

RESEARCH ARTICLE

MRTF-A mediates LPS-induced pro-inflammatory transcription by interacting with the COMPASS complex

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ABSTRACT

Chronic inflammation underscores the pathogenesis of a range of human diseases. Lipopolysaccharide (LPS) elicits strong pro-inflammatory responses in macrophages through the transcription factor NF-κB. The epigenetic mechanism underlying LPS-induced pro-inflammatory transcription is not fully understood. Herein, we describe a role for myocardin-related transcription factor A (MRTF-A, also known as MKL1) in this process. MRTF-A overexpression enhanced NF-κB-dependent pro-inflammatory transcription, whereas MRTF-A silencing inhibited this process. MRTF-A deficiency also reduced the synthesis of pro-inflammatory mediators in a mouse model of colitis. LPS promoted the recruitment of MRTF-A to the promoters of pro-inflammatory genes in an NF-κB-dependent manner. Reciprocally, MRTF-A influenced the nuclear enrichment and target binding of NF-κB. Mechanistically, MRTF-A was necessary for the accumulation of active histone modifications on NF-κB target promoters by communicating with the histone H3K4 methyltransferase complex (COMPASS). Silencing of individual members of COMPASS, including ASH2, WDR5 and SET1 (also known as SETD1A), downregulated the production of pro-inflammatory mediators and impaired the NF-κB kinetics. In summary, our work has uncovered a previously unknown function for MRTF-A and provided insights into the rationalized development of anti-inflammatory therapeutic strategies.

KEY WORDS: Pro-inflammatory transcription, MRTF-A, NF-κB, COMPASS, Epigenetics

INTRODUCTION

Macrophages are a group of heterogeneous cells that play a wide range of roles in maintaining internal homeostasis (Wynn et al., 2013). When inadvertently activated under stress conditions, however, macrophages can contribute to the pathogenesis of human diseases (Hotamisligil, 2006; Qian and Pollard, 2010; Weber et al., 2008). A paradigm of macrophage-dependent pathophysiological process is the initiation and perpetuation of chronic inflammation, which is considered to be pivotal to the disruption of the physiological integrity of tissues and organs. For instance, it has been demonstrated that human atherosclerotic lesions contain high numbers of macrophages (Gown et al.,

1986). Conversely, systemic depletion of macrophages stalls the development of atherosclerosis in mice (Stoneman et al., 2007).

Lipopolysaccharide (LPS) engages the pattern recognition receptor TLR4 to launch a strong pro-inflammatory reaction in macrophages. LPS-induced pro-inflammatory transcription is programmed by NF-κB, the master regulator of chronic inflammation (Smale, 2011). Accumulating evidence has helped to fuel the notion that NF-κB-dependent pro-inflammatory transcription is a function of chromatin structure modulated by the epigenetic machinery; chromatin wrapped with acetylated histones H3 and H4 and methylated histone H3 lysine 4 (H3K4) generally facilitates NF-κB-mediated transcriptional activation (Natoli, 2009). Recent investigations have given support to this model. For instance, Austenaa et al. have demonstrated that the H3K4 methyltransferase (HMT) MLL4 (also known as KMT2B or WBP7) is required for LPS-induced gene expression in macrophages (Austenaa et al., 2012). Wang et al., by contrast, have shown that another HMT (MLL1, also known as KMT2A) is indispensable for NF-κB-mediated transcription in response to TNF-α (Wang et al., 2012). Currently, it is not completely clear whether NF-κB is self-sufficient in terms of engaging various epigenetic factors in transcriptional regulation or whether a co-factor is necessary to act as a bridge between NF-κB and the epigenetic machinery.

Myocardin-related transcription factor A (MRTF-A, also known as MKL1) is known to function as a coactivator for SRF (Wang et al., 2002), Smad3 (Morita et al., 2007) and Sp1 (Luchsinger et al., 2011). Our previous study has found that MRTF-A can drive the transcription of adhesion molecules in vascular endothelial cells by directly interacting with the p65 subunit of NF-κB (Fang et al., 2011). The ability of MRTF-A to steer transcriptional events is derived in part from its extensive crosstalk with the epigenetic machinery, including histone modifying enzymes and chromatin remodeling proteins (Hanna et al., 2009; Lockman et al., 2007; Yang et al., 2013; Zhang et al., 2007; Zhou et al., 2009). Building on these findings, we hypothesized that MRTF-A could fine-tune LPS-induced NF-κB-dependent pro-inflammatory transcription in macrophages. Our data, as summarized here, support a role for MRTF-A as a key epigenetic coordinator in NF-κB-mediated pro-inflammatory transcription both *in vitro* and *in vivo*.

RESULTS

MRTF-A is necessary for LPS-induced pro-inflammatory transcription in macrophages

We have previously shown that MRTF-A interacts with NF-κB to activate ICAM-1 expression in vascular endothelial cells (Fang et al., 2011). Because NF-κB is the central mediator of LPS-induced pro-inflammatory transcription in macrophages, here, we

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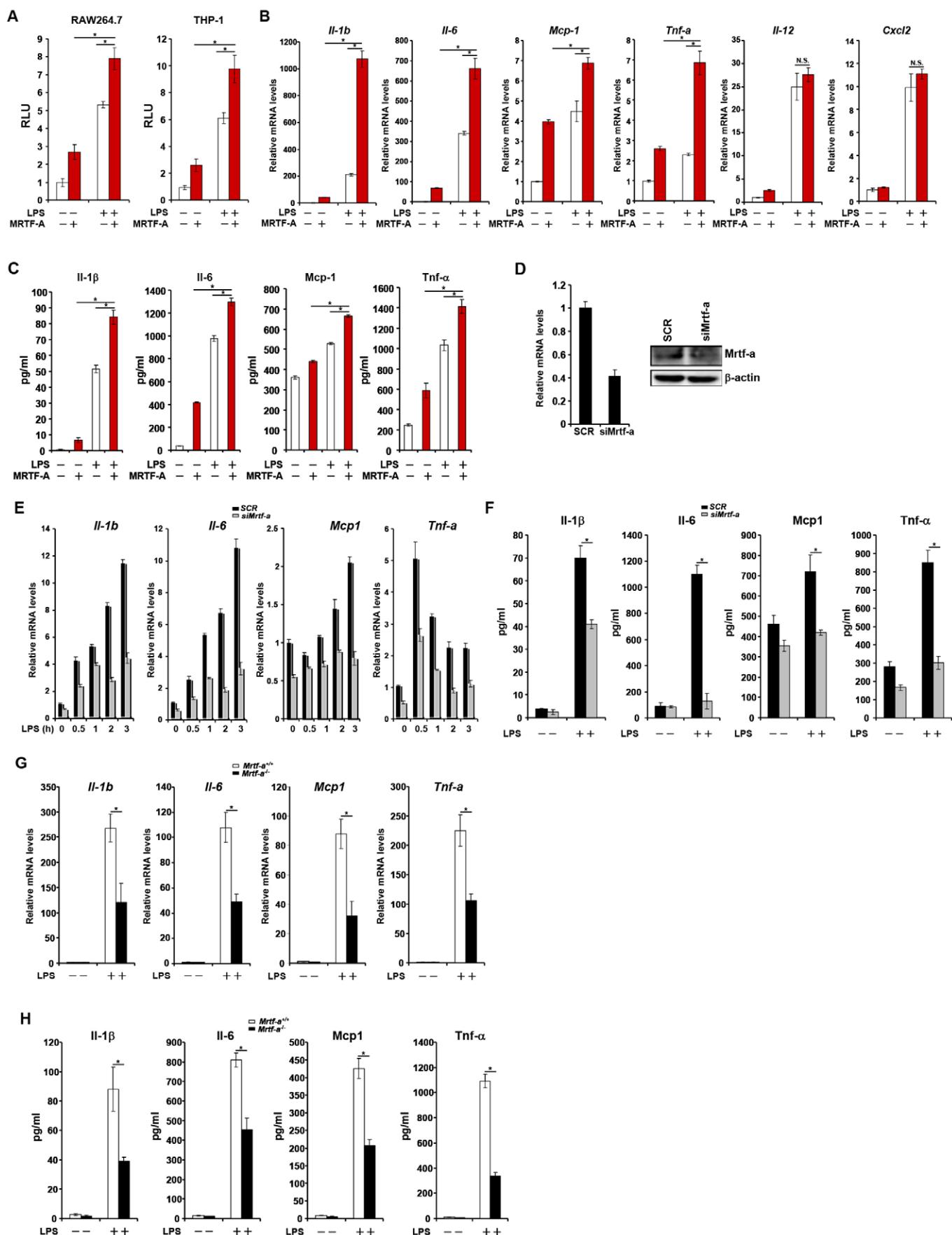


Fig. 1. See next page for legend.

Fig. 1. MRTF-A is necessary for LPS-induced pro-inflammatory transcription in macrophages. (A) A 2 \times B reporter construct was transfected into RAW264.7 or THP-1 cells with or without MRTF-A followed by treatment with LPS. Data are expressed as relative luciferase units (RLU). (B,C) An MRTF-A expression construct was transfected into RAW264.7 cells followed by LPS treatment for 3 hours. mRNA (B) and protein (C) levels of pro-inflammatory mediators were examined by qPCR and ELISA, respectively. (D) RAW264.7 cells were transfected with siRNA targeting MRTF-A (siMrtf-a) or control siRNA (SCR). mRNA and protein levels of MRTF-A were examined by qPCR and western blotting, respectively. (E,F) RAW264.7 cells were transfected with the indicated siRNA, followed by treatment with LPS. mRNA (E) and protein (F) levels of pro-inflammatory mediators were examined by qPCR and ELISA, respectively. (G,H) BMDMs from wild-type or MRTF-A-deficient mice were treated with or without LPS for 3 hours. mRNA (G) and protein (H) levels of pro-inflammatory mediators were examined by qPCR and ELISA, respectively. All data show the mean \pm s.d.; *P<0.05; N.S., non-significant.

have tackled the question of whether MRTF-A would also be necessary for this process. Overexpression of MRTF-A enhanced the κ B reporter activity in response to LPS treatment in both murine (RAW264.7) and human (THP-1) macrophages (Fig. 1A). Compared with MRTF-A, MRTF-B (also known as MKL2), another member of the MRTF family, was a much weaker activator of the κ B reporter (supplementary material Fig. S1A). Overexpression of MRTF-A also significantly upregulated the synthesis of endogenous pro-inflammatory mediators in macrophages treated with LPS, both at the mRNA level, as assayed by quantitative (q)PCR (Fig. 1B), and at the protein level, as measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 1C). By contrast, overexpression of a dominant-negative form of MRTF-A, which can interact with p65 but which lacks the transactivation domain (Fang et al., 2011), suppressed the activation of the κ B reporter (supplementary material Fig. S1B) and reduced the production of endogenous pro-inflammatory mediators (supplementary material Fig. S1C,D). Immunofluorescent staining revealed that treatment with LPS caused a rapid accumulation of MRTF-A along with the p65 subunit of NF- κ B in the nucleus of RAW264.7 cells (supplementary material Fig. S1E).

