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***SERPINB2* is regulated by dynamic interactions with pause-release proteins and enhancer RNAs**

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Abstract

The *SERPINB2* gene is strongly upregulated in inflammatory states. In monocytes, it can constitute up to 1% of total cellular protein. It functions in protection from proteotoxic stress and plays a role in angioedema. The purpose of this study was to define the roles of enhancer RNAs embedded in the SERPIN gene complex. We found that the upstream enhancer RNAs upregulated *SERPINB2* and the enhancer RNAs were expressed prior to those of *SERPINB2* mRNA. Studies of the *SERPINB2* promoter demonstrated the presence of an RNA polymerase II pause-inducing protein, NELF. Stimulation with LPS led to recruitment of the pause-releasing kinase P-TEFb and departure of the pause-inducing protein NELF. RNA immunoprecipitation revealed that NELF and the CDK9 component of P-TEFb bound to the enhancer RNAs after stimulation with distinct kinetics. Knock-down of the enhancer RNAs compromised stimulus induction of promoter and enhancer chromatin changes. Conversely, over-expression was associated with enhanced recruitment of c-JUN and increased expression of *SERPINB2* mRNA expression. This study is the first to associate enhancer RNAs with *SERPINB2* and is the first demonstration of acquisition of NELF binding by enhancer RNAs on chromatin.

Graphical Abstract

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Abbreviations

CDK9
DSIF
LPS
NELF
RNAPII

Keywords

eRNA; NELF; CDK9; P-TEFb; PAI-2; histone modifications

1.0 Introduction

SERPINB2 has been identified as the gene most highly upregulated in inflammatory bowel disease [1, 2]. We also identified it as upregulated in systemic lupus erythematosus (SLE) [3]. It is expressed in keratinocytes, monocytes, placenta, and adipocytes [4, 5] and is one of the most highly inducible genes in monocytes. It can comprise up to 1% of total cellular protein in monocytes [6, 7]. Recent data have implicated *SERPINB2*, also known as PAI-2 (plasminogen activator inhibitor-2), in type III angioedema and a new functional analysis revealed one of its key functions is to modulate proteotoxic stress [8, 9].

In spite of its highly dynamic expression, little is known about its regulation. *SERPINB2* has been implicated in Th2 responses where it is regulated by STAT6 [10]. Other recognized transcriptional regulators include NF κ B, AP-1 and C/EBP β [11, 12]. Histone deacetylase inhibitors modulate *SERPINB2* expression but the epigenetic control of expression has not been previously investigated [13]. Investigation of epigenetic regulation is critical given the aberrant expression of *SERPINB2* in many human disease states and its highly dynamic expression.

We identified a complex of non-coding RNAs (ncRNAs) within the *SERPIN* gene cluster on chromosome 18. These ncRNAs overlapped with known enhancer chromatin marks and we therefore investigated their role in the regulation of *SERPIN* family members in the vicinity. Enhancers represent regulatory regions capable of acting on promoters at a distance and recent evidence has demonstrated that enhancer RNAs (eRNAs) participate in chromatin looping that activates gene transcription [14, 15].

Most inducible genes are regulated at the level of pause-release and elongation [16–20]. Paused RNAPII is held in place by negative elongation factor (NELF), Gdown1, and DRB-sensitivity inducing factor (DSIF) [21–23]. Release from pausing requires recruitment of positive transcription elongation factor-b (P-TEFb), a complex of cyclin-dependent kinase 9 (CDK9) and cyclin T1. P-TEFb is typically sequestered in an inactive complex with 7SK snRNP, Hexim1, and MePCE proteins [24, 25]. To escape pausing, BRD4 recruits P-TEFb

by binding to acetylated histones [26, 27]. The mechanism by which P-TEFb is liberated from the 7SK complex is not completely understood but appears to involve several pathways [28–30]. The eRNAs may induce release of NELF from RNAPII [31], thereby linking enhancer function and pause–release of RNAPII. There have been few studies examining eRNA interactions with pause-release proteins at enhancers and promoters.

