THE ROLE OF SWI/SNF IN REGULATING SMOOTH MUSCLE DIFFERENTIATION

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This thesis is dedicated to the memory of my beloved father Jiagen Zhang and my grandparents Cijing Chen and Qinchen Zhang.

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Abstract

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THE ROLE OF SWI/SNF IN REGULATING SMOOTH MUSCLE DIFFERENTIATION

There are many clinical diseases involving abnormal differentiation of smooth muscle, such as atherosclerosis, hypertension and asthma. In these diseases, one important pathological process is the disruption of the balance between differentiation and proliferation of smooth muscle cells. Serum Response Factor (SRF) has been shown to be a key regulator of smooth muscle differentiation, proliferation and migration through its interaction with various accessory proteins. Myocardin Related Transcrition Factors (MRTFs) are important co-activators of SRF that induce smooth muscle differentiation. Elucidating the mechanism of how MRTFs and SRF discriminate between genes required to regulate smooth muscle differentiation and those regulating proliferation will be a significant step toward finding a cure for these diseases. We hypothesized that SWI/SNF ATP-dependent chromatin remodeling complexes, containing Brg1 and Brm, may play a role in this process. Results from western blotting and quantitative reverse transcription - polymerase chain reaction (qRT-PCR) analysis demonstrated that expression of dominant negative Brg1 or knockdown of Brg1 with silence ribonucleic acid (siRNA) attenuated expression of SRF/MRTF dependent smooth muscle-specific genes in primary cultures of smooth muscle cells.

Immunoprecipitation assays revealed that Brg1, SRF and MRTFs form a complex *in vivo* and that Brg1 directly binds MRTFs, but not SRF, *in vitro.* Results from chromatin immunoprecipitation assays demonstrated that dominant negative Brg1 significantly attenuated SRF binding and the ability of MRTFs to increase SRF binding to the promoters of smooth muscle-specific genes, but not proliferation-related early response genes. The above data suggest that Brg1/Brm containing SWI/SNF complexes play a critical role in differentially regulating expression of SRF/MRTF-dependent genes through controlling the accessibility of SRF/MRTF to their target gene promoters. To examine the role of SWI/SNF in smooth muscle cells *in vivo*, we have generated mice harboring a smooth muscle-specific knockout of Brg1. Preliminary analysis of these mice revealed defects in gastrointestinal (GI) development, including a significantly shorter gut in Brg1 knockout mice. These data suggest that Brg1-containing SWI/SNF complexes play an important role in the development of the GI tract.

B.Paul Herring, Ph.D, Chair

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List of Abbreviations

Brg1: Brahma-like gene

Brm: Brahma

cDNA: Complementary Deoxyribonucleic Acid

ChIP: chromatin immunoprecipitation

Co-IP: Co-immunoprecipitation.

DKO: double knockout

DMEM: Dulbecco's Modified Eagle Medium

DN-Brg1: dominate negative Brg1

ECM: extracellular matrix

EM: electron microscopy

FBS: fetal bovine serum

GI tract: gastrointestinal tract

HATs: histone acetyl transferases

HDACs: histone deacetylases

HE staining: Haemotoxylin-Eosin staining

Hox genes: Homeobox genes

IEGs: immediate early response genes

KLF: Kruppel-like factors

KO: knockout

MAFs: murine adult fibroblasts

MLCK: myosin light chain kinase

mRNA: messenger ribonucleic acid

MRTFA: Myocardin Related Transcription Factor A

MRTFB: Myocardin Related Transcription Factor B

MRTFs: Myocardin Related Transcription Factor Family

PBS: phosphate-buffered saline

PCNA: Proliferating Cell Nuclear Antigen

PEO: proepicardial organ

qRT-PCR: quantitative reverse transcription (RT)-Polymerase chain reaction (PCR)

SM: smooth muscle

SMCs: smooth muscle cells

SM MHC: smooth muscle myosin heavy chain

Shh: sonic hedgehog

siRNA: silence ribonucleic acid

SRF: Serum Response Factor

SWI/SNF complex: Switching defective (SWI) and Sucrose nonfermenting complex

TAD: transcription activation domain

TCF: the ternary complex factor family

TGFβ: transforming growth factor beta

TUNEL: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

Chapter I: Introduction

A. Smooth muscle development.

During development mesenchymal stem cells differentiate into precursor smooth muscle cells (SMCs), characterized by the expression of smooth muscle α-actin in the absence of other smooth muscle-specific proteins. The precursor SMCs further differentiate into mature contractile SMCs characterized by their elongated, spindle shape and high levels of smooth muscle-specific contractile proteins such as smMHC, calponin, caldesmon, SM22α and telokin (57, 111). The origins of the mesenchymal stem cells that give rise to smooth muscle cells are quite diverse. In the gut, stem cells were mainly from the splanchnic mesoderm, which is closely surrounding the endoderm of the primitive gut tube (5, 146); stem cells from ventral cranial neural tube are also a source of some gut SMCs (11). In the vascular system, smooth muscle cells arise from a variety of sources. For example, stem cells from cranial neural crest give rise to the SMC of the aortic arch, proepicardial organ (PEO) stem cells differentiate into coronary artery SMCs and progenitors cells within the endothelium are a source of SMCs in some vessels (64).

Within each smooth muscle tissue a complex cross-talk between epithelial or endothelial cells and smooth muscle precursor cells plays a critical role in organogenesis. For example, in the gut, sonic hedgehog (Shh) from the endoderm induces the expression of Bmp4 and Hoxd13 in the splanchnic mesoderm that expresses Shh receptor (Ptc) and subsequently regulate SMCs

differentiation (114). Homeobox (Hox) genes are expressed in both endoderm and mesoderm. The expression pattern of Hox genes along the gut plays an important role in determining the anterior-posterior patterning of the developing gut (Figure 1). Evidence also shows that the mesoderm can affect endoderm differentiation in that small intestine mesoderm grafted onto colon endoderm results in the development of a small intestinal-like epithelium rather than the normal colonic epithelium (45).

Generally there are three important determinants of SMC differentiation: biochemical factors, extracellular matrix (ECM) proteins and physical parameters (reviewed in (111), Figure 2). Besides the Shh and Hox genes discussed above, other biochemical factors including retinoic acid, TGFβ1, BMPs and Wnt signaling molecules are also important regulators of smooth muscle development (reviewed in (113)) (34). Heparin collagen type IV, as well as laminin in the ECM generally maintain SMC's in a differentiated state and decrease proliferation (reviewed in (111)). Stretch and shear stress also work as mechanical factors to promote smooth muscle differentiation (111).

B. Smooth muscle diseases.

SMCs are very dynamic even after differentiation. In many pathological states contractile SMCs can transform into a proliferative, synthetic state characterized by decreased expression of smooth muscle-specific contractile proteins, increased proliferation and increased synthesis of extracellular matrix proteins

(reviewed in (111) (104)). For example, the expression of smooth muscle contractile proteins is changed during the diseases of intestinal obstruction, idiopathic megacolon, obstructive bladder disease, atherosclerosis, hypertension and asthma (3, 53) (29, 52) (78). Understanding the mechanisms by which SMCs regulate the transformation between differentiation and proliferation under physiological and pathological conditions will be an important step toward treating and preventing these diseases. There are many different extracellular signaling molecules that can affect the phenotype of smooth muscle cells under pathological conditions. These include cytokines such as TGFβ and peptide hormones such as PDGFbb and Angiotensin II (58, 64, 125, 143). These hormones regulate intracellular signaling cascades that affect the activity of transcription factors that regulate the differentiation and proliferation of smooth muscle cells. For example TGFβ induces the expression of smooth muscle contractile proteins SM22α and sm α-actin in precursor cells, while PDGFbb and KLF4 inhibit this induction (56). KLF4 conditional knockout mice exhibit delayed attenuation of smooth muscle-specific contractile protein expression following vascular injury (144). Our lab and other groups have shown that a zinc finger transcription factor, GATA6 activates expression of smMHC and sm α-actin (141) and is down-regulated following vascular injury (87). Importantly, local injection of GATA6 into a balloon-injured carotid artery inhibited the dedifferentiation of SMCs and prevented lesion formation (87), demonstrating that pathological down regulation of important transcription activators is sufficient to alter the phenotype of smooth muscle cells. In a mouse model of chronic partial obstruction of the

small intestine, intestinal smooth muscle cells initially dedifferentiate and proliferate and subsequently the proliferation ceases, the cells begin to re-differentiate and then there is hypertrophy. During this process inhibitory factors such as KLF4 initially increase in the proliferating SMC while the transcription activators such as myocardin decrease. This pattern is then reversed during the hypertrophic phase (29). Together these studies suggest that the dynamic regulation of transcription activators and repressors regulates the phenotype of SMC under pathological conditions.

Recent studies have also demonstrated changes in the structure of chromatin within smooth muscle cells under pathological conditions. Histone deacetylase (HDACs) activity was decreased in the lung tissue obtained from patients with chronic obstructive pulmonary disease (COPD)(73). Histone acetyltransferases (HATs) and HDACs have been shown to regulate the proliferation of SMCs which is involved in atherosclerosis and restenosis (108). For example, the HDAC inhibitor TSA reduced vascular SMC proliferation through increasing expression of the cell cycle inhibitor p21 (102). In addition, deacetylation of histone H4 at the promoters of smooth muscle-specific genes has been associated with vascular injury (91). The ATP-dependent chromatin remodeling enzyme Brg1 has also been shown to be unregulated in vascular smooth muscle cells in primary atherosclerosis and in stent stenosis (148). Together these studies suggest that changes in chromatin structure likely act coordinately with changes in transcription factor expression to regulate the phenotype of smooth muscle cells

under physiological and pathological conditions.

C. Serum Response Factor and smooth muscle differentiation in health and disease.

Serum Response Factor (SRF), is a transcription activator that has been shown to play a central role in smooth muscle differentiation, proliferation and migration through regulating the expression of muscle-specific genes, immediate early genes (IEGs) and cytoskeletal genes (23, 130). Smooth muscle-specific genes that are regulated by SRF include sm α-actin, smooth muscle myosin heavy chain (MHC), myosin light chain kinase (MLCK), calponin, SM22α and telokin. IEGs are named so because of their rapid transcriptional response to serum or growth factor stimulation. There are two classes of SRF-dependent IEGs: early IEGs (including c-fos, Egr-1, Egr-2) and late IEGs (including SRF, vinculin) (Figure 1). SRF activates these multiple pathways, through its association with distinct accessory proteins. SM-specific genes (sm α-actin, MLCK, SM22α, telokin) are activated by SRF-myocardin, SRF-MRTFA, SRF-GATA6-CRP2 or SRF-Nkx3 complexes (18, 47, 134, 141). The early IEGs are regulated by Elk (ets)-SRF complexes (88, 137, 151) (Figure 3). The late IEGs that are actin/Rho-dependent are regulated by SRF-MRTFA complexes (121). SRF dimers bind the consensus sequence CC(A/T)$_6$GG (CArG box) in all SRF-dependent genes through the MADs domain of SRF. SRF is required for mammalian development as SRF null embryos do not form the mesoderm from which most smooth muscle cells arise, and exhibit decreased c-fos, egr1 and α-actin expression (7). SRF is

critical for the development of all muscle lineages. Cardiac-specific SRF knockout mice have defects in cardiac development and less expression of sm α-actin (101). Skeletal muscle-specific knockout of SRF in adult mice causes highly hypotrophic myofibers, immature muscle and low levels of skeletal α-actin (27). Smooth muscle-specific knockout of SRF in adult mice causes decreased smooth muscle contractile protein expression, resulting in decreased intestinal contractility and severe intestinal obstruction (4). SRF has also been reported to be involved in gastric ulcer and esophageal ulcer healing in rats (21, 22). SRF is up-regulated in epithelial, myofibroblast and smooth muscle cells in gastric ulcers and local injection of an SRF expression plasmid into rat gastric ulcers increased smooth muscle restoration and accelerated ulcer healing that was associated with increased expression of sm α-actin and smoothelin.

Several mechanisms have been shown to regulate SRF activity (23): phosphorylation-dependent changes in DNA binding; alternative RNA splicing; regulated nuclear translocation; and association with positive and negative cofactors. Of these, perhaps the best studied and most important mechanism that regulates SRF activity is its interaction with various negative or positive cofactor proteins (19, 94, 134, 151) (Figure 3). There are two major families of SRF cofactors: the ternary complex factor family (TCF, including Elk-1, SAP-1 and Net) and Myocardin-Related Transcription Factor Family (MRTFs, including myocardin, MRTFA, MRTFB)(see the more details below and reviews by (109), (107)). TCFs are activated by mitogen activated protein (MAP) kinase

phosphorylation and regulate early response gene expression. Myocardin constitutively activates SRF, while MRTFA and B are regulated by a Rho-actin signaling pathway (see review of (109)) (Figure 3). In addition to TCFs and MRTFs, several other factors also associate with SRF to regulate its activity including positive factors such as GATA and Nkx family members and negative factors including FHL2 and HOP (109).

D. Myocardin Related Transcription Factor Family and smooth muscle development.

Identification of MRTFs as important co-activators of SRF, that potently stimulate expression of smooth muscle-specific genes, has been pivotal in our understanding of smooth muscle differentiation. This family includes Myocardin, Myocardin Related Transcription Factor A (MRTFA, also known as MAL or Mkl1) and Myocardin Related Transcription Factor B (MRTFB, also known as Mkl2). Myocardin, MRTFA and MRTFB share a high degree of structural homology in several function domains (Figure 4)(107). An N-terminal REPEL domain is important for cytoskeletal actin binding; a basic and glutamine-rich region binds multiple factors, including SRF, SMAD1, HDAC, FOXO4; a SAP domain, named after SAF-A/B, Acinus, and PIAS, that may contribute to promoter binding specificity; a leucine zipper domain mediates dimerization of MRTFs; and a C-terminal transcription activation domain (TAD) (107).

Myocardin and MRTFA have been shown to upregulate the expression of smooth muscle-specific genes such as SM22α, SM-MHC, SM α-actin and telokin and Rho/Actin dependent genes such as SRF and vinculin, but not the MAPK/Elk dependent SRF target genes such as c-fos or Egr1 (30, 86, 94, 117, 143). MRTFA knockout mice have defects in mammary gland development: SM α-actin, MHC, MLCK and SM22α are all significantly down regulated in mammary myoepithelial cells from knockout mice (mammary myoepithelial cells resemble SMCs and express SM-specific genes also)(80, 128). Moreover, knockdown of MRTFA in rat aortic SMC in vitro decreased expression of smooth muscle-specific genes (143). Myocardin knockout mice have no vascular SMCs around their aorta or in the placental vasculature and die by embryonic day 10.5 (E10.5) due to placental vascular insufficiency (81). Neural crest-specific myocardin KO mice also exhibit vascular defects and die within three days of birth from patent ductus arteriosus associated with decreased contractile protein expression in smooth muscle cells of the aortic arch (67). Similarly MRTFB KO mice die between E17.5 and postnatal day 1 from cardiac outflow tract defects, resulting from defects in the differentiation of cardiac neural crest cells into smooth muscle cells (79). Results from these studies demonstrate that myocardin family members have distinct but partially overlapping roles in regulating smooth muscle differentiation *in vivo*.

Previous studies have shown that SRF has very weak or transient binding to SM-specific gene promoters in non-muscle cells, because these promoters are in a

closed or condensed chromatin landscape (91). Over-expression of myocardin in non-muscle cells was found to open chromatin and increase SRF binding to its target gene promoters (91). Myocardin has also been found to increase SRF binding to methylated histone (91). Since no evidence shows that myocardin itself has chromatin remodeling functions, myocardin must recruit a chromatin regulator to achieve this chromatin remodeling. Also in support of this proposal myocardin has been shown to induce histone acetylation, at least partially through interacting with the histone acetyl transferases (HATs), p300 (17). However, it is not clear if this would be sufficient to explain how myocardin can open the chromatin structure of smooth muscle-specific genes to facilitate SRF binding. Based on data discussed below we hypothesize that Brg1/Brm ATP-dependent chromatin remodeling enzymes may also contribute to this process.

E. Brg1/Brm ATP-dependent chromatin remodeling enzymes.

In eukaryotes, gene expression control can be achieved at several levels: chromatin structure, transcription, post-transcription, translation and post-translation. The regulation of chromatin accessibility to transcription factors and RNA polymerase is the first level of regulation. Chromatin structure is regulated by 2 groups of enzymes: one group that includes HATs, histone deacetylases (HDACs) and histone methyltranferases catalyze covalent modification of histones. A second group hydrolyzes ATP to change the contacts between histones and genomic DNA and thereby remodel nucleosomes. The two classes of chromatin modifying enzymes often cooperate to remodel chromatin structure

through sequentially or simultaneously binding to genes to facilitate both covalent modification of histones and ATP-dependent remodeling of nucleosomes (reviewed by (40, 54, 95)).

Four different classes of ATP-dependent chromatin remodeling complexes have been found and named after their unique ATPase subunits: SWI/SNF, ISWI, Mi-2 and Ino80. SWI/SNF is the most characterized complex in mammalian cells. Brg1 (Brahma related gene one) and Brm (Brahma) are the ATPase subunits of the SWI/SNF complex. The SWI/SNF remodeling complex has been shown to play an essential role in the differentiation of many tissues, including the neural system, T cells, liver, skeletal and cardiac muscle (46, 66, 89). A dominant negative Brg1 has been shown to block MyoD-mediated induction of skeletal muscle-specific genes (38) and Baf60c (a component of the SWI/SNF complex) is required for heart development. During skeletal muscle differentiation, the dominant transcription factor MyoD initially binds weakly to the myogenin promoter through its interaction with Pbx. MyoD then recruits SWI/SNF and SWI/SNF remodels the structure of the myogenic locus facilitating tight binding of MyoD to E boxes within the myogenin promoter, subsequently activating myogenin expression and skeletal muscle differentiation (38). The recruitment of SWI/SNF by MyoD is thus critical for skeletal muscle differentiation. MRTFs in smooth muscle cells are somewhat analogous to the MyoD family in skeletal muscle cells. Given this analogy and the requirement of MRTFs to recruit chromatin remodeling enzymes to facilitate SRF binding during smooth muscle

differentiation we thus proposed that MRTFs may recruit SWI/SNF to facilitate this process. This proposal leads me to develop the following hypothesis, which is the foundation for my research studies:

F. Hypothesis

SWI/SNF ATP-dependent chromatin remodeling enzymes containing either Brg1 or Brm as their catalytic subunits, play a critical role in the activation of SRF-dependent genes by MRTFs during smooth muscle development.

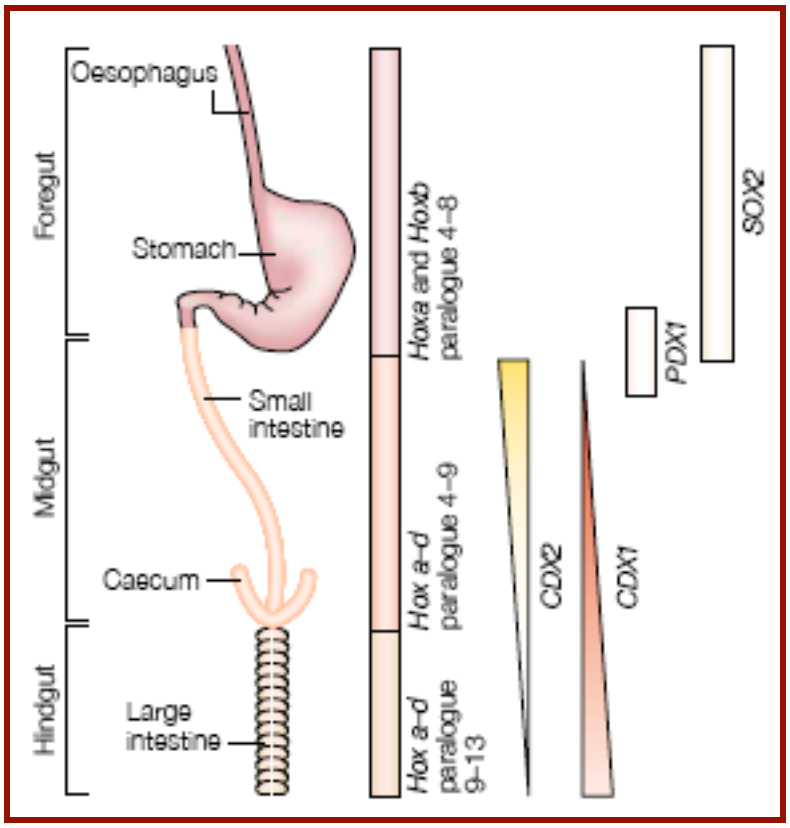


Figure 1. The gradient expression of transcription factors in GI tract development. (Adapted from Yuasa, et al, 2003 Nat Rev Cancer).

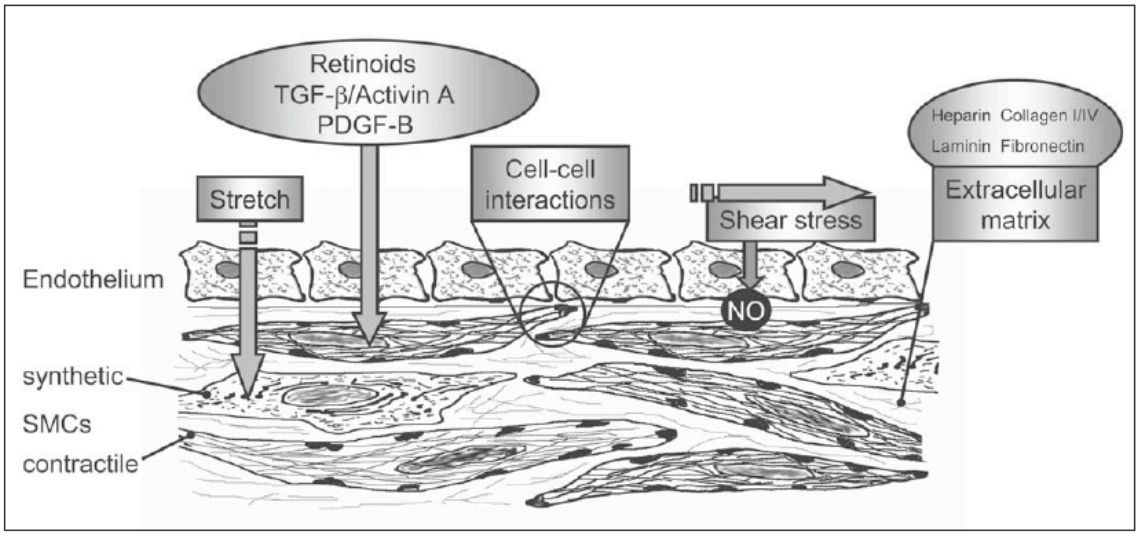


Figure 2. Three important determinants of SMC differentiation and phenotypic changes. 1). Biochemical factors: Retinoids, TGF-β, PDFGB et al. 2). Extracellular matrix (ECM proteins): Heparin, collagen, laminin, and fibronectin. 3). Physical parameters: stretch, shear stress. (Adapted from Rensen et al, 2007 Neth Heart J.)

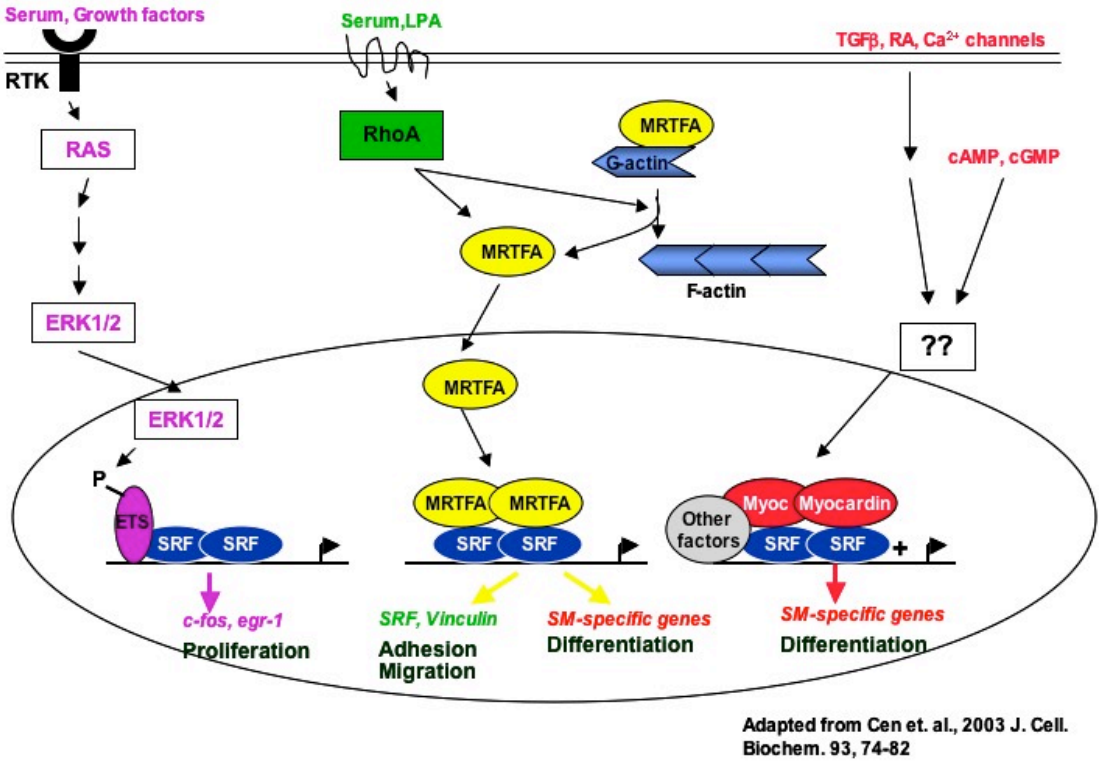


Figure 3. SRF/MRTFs target genes. (Adapted from Cen et al, 2003 J.Cell. Biochem)

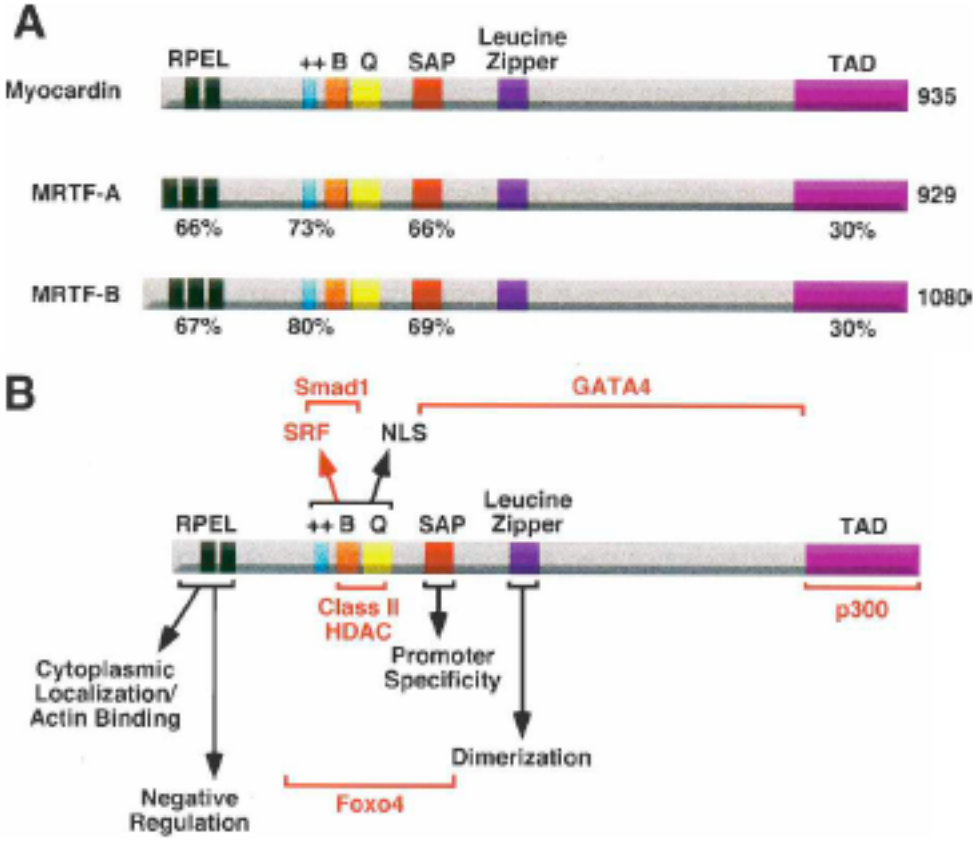


Figure 4. The structural domains and binding partners of the MRTF family. **A.** Structural domains of Myocardin, MRTFA and MRTFB. The similarity percentage between each domain relative to myocardin is shown. **B.** Binding partners of MRTF family. REPEL domain, basic and glutamine-rich region, SAP domain, Leucine zipper domain, transcription activation domain (TAD). (Adapted from G. C. Pipes et al, Genes Dev, 2006)

Chapter II: A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific gene expression

Abstract

Serum Response Factor (SRF) is a key regulator of smooth muscle differentiation, proliferation and migration. Myocardin Related Transcription Factor A (MRTFA) is a co-activator of SRF that can induce expression of SRF-dependent, smooth muscle-specific genes and actin/Rho-dependent genes, but not MAPK regulated growth response genes. How MRTFA and SRF discriminate between these sets of target genes is still unclear. We hypothesized that SWI/SNF ATP-dependent chromatin remodeling complexes, containing Brahma-related gene 1 (Brg1) and Brahma (Brm), may play a role in this process. Results from western blotting and qRT-PCR analysis demonstrated that dominant negative Brg1 blocked the ability of MRTFA to induce the expression of smooth muscle-specific genes, but not actin/Rho-dependent early response genes in fibroblasts. In addition, dominant negative Brg1 attenuated expression of smooth muscle-specific genes in primary cultures of smooth muscle cells. MRTFA over-expression did not induce expression of smooth muscle-specific genes in SW13 cells, which lack endogenous Brg1 or Brm. Reintroduction of Brg1 or Brm into SW13 cells restored their responsiveness to MRTFA. Immunoprecipitation assays revealed that Brg1, SRF and MRTFA form a complex *in vivo* and Brg1 directly binds MRTFA, but not SRF, *in vitro.* Results from chromatin immunoprecipitation assays demonstrated that dominant negative Brg1 significantly attenuated the ability of MRTFA to increase SRF binding to the

promoters of smooth muscle-specific genes, but not early response genes. Together these data suggest that Brg1/Brm containing SWI/SNF complexes play a critical role in regulating expression of SRF/MRTFA-dependent smooth muscle-specific genes but are not required for SRF/MRTFA-dependent early response genes.

Introduction

There are many diseases, such as atherosclerosis, hypertension and asthma that involve abnormal differentiation of smooth muscle cells. An important pathological process that occurs in these diseases is the disruption of the balance between differentiation and proliferation of smooth muscle cells (53, 104, 120, 140). Serum Response Factor (SRF) has been shown to play an essential role in regulating smooth muscle differentiation, proliferation and migration through its interaction with various accessory proteins (93). Smooth muscle-specific genes, such as SM α-actin, SM MHC, 130kDa MLCK, SM22α, and telokin, are activated by SRF-myocardin, SRF/MRTFA, SRF/GATA6/CRP2 or SRF/Nkx complexes (19, 26, 30, 43, 44, 82, 100, 106, 107, 138, 142, 143, 145, 151). The immediate early growth factor responsive genes, such as c-fos and Egr-1 are regulated by SRF/Elk (ets) complexes (88, 110, 119). The later early response genes, such as SRF itself and vinculin, that are actin/Rho-dependent, are regulated by SRF/MRTFA complexes (19, 94, 121). Myocardin Related Transcription Factor A (MRTFA, or Mkl1, MAL, BSAC) is a unique co-activator of SRF in that it is involved in the regulation of multiple SRF-dependent gene families (reviewed by (20)). MRTFA has been reported to induce SRF-dependent, smooth muscle-specific genes such as telokin, SM22α and SM α-actin and actin/Rho-dependent early response genes, but not proliferation related MAPK-dependent immediate early response genes (19, 43, 121, 143). It still remains a mystery how MRTFA can discriminate between different SRF-dependent genes. One possible mechanism could involve gene-specific

restriction of promoter access due to chromatin structure. In support of this model, it has been shown that there is very little SRF detectable at the CArG boxes of smooth muscle-specific genes in nonmuscle cells, whereas SRF binding can be readily detected at CArG boxes of early response genes such as c-fos (91). In addition, over-expression of myocardin in nonmuscle cells was found to lead to increased SRF binding to the promoters of smooth muscle-specific genes. In the current study we provide evidence supporting a role for ATP-dependent chromatin remodeling in regulating SRF binding to CArG boxes within promoters of smooth muscle-specific genes.

Since chromatin is highly condensed, the regulation of chromatin accessibility to transcription factors and RNA polymerase is an essential step in gene activation (40, 131). Although studies have shown that myocardin can recruit enzymes capable of modifying chromatin structure through covalent modification of histone tails (17, 35), no studies have examined how chromatin structure affects promoter access by MRTFA. In addition, the role of ATP-dependent chromatin remodeling enzymes in the regulation of smooth muscle differentiation is unknown. The SWI/SNF complex is the best characterized, mammalian, ATP-dependent chromatin remodeling complex (40). It is comprised of 7 to 11 components, which assemble into distinct complexes containing either Brg1 (Brahma-related gene 1) or Brm (Brahma) ATPase subunits. SWI/SNF remodeling complexes have been shown to play an essential role in the differentiation of neurons, T cells, erythrocytes, hepatocytes, adipocytes, skeletal

and cardiac muscle cells (16, 31, 32, 37, 69, 70, 105, 116, 122, 132, 133). Although, the role of Brg1 or SWI/SNF in smooth muscle development is largely unknown, Brg1 has been shown to be upregulated in vascular smooth muscle cells in primary atherosclerosis and in stent stenosis (148). A recent study has also demonstrated that Brg1 binding to CRP2 is critical for induction of smooth muscle-specific genes by CRP2 (24). During skeletal muscle differentiation it has been shown that the recruitment of Brg1 to MyoD, that is associated with DNA bound Pbx1, induces chromatin remodeling of the myogenin gene. This facilitates tight binding of MyoD to E boxes resulting in co-factor recruitment and transcription activation (38). By analogy, we propose that weak SRF binding to the CArG boxes of smooth muscle-specific genes may facilitate recruitment of MRTFA and that interaction of MRTFA with SWI/SNF may then remodel chromatin permitting tight binding of SRF.

Results from our study demonstrate that Brg1 is required for the induction of smooth muscle-specific gene expression but not for early response gene expression by MRTFA. Endogenous Brg1, SRF and MRTFA were found to form a complex in smooth muscle cells and tissue and Brg1 directly bound to MRTFA, but not SRF, *in vitro*. Chromatin immunoprecipitation assays revealed that SWI/SNF is required for MRTFA to increase SRF binding to the promoters of smooth muscle specific genes. Furthermore, expression of a dominant negative Brg1 in differentiated smooth muscle cells attenuated expression of smooth muscle specific genes. Together these data indicate that SWI/SNF plays a critical

role in regulating expression of SRF/MRTFA-dependent smooth muscle-specific genes but not SRF-dependent early response genes. SWI/SNF thus plays an important role in regulating the balance between the differentiation and proliferation roles of SRF.

Experimental Procedures

Cell culture and adenoviral transduction. An MRTFA cDNA image clone was purchased from Invitrogen (Clone ID: 682130) and moved to Adeno-X vector according to the manufacturer's protocol (BD Biosciences). Adenovirus encoding nuclear localized YFP (Yellow Fluorescent Protein) was used as negative control. B22 cells, which are NIH3T3 cells that express a tetracycline inducible dominant negative Brg1 (DN-Brg1, K798R mutant) (36), were obtained from Dr. Anthony N. Imbalzano (University of Massachusetts Medical School, Worcester, Massachusetts). By withdrawing tetracycline from the growth media of these cells, the expression of DN-Brg1 can be induced. B22 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech) containing 2 μg/ml tetracycline, 350 units/ml Hygromycin B, 75 μg/ml G418 and 10% fetal bovine serum (FBS). B22 cells were seeded into 6 well dishes at the density of 2×10^5cells per well in the medium either with or without tetracycline and grown for 24 hours prior to adenoviral transduction. Cells were incubated with adenovirus encoding MRTFA or YFP for 4 hrs at 37°C and then replaced with complete medium. 30-48 hrs after transduction, cells were harvested for protein, RNA or chromatin Immunoprecipitation analysis. SW13 and HeLa cells were obtained from ATCC and grown in high glucose DMEM containing 5 units/ml penicillin, 50 mg/ml streptomycin and 10% FBS. 24 hours before transduction, SW13 and HeLa cells were seeded at the density of 2.5×10^5 cells per well in 6 well dish. Cells were then transduced with adenovirus as described above for B22 cells. Primary mouse colon smooth muscle cells were prepared

from colons dissected from 4-week-old mice. The epithelial layer was removed and remaining smooth muscle layer was minced and digested with 1ml of tissue digestion buffer per organ ((0.4 units/ml Blendzyme #3 (Roche) in DMEM) at 37°C for 1-2 hours with shaking. The digested tissue is then passed through a cell sieve and the cells collected by centrifugation. Pelleted cells are washed in DMEM containing 10% FCS and penicillin/streptomycin and plated into dishes. After 4-5 days cells reached confluence, were trypsinized and replated at $7x10^4$ per well in 12 well plates. 12 hours after plating cells were transduced by DN-Brg1 or YFP control adenovirus. 72 hours after transduction, mRNA was harvested and the levels of SRF dependent genes were measured by quantitative real time RT-PCR.

Plasmids used and cell transfection. Human Brg1 and Brm cDNA and DN-Brg1 in pBABE retroviral expression plasmids were obtained from AddGene (124). MRTFA was cloned into pcDNA myc His (Invitrogen) for transfection and *in vitro* translation experiments. HA-SRF pShuttle was generated by cloning the human SRF cDNA into a modified pShuttle (Clontech) vector that includes an amino-terminal HA epitope tag. 12 hrs prior to transfection, cells were seeded at the density of 2.5×10^5 cells per well in 6 well plates. Plasmids were transfected into cells using Fugene 6 (Roche Applied Science): cells were washed once with phosphate-buffered saline (PBS) (pH 7.4) then 2 ml of complete medium was added to each well together with 2 μg plasmid DNA and 4 μl Fugene in 100 μl DMEM.

RNA analysis. RNA was extracted with Trizol reagent (Invitrogen). 1.2 μg RNA was used as template for reverse transcription (RT) using Superscript first strand cDNA synthesis kit (Invitrogen). cDNA was dissolved in 20 μl H_2O. The cDNA levels of specific genes were measured by quantitative real time PCR using SYBR green PCR master mix (Invitrogen) and a 7500 Real Time PCR system (Applied Biosystems) with gene specific primers (Table 1). 2 μl of 1:10 diluted cDNA was used to each reaction in 25 μl total volume. All PCR reactions were performed in duplicate.

Western blotting. Protein was extracted with RIPA lysis buffer. Protein concentrations were determined by using a BCA Protein Assay Kit (Pierce). 30 μg of proteins were fractionated on 7.5 or 15% SDS-polyacrylamide gels and transferred to nitrocellulose or polyvinyl difluoride membranes. Membranes were then probed with a series of antibodies. Antibodies used for western blotting were against: Brg1 (Upstate, 1:5,000), Brm (Abcam, 1:1000) MRTFA (Santa Cruz, C-19, 1:500), SRF (Santa Cruz, G20X, 1:6,000), α-actin (Sigma, 1:10,000), Egr-1 (Santa Cruz, 1:1,000), Flag tag (Sigma, M2, 1:5,000), HA tag (Covance, 1:3,000), myc tag (Invitrogen, 1:1000), MLCK (Sigma, clone K36, 1:10,000), SM α-actin (Sigma, clone 3A1, 1:10,000), SM22α (a gift from Dr. Len Adam, 1:6,000), telokin (1:6,000) (50), vinculin (Santa Cruz, 1:5,000), NMMHC IIA (a gift from Dr. Patricia Gallagher). Primary antibodies were detected using horseradish peroxidase conjugated secondary antibodies and visualized using chemiluminescence.

Co-immunoprecipitation (Co-IP). Co-IP assays were performed using a nuclear complex Co-IP kit, essentially as described by the manufacturer (Active Motif). 250 µg of nuclear protein extracts were incubated with 3 µg of anti-Brg1 antibody (Upstate), anti-SRF antibody (Santa Cruz, G20X), anti-MRTFA antibody (Santa Cruz, C-19; or ProteinTech), or appropriate IgG control in 500 µl of low salt IP buffer (Active Motif) overnight at 4°C. 60 µl of EZview protein A beads (Sigma) were added to the mixture and incubated for an additional hour with rocking. Beads were then washed 6 times with the low salt IP buffer. The immunoprecipitated proteins were dissolved in 35 µl of 2XSDS sample buffer and boiled for 5 minutes, prior to analysis by western blotting as described as above.

In vitro **transcription/translation.** Synthesis of proteins was carried out in a coupled transcription/translation system (Promega, Madison, WI) *in vitro*, programmed with 1µg of pShuttle-Brg1 (flag tag), pcDNAmyc/his-MRTFA (myc tag) and pShuttle-SRF (HA tag) plasmids. The TNT products were mixed together as combinations of: SRF with Brg1, MRTFA with Brg1, SRF with MRTFA, in 250 µl low salt IP buffer (Active Motif). Proteins were immunoprecipitated with 3 µg anti-SRF (Santa Cruz, G20X) or 15µl anti-MRTFA antiserum over night. A matching rabbit IgG or a MRTFA preimmune serum served as negative controls. The immunoprecipitated proteins were then incubated with EZ-view beads (Sigma) for 2 hours. Following 6 washes with IP buffer the beads were dissolved in SDS-sample buffer and subjected to Western blotting as described above.

Quantitive chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed according to the protocol of Upstate with minor modifications. Cells were fixed in 3.7% formaldehyde for 15 minutes at room temperature and harvested using cold PBS with protease inhibitors. After collecting cells by centrifugation, cell pellets were lysed using 1%SDS lysis buffer (200 µl / 1×10^6 cells). For each group, 1ml of lysate was sonicated for 7 x30 seconds at setting 2.25 on a Sonic Dismembranator (Fisher Scientific). 200 µl aliquots of chromatin were immunoprecipitated using 6µg of anti-SRF antibody (Santa Cruz, G20X), anti-H3Ac (Upstate) or rabbit IgG as negative control. The precipitated genomic DNA was purified and the presence of specific promoters was measured by real time quantitative PCR, using gene specific primers (Table 2).

Results

DN-Brg1 inhibits the ability of MRTFA to induce expression of smooth muscle specific genes, but not SRF-dependent early response genes. To determine the role of SWI/SNF mediated chromatin remodeling on the induction of genes by SRF/MRTFA, we utilized a previously characterized 3T3 cell line that inducibly expresses a dominant negative Brg1 (B22 cells, (36)). The dominant negative K798R mutant Brg1 blocks the function of SWI/SNF complexes containing Brg1 or Brm catalytic subunits. B22 cells were transduced with MRTFA or YFP adenovirus. 30 hours after transduction, cells were lysed and mRNA and protein expression analyzed by quantitative real-time RT-PCR and western blotting, respectively (Figures 5, 6). In agreement with previous reports (43, 82, 121, 135, 143) MRTFA induced the expression of several smooth muscle-specific genes in fibroblast cells (Figure 5, compare solid bars to open bars in control cells). In contrast, MRTFA did not significantly induce expression of the early response genes, c-fos, Egr-1 or vinculin, although it did result in a 2-fold increase in expression of SRF mRNA (Figure 6). As shown by western blotting (Figure 6) and more quantitatively by qRT-PCR (Figure 5) dominant negative Brg-1 significantly abrogated the ability of MRTFA to increase expression of telokin, SM22α, and calponin but not SM α-actin or any of the SRF-dependent early response genes examined. Conversely, DN-Brg1 augmented the ability of MRTFA to increase SRF mRNA expression and increased the basal expression of Egr-1 and c-fos mRNA (Figure 5).

MRTFA cannot induce smooth muscle-specific genes in SW13 cells that lack Brg1 and Brm. It is possible that DN-Brg1 inhibits the activity of more than just Brg1 and Brm containing SWI/SNF complexes, hence we also examined the role of Brg1 in a Brg1/Brm null cell system. The ability of MRTFA to induce gene expression in human adrenal carcinoma SW13 cells that lack endogenous Brg1/Brm was determined. Human cervical cancer HeLa cells that express Brg1 and Brm were used as control for these experiments. Results from western blot analysis showed that over-expression of MRTFA in SW13 cells could not induce the expression of most smooth muscle-specific genes, including 130kDa smMLCK, telokin, or SM22α, and could only weakly induce sm α-actin expression (Figure 7A). In contrast, MRTFA readily induced expression of these genes in HeLa cells. MRTFA induced expression of SRF and vinculin in SW13 cells as well as in HeLa cells. Although Egr-1 was not induced by MRTFA in either of these two cell types, the basal expression of Egr-1 appeared higher in SW13 cells compared with HeLa cells. Surprisingly, MRTFA was able to induce expression of c-fos in SW13 cells but not in HeLa cells (Figure 7A) and this induction was attenuated by reintroduction of Brg1 into the SW13 cells (Figure 7B). These results are consistent with the results obtained from the DN-Brg1 3T3 cell lines. To verify that the inability of MRTFA to induce smooth muscle-specific genes in SW13 cells is due to the lack of Brg1/Brm, we reintroduced Brg1 or Brm1 back into SW13 cells. Western blot analysis demonstrated that restoration of Brg1 or Brm expression could rescue the ability of MRTFA to induce the expression of smooth muscle-specific proteins in SW13 cells (Figure 7B).

DN-Brg1 attenuates smooth muscle gene expression in primary smooth muscle cells. The above results suggest that Brg1 or Brm is required for MRTFA to induce the expression of smooth muscle specific genes in non-smooth muscle cells. To determine if Brg1 is required for smooth muscle cells to maintain the expression of smooth muscle-specific markers, DN-Brg1 was over expressed in primary colon smooth muscle cells. Results from quantitative real-time RT-PCR demonstrated that dominant negative Brg-1 significantly decreased the expression of smooth muscle specific genes (telokin, SM MHC, calponin, SM22α and sm α-actin) and increased the expression of the SRF-dependent early response genes, such as egr1 and c-fos. The expression of SRF was not significantly changed (Figure 8).

Brg1 interacts with SRF and MRTFA in intact cells. We next determined if Brg1 could interact with MRTFA and/or SRF in intact cells. Co-immunoprecipitation (Co-IP) assays were performed from COS cells transduced with HA- tagged MRTFA and SRF adenoviruses. MRTFA, SRF and endogenous Brg1 were immunoprecipitated from the transduced cell extracts. Western blotting of immunoprecipitated proteins showed that MRTFA immunoprecipitates contained Brg1, MRTFA and SRF (Figure 9A). SRF immunoprecipitates contained MRTFA and SRF, and a very weak Brg1 signal. Similarly Brg1 immunoprecipitates contained Brg1 and MRTFA and a small amount of SRF. These data indicate that Brg1, MRTFA and SRF can form a complex i*n vivo*. To confirm this finding, we determined if the endogenous proteins can also be

detected in a complex in A10 smooth muscle cells (Figure 9B) or in bladder tissue (Figure 9C). Endogenous Brg1, SRF and MRTFA were immunoprecipitated from nuclear extracts of A10 cells or mouse bladder and western blot analysis revealed that Brg1 can be co-immunoprecipitated with SRF and MRTFA. These data indicate that the 3 proteins can form a complex in A10 smooth muscle cells and bladder tissue *in vivo (*Figure 9B,C).

Brg1 interacts with MRTFA but not SRF *in vitro*. To determine if Brg1 directly binds to MRTFA and/or SRF, *in vitro* binding assays were performed. Brg1, MRTFA and SRF were synthesized *in vitro* using a coupled *in vitro* transcription and translation system. pShuttle-Brg1 (Flag tag), pcDNA myc/his-MRTFA (myc tag) and pShuttle-SRF (HA tag) were utilized as templates for the transcription/translation reactions. *In vitro* synthesized proteins were incubated together and immunoprecipitation assays were used to identify the interacting proteins (Figure 10). Results from this analysis indicate that Brg1 could be co-precipitated with MRTFA but not with SRF, demonstrating that Brg1 can directly bind to MRTFA.

DN-Brg1 inhibits the ability of MRTFA to increase SRF binding to the promoters of smooth muscle-specific genes. To explore why Brg1 is required for MRTFA induced smooth muscle-specific gene expression, but not for expression of the early response genes, chromatin immunoprecipitation (ChIP) assays were performed. B22 cells were grown in the presence or absence of

tetracycline and transduced with adenovirus encoding MRTFA or YFP. ChIP assays were performed to examine SRF binding to SRF-dependent promoters as described in ‘ Experimental Procedures ’. Consistent with previous studies using myocardin (91), we found that MRTFA significantly increased SRF binding to the promoters of smooth muscle-specific genes, telokin, SM22α and SM α-actin, (Figure 11A). DN-Brg1 dramatically attenuated the ability of MRTFA to increase SRF binding to the telokin and SM22α promoters, but not the SM α-actin promoter (Figure 11B). In contrast, neither MRTFA nor DN-Brg1 affected the SRF binding to the promoters of the early response genes, SRF and c-fos (Figure 11A, B). In addition to increasing SRF binding to the telokin promoter, MRTFA also increased levels of acetylated histone H3 (Figure 11A, right panel). This increase in acetylated histone H3 on the telokin promoter was blocked by expression of DN-Brg1. In contrast, the small increase in acetylated H3 on the SM α-actin promoter was not blocked by DN-Brg1 (Figure 11B, right panel). Under control conditions (no DN-Brg1 expression), in YFP transduced B22 cells, there appeared to be more SRF bound to the SM α-actin, SRF, and cfos promoters as compared to the telokin and SM22α promoters (Figure 11C, left panel). Similarly there was more acetylated histone H3 associated with the SM α-actin promoter as compared to the telokin promoter under control conditions (Figure 11C, right panel).

Discussion

In this study, we found that Brg1 or Brm are required for the MRTFA-mediated induction of smooth muscle-specific genes, but not early response genes. DN-Brg1 attenuated the induction of smooth muscle-specific genes and reintroducing Brg1/Brm into SW13 cells restored their responsiveness to MRTFA. Brg1 appears to be required for MRTFA to increase the binding of SRF to the promoters of smooth muscle-specific genes, within intact chromatin. Brg1, MRTFA and SRF were found to form a complex in smooth muscle cells *in vivo* and Brg1 directly bound MRTFA but not SRF *in vitro*. Since previous studies (19, 94, 135), and data presented in Figure 6 have shown that MRTFA directly binds SRF *in vitro,* MRTFA can thus act as a bridge to connect Brg1 and SRF.

Results shown in Figures 5 and 6 demonstrate that DN-Brg1 blocked the MRTFA-mediated induction of telokin, SM22α and calponin but did not significantly block the induction of SM α-actin. However, from results shown in Figure 5 it is apparent that SM α-actin, but not other smooth muscle marker genes, was readily detectable in B22 3T3 cells prior to over-expression of MRTFA (YFP control group). This indicates that the SM α-actin locus is transcriptionally active in control cells and thus likely to be in an open chromatin conformation in these cells. In support of this hypothesis, there was significantly more SRF and acetylated histone H3 associated with the SM α-actin promoter as compared to the telokin promoter in control cells (Figure 11C). Thus it might be predicted that Brg1 containing SWI/SNF complexes may not be required to

further open the chromatin structure of the SM α-actin promoter in order for MRTFA to increase SRF binding to the promoter. This suggests a model in which Brg1 is required for MRTFA to increase binding of SRF to the promoters of transcriptionally silent genes but not to genes that are already transcriptionally active within native chromatin (Figure 12).

As the MRTFA regulated early response genes are also expressed in control cells, our model would predict that activation of these genes would also be independent of Brg1. This is consistent with data presented in Figure 1 showing that MRTFA-mediated induction of SRF was not inhibited by DN-Brg1. In addition, under control conditions there was markedly more SRF bound to the SRF promoter as compared to the telokin or SM22α promoters (Figure 11C). In our experiments only in SW13 cells did we detect any activation of vinculin by MRTFA (Figure 7A), although this activation was not altered by expression of Brg1 or Brm (Figure 7B), suggesting that it is independent of these proteins. In agreement with previous studies (121) we also observed no affects of MRTFA on expression of MAPK dependent SRF-target genes such as c-fos or egr1 (Figure 5). However, the basal expression of c-fos and Egr-1 is higher in cells expressing DN-Brg1 (Figure 5) and in cells lacking Brg1/Brm (SW13 cells compared to HeLa cells in Figure 7A). This observation is consistent with a previous study that showed that Brg1 represses c-fos expression (98). It is not known why Brg1 inhibits c-fos expression, though it might be predicted that it either results in increased binding of a repressor or it may alter the positioning of the

nucleosome, which has been shown to be located between key regulatory elements in the c-fos promoter (118). As Brg1 is specifically required for MRTFA to induce expression of smooth muscle-specific genes but not early response genes, then regulation of MRTFA/Brg1 interactions could provide a mechanism for a cell to switch between activation of these two groups of genes.

Previous studies showed that Brg1 knockout mice die during the periimplantation stage (15), however, Brm knockout mice develop normally, except that adult mice have higher body weight (112). These data suggest that Brg1 has non-redundant functions that cannot be replaced by Brm. However, in our experiments we found that either Brg1 or Brm were required for MRTFA to induce expression of smooth muscle-specific genes. This is in contrast to a recent study that demonstrated that Brg1 but not Brm is required for CRP-mediated induction of smooth muscle-specific genes in cardiac myocytes (24). Future analysis of smooth muscle-specific knockouts of Brg1 will be required to determine the specific role of Brg1 and Brm in smooth muscle cells *in vivo*.

Although Brg1 is ubiquitously expressed, Brg1 has been shown to selectively rather than broadly regulate gene expression (39). This can be explained, at least in part, by the recruitment of Brg1 to individual promoters through its interaction with specific transcription factors (6, 8, 9, 14, 24, 38, 49, 105, 115). Our results further support this idea as we found that Brg1, SRF and MRTFA form a complex in smooth muscle tissue *in vivo* and Brg1 directly interacts with

MRTFA but not SRF (Figure 10). This would allow Brg1 to be recruited to MRTFA-dependent SRF target genes but not to Elk-dependent SRF target genes. A recent study has also shown that CRP2 directly binds to Brg1 (24), and CRP2 has previously been shown to form a complex with SRF in some smooth muscle cell types (25). Thus, CRP2 may act similar to MRTFA to facilitate recruitment of Brg1 complexes to mediate stable SRF binding to the promoters of smooth muscle-specific genes.

It is likely that the ATP-dependent chromatin remodeling catalyzed by Brg1/Brm acts together with covalent histone modifications to permit stable SRF/MRTFA binding and transcription activation. Although SWI/SNF itself does not covalently modify histones its' remodeling of chromatin through nucleosome reorganization can facilitate the recruitment of other proteins that covalently modify histones. Previous studies have shown that over-expression of Myocardin, an MRTFA homologue, can induce histone acetylation through myocardin's interaction with the HAT, p300 (17). Histone acetylation has also been shown to promote the binding of SWI/SNF complexes to chromatin, as the acetylated histone tail can bind to the bromodomain of Brg1 (2). Thus MRTFA/p300 induced histone acetylation, may further stabilize Brg1 binding to the promoters of smooth muscle-specific genes (Figure 12). In a reciprocal fashion Brg1-induced changes in chromatin structure will facilitate increased binding of SRF/MRTFA complexes to the promoter and thus result in increased recruitment of p300. Together these interactions can then lead to transcription activation.

In summary, we propose a model in which the recruitment of MRTFA/Brg1 complexes to weakly bound SRF at the promoters of smooth muscle-specific genes, leads to chromatin remodeling which facilitates tight binding of SRF complexes and subsequent transcription activation (Figure 12, upper panels). In contrast, in cells grown under high serum conditions, SRF is tightly bound to the promoters of SRF-dependent early response genes. Brg1 is thus not required to permit MRTFA or MAPK-Elk signals to activate these genes (Figure 12, lower panels).

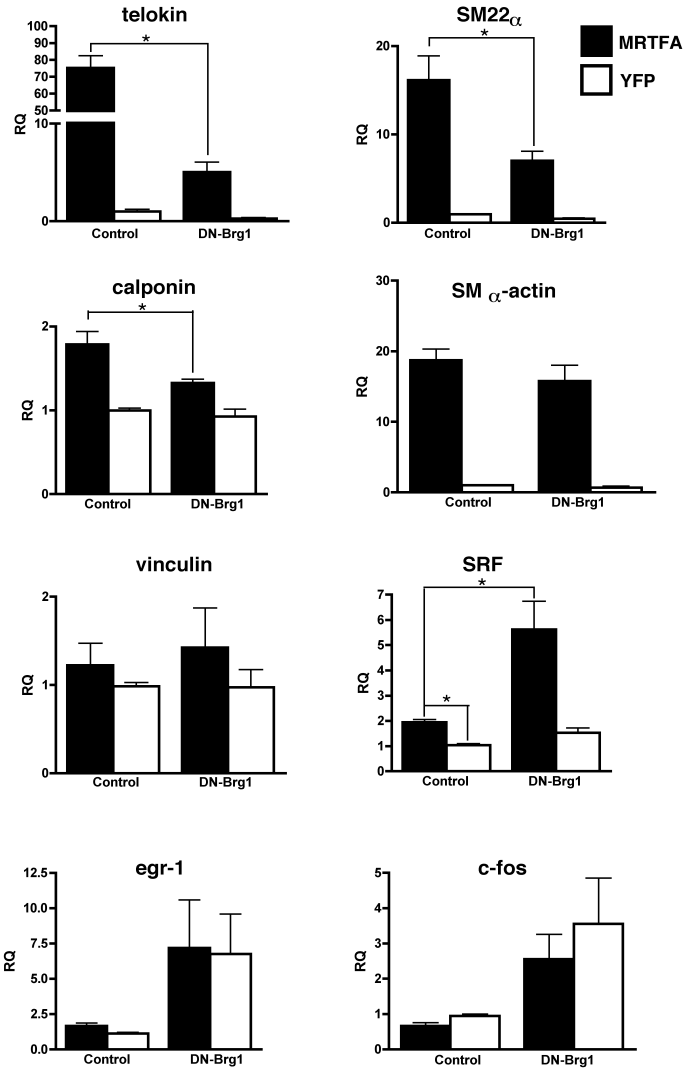


Figure 5. The expression of DN-Brg1 in 3T3 fibroblasts interferes with the induction of endogenous SRF-dependent smooth muscle-specific genes by MRTFA. Inducible DN-Brg1 3T3 (B22) cells were plated in media either with or without tetracycline. 24 hours later, cells were transduced with MRTFA (solid bars) or YFP (open bars) adenovirus. 30-48 hours following transduction cells were lysed for mRNA analysis. mRNA was isolated from cells and the levels of mRNA expression were measured by quantitative real time RT-PCR. Transcript levels was firstly normalized to acidic ribosomal phosphoprotein PO (RPLPO)

internal loading control and then normalized to their respective YFP control group. The $\Delta\Delta C_t$ method was used to calculate the relative quantity values (RQ) of gene expression levels. C_t is the threshold cycle where the amplification of template begins. $RQ=2^{-\Delta\Delta Ct}$ and $\Delta\Delta C_t = (C_{t\ experimental}-C_{t\ RPLPO})-(C_{t\ control}-C_{t\ RPLPO})$. Data presented are the mean±SEM of 8-9 samples obtained from 3 independent experiments. * Indicates statistical significance as determined by a student T test ($P<0.05$).

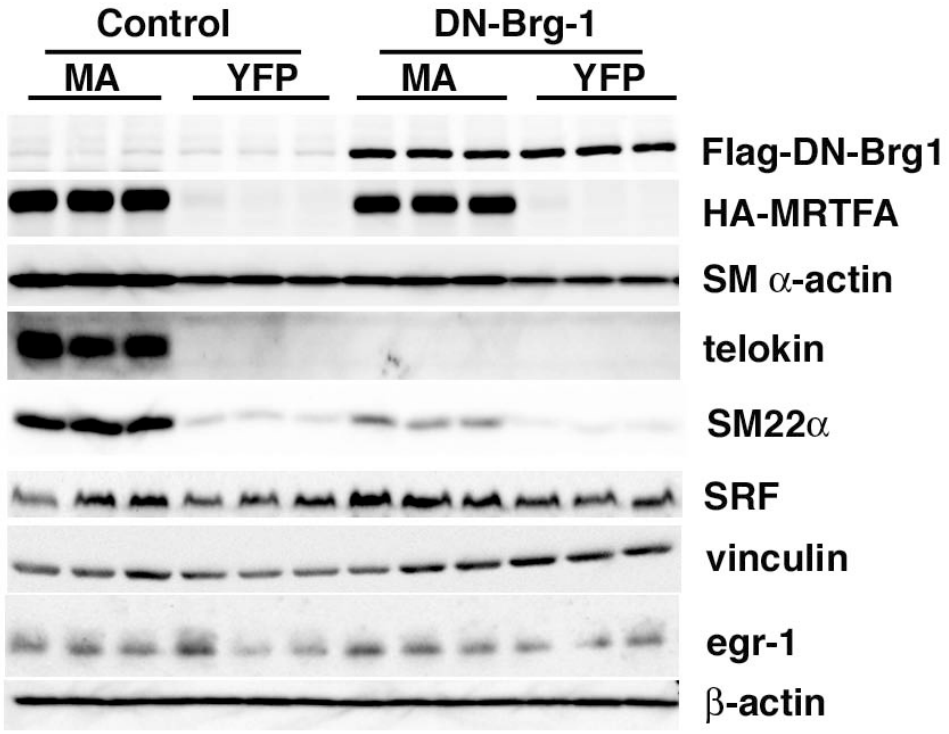


Figure 6. The expression of DN-Brg1 in 3T3 fibroblasts interferes with the induction of endogenous SRF-dependent smooth muscle-specific proteins by MRTFA. Inducible DN-Brg1 3T3 (B22) cells were plated in media either with or without tetracycline. 24 hours later, cells were transduced with MRTFA or YFP adenovirus. 30-48 hours following transduction cells were lysed using RIPA lysis buffer and protein expression analyzed by western blotting. 30 μg of protein were loaded in each lane.

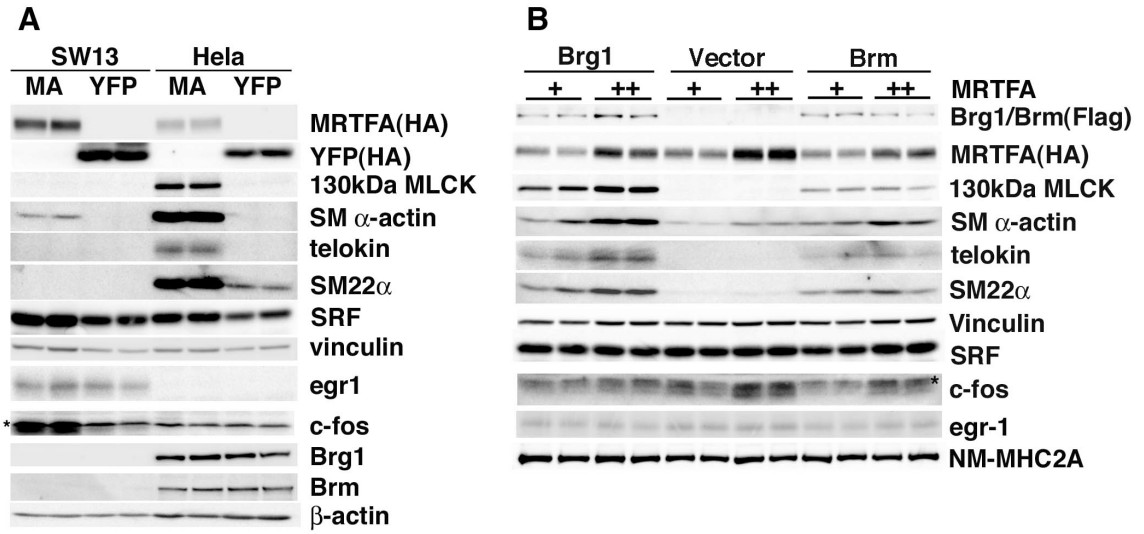


Figure 7. MRTFA cannot induce smooth muscle-specific gene expression in SW13 cells that lack Brg1/Brm1. A. SW13 and HeLa cells were transduced with MRTFA or YFP adenovirus, 48 hrs later, protein extracts were prepared and analyzed by western blotting. 30μg of protein were loaded onto each lane. * The upper band seen on the c-fos blots is a non-specific signal. β-actin was used as a loading control. **B.** WT-Brg1 or Brm-pBabe expression plasmids or empty vector control plasmids were transfected into SW13 cells in duplicate. After 24 hrs, cells were transduced with MRTFA adenovirus (low amount +, higher amount ++). 48 hours later cells were lysed by RIPA lysis buffer and analyzed by western blotting. NM-MHC2A serves as a loading control.

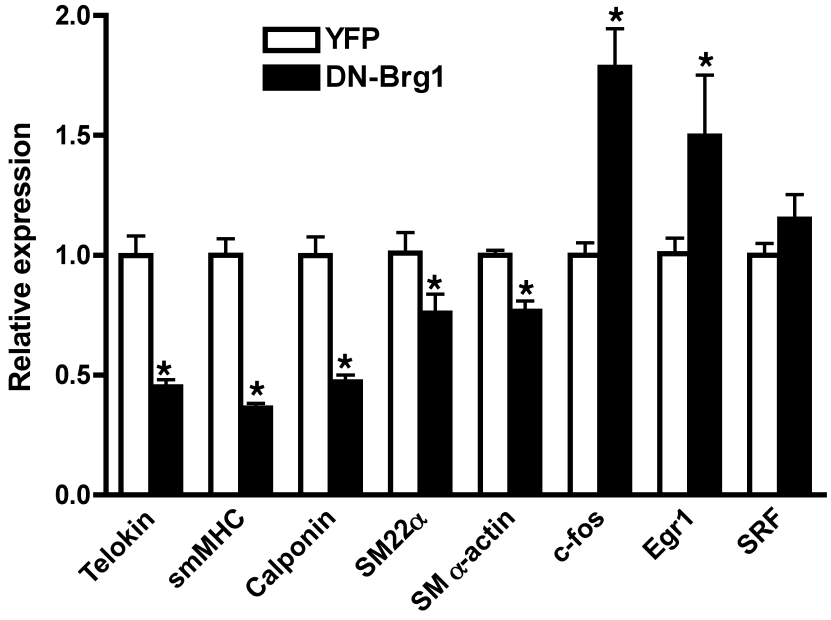


Figure 8. DN-Brg1 interferes with smooth muscle gene expression in primary smooth muscle cells. Primary colon smooth muscle cells were transduced by DN-Brg1 or YFP control adenovirus. 72 hours after transduction, mRNA was harvested and the levels of SRF dependent genes were measured by quantitative real time RT-PCR. Transcript level was calculated and presented as in figure 1. Data presented are the mean±SEM of 9 samples from 3 independent experiments. * Indicates statistical significance as determined by a student T test ($P<0.05$).

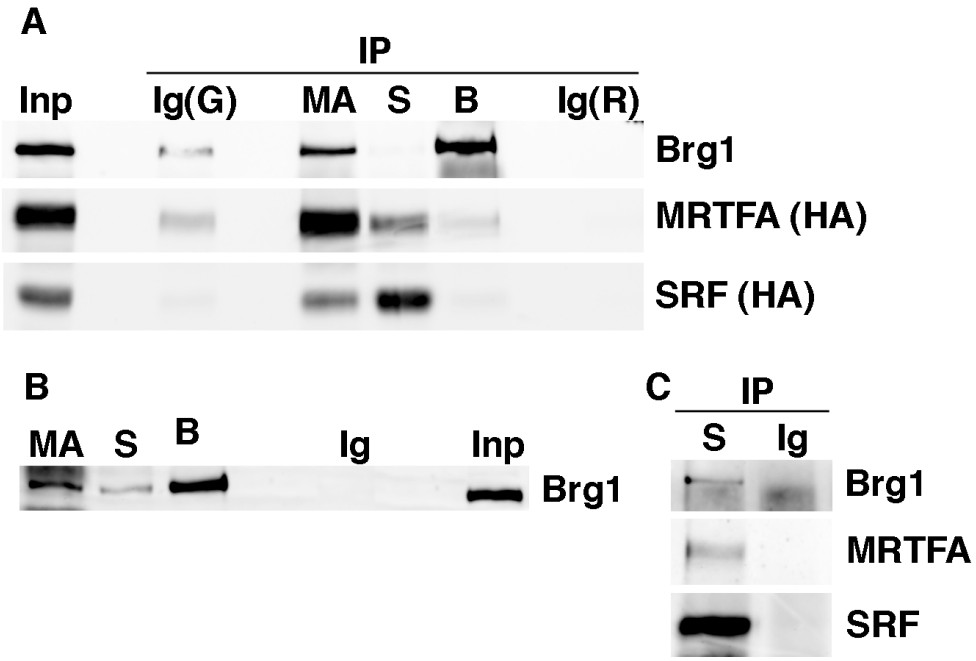


Figure 9. Brg1 forms a complex with SRF and MRTFA *in vivo*. A. Adenovirus encoding HA-tagged SRF and HA-tagged MRTFA were transduced into COS cells, 48 hours later, nuclear proteins were harvested using a hypotonic lysis/nuclease digestion protocol (Active Motif). Proteins were immunoprecipitated using MRTFA (MA), SRF (S) and Brg1 (B) antibodies and goat IgG (Ig(G)) or rabbit IgG (Ig(R)) as a negative control. (MRTFA is a goat antibody, SRF and Brg1 are rabbit antibodies.) Immunoprecipitated proteins were then identified by western blotting, using the antibodies indicated at the right of the blot. Inp- 10% of input. **B.** Co-immunoprecipitations were performed from nuclear extracts prepared from A10 cells. **C.** Mouse bladder tissues were diced

into small pieces and homogenized in a pre-chilled homogenizer prior to harvesting nuclear protein as described above for cells. Precipitated proteins were analyzed by western blotting using antibodies directed against endogenous, Brg1, MRTFA and SRF as described in Experimental Procedures.

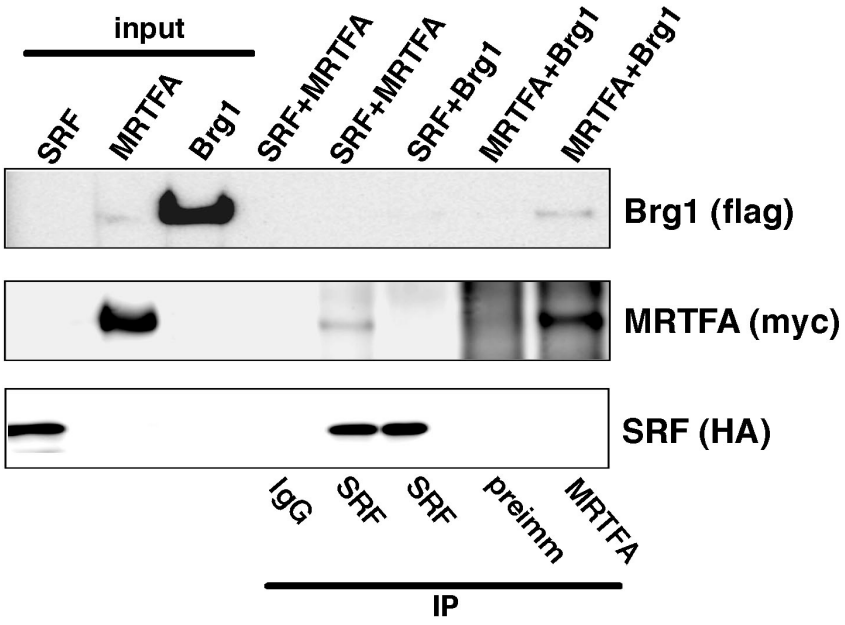


Figure 10. Brg1 binds MRTFA but not SRF *in vitro*. SRF, MRTFA and Brg1 proteins were synthesized by using *in vitro* transcription and translation, as described in experimental procedures. The proteins were then incubated together as shown at the top of the blot and proteins were immunoprecipitated using the antibodies indicted below the blot (IP) (preimm: preimmune serum from rabbit used to make MRTFA antibody). The presence of individual proteins in each immunoprecipitate was determined by western blotting using antibodies to each of their respective epitope tags (indicated at the right of the blot). 10% of the input of each IP was loaded at the left of the blot.

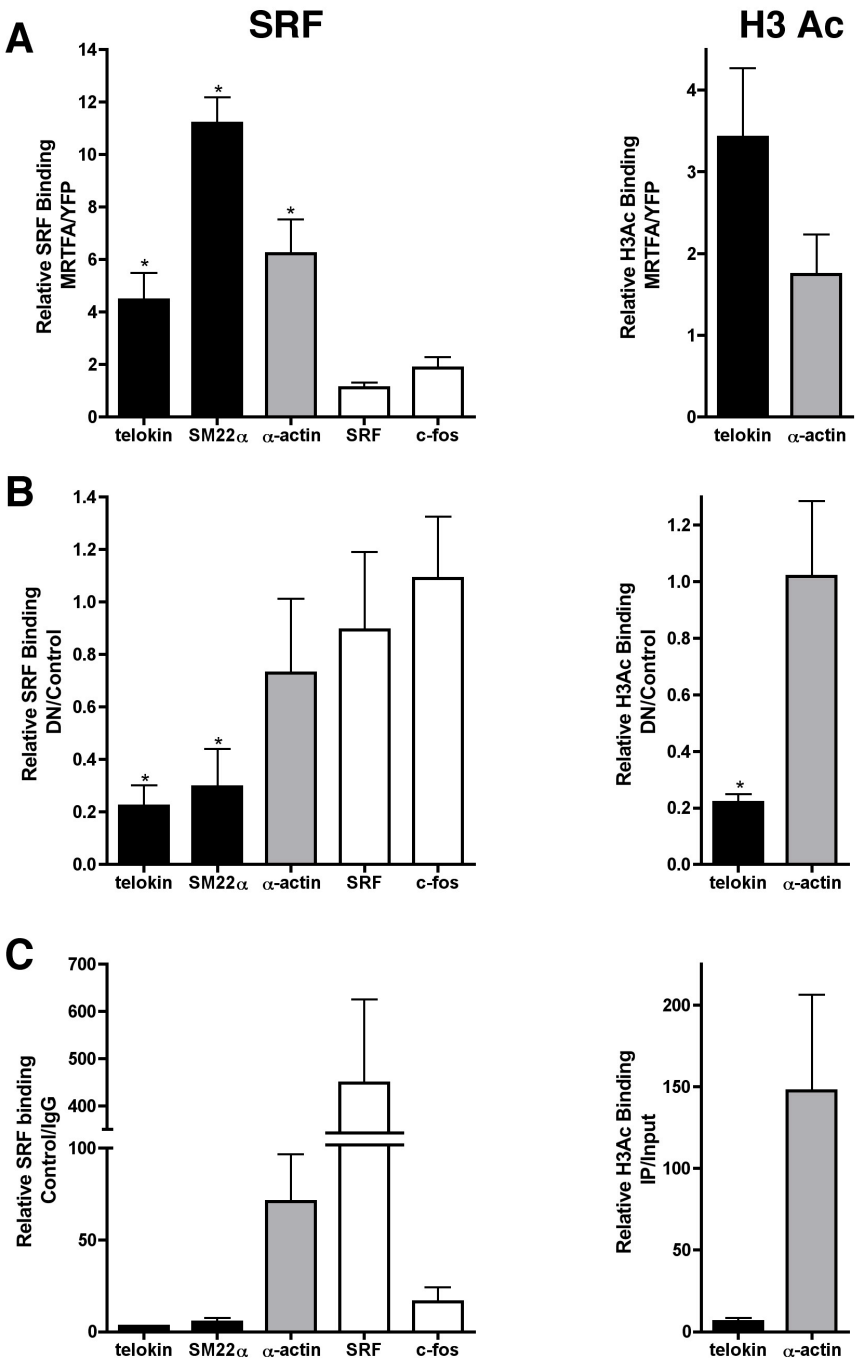


Figure 11. DN-Brg1 attenuates the ability of MRTFA to increase SRF binding to the promoters of smooth muscle-specific genes. B22 cells grown in the presence (control) or absence (+DN-Brg1) of tetracycline were transduced

with MRTFA or YFP control adenovirus. After 30 hrs, cells were fixed and harvested for chromatin immunoprecipitation assays. Chromatin was precipitated using an antibody against SRF (left panels), acetylated histone H3 (right panels) or using IgG negative control. The precipitated genomic DNA was purified and the presence of the promoters of SRF-dependent genes measured by real time quantitative PCR, using gene specific primers. **A.** The increase in SRF or H3Ac binding in samples transduced with MRTFA is indicated relative to those transduced with YFP. These data were calculated and normalized to input levels as follows: Relative SRF/H3Ac binding, $RQ=2^{-\Delta\Delta Ct}$, with $\Delta DC_t= (C_{t\ MRTFA}-C_{t\ input})-(C_{t\ YFP}-C_{t\ input})$ **B.** The relative inhibition of MRTFA induced SRF or H3Ac binding by DN-Brg1 is shown. This was calculated as follows: Relative SRF/H3Ac binding, $RQ=2^{-\Delta\Delta Ct}$, with $\Delta\Delta C_t= (C_{t\ DN\text{-}Brg1}-C_{t\ input})-(C_{t\ control}-C_{t\ input})$. SRF data shown in panels 'A' and 'B' are the mean±SEM of 7 samples obtained from 3 independent experiments. H3Ac data shown in panels 'A' and 'B' are the mean±SEM of 4 samples obtained from 2 independent experiments. A one sample t-test was performed and the asterisks indicates the results that are statistically different from 1 ($P<0.05$). **C.** The relative binding of SRF or H3Ac in control samples (no DN-Brg1, YFP transduced) is shown for each of the promoters indicated. Results were calculated as follows: Relative SRF/H3Ac binding, $RQ=2^{-\Delta ct}$, with $\Delta Ct =C_{t\ SRF\ IP\ group}-C_{t\ IgG\ IP\ group}$. Results presented are the mean±SEM of 4-5 samples.

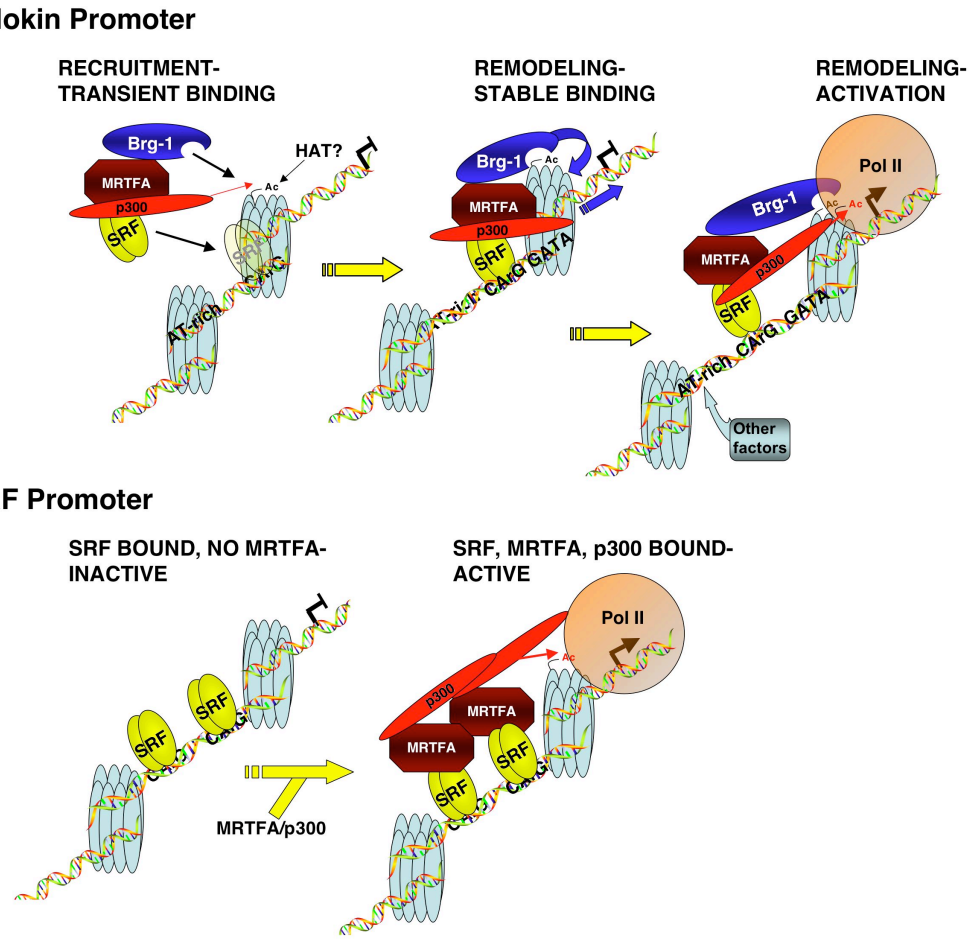


Figure 12. Proposed model describing the regulation of MRTFA/SRF activity by Brg1. Upper panels: The telokin promoter is used as an example to describe the activation of smooth muscle-specific promoters by MRTFA. In unstimulated non-muscle cells the promoter is in a condensed inactive chromatin conformation that only permits weak or transient SRF binding (Left panel). Upon increased nuclear MRTFA, MRTFA binds to Brg1 and recruits the MRTFA/Brg1/HAT (p300?) complex to the weakly bound SRF. The binding of Brg1 to acetylated histone tails may also play a role in this recruitment process. Once the MRTFA/Brg1/HAT complex is recruited to the promoter, Brg1 utilizes the energy derived from hydrolysis of ATP to remodel the chromatin structure,

permitting tight binding of SRF (Middle panel). Tightly bound SRF/MRTFA can then recruit additional co-activators such as p300, resulting in additional chromatin remodeling, facilitating binding of the RNA polymerase complex and subsequent activation of transcription (Right panel). Lower panels: On the promoters of MRTFA regulated early response genes, such as the SRF gene itself, SRF is tightly bound to the promoter under resting conditions (Left panel). MRTFA, therefore, does not require Brg1-mediated chromatin remodeling to be able to bind to the promoter, recruit co-activators and thereby activate transcription (Right panel).

Chapter III: The SWI/SNF chromatin remodeling complex regulates myocardin-induced smooth muscle-specific gene expression.

Abstract

Objective: Transcription regulatory complexes comprising myocardin and serum response factor (SRF) are critical for the transcriptional regulation of many smooth muscle-specific genes. However, little is known about the epigenetic mechanisms that regulate the activity of these complexes. In the current study, we investigated the role of SWI/SNF ATP-dependent chromatin remodeling enzymes in regulating the myogenic activity of myocardin. Methods and Results: We found that both Brg1 and Brm are required for maintaining expression of several smooth muscle-specific genes in primary cultures of aortic smooth muscle cells. Furthermore, the ability of myocardin to induce expression of smooth muscle-specific genes is abrogated in cells expressing dominant negative Brg1. In SW13 cells, that lack endogenous Brg1 and Brm1, myocardin is unable to induce expression of smooth muscle-specific genes. By contrast, reconstitution of wild type, or bromodomain mutant forms of Brg1 or Brm1 into SW13 cells restored their responsiveness to myocardin. SWI/SNF complexes were found to be required for myocardin to increase SRF binding to the promoters of smooth muscle-specific genes. Brg1 and Brm directly bind to the N-terminus of myocardin *in vitro* through their ATPase domains, and Brg1 forms a complex with SRF and myocardin *in vivo* in smooth muscle cells. Conclusion: These data demonstrate that the ability of myocardin to induce smooth muscle-

specific gene expression is dependent on its interaction with SWI/SNF ATP-dependent chromatin remodeling complexes.

Introduction

Smooth muscle cells are important contractile components of the cardiovascular system that regulate blood pressure and flow. Vascular smooth muscle cells modulate their phenotype in response to extracellular cues during the development and progression of a variety of diseases including atherosclerosis and hypertension. These diseases are associated with decreased expression of proteins required for the normal contractile function of smooth muscle cells (104). Understanding the mechanisms that control expression of contractile and regulatory proteins in smooth muscle cells is, therefore, an essential step toward determining how these processes are altered in pathological conditions.

The interaction of serum response factor (SRF) with the co-activator myocardin is a critical determinant of vascular smooth muscle development (81, 107). Myocardin null mice lack differentiated smooth muscle cells in the dosal aorta and placental vasculature and die around E10 (81). Myocardin is thus critically required for the differentiation of these populations of vascular smooth muscle cells. Myocardin does not bind directly to DNA, but interacts with genes via its binding to SRF through a basic domain and polyglutamine-rich (poly Q) domain in myocardin. Myocardin-bound SRF binds to CArG elements within the promoters of many smooth muscle-specific genes (92) and myocardin activates transcription of these genes through a strong transcriptional activation domain at its C-terminus (134). However, although myocardin is a potent activator of CArG box-containing cardiac and smooth muscle-specific genes, it poorly activates

SRF-dependent skeletal muscle-specific genes or early response genes such as c-fos or Egr-1 (107), yet how myocardin distinguishes smooth muscle-restricted genes from other SRF-dependent genes still remains elusive.

The dependence of myocardin on promoter bound SRF poses an interesting problem as there is little SRF bound to the promoters of smooth muscle-specific genes in nonmuscle cells (91), yet myocardin can induce expression of smooth muscle-specific genes in these cells (107). Myocardin also increases SRF binding to the promoters of smooth muscle-specific genes within intact chromatin (91), although the mechanism underlying this phenomena is unknown. We hypothesized that ATP-dependent chromatin remodeling may be required for these functions of myocardin, allowing it to increase SRF binding to the promoters of smooth muscle-specific genes within chromatin. Recent studies of ATP-dependent remodeling enzymes have highlighted crucial roles for these proteins in diverse developmental processes (39). Mammalian ATP-dependent chromatin-remodeling enzymes belong to the SNF2 family of DNA-dependent ATPases, which all have a helicase-like ATPase domain. The best-characterized mammalian ATP-dependent chromatin-remodeling complex is the SWI/SNF complex (39). Brahma (Brm) or Brahma-like gene 1 (Brg1) are the ATPase subunits of the SWI/SNF complex. These proteins have been shown to activate or repress expression of genes during myeloid differentiation, erythropoiesis, adipogenesis, skeletal muscle myogenesis, liver development and gliogenesis (reviewed in (39)). Recently we have also shown that Brg1 and Brm are required

for myocardin-related transcription factor A (MRTFA) to induce smooth muscle-specific genes in nonmuscle cells (149). However, as MRTFA knockout mice do not exhibit any major vascular defects (80) the importance of MRTFA-SWI/SNF interactions in vascular smooth muscle cells is not clear.

In the current study we found that Brg1 and Brm are essential for maintaining expression of smooth muscle-specific genes in vascular smooth muscle cells. Brg1/Brm are required for myocardin's ability to induce expression of smooth muscle-specific genes and to increase SRF binding to the promoters of these genes. We found that Brg1 forms a complex with myocardin and SRF *in vivo* and directly binds to myocardin *in vitro* through its ATPase domain. Together, our data demonstrate that SWI/SNF ATP-dependent chromatin remodeling complexes are required for differentiation of vascular smooth muscle cells.

Experimental Procedures

Mammalian expression and reporter gene assays. Expression plasmids encoding human Brg1 (pBJ5-Brg1) and the ATPase-defective variant of Brg1 (K798R) with an HA tag at their C-terminus were obtained from Dr. Gerald Crabtree (Stanford, CA) (75). Expression plasmids encoding human Brg1 and K798R mutants with C-terminal Flag tags (pBabe vectors) were obtained from Addgene (124). For adenoviral expression cDNAs were removed from the pBABE vectors and cloned into pShuttle (Clontech). Brg1 and Brm molecules lacking their C-termini (Brg1 truncated at amino acid 1414, Brm at 1344) were generated by removing Not I or Afl II fragments from the pShuttle constructs. Brg1 and Brm containing mutations in their bromodomains (Brg1 FN1506-7-AA, Brm FN1481-2-AA) were generated using the quick change mutagenesis kit (Stratagene) with the pShuttle vectors as templates. The mouse full-length SRF cDNA was cloned into a modified pShuttle vector (Clontech) encoding an N-terminal HA tag. The mouse myocardin (long form, equivalent to the cardiac enriched isoform) pcDNA3.1-myc/his vector was kindly provided by Dr. Eric N. Olson (Southwestern Medical Center, Dallas, TX). This vector was used to generate adenovirus in which the myocardin harbors an amino-terminal HA epitope tag. A truncated myocardin adenovirus (amino acids 80-935, equivalent to the smooth muscle enriched isoform) was also generated harboring an amino-terminal omni epitope tag. All promoter reporter genes were constructed by cloning fragments of promoters into the pGL2B luciferase vector (Promega, Madison, WI). The mouse telokin promoter-luciferase reporter gene used

includes nucleotides –190 to +181 (T370) of the telokin gene as described previously (63, 65). The SM22α-luciferase reporter gene includes nucleotides -475 to +61 of mouse SM22α (65). Plasmids were sequenced to verify the integrity of the insert. Transfection was carried out as previously described. The level of promoter activity was evaluated by measurement of the firefly luciferase activity relative to the internal control renilla luciferase activity using the Dual Luciferase Assay System essentially as described by the manufacturer (Promega, Madison, WI). A minimum of six independent transfections were performed and all assays were replicated at least twice. Results are reported as the mean ± SEM.

Mouse aortic SMC preparation and RNA interference. Primary mouse aortic smooth muscle cells were prepared from aorta dissected from 4-week-old mice essentially as described previously (147). For all experiments primary cells were trypsinized, and replated at 7 x 104 per well in 12-well plates. 12 h after plating, cells were transfected with pre-designed Dharmacon siRNA pools targeting Brg1 or Brm, as well as a control siRNA pool at final concentration of 50 nM. siRNA was delivered using Lipofactamine 2000 (Invitrogen) following the manufacturer's instructions. 36 h after transfection, mRNA was harvested and gene expression measured by quantitative real time reverse transcription (RT)-PCR as described below.

Reverse transcription and quantitative real time PCR (qRT-PCR). Total RNA was isolated with TRIzol reagent (Invitrogen). 1 µg of RNA was utilized as a template for reverse transcription with random hexamer primers. PCR was performed with 2 µl (of 1:10 dilution) of cDNA and SYBR green PCR master mix (Abgene) with respective gene-specific primers (See Table 1) using the following reaction conditions: 1 cycle: 50°C for 2 min; 1 cycle: 95°C for 2 min; 40 cycles: 95°C for 15 sec, 60°C for 40 sec; 1 cycle: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec. All PCR reactions were performed in duplicate on triplicate samples obtained from 2-3 independent experiments. Relative gene expression was converted with $2^{-\Delta\Delta ct}$ method against an internal control hprt or RPLPO house keeping gene as detailed in the Figure legends.

Adenovirus construction and cell infection. Adenovirus constructs were made using the Adeno-X vectors essentially followed the manufacturers instructions (BD Biosciences) as previously described (150). The recombinant adenovirus was packaged in HEK293 cells and amplified to obtain high titer stocks. 3T3 cells that inducibly express dominant-negative Brg1 following withdraw of tetracycline (B22 cells) were described previously (36). These cells were maintained in the complete growth media, comprising DMEM (Cellgro), 10% fetal bovine serum (Atlanta Biologicals), 2 mM glutamine (Invitrogen), 75 µg/ml G418, 350 U/ml hygromycin B (Fisher), and 2 µg/ml tetracycline (Fisher). Cells were washed twice with PBS before passage for experiments requiring growth in media lacking tetracycline (36). For adenoviral transduction, B22, SW13, 10T1/2 cells and

primary SMC were plated in 6-well plates at a density of 0.3-0.5x105 cells/well and grown overnight to 80% confluence (with or without tetracycline for B22 cells). The next day cells were transduced with adenovirus encoding HA-tagged YFP-NLS (Nuclear localized Yellow Fluorescent Protein) or HA-tagged myocardin or DN-Brg1 in 10% growth media for 4 hours at 37°C. The adenovirus was then aspirated and replaced with 10% growth media. These conditions resulted in close to 100% transduction of the cell lines and 50-60% transduction of the primary aortic cells. 48 hours following transduction, protein extracts or total cellular RNA were prepared from the transduced cells. The protein extracts were collected using RIPA buffer, containing a cocktail of protease inhibitors and protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical). The total cellular RNA was isolated using Trizol (Invitrogen) as described by the manufacturer.

Western blotting. Western blotting analysis was carried out essentially as described previously (62). Antibodies used in this study were against: β-actin (Sigma, 1:10,000), Brg1 (Upstate, 1:5000), Brm (Abcam, 1:1000), Egr-1 (Santa Cruz, 1:1,000), Flag tag (Sigma, M2, 1:5,000), HA tag (Covance, 1:3,000), c-fos (Santa Cruz, 1:1000), MLCK (Sigma, clone K36, 1:10,000), myosin light chain 20 (LC20, a gift from Dr. Patricia Gallagher, 1:5,000), SM α-actin (Sigma, clone 3A1, 1:10,000), SM22α (a gift from Dr. Len Adam, 1:6,000), SRF (Santa Cruz, G20X, 1:10,000), telokin (1:6,000)(51), vinculin (Santa Cruz, 1:5,000).

Quantitative chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were performed as described previously (147). Cross-linked chromatin was immunoprecipitated with 6 μg of anti-SRF (Santa Cruz), anti-Brg1 (Upstate) or rabbit IgG control and bound to ssDNA/protein A agarose beads (Sigma). The precipitated DNA was then purified and amplified by real time PCR for quantification of the target sequences using SYBR green PCR master mix (Abgene) with respective gene-specific primers as described previously (147).

Co-immunoprecipitation. For detecting protein-protein interaction *in vivo*, COS cells were transduced with adenovirus encoding HA-tagged SRF and HA-tagged myocardin. 24 hours after transduction nuclear protein was harvested from the COS cells. Co-immunoprecipitation (Co-IP) assays were performed using a nuclear complex Co-IP kit as described by the manufacturer (Active Motif). 250 μg of nuclear protein extracts were incubated with 3 μg of anti-Brg1 antibody (Upstate), anti-SRF antibody (Santa Cruz, G20X), anti-myocardin antibody (Santa Cruz, M16), or appropriate IgG controls in 500 μl low salt IP buffer (Active Motif), overnight at 4°C. 60 μl of EZview protein A beads (Sigma) were added to the mixture for an additional 1 hour with rocking and then wash 6 times with the low IP buffer. To examine myocardin/SRF/Brg1 complex formation in SMCs, rat aortic A10 smooth muscle cells were infected with adenovirus encoding myocardin for 3 days. Nuclear protein was then extracted and immuoprecipitated as described above. To examine protein-protein interactions *in vitro*, the TNT

Quick Coupled Transcription/Translation System (Promega) was employed to generate proteins *in vitro* programmed with 1 μg of pcDNA myc/hismyocardin (myc tag), pShuttle-Brg1 (flag tag), pShuttle Brm (flag tag) and pShuttle-SRF (HA tag) plasmid DNA, respectively, as templates. To define binding domains within Brg1, various Brg1 restriction fragments were subcloned into pET28 vector (Novagen, Madison, WI). These vectors were utilized as templates to produce T7 tagged protein *in vitro* as described above. 20 μl of each reaction product were incubated with 3μg SRF antibody (Santa Cruz, G20X) or myocardin antibody (Santa Cruz, M16) or appropriate IgG control in 250 μl low salt IP buffer (Active motif) overnight at 4°C and then protein A beads were added for an additional 2 hours. The immunoprecipitated proteins were subjected to Western blotting with antibodies against: Flag tag (Sigma, 1:5000), HA-tag (Covance, 1:3,000) or c-myc (Invitrogen, 1:5000), T7 tag (Novagen, 1:10000).

GST pull-down assays. To characterize the interaction between myocardin and Brg1 *in vitro*, various fragments of each of the proteins were expressed as fusion proteins in bacteria. Six myocardin deletion mutants were expressed as glutathione S-transferase (GST) fusion proteins. Deletion fragments were generated by polymerase chain reaction and then subcloned into pGEX4T (Amersham Pharmacia Biotech); all constructs were verified by DNA sequencing. The following fragments of myocardin were expressed: (1) NT-myocardin, encoding amino acids 1-587; (2) CT-myocardin, encoding amino acids 588-935; (3) deletion 1, encoding amino acids 1-220; (4) deletion 2, encoding amino acids

221-350; (5) deletion 3, encoding amino acids 351-474; and (6) deletion 4, encoding amino acids 475-587; Full-length SRF GST fusion protein was expressed as we described in our previous report9. Various Brg1 deletion mutants were expressed as fusion proteins with a His tag and T7 epitope tag at the amino terminus by polymerase chain reaction and subcloned into pET28 (Novagen, Madison, WI). The following fragments of Brg1 were expressed: deletion 5 encoding amino acids 767-931; deletion 6 encoding amino acids 932-1084; deletion 7 encoding amino acids1085-1246; deletion 8 encoding amino acids 1247-1477. T7 tagged Barx2 protein was produced in bacteria as we previously reported (60). *In vitro* binding experiments were carried out essentially as described previously (60). Briefly GST-myocardin fusion proteins were bound to glutathione-agarose beads and then incubated with bacterial lysates containing Brg1 truncation fusion proteins. After incubation at 4°C for 1 h, glutathione-agarose-bound protein complexes were washed three times in lysis buffer without bovine serum albumin. Glutathione-agarose-bound proteins were then analyzed by Western blotting with T7 antibody (1:10000, Novagen).

Results

Depletion of endogenous Brg1 and Brm in aortic SMCs attenuates expression of smooth muscle-specific genes. Previously we have shown that the ATP-dependent chromatin remodeling enzymes, Brahma-Related Gene 1 (Brg1) and Brahma (Brm) play an important role in balancing the ability of MRTFA to regulate expression of SRF-dependent smooth muscle-specific genes and immediate early genes (147). However, as MRTFA knockout mice have not been reported to exhibit any vascular defects (80) the importance of MRTFA-SWI/SNF interactions in vascular SMCs is not clear. We therefore examined the functional role SWI/SNF in vascular SMCs. siRNA-mediated knockdown of Brg1 or Brm in primary mouse aortic SMCs attenuated expression of telokin, calponin and smooth muscle myosin heavy chain (SM MHC), late markers of smooth muscle differentiation, by approximately 40%. In contrast, knockdown of Brg1 or Brm had a lesser effect on expression of the early markers of smooth muscle differentiation, SM22α or SM α-actin (Figure 13). Silencing Brg1 also led to a 30% reduction in expression of endogenous Brm although Brm knockdown did not affect Brg1 expression. This is not a result of cross reactivity of siRNA molecules as similar results were obtained with multiple siRNA duplexes, with DN-Brg1 and in Brg1 knockout cells (data not shown), suggesting that Brm expression is at least partially dependent on Brg1 in smooth muscle cells. Surprisingly, knockdown of both Brg1 and Brm together did not result in any further attenuation of smooth muscle-specific genes, as compared to knockdown of either protein alone (Figure 13). These data suggest that Brg1 and/or Brm are

important for maintaining expression of genes that are late differentiation markers of vascular SMCs.

DN-Brg1 represses activation of smooth muscle-specific genes by myocardin. As myocardin has been shown to be critical for vascular smooth muscle differentiation we next sought to determine if myocardin requires SWI/SNF to induce expression of smooth muscle-specific genes. For these experiments we utilized 10T1/2 embryonic fibroblasts and primary cultures of aortic smooth muscle cells, two well established systems in which myocardin has been shown to induce expression of smooth muscle-specific genes. To inhibit SWI/SNF activity, cells were transduced with adenovirus expressing an ATPase deficient mutant of Brg1 (K798R) that acts as a dominant negative (36). Consistent with previous reports, adenoviral-mediated expression of myocardin in 10T1/2 and primary aortic SMC cells resulted in induction of endogenous telokin, smMHC, calponin, SM22α and SM α-actin mRNA (Figure 14A, B). Expression of dominant negative Brg1 significantly attenuated the induction of telokin, smMHC, calponin, and SM22α by myocardin in 10T1/2 cells and aortic SMCs (Figure 14). In contrast, DN-Brg1 slightly augmented myocardin's ability to activate SRF itself and did not significantly affect the activation of c-fos in aortic SMC (Figure 14B). Similar results were also seen in NIH3T3 cells induced to express a dominant negative Brg1 (Figure 15). Interestingly DN-Brg1 also attenuated the ability of myocardin to induce the expression of the cardiac-specific ANF and cardiac α-actin genes in 10T1/2 cells (Figure 14A).

Previous reports have shown that transient over-expression of wild-type Brg1 can increase the activity of SWI/SNF-dependent reporter genes, while expression of a dominant negative Brg1 decreases the activity of these reporter genes through formation of inactive SWI/SNF complexes (33, 75). We therefore determined if DN-Brg1 could directly affect myocardin's ability to activate the telokin and SM22α promoters. Luciferase reporter assays revealed that over-expression of DN-Brg1 in NIH3T3 cells attenuated myocardin's activation of telokin and SM22α promoter reporter constructs (Figure 15). Taken together, data presented here demonstrate that dominant negative Brg1 can attenuate myocardin's ability to induce expression of many smooth muscle-specific genes in both nonmuscle and smooth muscle cells. By contrast, dominant negative Brg1 either did not affect or augmented myocardin's ability to activate other SRF target genes such as SRF itself or c-fos.

Induction of endogenous smooth muscle-specific genes by myocardin requires Brg1/Brm ATPase activity but does not require their bromodomains. To formally confirm that the ATPase activity of Brg1 and Brm is required to support myocardin's myogenic activity we utilized SW13 cells, an adenocarcinoma cell line that expresses no endogenous Brg1 or Brm (97) (Figure 16). In these cells myocardin fails to induce expression of most smooth muscle-specific proteins (Figure 16A, B, left four lanes). Only SM α-actin induction could be detected. Co-transfection of SW13 cells with myocardin together with wild type Brg1 or Brm expression plasmids restored myocardin's

ability to induce expression of smooth muscle-specific proteins such as SM22α, telokin, and smMLCK (Figure 16). In contrast, the dominant negative, ATPase deficient Brg1 could not restore myocardin's myogenic activity (Figure 16A, 'DN' lanes). Both Brg1 and Brm contain a bromodomain toward their c-termininal that has been shown to be able to interact with acetylated histones (127). To determine if Brg1/Brm-acteylated histone interactions are important for supporting myocardin's myogenic activity, mutant Brg1/Brm molecules were generated that either completely lacked the c-terminus, including the bromodomains or that harbored two specific amino-acid mutations within the bromodomain (Brg1 FN_{1506-7}-AA, Brm FN_{1481-2}-AA). Mutation of these residues has been previously shown to inhibit the binding of Brg1 to acetylated histones (123). These mutants were able to support myocardin's myogenic activity similar to the wild type molecules, indicating that the bromodomains of Brg1 or Brm are not required for this activity (Figure 16). These data also demonstrate that both Brm and Brg1 have similar abilities to support myocardin's myogenic activity (Figure 16B, compare the Brg1 WT lanes to the Brm WT lanes). Although identical amounts of myocardin plasmid were used in all co-transfections the resultant myocardin expression levels varied depending on which Brg1/Brm plasmid was co-transfected into the cells (Figure 16). This likely reflects differences in transfection efficiencies of specific plasmid mixtures. In general, the extent of induction of smooth muscle-specific genes by myocardin reflected the relative levels of myocardin expression. However, in cells transfected with the DN-Brg1 even high levels of myocardin failed to induce expression of smooth

muscle-specific genes (Figure 16A). This latter finding further demonstrates that the ATPase activity of Brg1 is required to support myocardin's myogenic activity.

DN-Brg1 inhibits myocardin's ability to increase SRF binding to the promoters of smooth muscle-specific genes. Previous studies have shown that in nonmuscle cells there is little SRF bound to the promoters of smooth muscle-specific genes but introduction of myocardin into these cells leads to increased SRF binding (91). Similarly we have previously shown that MRTFA increases SRF binding to the promoters of smooth muscle-specific genes and this process is attenuated by DN-Brg1 (147). We thus sought to determine if Brg1 is also required for myocardin to increase SRF binding to the promoters of smooth muscle-specific genes within intact chromatin. In agreement with previous reports (91), using quantitative ChIP assays we observed enhanced SRF binding to the telokin, SM22α and SM α-actin promoters but not the c-fos and SRF promoters following myocardin transduction into NIH 3T3 cells (Figure 17A). Expression of DN-Brg1 attenuated this myocardin-induced increase in SRF binding to the telokin and SM22α promoters without significantly affecting SRF binding to the SM a-actin, SRF and c-fos promoters (Figure 17B). ChIP assays in differentiated primary SMCs also revealed Brg1 binding to the promoters of smooth muscle-specific genes under control conditions (Figure 17C). In addition, expression of DN-Brg1 in differentiated SMCs significantly inhibited the binding of endogenous SRF to the promoters of the telokin and SM MHC gene but not the SRF or SM22α genes (Figure 17D). These data are consistent with expression

data shown in Figure 13 that demonstrate that the later markers of smooth muscle differentiation such as telokin and smMHC are most dependent on Brg1/Brm.

Brg1, myocardin and SRF form a complex *in vivo*. To determine if Brg1 interacts with myocardin/SRF complexes *in vivo,* immunoprecipitation assays were performed from COS cells transduced with adenoviruses encoding myocardin and SRF (Figure 18A). Brg1 immunoprecipitates were found to also contain myocardin and SRF. Similarly, SRF immunoprecipitates also contained Brg1 and myocardin. In rat aortic A10 SMCs, myocardin immunoprecipitates also contained SRF and Brg1 (Figure 18B). These data suggest that, either Brg1, myocardin and SRF exist in a single complex *in vivo* or that Brg1 binds to both myocardin and SRF. To distinguish these possibilities co-immunoprecipitation assays were performed using proteins generated *in vitro.* Results from these experiments demonstrated that Brg1 and Brm directly bind to myocardin but not to SRF (Figures 18C,D). When Brg1 or Brm were incubated with myocardin they could be co-immunoprecipitated with myocardin. In contrast, when Brg1 or Brm were incubated with SRF they were not co-immunoprecipitated with SRF. No endogenous SRF, myocardin and Brg1 were detected in the *in vitro* translation reaction system (data not shown).

The ATPase domain of Brg1 binds to N-terminus of myocardin. Co-immunoprecipitation assays using *in vitro* transcribed and translated myocardin

and fragments of Brg1 revealed that the region from amino acids 837 to 1446 of Brg1 binds to myocardin (Figure 19). To further resolve the myocardin binding site within this region, an additional series of Brg1 deletion mutants were generated, expressed in bacteria and used in GST-pulldown assays with the N-terminus of myocardin as bait (Figure 19). These assays demonstrate that the ATPase domain of Brg1 extending from amino acids 767-931 is sufficient to bind to myocardin. Interestingly this region is 94% identical between Brg1 and Brm, consistent with our observations that both of these molecules can bind to myocardin (Figure 18C,D). GST-pulldown assays were also performed to determine which portion of myocardin interacts with Brg1 (Figure 19). Data from these experiments demonstrated that the Brg1 interacts with the N-terminal portion of myocardin, with the region spanning the basic and poly Q domains (myo D2-GST in Figure 19E) having the highest apparent affinity. The GST-pulldown assays also confirm that Brg1 does not directly bind to SRF, while Barx2 can be readily detected bound to GST-SRF fusion proteins, as reported previously (60).

Discussion

Our data demonstrate that the ability of myocardin to induce expression of most smooth muscle-specific genes is regulated by the activity of the SWI/SNF ATP-dependent chromatin remodeling complex. We suggest a model in which myocardin associates with the SWI/SNF complex through direct binding to the Brg1 or Brm ATPase subunit. This association is required in order for myocardin to increase SRF binding to the promoters of smooth muscle-specific genes within intact chromatin, thereby leading to activation of these genes during differentiation of vascular smooth muscle cells.

Although Brg1/Brm containing SWI/SNF complexes are required for myocardin to induce expression of many smooth muscle-specific genes the induction of SM α-actin by myocardin was largely independent of SWI/SNF (Figures 13-16). In addition, in SW13 cells that lack Brg1 and Brm, myocardin was still able to induce SM α-actin expression (Figure 16A). This correlates with the relatively high basal levels of SM α-actin expression in many of these cells in the absence of added myocardin. Thus it is likely that the SM α-actin promoter is already in an active transcriptionally favorable conformation in the absence of myocardin. This may suggest that the SWI/SNF complex is dispensable for myocardin-induced activation of genes, such as SM α-actin, in cells in which these genes are already transcriptionally active. In contrast, SWI/SNF activity is required for myocardin to induce expression of genes that are otherwise transcriptionally silent in a given cell type. Similar to SM α-actin expression of SM22α in primary smooth muscle

cells was found to be largely independent of Brg1. This may also reflect the contribution of myocardin independent pathways in driving chromatin remodeling and transcription of the SM22α locus in smooth muscle cells. For example, both SM α-actin and SM22α can be induced by TGFβ in 10T1/2 cells by a myocardin independent pathway (1, 28). This model is, however, likely to be an over-simplification, as in primary cultures of mouse aortic smooth muscle cells we observed that the myocardin-induced increase in expression of smooth muscle-specific genes was at least partially attenuated by DN-Brg1 (Figure 14B). As all of the genes examined were expressed prior to myocardin over-expression, this may suggest that even if a gene is transcriptionally active, SWI/SNF induced changes in chromatin can further augment myocardin's myogenic activity.

Domain mapping experiments suggest that Brg1 and Brm interact with the region of myocardin that spans the SRF interaction domain (basic and poly Q region). Despite the overlapping binding sites, co-immunoprecipitation studies show that myocardin is present in a complex that includes both Brg1 and SRF within intact cells. The Brg1 binding site in myocardin is present in both the long, cardiac-selective isoform (1-935) and the shorter, smooth muscle-selective isoform (80-935). These results suggest that both cardiac- and smooth muscle-selective myocardin isoforms will interact with and be regulated by SWI/SNF. In support of this proposal, the myogenic activity of both the cardiac myocardin (Figure 15), and the smooth muscle myocardin (Figures 14,16) was attenuated by dominant negative Brg1. In addition, the ability of myocardin to induce expression of

cardiac-specific genes in 10T1/2 cells was also attenuated by DN-Brg1. These observations suggest that the myogenic activity of myocardin both in the heart and in vascular SMCs is regulated by SWI/SNF.

Results from experiments in which we reconstituted Brg1 or Brm expression in SW13 cells suggest that either wild-type Brg1 or Brm containing SWI/SNF complexes are equally effective at supporting myocardin's myogenic activity (Figure 16). In addition, knockdown of either Brg1 or Brm in aortic SMCs attenuated expression of smooth muscle-specific genes (Figure 13) and both Brg1 and Brm can directly bind to myocardin *in vitro* (Figure 18C and D). These data suggest that Brg1 or Brm containing SWI/SNF complexes may both play important roles in smooth muscle cells. In contrast to myocardin, the LIM domain protein CRP2 has recently been shown to interact specifically with Brg1, but not Brm, in order to induce expression of smooth muscle-specific genes in cardiomyocytes (24). Despite the ability of either Brg1 or Brm to support myocardin's myogenic activity in SW13 cells, knocking-down both Brg1 and Brm in smooth muscle cells did not result in any further attenuation of smooth muscle-specific genes as compared to knockout of either protein alone (Figure 13). The lack of an additive effect of the double knockdown, may suggest that Brg1- and Brm-containing SWI/SNF complexes act together in smooth muscle cells to regulate myocardin. This must however, be interpreted with caution as knockdown of Brg1 also attenuated expression of Brm. Further studies analyzing tissue specific single or double knockouts of Brg1 and Brm, *in vivo*, will be

required to clarify the role of individual SWI/SNF complexes in regulating smooth muscle differentiation.

In addition to ATP-dependent chromatin remodeling complexes, enzymes that covalently modify histones are important to mediate myocardin activation of smooth muscle-specific genes (17, 35). For example, myocardin has been shown to bind to p300 and to promote acetylation of histones associated with the promoters of smooth muscle-specific genes (17, 91). As the bromodomains of Brg1 and Brm are known to bind to acetylated histones we initially speculated that myocardin-recruited HAT activity may help recruit SWI/SNF to promote transcriptional activation of genes in smooth muscle cells. However, data showing that the bromodomains of Brg1 and Brm are not important for supporting myocardin's myogenic function argue against this proposal (Figure 16). Similarly, it is unlikely that direct DNA binding by the AT-hook domain of Brg1 or Brm is required to recruit myocardin to chromatin, as the C-terminal truncation of Brm that we analyzed also lacks this domain (12) yet was still able to support myocardin's myogenic activity (Figure 16).

In skeletal muscle, weak binding of MyoD to the myogenin promoter via MyoD interactions with Pbx, facilitates SWI-SNF recruitment through direct binding of MyoD and Brg1 (10, 38). Chromatin remodeling by SWI/SNF then facilitates tight binding of MyoD to the E box within the myogenin promoter, facilitating promoter activation and skeletal muscle cell differentiation. By analogy we propose a

model in which in undifferentiated SMC or in nonmuscle cells, SRF has a low binding affinity for CArG box elements in the promoters of smooth muscle-specific genes within intact chromatin. Little transcription activity of smooth muscle-specific genes such as telokin and SM-MHC, thus occurs in these cells. To induce smooth muscle differentiation, myocardin complexed with p300 and SWI/SNF, interacts with SRF weakly bound to the promoters of smooth muscle-specific genes. SWI/SNF binding to the promoter regions then leads to ATP-dependent chromatin remodeling and rearrangement of the nucleosomes that facilitates tight binding of SRF. This may also allow binding of additional activators to the adjacent DNA segments. These activators, together with the SRF/myocardin/p300 complex can then further modify chromatin to facilitate recruitment of general transcriptional factors, including RNA polymerase II, resulting in transcriptional activation of smooth muscle-specific genes.

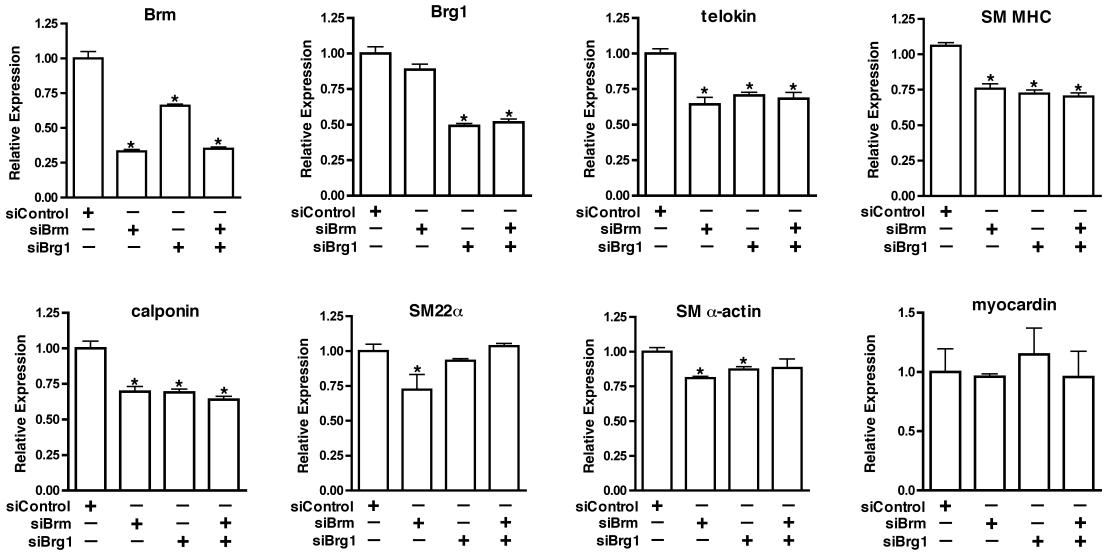


Figure 13. Effects of depletion Brg1 or Brm on expression of endogenous smooth muscle-specific genes. Mouse aortic smooth muscle cells were transfected with pre-designed Dharmacon siRNA smart pools targeting Brg1 or Brm, as well as a control siRNA, at final concentration of 50 nM. 36 h after transfection, mRNA was harvested and the levels of Brg1, Brm and smooth muscle marker genes were measured by quantitative real time RT-PCR as indicated in each panel. Data presented are the mean±SEM of 6 samples from 2 independent experiments and expression levels were normalized to an hprt internal control and are expressed relative to levels in siRNA control samples (set to 1). * Indicates statistical significance as determined by a student T test ($P<0.05$).

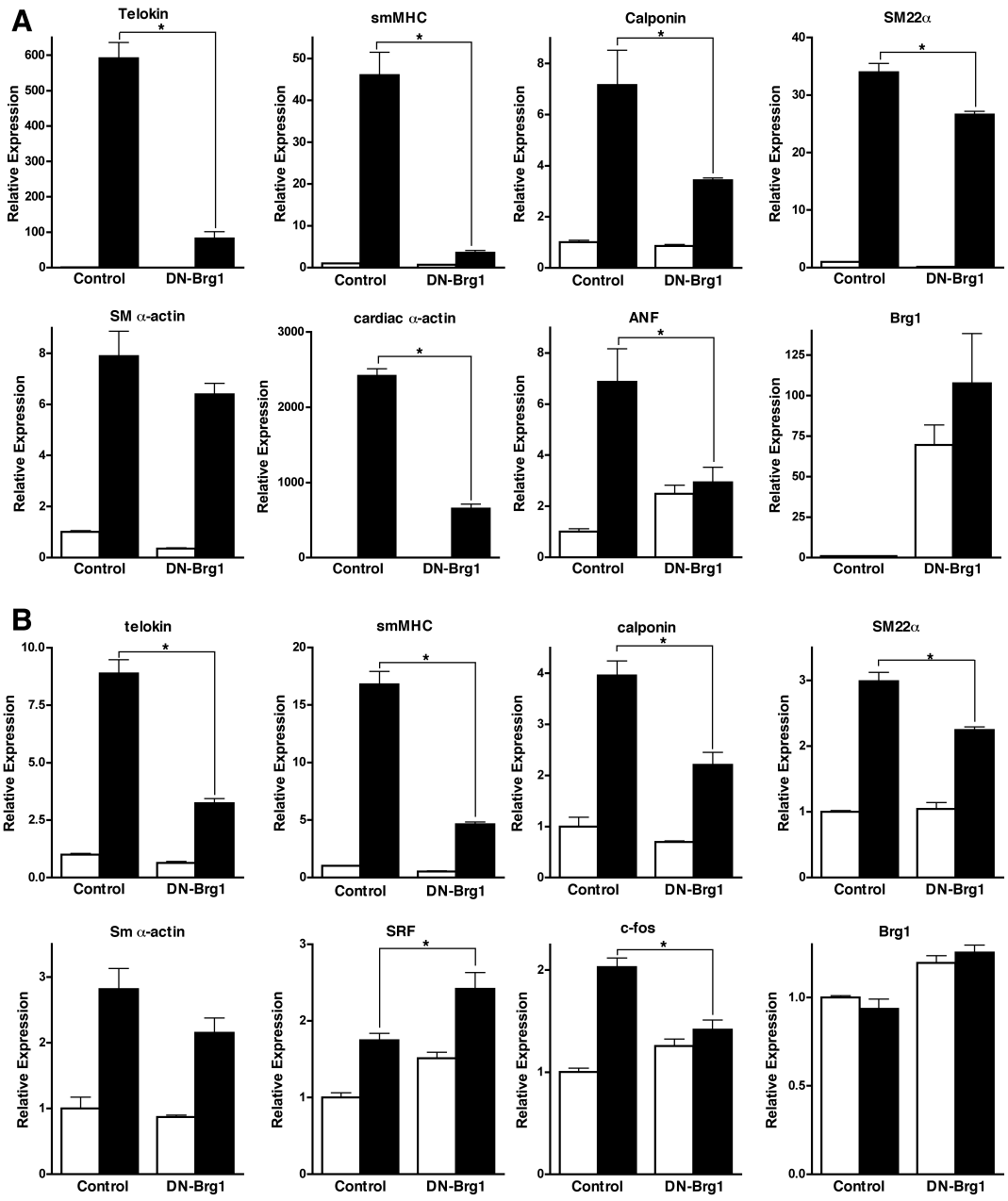


Figure 14. DN-Brg1 abrogates the induction of smooth muscle-specific genes by myocardin. A. 10T1/2 cells were transduced with DN-Brg1 or control YFP adenovirus together with myocardin (solid bars) or YFP (open bars) adenovirus. 48 hrs following transduction total RNA was harvested and analyzed by qRT-PCR. Transcript levels was firstly normalized to hprt internal loading

control and then normalized to their respective YFP control group. $RQ=2^{-\Delta\Delta Ct}$ and $\Delta\Delta C_t = (C_{t\ experimental}-C_{t\ hprt})-(C_{t\ control}-C_{t\ hprt})$. Data presented are the mean±SEM of 6 samples obtained from 2 independent experiments. **B.** Primary mouse aortic smooth muscle cells were transduced by DN-Brg1 or YFP control adenovirus with or without myocardin adenovirus as described in "A" (transduction efficiency of these cells was approximately 50-60%). 36-48 hours after transduction, cells were lysed, mRNA harvested and transcript levels analyzed as described above. Data presented are the mean±SEM of 3 samples obtained from one experiment. Similar results were obtained in a replicate experiment.

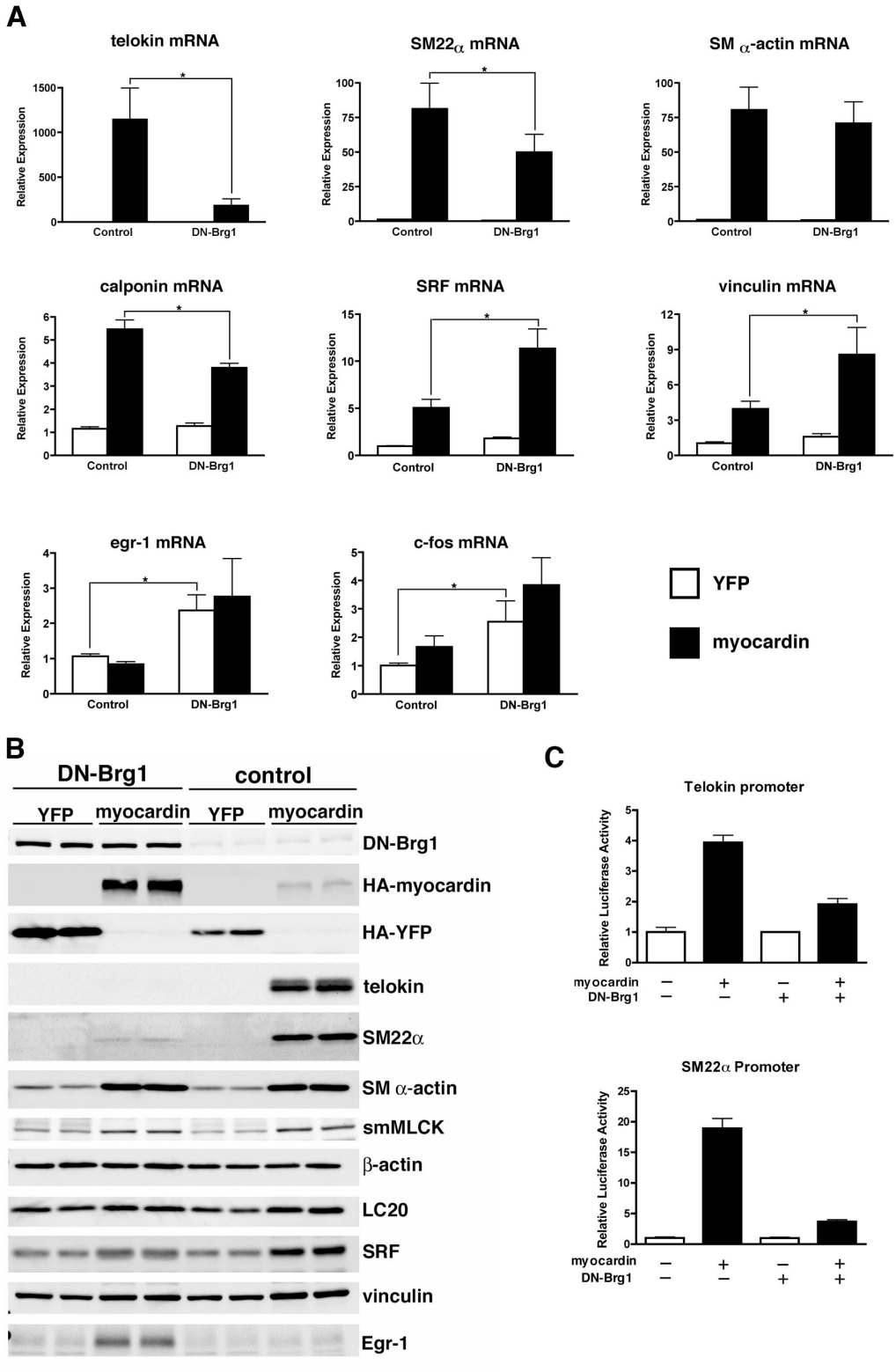


Figure 15. Dominant negative Brg1 blocks the induction of endogenous smooth muscle-specific genes by myocardin. A. B22 cells were grown in the

presence (control) or absence (DN-Brg1) of tetracycline for 24 hours. Cells were then transduced with HA-tagged myocardin (solid bars) or HA-tagged YFP (open bars) adenoviruses and maintained in their respective media for 30 hours. Total cellular mRNA was isolated, reverse transcribed and the levels of gene expression were measured by quantitative real time PCR. Transcript levels were first normalized to acidic ribosomal phosphoprotein PO (RPLPO) internal loading control and then normalized to their respective YFP control group. RQ=2-ΔΔCt and ΔΔCt = (Ct experimental-Ct RPLPO)-(Ct control-Ct RPLPO). Data presented are the mean±SEM of 8-9 samples obtained from 3 independent experiments. * Indicates statistical significance as determined by a student T test ($P<0.05$). **B.** B22 cells were treated as described in panel A except that protein extracts were prepared using RIPA lysis buffer and protein expression levels analyzed by Western blotting. **C**. Luciferase reporter assays were performed in B22 cells in presence (+, no tetracycline) or absence (−, with tetracycline) of DN-Brg1 (K798R) following transfection of myocardin or empty expression plasmids as indicated. Data presented are the mean±SEM of 6-9 samples obtained from 3 independent experiments. * Indicates statistical significance as determined by a student T test ($P<0.05$).

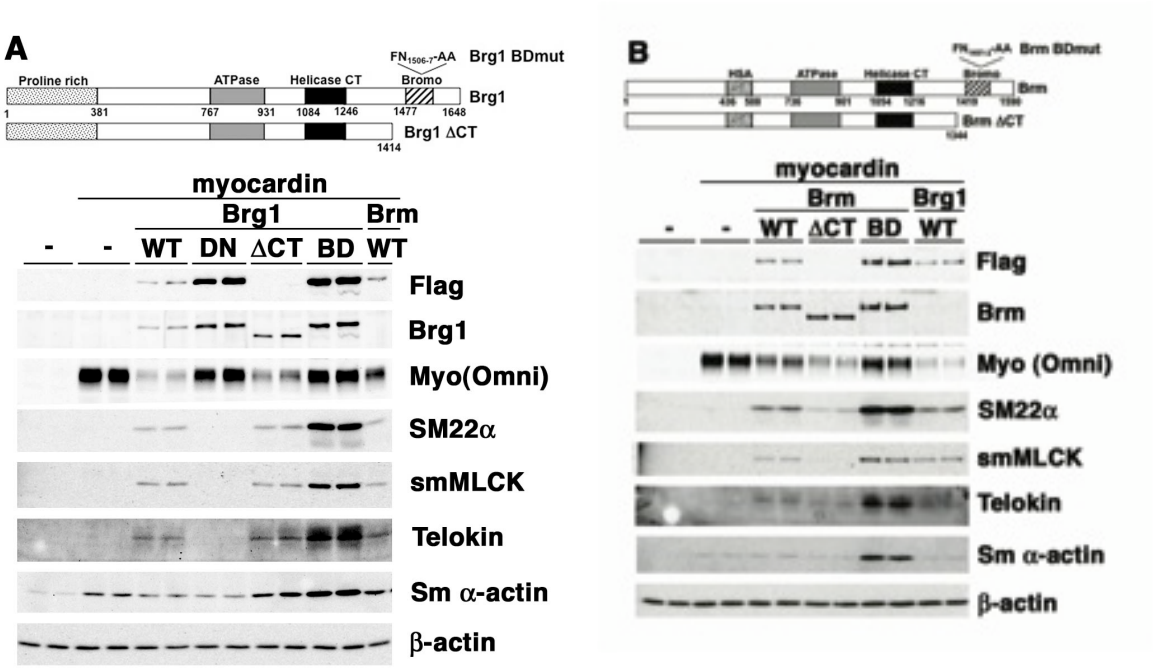


Figure 16. Re-introduction of wild type Brg1 or Brm, but not an ATPase deficient mutant, into SW13 cells restores myocardin's ability to induce expression of smooth muscle-specific genes. SW13 cells grown in 6-well plates were co-transfected with expression plasmids (1 μg) encoding either wild type Brg1 (WT) and Brm, DN-Brg1 (DN), CT-truncated Brg1 or Brm (DCT) or bromodomain mutant (BD) of Brg1 and Brm, together with myocardin expression plasmid (1 μg, except in the absence of the Brg/Brm plasmid where 2 μg were used), as indicated at the top of the panel (-, empty expression plasmid). 36 hours after transfection protein expression was analyzed by western blotting with the indicated antibodies. Exogenous Brg1 and Brm were detected with anti-Flag antibodies (Flag) and exogenous smooth muscle myocardin was detected with an omni-epitope tag antibody (Myo(Omni)). 30 μg of protein were loaded in each

lane. The blots shown are representative of data obtained from 2 separate experiments. The Brm WT sample shown in panel ‘A’ is the same sample as shown in the first WT lane of panel ‘B’. Similarly the WT Brg1 samples shown in panel ‘B’ are the same samples as those shown in panel ‘A’.

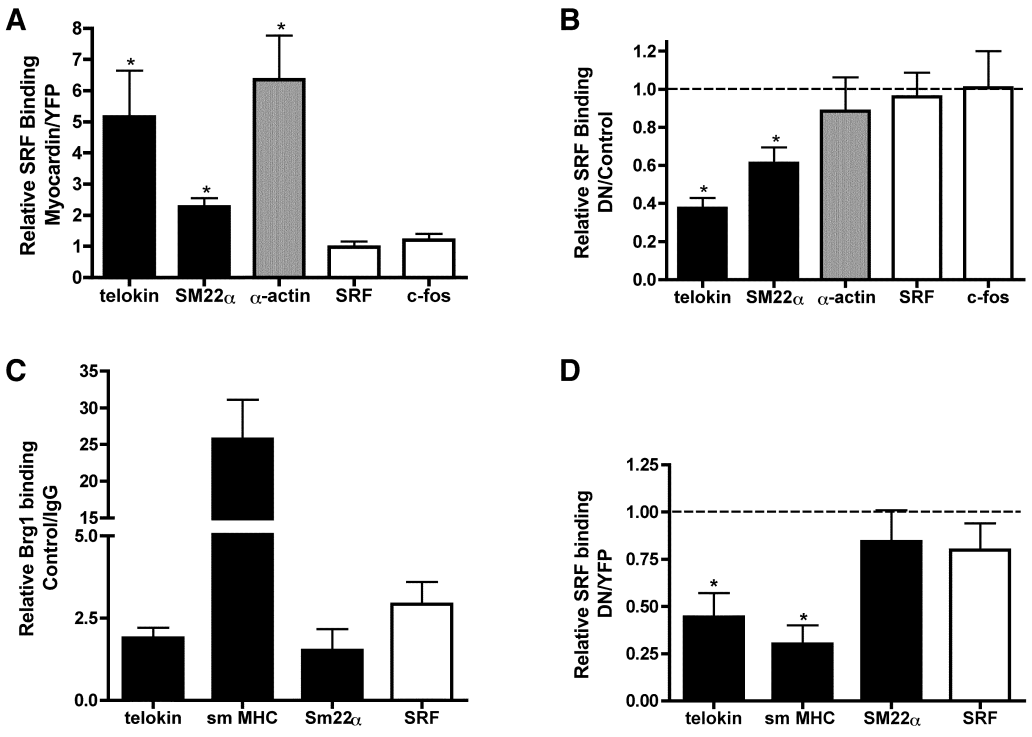


Figure 17. DN-Brg1 blocks the ability of myocardin to increase SRF binding to the promoters of smooth muscle-specific genes within chromatin. A-B. B22 cells (an NIH3T3 cells line that inducibly express DN-Brg1 in response to tetracycline withdrawal (36)) grown in the presence (control) or absence (induced DN-Brg1) of tetracycline were transduced with myocardin or YFP control adenovirus. After 30 hrs, cells were fixed and harvested for chromatin immunoprecipitation assays. Chromatin was precipitated using an antibody against SRF or using IgG negative control. The precipitated genomic DNA was purified and the presence of the promoters of SRF-dependent genes measured by real time PCR using gene specific primers (147). A. The increase in SRF binding in samples transduced with myocardin is indicated relative to those

transduced with YFP. These data were calculated and normalized to input levels as follows: Relative SRF binding=$2^{-\Delta\Delta Ct}$, with $\Delta\Delta C_t$= ($C_{t\ myocardin}$-$C_{t\ input}$)-($C_{t\ YFP}$-$C_{t\ input}$). B. The relative inhibition of myocardin induced SRF binding by DN-Brg1 is shown. This was calculated as follows: Relative SRF binding= $2^{-\Delta\Delta Ct}$, with $\Delta\Delta 4C_t$= ($C_{t\ DN\text{-}Brg1+Myocardin}$-$C_{t\ input}$)-($C_{t\ YFP+Myocardin}$-$C_{t\ input}$). Data shown in panels 'A' and 'B' are the mean±SEM of 7 samples obtained from 3 independent experiments. A one-way T test was performed and the asterisks indicate the results that are statistically different from 1 ($P<0.05$). **C-D.** Primary colon smooth muscle cells were prepared from 4 week old mice. The cells were transduced by DN-Brg1 or YFP control adenovirus. After 36 hours, cells were fixed and harvested for chromatin immunoprecipitation assays as above. **C.** The relative Brg1 binding to several SRF dependent genes in control primary smooth muscle cells is shown. The Brg1 binding to promoters were normalized to IgG control. The relative Brg1 binding was calculated as RQ=$2^{-\Delta Ct}$, with ΔC_t= $C_{t\ Brg1}$-$C_{t\ IgG}$. **D.** The relative inhibition of SRF binding by DN-Brg1 is shown. The inhibition of SRF binding by DN-Brg1 is calculated as, RQ=$2^{-\Delta\Delta Ct}$, with $\Delta\Delta C_t$= ($C_{t\ DN\text{-}Brg1}$-$C_{t\ input}$)-($C_{t\ YFP}$-$C_{t\ input}$). Data presented are the mean ±SEM of 4 samples.

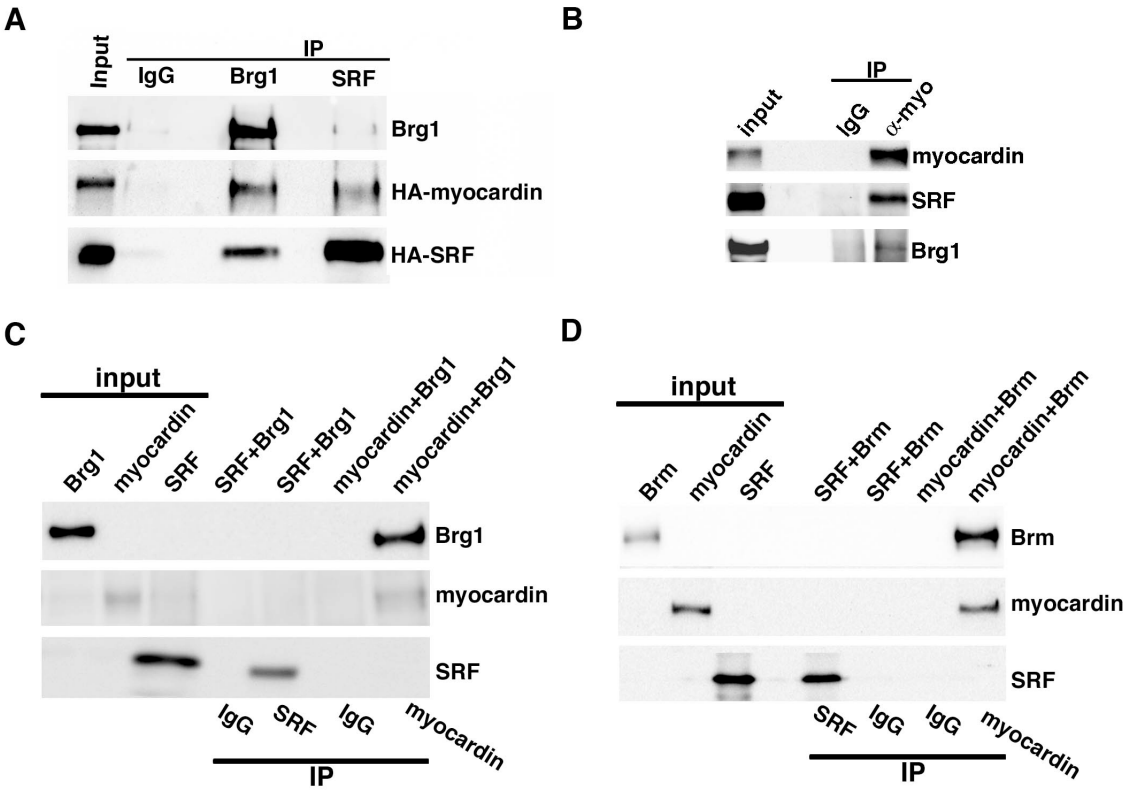


Figure 18. Myocardin, SRF and Brg1 form a complex *in vivo* and Brg1 binds directly to myocardin *in vitro*. A. COS cells were transduced with HA-tagged myocardin and HA-tagged SRF adenovirus. After 24 hours, nuclear protein was harvested and proteins were immunoprecipitated using Brg1, SRF or control IgG antibodies, as indicated. Immunoprecipitated proteins were identified by Western blotting using antibodies against Brg1 or the HA-epitope tags on myocardin and SRF as indicated at the right of the blot. **B.** A10 SMCs were transduced with omni-tagged myocardin for 24 hours. Nuclear protein was harvested and subsequently immunoprecipitated using myocardin or control IgG antibody. **C.** SRF, myocardin, and Brg1 or Brm (**D**) were transcribed and translated *in vitro*, the expressed proteins were then incubated together (SRF+Brg1/Brm or myocardin +Brg1/Brm) as indicated at the top of the blots. Protein mixtures were

immunoprecipitated with myocardin, SRF or IgG control antibodies, as indicated below the blots. The precipitated proteins were analyzed by western blotting, using antibodies indicated at the right of the blots. On all blots ‘input’ lanes represent 10% of the inputs that were mixed together and used for immunoprecipitates.

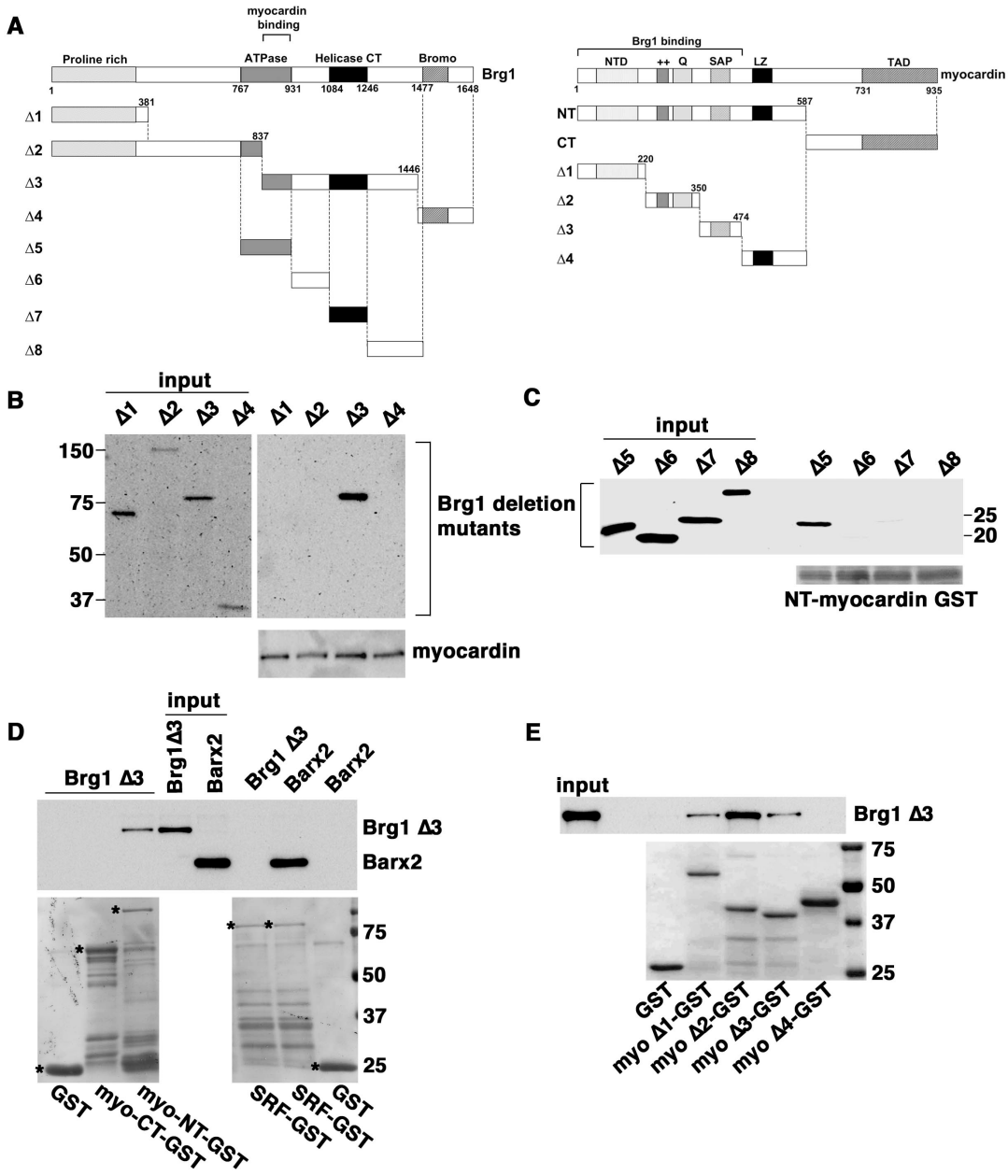


Figure 19. Brg1 ATPase domain binds to the amino-terminus of myocardin. **A.** Schematic illustration of the domain structures of Brg1 and myocardin indicating the location of the truncation mutants used for mapping studies. NTD: N-terminal domain, ++: basic domain, Q: poly Q domain, LZ: leuzine zipper domain, TAD: transcriptional activation domain. **B.** Myocardin and T7-tagged Brg1 truncation

mutants were transcribed and translated *in vitro*, the expressed proteins were then incubated and immunoprecipitated with myocardin antibody. The precipitated proteins were detected by western blotting, using an anti-T7 antibody. **C.** A GST-fusion protein comprising the N-terminus of myocardin was conjugated to GSH beads and incubated with T7-tagged Brg1 fragments expressed in bacteria. Bound proteins were identified by western blotting using a T7 antibody. **D.** GST-fused to NT- or CT-myocardin, SRF or GST alone were expressed in bacteria, conjugated to GSH beads and incubated with bacterial expressed T7-tagged Brg1Δ3 or Barx2 as indicated. A western blot of the GST-bound proteins is shown in the upper panel, the lower panels show the expression of the GST-fusion proteins (marked by an asterisks to the left of each protein). **E.** Myocardin-GST fusion proteins or GST alone was conjugated to GSH beads and incubated with *in vitro* translated T7-tagged Brg1Δ3. GST pull-down assay was performed as described above.

Chapter IV: The role of Brg1/Brm in smooth muscle development *in vivo*

Abstract

We have shown that Brahma-related gene 1 (Brg1) or Brahma (Brm), the ATP dependent chromatin remodeling enzymes in SWI/SNF complexes, are required for MRTFs myogenic induction function and for primary smooth muscle cells to maintain their high levels of smooth muscle contractile protein expression. However, our previous studies have only used cultured cells to explore the myogenic functions of Brg1 and Brm. The role of Brg1/Brm in smooth muscle development *in vivo* is still unknown. We have obtained or generated Brm global KO mice, smooth muscle-specific Brg1 KO mice and smooth muscle-specific Brg1KO/global Brm null (double KO) mice and analyzed the phenotypes of these mice. Results showed that both Brg1 SM-specific KO mice and Brm global KO mice had normal smooth muscle contractile protein expression, while Brg1/Brm double KO had significantly decreased levels of contractile proteins. These results suggest that Brg1 and Brm play redundant roles in regulating smooth muscle contractile protein expression during GI development *in vivo*. The other developmental defects in Brg1 KO, Brm KO and Brg1/Brm double KO mice will be discussed in the next chapter.

Introduction

As discussed in the last two chapters we have shown that SWI/SNF ATP-dependent chromatin remodeling complexes containing either Brahma-related gene 1 (Brg1) or Brahma (Brm) ATPase subunits are required to maintain SRF-dependent SM-specific contractile protein expression in primary colon, bladder and aorta SMCs. (147, 152). *In vitro,* in cultured SMC knockdown of Brg1 or Brm attenuated expression of smooth muscle-specific contractile proteins (152). This result suggests that either there are non-overlapping roles of Brg1 and Brm or that the combined total level of expression of Brg1 and Brm is required to maintain SRF-dependent SM-specific contractile proteins expression in SMCs. In support of this latter possibility, we also found that either Brg1 or Brm alone were sufficient to support the myogenic activity of two important SRF co-factors, MRTFA and myocardin. MRTFs cannot induce expression of SM-specific contractile proteins in SW13 cells that lack both Brg1 and Brm, while reintroducing either Brg1 or Brm alone into these cells restored the myogenic activity of MRTFs. This result suggests Brg1 and Brm have overlapping redundant roles in regulating the induction of SRF-dependent SM-specific contractile protein genes. Alternatively, it is possible that both Brg1 and Brm are required to maintain the differentiation status of SMCs, while either over-expressed Brg1 or Brm alone is sufficient to support the myogenic activity of over-expressed MRTFs in none muscle cells. These findings highlight the need to determine the role of Brg1 and Brm *in vivo* under physiological conditions of SM differentiation and development.

Brg1 and Brm are ubiquitously expressed in almost all tissues, including SM (data not shown). Brg1 KO mice die during the periimplantation stage before SM differentiation (15), thus the function of Brg1 in SM development cannot be determined from the global Brg1 KO mice. On the other hand Brm knockout mice develop normally, except that adult mice have higher body weight as compared to wild type littermates (112). However, no reports have examined the phenotype of smooth muscle tissues in the Brm null mice, although the phenotypes of these global knockout mice suggest that Brg1 and Brm have at least some specific non-redundant roles during mammalian development.

To fill the void in our knowledge of the role of Brg1 and Brm in SM development, and to specifically investigate the possible redundant role of these two molecules in supporting smooth muscle differentiation, we have obtained or generated Brm KO mice, smooth muscle-specific Brg1 KO mice and smooth muscle-specific Brg1 KO/global Brm null mice and analyzed the phenotypes of these mice. From this analysis we found that both Brg1 SM-specific KO mice and Brm global KO mice had normal smooth muscle contractile protein expression, while Brg1/Brm double KO mice showed severe gut defects associated with decreased contractile protein expression. These results suggest that Brg1 and Brm play redundant role in regulating smooth muscle contractile protein expression during GI development *in vivo*. They also demonstrate that the SWI/SNF complex plays a critical role in GI smooth muscle development.

Methods and Materials

Generation of smooth muscle specific Brg1 knockout mice. $Brg1^{flox/flox}$ mice (obtained from Dr. C-P Chang at Stanford), were bred with smMHCcre/eGFP mice (from Dr. Kotlikoff at Cornell) (139) in order to generate a smooth muscle-specific knockout of Brg1 mice. In smMHCCre/eGFP mice, cre recombinase is expressed under the control of the smooth muscle-specific smMHC promoter (139). $Brg1^{flox}$ mice were used on a mixed Sv129/C57B6 background. The male heterozygous $Brg1^{flox/+}$:smMHCCre/$eGFP^{-/+}$ mice were bred with female $Brg1^{flox/flox}$ mice to generate a smooth muscle-specific Brg1 knockout mice ($Brg1^{flox/-}$: smMHC-$Cre^{-/+}$)(Figure 1a). Because of a transient expression of Cre in the sperm of the $Brg1^{flox/+}$: smMHCCre-/+ mice(41) the floxed allele transmitted from these mice is recombined resulting in a global heterozygous null allele of Brg1 in all tissues. There are thus four possible genotypes of the offspring: $Brg1^{flox/-}$:smMHC-$Cre^{-/+}$ (SM-specific Brg1 KO with global heterozygous Brg1 background, in this paper, we use "Brg1 KO'' to refer to this genotype), $Brg1^{flox/-}$: smMHC-$Cre^{-/-}$ (global Brg1 heterozygous, which we use as a control for the KO mice), $Brg1^{flox/+}$: smMHC-$Cre^{-/+}$ (SM-specific Brg1 heterozygous) and $Brg1^{flox/+}$: smMHC-$Cre^{-/-}$ (wild type).

Generating Brg1/Brm double knockout mice.

Brg1/Brm double knockout mice were generated by crossing the $Brg1^{f/f}$ mice and smMHC-cre mice with Brm null mice as indicated in Figure 5A. Brm null mice

originally generated by Dr. M. Yaniv's group (112) have been obtained from Dr. Scott Bultman.

Genotyping

Mouse digits were cut and digested in 10 μl lysis buffer (25 mM NaOH and 0.2 mM EDTA) 95°C for 30 min. 100 μl of neutralization buffer (40 mM TrisHCl) was added and the resulting samples, containing genome DNA, were analyzed by PCR (95°C 2 min, 95°C 1 min, 58°C 30 sec and 73°C 1.5 min for 40 cycles, 73°C 10 min). Primers used were as indicated in table 4.

β-Galactosidase staining: β-Gal staining was performed as reported previously (129). Briefly, tissues were carefully dissected out and wash in cold phosphate-buffered saline (PBS). Then tissues were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS for 1 h on ice followed by washing with cold PBS. After washing, tissues were stained overnight with gentle rocking in X-Gal stain mix (0.5 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.2% Nonidet-P 40, 0.2% Tween 20, and 0.2% Triton X-100 in PBS). Next day, tissues were washed in PBS and fixed in 4.0% paraformaldehyde overnight. The fixed tissues were cleared with methyl-salacilate and photographed (Pixelink camera) under a dissecting microscope.

Immunofluorescence staining. Immunofluorescence staining was performed essentially as described previously (129). Tissues were dissected and incubated

in 20% sucrose overnight. After cryoprotection, tissues were frozen in embedding medium (O.C.T., Optimal Cutting Temperature, from Tissue-Tek) and 6-8 μm sections were obtained and stored at -80°C until use. For immunofluorescent analysis of protein expression sections were fixed in 3.7% formaldehyde and stained with primary antibodies: Brg1 (Santa Cruz, 1:100), SM1 and SM2 (61), telokin (50), cleaved caspase3 (Cell Signaling, 1:50), Ki67 (Dako, M7249, 1:25). Sm α-actin conjugated with FITC (Sigma, 1:500). Primary antibodies were detected using fluorescein-conjugated anti-rabbit, anti-rat or anti-mouse IgG.

Results

To verify that the smMHC Cre transgene mediates appropriate smooth muscle-specific Cre recombinase activity we crossed the smMHC-Cre mice with ROSA26^{floxstopflox}LAC (a Cre-dependent LAC reporter strain) mice. Analysis of β-galactosidase activity in these mice shows heterozygous Cre expression is sufficient to efficiently mediate recombination of the ROSA26 reporter allele (Figure 20B) in SM tissues. Importantly this recombination was also restricted to smooth muscle tissues with the exception of some expression in the cardiac atria. To confirm that the transgene also mediates SM-specific recombination of the Brg1 locus, we extracted genomic DNA from SM-specific Brg1 heterozygous mice (Brg1$^{flox/+}$: smMHC-Cre$^{-/+}$) and detected the recombined allele by PCR. Primers (112) that only amplify the recombined Brg1 locus were used to perform real time PCR. Results showed that Brg1 locus was only recombined in SM containing tissues (bladder, colon and ileum), but not in non-SM tissues (colon epithelium, skeletal muscle, heart, liver, kidney or lung, Figure 20C). To verify that the recombined allele also resulted in loss of Brg1 protein we analyzed expression of Brg1 protein in colon SMCs from Brg1 KO and littermate global heterozygous mice using immunohistochemistry. In the control global Brg1 heterozygous animals (Brg1$^{flox/+}$:smMHC-cre$^{-/+}$) there was abundant nuclear staining of Brg1 in colon SM cells, while in Brg1 KO mice (Brg1$^{flox/-}$:smMHC-cre$^{-/+}$) little or no Brg1 staining was detected in SMC (Figure 20D).

The expression of contractile proteins is not changed in Brg1 KO mice. We utilized immunofluorescent staining of tissue sections, real time RT-PCR analysis of mRNA expression and western blotting to evaluate the expression of smooth muscle-specific contractile proteins in Brg1 KO mice. Surprisingly, none of these approaches revealed any significant changes in the expression of smooth muscle-specific contractile proteins such as smooth muscle myosin, smooth muscle α-actin, calponin, SM22α, telokin or 130kDa MLCK (Figure 21A, B, C).

The expression of contractile proteins is not changed in Brm null mice. It has been reported that Brm null mice are essentially normal except for an increase in body and organ size (112). However, no published studies have examined SM-specific gene expression in $Brm^{-/-}$ mice. We thus used similar approaches to those described above to examine expression of smooth muscle-specific genes in these mice. From this analysis we did not find any significant changes in expression of contractile proteins, in colon and bladder from Brm null mice compared with wild type control littermates (Figure 22A, B, C). In contrast to previous reports (112), we also did not observe any compensatory changes in expression of Brg1 in the Brm KO mice (Figure 22A, B).

Contractile protein expression is decreased in Brg1/Brm double KO mice. As some of our *in vitro* data suggested that Brg1 and Brm might have redundant roles in regulating expression of smooth muscle contractile proteins. Brg1/Brm double KO mice ($Brg1^{flox/-}$:smMHC-$Cre^{-/+}$: $Brm^{-/-}$) (smooth muscle-specific Brg1

KO/global Brg1 het/ global Brm null mice) were generated and analyzed (Figure 23). Global Brg1 het/Brm null ($Brg1^{flox/-}$:smMHC-$Cre^{-/-}$: $Brm^{-/-}$) mice were used as controls for these experiments as these represent mice that only differ from the KO animals by the presence of Brg1 in their smooth muscle cells. Decreased expression of endogenous contractile proteins, including telokin, 130 kDa MLCK, SM22α, calponin, sm α-actin were found by western blotting, and immunofluorescence staining of the Brg1/Brm double KO mice as compared to the control mice (Figure 24A, B). These results were also confirmed by qRT-PCR analysis of mRNA expression (Figure 24C). Although we did not observe any decrease in the expression of the SM2 isoform of smMHC by western blot or immunofluorescence staining, we did observe a decrease in total smMHC mRNA expression.

Discussion

In this study, we found that Brg1 SM-specific knockout and Brm global knockout mice had normal SM-specific contractile proteins expression levels, whereas Brg1/Brm double knockout mice have attenuated contractile protein expression suggesting that Brg1 and Brm have overlapping or redundant functions in regulating SM differentiation *in vivo.*

The findings of the current study are consistent with our *in vitro* studies in which reintroducing either Brg1 or Brm alone restored the myogenic functions of SRF cofactors MRTFs in SW13 cells (Figures 7,16). Both these sets of data support the hypothesis that Brg1 and Brm have redundant roles in regulating the induction of SM contractile proteins. However, these results are not entirely consistent with experiments in which we found that depletion of either one of the two ATPases, Brg1 or Brm from primary cultures of SMCs, was sufficient to decrease SM marker expression (Figure 13). The apparent discrepancy between these data could be explained if there is a threshold level of SWI/SNF complexes that is required to support smooth muscle differentiation. *In vivo* it may be necessary to delete all four Brg1/Brm alleles in order for SWI/SNF activity to fall below this threshold. In contrast, *in vitro* it may be sufficient to extinguish expression of either Brg1 or Brm in order to fall below the critical threshold of SWI/SNF activity. Although we can only speculate on the reasons why SMCs would require different levels of SWI/SNF activity *in vitro* and *in vivo* it is quite

possible this reflects differences in the cell's environment and hormonal mileu or differences in the differentiation status of the cells.

The specific functions of Brg1 and Brm and whether these two molecules can compensate for each other remains controversial. For example reports have shown Brg1 and Brm to be both pro-proliferative or anti-proliferative. Previous studies showed that the proliferation, but not RA-induced differentiation, is inhibited in Brg1 heterozygous mutant F9 EC cells (126). The proliferation of Brm-/- embryonic fibroblasts is increased as a result of deficiency in cell contact induced G0/G1 arrest (112). In contrast, neither Brm nor Brg1 are essential for the proliferation and early differentiation of keratinocytes (71). The Reisman group has reported that lung specific knockout Brg1 mice had increased cleaved caspase-3, a cellular apoptotic marker in lung cells; carcinogen induced adenomas in Brg1 null lung tissue had increased proliferation marker Ki67 and Proliferating Cell Nuclear Antigen (PCNA). These data suggest that Brg1 and Brm have cell specific functions and Brm can compensate for some of Brg1's function in certain types of cells but not others. We will discuss more about the redundancy and specificity of these two molecules in next chapter.

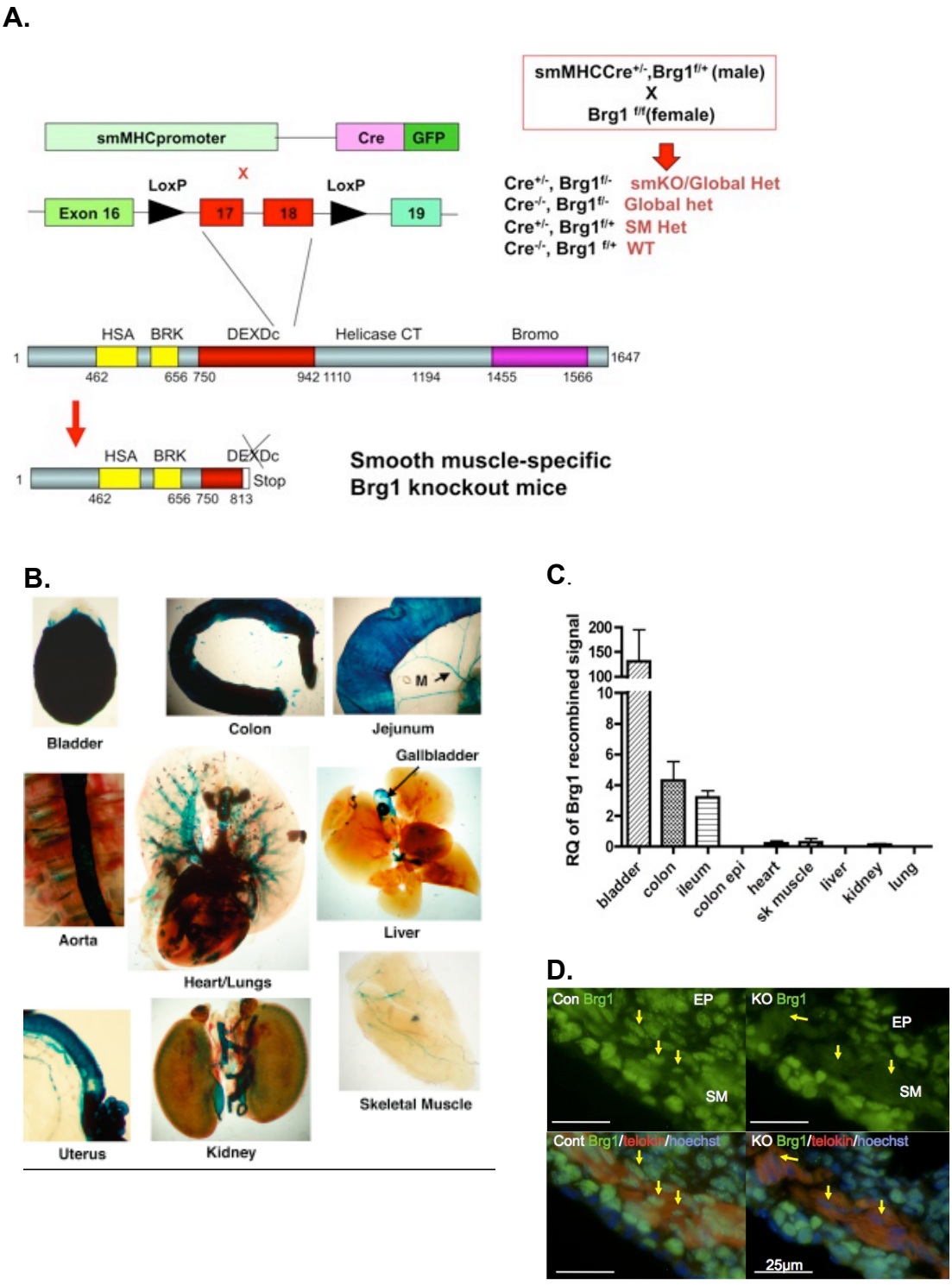
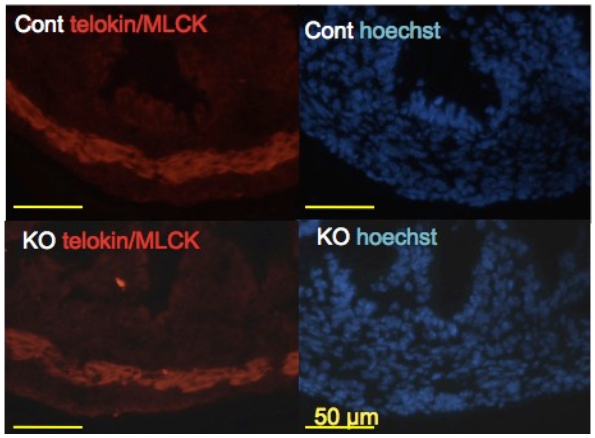


Figure 20. Generation of smooth muscle-specific Brg1 knockout mice. A. Brg1$^{flox/flox}$: smMHC-Cre$^{-/+}$ transgenic mice were crossed with Brg1$^{flox/flox}$: smMHC-

$Cre^{-/-}$ mice to generate SM-specific Brg1 knockout mice ($Brg1^{flox/-}$: smMHC-$Cre^{-/+}$). Global heterozygous Brg1 ($Brg1^{flox/-}$: smMHC-$Cre^{-/-}$) mice are used as control. **B.** β-galactosidase activity is smooth muscle-specific in smMHCcre ROSA26floxstopfloxLAC mice. **C.** Smooth muscle-specific recombination of the Brg1 locus. Genomic DNA was extracted from tissues of $Brg1^{flox/+}$: smMHC-$Cre^{-/+}$ (SM specific Brg1 heterozygous). Real time PCR was performing using primers that only amplify the recombined Brg1 locus. Levels of the recombined Brg1 locus were compared to a control single copy gene (telokin) within the same sample. Data shown are relative quantity of recombined Brg1 locus after normalization to telokin loading control. **D.** Immunofluorescent analysis of protein expression. Anti-Brg1-Fitc (green), MLCK/telokin-Rhodamine (red) and nuclear hoechst immunofluorescence staining of colon cryosections from 2-day-old Brg1 KO ($Brg1^{flox/-}$:smMHC-$Cre^{-/+}$) and littermate control ($Brg1^{flox/-}$:smMHC-$Cre^{-/-}$) mice.

A



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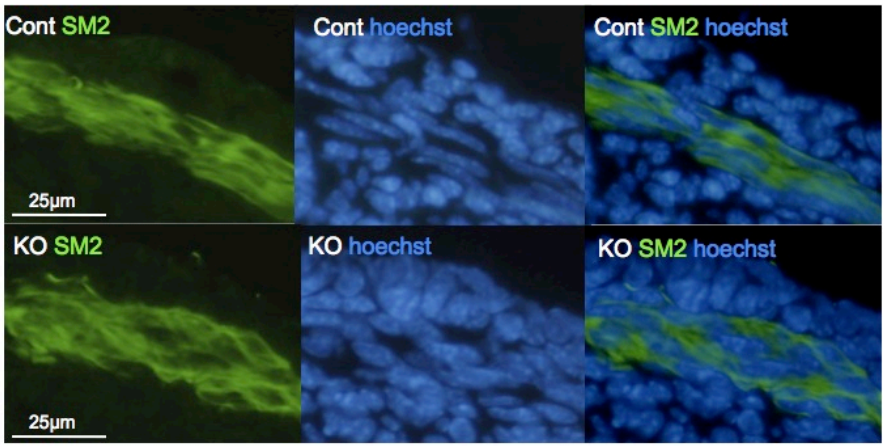


C.

Figure 21. Contractile proteins are not decreased in Brg1 KO mice. A, B. Colons from 4-day-old Brg1KO and control mice were dissected and 8 μM

cryosections were collected. MLCK/telokin-Rhodamine (red, **A**), SM2 (smMHC isoform2)-FITC (green, **B**), sm α-actin-FITC (green) and nuclear hoechst immunofluorescence staining were performed. The intensity of these SM markers in Brg1 KO colon was similar to control colon. **C.** Left panel**,** Epithelium layers were carefully peeled off colon, ileum and jejune from 3-week-old KO and littermate heterozygous control mice. The leftover smooth muscle layers were analyzed by western blot**.** Right panel, proteins extracted from hearts and bladders were also examined by western blot. “K”, Brg1KO; “C”, control.

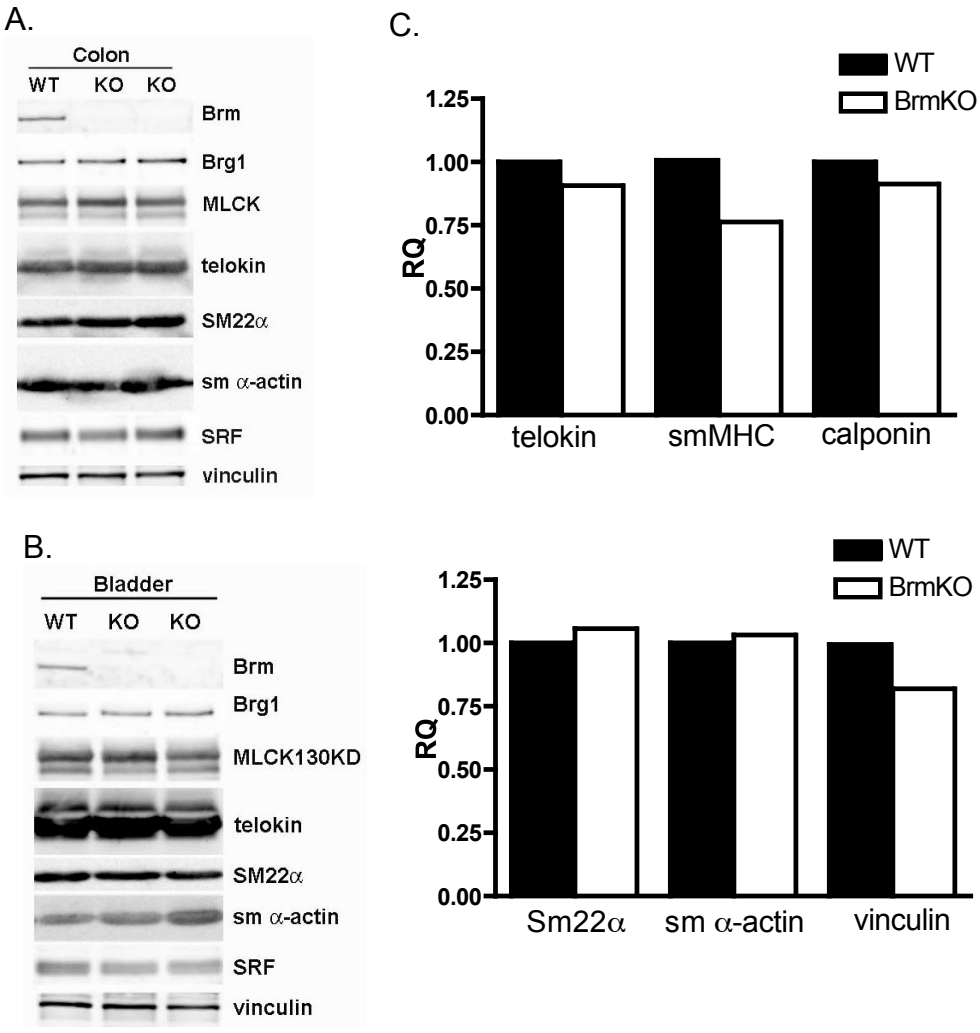


Figure 22. Contractile proteins are not decreased in Brm KO mice. A, B. Smooth muscle layer were dissected from colons and bladders of 3-week-old Brm null and WT control mice. Smooth muscle contractile proteins were examined by western blot analysis. There was no significant difference between Brm null and WT control mice. **C.** RNA was extracted from colons and bladders of newborn Brm null and control mice. Real time qRT-PCR was performed as described in materials and methods. No significant difference was found between KO and control samples.

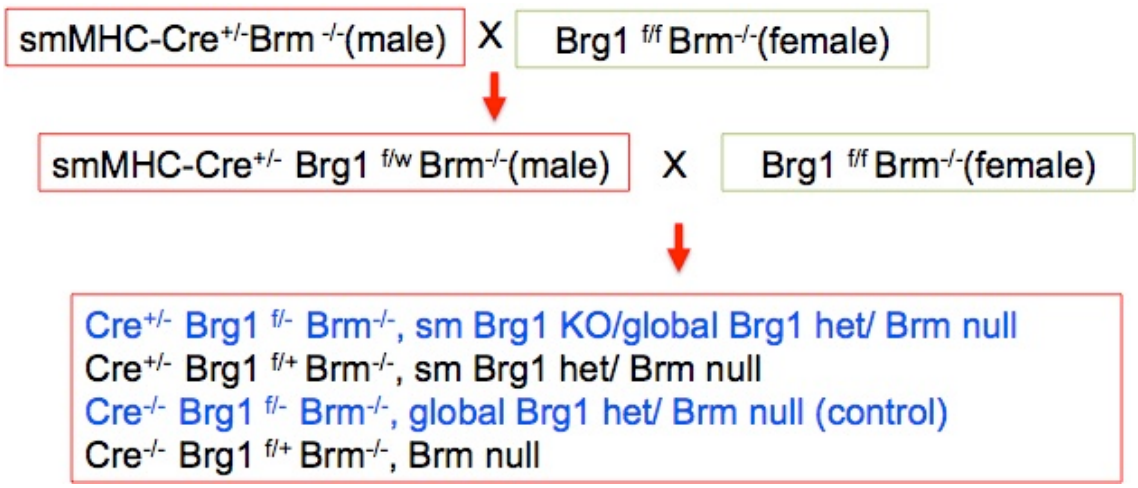


Figure 23. Generating smooth muscle-specific Brg1 KO on Brm null background. Male smMHC-Cre$^{+/-}$Brg1$^{f/w}$Brm$^{-/-}$ mice were crossed with female Brg1 $^{f/f}$ Brm$^{-/-}$ mice to generate Brg1/Brm double knockout (DKO, smMHC-Cre$^{+/-}$ Brg1$^{f/-}$Brm$^{-/-}$, SM-specific Brg1 KO, global Brg1 heterozygous on Brm null background). Littermate global Brg1 het/ Brm null mice (smMHC-Cre$^{-/-}$Brg1$^{flox/-}$ Brm$^{-/-}$) were used as controls.

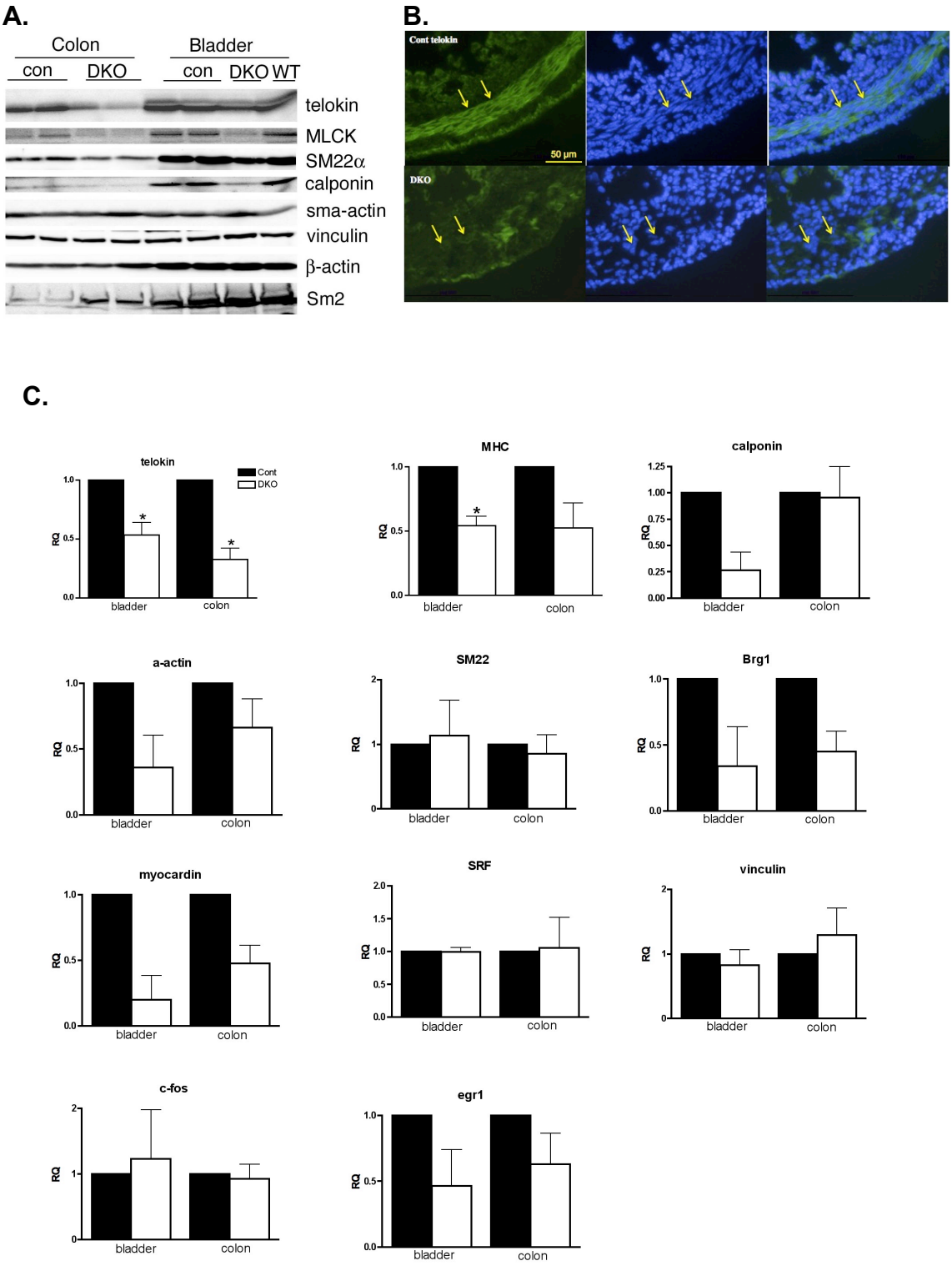


Figure 24. Contractile proteins are decreased in Brg1/Brm double KO mice.

A. Colon and bladder were dissected from 4-day-old DKO and control mice. Contractile proteins 130 kDa MLCK, telokin, calponin, SM22α were decreased in western blot analysis. Immunostaining in panel **B** also confirmed that telokin/MLCK (green) expression was down regulated in DKO colon. **C.** RNA was extracted from colon and bladder of newborn DKO mice and littermate control mice. qRT-PCR was performed.

Chapter V.

Understanding the GI phenotypes of smooth muscle-specific Brg1 KO and Brg1/Brm double KO mice.

Abstract

In the last chapter, we have shown the expression of contractile proteins was decreased in Brg1/Brm double knockout mice, but not in Brg1 smooth muscle specific or Brm global knockout mice. However, these results do not rule out that Brg1 or Brm KO mice may have the other smooth muscle related phenotypes. In this chapter, we show some interesting phenotypes in these knockout mice. We found that the colonic contractility of SM-specific Brg1 knockout mice was significantly impaired, probably because of the disorganization of smooth muscle cells in the circular SM layer. The colonic contractility was more severely affected in Brg1/Brm double KO mice, while it was normal in Brm KO mice. Brg1 KO and double KO mice had a shorter gut, which may be caused by decreased proliferation in SMCs after knocking out Brg1. This chapter further illustrates the important roles of Brg1 and Brm in smooth muscle development and provides more evidence that Brg1 can compensate for most, but not all, of the functions of Brm in tissue development *in vivo*.

Introduction

In Chapter IV, we showed that contractile protein expression did not change in SM-specific Brg1 KO mice or global Brm KO mice. However, SM-specific Brg1 KO in a Brm null background mice (double KO) resulted in decreased contractile protein expression in smooth muscle tissues. These results suggest that Brg1 and Brm have overlapping or redundant functions in regulating SM differentiation *in vivo*. The attenuated expression of contractile proteins in the double knockout mice also demonstrates that SWI/SNF activity is required for smooth muscle differentiation *in vivo*. It would be predicted that the attenuated contractile protein expression observed in the Brg1/Brm double knockout mice would result in impaired contractility and function of the GI tract. Brg1/Brm containing SWI/SNF chromatin remodeling complexes have also been reported to be involved in regulating many cellular functions, such as cell proliferation (126) and apoptosis (99) in addition to differentiation. In the current study we have thus examined the phenotype of smooth muscle-specific Brg1 KO, Brm KO, and Brg1/Brm double KO mice in order to fully characterize the role of SWI/SNF complexes in regulating development of the GI tract. The results of this study revealed that although Brg1 and Brm containing SWI/SNF complexes play redundant roles in regulating expression of contractile protein genes, Brg1 containing SWI/SNF complexes play an additional specific role in GI development perhaps through their regulation of smooth muscle cell proliferation.

Materials and methods

Contractile measurements of isolated colon rings. Colon was carefully dissected and immediately washed in cold PBS. Surrounding connective tissues and fat were gently removed and colon was cut into rings (1 cm axial length). T contractility of colon rings was measured as described previously for vascular rings (42, 76). Colon rings were mounted on "L-shaped" stainless steel supports and submerged in an 5ml organ bath with Krebs buffer saturated with 95% O_2 / 5% CO_2 and held at 37°C. The Krebs buffer contains 132mM NaCl, 25mM NaHCO3, 5mM KCl, 2.5mM CaCl2, 1.2mM NaH2PO4, 1.2mM MgCl2, 0.025mM EDTA, and 10mM glucose, pH7.4. 2.5g preload was empirically determined to result in the optimal length for most the rings tested. To assess smooth muscle function colon rings were contracted with 60 mMKCl or 0.1 μM carbachol.

Contractile measurements of isolated whole colon. Colon was removed from new born or 4 day old neonatal mice and washed in cold PBS. Colon basal spontaneous contractility and contractility stimulated by 60 mM KCl or 0.1 μM carbachol were measured using a video microscopy system as described previously (84). Briefly, 1 cm lengths of colon was mounted to two canulas which were fixed on the edge of an organ bath containing 10 ml 37°C Krebs buffer. One canula was connected to pressure regulator through which, 5mmHg pressure was applied to the inside of the colon. The diameter changes of the canulated colon were observed under a video microscope and quantitated using dimensional analysis software (DIAMTRAK 3+, Australia).

Immunofluorescence staining. Immunofluorescence staining was performed as described in Chapter IV.

Haemotoxylin-eosin staining. Cryosections were air dried in laminar flow hood at room temperature for 10 minutes. Then sections were fixed in MeOH: Acetone (50:50 dilution) for 30 seconds. Sections were stained in Haemotoxylin for 1 min, followed by 3 minutes rinsing in running tap water. Sections were then fixed in acid alcohol (70% EtOH/1% HCl) for 1.5-2 minutes. After rinsed in running tap water. Sections were stained in Eosin for 2 min. Sections were then destained and dehydrated through an ascending series of alcohols (75%, 80%, 90% and 100% EtOH for 2 minutes each). Finally sections were cleared in Xylene (Fisher) 1-2 min and cover-slipped using Permount (Fisher).

Electron microscopy: Colons were dissected and fixed in 2% Paraformaldehye-2% Glutaraldehyde in 0.1M Phosphate Buffer. Then the fixed samples were processed for transmission electron microscopy by the IU SOM electron microscopy facility (http://www.anatomy.iupui.edu/emcenter/index.html). 70-90nm sections were cut and placed on copper grids. Then the cut sections were stained with heavy metals, uranyl acetate and lead citrate for contrast. Sections were observed on electron microscope Tecnai BioTwin (FEI, Hillsboro, OR). Digital pictures were taken using an AMT CCD camera.

Smooth muscle cell counting. SM2 (smMHC isoform2) or sm α-actin-Fitc and nuclear hoechst immunofluorescence staining were performed on colon cryosections from Brg1 KO, DKO and control mice. Nuclei of SM2 or sm α-actin positive cells in the circular smooth muscle layer were counted and normalized to the area of circular SM layer, which was measured using the Imagetool software.

ECM supperarray. Colon of 3-month-old Brg1 KO and littermate control mice was dissected and the epithelium was carefully peeled off. RNA of was extracted with Trizol reagent (Invitrogen). Proteinase K and DNase treatments were performed to purify RNA. 0.5 μg RNA was used as template to make cDNA using RT^2 First Strand Kit (SABiosciences). cDNA in RT^2 SYBR Green/ROX qPCR Master Mix (SA Biosciences) was loaded to 96-well-plate with 84 preloaded ECM primers (RT^2 Profiler™ PCR Array Mouse Extracellular Matrix and Adhesion Molecules, SABioscience, PAMM-013A). 4-fold-difference between Brg1 KO and control colon was set as the threshold to determine significant changes.

Quantitative RT-PCR. Quantitative RT-PCR was performed as described in Chapter II.

Results

Neonatal lethality in Brg1/Brm double knockout mice. In contrast to either the Brg1 or Brm single knockout mice the Brg1/Brm double knockout mice exhibited neonatal lethality with no double knockout mice surviving more than 10 days. All of the double knockout mice analyzed exhibited intestinal defects such as enlarged stomach and/or enlarged small and large intestine (Figure 25E). These defects were specific to the double knockout mice as the global heterozygous Brg1; Brm null ($Brg1^{flox/-}$smMHC$cre^{-/-}$:$Brm^{-/-}$) mice did not display these abnormalities and survived beyond this neonatal period (data not shown). Although contractile protein expression in Brg1 KO mice was not significantly decreased as compared to control mice, some Brg1 KO mice accumulated gas in their intestines and eventually developed mega-colon (Figure 25B).

Colon from Brg1 KO and Brg1/Brm DKO but not Brm KO mice exhibited impaired contractility. The pathological phenotypes of Brg1 KO led us to measure the contractility of colon from smooth muscle-specific Brg1 KO mice ($Brg1^{flox/-}$smMHC-$Cre^{-/+}$). Isolated colonic rings from Brg1 KO mice stimulated to contract with either 60mM KCl or 0.1μm carbachol exhibited a marked impairment of force production as compared to rings obtained from littermate control mice ($Brg1^{flox/-}$smMHC-$Cre^{-/-}$) (Figure 25A).

Because smooth muscle-specific Brg1/Brm double knockout mice die around day 7 after birth at which time their colon is very small we were not able to measure

the contractility of colonic rings in the same manner as described above for the Brg1 KO mice. As an alternative approach we measured the contractility of canulated colonic segments using videomicroscopy. This analysis revealed that the normal basal spontaneous contractile activity of control colon (mainly in the proximal end) was completely absent in colon from Brg1/Brm double KO mice. To further quantitate these changes, the diameter of the colons from control ($Brg1^{flox/-}Brm^{-/-}$smMHC-$Cre^{-/-}$) and double KO ($Brg1^{flox/-}Brm^{-/-}$smMHC-$Cre^{+/-}$) mice were recorded over time and a representative plot is shown in Figure 25C. The increases and decreases of diameter of the colon correlate to the relaxation and contraction of circular SM layer, respectively. In colon from control mice, diameters spontaneously changed from 2100 μm to 1800 μm, while the diameter of colon from double KO mice remained constant at 2400 μm (Figure 25C). The larger resting diameter of the colon from double KO (2400 μm as compared to 2100 μm) is indicative of a dilated colon. The lack of *in vitro* motility within the colon is consistent with the *in vivo* phenotype of the double KO mice which all die 4 to 7 days after birth exhibiting dilated colon, stomach, small intestine and bladder (Figure 25D), indicative of impaired GI motility. Using the same method, we observed no differences in the contractility of colon from Brm KO mice as compared to colons from littermate Brm heterozygous and wild type control mice (data not show).

SMCs in the colon of Brg1 KO and Brg1/Brm double KO mice have altered alignment. Smooth muscle myosin heavy chain SM2 immunofluorescent

staining, and HE staining of colon from new born and 3-month-old Brg1 KO ($Brg1^{flox/-}$smMHC-$Cre^{-/+}$) and littermate control ($Brg1^{flox/-}$smMHC-$Cre^{-/-)}$ revealed a disorganization of the circular smooth muscle layer. The smooth muscle cells within the circular smooth muscle layer of the colon in Brg1 KO mice were not aligned as evenly as those of control mice (Figures 21A, B; 26A, B). The SMCs in KO colon appear to have lost the typical spindle shape of normal SMCs (Figure 21A,B; 26A,B). Similarly, the SMCs in the circular smooth muscle layer of colon from Brg1/Brm double KO mice were also disorganized (Figures 24B, 26E), whereas SMC in the colon of Brm KO mice appeared normal (Figure 26D). The disorganization of the smooth muscle layer observed in Brg1 KO and double KO mice maybe one of the causes of the decreased contractility of the colonic tissue.

To more closely examine the details of this SMC disorganization, we used electron microscopy. This analysis revealed that there was more space between circular SMCs in colon from Brg1 KO mice (Figure 26C). Furthermore, the borders of the circular smooth muscle cells were much more ruffled (or invaginated) in colon from KO mice as compared to controls (Figure 26C).

ECM proteins are not changed in Brg1 KO or DKO SM tissues. Since our initial analysis revealed that the shape of smooth muscle cells from Brg1 KO mice is altered we examined the possibility that the cells have aberrant expression of extracellular matrix (ECM) and adhesion molecules. For this

analysis an ECM qRT-PCR array was screened (Superarray, SABiosciences) using RNA extracted from colon of 3-week-old Brg1 KO and littermate control mice. Results showed that several genes were up-regulated (Adamts1, Emilin1, Icam1), while Sparc was down regulated (Figure 27A). However, we were not able to confirm these changes using qRT-PCR of multiple independent samples (Figure 27B). In this latter analysis we also examined genes which have been previously shown to be Brg1 target genes in other cell types, Bmp4, Col1a1, Col4a1, Col5a1, Gli1, Gli2, Itgam (48) (77), but no significant changes in expression of these genes were observed in colon from Brg1 KO mice (Figure 27C).

Gut is shorter in Brg1 KO and DKO mice. Although we did not observe any changes in the density of SMC in sections from the colon of Brg1 KO mice both the colon and small intestine were significantly shorter in these mice (Figure 28A). Body weight, body length (from nose to anus) and tibial length were also measured but not found to be any different between Brg1 KO, Brm KO, Brg1/Brm double KO and littermate control mice (Figure 28 B and data not shown). When colon and small intestine length were normalized to body weight, body length or tibial length, results showed that Brg1 KO and Brg1/Brm double KO colon and small intestine were significantly shorter than littermate controls (Figure 28B,D). However, neither colon nor small intestine length was changed in Brm KO mice compared with WT or heterozygous littermate controls (Figure 28B).

Shorter gut in Brg1 KO and DKO is probably caused by decreased proliferation but not by increased apoptosis. It has been reported that Brg1 regulates cell apoptosis and proliferation (99, 126, 136). We hypothesized that knocking out Brg1 in SMCs could increases apoptosis and/or decrease proliferation of SMCs resulting in a shorter gut. To evaluate apoptosis we performed immunofluorescent staining of cleaved caspase-3 on sections of colon obtained from newborn and 3-month-old Brg1 mice (Figure 29A) or colon from E19.5 and newborn Brg1/Brm double KO mice (Figure 29B). Although the epithelium layer had several cleaved caspase-3 positive cells in colon from both 3-month-old Brg1 KO and control mice, as expected (68), SMCs had no or rare cleaved caspase-3 positive staining in sections from all the groups of mice we examined with no differences detectable between Brg1 KO, Brg1/Brm double KO and control mice (Figure 29A, B). Similarly, phospho-H2AX staining was rarely detectable in SMC and not significantly different in colonic sections from Brg1 KO, Brg1/Brm double KO and control mice (data not shown).

To examine possible changes in SMC proliferation we performed immunofluorescent staining for Ki67. Ki67 is a commonly used cellular marker for proliferation (103). In the colon from 3-month-old Brg1 KO and control mice, Ki67 positive cells were found in the crypt epithelium above the submucosa layer, but not in SMCs as reported previously (103) (Figure 30A). However, in the colon from Brg1/Brm double KO mice at E19.5 and new born, Ki67 positive staining were found in both epithelium and SM layer (Figure 30B). Interestingly, we found

there were significant less Ki67 positive SMCs in the colonic circular SM layer from newborn double KO mice compared to littermate control mice, while current data showed there was no difference in E19.5 mice (Figure 30B, C). This is suggesting that the slower proliferation of colonic circular SMCs from DKO mice starts after birth, which is consistent with the observed decrease in colon length from after born DKO mice, but not apparent in embryonic DKO mice(data now shown). A similar analysis is currently in progress in tissues from Brg1 single KO mice. Furthermore, we will also investigate other markers of proliferation such as PCNA and a BrDU labeling assay to confirm these results.

Discussion

In this study, we found that both smooth muscle-specific Brg1 knockout mice and smooth muscle-specific Brg1 knockout mice on a Brm null background exhibit impaired GI contractility. This phenotype is more severe in the double knockout mice that exhibit neonatal lethality. This is consistent with the attenuated contractile protein expression seen in the GI tract of double but not single knockout mice. Together our data suggest that Brg1 and Brm-containing SWI/SNF complexes play redundant roles in regulating expression of smooth muscle contractile proteins during GI development. Whereas the pathological phenotype of the smooth muscle-specific Brg1 KO mice in the presence of normal levels of Brm suggests Brg1-containing SWI/SNF complexes play additional roles in GI development that cannot be performed by Brm-containing SWI/SNF complexes.

As SMCs are disorganized in the colon of Brg1 KO and Brg1/Brm double KO mice, but not Brm KO mice, this would suggest that this disorganization is a result of the specific loss of Brg1-containing SWI/SNF complexes. We have thus far been unable to identify the specific Brg1 target genes that result in this phenotype. Our initial results have ruled out a number of ECM and signaling proteins such as BMP4, Gli1, and Gli2. To better identify the Brg1-specific target genes we will need to perform a whole genome wide array analysis of smooth muscle cells isolated from Brg1 KO mice.

Our data showing a decreased intestinal length in smooth muscle-specific Brg1 knockout and Brg1/Brm double knockout but not Brm knockout mice suggest that this phenotype is likely a specific consequence of the loss of Brg1-containing SWI/SNF complexes. The decreased rate of SMC proliferation, in the absence of any changes in apoptosis, observed in the Brg1/Brm double KO mice could account for the shorter intestines in these mice. A similar analysis in the Brg1 KO mice is in progress to confirm that the defect in proliferation is a specific consequence of loss of Brg1. Loss of Brg1 could affect SMC proliferation in a number of ways. it has been reported that Brg1 affected cell cycle by regulating the expression of Cyclin A, E and p21 (59, 74). Also, Brg1 over expression in mesenchymal stem cells (MSCs) induced programmed cell death triggered by activated p53 pathway; however, this Brg1 induced apoptosis cannot be prevented by the anti-apoptotic regulator Rb.

Brg1 could also affect proliferation more indirectly through changes in nuclear structure. The loss of Brg1 may act to change gene expression through changing heterochromatin structure. Bourgo et al reported (13) that deletion of Brg1 in primary murine adult fibroblasts (MAFs) caused nuclear malformation. The normally discrete pericentometric heterochromatin domains were disorganized and dispersed; correspondingly, heterochromatin related modifications such as histone H3-trimethyl lysine 9 and H4-trimethyl lysine 20 were also dispersed, whereas the euchromatin related acetylation of histone H4 and histone H3 were unaffected. Interestingly, this heterochromatin disorganization was specific for

Brg1 depletion; Brm or SNF5 depletion had no such defects. Furthermore, Brg1 depleted MAFs also had remarkably increased micronuclei, while Brm deficient MAFs only had modestly increased micronuclei. Together these data suggest that Brg1, but not Brm, is the main factor that regulating heterochromatin organization. Chromatin disorganization can affect cell proliferation and genome stability (72) (83, 85), Consistently, Brg1 deficient MAFs showed decreased proliferation, which is likely due to aberrant mitotic cell division caused by disrupted chromatin structure. Further studies will be required to resolve which of these mechanisms result in the altered proliferation of GI smooth muscle cells.

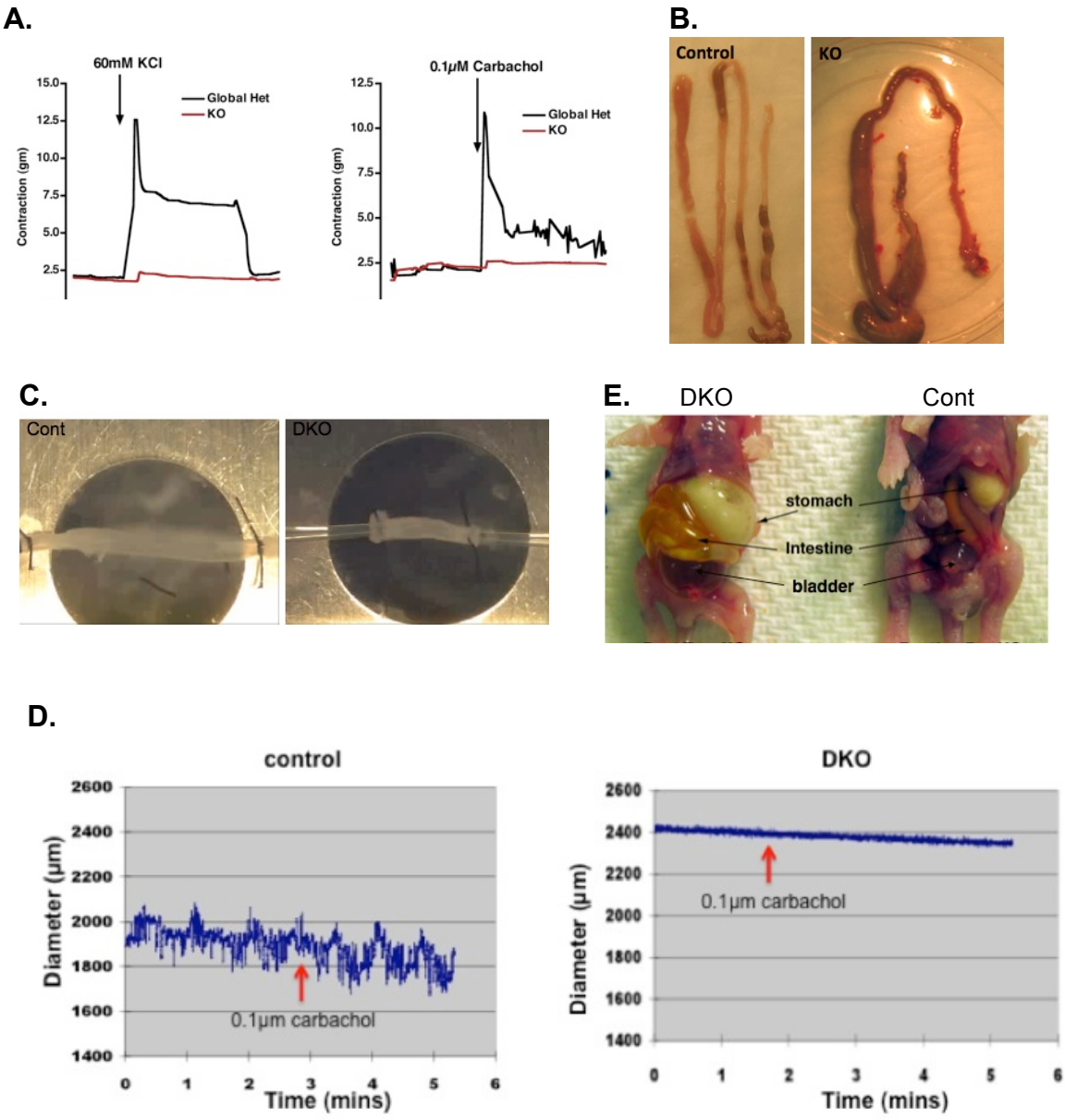


Figure 25. The contractility of the colon from Brg1 knockout mice is remarkably impaired. A. Left panel. Rings of colonic tissue were hung in a myograph and contractility measured in response to KCl induced membrane depolarization or the muscurinic agonist carbachol. The contractility of the colon from knockout mice is impaired. **B.** Some Brg1 KO mice developed megacolon in

proximal end and enlarged ileum. **C, D.** The contractility of colon was impaired in Brg1/Brm DKO mice**.** "**C**" is a video, showing that the spontaneous contraction of proximal end of colon from new born DKO mice was decreased; "**D**" is recording the spontaneous diameter changes in the video "**C**" and after stimulation by 0.1μm carbachol**. E.** Stomach, intestine and bladder were dilated in DKO mice.

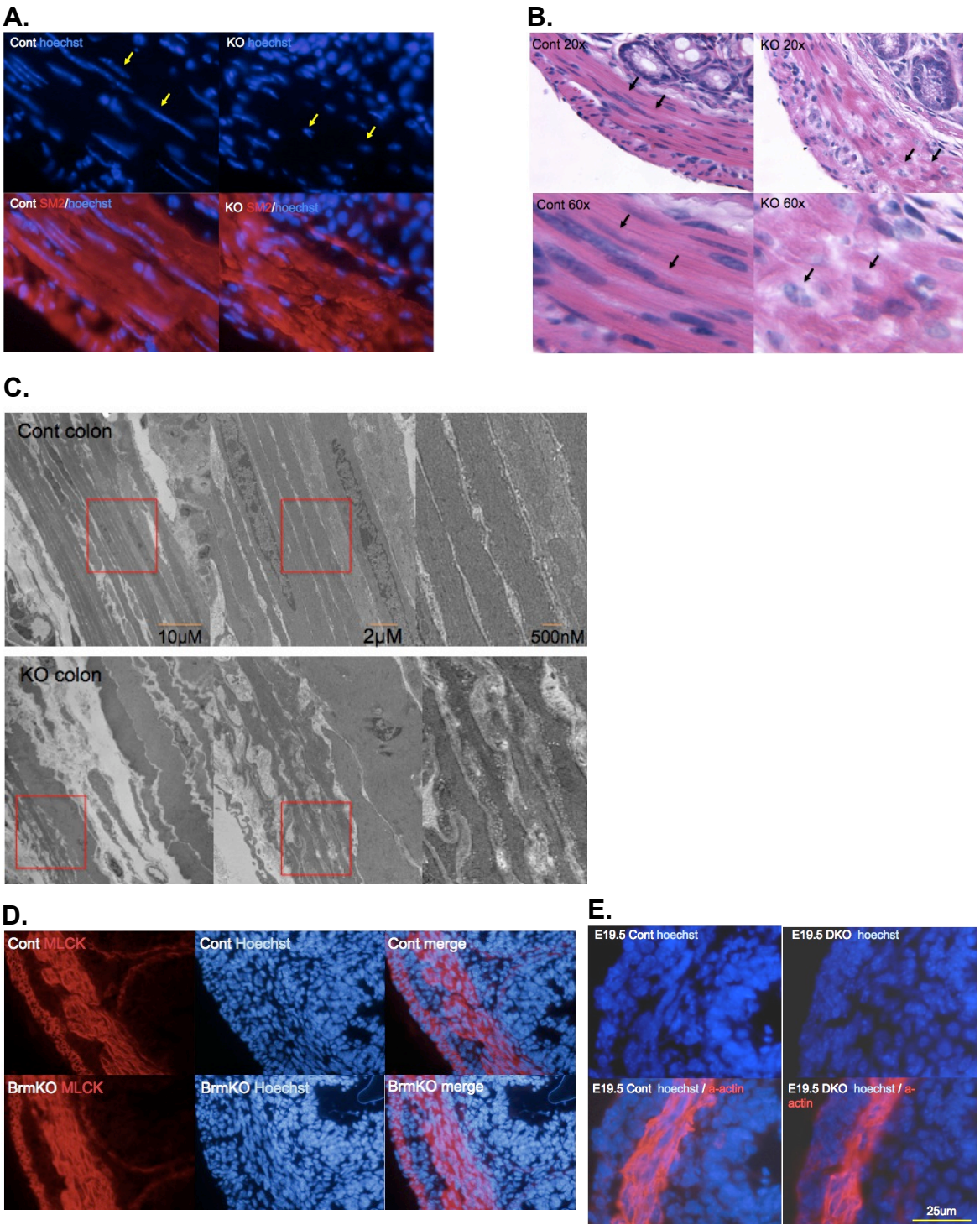


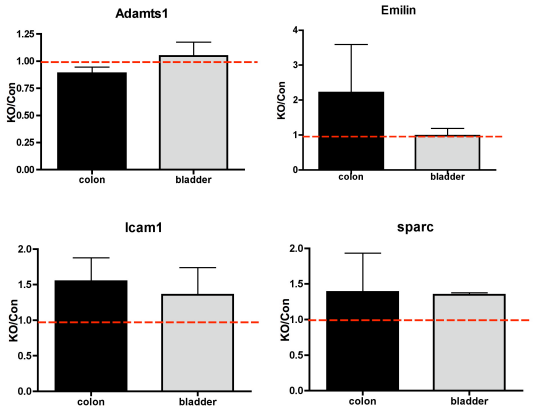
Figure 26. SMCs in the colon of Brg1 KO and Brg1/Brm double KO mice have altered alignment. A. SM2 immunofluorescent staining (red) and Hoechst nuclear staining (blue) of colon from 3-month-old Brg1 KO mice. **B.** HE staining

of colon from 3-month-old Brg1 KO mice. **C.** Ultra-structure of colon from 3-month-old Brg1 KO mice and littermate control using Electron Microscopy. **D.** MLCK immunofluorescent staining (red) and hoechst nuclear staining (blue) of colon from newborn Brm KO mice and WT littermates. **E.** sm α-actin immunofluorescent staining (red) and Hoechst nuclear staining (blue) of colon from E19.5 DKO mice.

A.

Gene Symbol	Fold Regulation	Ct KO/Cont
Adamts1	16.4111	29.8 / 34.0
Emilin1	987.4747	24.1 / 34.2
Icam1	7.3697	29.2 / 32.2
Sparc	-6.2784	27.6 / 25.1

B.



C.

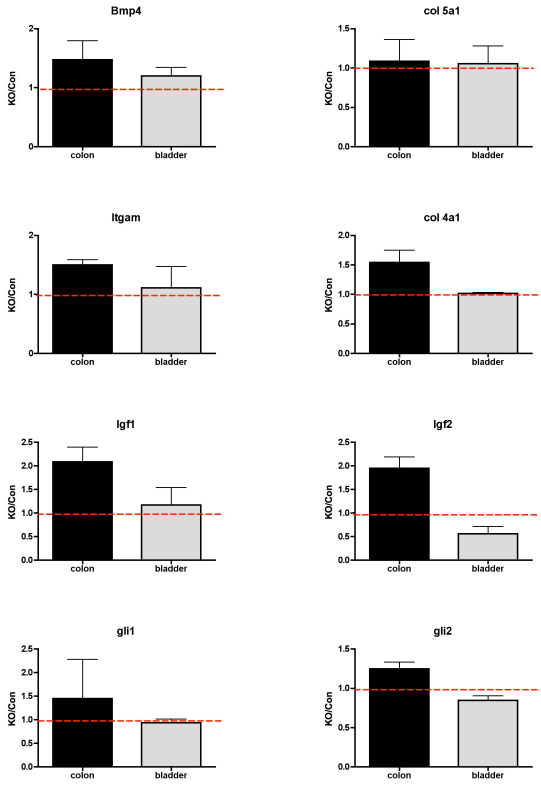


Figure 27. ECM proteins are not changed in Brg1 KO or DKO SM tissues. A. Supperarray results showed some extracellular matrix proteins increased in Brg1 KO colon (Adamts1, Emilin1, Icam1), while Sparc decreased. **B.** Quantitative RT-PCR results showed no changes of Adamts1, Emilin1, Icam1, Sparc in colon and bladder from Brg1 KO by using customized primers and didn't confirm the Supperarray results in "A". **C.** Quantitative RT-PCR results showed no changes of possible Brg1 target genes Bmp4, Col1a1, Col4a1, Col5a1, Igf1, Igf2, Gli1, Gli2 and Itgam, in the colon and bladder from Brg1 KO mice.

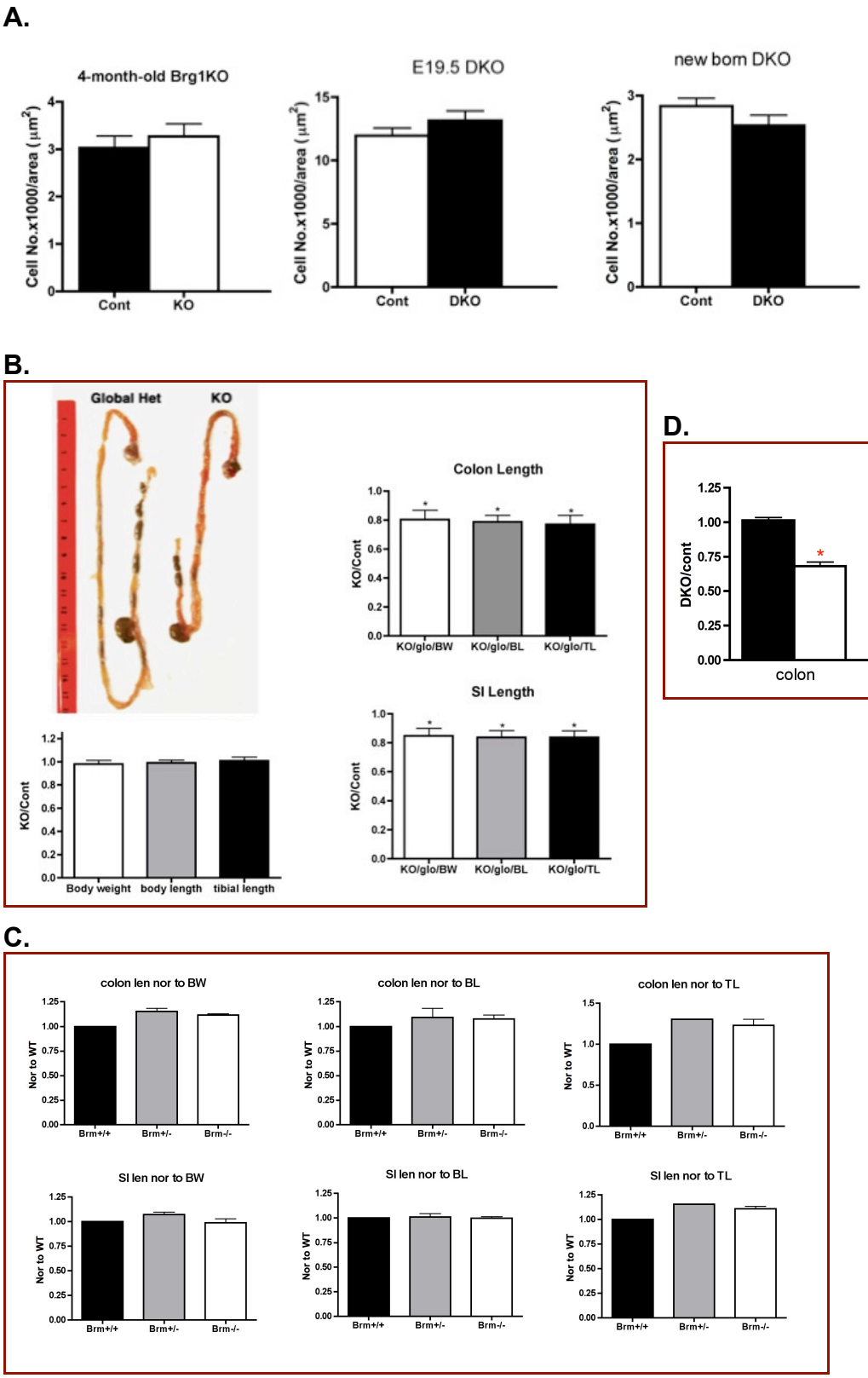
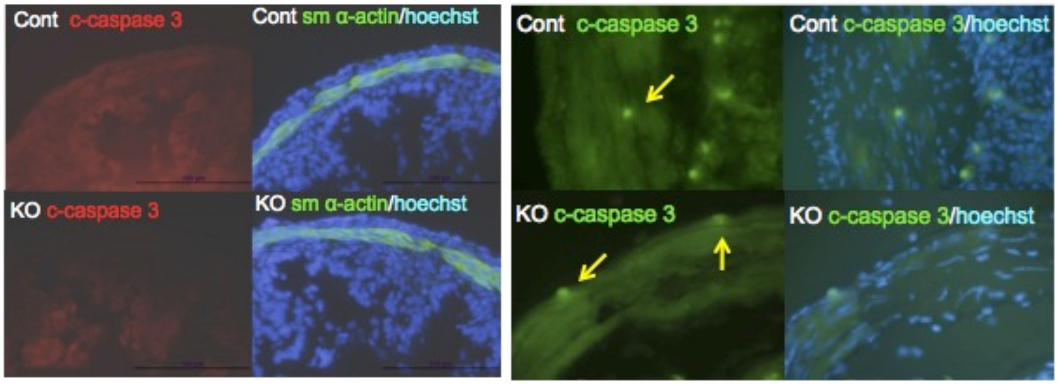


Figure 28. The gut of Brg1 KO and DKO mice is shorter than heterozygous

littermates. **A.** The density of SMCs in colonic circular layers from Brg1 KO and DKO mice was not decreased. **B.** The colon and small intestine (SI) of Brg1 KO mice were significantly shorter (about 20%) than the global heterozygous littermates after normalized to body length, tibial length or body weight. Body length, tibial length, or body weight of Brg1KO mice are not significantly different from littermate heterozygous controls. **C.** Gut length after normalizing to body length, tibial length or body weight, was not changed in Brm KO mice as compare to littermate WT or Brm heterozygous control mice. **D.** Colon and small intestine (SI) were shorter in DKO mice.

A.



B.

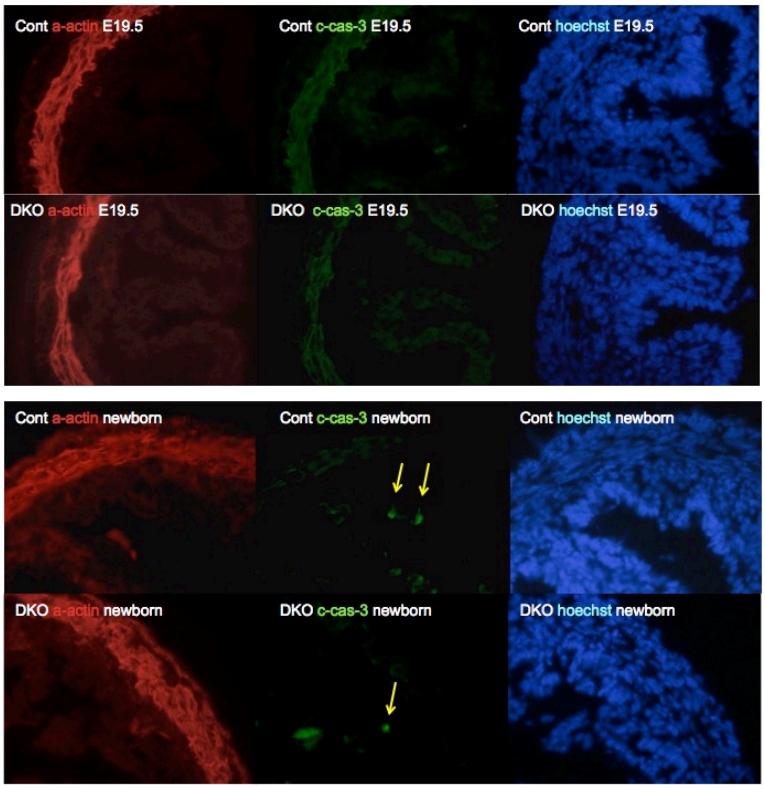


Figure 29. Activated caspase 3 expression is not increased in Brg1 KO or DKO colon. A. Cleaved caspase 3 immunofluorescent staining (red in left panel, green in right panel) and Hoechst nuclear staining (blue) in colon from new born (left panel) and 3-month-old Brg1 KO mice (right panel). Arrows are pointing to

cleaved caspase 3 positive SMCs. **B.** Cleaved caspase 3 immunofluorescent staining (green), sm α-actin (red) and Hoechst nuclear staining (blue) in colon from E19.5 (upper panel) and newborn DKO mice (lower panel).

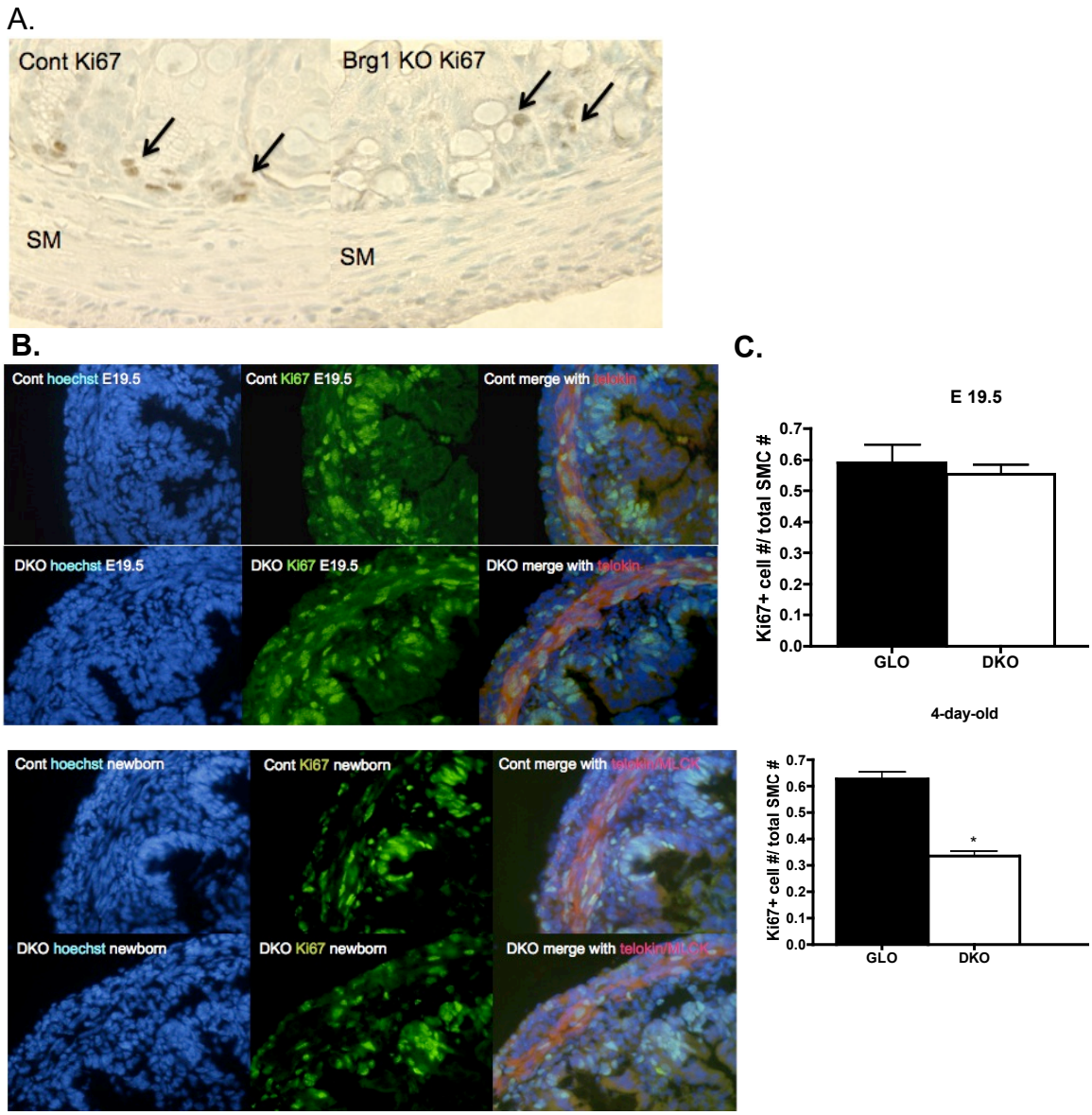


Figure 30. Ki 67 positive cells are decreased in the colon from new born DKO mice. **A.** Ki67 immunohistochemistry staining (brown) of colon from 3-month-old Brg1 KO mice. **B.** Ki67 immunofluorescent staining (green), telokin/MLCK (red) and Hoechst nuclear staining (blue) in colon from E19.5 (upper panel) and newborn DKO mice (lower panel). **C.** The percentage of Ki67

positive SMCs in the circular muscle layer of colon from E19.5 (upper panel) and newborn DKO mice (lower panel).

Table 1:

Primers used for quantitative RT-PCR primers

SM α-actin	F:	5'-CCA GAG TGG AGA AGC CCA GC-3'
	R:	5'-GGC TGT GCT GTC TTC CTC TTC AC-3'
Brg1	F:	5'-CCA TCC TGG AGC ACG AGG AGC-3'
	R:	5'-GGT CCA TGC GCA TGA ACA GAT C-3'
C-fos		Qiagen QuantiTect Primers
Calponin	F:	5'-CCAGCCAGGCTGGCATGACTGC-3'
	R:	5'-CCTTGTTGCTGCCCATCTGC-3'
Egr1	F:	5'-GAG CAC CTG ACC ACA GAG TC-3'
	R:	5'-CCA CAA AGT GTT GCC ACT GTT G-3'
Hprt	F:	5'-TGGCCCTCTGTGTGCTCAA-3'
	R:	5'-TGATCATTACAGTAGCTCTTCAGTCTGA
MRTFA	F:	5'-GCATTTCATGAGCAGAGAAGAAGCC-3'
	R:	5'-CTACCTTTGGGTAATTTACCTGGCC-3'
Myocardin	F:	5'-CAGCTACCCTGGGATGCACCAAACAC -3'
	R:	5'-GGGGCCTGGTTTGAGAGAAGAAACACC -3'
RPLPO (36B4)	F:	5'-GGA CCC GAG AAG ACC TCC TT-3'
	R:	5'-TGC TGC CGT TGT CAA ACA CC-3'
SM22α	F:	5'-CGA AGC CAG TGA AGG TGC CTG AGA AC-3'
	R:	5'-CCC AAA GCC ATT AGA GTC CTC TGC AC-3'
SRF	F:	5'-GTT CAT CGA CAA CAA GCT GC-3'
	R:	5'-CTG TCA GCG TGG ACA GCT CAT AG-3'
Telokin	F:	5'-GAC ACC GCC TGA GTC CAA CCT CCG-3'
	R:	5'-GGC TTT TCC TCA GCA ACA GCC TCC-3'
Vinculin		Qiagen QuantiTect Primers
Adamts1	F:	5'-CTG GGC AAG AAA TCT GAT GA-3'
	R:	5'-AAG CAC AGC CAC AGT TTA TCA-3'
Emilin1	F:	5'- TGT GCC TAG GGT AGC ATT TTC-3'
	R:	5'- GAG GCT GAA GAC GCC CAG AG-3'
Icam1	F:	5'- GTG GTG AAG TCT GTC AAA CAG GAG-3'
	R:	5'- ATT CCT GGT GAC ATT CCC ATG-3'
Sparc	F:	5'- TGA GAA TGA GAA GCG CCT GGA-3'
	R:	5'- AAG GGG GTA ATG GGA GGG GTG-3'

Table2:

Primers used in ChIP Assays

SM α-actin	F:	5'AGCAGAACAGAGGAATGCAGTGGAA3'
	R:	5'CCTCCCACTCGCCTCCCAAACAAGGA3'
c-fos	F:	5'CGGTTCCCCCCCTGCGCTGCACCCTC3'
	R:	5'AGAACAACAGGGACCGGCCGTGGAAA3'
SM22α	F:	5'GACCCCCGCAGCATCTC3'
	R:	5'GACACCAAGTTGGAGCAGTCTGT3'
SRF	F:	5'TGACAGCAGCGAGTTCGGTAT3'
	R:	5'GGCACCTAGGCTCCCCATTT3'
Telokin	F:	5'GGGCCCAGTCAGCAATAAGTC3'
	R:	5'CTGTGCTTCAACTCCCATAAAAGG3'

Note: the ChIP primer sequences for SM α-actin and c-fos are from McDonald et al, *J. Clinical Investigation*, 2006.

Table 3:

Primers used for mouse genotyping			Amplicon (bp)
Brg1 Flox	TG57:	GCCTTGTCTCAAACTGATAAG	387
	TH185:	GTCATACTTATGTCATAGCC	
Brg1 WT	TG57:	GCCTTGTCTCAAACTGATAAG	241
	TH185:	GTCATACTTATGTCATAGCC	
Brm WT	F:	CCTGAGTCATTTGCTATAGCCTGTG	700
	R1:	CTGGACTGCCAGCTGCAGAG	
Brm KO	F:	CCTGAGTCATTTGCTATAGCCTGTG	310
	R2:	CATCGCCTTCTATCGCCTTC	
Cre	F:	CATTTGGGCCAGCTAAACAT	450
	R:	CCCGGCAAAACAGGTGTTA	
ROSA26R	Rs1:	AAAGTCGCTCTGAGTTGTTAT	275
	Rs2:	GCGAAGAGTTTGTCCTCAACC	
ROSA WT	Rs1:	AAAGTCGCTCTGAGTTGTTAT	600
	Rs3:	GGAGCGGGAGAAATGGATATG	

Notes:

1. The primers sequences for Brg1 and Brm are from Bultman, et al, *Mol Cell*, 2000.
2. ROSA26R and cre primers are obtained from Conway lab in Indiana University.

Discussion and Future Directions

In my thesis project, I have studied the roles of Brg1 and Brm in regulating smooth muscle differentiation and development *in vitro* and *in vivo*. Furthermore, the overlapping and non-overlapping functions of Brg1 and Brm were investigated. I have shown that (1) Brg1/Brm are required for the induction of SM-specific genes by MRTFs and Brg1/Brm containing SWI/SNF chromatin remodeling complexes have overlapping functions in supporting the myogenic activity of the MRTFs in non-muscle cells. However, Brg1 and Brm may play non-overlapping roles in maintaining high expression levels of smooth muscle-specific genes in differentiated SMCs. Brg1 and Brm regulated expression of contractile protein genes through regulating SRF and MRTF binding. (2) Brg1 and Brm play redundant role in regulating smooth muscle contractile proteins expression *in vivo*. Both Brg1 and Brm single KO mice had normal smooth muscle contractile proteins expression levels. However, Brg1/Brm double KO mice had significant decreased expression of smooth muscle contractile proteins. (3) Brg1, not Brm, has indispensible roles in regulating the length of GI tract and smooth muscle cell morphology, GI contractility and nutrition absorption. Interestingly, Brg1/Brm double KO mice have more severe gut defects than Brg1 single KO mice, although Brm single KO mice have no obvious gut defects. This suggests that Brg1 can compensate for most functions of Brm in regulating gut proliferation and differentiation, but that Brm cannot fully compensate for the lack of Brg1. These specific functions of Brg1 are still an unsolved puzzle, as we have not yet identified the specific Brg1 target genes or pathways that they regulate. To begin

to address this issue we explored the possible mechanisms that could result in the shorter intestines seen in Brg1 single and Brg1/Brm double KO mice. These preliminary analyses suggest that Brg1 may affect the proliferation, but not apoptosis of developing SMCs.

We observed a disorganization of SMCs in the circular layer of colons from Brg1 single and Brg1/Brm double KO mice. We initially postulated that this might reflect a change in the expression of cell-cell or cell-matrix adhesion molecules. However, examination of expression of genes encoding 84 different ECM proteins failed to show any significant changes in expression of these genes (Figure 27). Future experiments will use a more comprehensive whole genome wide array analysis in order to identify the genes whose expression is changed by specific deletion of Brg1 from smooth muscle cells.

Brg1 single and Brg1/Brm double KO mice develop gut defects including shorter gut, decreased contractility and megacolon. These defects mimic those seen in some human GI tract diseases, such as short gut syndrome, intestinal pseudo-obstruction and idiopathic megacolon. The molecular mechanisms resulting in these diseases, especially the role of chromatin remodeling, are largely unknown. It is widely appreciated that chromatin epigenetic regulation plays an important role in human diseases, including cardiovascular and skeletal muscle related diseases (55) (90, 96). Based on my thesis findings it is thus tempting to speculate that changes in the expression of Brg1 may contribute to the pathology

of human GI tract diseases. Future studies will be aimed at examining the expression of Brg1 and Brm in animal intestinal disease models, such as chronic partial obstruction of small intestine (29).

In the introduction, I discussed the close and complex cross-talk between epithelial and smooth muscle layers during GI tract development that involves many signaling pathways. Consequently although we knocked out Brg1 only in smooth muscle cells, it is reasonable to hypothesize that the aberrant smooth muscle in Brg1 single and Brg1/Brm double KO mice may affect the differentiation, morphology or functions of the epithelial cells. In terms of gross morphology, based on H&E staining result (in Figure 26B) we did not observe any dramatic changes in the colonic epithelium from Brg1, Brm or Brg1/Brm knockout mice. However, the phenotype of the epithelial cells needs to be examined more specifically to rule out possible changes in differentiation of epithelium in these KO mice.

Smooth muscle containing organs include not only the GI tract but also many other organs including the urinary tract, reproductive tract and vasculature system. In my *in vitro* studies, I observed similar roles for Brg1 and Brm in SMCs isolated from colon, bladder and aorta. However, in my *in vivo* studies, I have currently focused only on defects in the gut, largely because GI defects are the most obvious phenotypes observed in the KO mice. However, since there are many similarities in the differentiation of GI, urinary, reproductive and vascular

SMCs, it is quite possible that these other organ systems may also exhibit defects in our KO mice. In the future, we will particularly focus on extending our studies to the urinary and vasculature system of these KO mice. In the Brg1/Brm double KO mice we have observed enlarged bladders suggesting that the smooth muscle in this urinary organ may be similarly affected to the GI smooth muscle. Currently, we have not observed obvious bleeding or heart defects reflecting the defects of the vascular development in embryo or adult stages of Brg1, Brm or double KO mice. However, further studies will be required to more carefully assess the function of the vasculature in the KO mice. In addition, it is possible that the pathological remodeling that occurs in vascular diseases or following vascular injury may be altered in the KO mice. As discussed in the introduction, in vascular diseases such as atherosclerosis, hypertension or animal vascular injury models, the affected SMCs are undergoing dramatic phenotypic changes (104) (144) from contractile to synthetic phenotypes and back; correspondingly, the expression levels of contractile proteins are also dynamically changing. In my thesis study, I have showed that Brg1 and Brm play important roles in regulating colon SMC genes expression, proliferation and phenotype changes. It is thus very likely that Brg1 and/or Brm may be involved in the pathology process of vascular diseases. To test this hypothesis I would propose to perform a carotid artery injury (144) in Brg1 or Brm single KO mice and examine the subsequent remodeling of the carotid artery in these mice as compared to control mice.

In summary, studies described in my thesis are the first to demonstrate a role of Brg1/Brm containing SWI/SNF chromatin remodeling complexes in regulating smooth muscle differentiation and development. Our unique SM cell-specific Brg1 knockout mouse may also provide a new disease model to study intestinal smooth muscle related diseases and thus contribute to a better understanding of these diseases that will aid in developing better therapeutic strategies.

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Curriculum Vitae

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Education

1995-2000	B.Sc.	Dentistry, Shanxi Medical University, P.R.China
2000-2003	M.S.	Physiology, Capital University of Medical Sciences
2003-2009	Ph.D.	Cellular and Integrative Physiology, Indiana University

Honors

1999	Outstanding Students Award, Shanxi Medical University
2006	Honorable mention, Sigma Xi Competition, Indiana University
2007	Second place, Sigma Xi Competition, Indiana University
2007	Stier Fellowship for Excellent Research, Indiana University
2009	Best Poster Awards in 2009 ASBMB Annual Meeting

Grant Support

2007	Fortune Fry Foundation Fellowship
2007 to 2009	American Heart Association Fellowship

Memberships

2005-present	Member of Sigma Xi Society
2006-present	Member of American Association of Anatomists
2007-present	Member of Advancing Science, Serving Society
2008-present	Member of The American Physiological Society

Publications

1. **Min Zhang**, Jiliang Zhou, B. Paul Herring. The role of SWI/SNF in regulating smooth muscle differentiation and development. (Manuscript in preparation)
2. Jiliang Zhou, **Min Zhang** (co-first author), Hong Fang, Omar El-Mounayri, Jennifer M. Rodenberg, Anthony N. Imbalzano and B. Paul Herring. The SWI/SNF Chromatin Remodeling complex Regulates Myocardin-induced smooth Muscle-Specific Gene Expression. Arterioscler. Thromb. Vasc. Biol., 29: 921-928, 2009.
3. **Min Zhang**, Hong Fang, Jiliang Zhou, B. Paul Herring. A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific gene expression. J. Biol. Chem., 2007 282, 25708–25716
4. **Min Zhang**, RuiYao Qu. The Research of NO and VIP as well as its Relationship with Gut Electrical Activity and Motility (Review). World Journal of Gastroenterology, 2003 11(7):1059-1063
5. **Min Zhang**, RuiYao Qu. The Research of the Effects of nNOS and VIP to the Gut Electrical Activity and Motility In Rat PiXu Disease Model. Journal of Capital University of Medical Sciences, 2003 Vol.24 No.3

Abstracts

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2. **Min Zhang**, Hong Fang, Jiliang Zhou and Paul Herring. A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific genes. Weinstein Cardiovascular Development Conference 2007.
3. **Min Zhang**, Hong Fang, Jiliang Zhou and Paul Herring. A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific genes. Experimental Biology 2007.
4. B. Paul Herring**,** Jiliang Zhou, **Min Zhang**, Hong Fang, and Jennifer Rodenberg. Regulation of smooth muscle differentiation by ATP-dependent chromatin remodeling enzymes. ASBMB conference on Transcription Regulation 2006.
5. Feng Yin, Jiliang Zhou, **Min Zhang**, April Hoggatt and Paul Herring. Transcriptional regulation of the *MYLK* gene. CSHL conference on Transcription 2005.

Oral Presentations

1. Paul Herring, **Min Zhang**, Jiliang Zhou. The role of SWI/SNF in regulating smooth muscle differentiation. Experimental Biology 2009.
2. **Min Zhang**, Jiliang Zhou, Hong Fang and Paul Herring. The role of Brg1/Brm chromatin remodeling enzymes in the regulation of smooth muscle differentiation and development. Transcriptional Regulation by Chromatin and RNA Polymerase II meeting 2008.

Future interests

- Muscle development and differentiation
- The mechanism regulating muscle related diseases