By contrast, when we reduced the expression of MRTF-A in RAW264.7 cells with small interfering RNA (siRNA; Fig. 1D), we noticed a decrease in LPS-induced synthesis of pro-inflammatory mediators (Fig. 1E,F). Several additional lines of evidence also lent support to the model wherein MRTF-A enhances LPS-induced pro-inflammatory transcription. First, bone-marrow-derived macrophages (BMDM) isolated from MRTF-A-deficient mice (*Mrtf-a*^{-/-}) produced less mRNA (Fig. 1G) and protein (Fig. 1H) of pro-inflammatory mediators in response to LPS treatment compared with that produced by wild-type (*Mrtf-a*^{+/+}) BMDMs. Similar observations were made in murine embryonic fibroblast (MEF) cells (supplementary material Fig. S1F,G). Second, LPS-induced κ B reporter activity was lost in MRTF-A-deficient MEF cells but could be recovered once ectopic MRTF-A was introduced (supplementary material Fig. S1H). Collectively, these data suggest that MRTF-A is necessary for LPS-induced pro-inflammatory transcription in macrophages.

MRTF-A deficiency attenuates colitis in mice

Next, we evaluated the role of MRTF-A in a mouse model of inflammatory disease, namely dextran sodium sulfate (DSS)-induced colitis. DSS caused weight loss (Fig. 2A) and shortening

(Fig. 2B) of the colon in wild-type mice, both of which were attenuated in MRTF-A-knockout mice. In addition, there was reduced intestinal edema, fewer inflammatory infiltrates and better preservation of intestinal structure in knockout mice, which collectively resulted in an improved pathology score (Fig. 2C). Finally, compared with wild-type mice, the intestines of knockout mice produced fewer pro-inflammatory mediators both at the mRNA (Fig. 2D) and protein (Fig. 2E) levels. Therefore, MRTF-A might play a role in modulating the inflammatory response *in vivo*.

Reciprocal interaction between MRTF-A and p65

Because the p65 subunit of NF- κ B is the chief orchestrator of LPS-induced pro-inflammatory transcription, we assessed the possibility that there might be a functional interaction between MRTF-A and p65. Chromatin immunoprecipitation (ChIP) assays revealed that MRTF-A was recruited to the promoters of the *Il-1b*, *Il-6*, *Mcp-1* and *Tnf-a* genes with a similar kinetics to the recruitment of p65 (Fig. 3A). Furthermore, LPS enhanced the formation of an MRTF-A-p65 complex on the promoters as measured using the Re-ChIP assay (Fig. 3B). Reducing the expression of p65 with siRNA (Fig. 3C) significantly reduced the recruitment of MRTF-A in response to LPS treatment (Fig. 3D), indicating that MRTF-A relies on p65 to participate in LPS-induced pro-inflammatory transcription.

To test the hypothesis that MRTF-A might reciprocally influence the activity of p65, we knocked down endogenous MRTF-A with siRNA in RAW264.7 cells. Interestingly, the affinity of p65 for its target genes was reduced (Fig. 3E). Consistently, in MRTF-A-deficient MEF cells, LPS-induced binding of p65 to target genes was reduced as opposed to that of wild-type MEF cells (supplementary material Fig. S2B). To further verify these observations, we also performed immunofluorescent staining and gel shift assays. Indeed, in the absence of MRTF-A, there was less p65 accumulation in the nucleus (supplementary material Fig. S2C,D). Thus, these data suggest that while p65 recruits MRTF-A to its target promoters, MRTF-A is able to actively influence the activity of p65.

MRTF-A deficiency is associated with a repressed chromatin structure

Previous studies have indicated that MRTF-A delegates transactivation to the epigenetic machinery (Fang et al., 2011; Hanna et al., 2009; Yang et al., 2013; Zhang et al., 2007). Therefore, we examined whether the attenuation of transcriptional activation of NF- κ B target genes as a result of MRTF-A silencing could be correlated with a repressed chromatin structure. MRTF-A knockdown in RAW264.7 cells led to a decrease in the enrichment of acetylated histone H3 (AcH3; supplementary material Fig. S3A) and acetylated histone H4 (AcH4; supplementary material Fig. S3B) on the gene promoters. Similarly, we noted a reduction in the accumulation of AcH3 (supplementary material Fig. S3C) and AcH4 (supplementary material Fig. S3D) in MRTF-A-deficient MEF cells in comparison to wild-type MEF cells. Moreover, dimethylated histone H3K4 (H3K4me2; Fig. 4A,C) and trimethylated H3K4 (H3K4me3; Fig. 4B,D) were erased from the NF- κ B target promoters when MRTF-A was silenced. Finally, the enrichment of BRG1 and BRM (also known as Brahma or SMARCA2), two core components of the mammalian chromatin remodeling complex known to participate in LPS-induced transactivation of pro-inflammatory mediators (Ramirez-Carrozzi

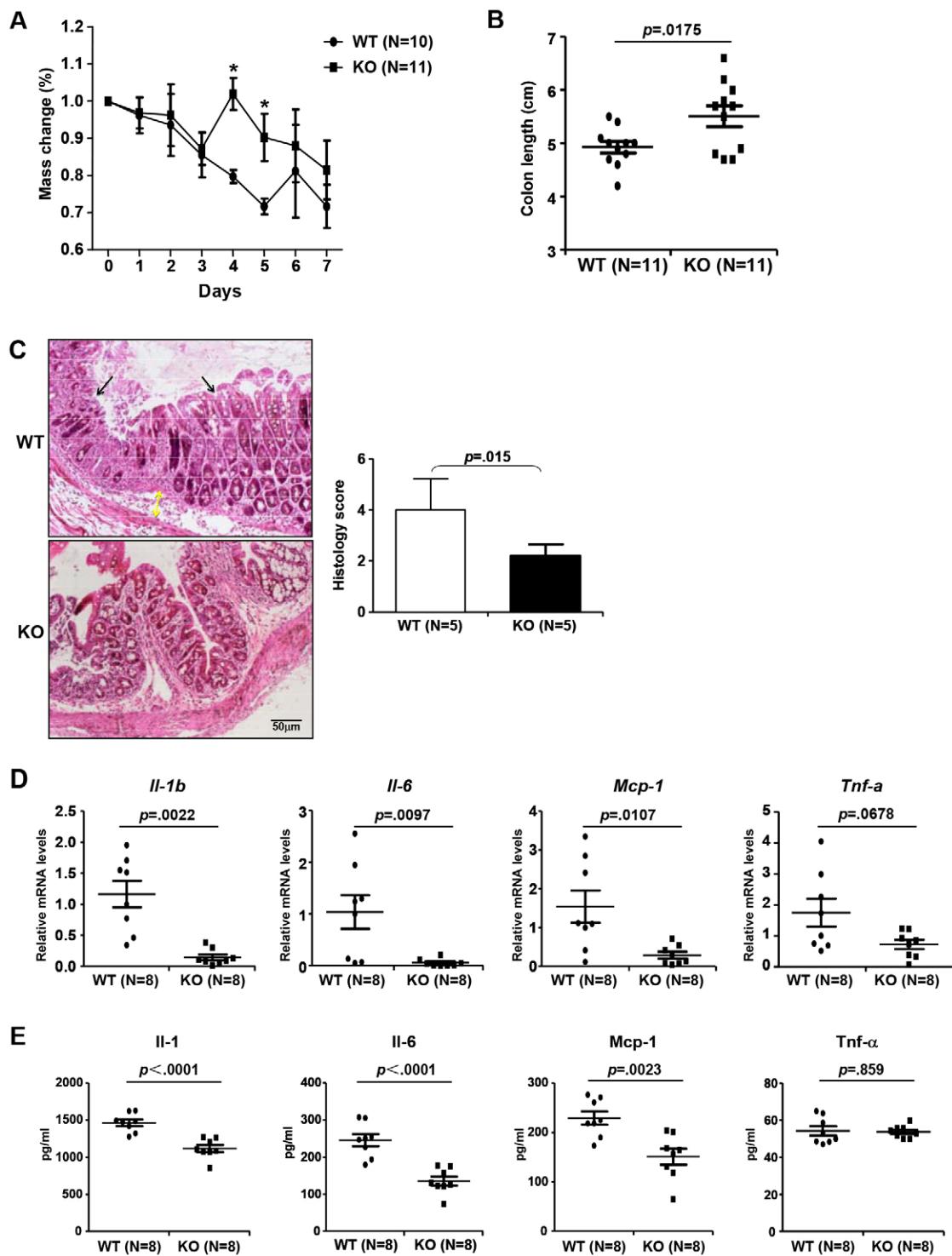


Fig. 2. MRTF-A-knockout mice are less susceptible to DSS-induced colitis. Age- and sex-matched wild-type (WT) and MRTF-A knockout (KO) mice were induced to develop colitis as described in Materials and Methods. (A) Changes in weight are shown. **P*<0.05. (B) Colon length is shown. (C) Left, representative H&E staining of colon. Black arrows, loss of intestinal crypt; yellow arrows, intestinal edema. Scale bar: 50 μ m. Right, the histology score of H&E-stained colon sections is shown. (D,E) Intestinal levels of pro-inflammatory mediators were measured by qPCR (D) and ELISA (E). All quantitative data show the mean \pm s.d.

et al., 2006), was also significantly reduced in RAW264.7 cells without MRTF-A (supplementary material Fig. S3E,F). Taken together, these data confirm our hypothesis that MRTF-A might modulate LPS-induced pro-inflammatory transactivation by altering the chromatin structure.

