

Supplemental information

The circular RNA Ataxia Telangiectasia Mutated regulates oxidative stress in smooth muscle cells in expanding abdominal aortic aneurysms

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Table S1. Summary of all statistically significant up-and downregulated circRNAs as of array analysis (excel spreadsheet).

Table S2. Full output of Reactome pathway analysis (excel spreadsheet).

Table S3. Full output of Gene ontology analysis (excel spreadsheet).

Table S4. List and features of all patient samples used in this study (excel spreadsheet).

Table S5. List of PCR oligonucleotides and Taqman assays used in this study.

Primer ID	Sequence (5'-->3')	Company
ATM_1_F	CGTGGCTAACGGAGAAAAGA	ThermoFisher
ATM_1_R	ACTGCACTCGGAAGGTCAAA	ThermoFisher
circNRIP1_F	TCCGGATGACATCAGAGCTA	ThermoFisher
circNRIP1_R	TGTGCATCTTCTGGCTGTGT	ThermoFisher
circSLIT33_F (hsa_circ_0074930)	GCACGTGCAGCAATAACATC	ThermoFisher
circSLIT33_R (hsa_circ_0074930)	ATCTTCGGCATGTGGTTGA	ThermoFisher
hsa_circ_0003218_F (cBMPR2)	CCGTTTCTGCTGTTGTAGCA	ThermoFisher
hsa_circ_0003218_R (cBMPR2)	CCTATCCCAAGGTCTTGCTG	ThermoFisher
hsa_circ_0003641_F (cATM)	TGGTGCTATTTACGGAGCTG	ThermoFisher
hsa_circ_0003641_R (cATM)	ACGGCAGCAGATAAGCAGAT	ThermoFisher
hsa_circ_0005615_F (cNFATC3)	CACCCTTTACCTGGAGCAAA	ThermoFisher
hsa_circ_0005615_R (cNFATC3)	TGGTAAGCAAAGTGGTGTGG	ThermoFisher
hsa_circ_0005660_F (cNFIX)	CAGCCACATCACATTGGAGT	ThermoFisher
hsa_circ_0005660_R (cNFIX)	TGCAGGTTGAACCAGGTGTA	ThermoFisher
hsa_circ_0008068_F	TGATGAAGCTTTGCGAAGAA	ThermoFisher
hsa_circ_0008068_R	TATGCCCTTCTTGAAATTACG	ThermoFisher
hsa_circ_0016661_R (cENAH)	GAATCCAGTTGAGCCACCAG	ThermoFisher
hsa_circ_0016661_F (cENAH)	CCATCCCAAGAAGAATTGGA	ThermoFisher
hsa_circ_0024824_F (cSTGAL4)	TCTCAACCCCTTCTTCATGG	ThermoFisher
hsa_circ_0024824_R (cSTGAL4)	GCTGACCATGTTTCTCAGCA	ThermoFisher
hsa_circ_0042103_F (cMYOCD)	TTCCTGTGGATTCTGCTGTG	ThermoFisher
hsa_circ_0042103_R (cMYOCD)	GGAATTCAGCTGGACGTTTC	ThermoFisher
hsa_circ_0073492_F (cPAM)	CTCGAGCCAGCATGGATAC	ThermoFisher
hsa_circ_0073492_R (cPAM)	TTATGACTCCGGAATGACAGG	ThermoFisher
hsa_circ_0075671_F (cJARID)	CATCCCAAGTGTCTCCACT	ThermoFisher

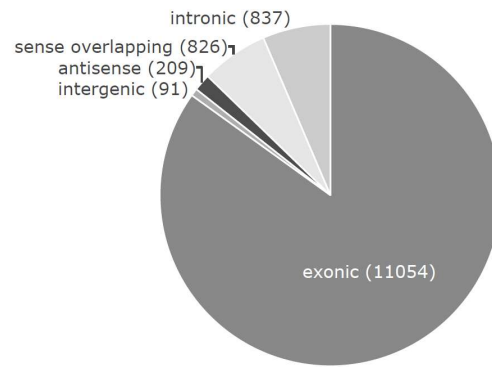
<i>hsa_circ_0075671_R (cJARID)</i>	ATGCTGCCTCTTCTGGGAAT	ThermoFisher
<i>RPLPO_human_Fwd</i>	ATGGCAGCATCTACAACCCT	ThermoFisher
<i>RPLPO_human_Rev</i>	TTGGGTAGCCAATCTGCAGA	ThermoFisher
Taqman Assay	Assay ID	Company
<i>MYOCD</i>	Hs00538076_m1	ThermoFisher
<i>NEAT1</i>	HS03453535_S1	ThermoFisher
<i>NFATC3</i>	Hs01001566_m1	ThermoFisher
<i>NFIX</i>	Hs00958846_m1	ThermoFisher
<i>NRIP1</i>	Hs00940781	ThermoFisher
<i>RPLPO</i>	HS99999902	ThermoFisher
<i>RPLPO</i>	HS00420895_gH	ThermoFisher
Custom Taqman Assay	Assay details	Sequence
<i>circATM</i>	Fwd	TGGTGCTATTTACGGAGCTG
	Rev	ACGGCAGCAGATAAGCAGAT
	Probe	TGTGTTCTGAAATTGTGAACCA
<i>circBMPR2</i>	Fwd	TGGAACATACCGTTTCTGCT
	Rev	CCTATCCCAAGGTCTTGCTG
	Probe	CCACTCACTTCGCAGAATCA
<i>circNFIX</i>	Fwd	CAGCCACATCACATTGGAGT
	Rev	TCTTGAAGTACTTGCCTTCC
	Probe	TACACCTGGTTCAACCTGC
<i>circNRIP1</i>	Fwd	CTCCGGATGACATCAGAGCT
	Rev	TCTGGCTGTGTTTCTCCAA
	Probe	GCTCAGAGCTTGAGACAGA
<i>circNFATC3</i>	Fwd	GTTTCTTTCAAGTTCCTTACCCT
	Rev	TGGTAAGCAAAGTGGTGTGG
	Probe	CCTGGCCACACCCCTATATT
<i>circMYOCD</i>	Fwd	TTCTGTGGATTCTGCTGTG
	Rev	GGAATTCAGCTGGACGTTTC
	Probe	GGACCCAGGAACAACCTGGCTAACCA

Table S6. Template file for digital PCR data analysis (*excel spreadsheet*).

Table S7. List of oligonucleotides for silencing and *in situ* hybridization.

siRNA ID	Cat. Number	Company
Human <i>ATM</i> esirna1	EHU089521	Sigma Aldrich
Custom made siRNA	Sequence	Company
<i>sicATM_2</i>	AGTGGTTAGACAGTGATGTGT	Sigma Aldrich
<i>sicATM_1</i>	GGTTAGACAGTGATGTGTGTT	Sigma Aldrich
ISH probes	Details	Company
Sense_ <i>cATM</i>	DIG: 5' DIG-AGTGGTTAGACAGTGATGTGT-DIG 3'	Exiqon

