# REGULATION OF SRF ACTIVITY BY THE ATP-DEPENDENT CHROMATIN REMODELING ENZYME, CHD8

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I would like to dedicate this dissertation to my adoring husband, Eric, without whom I would not be where I am today. Through his love and support, he was my pillar of strength throughout my graduate work, and for this I am eternally thankful.

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ABSTRACT

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REGULATION OF SRF ACTIVITY BY THE ATP-DEPENDENT CHROMATIN REMODELING ENZYME, CHD8

Under normal conditions, smooth muscle cells do not replicate, or proliferate, and provide a means of contraction for many internal organs, including blood vessels and the gut. However, under abnormal or disease conditions, such as congenital heart disease and cancer, smooth muscle cells acquire the ability to replicate, to make extracellular matrix proteins and to migrate. Thus, determining how smooth muscle cells regulate these processes is crucial to understanding how the cells can switch between normal and diseased states. Serum response factor (SRF) is a widely expressed protein that plays a key role in the regulation of smooth muscle differentiation, proliferation and migration. It is generally accepted that one way that SRF can distinguish between these functions is through pathway-specific co-factor interactions. A novel SRF co-factor, chromodomain helicase DNA binding protein 8 (CHD8), was originally isolated from a yeast two-hybrid assay. CHD8 is widely expressed in adult tissues including smooth muscle. Data from *in vitro* binding assays indicate that the N-terminus of CHD8 can interact directly with the MADS domain of SRF. Co-immunoprecipitation assays verified the ability of these two proteins to interact within cells. Adenoviral-mediated shRNA knockdown of CHD8 in smooth muscle cells resulted in statistically significant 10-20% attenuation of

expression of SRF-dependent, smooth muscle-specific genes. Similar experiments revealed that knockdown of CHD8 did not affect the SRF-dependent induction of immediate early genes required to promote proliferation. In contrast, knockdown of CHD8 in A10 vascular smooth muscle cells resulted in a marked induction in of apoptosis, characterized by increases in apoptotic markers such as phospho-H2A.X, cleaved PARP and activated caspase-3. These data suggest that CHD8 may play a specific role in modulating SRF's activity toward anti-apoptotic genes, thereby regulating smooth muscle cell survival.

B. Paul Herring, Ph.D., chair

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### i. Overview of Smooth Muscle

Smooth muscle cells surround the walls of the body's hollow organs such as the blood vessels, the gastrointestinal tract, the genitourinary tracts, and the airways. As a tissue system, smooth muscle provides the contractile force to regulate flow of materials through the hollow organs. For instance, in the blood vessels smooth muscle contraction and relaxation helps to regulate both the blood flow and the blood pressure. Smooth muscle in the gastrointestinal tract is important for regulating the movement of a food bolus for excretion. Similarly, smooth muscle that lines the bladder and urinary tract plays a vital role in the expulsion of urine. The female reproductive tract, where smooth muscle allows for the contraction and relaxation of the uterus during both menstruation and childbirth, provides another example of the important function of smooth muscle. Finally, smooth muscle is critical for the function of the airways, where it helps to regulate the intake and expulsion of air.

Smooth muscle on the cellular level is extremely complex. The differentiated smooth muscle cell contains a network of contractile and regulatory proteins, such as actin and myosin (Owens, 2007). Smooth muscle cells are

spindle shaped, and under normal conditions the smooth muscle cell is largely quiescent. However, this differentiated smooth muscle cell has the uncanny ability to change from this quiescent, contractile phenotype under normal conditions into a more proliferative, migratory and synthetic phenotype under pathological or wound-healing conditions. This pathological state is often termed "de-differentiated," as cells lose their spindle shape, have multiple protrusions and down-regulate expression of many contractile proteins (Owens, 2007).

ii. Overview of Smooth Muscle Differentiation

During mammalian development, or embryogenesis, embryonic stem cells give rise to all of the body's tissues and cells. One of the main processes of embryogenesis is gastrulation, during which cells form the three main germ layers of the ectoderm, the mesoderm and the endoderm. The ectoderm is the outermost layer, the mesoderm is the middle layer, and the endoderm in the innermost layer. As gastrulation and development continue, the multi-potential cells in the embryo begin to move in various directions and manners to form different organ systems in the body (Leptin, 2005). As a general rule, the ectoderm forms the nervous system, the sense organs, the skin (epidermis) and associated structures and the pituitary gland (Solomon, 1993). Systems such as the skeleton (bone and cartilage), the muscles (skeletal, smooth and cardiac), the circulatory system, the excretory system, the reproductive system, the inner layer of the skin (dermis), the outer layers of the digestive system and the

respiratory system are all derived from the mesoderm germ layer (Solomon, 1993). The lining of the digestive tract and the respiratory system arise from the endoderm. Thus, much of the body's smooth muscle is developed from the mesoderm, while a small amount is also derived from the endoderm.

Looking more closely at the development of the vascular smooth muscle, three main origins of vascular smooth muscle cells become apparent: *(a)* the cranial neural crest population, *(b)* the proepicardium population and *(c)* the endothelial progenitor cell population (Hirschi and Majesky, 2004). During embryogenesis, bone morphogenetic protein (BMP) signaling creates a gradient, allowing for the formation of the neural crest at the mediolateral border between the neural plate and the epidermis (Hirschi and Majesky, 2004). These cells then give rise to various types of cells, including smooth muscle cells. More specifically, the cranial neural crest cells, located anterior to somite 5, give rise to the vascular smooth muscle cells of the aortic arch arteries, the pulmonary artery and the ductus arteriosus (Hirschi and Majesky, 2004).

Whereas the cranial neural crest gives rise to the smooth muscle cells of the aortic arch and pulmonary artery, the stem cells of the proepicardial organ (PEO) differentiate into the smooth muscle cells of the coronary vessels (Hirschi and Majesky, 2004). The PEO structure is transient and establishes contact with the heart, which leads to epicardial layer formation (Hirschi and Majesky, 2004). Furthermore, cells in this population undergo the epithelial-to-mesenchymal transition in response to signals that are released from the myocardium (Hirschi

and Majesky, 2004), thereby allowing them to become migratory and to form the precursors of the smooth muscle cells in the coronary vessels.

The final group of stem cells from which smooth muscle cells are derived is the endothelial progenitor cell subset. Studies have illustrated that depending on the conditions present in the environment, these stem cells can form either endothelial cells or smooth muscle cells. More specifically, smooth muscle cells are derived from this population in the presence of PDGF-BB (Hirschi and Majesky, 2004; Yamashita et al., 2000).

In the adult, much of the remodeling and formation of blood vessels has been thought to occur from the proliferation and migration of smooth muscle cells that are already present. However, recent studies have suggested that adults can differentiate smooth muscle cells from various populations (Yamashita et al., 2000): *(a)* bone marrow-derived stem cells, *(b)* hematopoietic stem cells and *(c)* circulating stem cells. The bone marrow has been thought to harbor both hematopoietic stem cells and mesenchymal stem cells. Yet, recently it has been illustrated that the bone marrow might also contain a population of vascular smooth muscle progenitor cells (Yamashita et al., 2000). As for the hematopoietic stem cells, studies show contradictory results as to whether this population contributes to vascular smooth muscle regeneration after injury (Sata et al., 2002), or whether this population contributes to the regeneration of endothelial cells post-injury (Goodell et al., 1996; Hirschi and Majesky, 2004; Jackson et al., 2001). In addition, various studies have demonstrated that progenitor cells within the mononuclear fraction of the blood have the potential to

give rise to smooth muscle cells, wherein each group was able to show evidence of smooth muscle alpha actin (SM-α-actin) expression from cultured cells fractionated from blood (Hillebrands et al., 2001; Hirschi and Majesky, 2004; Simper et al., 2002).

In general, cellular differentiation can be defined as the process during development where multi-potential cells acquire the cell-specific attributes that discriminate them from other cell types (Owens et al., 2004). As Owens, et al., describes the actual process of cellular differentiation can be divided into three main components: *(a)* activation of specific genes that are required for differentiation of a cell, *(b)* control of expression of these specific genes at certain times and quantities and *(c)* regulation of overall gene expression via the microenvironmental signals that regulate the cell's lineage, which includes transcription factors and epigenetics (Owens et al., 2004).

In the case of smooth muscle cells, a differentiated smooth muscle cell can be identified by the expression of specific genes, or smooth muscle markers. These markers include: smooth muscle alpha and gamma actin (SM-α-actin and SM-γ-actin), smooth muscle myosin heavy chain (SM-MHC), telokin, SM22α, calponin, caldesmon, metavinculin, smoothelin and 130 kD myosin light chain kinase (MLCK). One important characteristic of the promoters of most of these smooth muscle marker genes is that they contain at least one CArG element, which is the consensus sequence for the transcription factor serum response factor (SRF). However, many other factors also play a role in the regulation of these smooth muscle markers as detailed below.

### iii. Mechanisms of Smooth Muscle Differentiation

Much is still to be elucidated about how smooth muscle cells differentiate from the multi-potential cells throughout development. However, several studies have investigated this differentiation phenomenon, and various factors and pathways have been described as being involved in the process of deriving a smooth muscle cell. One well-described example of a factor and its signaling pathways being implicated in smooth muscle cell differentiation is transforming growth factor β1 (TGFβ1).

TGFβ1 is a cytokine that signals through multiple membrane receptors and intracellular pathways. Knockout models of either TGFβ1, TGFβ type II receptor, activin receptor-like kinase 1 (Alk1), endogelin or SMAD5 are all embryonic lethal (Bourdeau et al., 1999; Chang et al., 1999; Dickson et al., 1995; Oh et al., 2000; Oshima et al., 1996; Sinha et al., 2004; Urness et al., 2000). At least 50% of the TGFβ1 and TGFβ type II receptor null mice die in utero from defects in the yolk sac vasculature by embryonic day 11.5. Alk1, endoglin and SMAD5 knockouts succumb to intrauterine death around midgestation due to hemorrhaging from dilated and fragile vessels. Further highlighting the importance of TGFβ intracellular signaling specifically in smooth muscle differentiation, knockout of either endogelin or Alk1 lead to a loss of smooth muscle cells that coat the dorsal aorta (Li et al., 1999; Oh et al., 2000; Sinha et al., 2004).

TGFβ has the ability to up-regulate the expression of several smooth muscle markers, including SM22α, SM-α-actin and $h_1$-calponin during myofibroblast differentiation (Bjorkerud, 1991; Sinha et al., 2004). Inactivating TGFβ1, the TGFβ type II receptor, SMAD2 and SMAD3 in an embryonic stem cell – embroid body model of smooth muscle differentiation attenuated expression of many smooth muscle markers including SM MHC, SM- α-actin and SM22α (Sinha et al., 2004). In addition, the SM-α-actin promoter was found to be regulated by both SMAD2 and SMAD3; whereas, SM-MHC only required SMAD2 for proper transcription (Sinha et al., 2004). The authors note that this difference in requirement of SMADs could be due to the fact that SM-α-actin is also expressed in the myofibroblast and other cell types, indicating that perhaps SMAD2 is more important for regulation of smooth muscle cell differentiation (Sinha et al., 2004). Overall, these findings therefore indicate that TGFβ1 signaling, specifically through SMAD2 and SMAD3, has an important role in the development of smooth muscle cells.

Another factor implicated in smooth muscle cell differentiation is GATA6. GATA6 is a member of the GATA family zinc-finger transcription factors. Studies have shown that GATA6 is the only member of this family to be expressed in vascular smooth muscle cells (Lepore et al., 2005). Targeted knockout of GATA6 causes embryonic lethality at embryonic day 6.5 from a defect in visceral endoderm formation prior to vascular smooth muscle development (Lepore et al., 2005; Morrisey et al., 1998). Even though GATA6 is not expressed in all types of smooth muscle, studies have shown that GATA6 weakly activates the SM-MHC

promoter and that GATA6 in combination with cysteine-rich protein 2 (CRP2) activates serum response factor-dependent smooth muscle marker transcription (Chang et al., 2003; Wada et al., 2002; Wada et al., 2000). These findings all indicate that GATA6 has a role in regulating the differentiation of smooth muscle cells; however, this type of regulation may be restricted to vascular smooth muscle.

iv. SRF's Role in Smooth Muscle Differentiation

In 1984, Greenberg and colleagues found that cFos transcription in quiescent cells could be stimulated by serum (Chai and Tarnawski, 2002; Greenberg and Ziff, 1984). Upon further investigation, it was determined that serum was able to activate the cFos promoter due to the presence of a specific DNA element upstream of the transcriptional start site in the promoter. This element has the general consensus sequence $CC(A/T)_6GG$ and was named Serum Response Element (SRE) (Chai and Tarnawski, 2002). Treisman later identified the transcription factor that bound to this specific sequence as a dimer and named it Serum Response Factor (SRF) (Chai and Tarnawski, 2002; Treisman, 1986). The full-length mRNA transcript of SRF contains seven exons; however, due to alternative RNA splicing, it has been shown that four isoforms of SRF exist in the mouse: *(a)* SRF-L, or full-length SRF, *(b)* SRF-M, which lacks exon 5, *(c)* SRF-S, which lacks exons 4 and 5, and *(d)* SRF-I, which contains only exons 1,2,6, and 7 (Chai and Tarnawski, 2002; Kemp and Metcalfe, 2000).

The resulting SRF proteins are 67, 62, or 48 kDa, depending on the isoform. It is important to note that the SRF-L isoform corresponds to the dominant isoform found in the human, and the SRF-M isoform functions as a dominant negative of the full-length wild type (Belaguli et al., 1999; Chai and Tarnawski, 2002). SRF contains three major conserved domains (***Figure 1***): *(a)* MADS box, which serves as a DNA-binding motif, *(b)* several phosphorylation sites, which allow for post-translational regulation of SRF, and *(c)* transactivation domain, which is located at the C-terminus of SRF.

Expression of SRF in the chicken and mouse provide strong evidence for a role of SRF in smooth muscle differentiation. In the adult chicken, SRF expression is only detected in tissues of mesodermal and neuroectodermal origin (Arsenian et al., 1998; Croissant et al., 1996). During gastrulation in the chicken, SRF mRNA is localized to the primitive streak, the neural groove, the lateral plate and the precardiac splanchnic mesoderm, the myocardium and the somites (Arsenian et al., 1998; Croissant et al., 1996). In an adult mouse the highest levels of SRF mRNA can be seen in the skeletal, cardiac and smooth muscle tissues. During development SRF mRNA is highly expressed in the medial smooth muscle layer of the vessels, the myocardium of the heart and the myotomal portions of the somites (Arsenian et al., 1998; Belaguli et al., 1997).

As a transcription factor, SRF is involved in multiple pathways that regulate disparate processes. SRF is critical for the differentiation of skeletal, cardiac and smooth muscle lineages (Owens et al., 2004; Pipes et al., 2006). SRF regulates smooth muscle differentiation through its interaction with various

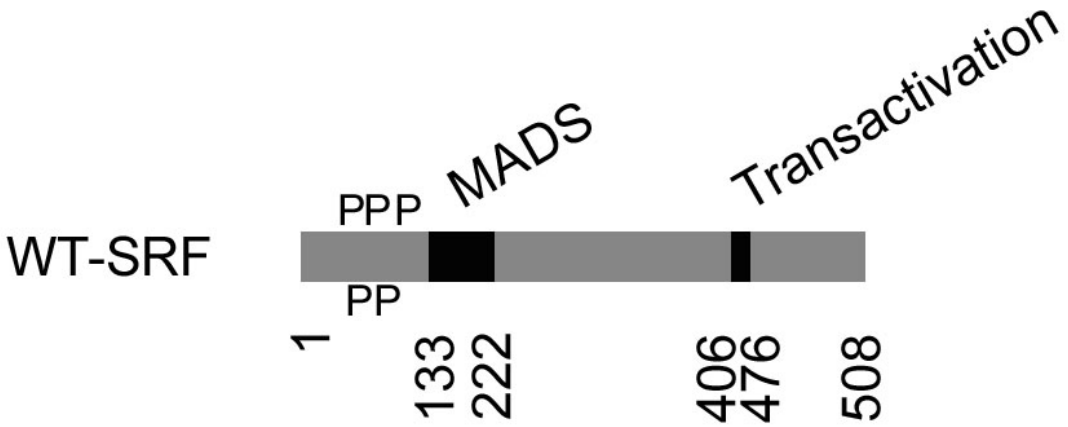


***Figure 1. Schematic of functional domains of serum response factor (SRF).*** SRF contains a MADS domain, which serves as a DNA-binding motif; several phosphorylation sites, which allow for post-translational regulation of SRF; and a transactivation domain, which is located at the C-terminus of SRF.

coactivators and/or cofactors such as myocardin, the myocardin family members MKL1 (MRTFA) and MRTFB (Behrens and Lustig, 2004; Wang et al., 2001), Mhox (Owens et al., 2004), Nkx3.1, Nkx3.2, Nkx2.5 (Carson et al., 2000; Nishida et al., 2002; Phiel et al., 2001), Barx2, Barx1b (Herring et al., 2001; Nakamura et al., 2001) and GATA6/CRP2 (Chang et al., 2003).

The members of the myocardin family of SRF coactivators are very powerful activators of the smooth muscle differentiation program (Pipes et al., 2006). The expression of myocardin itself is restricted to cardiac and smooth muscle-specific genes (Wang et al., 2001). By forming a ternary complex with SRF and CArG elements, myocardin stimulates transcription of CArG-dependent muscle specific genes (Pipes et al., 2006). The interaction of myocardin with SRF is crucial because myocardin itself does not bind directly to DNA, whereas SRF does. The importance of myocardin in vascular smooth muscle development was demonstrated by analysis of myocardin knockout mice, which exhibited a lack of differentiated vascular smooth muscle cells and consequently died during embryonic development (Chen et al., 2003).

In addition, myocardin related transcription factor A (MRTFA), which is expressed in a wider array of tissues, can also activate smooth muscle gene expression when over-expressed in fibroblasts, indicating that MRTFA may also regulate smooth muscle differentiation via interactions with SRF (Al-Aynati et al., 2004; Pipes et al., 2006; Wang et al., 2002). In support of this proposal siRNA mediated knockdown of MRTFA in rat vascular smooth muscle cells attenuated expression of CArG-dependent smooth muscle marker genes (Yoshida et al.,

2007). However, MRTFA knockout mice do not exhibit defects in smooth muscle cells although expression of smooth muscle-specific genes was attenuated in myoepithelial cells of the mammary gland (Li et al., 2006). The discordance between these *in vivo* and *in vitro* data suggest that myocardin family members may have partially redundant functions such that one family member may be able to compensate for the loss of another family member *in vivo*.

As with MRTFA, myocardin related transcription factor B (MRTFB) is also more widely expressed than myocardin (Du et al., 2003), and inactivation of both MRTFA and MRTFB is required to block Rho-A-dependent SRF target gene activation (Cen et al., 2003; Li et al., 2005). However, unlike MRTFA, despite its potent transcriptional activation domain, MRTFB is only a weak coactivator (Li et al., 2005; Wang et al., 2002). In support of the idea that MRTFB also plays a role in regulating smooth muscle differentiation, MRTFB knockout mice are embryonic lethal at embryonic day 13.5 to 14.5 (Li et al., 2005; Oh et al., 2005; Wei et al., 2007). Mice deficient of MRTFB displayed defective brachial arch arteries and cardiac outflow tracts. MRTFB null mice also demonstrated a lack of SM-$\alpha$-actin in vascular smooth structures derived from neural crest cells (Li et al., 2005; Oh et al., 2005), indicating that MRTFB plays a role in smooth muscle cell differentiation. However, even though MRTFB is expressed in the embryonic heart and peripheral vasculature, these structures are normal in MRTFB knockout mice and only secondary vascular structures such as the brachial arch arteries and cardiac outflow tracts were affected (Li et al., 2005; Oh et al., 2005). Additional defects in MRTFB null mice include abnormal liver and portal vascular

development (Wei et al., 2007). Nonetheless, since myocardin knockout mice are still embryonic lethal at embryonic day 10.5 when MRTFB is present, MRTFB cannot compensate for myocardin at this stage (Li et al., 2005; Oh et al., 2005).

Another factor that affects how SRF regulates the differentiation of smooth muscle cells is Mhox. Mhox has been shown to dramatically increase SRF binding to the CArG element in the SM-α-actin promoter (Hautmann et al., 1997; Owens et al., 2004). This data has indicated that a homeodomain region in near proximity to one of the CArG elements in the SM-α-actin promoter is important for regulating SRF's activity. A further piece of evidence for this idea is that the over-expression of Mhox stimulated the SM-α-actin promoter in cultured smooth muscle cells (Owens et al., 2004). In addition, studies have suggested a role for Mhox in angiotensin II-mediated smooth muscle differentiation. Angiotensin II is capable of increasing expression of Mhox, which then subsequently enhances binding of SRF to either of the CArG elements in the SM-α-actin promoter (Owens et al., 2004).

Nkx, Barx and GATA6 are additional factors that have been shown to regulate SRF and smooth muscle cell differentiation through forming a ternary complex with SRF. Both Nkx and Barx are homeodomain-containing proteins that may function in a similar fashion to Mhox; whereas, GATA6 is a zinc finger protein. A study by Nishida, et al., illustrated that a trimeric complex of GATA6, Nkx3.2 and SRF specifically activated smooth muscle markers such as SM22α and caldesmon, but not the proliferation factor cFos (Nishida et al., 2002; Owens et al., 2004).