MRTF-A recruits the COMPASS proteins to activate LPS-induced pro-inflammatory transcription

The complex associated with SET1 (COMPASS) has been shown to mediate histone H3K4 methylation in mammals (Shilatifard, 2012). Having observed that MRTF-A deficiency was associated

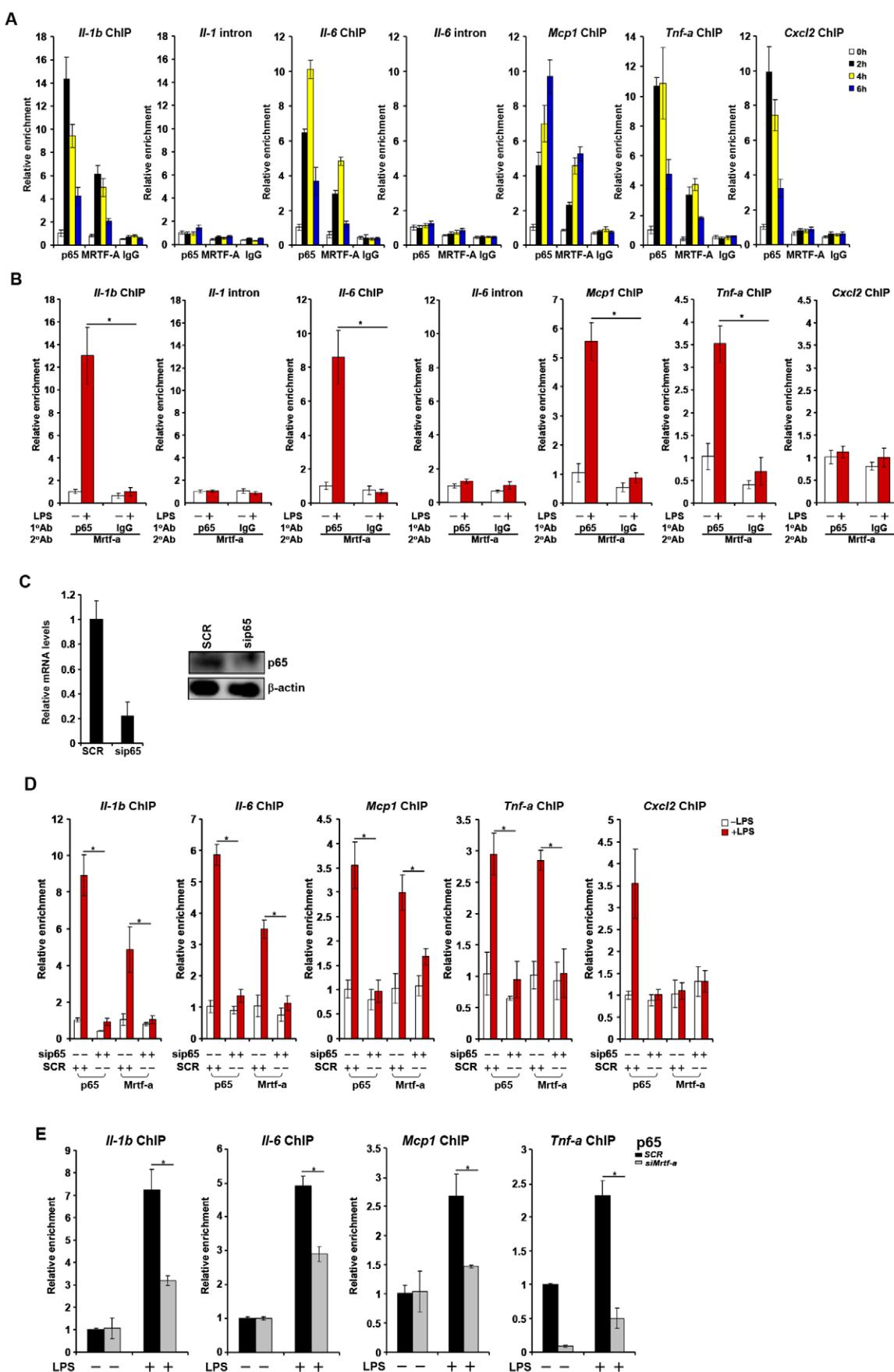


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Fig. 3. LPS promotes the interplay between MRTF-A and the p65 subunit of NF-κB. (A) RAW264.7 cells were treated with LPS and harvested at the indicated time points. ChIP assays were performed with the indicated antibodies. (B) RAW264.7 cells were treated with LPS for 3 hours. Re-ChIP assays were performed with the indicated antibodies (Ab). (C) RAW264.7 cells were transfected with control siRNA (SCR) or siRNA targeting p65 (sip65). mRNA and protein levels of p65 were examined by qPCR (left) and western blotting (right), respectively. (D) RAW264.7 cells were transfected with the indicated siRNAs, followed by treatment with LPS. ChIP assays were performed with the p65 or MRTF-A antibodies, as indicated. (E) RAW264.7 cells were transfected with the indicated siRNAs followed by treatment with LPS. ChIP assays were performed with antibody against p65. All quantitative data show the mean \pm s.d.; *P<0.05.

with the disappearance of H3K4me2 and H3K4me3 from the promoter regions, we decided to examine whether MRTF-A could recruit COMPASS to activate LPS-induced pro-inflammatory transcription. Indeed, there was an interaction between MRTF-A and COMPASS proteins as confirmed by immunoprecipitation (Fig. 5A). In LPS-treated macrophages, we observed significant recruitment of several COMPASS components, including ASH2, WDR5 and SET1 (also known as SETD1A), with a similar kinetics to the recruitment of MRTF-A and p65 (Fig. 5B). More importantly, Re-ChIP data suggested that MRTF-A could form a complex with ASH2 (Fig. 5C), WDR5 (Fig. 5D) and SET1 (Fig. 5E) on the gene promoters in response to LPS stimulation. By contrast, depletion of MRTF-A significantly reduced the binding of COMPASS proteins (Fig. 5F), indicating that MRTF-A is responsible for COMPASS recruitment. Finally, coexpression of MRTF-A and COMPASS proteins synergistically activated the κB reporter activity (Fig. 5G).

Recently, Tang et al. have shown that there is a crosstalk between histone acetylation (mediated by p300) and histone methylation (mediated by COMPASS) in the regulation of p53-dependent transcription (Tang et al., 2013). Combining this report with our own observation that MRTF-A silencing was met with a decrease in both histone acetylation and methylation, we were prompted to propose that MRTF-A might be responsible for the communication between p300 and COMPASS. Indeed, in RAW264.7 cells treated with LPS, p300 could interact with ASH2, WDR5 and SET1 on the promoters of several NF-κB target genes; siRNA-mediated MRTF-A knockdown markedly disrupted these interactions (supplementary material Fig. S3G). Similarly, the interaction between p300 and COMPASS following LPS treatment was less prominent in MRTF-A-deficient MEF cells than in wild-type MEF cells (supplementary material Fig. S3H).

Because NF-κB appeared to be essential for MRTF-A occupancy on the pro-inflammatory promoters (Fig. 3D), a reasonable question to ask would be whether NF-κB is equally important for altering the chromatin structure and for COMPASS recruitment. To test this hypothesis, we depleted the p65 subunit of NF-κB using siRNA in RAW264.7 cells and then examined the binding of modified histones and COMPASS proteins in ChIP assays. Indeed, loss of p65 binding as a result of p65 knockdown (Fig. 6A) suppressed the deposition of acetylated histones H3 (Fig. 6B) and H4 (Fig. 6C) and methylated H3K4 (Fig. 6D,E), while simultaneously blocking the occupancies of ASH2 (Fig. 6F) and WDR5 (Fig. 6G) on the pro-inflammatory promoters. Therefore, we conclude that MRTF-A might enhance LPS-mediated pro-inflammatory transcription at least in part by recruiting COMPASS to the NF-κB target promoters and by coordinating the dialogue between p300 and COMPASS.

COMPASS is necessary for LPS-induced pro-inflammatory transcription

Finally, we sought to determine the role of COMPASS in LPS-induced pro-inflammatory transcription. Depletion of individual components of COMPASS (Fig. 7A–C for knockdown efficiencies) abrogated the induction of pro-inflammatory mediators by LPS (Fig. 7D,E). In keeping with the downregulation of transactivation, the binding of the p65 subunit of NF-κB to its target promoters was, to varied extent, reduced in the absence of COMPASS proteins (Fig. 7F), indicating that COMPASS is required for optimal chromatin positioning of NF-κB.

DISCUSSION

Chromatin is the hub where different signals and inputs are being integrated and processed to guide transcription in mammalian cells. Recent investigations have given rise to the model wherein NF-κB-dependent pro-inflammatory transcription is spatiotemporally dictated by chromatin organization (Natoli, 2011). We provide new evidence here that MRTF-A is a co-factor of NF-κB in macrophages, involved in regulating a histone H3K4 methyltransferase complex to mediate LPS-induced pro-inflammatory transcription.

We find here that MRTF-A is both necessary and sufficient to potentiate the transactivation of several pro-inflammatory mediators in cultured cells. We also find that MRTF-A deficiency attenuates inflammation in a mouse model of colitis, which is consistent with several previous reports showing that MRTF-A ablation alleviates pulmonary and cardiac injury and fibrosis (Small et al., 2010; Zhou et al., 2013). This could potentially be interpreted as impaired macrophage activation, supported by the *in vitro* observation. Alternatively, our previous investigation has assigned a role for MRTF-A in mediating the interaction between endothelial cells and circulating innate immune cells (Fang et al., 2011). Given that the recruitment of circulating immune cells plays an important role in the pathogenesis of colitis (Sans et al., 1999), the diminished inflammatory response in MRTF-A-deficient mice could also be explained by a reduction in leukocyte adhesion and/or chemotaxis. In addition, NF-κB has been shown to program macrophage polarization, favoring a pro-inflammatory M1 phenotype (Tugal et al., 2013). Mounting evidence supports a role for MRTF-A in directing differentiation/trans-differentiation in smooth muscle cells (Hinson et al., 2007), skeletal muscle cells (Selvaraj and Prywes, 2003), epithelial cells (Morita et al., 2007), megakaryocytes (Gilles et al., 2009) and fibroblast cells (Crider et al., 2011). We therefore speculate that MRTF-A deficiency could potentially skew the macrophages to an anti-inflammatory M2 phenotype. Clearly, further studies are warranted to reconcile these different scenarios and delineate a more definitive role for MRTF-A.

