DNA Methylation of a GC Repressor Element in the Smooth Muscle Myosin Heavy Chain Promoter Facilitates Binding of the Notch-Associated Transcription Factor, RBPJ/CSL1

Julian M. Rozenberg, Daniel B. Tesfu, Srilaxmi Musunuri, Joan M. Taylor, Christopher P. Mack

Objective—The goal of the present study was to identify novel mechanisms that regulate smooth muscle cell (SMC) differentiation marker gene expression.

Approach and Results—We demonstrate that the CArG-containing regions of many SMC-specific promoters are imbedded within CpG islands. A previously identified GC repressor element in the SM myosin heavy chain (MHC) promoter was highly methylated in cultured aortic SMC but not in the aorta, and this difference was inversely correlated with SM MHC expression. Using an affinity chromatography/mass spectroscopy–based approach, we identified the multifunctional Notch transcription factor, notch-shuffling signal binding protein for immunoglobulin κ J region (RBPJ), as a methylated GC repressor–binding protein. RBPJ protein levels and binding to the endogenous SM MHC GC repressor were enhanced by platelet-derived growth factor-BB treatment. A methylation mimetic mutation to the GC repressor that facilitated RBPJ binding inhibited SM MHC promoter activity as did overexpression of RBPJ. Consistent with this, knockdown of RBPJ in phenotypically modulated human aortic SMC enhanced endogenous SMC marker gene expression, an effect likely mediated by increased recruitment of serum response factor and Pol II to the SMC-specific promoters. In contrast, the depletion of RBPJ in differentiated transforming growth factor-β–treated SMC inhibited SMC-specific gene activation, supporting the idea that the effects of RBPJ/Notch signaling are context dependent.

Conclusions—Our results indicate that methylation-dependent binding of RBPJ to a GC repressor element can negatively regulate SM MHC promoter activity and that RBPJ can inhibit SMC marker gene expression in phenotypically modulated SMC. These results will have important implications on the regulation of SMC phenotype and on Notch-dependent transcription. (Arterioscler Thromb Vasc Biol. 2014;34:2624-2631.)

Key Words: epigenetics • muscle, smooth • RBPJ protein, human • serum response factor

It has been well established that serum response factor (SRF) and the myocardin family of SRF cofactors mediate smooth muscle cell (SMC)–specific transcription by interacting with conserved CArG elements within the promoters of the SMC differentiation marker genes. However, because these transcription factors are expressed in many non-SMC subtypes, it is clear that additional mechanisms are also critical for the overall pattern of SMC-specific gene expression observed in vivo.

One mechanism that is likely to be important in the regulation of SMC-specific transcription is the modification of chromatin structure. Histone modifications that favor gene expression (ie, H3 and H4 acetylation, H3K4 methylation, and H3K9 dimethylation/acylation) have been observed at the SMC-specific promoters in SMC. In addition, we and others have shown that the myocardin factors can facilitate chromatin modification by recruiting histone-modifying enzymes.

Another epigenetic mechanism that has received less attention in regard to its effects on SMC-specific transcription is DNA methylation. This epigenetic mark is predominantly associated with gene silencing and has been shown to be important for a wide variety of cellular functions, including genomic imprinting, X-inactivation, cellular differentiation, and carcinogenesis. DNA methylation is catalyzed by a family of DNA methyltransferases and typically occurs on cytosines at the 5 position of the pyrimidine ring and in the context of CpG dinucleotides (C followed by G). The majority of CpGs within the genome are dispersed and methylated. However, many gene promoters, especially those of highly expressed house-keeping genes, contain regions of high CpG content known as CpG islands that are typically unmethylated. Methylated cytosines are thought to suppress gene expression by sterically inhibiting transcription factor binding to cis regulatory elements or by associating with methyl binding
domain-containing proteins (MBDs1–4, MeCP2, Kaiso) that recruit additional transcription repressors. Interestingly, DNA regions that are heavily methylated are also associated with high levels of trimethyl H3K9, and these negative chromatin marks act cooperatively by the reciprocal recruitment of their respective methyltransferases.13

On the basis of our previous demonstration that H3K9 methylation status at the CArG-containing regions of the SMC-specific promoters was an important determinant of SMC differentiation marker gene expression,7 we hypothesized that DNA methylation may also play a role. Our results indicate that methylation of a GC repressor in the SM myosin heavy chain (MHC) promoter inversely correlates with SM MHC expression in aortic SMC, that the methylated GC repressor recruits the multifunctional transcription factor recombination signal binding protein for immunoglobulin κ J region (RBPJ)/CSL-1, and that RBPJ can inhibit SMC marker gene expression in phenotypically modulated human aortic SMC.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

SM MHC Expression in SMC Inversely Correlates With Promoter Methylation

To begin to examine whether DNA methylation plays a role in the regulation of SMC-specific gene expression, we searched for CpG islands within the SMC differentiation marker gene promoters using the formal definition (sequence of ≥200 bp in length, a GC content of >50%, and an observed:expected CpG ratio >60%). In both human and mouse, the CARG-containing regions shown to be important for SM MHC, SM22, and calponin expression were imbedded within CpG islands, suggesting that methylation might regulate the SRF/myocardin factor–dependent activation of these genes. We used standard bisulfite sequencing to measure CpG island methylation within the SM22 and SM MHC promoters in primary mouse aortic SMC cultures and in mouse 10T1/2 cells, a pluripotent line frequently used as a SMC precursor. As shown in Figure 1A, the CpG island within the SM22 promoter was highly methylated in 10T1/2 cells but almost completely unmethylated in SMC. This result correlates well with SM22 expression levels.

Figure 1. A GC repressor element in the mouse smooth muscle (SM) myosin heavy chain (MHC) promoter is methylated in cultured SMC. Schematic of CpG island methylation within the CArG-containing regions of the SM22 (A) and SM MHC (B) promoters as determined by bisulfite sequencing of genomic DNA isolated from 10T1/2 cells, mouse aortic SMC cultures, and mouse aorta. C, Western blot for SM MHC expression in the indicated cells and tissues. D, The indicated radiolabeled mouse SM MHC GC repressor probes were incubated with nuclear extracts prepared from mouse aortic SMC treated for 24 hours with vehicle, platelet-derived growth factor –BB (20 ng/mL), or transforming growth factor (TGF)-β (1 ng/mL), and from control (Ctl) or injured (Inj) femoral arteries. After 30 minutes, reactions were run on a 5% nondenaturing polyacrylamide gel, which was then dried and exposed to film.
in these cell types and suggests that promoter methylation may play a role in the regulation of SM22 expression. The CpG island within the proximal SM MHC promoter was also highly methylated in 10T1/2 cells (Figure 1B). However, we also detected significant methylation of the SM MHC promoter in SMC. Because the SM MHC gene is the first differentiation marker to be downregulated in phenotypically modulated SMC, we hypothesized that the observed methylation of the SM MHC promoter reflected phenotypic modulation of our cultured SMC. To test this idea, we measured SM MHC promoter methylation in freshly isolated aortic media after removal of the adventitial and endothelial cell layers by collagenase/elastase digestion and microdissection. The SM MHC CpG island was completely unmethylated in aortic medial SMC in vivo, and this result correlated with much stronger expression of SM MHC as measured by Western blotting of lysates prepared from the same samples (Figure 1C).