Another piece of evidence for SRF's involvement in smooth muscle differentiation can be obtained from the study of SRF-null mice. Global SRF knockout was embryonic lethal by embryonic day 12.5 (Arsenian et al., 1998). By embryonic day 7.5, the $SRF^{-/-}$ embryos were reduced in size compared to their heterozygous littermates and displayed an absence of a primitive streak (Arsenian et al., 1998). In addition, the $SRF^{-/-}$ embryos form a misfolded endoderm and ectoderm and fail to form the mesodermal layer, indicating that SRF is critical for formation of the mesoderm, from which most smooth muscle is derived (Arsenian et al., 1998). The absence of a mesodermal layer was confirmed by lack of Brachyury expression, a major mesoderm marker (Arsenian et al., 1998). The study also demonstrated decreased expression of SRF-dependent genes, such as cFos and Egr1, in the $SRF^{-/-}$ embryos (Arsenian et al., 1998). Since expression levels were reduced but not obliterated, this suggests that SRF is important for proliferation, but regulation of transcription of these genes is also controlled by additional transcription factors. In addition to SRF-dependent proliferation genes, the lack of SRF affects the expression of smooth muscle markers as well. At embryonic day 7.5 to 8.5, $SRF^{-/-}$ embryos show drastic decreases in expression of both smooth muscle $\alpha$- and $\gamma$-actin (Arsenian et al., 1998).

In order to study the role of SRF in smooth muscle development without the issue of embryonic lethality, SRF knockout studies have also been conducted at the adult stage by employing a Cre-Lox conditional recombination model. Two independent studies created smooth muscle-specific, conditional SRF knockout

models by crossing mice with a floxed SRF allele with transgenic mice with SM-CreER$^{T2}$, which was under the control of the smooth muscle-specific promoter SM22$\alpha$ and was tamoxifen-inducible (Angstenberger et al., 2007; Mericskay et al., 2007). Within 2-4 days post-induction of SRF knockout, the mutant mice became lethargic, had a distended abdomen, and began to stop eating food and excreting feces (Angstenberger et al., 2007); and by days 13 and 20 post-induction, the mutant mice had severe dilation of both the small and large intestines (Mericskay et al., 2007). Both studies indicated that the mutant mice die before their wild-type counterparts, and one study noted that there was 100% lethality of mutant mice by 16 days post-induction. Upon further investigation, the studies found that the SRF knockout mice had quickly developed chronic intestinal pseudo-obstruction (CIPO, also known as megacolon), which is characterized by chronic intestinal dilation and defective peristalsis due to a loss of contractile proteins (Angstenberger et al., 2007; Mericskay et al., 2007). This decrease in intestinal contraction was the reason for the observed lack of feces excretion in the mice. As for the reduced expression of contractile proteins, the studies illustrated that the loss of SRF greatly affected expression of SM-$\alpha$-actin, SM-$\gamma$-actin and SM-MHC, which are all members of the core components of the contractile apparatus in smooth muscle cells (Mericskay et al., 2007). The expression of SM22$\alpha$, $h_1$-calponin, SM-MLCK and telokin were also decreased, but the expression of proliferation gene cFos was unchanged between knockouts and controls (Mericskay et al., 2007). Therefore, the loss of SRF severely affected the ability of the smooth muscle cells to remain in a differentiated state.

Similarly, loss of SRF has been shown to play a role in obstructive bladder disease due to its regulation of smooth muscle (Levin et al., 2000; Owens et al., 2004). SRF also appears to be crucial in the upper gastrointestinal tract where studies indicate that it is required for esophageal and gastric ulcer healing (Chai et al., 2004; Chai et al., 2007).

Another clinical area that SRF plays a large role in is cardiovascular disease (CVD). According to the American Heart Association, CVD is the number one killer in the United States to date. A recent study demonstrates that inactivation of SRF in the adult heart results in dilated cardiomyopathy, or an enlarged heart, which then leads to death only eight to ten weeks post-SRF loss as a result of heart failure (Parlakian et al., 2005). In a fashion similar to this role of SRF, studies have also linked aberrant SRF function to atherosclerosis and hypertension due to its ability to regulate proliferation, migration and differentiation of vascular smooth muscle cells (Owens et al., 2004). To further support the idea of SRF's importance in vascular development, Holtz, et al., note that loss of SRF severely impairs proper remodeling of the vessels in the vasculature when SRF was specifically ablated from endothelial cells using an endothelial cell-specific promoter Tie2-Cre system (Holtz and Misra, 2008). Specifically, mutant embryos, or $Tie2Cre^{+/-}SRF^{f/f}$, exhibited cerebrovascular hemorrhaging and blood pooling starting at embryonic day 11.5, which progressed during development and became lethal by embryonic day 14.5 (Holtz and Misra, 2008). Further investigation revealed that the hearts of mutant embryos were smaller than those of wild type embryos, and the muscular region

of the interventricular septum in the mutants was reduced in size (Holtz and Misra, 2008). The vessels in the mutant embryos were also enlarged, most likely due to edema resulting from vascular insufficiency (Holtz and Misra, 2008). Thus, SRF is not only important in the development and differentiation of vascular smooth muscle cells, but it also has an important function in the regulation of endothelial cells during vascular development and remodeling.

B. SRF's Regulation of Proliferation, Motility, Apoptosis and Other Processes

As previously mentioned, SRF is involved in several pathways that regulate disparate processes. In addition to muscle differentiation, SRF has been implicated in the regulation of proliferation (Chai and Tarnawski, 2002; Johansen and Prywes, 1994), motility (Chai and Tarnawski, 2002; Hill et al., 1995) and apoptosis (Schratt et al., 2004; Vickers et al., 2004).

The major pathway involved in SRF's regulation of proliferation is the MAPK pathway. In the MAPK pathway, growth factors or serum stimulate the phosphorylation of ternary complex factor (TCF), which interacts with SRF. Upon this phosphorylation, transcription of immediate early genes (IEGs), such as cFos and Egr1, is up-regulated in order to regulate proliferation (Treisman, 1994). A recent study also documented the involvement of factors such as cFos, SRF, Elk-1 and myocardin during intestinal obstruction in a murine model. The authors of this study demonstrated that in response to obstruction, myocardin expression in smooth muscle cells of the small intestine initially decreased; whereas, cFos expression initially increased due to increased SRF/Elk-1 binding to the cFos promoter (Chen et al., 2008). After a period of time, expression of both myocardin and cFos returned to control levels, and hypertrophy became apparent (Chen et al., 2008). This data indicates that initially the smooth muscle cells in the small intestine are losing a differentiated phenotype and gaining the ability to proliferate, which is then followed by hypertrophy of the smooth muscle

layer of the small intestine once the cells begin to re-differentiate into smooth muscle cells.

Signaling through the RhoA pathway activates SRF independently of MAPK/TCF signaling. The key protein in this signaling cascade is the myocardin family member MRTFA (MKL1, megakaryoblastic leukemia-1). MRTFA interacts with SRF in order to stimulate transcription factors such as SRF itself, vinculin, and junB (Cen et al., 2004). Upregulation of these factors allows SRF to regulate processes such as differentiation, migration, and adhesion.

Although not as well studied, SRF has also been found to play a role in regulating apoptosis. SRF has been shown to be required for differentiation-dependent expression of the anti-apoptotic factor Bcl-2 (Schratt et al., 2004). In this study, Schratt, et al., notes that embryonic stem cells that are devoid of SRF display increased apoptosis. SRF affects apoptosis through regulating transcription of Bcl2 by activating its promoter through binding at the CArG region (Schratt et al., 2004). Similarly, Vickers, et al., demonstrated that SRF is necessary for another anti-apoptotic factor's transcription, Mcl-1, through its interaction with the transcription factor Elk-1, as well as its own binding to the promoter (Vickers et al., 2004).

Due to its regulation of proliferation, motility and apoptosis, SRF could potentially have a role in cancer progression. Several studies have demonstrated that an aberrant $MAPK^{erk}$ pathway leads to various forms of cancer through the upregulation of transcription factors, which causes increased proliferation (Fang and Richardson, 2005; Lu et al., 2005; Ludes-Meyers et al., 2001; Wang and

Olson, 2004). Unlimited replication, or proliferation, and the ability to evade apoptosis are two hallmarks of cancer and cancer progression.

## C. Smooth Muscle Cell Phenotypic Modulation

Since cardiovascular disease remains the number one killer of Americans, understanding the molecular mechanisms of such pathology is imperative to finding therapies and potential cures. The main school of thought is that smooth muscle cells found in the intima following vascular injury arise from pre-existing medial smooth muscle cells that migrate and undergo phenotypic modulation (Owens et al., 2004; Ross and Glomset, 1976a; Ross and Glomset, 1976b). However, it has also been suggested that since both cultured adventitial fibroblasts and cultured endothelial cells can be induced to express smooth muscle markers such as SM-$\alpha$-actin and SM-MHC (Frid et al., 2002; Owens et al., 2004), perhaps they contribute to the smooth muscle cell population in the intima during vascular injury. There has also been recent compelling evidence that this intimal smooth muscle cell population is derived from bone marrow-derived cells (Owens et al., 2004; Remy-Martin et al., 1999; Simper et al., 2002). However, no matter the origin of this population of smooth muscle cells during vascular injury, several studies have focused on factors involved in regulating this process on the molecular level.

### i. Kruppel-like Factors (KLFs)

One family of transcription factors widely studied in their role of the phenotypic modulation of smooth muscle cells is the kruppel-like factors (KLFs).

The KLFs are a group of zinc-finger DNA-binding transcription factors that have three main distinguishing features (Haldar et al., 2007): *(a)* three C-terminal $Cysteine_2/Histidine_2$ containing zinc fingers (Bieker, 1996; Turner and Crossley, 1999), *(b)* the highly conserved sequence TGEKP(Y/F)X between zinc fingers (Dang et al., 2002) and *(c)* the ability to bind the consensus CACCC or the GT box (Bieker, 1996; Dang et al., 2002). In mammals, 17 KLFs numbered 1 through 17 have been identified and have functions in the heart, skeletal muscle and smooth muscle.

With respect to smooth muscle, KLFs 4,5,13 and 15 have been demonstrated to have a role in the phenotypic changes of vascular smooth muscle cells during injury. Under basal conditions in the adult, KLF4 expression is extremely low; however, after vascular injury, KLF4 expression is drastically increased and peaks within 1-2 hours post-injury but returns to basal levels after 24 hours (Haldar et al., 2007). Various studies have implicated KLF4 in promoting the de-differentiation of a smooth muscle cell during vascular injury. For example, Owens, et al., showed that knockdown of KLF4 partially regulates the repressive effects of PDGF-BB on smooth muscle cell gene expression (Haldar et al., 2007; Liu et al., 2005), which could be a result of the repressive effect of KLF4 on myocardin's activity (Du et al., 2003; Haldar et al., 2007; Parmacek, 2007; Pipes et al., 2006; Wang et al., 2003). Conditional knockout of KLF4 results in a delay in the down-regulation of smooth muscle markers following vascular injury demonstrating an important role of KLF4 in this phenotypic modulation (Yoshida et al., 2008). Consistent with these data KLF4

has been shown to attenuate expression of TGFβ1-dependent increases in SM22α and SM-α-actin (Haldar et al., 2007).

KLF5 is highly expressed in fetal smooth muscle, but is nearly undetected in basal conditions of adult smooth muscle cells. However, in a fashion similar to KLF4, KLF5 expression after vascular injury or angiotensin II treatment is dramatically increased, which may be regulated by the intracellular signaling of survivin and/or the MAPK pathways (Haldar et al., 2007; Hoshino et al., 2000; Nagai et al., 2005; Sakamoto et al., 2003). Studies indicate that the highest expression of KLF5 post-injury is in the neointimal smooth muscle cells (Haldar et al., 2007). Global knockout of KLF5 is embryonic lethal, but $KLF5^{-/+}$ mice are viable (Haldar et al., 2007; Shindo et al., 2002). Post-vascular injury, these mice have reduced neointimal formation, smooth muscle proliferation and perivascular fibrosis, suggesting that similar to KLF4, KLF5 has a role in vascular remodeling after injury. Furthermore, over-expression of KLF5 increases expression of factors such as PDGF-A and B, PAI-1, iNOS and VEGF-R (Haldar et al., 2007; Nagai et al., 2005) that are increased following vascular injury. Importantly, KLF5 has been shown to regulate smooth muscle through its interaction with chromatin remodeling factors. For instance, interaction with p300 allows for increased activity of KLF5, which is partially mediated by the acetylation of KLF5 itself (Haldar et al., 2007; Miyamoto et al., 2003). In addition, HDAC1 can interact with KLF5 to inhibit KLF5 from binding to DNA, and myeloid leukemia associated oncoprotein SET can inhibit KLF5 activity by negatively regulating

KLF5's ability to bind DNA, to transactivate target promoters and to exhibit pro-proliferative effects (Haldar et al., 2007; Miyamoto et al., 2003).

Another Kruppel-like factor involved in smooth muscle regulation is KLF13. While KLF13 is well described for its activity in cardiac muscle, KLF13 is much less studied than other KLFs with regard to smooth muscle. However, one study has investigated it role in smooth muscle phenotypic modulation. In this study, the authors report that KLF13, or basic transcription element-binding protein (BTEB 3), can activate the minimal promoter of SM22$\alpha$, which is a smooth muscle-specific gene (Martin et al., 2003); yet, further investigation is required to elucidate the full function of KFL13 in smooth muscle.

A final KLF that has been shown to be involved in smooth muscle phenotypic modulation is KLF15. In contrast to KLF4 and KLF5, KLF15 is highly expressed at basal levels in the adult; however, upon vascular injury or pro-proliferative signals, KLF15 expression is dramatically reduced (Haldar et al., 2007). Over-expression of KLF15 leads to a potent inhibition of proliferation in cultured smooth muscle cells, and KLF15$^{-/-}$ mice display exaggerated neointimal response to vascular injury (Haldar et al., 2007). Thus, KLF15 opposes the role of KLF5, and it is possible that a balance between these factors is crucial in the mechanistic response of smooth muscle cells to vascular injury.

ii. Platelet Derived Growth Factor (PDGF)

Promotion of proliferation and migration of smooth muscle cells accompanied by the downregulation of smooth muscle markers can be regulated by platelet derived growth factor (PDGF). PDGF is a powerful chemoattractant that is produced by activated platelets and lesion macrophages (Ferns et al., 1991; Heldin et al., 1998; Owens et al., 2004). PDGF is expressed as a dimer of the isoforms PDGF-A and PDGF-B, which are connected by disulfide bonds (Heldin and Westermark, 1990; Owens et al., 2004). Similarly, PDGF has two receptors: PDGFR-$\alpha$ and PDGFR-$\beta$. These receptors are tyrosine kinases and can be activated by PDGF-A alone, or either PDGF-A or PDGF-B, respectively (Owens et al., 1996). Several studies have investigated the function of PDGF and its receptors as related to smooth muscle. Global knockout of either PDGF isoform or either PDGF receptor is embryonic lethal (Bostrom et al., 1996; Levin et al., 2000; Owens et al., 2004; Soriano, 1994; Stenmark et al., 2000), but $ApoE^{-/-}$ mice where both PDGFR-$\alpha$ and PDGFR-$\beta$ have been inhibited exhibit decreased recruitment of smooth muscle cells in the neointimal region of an atherosclerotic lesion (Sano et al., 2001). Additional studies using cultured embryonic liver cells deficient in PDGF-B introduced into lethally irradiated $ApoE^{-/-}$ mice found that the presence of smooth muscle cells in the fibrous cap in an atherosclerotic lesion of the $ApoE^{-/-}$ mice was delayed (Kozaki et al., 2002; Owens et al., 2004). Similar results were seen in an analogous model where both PDGF receptors were inhibited with synthetic compounds (Kozaki et al.,

2002; Owens et al., 2004). Thus, it appears that inhibition of either PDGF and/or PDGF signaling attenuates but does not block smooth muscle cell migration and proliferation in a vascular lesion.

iii. Matrix metalloproteinases (MMPs)

Smooth muscle cells and macrophages produce endopeptidases called matrix metalloproteinases (MMPs). These MMPs are thought to play a role during the degradation and remodeling of the extracellular matrix of an atherosclerotic plaque (Galis and Khatri, 2002; Owens et al., 2004). Several factors have been linked to the regulation of MMP expression *in vivo* in response to vascular injury in order to aid in the phenotypic modulation of smooth muscle cells. However, it is ultimately the balance between the expression of MMPs, the expression of their inhibitors (tissue inhibitor of metalloproteinase, or TIMPs) and the production of matrix by smooth muscle cells that determines the stability of a plaque (Owens et al., 2004). For example, in the arteries of healthy individuals, a normal ratio of constitutively expressed MMP-2 (72-kDa gelatinase) and TIMP-1/TIMP-2 exists, by which the matrix is maintained (Galis and Khatri, 2002; Owens et al., 2004). However, as an atherosclerotic lesion begins and continues to develop, this normal ratio begins to favor the MMPs. This change in ratio is most likely due to increased expression of MMP-3 (stromelysin) and MMP-9 (92-kDa gelatinase) (Galis and Khatri, 2002; Owens et al., 2004). Interestingly, a study by Galis, et al., demonstrated that the smooth muscle cells in the shoulder

region of an atherosclerotic plaque expressed high levels of both MMP-3 and MMP-9, which indicated to the authors that these MMPs may aid in the destabilization and rupture of a plaque as well as the recruitment and migration of smooth muscle cells to form a plaque (Galis et al., 1994; Owens et al., 2004). Supporting this proposal, MMP-9-deficient mice have reduced intimal smooth muscle cell hyperplasia and decreased late lumen loss following vascular injury (Galis et al., 2002). Overall, the MMPs appear to have a critical role in the phenotypic modulation of smooth muscle cells during vascular injury; however, much remains to be elucidated about the detailed mechanism of environmental cues that regulate the MMPs.

iv. Transforming Growth Factor Beta-1 (TGFβ1)

As previously described, TGFβ has the ability to induce expression of smooth muscle markers such as SM-α-actin and SM-MHC during differentiation. In addition to this role in differentiation, TGFβ can also regulate the phenotype of smooth muscle cells during vascular injury. For instance, in the vascular balloon injury model, studies note that TGFβ expression is dramatically increased within 6 to 24 hours post-injury (Majesky et al., 1991; Owens et al., 2004). By over-expressing TGFβ, the formation of neointima, the deposition of matrix and the proliferation of smooth muscle cells is drastically decreased (Smith et al., 1999), which further illustrates a role for TGFβ in regulating smooth muscle cells during vascular injury. Furthermore, evidence supports the idea that TGFβ signaling

has a protective effect against atherosclerotic plaque formation in that the smooth muscle cells of advanced plaques tend to have mutations in the TGFβ receptor type II, which decreases the sensitivity of these cells to TGFβ (Massari and Murre, 2000; Owens et al., 2004). Similarly, patients with unstable angina are inclined to have decreased levels of circulating plasma TGFβ (Grainger et al., 1995; Owens et al., 2004). Another study demonstrated that inactivating TGFβ1, TGFβ2 or TGFβ3 all lead to an accelerated development of atherosclerotic plaques by 15 weeks post-treatment, and this was accompanied by the increase in the presence of inflammatory cells along with a decrease in the amount of collagen (Mallat et al., 2001). This data thereby indicates that TGFβ normally functions to decrease inflammation while contributing to matrix production of smooth muscle cells, which may lead to plaque stabilization.

v. Apoptosis and Senescence in Modulation of Smooth Muscle Cell Phenotype during Vascular Remodeling

Another level of regulation of smooth muscle cell phenotypic modulation can be seen when examining the role of apoptosis, or programmed cell death, in vascular remodeling. Indeed several studies have illustrated the importance of apoptosis during vascular wall remodeling in multiple cardiovascular diseases including hypertension, restenosis, aneurysms and atherosclerosis (Bennett et al., 1995; Bochaton-Piallat et al., 1995; Hamet et al., 1995; Korshunov and Berk, 2008; Lopez-Candales et al., 1997). For example, one study demonstrated that

compared to symptomatic atherosclerotic plaques, asymptomatic plaques have decreased expression of the antiapoptotoic factors cIAP2, xIAP and survivin, but the plaques have higher expression of SM-MHC, proliferating cell nuclear antigen (PCNA) and p50 subunit of nuclear factor-κB (NF-κB) (Korshunov and Berk, 2008; Moran and Agrawal, 2007).

Recent studies have also determined that MMPs have a role in propagating vascular smooth muscle apoptosis during vascular remodeling. The collagen fragments released by MMPs are thought to mediate the calpain-dependent inactivation of the antiapoptotic factors xIAP (Korshunov and Berk, 2008; von Wnuck Lipinski et al., 2006b). However, another study shows that vascular smooth muscle cells appear to be protected from apoptosis by degraded collagen though αvβ3 integrin-mediated activation of NFκB and IAPs (Korshunov and Berk, 2008; von Wnuck Lipinski et al., 2006a). Despite the discrepancy in results, both studies illustrate the importance of the extracellular matrix in regulating vascular smooth muscle cell apoptosis.

In addition to the vital role of the extracellular matrix in the phenotypic modulation of smooth muscle cells during vascular remodeling, other studies indicate that oxidative stress and inflammation have roles in this process. For example, during neointima formation and transplant vasculopathy, a NFκB-dependent gene, *A20*, was shown to have dual roles. Not only did *A20* have anti-inflammatory and antiapoptotic effects on medial vascular smooth muscle cells (increases in p21 and p27), but it also had sensitizing effects of neointimal vascular smooth muscle cells to apoptosis through a nitric-oxide-synthase-

dependent pathway (Daniel et al., 2006; Korshunov and Berk, 2008; Patel et al., 2006).

