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MRTF-A steers an epigenetic complex to activate endothelin-induced pro-inflammatory transcription in vascular smooth muscle cells

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ABSTRACT

Endothelin (ET-1) was initially identified as a potent vasoconstrictor contributing to the maintenance of vascular rhythm. Later studies have implicated ET-1, when aberrantly up-regulated within the vasculature, in a range of human pathologies associated with disruption of vascular homeostasis. ET-1 has been shown to invoke strong pro-inflammatory response in vascular smooth muscle cells (VSMCs); the underlying mechanism, however, remains elusive. Here, we report that the transcriptional modulator MRTF-A mediates the activation of pro-inflammatory mediators by ET-1 in VSMCs. ET-1 increased nuclear enrichment and activity of MRTF-A in cultured VSMCs. MRTF-A silencing attenuated ET-1 induced synthesis and release of pro-inflammatory mediators including IL-6, MCP-1 and IL-1 likely as a result of diminished NF-κB activity. In addition, MRTF-A was indispensible for the accumulation of active histone modifications on the gene promoters. Of intrigue, MRTF-A interacted with and recruited ASH2, a component of the mammalian histone methyltransferase complex, to transactivate pro-inflammatory genes in response to ET-1 treatment. The chromatin remodeling proteins BRG1 and BRM were also required for ET-1-dependent induction of pro-inflammatory mediators by communicating with ASH2, a process dependent on MRTF-A. In conclusion, our data have identified a novel epigenetic complex responsible for vascular inflammation inflicted by ET-1.

INTRODUCTION

Vascular homeostasis is maintained by a dynamic interplay between humoral factors and vascular cells. Endothelin (ET-1) is a vasoactive substance that potently triggers constriction of vessels playing an essential role in guarding the integrity of the vasculature (1). However, elevated ET-1 levels are synonymous with a diverse range of cardiovascular diseases including atherosclerosis, cardiac hypertrophy and pulmonary hypertension by inflicting injuries to the vascular cells (2–5). Among the detrimental effects exerted by ET-1 on the vasculature are excessive accumulation of reactive oxygen species (6), promotion of vascular fibrosis (7), micro-vascular leakage (8) and chronic inflammation (9).

Chronic inflammation is considered a major culprit for the disruption of vascular homeostasis. ET-1 blockade with selective receptor antagonists has been demonstrated to alleviate vascular inflammation in animal models and in humans (10,11). When exposed to endothelin, vascular smooth muscle cells (VSMCs) up-regulate the production of a series of pro-inflammatory mediators including interleukin 6 (IL-6) (12), MCP-1 (13) and IL-1 (14). The transcription factor NF-κB is believed to be responsible for the transcriptional activation of pro-inflammatory genes by ET-1 as nuclear factor kappa B (NF-κB) inhibition prevented ET-1-induced vascular inflammation (15). An intriguing mystery remains unanswered regarding NF-κB-dependent pro-inflammatory transcription is the mobilization of the epigenetic machinery. During transcriptional activation, chromatin undergoes active remodeling characterized by the movement of nucleosomes and the alteration of histone modifications. Typically, transcriptionally active chromatin is marked with the enrichment of acetylated histones H3 and H4 and methylated lysine 4 of histone H3 (H3K4) (16). For instance, Wu *et al.* have recently reported that the H3K4 methyltransferase MLL1 is required for tu-

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mour necrosis factor alpha (TNF-α) stimulated transcription downstream of NF-κB by promoting tri-methylation of H3K4 (17).

Our previous investigation has implicated myocardin-related transcription factor A, or MRTF-A, as a *de novo* co-factor for NF-κB functioning to orchestrate signature histone modifications on the promoter region of ICAM-1 gene, an NF-κB target, in endothelial cells (18). MRTF-A is known to coordinate various components of the epigenetic machinery to modulate transcription (19–21). Thus, a tempting hypothesis would be that MRTF-A participates in ET-1-induced transactivation of pro-inflammatory genes in VSMCs by bridging the epigenetic machinery to NF-κB. Our data as presented here place MRTF-A at the center of an epigenetic complex that mediates endothelin-induced pro-inflammatory transcription and as such shed new light on the development of interventional strategies against ET-1-induced pathologies.

MATERIALS AND METHODS

Cell culture

Rat VSMCs (A10, ATCC) and human adrenal carcinoma cells (SW13, ATCC) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). Human primary aortic smooth muscle cells (HASMC) were maintained in specialized medium supplied by the vendor (Lifeline Cell Technology, Frederick, MD, USA). Mouse embryonic fibroblast (MEF) cells were isolated from wild type (WT) or MRTF-A knockout mice (22). Recombinant endothelin-1 (ET-1) was purchased from Sigma.

Plasmids, transient transfection and luciferase assay

Promoter constructs for IL-1, IL-6, MCP-1 and TNF-α (23), the κB reporter (24) and expression constructs for MRTF-A (25), ASH2 (26), BRG1 and BRM (27) have been previously described. Small interfering RNA (siRNA) for MRTF-A, BRG1 and BRM have been described (18,23). siRNA for human ASH2, GCCUGGUAUUUUGAAAUCA and for rat Ash2, CCCTAGCAGATCCATGCTT. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) essentially as previously described (28). Luciferase activities were assayed 24–48 h after transfection using a luciferase reporter assay system (Promega). Experiments were repeated at least three times.

RNA isolation and real-time polymerase chain reaction (PCR)

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen). Real-time PCR reactions were performed on an ABI Prism 7500 system. Primers and Taqman probes were purchased from Applied Biosystems. Experiments were repeated at least three times.

Protein extraction, immunoprecipitation and western blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor tablet (Roche). Nuclear proteins were prepared with the NE-PER Kit (Pierce) following manufacturer's recommendation. Specific antibodies or pre-immune immunoglobulin Gs (IgGs) (P.I.I.) were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complex was released by boiling with 1× sodium dodecyl sulphate electrophoresis sample buffer. Western blot analyses were performed with anti-α-tubulin (Millipore), anti-MRTF-A, anti-Lamin B (Santa Cruz) and anti-ASH2 (Bethyl Laboratories) antibodies.

Immunofluorescence microscopy

Cells were fixed with 4% formaldehyde, blocked with 5% bovine serum albumin and incubated with primary antibodies overnight. After several washes with phosphate buffered saline, cells were incubated with FITC-labeled secondary antibodies (Jackson) for 30 min. DAPI (Sigma) was added and incubated with cells for 5 min prior to observation. Immunofluorescence was visualized on a co-focal microscope (LSM 710, Zeiss). For quantification, cells in triplicate wells from three independent experiments were counted for each condition; for each well, up to 10 fields with ∼5 cells/field were counted.

Enzyme-linked immune absorbance assay (ELISA)

ELISA was performed using supernatant collected from cultured vascular endothelial cells or rat pulmonary artery homogenates according to manufacturer's instructions (USCN Life Sciences).

Chromatin immunoprecipitation (ChIP) and Re-ChIP

ChIP and Re-ChIP assays were performed essentially as described before (29). Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-MRTF-A (Santa Cruz, SC-32909), anti-BRG1 (Santa Cruz, SC-10768), anti-BRM (Santa Cruz, SC-6450), anti-acetyl H3 (Millipore, 06–599), anti-acetyl H4 (Millipore, 06-598), anti-dimethyl (Millipore, 07-030), anti-trimethyl H3K4 (Millipore, 07-473), anti-ASH2 (Bethyl Laboratories, A300-489A) or pre-immune IgG. Precipitated genomic DNA was amplified by real-time PCR with primers listed in Supplementary Table S1.

