Published in final edited form as:

Eur J Immunol. 2016 October ; 46(10): 2322–2332. doi:10.1002/eji.201646315.

Acetylation impacts Fli-1 driven regulation of granulocyte colony stimulating factor (G-CSF)

Mara L. Lennard Richard¹, Danielle Brandon¹, Ning Lou^{1,3}, Shuzo Sato¹, Tomika Caldwell¹, Tamara K. Nowling¹, Gary Gilkeson^{1,2}, and Xian K. Zhang^{1,2}

¹Department of Medicine, Division of Rheumatology & Immunology, Medical University of South Carolina, Charleston, SC, USA

²Medical Research Service, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC, USA

³Jinan Central Hospital, Shandong University, Jinan, Shangdong, China

Abstract

Fli-1 has emerged as a critical regulator of inflammatory mediators, including MCP-1, CCL5, and IL-6. The cytokine, granulocyte colony stimulating factor (G-CSF) regulates neutrophil precursor maturation and survival, and activates mature neutrophils. Previously, a significant decrease in neutrophil infiltration into the kidneys of Fli-1^{+/-} lupus-prone mice was observed. In this study, a significant decrease in G-CSF protein expression was detected in stimulated murine and human endothelial cells when expression of Fli-1 was inhibited. The murine G-CSF promoter contains numerous putative Fli-1 binding sites and several regions within the proximal promoter are significantly enriched for Fli-1 binding. Transient transfection assays indicate that Fli-1 drives transcription from the G-CSF promoter and mutation of the Fli-1 DNA binding domain resulted in a 94% loss of transcriptional activation. Mutation of a known acetylation site, led to a significant increase in G-CSF promoter activation. The histone acetyltransferases p300/CBP and p300/CBP associated factor (PCAF) significantly decrease Fli-1 specific activation of the G-CSF promoter. Thus, acetylation appears to be an important mechanism behind Fli-1 driven activation of the G-CSF promoter. These results further support the theory that Fli-1 plays a major role in the regulation of several inflammatory mediators, ultimately affecting inflammatory disease pathogenesis.

Keywords

Cytokines; G-CSF; Fli-1; Gene regulation; Inflammation; Transcriptional factors

Please direct correspondence to: Dr. Xian K. Zhang, Division of Rheumatology and Immunology, 96 Jonathan Lucas St. Suite 912, MSC 637, Medical University of South Carolina, Charleston SC 29425-6370, USA. Phone Number: (843)-789-6800 Fax Number: (843)-876-5131, zhangjomusc.edu.

Author contributions – MLR and XKZ conceived the study, designed the experiments and prepared the manuscript, MLR designed all of the primers, obtained or cloned the constructs used, and performed all of the transfection experiments, the LPS stimulated ChIP assay, and mRNA analysis, DB and NL performed the siRNA experiments and ELISA, SS and TC performed the ChIP assay, TKN and GG contributed supplies, made suggestions regarding the experimental design and assisted in editing the final manuscript.

Conflict of Interest: The authors declare no commercial or financial conflict of interest.

Introduction

Granulocyte colony stimulating factor (G-CSF) is a glycoprotein and cytokine that can induce differentiation in myeloid leukemia cells [1, 2] and has been well characterized as a regulator of neutrophil precursor maturation and survival, and an activator of mature neutrophils [2–4]. G-CSF knockout mice appear to be healthy overall, but suffer from neutropenia with depleted peripheral blood neutrophils and the availability for rapid mobilization of neutrophils is significantly decreased. Additionally, these mice have a diminished capacity to handle *Listeria* infection [3]. Stimulation of mature human neutrophils was shown to cause up-regulation of interferon α (IFN α), suggesting the potential of a regulatory feedback loop for the production of neutrophils, since IFN α was shown to inhibit neutrophil colony formation [2, 5]. Synergistic interactions between G-CSF and other cytokines, including IL-4 and IL-6, were observed, with complex interactions resulting in the enrichment of neutrophilic progenitor cells [6] and promotion of hematopoietic colony formation [2, 7]. G-CSF has been shown to work with the CXC chemokine CXCL2 to mobilize neutrophils in the bone marrow during acute inflammation [8]. G-CSF mRNA and protein expression levels dramatically increase upon stimulation by LPS in a variety of cell types including endothelial cells [4, 9, 10]. G-CSF is routinely used in clinical settings to treat patients with neutropenia and as a therapeutic agent for those undergoing chemotherapy, by aiding in the mobilization of hematopoietic stem cells [4].

As a cytokine, G-CSF can act both as anti-inflammatory, activating neutrophils in defense of a bacterial infection [3, 11–13], and proinflammatory intensifying the inflammatory response, leading to increased vascular and tissue damage and ultimately inducing chronic inflammation in autoimmune diseases, such as chronic inflammatory arthritis and immune complex-mediated arthritis [10, 11, 14–16]. Therefore a delicate balance must be maintained in the expression and regulation of G-CSF. A previous study indicated that the primary regulatory region of the murine G-CSF promoter is from –2900bp to the transcription start site, with the highest amount of activity located between –289bp and –53bp [17]. Three specific regulatory regions were initially identified within the murine promoter, a CSF box similar to an NF- κ B binding element also located within GM-CSF and IL-3 genes, an octamer binding motif, and a third region with unknown function [17]. Three binding sites for the C/EBP (NF-IL6) transcription factor were also identified [9], one of the sites is located adjacent to the CSF box. The NF- κ B p65 transcription factor, but not p50 or a heterodimer of the two, was shown to activate transcription and bind to the CSF region of the G-CSF promoter in human embryonic lung fibroblasts, but not in Jurkat T-cells [18]. Interestingly, the C/EBP binding site was required for NF- κ B p65 transactivation and the two proteins (p65 and C/EBP) were shown to form a ternary complex when binding to the G-CSF promoter [18]. C/EBP binding to the site is induced by LPS stimulation, although binding and transcriptional activation were shown to be attenuated by the heat shock response and more specifically, heat shock factor 1 (HSF1)[9]. Stimulation of murine macrophages with LPS also was shown to increase binding of the Oct2 transcription factor to the proximal promoter of G-CSF, causing an induction of G-CSF mRNA and protein expression. Treatment of these cells with rapamycin leads to a reduction in Oct2 protein levels and binding to the G-CSF promoter [10].

G-CSF plays a role in the inflammatory response and has been implicated in the development of inflammatory diseases, including chronic inflammatory arthritis, rheumatoid arthritis, immune complex mediated arthritis and systemic lupus erythematosus (SLE) [10, 11, 14–16, 19, 20]. In patients with SLE, a subset of neutrophils referred to as low-density granulocytes (LDGs), produce more proinflammatory cytokines and exhibit increased cytotoxicity of endothelial cells. Additionally, stimulation of these LDGs with G-CSF causes a significant increase in IFN α and an induction of the type I IFN signature [15]. Endothelial cell dysfunction and the increase in type I IFN signature are mechanisms involved in the development of atherosclerosis and cardiovascular disease, a major health complication of patients with SLE [21–23].