Not only has apoptosis been shown to have a critical role in vascular smooth muscle cell phenotypic modulation during vascular remodeling, but senescence has also been shown to have a role in this process, specifically in atherosclerosis. The state of cellular senescence is defined as irreversible cell cycle arrest and the exhaustion of replicative potential (Gorenne et al., 2006; Hayflick, 1965). Vascular smooth muscle cells undergoing senescence have an enlarged, flattened and stellate morphology with increased presence of cytoplasmic vacuoles and lysosomes (Goldstein, 1990; Gorenne et al., 2006). In addition, these senescent cells have shorter telomeres and express high levels of markers of senescence, such as senescence-associated β-galactosidase (SAβG), and cell cycle regulators, such as p16 and p21 (Gorenne et al., 2006). Importantly, all of these characteristics of senescence have been demonstrated to be present in the medial vascular smooth muscle cells of atherosclerotic plaques (Bauriedel et al., 1999; Bennett et al., 1995; Gorenne et al., 2006; Mosse et al., 1985; Ross et al., 1984).

## D. Chromatin Remodeling Enzymes' Roles in Development and Differentiation

### i. Introduction to Types of Chromatin Remodeling Enzymes

Chromatin is defined as the entire complex of a cell's DNA and associated proteins (Berg, 2002). The building blocks of chromatin are the repeating nucleosomes, which consist of the histone octamer wrapped by approximately 145 base pairs of DNA and approximately 55 base pairs of linker DNA between nucleosomes. The histone octamer consists of four major histones: H2A, H2B, H3, and H4. Each of these histones forms a dimer within the octamer. Histone H1 is found in the linker DNA in front of the octamer (Berg, 2002).

*In vivo* chromatin exists in two states: euchromatin and heterochromatin. Euchromatin is classified as transcriptionally active, or open chromatin. This type of chromatin has irregularly spaced nucleosomes, and it is relatively accessible to nucleases and exhibits nuclease-hypersensitve sites that indicate the presence of sequence-specific DNA-binding proteins, or transcription factors (Gaszner and Felsenfeld, 2006). Another descriptive characteristic of euchromatin is the presence of highly acetylated histones; hence enzymes that acetylate histones, the histone acetyl transferases (HATs), aid in the propagation of euchromatin. Heterochromatin, on the other hand, is transcriptionally inactive, or closed, condensed chromatin. Heterochromatin has significantly reduced sensitivity to nuclease digestion as compared to euchromatin (Gaszner and Felsenfeld, 2006). In contrast to euchromatin, histones of heterochromatin are

mostly deacteylated, while instead they are hypermethylated at residues such as histone H3 at Lysine 9 and Lysine 27. In addition to inactivating histone modifications, such as methylation, heterochromatin also displays extensive CpG DNA methylation, indicating inaccessible regions of DNA (Gaszner and Felsenfeld, 2006). Whereas euchromatin is propagated by HATs, heterochromatin is spread through the activity of histone and DNA methyl transferases (HMTs and DMTs), as well as histone deacetylase complexes (HDACs).

Since its conception, the idea of chromatin remodeling has evolved into multiple definitions. In general chromatin remodeling can be defined as any event that alters the structure of a chromatin region to change histone-DNA interactions (Aalfs and Kingston, 2000; Lusser and Kadonaga, 2003). To accomplish this alteration of chromatin structure, two main mechanisms exist. In the first, ATP-dependent remodeling complexes use ATP as an energy source to rearrange nucleosomes (Aalfs and Kingston, 2000). Secondly, remodeling complexes covalently modify histone tails to change DNA-histone interactions and to alter the recruitment of other proteins to the nucleosomes (Aalfs and Kingston, 2000).

Processes such as DNA transcription, replication, recombination, and repair rely on this rearrangement of chromatin structure. For example, the promoter of a specific gene must be accessed by various transcription factors and the basal transcription machinery in order to initiate transcription. Chromatin remodeling complexes thus alter the structure of the chromatin to allow access to

these factors required for gene transcription. Whether a specific transcription factor binds to DNA at its consensus sequence and then recruits the chromatin remodeling complex to remodel chromatin allowing access to the basal transcription machinery, or whether the chromatin remodeling complex changes the conformation and then allows for the transcription factor to bind DNA largely remains to be elucidated. Initial observations suggest that this is likely to be different for each transcription factor analyzed and likely also gene specific. In general chromatin remodeling involves multiple steps, which might include numerous transcription factors, and possibly even several, distinct chromatin remodelers (Aalfs and Kingston, 2000).

Covalent modification of histones and the subsequent recruitment of specific binding proteins has led to the histone code hypothesis. This hypothesis states that specific post-translational covalent modifications of the histones' tails, including phosphorylation, ubiquitination, sumoylation, acetylation and methylation, function to recruit unique proteins either alone or in combination with other modifications, such as DNA modifications that regulate gene transcription, replication and repair (Jenuwein and Allis, 2001). For example, many regulatory proteins contain tandem bromodomains. The bromodomain preferentially binds to diacetylated histone tails at lysine residues on histone H3 or H4 (Jenuwein and Allis, 2001). Binding of such factors is thought to stabilize the chromatin structure and aid in activation of transcription. Whereas the bromodomain is recruited to acetylated histone tails, the chromodomain recognizes methylated histone tails. However, whether this recruitment supports transcriptional

activation or inactivation depends on the number of chromodomains within the regulatory protein. For instance, heterochromatin protein 1 (HP1) contains a single chromodomain and is recruited to the inactivating methylations such as methylated H3 at lysine 9 or 27 (Jenuwein and Allis, 2001). On the other hand, two tandem chromodomains are recruited to methylations on H3 at lysine 4, which are characteristic of sites of transcriptional activation (Jenuwein and Allis, 2001). In response to DNA double strand breaks, H2A.X – a variant of histone H2 – is phosphorylated and this serves as a signal to recruit regulatory proteins involved in DNA repair (Escargueil et al., 2008).

ii. ATP-dependent Chromatin Remodeling Enzymes

As mentioned in the previous section, a multitude of chromatin remodeling enzymes exist, but structurally three main categories exist: *(a)* DNA-modifying enzymes that methylate CpG-rich sequences, *(b)* histone-modifying enzymes that covalently modify histones by methylation, acetylation, phosphorylation, or ubiquitination, and *(c)* ATP-dependent chromatin remodeling complexes that reorganize nucleosomes (Sif, 2004).

The ATP-dependent chromatin remodeling enzymes all belong to the SNF2 family of DNA-dependent ATPases, meaning that they all have a helicase-like ATPase domain (de la Serna et al., 2006). Many of these enzymes are highly conserved across species and have numerous subfamilies. These subfamilies, or subclasses, are determined by the functional domains of each

enzyme. There are three major subclasses: the SWI/SNF family, the SWI (ISWI) family and the CHD family (de la Serna et al., 2006). The SWI/SNF family is characterized by the ability to bind to acetylated histones via their bromodomain. This group includes proteins such as brahma (BRM), brahma-like gene 1 (Brg1) and Snf2. The SWI (ISWI) family of proteins, on the other hand, contain not a bromodomain, but a SANT domain. The SANT domain is thought to bind to modified histones, and this family includes Isw1, Isw2, SNF2H and SNF2L. The third subclass, or CHD class, is the chromodomain and helicase-like domain family. As the name implies, this subclass is characterized by its two amino-terminal chromodomains that recognize methylated histones. This group contains the CHD proteins one through nine.

As an example of ATP-dependent chromatin remodeling, Brg1 and Brm1, the ATPase subunits of the SWI/SNF ATP-dependent chromatin remodeling complexes, will be briefly discussed. Recently our laboratory has demonstrated that Brg1 is required for MRTFA, a myocardin related transcription factor, to induce expression of smooth muscle specific genes in nonmuscle cells, but it does not affect expression of SRF-MRTFA-dependent immediate early genes (Zhang et al., 2007). MRTFA is unable to induce expression of smooth muscle specific genes in SW13 cells that lack Brg1 or in 3T3 cells expressing dominant negative Brg1. Reintroduction of Brg1 or Brm1 into SW13 restores their responsiveness to MRTFA (Zhang et al., 2007). In addition, Brg1 is necessary for MRTFA to increase binding of SRF to the promoters of smooth muscle genes in primary smooth muscle cells. These findings support the idea that ATP-

dependent chromatin remodeling enzymes, specifically Brg1 in this case, might stabilize active chromatin and allow for transcription factor binding to the promoters of smooth muscle-specific genes in order to activate transcription in smooth muscle cells.

iii. CHD Family and CHD8

Since the CHD family currently has nine members that are defined by their chromodomains, it is important to define a chromodomain. The chromodomain can be classified as a conserved region of approximately fifty amino acids that functions to bind DNA, RNA or methylated histones (Akhtar et al., 2000; Bouazoune et al., 2002; Cowell and Austin, 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007). Additionally, common to all CHD family members is the ATPase/helicase domain, which is very similar to the SWI2/SNF2 ATPase. Even though the chromodomain is the major domain that identifies these nine proteins, they can be further divided into subcategories based on their additional domains that are characteristic of their subfamily's function.

The first subfamily of CHD proteins includes CHD1 and CHD2. This subfamily is defined by the presence of a DNA-binding domain within the C-terminus, which preferentially binds to AT-rich regions of DNA (Delmas et al., 1993; Hall and Georgel, 2007; Marfella and Imbalzano, 2007; Stokes and Perry, 1995; Woodage et al., 1997). CHD1 has been the most described CHD protein to date. Studies have illustrated that CHD1 interacts with a number of

transcription factors. For instance, Kelley, et al., demonstrated that CHD1 colocalizes with the HMG box-containing protein and chromatin transcription coactivator, SSRP1 (Hall and Georgel, 2007; Kelley et al., 1999). This data provides evidence of CHD members' ability to associate with chromatin. In addition, Tai, et al., demonstrated via yeast 2-hybrid assays that CHD1 interacts with the transcriptional corepressor NCoRand, as well as with HDAC1. The interaction with HDAC1 indicated that CHD1 could be recruited by specific histone modifications (Hall and Georgel, 2007; Tai et al., 2003). CHD1 has been shown to interact with the histone acetyltransferase complexes SAGA and SILK to regulate transcription (Hall and Georgel, 2007; Pray-Grant et al., 2005). In addition, Pray-Grant, et al., determined that one of CHD1's two chromodomains was required for interaction specifically with histone H3 that is methylated at lysine 4 (Pray-Grant et al., 2005). Another function of CHD1 is to aid in transcriptional initiation and elongation by interacting with Paf1, a cofactor of polymerase II, or Rtf1, a regulator of transcriptional elongation, at regions of actively transcribed genes (Hall and Georgel, 2007; Simic et al., 2003).

The next subset of the CHD family includes CHD3 (a.k.a. Mi-2$\alpha$) and CHD4 (a.k.a. Mi-2$\beta$). These two members of the CHD family differ from the proteins in the first subset in that they lack the C-terminal DNA binding domain but have paired PHD (plant homeo domain) zinc-finger-like domains in the N-terminus (Marfella and Imbalzano, 2007; Woodage et al., 1997). These two CHD family members have been implicated as being the central components of the nucleosome-remodeling and histone deacetylase NuRD complex (Hall and

Georgel, 2007). As a member of the complex, CHD3 interacts with the histone deactylases HDAC1 and HDAC2, and CHD4 binds these two proteins as well as Brg1, a component of the SWI/SNF remodeling complex (Bowen et al., 2004; Hall and Georgel, 2007; Shimono et al., 2003).

Much less is known about the members of the third and final subset of CHD proteins. This subfamily includes CHD5-CHD9, which is defined by the presence of BRK domains (Marfella and Imbalzano, 2007). CHD6 has been demonstrated to interact with polymerase II, and it is present at sites of mRNA synthesis, suggesting a role in RNA activation and/or transcription (Hall and Georgel, 2007; Lutz et al., 2006). Mutations in CHD7 appear to be critical for the development of CHARGE syndrome (coloboma of the eye, heart defects, atresia of the choanae, retardation of growth and/or development, genital and/or urinary abnormalities and ear abnormalities and deafness) (Hall and Georgel, 2007). CHD7 has also been implicated in the development of the human foregut (Brunner and van Bokhoven, 2005; Hall and Georgel, 2007). CHD9 (a.k.a. CReMM) has been shown to play a role in osteoblast development (Benayahu et al., 2007; Shur et al., 2006). As CHD8 is the main focus of this thesis dissertation, its known functions are now described in more detail.

Sakamoto, et al., originally discovered duplin, which is an N-terminal splice variant of CHD8, as a component of the wnt signaling pathway. Initially, duplin was cloned through its ability to bind dishevelled (Dvl) in a yeast two-hybrid screen; however, after further investigation they determined that duplin bound directly to the Armadillo repeats of β-catenin, rather than to Dvl (Sakamoto

et al., 2000). Duplin's interaction with β-catenin within the nucleus of intact cells was found to inhibit wnt signaling by preventing β-catenin from binding to TCF4 (Sakamoto et al., 2000). Duplin was also found to be able to inhibit β-catenin's ability to induce axis duplication in Xenopus (Sakamoto et al., 2000). Recently, Thompson, et al., also demonstrated direct binding of CHD8 to β-catenin and that this interaction required the Armadillo repeats of β-catenin (Thompson et al., 2008). They also showed that CHD8 localized to the promoter of β-catenin target genes Axin2, Dkk1 and Nkd3 and functioned to inhibit transcription of these genes under normal conditions, as loss of CHD8 caused modest, but significant increases in their transcription (Thompson et al., 2008).

Further studies from the Kikuchi group determined that the nuclear localization of duplin itself was essential for its inhibitory effects on β-catenin signaling. Kobayashi, et al., found that importin α bound to duplin at the basic amino acid clusters located from amino acids 500-584 of duplin (Kobayashi et al., 2002). Importantly, when this region of duplin was deleted, the authors noted that duplin was no longer expressed in the nucleus, rather it remained in the cytoplasm. In addition, deletion of duplin's amino acids 500-584 allowed for binding to β-catenin; however, this construct could no longer prevent wnt-dependent activation of TCF-dependent transcriptional targets (Kobayashi et al., 2002). Hence, this data illustrates that duplin must be located in the nucleus to inhibit β-catenin signaling and that duplin's interaction with importin α is most likely critical for this location.

Yamashina, et al., conducted a yeast two-hybrid screen of a mouse cDNA library, using the C-terminal half of duplin as bait (duplin amino acids 482-749). As a result, the authors identified PIAS3, a SUMO E3 ligase and negative regulator of STAT3 (Yamashina et al., 2006). This interaction was confirmed to occur within intact cells and was localized to the nucleus (Yamashina et al., 2006). Since PIAS3 has the ability to sumoylate proteins, they investigated whether duplin could be sumoylated and found that $Lys^{609}$ of duplin was indeed sumoylated; however, this modification had no effect on duplin's function in the wnt signaling pathway (Yamashina et al., 2006). In contrast, duplin inhibited LIF-dependent STAT3 activity without preventing the phosphorylation and nuclear transportation of STAT3 (Yamashina et al., 2006). Using electromobility shift assays (EMSA), it was shown that duplin inhibited STAT3 transcriptional activity by preventing STAT3 from binding to DNA, and duplin itself was also shown to directly interact with STAT3. Yet, although both duplin and STAT3 can be sumoylated, duplin regulates STAT3 independent of this modification (Yamashina et al., 2006).

Since duplin was shown to play a role in the wnt signaling pathway which is critical in development, a duplin knockout model was developed to determine whether duplin was also important in development. It is important to note that although the previous studies with duplin may not necessarily translate into the same results for full-length CHD8 due to differences in functional domains, this knockout model would result in the loss of both duplin and CHD8 (Nishiyama et al., 2004). Homozygous duplin knockouts were viable until embryonic day 8.5

and were completely resorbed by embryonic day 9.5 (Nishiyama et al., 2004). The duplin$^{-/-}$ embryos failed during gastrulation and lacked a primitive streak and mesodermal layer (Nishiyama et al., 2004). In addition, these duplin$^{-/-}$ embryos exhibited massive amounts of apoptosis as visualized by TUNEL staining (Nishiyama et al., 2004).

CHD8 has also been shown to function independent of wnt signaling to regulate boundary elements and to have an insulator function through an interaction with CTCF (Ishihara et al., 2006). This study found that CHD8 bound directly to the zinc finger region of the chromatin insulator CTCF through the Brk domains of CHD8. Through this interaction, CHD8 was able to locate to known CTCF binding sites, such as the differentially methylated region (DMR) of H19 and the promoters of BRCA1 and c-myc, as visualized by chromatin immunoprecipitation assays (Ishihara et al., 2006). CHD8 through its interaction with CTCF was found to be required for the imprinted expression of IGF2 and to be essential for the insulation of the H19 DMR region (Ishihara et al., 2006). CHD8 was also shown to participate in the prevention of spreading of CpG methylation or condensed chromatin in the BRCA1 and c-myc promoters, respectively (Ishihara et al., 2006). Thus, these studies revealed a direct role of CHD8 in chromatin remodeling. Furthermore, Thompson, et al., demonstrated that CHD8 has ATPase activity and mediates ATP-dependent remodeling of nucleosomes (Thompson et al., 2008).

## E. Rationale

It is widely accepted that one mechanism by which SRF can distinguish between different pathways is through pathway-specific cofactor interactions. In order to determine whether SRF was regulated by additional cofactors, our laboratory conducted a yeast two-hybrid screen, using SRF as bait. From this screen we identified the N-terminus of chromodomain helicase DNA binding protein 8 (CHD8). As described above, CHD8 is an ATP-dependent chromatin remodeling enzyme. Our laboratory has previously demonstrated that another ATP-dependent chromatin remodeling enzyme, Brg1, regulates SRF's activity in smooth muscle cells. It is also well known that SRF is crucial for smooth muscle development and plays an important role under physiological and pathological conditions. This dissertation, thus, focuses on this newly identified SRF cofactor, CHD8, and determines its role in transcription regulation in smooth muscle cells.

## F. Hypothesis

CHD8 regulates the chromatin structures of SRF-dependent genes to affect the phenotype of vascular smooth muscle cells.

# CHAPTER II

## Methods

### A. RNase Protection Assay

The RPA III™ protocol from Ambion was used to perform ribonuclease protection assays. Briefly, a probe was designed to span the alternatively spliced exons that give rise to duplin and CHD8 such that the duplin mRNA will protect a 318 nt fragment of the probe; whereas, CHD mRNA will protect a 150 nt fragment. Mouse brain mRNA was used as a template for RT-PCR and the resultant fragment was cloned, sequenced and used as the probe for RNase protection analysis. Twenty-five µg of RNA from each mouse tissue were subject to RNase protection analysis.

### B. GST-pull Down Assays

A series of SRF deletion constructs fused to GST, which were described previously (Herring et al., 2001), were utilized. In addition, a series of CHD8 truncations were constructed in the pET vector to perform the reverse experiment; CHD8 truncations employed in experiments are indicated in the figure legends. As it was previously shown that the N-terminal fragment of CHD8 is sufficient to bind to SRF, further deletions of this region were be generated by PCR and cloned into pET28, which has a T7 epitope tag. Bacterial proteins were

isolated, and GST-pull down assays were conducted as previously described (Herring et al., 2001). Binding interactions were visualized using standard western blot techniques with primary antibodies against GST, T7 for pET vector fusion proteins.

C. Co-immunoprecipitation Assays

All coIP assays from mammalian cells were performed using the Active Motif® Nuclear coIP Kit, according to the protocol suggested by the manufacturer with a few minor adjustments. Lysates were precleared for one hour with 50 μL of EZ view Protein A Sepharose (Sigma) beads (PAS) in 900 μL of IP wash buffer. PAS beads were washed twice for 5 minutes in IP wash buffer with 1 mg/ml BSA and once for 5 minutes in wash buffer without BSA prior to addition to the antibody-lysate mixture. Both SRF and CHD8 were immunoprecipitated from 500 μg of nuclear extract with approximately 5 μg of protein-specific antibody or IgG control. All conditions during the co-immunoprecipitation were low stringency. Antibodies used for immunoprecipitation included SRF (Santa Cruz) and CHD8 (Proteintech). Standard western blot techniques were used to visualize the presence of CHD8 (Bethyl antibody) or SRF. A10 vascular smooth muscle cells were utilized for collection of nuclear lysate. All experiments were repeated at least three times using independent nuclear lysates.

D. Cell Culture

All primary mouse cells and 10T1/2 mouse fibroblasts were cultured in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, and 50 Units/ 50 μg penicillin/streptomycin. A10 vascular smooth muscle cells (VSMCs) were cultured in DMEM supplemented with 20% FBS, 2 mM L-Glutamine, and 50 Units/ 50 μg penicillin/streptomycin. A10 cells were passaged every other day and plated at 1:3 dilutions until passage 23.

E. Adenovirus Construction and Cell Transduction

A previously published shRNA sequence directed to CHD8 was cloned into the AdenoX vector (BD Bioscience) (Ishihara et al., 2006). The virus was packaged and harvested in HEK cells, according to the manufacturer's protocol. Primary mouse colon, bladder or aortic SMC were be plated at a density of $5x10^4$ cells per well in 12-well plates and transduced with either CHD8 shRNA or control shRNA virus as previously described (El-Mounayri et al., 2005; Yin et al., 2006; Zhou and Herring, 2005). A minimum of triplicate wells of cells were infected with each virus, and all experiments were be repeated at least three times using independent preparations of smooth muscle cells.

F. Serum Starvation/Stimulation During Adenoviral Infection

Mouse passage 1 colon SMCs were transduced as described above. After 24 hours of transduction, media was replaced with DMEM supplemented with 0.5% FBS. After 24 hours in low serum media, the media on half of the samples was replaced with DMEM supplemented with 10% FBS. Cells were incubated for 30 minutes or 2 hours to stimulate SRF-dependent growth factor responsive genes. RNA was isolated after this time point for analysis of effects on SRF-dependent gene expression by quantitative RT-PCR.