Statistical analysis

One-way ANOVA with post hoc Scheffe analyses were performed using an SPSS package. *P*-values smaller than 0.05 were considered statistically significant and designated *.

RESULTS

MRTF-A is essential for ET-1 induced transcription of pro-inflammatory mediators in VSMCs

To examine whether MRTF-A plays a role in ET-1-induced transcription of pro-inflammatory genes in VSMCs, we first transfected promoter-luciferase fusion constructs for IL-6, IL-1, MCP-1 and TNF-α into a rat VSMC line (A10) with or without MRTF-A. As shown in Figure 1A, MRTF-A by itself up-regulated the promoter activities of pro-inflammatory genes; more importantly, MRTF-A enhanced the activation by ET-1. To verify whether the same observation would hold true for NF-κB-dependent transcription in general, we transfected a reporter construct driven by two copies of the consensus κB recognition element into A10 cells (Figure 1B); overexpression of MRTF-A significantly potentiated NF-κB-dependent transcription in the presence of ET-1. In support of this observation, ET-1 was less potent in stimulating NF-κB activity in MRTF-A deficient (KO) MEF cells than in WT MEF cells (Supplementary Figure S1A and B).

On the other hand, silencing of MRTF-A by siRNA in A10 (Figure 1C; Supplementary Figure S1C for knockdown efficiency) and HASMC (Figure 1D; Supplementary Figure S1D for knockdown efficiency) blocked the induction of pro-inflamamtory mediator messages. As a control, depletion of MRTF-A did not alter the expression of endothelin receptors (ETA/ETB, Supplementary Figure S1E). ELISA assay confirmed that MRTF-A knockdown also down-regulated protein levels of pro-inflammatory mediators in HASMCs (Figure 1E). Again, less Il-6 and Mcp-1 were synthesized in KO MEF cells in the presence of ET-1 than in WT MEF cells (Supplementary Figure S1F).

Having established that MRTF-A could indeed participate in ET-1-induced pro-inflammatory transcription, we next probed the potential effect of ET-1 on MRTF-A in VSMCs. Treatment of ET-1 led to a rapid accumulation of MRTF-A in the nucleus as evidenced by Western (Figure 2A) and immunofluorescence staining (Figure 2B). In addition, ET-1 promoted the binding of MRTF-A on the promoters, but not introns, of pro-inflammatory genes (Figure 2C). Finally, there was a significant increase in the formation of a MRTF-A/NF-κB complex on the gene promoters, but not on the intronic regions, in the presence of ET-1 (Figure 2D). Two additional lines of evidence support our model that MRTF-A activates the transcription of pro-inflammatory mediators in response to ET-1 stimulation through NF-κB. First, overexpression of a constitutively active form of IκB (IκB SR) completely blocked the transactivation by MRTF-A (Supplementary Figure S2A). Furthermore, mutation of the κB response element in the promoter constructs also abrogated MRTF-A-induced transactivation (Supplementary Figure S2B). Collectively, these data establish a model in which ET-1 augments the activity of MRTF-A, which in turn mediates the transactivation of pro-inflammatory genes in VSMCs.

MRTF-A is responsible for key histone modifications

One of the mechanisms by which MRTF-A activates transcription is the alterations of histone modifications via interaction and recruitment of epigenetic factors (18,19,21,25). Therefore, we checked to see whether MRTF-A could influence the chromatin structure surrounding the promoter regions of the pro-inflammatory mediator genes. ET-1 treatment triggered an increase in the levels of histone H3 acetylation (AcH3), H4 acetylation (AcH4), histone H3 lysine 4 dimethylation (H3K4Me2) and trimethylation (H3K4Me3) on the promoter regions of IL-6, IL-1 and MCP-1 (Figure 3A–D). RNAi-mediated knockdown of MRTF-A attenuated the accumulation of these histone marks on all three promoters. In keeping with these observations, MRTF-A KO MEF cells treated with ET-1 exhibited less prominent enrichment of active histone marks on the promoters of pro-inflammatory genes than WT MEF cells (Supplementary Figure S3A–D). Thus, MRTF-A is responsible for the deposition of modified histones synonymous with transcriptional activation in response to ET-1 treatment in VSMCs.

MRTF-A recruits ASH2 to the promoter regions of the pro-inflammatory mediator genes

In eukaryotes, H3K4 methylation is catalyzed by a conserved protein aggregate called COMPASS/COMPASS-like complex (30). Through proteomic screening, we identified ASH2, a key component of the COMPASS/COMPASS-like complex, as a novel binding partner for MRTF-A (Fang and Xu, manuscript under consideration elsewhere). Immunoprecipitation assay using HASMC whole cell lysates confirmed the interaction between MRTF-A and ASH2 (Figure 4A). Similar to MRTF-A, binding of ASH2 to the promoter regions of pro-inflammatory mediators was also stimulated by ET-1 (Figure 4B). Of key significance, ET-1 promoted the interaction between ASH2 and MRTF-A on the gene promoters (Figure 4C). ASH2 binding was abolished in the absence of MRTF-A (Figure 4D), confirming the essential role for MRTF-A in recruiting ASH2.

Next, we assessed the functional relevance of ASH2 in ET-1-induced pro-inflamamtory transcription. Overexpression of ASH2 synergized with MRTF-A (Figure 5A, Supplementary Figure S4A) and ET-1 (Figure 5B, Supplementary Figure S4B) to activate transcription in reporter assays, indicating that ASH2 may play a role in NF-κB-dependent transcription in cells. In contrast, ASH2 elimination by RNAi (Supplementary Figure S4C and D for validation) dampened the synthesis of pro-inflammatory mediators in A10 cells (Figure 5C) and HASMCs (Figure 5D and E). Together, these data suggest that MRTF-A mediates ET-1-induced pro-inflammatory transcription in VSMCs by enlisting ASH2.

BRG1 and BRM are indispensible for ET-1-induced transcription of pro-inflammatory mediators in VSMCs

BRG1 and BRM have been shown to facilitate MRTF-A to activate contractile genes in VSMCs (20) and the ET-1 gene in endothelial cells (25). We asked whether BRG1/BRM could be part of the MRTF-A-centered epigenetic complex that contributes to ET-1-induced transcription of pro-inflammatory mediators in VSMCs. Indeed, BRG1 and

