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Supplementary Information for

The long noncoding RNA NEAT1 (nuclear paraspeckle assembly transcript 1) is critical for phenotypic switching of vascular smooth muscle cells

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Supplementary Information

DETAILED MATERIAL AND METHODS

Rat balloon injury model. Rat balloon angioplasty was carried out as previously described (1-3). Briefly, male Sprague-Dawley rats (350 g; Taconic Farms, Germantown, NY) were anesthetized with xylazine 4.6 mg/kg and ketamine 70 mg/kg via intraperitoneal injection. Following a midline cervical incision and muscular tissues separation, the left common carotid artery was exposed and blunt dissection was performed alongside the artery by dull forceps to expose the carotid artery bifurcation into the internal/external branches. Blood flow cessation was achieved by arterial clamps and a small arteriotomy was made in the external carotid artery. A 2F Fogarty balloon embolectomy (Edwards) was inserted through the small cut and passed into the common carotid artery. After balloon inflation at 1.6-2.0 atm of pressure, the catheter was partially withdrawn and re-inserted 3 times. A permanent ligation was then placed in the external carotid artery, and the blood flow in the common carotid artery and its internal branch was restored by releasing arterial clamps. The right intact carotid artery served as a contralateral control. Post injury 3, 7 or 14 days, right control and left injured common carotid artery were harvested either for total RNA extraction or OCT (optimum cutting temperature) embedding as described below. The use of experimental rats for arterial injury procedures, including BSL-2 viral work was approved by the IACUC and Biosafety committees at Augusta University.

Quantitative real time reverse transcription-PCR (qRT-PCR) analysis. Total RNA from rat intact right carotid artery or balloon injured left common carotid artery was extracted by TRIzol reagent (Invitrogen) as previously described (2). 0.5-1 μg of RNA was utilized as a template for RT with random hexamer primers using the High Capacity RNA-to-cDNA Kit (Invitrogen). qRT-PCR was performed with respective gene-specific primers as we previously reported otherwise were listed in Table S1 (2, 4, 5). All samples were amplified in duplicate and every experiment was repeated independently 2 times with triplicates of samples each time. Relative gene expression was converted using the $2^{-\triangle\triangle CT}$ method against the internal control house-keeping gene hypoxanthine phosphorribosyltransferase 1 (HPRT) for mouse, human and rat. $\triangle\triangle CT = (CT_{\text{experiment gene}} - CT_{\text{experiment HPRT}}) - (CT_{\text{control gene}} - CT_{\text{control HPRT}})$. The relative gene expression in control group was set to 1.

Cell culture and PDGF-BB treatments. Rat arterial tissues were harvested and primary rat aortic SMCs were prepared and cultured as described in our previous reports (2, 4, 5). Briefly, male Sprague-Dawley rats (200-250 grams; Taconic Farms, Germantown, NY) were euthanized by CO_2 and the aorta was dissected to remove adhering peri-adventitial tissue and the endothelium denuded with a catheter. The aorta was then digested with a Blend enzyme III solution (Roche, 0.5U/ml) for 10 min at 37°C followed by dissection to remove the adventitial layer, then the remaining medial layer was minced into small pieces for a second digestion with Blend enzyme III for 2 hours at 37°C. Following removal of digestion solution and re-suspending in 10% FBS DMEM medium, cells were gently liberated with a pipette and transferred into culture dishes. For PDGF-BB treatment experiments, rat primary SMCs were grown to 80-90% confluence and serum-starved for 2 days and then treated with recombinant rat PDGF-BB (25 ng/ml platelet-derived growth factor BB, Calbiochem) for 8 or 24 hours. Cells treated with vehicle served as control. Subsequently the cultured cells were rinsed with PBS and total RNA was harvested for measuring gene expression by qRT-PCR. Human coronary artery SMCs (HCASMCs) were purchased from ATCC (catalogue number: PCS-100-021) and cultured following the vendor's protocol. All primary SMCs were used before passage 7.