G. TGFβ-induced Myofibroblast Differentiation

10T1/2 cells were plated in the afternoon at a density of $3x10^5$ cells per well in a standard 6-well format. After an overnight incubation at 37˚C with 5% $CO_2$, cells were transduced as previously described in the methods with adenoviral vectors for shRNA targeting CHD8 as well as an shRNA control. However, instead of adding fresh full-serum media after the initial 4-hour incubation, media supplemented with 0.5% FBS was added after the virus was aspirated. Following a 24-hour period at 37˚C with 5% $CO_2$, cells were either given fresh 0.5% FBS media, or treated with TGFβ1 at a final concentration of 2 ng/mL under low serum conditions. Cells were treated for 24 hours and then collected for analysis of gene expression by quantitative RT-PCR.

H. Quantitative RT-PCR (qRT-PCR)

For the RT-step, 0.5-1 μg of RNA was reverse-transcribed to cDNA using random hexamer primers (Invitrogen Superscript First-Stand Kit). A 1:10 dilution of the resulting cDNA was used in a quantitative PCR reaction, following the methods for a 25 μL, 2-step cycling reaction from the ABgene qPCR with SYBR green kit. Sequence-specific primers for SRF and its targets included the following: *CHD8* sense CCAGCTCCAGCTCCAGCAC; anti-sense CCTGCAGTAGCAGCAACTCAG; *SRF* sense GTTCATCGACAACAAGCTGC; anti-sense CTGTCAGCGTGGACAGCTCATAG; *cyclin D1* sense GCCAGAGGCGGATGAGAACAAGC; anti-sense GGTCACACTTGATGAC TCTGG; *EGR1* sense GAGCACCTGACCACAGAGTC; anti-sense CCACAAAGTGTTGCCACTGTTG; *α-actin* sense CCAGAGTGGAGAAA GCCCAGC; anti-sense GGCTGTGCTGTCTTCCTCTTCAC; *SM22α* sense CGAAGCCAGTGAAGGTGCCTGAGAAC; anti-sense CCCAAAGCCATTAG AGTCCTCTGCACTGC; *telokin* sense GACACCGCCTGAGTCCAACCTCCG; anti-sense GGCTTTTCCTCAGCAACAGCCTCC; *cMYC* sense CCACC AGCAGCGACTCTGAA; anti-sense CTGTGCGGAGGTTTGCTGTG; *36B4* sense GGACCCGAGAAGACCTCCTT; anti-sense TGCTGCCGTTGTCAAACACC; and Birc5/survivin sense CTGGCCCTTCCTGGAGGA; anti-sense CTCGGTAGG GCAGTGGATGA. Primers for cFos and vinculin were obtained from Qiagen (QT00147308 and QT00158319, respectively). Each of these primer sets spans introns in their respective genes.

I. Induction of Apoptosis

A10 vascular SMCs were plated at $5x10^4$ cells per well in 12-well plates transduced in quadruplicate with either CHD8 shRNA, SRF shRNA, or control shRNA virus as previously described (El-Mounayri et al., 2005; Yin et al., 2006; Zhou and Herring, 2005). After 72 hours of incubation at 37˚C with 5% $CO_2$, cells were treated with 0.2 mM $H_2O_2$ for 6 hours. Media was collected, and wells were washed twice with PBS, which was added to the collected media. The pooled media and washes were spun at 2,000 rpm for two minutes at room temperature to accumulate any non-adherent cells. The PBS and media were then aspirated and the pellet was washed once with PBS. In the meantime, 100 μL of RIPA lysis buffer was added to each well, and the cells were incubated on ice for 10 minutes. Cells were then scraped into the corresponding tube with the cell pellet and incubated on ice an additional 5 minutes. Tubes were then spun at 14,000 rpm for 2 minutes at 4˚C, and resulting supernatants were transferred to a fresh tube. BCA assays were performed to ensure equal loading during protein separation by SDS-PAGE. Standard western blot techniques were employed to visualize changes in protein expression.

J. Western Blotting

For apoptosis studies, 20 μg of protein were loaded for each sample during SDS-PAGE. Antibodies used include anti-SRF (Santa Cruz), anti-CHD8 (Bethyl), anti-phospho-H2A.X (Upstate), anti-caspase-3 (Cell Signaling), anti-cleaved-caspase-3 (Cell Signaling), anti-PARP (Santa Cruz), anti-GAPDH (Novus), anti-Bcl-2 (BD Biosciences), anti-Bcl-XL (Cell Signaling), anti-Mcl-1 (Abcam), and anti-non-muscle-MHCIIb (Covance). For GST Pull Down assays, antibodies used included anti-T7 (Novagen). GST was visualized via ponceau staining. Secondary antibodies were used accordingly and included goat-anti-rabbit-HRP and goat-anti-mouse-HRP.

# CHAPTER III

## CHD8 binds SRF, promotes the expression of smooth muscle-specific genes and protects smooth muscle cells from apoptosis

### A. Summary

Serum response factor (SRF) is a widely expressed protein that plays a key role in the regulation of smooth muscle differentiation, proliferation and migration. It is generally accepted that one mechanism by which SRF can distinguish between stimulating differentiation, proliferation or migration is through pathway-specific cofactor interactions. A novel SRF cofactor, chromodomain helicase DNA binding protein 8 (CHD8), was isolated from a yeast two-hybrid screen using SRF as bait. CHD8 is highly expressed in adult smooth muscle tissues. Co-immunoprecipitation assays from cells demonstrated binding of the full-length SRF and endogenous CHD8 within cells. Data from GST-pull down assays indicate that the N-terminus of CHD8 can interact directly with the MADS domain of SRF. Adenoviral-mediated knockdown of CHD8 in smooth muscle cells resulted in statistically significant 10-20% attenuation of expression of SRF-dependent, smooth muscle-specific genes. Knockdown of CHD8 did not affect the SRF-dependent induction of immediate early genes by serum. In contrast, knockdown of either SRF or CHD8 in A10 vascular smooth muscle cells resulted in a marked induction of apoptosis, characterized by increased phospho-H2A.X, cleaved PARP and activated caspase-3. These data

suggest that CHD8 may play a specific role in modulating SRF activity toward anti-apoptotic genes, thereby promoting smooth muscle cell survival.

B. Introduction

SRF is a broadly expressed transcription factor that regulates disparate processes under both physiological and pathological conditions (Miano, 2003). It has been implicated in a number of signaling pathways including the ras-raf-ERK-MAPK-cascade (Chai and Tarnawski, 2002; Johansen and Prywes, 1994), the RhoA small GTPase cascade (Chai and Tarnawski, 2002; Hill et al., 1995) and the muscle differentiation pathway (Miano et al., 2007; Owens et al., 2004; Pipes et al., 2006). In the MAPK pathway, growth factors or serum stimulate the phosphorylation of the ternary complex factor (TCF) family of proteins, such as Elk1, which interact with SRF. Upon this phosphorylation, transcription of immediate early genes (IEGs), such as cFos and Egr1, is up-regulatedd in order to promote proliferation (Treisman, 1994). RhoA signaling stimulates production of filamentous actin and activates the dissociation of cytoplasmic MKL1 from globular actin, leading to nuclear accumulation and activation of MKL1 (Miralles et al., 2003). Mkl1 then interacts with SRF in order to stimulate transcription of genes such as SRF itself, vinculin, and junB (Selvaraj and Prywes, 2004). Upregulation of these factors allows SRF to regulate processes such as differentiation, migration, and adhesion. SRF is also critical for the differentiation of skeletal, cardiac and smooth muscle lineages (Miano et al., 2007; Owens et al., 2004; Pipes et al., 2006). SRF regulates smooth muscle differentiation through its interaction with coactivators such as myocardin and the myocardin family members MKL1 (MRTFA) and MRTFB (Cen et al., 2004; Wang et al., 2001). In addition to its association with cofactors, the activity of SRF is also

regulated through modulation of nuclear translocation (Camoretti-Mercado et al., 2000; Chai and Tarnawski, 2002), phosphorylation-dependent changes in DNA binding (Chai and Tarnawski, 2002; Manak and Prywes, 1991) and alternative RNA splicing (Belaguli et al., 1999; Chai and Tarnawski, 2002; Kemp and Metcalfe, 2000). Global SRF knockout mice are embryonic lethal due to a failure in gastrulation. The $SRF^{-/-}$ embryos form a misfolded endoderm and ectoderm and fail to form the mesodermal layer, indicating that SRF is critical for formation of the mesoderm from which most muscle is derived (Arsenian et al., 1998). To circumvent this embryonic lethality a number of groups have specifically ablated SRF in different muscle tissues. These studies have shown that SRF is critical for the development of each muscle lineage (Angstenberger et al., 2007; Charvet et al., 2006; Mericskay et al., 2007; Niu et al., 2005). Using a conditional smooth muscle-specific knockout system SRF deletion in adult mice quickly resulted in the development of chronic intestinal pseudo-obstruction (CIPO) (Angstenberger et al., 2007; Mericskay et al., 2007). The CIPO was characterized by chronic intestinal dilation and defective peristatlsis due to a loss of contractile proteins, indicating that the expression of many contractile proteins is dependent upon SRF in adult mice.

In an attempt to identify other cofactors that regulate SRF activity we conducted a yeast two-hybrid screen, using SRF as bait (Herring et al., 2001). From this screen we identified the homeodomain protein Barx2 (Herring et al., 2001) and a cDNA encoding the N-terminus of chromodomain helicase DNA-binding protein 8 (CHD8). CHD8 is a member of the CHD family of ATP-

dependent chromatin remodeling proteins. CHD proteins, such as CHD8, contain two tandem chromodomains, an ATPase domain and a helicase domain. In addition to these domains, CHD8 contains two homeodomain-like domains. Duplin, an N-terminal splice variant of CHD8 orginally discovered as a β-catenin-binding protein (Sakamoto et al., 2000), contains only the first of the two chromodomains. Chromodomains are regions of 40-50 amino acids that are involved in chromatin remodeling and gene regulation (Cavalli and Paro, 1998). These domains can serve as protein interaction modules, RNA-binding modules, or DNA-binding modules (Akhtar et al., 2000; Bouazoune et al., 2002; Cowell and Austin, 1997). The chromodomains in CHD8 have been shown to specifically interact with histone H3 trimethylated at lysine 4 (Yuan et al., 2007). CHD8 has been shown to exhibit ATP-dependent chromatin remodeling activity and to repress β-catenin target genes (Thompson et al., 2008). CHD8 has also been shown to bind to the insulator binding protein CTCF (Ishihara et al., 2006), and the CHD8/CTCF complex has been shown to play an important role in the epigentic regulation of insulator sites. Duplin/CHD8 knockout mice (both duplin and CHD8 were knocked-out) are embryonic lethal and failed to form a primative streak or the mesodermal layer during gastrulation, indicating that CHD8/duplin play a critical role in early development (Nishiyama et al., 2004).

In the current study we demonstrate that CHD8 interacts with SRF and determined the effects of CHD8 on SRF activity in smooth muscle cells. Results from these studies suggest that CHD8 modestly affects expression of SRF-

dependent genes characteristic of differentiation, whereas CHD8 has a more significant role in protecting smooth muscle cells from apoptosis.

C. Results

i. CHD8 is more widely and abundantly expressed than duplin

From a yeast two-hybrid screen of a mouse intestinal cDNA library (Herring et al., 2001) we isolated 2 cDNA clones that encoded the amino terminal 529 amino acids of CHD8/duplin (***Figure 3***). These cDNA were used to rescreen lambda gt11 cDNA library made from mouse intestine. From this screen, 3 additional cDNAs were isolated. One of these extended further 3' and included sequence encoding amino acids 282 - 770 of CHD8. Sequences of these clones together with the sequence from image clone #683 (Invitrogen) were used to compile the full-length mouse CHD8 sequence. This sequence was found to correspond to the previously annotated mouse CHD8 sequence NM_201637.2. Duplin, originally discovered in *Rattus norvegicus* (NM_022933) (Sakamoto et al., 2000), and CHD8 are alternatively spliced transcripts of the CHD8 gene. Both transcripts are identical in their first 8 exons; however, they diverge in exon 9 (***Figure 2A and B***). CHD8 transcripts utilize an alternative splice donor site in exon 9 such that the stop codon encoded by the 3' portion of exon 9 is removed when the alternative donor site is spliced to exon 10. The most 3' cDNA that we isolated from our mouse intestinal cDNA library includes the exon 9-10 splicing pattern characteristic of CHD8 rather than duplin.

To further evaluate the expression of duplin and CHD8, RNase protection assays (RPA) were performed. A probe was constructed based on the *Mus*

*musculus* sequence difference between the isoforms in exon 9 (***Figure 2A and B***). Results from this anlaysis show that CHD8 is the predominant isoform expressed in all mouse tissues and duplin could only be detected at low levels in brain (***Figure 2C and D)***. The apparent lack of CHD8 in rat aortic A10 cells reflects species differences in the rat and mouse sequences that prevented its detection in this assay, as CHD8 can be readily detected by RT-PCR and western blotting in these cells (***Figure 3D***). As CHD8 is the only isoform detected in smooth muscle tissues and a cDNA encoding this isoform was isolated from mouse intestine the remainder of the study was focussed on CHD8.

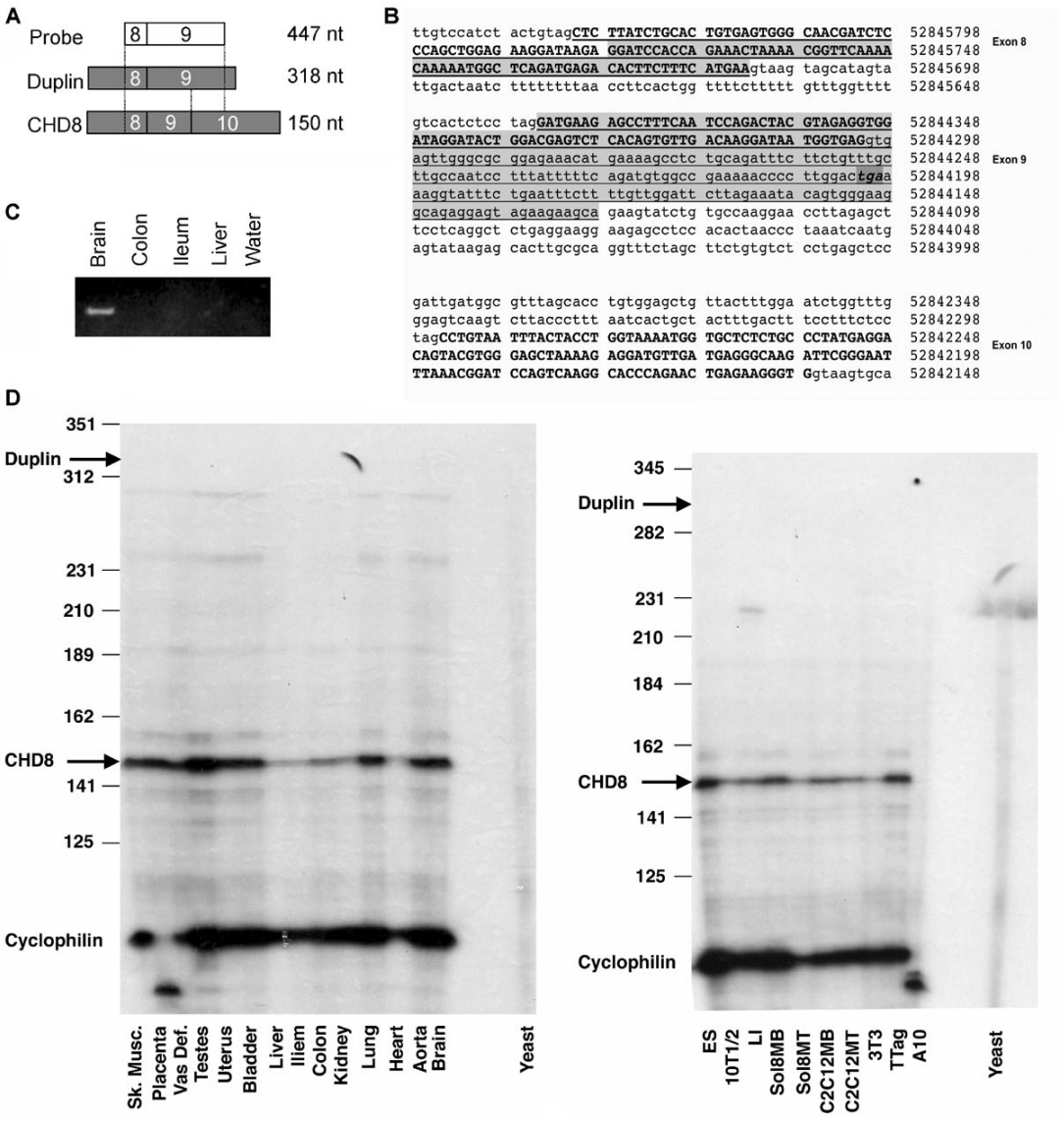


***Figure 2***. ***CHD8 is more ubiquitously expressed than duplin, its N-terminal splice variant.*** ***A.*** The probe used for RNase protection assays was designed using exons 8 and 9 of duplin as shown in panel 'B'. ***B.*** Genomic sequence encompassing CHD8 exons 8-10 as annotated from BLAT on 7-28-08 (only the partial sequence of the introns is shown). Exons of full-length CHD8 are shown in bold, exons included in duplin are underlined and the shaded region is the probe used for RNase protection assays. ***C.*** The probe shown in panel A was isolated by RT-PCR from RNA isolated from mouse brain. An ethidium bromide stained gel of the RT-PCR products are shown. ***D.*** Ribonuclease Protection Assays were conducted to determine the amount of full-length CHD8 mRNA relative to its splice variant, Duplin in various mouse tissues (left panel) and cell lines (right panel). Cyclophilin was used as an internal control; yeast RNA was used as a negative control (Yeast). Abbreviations are as follows: Sk. Musc. = skeletal muscle, Vas Def. = vas deferens, 10T1/2 = 10T1/2 mouse fibroblasts, LI = mouse colon smooth muscle cell line , A10 = A10 rat aortic smooth muscle, TTag = T-antigen derived colonic tumor cells, 3T3 = NIH 3T3 mouse fibroblasts, MB = myoblast, and MT = myotubes.

ii. CHD8 binds the MADS domain of SRF

To confirm our yeast two-hybrid result and to map the binding domains of SRF and CHD8, GST pull-down assays were performed. (***Figure 3A***) Results from this analysis demonstrate that CHD8 binds to the MADS domain of SRF, and SRF binds to two regions in the N-terminus of CHD8 between amino acid residues 282-773 (***Figure 3B-C***). SRF binds to a region between 282-483 of CHD8, and it also binds to the first of the two chromodomains of CHD8 – but this binding to the chromodomain is not required for binding to the 282-483 region. In order to confirm that CHD8 and SRF interact with each other within intact cells, co-immunoprecipitation (coIP) assays were performed in A10 cells transduced with HA-SRF adenovirus. Western blot analysis revealed that endogenous CHD8 co-immunoprecipitated with SRF in these smooth muscle cells (***Figure 3D***).