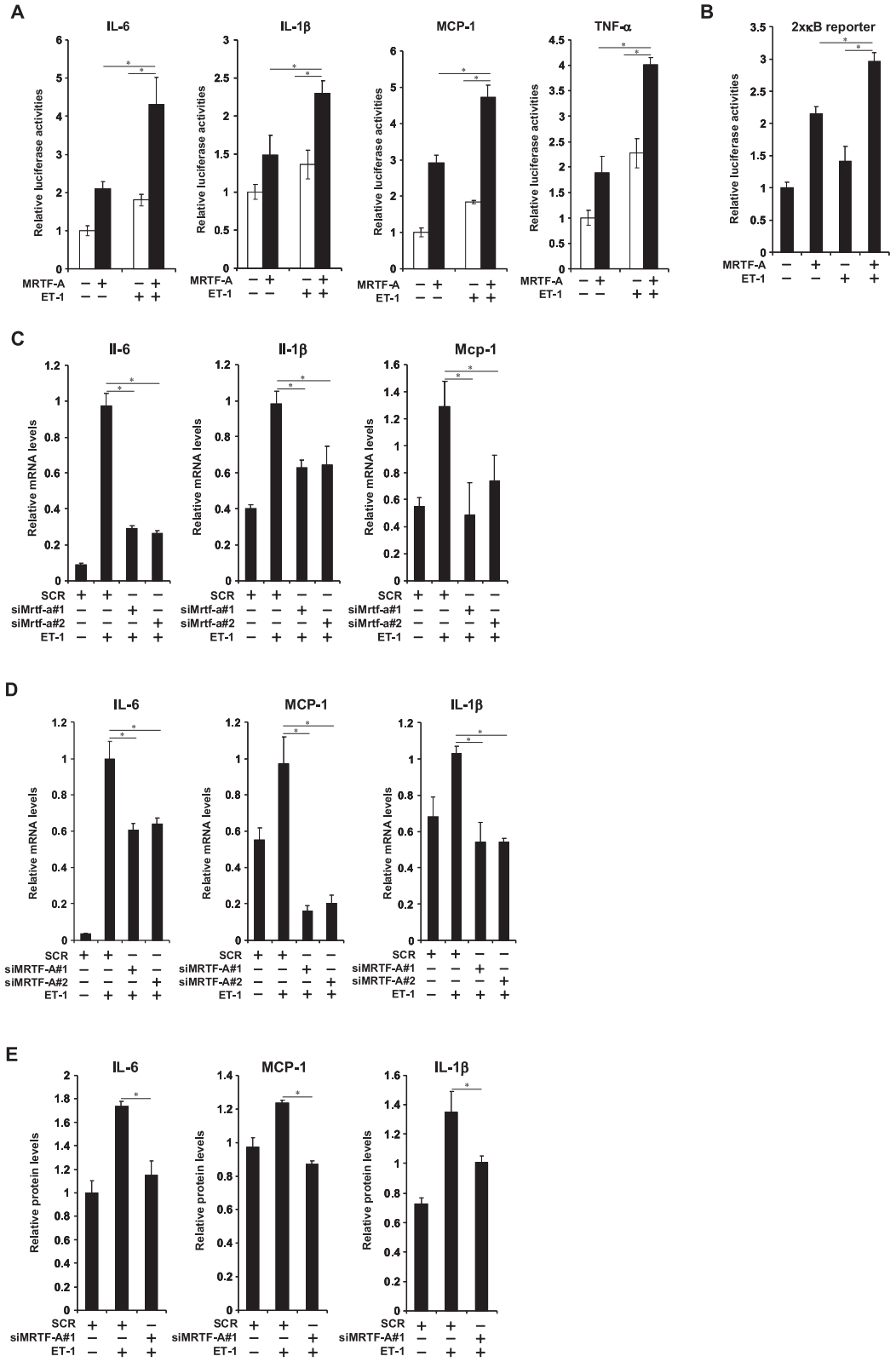

Figure 1. MRTF-A is essential for ET-1-induced transcription of pro-inflammatory mediators in VSMCs. (**A**) MRTF-A is co-transfected with indicated reporter constructs into A10 cells followed by treatment with ET-1 (1 nM). Data are expressed as relative luciferase activities. (**B**) MRTF-A is co-transfected with an NF-κB reporter construct into A10 cells followed by treatment with ET-1 (1 nM). Data are expressed as relative luciferase activities. (**C**) A10 cells were transfected with scrambled sequence (SCR) or siRNA targeting MRTF-A (siMrtf-a) followed by treatment with ET-1 (1 nM). Expression of pro-inflammatory mediators was measured by qPCR. (**D** and **E**) HASMCs were transfected with SCR or siMRTF-A followed by treatment with ET-1 (1 nM). mRNA (D) and protein (E) levels of pro-inflammatory mediators were measured by qPCR and ELISA. All experiments were repeated at least three times. *, $P < .05$.

