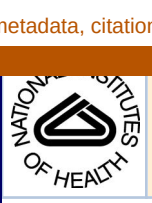

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DNA methylation of a GC repressor element in the SM MHC promoter facilitates binding of the Notch-associated transcription factor, RBPJ/CSL1

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Abstract

Objective—The goal of the present study was to identify novel mechanisms that regulate 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 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, RBPJ/CSL1, as a methylated GC-repressor binding protein. RBPJ protein levels and binding to the endogenous SM MHC GC repressor were enhanced by PDGF-BB treatment. A methylation mimetic mutation to the GC repressor that facilitated RBPJ binding inhibited SM MHC promoter activity as did over-expression 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 SRF and Pol II to the SMC-specific promoters. In contrast, depletion of RBPJ in differentiated TGF-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.

Keywords

Smooth muscle; serum response factor; epigenetics; Notch; RBPJ

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Disclosures – none

INTRODUCTION

It has been well established that SRF and the myocardin family of SRF co-factors mediate SMC-specific transcription by interacting with conserved CArG elements within the promoters of the SMC differentiation marker genes [1]. However, since these transcription factors are expressed in many non-SMC sub-types [2], 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 modification of chromatin structure. Histone modifications that favor gene expression (i.e. H3 and H4 acetylation, H3K4 methylation, H3K9 demethylation/acetylation) have been observed at the SMC-specific promoters in SMC [3–7]. In addition, we and others have shown that the myocardin factors can facilitate chromatin modification by recruiting histone modifying enzymes [5, 8–12].

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 (see [13] for review). 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 tri-methyl H3K9, and these negative chromatin marks act cooperatively by the reciprocal recruitment of their respective methyltransferases (see [14] for review).

Based upon 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 [8], we hypothesized that DNA methylation may also play a role. Our results indicate that methylation of a GC repressor in the SM MHC promoter inversely correlates with SM MHC expression in aortic SMC, that the methylated GC repressor recruits the multifunctional transcription factor RBPJ/CSL-1, and that RBPJ can inhibit SMC marker gene expression in phenotypically modulated human aortic SMC.

MATERIELS AND METHODS

Detailed methods can be found in the on-line 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 at least 200 bp in length, a GC content of greater than 50%, and an observed-to-expected CpG ratio greater than 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 bisulphite 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 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 (Fig 1b). However, we also detected significant methylation of the SM MHC promoter in SMC. Since the SM MHC gene is the first differentiation marker to be down-regulated 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 following 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 (Fig 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 down-stream of CArG2 that had been previously characterized as a repressor element [15]. Deletion of a similar but not identical GC repressor within the SM22 promoter prevented the down-regulation of SM22 promoter activity that occurs following vessel injury [16, 17]. The GC repressor within the SM MHC promoter was also fully methylated in mouse EC (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 (Fig 1d, compare lanes 1 and 4). In addition, complex binding to the methylated repressor was increased when extracts were prepared from cells treated with PDGF-BB, an agonist known to down-regulate SMC differentiation marker gene expression. In contrast, treatment of cells with TGF-b whih activates SMC

differentiation marker gene expression had little effect on complex formation. To better 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 (Fig 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 non-specific (Fig 2 and data not shown).

The methylated SM MHC GC repressor binds RBPJ

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 spec analysis. Each of three 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 (Fig 2a; compare lanes 1 and 5). Moreover, addition of an RBPJ antibody to gel shift reactions resulted in a complete super-shift 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 EC and SMC (see [18] for review). 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 has been shown to inhibit gene expression by recruiting HDACs and other transcriptional repressors. NICD binding to RBPJ displaces the repressive factors (resulting in derepression) but also aids in the recruitment of additional transcription activators. Although Notch signaling has been shown to be required for SMC differentiation of neural crest cells, epicardial cells, or Tie1-expressing progenitor cells [19–21], the direct effects of Notch/RBPJ signaling on SMC differentiation marker gene expression are relatively weak [22–24], are significantly complicated by feedback inhibition mediated by the Notch target genes, Hes and Hey (see [25] for review), and are context dependent with several studies showing both positive and negative effects [22–24, 26–28].