Several CpGs within the SM MHC promoter were almost completely methylated in cultured SMC. One of these was present within a GC-rich sequence just downstream of CArG2 that had been previously characterized as a repressor element. Deletion of a similar but not identical GC repressor within the SM22 promoter prevented the downregulation of SM22 promoter activity that occurs after vessel injury. The GC repressor within the SM MHC promoter was also fully methylated in mouse endothelial cells (data not shown) perhaps suggesting that this mechanism is important for the repression of SM MHC expression in other cell types in the vessel wall.

Because the effects of promoter methylation are thought to be mediated by alterations in protein–DNA binding, we performed gel shift analyses with methylated and unmethylated mouse SM MHC GC repressor probes and nuclear extracts isolated from SMC cultures. Importantly, the major protein complex that bound to the methylated GC repressor did not bind to the unmethylated probe (Figure 1D, compare lanes 1 and 4). In addition, complex binding to the methylated repressor was increased when extracts were prepared from cells treated with platelet-derived growth factor (PDGF)-BB, an agonist known to downregulate SMC differentiation marker gene expression. In contrast, treatment of cells with transforming growth factor (TGF)-β that activates SMC differentiation marker gene expression had little effect on complex formation. To examine the formation of this methylation-dependent complex in vivo, we performed gel shift assays using nuclear extracts prepared from control and wire-injured carotid arteries. A similar methylation-dependent binding complex was detected and was significantly increased in extracts prepared from injured arteries (Figure 1D, compare lanes 7 and 8). We initially hypothesized that this complex contained one of the known methylated DNA-binding proteins, but we were unable to supershift this band with antibodies to these factors. Several additional bands were detected in some gel shift assays, but these were either not methylation-dependent or were mostly nonspecific (Figure 2, data not shown).

RBPJ Binds the Methylated SM MHC GC Repressor

We next used agarose beads conjugated to the methylated GC repressor to affinity purify the methylation-dependent protein-binding complex from SMC nuclear extracts. Proteins that precipitated with the methylated, but not the unmethylated probe, were cut out of SDS-PAGE gels and sent for mass spectrometric analysis. Each of 3 separate experiments identified the multifunctional Notch transcription factor, RBPJ, as a methylated GC repressor–binding protein. Additional gel shifts demonstrated that the major complex that bound the methylated GC repressor was similar to that formed when using a consensus RBPJ oligonucleotide probe (Figure 2A; compare lanes 1 and 5). Moreover, addition of an RBPJ antibody to gel shift reactions resulted in a complete supershift providing conclusive evidence for the presence of RBPJ in this complex (Figure 2A; compare lanes 1 and 2). As shown in Figure 2B, RBPJ binding to the methylated GC repressor was specific in that it was completely abolished by addition of cold methylated probe (or the consensus RBPJ element) but not by cold unmethylated probe.

Notch signaling plays an important role in vascular development and maintenance by regulating cell fate decisions in both endothelial cells and SMC. Activation of the integral membrane Notch receptors by Delta-like or Jagged ligands results in proteolytic cleavage of the receptor by γ-secretase, release of the Notch intracellular domain (NICD), and translocation of the NICD to the nucleus where it interacts with RBPJ. In the absence of NICD, RBPJ binds a consensus site (GTGGGA) within the promoters of Notch target genes, and this result correlated with much stronger expression of SM MHC as measured by Western blotting of lysates prepared from the same samples (Figure 1C).

Because the effects of promoter methylation are thought to be mediated by alterations in protein–DNA binding, we performed gel shift analyses with methylated and unmethylated mouse SM MHC GC repressor probes and nuclear extracts isolated from SMC cultures. Importantly, the major protein complex that bound to the methylated GC repressor did not bind to the unmethylated probe (Figure 1D, compare lanes 1 and 4). In addition, complex binding to the methylated repressor was increased when extracts were prepared from cells treated with platelet-derived growth factor (PDGF)-BB, an agonist known to downregulate SMC differentiation marker gene expression. In contrast, treatment of cells with transforming growth factor (TGF)-β that activates SMC differentiation marker gene expression had little effect on complex formation. To examine the formation of this methylation-dependent complex in vivo, we performed gel shift assays using nuclear extracts prepared from control and wire-injured carotid arteries. A similar methylation-dependent binding complex was detected and was significantly increased in extracts prepared from injured arteries (Figure 1D, compare lanes 7 and 8). We initially hypothesized that this complex contained one of the known methylated DNA-binding proteins, but we were unable to supershift this band with antibodies to these factors. Several additional bands were detected in some gel shift assays, but these were either not methylation-dependent or were mostly nonspecific (Figure 2, data not shown).

RBPJ Binds the Methylated SM MHC GC Repressor

We next used agarose beads conjugated to the methylated GC repressor to affinity purify the methylation-dependent protein-binding complex from SMC nuclear extracts. Proteins that precipitated with the methylated, but not the unmethylated probe, were cut out of SDS-PAGE gels and sent for mass spectrometric analysis. Each of 3 separate experiments identified the multifunctional Notch transcription factor, RBPJ, as a methylated GC repressor–binding protein. Additional gel shifts demonstrated that the major complex that bound the methylated GC repressor was similar to that formed when using a consensus RBPJ oligonucleotide probe (Figure 2A; compare lanes 1 and 5). Moreover, addition of an RBPJ antibody to gel shift reactions resulted in a complete supershift providing conclusive evidence for the presence of RBPJ in this complex (Figure 2A; compare lanes 1 and 2). As shown in Figure 2B, RBPJ binding to the methylated GC repressor was specific in that it was completely abolished by addition of cold methylated probe (or the consensus RBPJ element) but not by cold unmethylated probe.

Notch signaling plays an important role in vascular development and maintenance by regulating cell fate decisions in both endothelial cells and SMC. Activation of the integral membrane Notch receptors by Delta-like or Jagged ligands results in proteolytic cleavage of the receptor by γ-secretase, release of the Notch intracellular domain (NICD), and translocation of the NICD to the nucleus where it interacts with RBPJ. In the absence of NICD, RBPJ binds a consensus site (GTGGGA) within the promoters of Notch target genes, and this result correlated with much stronger expression of SM MHC as measured by Western blotting of lysates prepared from the same samples (Figure 1C).

Because the effects of promoter methylation are thought to be mediated by alterations in protein–DNA binding, we performed gel shift analyses with methylated and unmethylated mouse SM MHC GC repressor probes and nuclear extracts isolated from SMC cultures. Importantly, the major protein complex that bound to the methylated GC repressor did not bind to the unmethylated probe (Figure 1D, compare lanes 1 and 4). In addition, complex binding to the methylated repressor was increased when extracts were prepared from cells treated with platelet-derived growth factor (PDGF)-BB, an agonist known to downregulate SMC differentiation marker gene expression. In contrast, treatment of cells with transforming growth factor (TGF)-β that activates SMC differentiation marker gene expression had little effect on complex formation. To examine the formation of this methylation-dependent complex in vivo, we performed gel shift assays using nuclear extracts prepared from control and wire-injured carotid arteries. A similar methylation-dependent binding complex was detected and was significantly increased in extracts prepared from injured arteries (Figure 1D, compare lanes 7 and 8). We initially hypothesized that this complex contained one of the known methylated DNA-binding proteins, but we were unable to supershift this band with antibodies to these factors. Several additional bands were detected in some gel shift assays, but these were either not methylation-dependent or were mostly nonspecific (Figure 2, data not shown).