The Ets family of transcription factors regulate cellular functions from tumor progression to the immune response and lymphoid cell development; one member of this gene family is friend leukemia insertion site 1 (Fli-1) [24–26]. Transcription factors in the Ets family traditionally bind to a short core consensus DNA binding motif GGAA/T through a winged helix turn helix domain [25, 27, 28] and are capable of regulating transcription through activation and repression [25, 29]. The well conserved DNA binding domain, consists of an α helical region, a basic region, and three conserved tryptophan residues [30]. Mutation of the third tryptophan abolishes DNA binding of Ets1 to the enhancer within Maloney sarcoma virus and mutation of this conserved amino acid abolishes binding of Fli-1 to the Ets binding domain and reduces activity within the collagen α 2(I) promoter [30–32]. Increasing evidence has demonstrated that the Fli-1 transcription factor is a key regulator of a number of inflammatory cytokines and chemokines [33–37], suggesting that Fli-1 may be more important in inflammation than previously recognized. A critical role for Fli-1 was demonstrated in the pathogenesis of SLE and the development of lupus nephritis [37–43]. Fli-1 effects the infiltration of a variety of inflammatory cells, including neutrophils, into the kidneys of lupus prone mice [37, 44]. In addition, two separate SNPs located in the intergenic region between Ets1 and Fli-1 were recently identified in a genome wide association study as having an association in rheumatoid arthritis within the Japanese and European populations [45, 46], further emphasizing the importance of Fli-1 in the inflammatory response.

Since G-CSF plays a dual role in inflammation and disease, strict regulation of this cytokine is necessary to maintain proper functioning. Based on our recent findings that Fli-1 critically regulates several chemokines and cytokines and the role G-CSF plays in neutrophil activation and the inflammatory response, this study was designed to determine if the Fli-1 transcription factor was involved in the regulation of G-CSF. The results herein demonstrate that Fli-1 regulates transcription of G-CSF, binding to the promoter and affecting protein expression in endothelial cells and acetylation is an important mechanism behind Fli-1-mediated regulation of the G-CSF promoter.

Results

Reduced Fli-1 expression results in decreased G-CSF protein expression in endothelial cells

In previous reports, we have demonstrated that a decrease of Fli-1 in endothelial cells results in a significant reduction of the expression levels, both mRNA and protein, of several inflammatory mediators [35–37]. G-CSF plays a role in the regulation of neutrophils and in patients with SLE a subset of the neutrophil population expresses increased inflammatory cytokines and cytotoxicity of endothelial cells [15]. Therefore, we chose to investigate the effect of Fli-1 on G-CSF expression in endothelial cells. Specific knockdown of Fli-1 was accomplished through transfection of both the murine MS1 endothelial cell line and primary human umbilical vein endothelial cells (HUVECs) with Fli-1 specific siRNA or control, non-specific siRNA. A significant decrease in Fli-1 protein expression was observed after transfection with siRNA (Figure 1A). Twenty four hours after transfection with siRNA, the endothelial cells were stimulated with LPS (1 µg/ml). MS1 endothelial cells were harvested 0, 6 and 24 hours after stimulation, mRNA extracted using TRIzol reagent and real-time PCR performed to determine the effect of siRNA knockdown on mRNA expression. As indicated in Figure 1B, there was no difference in G-CSF mRNA expression prior to LPS stimulation. The average Ct values for the negative control and Fli-1-specific siRNA were near 40 prior to stimulation. Ct values for both sample sets decreased upon LPS stimulation, yet clear differences were observed in the samples transfected with Fli-1 siRNA 6 and 24 hours after LPS stimulation; G-CSF mRNA expression was reduced upon Fli-1 knockdown (Figure 1B). Cell supernatants were collected prior to and after stimulation (0hrs), 6, 12, and 24 hours and the G-CSF protein concentrations measured. An increase in the G-CSF protein concentration 24 hours after stimulation with LPS was observed for the control siRNA transfected MS1 cells (Figure 1C). The G-CSF protein concentration level prior to LPS stimulation was not detectable. A statistically significant decrease in G-CSF protein concentration was measured 6, 12, and 24 hours post-LPS stimulation in MS1 cells transfected with Fli-1 specific siRNA compared to controls (Figure 1C). To investigate if Fli-1 affects G-CSF in human cells in a similar manner, primary HUVECs were transfected with siRNA and Fli-1 protein levels were inhibited after knockdown with Fli-1 specific siRNA (Figure 1D). A similar statistically significant decrease in G-CSF protein expression was detected in HUVEC cells transfected with Fli-1 specific siRNA 6 and 24 hours after LPS stimulation (Figure 1E).

Fli-1 binds primarily to the proximal promoter of G-CSF

Sixty putative Ets binding sites were identified within a 2900bp region of the G-CSF promoter and 16 primer pairs were designed to capture these sites using ChIP assay (Figure 2A and Supporting Information Table 1). Binding of Fli-1 was tested by ChIP assay in the MS1 endothelial cell line; the crosslinked protein-DNA complexes were amplified by real-time PCR to calculate the amount of DNA precipitated. Several sites within the proximal promoter showed enrichment for Fli-1 (Supporting Information Table 2). Figure 2B shows the four sites (ChIP 3, 4, 6, 7) within the proximal promoter region with the highest enrichment for the Fli-1 specific antibodies compared to normal IgG controls. The regions of the promoter covered by the ChIP4 and ChIP7 primers showed the highest fold change

(Fli-1/IgG), while more distal regions ChIP13, ChIP14 and ChIP 16 showed no enhancement. No binding was observed for the ChIP11 primer set. The effect of LPS stimulation after 24 hours on Fli-1 binding was also examined by ChIP assay. Eleven out of sixteen of the primer pairs showed increased binding after stimulation with LPS, although the overall binding results remained consistent with the proximal region of the promoter showing the majority of the enrichment for Fli-1 (Supporting Information Table 3). In this experiment, binding to the ChIP5 region increased and the ChIP4 and ChIP7 regions still showed the highest fold change.

Dose dependent transcriptional activation of the G-CSF promoter is mediated by Fli-1

To investigate whether Fli-1 activates transcription from the G-CSF promoter, transient transfection assays were performed in NIH3T3 cells, which contain undetectable amounts of endogenous Fli-1 (Figure 3B, lanes 1 and 2). A 2946bp fragment of the G-CSF promoter, upstream of the transcription start site and covering a previously defined region of the promoter that includes several potential enhancing elements [17], was cloned into the pGL3 basic vector to determine transcriptional activation. As increasing amounts of Fli-1 were added, the transcriptional activation from the G-CSF promoter increased significantly, in a dose-dependent manner, and as little as 25ng of Fli-1 was needed to increase the transcriptional activation from the G-CSF promoter over the reporter construct alone (Figure 3A). All of the concentrations of Fli-1 transfected into the cells led to a statistically significant increase in G-CSF promoter activation. The expression of Fli-1 protein corresponding to the amount of Fli-1 expressing plasmids was confirmed (Figure 3B).