A



B

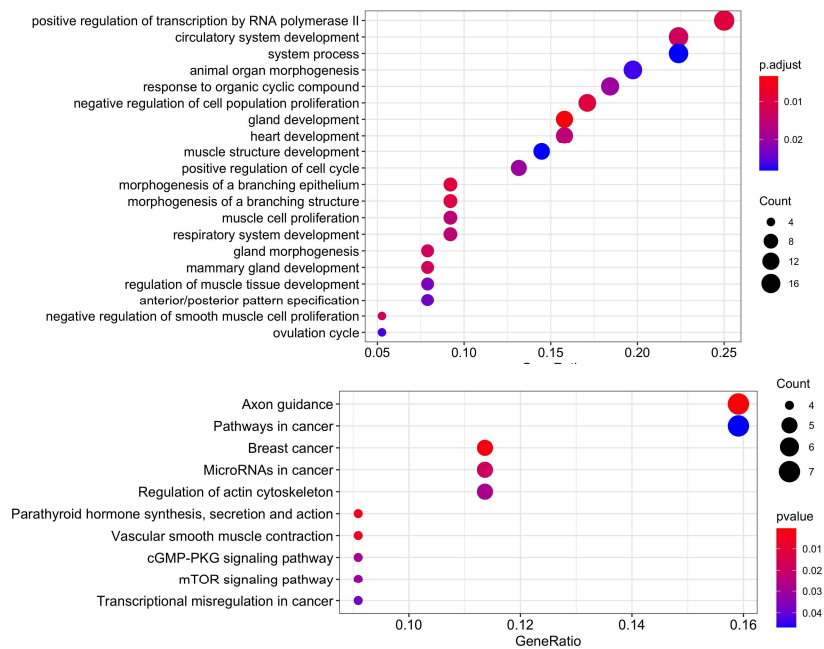


Figure S1. Differentially expressed circRNAs in eAAA vs CTRL patients. **A.** Pie chart illustrating the proportion of exonic, intronic, sense-overlapping and antisense circRNAs in all (13.617) circRNAs covered by one array chip. Absolute numbers are further indicated for each group. **B.** Top: Gene Ontology (GO) and pathway enrichment analysis relative to linear mRNA counterparts of differentially expressed exonic circRNAs. Top significantly enriched (adjusted p value < 0.05) GO-terms/ pathways are shown. Abbr: eAAA: elective AAA; CTRL: control.

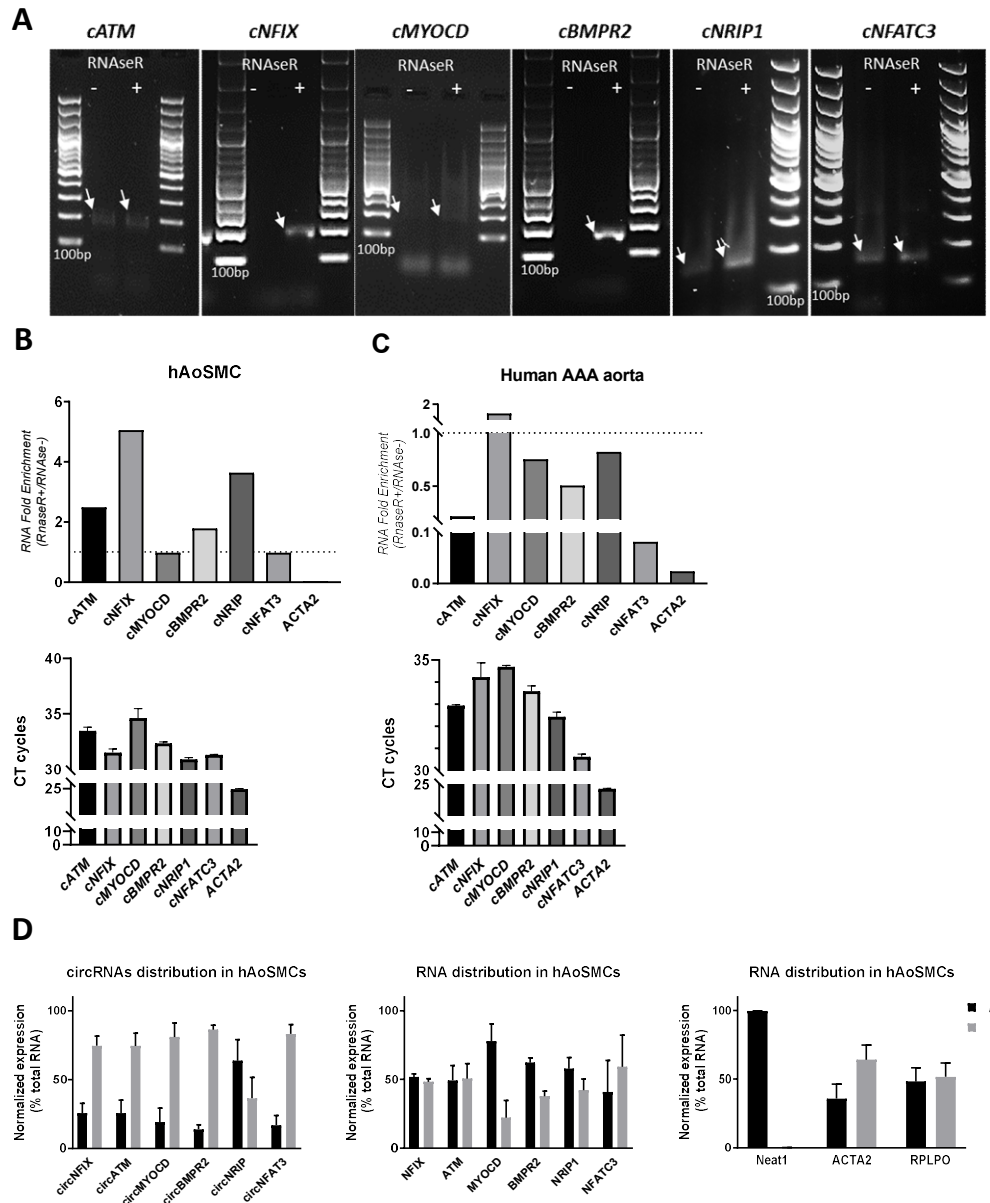


Figure S2. Validation of circular junctions in human aortic smooth muscle cells and human abdominal aortic aneurysm tissue specimens. **A.** circRNAs amplicons obtained from PCR on hAoSMCs cDNA were cloned and submitted to Sanger sequencing. Enrichment upon RNaseR treatment is shown (lane labelled with +). **B and C,** top: RNA fold enrichment upon RNaseR treatment in hAoSMCs and AAA tissue. Fold enrichment was calculated by comparing CT values in RNaseR+ vs RNaseR- and expressed as $2^{-\Delta\Delta CT} \times 100$, with $\Delta\Delta CT = CT_{RNaseR+} - CT_{RNaseR-}$. Targets displaying values >1 are considered enriched. Bottom: average CT cycles of the six circRNA targets compared to housekeeping gene (*ACTA2*) in untreated conditions. **D.** Subcellular localization of circRNAs (left) and respective linear counterpart (middle) in AoSMCs as quantified by qRT-PCR. Nuclear (abbr.: N) and cytoplasm (abbr.: C) purity was monitored by measuring *ACTA2*/*RPLPO* or *NEAT1*, respectively (right). Expression levels are indicated as percentage of total RNA. Data are represented as mean \pm SEM; N=3. Abbr.: hAoSMCs= human aortic smooth muscle cells; AAA= abdominal aortic aneurysm.