RBPJ Binds the Methylated SM MHC GC Repressor

We next used agarose beads conjugated to the methylated GC repressor to affinity purify the methylation-dependent protein-binding complex from SMC nuclear extracts. Proteins that precipitated with the methylated, but not the unmethylated probe, were cut out of SDS-PAGE gels and sent for mass spectrometric analysis. Each of 3 separate experiments identified the multifunctional Notch transcription factor, RBPJ, as a methylated GC repressor–binding protein. Additional gel shifts demonstrated that the major complex that bound the methylated GC repressor was similar to that formed when using a consensus RBPJ oligonucleotide probe (Figure 2A; compare lanes 1 and 5). Moreover, addition of an RBPJ antibody to gel shift reactions resulted in a complete supershift providing conclusive evidence for the presence of RBPJ in this complex (Figure 2A; compare lanes 1 and 2). As shown in Figure 2B, RBPJ binding to the methylated GC repressor was specific in that it was completely abolished by addition of cold methylated probe (or the consensus RBPJ element) but not by cold unmethylated probe.

Notch signaling plays an important role in vascular development and maintenance by regulating cell fate decisions in both endothelial cells and SMC. Activation of the integral membrane Notch receptors by Delta-like or Jagged ligands results in proteolytic cleavage of the receptor by γ-secretase, release of the Notch intracellular domain (NICD), and translocation of the NICD to the nucleus where it interacts with RBPJ. In the absence of NICD, RBPJ binds a consensus site (GTGGGA) within the promoters of Notch target genes, and this result correlated with much stronger expression of SM MHC as measured by Western blotting of lysates prepared from the same samples (Figure 1C).
dependent with several studies showing both positive and negative effects.21–23,25–27

Characterization of RBPJ Binding to Methylated DNA
We noted that the reverse strand of the GC-rich element (GCGGGGA) differed from the consensus core RBPJ-binding site by only 1 base pair, a thymine to cytosine substitution. Additional gel shift assays with hemimethylated probes demonstrated that only methylation of the reverse strand was required for RBPJ binding (Figure 3A). Because the pyrimidine ring of thymine is constitutively methylated at the 5' position (Figure 3B), we hypothesized that methylation of cytosine conferred enough structural similarity between these nucleotides to facilitate RBPJ binding. Supporting this idea, a GC repressor containing a cytosine to thymine substitution was shown to interact with RBPJ (Figure 3C, lane 3). Conversely, RBPJ did not bind well to a consensus sequence in which the thymine was replaced by cytosine, but did bind strongly to a consensus probe in which the thymine was replaced with a methylated cytosine (Figure 3C, compare lanes 7 and 6).

The crystal structure of RBPJ bound to its consensus DNA element has been solved.28,29 Several conserved polar amino acids in the RBPJ DNA-binding pocket (especially Arg 65) were shown to interact with the 3 central guanine residues at least partially explaining the specificity of RBPJ binding to the consensus sequence. Interestingly, no such interactions were detected for the consensus thymine supporting our contention that binding specificity at this residue is mediated by the presence of a methyl group at the pyrimidine 5' position. Although Glu63 is the nearest amino acid to this thymine residue, the 5' methyl group is positioned closer to the carbon backbone of the glutamate side chain than to the polar head group. As shown in Figure 3D, a conservative Glu63Asp mutation almost completely inhibited RBPJ binding to the consensus probe and the same probe in which the thymine was replaced by methylated cytosine. Taken together, these data strongly suggest that the positioning of the carboxyl group of Glu63 is critical for the formation of a methyl binding pocket that stabilizes the RBPJ–DNA interaction.

RBPJ Negatively Regulates SM MHC Promoter Activity
We next used several gain-of-function/loss-of-function approaches to examine the effects of RBPJ on SMC-specific promoter activity. As shown in Figure 4A, overexpression of RBPJ in 10T1/2 cells significantly inhibited the activities of multiple SMC-specific promoters in the presence of myocardin. We also established SMC cultures from RBPJlox/+lox mice and used adenoviral-meditated expression of Cre recombinase to reduce RBPJ expression and DNA binding in these cells by ~85% (Figure 4B). Importantly, SM MHC promoter activity in SMC overexpressing myocardin was significantly higher in Cre versus LacZ-infected SMC (Figure 4C). Although these results strongly suggest that RBPJ can function as an inhibitor in these contexts, the lack of reagents to alter the methylation of specific CpGs has made it difficult to study the functional effects of this modification. However, our ability to promote RBPJ binding to the GC repressor by a thymine substitution that mimics cytosine methylation (Figure 3C) allowed us to assess the functional significance of this interaction. A cytosine to thymine substitution within the GC repressor significantly attenuated myocardin-dependent SM MHC promoter activity supporting our hypothesis that methylation-dependent binding of RBPJ to the GC repressor inhibits SM MHC promoter activity. Chromatin immunoprecipitation (ChIP) assays in our mouse SMC cultures demonstrated that RBPJ binds to the endogenous SM MHC GC repressor (Figure 5A), and in excellent agreement with our gel shift assays, that this interaction was enhanced by PDGF-BB treatment. As shown in Figure 5B, PDGF-BB treatment increased RBPJ protein levels in both SMC and 10T1/2 cells. Given that methylation of the GC repressor is already high in our cultured SMC, this result likely explains the increase in RBPJ binding to the GC repressor in PDGF-BB–treated cells.

RBPJ Has Dual Effects on SMC-Specific Expression
The ability of Notch to stimulate SMC differentiation has been attributed to the recruitment of the NICD to RBPJ binding sites within the SMC-specific promoters.21–23 However, our demonstration that RBPJ binds to methylated DNA, is upregulated
by PDGF-BB and vessel injury, and can inhibit SMC-specific promoter activity, led us to hypothesize that RBPJ can also function as a repressor in phenotypically modulated SMC. To begin to test this idea, we used siRNA to knockdown RBPJ in a human aortic SMC line that exhibits low levels of the SMC marker genes under basal growth conditions but high levels when treated with TGF-β. As shown in Figure 6A, RBPJ bound to the CARG-containing regions of the SM MHC, SM α-actin, calponin, and SM22 genes in human aortic SMC and treatment of these cells with siRNA significantly inhibited RBPJ binding in this model. Importantly, depletion of RBPJ under growth conditions resulted in an increase in SMC marker gene mRNA levels, suggesting that RBPJ functions as a repressor in this context (Figure 6B, undiff). Given the positioning of the GC repressor and other RBPJ-binding sites to CARG elements in the SM MHC and other promoters,22,23 we postulated that RBPJ might interfere with SRF binding under these conditions. Indeed, ChIP assays demonstrated that SRF binding to the SMC promoters was increased in RBPJ-depleted cells (Figure 6C) even though SRF protein levels were not affected (Figure I in the online-only Data Supplement). Moreover, the increase in SMC marker gene mRNA in RBPJ knockdown cells was completely inhibited by codepletion of SRF (Figure 6D). As expected, the positive effects of RBPJ depletion on SMC-specific gene expression and SRF binding were accompanied by the increased recruitment of RNA polymerase II.