Deletion of the G-CSF promoter reveals important regions for Fli-1 activation

ChIP assay results indicate that Fli-1 likely binds preferentially to the proximal portion of the G-CSF promoter (from -1275bp, the ChIP7 region, to the transcription start site). Therefore, a series of deletion constructs were designed to systematically remove portions of the G-CSF promoter in order to determine regions important for Fli-1 binding and activation. A schematic diagram of the G-CSF promoter, the location of the ChIP primers, and the deletion constructs can be found in Figure 4A and Supporting Information Table 1. Deletion of the distal half of the promoter leads to a 43% reduction in Fli-1 activation when compared to the full-length promoter. In some instances, removal of a portion of the promoter led to increased activity in response to Fli-1 (regions B and D), which suggests the presence of transcriptional repressors that may bind to the promoter. The most dramatic decreases in activity were seen for regions C, F, and G (Figure 4B). Region C corresponds to the removal of ChIP 6 and 7, and F the removal of ChIP3 and 4, which are the regions that showed the strongest enrichment for Fli-1 binding (Figure 2B and Supporting Information Tables 2 and 3). An additional eight putative Ets binding sites are removed in region G, which results in a further loss of transcriptional activity (Figure 4B).

The Fli-1 DNA binding domain is critical for activation of the G-CSF promoter

Fli-1 was previously shown to activate transcription from the MCP-1 promoter through primarily indirect binding and directly binding to the CCL5 promoter [34, 35]. Therefore, we next investigated whether Fli-1 activation of transcription from the G-CSF promoter is through direct binding. A Fli-1 expression construct with a single amino acid change in the

DNA binding domain demonstrated to prevent binding [31, 32], was transfected into the NIH3T3 cells along with the full-length G-CSF promoter construct. Mutation of the Fli-1 DNA binding domain led to a statistically significant reduction in activation from the G-CSF promoter when compared to wild-type Fli-1. Overall, 94% of Fli-1 driven activation was lost upon mutation of the DNA binding domain of Fli-1 (Figure 5A). A representative western blot of the transfected cell lysates was run to demonstrate that similar amounts of Fli-1 and the DNA binding mutant protein were expressed (Figure 5B).

Acetylation plays a role in Fli-1 driven activation of the G-CSF promoter; PCAF and p300 are involved

To gain a better understanding of the mechanisms behind the regulation of the G-CSF promoter by Fli-1, we utilized a Fli-1 expression construct that contains a mutation (lysine to arginine) at amino acid 380 within the Fli-1 protein. Mutation of this site prevents acetylation of the protein and was previously described to nullify Fli-1 driven repression of the collagen promoter [47]. Figure 6A clearly demonstrates that preventing acetylation of the aa380 site, leads to a statistically significant increase in Fli-1 driven activation of the G-CSF promoter. Proteins for the wild-type Fli-1 and acetylation mutant were expressed at similar levels as shown in the western blot (Figure 6B). These results indicate that acetylation is one mechanism behind Fli-1 driven activation of the G-CSF promoter. In order to further investigate the role acetylation plays, we next tested the impact of the acetyltransferases PCAF and p300 previously shown to interact with Fli-1 [47, 48]. As illustrated in Figure 6C, transfection of Fli-1 into the NIH3T3 cells along with the G-CSF promoter leads to a characteristic increase in promoter activation. When PCAF and p300 were transfected into the cells along with the G-CSF promoter construct, a slight, but significant increase was observed for the G-CSF promoter with the addition of PCAF; no difference was detected with p300. A statistically significant decrease in G-CSF promoter activation was detected when Fli-1 was co-transfected with PCAF or p300 (Figure 6C). To assess whether the observed effects of PCAF and p300 were related to the acetylation site at aa380, co-transfection between these constructs and the Fli-1 acetylation mutant were performed. As shown in Figure 6D, no difference in transcriptional activation was detected upon co-transfection of the Fli-1 acetylation mutant and the acetyltransferases PCAF and p300, indicating their primary mode of action is through the acetylation site at aa380 (Figure 6D).

Discussion

Our previous studies have indicated that the Fli-1 transcription factor plays a critical role in the regulation of several inflammatory chemokines and cytokines [34–37] and specifically affects the infiltration of inflammatory cells, including neutrophils, into the kidneys of lupus prone mice [37, 44]. The maturation, survival and activation of neutrophils are dependent on regulation from the cytokine G-CSF [2, 3]. G-CSF requires tight regulation to maintain an adequate balance between anti- and proinflammatory roles within the immune system [3, 10–15, 49]. Here, for the first time, we demonstrate that the Fli-1 transcription factor regulates the expression of the G-CSF cytokine, in human and murine endothelial cells. Specific knockdown of the Fli-1 gene leads to a significant decrease in G-CSF protein

expression in both murine MS1 endothelial cells and HUVEC cells. Fli-1 activates transcription of the G-CSF gene primarily by binding directly to the proximal region of the promoter. Additionally, we show that acetylation of the Fli-1 protein affects the ability of Fli-1 to regulate transcription of G-CSF. Ultimately, this data provides a previously unknown, key mechanism for the direct regulation of the cytokine G-CSF by the Fli-1 transcription factor.

The proximal region of the G-CSF promoter has been shown to be of primary importance to G-CSF gene transactivation [17]. Consistent with this finding, our results indicate that Fli-1 activates transcription primarily through the proximal region of the G-CSF promoter. The core region of the G-CSF promoter was previously identified to be between -289 to -53bp [17] and our results show enrichment for Fli-1 within this region (ChIP1 and 2, Supporting Information Tables 2 and 3). At least two putative Fli-1 binding sites are located within 10bp of the known CSF box (NF- κ B binding site) and the octamer binding site [17], suggesting the potential for protein-protein interactions with these factors. Fli-1 interacts with the p65 subunit of NF- κ B on the MCP-1 promoter [34] and p65 activates G-CSF in human lung fibroblasts [18], suggesting that these two proteins may work together to activate G-CSF in endothelial cells. Despite the clear importance of the -289 to -53bp region of the G-CSF promoter, Fli-1 exhibited the most significant enrichment for the ChIP 4 and 7 primer sets (Figure 2B). Deletion of the distal region of the G-CSF promoter resulted in a 43% loss of activity, indicating the likelihood that factors other than Fli-1 regulate transcription within this portion of the promoter (Figure 4). Surprisingly, removal of ChIP7 (region B), saw an increase in transcriptional activity, indicating the possible presence of transcriptional repressors within this region. Combined, our results suggest that regions corresponding to ChIP 2, 3, and 6 are most likely to bind Fli-1 (Figure 4). While these studies narrow down the potential Fli-1 binding sites, there are still 16 – 18 putative sites that Fli-1 could bind, making it difficult to pinpoint the specific sites occupied by Fli-1.