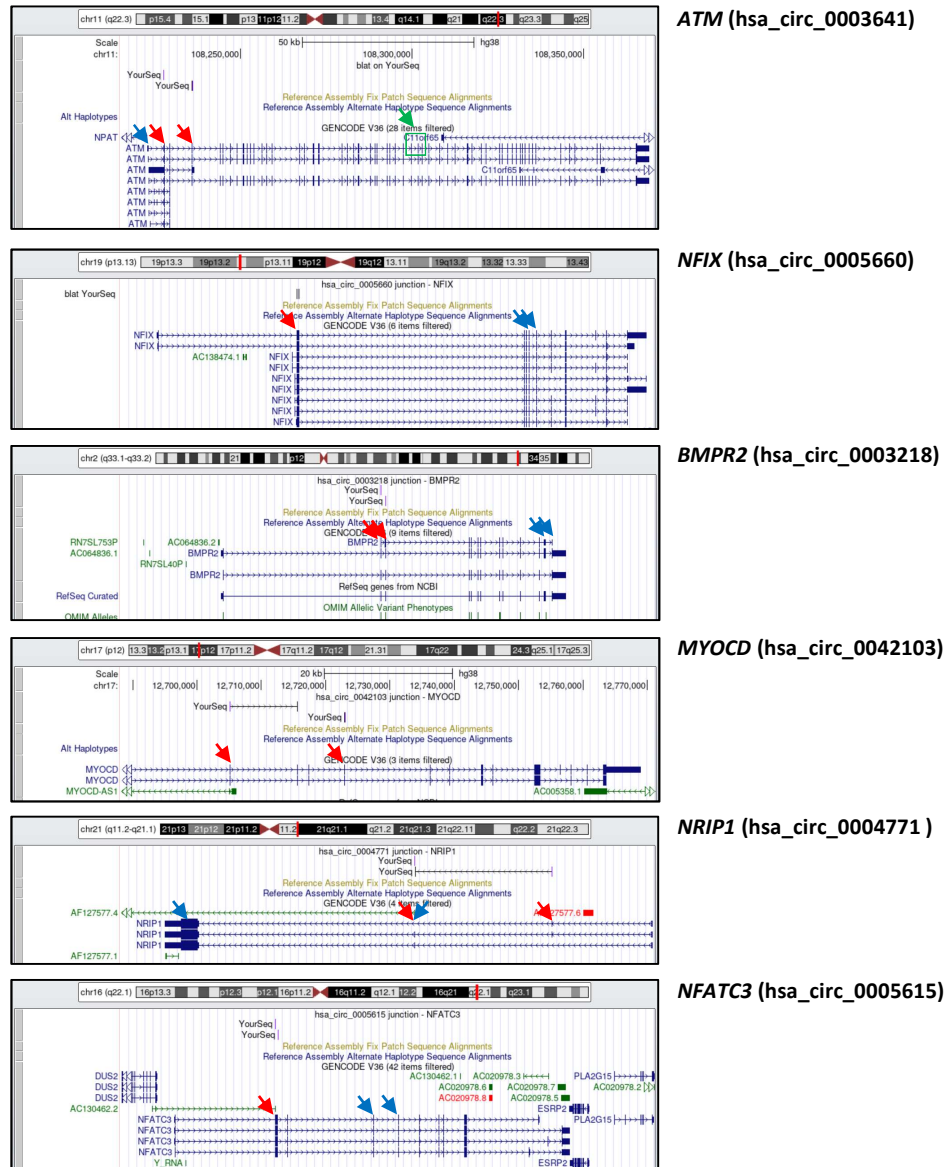


Figure S3. Genome Browser view of host gene loci of validated circRNA targets. Red arrows indicate exons involved in backsplicing. Taqman assays and siRNAs targeting circRNAs were designed on the backsplicing junction. Taqman assays/ primers for detection of linear transcripts map on blue arrows. SiRNAs target sites of linear transcripts are indicated by green arrows.

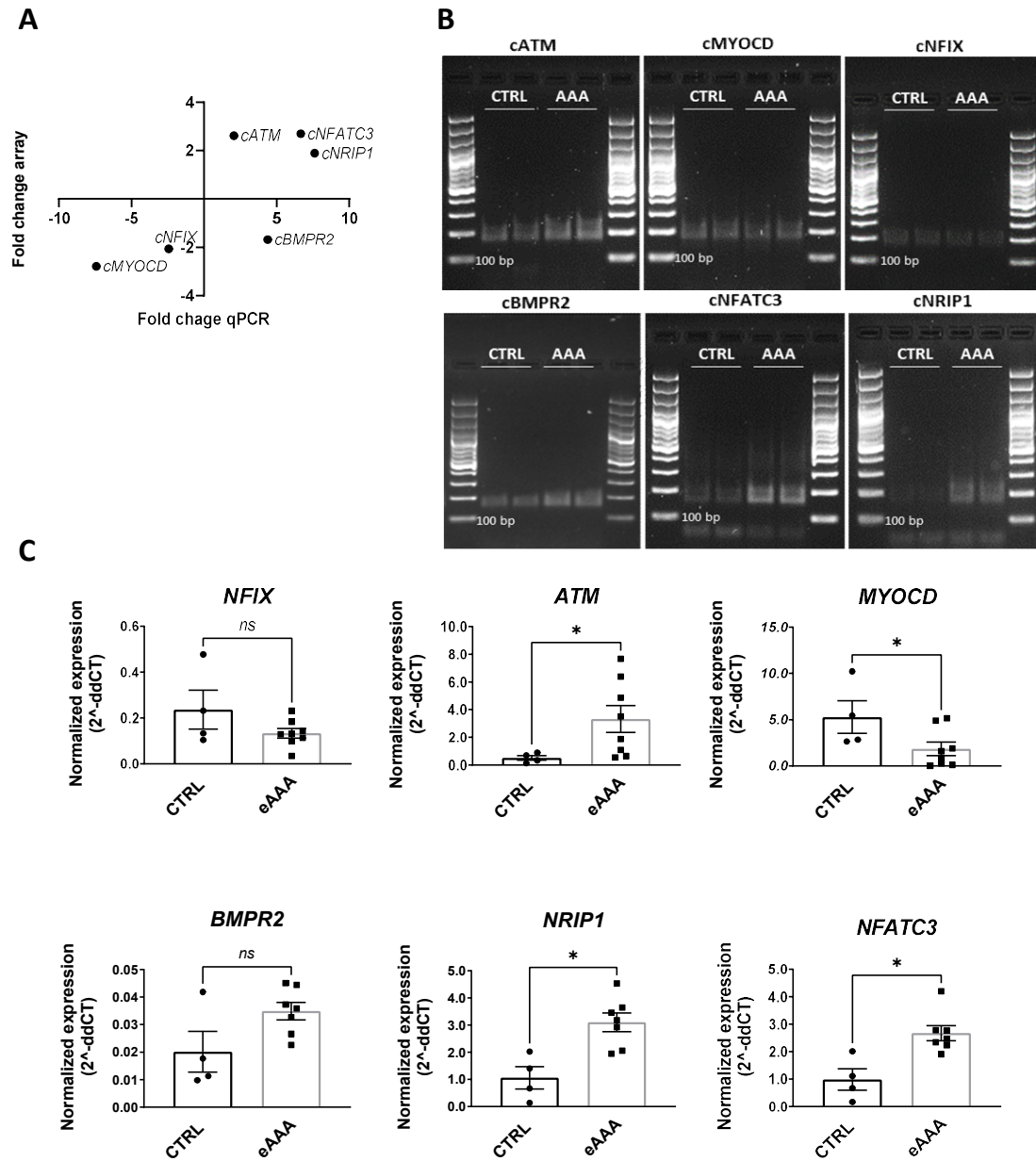


Figure S4. Expression of circRNAs chosen for validation and of their linear counterparts in eAAA vs CTRL patients. **A.** Correlation plot comparing array vs. qPCR results relative to the six circRNA targets chosen for validation. Array vs qPCR fold changes are plotted. Statistics: Pearson r ; R squared: 0.5546; p value (two-tailed): 0.0894. **B.** circRNAs amplicons obtained from qPCR with Taqman assays on eAAA tissue-derived cDNA. Amplification products from one representative CTRL and one representative eAAA sample are shown in duplicate. **C.** *NFIX*, *ATM*, *MYOCD*, *BMPR2*, *NRIP1* and *NFATC3* mRNA levels were determined by qRT-PCR and compared in eAAA vs CTRL patients. $2^{-\Delta\Delta CT}$ are plotted. Data are represented as mean \pm SEM. Statistics: Unpaired T-test. P values < 0.05 are considered significant. Abbr.: CTRL=control; eAAA: elective AAA.