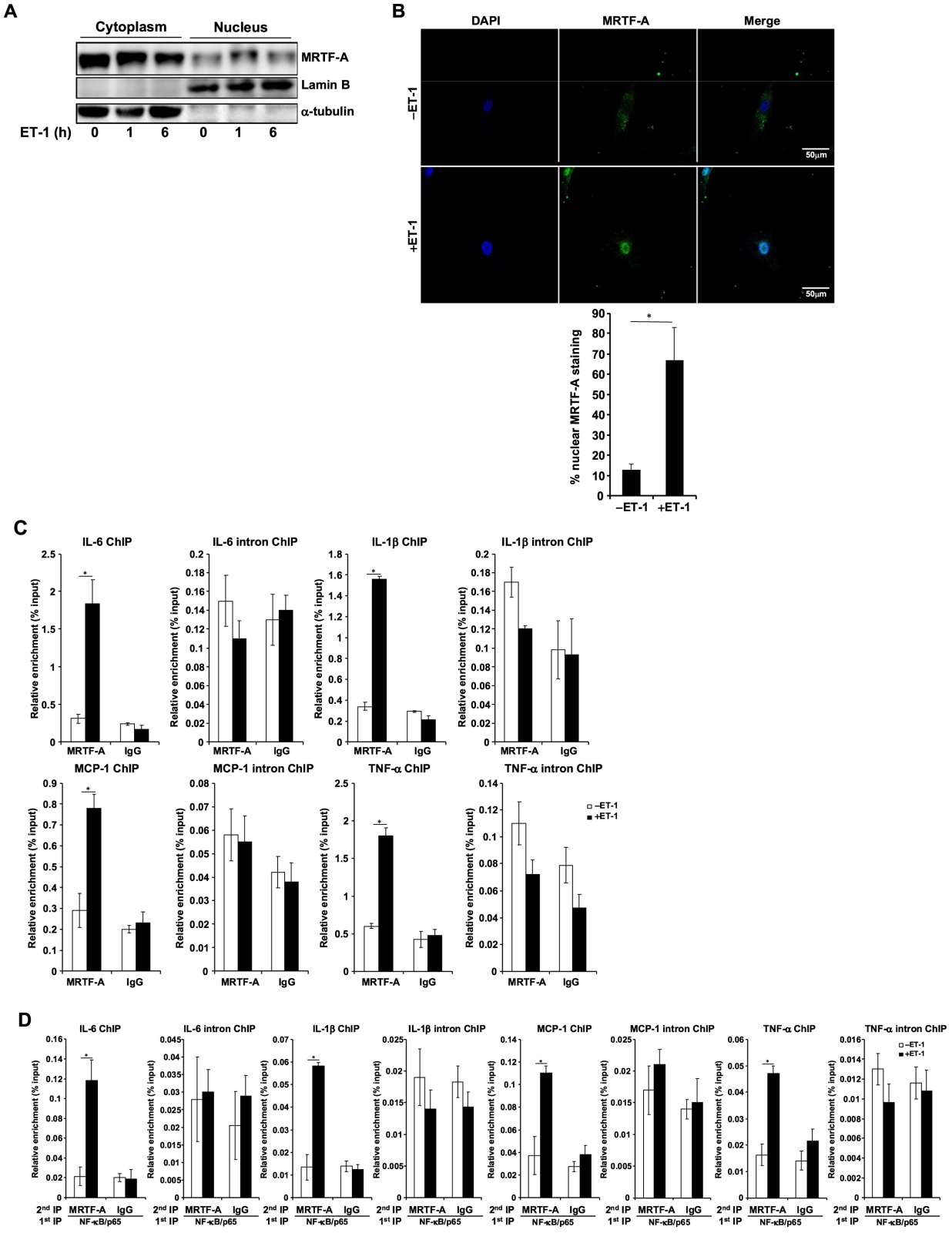

Figure 2. ET-1 activates MRTF-A in VSMCs. (**A**) A10 cells were treated with ET-1, harvested at indicated time points and fractionated. Cytoplasmic and nuclear MRTF-A were probed by Western. (**B**) HASMCs were treated with or without ET-1 for 3 h. Subcellular MRTF-A was visualized by immunofluorescence staining. The nuclei were counterstained with DAPI. Nuclear MRTF-A staining was quantified by Image J and expressed as percentage of overall MRTF-A staining. (**C**) HASMCs were treated with or without ET-1 (1 nM). ChIP assays were performed with anti-MRTF-A or IgG. (**D**) HASMCs were treated with ET-1 (1 nM) and harvested at different time points. Re-ChIP assays were performed with indicated antibodies. All experiments were repeated at least three times. *, $P < .05$.

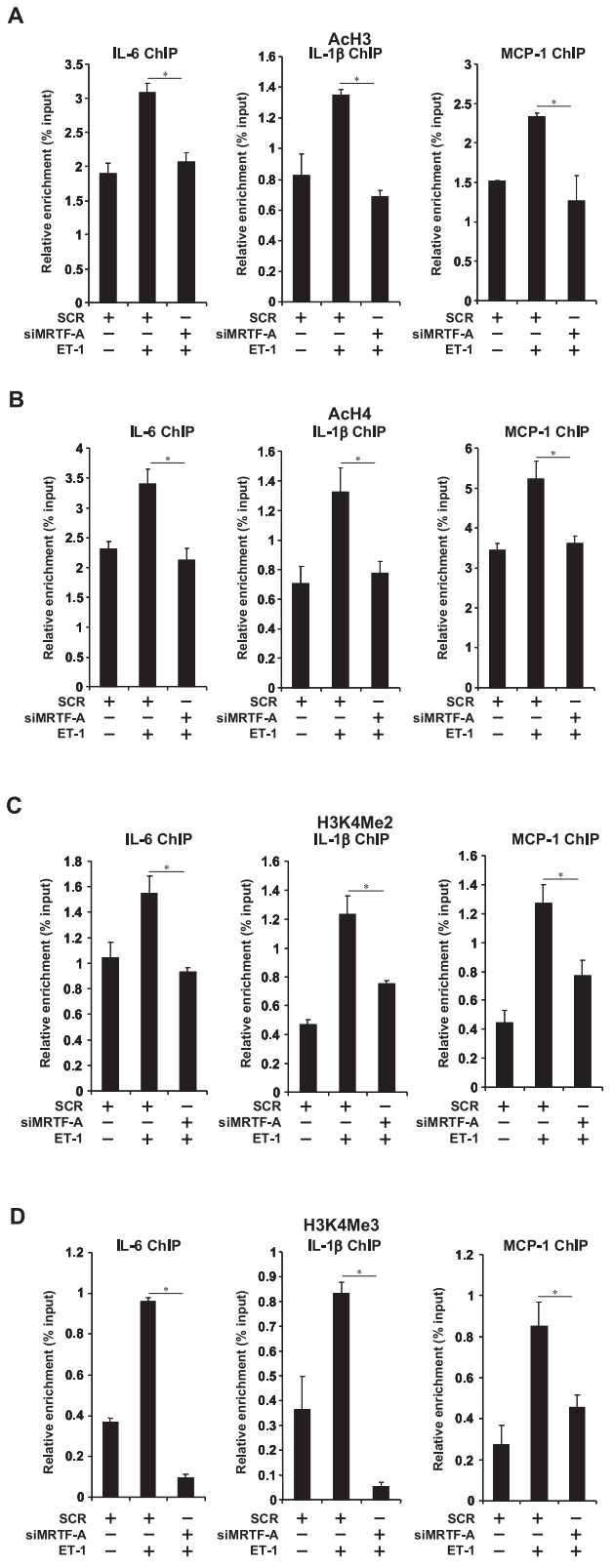

Figure 3. MRTF-A is responsible for key histone modifications surrounding the promoter regions of the pro-inflammatory mediator genes. HASMCs were transfected with SCR or siMRTF-A followed by treatment with ET-1 (1 nM). ChIP assays were performed with anti-acetyl H3 (**A**), anti-acetyl H4 (**B**), anti-H3K4Me2 (**C**) and anti-H3K4Me3 (**D**). All experiments were repeated at least three times. *, $P < .05$.

BRM significantly enhanced promoter activities of NF-κB target genes in the presence MRTF-A and ET-1 in reporter assays (Figure 6A and B, Supplementary Figure S5A and B). A BRG1/MRTF-A complex and a BRM/MRTF-A complex were readily detectable on the gene promoters in VSMCs treated with ET-1 (Figure 6C). In keeping with a previous observation made on the ET-1 promoter (25), the occupancies of BRG1 and BRM on the gene promoters were abrogated by MRTF-A silencing (Figure 6D and E). Depletion of BRG1 and/or BRM by siRNA (Supplementary Figure S5C and D for validation) attenuated the induction of pro-inflammatory mediators by ET-1 in HASMCs. Therefore, ET-1 could promote the incorporation of BRG1 and BRM into the MRTF-A-containing activation complex to stimulate pro-inflammatory transcription in VSMCs.

MRTF-A mediates the crosstalk between ASH2 and BRG1/BRM

Numerous previous investigations have demonstrated that BRG1 and BRM could forge a crosstalk with histone modifying enzymes (23,25,27,29,31–33). Silencing of BRG1/BRM suppressed the enrichment of acetylated histones (Supplementary Figure S6A and B) on the promoter regions of pro-inflammatory mediator genes. BRG1/BRM knockdown also reduced the levels of dimethylated H3K4 (Figure 7A) and trimethylated H3K4 (Figure 7B), raising the possibility that BRG1/BRM could communicate with ASH2 in response to ET-1 stimulation. Indeed, BRG1 and BRM were present in the immune complex precipitated by anti-ASH2 antibody (Figure 4A). More importantly, treatment with ET-1 led to the appearance of a BRG1/ASH2 complex (Figure 7C) and a BRM/ASH2 complex (Figure 7D) on the gene promoters. Depletion of BRG1/BRM significantly down-regulated ASH2 occupancy (Figure 7E). Adding support to the physical association between BRG1/BRM and ASH2 on the promoters of pro-inflammatory genes, we showed that in reporter assays, ASH2 failed to enhance ET-1-induced NF-κB activity in a BRG1/BRM-null cell line (SW-13) unless ectopic BRG1 and BRM were introduced (Figure 7F, Supplementary Figure S7A). Silencing BRG1/BRM in ET-1-stimulated HASMCs did not, however, significantly alter the interaction between ASH2 and MRTF-A (Supplementary Figure S7B).