Probe synthesis for RNA-FISH (fluorescence in situ hybridization). Rat brain or human Hela cell genomic DNA was utilized as templates for PCR to obtain a portion of rat (327bp) or human (416bp) NEAT1, respectively. The sequence of the primers used for PCR was listed in Table S1. Subsequently the PCR products were ligated into a blunt PCR cloning vector (Agilent Technologies) and verified by Sanger sequencing. Digoxigenin labelled sense or antisense RNA probes were then synthesized by MAXIscript T3 and T7 *in vitro* transcription kit (Ambion, Austin, TX) using the T3 and T7 promoters in the blunt vector. FISH assay were performed essentially following the protocol previously published (6). Briefly, rat control and injured carotid arteries were harvested post-injury day 7, embedded in OCT and sectioned at 7 um thickness. These sections then were fixed in 4% paraformaldehyde, permeabilized in a 1:1 solution of acetone and methanol, and then hybridized with digoxigenin-labelled NEAT1 sense or antisense RNA probes. After peroxidase quenching and blocking, the hybridized sections were incubated with peroxidase-conjugated anti-digoxigenin antibody (Roche, Indianapolis, IN, USA) and revealed using SuperGlo^{TM} Green Immunofluorescence Amplification Kits (Fluorescent Solutions, Augusta, GA, USA). Nuclei were then counter-stained with DAPI (Invitrogen, Carlsbad, CA, USA). Images were collected using the confocal microscopy (LSM780 upright, Zeiss) at the imaging core of Augusta University.

RNA-FISH/IF (immunofluorescence). Discarded human carotid endarterectomy specimen were collected by the Department of Vascular Surgery at Augusta University. Following FISH assay carried out as described above, IF was performed as previously reported (3, 7, 8). Briefly, after goat serum (10%, Invitrogen) blocking for 30 minutes, sections were then incubated with anti SM α-actin (Sigma, mouse, 1:500) antibodies. The sections were then stained with secondary antibody (568nm anti-mouse secondary antibody, 1:250 dilution, Invitrogen) diluted in blocking buffer for 1 hour at room temperature. Sections were immersed with mounting medium (ProLong Gold anti-fade reagent with DAPI, Invitrogen) to visualize nuclei. Sections after stained with fluorescence-labeled secondary antibody were imaged using confocal microscopy (LSM780 upright, Zeiss) as described above. Three specimens from three patients were analyzed by the RNA-FISH/IF assay. The numbers of RNA-FISH NEAT1 foci per cell with or without PDGF-BB treatment in HCASMCs were manually counted at least 5 random fields from 3 biological samples each group. To determine NEAT1 co-localization with WDR5 or SRF in VSMCs, HCASMCs were plated on cell culture chamber slides (Nunc, Lab-Tek chamber slide) and then treated with or without PDGF-BB (25ng/ml) for 24 hours. Subsequently RNA-FISH/IF was carried out as described above using anti-SRF (Santa Cruz, G-20X, sc-335, rabbit, 1:150) antibody or WDR5 antibody (Bethyl Laboratories, A302-403A, rabbit, 1:30).

Aortic tissue preparation and aortic organ culture. Rat aorta was dissected to remove adhering tissue and denuded with a catheter. The aortic tissue was then cut into 2 mm cylindrical segments and cultured in 10% FBS DMEM for 48 or 60 hours at 37 °C in a humidified chamber (5% CO_2). The freshly isolated tissues or cultured vessels were then harvested with TRIzol for total RNA to evaluate gene expression by real time PCR as described above.

siRNA transfection. Scrambled control siRNA, siRNA targeting human NEAT1 (#1, siRNA ID: n272458; sense sequence (5'->3'): GACCGUGGUUUGUUACUAUtt; #2, siRNA ID: n272460; sense sequence (5'->3'): AACUUUACUUCGUUAGAUUtt) were purchased from Ambion. siRNA targeting human SFPQ (#1, catalogue number: J-006455-06 and #2, catalogue number: J-006455-08) and siRNA targeting human WDR5 (ON-TARGETplus, catalogue number: LU-013383-00) was purchased from Dharmacon. Delivery siRNA into primary human coronary artery SMCs was done using Neon transfection system (Invitrogen) essentially

following the manufacturer's protocol and as described in our previous report (2). Briefly, HCASMCs were detached from dish by trypsin digestion. Following a wash with PBS, cells were then suspended in R buffer and mixed with siRNA duplexes (100 nM). Finally, electroporation was performed with the parameters of 1100V, 2 pulses of 20 milli-second width. After electroporation, the cells then were plated in 10% FBS antibiotic-free medium for 48 hours and harvested to extract total RNA for RNA sequencing or qRT-PCR to validate the knock-down efficacy.