Characterization of RBPJ binding to methylated DNA

We noted that the reverse strand of the GC-rich element (GCGGGA) differed from the consensus core RBPJ binding site by only one base-pair, a thymine to cytosine substitution. Additional gel shift assays with hemi-methylated probes demonstrated that only methylation

of the reverse strand was required for RBPJ binding (Fig 3a). Since the pyrimidine ring of thymine is constitutively methylated at the 5′ position (Fig 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 (Fig 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 (Fig 3c, compare lanes 7 and 6).

The crystal structure of RBPJ bound to its consensus DNA element has been solved [29, 30]. 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, over-expression 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 $RBPJ^{flox/flox}$ mice and used adenoviral-mediated expression of Cre recombinase to reduce RBPJ expression and DNA binding in these cells by approximately 85% (Fig 4b). Importantly, SM MHC promoter activity in SMC over-expressing myocardin was significantly higher in Cre versus LacZ infected SMC (Fig 4c). Although these results strongly suggest that RBPJ can function as an inhibitor in these contexts, the lack of reagents to the 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 (see 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 (Fig 5a), and in excellent agreement with our gel shift assays, that this interaction was enhanced by PDGF-BB treatment. As shown in Fig 5b, PDGF-BB treatment increased RBPJ protein levels in both SMC and 10T1/2 cells. Given that methylation of the GC repressor is already

very 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 [22–24]. However, our demonstration that RBPJ binds to methylated DNA, is up-regulated 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 very 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 binds 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 [23, 24], 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 (Fig 6c) even though SRF protein levels were not affected (suppl Figure I). Moreover, the increase in SMC marker gene mRNA in RBPJ knockdown cells was completely inhibited by co-depletion of SRF (Fig 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. While 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 (Fig 6e) and H3K9 acetylation (Fig 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 TGF-β-treated cells a result consistent with the requirement of Notch signaling for SMC differentiation in vivo[19–21]. It is likely that inhibition of NICD 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 NICD and the positive transcriptional effects of Notch signaling.

Although DNA methylation can promote long-term and heritable gene inactivation [31], its role in the regulation of cell-type-specific gene expression is less clear [31–38] and may be related to the number and methylation status of CpGs within a particular promoter [38–40]. Since de novo DNA methylation is catalyzed by DNA methyltranferases 3a and 3b, it will be important to identify the mechanisms by which these enzymes are recruited to the SM MHC 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 MHC expression [8], and we are currently investigating whether this histone modification precedes and/or promotes SM MHC promoter methylation. Another interesting question is whether changes in DNA methylation regulate SMC-specific gene expression more acutely. We observed modest reductions in SM MHC promoter methylation during TGF-β-induced up-regulation of SM MHC expression in 10T1/2 cells (see suppl Fig II). However, the known heterogeneity in these cells is a confounding factor and additional experiments in the subset of cells that up-regulate SM MHC expression will likely be required to demonstrate that SM MHC 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 [41, 42] suggesting that DNA methylation (like histone methylation) is more dynamic than previously thought [43]. 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 [44] while SM22 methylation was decreased in multipotential adventitial cells that were induced to differentiate into SMC [45]. Hu et al. has also shown that SM α-actin expression in fibroblasts inversely correlated with DNA methylation near the SM α-actin transcription start site [46].