One major finding in this report is that the interplay between MRTF-A and the p65 subunit of NF-κB appears to extend both ways. MRTF-A depends on p65 to gain access to the chromatin, while, at the same time, actively influencing the nuclear accumulation and target-binding affinity of p65 (Fig. 3; supplementary material Fig. S2). The ability of MRTF-A to modulate the chromatin positioning of p65 could be easily attributed to the fact that MRTF-A interacts with histone-modifying enzymes (COMPASS, Fig. 5) and remodeling proteins (Brg1 and Brm, supplementary material Fig. S3) and in so doing creates a ‘friendly’ conformation of chromatin for p65.

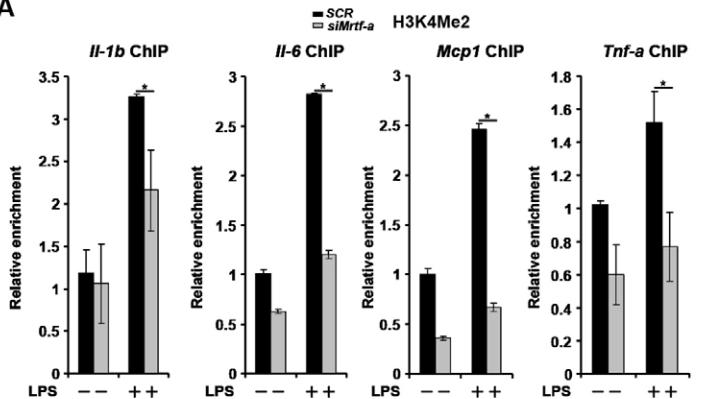
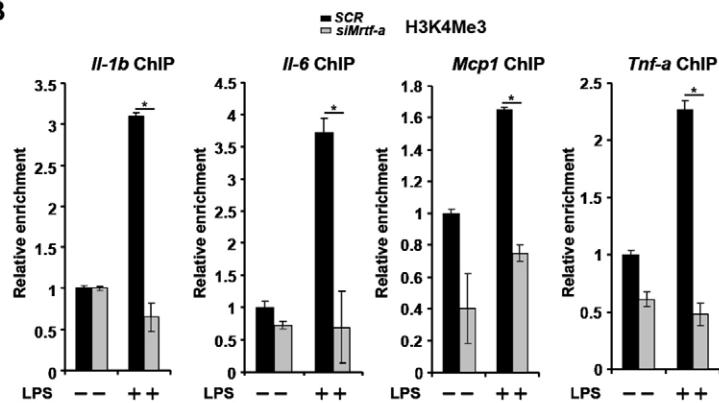
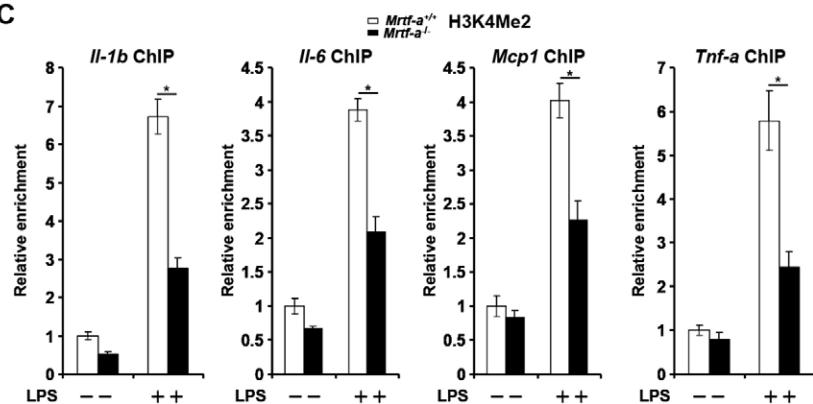
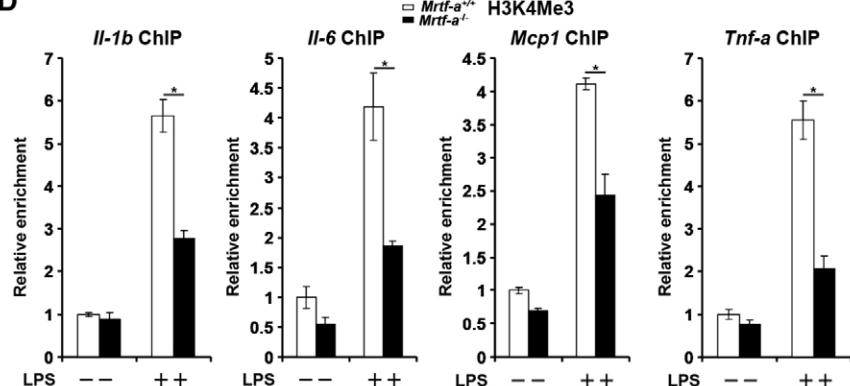
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Fig. 4. MRTF-A is responsible for active chromatin structure in LPS-induced pro-inflammatory transcription. (A,B) RAW264.7 cells were transfected with control siRNA (SCR) or siRNA targeting MRTF-A (siMrtf-a), as indicated, followed by treatment with LPS. ChIP assays were performed with antibody against dimethylated H3K4 (A) or trimethylated H3K4 (B). (C,D) Wild-type or MRTF-A-knockout MEF cells were treated with LPS for 3 hours and a ChIP assay was performed with antibody against dimethylated H3K4 (C) or trimethylated H3K4 (D). All data show the mean \pm s.d.; * $P < 0.05$.

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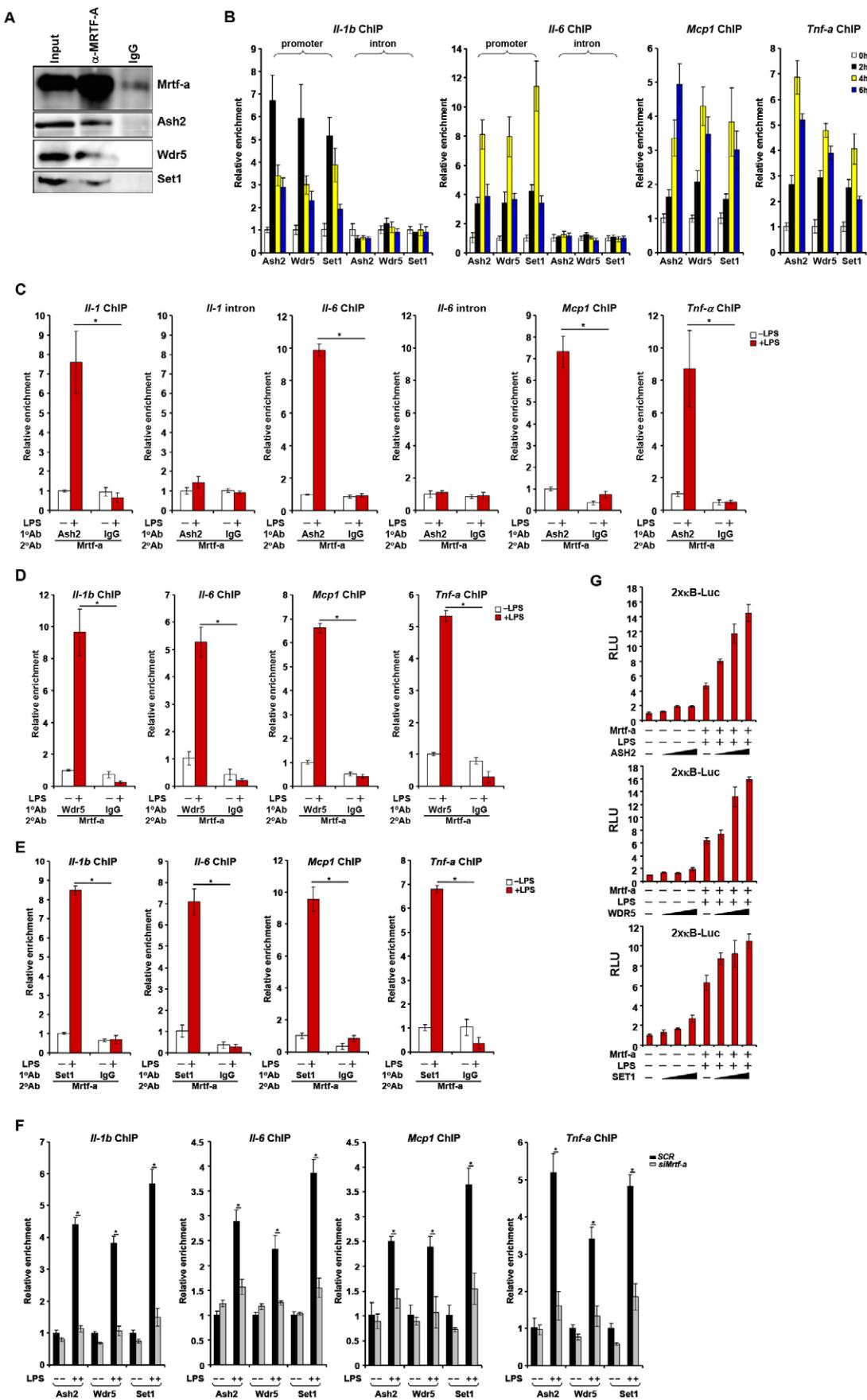


Fig. 5. MRTF-A recruits COMPASS to the promoters of pro-inflammatory genes.

(A) Co-immunoprecipitation assays were performed using whole-cell lysates from RAW264.7 cells, with the indicated antibodies.

(B) RAW264.7 cells were treated with LPS and harvested at the indicated time-points. ChIP assays were performed with the indicated antibodies.

(C–E) RAW264.7 cells were treated with or without LPS for 3 hours. Re-ChIP assays were performed with the indicated antibodies (Ab).