Next generation sequencing and pathway enrichment analysis. Differential gene expression analysis was performed using RNA-seq at the Genome Technology Access Center at Washington University. After transfection with scrambled control or NEAT1 silencing RNA duplex (#1) for 48 hours, HCASMCs were harvested to extract total RNA using TRIzol reagent (Invitrogen) for RNA-seq. Library was prepared using Ribo-Zero gold rRNA removal kit and paired-end sequencing of 100 bases length was performed on a HiSeq 2500 system (Illumina). Adaptor sequence and low-quality reads were removed using Trimmomatic 0.32 (9). Pass-filtered reads were then mapped to Ensembl human reference genome GRCh38 using TopHat version 2.1.0 (10). The genes annotated in Ensembl GRCh38 were quantified with HTSeq 0.6.1 (11) and only those with count ≥ 10 in at least 3 samples were considered as expressed genes and used for subsequent analysis. Differential expression analysis was performed with the R package DESeq2 (12). Cutoff values of fold change greater than 2 and FDR less than 0.01 were considered significant between control and silencing NEAT1 groups. g:Profiler was used to conduct Gene Ontology (GO) and KEGG pathway enrichment analysis for the differentially expressed genes (13) and functional categories with p-value <0.05 were considered significant. Fragments Per Kilobase of exon per Million fragments mapped (FPKM), which corrects for variations in contig length and read depth between samples, was calculated from raw counts for each sample (14) and was used for heatmap visualization. Heatmaps were generated with heatmap.2 function of gplots package in R (https://www.r-project.org). The RNA-seq data generated in this study have been deposited in the Sequences Read Archive at the NCBI under accession number SRP154294.

Adenoviral construction and cell infection. Adenovirus encoding mouse NEAT1 V1 was generated as described previously (4, 5, 7). PCR was carried out using primers harboring BglII and NotI restriction enzyme sites (primer sequences were listed in the Table S1) and mouse brain genomic DNA as a template. The PCR product was then sub-cloned into AdTrack shuttle and verified by Sanger sequencing (Genewiz). As this vector contains an independent cytomegalovirus promoter-driven transcription cassette for green fluorescent protein (GFP) in addition of NEAT1, the efficiency of transduction can be directly monitored by visualization of GFP expression. Transferring the NEAT1 and GFP expression cassettes into AdEasy viral backbone, viral packaging and cell infection was performed as we previously reported (2-4). All new reagents described in this study have been deposited with the non-profit plasmid distribution service Addgene.

Protein extraction and Western blotting. Protein lysate was extracted from adenovirus infected HCASMCs as previously described (2, 3). Briefly, after rinsed with PBS, cells were harvested by RIPA buffer (Fisher) with 1% proteinase inhibitor cocktail (Pierce) and 1% PMSF to extract protein. Following sonication and centrifugation, protein lysate was quantified by BCA assay and then loaded in a 6-9% SDS-PAGE gel at 5-10 μg per lane. Antibodies used in this study were: α-tubulin (Cell Signaling, #2144, rabbit, 1:5000), β-actin (Sigma, A5316, mouse, 1:5000), calponin (Santa Cruz, N-15R, sc-16604-R, 1:1000), Hic-5 (BD, 611164, mouse 1:5000), MLCK (Sigma, clone K36, M7905, 1:5000), NONO (Bethyl Laboratories, A300-587A, rabbit, 1:5000), PSPC1 (Dundee Cell Products, AB1013, rabbit, 1:5000), SFPQ

(Sigma, P2860, mouse, 1:500), SM22α (Abcam, ab10135, goat, 1:2000), SM α-actin (Sigma, A2547, mouse, 1:4000), SM MHC (Biomedical Technologies Inc, BT-562, rabbit, 1:2000), WDR5 (Bethyl Laboratories, A310-880A, rabbit, 1:2000). Images were acquired by ImageQuant LAS 4000 Imaging Station (GE) and band densities were quantified using the ImageQuant TL software (GE).