We chose to investigate the interaction of eRNAs at the promoter of the SERPIN complex because it offers a unique opportunity to compare related but divergently regulated genes. Recognition of regulatory facets of ncRNAs and their interactions with transcriptional regulatory proteins has been increasing [32]. A novel finding is the interaction of CDK9 and NELF with the eRNAs.

2.0 Materials and Methods

2.1 Cells, transfections and reagents

Primary monocytes from healthy human donors were obtained from the Center for AIDS Research Core Facility at the University of Pennsylvania under an IRB approved protocol. They were purified by elutriation and adherence and were more than 90% pure by flow cytometry. The SLE samples reported in Figure 1 were obtained as part of a previously reported study under a separate IRB-approved protocol [33, 34]. MonoMac6 cells were used as a monocyte cell line, and K562 was used as an early hematopoietic stem cell line. Both were maintained in RPMI with 10% cosmic calf serum (with OPI supplement (Sigma-Aldrich, St. Louis, MO) for MonoMac6 cells). MonoMac6 cells were obtained from the German source and thawed from the original source vials every 2–3 months. K562 was obtained from ATCC and the phenotype was validated periodically by flow cytometry. HEK293 cells were cultured in DMEM with 10% cosmic calf serum and were obtained from ATCC and thawed fresh for these experiments. The SLE patient RNA samples have been previously reported [3, 33], although these analyses are new. Transfection of cells was performed by electroporation with the Amaxa Cell Line Lonza Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD) or Lipofectamine 2000 (ThermoFisher, Waltham, MA). HPLC-purified LPS and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO).

For knock-down assays, 2 million cells were transfected with 500nM antisense oligonucleotide using nucleofection. For over-expression, 2 million cells were transfected with 5µg plasmid using nucleofection. LPS treatment utilized 1 µg for one hour unless otherwise specified. Each assay was performed using three separate experiments minimally.

5,6-Dichloro-1-β-D-ribofuranosy Benzimidazole (DRB) is a CDK9 inhibitor from Sigma-Aldrich. The iBET (Life science Research, Billerica, Massachusetts) inhibitors targets bromodomains. C646 (Santa Cruz, Dallas, Texas) is an inhibitor of dynamic histone acetylation. SP600125 (Calbiochem, Darmstadt, Germany) is a JNK inhibitor. U0126 (Cell Signaling, Danvers, MA) is an MEK1/2 inhibitor. SB203580 is a p38-MAPK inhibitor (Cell Signaling, Danvers, MA). JSH-23 is an NFκB inhibitor from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 RNA extraction and qRT-PCR

Cells were harvested and total RNA was extracted by TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) and the Direct-zol™ RNA MiniPrep Kit (Zymo Research Irvine, CA). RNA was reverse transcribed using the Clontech Advantage RT for PCR kit (Clontech, Mountain View, CA). RNA levels were detected by real-time PCR using the TaqMan 7900. Transcript levels were normalized to the 18S or β -actin signal. ncRNAs were detected with custom SYBR green primers. Primer sequences are listed below. Commercially available primers for *SERPINB2*, *SERPINB7* and *SERPINB10* were purchased from Applied Biosystems (Foster City, CA).