(F) RAW264.7 cells were transfected with control siRNA (SCR) or siRNA targeting MRTF-A (siMrtf-a), as indicated, followed by treatment with LPS. ChIP assays were performed with the indicated antibodies.

(G) A 2 \times kB reporter construct was transfected into RAW264.7 cells with the indicated expression constructs, followed by treatment with LPS. Data show relative luciferase units (RLU). All quantitative data show the mean \pm s.d.; *P<0.05.

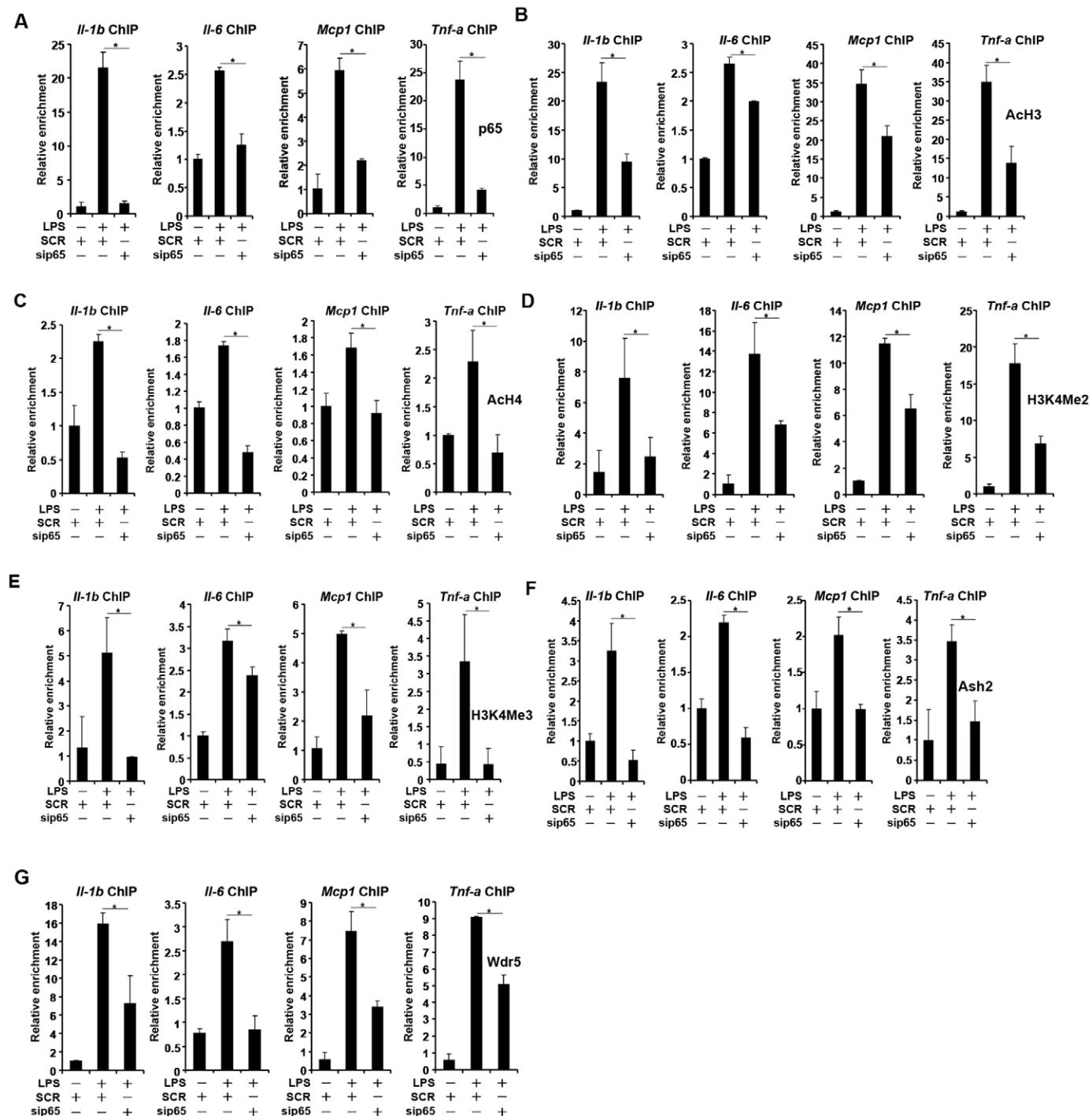


Fig. 6. The p65 subunit of NF- κ B is necessary for maintaining an active chromatin structure at pro-inflammatory genes in response to LPS stimulation. (A–G) RAW264.7 cells were transfected with control siRNA (SCR) or siRNA targeting p65 (sip65), as indicated, followed by treatment with LPS. ChIP assays were performed with antibodies against p65 (A), acetylated H3 (B), acetylated H4 (C), dimethylated H3K4 (D), trimethylated H3K4 (E), Ash2 (F) and Wdr5 (G). All data show the mean \pm s.d.; *P<0.05.

to bind. This model is further supported by our previous observation that silencing of Brg1 or Brm impedes the binding of p65 to the promoters of adhesion molecules (Fang et al., 2013) and our current observation that silencing the COMPASS complex disrupts the binding kinetics of p65 on the promoters of pro-inflammatory mediators (Fig. 7). By contrast, the depletion of p65 negatively impacted the binding of the

COMPASS complex and the deposition of acetylated and methylated histones (Fig. 6). It is of particular importance to note that our data also suggest that MRTF-A could directly regulate the nuclear localization of p65. Thus, these data, when taken together, seem to argue that epigenetic modulation of pro-inflammatory transcription takes place in a feed-forward manner – co-factors (e.g. MRTF-A) facilitate the translocation of

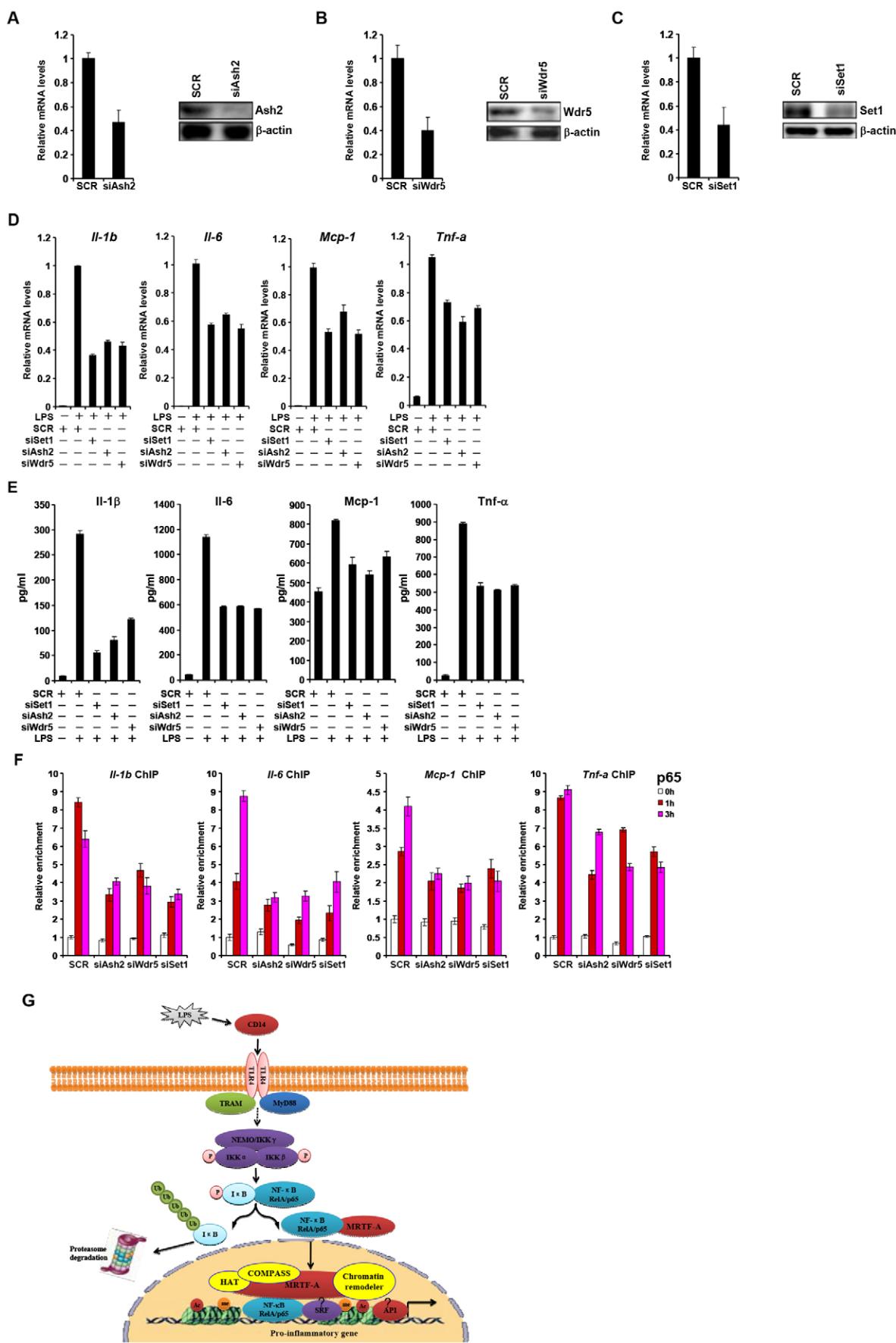


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Fig. 7. COMPASS is necessary for LPS-induced pro-inflammatory transcription in macrophages. (A–C) RAW264.7 cells were transfected with control siRNA (SCR) or siRNA targeting Ash2 (siAsh2), Wdr5 (siWdr5) or Set1 (siSet1). mRNA and protein levels of Ash2 (A), Wdr5 (B) and Set1 (C) were examined by qPCR and Western. (D–F) RAW264.7 cells were transfected with the indicated siRNAs followed by treatment with LPS. mRNA (D) and protein (E) levels of pro-inflammatory mediators were examined by qPCR and ELISA, respectively. (F) ChIP assay was performed with antibody against p65. All quantitative data show the mean \pm s.d.; *P<0.05. (G) A schematic model depicting LPS-induced MRTF-A-dependent epigenetic regulation of pro-inflammatory transcription. TLR4, Toll-like receptor 4; IKK, inhibitor of nuclear factor kappa-B kinase; SRF, serum response factor; TRAM, TRIF-related adaptor molecule; AP-1, activator protein 1; MyD88, myeloid differentiation factor 88; Ub, ubiquitylation.

sequence-specific transcription factors (e.g. p65). The latter then recruit co-factors to DNA in a context-dependent manner. Consequently, co-factors enlist histone-modifying enzymes and chromatin remodelers to reorganize the chromatin structure, thus allowing more transcription factors to bind to chromatin (Natoli, 2009).