In contrast, depletion of RBPJ from TGF-β-treated human aortic SMC had an inhibitory effect on SMC-specific transcription. Although SRF binding in these cells (which was much stronger than that observed under growth conditions) was mostly unchanged, RBPJ depletion strongly attenuated the presence of RNA polymerase II, suggesting that recruitment of transcriptional cofactors or chromatin-modifying enzymes was affected. In support of this idea, additional ChIP assays demonstrated that Notch3 binding (Figure 6E) and H3K9 acetylation (Figure 6F) were decreased in RBPJ-depleted cells under these conditions.

**Discussion**

The current studies indicate that methylation-dependent recruitment of RBPJ to a GC repressor element inhibits SM MHC promoter activity and that RBPJ can inhibit SMC differentiation marker gene expression in phenotypically modulated SMC by inhibiting SRF binding to the CARG-containing regions of the SMC-specific promoters. Interestingly, RBPJ was required for full SMC marker gene activation in TGFB-β-treated cells, a result consistent with the requirement of Notch signaling for SMC differentiation in vivo.18–20 It is likely that inhibition of NIDC recruitment under these conditions decreased positive chromatin remodeling and Pol II binding leading to reductions in SMC marker gene expression. Taken together, our results support the current model of Notch signaling in which RBPJ inhibits gene expression under unstimulated conditions but is required for recruitment of the NIDC and the positive transcriptional effects of Notch signaling.

Although DNA methylation can promote long-term and heritable gene inactivation,20 its role in the regulation of cell-type-specific gene expression is less clear25–37 and may be related to the number and methylation status of CpGs within a particular

---

**Figure 4.** Recombination signal binding protein for immunoglobulin κ J region (RBPJ) inhibits smooth muscle (SM) myosin heavy chain (MHC) promoter activity. A, 10T1/2 cells were transfected with SM22, SM α-actin, SM22 or minimal thymidine kinase (TK) reporter/luciferase constructs +/−myocardin and +/−RBPJ expression vectors. The total amount of expression vector in each well was equalized by addition of empty vector (ev). Luciferase activity was measured at 48 hours and is expressed relative to promoter activity plus empty vector only (Con). *P<0.05 vs control. **P<0.05 vs plus myocardin. B, Western blot and gel shift analyses demonstrating RBPJ knockdown in our RBPJflox/flox SMC model. C, A mutation (C to T) within the GC repressor that facilitates RBPJ binding was made in the context of the SM MHC promoter shown. SMC isolated from RBPJflox/flox mice were treated with Cre- or LacZ-expressing adenovirus for 48 hours and then transfected with the wild-type (Wt) and T mutant constructs. Luciferase activity was measured at 48 hours. *P<0.05 vs Wt. **P<0.05 vs LacZ.

---

**Figure 5.** Recombination signal binding protein for immunoglobulin κ J region (RBPJ) binds the endogenous smooth muscle (SM) myosin heavy chain (MHC) repressor. A, Chromatin immunoprecipitation assays for RBPJ were performed in control and platelet-derived growth factor (PDGF)-BB–treated SMC using primers spanning the GC repressor-containing region of the SM MHC promoter. B, Western blot for RBPJ expression in mouse aortic SMC and 10T1/2 cells treated with PDGF-BB and transforming growth factor (TGF)-β. The chromatin immunoprecipitation and Western Blot results shown are representative of ≥3 independent experiments.
Because de novo DNA methylation is catalyzed by DNA methyltransferases 3a and 3b, it will be important to identify the mechanisms by which these enzymes are recruited to the SM HMC promoter in cultured SMC. We have previously shown that H3K9 methylation at the CARG-containing regions of the SMC-specific promoters was associated with decreased SM HMC expression, and we are currently investigating whether this histone modification precedes or promotes SM HMC promoter methylation. Another interesting question is whether changes in DNA methylation regulate SMC-specific gene expression more acutely. We observed modest reductions in SM HMC promoter methylation during TGF-β–induced upregulation of SM HMC expression in 10T1/2 cells (Figure II in the online-only Data Supplement). However, the known heterogeneity in these cells is a confounding factor and additional experiments in the subset of cells that upregulate SM HMC expression will likely be required to demonstrate that SM HMC promoter methylation is responsive to TGF-β. The failure to identify a robust DNA demethylation regulatory pathway has also hindered our understanding of the temporal control of DNA methylation. Enzymes that facilitate the demethylation reaction have been identified, suggesting that DNA methylation (like histone methylation) is more dynamic than previously thought. Several studies provide support for the idea that SMC differentiation marker gene expression is regulated by DNA methylation. For example, the decrease in SM22 expression observed in human SMCs treated with high phosphate was associated with increased SM22 promoter methylation, whereas SM22 methylation was decreased in multipotential adventitial cells that were induced to differentiate into SMC. Hu et al have also shown that SM α-actin expression in fibroblasts inversely correlated with DNA methylation near the SM α-actin transcription start site.

Figure 6. recombination signal binding protein for immunoglobulin κ J region (RBPJ) inhibits smooth muscle cell (SMC) differentiation marker gene expression in phenotypically modulated human SMC. A. Chromatin immunoprecipitation (ChIP) assays for RBPJ binding to the indicated promoters in control and RBPJ knockdown human aortic SMC. B. RNA was isolated from control and RBPJ knockdown human aortic SMC that were maintained in growth media (undiff) or treated with transforming growth factor (TGF)-β. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for the indicated SMC marker genes and GAPDH. Results of RT-PCR using 5 and 45 ng of RNA from TGF-β–treated cells are shown for quantification. C. ChIP assays for SRF and RNA Pol II binding in control and RBPJ knockdown SMC under growth or TGF-β–treated conditions. D. RT-PCR of SMC marker gene expression in RBPJ knockdown, SRF knockdown, and RBPJ/ SRF double knockdown human SMC under growth conditions. E. Notch3 binding and H3K9 acetylation at the SMC-specific promoters were measured by ChIP assay in control and RBPJ knockdown human aortic SMC treated with TGF-β. The RT-PCR and ChIP results shown are representative of ≥3 independent experiments.
Structural analyses of several zinc finger transcription factors that bind to methylated DNA identified a 5mCytosine-Arg-Guanine triad that mediates this interaction.\(^4\) and our data strongly support this mechanism. In this model, hydrogen bonding of Arg65 with the guanine residue at position 3 of the consensus RBPJ-binding sequence promotes van der Waals contacts between the Arg65 guanidino carbon moiety and the methyl group of the nucleotide at position 2 (whether 5mC or T). Our data also suggest that Glu63 is important for RBPJ binding to DNA, a result in excellent agreement with a recent human genetic study that identified a Glu63Gly mutation in RBPJ that was causal for Adams–Oliver syndrome, a disease that affects limb and cranium formation.\(^4\) Interestingly, the methylated DNA-binding proteins, Kaiso and Zfp57, have similarly positioned Glu residues\(^5\) further supporting the idea that Glu63 is critical for RBPJ binding to the methylated pyrimidine ring.