For ChIP PCR assay	
SERPINB2-Promoter	FWD: 5'-TCACTCAAAGGACACAGATCAA-3' Probe: 5'-/56-FAM/ACCAGTCATTACCATGTCTGAACTGT/36-TAMSp/-3' REV: 5'-GCAATGCTCCTCTGAGAGTT-3'
SERPINB2 Enhancer1	FWD: 5'-CCCATAATCAGAAAGTCAGGTAAGG-3' Probe: 5'-/56-FAM/TCACTTACTGTGTGTCAGGGCACT/36-TAMSp/-3' REV: 5'-TGACTGCATACTGATGTTTCCC-3'
SERPINB10 promoter	FWD: 5'-CTGTGCATCCTTTGCTCAATC-3' Probe: 5'-/56-FAM/ACCTCTCTCAGCTTCAGTTTCTTAGCAGA/36-TAMSp/-3' REV: 5'-CAGTGTGGAAAGACCCTTAGA-3'
GAPDH	FWD: 5'-CGGTGCGTGCCCGATT-3' Probe: 5'-/56-FAM/ACCAGGCGGCTGCGGAAAAAA/36-TAMSp/-3' REV: 5'-CCCTACTTTCTCCCGCTTT-3'
ncRNA SYBR primers	
772	FWD: 5'-GCTTTTGAGACCCTTTGGCTATGA-3' REV: 5'-CCAGGCAATGGTAACATCAAAACA-3'
774	FWD: 5'-CAGGTTACAATGCACCTTGTCTGG-3' REV: 5'-GGATTTTGTATGTGGTGTGTCATGGA-3'
775	FWD: 5'-AACCTGGAACAATTGCACTGAGTA-3' REV: 5'-ACTCCCGACCTCAGGTGATT-3'
571	FWD: 5'-ACACTCCCTAACAAAGCTGAC-3' REV: 5'-CGAGAGACAGGGCAAAGAAA-3'
477	FWD: 5'-TGCTCTTCACTATGGCTCTTAC-3' REV: 5'-GTCAGCGTTGTTGTGTCAGTTTAC-3'
896	FWD: ATCCACCTCTTCCGACT REV: CAGTGACGCTGAATGAATGAATAC
ACTIN	FWD: AAGGGACTTCCTGTAACAATGCA

For ChIP PCR assay	
	REV: CTGGAACGGTGAAGGTGACA
For luciferase constructs (PGL3-TK)	
775	FWD: 5'-AAATCGATAAGGATCGCTAAGCTGACTGTGTGGAG-3' REV: 5'-AAGGGCATCGGTCGACAGCCTCCCAAGTAGCTG-3'
172	FWD: 5'-AAATCGATAAGGATCGTCTCAACAACATCGACTGC-3' FWD: 5'-AAGGGCATCGGTCGATTCAAACGTCTCCACAGGA-3'
For over-expression constructs (PCDNA3.1+)	
774	FWD: CAGTGACGCTGAATGAATGAATAC REV: GCTCTAGAGCCTGAGGAATTTTGTAAAGCA
775	FWD: 5'-CGCGGATCCACCCCTACTCCTCAGCCTCTCT-3' REV: 5'-GCTCTAGATGTACCACCACACCTGGCTAAG-3'
477	FWD: 5'- CGGGATCC CACTGGTGGTTCACTGGTGAAT-3' REV: 5'- GCTCTAGAAGTTTGTCTAGCGGTTTGTGAGT-3'
Knock-down Antisense oligo	
774ASO	5'- A *G *A * A *A *C * T *C *C * C *T *T * G *C *C * A *C *C * C *A -3
775ASO1	5'-G *G *T * T *T *G * C *A *G * G *T *C * C *C *A * G *G *T * T *C -3'
775ASO2	5'-G *T *G *C *C *C *G *C *T *T *T *C *C *T *A *C *T *C *A *G *T -3'

2.3 RNA immunoprecipitation and ChIP assays

The RNA immunoprecipitation (RNA-IP) assay was modified as described by Niranjanakumari [35] and Chand [36]. Briefly, 20–40 million cells were crosslinked using 1% formaldehyde for 10 min and quenched with glycine. The fixed cells were lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.05% SDS, 1mM EDTA, 150mM NaCl) and sonicated. The precleared lysates were incubated with antibodies and protein A agarose at 4°C. Immune complexes were washed and reverse cross-linked. Direct-zol™ RNA MiniPrep Kit was used to extract the immunoprecipitated RNA. Chromatin immunoprecipitation assay (ChIP) experiments were performed as previously described [37, 38]. The antibodies to CDK9 (C-20), c-JUN (H-79) and NELF-A (A-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to H3K4me3 and H3K27ac were from Active Motif (Carlsbad, CA). Antibodies to H3K4me1 and H3K4me2 were from Millipore (Darmstadt, Germany). GST (Invitrogen, Camarillo, CA) or IgG (Cell Signaling, Boston, MA) were used as negative control antibodies. Signals are reported as units normalized to 10% input according to the formula 2^{Δ} (10%input CT-Sample CT).