Mouse breeding and genotyping. NEAT1 knock out mice were generated as previously described in details (15). Heterozygous NEAT1 mice were intercrossed to generate NEAT1 knock out experimental mice for carotid artery ligation injury. This breeding strategy yielded 25% progeny with NEAT1 null mice and 25% wild-type mice as control littermates. Genotyping of these mice was performed as previously described using the primers listed in Table S1 (15). The use of experimental animals has been approved by the IACUC at Augusta University in accordance with NIH guidelines.

Mouse carotid artery ligation model. Mouse carotid artery ligation was performed as previously described (2, 5). Briefly, three-month old gender-matched NEAT1 KO mice and their littermate control mice were anesthetized by intraperitoneal injection of a mixed solution of xylazine (5mg/kg body weight) and ketamine (80mg/kg body weight). The left common carotid artery was dissected and completely ligated just proximal to the carotid bifurcation. The right carotid artery served as an uninjured contralateral control. Post injury 21 days, carotid artery tissues were harvested to embed transversely in paraffin for histological analysis as described below. 7 mice per genotype were analyzed in this study.

Sectioning, HE (hematoxylin/eosin) staining and IF. Cross-sections of carotid arteries (7μm thickness) were prepared from the ligature to the aortic arch. Morphometric analysis was performed using 6 sections from each artery and these sections were located at around 250μm proximal to the ligature. HE staining was performed following standard protocol as previously described (2, 5) and images were acquired using an Olympus BX41 upright microscope. For IF, sections were deparaffinized and antigen retrieval was done by using microwave to heat at 98°C for 5 minutes in citric acid buffer (10mM, pH6.0). After goat serum (10%, Invitrogen) blocking for 30 minutes, sections were then incubated with anti-SM α-actin (Sigma, A2547, mouse, 1:200) and anti-Ki-67 (Thermo Scientific, RM-9106, rabbit, 1:30) antibodies. Subsequently sections were stained with secondary antibody (488nm anti-mouse secondary antibody or 568nm anti-rabbit secondary antibody, 1:250, Invitrogen) diluted in blocking buffer for 1 hour at room temperature. Sections were then immersed with mounting medium (ProLong Gold anti-fade reagent with DAPI, Invitrogen) to stain nuclei. Images were collected by using a confocal microscopy (LSM 780 upright, Zeiss). The areas of intima and media were measured by ImageJ software (NIH).

SMC proliferation assay. Proliferation of HCASMCs was measured by cell proliferation WST-1 kit (Roche) in 96-well format as previously described (2, 3). Following silencing or over-expression of NEAT1, HCASMCs were maintained in 0.2% FBS medium for additional 24 hours to allow growth arrest. Cells were then treated with 10% FBS full medium or 25 ng/ml PDGF-BB as indicated in figures. After 24 hours the rates of proliferation were determined with incubation of 10 μl/well WST-1 for 4 hours, and then measured the absorbance at 480 nm with a plate reader. For SMC counting, NEAT1 over-expressing or knock-down HCASMCs were seeded in 60mm dish at equal density and then cell numbers were manually counted at the days as indicated in figures by a hemocytometer.

Assessment of SMC migration by Boyden chamber assay. Boyden chamber assays were carried out as previously described (2-4). Briefly, HCASMCs were grown in medium containing

10% FBS for 48 hours post infection with NEAT1 or post electroporation 48 hours with silencing NEAT1 duplex. Subsequently, the treated cells were trypsinized and seeded into Boyden chambers (PET track-etched, 8-um pores, 24-well format; Becton Dickinson) in serum-free DMEM medium. Chambers were then immersed in 10% FBS medium or 25 ng/ml PDGF-BB for 5 hours. The top-side of the membranes was swabbed to remove cells, and then cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde, stained with DAPI to visualize nuclei, and counted under fluorescence microscopy. Five identically located fields per membrane were averaged for quantification of migrated cell numbers.