Our data also implicate MRTF-A as a coordinator of the crosstalk between histone acetyltransferases (HATs) and HMTs (supplementary material Fig. S3). It is not entirely clear at this point how this is achieved. The most straightforward explanation is that MRTF-A interacts with p300 and COMPASS and, as such, could act as a mediator. It is, however, equally plausible that MRTF-A might rely on other means to broker this dialogue. For instance, both HATs and HMTs are known to prefer certain chromatin structure to exert their effects (Holbert et al., 2007; Krajewski and Reese, 2010). Therefore, MRTF-A could, independently of its interaction with COMPASS and p300, recruit Brg1 or Brm to reorganize the chromatin to establish a suitable chromatin environment for HATs and HMTs to engage on. Hopefully ChIP-seq analyses of the binding patterns of MRTF-A, Brg1, Brm, COMPASS and p300 in the context of LPS-induced pro-inflammatory conditions would shed more light on the mechanism whereby MRTF-A helps different histone-modifying enzymes to navigate the genome.

An interesting observation in the present study is that both primary response genes (e.g. *Tnf-α*) and secondary response genes (e.g. *Il-6*) seem to be affected by the NF-κB–MRTF-A–COMPASS complex. However, cautions need to be taken in interpreting these data. Chromatin structure is determined cumulatively by the presence of different histone modifications, remodeling proteins and histone variants. Although the COMPASS complex might bind to both early- and late-response genes, the kinetics are quite different (Fig. 5). In addition, the presence of COMPASS does not necessarily mean that it is the rate-limiting factor for the transcription of its target genes. Instead, its role needs to be evaluated in the context of other histone-modifying enzymes (e.g. p300) and chromatin remodelers (e.g. Brg1) that can also be recruited by MRTF-A. Further complicating the role of COMPASS in the current model is the fact that the action of COMPASS can be antagonized by demethylases, which are not examined in the current report but are nonetheless involved in NF-κB-dependent transcription (Fuchs, 2013). Again, more investigations are needed to more clearly define the role of COMPASS in pro-inflammatory transcription.

In essence, our report indicates that the NF-κB–MRTF-A interaction is responsible for the epigenetic regulation of pro-inflammatory transcription induced by LPS (Fig. 7G). However, it is paramount not to overstate this conclusion, because there are still several unsolved issues regarding the current model. First, it

cannot be ignored at this point that other transcription factors (e.g. SRF and AP-1) might also participate in this process by modulating chromatin structure and/or influencing the access of NF-κB–MRTF-A to chromatin. In fact, our previous report has suggested such a role for SRF in the regulation of ICAM-1 activation in endothelial cells (Fang et al., 2011). Second, the current study focuses entirely on the proximal promoter region, whereas the role of distal regulatory elements is left unaddressed. Numerous investigations have established that epigenetic events taking place at enhancers are key to inflammation-related transcription (Arvey et al., 2014; Ghisletti et al., 2010; Li et al., 2013). It is possible that different isoforms of MRTF-A might interact with different sites to drive pro-inflammatory transcription. Finally, MRTF-A activity is impacted by the reorganization of actin filaments as a result of RhoA activation (Olson and Nordheim, 2010). Several reports have suggested that RhoA signaling might be transmitted to the nucleus to influence differential recruitment of epigenetic factors and gene expression (Helms et al., 2007; Kim et al., 2005; Ling and Lobie, 2004). The question as to whether MRTF-A serves as the moderator linking cytoskeletal dynamics and epigenetic regulation of pro-inflammatory genes is certainly worth further investigation.

In summary, our data, as reported here, reaffirm the role of MRTF-A as a key epigenetic orchestrator regulating LPS-induced NF-κB-dependent pro-inflammatory transcription in macrophages. A lingering question of the current report is whether the proposed model is cell-specific (i.e. restricted to macrophages). MRTF-A has been implicated in the regulation of smooth muscle cell phenotypic modulation and epithelial-to-mesenchymal transition (EMT), both of which involve extensive chromatin reorganization of MRTF-A target genes (Alexander and Owens, 2012; Tam and Weinberg, 2013). Tissue-specific animal models of MRTF-A deficiency would help clarify this issue and enable us to better understand the cell-autonomous role of MRTF-A for the ultimate goal of rationalized development of MRTF-A-targeting therapeutic strategies.

MATERIALS AND METHODS

Cell culture

Murine RAW264.7 macrophages (ATCC) and human THP-1 monocytic/macrophage-like cells (ATCC) were maintained in DMEM supplemented with 10% FBS. Murine bone-marrow-derived macrophages (BMDM) were isolated and cultured as described previously (Xu et al., 2012). Mouse embryonic fibroblast (MEF) cells were isolated from wild-type and MRTF-A-knockout mice as described previously (Sun et al., 2006). LPS was purchased from Sigma.

Plasmids, transfection and reporter assay

Expression constructs for MRTF-A (Cen et al., 2003), ASH2, WDR5 and SET1 (Wu et al., 2008), as well as the κB reporter (Rosette and Karin, 1995) have been described previously. siRNA sequences are listed in supplementary material Table S1. Transient transfections were performed with Lipofectamine LTX (Invitrogen). An EGFP expression construct was included in each well to monitor transfection efficiency. Luciferase activities were assayed at 24–48 hours after transfection using a luciferase reporter assay system (Promega). Luciferase activities were normalized to both protein concentration and GFP fluorescence. Experiments were routinely performed in triplicate wells and repeated three times.

Animal studies

All animal protocols were approved by the intramural Ethics Committee on Animal Studies and performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory

Animals. MRTF-A-deficient mice have been described previously (Sun et al., 2006). To induce colitis, 6–8-week-old MRTF-A-deficient mice and their littermates were given 2% (w/v) DSS (MP Biomedicals) in their drinking water for 7 days.

Protein extraction, immunoprecipitation, western blotting and gel shift assay

Whole-cell lysates were obtained by resuspending cell pellets in RIPA buffer with freshly added protease inhibitor tablet (Roche). Nuclear proteins were prepared with the NE-PER Kit (Pierce) following the manufacturer's recommendations. Specific antibodies or pre-immune IgG were added to and incubated with cell lysates overnight before the solutions were incubated with Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complexes were released by boiling with 1× SDS electrophoresis sample buffer. Western analyses were performed with antibodies against β-actin (Sigma), MRTF-A, p65, lamin B, α-tubulin (Santa Cruz), ASH2, WDR5 and SET1 (Bethyl Laboratories). Electrophoresis mobility shift assay (EMSA) was performed essentially as described previously (Yang et al., 2013).

ChIP and Re-ChIP assay

ChIP assays were performed essentially as described previously (Chen et al., 2013; Fang et al., 2013; Tian et al., 2013). Aliquots of lysates containing 200 µg of nuclear protein were used for each immunoprecipitation reaction with antibodies against MKL1, p300, p65, Brg1, Brm (Santa Cruz), ASH2, WDR5, SET1 (Bethyl Laboratories), acetylated H3, acetylated H4, acetylated H3K9, acetylated H3K14, acetylated H3K18, acetylated H3K27, dimethylated H3K4 and trimethylated H3K4 (Millipore/Upstate). For Re-ChIP, immune complexes were eluted with the elution buffer (1% SDS, 100 mM NaCO₃), diluted with the Re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1), and subjected to immunoprecipitation with a second antibody of interest. Precipitated genomic DNA was amplified by real-time PCR with the primers listed in supplementary material Table S2.

RNA extraction and real-time PCR

RNA was extracted using an 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 STEPONE Plus (Life Tech) with primers and Taqman probes purchased from Applied Biosystems.

Histology

Immunohistochemistry was performed as described previously (Xu et al., 2011). Paraffin sections of colon were processed by standard techniques. Longitudinal sections of 5-mm thick were stained with hematoxylin and eosin (H&E). Pictures were taken using an Olympus IX-70 microscope and examined by two independent pathologists using a scoring system as described previously (Zaki et al., 2011).

Immunofluorescence microscopy

Formaldehyde-fixed cells were blocked with 5% bovine serum albumin (BSA), and incubated with the indicated primary antibodies overnight. After several washes with PBS, cells were incubated with FITC-labeled secondary antibodies (Jackson) for 30 minutes. DAPI (Sigma) was added and incubated with cells for 5 minutes prior to observation. Immunofluorescence was visualized on a confocal microscope (LSM 710, Zeiss). For quantification, 30 cells were counted in triplicate culture dishes for each condition.

ELISA

Supernatants containing pro-inflammatory mediators were collected from cultured cells or tissue lysates and ELISA was performed to measure IL-1, IL-6, MCP-1 and TNF-α using commercially available kits (Ray Biotech, Norcross, GA).

Statistical analysis

Two-tailed *t*-test (for experiments involving two groups) or one-way ANOVA with post-hoc Scheffe analyses (for experiments involving at

least three groups) were performed using an SPSS package. *P* values smaller than 0.05 were considered statistically significant.

Competing interests

The authors declare no competing interests.

Author contributions

Y.X. conceived of the project; F.F., L.M.Y., X.Y.W. and Y.X. designed experiments; F.F., L.M.Y., X.Y.W., P.L., X.D., X.Y.W., H.H.X. and M.M.F. performed the experiments and analyzed the data; Y.X. wrote the manuscript with input from the rest of the authors.