2.4 Luciferase reporter assay

The pGL3-TK vector and the TK-A7 positive control were gifts from the Shiekhatar lab and used to test enhancer function by transient transfection [39]. The TK-A7 control regulates the UBE2VI gene. The inserts were amplified from genomic DNA and subcloned into the BamHI and SalI sites. HEK 293 and K562 cells were transfected with Lipofectamine 2000 (Invitrogen). The luciferase assay was performed in 96-well white plates using the Dual-Luciferase reporter assay kit (Promega, Madison, WI) according to the manufacturer's protocol. 5×10^4 cells were transfected with 0.2 μ g of plasmid and 0.01 μ g pRL renilla reporter vector. Luciferase and renilla signals were measured at 24 hours.

3.0 Theory

Pause-release of RNAPII appears to be a key regulatory mechanism particularly for genes involved in inflammatory responses [16, 18]. NELF and CDK9 (P-TEFb) are known to regulate RNAPII pausing. This study examined pause-release at two related genes that are divergently regulated and specifically examined interactions of eRNAs with the pause release machinery. eRNA behaviors with pause release proteins have been infrequently examined and our study addressed a major knowledge gap in a biologically relevant system.

4.0 Results

4.1 Non-coding RNA and mRNA expression

The rationale for this study builds on an observation made using monocytes from patients with systemic lupus erythematosus (SLE). A cluster of RNAs, not previously annotated, was found approximately 21kb upstream of the *SERPINB2* gene in SLE monocytes [3]. Very low levels were seen in control monocytes. We subsequently identified that the ncRNAs were inducible with LPS. These RNAs did not appear to be translatable except as small peptides and were considered to be ncRNAs. Near the *SERPINB2* locus were other Clade B SERPIN family members, *SERPINB3*, *SERPINB4*, *SERPINB8*, *SERPINB10*, *SERPINB7* and *SERPINB11*. *SERPINB2* was the closest gene to the identified ncRNAs and the 3' end of the *SERPINB7* gene was 50kb upstream of *SERPINB2* and the first exon of *SERPINB10* was downstream of *SERPINB2* (Figure 1A). The RNAs (772, 774, 775) formed a cluster near an enhancer, defined by H3K27ac in UCSC Genome Browser. 774 is centered on the H3K27ac peak, 775 is at the proximal edge of the H3K27ac peak and 772 is at the distal shores of the H3K27ac peak. We will refer to this region as Enhancer 1. Examination of UCSC Genome Browser revealed limited tissue expression of the eRNAs. Using the criteria of H3K27ac peaks, a second enhancer (Enhancer 2) was found just 8kb upstream of the *SERPINB2* promoter. Another ncRNA (571) was found 4kb upstream of the transcription start site of *SERPINB2* and was not embedded in enhancer-like chromatin.

We hypothesized that the RNAs participated in the regulation of the highly inducible *SERPINB2* gene. To determine whether chromatin alterations were also found at this locus in this inflammatory disease, we examined H3K4me3 at the *SERPINB2* promoter using previously generated ChIP-seq data on patients with SLE. We found higher H3K4me3 both downstream and upstream of the transcription start site in SLE patients (Figure 1B).

Additionally, mRNA for *SERPINB2* was higher in SLE patients (Figure 1C). These analyses support efforts to understand the regulatory dynamic of *SERPINB2*. It is the most highly upregulated gene in inflammatory bowel disease, strongly upregulated in SLE, and sequence variants are associated with multiple human diseases [1, 2, 40, 41]. Our lack of understanding of the regulatory landscape is a significant knowledge gap.