Given that MRTF-A could interact with both ASH2 and BRG1/BRM, we hypothesized that MRTF-A might function as a moderator bridging the interplay between ASH2 and BRG1/BRM. As shown in Figure 8A, the BRG1/ASH2 complex established on the promoters of pro-inflammatory genes in the presence of ET-1 collapsed when MRTF-A was depleted by siRNA. Similarly, the loss of MRTF-A also crippled the formation of a BRM/ASH2 complex in Re-ChIP assays (Figure 8B). Furthermore, the synergy between BRG1/BRM and ASH2 in stimulating NF-κB-dependent transcription in WT MEF cells was lost in MRTF-A KO cells (Figure 8C, Supplementary Figure S8). Collectively, these data suggest that MRTF-A coordinates the communication between BRG1/BRM and ASH2 to mediate ET-1-induced pro-inflammatory transcription in VSMCs.

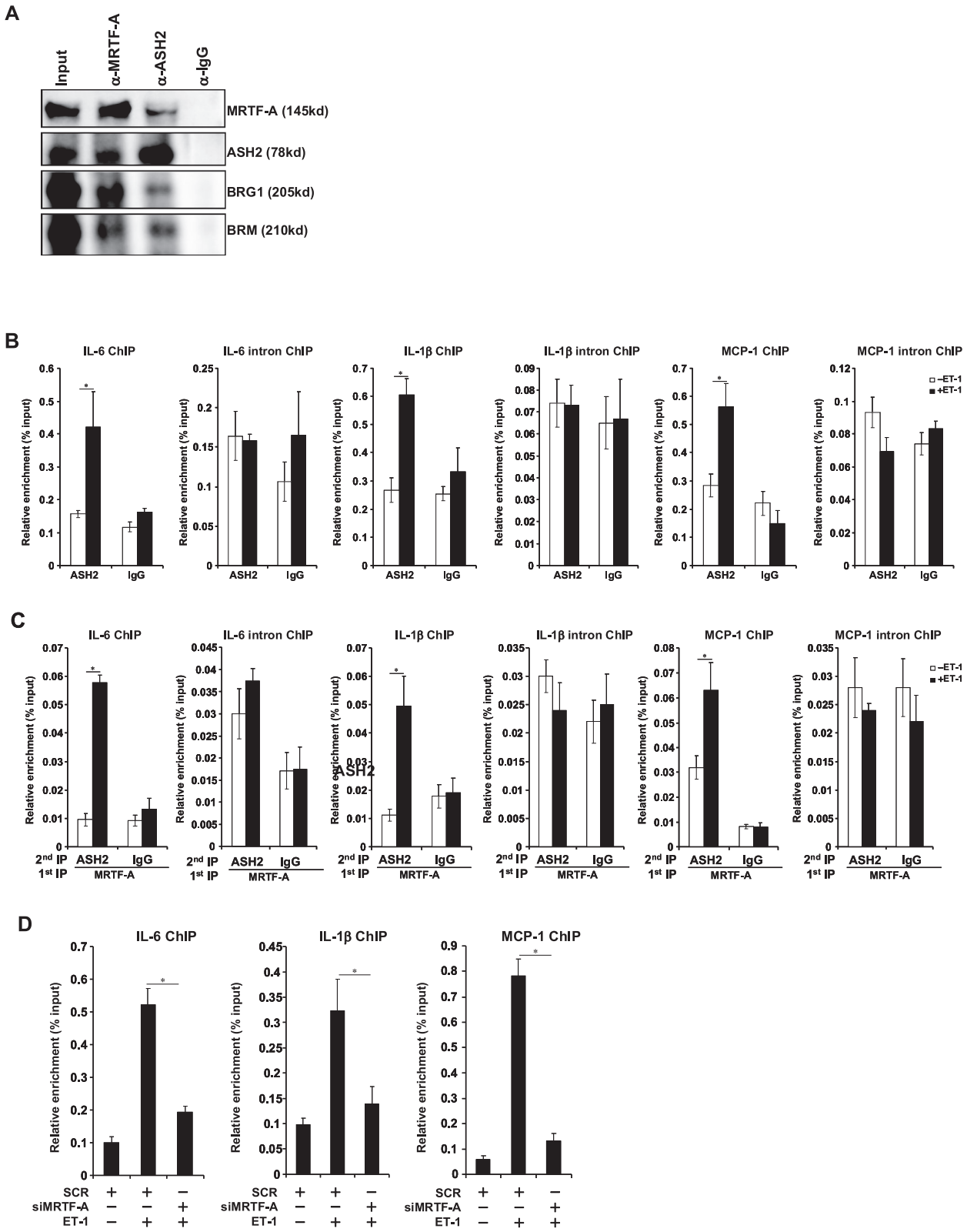

Figure 4. MRTF-A recruits ASH2 to the promoter regions of the pro-inflammatory mediator genes. (**A**) Immunoprecipitation was performed with indicated antibodies using HASMC lysates. (**B** and **C**) HASMCs were treated with or without ET-1 (1 nM). ChIP (B) and Re-ChIP (C) assays were performed with anti-ASH2, anti-MRTF-A or IgG. (**D**) HASMCs were transfected with SCR or siMRTF-A followed by treatment with ET-1 (1 nM). ChIP assays were performed with anti-ASH2. All experiments were repeated at least three times. *, $P < .05$.

