Smooth Muscle Calponin: An Unconventional CArG-Dependent Gene that Antagonizes Neointimal Formation

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Abstract

Objective—Smooth muscle calponin (*CNN1*) contains multiple, conserved intronic CArG elements that bind serum response factor (SRF) and display enhancer activity in vitro. The objectives here were to evaluate these CArG elements for activity in transgenic mice and determine the effect of human CNN1 on injury-induced vascular remodeling.

Methods and Results—Mice carrying a lacZ reporter under control of intronic CArG elements in the human *CNN1* gene failed to show smooth muscle cell (SMC)-restricted activity. However, deletion of the orthologous sequences in mice abolished endogenous *Cnn1* promoter activity suggesting their necessity for in vivo *Cnn1* expression. Mice carrying a 38-kilobase bacterial artificial chromosome (BAC) harboring the human *CNN1* gene displayed SMC- restricted expression of the corresponding CNN1 protein as measured by immunohistochemistry and Western blotting. Extensive BAC recombineering studies revealed the absolute necessity of a single intronic CArG element for correct SMC-restricted expression of human CNN1. Over-expressing human *CNN1* suppressed neointimal formation following arterial injury. Mice with an identical BAC carrying mutations in CArG elements that inhibit human CNN1 expression, showed outward remodeling and neointimal formation.

Conclusions—A single intronic CArG element is necessary but insufficient for proper CNN1 expression in vivo. CNN1 over-expression antagonizes arterial injury-induced neointimal formation.

Keywords

calponin; transgenic mice; smooth muscle; serum response factor; transcription

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Smooth muscle cells (SMC) are essential constituents of the vessel wall that arise through complex cell-cell and cell-matrix signaling events at multiple sites during mouse embryogenesis [1]. SMC confer structural stability to endothelial cell-lining nascent blood vessels and, later in life, provide further structural support through formation of lamellar units and the elaboration of various extracellular matrix proteins. SMC also control the caliber of the vessel wall, and thus the flow of blood, through the coordinate action of many cytoskeletal and contractile proteins. As such, SMC in the normal adult vessel wall exhibit a quiescent, non-motile phenotype conducive for contraction and structural support [2]. This so-called contractile phenotype is compromised in a variety of vascular disorders including

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atherosclerosis, transplant arteriopathy, hypertension, and vein graft failure following coronary artery bypass graft surgery [3]. Such phenotypic adaptation involves attenuated expression of numerous cytoskeletal and contractile genes, the acquisition of a growth/ migratory state, and/or the transdifferentiation to other cell types, all of which contribute substantively to the pathogenesis of vascular disease. Hence, there has been enormous interest in elucidating the definition of vascular SMC phenotypes and, more importantly, the molecular circuitry governing these phenotypes.

One of the principal ways in which the normal adult vascular SMC phenotype is established is through the coordinate transcriptional activation of many contractile genes that are highly specific to these cells. Work over the last 20 years has uncovered a transcription factor binding code (TFBC) common to most SMC contractile genes. This binding code, known as a CArG box [4], is 10 base pairs in length and conforms to either a high affinity binding sequence of $CC(A/T)_6GG$ or to any one of more than 1,100 permutations of this consensus sequence [5]. The aggregate collection of functional CArG boxes in the genome, known as the CArGome [6], bind the serum response factor (SRF) transcription factor with varying affinity [7]. SRF is a widely expressed transcription factor that controls a variety of genes linked to the contractile apparatus and the actin cytoskeleton [5]. There is only one SRF-like gene in mammalian genomes; however, myocyte enhancer factor 2 proteins share homology both in functional protein domains and DNA binding sequences, though SRF and MEF2 do not compete for one another's binding site [8,9]. Genetic inactivation of SRF in developing vascular SMC results in attenuated expression of contractile genes and a reduction in the recruitment of nascent SMC to the dorsal aorta, both of which likely contribute to mid-gestation arrest of the mouse [10]. Indeed, every cell type in which SRF has been inactivated displays defective local homeostasis with death of the animal as a frequent endpoint [11]. Thus, CArG-SRF is viewed as a critical mediator of diverse cellular activities, including those linked to normal vascular SMC physiology.

Because SRF displays broad expression across essentially every cell type, including anucleated cells such as platelets [12], the ability to orchestrate specific programs of gene expression hinges upon its interaction with a growing number of cofactors. One such cofactor is myocardin (MYOCD), a cardiomyocyte- and SMC-restricted protein that powerfully activates a subset of SRF-dependent genes [13]. Myocardin transcripts are expressed abundantly in adult vascular SMC, but invariably decrease upon cell culture where SMC phenotypically adapt to a less contractile state [14]. Forced expression of MYOCD is sufficient to activate CArG-containing SMC contractile genes [14-16] and functional SMC-like contraction [17], so long as SRF is present [18]. Thus, the CArG-SRF-MYOCD triad constitutes the major transcriptional switch for establishment of a functional SMC contractile phenotype. Other molecular switches exist in a supporting role, including the recently discovered microRNA143/145 gene that promotes MYOCD-dependent SMC contractile gene expression by regulating a network of transcription factors and signaling proteins [19-23].