The 774, 775 ncRNAs cluster in a single region with chromatin characteristics of an enhancer. 772 lies just at the distal edge of the enhancer chromatin. We tested the DNA corresponding to the 775 ncRNA in an enhancer assay by sub-cloning it downstream of a minimal thymidine kinase (TK) promoter. The DNA corresponding to the 775 ncRNA location was capable of augmenting luciferase production in this transient transfection assay in two different cell types (K562 and HEK293), thereby assigning it as a probable enhancer (Figure 2A, B). For this reason, we will refer to the ncRNAs subsequently as eRNAs. Enhancer RNAs are predicted to have chromatin localization. To validate the remaining eRNAs, we used qRT-PCR from three different cell types to demonstrate tissue specificity and analyzed eRNA sub-cellular localization by fractionating cells. The eRNAs were found predominantly in monocytes and in the chromatin and nucleoplasm fractions predominantly (Figure 2C, D). This demonstration of enhancer activity of the DNA and chromatin localization support the assignment of the ncRNAs as eRNAs.

To understand the relationship of the RNAs to the various SERPIN family members, we treated primary monocytes with LPS and examined the effect on the ncRNAs and SERPIN family mRNAs, measured by qRT-PCR. We used a series of inhibitors to further characterize the regulation of expression of each of these RNAs. DRB is a CDK9 inhibitor, iBET targets bromodomains, C646 inhibits dynamic histone acetylation, SP600125 is a JNK inhibitor, U0126 is an MEK1/2 inhibitor, SB203580 is a p38-MAPK inhibitor and JSH-23 is an NF κ B inhibitor. RNAs 772, 774, and 775 form a cluster 21kb upstream of *SERPINB2* in enhancer-like chromatin (Enhancer 1). Their pattern of expression closely paralleled that of *SERPINB2* (Figure 2E, F) but not *SERPINB10* (Figure 2G). *SERPINB10* is modestly LPS-inducible but not inhibitable. Thus, *SERPINB2* exhibits a pattern consistent with that seen for the 772, 774 and 775 eRNAs. The 571 RNA, which lies near enhancer 2 but not within enhancer 2, had a slightly different pattern, with less induction by LPS. Based on comparable patterns of induction and inhibition, we tentatively assigned the 772, 774, and 775 RNAs to the *SERPINB2* regulon.

We wished to define the kinetics of LPS induction of expression in our system. Expression of eRNAs typically precedes that of the target genes and eRNA expression is essential for function and parallels that of the target mRNA [42–44]. Therefore, we performed careful kinetic analysis of eRNA and mRNA expression after LPS stimulation at early time points to define the order of transcript appearance. Expression of 772, 774 and 775 preceded *SERPINB2* and *SERPINB10* expression after LPS stimulation (Figure 3A). RNA 571 does not lie within an enhancer and *SERPINB10* is not LPS-inducible in MonoMac6 cells (in contrast to the slight inducibility seen in primary monocytes in Figure 1), thus representing controls. 477 and 896 are eRNAs that are not LPS inducible and their expression is constant throughout the time course. GAPDH is a housekeeping gene not inducible with LPS. This

kinetic analysis supports our definition of 772, 774, and 775 as eRNAs because their expression occurs temporally before *SERPINB2* mRNA.

The mechanistic role of eRNAs is incompletely understood and we therefore examined whether manipulation of eRNA levels in our system would modulate mRNA expression. 772 is located outside the peak of H3K27ac, demarking the limits of the enhancer. Therefore, we pursued a mechanistic analysis of 774 and 775. The 571 RNA is not thought to represent an eRNA based on chromatin characteristics and was not examined. We knocked down 774 and 775 as *SERPINB2*-specific eRNAs. Knock-down was validated by qRT-PCR of the target eRNAs. Knock-down of 774 and 775 led to inhibition of *SERPINB2* mRNAs but not *SERPINB10*, which is not co-regulated with *SERPINB2* (Figure 3B). An anti-sense oligonucleotide to GFP (AGFP) was used as an off-target control. Statistical analyses compared eRNA antisense treatment with GFP antisense treatment. We then over-expressed 774 and 775 to determine whether they could regulate SERPIN gene expression when provided in *trans*. Each over-expression construct was validated as expressing the correct RNA species (Figure 3C). Over-expression of both 774 and 775 led to higher *SERPINB2* RNA but not the distinctly regulated *SERPINB10*. An off-target, control RNA, 477, had no effect. 477 is a ncRNA from a regulatory region at different locus that is not LPS inducible. Therefore, 774 and 775 appeared to be capable of regulating *SERPINB2* expression.