LPS stimulation of both murine (MS1) and human (HUVEC) endothelial cells caused an increase in G-CSF protein expression, which is consistent with previous reports [9, 10], while specific knockdown of Fli-1 through siRNA triggered a significant decrease in G-CSF protein expression (Figure 1). LPS stimulation improved binding of C/EBP and Oct2 to the G-CSF promoter [9, 10] and ChIP assay results from this study indicate that LPS increases Fli-1 binding to proximal promoter regions (Supporting Information Table 3). Thus it appears that the induction of neutrophils as part of the inflammatory response to bacterial infection is largely dependent on the increased binding of several transcription factors to the G-CSF promoter. Further evidence of the importance of Fli-1 binding to the G-CSF promoter was discovered upon mutation of the Fli-1 DNA binding domain, which resulted in a 94% loss in G-CSF promoter activation (Figure 5). These results indicate that Fli-1 binding directly to the G-CSF promoter is a key method for regulation of this cytokine gene.

To further elucidate the mechanisms behind the activation of G-CSF by Fli-1, we analyzed whether acetylation of Fli-1 may be involved. Fli-1 can act both as an activator or repressor [25, 29] and repression of the human α 2(I) collagen promoter by Fli-1 is directly affected by acetylation of the Fli-1 protein. Acetylation at aa380 leads to dissociation of Fli-1 from the promoter and a decrease in Fli-1 driven repression [47]. Mutation of the acetylation site at

aa380, leads to a significant increase in Fli-1 activation of the G-CSF promoter (Figure 6). These results strongly suggest that acetylation, particularly at the aa380 lysine residue, is an important mechanism behind Fli-1 driven activation of the G-CSF promoter. Similar to the collagen promoter, it may be that acetylation leads to dissociation of Fli-1 from the promoter, thus inhibiting acetylation leads to increased activation of G-CSF by Fli-1. It appears that similar mechanisms are involved in the Fli-1 mediated regulation of G-CSF as those involved in the regulation of the collagen promoter in dermal fibroblasts; yet, since Fli-1 represses collagen and activates the G-CSF promoter, opposing results are achieved.

Our results seem to indicate a fundamental importance on the direct binding of Fli-1 to the G-CSF promoter, which may be influenced by both acetylation of the Fli-1 protein and LPS stimulation. To our knowledge, this is the first report on Fli-1 mediated cytokine activation that points to acetylation as a key mechanism of activation. In addition, protein-protein interactions with other transcription factors may also play a role in Fli-1 mediated activation, given the proximity of several Ets binding sites to known NF- κ B and octamer binding motifs [17]. Previous studies have reported the significance of several of these mechanisms individually in Fli-1 mediated gene regulation [34, 35, 47, 48], this study suggests that all of these mechanisms may be important for G-CSF promoter activation. It is becoming increasingly evident that the Fli-1 transcription factor is a major regulator of inflammatory chemokines and cytokines [34–37, 44].

The infiltration and activation of neutrophils and endothelial cell dysfunction are involved in the progression of a variety of inflammatory diseases, including atherosclerosis and cardiovascular disease. Fli-1 affects neutrophil infiltration into kidneys of lupus prone mice [37, 44] and we report here that Fli-1 regulates transcription of G-CSF, which activates mature neutrophils. Additionally, we have found that Fli-1 regulates the chemokine, CXCL2 (Lennard Richard et al., unpublished observations), which triggers the mobilization and release of neutrophils from bone marrow. Thus, suggesting that Fli-1 is a critical regulator for the coordinated activation of neutrophils and ultimately the development of a variety of inflammatory diseases from cardiovascular disease to rheumatoid arthritis and SLE. Uncovering the specific mechanisms behind Fli-1 mediated activation of inflammatory chemokines and cytokines, may lead to better targets for treatment of these and other diseases.

Materials and Methods

Cell lines

The murine endothelial MS1 cell line was purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech Inc., Manassas, VA) with 5% fetal bovine serum. The human umbilical vein endothelial cells were purchased from LONZA (Allendale, NJ), maintained in Endothelial Growth Media-2 (EGM-2) according to the manufacturer's instructions and used for siRNA transfection. The mouse embryonic fibroblast cell line, NIH3T3, grown in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin was used for all transient transfection experiments since it is a commonly used cell line for DNA transfection and the expression

level of Fli-1 in these cells is not detectable, eliminating any issues that may arise with endogenous Fli-1 expression. All of the cells were maintained at 37°C with 5% CO₂.

siRNA Transfection

siRNA transfections were carried out using Fli-1 specific and negative control siRNA purchased from Invitrogen (Life Technologies). Lipofectamine (Invitrogen/Life Technologies) was used to transfect both the MS1 endothelial cell line and the HUVEC cells following the manufacturer's instructions. The transfected cells were cultured for 24hrs in the appropriate media and stimulated by adding 1µg of LPS (Sigma-Aldrich, St. Louis, MO) to each well and incubated at 37°C for up to 24 hours. The supernatants were collected at the following time points after stimulation (0, 6, 12 and 24 hours) and G-CSF cytokine production measured by ELISA.

RNA Extraction and real-time PCR

Total RNA was extracted from cells using the TRIzol reagent (Ambion, Life Technologies) following the manufacturer's instructions. DNase I digestion was performed using the on-column treatment described in the RNeasy Mini Kit (Qiagen, Germantown, MD). First strand synthesis with equivalent amounts of RNA was completed using the RT² First Strand Kit (Qiagen) prior to real-time PCR. The RT² SYBR Green Mastermix and primers to the murine granulocyte CSF3 (Accession Number NM_009971.1) and GAPDH housekeeping gene (Accession Number NM_008084) were used to carry out real-time PCR analysis. Relative mRNA expression and standard deviations were calculated using the comparative Ct method (2^{-Ct}) as described by the Applied Biosystems manual (Life Technologies).

ELISA

G-CSF protein concentrations in the supernatants collected from the MS1 and HUVEC cells were measured by ELISA using the DuoSet® ELISA Development System for Mouse and Human G-CSF (R&D Systems Inc.) following the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP) Assay

A Fli-1 rabbit polyclonal antibody was used for the ChIP assay, which was carried out as in previous studies [35, 37, 50, 51]. Chromatin was isolated from cross-linked MS1 endothelial cells that were immunoprecipitated by antibodies specific for Fli-1 or rabbit IgG as a control. The G-CSF promoter was screened for putative Ets-1 DNA binding sites (EBSs) through visual inspection of the 2946bp promoter and utilizing the MatInspector software tool (Genomatix, Ann Arbor, MI [52]). Sixteen primer pairs were designed to cover the 60 putative EBSs identified and can be found in Supporting Information Table 1. RT-PCR (real-time PCR) using these primers to amplify genomic fragments from the immunoprecipitated DNA was performed and gel electrophoresis used to visualize the results. Fold change was calculated using the comparative Ct method $2^{-(\Delta Ct)}$ where delta Ct was the difference between IgG and Fli-1 Ct values. ChIP assay with LPS stimulation was performed as described above, two sterile petri dishes of MS1 endothelial cells were used, one stimulated with LPS (1µg/ml). Cells were harvested for ChIP assay 24 hours after LPS stimulation.