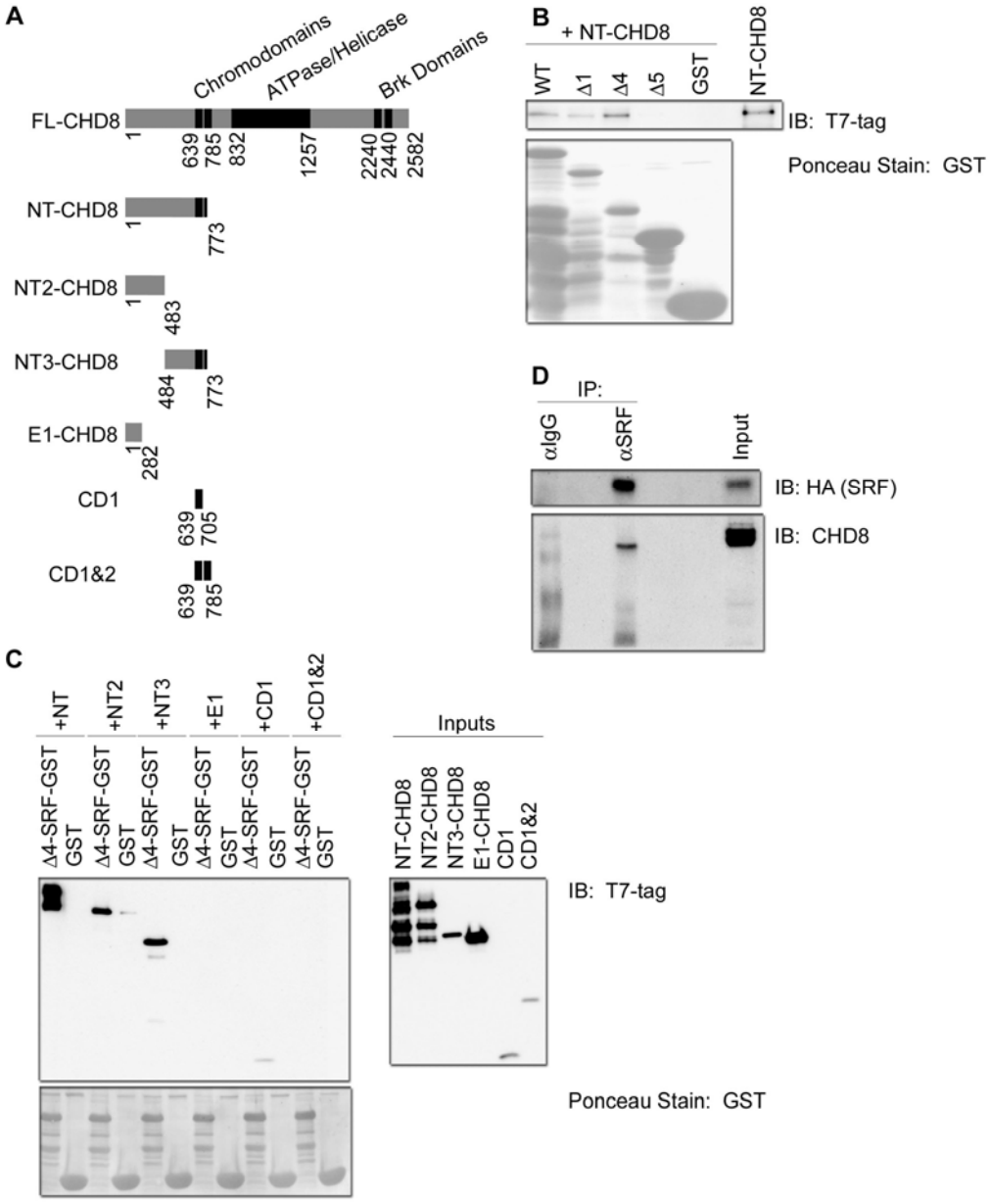


***Figure 3. SRF and CHD8 interact both in vitro and in vivo. A.*** Schematic of CHD8's structural domains and truncations used in experiments. Note that NT-CHD8 corresponds to the structure of duplin, described in previous sections. ***B.*** GST-WT-SRF, GST-SRF-Δ1, GST-SRF-Δ4, GST-SRF-Δ5 or GST alone were bound to glutathione beads and incubated with NT-CHD8 pET bacterial lysate to determine the minimal portion of SRF that binds to CHD8. ***C.*** GST-SRF-Δ4 was incubated with bacterial lysate of various truncations of CHD8. Following washing the glutathione bead bound proteins were analyzed by western blotting. Presence of GST proteins was confirmed by ponceau staining. n = 2 ***D.*** Proteins were immunoprecipitated with a SRF-specific antibody (Santa Cruz) or an IgG control from nuclear lysates of A10 cells that were transduced with HA-SRF AdenoX at an MOI of 100. Western blot analysis of the immunoprecipitated proteins was conducted using the primary antibodies specific for CHD8 (Bethyl) and HA-tag (Covance).

iii. CHD8 knockdown attenuates expression of SRF-dependent genes in smooth muscle cells

In order to investigate the function of CHD8 in smooth muscle, CHD8 was knocked-down in primary mouse smooth muscle cells, using an adenoviral-encoded shRNA and the subsequent effects on expression of SRF-dependent genes were determined by quantitative RT-PCR. Using this approach we obtained an approximately 75% knockdown of CHD8 in primary cells isolated from mouse colon, bladder and aorta (***Figure 4***). In both bladder and colon smooth muscle cells, loss of CHD8 caused a small but significant 10-20% attenuation in expression of differentiation genes, such as telokin, smooth muscle-MHC, SM22$\alpha$, calponin, and SM $\alpha$-actin (colon only) (***Figure 4A and B***). In aortic SMC CHD8 knockdown did not result in any significant changes in the expression of smooth muscle-specific genes (***Figure 4C***). Expression of SRF and cFos were more variable, with neither being changed following CHD8 knockdown in bladder cells, whereas both were attenuated in aortic cells and cFos but not SRF was attenuated in colonic smooth muscle cells. In addition, vinculin expression was significantly attenuated by CHD8 knockdown in each of the smooth muscle cell types.

Because of the variable affects of CHD8 knockdown on SRF-dependent early response genes we further determined whether CHD8 affected the serum stimulation of immediate early genes in primary colon smooth muscle cells. Knockdown of CHD8 was found not to affect the ability of serum to induce

expression of cFos or SRF (***Figure 5***). This data suggest that CHD8 is not required for serum induction of SRF-dependent growth/proliferation genes in colonic smooth muscle cells.

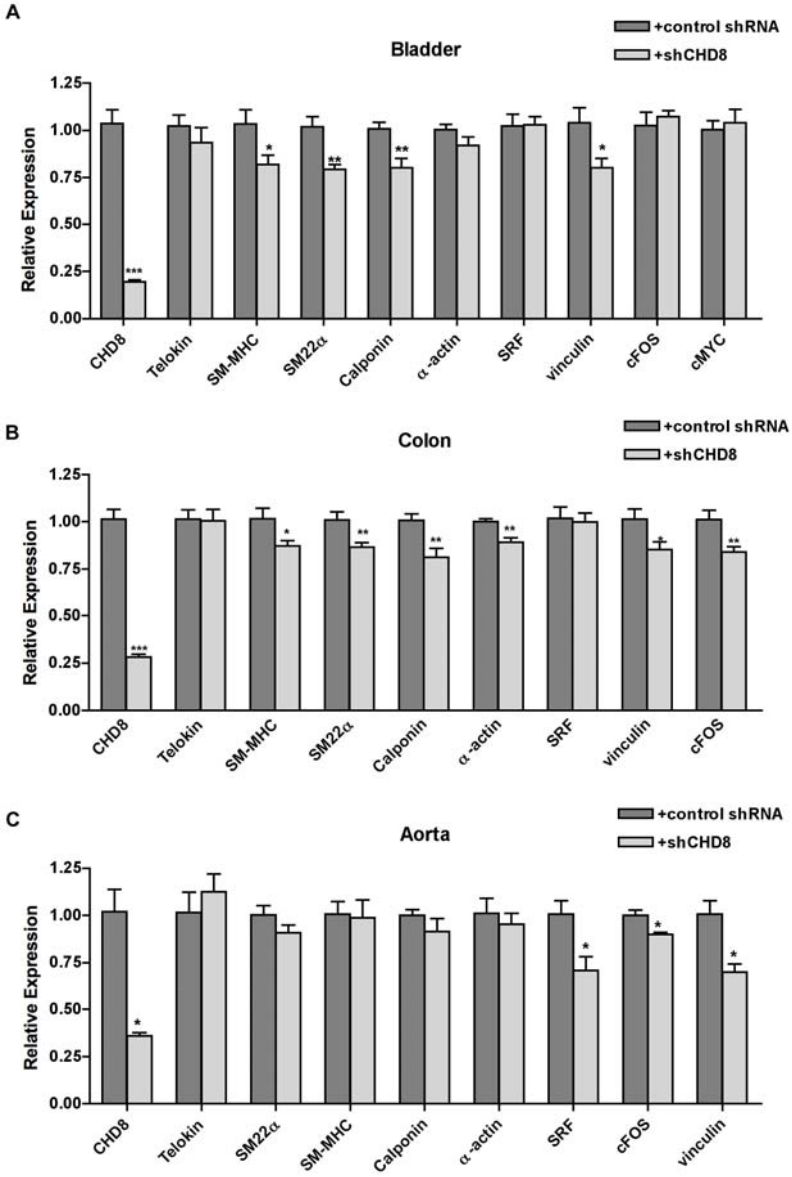


***Figure 4. Knockdown of CHD8 causes reduced expression of SRF-dependent genes.*** Passage 1 primary mouse smooth muscle cells were plated at a density of $5x10^4$ cells per well in 12-well plates. After 24 hours cells were transduced with an adenovirus containing either CHD8-specific shRNA or control shRNA. 72 hours after transduction, RNA was isolated using Trizol (Invitrogen). Quantitative RT-PCR was conducted using sequence-specific primers for the transcripts indicated ***A.*** In bladder SMC, loss of CHD8 affects SM-MHC, SM22$\alpha$, and vinculin by causing a significant decrease in transcript expression. All data are n = 17, except cFos where n = 13 and cMYC where n = 4. ***B.*** In colon SMC, loss of CHD8 causes a significant decrease in SM22$\alpha$, vinculin, and cFos transcript expression. All transcripts are n = 14. ***C.*** In aorta SMC, loss of CHD8 causes a significant decrease in SRF, cFos and vinculin transcripts. All transcripts are n = 4. For all cell types: *** $p < 0.0001$ versus control shRNA, ** $p < 0.001$ versus control shRNA and * $p < 0.05$ versus control shRNA.

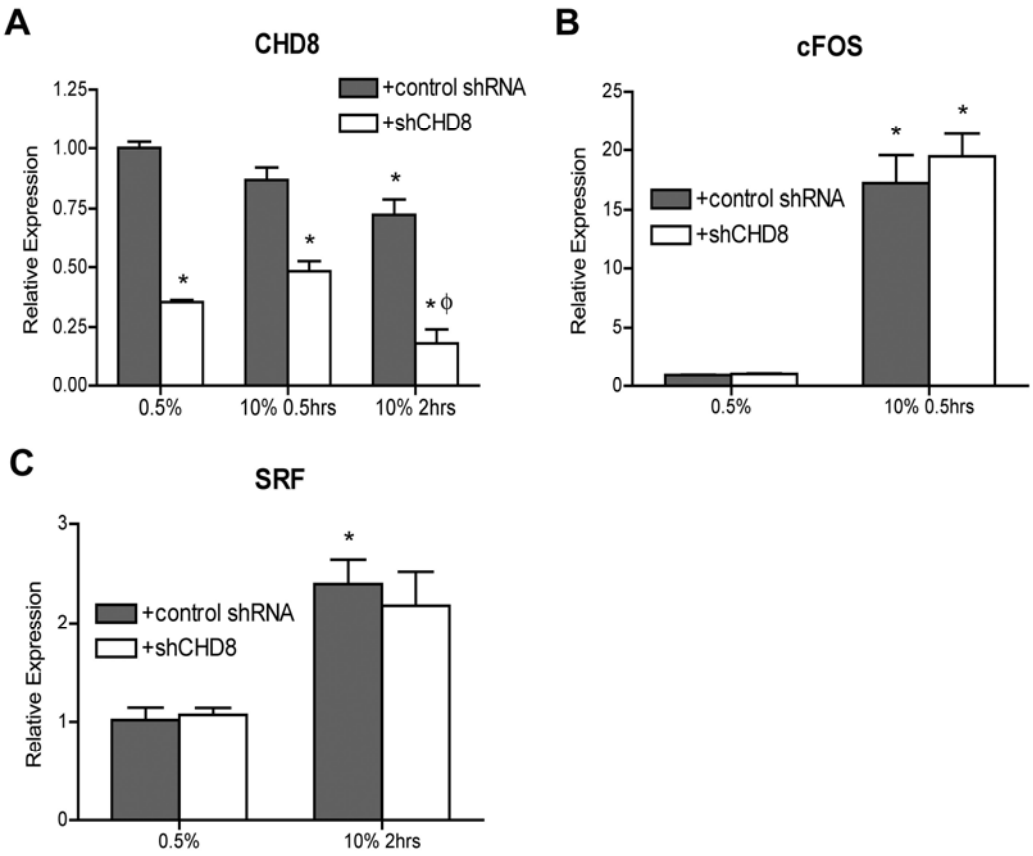


***Figure 5. Knockdown of CHD8 does not affect serum stimulation of immediate early or late early genes.*** Passage 1 primary mouse colonic smooth muscle cells were transduced with an adenovirus containing either CHD8-specific shRNA or control shRNA as described in ***Figure 4***. 24 hours after transduction, media was replaced with 0.5% FBS supplemented DMEM. Cells were serum starved for 48 hours. 10% FBS supplemented DMEM was then given to the cells for 0.5 or 2 hours. Cells kept in 0.5% media were used as controls. RNA was then isolated using Trizol (Invitrogen) and analyzed by quantitative real time RT-PCR. Results indicate that loss of CHD8 ***(A)*** did not affect serum stimulation of cFos ***(B)*** or SRF ***(C)***. n = 4. * $p < 0.05$ versus +shRNA control 0.05%. $\phi$ $p < 0.05$ versus +shRNA control 10% 2hrs.

iv. CHD8 does not affect induction of smooth muscle markers during myofibroblast differentiation

SRF is important for both smooth muscle and myofibroblast differentiation, hence, we next determined if CHD8 is required for myofibroblast differentiation. We utilized TGFβ treated 10T1/2 cells as a model for myofibroblast differentiation. We found that knockdown of CHD8 in this system did not prevent TGFβ-dependent induction of smooth muscle markers such as SM22α and SM α-actin (***Figure 6***).

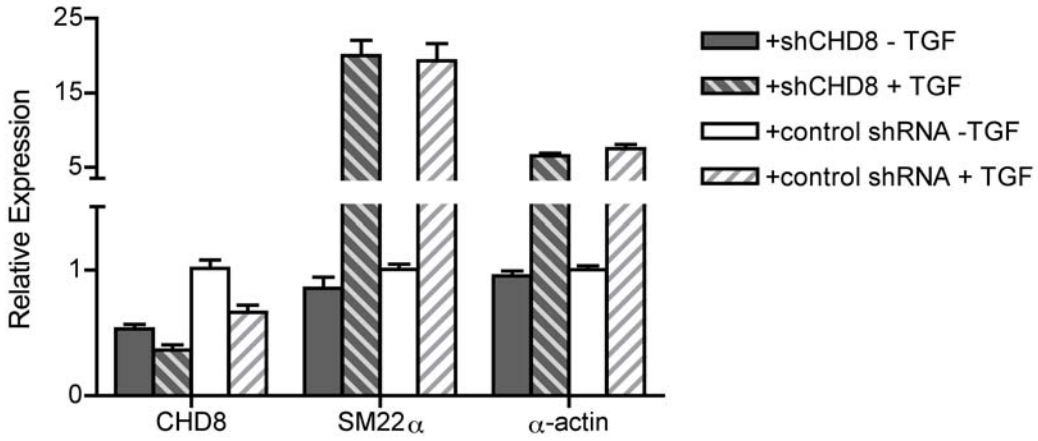


***Figure 6. Loss of CHD8 does not affect TGFβ-dependent stimulation of myofibroblast differentiation.*** 10T1/2 cells were plated at a density of 3 x $10^5$ cells per well in a standard 6-well plate. After an overnight incubation at 37˚C with 5% $CO_2$, cells were transduced with adenoviral vectors for shRNA targeting CHD8 as well as an shRNA control as described in ***Figure 4***. However, instead of adding fresh 10% serum media after the initial 4-hour incubation, media supplemented with 0.5% FBS was added after the virus was aspirated. 24 hours later cells were either given fresh 0.5% FBS media containing 1 μl/mL DMSO or TGFβ1 at a final concentration of 2 ng/mL. Cells were treated for 24 hours and then RNA extracted for analysis of gene expression by quantitative RT-PCR.

v. Loss of CHD8 induces apoptosis in A10 VSMC

Previous studies have linked SRF to apoptosis through its ability to activate the transcription of anti-apoptotic Bcl2 family members. SRF has been shown to be required for differentiation-dependent expression of Bcl-2 through a CArG sequence in the Bcl-2 promoter (Schratt et al., 2004). In addition, Vickers, et al., demonstrated that SRF is necessary for Mcl-1 transcription through its interaction with the transcription factor Elk-1 (Vickers et al., 2004). In order to determine if CHD8 plays a role in regulating apoptosis in smooth muscle cells we knocked down expression of CHD8 or SRF in A10 VSMC and induced apoptosis with $H_2O_2$. Analysis of markers of apoptosis such as phospho-H2A.X, caspase-3 activation and PARP cleavage, in these cells, revealed that knockdown of either SRF or CHD8 induced apoptosis in the absence of any additional stimuli (***Figure 7A***). Induction of apoptosis by $H_2O_2$ to mimic free radicals created during vascular injury further increased the levels of apoptosis in the knockdown cells, although $H_2O_2$ alone had very little affect in control cells. Surprisingly, neither SRF nor CHD8 depletion appeared to affect the expression of Bcl-2 (***Figure 7B***). While loss of SRF caused a small reduction in Mcl-1 expression, loss of CHD8 did not significantly affect its expression (***Figure 7B***). In addition, even though both SRF and CHD8 depletion cause apoptosis-dependent cleavage of Bcl-XL, its total expression appears unchanged (***Figure 7B***). These data indicate that CHD8 plays an anti-apoptotic role in A10 VSMC, but this is not due to its

regulation of expression of the known SRF-regulated anti-apoptotic genes in the Bcl2 family.

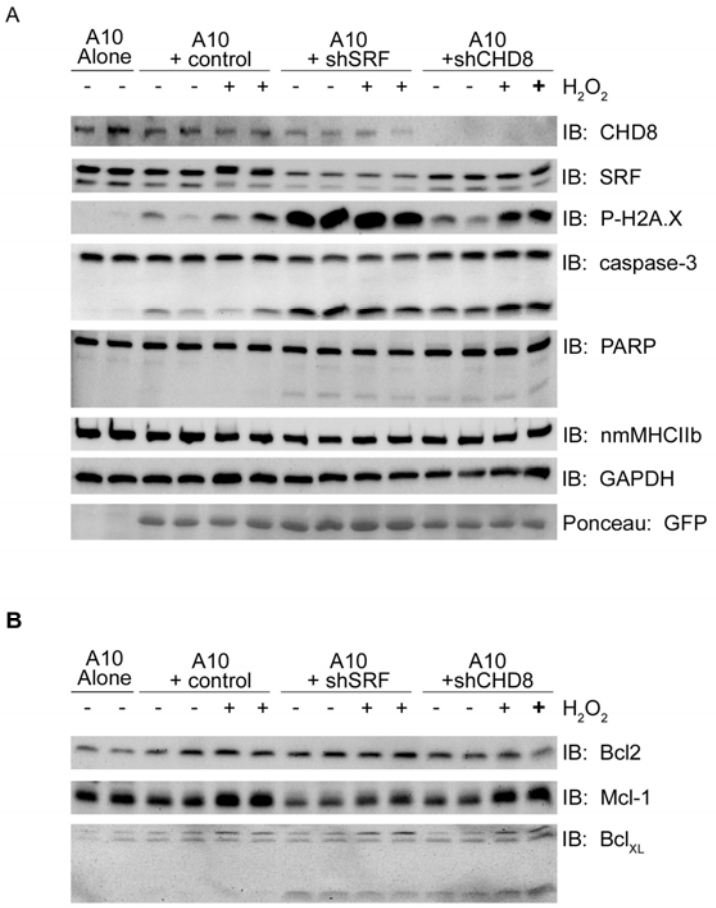


***Figure 7. CHD8 imparts a pro-survival effect on A10 vascular smooth muscle cells.*** A10 vascular SMCs were plated at $5x10^4$ cells per well in 12-well plates and transduced in quadruplicate with either CHD8 shRNA, SRF shRNA, or control shRNA adenovirus as previously described (El-Mounayri et al., 2005; Yin et al., 2006; Zhou and Herring, 2005). After 72 hours, cells were treated with 0.2 mM $H_2O_2$ for 6 hours. Both adherent and detached cells were washed twice with PBS, and lysed in 100 μL of RIPA lysis buffer. 20 μg of protein from each extract were separated on SDS-PAGE and analyzed by western blotting. ***A.*** Loss of CHD8 causes apoptosis as visualized by increased expression of apoptotic markers phospho-H2A.X, activated caspase-3 and cleaved PARP. NmMHCIIb and GAPDH serve as loading controls. Levels of apoptosis are similar to those in parallel knockdown of SRF. ***B.*** Knockdown of SRF appears to slightly decrease expression of Mcl-1; whereas, CHD8 knockdown did not consistently affect Mcl-1 expression.

