we found that S1P-mediated stimulation of SMC gene expression was dependent on the presence of mDia1 and mDia2, suggesting that the S1P-RhoA-mDia signaling axis may be required for maintenance of inner ear fluid homeostasis by the *stria vascularis*. Further structural and functional analysis of the *stria vascularis* in the absence or

disruption of mDia signaling could prove valuable to the development of therapeutic approaches for DFNA1 and DFN2.

The absence of other vascular phenotypes associated with mDia1 or mDia2 may be due to inadequate *in vivo* animal models. There has been no indication of vascular abnormalities in mDia1-null mice, but such a phenotype may be masked by compensatory upregulation of mDia2 [190, 191]. In support of this, we and others have found that cells in which mDia1 has been deleted or silenced exhibit increased expression of mDia2 [127]. The generation of mDia2 and mDia1/2 double-knockout mice is required to better understand the role of these DRFs in vascular development. To our knowledge, the only study investigating the *in vivo* role of mDia2 during development has been conducted in zebrafish through the use of antisense morpholino oligonucleotides (MOs), which ablate mDia2 expression by blocking translation [222]. MOs targeted to mDia2 induced gastrulation defects which were attributed to abnormal actin filament assembly and cell movement at the leading edge of the blastoderm. These findings are in agreement with a plethora of studies indicating that mDia2 regulates cell motility by controlling the formation of actin stress fibers, filopodia, and lamellipodia [223-226]. These early developmental defects have unfortunately made it difficult to access the role of mDia2 in SMC differentiation in zebrafish. It is reasonable that a similar phenotype in mice may occur following deletion of mDia2, and for these reasons it will be imperative to specifically target mDia2 in SMCs using the Cre/LoxP recombination system.

There are several extant mouse models that express Cre recombinase under the control of an SMC-specific promoter, including SM22 and SM-MHC. However, their use in SMC-specific knockout studies has been limited because of low excision efficiencies, lack of

SMC specificity during development, and embryonic lethality (see [227] for commentary). To overcome these obstacles, our lab has obtained mice containing a fusion protein of Cre recombinase and the modified estrogen receptor domain under the control of the SM-MHC promoter (SM-MHC-Cre-ER) [228]. Treatment of these adult mice with tamoxifen resulted in robust recombination in SMCs but not in other cell types, including skeletal and cardiac muscle cells [228]. It will also be interesting to identify the role of mDia1 or mDia2 in the differentiation of variant SMC lineages. For example, using the Wnt1-Cre-ER mouse, we could selectively ablate mDia2 expression in neural crest cells, and assess the impact of this deletion on SMC differentiation from this particular embryonic origin [229].

Although they lack vascular abnormalities, mDia1-null mice do develop an age-dependent myeloproliferative phenotype that resembles human myeloproliferative and myelodysplastic syndromes (MPS, MDS) [191]. While these data suggest that mDia1 inhibits proliferation of myeloid cells, a separate study found that increased levels of mDia1 stimulated cell proliferation in melanocytes [230]. Thus, the role of mDia1 in cell proliferation may be cell-type specific, a possibility that warrants further investigation. Given their importance in regulating cell migration, proliferation and differentiation, it is worthwhile to investigate how the DRFs might contribute to the initiation and progression of specific cancers. A recent study discovered that depletion of SRF or MRTF in MDA-MB-231 breast carcinoma and B16F2 melanoma cells results in reduced cell adhesion, spreading, invasion and motility in culture [231]. Furthermore, tumor cells lacking SRF or MRTF were unable to colonize the lung from the bloodstream. These data suggest that modulation of gene expression by the RhoA-actin-MRTF-SRF signaling pathway is an important component of cytoskeletal remodeling and metastasis. Since we have shown that DRFs can

regulate the SRF-MRTF signaling pathway, it will be interesting to address whether deletion of mDia1 or mDia2 can similarly impede cancer cell motility. Interestingly, several formins have already been implicated in tumor progression and metastasis in colorectal carcinomas, leiomyosarcomas, and osteosarcomas [232-235]. Smooth muscle neoplasms occur in almost 25% of women in the United States, and are the cause of approximately one-third of hysterectomies performed each year [236]. Altered SRF binding to the promoters of certain SMC-specific genes have been closely linked to the progression of these neoplasms [236], suggesting that upstream RhoA or mDia signaling may also contribute to their pathogenesis. Given their unique ability to control cell migration by regulating the actin cytoskeleton and gene expression, developing therapeutic strategies to target the DRFs in cancer may prove to be beneficial.

Conclusion

The studies detailed in this dissertation identify the DRFs as dynamic regulators of SMC differentiation. However, additional studies are required to further characterize the molecular mechanisms that govern DRF activity and to determine the role of these factors in nucleoskeletal dynamics. Since existing reports have primarily utilized biochemical and cellular techniques to study the DRFs, the development of small animal models to study these proteins *in vivo* remains an outstanding need in the field. Such models may well identify new roles for the DRFs in the pathogenesis of diseases such as atherosclerosis, restenosis, deafness, and cancer. Given a better understanding of how the DRFs contribute to these pathologies, development of small molecular compounds that can alter GTPase binding or intramolecular interactions could prove a valuable therapeutic approach.

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