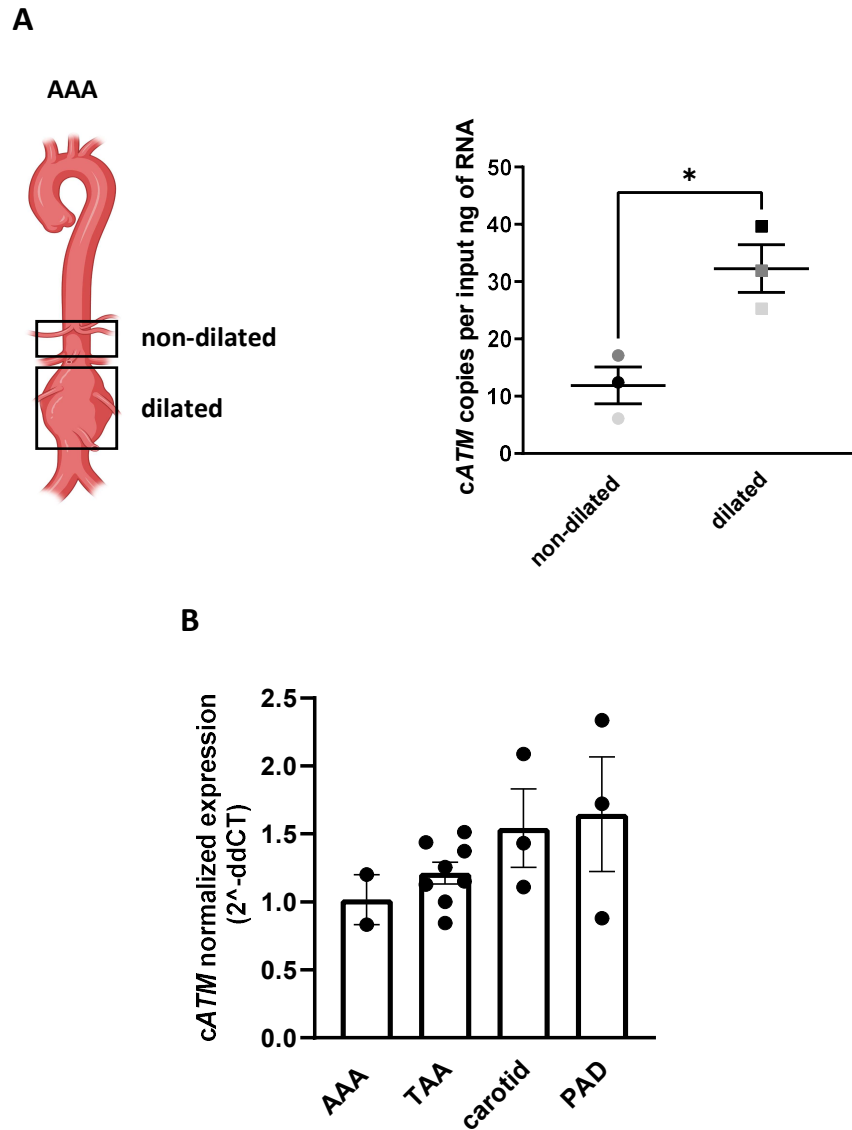


Figure S5. Expression of *cATM* in vascular tissue. A. *cATM* abundance was quantified by digital PCR in tissue from patients undergoing open repair surgeries, in which both the upstream non-aneurysmal aortic segment (non-dilated control, N=3) and the aneurysmal aortic portion (dilated AAA, N=3) were collected. Different colors indicate different pairs (statistics: paired T-test; P values < 0.05 are considered significant; data are represented as mean \pm SEM). B. *cATM* expression was assessed by qRT-PCR in different diseased human vascular tissue specimens, including abdominal aortic aneurysm (N=2), thoracic aortic aneurysm (N=8), carotid artery (N=3) and peripheral artery disease (N=3). Data are represented as mean \pm SEM.

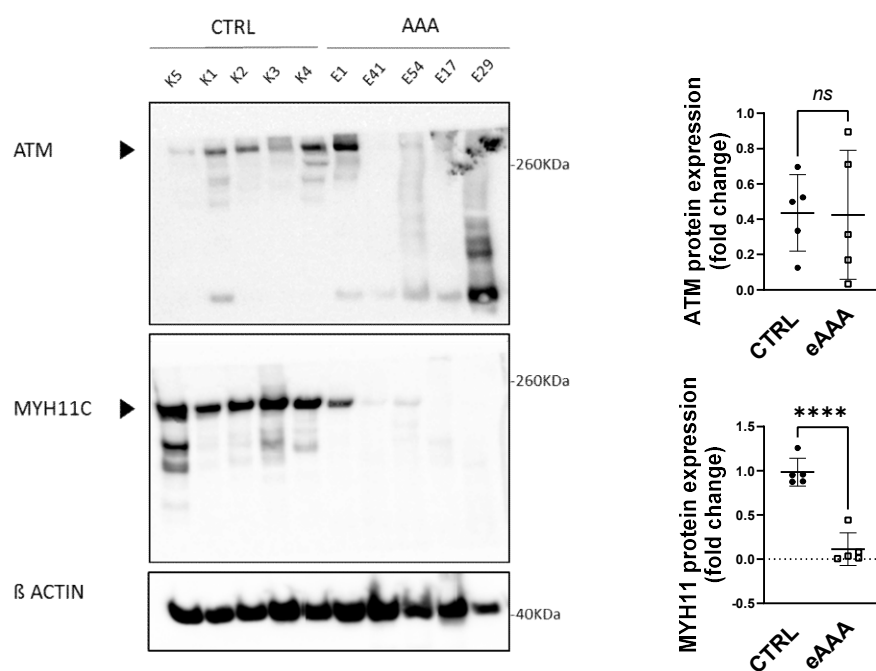


Figure S6. ATM protein expression in eAAA vs CTRL patients. WB showing ATM protein in AAA (N=5) vs CTRL (N=5) patients. Black arrows indicate expected molecular weights. Plots on the right show blots quantification (norm.: β actin). Statistics: unpaired T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM. Abbr.: CTRL= control; eAAA: elective AAA.