To identify possible targets of CHD8 that could be regulating apoptosis in A10 smooth muscle cells we performed a PCR based array screen of 84 apoptosis related genes (SABiosciences catalog #PARN-012A). Results from this screen revealed a greater than two fold decrease in the expression of survivin (Birc5) in CHD8 knockdown cells (***Table 1***). These results were confirmed using qPCR with primers specific for Birc5/survivin, which also indicated a significant decrease in Birc5/survivin expression as a result of CHD8 knockdown (***Figure 8***). These data suggest that CHD8 may be important for regulating expression of Birc5/survivin and that attenuated expression of Birc5/survivin may be sufficient to induce apoptosis in A10 vascular smooth muscle cells.

| Symbol | Name | Pro- or anti-apoptotic | Fold Change: +shCHD8 v. +control |
|---|---|---|---|
| Aven_predicted | apoptosis, caspase activation inhibitor (predicted) | Anti | 2.75 |
| Birc5 | Baculoviral IAP repeat-containing 5, survivin | Anti | -2.2 |
| Card10_predicted | Caspase recruitment domain family, member 10 (predicted) | Anti | -4.67 |
| Casp1 | Caspase 1 | Pro | 3.73 |
| Casp7 | Caspase7 | Pro | -2.63 |
| Il10 | Interleukin 10 | Pro | -2.96 |
| Pycard | PYD and CARD domain containing | Pro | 2.24 |
| Faslg | Fas ligand (TNF superfamily, member 6) | Pro | 3.07 |
| Trp63 | Transformation related protein 63 | Pro | 2.57 |

***Table 1. Changes in apoptotic genes as a result of loss of CHD8.*** The rat apoptosis array from SuperArray (SABiosciences catalog #PARN-012A) was used to compare CHD8 knockdown to control A10 cells. Of the 84 key apoptosis genes analyzed on the array, those that showed a 2-fold or greater change between control shRNA and CHD8 shRNA treated cells are shown. A positive number for fold change indicates an up-

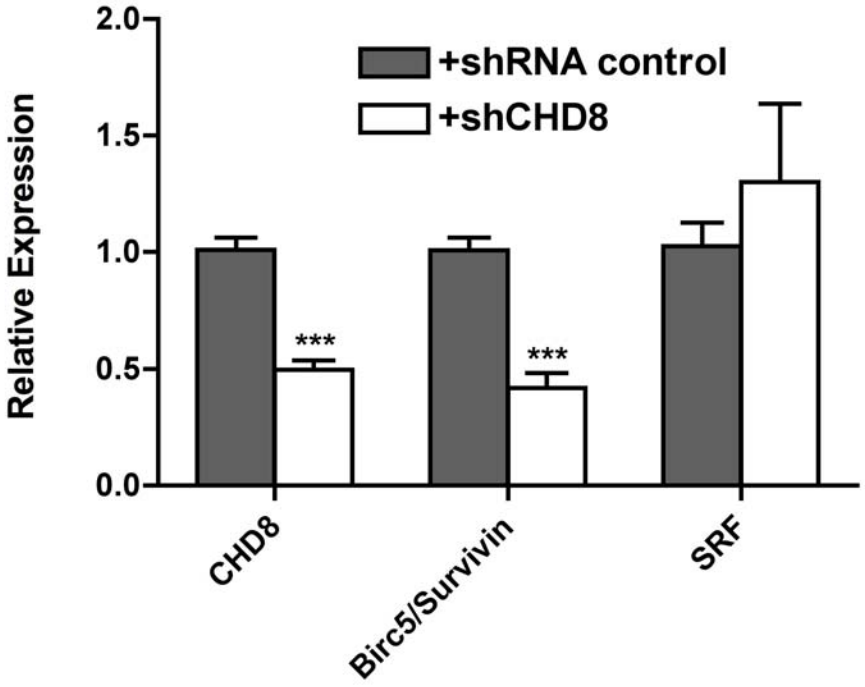


***Figure 8. Loss of CHD8 causes attenuated expression of Birc5/survivin.*** A10 VSMC were plated and infected as described in ***Figure 7***. qPCR with primers specific to Birc5/survivin was conducted in order to confirm a reduction in Birc5/survivin expression as a result of CHD8 knockdown in A10 cells; whereas, SRF transcript levels remained the same. n = 8. *** $p < 0.0001$

D. Discussion

Previous studies have demonstrated that SRF plays important roles in regulating genes required for differentiation, proliferation, migration and apoptosis (Chai and Tarnawski, 2002; Hill et al., 1995; Johansen and Prywes, 1994; Miano et al., 2007; Owens et al., 2004; Pipes et al., 2006; Schratt et al., 2004; Vickers et al., 2004). In the current study we found that SRF associates with the chromatin remodeling enzyme CHD8 in smooth muscle cells. CHD8 appears to moderately affect SRF's ability to regulate the genes involved in smooth muscle differentiation or migration and has no significant affect on the serum stimulation of SRF-dependent proliferation genes (***Figures 4 and 5***). In contrast, knockdown of CHD8 mimics knockdown of SRF in inducing apoptosis in smooth muscle cells (***Figure 7***).

Both CHD8 and its alternatively spliced product, duplin have been shown to directly interact with the Armadillo repeats of β-catenin in order to negatively regulate β-catenin-dependent TCF/LEF transcriptional activation (Sakamoto et al., 2000; Thompson et al., 2008). In the current study, we found that loss of CHD8 led to induction of apoptosis in A10 vascular smooth muscle cells. Even if this loss of CHD8 also resulted in de-repression of β-catenin, this could not explain the increase in apoptosis, as an active β-catenin signaling pathway is characteristic of proliferation and growth, not death.

Duplin has also been shown to negatively regulate STAT3 signaling (Yamashina et al., 2006). PIAS3, a SUMO E3 ligase that inhibits STAT3

signaling, was found to bind duplin. By binding to STAT3, duplin did not prevent STAT3 from translocating to the nucleus, but it was capable of inhibiting LIF-dependent binding of STAT3 to DNA, which subsequently prevented LIF/STAT3-dependent transcriptional activation (Yamashina et al., 2006). Even if CHD8 plays a similar role to duplin in repressing STAT3 signaling it is also unlikely that de-repression of this pathway would induce apoptosis. In contrast to duplin's inhibitory role in β-catenin and STAT signaling we found that CHD8 plays a positive role in smooth muscle cells. Loss of CHD8 modestly attenuated transcription of SRF-dependent target genes involved in differentiation (***Figure 4***), indicating that CHD8 would normally function to positively regulate transcription of these genes, possibly by aiding in recruiting SRF to DNA through CHD8's chromodomains interacting with histone H3 K4diMe.

Previously, CHD8 was shown to interact with the chromatin insulator CTCF. Through this interaction, CHD8 was able to function in the epigenetic regulation of the reciprocal transcription of H19 and IGF2, as well as to participate in preventing the spread of CpG methylation or condensed chromatin adjacent to the BRCA1 and c-myc promoters, respectively (Ishihara et al., 2006). The ability of CHD8 to participate in chromatin insulation and preventing the spread of CpG methylation may be important for maintaining SRF-dependent differentiation genes in an active chromatin confirmation. This could explain why CHD8 knockdown attenuates expression of these genes in smooth muscle cells (***Figure 4***). Alternatively CHD8 could affect expression of differentiation genes through its role as an ATP-dependent chromatin remodeling enzyme (Thompson

et al., 2008). In support of this proposal CHD8 has been shown to be present in complexes that also include the SWI/SNF chromatin remodeling complex (Thompson et al., 2008), and we have previously shown that the SWI/SNF complex plays a critical role in the induction of smooth muscle differentiation genes by the myocardin family of transcription activators (Zhang et al., 2007). Additional studies will be required to resolve these possibilities.

Loss of CHD8 resulted in a marked increase in apoptosis of A10 vascular smooth muscle cells, indicating that CHD8 normally plays an important survival function in these cells. This is consistent with the massive apoptosis that was observed in global CHD8 knockout embryos (Nishiyama et al., 2004). Studies have also demonstrated a role of SRF in regulating cell survival as embryonic stem cells that are devoid of SRF display increased amounts of apoptosis (Schratt et al., 2004). In this study the pro-survival activity of SRF was suggested to result from its ability to regulate differentiation-dependent transcription of the anti-apoptotic factor Bcl-2. SRF has also been shown to regulate expression of the anti-apoptotic factor Mcl-1 in both HeLa cells and EcR293, transformed human embryonic kidney cells (Vickers et al., 2004). In contrast to these studies, we did not observe any change in expression of Bcl-2 following knockdown of SRF or CHD8 in A10 vascular smooth muscle cells (***Figure 7***). Although we did observe a small decrease in Mcl-1 expression following SRF knockdown, knockdown of CHD8 did not significantly alter Mcl1 expression (***Figure 7***). However, results from a PCR microarray screen revealed that knock down of CHD8 significantly attenuated expression of the anti-apoptotic

protein survivin (Birc5) (***Table 1***), which was confirmed with qPCR (***Figure 8***). These data thus suggest that CHD8 may protect vascular smooth muscle cells from apoptosis by regulating expression of Birc5/survivin. Previous studies have shown that in a rabbit balloon injury model, Birc5/survivin is up-regulated 4 to 7 days post-injury in the medial smooth muscle cells, and Birc5/survivin expression peaked in the neointima and media at day 14 post-injury (Blanc-Brude et al., 2002). Importantly, Blanc-Brude, et al., demonstrated that after stimulating smooth muscle cells with serum, PDGF-AB or HB-EGF, Birc5/survivin expression was increased by 16-fold, 13-fold and 9-fold, respectively, with peak levels detected at 20 to 24 hours post-stimulation (Blanc-Brude et al., 2002). Together these data suggest that SRF/CHD8 mediated induction of Birc5/survivin, following vascular injury, may help protect medial smooth muscle cells from apoptosis.

In addition, apoptosis of vascular smooth muscle cells has been shown to induce plaque rupture, coagulation, vessel remodeling and calcification (Clarke et al., 2008). Hence the increased expression of survivin in intimal smooth muscle cells may play a critical role in promoting plaque stability. Since SRF plays a pleotropic role in pathways that regulate smooth muscle differentiation, migration, proliferation and apoptosis it is not an ideal target for therapeutic intervention. In contrast, as CHD8 only modest affects transcription of differentiation genes whereas it has prominent anti-apoptotic activity, perhaps CHD8 could provide a novel target for therapeutic intervention.

# CHAPTER IV

## Discussion and Future Studies

Overall, the data presented here indicates that CHD8 plays an important role in smooth muscle cell survival, possibly through the regulation of Birc5/survivin expression where loss of CHD8 attenuates Birc5/survivin expression. As CHD8 is a chromatin remodeling enzyme (Thompson et al., 2008) and we have previously shown that Brg1, another chromatin remodeler, can regulate SRF through its interaction with MRFTA (Zhang et al., 2007), it is credible to believe that CHD8 might also function in similar fashion. In addition, since CHD8 knockout mice are embryonic lethal as a result of massive apoptosis and a malfunction during gastrulation (Nishiyama et al., 2004), our results may provide important insights into the mechanisms underlying this apoptosis.

Several studies have illustrated the importance of apoptosis during vascular wall remodeling in multiple cardiovascular diseases (Bennett et al., 1995; Bochaton-Piallat et al., 1995; Hamet et al., 1995; Korshunov and Berk, 2008; Lopez-Candales et al., 1997) such as hypertension, restenosis, aneurysms and atherosclerosis. For example, studies demonstrate that compared to symptomatic atherosclerotic plaques, asymptomatic plaques have higher expression of SM-MHC, but decreased expression of the antiapoptotoic factors cIAP2, xIAP and survivin (Korshunov and Berk, 2008; Moran and Agrawal, 2007). In addition, increased expression of survivin has been shown to promote neointima formation following vascular injury (Blanc-Brude et al., 2002).

With regard to the mechanism by which CHD8 regulates apoptosis, we currently hypothesize that CHD8 regulates the transcription of SRF-dependent pro- or anti-apoptotic genes. In order to determine whether CHD8 does affect the transcription of apoptotic genes, I utilized the rat apoptosis RT$^2$Prolifer™ PCR Array from SuperArray (SABiosciences catalog #PARN-012A). Upon comparing samples obtained from A10 cells transduced with shRNA control to shRNA specific for CHD8, a total of nine factors out of the 84 tested, were found to change by 2-fold or more following knockdown of CHD8. Genes that were up-regulated by loss of CHD8 include Aven, caspase-1, Pycard, Fas ligand and Trp63 (***Table 1***). Genes that were down-regulated after loss of CHD8 include Birc5/survivin, Card10, caspase-7 and interleukin-10 (***Table 1***). After conducting a literature search, Birc5/survivn appears to be the most exciting potential target for further examination in that Blanc-Brude, et al., have previously demonstrated a role for Birc5/survivin in the regulation of smooth muscle cells during vascular injury (Blanc-Brude et al., 2002). Under basal conditions, Birc5/survivin expression is usually low, and Birc5/survivin is only expressed in the G2/M phase of the cell cycle in a cycle-regulated manner (Li et al., 1998). However, using a rabbit balloon injury model, studies have illustrated that 4 to 7 days post-injury Birc5/survivin is up-regulated in the medial smooth muscle cells, and Birc5/survivin expression peaked in the neointima and media at day 14 post-injury (Blanc-Brude et al., 2002). In addition, inhibition of survivin was found to suppress neointima formation following vascular injury. As our studies suggest that CHD8 is required for survivin expression we might predict that conditional

smooth muscle-specific CHD8 knockout mice may thus be protected from vascular injury.

Importantly, Blanc-Brude, et al., also demonstrated that after stimulating smooth muscle cells with serum, PDGF-AB or HB-EGF, Birc5/survivin expression was increased by 16-fold, 13-fold and 9-fold, respectively, with peak levels detected at 20 to 24 hours post-stimulation (Blanc-Brude et al., 2002). The fact that Birc5/survivin expression was up-regulated in smooth muscle cells due to serum stimulation could provide a link between survivin expression and SRF. In addition, the Blanc-Brude, et al., study illustrated that forced Birc5/survivin expression in smooth muscle cells suppressed apoptosis; whereas, inhibition of Birc5/survivin by a phosphorylation-defective mutant enhanced apoptosis induced by $C_2$ ceramide or TNF$\alpha$/CHX as visualized by caspase-3 and caspase-7 activation (Blanc-Brude et al., 2002). This data could help explain why loss of CHD8 in A10 cells caused caspase-3 activation in my current study, as my data indicates that loss of CHD8 reduces the levels of Birc5/survivin.

To extend and confirm these initial findings, I have designed RT-PCR primers for Birc5/survivin and have indeed confirmed that loss of CHD8 in A10 cells causes a significant decrease in Birc5/survivin expression (***Figure 8***). Additional experiments would need to be conducted to knockdown Birc5/survivin in order to determine whether this reduction in expression is sufficient to induce apoptosis specifically in A10 cells, though studies by Blanc-Brude, et al., indicate that it would be sufficient (Blanc-Brude et al., 2002). At this time, however, it is not clear how CHD8 regulates survivin expression. Two possible mechanisms

can be envisioned, either through direct activation of the promoter via SRF/CHD8 complexes or perhaps through inhibiting the methylation of the promoter through CHD8/CTCF interactions (***Figure 9***).

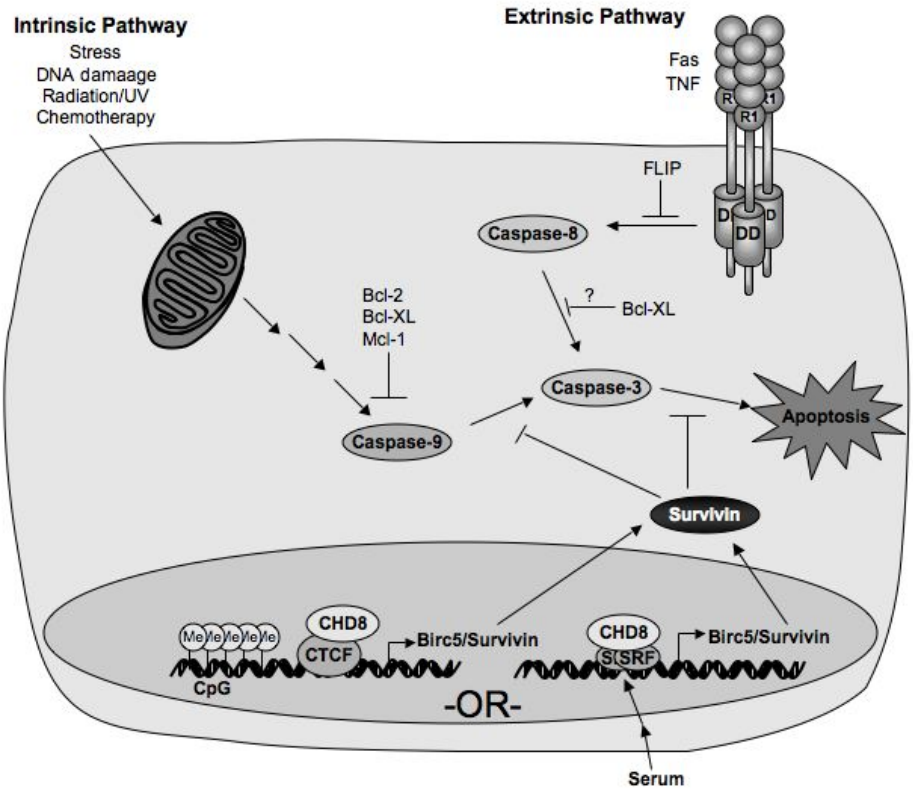


***Figure 9. Proposed model of CHD8's regulation of apoptosis through mediation of survivin expression.*** Two possibilities for CHD8's regulation of survivin expression exist: *(a)* by interacting with SRF to mediate transcription up-regulatedd by serum and *(b)* by interacting with CTCF to prevent the spread of CpG methylation.

Several experiments could be conducted to distinguish between these possibilities. Expression of Birc5/survivin first needs to be examined in A10 cells where SRF has been knocked-down to determine if SRF is required to regulate survivin expression. If loss of either SRF or CHD8 causes the same changes in survivin transcription, this would support the model in which CHD8 is acting through its interaction with SRF. In addition, in A10 VSMCs serum deprivation-stimulation assays similar to those conducted in ***Figure 5*** would be utilized under parallel CHD8 and SRF knockdown conditions to determine whether loss of either CHD8 or SRF prevents the upregulation of Birc5/survivin expression in response to serum. Again, this would help to provide a link between CHD8, SRF Birc5/survivin and apoptosis. Chromatin immunoprecipitation (ChIP) assays would be used to determine whether CHD8 and SRF are physically located at the Birc5/survivin promoter. Similarly, in order to elucidate if CHD8 is regulating Birc5/survivin expression via interaction with CTCF rather than SRF, ChIP assays would also be utilized to illustrate the presence of CTCF and CHD8 at the promoter. Additionally, we would compare the affects of knockdown of CTCF to determine if this also affects surviving expression. If CHD8 is regulating Birc5/survivin through its interaction with CTCF, then further experiments would be required in order to determine if this interaction is functioning to prevent the spread of CpG methylation. To such ends, either bisulfate sequencing or methyl-sensitive restriction enzyme analysis could be employed in control shRNA versus CHD8 shRNA infected A10 cells. Initially, experiments will focus specifically on Birc5/survivin, as it appears to be the most logical target; however, the other

genes identified by our array as being affected by loss of CHD8 will also be examined in further detail in a similar fashion as described for Birc5/survivin.

In addition to playing a role in SMCs, Birc5/survivin has been demonstrated to be highly expressed in various forms of cancer. For instance, Kawasaki, et al., found that 53.2% of colorectal tumors expressed high levels of Birc5/survivin and correlated with a poor prognosis (Kawasaki et al., 1998). Birc5/survivin can inhibit apoptosis by preventing caspase-3 and caspase-7 activation in the Fas ligand-dependent apoptotic pathway (Asanuma et al., 2004; Conway et al., 2002; Tamm et al., 1998). It would thus be interesting to determine whether CHD8 is also highly expressed in colon cancer cells. If CHD8 is highly expressed in these cancerous cells, studies could then be conducted to knockdown CHD8 in the colon cancer cells to determine if Birc5/survivin expression subsequently decreases and allows for the cells to become more susceptible to apoptosis.

Although I have shown that CHD8 knockdown attenuates survivin expression, it is possible that this alone is not sufficient to result in apoptosis. Alternatively it is possible that CHD8 knockdown induces apoptosis through promoting anoikis (or detachment-mediated apoptosis). In this model it would be predicted that CHD8 is regulating expression of SRF-dependent genes that are required for cells to attach to the extracellular matrix. Likely candidate target genes would include the integrins, at least some of which have been previously shown to be regulated by SRF, such as alpha-1, alpha-5, alpha-9 and beta-1 (Miano et al., 2007). Expression of these integrins would be examined after

CHD8 and SRF knockdown in parallel in order to determine if CHD8 is affecting their expression. ChIP assays would determine whether CHD8 is located at the promoters of the integrins affected. Additionally, adhesion experiments would be conducted in CHD8 and SRF knockdown cells in the presence and absence of zVAD-FMK, a global caspase inhibitor that functions by irreversibly binding to the catalytic region of the caspases. If loss of CHD8 causes apoptosis through anoikis, then one would predict that in the zVAD-treated CHD8 or SRF knockdown cells, although apoptosis is inhibited, the cells would still detach from the cell culture plate in a more significant number than the control cells.

As we initially identified CHD8 as an SRF associated protein, our first line of thought led us to believe that CHD8 would regulate SRF's activity in proliferation, migration, or differentiation. In contrast to the laboratory's previous dramatic results with loss of the Brg1 ATP-dependent chromatin remodeling enzyme, loss of CHD8 caused only a modest attenuation in transcription of SRF-dependent genes involved in differentiation, and did not affect serum stimulation of immediate early genes that are involved in proliferation. A possible explanation of these results is that perhaps the remaining amounts of CHD8 in this *in vitro* knockdown model are still capable of allowing for regular function of SRF signaling. Perhaps the effects of the loss of CHD8 would be more dramatic if CHD8 was completely ablated. This could be achieved in an *in vivo* model, such as a smooth muscle-specific conditional CHD8 knockout mouse. To generate these mice I would generate a floxed CHD8 allele in which exons 2 and 3 of CHD8 are flanked by loxP sites. This would generate a recombined allele in

which exon 1 is spliced to exon 4 resulting in incorporation of a stop codon at the very beginning of the aberrantly sliced exon 4. As I have shown that the protein product of exon 1 of CHD8 does not bind to SRF or have any other know functional domains, then the resulting product of such a recombined allele should have no function and will not interfere with normal SRF signaling in cells. In order to make the mice conditional and smooth muscle-specific, our lab could use a tamoxifen-regulated SM22$\alpha$-promoter-driven cre mouse line.

Another possible explanation as to why loss of CHD8 only modestly affected transcription of SRF-dependent differentiation genes is that perhaps another member, or members, of the CHD subfamily CHD5-CHD9 could be compensating for the function of CHD8. In order to determine whether this is true, it will be first necessary to examine expression of the other CHD proteins in A10 cells and smooth muscle tissues. Specific primers would need to be designed to distinguish between each CHD protein, and then mRNA expression could be analyzed via quantitative RT-PCR. Screening could also be conducted at the protein level using protein-specific antibodies for each CHD member, which would be visualized from nuclear fractions of A10 cells and smooth muscle tissue lysates by Western blotting techniques. Specific smooth muscle tissues of interest would include bladder, colon and aorta. If any of the CHD members 5-9 are expressed in one or all of these smooth muscle types, the next step would be to determine whether SRF forms a complex with any of the members. To this end, coIP assays would need to be conducted similar to those in this study when examining CHD8 and SRF interactions. With each protein found to interact with

SRF, subsequent co-knockdown assays would need to be conducted with CHD8. To do so, shRNA adenoviral constructs would be cloned from either previously published or newly constructed sequences that target each CHD protein. After viral particle packaging and amplification and harvesting, each construct would be co-infected with the shRNA specific for CHD8. In addition, combinations of multiple CHD proteins would be conducted if necessary (e.g. CHD8 shRNA + CHD7 shRNA + CHD9 shRNA). Quantitative RT-PCR would be used to examine effects of CHD8 knockdown alone versus the combinations on SRF-dependent genes.