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Supplementary material

Supplementary material available online at
<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.152314/-DC1>

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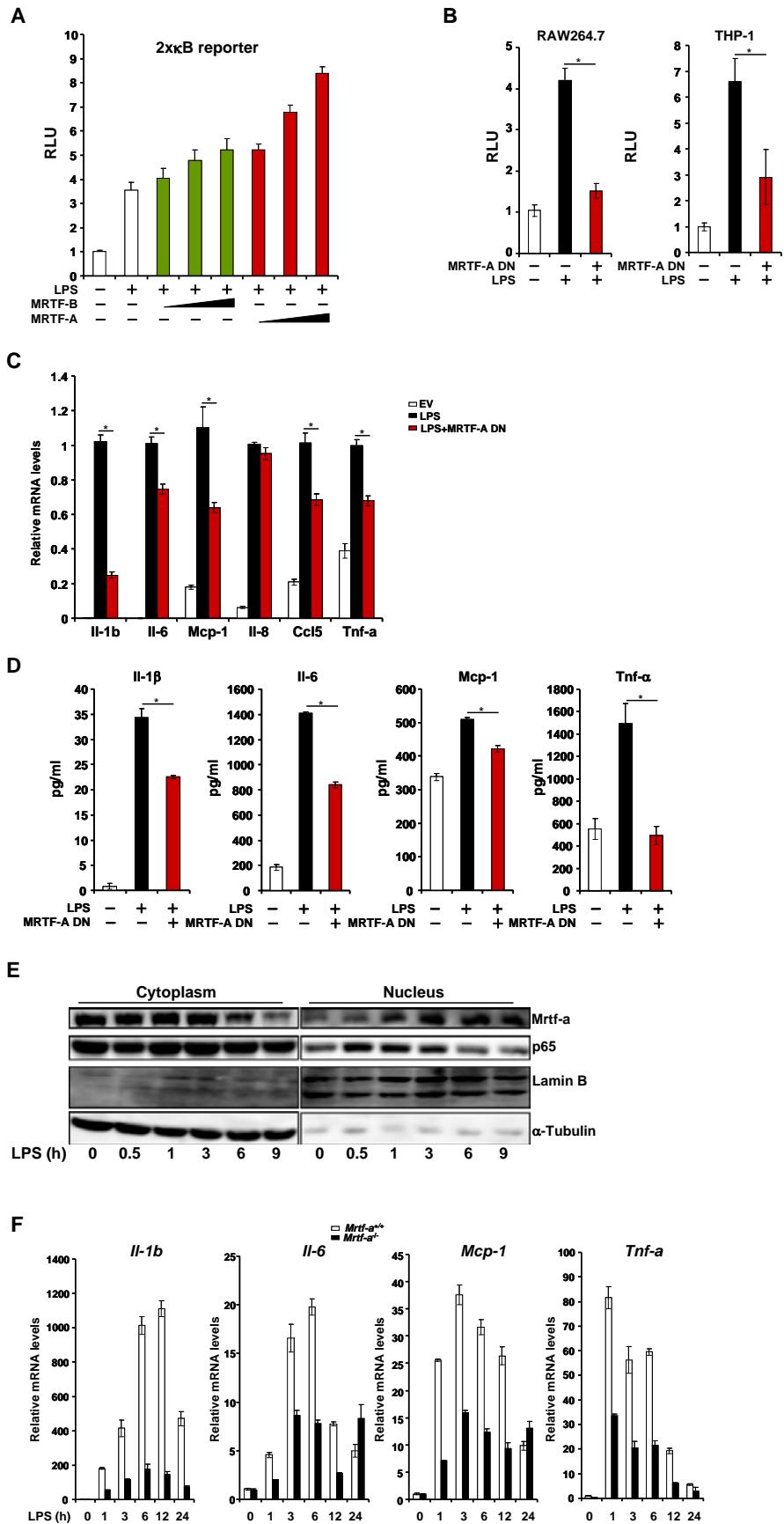
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Supplementary Figures: **3**

Supplementary Tables: **2**



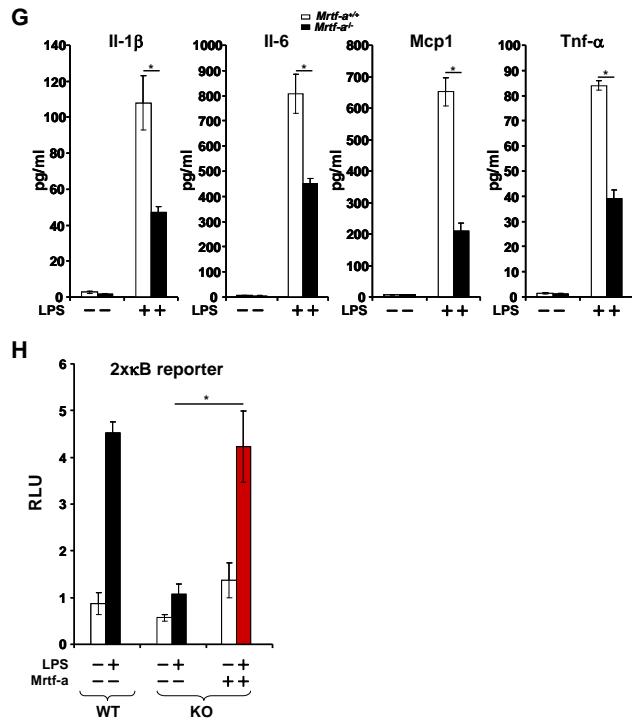


Fig.S1: (A) A 2x κ B reporter construct was transfected into RAW264.7 cells with or without MRTF-A/MRTF-B followed by treatment with LPS. Data are expressed RLU. (B) A 2x κ B reporter construct was transfected into RAW264.7 cells with or without dominant negative (DN) MRTF-A followed by treatment with LPS. Luciferase activities are expressed as RLU. (C, D) MRTF-A DN construct was transfected into RAW264.7 cells followed by treatment with LPS. Message RNA (C) and protein (D) levels of pro-inflammatory mediators were examined by qPCR and ELISA. (E) RAW264.7 cells were treated with LPS and harvested at indicated time points for cytoplasmic/nuclear fractionation. Western blotting was performed with indicated antibodies. (F, G) MEFs from wild type or MRTF-A deficient mice were treated with or without LPS for 3 hours. Message RNA (F) and protein (G) levels of pro-inflammatory mediators were examined by qPCR and ELISA. (H) A 2x κ B reporter construct was transfected into WT or MRTF-A KO MEF cells with or without MRTF-A followed by treatment with LPS. Data are expressed RLU. *, p<.05. All quantitative data show the mean \pm S.D.

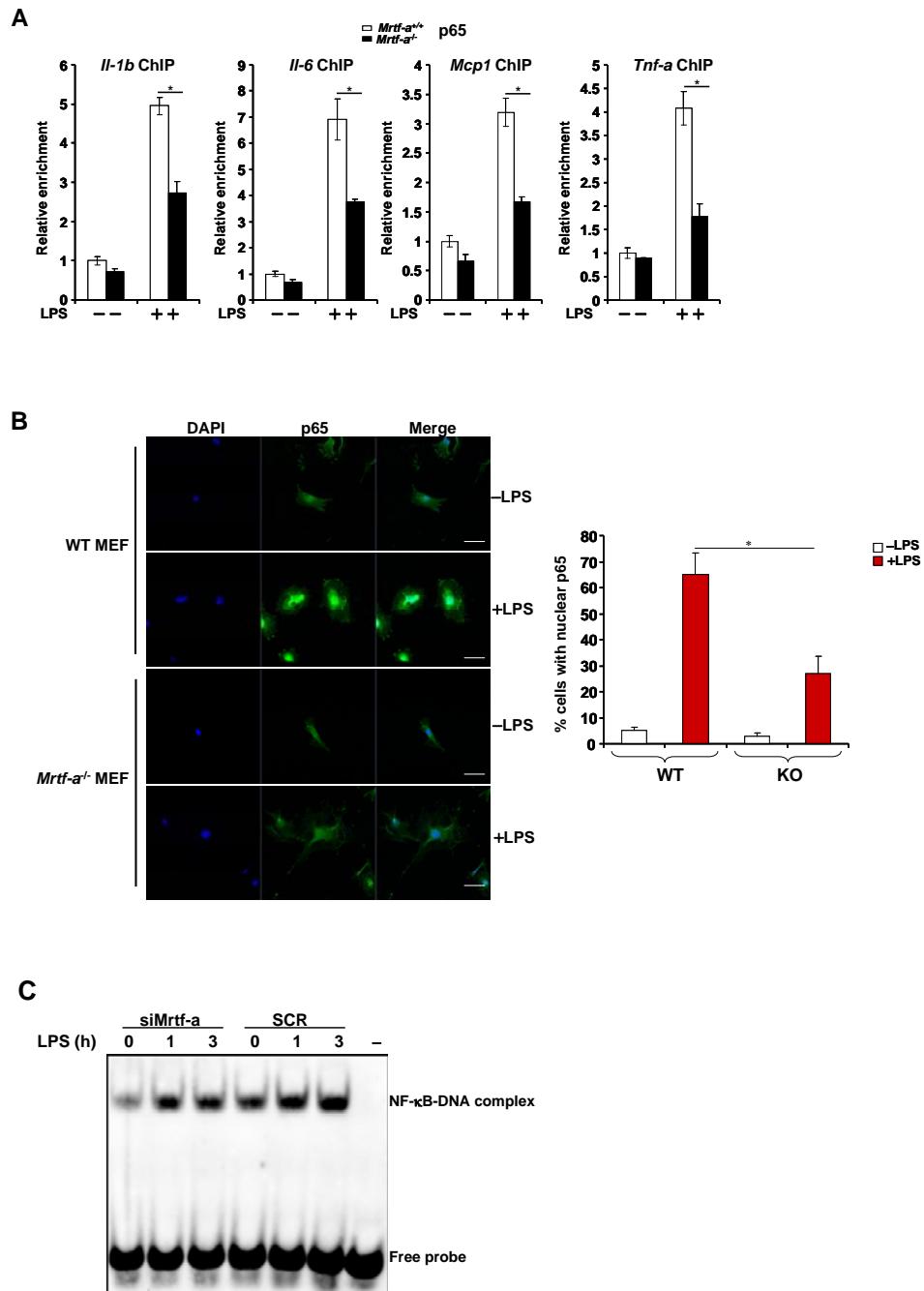
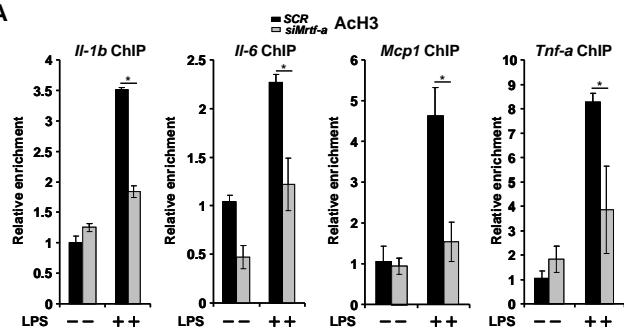
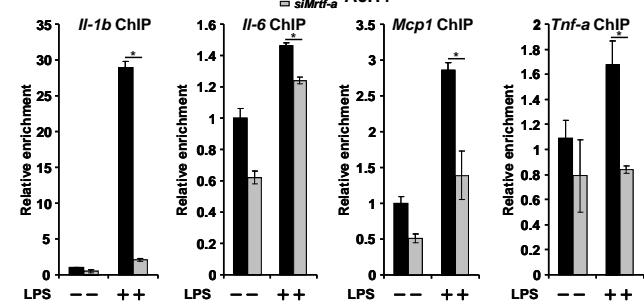
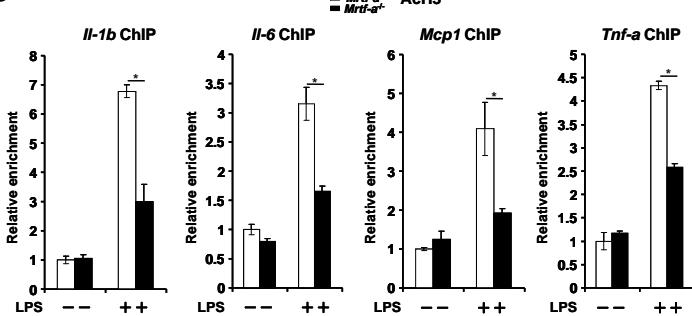
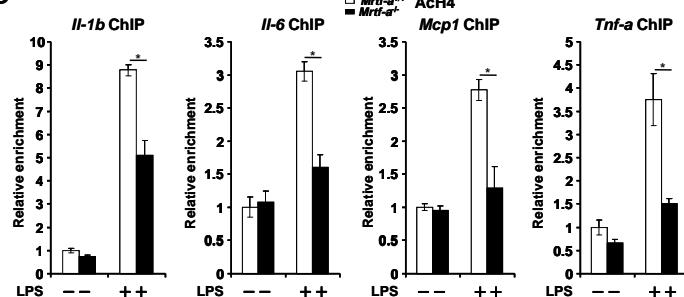
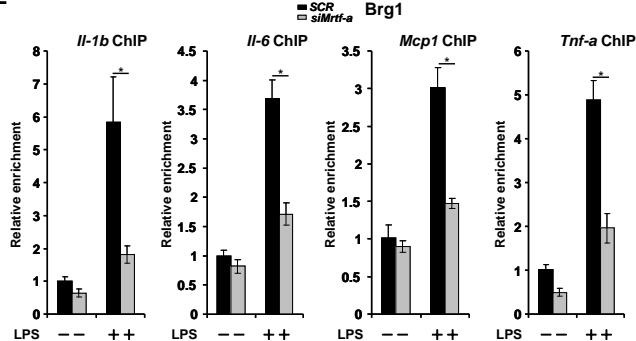