Reporter and Expression Constructs

The full-length (2946bp) murine G-CSF promoter was cloned from genomic DNA obtained from a B6 mouse. The forward primer contains a *KpnI* enzyme site and the reverse primer, which was modified from a previously described primer [10], contains an *XhoI* enzyme site for cloning into the pGL3 basic vector (Promega, Madison, WI). The sequences for the forward and reverse primers can be found in Supporting Information Table 4. The full-length promoter sequence was PCR amplified using the following program: 94°C for 3min.; thirty cycles of 94°C for 30sec, 59°C for 30sec, 72°C for 1min; 72°C for 10min; with final incubation at 4°C. The G-CSF promoter was subsequently cloned into the pGL3 basic vector upstream of the Luciferase reporter gene. Seven G-CSF promoter constructs (designated Regions A – G) were also designed to exclude putative EBSs within the proximal region of the promoter. Details on the specific sequences for these primers can be found in Supporting Information Table 2. The PCR programs for amplifying the deleted regions were as follows: For Regions A, D, F, and G –94°C for 3min.; thirty cycles of 94°C for 30sec, 60°C for 30sec, 72°C for 1min; 72°C for 10min; with a final hold at 4°C; and for Regions B,C and E –94°C for 3min.; thirty cycles of 94°C for 30sec, 58°C for 30sec, 72°C for 1min; 72°C for 10min; with a final hold at 4°C. The deletion constructs were all directionally cloned into the pGL3 basic vector upstream of the Luciferase reporter gene. The enzymes used in the cloning process were purchased from New England BioLabs Inc. (Ipswich, MA). Sequences for all of the promoter constructs were confirmed by DNA sequencing (Genewiz, South Plainfield, NJ). Several expression vectors were used in these studies and are described below. The mouse Fli-1 gene cloned into the pcDNA3.0 expression vector (Life Technologies) contains a 5' kozak sequence and Flag tag and has been described previously [35]. The following expression vectors were generously provided by Dr. Maria Trojanowska (Boston University, School of Medicine Arthritis Center, Boston, MA): The human Fli-1 DNA binding domain mutant, cloned into the pSG5 expression vector (Agilent Technologies, Santa Clara, CA), which contains a single amino acid mutation (Tryptophan 321 to Arginine) preventing Fli-1 from binding to DNA; the Fli-1 acetylation mutant, also in pSG5 (with Lysine 380 replaced by Arginine); and the PCAF expression vector, all of which have been described previously [32, 35, 47]. The pcDNA/p300/CBP (p300) was a gift from Tso-Pang Yao (Addgene plasmid #32908) and was obtained through Addgene (Cambridge, MA) and has been described in the following publications [53].

DNA Transfection

For all of the transient transfection studies, 24 hours prior to transfection 4×10^5 NIH3T3 cells were seeded in 6 well plates. The cells were transfected using the manufacturer's instructions for the Fugene 6 transfection reagent (Promega). The reporter constructs were transfected into the cells at a concentration of 2µg (pGL3/basic, pGL3/GCSF, pGL3/GCSFRegionA, pGL3/GCSFRegionB, pGL3/GCSFRegionC, pGL3/GCSFRegionD, pGL3/GCSFRegionE, pGL3/GCSFRegionF, and pGL3/GCSFRegionG) for all experiments. All expression constructs (pcDNA3.0, pcDNA/Fli1, pSG5, pSG5/Fli1, pSG5/Fli1DNAbindMut, pSG5/AceMut, pCX/PCAF, and pcDNA/p300) were transfected into the cells at equimolar concentrations. The pcDNA/Fli1 expression construct was transfected into the cells in increasing concentrations (0.025µg, 0.05µg, 0.1µg, 0.2µg, 0.25µg, 0.5µg, and 1µg) for the dose response study. In the deletion experiment, the acetylation mutant experiment, and the

acetyltransferase/deacetylase experiments 0.5µg of the pcDNA/Fli1, pSG/Fli1, pSG/Fli1Mut, pSG/Fli1AceMut expression constructs were transfected into the cells. In the DNA binding domain mutant experiment 2µg of the pSG/Fli1 construct was used. In the acetyltransferase experiments three times as much or 1.5 µg of the PCAF and p300 expression constructs were transfected into the cells, based on previous results [47]. In all cases empty expression vectors were used such that an equivalent amount of total DNA was transfected into the cells. A *Renilla* luciferase construct pRL/TK (Promega) at a concentration of 200ng was used to control for transfection efficiency. The NIH3T3 cells were harvested 48 hours after the transfection experiment was completed.

Luciferase Reporter Assays

The Dual-luciferase reporter assay system (Promega) was utilized to determine the amount of luciferase activity for each experiment. Luminescence was measured on the Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific, Waltham, MA). The *Renilla* luciferase construct was used to normalize the activity for all transfections. The values reported are mean $\pm$ standard error for fold activation based on the activity compared to the empty pGL3/basic vector.

Western blotting

Western blot analysis was performed on cell lysates collected from the siRNA transfections and the luciferase assay. Twenty micrograms of the cell lysates were run on a 4–15% Criterion TGX Precast gel (Bio-Rad, Hercules, CA) for two hours at 120V. Transferred proteins were probed with both a Fli-1 polyclonal antibody described previously [37] and an antibody to β -actin (Cell Signaling, Beverly, MA). The results were visualized using the Odyssey Imaging System (LI-COR, Lincoln, NE).

Statistics

For the transient transfection studies and relative mRNA expression, statistical significance was calculated using a two tailed Student's *t*-test based on observed variances (unequal or equal). For other experiments where significance is reported a one tailed Student's *t*-test with Welch's correction was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Mercedes Zapata-Garcia for her technical support and Dr. Maria Trojanowska (Boston University, School of Medicine Arthritis Center, Boston, MA) who provided several expression vectors. This research study was supported by the National Institute of Health grant (NIAMS, AR056670 to X.K.Z.). M.L.R is a recipient of Careers in Immunology Fellowships from American Association of Immunologists (AAI) in 2015.