Several recent studies have used ChIP seq analyses to identify RBPJ and Notch-binding sites on a genome-wide level in T-lymphoblastic leukemia cells, mouse E13.5 neural cortices, and its consequences.\(^2\) This work was supported by National Institutes of Health grants HL070953 and HL109607 (C.P. Mack).

###Sources of Funding

This work was supported by National Institutes of Health grants HL070953 and HL109607 (C.P. Mack).

###Disclosures

None.

###References


These results have important implications on the regulation of SMC-specific and Notch/RBPJ–dependent gene expression.

**Significance**

We show that methylation of a GC repressor in the smooth muscle (SM) myosin heavy chain promoter inversely correlates with SM MHC expression in aortic SMC, that the methylated GC repressor recruits the multifunctional transcription factor recombination signal binding protein for immunoglobulin κ J (RBPJ) and that RBPJ can inhibit SMC marker gene expression in phenotypically modulated human aortic SMC. These results have important implications on the regulation of SMC-specific and Notch/RBPJ–dependent gene expression.
DNA Methylation of a GC Repressor Element in the Smooth Muscle Myosin Heavy Chain Promoter Facilitates Binding of the Notch-Associated Transcription Factor, RBPJ/CSL1

Julian M. Rozenberg, Daniel B. Tesfu, Srilaxmi Musunuri, Joan M. Taylor and Christopher P. Mack

Arterioscler Thromb Vasc Biol. 2014;34:2624-2631; originally published online October 16, 2014;
doi: 10.1161/ATVBAHA.114.304634

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/12/2624

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/10/16/ATVBAHA.114.304634.DC1
http://atvb.ahajournals.org/content/suppl/2015/06/25/ATVBAHA.114.304634.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure Legends

Suppl Fig I. SRF protein levels were not affected by RBPJ knockdown. Western blot for RBPJ and SRF in control and RBPJ siRNA treated human aortic SMC under growth and TGF-β-treated conditions.

Suppl Fig II. Effects of TGF-β on methylation of the CpG island within the SM MHC promoter. Genomic DNA samples from 10T1/2 cells treated with vehicle or TGF-β (1 ng/ml) were subjected to bisulphite sequencing. Methylation of specific CpGs are expressed as a percentage of full methylation at that site.

Suppl Fig III. An RBPJ/NICD1 complex can form on a methylated binding site. The indicated RBPJ consensus probe variant was combined with 1 μL of in vitro translated RBPJ and 0 - 3 μL of in vitro translated NICD1.

Suppl Fig IV. The SM22 GC repressor does not bind RBPJ. Methylated and unmethylated radiolabeled GC repressor probes from the SM MHC and SM22 GC promoters were combined with SMC nuclear extracts. After 30 min, reactions were run on a 5% non-denaturing polyacrylamide gel which was then dried and exposed to film.
Suppl Figure I

RBPJ siRNA

α RBPJ

α SRF

α Histone H3

Undiff  TGF-β

-  +  -  +
Suppl Fig III

<table>
<thead>
<tr>
<th>Probe</th>
<th>Cons RBPJ</th>
<th>Cons RBPJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NICD1</td>
<td>0 3 1 .3 .1</td>
<td>0 3 1 .3 .1</td>
</tr>
</tbody>
</table>

- **RBPJ + NICD1**
- **ns**
- **RBPJ**
<table>
<thead>
<tr>
<th>GC rep Meth</th>
<th>MHC</th>
<th>SM22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Suppl Fig IV

RBPJ
Materials and Methods

Antibodies and reagents
The following antibodies were used: SRF (Santa Cruz, sc-335), SM MHC (Abcam, 53219), SM α-actin (Sigma, A5228), Calponin (LifeSpanBio, 49769), SM22 (Santa Cruz, sc-271719), GAPDH (Santa Cruz, sc-25778), RBPJ for Westerns (Cell Signaling, 5313), RBPJ for supershifts (CosmoBioCo, K0043), RBPJ for ChIP assays (a kind gift from Raymond McDonald (University of Texas Southwestern, Dallas), acetylated H3K9 (Abcam, 12178), Notch3 (Santa Cruz sc-5593) RNA Polymerase II (Millipore, 05-952).

Cell culture and treatments
Human aortic SMC were purchased from Lonza and were maintained in Lonza Sm-GM-2 smooth muscle growth media containing 5% FBS. Multi-potential 10T1/2 cells were cultured as described previously 1. RBPJ flox/flox mice were kindly provided by Raphael Kopan (Washington University, MO, USA) with permission of Tasuku Honjo (Kyoto University, Japan) and have been described previously 2. Aortic SMC were isolated from 4-6 week old male C57/Bl6 and RBPJ flox/flox mice by enzymatic digestion with elastase and collagenase. The adventitial and endothelial cell layers were removed mechanically. SMC were maintained in DMEM:F12 (1:1) supplemented with 10% FBS and 1% penicillin-streptomycin and were typically used between passages 5 and 10. For Cre-dependent knockout experiments, cells were treated with Cre or LacZ expressing adenovirus for 48 h. In some experiments cells were serum starved for 24 h and then treated with PDGF-BB (20ng/ml) or TGF-β (1ng/ml) for 24h. For harvesting protein, cells were lysed in RIPA buffer plus protease and phosphatase inhibitors (15 mM Hepes pH7.2, 140 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS).

siRNA knockdowns
Cells were transfected with siRNAs for RBPJ (siRNApool M-007772-00 from siGENOME), SRF (UAAUACUCAUGGCAAACAU[dT][dT] and AUGUUUGCCAUGAGUAUUA[dT][dT]), or GFP (GGUGCGCUCCUGACGGCACC and GGCUACGUCAGAGCCGACC) using Dharmafect (Dharmacon) as per manufacturer's protocol. When co-depleting RBPJ and SRF the total amount of siRNA was equalized by addition of GFP siRNA. Cells were re-transfected the following day using the same protocol. Cells were then either maintained in Sm-GM-2 growth media for 40h or serum starved for 16h and then treated with TGF-β (1ng/ml) for 24h.

Luciferase reporter assays
The SM α-actin, SM22, and minimal thymidine kinase promoters used have been previously described 1. The SM MHC promoter from -1262 to +132 bp was amplified from mouse genomic DNA and cloned into a CpG-less luciferase vector that was a kind gift of Michael Rehli (University of Regensburg, Germany). C to T mutations of the GC repressor element where introduced by PCR. For tranfections, cells were maintained in 48 well dishes in 10% serum media and transfected 24 h after plating at 70-80% confluency using LT-1 transfection reagent (Mirus) per protocol. Luciferase activity was measured 48 h post-transfection and is expressed relative to activity of the promoterless vector. In some experiments, luciferase reporters were co-transfected with a myocardin expression plasmid or an empty expression vector as a control. Luciferase results are presented as means and standard errors from at least 3 independent transfections.