Quantitative Chromatin immunoprecipitation (qChIP) assays. After electroporation with silencing RNA control or NEAT1 duplex for 48 hours, HCASMCs were fixed with formaldehyde, and ChIP assay was performed by using anti-SRF (Santa Cruz, G-20X, sc-335, rabbit), anti-Histone H3 tri-methyl K4 (H3K4me3, Abcam, ab8580, rabbit), anti-Histone H3 acetyl K9 (H3K9ac, Abcam, Ab4441, rabbit), anti-Histone H3 tri-methyl K27 (H3K27me3, Abcam, ab6002, rabbit) antibodies as described by the manufacturer (Active Motif) and in our previous reports (5, 16). ChIP assay was repeated 3 times with duplicates each time and each IP was duplicated. Equal amount of chromatin from silencing control and silencing NEAT1 groups were used for each IP. The genomic DNA purified from individual antibody precipitated genomic DNA or from input were amplified by qPCR. Primers for quantitative evaluation of enrichment of the smooth muscle-specific gene promoter CArG region were listed in Table S1. Data were expressed as relative binding by using the $2^{-\triangle\triangle CT}$ method against the silencing control samples (set to 1) where $\triangle\triangle CT = (CT_{\text{IP silencing NEAT1}} - CT_{\text{input silencing NEAT1}}) - (CT_{\text{IP silencing control}} - CT_{\text{input silencing control}})$.

RNA-protein pull-down assay. RNA-protein pull-down assays were carried out using the Pierce magnetic RNA-Protein pull-down kit essentially following the protocol provided by the manufacturer (Thermo Scientific). A pGEMTE vector containing full-length human NEAT1 V1 sequence (provided by Dr. David Spector, Cold Spring Harbor Laboratory) (17) was utilized to synthesize sense or antisense probes in vitro by using T7 or SP6 promoter, respectively. Subsequently the RNA probes were labeled with biotinylated cytidine bisphosphate and captured by streptavidin magnetic beads. Nuclear proteins were extracted from HCASMCs treated with or without 25 ng/ml PDGF-BB for 24 hours and then incubated with the RNA probe magnetic beads. The RNA bound proteins were eluted for western blotting as described above.

RNA immunoprecipitation (RIP). RIP was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit according to the manufacturer's instructions (Millipore, USA). Briefly, HCASMCs were first starved for 24 hours with serum-free medium and then either were maintained in serum-free medium (control) or stimulated with 25 ng/ml PDGF-BB. After 24 hours of stimulation cells were lysed and incubated with WDR5 antibody (Bethyl Laboratories, A302-429A, rabbit, 6 ug) or control IgG (5 ug) conjugated with magnetic beads (50 ul) overnight. The beads, protein, and mRNA complexes were immunoprecipitated and then magnetically separated. The mRNAs were purified and were quantified by qRT-PCR using human NEAT1 primer listed in Table S1A. Fold enrichment of WDR5 relative to IgG control is calculated as previously described (18).

Statistical analysis. Data are expressed as means ± SE, and statistical analysis using unpaired *t* test (two group comparison) or one-way ANOVA test (multiple group comparison) was done with Prism software (GraphPad). Differences with p values < 0.05 were considered significant.

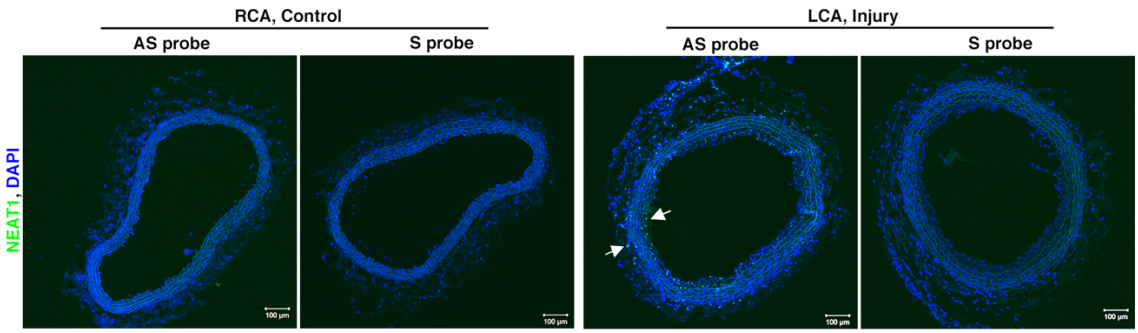

Figure S1. NEAT1 expression is induced in balloon injured rat carotid arteries (related to figure 1B). Low magnification images are shown to demonstrate that NEAT1 expression is induced in neointima and adventitial area of injured vessels. Rat control right common carotid artery (RCA) or left common carotid (LCA) 7-day post injury by a balloon dilation were sectioned for RNA-FISH to visualize NEAT1 (green, AS) as indicated by arrows. Cell nuclei were counter-stained with DAPI (blue). The section hybridized with NEAT1 sense (S) probe was used as a negative control.