Using an unbiased SILAC-based biochemical screen, Bartels et. al. were the first to show that RBPJ interacted with methylated DNA [47]. Our data strongly confirm that RBPJ binds to the sequence, G(Cm/T)GGGA, and we have significantly extended this finding by providing functional significance to RBPJ's interaction with methylated DNA and by identifying SM MHC as an in vivo target for this mechanism. It is currently unclear whether RBPJ interacts with other gene promoters through a methylation-dependent mechanism or whether RBPJ binding to a methylated versus unmethylated sequence results in different transcriptional outcomes. Our gel shift data indicate that the methylated GC repressor can support the formation of a RBPJ/Notch complex in vitro (suppl Fig III), but it is unknown whether RBPJ's interaction with repressive cofactors is affected. Defining the relationship between multiple RBPJ binding sites within a single promoter will also be important. As shown in suppl Figure IV, we did not detect significant binding of RBPJ to the SM22 GC repressor in gel shift assays most likely because it does not conform to the consensus RBPJ binding site even when methylated. Recent studies indicated that the effects of the SM22 GC repressor are mediated by the pluripotency factor, KLF4 [17], and it may be interesting to test whether KLF4 binding to this element is affected by DNA methylation status.

Structural analyses of several zinc finger transcription factors that bind to methylated DNA identified a 5mCytosine-Arg-Guanine triad that mediates this interaction (see [48] for review), 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 casual for Adams-Oliver syndrome, a disease that affects limb and cranium formation [49]. Interestingly, the methylated DNA binding proteins, Kaiso and Zfp57, have similarly positioned Glu residues [48] 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 C2C12 cells [50–52]. Although Notch/RBPJ binding to the SMC marker gene promoters was not detected in these assays, several interesting findings are worth noting. Many RBPJ-only and Notch-only binding sites were identified suggesting that these transcription factors have independent effects and perhaps that Notch can be recruited to DNA by transcription factors other than RBPJ. In addition, Notch/RBPJ binding was a poor predictor of gene activation. In fact, only 3% of the genes that were shown to bind Notch exhibited significant expression changes upon Notch activation strongly suggesting that additional transcription mechanisms are important for regulating Notch/RBPJ-dependent gene expression. Finally, bioinformatic analysis of over-represented sequences in the RBPJ ChIP seq data set from neuronal cells failed to identify the consensus RBPJ element, but did identify a GC rich sequence similar to the GC repressor. Additional studies that also incorporate methylation status and changes in gene expression in knockout cells will be required to better characterize the extent to which promoter methylation regulates Notch/RBPJ-dependent gene activation and/or repression.

In summary, our results indicate that RBPJ can inhibit SMC marker gene expression in phenotypically modulated SMC and that methylation-dependent recruitment of RBPJ may facilitate this repressive mechanism. These results have significant implications on our understanding of RBPJ/Notch-dependent regulation of cardiovascular development and disease and support further characterization of this mechanism and its consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and acronyms

SRF	serum response factor
MRTF	myocardin-related transcription factor
SMC	smooth muscle cell
EC	endothelial cell
MHC	myosin heavy chain
RBPJ	recombination signal binding protein for immunoglobulin kappa J region
NICD	Notch intracellular domain
PDGF	platelet-derived growth factor
TGF	transforming growth factor
ChIP	chromatin immunoprecipitation

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Significance

We show for the first time that methylation of a GC repressor in the SM MHC promoter inversely correlates with SM MHC expression in aortic SMC, that the methylated GC repressor recruits the multifunctional transcription factor RBPJ/CSL-1, 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.