Fig.S2: (A) WT or MRTF-A KO MEF cells were treated with LPS for 3 hours and ChIP assay was performed with anti-p65. (B) WT or MRTF-A KO MEF cells were treated with LPS for 1 hour and immunofluorescence staining was performed with anti-p65. Scale bar, 20 μ m (C) RAW264.7 cells were transfected with indicated siRNAs followed by treatment with LPS. Gel shift assay was performed as described under *Methods*. *, $p < .05$. All quantitative data show the mean \pm S.D.

A**B****C****D****E**

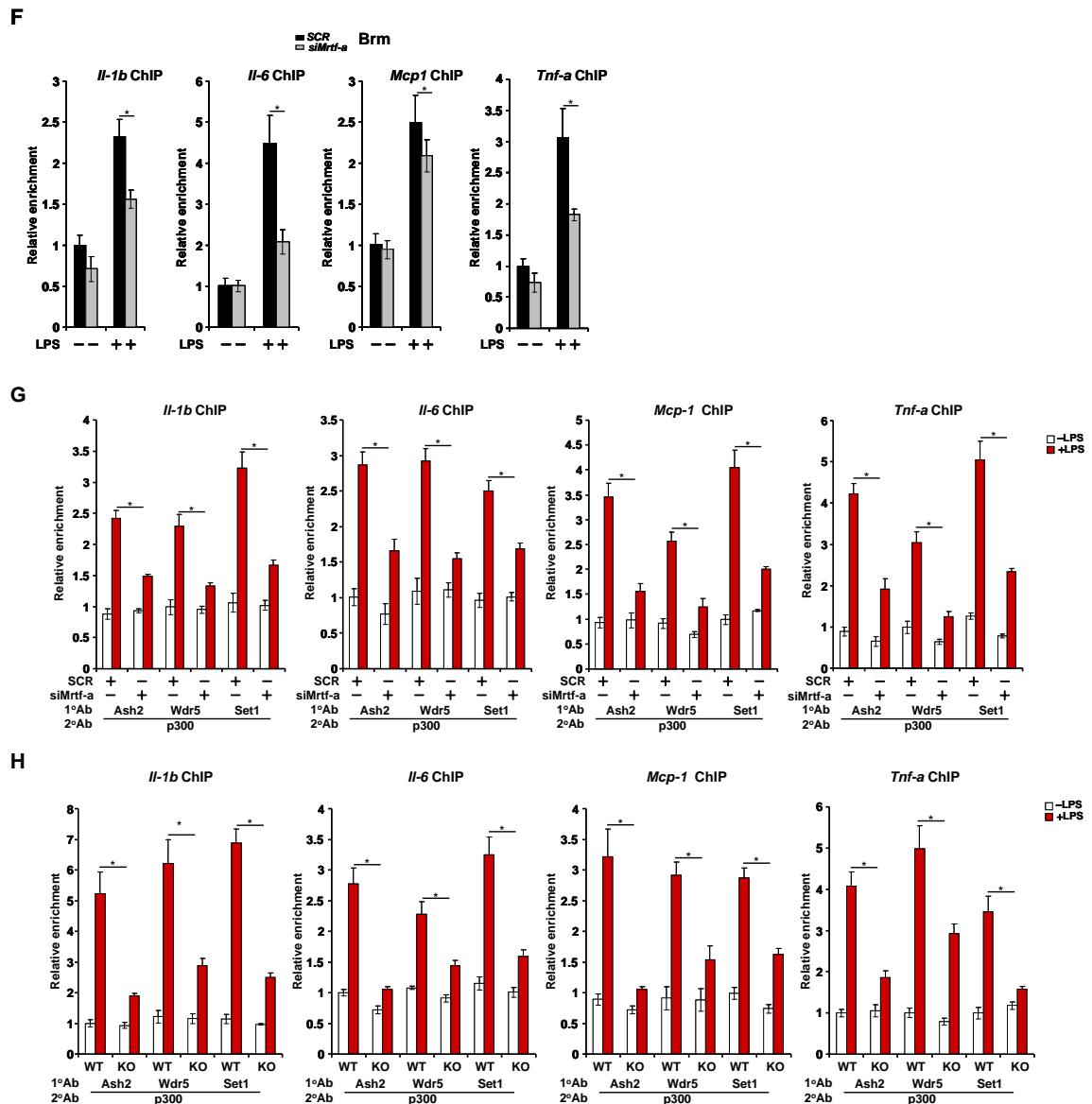


Fig.S3: (A, B) RAW264.7 cells were transfected with indicated siRNAs followed by treatment with LPS. ChIP assays were performed with anti-acetyl H3 (A) or anti-acetyl H4 (B). (C, D) WT or MRTF-A KO MEF cells were treated with LPS for 3 hours and ChIP assay was performed with anti-acetyl H3 (C) or anti-acetyl H4 (D). (E, F) RAW264.7 cells were transfected with indicated siRNAs followed by treatment with LPS. ChIP assays were performed with anti-Brg1 (E) or anti-Brm (F). (G) RAW264.7 cells were transfected with indicated siRNAs followed by treatment with LPS. Re-ChIP assays were performed with indicated antibodies. (H) WT or MRTF-A KO MEF cells were treated with LPS for 3 hours. Re-ChIP assays were performed with indicated antibodies. *, $p < .05$. All quantitative data show the mean \pm S.D.

Table 1: siRNA sequences

Mouse Mrtf-a	CATGGAGCTGGTGGAGAAGAA
Mouse p65	UGUGUCCAUUGUCUCACUC
Mouse Ash2	CGAGTCTGTTAGCCCTACAT
Mouse Wdr5	GCCGTTCATTTCAACCGTGAT
Mouse Set1	CAGCATATTATGAAAGCTGGA

Table 2: ChIP Real-time qPCR primers

Mouse Il-1	Forward: 5'-AACGGAGGAGGCCGTTGATATG -3'
	Reverse: 5'-AGAGCATCTTCCTAATGC-3'
Mouse Il-1 intron	Forward: 5'-AACGTCTGTGTCCGTGTG-3'
	Reverse: 5'-ACTCTATCCAGGGATTTAG-3'
Mouse Il-6	Forward: 5'- AGCTCATTCTGCTCTG-3'
	Reverse: 5'-AGATTGCACAATGTGACGTCG-3'
Mouse Il-6 intron	Forward: 5'-AAGGTCAAGACTAGACTGTG-3'
	Reverse: 5'-ATCCCCACCTAACGAAACGAATAG-3'
Mouse Mcp-1	Forward: 5'-CGTGGGAAAATCCAGTATTAAATG-3'
	Reverse: 5'-CAAATGTATCACCATGCAAATATGC-3'
Mouse Tnf	Forward: 5'-TGAGTTGATGTACCGCAGTCAAGA-3'
	Reverse: 5'-AGAGCAGCTTGAGAGTTGGGAAGT-3'
Mouse Cxcl2	Forward: 5'-CAACAGTGTACTTACGCAGACG-3'
	Reverse: 5'-CTAGCTGCCTGCCTCATTCTAC-3'