If it is determined that one or more combination causes a more significant effect on SRF targets, then ChIP assays will be conducted to examine the presence of CHD8 and the newly identified CHD member at the promoters of the genes affected. In addition, as CHD8 has been shown to bind to histone H3 K4diMe and this modification is associated with transcription activation, I would also perform ChIP assays to examine the presence of this modified histone at the promoters of CHD8 target genes. Previous data also suggested that CHD8 was part of a large complex containing WDR5 and MLL1 (Dou et al., 2005). MLL1 is a histone methyltransferase that methylates histone H3 at K4. It is thus possible that this histone modification may not only help recruit CHD8 to the promoters of genes but that the CHD8 complex may also promote this activating histone modification. In this latter scenario it would be expected that CHD8 knockdown may decrease dimethylated H3 K4 at the promoters of its target genes.

In a separate study CHD8 was shown to be a member of a complex that was approximately 900 kD and contained WDR5, β-catenin and but not MLL1 (Thompson et al., 2008). This finding together with previous studies showing CHD8 as part of a complex with CTCF (Ishihara et al., 2006) and our studies showing that CHD8 can be found in a complex with SRF suggest that perhaps CHD8 is a member of multiple complexes. In other words, maybe not all CHD8 is associated with β-catenin, CTCF, WDR5, MLL1 and SRF in one large complex, but perhaps multiple large complexes exist under specific conditions and in different cell types. In order to determine whether other factors might be part of the CHD8/SRF complex in smooth muscle, coIP assays would be conducted to examine the presence of proteins such as CTCF and β-catenin in SRF immunoprecipitates. If another protein is found in the complex, ChIP assays could be conducted to determine whether this new protein is also located at the promoters of the CHD8/SRF-dependent genes. Luciferase assays could also be conducted to examine the effects of this protein on the activation of promoters of SRF-dependent genes, alone and in combination with SRF and/or CHD8. In addition, as described above, knockdown and co-knockdown with CHD8 could be performed in order to determine the affects of the protein on the transcription of SRF-dependent genes.

In summary, our current studies identify a novel function for chromodomain helicase DNA-binding protein 8 (CHD8) in protecting vascular smooth muscle cells from apoptosis. We have also shown that CHD8 attenuates expression of SRF-dependent differentiation genes in smooth muscle cells.

Even though we have begun to investigate the mechanism of these functions of CHD8, there is still much to be elucidated about the role of CHD8/SRF interactions in smooth muscle cells.

# REFERENCES


Aalfs, J. D., and Kingston, R. E. (2000). What does 'chromatin remodeling' mean? *Trends Biochem Sci*. *25*, 548-555.

Akhtar, A., Zink, D., and Becker, P. B. (2000). Chromodomains are protein-RNA interaction modules. *Nature*. *407*, 405-409.

Al-Aynati, M. M., Radulovich, N., Riddell, R. H., and Tsao, M. S. (2004). Epithelial-cadherin and beta-catenin expression changes in pancreatic intraepithelial neoplasia. *Clin Cancer Res*. *10*, 1235-1240.

Angstenberger, M., Wegener, J. W., Pichler, B. J., Judenhofer, M. S., Feil, S., Alberti, S., Feil, R., and Nordheim, A. (2007). Severe intestinal obstruction on induced smooth muscle-specific ablation of the transcription factor SRF in adult mice. *Gastroenterology*. *133*, 1948-1959.

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

Asanuma, K., Tsuji, N., Endoh, T., Yagihashi, A., and Watanabe, N. (2004). Survivin enhances Fas ligand expression via up-regulation of specificity protein 1-mediated gene transcription in colon cancer cells. *J Immunol*. *172*, 3922-3929.

Bauriedel, G., Hutter, R., Welsch, U., Bach, R., Sievert, H., and Luderitz, B. (1999). Role of smooth muscle cell death in advanced coronary primary lesions: implications for plaque instability. *Cardiovasc Res*. *41*, 480-488.

Behrens, J., and Lustig, B. (2004). The Wnt connection to tumorigenesis. *Int J Dev Biol*. *48*, 477-487.

Belaguli, N. S., Schildmeyer, L. A., and Schwartz, R. J. (1997). Organization and myogenic restricted expression of the murine serum response factor gene. A role for autoregulation. *J Biol Chem*. *272*, 18222-18231.

Belaguli, N. S., Zhou, W., Trinh, T. H., Majesky, M. W., and Schwartz, R. J. (1999). Dominant negative murine serum response factor: alternative splicing within the activation domain inhibits transactivation of serum response factor binding targets. *Mol Cell Biol*. *19*, 4582-4591.

Benayahu, D., Shacham, N., and Shur, I. (2007). Insights on the functional role of chromatin remodelers in osteogenic cells. *Crit Rev Eukaryot Gene Expr*. *17*, 103-113.

Bennett, M. R., Evan, G. I., and Schwartz, S. M. (1995). Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J Clin Invest*. *95*, 2266-2274.

Berg, J. M., John L. Tymoczo, and Lubert Stryer, ed. (2002). Biochemistry, Fifth edn (New York: W.H. Freeman and Company).

Bieker, J. J. (1996). Isolation, genomic structure, and expression of human erythroid Kruppel-like factor (EKLF). *DNA Cell Biol*. *15*, 347-352.

Bjorkerud, S. (1991). Effects of transforming growth factor-beta 1 on human arterial smooth muscle cells in vitro. *Arterioscler Thromb*. *11*, 892-902.

Blanc-Brude, O. P., Yu, J., Simosa, H., Conte, M. S., Sessa, W. C., and Altieri, D. C. (2002). Inhibitor of apoptosis protein survivin regulates vascular injury. *Nat Med*. *8*, 987-994.

Bochaton-Piallat, M. L., Gabbiani, F., Redard, M., Desmouliere, A., and Gabbiani, G. (1995). Apoptosis participates in cellularity regulation during rat aortic intimal thickening. *Am J Pathol*. *146*, 1059-1064.

Bostrom, H., Willetts, K., Pekny, M., Leveen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellstrom, M., Gebre-Medhin, S., Schalling, M.*, et al.* (1996). PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell*. *85*, 863-873.

Bouazoune, K., Mitterweger, A., Langst, G., Imhof, A., Akhtar, A., Becker, P. B., and Brehm, A. (2002). The dMi-2 chromodomains are DNA binding modules important for ATP-dependent nucleosome mobilization. *Embo J*. *21*, 2430-2440.

Bourdeau, A., Dumont, D. J., and Letarte, M. (1999). A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest*. *104*, 1343-1351.

Bowen, N. J., Fujita, N., Kajita, M., and Wade, P. A. (2004). Mi-2/NuRD: multiple complexes for many purposes. *Biochim Biophys Acta*. *1677*, 52-57.

Brunner, H. G., and van Bokhoven, H. (2005). Genetic players in esophageal atresia and tracheoesophageal fistula. *Curr Opin Genet Dev*. *15*, 341-347.

Camoretti-Mercado, B., Liu, H. W., Halayko, A. J., Forsythe, S. M., Kyle, J. W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J. E*., et al.* (2000). Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. *J Biol Chem*. *275*, 30387-30393.

Carson, J. A., Fillmore, R. A., Schwartz, R. J., and Zimmer, W. E. (2000). The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3-1, and serum response factor. *J Biol Chem*. *275*, 39061-39072.

Cavalli, G., and Paro, R. (1998). Chromo-domain proteins: linking chromatin structure to epigenetic regulation. *Curr Opin Cell Biol*. *10*, 354-360.

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

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

Chai, J., Baatar, D., and Tarnawski, A. (2004). Serum response factor promotes re-epithelialization and muscular structure restoration during gastric ulcer healing. *Gastroenterology*. *126*, 1809-1818.

Chai, J., Norng, M., Tarnawski, A. S., and Chow, J. (2007). A critical role of serum response factor in myofibroblast differentiation during experimental oesophageal ulcer healing in rats. *Gut*. *56*, 621-630.

Chai, J., and Tarnawski, A. S. (2002). Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. *J Physiol Pharmacol*. *53*, 147-157.

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

Chang, H., Huylebroeck, D., Verschueren, K., Guo, Q., Matzuk, M. M., and Zwijsen, A. (1999). Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development*. *126*, 1631-1642.

Charvet, C., Houbron, C., Parlakian, A., Giordani, J., Lahoute, C., Bertrand, A., Sotiropoulos, A., Renou, L., Schmitt, A., Melki, J.*, et al.* (2006). New role for serum response factor in postnatal skeletal muscle growth and regeneration via the interleukin 4 and insulin-like growth factor 1 pathways. *Mol Cell Biol*. *26*, 6664-6674.

Chen, J., Chen, H., Sanders, K. M., and Perrino, B. A. (2008). Regulation of SRF/CArG-dependent gene transcription during chronic partial obstruction of murine small intestine. *Neurogastroenterol Motil*. *20*, 829-842.

Chen, W. F., Huang, M. H., Tzang, C. H., Yang, M., and Wong, M. S. (2003). Inhibitory actions of genistein in human breast cancer (MCF-7) cells. *Biochim Biophys Acta*. *1638*, 187-196.

Clarke, M. C., Littlewood, T. D., Figg, N., Maguire, J. J., Davenport, A. P., Goddard, M., and Bennett, M. R. (2008). Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res*. *102*, 1529-1538.

Conway, E. M., Pollefeyt, S., Steiner-Mosonyi, M., Luo, W., Devriese, A., Lupu, F., Bono, F., Leducq, N., Dol, F., Schaeffer, P.*, et al.* (2002). Deficiency of survivin in transgenic mice exacerbates Fas-induced apoptosis via mitochondrial pathways. *Gastroenterology*. *123*, 619-631.

Cowell, I. G., and Austin, C. A. (1997). Self-association of chromo domain peptides. *Biochim Biophys Acta*. *1337*, 198-206.

Croissant, J. D., Kim, J. H., Eichele, G., Goering, L., Lough, J., Prywes, R., and Schwartz, R. J. (1996). Avian serum response factor expression restricted primarily to muscle cell lineages is required for alpha-actin gene transcription. *Dev Biol*. *177*, 250-264.

Dang, D. T., Zhao, W., Mahatan, C. S., Geiman, D. E., and Yang, V. W. (2002). Opposing effects of Kruppel-like factor 4 (gut-enriched Kruppel-like factor) and Kruppel-like factor 5 (intestinal-enriched Kruppel-like factor) on the promoter of the Kruppel-like factor 4 gene. *Nucleic Acids Res*. *30*, 2736-2741.

Daniel, S., Patel, V. I., Shrikhande, G. V., Scali, S. T., Ramsey, H. E., Csizmadia, E., Benhaga, N., Fisher, M. D., Arvelo, M. B., and Ferran, C. (2006). The universal NF-kappaB inhibitor a20 protects from transplant vasculopathy by differentially affecting apoptosis in endothelial and smooth muscle cells. *Transplant Proc*. *38*, 3225-3227.

de la Serna, I. L., Ohkawa, Y., and Imbalzano, A. N. (2006). Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. *Nat Rev Genet*. *7*, 461-473.

Delmas, V., Stokes, D. G., and Perry, R. P. (1993). A mammalian DNA-binding protein that contains a chromodomain and an SNF2/SWI2-like helicase domain. *Proc Natl Acad Sci U S A*. *90*, 2414-2418.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S., and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development*. *121*, 1845-1854.

Dou, Y., Milne, T. A., Tackett, A. J., Smith, E. R., Fukuda, A., Wysocka, J., Allis, C. D., Chait, B. T., Hess, J. L., and Roeder, R. G. (2005). Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF. *Cell*. *121*, 873-885.

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

El-Mounayri, O., Triplett, J. W., Yates, C. W., and Herring, B. P. (2005). Regulation of smooth muscle-specific gene expression by homeodomain proteins, Hoxa10 and Hoxb8. *J Biol Chem*. *280*, 25854-25863.

Escargueil, A. E., Soares, D. G., Salvador, M., Larsen, A. K., and Henriques, J. A. (2008). What histone code for DNA repair? *Mutat Res*. *658*, 259-270.

Fang, J. Y., and Richardson, B. C. (2005). The MAPK signalling pathways and colorectal cancer. *Lancet Oncol*. *6*, 322-327.

Ferns, G. A., Raines, E. W., Sprugel, K. H., Motani, A. S., Reidy, M. A., and Ross, R. (1991). Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science*. *253*, 1129-1132.

Frid, M. G., Kale, V. A., and Stenmark, K. R. (2002). Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ Res*. *90*, 1189-1196.

Galis, Z. S., Johnson, C., Godin, D., Magid, R., Shipley, J. M., Senior, R. M., and Ivan, E. (2002). Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. *Circ Res*. *91*, 852-859.

Galis, Z. S., and Khatri, J. J. (2002). Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res*. *90*, 251-262.

Galis, Z. S., Sukhova, G. K., Lark, M. W., and Libby, P. (1994). Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest*. *94*, 2493-2503.

Gaszner, M., and Felsenfeld, G. (2006). Insulators: exploiting transcriptional and epigenetic mechanisms. *Nat Rev Genet*. *7*, 703-713.

Goldstein, S. (1990). Replicative senescence: the human fibroblast comes of age. *Science*. *249*, 1129-1133.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. *183*, 1797-1806.

Gorenne, I., Kavurma, M., Scott, S., and Bennett, M. (2006). Vascular smooth muscle cell senescence in atherosclerosis. *Cardiovasc Res*. *72*, 9-17.

Grainger, D. J., Kemp, P. R., Metcalfe, J. C., Liu, A. C., Lawn, R. M., Williams, N. R., Grace, A. A., Schofield, P. M., and Chauhan, A. (1995). The serum concentration of active transforming growth factor-beta is severely depressed in advanced atherosclerosis. *Nat Med*. *1*, 74-79.

Greenberg, M. E., and Ziff, E. B. (1984). Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature*. *311*, 433-438.

Haldar, S. M., Ibrahim, O. A., and Jain, M. K. (2007). Kruppel-like Factors (KLFs) in muscle biology. *J Mol Cell Cardiol*. *43*, 1-10.

Hall, J. A., and Georgel, P. T. (2007). CHD proteins: a diverse family with strong ties. *Biochem Cell Biol*. *85*, 463-476.

Hamet, P., Richard, L., Dam, T. V., Teiger, E., Orlov, S. N., Gaboury, L., Gossard, F., and Tremblay, J. (1995). Apoptosis in target organs of hypertension. *Hypertension*. *26*, 642-648.

Hautmann, M. B., Thompson, M. M., Swartz, E. A., Olson, E. N., and Owens, G. K. (1997). Angiotensin II-induced stimulation of smooth muscle alpha-actin expression by serum response factor and the homeodomain transcription factor MHox. *Circ Res*. *81*, 600-610.

Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res*. *37*, 614-636.

Heldin, C. H., Ostman, A., and Ronnstrand, L. (1998). Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta*. *1378*, F79-113.

Heldin, C. H., and Westermark, B. (1990). Platelet-derived growth factor: mechanism of action and possible in vivo function. *Cell Regul*. *1*, 555-566.

Herring, B. P., Kriegel, A. M., and Hoggatt, A. M. (2001). Identification of Barx2b, a serum response factor-associated homeodomain protein. *J Biol Chem*. *276*, 14482-14489.

Hill, C. S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell*. *81*, 1159-1170.

Hillebrands, J. L., Klatter, F. A., van den Hurk, B. M., Popa, E. R., Nieuwenhuis, P., and Rozing, J. (2001). Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. *J Clin Invest*. *107*, 1411-1422.

Hirschi, K. K., and Majesky, M. W. (2004). Smooth muscle stem cells. *Anat Rec A Discov Mol Cell Evol Biol*. *276*, 22-33.

Holtz, M. L., and Misra, R. P. (2008). Endothelial-specific ablation of serum response factor causes hemorrhaging, yolk sac vascular failure, and embryonic lethality. *BMC Dev Biol*. *8*, 65.

Hoshino, Y., Kurabayashi, M., Kanda, T., Hasegawa, A., Sakamoto, H., Okamoto, E., Kowase, K., Watanabe, N., Manabe, I., Suzuki, T.*, et al.* (2000). Regulated expression of the BTEB2 transcription factor in vascular smooth muscle cells: analysis of developmental and pathological expression profiles shows implications as a predictive factor for restenosis. *Circulation*. *102*, 2528-2534.

Ishihara, K., Oshimura, M., and Nakao, M. (2006). CTCF-dependent chromatin insulator is linked to epigenetic remodeling. *Mol Cell*. *23*, 733-742.

Jackson, K. A., Majka, S. M., Wang, H., Pocius, J., Hartley, C. J., Majesky, M. W., Entman, M. L., Michael, L. H., Hirschi, K. K., and Goodell, M. A. (2001). Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. *107*, 1395-1402.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. *Science*. *293*, 1074-1080.

Johansen, F. E., and Prywes, R. (1994). Two pathways for serum regulation of the c-fos serum response element require specific sequence elements and a minimal domain of serum response factor. *Mol Cell Biol*. *14*, 5920-5928.

Kawasaki, H., Altieri, D. C., Lu, C. D., Toyoda, M., Tenjo, T., and Tanigawa, N. (1998). Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res*. *58*, 5071-5074.

Kelley, D. E., Stokes, D. G., and Perry, R. P. (1999). CHD1 interacts with SSRP1 and depends on both its chromodomain and its ATPase/helicase-like domain for proper association with chromatin. *Chromosoma*. *108*, 10-25.

Kemp, P. R., and Metcalfe, J. C. (2000). Four isoforms of serum response factor that increase or inhibit smooth-muscle-specific promoter activity. *Biochem J*. *345 Pt 3*, 445-451.

Kobayashi, M., Kishida, S., Fukui, A., Michiue, T., Miyamoto, Y., Okamoto, T., Yoneda, Y., Asashima, M., and Kikuchi, A. (2002). Nuclear localization of Duplin, a beta-catenin-binding protein, is essential for its inhibitory activity on the Wnt signaling pathway. *J Biol Chem*. *277*, 5816-5822.

Korshunov, V. A., and Berk, B. C. (2008). Smooth muscle apoptosis and vascular remodeling. *Curr Opin Hematol*. *15*, 250-254.

Kozaki, K., Kaminski, W. E., Tang, J., Hollenbach, S., Lindahl, P., Sullivan, C., Yu, J. C., Abe, K., Martin, P. J., Ross, R*., et al.* (2002). Blockade of platelet-derived growth factor or its receptors transiently delays but does not prevent fibrous cap formation in ApoE null mice. *Am J Pathol*. *161*, 1395-1407.

Lepore, J. J., Cappola, T. P., Mericko, P. A., Morrisey, E. E., and Parmacek, M. S. (2005). GATA-6 regulates genes promoting synthetic functions in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. *25*, 309-314.

Leptin, M. (2005). Gastrulation movements: the logic and the nuts and bolts. *Dev Cell*. *8*, 305-320.

Levin, R. M., Haugaard, N., O'Connor, L., Buttyan, R., Das, A., Dixon, J. S., and Gosling, J. A. (2000). Obstructive response of human bladder to BPH vs. rabbit bladder response to partial outlet obstruction: a direct comparison. *Neurourol Urodyn*. *19*, 609-629.

Li, D. Y., Sorensen, L. K., Brooke, B. S., Urness, L. D., Davis, E. C., Taylor, D. G., Boak, B. B., and Wendel, D. P. (1999). Defective angiogenesis in mice lacking endoglin. *Science*. *284*, 1534-1537.

Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C., and Altieri, D. C. (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*. *396*, 580-584.

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

Li, S., Chang, S., Qi, X., Richardson, J. A., and Olson, E. N. (2006). Requirement of a myocardin-related transcription factor for development of mammary myoepithelial cells. *Mol Cell Biol*. *26*, 5797-5808.

Liu, Y., Sinha, S., McDonald, O. G., Shang, Y., Hoofnagle, M. H., and Owens, G. K. (2005). Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. *J Biol Chem*. *280*, 9719-9727.

Lopez-Candales, A., Holmes, D. R., Liao, S., Scott, M. J., Wickline, S. A., and Thompson, R. W. (1997). Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms. *Am J Pathol*. *150*, 993-1007.

Lu, C., Shen, Q., DuPre, E., Kim, H., Hilsenbeck, S., and Brown, P. H. (2005). cFos is critical for MCF-7 breast cancer cell growth. *Oncogene*. *24*, 6516-6524.

Ludes-Meyers, J. H., Liu, Y., Munoz-Medellin, D., Hilsenbeck, S. G., and Brown, P. H. (2001). AP-1 blockade inhibits the growth of normal and malignant breast cells. *Oncogene*. *20*, 2771-2780.

Lusser, A., and Kadonaga, J. T. (2003). Chromatin remodeling by ATP-dependent molecular machines. *Bioessays*. *25*, 1192-1200.

Lutz, T., Stoger, R., and Nieto, A. (2006). CHD6 is a DNA-dependent ATPase and localizes at nuclear sites of mRNA synthesis. *FEBS Lett*. *580*, 5851-5857.

Majesky, M. W., Lindner, V., Twardzik, D. R., Schwartz, S. M., and Reidy, M. A. (1991). Production of transforming growth factor beta 1 during repair of arterial injury. *J Clin Invest*. *88*, 904-910.

Mallat, Z., Gojova, A., Marchiol-Fournigault, C., Esposito, B., Kamate, C., Merval, R., Fradelizi, D., and Tedgui, A. (2001). Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res*. *89*, 930-934.

Manak, J. R., and Prywes, R. (1991). Mutation of serum response factor phosphorylation sites and the mechanism by which its DNA-binding activity is increased by casein kinase II. *Mol Cell Biol*. *11*, 3652-3659.

Marfella, C. G., and Imbalzano, A. N. (2007). The Chd family of chromatin remodelers. *Mutat Res*. *618*, 30-40.

Martin, K. M., Ellis, P. D., Metcalfe, J. C., and Kemp, P. R. (2003). Selective modulation of the SM22alpha promoter by the binding of BTEB3 (basal transcription element-binding protein 3) to TGGG repeats. *Biochem J*. *375*, 457-463.

Massari, M. E., and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol*. *20*, 429-440.

Mericskay, M., Blanc, J., Tritsch, E., Moriez, R., Aubert, P., Neunlist, M., Feil, R., and Li, Z. (2007). Inducible mouse model of chronic intestinal pseudo-obstruction by smooth muscle-specific inactivation of the SRF gene. *Gastroenterology*. *133*, 1960-1970.

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

Miano, J. M., Long, X., and Fujiwara, K. (2007). Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *Am J Physiol Cell Physiol*. *292*, C70-81.

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

Miyamoto, S., Suzuki, T., Muto, S., Aizawa, K., Kimura, A., Mizuno, Y., Nagino, T., Imai, Y., Adachi, N., Horikoshi, M., and Nagai, R. (2003). Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and the oncogenic regulator SET through interaction and acetylation on the DNA-binding domain. *Mol Cell Biol*. *23*, 8528-8541.

Moran, E. P., and Agrawal, D. K. (2007). Increased expression of inhibitor of apoptosis proteins in atherosclerotic plaques of symptomatic patients with carotid stenosis. *Exp Mol Pathol*. *83*, 11-16.

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

Mosse, P. R., Campbell, G. R., Wang, Z. L., and Campbell, J. H. (1985). Smooth muscle phenotypic expression in human carotid arteries. I. Comparison of cells from diffuse intimal thickenings adjacent to atheromatous plaques with those of the media. *Lab Invest*. *53*, 556-562.

Nagai, R., Suzuki, T., Aizawa, K., Shindo, T., and Manabe, I. (2005). Significance of the transcription factor KLF5 in cardiovascular remodeling. *J Thromb Haemost*. *3*, 1569-1576.

Nakamura, M., Nishida, W., Mori, S., Hiwada, K., Hayashi, K., and Sobue, K. (2001). Transcriptional activation of beta-tropomyosin mediated by serum response factor and a novel Barx homologue, Barx1b, in smooth muscle cells. *J Biol Chem*. *276*, 18313-18320.

Nishida, W., Nakamura, M., Mori, S., Takahashi, M., Ohkawa, Y., Tadokoro, S., Yoshida, K., Hiwada, K., Hayashi, K., and Sobue, K. (2002). A triad of serum response factor and the GATA and NK families governs the transcription of smooth and cardiac muscle genes. *J Biol Chem*. *277*, 7308-7317.

Nishiyama, M., Nakayama, K., Tsunematsu, R., Tsukiyama, T., Kikuchi, A., and Nakayama, K. I. (2004). Early embryonic death in mice lacking the beta-catenin-binding protein Duplin. *Mol Cell Biol*. *24*, 8386-8394.

Niu, Z., Yu, W., Zhang, S. X., Barron, M., Belaguli, N. S., Schneider, M. D., Parmacek, M., Nordheim, A., and Schwartz, R. J. (2005). Conditional mutagenesis of the murine serum response factor gene blocks cardiogenesis and the transcription of downstream gene targets. *J Biol Chem*. *280*, 32531-32538.

Oh, J., Richardson, J. A., and Olson, E. N. (2005). Requirement of myocardin-related transcription factor-B for remodeling of branchial arch arteries and smooth muscle differentiation. *Proc Natl Acad Sci U S A*. *102*, 15122-15127.

Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S., and Li, E. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A*. *97*, 2626-2631.

Oshima, M., Oshima, H., and Taketo, M. M. (1996). TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol*. *179*, 297-302.

Owens, G. K. (2007). Molecular control of vascular smooth muscle cell differentiation and phenotypic plasticity. *Novartis Found Symp*. *283*, 174-191; discussion 191-173, 238-141.

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

Owens, G. K., Vernon, S. M., and Madsen, C. S. (1996). Molecular regulation of smooth muscle cell differentiation. *J Hypertens Suppl*. *14*, S55-64.

Parlakian, A., Charvet, C., Escoubet, B., Mericskay, M., Molkentin, J. D., Gary-Bobo, G., De Windt, L. J., Ludosky, M. A., Paulin, D., Daegelen, D*., et al.* (2005). Temporally controlled onset of dilated cardiomyopathy through disruption of the SRF gene in adult heart. *Circulation*. *112*, 2930-2939.

Parmacek, M. S. (2007). Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation. *Circ Res*. *100*, 633-644.

Patel, V. I., Daniel, S., Longo, C. R., Shrikhande, G. V., Scali, S. T., Czismadia, E., Groft, C. M., Shukri, T., Motley-Dore, C., Ramsey, H. E*., et al.* (2006). A20, a modulator of smooth muscle cell proliferation and apoptosis, prevents and induces regression of neointimal hyperplasia. *Faseb J*. *20*, 1418-1430.

Phiel, C. J., Gabbeta, V., Parsons, L. M., Rothblat, D., Harvey, R. P., and McHugh, K. M. (2001). Differential binding of an SRF/NK-2/MEF2 transcription factor complex in normal versus neoplastic smooth muscle tissues. *J Biol Chem*. *276*, 34637-34650.

Pipes, G. C., Creemers, E. E., and Olson, E. N. (2006). The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes Dev*. *20*, 1545-1556.

Pray-Grant, M. G., Daniel, J. A., Schieltz, D., Yates, J. R., 3rd, and Grant, P. A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature*. *433*, 434-438.

Remy-Martin, J. P., Marandin, A., Challier, B., Bernard, G., Deschaseaux, M., Herve, P., Wei, Y., Tsuji, T., Auerbach, R., Dennis, J. E*., et al.* (1999). Vascular smooth muscle differentiation of murine stroma: a sequential model. *Exp Hematol*. *27*, 1782-1795.

Ross, R., and Glomset, J. A. (1976a). The pathogenesis of atherosclerosis (first of two parts). *N Engl J Med*. *295*, 369-377.

Ross, R., and Glomset, J. A. (1976b). The pathogenesis of atherosclerosis (second of two parts). *N Engl J Med*. *295*, 420-425.

Ross, R., Wight, T. N., Strandness, E., and Thiele, B. (1984). Human atherosclerosis. I. Cell constitution and characteristics of advanced lesions of the superficial femoral artery. *Am J Pathol*. *114*, 79-93.

Sakamoto, H., Sakamaki, T., Kanda, T., Hoshino, Y., Sawada, Y., Sato, M., Sato, H., Oyama, Y., Nakano, A., Takase, S.*, et al.* (2003). Smooth muscle cell outgrowth from coronary atherectomy specimens in vitro is associated with less time to restenosis and expression of a key Transcription factor KLF5/BTEB2. *Cardiology*. *100*, 80-85.

Sakamoto, I., Kishida, S., Fukui, A., Kishida, M., Yamamoto, H., Hino, S., Michiue, T., Takada, S., Asashima, M., and Kikuchi, A. (2000). A novel beta-catenin-binding protein inhibits beta-catenin-dependent Tcf activation and axis formation. *J Biol Chem*. *275*, 32871-32878.

Sano, H., Sudo, T., Yokode, M., Murayama, T., Kataoka, H., Takakura, N., Nishikawa, S., Nishikawa, S. I., and Kita, T. (2001). Functional blockade of platelet-derived growth factor receptor-beta but not of receptor-alpha prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. *Circulation*. *103*, 2955-2960.

Sata, M., Saiura, A., Kunisato, A., Tojo, A., Okada, S., Tokuhisa, T., Hirai, H., Makuuchi, M., Hirata, Y., and Nagai, R. (2002). Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med*. *8*, 403-409.

Schratt, G., Philippar, U., Hockemeyer, D., Schwarz, H., Alberti, S., and Nordheim, A. (2004). SRF regulates Bcl-2 expression and promotes cell survival during murine embryonic development. *Embo J*. *23*, 1834-1844.

Selvaraj, A., and Prywes, R. (2004). Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent. *BMC Mol Biol*. *5*, 13.

Shimono, Y., Murakami, H., Kawai, K., Wade, P. A., Shimokata, K., and Takahashi, M. (2003). Mi-2 beta associates with BRG1 and RET finger protein at the distinct regions with transcriptional activating and repressing abilities. *J Biol Chem*. *278*, 51638-51645.

Shindo, T., Manabe, I., Fukushima, Y., Tobe, K., Aizawa, K., Miyamoto, S., Kawai-Kowase, K., Moriyama, N., Imai, Y., Kawakami, H., *et al.* (2002). Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med*. *8*, 856-863.

Shur, I., Socher, R., and Benayahu, D. (2006). In vivo association of CReMM/CHD9 with promoters in osteogenic cells. *J Cell Physiol*. *207*, 374-378.

Sif, S. (2004). ATP-dependent nucleosome remodeling complexes: enzymes tailored to deal with chromatin. *J Cell Biochem*. *91*, 1087-1098.

Simic, R., Lindstrom, D. L., Tran, H. G., Roinick, K. L., Costa, P. J., Johnson, A. D., Hartzog, G. A., and Arndt, K. M. (2003). Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. *Embo J*. *22*, 1846-1856.

Simper, D., Stalboerger, P. G., Panetta, C. J., Wang, S., and Caplice, N. M. (2002). Smooth muscle progenitor cells in human blood. *Circulation*. *106*, 1199-1204.

Sinha, S., Hoofnagle, M. H., Kingston, P. A., McCanna, M. E., and Owens, G. K. (2004). Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am J Physiol Cell Physiol*. *287*, C1560-1568.

Smith, J. D., Bryant, S. R., Couper, L. L., Vary, C. P., Gotwals, P. J., Koteliansky, V. E., and Lindner, V. (1999). Soluble transforming growth factor-beta type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circ Res*. *84*, 1212-1222.

Solomon, E. P., Linda R. Berg, Diana W. Martin and Claude Villee (1993). Biology, Third edn (New York: Saunders College Publishing).

Soriano, P. (1994). Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev*. *8*, 1888-1896.

Stenmark, K. R., Bouchey, D., Nemenoff, R., Dempsey, E. C., and Das, M. (2000). Hypoxia-induced pulmonary vascular remodeling: contribution of the adventitial fibroblasts. *Physiol Res*. *49*, 503-517.

Stokes, D. G., and Perry, R. P. (1995). DNA-binding and chromatin localization properties of CHD1. *Mol Cell Biol*. *15*, 2745-2753.

Tai, H. H., Geisterfer, M., Bell, J. C., Moniwa, M., Davie, J. R., Boucher, L., and McBurney, M. W. (2003). CHD1 associates with NCoR and histone deacetylase as well as with RNA splicing proteins. *Biochem Biophys Res Commun*. *308*, 170-176.

Tamm, I., Wang, Y., Sausville, E., Scudiero, D. A., Vigna, N., Oltersdorf, T., and Reed, J. C. (1998). IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res*. *58*, 5315-5320.

Thompson, B. A., Tremblay, V., Lin, G., and Bochar, D. A. (2008). CHD8 is an ATP-dependent chromatin remodeling factor that regulates beta-catenin target genes. *Mol Cell Biol*. *28*, 3894-3904.

Treisman, R. (1986). Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum factors. *Cell*. *46*, 567-574.

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

Turner, J., and Crossley, M. (1999). Basic Kruppel-like factor functions within a network of interacting haematopoietic transcription factors. *Int J Biochem Cell Biol*. *31*, 1169-1174.

Urness, L. D., Sorensen, L. K., and Li, D. Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet*. *26*, 328-331.

Vickers, E. R., Kasza, A., Kurnaz, I. A., Seifert, A., Zeef, L. A., O'Donnell, A., Hayes, A., and Sharrocks, A. D. (2004). Ternary complex factor-serum response factor complex-regulated gene activity is required for cellular proliferation and inhibition of apoptotic cell death. *Mol Cell Biol*. *24*, 10340-10351.

von Wnuck Lipinski, K., Keul, P., Ferri, N., Lucke, S., Heusch, G., Fischer, J. W., and Levkau, B. (2006a). Integrin-mediated transcriptional activation of inhibitor of apoptosis proteins protects smooth muscle cells against apoptosis induced by degraded collagen. *Circ Res*. *98*, 1490-1497.

von Wnuck Lipinski, K., Keul, P., Lucke, S., Heusch, G., Wohlschlaeger, J., Baba, H. A., and Levkau, B. (2006b). Degraded collagen induces calpain-mediated apoptosis and destruction of the X-chromosome-linked inhibitor of apoptosis (xIAP) in human vascular smooth muscle cells. *Cardiovasc Res*. *69*, 697-705.

Wada, H., Hasegawa, K., Morimoto, T., Kakita, T., Yanazume, T., Abe, M., and Sasayama, S. (2002). Calcineurin-GATA-6 pathway is involved in smooth muscle-specific transcription. *J Cell Biol*. *156*, 983-991.

Wada, H., Hasegawa, K., Morimoto, T., Kakita, T., Yanazume, T., and Sasayama, S. (2000). A p300 protein as a coactivator of GATA-6 in the transcription of the smooth muscle-myosin heavy chain gene. *J Biol Chem*. *275*, 25330-25335.

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

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

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

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

Wei, K., Che, N., and Chen, F. (2007). Myocardin-related transcription factor B is required for normal mouse vascular development and smooth muscle gene expression. *Dev Dyn*. *236*, 416-425.

Woodage, T., Basrai, M. A., Baxevanis, A. D., Hieter, P., and Collins, F. S. (1997). Characterization of the CHD family of proteins. *Proc Natl Acad Sci U S A*. *94*, 11472-11477.

Yamashina, K., Yamamoto, H., Chayama, K., Nakajima, K., and Kikuchi, A. (2006). Suppression of STAT3 Activity by Duplin, Which Is a Negative Regulator of the Wnt Signal. *J Biochem (Tokyo)*. *139*, 305-314.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., and Nakao, K. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature*. *408*, 92-96.

Yin, F., Hoggatt, A. M., Zhou, J., and Herring, B. P. (2006). 130-kDa smooth muscle myosin light chain kinase is transcribed from a CArG-dependent, internal promoter within the mouse mylk gene. *Am J Physiol Cell Physiol*. *290*, C1599-1609.

Yoshida, T., Gan, Q., Shang, Y., and Owens, G. K. (2007). Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to their promoters. *Am J Physiol Cell Physiol*. *292*, C886-895.

Yoshida, T., Kaestner, K. H., and Owens, G. K. (2008). Conditional deletion of Kruppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. *Circ Res*. *102*, 1548-1557.

Yuan, C. C., Zhao, X., Florens, L., Swanson, S. K., Washburn, M. P., and Hernandez, N. (2007). CHD8 associates with human Staf and contributes to efficient U6 RNA polymerase III transcription. *Mol Cell Biol*. *27*, 8729-8738.

Zhang, M., Fang, H., Zhou, J., and Herring, B. P. (2007). A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific gene expression. *J Biol Chem*. *282*, 25708-25716.

Zhou, J., and Herring, B. P. (2005). Mechanisms responsible for the promoter-specific effects of myocardin. *J Biol Chem*. *280*, 10861-10869.

# CURRICULUM VITAE

Jennifer Marie Rodenberg

## EDUCATION

| | |
|---|---|
| 2003-2008 | Ph.D., Department of Cellular and Integrative Physiology, Indiana University, Indianapolis, IN. |
| 1999-2003 | Bachelor of Arts in Chemistry, *Cum Laude*, Hanover College, Hanover, IN. |
| 1995-99 | Noblesville High School, Noblesville, IN.<br>*Valedictorian of graduating class* |

## THESIS WORK

Title: "Regulation of SRF Activity by the ATP-dependent chromatin remodeling enzyme, CHD8."
Advisor: B. Paul Herring, PhD
Major Area of Study: Smooth Muscle Development and Regulation
Minor Area of Study: Cancer Biology

## MAJOR RESEARCH INTERESTS

Mechanisms and Regulation of Cancer
Cancer Prevention
Breast Cancer, Ovarian Cancer, Uterine Cancer, Endometrial cancer

## HONORS

| | |
|---|---|
| 2008 | Sigma Xi Research Competition-Honorable Mention, Indiana University School of Medicine Chapter |
| 2006 | Sigma Xi Research Competition-First Place "Judy Boyd-White Award," Indiana University School of Medicine Chapter |
| 2005 | Sigma Xi Research Competition-Honorable Mention, Indiana University School of Medicine Chapter |
| 2002 | Mortar Board |
| 2001 | Phi Sigma Iota Honors Society |
| 2000 | Alpha Lambda Delta/Phi Eta Sigma Honors Society |
| 1999-2003 | Crowe/Long Scholarship and Music Scholarship |
| 1998 | International Science and Engineering Fair Participant |
| 1998-1999 | National Honors Society |

SPECIAL COURSES

Preparing Future Faculty (PFF) Scholar-completed May 2008

RESEARCH EXPERIENCE

| | |
|---|---|
| 2002-2003 | Department of Chemistry, Hanover College, Robert C. Evans, Ph.D. (advisor)-senior year independent study project |
| 2003-2008 | Department of Cellular and Integrative Physiology, Indiana University School of Medicine, B. Paul Herring, Ph.D. (advisor) |

WORK EXPERIENCE

| | |
|---|---|
| 2002 | Summer Internship at Roche Diagnostics: Research & Development; Patient Care/Diabetes Care; Analytical Chemistry Department |
| 2001-2002 | Science Support Assistant, Hanover College, IN |
| 2001 | Public Relations Person for Hanover College Music Department |
| 2000-2002 | CVS Pharmacy: Pharmacy Service Associate, working towards Pharmacy Technician. Noblesville, IN. |
| 1999-2001 | German Tutor, Hanover College, IN |

PUBLICATIONS

*Manuscripts:*

1. *Rodenberg, Jennifer M.,* Touw, Ketrija and Herring, B. Paul. "Regulation of SRF Activity by the ATP-dependent Chromatin Remodeling Enzyme, CHD8." (*In Preparation*)
2. Jiliang Zhou, Min Zhang, Hong Fang, Ketrija Touw, Omar El-Mounayri, *Jennifer M Rodenberg*, Anthony N Imbalzano, and B. Paul Herring. "The SWI/SNF chromatin remodeling complex is essential for myocardin-induced smooth muscle-specific gene expression." *Circulation Research*. 2008. (*Under revision*)

*Abstracts:*

1. *Rodenberg, Jennifer M.* and Herring, B. Paul "A Role for Duplin in SRF-mediated Signaling." Indiana University School of Medicine, Department of Cellular and Integrative Physiology, Indianapolis, IN 46237. American Society for Cell Biology, 45th Annual Meeting 2005, San Francisco, CA.

PRESENTATIONS

02/2005 Research in Progress Seminar “Duplin: A Link Between Signaling Pathways?” Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN

05/2005 Sigma Xi Indiana University Medical Center Chapter Competition “Duplin: A Link Between Signaling Pathways?”

08/2005 Poster Session at Department of Cellular and Integrative Physiology Annual Retreat “A Role for Duplin in SRF-mediated Signaling.” Indiana University School of Medicine, Indianapolis, IN

12/2005 Poster Session at American Society for Cell Biology, 45th Annual Meeting, San Francisco, CA: “A Role for Duplin in SRF-mediated Signaling.”

04/2006 Research in Progress Seminar “A Role for Duplin in SRF Signaling.” Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN

05/2006 Scientific Poster Session at Indiana University Cancer Center Annual Cancer Research Day: "A Role for Duplin in SRF-mediated Signaling." Indiana University School of Medicine, Indianapolis, IN

06/2006 Sigma Xi Indiana University Medical Center Chapter Competition “A Role for Duplin in SRF-mediated Signaling”

09/2006 Poster Session at Department of Cellular and Integrative Physiology Annual Retreat “Regulation of SRF Activity by the ATP-dependent Chromatin Remodeling Enzyme, CHD8.” Indiana University School of Medicine, Indianapolis, IN

09/2006 Thesis Proposal Seminar for advancement to candidacy at Department of Cellular and Integrative Physiology. “Regulation of SRF Activity by the ATP-dependent Chromatin Remodeling Enzyme, CHD8.” Indiana University School of Medicine, Indianapolis, IN

## PROFESSIONAL SOCIETIES

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|---|---|
| 2008-09 | American Physiological Society |
| 2005-06 | American Society for Cell Biology |
| 2005-06 | Sigma Xi |

## MEETINGS/CONFERENCES ATTENDED

| | |
|---|---|
| 12/2005 | American Society for Cell Biology, 45$^{th}$ Annual Meeting, San Francisco, CA |

## SCIENTIFIC SKILLS

| | |
|---|---|
| Cell culture (lines and primary) | RNA isolation |
| Immunofluorescence | qRT-PCR; PCR |
| Western blotting | RPA |
| Plasmid construction/cloning | GST pull-down |
| Co-immunoprecipitation | Chromatin-immunoprecipitation |
| Propidium Iodide analysis of cell cycle | Adenovirus production |

## LANGUAGES

English (first language)
German (second language; write and speak; semi-fluent)