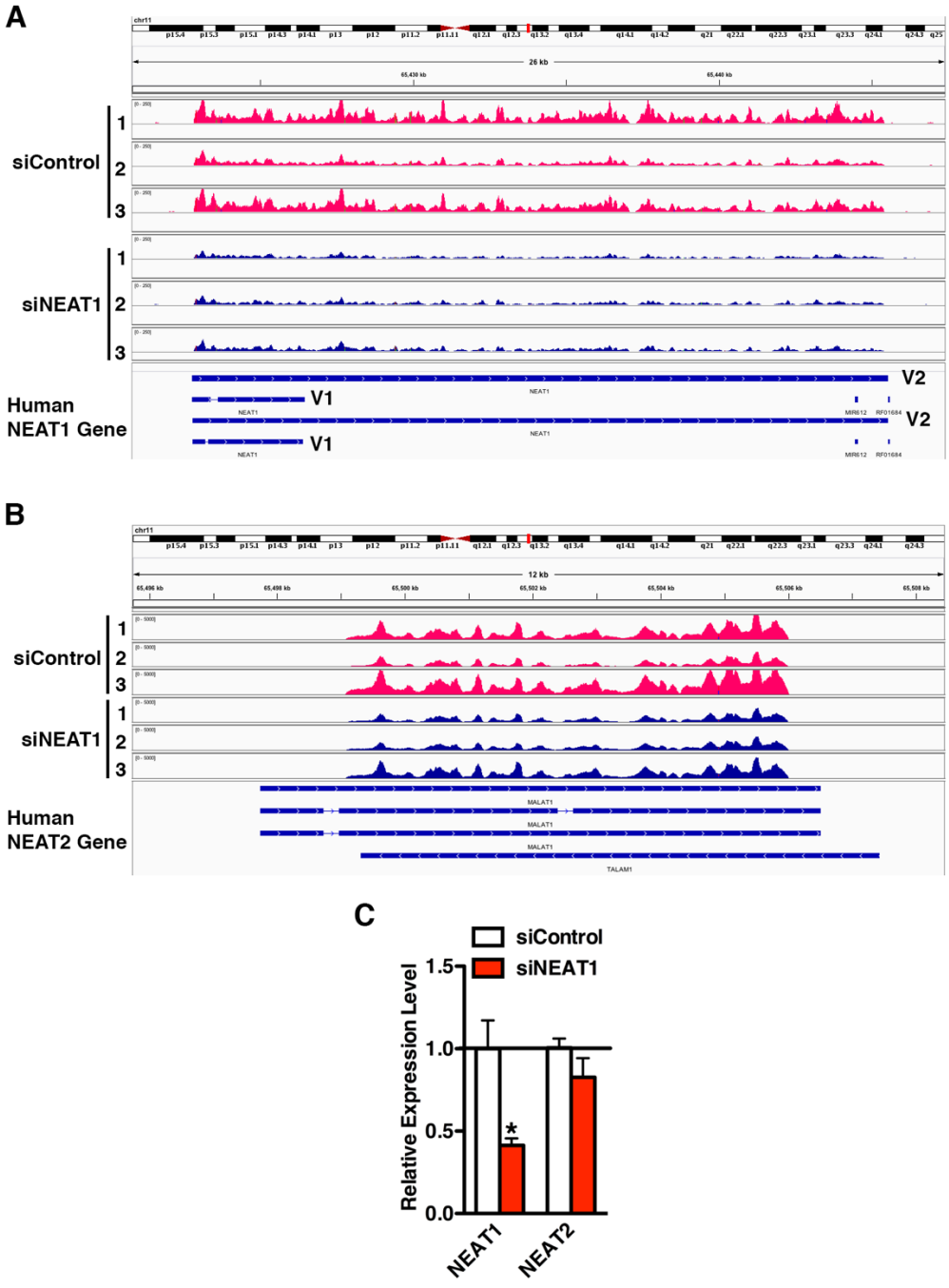

Figure S2. Silencing NEAT1 specifically down-regulates expression of NEAT1 V1 and V2 but not its adjacent lncRNA NEAT2 (related to figure 3A). A. RNA-seq reads alignment to NEAT1 or NEAT2 gene (**B**) between silencing control and silencing NEAT1 groups in HCASMCs is displayed using Integrative Genomics Viewer (IGV). The Y axis represents the number of reads at each nucleotide position of the genome. **C.** The relative expression of NEAT1 or NEAT2 between silencing control (set to 1) and silencing NEAT1 groups was plotted based on the calculation of FPKM by RNA-seq. N=3. *<0.05.

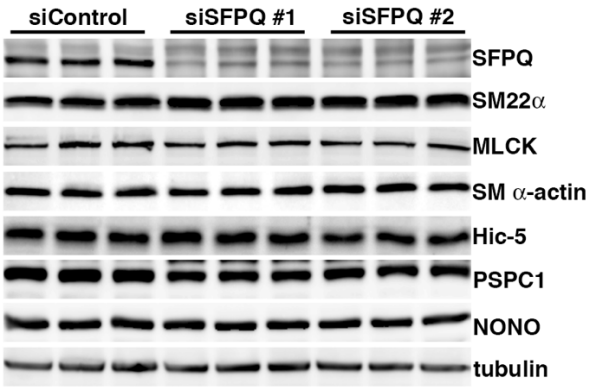

Figure S3. Silencing SFPQ has no effects on SM-specific gene expression (related to figure 3E). HCASMCs were electroporated with control silencing RNA duplex or 2 different silencing RNA duplexes against SFPQ. 72 hours post electroporation cells were harvested for western blotting using the antibodies as indicated. Knocking down of the paraspeckle protein SFPQ has no effects on SM-specific gene expression.

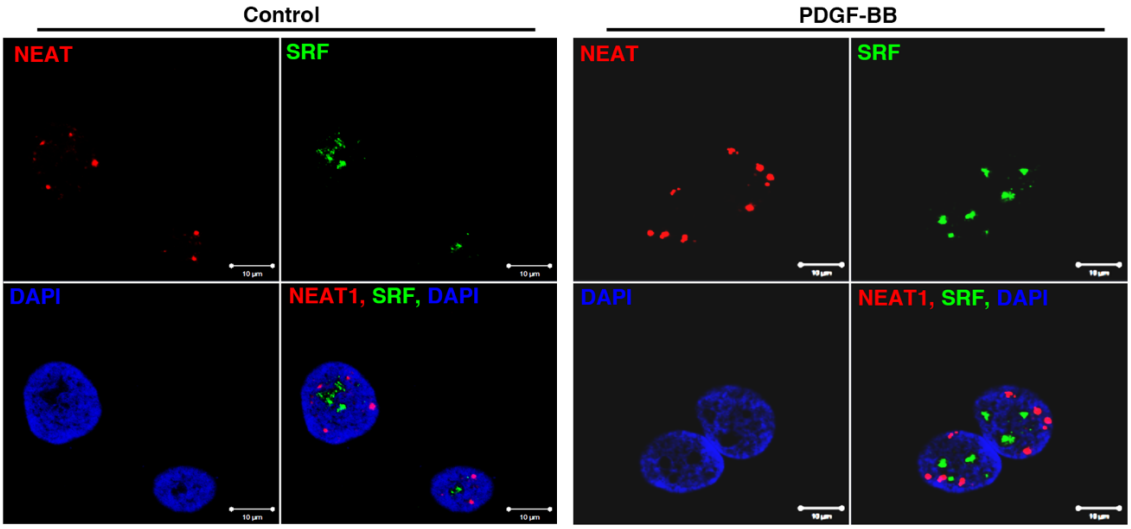

Figure S4. NEAT1 does not co-localize with SRF in VSMCs (related to figure 6E). RNA-FISH/IF was performed to determine the co-localization of NEAT1 (red) and SRF (green) with or without PDGF-BB treatment (25ng/ml, 24 hours). Nucleus was counter stained with DAPI (blue).