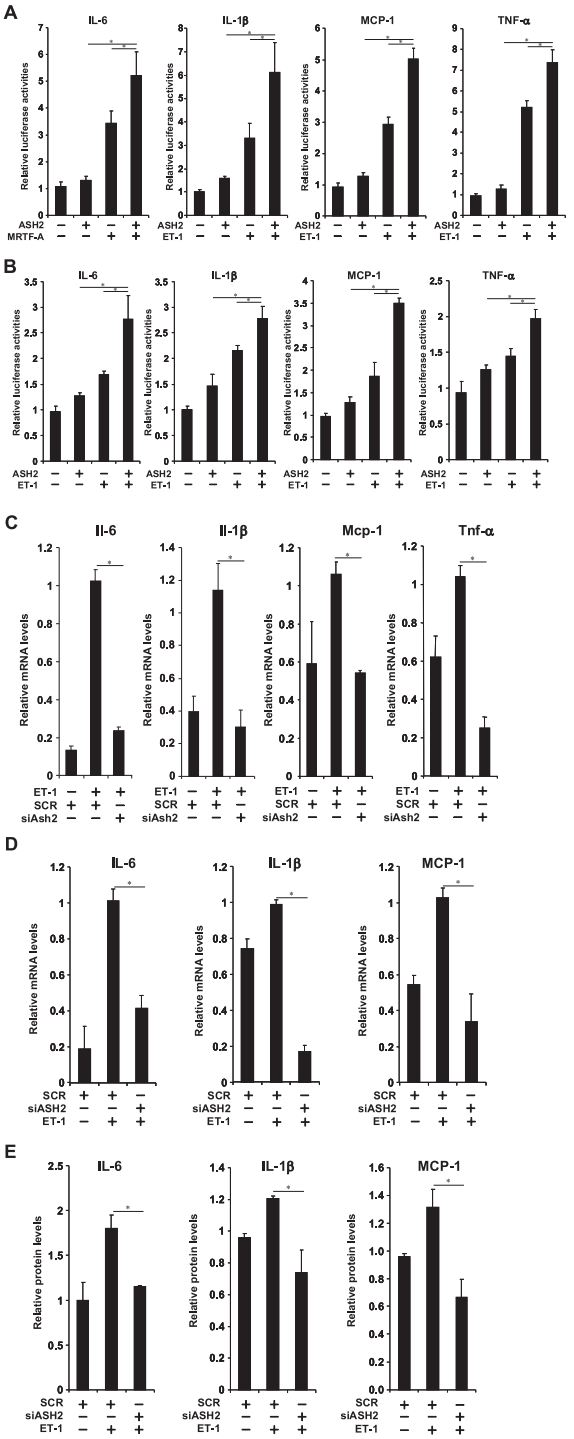

Figure 5. ASH2 is necessary for ET-1-induced transcription of pro-inflammatory mediators in VSMCs. (**A**) A10 cells were transfected with indicated promoter constructs with or without expression construct for ASH2 or MRTF-A. Data are expressed as relative luciferase activities. (**B**) A10 cells were transfected with indicated promoter constructs with or without expression construct for ASH2 followed by treatment with ET-1 (1 nM). Data are expressed as relative luciferase activities. (**C**) A10 cells were transfected with SCR or siAsh2 followed by treatment with ET-1. Expression of pro-inflammatory mediators was measured by qPCR. (**D** and **E**) HASMCs were transfected with SCR or siASH2 followed by treatment with ET-1 (1 nM). mRNA (D) and protein (E) levels of pro-inflammatory mediators were measured by qPCR and ELISA. All experiments were repeated at least three times. *, $P < .05$.

DISCUSSION

MRTF-A was initially identified as a co-factor for SRF driving the transcription of muscle-specific genes (34). While forced expression of MRTF-A in non-muscle cells up-regulate the expression of a panel of SMC signature genes, MRTF-A has been found to be dispensable in the maintenance of SMC phenotype *in vivo* as MRTF-A-null mice exhibit no overt vascular disorder under physiological conditions (35,36). Meanwhile, an emerging consensus seems to indicate that MRTF-A might function as a stress protein coordinating cellular response to injurious signals (37). Here, we report that MRTF-A programs ET-1-induced pro-inflammatory response in VSMCs by committing the epigenetic machinery.

Several recent investigations have implicated histone H3K4 methylation in the regulation of pro-inflammatory transcription (17,38–42). The mammalian H3K4 methyltransferase (COMPASS/COMPASS-like) complex operates in a modular fashion. Except for the enzymatic component, six of which have been identified so far including MLL1, MLL2, MLL3, MLL4, SETD1A and SETD1B, most constituents, such as ASH2, are shared among different complexes (30). Not possessing the SET domain for catalyzing H3K4 methylation, ASH2 nonetheless is essential for calibrating the activity of the COMPASS/COMPASS-like complex and for ensuring proper H3K4 methylation (43). Our data clearly show that when ASH2 is silenced in cells, the induction of several pro-inflammatory mediators by ET-1 is completely gone (Figure 5), whereas earlier reports have demonstrated an attenuation instead of a loss in the expression of these pro-inflammatory genes in MLL1 (17) and MLL4 (39) deficient cells, indicating that ASH2 plays a non-redundant role in programming cellular pro-inflammatory transcription. ChIP-seq in drosophila has established a role for Ash2 in RNA polymerase II pausing (44). Due to the limits of single-gene based analyses as performed in the current report, it remains an open question how ASH2 operates on a genome-wide scale to influence the synthesis of pro-inflammatory mediators in VSMCs.

Consistent with previous reports that BRG1 and BRM are essential for liposaccharide (LPS) (31) and palmitate (23) induced transactivation of pro-inflammatory mediators, BRG1 and BRM are also required for ET-1-induced production of pro-inflammatory cytokines/chemokines (Figure 6). Of intrigue, the activity of BRG1/BRM to modulate transcription seems to at least partly depend on their ability to influence histone modifications and, in particular, to interact with ASH2 (Figure 7). The crosstalk between ASH2 and the histone remodeling proteins creates a defining distinction that can separate BRG1 from BRM. For instance, whereas both BRG1 and BRM regulate IL-6 transactivation, only BRG1 is dispensable for IL-1 transactivation, which is reflected by the observation that both BRG1 and BRM interact with ASH2 on the IL-6 promoter but only the BRG1/ASH2 complex is detectable on the IL-1 promoter. Reciprocally, the capability of ASH2 to participate in ET-1-induced pro-inflammatory transcription demands the availability of BRG1/BRM although BRG1/BRM seemed to be indispensible for the ASH2-MRTF-A interaction (Supplementary Figure S7). There-

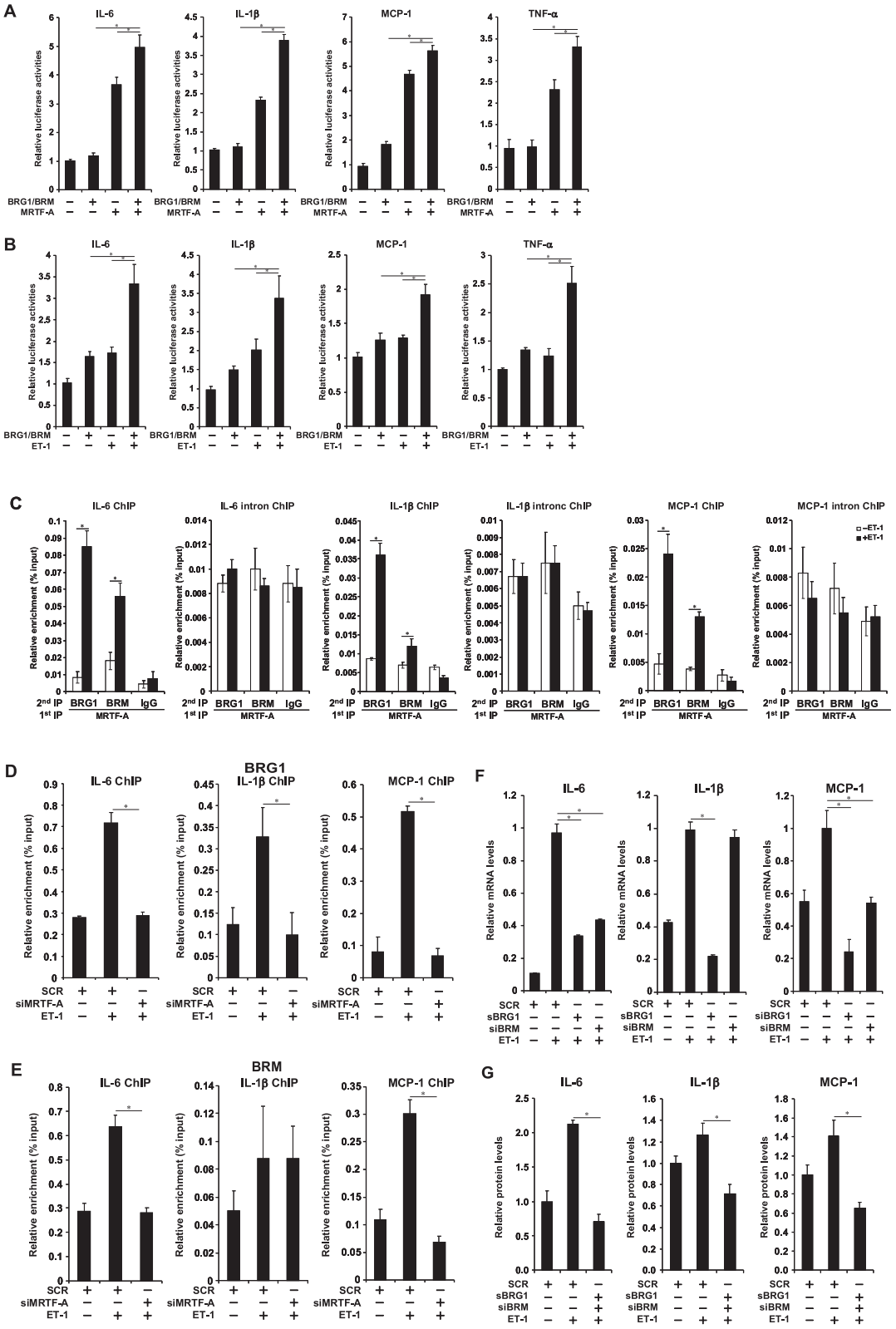

Figure 6. MRTF-A relies on BRG1 and BRM to activate ET-1-induced transcription of pro-inflammatory mediators. (**A**) A10 cells were transfected with indicated promoter constructs with or without expression construct for BRG1/BRM or MRTF-A. Data are expressed as relative luciferase activities. (**B**) A10 cells were transfected with indicated promoter constructs with or without expression construct for BRG1/BRM followed by treatment with ET-1 (1 nM). Data are expressed as relative luciferase activities. (**C**) HASMCs were treated with or without ET-1 (1 nM). Re-ChIP assays were performed with anti-BRG1, anti-BRM, anti-MRTF-A or IgG. (**D** and **E**) HASMCs were transfected with SCR or siMRTF-A followed by treatment with ET-1 (1 nM). ChIP assays were performed with anti-BRG1 (D) or anti-BRM (E). (**F** and **G**) HASMCs were transfected with indicated siRNAs followed by treatment with ET-1 (1 nM). mRNA (F) and protein (G) levels of pro-inflammatory mediators were measured by qPCR and ELISA. All experiments were repeated at least three times. *, $P < .05$.

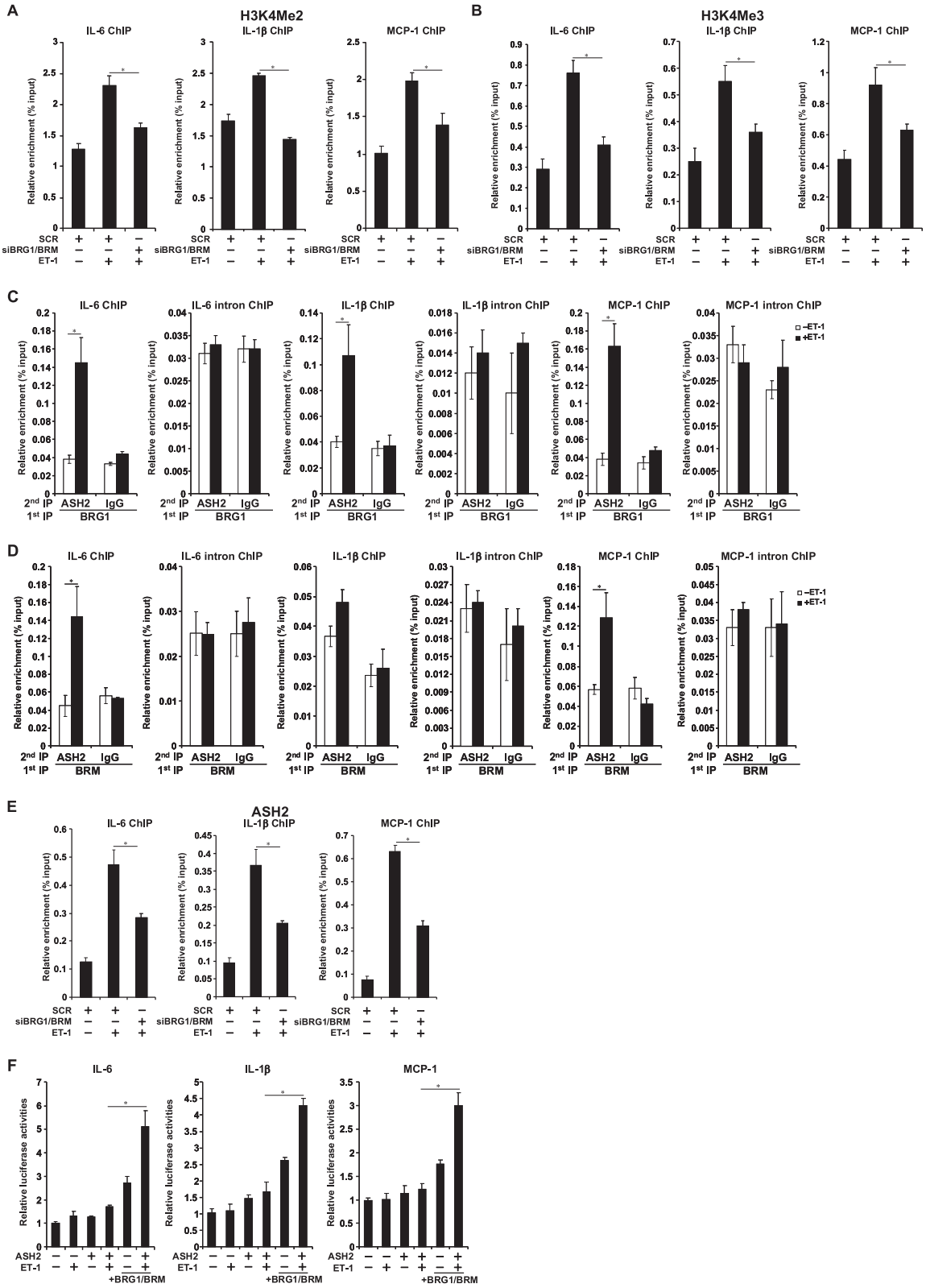

Figure 7. ET-1 promotes the crosstalk between BRG1/BRM and ASH2. (**A** and **B**) HASMCs were transfected with indicated siRNAs followed by treatment with ET-1 (1 nM). ChIP assays were performed with anti-H3K4Me2 (A) or anti-H3K4Me3 (B). (**C** and **D**) HASMCs were treated with or without ET-1 (1 nM). Re-ChIP assays were performed with indicated antibodies. (**E**) HASMCs were transfected with indicated siRNAs followed by treatment with ET-1 (1 nM). ChIP assays were performed with anti-ASH2. (**F**) SW-13 cells were transfected with indicated reporter constructs along with indicated expression constructs followed by treatment with or without ET-1 (1 nM). Data are expressed as relative luciferase activities. All experiments were repeated at least three times. *, $P < .05$.