DNA affinity chromatography
Mouse aortic SMC nuclear extracts were combined with 150 nmoles of annealed biotinylated GC repressor oligos (methylated or unmethylated) and 150nmoles of 20 mer random oligos in
binding buffer (10mM Hepes pH7.4, 8% glycerol, 150 mM NaCl, 1mM MgCl₂, 0.05% Triton x100, 1.3 mM dithiotheritol). After 10 min of rotation at room temperature, 100 ul of 33% streptavidin agarose (Sigma) was added and reactions were incubated for an additional 30 min. Pelleted beads were washed 2x with wash buffer (KCl 75mM, Hepes 5mM pH7.4, MgCl₂ 0.5mM, glycerol 4%, Tween 20 0.05%, DTT 1mM) and bound proteins were eluted with 500mM NaCl. Eluted samples were subjected to a second round of affinity purification as above except that the binding buffer also contained 1 mM spermine and 1 mM spermidine. Eluted proteins were run on an SDS-PAGE gel and Coomassie stained bands were sent to the UNC mass spec core facility for identification. See on-line supplement for oligo sequences.

Gel shift assays
RBPJ clone BC051387 was purchased from OriGene and cloned into mammalian expression pcDNA3 vector (Invitrogen) containing an N-terminal flag epitope tag. RBPJ mutations were generated by PCR. The NICD1 expression construct was a kind gift from Stephen Blacklow (Harvard University, Boston). RBPJ, SRF, and NICD proteins were in vitro translated using the TnT kit from Promega according to protocol. Nuclear extracts were prepared from mouse aortic SMC and frozen and ground carotid artery samples using the NE-Per nuclear extraction kit from Thermo Scientific according to the manufacturer's instructions. Binding reactions were incubated for 30 min at RT and contained 5 µg of nuclear extract or 4µL of TnT reaction mix, 0.12 pmols of ³²P-labeled oligonucleotide probe, and 2.5 pmols of random oligos in binding buffer (10mM Hepes pH7.4, 8% glycerol, 150 mM NaCl,1mM MgCl₂, 0.05% Triton X100, 1mM dithiotheritol). For supershifts, 3µl of anti-RBPJ antibody was added during incubation. Samples where run on native gel, dried and exposed to film. Probe sequences used were as follows (RBPJ binding sites in bold): SM MHC GCrep – GGCCTGGC[G5Me-dC]GGGAGACAACC; Methylated SM MHC GCrep - GGCCTGGG[C5Me-dC]GGGAGACAACC; GCrep C to T mutation – GGCCTGGG[C5Me-dC]GGGAGACAACC; consensus RBPJ – GGAATGTGCGGGAAGAAAG; consensus RBPJ T to C – GGAATGTGCGGGAAGAAAG; consensus RBPJ T to CMe - GGAATGTG[C5Me-dC]GGGGAAGAAAG; SM22 GCRep – TTTTCCCGGCGCGGCGCTCACG; Methylated SM22 GCRep - TTTTCC[C5Me-dC] GGC[C5Me-dC]GCCCTCACG.

DNA affinity chromatography
Mouse aortic SMC nuclear extracts were combined with150 nmoles of biotinylated GC repressor oligos (methylated and unmethylated) and 150nmoles of 20 mer random oligos in binding buffer (10mM Hepes pH7.4, 8% glycerol, 150 mM NaCl, 1mM MgCl₂, 0.05% Triton x100, 1.3 mM dithiotheritol). After 10 min of rotation at room temperature, 100 ul of 33% streptavidin agarose (Sigma) was added and reactions were incubated for an additional 30 min. Pelleted beads were washed 2x with wash buffer (KCL 75mM, Hepes 5mM pH7.4, MgCl₂ 0.5mM, glycerol 4%, Tween 20 0.05%, DTT 1mM) and bound proteins were eluted with 500mM NaCl. Eluted samples were subjected to a second round of affinity purification as above except that the binding buffer also contained 1 mM spermine and 1 mM spermidine. Eluted proteins were run on an SDS-PAGE gel, and Coomassie stained bands were sent to the UNC mass spec core facility for identification. Oligos used were as follows; Methylated GC repressor - biotinGGTTGTCTCC[C5Me-dC]GCCCAGGCC; GC repressor - biotin-GGTTGTCTCCGCGCCAGGCC. Random oligos - TNNNNNNNNNNNNNNNNNNA.

Bisulfite conversion and sequencing.
Genomic DNA was extracted from cells and frozen and ground aortae using the QiaAmp kit from Qiagen. Bisulfite conversion was done using the MethylCode bisulfite conversion kit from Invitrogen. Following PCR amplification, products were either sequenced directly or were subcloned into a vector for transformation and sequencing of individual clones.
**Chromatin immunoprecipitation**

The SimpleChip Kit from Cell Signaling was used according the manufacturer's instruction. 1% of starting material was used to control equal quantity of DNA in reactions from different samples. DNA was extracted using ZymoResearch ChIP DNA clean and concentrator kit according to manufacturer instruction, eluted in 20 µl and 1 µl was used in PCR reactions. Serial dilution of mixed input samples was used as a calibration curve to estimate enrichment of specific DNA sites in ChIP reactions. ChIP primer sequences are listed in Table I.

**RT PCR**

RNA was prepared from human SMC using the RNeasy kit from Qiagen. RNA was quantified by the Nanodrop method and RT-PCR was performed on 15 ng of RNA using the Verso 1-Step RT-PCR Kit from Thermo Scientific. RT-PCR products were separated on agarose gel and five and 15ng of RNA from TGF-β treated samples were used to generate a quantification curve. All primers sequences were designed around exon junctions and are listed in Table II.

**Statistical comparisons**

Data are expressed as mean ± SEM. Two group comparisons were analyzed by the two-tailed Student’s t test for independent samples.