Table S1. List of oligonucleotides used in the study

A. Primers used for quantitative RT-PCR (F: forward; R: reverse)

Gene Name	*Species*	*Sequence (5'-3')*
NEAT1	Rat	F: TCTCACTCTTCCCTACTCTTCC
	Rat	R: CACACGGGCTCTACATCTTC
NEAT1	Human, V1 and V2	F: CTTCCTCCCTTTAACTTATCCATTC
	Human, V1 and V2	R: CTCTTCCTCCACCATTACCAACAATAC
NEAT1	Human, V2	F: CAGTTAGTTTATCAGTTCTCCCATCCA
	Human, V2	R: GTTGTTGTCGTCACCTTTCAACTCT
NEAT1	Mouse, V1 and V2	F: TGGTCTCACTCTTCCCTACTC
	Mouse, V1 and V2	R: CCACCTTTACCAAAATGCCG
NEAT2	Human	F: GTGATGCGAGTTGTTCTCCG
	Human	R: CTGGCTGCCTCAATGCCTAC
SRF	Human	F: GATGGAGTTCATCGACAACAAGCTG
	Human	R: CCCTGTCAGCGTGGACAGCTCATA
MKL1	Human	F: TGTGTCTCAACTTCCGATGG
	Human	R: TTCACCTTTGGCTTCAGCTC
MKL2	Human	F: GCAACTGCTGCACAAATACC
	Human	R: TTGATAAAGGGCTGCTGGAC
SM22α	Mouse/Rat/Human	F: TGACATGTTCCAGACTGTTGACCTCT
	Mouse/Rat/Human	R: CTTCATAAACCAGTTGGGATCTCCAC
calponin	Human	F: GTCCACCCTCCTGGCTTT
	Human	R: AAACTTGTTGGTGCCCATCT
Hic-5	Mouse/Rat/Human	F: GCCTCTGTGGCTCCTGCAATAAAC
	Mouse/Human	R: CTTCTCGAAGAAGCTGCTGCCTC
myocardin	Human	F: ATTCAGCTACCTAGGGATGCACCAAG
	Human	R: GGCCTGGTTTGAAAGAAGAGACACC
HPRT	Mouse/Rat/Human	F: TCTTTGCTGACCTGCTGGATTACA
	Mouse/Rat/Human	R: AGTTGAGAGATCATCTCCACCAAT

B. Primers used for cloning NEAT1 fragment to synthesize probe for FISH

Primer Name	*Sequence (5'-3')*
Rat NEAT1	F: GTGGACCCTTGGGTGGGTTCAC
Rat NEAT1	R: ACGGCCCCTCCAGCTGGAAGC
Human NEAT1	F: GGCTCGAGGGGCCATCAGCTTTG
Human NEAT1	R: AGCACAACACAATGACACCC

C. Primers for quantitative ChIP assay

Primer Name	*Sequence (5'-3')*
Human calponin CArG region	F: GAGACAGATCCCAGCGCCGCCCTCCT
	R: AAGCAAACCGGCCCTTATATAGCGTG
Human SM22α CArG region	F: CCCTCTCTCCAGCCAGACT
	R: CCCCAGTGACTCCACACAG

D. Primers for cloning mouse NEAT1 V1 to generate adenovirus

Primer Name	*Sequence (5'-3')*
M Neat1	F (BglII): AAGATCTAGGAGTTAGTGACAAGGAGGGCTCGC
	R(Not1): AGCGGCCGCGAAGCTTCAATCTCAAACCTTTATTTTGCT

E. Primers for NEAT1 knockout mouse genotyping

Primer Name	*Sequence (5'-3')*
Primer 1	F: GCCATTCAGGCTGCGCAACTG
Primer 2	R: AGCAGGGATAGCCTGGTCTT
Primer 3	R: CTAGTGGTGGGGAGGCAGT

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