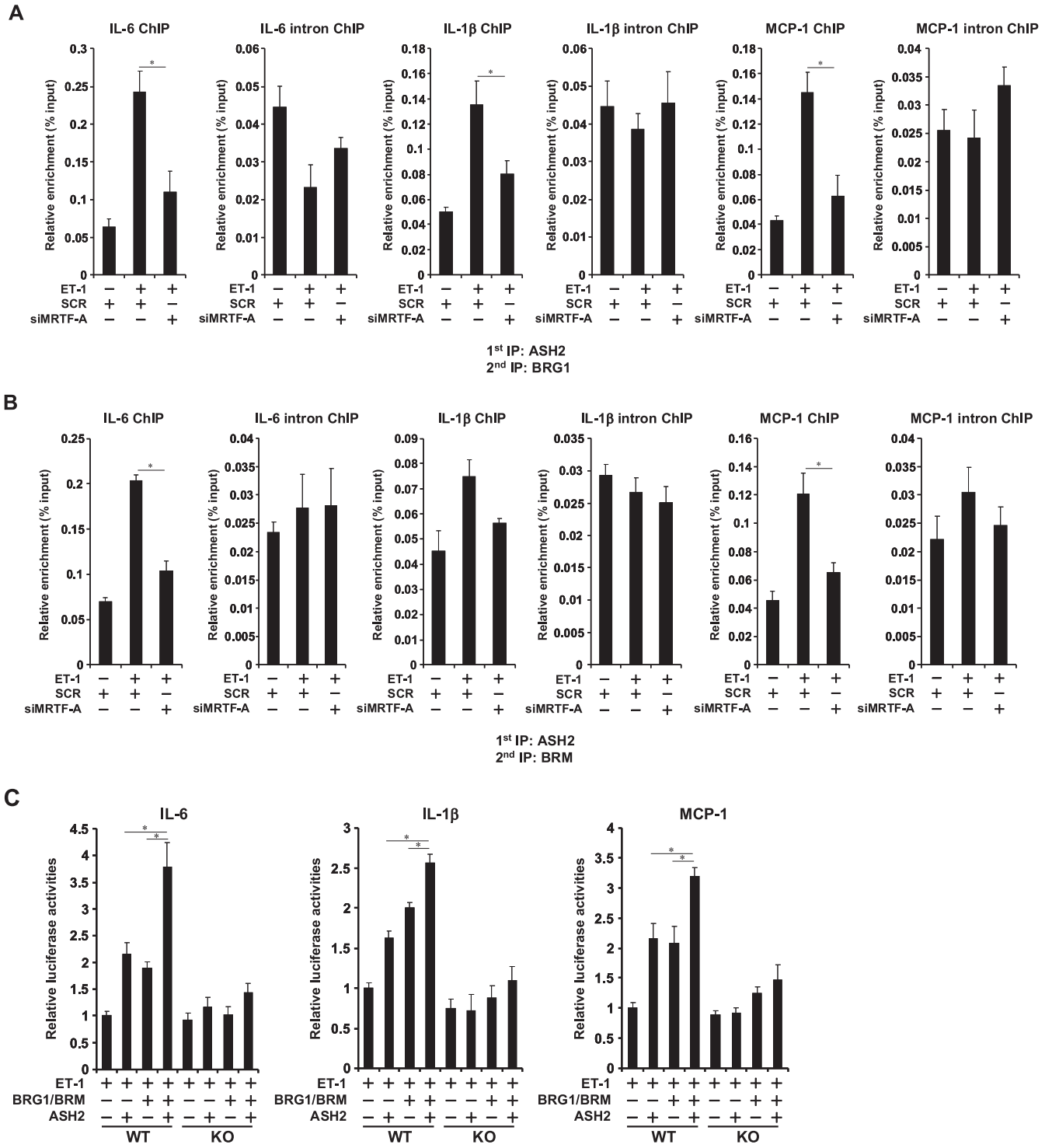

Figure 8. MRTF-A is essential for the crosstalk between BRG1/BRM and ASH2. (**A** and **B**) HASMCs were transfected with indicated siRNAs followed by treatment with or without ET-1 (1 nM). Re-ChIP assays were performed with indicated antibodies. (**C**) WT or MRTF-A deficient (KO) MEF cells were transfected with indicated reporter constructs along with indicated expression constructs followed by treatment with ET-1 (1 nM). Data are expressed as relative luciferase activities. All experiments were repeated at least three times. *, $P < .05$.

fore, our data seem to suggest that in order for the ASH2-containing H3K4 methyltransferase complex to be safely loaded on the chromatin at least two prerequisites have to be met. First, a transcription factor/co-factor (e.g. MRTF-A) has to guide the complex to a specific region of DNA. Second, the chromatin structure has to be made accessible by remodeling proteins (e.g. BRG1/BRM). It will be of great interest to examine, using ChIP-seq technique, the distributions of MRTF-A, ASH2 and BRG1/BRM on the genome and how the interplays between these proteins dictate transcriptional outcomes.

VSMCs are known to transition between different phenotypes with concomitant changes in gene expression profiles (45). In particular, VSMCs can assume a pro-inflammatory phenotype when exposed to certain humoral factors, such as ET-1 (9). In agreement with previous findings that MRTF-A modulates transcription by forging extensive dialogues with the epigenetic machinery, the data set presented here portrays MRTF-A as a central coordinator of ET-1-induced pro-inflammatory transcription in VSMCs. Since the genes analyzed here are all targets for NF-κB and since MRTF-A is essential for bringing ASH2 and BRG1/BRM together to activate the NF-κB reporter (Figure 8), an implicit conclusion would be that MRTF-A might help establish a transcriptionally friendly chromatin structure to sustain NF-κB-dependent transcription. Indeed, our preliminary ChIP data indicate that in MRTF-A deficient cells, the affinity of NF-κB for its target genes is dampened. Future investigations will have to determine to what extent NF-κB-dependent transcription is a function of MRTF-A-related activity.

Recently, Wang *et al.* have reported that MRTF-A mediates the anti-inflammatory effect by BMP (46). There are several possibilities that could explain the discrepancy between the conclusion drawn by our study and that by Wang *et al.* First, we have used aortic smooth muscle cells while pulmonary smooth muscle cells were used by Wang *et al.* These two types of smooth muscle cells exhibit great heterogeneity in terms of biochemistry (47) and response to humoral factors (48), indicating that the effects observed might be cell type-specific. Second, Wang *et al.* have used TNF-α instead of ET-1 to treat cells. Although both treatments can lead to NF-κB activation, the signaling pathways and therefore the co-factors involved are different, which may lead to distinct outcomes. Third, we have found that MRTF-A interacts with NF-κB via its N-terminal Q domain (18) and our data as presented here show that MRTF-A directly interact with NF-κB on the promoters of pro-inflammatory genes (Figure 2D). Wang *et al.*, on the other hand, have proposed that the C-terminus of MRTF-A is responsible for antagonizing NF-κB activity, indicating that MRTF-A might inhibit p65 activity indirectly. Clearly, further studies are warranted to clarify this issue.

In summary, our data have identified a novel epigenetic complex responsible for vascular inflammation inflicted by ET-1. Small-molecule peptide that blocks the interaction between calcineurin and NF-AT has been successfully exploited in the treatment of pathological hypertrophy (49). If the current report is to be validated by future studies, then a similar strategy can be employed to develop therapeutic solutions for ET-1-related vascular pathologies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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