**References**


<table>
<thead>
<tr>
<th>HuMHC GCrep for</th>
<th>GGGCGGGAGACAAACCCAAAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuMHC GCrep rev</td>
<td>GGAAGGCCACTCGGCACCAT</td>
</tr>
<tr>
<td>HuSMA for</td>
<td>AGCAGAACAGAGGAATGCAGGGGAAGAG</td>
</tr>
<tr>
<td>HuSMA rev</td>
<td>CCTCCACTCGGCTTCCAAACAAAGG</td>
</tr>
<tr>
<td>HuSM22 for</td>
<td>CCCGCTAGCTGTCCAACCT</td>
</tr>
<tr>
<td>HuSM22 rev</td>
<td>CTGGTGAGGAGGTTAAAGG</td>
</tr>
<tr>
<td>HuCalponin for</td>
<td>CCCCTAGGTGGAAACATG</td>
</tr>
<tr>
<td>HuCalponin rev</td>
<td>TTCCCTCCTCACAGTTGGTTG</td>
</tr>
<tr>
<td>MoMHC GCrep for</td>
<td>CTGGCGGGGAGACAAACCCAAAA</td>
</tr>
<tr>
<td>MoMHC GCrep rev</td>
<td>CTGGCGGGGAGACAAACCCAAAA</td>
</tr>
</tbody>
</table>

Table I – ChIP primer sequences
<table>
<thead>
<tr>
<th>Gene</th>
<th>RNA forward</th>
<th>RNA reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calponin</td>
<td>AACAACTTCATGGACGGCCT</td>
<td>TCTCCAGCTGGTGCCAATTTT</td>
</tr>
<tr>
<td>SM22</td>
<td>GTGCATTTCAGGCAGGCTCTC</td>
<td>GCACTATGATCCACTCCACCA</td>
</tr>
<tr>
<td>SM MHC</td>
<td>GCGTGTTGGTCAACCCCTAT</td>
<td>TGTTTTTCTGACCCTTGGAGA</td>
</tr>
<tr>
<td>SMA</td>
<td>GCTTTTCAGCTTCCCTGAACACC</td>
<td>TGTTTTTCGTCACCACGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AATGGGCAGCCGGTTAGGAAA</td>
<td>GCGCCCAATACGACCAAATC</td>
</tr>
</tbody>
</table>

Table II – RT PCR primer sequences
Supplemental Figure Legends

Suppl Fig I. SRF protein levels were not affected by RBPJ knockdown. Western blot for RBPJ and SRF in control and RBPJ siRNA treated human aortic SMC under growth and TGF-β-treated conditions.

Suppl Fig II. Effects of TGF-β on methylation of the CpG island within the SM MHC promoter. Genomic DNA samples from 10T1/2 cells treated with vehicle or TGF-β (1ng/ml) were subjected to bisulphite sequencing. Methylation of specific CpGs are expressed as a percentage of full methylation at that site.

Suppl Fig III. An RBPJ/NICD1 complex can form on a methylated binding site. The indicated RBPJ consensus probe variant was combined with 1 μL of in vitro translated RBPJ and 0 - 3 μL of in vitro translated NICD1.

Suppl Fig IV. The SM22 GC repressor does not bind RBPJ. Methylated and unmethylated radiolabeled GC repressor probes from the SM MHC and SM22 GC promoters were combined with SMC nuclear extracts. After 30 min, reactions were run on a 5% non-denaturing polyacrylamide gel which was then dried and exposed to film.
Suppl Figure I

RBPJ siRNA

Undiff  TGF-β

-  +  -  +  -  +

α RBPJ

α SRF

α Histone H3
Suppl Fig IV

<table>
<thead>
<tr>
<th>GC rep Meth</th>
<th>MHC</th>
<th>SM22</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ns RBPJ
Materials and Methods

Antibodies and reagents
The following antibodies were used: SRF (Santa Cruz, sc-335), SM MHC (Abcam, 53219), SM \( \alpha \)-actin (Sigma, A5228), Calponin (LifeSpanBio, 49769), SM22 (Santa Cruz, sc-271719), GAPDH (Santa Cruz, sc-25778), RBPJ for Westerns (Cell Signaling, 5313), RBPJ for supershifts (CosmoBioCo, K0043), RBPJ for ChIP assays (a kind gift from Raymond McDonald (University of Texas Southwestern, Dallas), acetylated H3K9 (Abcam, 12178), Notch3 (Santa Cruz sc-5593) RNA Polymerase II (Millipore, 05-952).

Cell culture and treatments
Human aortic SMC were purchased from Lonza and were maintained in Lonza Sm-GM-2 smooth muscle growth media containing 5% FBS. Multi-potential 10T1/2 cells were cultured as described previously 1. RBPJ \textit{flox/flox} mice were kindly provided by Raphael Kopan (Washington University, MO, USA) with permission of Tasuku Honjo (Kyoto University, Japan) and have been described previously 2. Aortic SMC were isolated from 4-6 week old male C57/Bl6 and RBPJ \textit{flox/flox} mice by enzymatic digestion with elastase and collagenase. The adventitial and endothelial cell layers were removed mechanically. SMC were maintained in DMEM:F12 (1:1) supplemented with 10% FBS and 1% penicillin-streptomycin and were typically used between passages 5 and 10. For Cre-dependent knockout experiments, cells were treated with Cre or LacZ expressing adenovirus for 48 h. In some experiments cells were serum starved for 24 h and then treated with PDGF-BB (20ng/ml) or TGF-\( \beta \) (1ng/ml) for 24h. For harvesting protein, cells were lysed in RIPA buffer plus protease and phosphatase inhibitors (15 mM Hepes pH7.2, 140 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS).

siRNA knockdowns
Cells were transfected with siRNAs for RBPJ (siRNApool M-007772-00 from siGENOME), SRF (UAUACAUACCGAAAC[dt][dt] and AUGUUUGCCAUGAUUAA[dt][dt]), or GFP (GGUGCCUCUCCUGACGUAGCC and GCCUACGUCAGGAGCGCACC) using Dharmafect (Dharmacon) as per manufacturer’s protocol. When co-depleting RBPJ and SRF the total amount of siRNA was equalized by addition of GFP siRNA. Cells were re-transfected the following day using the same protocol. Cells were then either maintained in Sm-GM-2 growth media for 40h or serum starved for 16h and then treated with TGF-\( \beta \) (1ng/ml) for 24h.

Luciferase reporter assays
The SM \( \alpha \)-actin, SM22, and minimal thymidine kinase promoters used have been previously described 1. The SM MHC promoter from -1262 to +132 bp was amplified from mouse genomic DNA and cloned into a CpG-less luciferase vector that was a kind gift of Michael Rehli (University of Regensburg, Germany). C to T mutations of the GC repressor element where introduced by PCR. For tranfections, cells were maintained in 48 well dishes in 10% serum media and transfected 24 h after plating at 70-80% confluency using LT-1 transfection reagent (Mirus) per protocol. Luciferase activity was measured 48 h post-transfection and is expressed relative to activity of the promoterless vector. In some experiments, luciferase reporters were co-transfected with a myocardin expression plasmid or an empty expression vector as a control. Luciferase results are presented as means and standard errors from at least 3 independent transfections.

DNA affinity chromatography
Mouse aortic SMC nuclear extracts were combined with 150 nmoles of annealed biotinylated GC repressor oligos (methylated or unmethylated) and 150nmoles of 20 mer random oligos in
binding buffer (10 mM Hepes pH 7.4, 8% glycerol, 150 mM NaCl, 1 mM MgCl2, 0.05% Triton x 100, 1.3 mM dithiotheritol). After 10 min of rotation at room temperature, 100 ul of 33% streptavidin agarose (Sigma) was added and reactions were incubated for an additional 30 min. Pelleted beads were washed 2x with wash buffer (KCl 75 mM, Hepes 5 mM pH 7.4, MgCl 0.5 mM, glycerol 4%, Tween 20 0.05%, DTT 1 mM) and bound proteins were eluted with 500 mM NaCl. Eluted samples were subjected to a second round of affinity purification as above except that the binding buffer also contained 1 mM spermine and 1 mM spermidine. Eluted proteins were run on an SDS-PAGE gel and Coomassie stained bands were sent to the UNC mass spec core facility for identification. See on-line supplement for oligo sequences.

**Gel shift assays**

RBPJ clone BC051387 was purchased from OriGene and cloned into mammalian expression pcDNA3 vector (Invitrogen) containing an N-terminal flag epitope tag. RBPJ mutations were generated by PCR. The NICD1 expression construct was a kind gift from Stephen Blacklow (Harvard University, Boston). RBPJ, SRF, and NICD proteins were in vitro translated using the TnT kit from Promega according to protocol. Nuclear extracts were prepared from mouse aortic SMC and frozen and ground carotid artery samples using the NE-Per nuclear extraction kit from Thermo Scientific according to the manufacturer's instructions. Binding reactions were incubated for 30 min at RT and contained 5 µg of nuclear extract or 4 µL of TnT reaction mix, 0.12 pmoles of 32P-labeled oligonucleotide probe, and 2.5 pmoles of random oligos in binding buffer (10 mM Hepes pH 7.4, 8% glycerol, 150 mM NaCl, 1 mM MgCl2, 0.05% Triton X 100, 1 mM dithiotheritol). For supershifts, 3 µL of anti-RBPJ antibody was added during incubation. Samples where run on native gel, dried and exposed to film. Probe sequences used were as follows (RBPJ binding sites in bold): SM MHC GCrep – GGCTGGGCGGGAGACAACC; Methylated SM MHC GCrep - GGCTGGGG[G5Me-dC]GGGGAGACAACC; consensus RBPJ – GGAATGTGCAGAAAAAGAAG; consensus RBPJ T to C – GGAATGTGGGAGAAAAAGAAG; consensus RBPJ T to C Me – GGAATGT[G5Me-dC]GGGAGAAAAAGAAG; SM22 GCrep – TTTTCCCGCCGCCCCTCAGC; Methylated SM22 GCrep - TTTTCC[G5Me-dC]GGC[G5Me-dC]GGCCCTCAGC.

**DNA affinity chromatography**

Mouse aortic SMC nuclear extracts were combined with 150 nmoles of biotinylated GC repressor oligos (methylated and unmethylated) and 150 nmoles of 20 mer random oligos in binding buffer (10 mM Hepes pH 7.4, 8% glycerol, 150 mM NaCl, 1 mM MgCl2, 0.05% Triton x 100, 1.3 mM dithiotheritol). After 10 min of rotation at room temperature, 100 ul of 33% streptavidin agarose (Sigma) was added and reactions were incubated for an additional 30 min. Pelleted beads were washed 2x with wash buffer (KCl 75 mM, Hepes 5 mM pH 7.4, MgCl 0.5 mM, glycerol 4%, Tween 20 0.05%, DTT 1 mM) and bound proteins were eluted with 500 mM NaCl. Eluted samples were subjected to a second round of affinity purification as above except that the binding buffer also contained 1 mM spermine and 1 mM spermidine. Eluted proteins were run on an SDS-PAGE gel, and Coomassie stained bands were sent to the UNC mass spec core facility for identification. Oligos used were as follows: Methylated GC repressor - biotinGGTTGTCTCC[G5Me-dC]GGCCAGGCC; GC repressor - biotin-GGTTGTCTCCCGCCAGGCC. Random oligos - TNNNNNNNNNNNNNNNNNNA.

**Bisulfite conversion and sequencing.**

Genomic DNA was extracted from cells and frozen and ground aortae using the QiaAmp kit from Qiagen. Bisulfite conversion was done using the MethylCode bisulfite conversion kit from Invitrogen. Following PCR amplification, products were either sequenced directly or were subcloned into a vector for transformation and sequencing of individual clones.
Chromatin immunoprecipitation
The SimpleChip Kit from Cell Signaling was used according the manufacturer's instruction. 1% of starting material was used to control equal quantity of DNA in reactions from different samples. DNA was extracted using ZymoResearch ChIP DNA clean and concentrator kit according to manufacturer instruction, eluted in 20 μl and 1 μl was used in PCR reactions. Serial dilution of mixed input samples was used as a calibration curve to estimate enrichment of specific DNA sites in ChIP reactions. ChIP primer sequences are listed in Table I.

RT PCR
RNA was prepared from human SMC using the RNeasy kit from Qiagen. RNA was quantified by the Nanodrop method and RT-PCR was performed on 15 ng of RNA using the Verso 1-Step RT-PCR Kit from Thermo Scientific. RT-PCR products were separated on agarose gel and five and 15ng of RNA from TGF-β treated samples were used to generate a quantification curve. All primers sequences were designed around exon junctions and are listed in Table II.

Statistical comparisons
Data are expressed as mean ± SEM. Two group comparisons were analyzed by the two-tailed Student’s t test for independent samples.

References

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuMHC GCrep for</td>
<td>GGGCGGGAGACAACCCAAAA</td>
</tr>
<tr>
<td>HuMHC GCrep rev</td>
<td>GGAAGGCCACTCGGCACCAT</td>
</tr>
<tr>
<td>HuSMA for</td>
<td>AGCAAGACAGAGGAGATGCAGTGGAAGAG</td>
</tr>
<tr>
<td>HuSMA rev</td>
<td>CCTCCACTCGGTTCCAAAACAAGGAGC</td>
</tr>
<tr>
<td>HuSM22 for</td>
<td>CCCGCTAGACTGCTCCAAC</td>
</tr>
<tr>
<td>HuSM22 rev</td>
<td>CTTGGGTAGGGGTTTAAAGG</td>
</tr>
<tr>
<td>HuCalponin for</td>
<td>CCCCTAGTGGAACACAATG</td>
</tr>
<tr>
<td>HuCalponin rev</td>
<td>TTCCCTCTCATGTTTTTTG</td>
</tr>
<tr>
<td>MoMHC GCrep for</td>
<td>CTGCGGGAGGACCATATTAGTCAGGG</td>
</tr>
<tr>
<td>MoMHC GCrep rev</td>
<td>CTTGGCGGGAGACAACCCAAAAAGGCCA</td>
</tr>
</tbody>
</table>

Table I – ChIP primer sequences
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calponin</td>
<td>AACAACTTCATGGACGGCCT</td>
<td>TCTCCAGCTGGTGCCAATTT</td>
</tr>
<tr>
<td>SM22</td>
<td>GTGCATTTCAGGCAGGGCTCTC</td>
<td>GCACTATGATCCACTCCACCA</td>
</tr>
<tr>
<td>SM MHC</td>
<td>GCCGTGGGTGGTCAACCCCTAT</td>
<td>TGTCTTTCTTGGCCCTTGTTGGA</td>
</tr>
<tr>
<td>SMA</td>
<td>GCTTTTCAGCTTCCTGAACACC</td>
<td>TGTCGCTTCTGACCCACGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AATGGGCAGCCGTAGGAAA</td>
<td>GCGCCCAATACGACCAAATC</td>
</tr>
</tbody>
</table>

Table II – RT PCR primer sequences