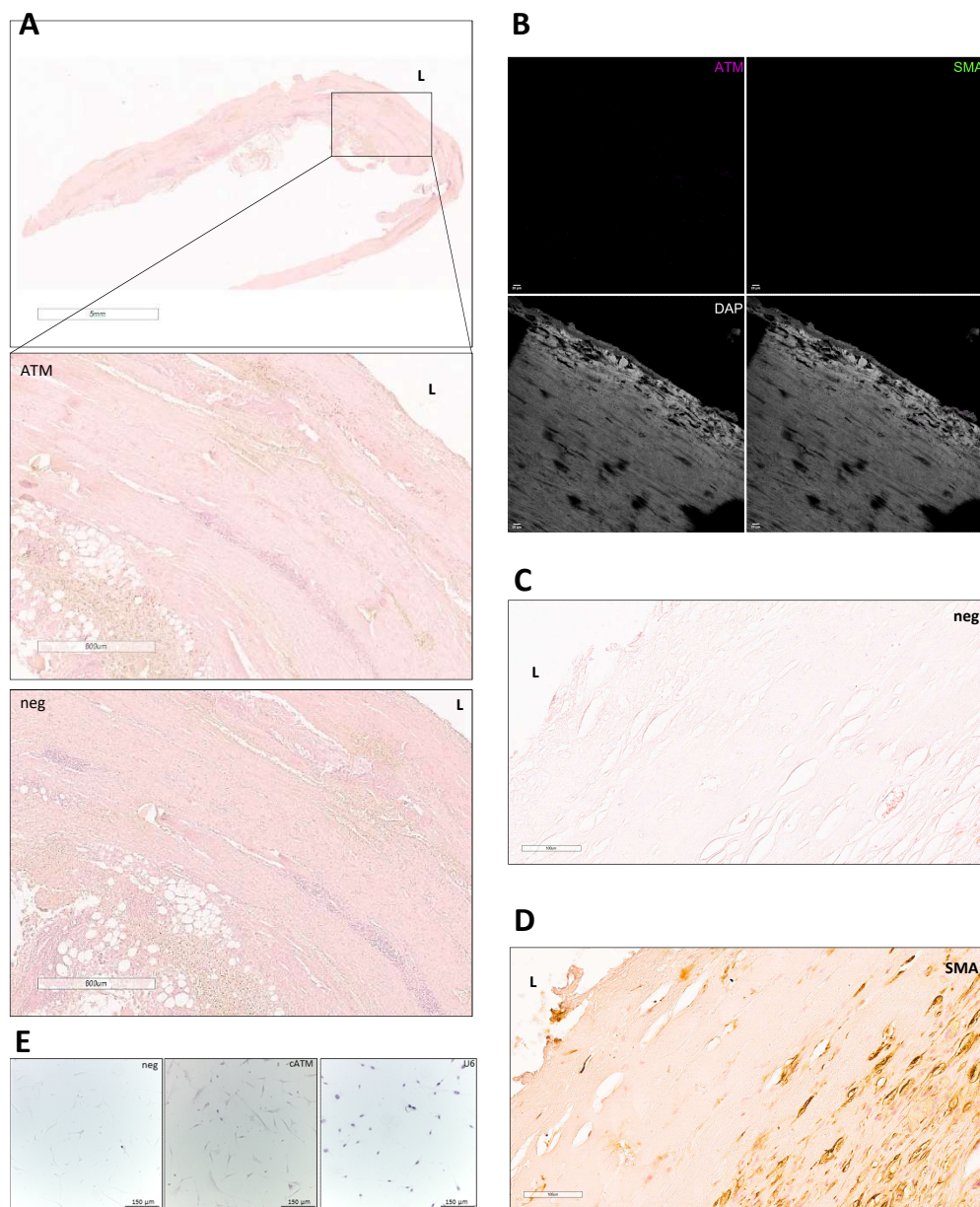


Figure S7. ATM protein and cATM staining in human eAAA specimens and AoSMCs. A. ATM immunohistochemistry in AAA patient section (top) and relative negative control. B. ATM and SMA IF negative control. C. cATM ISH negative control and (D) SMA IHC staining were performed in consecutive slides. E. ISH in human aortic SMCs negative control (left), cATM (middle) and U6 positive control (right) signal. L= lumen; neg= negative control

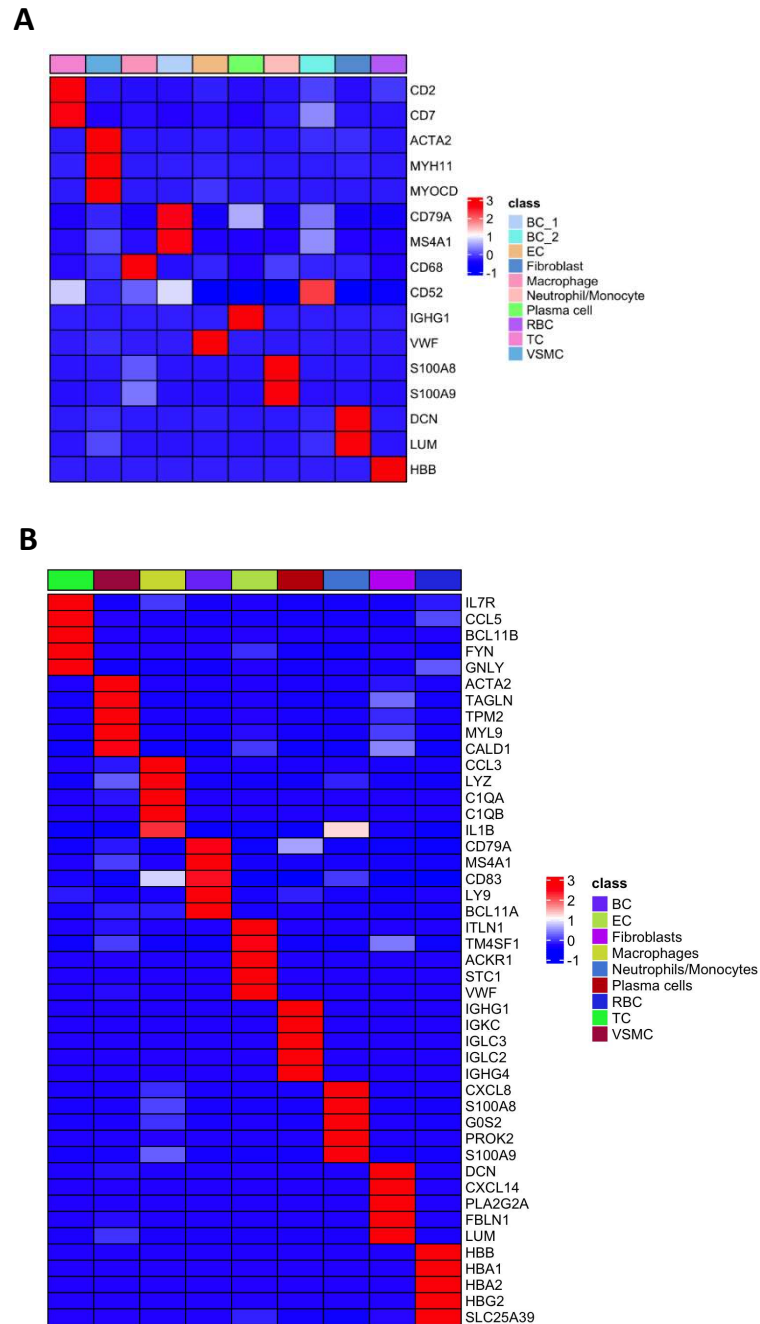


Figure S8. Clusters identified by single-cell RNA sequencing of human AAA specimens. A. Heat maps showing the main gene markers employed for cell clusters labeling and **(B)** summarizing the top five genes for each of the nine identified cell clusters (listed on the right). Color represents the average expression level (blue=low, red= high). Abbr.: BC=B cells; EC=endothelial cells; RBC=red blood cells; TC=T cells; VSMC=vascular smooth muscle cells.

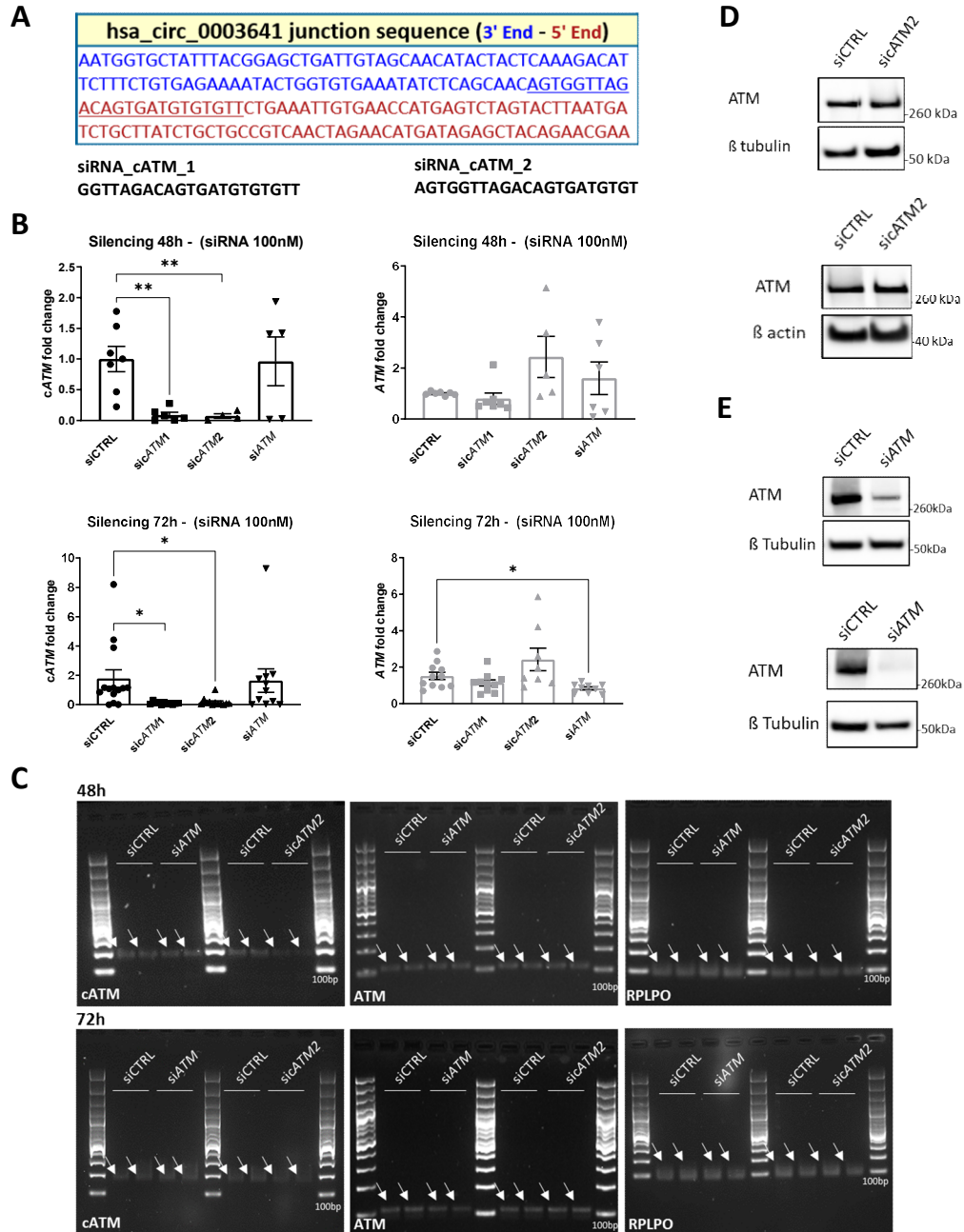


Figure S9. *cATM* KD in hAoSMCs. **A.** *cATM* (hsa_circ_0003641) siRNA design. The underlined sequence indicates the region covered by two alternative siRNA (siATM1 and siATM2), targeting the backsplicing junction. Different colors indicate different exons. **B** and **C.** qRT-PCR and gel electrophoresis of *cATM*, *ATM* and *RPLPO* amplicons upon *cATM* or *ATM* KD (100nM siRNA) in control hAoSMCs (statistics: unpaired T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM). **D.** *ATM* protein levels are not affected by *cATM* KD in both CTRL (top) and AAA-derived AoSMCs (bottom) at 72h, while they are significantly decreased upon *ATM* KD (**E**) in both CTRL (top) and AAA-derived AoSMCs (bottom).

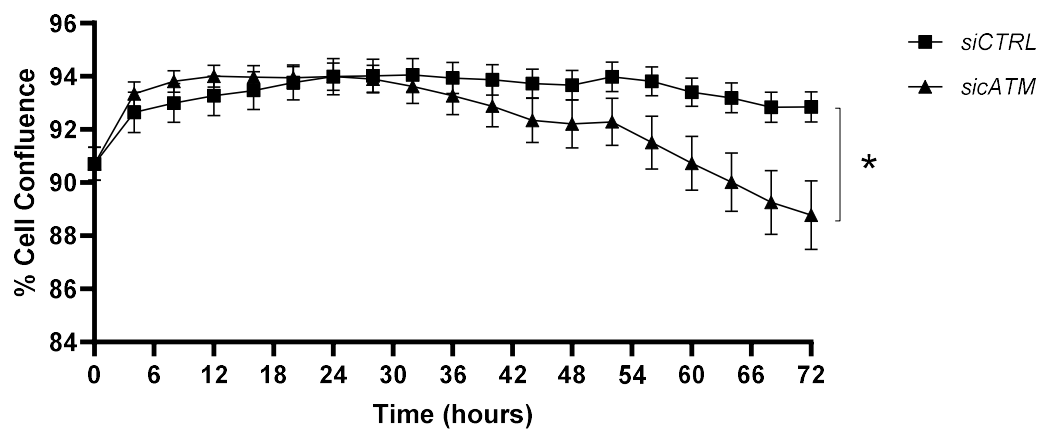


Figure S10. Effects of *cATM* KD in *hAoSMCs* proliferation. *cATM* was silenced in CTRL *AoSMCs* and proliferation monitored by live cell imaging. Statistically significant variations in cell confluence were assessed at 72h (statistics: Multiple T-test; p values < 0.05 are considered significant; data are represented as mean \pm SD).

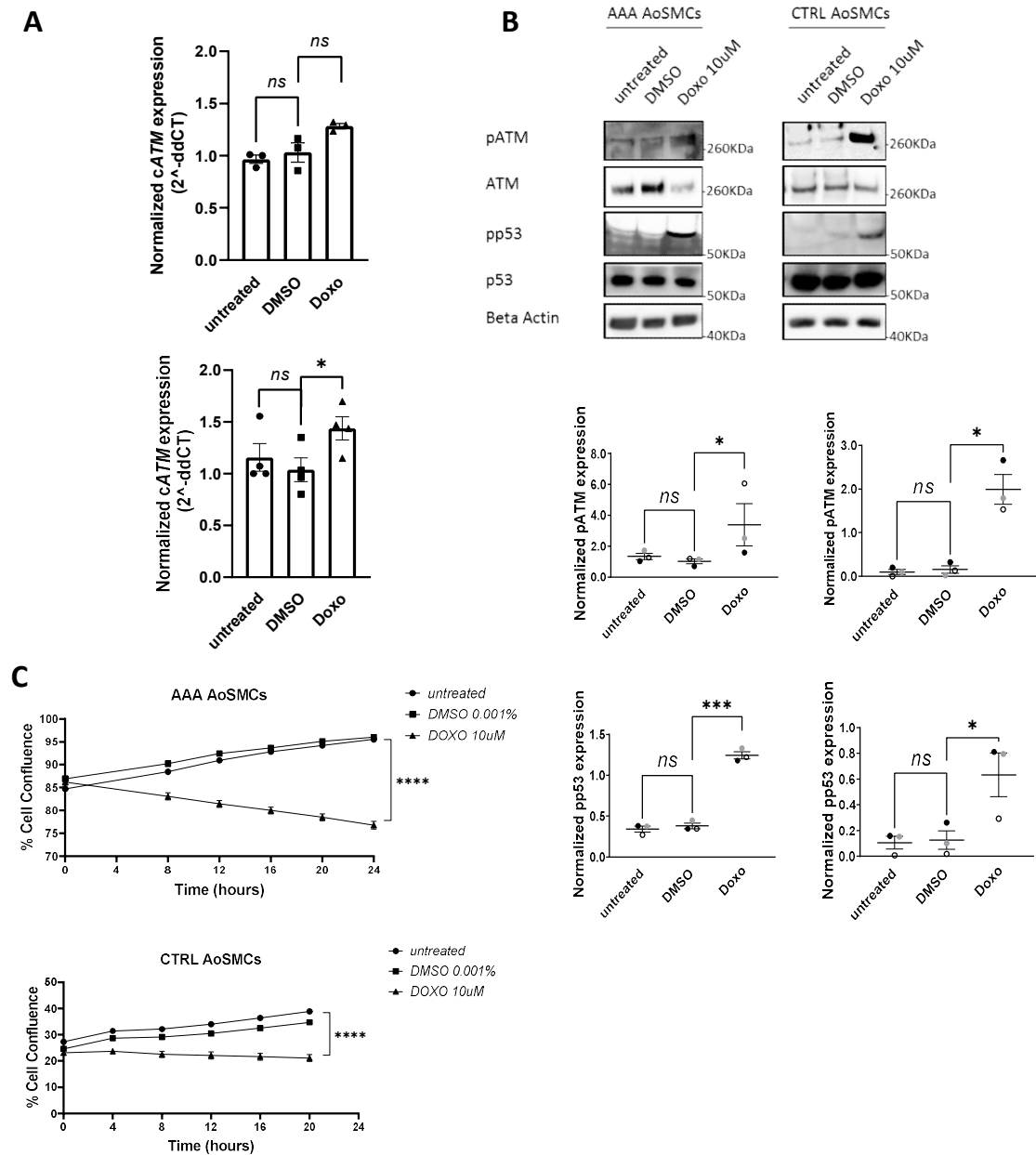


Figure S11. DMSO does not affect cATM expression, phosphorylation of ATM/p53 or proliferation of AoSMCs. A. Expression of cATM was assessed after treatment of AAA (top) and CTRL (bottom) AoSMCs with doxorubicin. Administration of 0.001% DMSO did not significantly impact cATM expression, phosphorylation of ATM and p53 (**B**, quantification of blots on bottom-left for AAA and on bottom-right for CTRL AoSMCs) and AoSMCs proliferation dynamics (**C**). (For panel A: statistics: Unpaired T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM. For panel B: statistics: Paired ratio T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM. For panel C: statistics: Multiple T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM).

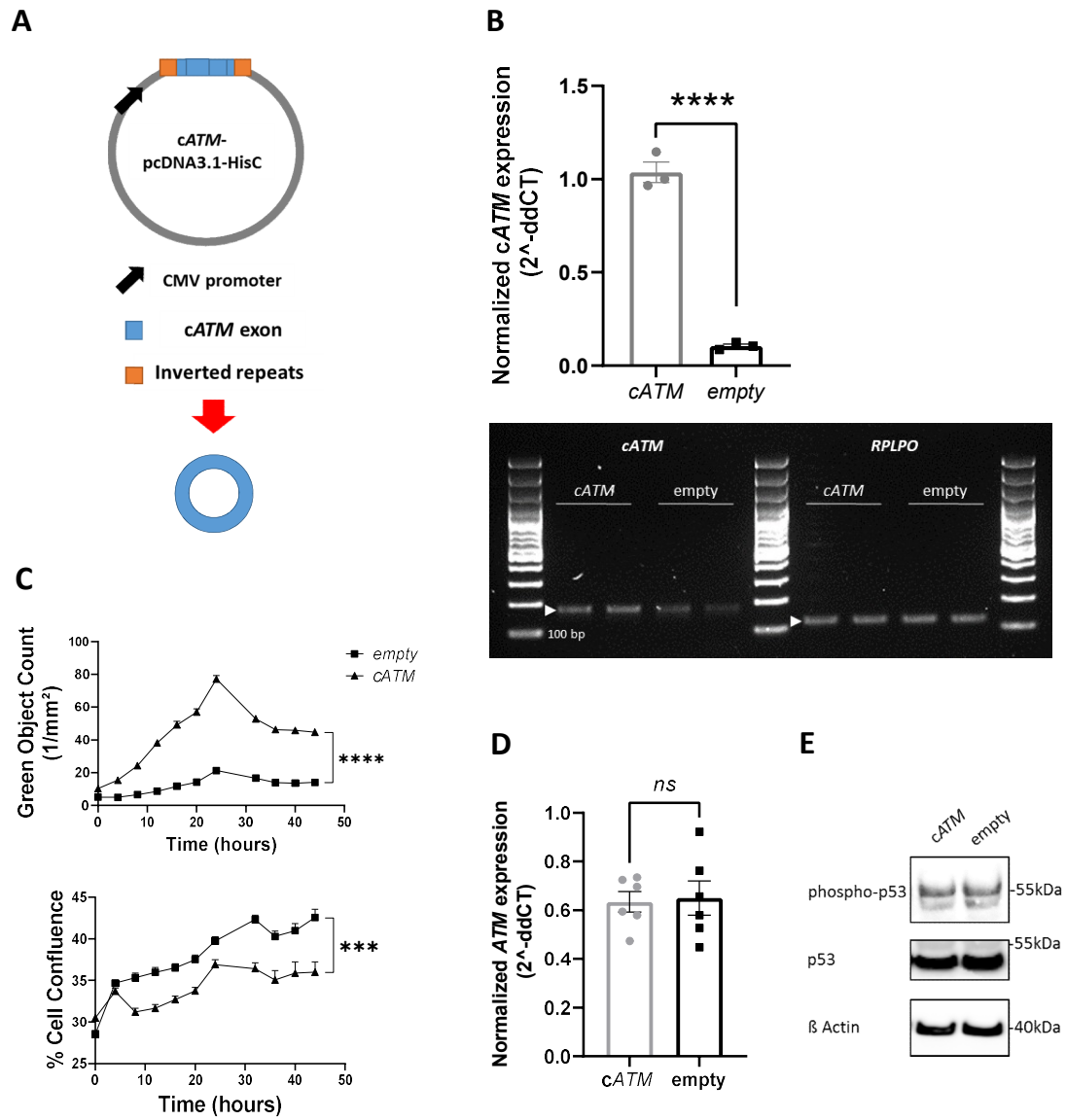


Figure S12. Effects of *cATM* overexpression in *hAoSMCs*. **A.** Schematic representation of *cATM* construct. **B.** *cATM* was overexpressed in CTRL *AoSMCs* and amplification products obtained from qPCR on cDNA synthesized from transfected cells were run on a gel and sequenced (statistics: unpaired T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM). **C.** Apoptosis and proliferation were monitored by live cell imaging (statistics: Multiple T-test; p values < 0.05 are considered significant; data are represented as mean \pm SEM). **D.** *ATM* mRNA expression and **(E)** phosphorylation of p53 were monitored upon *cATM* overexpression.

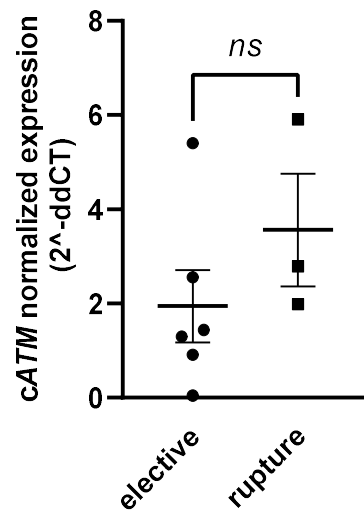


Figure S14. Expression profile of *cATM* in ruptured AAA vs. elective patients' serum samples. *cATM* levels were determined by qRT-PCR in serum samples collected from elective (N=6) vs ruptured (N=3) AAA patients. 2^{-ddCT} are plotted. statistics: T-test (two-tailed); p values < 0.05 are considered as significant; data are represented as mean \pm SEM.

Figure S15. Sanger sequencing of the circular junctions.

hsa_circ_0003641 (cATM)

AATGGTGCTATTTACGGAGCTGATTGTAGCAACATACTACTCAAAGACATTCTTTCTGTGAGAAAATACTG
GTGTGAAATATCTCAGCAACAGTGGTTAGACAGTGATGTGTGTTCTGAAATTGTGAACCATGAGTCTAGT
ACTTAATGATCTGCTTATCTGCTGCCGTCAACTAGAACATGATAGAGCTACAGAACGAA

>PRIMER FWD5

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>PRIMER REV5

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AGCACCAAATCTGTGCTTTTTCTGTGGGGTTTCKGGTGWAWWWCRCMGSAGGGTTTCGAAAGATCGC
GCTGACCCCCCTTTGTGGTCCCTGTGCGTAAA

hsa_circ_0005660 (cNFIX)

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ACTCACAACCTACTATATTCTCTCCTTGCTGCCCACACTTGTCAACAGGGACGTGTGTGAC

> PRIMER REV14

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GTGCTGGCCACAKGCCWGTGTCGTTCTCCTGTCTGCCGGCCACTTGTAGATAAACATGGACGTGTGT
GAASAACCAGCGTCCAGGACGATGCCATACTGSGGGGGAGGGGGAGGSAGWCG

> PRIMER FWD14

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TACTATATTCTCTCCTTGCTGCCCACACTTGTCAACAGGGACGTGTGTGAC

hsa_circ_0003218 (cBMPR2)

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>PRIMER FWD7

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>PRIMER REV7

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hsa_circ_0042103 (cMYOCD)

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> PRIMER FWD9

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ACTARTGCKTATTTTTACGGAAACGCWTTGTTTCACCGAAT

> PRIMER REV9

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TCGAWC

hsa_circ_0004771 (cNRIP1)

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PRIMER FWD2_HW

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CACAAA

PRIMER REV2_HW

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GGA

hsa_circ_0005615 (cNFATC3)

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PRIMER FWD5_HW

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PRIMER REV5_HW

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DOWNSTREAM EXON

UPSTREAM EXON

VALIDATED SEQUENCE (SANGER)

Figure S16. *cATM-pcDNA3.1-HisC* - 2669bp.

LINE repeat

MLLT3/AF9 intron 4 ([chr9:20414651+20415428](#))

ATM intron

Simple repeat

ATM exons

Splicing sites

EcoRI

XhoI

GAGCGCGACGTAATACGACTCACTATAGGGCGAATTGAGTGAAGGCCGTCAAGGCCGCATGAATTCCTTTCTTAATC
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TTATACAGCATAAAAATAATTGCTTTTTATCTCAAATTGAGAATTACTTTATTTCTTTGGGGGGGTTTTATGATTCCT
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AATCTTGTTCTATAGCTGGGTGTTCCCAATAACAATCTGTGCTTGTCTCCCTGTTGTTTATGTTTATTCTGGTCTTG
GTCTCTTAAGTACCTTTCCATTCTTTTATTCTAGTAACCTGTTTATCCTCCAGGACCTAATTCAGATGTTACTTCT
TTATGAATTTTTGAATTATTCTTCGCCCCATGCTCCCAACAGAATTAATAGTTCCCGTCTTTTGGTTGTCAACATCTAT
CAATCATAGATACGTCATGTTATTAAGGGTGGGGTCCATGTGTTATTCATCCTTGATCTTCTAGGACAAGCAGTA
GTTAACATTTGTTGAATTGAAATGTTTTAAACTCCATGCTTCAGATGATTAAGAAACTCGAGCTGGGCCTCATGGG
CCTTCACTTCACTGCCCGCTTCCAGTCGGGAAACCTGTCGTGCCA

Figure S17. Cloning strategy.

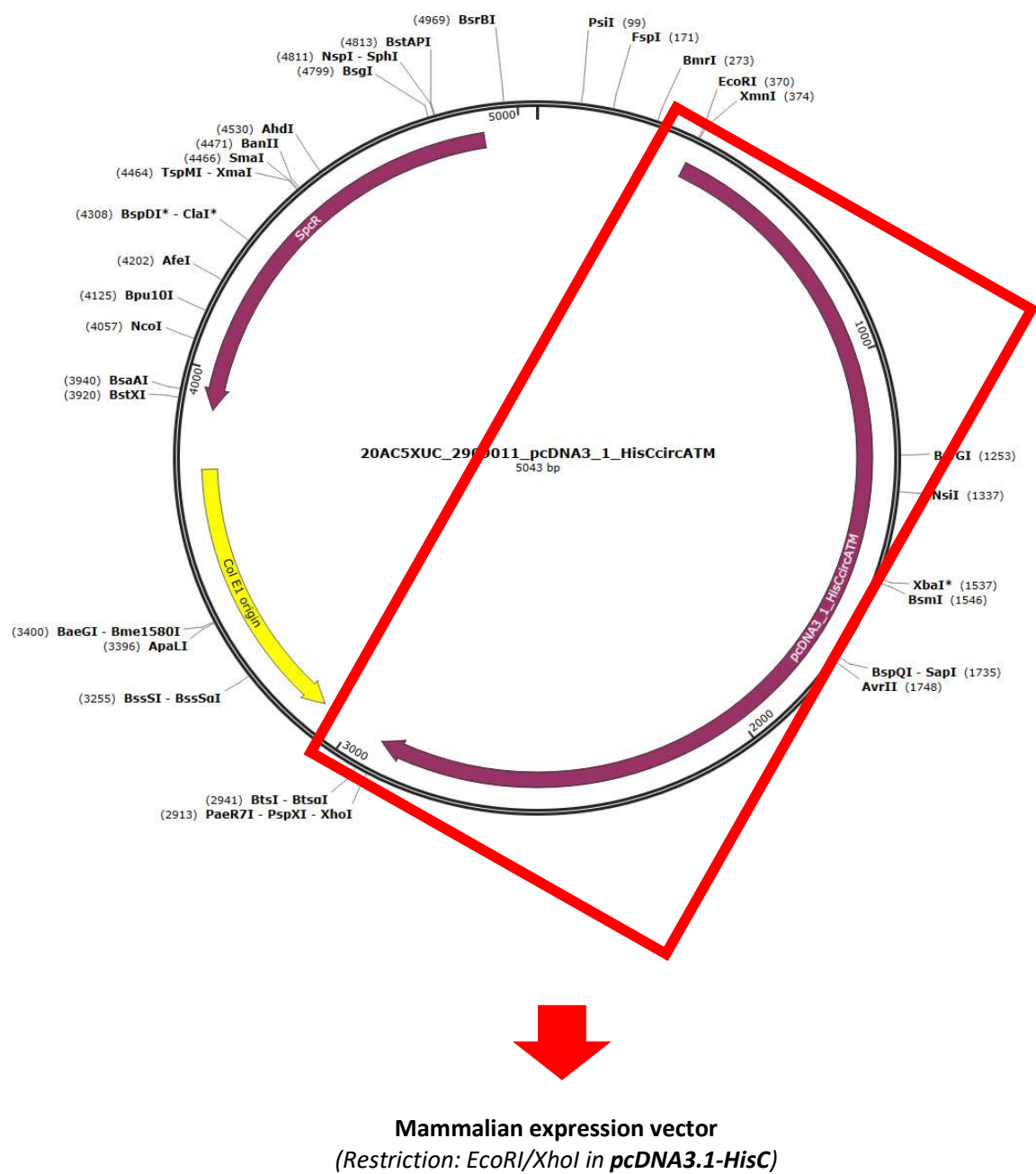


Figure S18. qPCR amplicon Sanger sequencing of cATM-pcDNA3.1-HisC-transfected AoSMCs.

hsa_circ_0003641 junction sequence (3' End - 5' End of circRNA)
AATGGTGCTATTTACGGAGCTGATTGTAGCAACATACTACTCAAAGACATTCTTTCTGTGAGAAAATAC TGGTGTGAAATATCTCAGCAACAGTGGTTAG ACAGTGATGTGTGTTCTGAAATTGTGAACCATGAGTCT AGTACTTAATGATCTGCTTATCTGCTGCCGTCAACTAGAACATGATAGAGCTACAGAACGAA

in grey: SANGER VALIDATED

>cATM_c30 B1+cATM_fwd

NHNWNTCAAGANMMNTTCTTTCTGTGNNAAATACTGGNTGTGAAATATCTCAGCAACAGTGGTTAGACAGTNM
YGN

>cATM_c30 B1+cATM_rev

NNTTTNNGANCACACATCACTGTCTAACCACTGTTGCTGAGATATTTACACCAGTATTTTCTCACAGAAAGAATGT
CTTGAGTAGTATGTTGCTACAATCAGCTCCGTAAATAGCACCNMM

>cATM_c30 B2+cATM_fwd

NAAGANTTCTTTCTGTGAGAAATACTGGTGTGAAATATCTCAGCAACAGTGGTTAGACAGTGATGTGTGTTCTGAA
ATTGTGAACCATGAGTCTAGTACTTAATGATCTGCTTATCTGCTGCCGTN

>cATM_c30 B2+cATM_rev

NNAATTCNGAACACACATCACTGTCTAACCACTGTTGCTGAGATATTTACACCAGTATTTTCTCACAGAAAGAAT
GTCTTGAGTAGTATGTTGCTACAATCAGCTCCGTAAATAGCACCAAANCNT