4.2 Enhancer RNAs regulate target gene histone modifications

Enhancer RNAs have been hypothesized to regulate gene expression via diverse potential mechanisms. We tested whether they regulated histone modifications. To establish the landscape, we defined chromatin at the *SERPINB2* locus using ChIP-seq from LPS-treated primary monocytes and control monocytes. LPS treatment led to increased H3K27ac at Enhancer 1 and increased H3K4me2 at both Enhancer 1 and promoter regions (Figure 4A). H3K27ac is a classic mark for enhancers and H3K4me2 is a mark that localizes to both promoters and enhancers. These data demonstrate the expected changes related to LPS stimulation. Figure 4B demonstrates a CD14 monocyte track from enhanceratlas.org displaying chromatin loops between the enhancers and the promoter. To define effects related to eRNAs, we used anti-sense oligonucleotides to knock-down 774 and 775. We examined chromatin marks of gene activation in MonoMac6 cells at baseline and after treatment with LPS (Figure 4C–F). We compared the effects of 774 and 775 knock-down with GFP knock-down. Non-transfected cells were included as assay controls for each experiment but are not shown to streamline the figures. LPS was associated with increased H3K4me1, H3K4me2, H3K4me3 and H3K27ac at the *SERPINB2* promoter and at Enhancer 1 (compare AGFP and LPS-GFP). The GAPDH promoter was unaffected by LPS stimulation and serves as a control. The anti-sense oligonucleotides to 774 and 775 were associated with diminished acquisition of the chromatin marks seen with LPS stimulation at Enhancer 1. The effect was limited to LPS-stimulated cells and was not observed in resting cells. Knock-down of 774 and 775 primarily affected H3K4me3 and H3K27ac at the promoter. These data demonstrate that eRNAs regulate histone modifications not just at the enhancer but also at their target gene promoter. In a model where chromatin looping is facilitated by eRNAs, chromatin modifying enzymes would be at the nexus of enhancer, eRNAs, transcription factors, and the promoter.

4.3 Enhancer RNAs regulate pause-release of RNAPII

An emerging mechanism by which eRNAs are thought to function involves binding to the NELF protein that regulates RNAPII pausing [22, 45]. To examine whether NELF and its major kinase are localized to the promoter and enhancer of *SERPINB2*, we performed ChIP studies for NELF and CDK9 (Figure 5). The goal was to define the presence of pause-release proteins on chromatin in unstimulated and stimulated settings. NELF in the paused state binds the nascent mRNA and RNAPII [22]. CDK9 is a component of P-TEFb, which phosphorylates NELF and RNAPII serine 2, releasing RNAPII from its paused state. At the *SERPINB2* promoter and enhancer, CDK9 recruitment occurred rapidly after stimulation, peaking at 10 minutes (Figure 5A). Release of NELF from the promoter and enhancer was much slower (Figure 5B). *GAPDH* is a housekeeping gene, not regulated by pause-release, and neither CDK9 nor NELF were altered by stimulation at the *GAPDH* promoter. These data support an RNAPII pause-release mechanism regulating both *SERPINB2* mRNA and eRNA transcription.