Formal proof of an SRF target gene's dependence on CArG elements for normal expression requires rigorous analysis in transgenic mice. Such analyses have been done to show CArG-dependent regulation of the *Tagln* [24,25], *Acta2* [26], *Myh11* [27], *Telokin* [28], *Kcnmb1* [29], and *Csrp1* [30] genes. The mouse SM calponin gene (*Cnn1*) contains multiple intronic CArG boxes that display enhancer activity in vitro [31]. These intronic CArG elements are completely conserved in sequence and space within the human *CNN1* gene. We previously reported SMC-restricted expression of human CNN1 during development and in post-natal tissues using BAC transgenic mice. The importance of intronic CArG elements, however, was not investigated [32]. Here, we report that CArG-containing intron 1 sequences within the *CNN1* gene are insufficient for directing proper transgene expression in SMC lineages,

although orthologous sequences are necessary in the context of a *Cnn1* knockout mouse. BAC transgenic mice with various CArG element mutations support the gene knockout phenotype and provide strong evidence for a critical role of a single intronic CArG element in the control of *CNN1* expression in vivo. Finally, we make the unanticipated observation that over-expression of human CNN1 confers resistance to outward remodeling and neointimal formation following arterial injury.

Materials and Methods

For an expanded Materials and Methods section, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Animals

Transgenic and *Cnn1* knockout mice were generated through standard methods and were handled in accordance with the University of Rochester's institutional animal care and use committee. Partial ligation injury of the carotid artery and mouse genotyping were done as described in the supplemental material. All mice were provided water and food *ad libitum*.

Bioinformatics

The human and mouse CNN1 genes were subjected to comparative genomics analyses using the visualization tools for alignment (VISTA, http://genome.lbl.gov/vista/index.shtml) and the basic local alignment search tool. Sequence motifs for CArG elements were generated with a sequence logo tool.

Expression assays

CNN1 detection was done by Western blotting and immunohistochemistry of various tissues using an antibody specific for the human antigen. Total RNA isolated from injured or non-injured carotid arteries was assessed for human and mouse *CNN1* expression by quantitative RT-PCR.

Luciferase assay

An upstream CArG-containing region was cloned into the pGL3 basic plasmid and transfected into cells in the presence or absence of either an SRF or myocardin expression plasmid and luciferase activity determined by luminometry.

Results

CNN1 Harbors Conserved Intronic CArG-Rich Regions

Functional TFBC are often identical in sequence and genomic position across multiple species. We routinely use the VISTA program [33] to compare orthologous gene sequences for conservation and TFBC discovery. A VISTA plot of the smooth muscle calponin locus (*CNN1*) shows 2 modules of high sequence identity within the first intron (Figure 1). Each module contains 2 conserved CArG elements (C2-C5, Figure 1). In a previous analysis of the mouse *Cnn1* gene, we showed that 3 of the conserved intronic CArG elements (C2, C4, and C5) bind SRF and display in vitro enhancer activity to varying degrees [31] based on the known sequence binding rules associated with CArG-SRF [34]. C2 represents a perfect consensus CArG box and binds SRF avidly whereas C4 and C5 deviate from the consensus CArG box by 1 bp and bind SRF weakly [31]. Because our prior study was confined to in vitro analyses only [31], we set out here to evaluate these CArG elements in the context of transgenic mice.

Intronic CArG Boxes are Insufficient, but Necessary, for Correct CNN1 Expression In Vivo

Smooth muscle calponin is transiently expressed in the heart during mouse embryogenesis but then becomes restricted to adult SMC lineages [35,36]. Based on our previous in vitro analysis of 3 intronic CArG elements [31], we surmised that intron 1 of human *CNN1*, whose CArG elements are 100% conserved with those in mice (data not shown), would orchestrate correct spatiotemporal expression of a lacZ reporter in transgenic mouse embryos. Surprisingly, out of 44 independent founder mice, 22 failed to display any detectable beta galactosidase staining and of the remaining 22, none exhibited correct cardiovascular-restricted activity (Supplemental Figure I). We then replaced exon 1, intron 1, and exon 2 of the mouse *Cnn1* gene with a lacZ reporter and removed the neomycin cassette to assess beta galactosidase staining in mice lacking all intronic CArG boxes (Figure 2A). Southern blotting (Figure 2B), quantitative RT-PCR (Figure 2C), and long and accurate PCR (data not shown) validated correct targeting of the *Cnn1* gene. No evidence of lacZ activity was observed in heterozygous embryonic (data not shown) or adult tissues (Figure 2D). Further, we have been unable to generate homozygous null mice despite a previous report of viable *Cnn1* knockout mice using a different targeting strategy [37]. The basis for this result is unknown and will be pursued in future studies. Because the lacZ reporter can be silenced [38], we determined whether the absence of beta galactosidase staining in our *Cnn1* heterozygous mice resulted from methylation of lacZ sequences; however, we found no evidence of methylated lacZ sequences (data not shown). Collectively, these results suggest that intronic CArG elements within smooth muscle calponin are necessary, but insufficient, for in vivo promoter/enhancer activity.

SMC-Restricted Expression of Human CNN1 in Mini-BAC Transgenes

We previously demonstrated correct spatio-temporal expression of human CNN1 protein derived from a 103-kb BAC transgene [32]. To determine whether shorter versions of the original BAC could direct similar patterns of staining, we trimmed the 103-kb BAC to 38-kb and 19-kb lengths (Figure 3, top). Several lines of mice carrying each of these mini-BAC transgenes replicated SMC-specific CNN1 staining in such SMC-rich tissues as aorta, bladder, distal esophagus, and vessels of both cardiac and skeletal muscle (red stain in Figure 3). We also noted strong staining for human CNN1 protein in uterus, bronchiolar SMC of the lung, and blood vessels in lung, kidney, and spleen (Supplemental Figure II). This staining was specific since non-transgenic littermates and non-SMC tissues (Figure 3 and Supplemental Figure II) showed no detectable CNN1 immunoreactivity. Substituting the CNN1 antibody with a non-immune IgG control also revealed the absence of specific staining (Supplemental Figure III). These results demonstrate that as little as 19-kb of BAC sequence is sufficient to direct the restricted expression of CNN1 in essentially all vascular and visceral SMC lineages of adult tissues.

A Single Intronic CArG Box is Necessary for Human CNN1 Expression in Transgenic Mice

We next generated a series of transgenic mouse lines with point mutated intronic CArG elements using a BAC recombineering strategy [39] (Figure 4A). Replacing ~1-kb of intronic sequence comprising all 4 intronic CArG elements (C2-C5, Figure 1) with a *galK* selectable marker (m1BAC38) completely abolished CNN1 protein staining in the aorta and reduced visceral SMC staining in the bladder (Figure 4B). Western blotting confirmed these expression changes (Figure 4C). Upon counter-selection, wherein the *galK* cassette is replaced with wildtype human *CNN1* BAC sequences containing point mutated CArG elements (m2BAC38), similar loss in CNN1 staining was observed indicating that attenuated SMC-specific CNN1 expression stems from loss in functional CArG elements. When only the consensus intronic CArG element (C2, Figure 1) was mutated (m3BAC38), there remained a dramatic decrease in CNN1 staining both in aorta and bladder (Figure 4C). These results were further supported by Western blotting studies that showed reduced

human CNN1 protein in aorta, bladder, and stomach of several m3BAC38 mouse lines (Supplemental Figure IV). Finally, we examined the expression of human CNN1 in embryonic day 12.5 embryos. These results demonstrated expected human CNN1 expression in the developing heart and aortic SMC of wildtype BAC38 mice, but loss of staining in m3BAC38 mice (Supplemental Figure V). Taken together, these results establish a necessary role for a single intronic CArG element (C2) for the expression of human CNN1 in mouse tissues. These findings are consistent with the loss in lacZ activity upon deletion of endogenous mouse *Cnn1* CArG elements (Figure 2D).

Identification and Functional Analysis of a Conserved CArG Box Upstream of the *CNN1* Locus

Comparative sequence analysis revealed a previously undetected CArG element located ~3-kb upstream of the *CNN1* gene (Figure 1 and Figure 5A). This CArG element falls within a block of conserved sequence containing several putative CREB-binding sites (Figure 5A). We first determined the activity of this CArG element in cultured cells. A luciferase reporter displayed SRF- and Myocardin-dependent transactivation, which was reduced upon mutation of the CArG element (Figure 5B). A ChIP assay showed enriched SRF binding to the upstream CArG element in human SMC (inset, Figure 5B). We next used BAC recombineering to replace the CArG/CREB-containing island of sequence homology with the *galK* cassette (m5BAC38) or counter-selected and re-introduced wildtype sequences with a point mutated CArG box (m6BAC38) (Figure 5C). Each transgene was then introduced into the mouse genome to evaluate the role of this upstream, conserved CArG-containing region in human CNN1 expression. Results showed that loss of the entire conserved sequence block (m5BAC38) or mutation of just the CArG element (m6BAC38) had no effect on CNN1 expression in aortic SMC as well as vessels of the heart and skeletal muscle (Figure 5D). Moreover, there was no change in expression of CNN1 in vascular and visceral SMC of other organs (Supplemental Figure VI). Thus, we conclude that an upstream functional CArG element (and putative CREB binding sites) is dispensable for CNN expression in vivo.

Human CNN1 Antagonizes Neointimal Formation in a CArG-Dependent Manner

Most SMC differentiation proteins, including CNN1, are down-regulated during atherogenesis or following various mechanical injuries to the vessel wall [3]. We performed partial ligation injury of the carotid artery in wildtype and pan-CArG mutant BAC38 mice to ascertain whether human CNN1 would be subject to the same negative regulatory cues accompanying arterial injury as endogenous mouse SMC differentiation markers [3]. The pan-CArG mutant BAC38 mouse was engineered to have all 5 CArG elements defined in Figure 1 mutated; we refer to this transgenic line as m8BAC38. Similar to the m3BAC38 mouse, m8BAC38 mice displayed virtually no expression of human CNN1 in adult aorta, bladder, and vascular SMC of brain, heart, kidney, and spleen (Supplemental Figure VII). Further, there was a complete absence of CNN1 immunostaining in the uninjured (data not shown) and injured (Figure 6Ad) carotid artery. In contrast, wildtype BAC38 animals exhibited strong CNN1 expression in medial SMC of the injured carotid artery (Figure 6Ac). Results from qPCR studies revealed similar reductions in the mRNA expression of human *CNN1* and endogenous mouse *Cnn1* 7 days after carotid ligation injury (Supplemental Figure VIII). Interestingly, there was a notable absence of neointimal formation in wildtype BAC38 animals as compared to the m8BAC38 controls (Figure 6A, panels c versus d). This was also evident in an independent, human CNN1-expressing transgenic line suggesting the phenomenon was not simply a result of the site-of-integration or some genomic perturbation (Supplemental Figure IX). Both vessel wall area (Figure 8B) and circumference (Figure 8C) were significantly higher in m8BAC38 mice suggesting that human CNN1 protein imparts resistance to both outward remodeling and neointimal formation following injury. To begin

to understand the basis for this unexpected phenotype, we evaluated the growth fraction of medial SMC 7 days after injury by Ki-67 staining; there was no difference in medial SMC growth rate (Supplemental Figure X). A summary of the 70 transgenic mice studied in this report is provided in Supplemental Table II.

Discussion

Vascular SMC are defined by a molecular signature of gene expression that includes an array of CArG-dependent cyto-contractile genes governed directly by the SRF-MYOCD transcriptional switch. The importance of SRF and MYOCD in the control of SMC gene expression has been demonstrated through gene knockout experiments [10,40,41]. Linking specific SRF-binding CArG elements to the activity of a gene's promoter or enhancer requires in depth analyses in transgenic mice. Such transgenic studies have demonstrated the sufficiency of 1 or more CArG elements in recapitulating correct spatial and temporal patterns of SMC-specific gene expression [42,43]. However, the smooth muscle isoform of calponin (*Cnn1*), which is expressed transiently in the developing heart before emerging as a highly restricted marker for adult SMC lineages [35,36], has been a rather unconventional SMC-specific gene. For example, while such SMC genes as *Acta2* [44], *Actg2* [45], *Tagln1* [24], and *Kcnmb1* [29] contain 2 or more conserved CArG elements in the immediate vicinity of their transcription start sites, no such CArG elements are present near *Cnn1*. Rather, several conserved CArG elements exist within the first intron of both human and mouse smooth muscle calponin. Each intronic CArG element binds SRF and displays variable enhancer activity in vitro [31]. However, as reported here, inclusion of human *CNN1* intronic CArG elements in a lacZ reporter fails to duplicate the cardiovascular-restricted expression of *Cnn1* in embryonic mice. On the other hand, removing the endogenous intronic CArG elements by way of gene targeting completely suppressed lacZ activity in developing mouse embryos and postnatal tissues that otherwise would exhibit high-level *Cnn1* expression. These results suggest that intronic CArG sequences in smooth muscle calponin require strict positional interactions with other genomic modules. A less likely explanation is there are salient differences in human *CNN1* intronic sequences that influence CArG element functionality in transgenic mice.

The insufficiency of 4 conserved CArG elements in the first intron of human SMC calponin to direct cardiovascular restricted activity of a lacZ reporter is unprecedented as every other SMC-restricted gene whose regulatory sequences have been examined in vivo, display at least partial duplication of the endogenous gene's pattern of expression. This is true for *Actg2* [46], *Tagln1* [47], *Acta2* [26], *Myh11* [27], *Telokin* [48], *Kcnmb1* [29], and *Csrp1* [30]. As reported here, the SMC calponin's unconventional in vivo regulation necessitated an alternative approach to solve this gene's in vivo expression control. Several years ago, we adopted a BAC transgenic strategy because BAC cloning vectors accommodate large (up to 350-kb) genomic sequences that are likely to contain most, if not all, regulatory elements controlling a gene's expression profile [49]. Another advantage of using BACs to elucidate the control of gene expression is the preservation of the native genomic landscape versus the out-of-genomic context that is evident when utilizing a surrogate reporter such as the bacterial lacZ gene [50]. We reported previously on the identification of a 103-kb BAC harboring an unadulterated (*i.e.*, no lacZ or GFP reporter introduced) human *CNN1* gene that we subsequently integrated into the mouse genome for transgenic studies. We found that human CNN1 expression reproduced the endogenous mouse gene's pattern of expression in both embryonic and postnatal tissues [32]. In the present report, we have trimmed the original BAC down to 38-kb and demonstrate the same pattern of human CNN1 expression as documented previously. We also provide evidence for a 19-kb BAC showing the same SMC-specific staining of adult tissues, including the vasculature. These results indicate that the *CNN1* gene is not under the remote control of distal regulatory elements as we initially theorized

based on results of other reports [50], and that all regulatory control elements for tissue-restricted expression of CNN1 are contained within a relatively small genomic interval.

Evidence is provided here to support a vital role for a single intronic CArG element (C2, Figure 1) in directing the complete expression profile of CNN1 in both embryonic and adult SMC lineages. This analysis, however, required that C2 be in its native genomic location within a BAC since its presence in the context of a lacZ reporter failed to direct SMC-specific lacZ activity. The results of the m3BAC38 (consensus C2 mutant) also indicate that the 3 other intronic and upstream CArG elements are insufficient for CNN1 expression within the 38-kb BAC. This result is reminiscent of single CArG element functionality within other SMC-restricted genes. For example, the *Tagln1* promoter contains 2 closely-spaced CArG elements originally referred to as CArG-near and CArG-far; disruption of CArG-near completely inhibited muscle-specific activity of a lacZ reporter whereas mutating CArG-far had no effect whatsoever [24]. The *Acta2* proximal promoter has 2 similarly positioned CArG elements but only 1 (CArG-B) directs all cell-restricted activity of a reporter in transgenic mice [26]. Finally, the *Actg2* proximal promoter has 4 CArG elements, but only 1 of these CArG elements is necessary for full activity in vitro [51,52]. An obvious question, therefore, is what function do extra CArG elements serve in these and other multi-CArG-containing regulatory sites? One idea is that multiple SRF-binding CArG elements "bridge" myocardin homooligomers to enhance transcriptional activity [16]. There is clear evidence for this model in such multi-CArG genes as *Actg2, Myh11*, and *Tagln1* [16,52]. Moreover, single CArG-containing SMC genes such as *Csrp1* and *Telokin* are activated by myocardin [16,53], but the level of activation may not be as strong as that of multi-CArG containing genes. Another possible function for multiple CArG elements in a gene locus may be related to sequestration of inactive SRF that would be on "reserve" for rapid deployment when active SRF over a dominant CArG element (such as C2 in the *CNN1* locus) is depleted. There may also be other functions of SRF-bound CArG elements unrelated to transcription. For example, the orthologous SRF gene in yeast (*Mcm1*) has been implicated to play a role in DNA replication [54]. A full understanding of CArG-SRF will require identification and functional characterization of the CArGome using both informatics and a variety of wet-lab assays, including ChIP-Seq and RNA-Seq.

A rationale for the use of BAC clones carrying human DNA sequences is to ascertain whether human genes respond to perturbations in mouse physiology in the same manner as the orthologous mouse gene. Here, we were interested to determine whether the expression of human CNN1 would be attenuated in the neointima following arterial injury where many SMC markers, including CNN1, are down-regulated. As expected, we found that human *CNN1* mRNA expression was reduced to a similar extent as the endogenous *Cnn1* transcript. Surprisingly, there was little to no evidence for neointimal formation in two independent lines of transgenic mice with WT BAC38-mediated human CNN1 expression. In contrast, the same BAC vector carrying mutations in C1-C5 where no human CNN1 expression was manifest, phenocopied both the outward remodeling and neointimal tissue seen in normal FVB mice. There are reports of CNN1 displaying tumor suppressor activity in the setting of cancer [55] and at least 1 report exists demonstrating SMC growth suppression upon expression of CNN1 in vitro [56]. However, the medial SMC growth rate was no different between WT BAC38 and the m8BAC38 transgenic mice 7 days after injury. The latter findings suggest there may be differences in the growth fraction over time following injury or some other mechanisms of action are at play, including altered SMC migration. Whatever the mechanism is, if similar protective findings are found in other species and vascular disease processes, the *CNN1* gene may represent a novel target of intervention for the treatment of vascular occlusive disorders.

In summary, we have shown through BAC transgenic and gene disruption studies that a consensus intronic CArG element appears necessary for smooth muscle calponin expression in vivo, despite the presence of 4 additional CArG boxes. However, intron 1 sequences taken out of their normal genomic landscape are insufficient for driving SMC-specific expression of a reporter gene suggesting that there may be unique structural requirements for the SRF-bound consensus CArG element to mediate transcription optimally in vivo. Future work should examine more deeply the sufficiency of the consensus intronic CArG element for CNN1 expression in the context of a BAC as well as the remodeling phenotype seen upon over-expression of human (or rodent) CNN1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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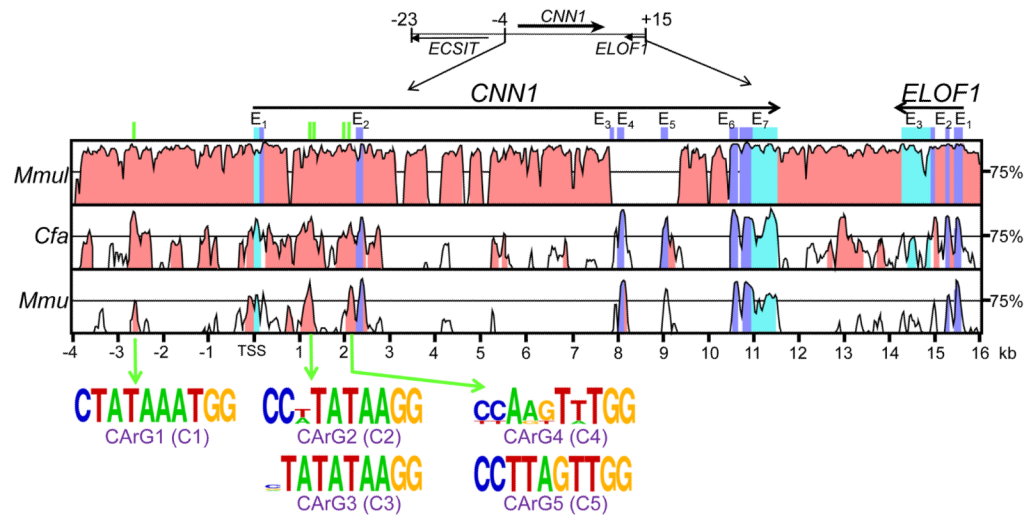
**Figure 1. Conservation of CArG sequences across the human *CNN1* gene**
(top) 38-kb BAC illustrated with nucleotide coordinates relative to the transcription start site (TSS) of the *CNN1* gene used throughout this study. Note portions of two other genes (*ECSIT* and *ELOF1*). Below this schematic is a VISTA plot showing the human *CNN1* gene structure with translated and untranslated exons (E) numbered as dark blue and lighter blue boxes, respectively, and 5 CArG elements schematized with green vertical lines (top). The height of exonic (blue) and intronic/intergenic (pink) peaks represents the percent nucleotide sequence homology between the indicated species. Each of the 3 species' plots depicts nucleotide sequence homology with human as the base sequence labeled from -4 to 16 kb at bottom (compare with 38-kb BAC at top). (bottom) Conservation of each of the 5 CArG elements is shown in Sequence Logos [57]. Note that each CArG element falls within a broader region of non-coding sequence homology (pink peaks).
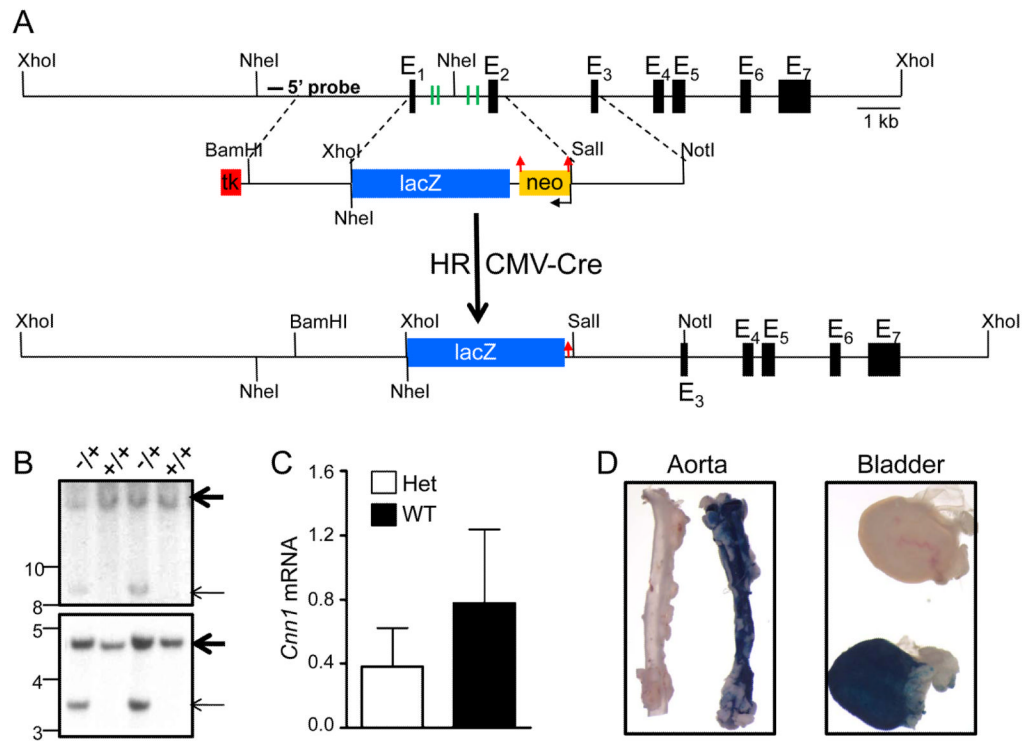
Figure 2. Targeting mouse *Cnn1* locus with lacZ reporter
A, Schematic showing endogenous mouse *Cnn1* gene structure (top), targeting vector with homology arms to the 5′ promoter and 3′ intron 2 sequences (dotted lines to middle), and final targeted *Cnn1* locus following homologous recombination (HR) and breeding of heterozygous mice to a CMV-Cre mouse to excise the neo cassette (bottom). Note 5′ external probe used in Southern blot, labeled restriction enzyme sites, and the deletion of all 4 CArG elements (green vertical lines in intron 1) following HR. B, Southern blotting of genomic DNA digested either with *XhoI* (upper blot) or *NheI* (lower panel) from the indicated genotype using the 5′ probe. The large arrow indicates the wildtype band and the smaller arrow points to the band in correctly targeted mice (see restriction sites in panel A). Numbers to left represent size of DNA ladder (in kilobases). C, Quantitative RT-PCR of total RNA obtained from aorta of heterozygous and wildtype mice (n=4). D, Beta galactosidase staining of indicated adult tissues from *Cnn1*^{-/+} mice (lacking blue stain) or a positive control mouse (*Sm22-lacZ*).
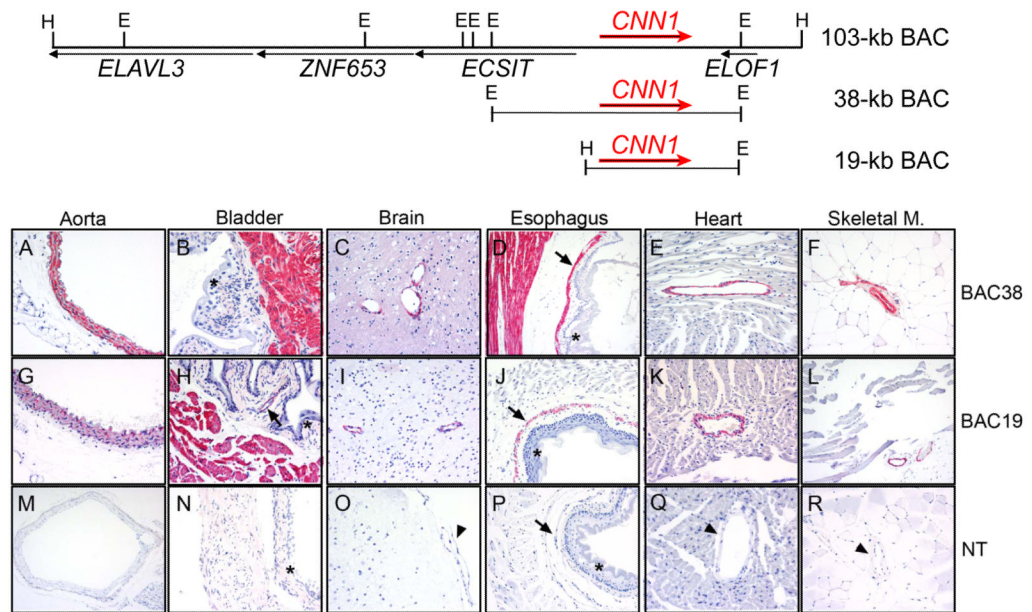
Figure 3. BAC trimming retains SMC-specific staining of human CNN1 in the mouse
Schematics of original 103-kb BAC harboring human *CNN1* and other gene loci as well as restriction digested mini-BACs. Multiple tissues were analyzed for human CNN1 protein expression (red) in BAC38 (panels A-F), BAC19 (panels G-L) or non-transgenic (NT) littermate control (panels M-R) mice by immunohistochemistry. Note complete absence of immunostaining in latter tissues. Asterisks in Bladder (panels B, H, N) and Esophagus (panels D, J, P) represent CNN1 negative epithelial cells. Arrows in Esophagus point to SMC in the lamina propria. Arrowheads in Bladder (H), Brain (O), Heart (Q), and Skeletal Muscle (R) indicate blood vessels. Original magnification of all images was 400x.
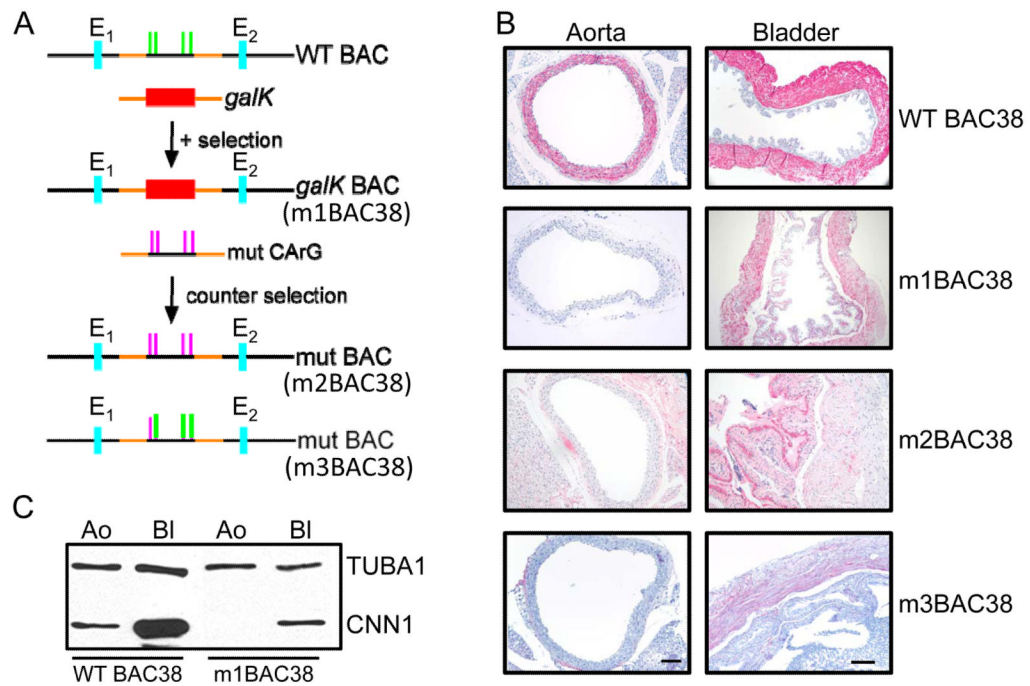
Figure 4. Necessary role for a single intronic CArG box in the expression of human CNN1
(A) Schematic illustrating steps in BAC recombineering for the generation of transgenic mice carrying various mutations within the first intron of the human *CNN1* gene. The green vertical lines in intron 1 represent the 4 CArG boxes flanked by sequences (orange) used to create homology arms in the *galK* cassette. Positive selection in bacteria facilitates homologous recombination and a mutant BAC38 transgene that carries the *galK* cassette in place of intronic sequences encompassing all 4 CArG boxes (m1BAC38). In the counter-selection step, m1BAC38 undergoes homologous recombination with the same homology arms as above plus original intronic sequences carrying point mutations (pink vertical lines) in each of the 4 CArG elements (m2BAC38). Similar counter-selection of m1BAC38 was done to generate a single CArG mutant (pink vertical line representing the consensus CArG box, C2; Figure 1) with the remaining 3 CArG boxes intact (green vertical lines). See extended methods for more details about BAC recombineering. (B) Indicated BAC transgenic lines from aorta and bladder immunostained for human CNN1 protein (red). Scale bar is 40 μm under bladder sections and 100 μm under aorta sections. (C) A representative Western blot of aorta (Ao) and bladder (Bl) protein extracts from indicated transgenic lines probed simultaneously with antibodies to human CNN1 or alpha tubulin. See also Supplemental Figure IV.
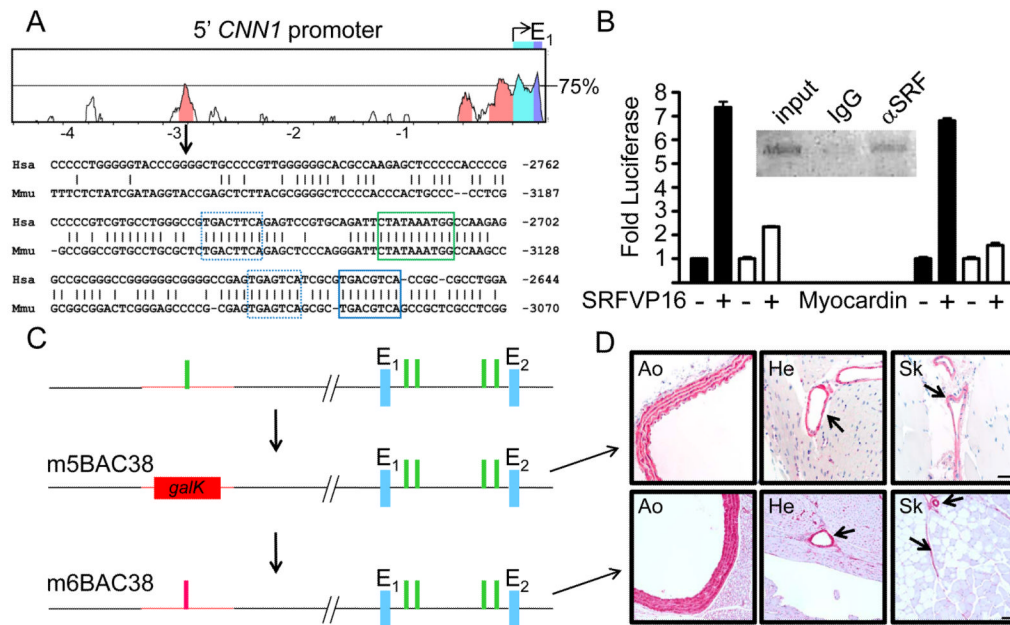
Figure 5. A distal upstream CArG box is not necessary for human CNN1 protein expression
(A) VISTA plot illustrating position of conserved sequence block (mouse versus human) containing a CArG-like element (green rectangle) as well as a perfect consensus CREB element (blue rectangle) and two CREB-like elements (dotted blue rectangles). (B) Luciferase assay in PAC1 SMC line co-transfected with a ~3-kb promoter containing either the wildtype CArG box (filled bars) or a mutant CArG box (open bars) in the absence or presence of SRF-VP16 or Myocardin. Similar results were seen in the C_2C_{12} muscle cell line (data not shown). The inset shows SRF enrichment at the CArG site in human coronary artery SMC. (C) Schematic illustrating BAC recombineering strategy for generating mutant upstream CArG element in context of BAC38-kb transgene. (D) Immunostaining for human CNN1 protein in aorta (Ao), heart (He), and skeletal muscle (Sk) of m5BAC38 (top row) or m6BAC38 (bottom row) transgenic mice. Arrows point to blood vessels. Scale bar is 50 μm (upper row) or 100 μm (lower row).
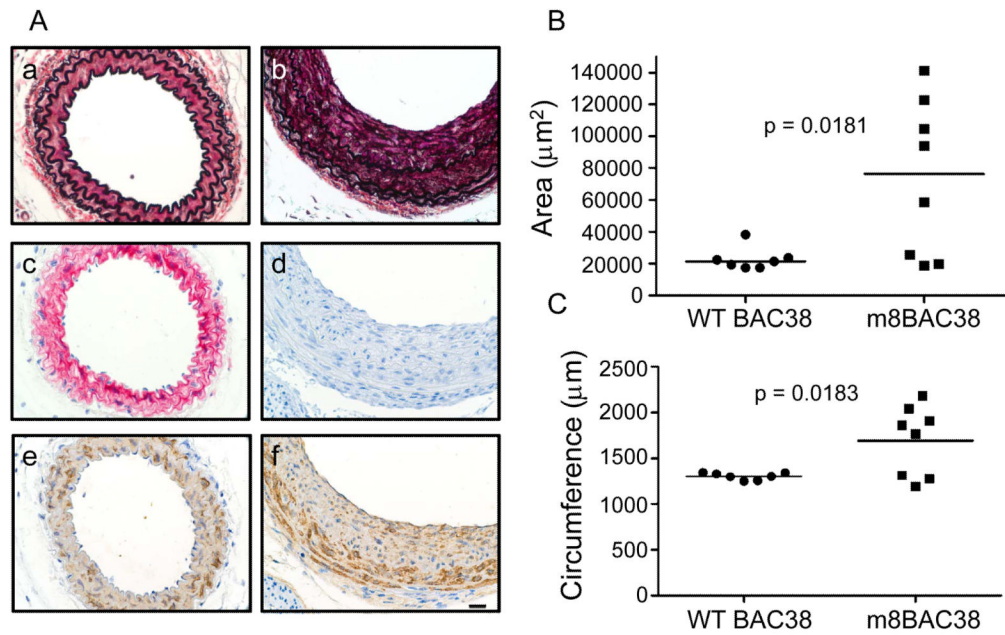
Figure 6. Overexpression of human CNN1 prevents neointimal formation and outward remodeling of the injured vessel wall
(A) Representative serial cross-sections of injured carotid artery of WT BAC38 (panels a, c, e) or m8BAC38 (panels b, d, f) mice 3 weeks after partial ligation injury were stained with Masson Trichrome (a, b) or with antibodies to human CNN1 (c, d), or ACTA2 (e, f). Note the thickened neointima and attending reduction in ACTA2 immunostaining in the m8BAC38 mouse. Scale bar in lower right of panel f is 30 μm for all images. Quantitative analysis showed a statistically significant increase in vessel wall area (B) and vascular circumference (C) in the m8BAC38 group of mice.