Abbreviations

CBP CREB binding protein

CCL5	C-C motif ligand 5 or RANTES
C/EBP	CCAAT enhancer binding protein
EBS	Ets binding site
Ets	E26 transformation-specific family of transcription factors
Fli-1	friend leukemia insertion site 1
LDG	low-density granulocyte
PCAF	p300/CBP associated factor
SLE	systemic lupus erythematosus

References

1. Nicola NA, Metcalf D, Matsumoto M, Johnson GR. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. *J Biol Chem.* 1983; 258:9017–9023. [PubMed: 6190815]
2. Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. *Blood.* 1991; 78:2791–2808. [PubMed: 1720034]
3. Lieschke GJ, Grail D, Hodgson G, Metcalf D, Stanley E, Cheers C, Fowler KJ, Basu S, Zhan YF, Dunn AR. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood.* 1994; 84:1737–1746. [PubMed: 7521686]
4. Bendall LJ, Bradstock KF. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev.* 2014; 25:355–367. [PubMed: 25131807]
5. Shirafuji N, Matsuda S, Ogura H, Tani K, Kodo H, Ozawa K, Nagata S, Asano S, Takaku F. Granulocyte colony-stimulating factor stimulates human mature neutrophilic granulocytes to produce interferon-alpha. *Blood.* 1990; 75:17–19. [PubMed: 1688496]
6. Sonoda Y, Okuda T, Yokota S, Maekawa T, Shizumi Y, Nishigaki H, Misawa S, Fujii H, Abe T. Actions of human interleukin-4/B-cell stimulatory factor-1 on proliferation and differentiation of enriched hematopoietic progenitor cells in culture. *Blood.* 1990; 75:1615–1621. [PubMed: 1691665]
7. Rennick D, Jackson J, Yang G, Wideman J, Lee F, Hudak S. Interleukin-6 interacts with interleukin-4 and other hematopoietic growth factors to selectively enhance the growth of megakaryocytic, erythroid, myeloid, and multipotential progenitor cells. *Blood.* 1989; 73:1828–1835. [PubMed: 2469502]
8. Wengner AM, Pitchford SC, Furze RC, Rankin SM. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood.* 2008; 111:42–49. [PubMed: 17928531]
9. Zhang L, Yang M, Wang Q, Liu M, Liang Q, Zhang H, Xiao X. HSF1 regulates expression of G-CSF through the binding element for NF-IL6/CCAAT enhancer binding protein beta. *Mol Cell Biochem.* 2011; 352:11–17. [PubMed: 21455770]
10. Chou YY, Gao JI, Chang SF, Chang PY, Lu SC. Rapamycin inhibits lipopolysaccharide induction of granulocyte-colony stimulating factor and inducible nitric oxide synthase expression in macrophages by reducing the levels of octamer-binding factor-2. *FEBS J.* 2011; 278:85–96. [PubMed: 21114628]
11. Haley M, Parent C, Cui X, Kalil A, Fitz Y, Correa-Araujo R, Natanson C, Danner RL, Banks SM, Eichacker PQ. Neutrophil inhibition with L-selectin-directed MAb improves or worsens survival dependent on the route but not severity of infection in a rat sepsis model. *J Appl Physiol.* 1985; 2005; 98:2155–2162.

12. Boneberg EM, Hartung T. Molecular aspects of anti-inflammatory action of G-CSF. *Inflamm Res*. 2002; 51:119–128. [PubMed: 12005202]
13. Root RK, Dale DC. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor: comparisons and potential for use in the treatment of infections in nonneutropenic patients. *J Infect Dis*. 1999; 179(Suppl 2):S342–S352. [PubMed: 10081506]
14. Lawlor KE, Campbell IK, Metcalf D, O'Donnell K, van Nieuwenhuijze A, Roberts AW, Wicks IP. Critical role for granulocyte colony-stimulating factor in inflammatory arthritis. *Proc Natl Acad Sci U S A*. 2004; 101:11398–11403. [PubMed: 15272075]
15. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, McCune WJ, Kaplan MJ. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol*. 2010; 184:3284–3297. [PubMed: 20164424]
16. Christensen AD, Haase C, Cook AD, Hamilton JA. Granulocyte colony-stimulating factor (G-CSF) plays an important role in immune complex-mediated arthritis. *Eur J Immunol*. 2016; 46:1235–1245. [PubMed: 26848119]
17. Nishizawa M, Tsuchiya M, Watanabe-Fukunaga R, Nagata S. Multiple elements in the promoter of granulocyte colony-stimulating factor gene regulate its constitutive expression in human carcinoma cells. *J Biol Chem*. 1990; 265:5897–5902. [PubMed: 1690717]
18. Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA, Shannon MF. Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood*. 1994; 83:2469–2479. [PubMed: 7513199]
19. Cornish AL, Campbell IK, McKenzie BS, Chatfield S, Wicks IP. G-CSF and GM-CSF as therapeutic targets in rheumatoid arthritis. *Nat Rev Rheumatol*. 2009; 5:554–559. [PubMed: 19798030]
20. Eyles JL, Roberts AW, Metcalf D, Wicks IP. Granulocyte colony-stimulating factor and neutrophils--forgotten mediators of inflammatory disease. *Nat Clin Pract Rheumatol*. 2006; 2:500–510. [PubMed: 16951705]
21. Denny MF, Thacker S, Mehta H, Somers EC, Dodick T, Barrat FJ, McCune WJ, Kaplan MJ. Interferon-alpha promotes abnormal vasculogenesis in lupus: a potential pathway for premature atherosclerosis. *Blood*. 2007; 110:2907–2915. [PubMed: 17638846]
22. Thacker SG, Berthier CC, Mattinzoli D, Rastaldi MP, Kretzler M, Kaplan MJ. The detrimental effects of IFN-alpha on vasculogenesis in lupus are mediated by repression of IL-1 pathways: potential role in atherogenesis and renal vascular rarefaction. *J Immunol*. 2010; 185:4457–4469. [PubMed: 20805419]
23. Mak A, Kow NY. Imbalance between endothelial damage and repair: a gateway to cardiovascular disease in systemic lupus erythematosus. *Biomed Res Int*. 2014; 2014:178721. [PubMed: 24790989]
24. Dittmer J. The biology of the Ets1 proto-oncogene. *Mol Cancer*. 2003; 2:29. [PubMed: 12971829]
25. Sementchenko VI, Watson DK. Ets target genes: past, present and future. *Oncogene*. 2000; 19:6533–6548. [PubMed: 11175369]
26. Watson DK, Smyth FE, Thompson DM, Cheng JQ, Testa JR, Papas TS, Seth A. The ERGB/Fli-1 gene: isolation and characterization of a new member of the family of human ETS transcription factors. *Cell Growth Differ*. 1992; 3:705–713. [PubMed: 1445800]
27. Hsu T, Trojanowska M, Watson DK. Ets proteins in biological control and cancer. *J Cell Biochem*. 2004; 91:896–903. [PubMed: 15034925]
28. Karim FD, Urness LD, Thummel CS, Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA, Gunther CV, Nye JA, et al. The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev*. 1990; 4:1451–1453. [PubMed: 2253872]
29. Li R, Pei H, Watson DK. Regulation of Ets function by protein - protein interactions. *Oncogene*. 2000; 19:6514–6523. [PubMed: 11175367]
30. Wang CY, Petryniak B, Ho IC, Thompson CB, Leiden JM. Evolutionarily conserved Ets family members display distinct DNA binding specificities. *J Exp Med*. 1992; 175:1391–1399. [PubMed: 1569404]