To define the effects of the eRNAs, we over-expressed 774 and 775 and treated cells with LPS. We hypothesized that the eRNAs would serve as a “sink” for NELF, leading to eviction from the promoter. LPS treatment was associated with loss of NELF at the enhancer and promoter (compare PC with LPS-PC), consistent with 5B. Over-expression of 774 and 775 led to diminished NELF at the promoter without LPS stimulation (Figure 5C). This suggested that one of the roles of the eRNAs might be in the modulation of NELF binding on chromatin. NELF binds the short mRNA produced by stalled RNAPII. We hypothesized that eRNAs can compete for NELF binding. Eviction of the pause-inducing NELF protein from the promoter of *SERPINB2* would be expected to enhance elongation and increase transcript abundance, as was seen in Figure 2 with 774 and 775 over-expression. We next performed the complementary experiment, knocking down the eRNAs (Figure 5D). Here, we saw relative preservation of NELF on DNA after LPS stimulation, although the results varied with different oligonucleotides.

We hypothesized that eRNA over-expression could compete for NELF binding, leading to dissociation from the nascent transcript. To further investigate the role of the eRNA, we defined RNA binding by NELF and CDK9 (Figure 6). We used RNA immunoprecipitation (RNA-IP) to define the enrichment of RNAs bound by NELF and CDK9 before and after LPS. In addition to the eRNAs, we quantitated binding of the stalled mRNA (B2+80), an irrelevant eRNA from another chromosome (896), and the promoter RNA species 571. After LPS, both CDK9 and NELF had greater association with the eRNAs but not the irrelevant RNA (896) from a regulatory region on another chromosome nor the upstream RNA, 571. Binding to the *SERPINB2* stalled mRNA (B2+80) was minimally impacted by LPS stimulation supporting a specific interaction with eRNAs. CDK9 binding to eRNAs was coincident with the appearance on chromatin (Figure 5A) at 10 minutes of stimulation. In contrast, NELF bound the eRNAs at baseline with a gradual increase up to 30 minutes of stimulation. Eviction from chromatin, as detected by the ChIP assay, showed removal first detected at 30 minutes and progression at 60 minutes. Therefore, eRNA binding preceded eviction from chromatin. RNA-IP with antibodies to H4ac demonstrated that this complex

was likely anchored to chromatin. These data place NELF and CDK9 engaging eRNAs on chromatin.

4.4 Histone modifications impact eRNA production

Our studies thus far focused on how eRNAs regulate *SERPINB2* transcription. An equally important question is how eRNA expression itself is regulated. Figure 2 demonstrated that LPS signaling pathway inhibitors could regulate eRNA expression supporting an overall transcription program comparable to that of mRNAs. C464, which inhibits de novo histone acetylation, DRB, which inhibits P-TEFb, and iBET, which inhibits bromodomain proteins were examined for their effects on LPS-inducible eRNA transcript abundance to define the role of chromatin in regulating eRNA expression. Each of the inhibitors targeted a distinct pathway. The inhibitors were associated with lower levels of *SERPINB2* mRNA and eRNA abundance after LPS stimulation in primary monocytes (Figure 7A, B, C). *SERPINB10*, much less LPS inducible, exhibited a paradoxical increase with the inhibitors. These data suggest that the same chromatin modifications regulate eRNA and mRNA expression. Three MAP kinase inhibitors in Figure 2 revealed a role for MAP kinases in regulating both eRNAs and mRNAs. We therefore examined whether eRNA over-expression altered c-JUN binding, a key transcription factor downstream of the MAP kinase pathway. Over-expression of 775 led to increased c-JUN binding at the enhancer but not at the promoter (Figure 7D). Accumulation of eRNAs may serve to recruit or stabilize transcription factors at enhancers.

5.0 Discussion

The precise physiologic function of SERPINB2 has been hotly debated because it is found predominantly as a 47 kDa cytosolic protein with minimal secretion as an extracellular glycosylated 60 kDa protein [4, 46–48]. Studies examining the function of SERPINB2 have focused on inflammation, malignancy and allergy [4, 6, 49–51]. Its recently defined role in the misfolded protein response may tie together these disparate effects. Several studies have identified that overexpression of SERPINB2 protects from TNF-induced apoptosis [52–55]. In inflammatory disease, it may therefore offer a protective role.