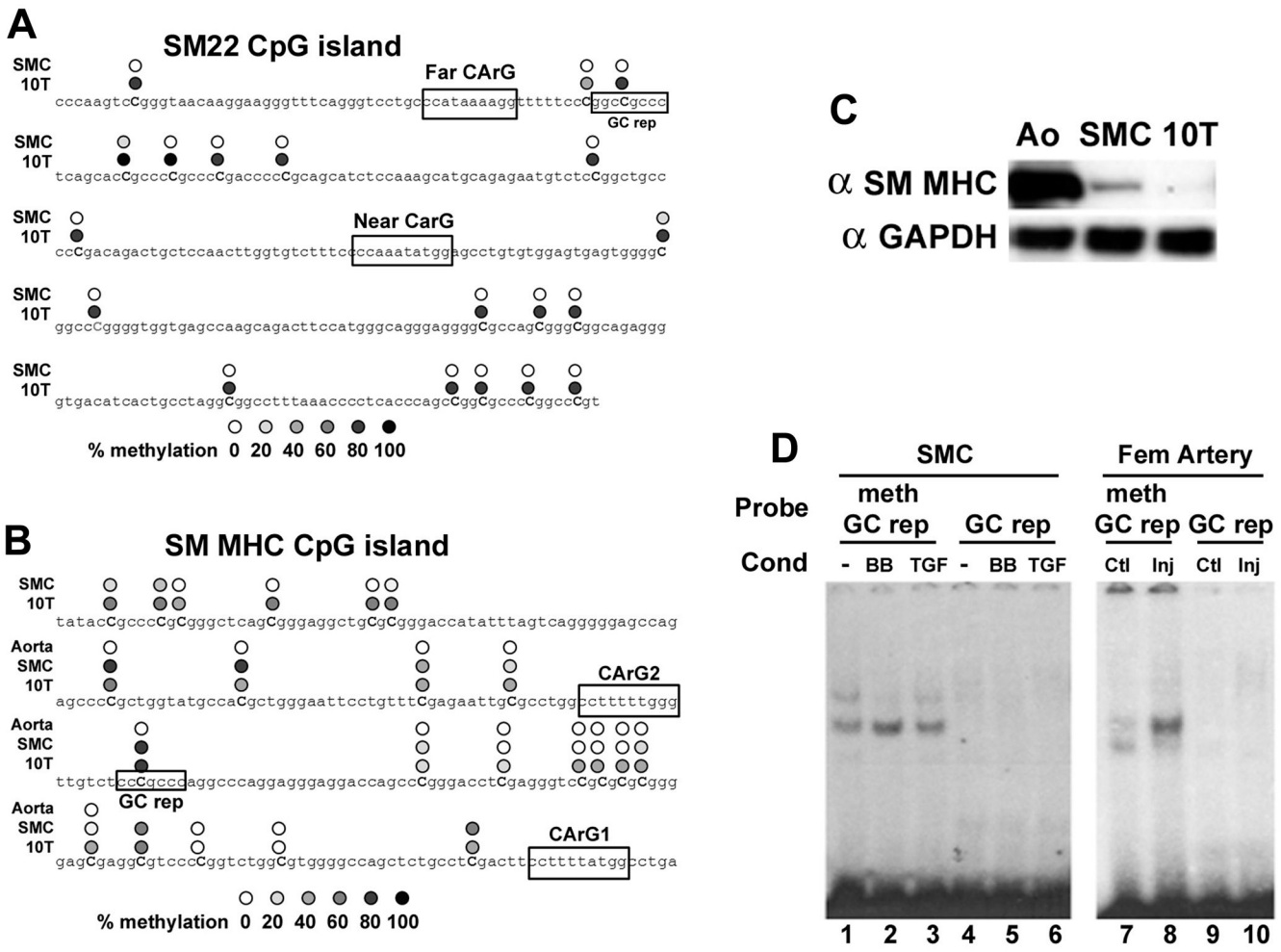

Figure 1. A GC repressor element in the mouse SM MHC promoter is methylated in cultured SMC

Schematic of CpG island methylation within the CArG-containing regions of the SM22 **(A)** and SM MHC **(B)** promoters as determined by bisulphite 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 24h with vehicle, PDGF-BB (20ng/ml), or TGF-β (1ng/ml) and from control (Ctl) or injured (Inj) femoral arteries. After 30 min, reactions were run on a 5% non-denaturing polyacrylamide gel which was then dried and exposed to film.

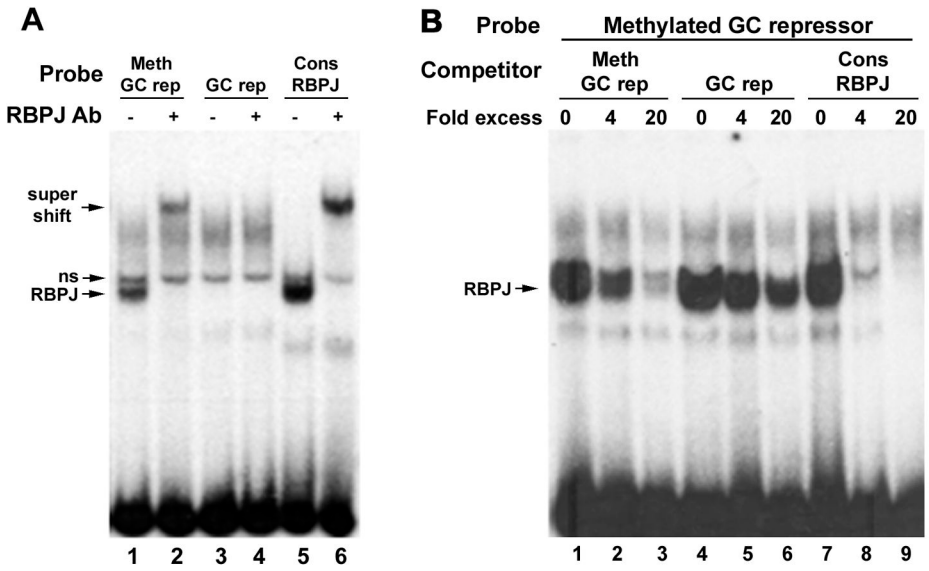

Figure 2. The methylated SM MHC GC repressor binds RBPJ

A) The indicated oligonucleotide probes were incubated with SMC nuclear extracts +/− an antibody for RBPJ. After 30 min, reactions were run on a 5% non-denaturing polyacrylamide gel which was then dried and exposed to film. ns, non-specific band. **B)** The indicated non-radiolabeled competitor oligonucleotide was added in increasing concentrations to gel shift reactions containing the methylated GC repressor probe and SMC nuclear extracts.

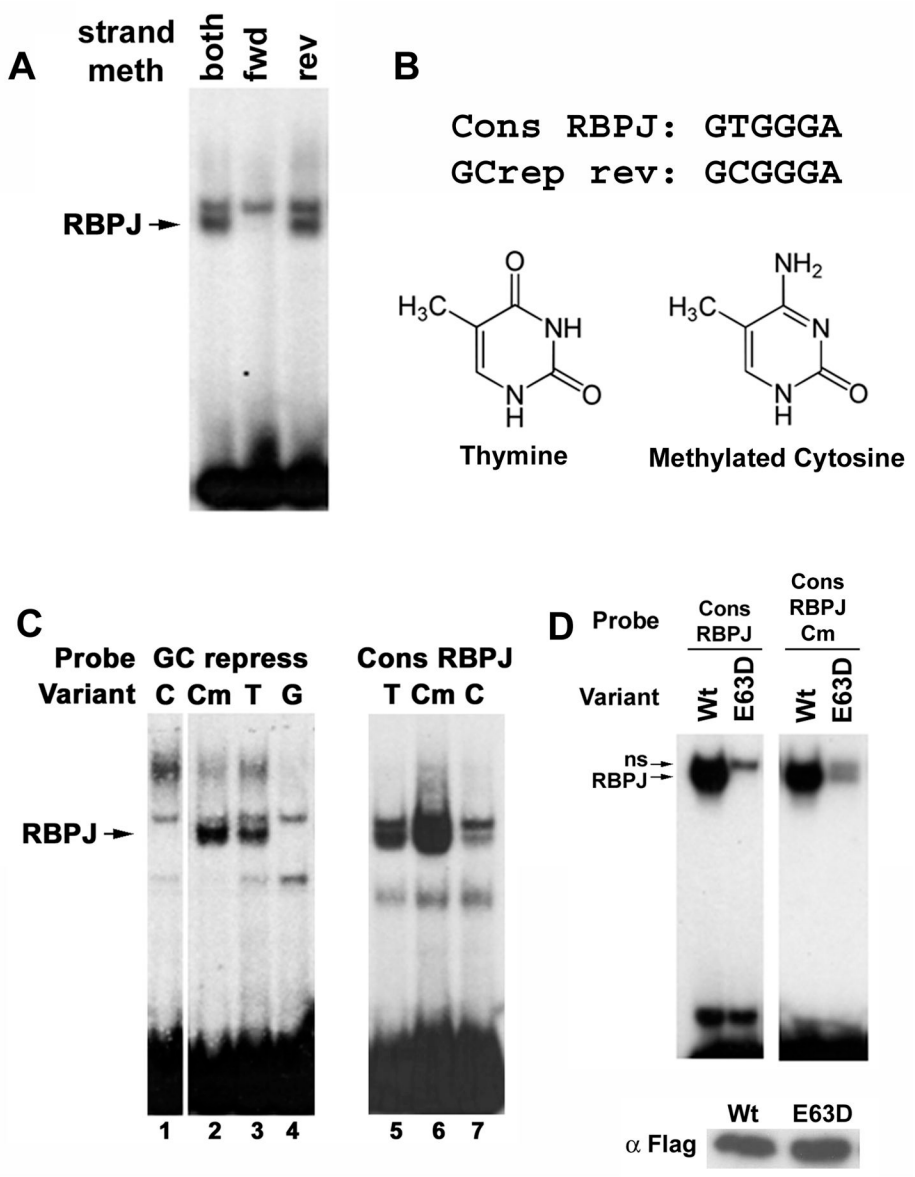

Figure 3. Analysis of the RBPJ-DNA binding complex

A) Gel shift analysis of RBPJ binding to fully and hemi-methylated GC-repressor probes. **B)** Comparison between consensus and GC repressor RBPJ binding sites and thymine and methylated-cytosine structures. **C)** Gel shift reactions using the indicated variant of the GC repressor or consensus RBPJ probes. Please see Materials and Methods for full oligonucleotide sequences. **D)** Wt and E63D RBPJ variants were expressed in vitro and used in gel shift reactions with Wt and methylated (T to Cm) consensus RBPJ probes. Note equal expression of each RBPJ variant as measured by Western blotting for the flag epitope.

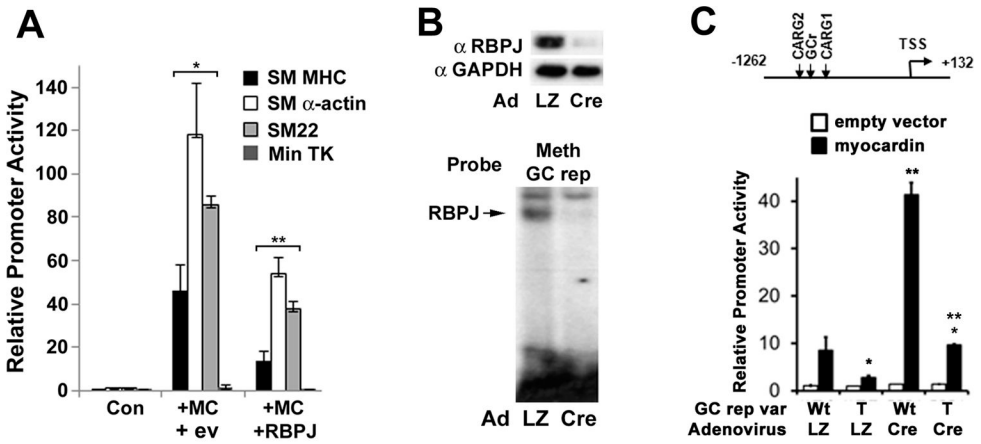

Figure 4. RBPJ inhibits SM MHC promoter activity

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 h and is expressed relative to promoter activity plus empty vector only (Con). * $p<0.05$ versus control. ** $p<0.05$ versus plus myocardin. **B)** Western blot and gel shift analyses demonstrating RBPJ knockdown in this 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 $RBPJ^{flox/flox}$ mice were treated with Cre- or LacZ-expressing adenovirus for 48 h and then transfected with the Wt and T mutant constructs. Luciferase activity was measured at 48 h. * $p<0.05$ versus Wt. ** $p<0.05$ versus LacZ.

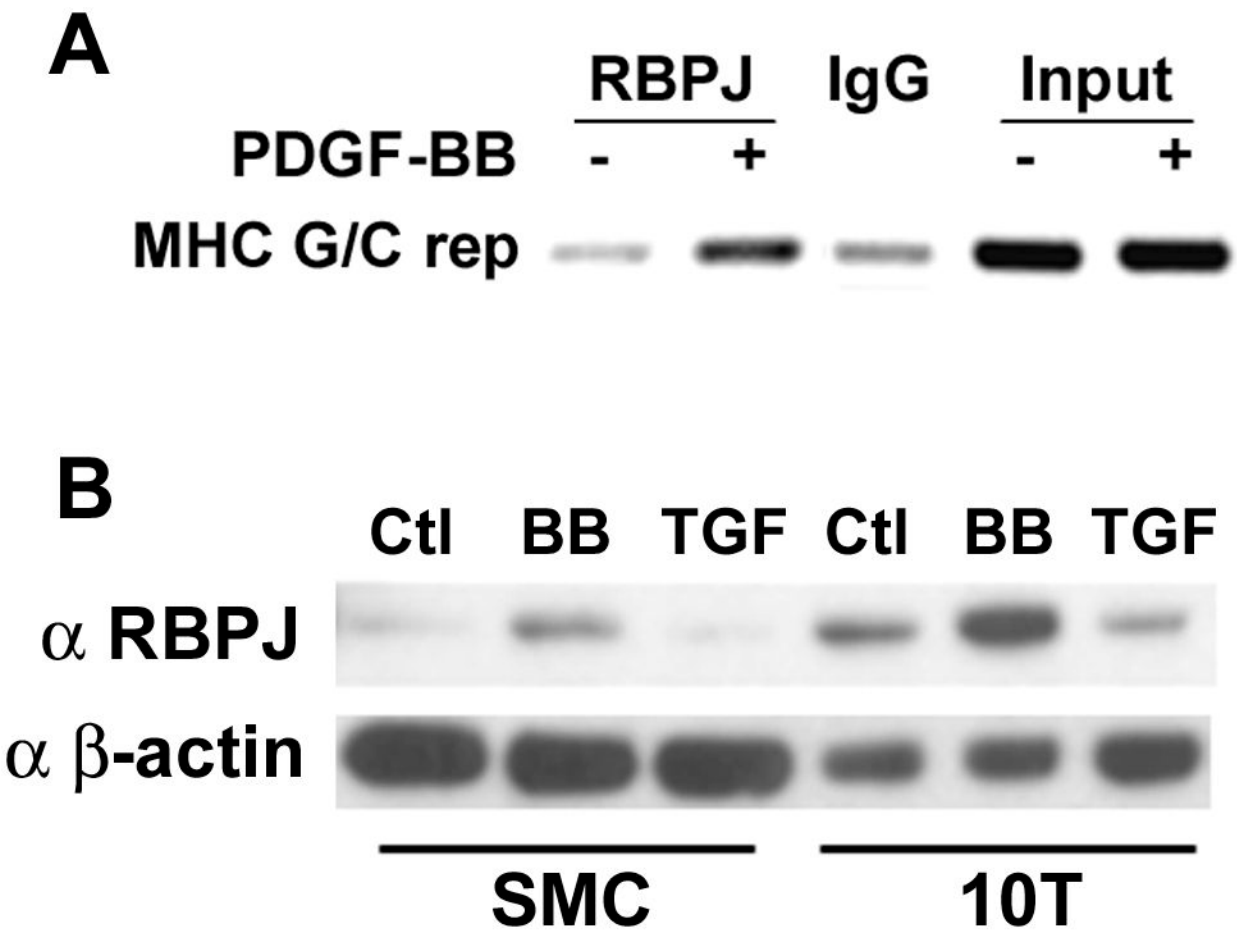

Figure 5. RBPJ binds the endogenous SM MHC repressor

A) Chromatin immunoprecipitation assays for RBPJ were performed in control and 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 TGF-β. The ChIP and Western Blot results shown are representative of at least three independent experiments.

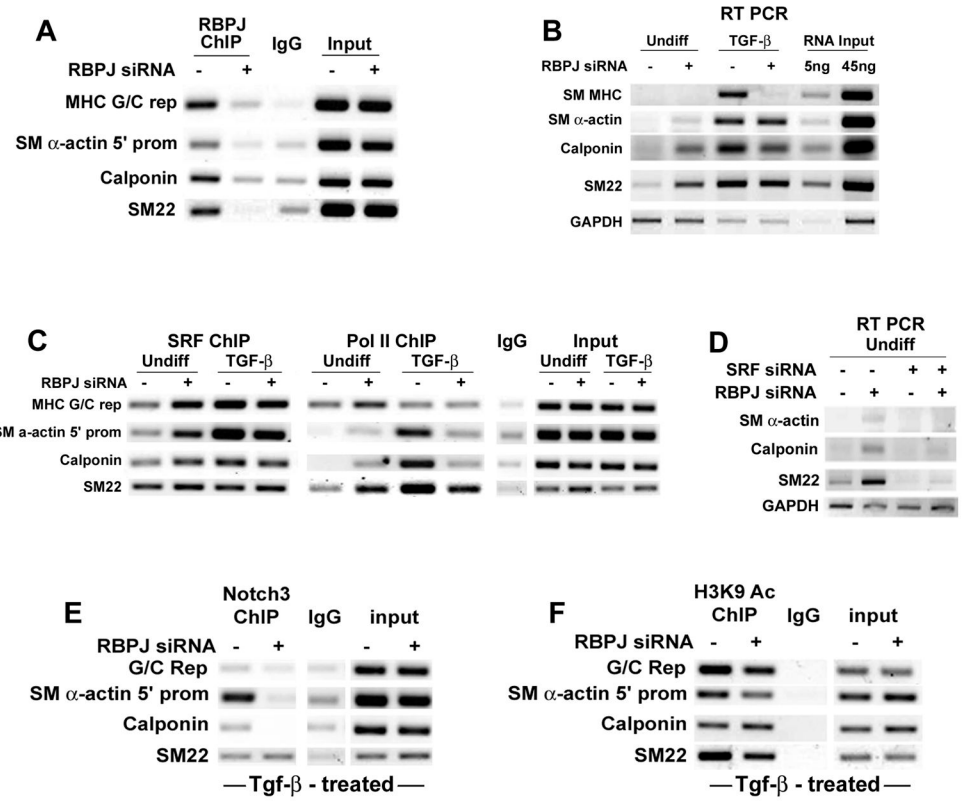

Figure 6. RBPJ inhibits SMC differentiation marker gene expression in phenotypically modulated human SMC

A) 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 TGF-β. Semi-quantitative RT PCR was performed for the indicated SMC marker genes and GAPDH. Results of RT-PCR using 5 and 45ng 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. Notch3 binding **(E)** and H3K9 acetylation **(F)** 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 at least 3 independent experiments.