31. Bailly RA, Bosselut R, Zucman J, Cormier F, Delattre O, Roussel M, Thomas G, Ghysdael J. DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. *Mol Cell Biol.* 1994; 14:3230–3241. [PubMed: 8164678]
32. Czuwara-Ladykowska J, Shirasaki F, Jackers P, Watson DK, Trojanowska M. Fli-1 inhibits collagen type I production in dermal fibroblasts via an Sp1-dependent pathway. *J Biol Chem.* 2001; 276:20839–20848. [PubMed: 11278621]
33. Ichimura Y, Asano Y, Akamata K, Takahashi T, Noda S, Taniguchi T, Toyama T, Aozasa N, Sumida H, Kuwano Y, Yanaba K, Tada Y, Sugaya M, Sato S, Kadono T. Fli1 deficiency contributes to the suppression of endothelial CXCL5 expression in systemic sclerosis. *Arch Dermatol Res.* 2014; 306:331–338. [PubMed: 24292093]
34. Lennard Richard ML, Nowling TK, Brandon D, Watson DK, Zhang XK. Fli-1 controls transcription from the MCP-1 gene promoter, which may provide a novel mechanism for chemokine and cytokine activation. *Mol Immunol.* 2015; 63:566–573. [PubMed: 25108845]
35. Lennard Richard ML, Sato S, Suzuki E, Williams S, Nowling TK, Zhang XK. The Fli-1 transcription factor regulates the expression of CCL5/RANTES. *J Immunol.* 2014; 193:2661–2668. [PubMed: 25098295]
36. Sato S, Lennard Richard M, Brandon D, Jones Buie JN, Oates JC, Gilkeson GS, Zhang XK. A critical role of the transcription factor fli-1 in murine lupus development by regulation of interleukin-6 expression. *Arthritis Rheumatol.* 2014; 66:3436–3444. [PubMed: 25155007]
37. Suzuki E, Karam E, Williams S, Watson DK, Gilkeson G, Zhang XK. Fli-1 transcription factor affects glomerulonephritis development by regulating expression of monocyte chemoattractant protein-1 in endothelial cells in the kidney. *Clin Immunol.* 2012; 145:201–208. [PubMed: 23108091]
38. Georgiou P, Maroulakou I, Green J, Dantis P, Romanospica V, Kottaridis S, Lautenberger J, Watson D, Papas T, Fischinger P, Bhat N. Expression of ets family of genes in systemic lupus erythematosus and Sjogren's syndrome. *Int J Oncol.* 1996; 9:9–18. [PubMed: 21541474]
39. Zhang XK, Papas TS, Bhat NK, Watson DK. Generation and characterization of monoclonal antibodies against the ERGB/FLI-1 transcription factor. *Hybridoma.* 1995; 14:563–569. [PubMed: 8770644]
40. Zhang XK, Gallant S, Molano I, Moussa OM, Ruiz P, Spyropoulos DD, Watson DK, Gilkeson G. Decreased expression of the Ets family transcription factor Fli-1 markedly prolongs survival and significantly reduces renal disease in MRL/lpr mice. *J Immunol.* 2004; 173:6481–6489. [PubMed: 15528390]
41. Mathenia J, Reyes-Cortes E, Williams S, Molano I, Ruiz P, Watson DK, Gilkeson GS, Zhang XK. Impact of Fli-1 transcription factor on autoantibody and lupus nephritis in NZM2410 mice. *Clin Exp Immunol.* 2010; 162:362–371. [PubMed: 20731671]
42. Bradshaw S, Zheng WJ, Tsoi LC, Gilkeson G, Zhang XK. A role for Fli-1 in B cell proliferation: implications for SLE pathogenesis. *Clin Immunol.* 2008; 129:19–30. [PubMed: 18692443]
43. Molano I, Mathenia J, Ruiz P, Gilkeson GS, Zhang XK. Decreased expression of Fli-1 in bone marrow-derived haematopoietic cells significantly affects disease development in Murphy Roths Large/lymphoproliferation (MRL/lpr) mice. *Clin Exp Immunol.* 2010; 160:275–282. [PubMed: 20015093]
44. Sato S, Zhang XK. The Friend leukaemia virus integration 1 (Fli-1) transcription factor affects lupus nephritis development by regulating inflammatory cell infiltration into the kidney. *Clin Exp Immunol.* 2014; 177:102–109. [PubMed: 24580413]
45. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, Kawaguchi T, Stahl EA, Kurreeman FA, Nishida N, Ohmiya H, Myouzen K, Takahashi M, Sawada T, Nishioka Y, Yukioka M, Matsubara T, Wakitani S, Teshima R, Tohma S, Takasugi K, Shimada K, Murasawa A, Honjo S, Matsuo K, Tanaka H, Tajima K, Suzuki T, Iwamoto T, Kawamura Y, Tanii H, Okazaki Y, Sasaki T, Gregersen PK, Padyukov L, Worthington J, Siminovitch KA, Lathrop M, Taniguchi A, Takahashi A, Tokunaga K, Kubo M, Nakamura Y, Kamatani N, Mimori T, Plenge RM, Yamanaka H, Momohara S, Yamada R, Matsuda F, Yamamoto K. Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. *Nat Genet.* 2012; 44:511–516. [PubMed: 22446963]

46. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, Yoshida S, Graham RR, Manoharan A, Ortmann W, Bhangale T, Denny JC, Carroll RJ, Eyler AE, Greenberg JD, Kremer JM, Pappas DA, Jiang L, Yin J, Ye L, Su DF, Yang J, Xie G, Keystone E, Westra HJ, Esko T, Metspalu A, Zhou X, Gupta N, Mirel D, Stahl EA, Diogo D, Cui J, Liao K, Guo MH, Myouzen K, Kawaguchi T, Coenen MJ, van Riel PL, van de Laar MA, Guchelaar HJ, Huizinga TW, Dieude P, Mariette X, Bridges SL Jr, Zhernakova A, Toes RE, Tak PP, Miceli-Richard C, Bang SY, Lee HS, Martin J, Gonzalez-Gay MA, Rodriguez-Rodriguez L, Rantapaa-Dahlqvist S, Arlestig L, Choi HK, Kamatani Y, Galan P, Lathrop M, consortium, R., consortium, G. Eyre S, Bowes J, Barton A, de Vries N, Moreland LW, Criswell LA, Karlson EW, Taniguchi A, Yamada R, Kubo M, Liu JS, Bae SC, Worthington J, Padyukov L, Klareskog L, Gregersen PK, Raychaudhuri S, Stranger BE, De Jager PL, Franke L, Visscher PM, Brown MA, Yamanaka H, Mimori T, Takahashi A, Xu H, Behrens TW, Siminovitch KA, Momohara S, Matsuda F, Yamamoto K, Plenge RM. Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature*. 2014; 506:376–381. [PubMed: 24390342]
47. Asano Y, Czuwara J, Trojanowska M. Transforming growth factor-beta regulates DNA binding activity of transcription factor Fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. *J Biol Chem*. 2007; 282:34672–34683. [PubMed: 17884818]
48. Asano Y, Trojanowska M. Fli1 represses transcription of the human alpha2(I) collagen gene by recruitment of the HDAC1/p300 complex. *PLoS One*. 2013; 8:e74930. [PubMed: 24058639]
49. Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol*. 2005; 175:7085–7091. [PubMed: 16301609]
50. Zhang XK, Moussa O, LaRue A, Bradshaw S, Molano I, Spyropoulos DD, Gilkeson GS, Watson DK. The transcription factor Fli-1 modulates marginal zone and follicular B cell development in mice. *J Immunol*. 2008; 181:1644–1654. [PubMed: 18641300]
51. Jackers P, Szalai G, Moussa O, Watson DK. Ets-dependent regulation of target gene expression during megakaryopoiesis. *J Biol Chem*. 2004; 279:52183–52190. [PubMed: 15466856]
52. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics*. 2005; 21:2933–2942. [PubMed: 15860560]
53. Zhao X, Sternsdorf T, Bolger TA, Evans RM, Yao TP. Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. *Mol Cell Biol*. 2005; 25:8456–8464. [PubMed: 16166628]

Figure 1. Reduced G-CSF mRNA and protein expression in endothelial cells after treatment with Fli-1 siRNA

(A–E) Endothelial cells (murine MS1(A–C) and HUVECs (D–E)) were transfected with Fli-1 specific or negative control siRNA and were stimulated with LPS. (A) Western blot results showing the specific knockdown of Fli-1 in MS1 cells. Arrows are used to denote Fli-1 and β -actin as a loading control. (B) G-CSF mRNA expression in MS1 cells after siRNA knockdown of Fli-1 and LPS stimulation (0, 6 and 24 hours). Relative expression was determined using the comparative Ct method for G-CSF compared to the GAPDH housekeeping gene prior to stimulation. Data presented are mean (2^{-Ct}) + standard deviation range, samples performed in triplicate (n=3). (C). G-CSF concentrations in murine MS1 cells were measured by ELISA 0, 6, 12, or 24 hours after stimulation. (D) Western blot results showing specific knockdown of Fli-1 in HUVECs. Arrows denote the Fli-1 and β -actin (loading control) bands. (E). The G-CSF concentrations in HUVECs were measured using ELISA 0, 6, and 24 hours after stimulation. (A and D) Blots are representative of three independent experiments. (B.) The experiment was performed in triplicate n=3. C and E) Data are shown as mean $\pm$ SD and are representative of three separate experiments (n=12). * $p < 0.05$ and ** $p < 0.01$; Student's *t*-test.

Figure 2. Fli-1 binds to the proximal region of the G-CSF promoter

(A) Schematic diagram showing the location of the 60 putative EBS based on the MatInspector Software and the primers designed for ChIP, including distance from the transcription start site (TSS), can be found in panel A. (B) Representative agarose gel results for several primer pairs on the G-CSF promoter. Fold change was determined using the comparative Ct method (n=3) and the numerical values are listed to the right of the gel images. Input represents 1% of the total crosslinked chromatin prior to immunoprecipitation. Data are representative of four independent experiments.

Figure 3. Fli-1 drives transcription from the G-CSF promoter

(A) Graph depicting the results of the luciferase assay for the G-CSF promoter in the NIH3T3 cell line. Transfections were carried out using increasing amounts of Fli-1 (0.025µg, 0.05µg, 0.1µg, 0.2µg, 0.25µg, 0.5µg, to 1µg). Values are shown as fold activation over the empty vector control and are the mean + standard error for three replicate experiments (n=9). ** p-value of < 0.01; Student's t-test. (B) Western blot representative of three replicate experiments illustrating Fli-1 protein concentrations after transfection into the

NIH3T3 cells. Arrows are used to denote the Fli-1 and β -actin loading control bands; the molecular weight marker is shown on the left.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Deletion of the G-CSF promoter further identifies regions important for Fli-1 binding (A) Schematic diagram that depicts the location of specific ChIP primers and the design of the deletion constructs. Transient transfections were performed in NIH3T3 cells and the results are shown in panel. (B) Percent activation is compared to Fli-1 driven activation from the full length promoter and indicated by the numbers above each region. The results shown are mean + standard error for three replicate experiments (n=9).

Figure 5. Fli-1 regulates G-CSF expression by directly binding to the promoter

(A) Graph showing the luciferase assay results when adding equivalent amounts of Fli-1 and the Fli-1 DNA binding mutant to NIH3T3 cells. All values shown are fold activation over the empty vector control and are the mean + standard error for three replicate experiments (n=9). ** p-value of < 0.01; Student's t-test. (B) Western blot, representative of three experiments illustrating Fli-1 protein concentrations after transfection into the NIH3T3 cells. Arrows are used to denote the Fli-1 and β -actin loading control bands on the left and the approximate location of the 50Kd and 37Kd molecular weight marker bands is labeled on the right.

Figure 6. The role of acetylation in Fli-1 driven activation of the G-CSF promoter
 (A) Luciferase assay data illustrating activation from the G-CSF promoter for Fli-1 and a Fli-1 acetylation mutant (aa380). Equivalent amounts of Fli-1 and the Fli-1 acetylation mutant were transfected into NIH3T3 cells and luciferase activity measured after 48 hours.
 (B) Western blot, representative of three experiments illustrating Fli-1 protein concentrations after transfection into the NIH3T3 cells. Arrows are used to denote the Fli-1 and β -actin loading control bands on the left and the approximate location of the 50Kd and 37Kd molecular weight marker bands is labeled on the right. (C) Luciferase assay results from co-

transfecting Fli-1 (0.5 µg) with the histone acetyltransferases (PCAF and p300) at concentrations of 1.5 µg. (D) Luciferase assay results from co-transfecting the Fli-1 acetylation mutant along with PCAF and p300. The concentrations transfected are the same as in panel (C). Equivalent amounts of DNA were transfected in all cases with the difference being made up by empty expression vectors. For panels A, C and D all values shown are fold activation over the empty vector control and are the mean + standard error for three replicate experiments (n=9). ** p-value of < 0.01; Student's t-test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript