Long Noncoding RNA MANTIS Facilitates Endothelial Angiogenic Function

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BACKGROUND: The angiogenic function of endothelial cells is regulated by numerous mechanisms, but the impact of long noncoding RNAs (lncRNAs) has hardly been studied. We set out to identify novel and functionally important endothelial lncRNAs.

METHODS: Epigenetically controlled lncRNAs in human umbilical vein endothelial cells were searched by exon-array analysis after knockdown of the histone demethylase JARID1B. Molecular mechanisms were investigated by RNA pulldown and immunoprecipitation, mass spectrometry, microarray, several knockdown approaches, CRISPR-Cas9, assay for transposase-accessible chromatin sequencing, and chromatin immunoprecipitation in human umbilical vein endothelial cells. Patient samples from lung and tumors were studied for MANTIS expression.

RESULTS: A search for epigenetically controlled endothelial lncRNAs yielded lncRNA n342419, here termed MANTIS, as the most strongly regulated lncRNA. Controlled by the histone demethylase JARID1B, MANTIS was downregulated in patients with idiopathic pulmonary arterial hypertension and in rats treated with monocrotaline, whereas it was upregulated in carotid arteries of *Macaca fascicularis* subjected to atherosclerosis regression diet, and in endothelial cells isolated from human glioblastoma patients. CRISPR/Cas9-mediated deletion or silencing of MANTIS with small interfering RNAs or GapmeRs inhibited angiogenic sprouting and alignment of endothelial cells in response to shear stress. Mechanistically, the nuclear-localized MANTIS lncRNA interacted with BRG1, the catalytic subunit of the switch/sucrose nonfermentable chromatin-remodeling complex. This interaction was required for nucleosome remodeling by keeping the ATPase function of BRG1 active. Thereby, the transcription of key endothelial genes such as *SOX18*, *SMAD6*, and *COUP-TFII* was regulated by ensuring efficient RNA polymerase II machinery binding.

CONCLUSION: MANTIS is a differentially regulated novel lncRNA facilitating endothelial angiogenic function.

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Clinical Perspective

What Is New?

- MANTIS is a long noncoding RNA strongly regulated by histone demethylase JARID1B.
- MANTIS maintains endothelial cell function.
- It interacts with the nucleosome-remodeling protein BRG1.
- It maintains ATPase activity of BRG1 by stabilizing the switch/sucrose nonfermentable complex.
- MANTIS promotes transcription of SOX18, SMAD6, and COUP-TFII by enabling RNA polymerase II binding to the transcriptional start sites.
- MANTIS is downregulated during pulmonary arterial hypertension but induced in tumor endothelium.

What Are the Clinical Implications?

- MANTIS is a novel long noncoding RNA that positively affects endothelial angiogenic function.
- Altering MANTIS expression could be exploited to control the angiogenic process in situations of excessive or insufficient angiogenesis.

The endothelium forms the central vascular barrier to maintain vessel function and integrity. Healthy endothelium prevents thrombus formation, leukocyte adhesion, and smooth muscle cell proliferation, whereas activated endothelium has opposing properties.[1] Understanding how endothelial cells maintain or change their phenotype is key to preventing vascular disease development. Important aspects in this regulation are epigenetic mechanisms, but our understanding of vascular epigenetics is still in its beginning.

Epigenetic mechanisms regulating gene expression involve chromatin modifications without associated DNA sequence alterations. For example, trimethylation at histone 3 lysine 4 (H3K4) in promoter regions is generally associated with gene expression.[2,3] The H3K4 lysine-specific demethylase 5B (JARID1B) has been shown to maintain normal endothelial gene expression by limiting expression of gene repressors.[4] Several chromatin-modifying complexes have been found to be important for endothelial gene expression. In human umbilical vein endothelial cells (HUVECs), for example, polycomb repressor complex-2 (PRC2) regulates gene expression of *Ten-eleven translocation-1*.[5] Moreover, the catalytic subunit of the switch/sucrose nonfermentable (SWI/SNF) complex, Brahma related gene-1 (BRG1), is recruited to the endothelial nitric oxide synthase promoter under hypoxic conditions,[6] and to the *Selectin E* promoter under resting conditions.[7] Knockout of BRG1 or loss of PRC2 function results in mouse embryonic lethality,[8,9] highlighting the importance of epigenetic modifiers for vascular development. Moreover, BRG1 has been identified as a central regulator of gene expression, eg, of *Chicken Ovalbumin Upstream Promoter Transcription-Factor-2* (*COUP-TFII*) in vascular cells.[10]

Epigenetic functions are also mediated by RNAs. Only a small portion of the human genome carries protein-coding potential; the majority is differentially and dynamically transcribed to produce noncoding RNAs, of which the majority are long noncoding RNAs (lncRNAs).[11] LncRNA is the most diverse, plastic, and poorly understood class of noncoding RNA. It is broadly defined as transcribed, but not translated, RNA molecules >200 nucleotides in length.[12] Many lncRNAs are associated with epigenetic factors, where they recruit chromatin-modifying complexes, such as PRC2, to target sites. Through this and other mechanisms, lncRNAs influence transcriptional activation or repression, depending on the interaction partner.[11] So far, only a few lncRNAs have been shown to contribute to vascular disease and endothelial cell integrity.[13] This contrasts with the important role of the endothelium in vascular biology. In the present study, we set out to identify epigenetically controlled endothelial lncRNAs possessing relevant and novel functions. Among them, we focused on the unreported lncRNA n342419, which we named MANTIS, and establish this as a disease-relevant element controlling vascular transcription factor expression through BRG1.

METHODS

All methods are described in detail in the online-only Data Supplement.

Primers

The primers for quantitative real-time polymerase chain reaction are listed in online-only Data Supplement Table I. The primers for chromatin immunoprecipitation are listed in online-only Data Supplement Table II.

Statistics

Unless otherwise indicated, data are given as means±standard error of mean. Calculations were performed with Prism 5.0 or BiAS.10.12. The latter was also used to test for normal distribution and similarity of variance. In case of multiple testing, Bonferroni correction was applied. For multiple group comparisons, analysis of variance followed by post hoc testing was performed. Individual statistics of dependent samples were performed by paired *t* test, of unpaired samples by unpaired *t* test, and, if not normally distributed, by Mann-Whitney test. *P* values of <0.05 were considered as significant. Unless otherwise indicated, n indicates the number of individual experiments.

Study Approval

The study protocol for tissue donation from patients who have human idiopathic pulmonary hypertension was approved by the ethics committee (Ethik Kommission am Fachbereich

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Humanmedizin der Justus Liebig Universität Giessen) of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with Good Clinical Practice/International Conference on Harmonisation guidelines. Written informed consent was obtained from each individual patient or the patient's next of kin (AZ 31/93, 10/06, 58/15).[14]

Studies for human glioblastoma were covered by an ethics statement according to the guidelines of the University of Frankfurt, whose approval number for autopsy material is GS-249/11 and for resection material, GS-04/09.

Animal experiments regarding severe combined immunodeficiency mice were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. The University Animal Care Committee and the Federal Authorities for Animal Research (Darmstadt, Germany) approved the study protocol.

Animal studies regarding rats were performed according to the guidelines of the University of Giessen and were approved by the local authorities (GI 20/10 Nr.44/2013).

Experiments on adult male cynomolgus monkeys were approved by the Institutional Care and Use Committee of the University of Iowa as approved experiments in this study.[15]

RESULTS

MANTIS Is a JARID1B-Suppressed lncRNA Downregulated in Human Idiopathic Pulmonary Arterial Hypertension

To identify epigenetically controlled lncRNAs, the impact of the knockdown of the histone demethylase JARID1B on endothelial RNA expression was determined by Exon arrays. JARID1B is one of the highest expressed histone demethylases in HUVECs.[4] The expression of several lncRNAs was altered by depletion of JARID1B with small interfering RNAs (siRNAs) (Figure 1A, online-only Data Supplement Table III, online-only Data Supplement Figure IA), of which n342419 and n406914 were most consistently regulated (online-only Data Supplement Figure IB). Expression of n342419 was much higher than that of n406914 in HUVECs (online-only Data Supplement Figure IC), and the RNA was also most strongly induced by JARID1B knockdown (online-only Data Supplement Figure IB) and dependent on histone modification changes rather than direct transcriptional repression (online-only Data Supplement Figure ID).

On the basis of these features, we decided to further focus on this uncharacterized lncRNA, which is genomically located as intronic antisense to *Annexin A4* (*ANXA4*) (online-only Data Supplement Figure IE). Given the multiple cryptic names of n342419, ie, ANXA4-AS, uc002sft.1, and AK125871, we decided to name this lncRNA MANTIS based on its secondary structure. No indication of peptide-coding potential was found with open reading frame (ORF) determination by the RiboTaper[16] method and by the coding potential assessment tool[17] (online-only Data Supplement Figure IF and IG). In contrast to the positive control DYR, artificial in vitro translation suggested that MANTIS does not encode a protein with a molecular weight >10 000 Dalton (online-only Data Supplement Figure IH). None of the in silico predicted ORFs within the MANTIS sequence were identified in searches of a HEK Proteome with the PRIDE database (PXD000705, online-only Data Supplement Tables IV and V). A search for micropeptides after overexpression of MANTIS in HUVECs with subsequent liquid chromatography-tandem mass spectrometry without trypsination showed that none of the micropeptides found had any similarity to potential MANTIS ORFs, whereas the positive control yielded micropeptides encoded from GFP plasmid (online-only Data Supplement Tables VI and VII). A second experiment using MANTIS overexpressing HUVECs, this time with trypsination before liquid chromatography-tandem mass spectrometry, revealed no micropeptides with similarity to potential MANTIS ORFs, suggesting that MANTIS is noncoding (online-only Data Supplement Tables VIII and IX), thereby also minimizing the lack of detection by mass spectrometry, which does not exclude the presence of micropeptides. The expression of MANTIS was not restricted to HUVECs and even not to endothelial cells; MANTIS could also be detected in aortic, lymphatic, and pulmonary artery endothelial cells, in human smooth muscle cells isolated from different arteries, fibroblasts, MCF-7 cells, and THP-1 monocytes (online-only Data Supplement Figure II).

It is important to note that the expression of MANTIS was also altered in the disease context. In lungs from patients with end-stage idiopathic pulmonary arterial hypertension (IPAH), an isolated small-vessel disease accompanied by endothelial dysfunction, endothelial apoptosis, and proliferation,[18] MANTIS was downregulated, whereas JARID1B was upregulated (Figure 1B). A similar regulation pattern was also found in pulmonary artery endothelial cells (online-only data Supplement Figure IIA). Conversely, during vascular regeneration, JARID1B was downregulated and MANTIS was induced, as observed in the atherosclerosis regression phase in samples obtained from a *M. fascicularis* high-fat feeding study[15] (Figure 1C). In endothelial cells isolated from glioblastoma or adjacent healthy brain tissue, MANTIS was increased, whereas JARID1B was decreased (Figure 1D), which was also shown for MANTIS by RNA in situ hybridization using RNAscope (Figure 1E). Similarly, laminar flow induced MANTIS, whereas it decreased JARID1B expression (Figure 1F). To identify a functional role of MANTIS, loss-of-function approaches were used in the cell culture system with HUVECs. Because full-length MANTIS is only partially conserved beyond primates, studying its physiological importance by genetic knockout in mice is difficult. Therefore, we determined the capac-

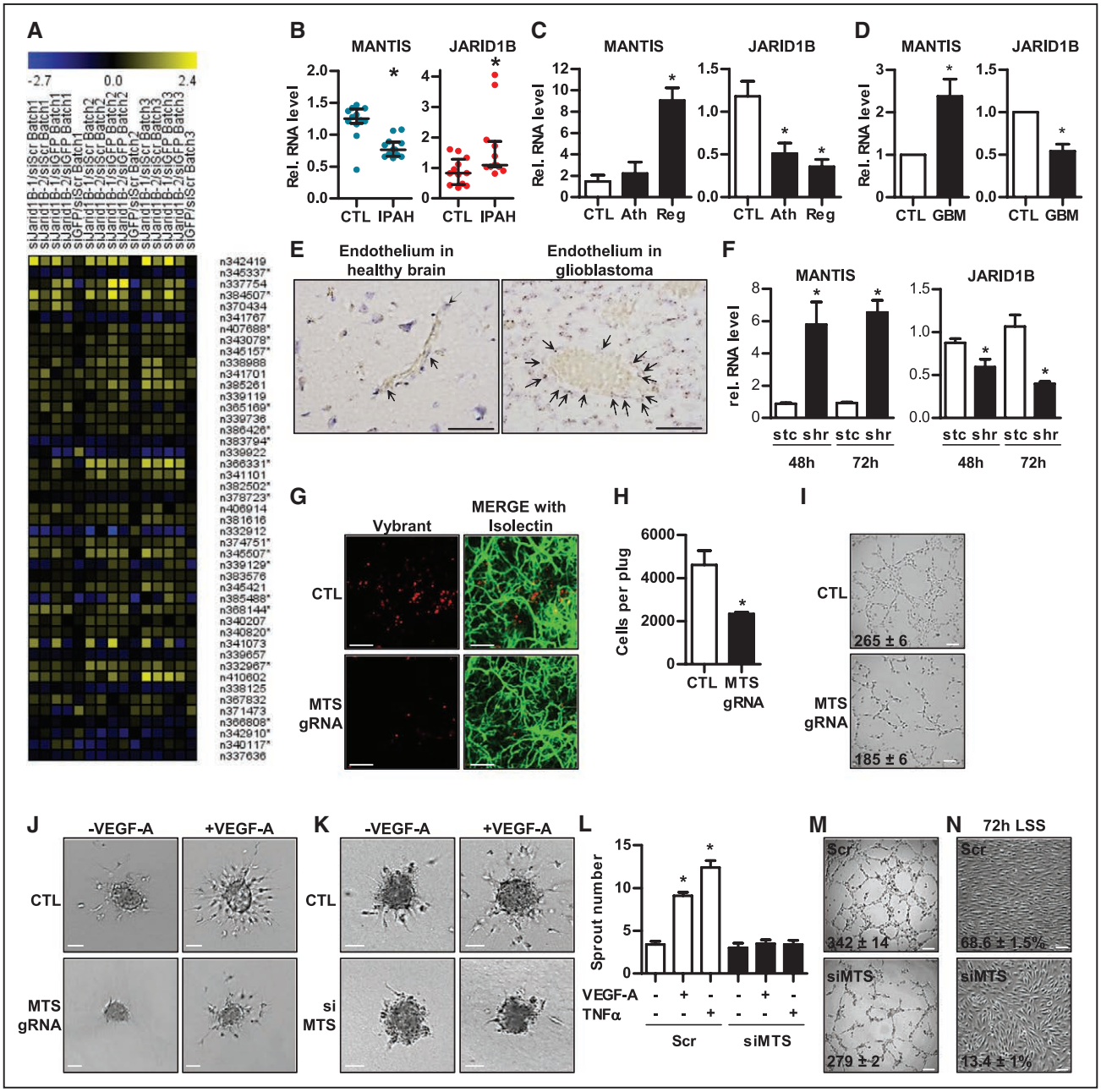

Figure 1. Endothelial angiogenic capacity is dependent on lncRNA MANTIS.

A, Affymetrix Exon-array heatmap comparing siJARID1B-1/siScr, siJARID1B-2/siScr, siJARID1B-1/siGFP, siJARID1B-2/siGFP, and siScr/siGFP levels of HUVEC batches 1 to 3. Scale bar shows color code from –2.7 (blue) to 2.4 (yellow) log2 fold change. lncRNAs marked by an asterisk revealed >1 noncode accession numbers. See online-only Data Supplement Table III and online-only Data Supplement Figure IA for all lncRNA names. **B,** qRT-PCR of MANTIS and JARID1B in lungs from control donors (CTL) or patients with IPAH. n=12. Median with interquartile range is shown, and Mann-Whitney test was used. **C**, qRT-PCR of MANTIS and JARID1B in monkey vessels treated either with a normal diet (CTL), a high-fat diet (Ath), or a high-fat diet and a subsequent recovery phase (Reg). n=3. One-way ANOVA, Bonferroni. **D**, qRT-PCR of MANTIS and JARID1B from endothelial cells isolated from glioblastoma (GBM) or adjacent healthy control (CTL) tissue. n=5. Paired *t* test. **E**, RNA in situ hybridization of endothelium of healthy brain or glioblastoma with RNAscope. Scale bar indicates 50 μm. Arrows point to dots indicating MANTIS RNA. **F**, qRT-PCR measurements relative to β-Actin of MANTIS and JARID1B after laminar flow exposure (shr, 20 dyn/cm^2) for 48 hours or 72 hours in HUVECs as indicated. Static (stc) samples served as control. n=4. Unpaired *t* test. **G**, CRISPR/Cas9 MANTIS guide RNAs (MTS gRNA) and control cells (CTL) after in vivo matrigel angiogenesis assay in mice. HUVECs were embedded in matrigel, stained with Vybrant dil (red), and injected. Isolectin GS-IB4 Alexa 647 conjugated stained vessels (green). Images were taken by light sheet microscopy 26 days after injection. Representative pictures are shown. Scale bar indicates 200 μm. **H**, Quantification of cells per plug as shown in **G** 26 days after injection. n=3. Unpaired *t* test. **I,** Tube formation assay performed with MTS gRNA and CTL. Numbers indicate number of tubes ± SEM. n=3. Scale bar indicates 200 μm. **J**, Spheroid outgrowth assay with MANTIS (*Continued*)

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ity of HUVECs with or without CRISPR/Cas9-mediated deletion of MANTIS to integrate into the vascular network of matrigels injected in severe combined immunodeficiency mice. Importantly, deletion of *MANTIS* resulted in a >50% reduction of the capacity of HUVEC to be retained in this model (Figure 1G and 1H, online-only Data Supplement Figure IIB and IIC). In addition, CRISPR/Cas9-mediated knockout of MANTIS in HUVECs greatly attenuated tube formation and sprouting (Figure 1I and 1J).

Because genetic deletion of *MANTIS* greatly reduced cellular health, siRNAs were used as a less drastic knockdown strategy. Of the 3 different siRNAs used, all reduced *MANTIS* expression, albeit siRNA-1 being most effective (online-only Data Supplement Figure IID). Therefore, siRNA-1 was used for the subsequent experiments. MANTIS siRNA-mediated depletion reduced endothelial sprouting (Figure 1K and 1L, online-only Data Supplement Figure IIE and IIF) and tube formation (Figure 1M), also in pulmonary artery endothelial cells (online-only Data Supplement Figure IIG). Moreover, it attenuated migration of HUVECs in Boyden-Chamber assays (online-only Data Supplement Figure IIH). In a competition-like spheroid outgrowth assay, siMANTIS-transfected cells were underrepresented in the tip cell position (online-only Data Supplement Figure III and IIJ). Also, the ability of HUVECs to properly orientate toward the direction of flow was lost after the knockdown of the lncRNA (Figure 1N).

The regulation of MANTIS by JARID1B was further studied by chromatin immunoprecipitation experiments. JARID1B bound H3K4me3-rich regions near the transcriptional start site (TSS) of MANTIS (online-only Data Supplement Figure IIK). Knockdown of JARID1B increased H3K4me3 close to the TSS of MANTIS (online-only Data Supplement Figure IIL), whereas other family members such as JARID1A and JARID1C did not regulate MANTIS (online-only Data Supplement Figure IIM).

Together, these data suggest that the lncRNA MANTIS could be of importance during IPAH and glioblastoma and that its control by Jarid1B is essential to preserve multiple aspects of normal endothelial cell functions in culture and in a mouse in vivo model.

MANTIS Interacts With the SWI/SNF Chromatin-Remodeling Complex Subunit BRG1

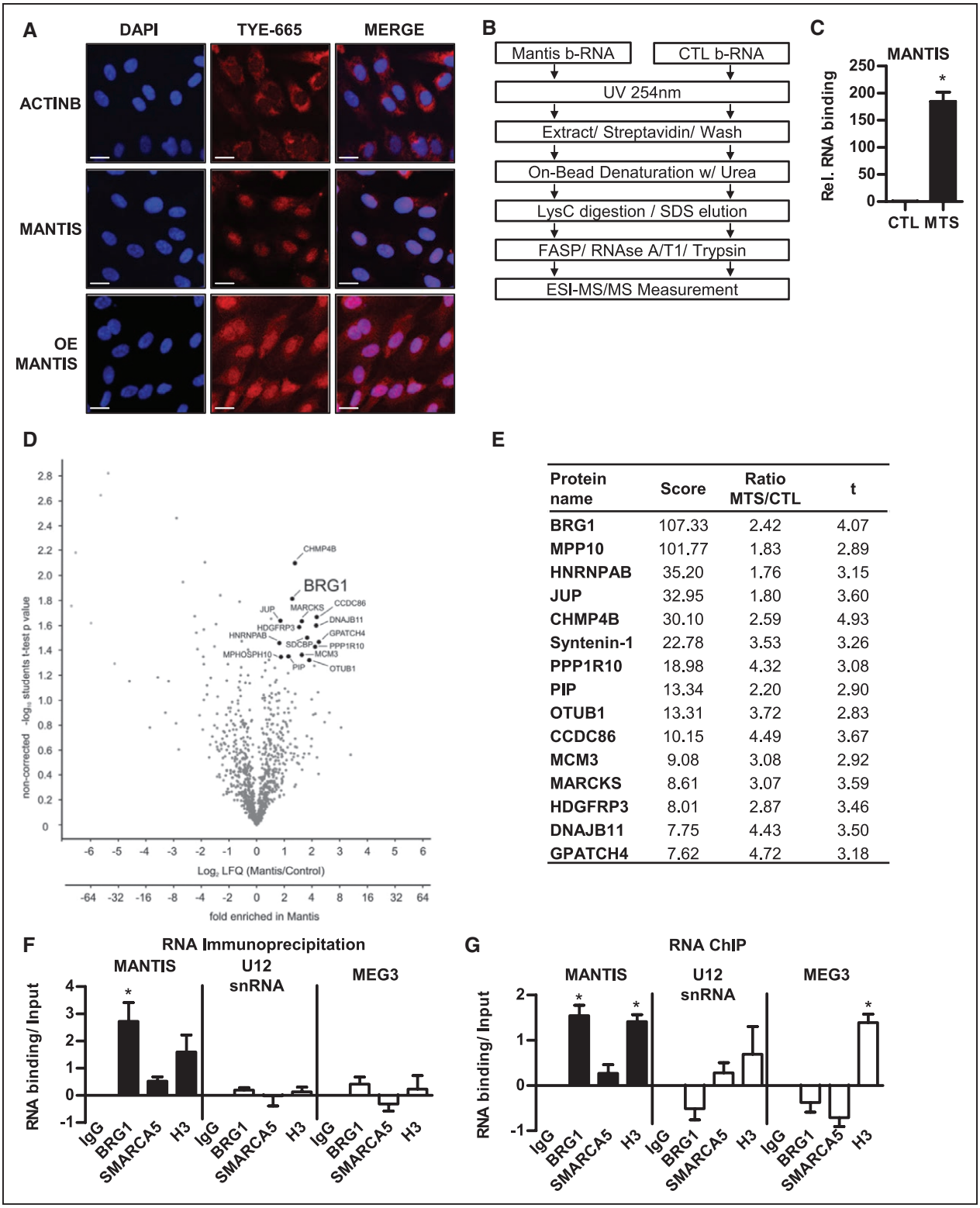
To get insights into the molecular mechanism by which MANTIS controls endothelial cell functions, we first evaluated the intracellular localization of MANTIS in HUVECs. RNA-fluorescence in situ hybridization demonstrated that the endogenous MANTIS RNA is localized in the nucleus, whereas the negative control ACTINB mRNA was predominantly localized in the cytosol. Also, after overexpression, MANTIS was retained in the nucleus (Figure 2A).

To identify candidates as interaction partners of MANTIS, RNA-pulldown experiments with 3′-biotinylated MANTIS lncRNA or 3′-biotinylated pcDNA3.1+ negative control RNA with nuclear extracts from HUVECs were performed (Figure 2B). As expected, MANTIS was strongly enriched in the 3′-biotinylated MANTIS pulldowns in comparison with the controls (Figure 2C). Electrospray ionization mass spectrometry of the samples identified several coprecipitating proteins as candidates for interaction (Figure 2D and 2E, online-only Data Supplement Table X), with BRG1 having the highest score. The ATPase BRG1 is a known regulator of endothelial cell functions and has been shown to be increased in humans with thoracic aortic aneurysms and to impact on proliferation, apoptosis, and myocardin-specific gene regulation in vascular smooth muscle cells.[19,20] Importantly, reverse RNA immunoprecipitation and RNA chromatin immunoprecipitation all confirmed MANTIS as an interaction partner of BRG1. The interaction of MANTIS with BRG1 was specific: MANTIS was not pulled down by SMARCA5, the catalytic subunit of the ISWI chromatin-remodeling complex,[21] or IgG (Figure 2F and 2G). BRG1 also did not coprecipitate with MEG3 lncRNA or U12 snRNA. These data show that MANTIS and the SWI/SNF catalytic subunit BRG1 are interacting. Potentially, this interaction mediates the physiological effects of MANTIS.

MANTIS Is Required for the Gene Expression of SOX18, SMAD6, and COUP-TFII

A key function of lncRNAs associated with chromatin-remodeling complexes is the regulation of transcriptional

Figure 1 Continued. gRNA and CTL. Cells treated with or without VEGF-A are shown. Scale bar indicates 50 μm. **K**, Spheroid outgrowth assay after siMTS. Scr served as negative control. Cells treated ± VEGF-A are shown. Scale bar indicates 50 μm. **L**, Quantification of sprout numbers from the spheroid outgrowth assays seen in **K** or online-only Data Supplement Figure IIE. n=10. One-way ANOVA, Bonferroni. **M**, Tube formation assay after siMTS. Scr served as negative control. Numbers indicate number of tubes ± SEM. n=3. Scale bar indicates 200 μm. **N,** Images of cells treated with scrambled or siMTS after 72 h of laminar flow (LSS, 20 dyn/cm^2). Numbers indicate number of cells orientated in direction of the flow ± SEM. n=4. Scale bar indicates 100 μm. All qRT-PCR data are relative to β-actin. Error bars are defined as mean ±SEM. **P*<0.05. ANOVA indicates analysis of variance; HUVEC, human umbilical vein endothelial cell; IPAH, idiopathic pulmonary arterial hypertension; lncRNA, long noncoding RNA; qRT-PCR, quantitative real-time polymerase chain reaction; Scr, scrambled; SEM, standard error of the mean; siMTS, MANTIS siRNA; and VEGF-A, vascular endothelial growth factor A.



Protein name	Score	Ratio MTS/CTL	t
BRG1	107.33	2.42	4.07
MPP10	101.77	1.83	2.89
HNRNPAB	35.20	1.76	3.15
JUP	32.95	1.80	3.60
CHMP4B	30.10	2.59	4.93
Syntenin-1	22.78	3.53	3.26
PPP1R10	18.98	4.32	3.08
PIP	13.34	2.20	2.90
OTUB1	13.31	3.72	2.83
CCDC86	10.15	4.49	3.67
MCM3	9.08	3.08	2.92
MARCKS	8.61	3.07	3.59
HDGFRP3	8.01	2.87	3.46
DNAJB11	7.75	4.43	3.50
GPATCH4	7.62	4.72	3.18

Figure 2. The nuclear localized MANTIS lncRNA interacts with the SWI/SNF complex member BRG1.
A, RNA fluorescence in situ hybridization (FISH) of HUVECs with TYE-665–modified probes against ACTINB and MANTIS. DAPI was used to stain nuclei. MANTIS overexpression (OE) samples were treated with an additional overexpression of pcDNA3.1+MANTIS for 48 hours before FISH. Scale bar indicates 20 μm. **B**, Scheme of RNA pulldown assay with subsequent preparation for mass spectrometric measurements. b-RNA indicates biotinylated RNA. **C**, qRT-PCR after RNA pulldown assay by measuring the amount of MANTIS RNA (MTS) in the eluates relative to the negative control RNA (CTL). n=4. Paired *t* test. **D**, Volcano plot of log2 ratio of MANTIS versus control interaction partner proteins after RNA pulldown assay and ESI-MS/MS measurements. n=3. Noncorrected $-\log_{10}$ Student *t* test. LFQ indicates label-free quantification. **E**, Proteins (*Continued*)

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events.[22] Thus, microarrays were used to identify the impact of MANTIS knockdown on endothelial gene expression. Depletion of MANTIS with LNA-GapmeRs led to downregulation of a high number of angiogenesis-related mRNAs, among them SRY (Sex Determining Region Y)-Box 18 (SOX18), Mothers against decapentaplegic homologue 6 (SMAD6), and COUP-TFII, and on upregulation of stress-induced genes like interleukin 6 and superoxide dismutase 2 (Figure 3A, online-only Data Supplement Table XI); however, genes neighboring the MANTIS locus in the genome (ANXA4, AAK1, GCML1, snRNP27) were not affected, excluding a *cis*-regulatory activity of MANTIS in HUVEC (Figure 3B). To confirm these results, quantitative real-time polymerase chain reaction after depletion of MANTIS with either LNA-GapmeRs, siRNAs, or CRISPR/Cas9 deletion mutants was performed. All silencing approaches confirmed the data obtained with the microarrays (Figure 3C and 3D, online-only Data Supplement Figure IIIA through IIIF). Gene ontology (GO) analyses for genes downregulated in response to MANTIS knockdown yielded angiogenesis as the most significantly affected GO term (Figure 3E, online-only Data Supplement Tables XII and XIII). Indeed, it has been reported previously that *SOX18*, *SMAD6*, and *COUP-TFII* are key endothelial genes important for angiogenesis and other endothelial features mentioned in reference 23.

On this basis, mRNA data were validated on the protein level. Western blot analyses revealed that both, LNA-GapmeRs and siRNAs, as well, not only reduced MANTIS mRNA, but also SOX18, SMAD6, and COUP-TFII protein levels in HUVECs (Figure 3F through 3I). Given the potential function of SOX18 for lymphatic and of COUP-TFII for venous specification, additional cell types were studied. Downregulating MANTIS, however, yielded similar responses in human dermal lymphatic endothelial cells, aortic arterial and pulmonary artery endothelial cells, and even vascular smooth muscle cells (online-only Data Supplement Figure IIIG through IIIL).

The fact that SOX18, SMAD6, and COUP-TFII were also downregulated in the samples of patients with IPAH in which also MANTIS was downregulated, might suggest that this interaction also occurs in the disease context (Figure 3J through 3L), because, in endothelial cells isolated from glioblastoma, SOX18, SMAD6, and COUP-TFII were increased (online-only Data Supplement Figure IIIM through IIIO).

To establish a link between these MANTIS target genes and endothelial angiogenic capacity, siRNA-based knockdown was performed (Figure 4A). Downregulation of either of SMAD6, COUP-TFII or SOX18 all attenuated endothelial sprouting in the spheroid outgrowth assay (Figure 4B through 4D). However, overexpression of SMAD6, COUP-TFII, or SOX18 after MANTIS knockdown (online-only Data Supplement Figure IVA and IVB) failed to normalize endothelial sprouting capacity (online-only Data Supplement Figure IVC and IVD). This might suggest that several factors acting in concert are affected by MANTIS or that technical difficulties arising from multiple transfections and from the great size of the MANTIS plasmid render these studies infeasible. To address the second aspect, we searched for a fragment of MANTIS with RegRNA2.0,[24] which might be sufficient to rescue endothelial function after MANTIS knockdown. A small part of ≈450 nt of MANTIS Exon 3 contains an Alu element, which could be of functional importance (online-only Data Supplement Figure IE). Overexpression of this element (MANTIS-mut) indeed partially rescued the sprouting in response to vascular endothelial growth factor A (Figure 4E through 4H). Thus, MANTIS maintains endothelial angiogenic capacity through an induction of SMAD6, COUP-TFII, and SOX18, and this effect is potentially mediated by the Alu element in Exon 3. Interestingly, a corresponding SINE B1 (short interspersed nucleotide element) was also found in mice and rats at a similar position within the Intron of ANXA4. In CD31+ positive cells isolated from rats exposed to monocrotaline, a model system frequently used to study pulmonary arterial hypertension,[25] expression of this putative homologue MANTIS fragment was downregulated, tendentially together with SOX18, SMAD6, and COUP-TFII (Figure 4I). This might suggest that this lncRNA is conserved from humans to rats and that the Alu element is important in regulating IPAH.

MANTIS and BRG1 Facilitate RNA Polymerase II Binding by Reducing Heterochromatin

BRG1 functions as an ATP-dependent helicase in the SWI/SNF chromatin-remodeling complex.[26] Interestingly, it has been shown that BRG1 promotes *COUP-TFII* gene expression in vascular endothelium during mu-

Figure 2 Continued. enriched after RNA pulldown assay, their score, ratio MANTIS/CONTROL (MTS/CTL), and *t* value. **F**, MANTIS lncRNA (**Left**), U12 snRNA (**Middle**), and MEG3 lncRNA (**Right**) binding to complexes RNA immunoprecipitated with IgG, anti-BRG1, anti-SMARCA5, and anti-H3 were measured with qRT-PCR. The binding was analyzed relative to the input. Log2 values are shown. n=6. One-way ANOVA, Bonferroni. **G**, MANTIS lncRNA (**Left**), U12 snRNA (**Middle**), and MEG3 lncRNA (**Right**) binding to complexes RNA-chromatin immunoprecipitated with IgG, anti-BRG1, anti-SMARCA5, and anti-H3 were measured with qRT-PCR. The binding was analyzed relative to the input. Log10 values are shown. n=6. One-way ANOVA, Bonferroni. Error bars are defined as mean ± SEM. **P*<0.05. ANOVA indicates analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; HUVEC, human umbilical vein endothelial cell; lncRNA, long noncoding RNA; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; and SWI/SNF, switch/sucrose nonfermentable.

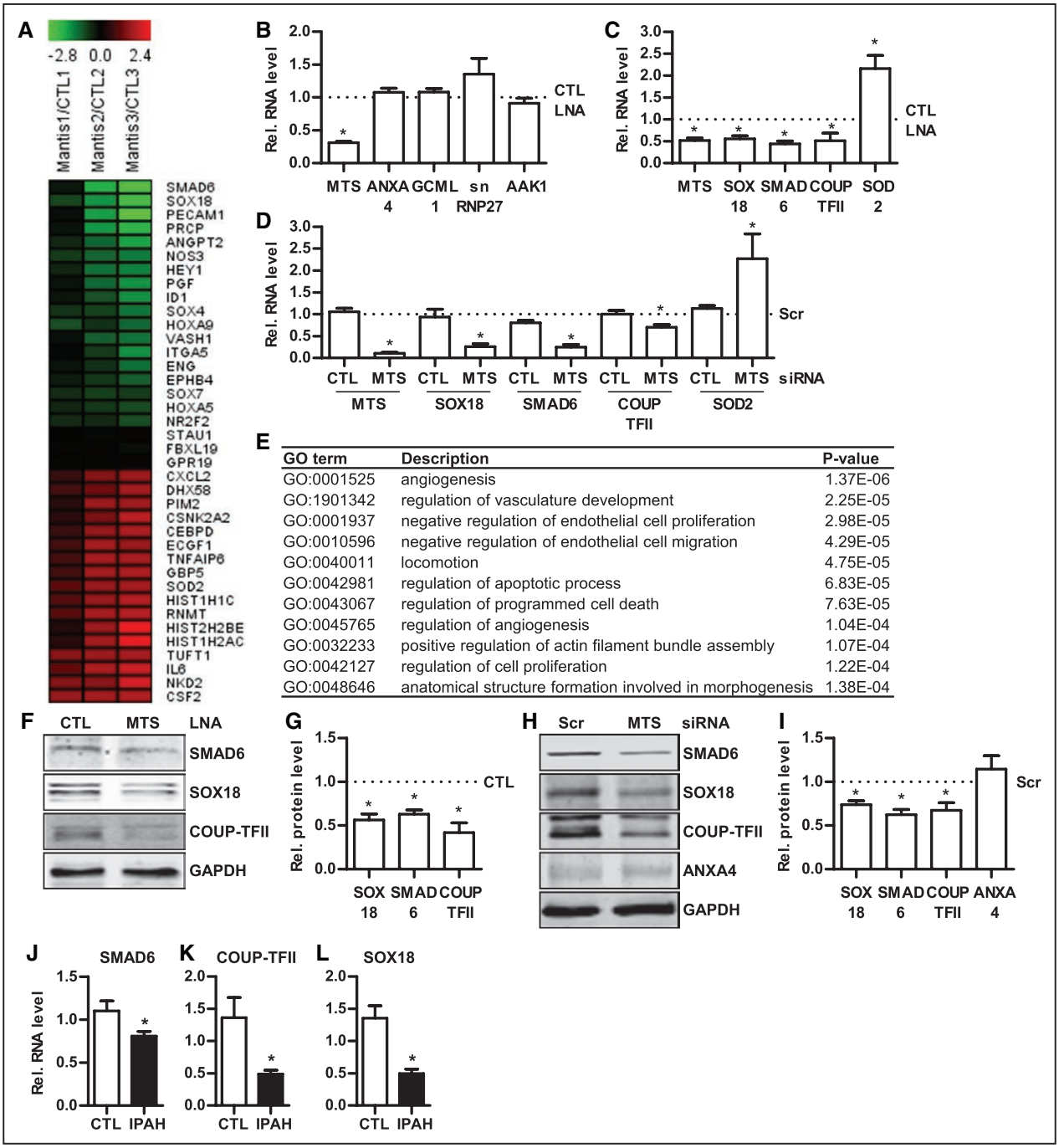

Figure 3. LncRNA MANTIS is required for SMAD6, COUP-TFII, and SOX18 expression.
A, Illumina Bead-Chip Array heat map comparing gene expression after MANTIS LNA-GapmeR versus Control LNA-GapmeR treatments for 48 hours. Scale bar shows color code from –2.8 (green) to 2.4 (red) log2 fold change. Only representative genes were shown. **B**, qRT-PCR after LNA-GapmeR knockdown of MANTIS lncRNA. Expression levels of MANTIS, ANXA4, GCML1, snRNP27, and AAK1 are shown. CTL LNA served as negative control and was set to 1. n=4, Paired *t* test. **C**, qRT-PCR after LNA-GapmeR based knockdown of MANTIS lncRNA. CTL served as negative control and was set to 1. Expression levels of MANTIS, SOX18, SMAD6, COUP-TFII, and SOD2 are shown. n=6. Paired *t* test. **D**, qRT-PCR after knockdown of MANTIS lncRNA with siRNA-1. Scrambled siRNA (Scr) and MANTIS siRNA-specific control siRNA (CTL) served as negative controls. Expression levels of MANTIS, SOX18, SMAD6, COUP-TFII, and SOD2 are shown. Scr was set to 1. n=7. One-way ANOVA, Bonferroni. **E**, Gene ontology (GO) analyses made with Gorilla using all significantly downregulated genes after MANTIS knockdown found in the array. **F**, Representative Western blot of HUVECs treated either with control or MANTIS LNA-GapmeRs. SMAD6, SOX18, and COUP-TFII antibodies were used. GAPDH served as control. **G**, Quantification of blots shown in **F**. CTL was set to 1. n=3, Paired *t* test. **H**, Representative Western blot of HUVECs treated either with control or MANTIS siRNA-1. SMAD6, SOX18, COUP-TFII, and ANXA4 antibodies were used. GAPDH served as control. **I**, Quantification of blots shown (*Continued*)

rine embryonic development by customizing its promoter for the transcriptional machinery.[10] To address the question whether MANTIS contributes to BRG1 nucleosome-remodeling activity, an Assay for Transposase Accessible Chromatin with subsequent DNA Sequencing (ATAC-Seq) was performed. The protein level of BRG1 was not changed by MANTIS depletion (Figure 5A). ATAC-Seq analysis after MANTIS knockdown revealed less open chromatin at the transcriptional start sites (TSS) of *SMAD6*, *SOX18*, and *COUP-TFII*, whereas the *MANTIS* TSS served as control (Figure 5B, online-only data Supplement Figure VA, online-only data Supplement Table XIV). The decrease in open chromatin at the TSS of *SMAD6*, *SOX18*, and *COUP-TFII* could be detected by Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) (Figure 5C), which is an alternative to DNase I.[27] To characterize the nucleosome occupancy, micrococcal nuclease (MNase) digestion was performed. MNase cuts preferentially linker DNA, whereas the nucleosomal DNA is partially protected because it is insensitive for MNase digestion.[27] As expected, MNase digestion resulted in increased nucleosome formation at the TSS of *SMAD6*, *SOX18*, and *COUP-TFII* (Figure 5D). Because BRG1 is involved in nucleosome remodeling, MANTIS may impact the compaction of chromatin and thus provides access of RNA polymerase II to the DNA. To demonstrate this, chromatin immunoprecipitation experiments of H3K27me3, heterochromatin protein 1α (HP1α), histone 3 (H3), and histone 4 (H4) were performed. On the *GAPDH* promoter no difference was observed, whereas on *SOX18* TSS, *SMAD6* TSS, and *COUP-TFII* TSS, H3K27me3 binding was increased by depletion of MANTIS (Figure 5E). For *SOX18* and *SMAD6* TSS, an additional increase of H3, H4, and HP1α binding was observed, which was only partially seen for *COUP-TFII* (online-only data Supplement Figure VB through VD). Consequently, *MANTIS* depletion decreased RNA polymerase II binding near the TSS of *SMAD6*, *SOX18*, and *COUP-TFII*, but not near the *GAPDH* TSS or further upstream of the promoters of *SMAD6*, *SOX18*, and *COUP-TFII* (Figure 5F). Importantly, and causative for the previous results, depletion of MANTIS resulted in a decreased binding of BRG1 near the TSS of *SMAD6*, *SOX18*, and *COUP-TFII* (Figure 5G), substantiating the concept that the nucleosome-remodeling activity of BRG1 on the *SMAD6*, *COUP-TFII*, and *SOX18* TSS is dependent on MANTIS, which may assist proper chromatin decompaction and thus may prepare the promoters for the recruitment of the RNA polymerase II transcriptional machinery.

By Stabilizing the Interaction Between BRG1 and BAF155, MANTIS Is Required for the ATPase Function of SWI/SNF

It is thought that the remodeling activity of BRG1 is stimulated by BAF155.[28] To understand the functional role of MANTIS within the SWI/SNF complex, the interactions of BRG1 with its interaction partners BAF155 and BAF53A were studied by proximity ligation assays (Figure 6A). The binding of BRG1 to BAF155 was significantly reduced by depletion of MANTIS (Figure 6B), whereas the interaction for BAF53A was not changed and LIS1 served as negative control. Vice versa, overexpression of *MANTIS* or the *MANTIS* Alu element mutant, but not C- or N-terminal *MANTIS* deletion mutants, was sufficient to increase the interaction between BRG1 and BAF155 in the proximity ligation assay (Figure 6C and 6D). Importantly, the reduced interaction of BRG1 to BAF155 after knockdown of *MANTIS* was not a consequence of decreased BAF155 or BRG1 protein expression as determined by Western blot analysis (Figure 6E). Given that *MANTIS* depletion decreased the interaction of BRG1 with BAF155 and also decreased the ability of BRG1 to bind on its target gene promoters, we measured the ATPase activity of BRG1 after the protein was immunoprecipitated from cells with and without MANTIS depletion. Importantly, BRG1 ATPase activity was drastically decreased by MANTIS knockdown (Figure 6F). These findings indicate that MANTIS improves the ATPase activity of BRG1 by stabilizing its interaction with BAF155.

DISCUSSION

In the present study, we identified the epigenetically regulated and previously unstudied lncRNA MANTIS (n342419) to be upregulated by knockdown of JARID1B and in vascular pathologies, whereas in IPAH MANTIS was repressed. Depletion of MANTIS reduced the endothelial angiogenic function ex vivo and in vivo. Mechanistically, MANTIS acts in *trans* through the SWI/SNF chromatin-remodeling factor BRG1 for which it facilitates the interaction with angiogenic genes and with the BRG1 stimulating factor BAF155. Thus, with the present work, we establish a putative link of a lncRNA acting through a novel mechanism of transacting lncRNAs in essential epigenetic regulatory mechanisms in endothelial cells, which is misregulated in patients with IPAH and glioblastoma (Figure 7). In contrast to *cis*-acting lncRNAs, which are often found to act on neigh-

Figure 3 Continued. in **H**. Scr was set to 1. n=4. Paired *t* test. **J** through **L,** qRT-PCR of SMAD6 (**J**), COUP-TFII (**K**), and SOX18 (**L**) in lungs from control donors (CTL) or patients with IPAH. n=12. Unpaired *t* test. All qRT-PCRs are relative to ß-Actin. Error bars are defined as mean ± SEM. **P*<0.05. CTL indicates control; HUVEC, human umbilical vein endothelial cell; IPAH, idiopathic pulmonary arterial hypertension; lncRNA, long noncoding RNA; MTS, MANTIS; qRT-PCR, quantitative real-time polymerase chain reaction; Scr, scrambled; SEM, standard error of the mean; and SOD2, superoxide dismutase 2.

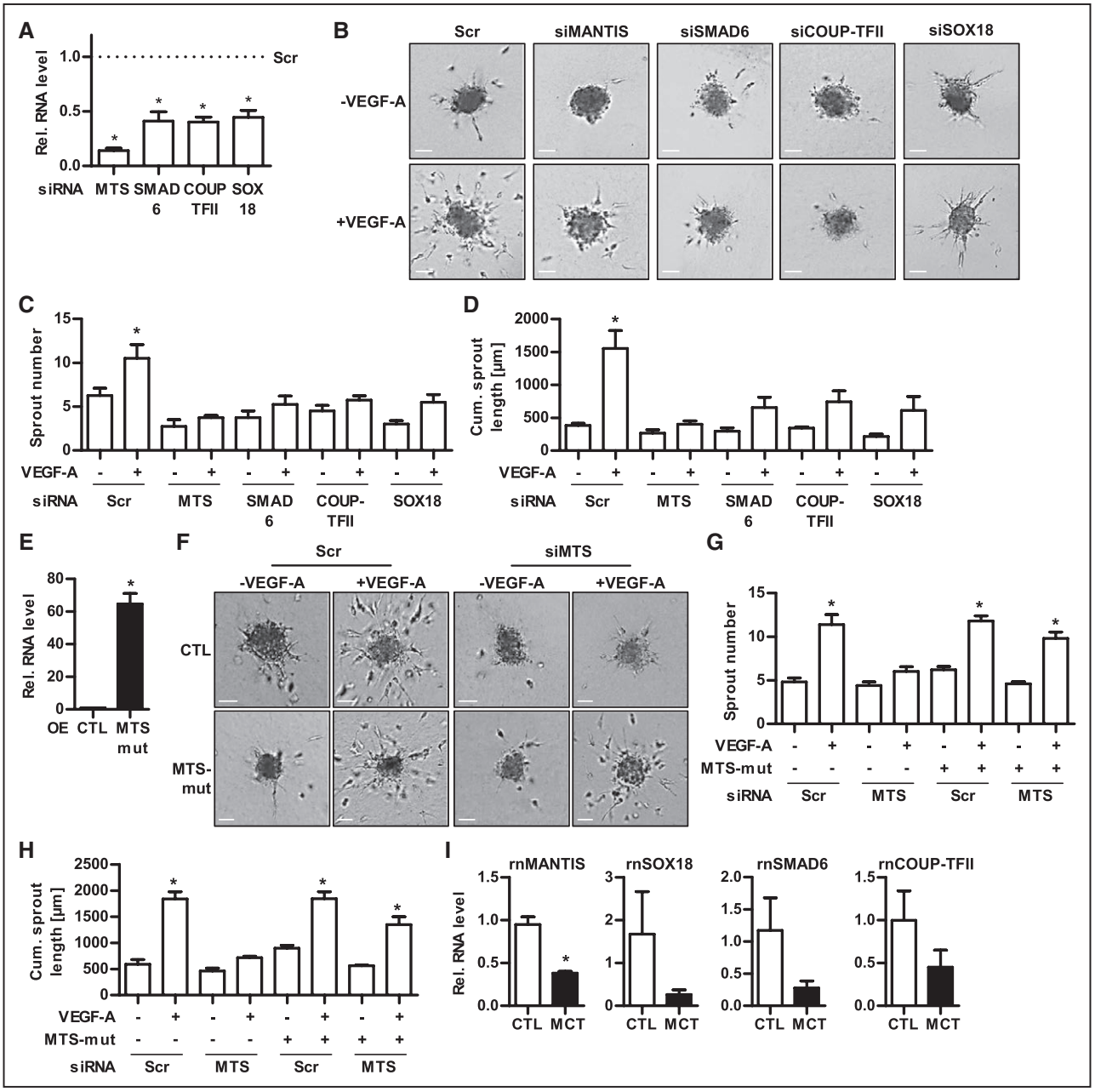

Figure 4. SMAD6, SOX18, or COUP-TFII knockdown resemble MANTIS-deficient phenotype.
A, qRT-PCR measurements after siRNA-based knockdown for 48 hours of MANTIS lncRNA (MTS), SMAD6, COUP-TFII, or SOX18. Scrambled siRNA (Scr) served as negative control and was set to 1. Expression levels of MANTIS (MTS), SMAD6, COUP-TFII, and SOX18 relative to β-ACTIN are shown. n=9. Paired *t* test. **B**, Spheroid outgrowth assay after MANTIS, SMAD6, COUP-TFII, or SOX18 siRNA-based knockdown for 48 hours. Scrambled siRNA (Scr) served as negative control. Cells treated with or without VEGF-A (±VEGF-A) are shown. Scale bar indicates 50 µm. **C, D**, Quantifications of sprout numbers (**C**) and cumulative sprout length (**D**) from the spheroid outgrowth assays shown in **B**. n=4. One-way ANOVA, Bonferroni. **E**, qRT-PCR measurements of MANTIS-mutant (MTS-mut) after overexpression (OE) of HUVEC with CTL or MTS-mut. n=6. Paired *t* test. **F**, Spheroid outgrowth assay after Scrambled (Scr) or MANTIS siRNA knockdown (siMTS) with subsequent overexpression of either CTL or MTS-mut for 24 hours. Cells treated with or without VEGF-A (±VEGF-A) are shown. Scale bar indicates 50 µm. **G, H**, Quantification of sprout numbers (**G**) and cumulative sprout length (**H**) from the spheroid outgrowth assays shown in **F**. n=5. One-way ANOVA, Bonferroni. **I,** qRT-PCR of MANTIS, SOX18, SMAD6, and COUP-TFII in PECAM-positive lung endothelial cells isolated from rats treated with saline (CTL) or monocrotaline (MCT). n=3. rn indicates *rattus norvegicus*. Unpaired *t* test. Error bars are defined as mean ± SEM. *P<0.05. ANOVA indicates analysis of variance; HUVEC, human umbilical vein endothelial cell; PECAM, platelet endothelial cell adhesion molecule; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; and VEGF-A, vascular endothelial growth factor A.

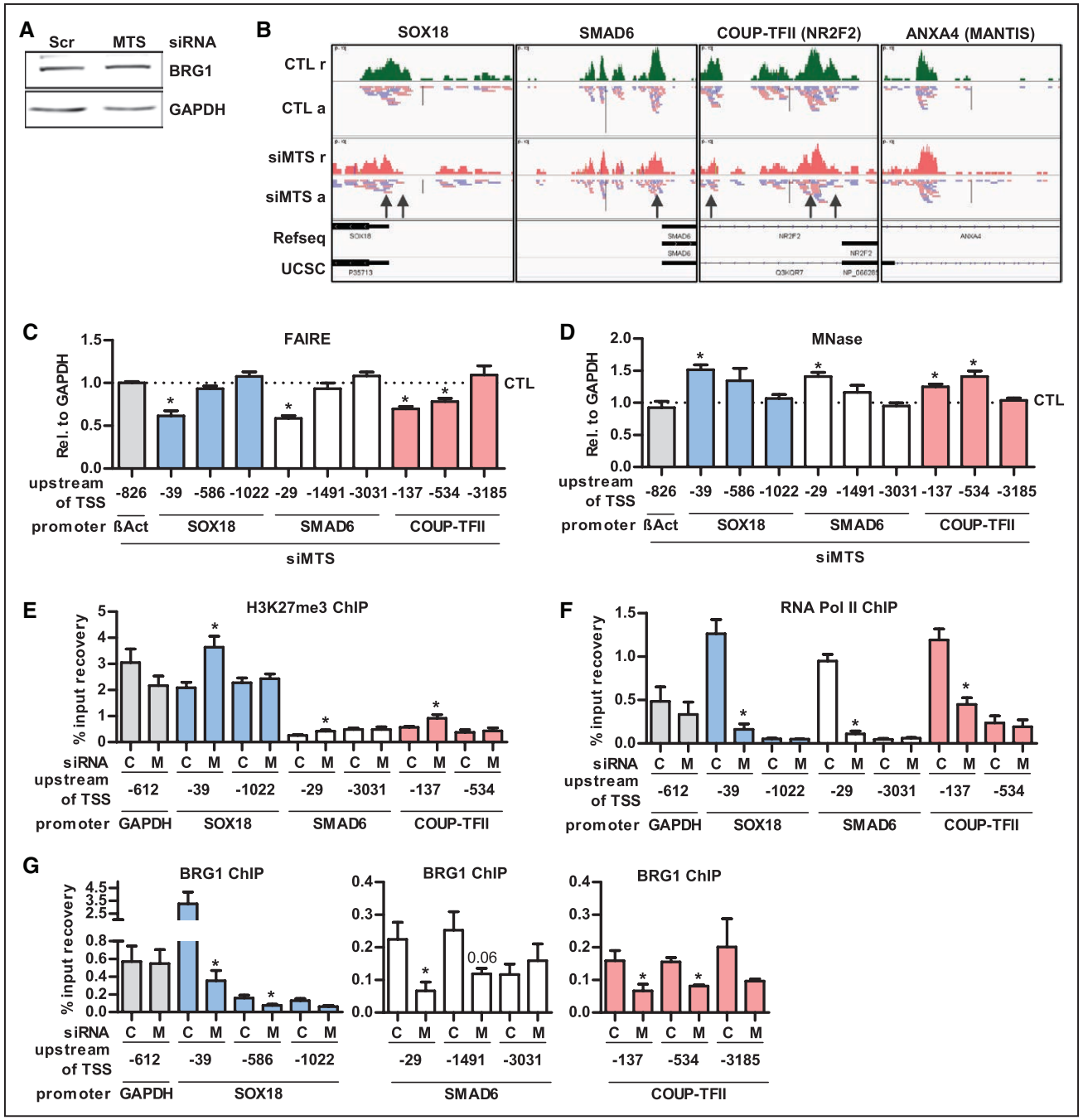

Figure 5. MANTIS facilitates nucleosome remodeling by BRG1.

A, Representative Western blot of HUVECs treated either with scrambled (Scr) or with MANTIS siRNA-1 (MTS) for 48 hours. GAPDH served as loading control. **B**, ATAC-Seq profiles of genomic loci of SOX18, SMAD6, COUP-TFII, and ANXA4 after transfection of HUVECs with scrambled (CTL) or MANTIS siRNA (siMTS) underlined with Refseq and University of California, Santa Cruz annotation. For all, number of reads ranges from 0 to 13. r indicates reads; and a, alignment. Arrows indicate regions of strong differences between CTL and siMTS. **C, D**, qPCR of indicated genomic loci relative to GAPDH after transfection of HUVEC with scrambled (CTL) or MANTIS siRNA (siMTS) with subsequent FAIRE (**C**) or Mnase (**D**). Numbers indicate nucleotide positions upstream of the TSS. CTL was set to 1. n=4, paired *t* test. **E** through **G**, ChIP of HUVECs transfected with scrambled (C) or MANTIS siRNA (M) with H3K27me3 (**E**, n=6), RNA Pol II (**F**, n=3), and BRG1 (**G**, n=4) followed by qPCR for GAPDH promoter, Sox18 promoter regions at the transcription start site (TSS, –39 nt) or 586 nt and 1022 nt, SMAD6 promoter regions at the transcription start site (TSS, –29 nt) or 1491 nt and 3031 nt, and COUP-TFII promoter regions at the transcription start site (TSS, –137 nt) or 534 nt and 3185 nt. Numbers indicate nucleotide positions upstream of the TSS. Unpaired *t* test. Error bars are defined as mean ± SEM. **P*<0.05. ChIP indicates chromatin immunoprecipitation; FAIRE, Formaldehyde-Assisted Isolation of Regulatory Elements; HUVEC, human umbilical vein endothelial cell; MNase, micrococcal nuclease; nt, nucleotide; qPCR, quantitative polymerase chain reaction; RNA Pol II, RNA polymerase II; SEM, standard error of the mean; and TSS, transcriptional start site.

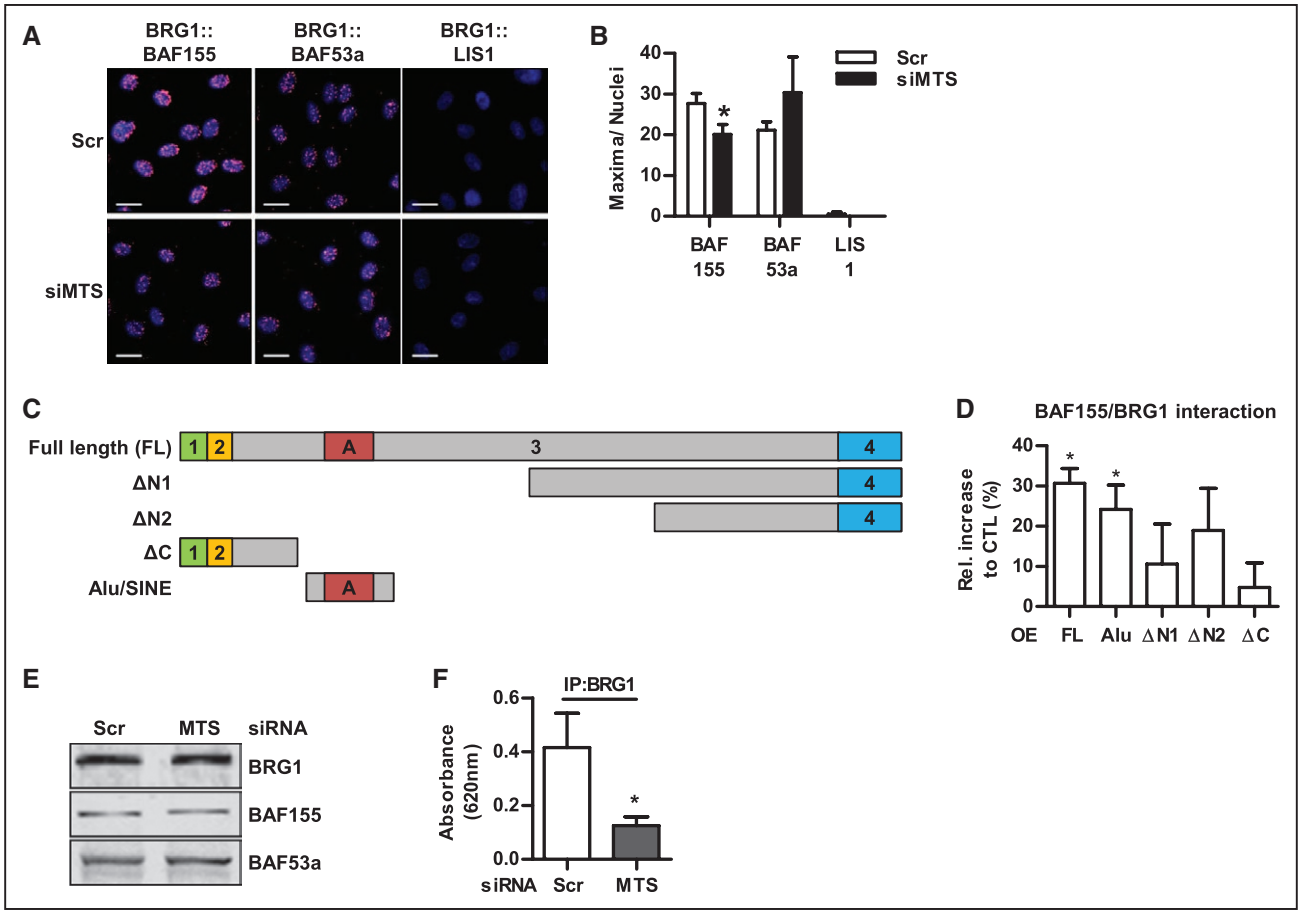

Figure 6. MANTIS improves ATPase activity of BRG1 by stabilizing its interaction with BAF155.
A, Proximity ligation assay (PLA) of HUVECs transfected with scrambled (Scr) or MANTIS siRNA-1 (siMTS) for BRG1 with BAF155, BAF53a, or LIS1. LIS1 served as negative control. Red dots indicate polymerase-amplified interaction signals. Scale bar indicates 20 μm. **B**, Quantifications of PLA shown in **A**. n=3, Unpaired *t* test. Maxima indicate number of dots originating from polymerase-amplified interaction signal. **C**, Scheme of different MANTIS mutants used in **D**. Numbers indicate Exon number; A, Alu element. **D**, Relative increase of BRG1/BAF155 interaction from a PLA after overexpression of MANTIS mutants. n=6, Unpaired *t* test. **E**, Representative Western blot of HUVECs treated either with scrambled (Scr) or with MANTIS siRNA-1 (MTS) for 48 hours for BRG1, BAF155, and BAF53a. **F**, ATPase activity assay (absorbance at 620 nm) after BRG1 immunoprecipitation of HUVECs transfected with scrambled (Scr) or MANTIS siRNA-1 (MTS) for 48 hours. n=5, Paired *t* test. Error bars are defined as mean ± SEM. **P*<0.05. HUVEC indicates human umbilical vein endothelial cell.

boring gene expression by influencing RNA stability or local epigenetic processes, transacting lncRNAs typically function in miRNA sponging or regulating epigenetics in the form of scaffolding epigenetic complexes.[11,29] The involvement of the transacting lncRNA MANTIS in ATPase activity modulation therefore represents a novel and essential epigenetic regulatory mechanism in endothelial cells.

Expression of *Brg1* in *Tie2*+ cells is critical for mouse embryonic development as yolk sac–derived blood cells from *Brg1*$^{fl/fl}$*:Tie2-Cre*$^{+/0}$ embryos underwent apoptosis at embryonic day 9.5.[30] Moreover, after knockout, the yolk sac vessels exhibited failure to interconnect, which lead to dead-end vascular termini reflecting that sprouting or pruning progression failed. This supports the potential in vivo function of the proangiogenic lncRNA MANTIS. The endothelial BRG1 knockout vascular morphology could be rescued by LiCl treatment,[31] highlighting, on the one hand, the stabilization of the Wnt signaling molecule β-catenin, but, on the other hand, the importance of RNA.[32] Also the BAF155 homologue *Srg3*$^{-/-}$*Tg*$^{+}$ mouse embryo yolk sacs showed poorly developed vasculatures accompanied by reduced expression of many angiogenesis-related genes.[33] This strengthens our hypothesis that the BRG1-BAF155 axis is required for angiogenesis-related gene expression. In this axis, our data suggest that MANTIS supports this interaction. We furthermore identified MANTIS lncRNA as dependent on the histone demethylase JARID1B. JARID1B, which is known to have a great overlap on target genes with polycomb proteins in embryonic stem cells, has been shown to function in the control of developmental processes like retina and eye development, neural differentiation, and respiratory failure.[4,34] Devel-

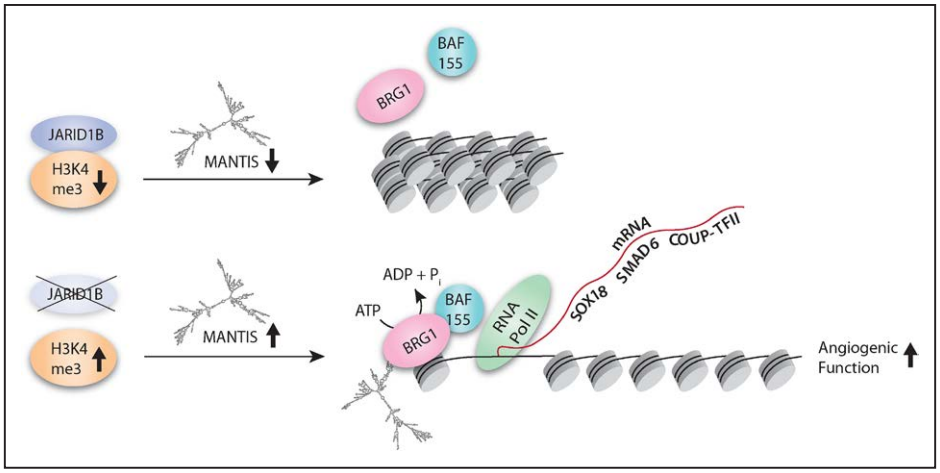

Figure 7. Model of mechanism of action for the lncRNA MANTIS.
Upper, MANTIS lncRNA expression is controlled by the histone demethylase JARID1B. Because of the limited expression of MANTIS, BRG1 and BAF155 assembly is decreased, leading to more heterochromatin formation at the TSS of SOX18, SMAD6, and COUP-TFII, limiting RNA Pol II binding and transcription of those genes. **Lower,** In case of JARID1B knockdown, H3K4me3 levels arise at the TSS of MANTIS, allowing more MANTIS expression. MANTIS interacts with BRG1, allowing increased binding of BAF155, which leads to a higher ATPase activity of BRG1 and euchromatin formation at the TSS of SOX18, SMAD6, and COUP-TFII allowing RNA Pol II binding and thereby transcription of SOX18, SMAD6, and COUP-TFII, which leads to increased angiogenic function. lncRNA indicates long noncoding RNA; RNA Pol II, RNA polymerase II; and TSS, transcriptional start site.

opmental importance was also shown for the knockout mice of the PRC2 components Suz12[9] and the endothelium-specific deletion of Jarid2, which led to cardiac defects.[35] MANTIS could serve here as a stimulus of BRG1 function to compensate the loss of JARID1B.

We identify the endothelial genes *SOX18*, *SMAD6*, and *COUP-TFII* as targets of MANTIS. These genes are known to be important in angiogenesis,[23,36,37] eg, (1) SOX18 induced adipose-derived stromal cells with an endothelial phenotype involved in vascular patterning,[36] (2) *SMAD6* gene knockout led to cardiovascular defects,[38] and (3) deletion of *COUP-TFII* was embryonically lethal and caused impaired angiogenesis, abnormal heart development, and aberrant formations of the vasculature.[39] We explored how MANTIS collectively regulated these angiogenesis-related genes. BRG1 is known to be a critical regulator of *COUP-TFII* expression in the cardiovascular system.[10,20] Our data confirm this finding, but also demonstrate that BRG1 acts on *SOX18* and *SMAD6*. MANTIS directly interacts with BRG1, and increases its ATPase activity by promoting BAF155 interaction. Genome-wide effects could be identified by endothelial ATAC-Seq. Thus, MANTIS appears to keep the SWI/SNF complex intact and preserve the catalytic activity of BRG1. Indeed, loss of BRG1 binding on target gene promoters consistently correlated with a decrease of mRNA expression of these target genes after MANTIS knockdown. Even in the rat MCT model, where the putative homologous MANTIS in the form of its regulatory SINE B1 was reduced, the expression of these target genes tended to be reduced. Together with endothelial-specific deletion of BRG1 leading to amelioration of pulmonary hypertension,[40] this finding suggests a critical role of the MANTIS-BRG1 axis in endothelial dysfunction, and offers a potential therapeutic option for pulmonary hypertension and glioblastoma. In case of pulmonary hypertension, several structural changes in the pulmonary arteries (loss of the distal pulmonary vasculature (vascular pruning), development of neointima and plexiform lesions, and remodeling of the distal pulmonary arteries contribute to the development and progression. Moreover, Masri et al[41] demonstrated that cells from the IPAH patients are impaired in their ability to form tube-like structures in culture, and this may be responsible for their inability to reconstitute the lost distal pulmonary vascular bed. This phenotype copies the results of MANTIS knockdown in endothelial cells. Quite the opposite situation is present in glioblastoma. In the endothelium of these tumors, expression of MANTIS is high, and a highly angiogenic situation is present in glioblastoma. Vessels show defective endothelium and abnormal morphology by, eg, dilatation, disorganization, and high permeability, which is a consequence of high levels of vascular endothelial growth factor A.[42,43] On this basis, it is attractive to speculate that MANTIS could be exploited to alter angiogenesis in patients.

Upregulation of MANTIS in Macaca aortae in the regression phase after atherosclerotic diet could imply an involvement of this lncRNA in vascular regeneration. Because MANTIS depletion affected several angiogenesis-relevant genes, it is unclear whether other target genes from the microarray experiment are directly dependent on MANTIS and the SWI/SNF complex.

The interaction between BRG1 and BAF155 has been demonstrated to increase ATPase activity of BRG1.[28] BAF155, however, was not identified as an interaction partner of MANTIS. Therefore, we speculate that MANTIS enhances the affinity of BRG1 to bind to BAF155 through binding to another so-far-unknown BAF155 recruiting factor. Such a protein could be GPATCH4, whose G-Patch domain is known to mediate RNA-protein interactions. Another possibility involves structural changes of BRG1 after MANTIS binding to enhance BAF155 binding affinity or to allow amino acid modifications. These could in turn enhance BRG1s affinity to bind to BAF155, possibly by the action of the MANTIS Alu element. In respect to BRG1 ATPase function, it has been reported that the lncRNA *EVF2* directly inhibits the ATPase and chromatin-remodeling activity of BRG1.[44] A similar inhibiting function on BRG1 could be found for the lncRNA Myheart (Mhrt). Mhrt was identified to inhibit myopathy and chromatin remodelers.[45] In ventricles of mouse hearts, Mhrt interacts with the helicase domain of BRG1, thereby repressing chromatin target recognition of BRG1. It is notable that BRG1 itself represses the expression of Mhrt. Although EVF2 and Mhrt were predominantly studied in mice, and assuming that their function is conserved and that gene expression of them is given to a certain extent in the individual cells, they could represent counterparts to the BRG1 promoting lncRNA MANTIS. However, one can speculate that the SWI/SNF complex assembly is different from cell type to cell type. The current report of a facilitation of ATPase function is, however, novel. MANTIS appears to mediate this effect through a scaffolding function in SWI/SNF.

Taken together, these findings suggest that MANTIS lncRNA plays a significant and unique role for endothelial cell function by acting as a scaffolding lncRNA within a chromatin-remodeling complex, mediating and directing efficient key endothelial gene transcription.

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DISCLOSURES

None.

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FOOTNOTES

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