Our data significantly increase our understanding of the regulation of *SERPINB2* expression by demonstrating that pause-release of RNAPII is a key regulatory mechanism. This transcriptional regulatory strategy has been identified in approximately 30% of genes, however, it may be more common in genes involved in inflammatory responses [16, 18]. In addition to identifying NELF and CDK9 (P-TEFb) as key proteins interacting on the promoter, we identified an interaction between eRNAs and the pause-release machinery. This type of interaction has been infrequently described in detail. A recent study found that CBP, often found at enhancers, is activated by binding eRNAs and drives increased H3K27ac [42]. Enhancer RNAs have been previously described as luring NELF away from paused RNAPII at neuronal genes [31]. Other studies have focused on the biogenesis of eRNAs [56]. Yet, there is little consensus on the function of eRNAs even from the standpoint of chromosome looping with some studies supporting a role as facilitators of chromatin looping and others not [43, 57, 58]. One study suggested that eRNA expression and mRNA expression was not necessarily concordant at the single cell level [59].

Therefore, there are key knowledge gaps in our understanding of enhancer function and eRNA function. This study is one of the first to provide detailed kinetic and binding analysis at a locus of specific medical interest.

NELF in the paused state binds a short mRNA tail and RNAPII. Our data support a model where the eRNAs compete for NELF binding, leading to release from pausing (Figure 8). A similar mechanism has been shown in HIV [60, 61]. Our careful kinetic analysis allowed us to demonstrate that CDK9 recruitment to the promoter and NELF binding to the eRNAs preceded NELF eviction after LPS. This has important functional implications. It supports a model where eRNA binding is functionally relevant to release from pausing and regulated by an enhancer. In our model (Figure 8), P-TEFb (CDK9) appears on chromatin within 10 minutes of stimulation, coincident with CDK9 binding to eRNAs. A precondition for this in our current conceptualization is that P-TEFb has been liberated from 7SK snRNP through undefined mechanisms. P-TEFb is thought to dephosphorylate DSIF as part of liberation of NELF [62]. Our data suggest that eRNA binding may replace 7SK snRNP binding for CDK9. In our model, eRNAs also bind to NELF as an intermediate step from 10–60 minutes after LPS stimulation followed by gradual liberation of NELF from the promoter. The first detectable increase in eRNA abundance is at 60 minutes post-stimulation followed by increased mRNA, first detected at 90 minutes post-stimulation. We hypothesize that the eRNA acts as a type of glue for this macromolecular interaction. Thus, over-expression of the eRNAs in *trans* can elicit chromatin effects and transcription factor binding by facilitating a favorable structure.

In spite of the great advances in our understanding of *SERPINB2*, this study does have some limitations. Our studies were performed primarily in MonoMac6 cells. Cell lines provide a homogeneous population for study, however, they often exhibit alterations to their epigenome as well as to chromosomal structure. A second limitation of this study is that we focused on a single stimulus, namely LPS. It may be that alternative stimuli utilize a different regulatory strategy. In spite of these limitations, our study has made a major contribution not only to the understanding of the regulation of *SERPINB2* but also adds to the growing body of literature on the role of eRNAs at the promoter.

6.0 Conclusions

Our data suggest a model where the chromatin environment may favor eRNA expression and the eRNAs may serve to stabilize an architecture as well as preserve/induce a favorable histone landscape for transcription, although we have no direct data on this. Our data specifically demonstrate that the eRNAs can regulate histone modifications both at the enhancer and at the promoter. Manipulation of eRNA levels led to altered mRNA expression, altered interactions of pause-release proteins on chromatin and even transcription factor binding. It is clear that transcription is not regulated according to a single paradigm, however, this study of *SERPINB2* clearly expands our understanding of enhancer-promoter interactions and serves as a case-study for eRNA interactions with pause-release proteins.

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Glossary

Pause-release

This mechanism of transcriptional regulation causes RNA polymerase II to halt 60–80bp downstream of the transcription start site and remain until released for productive elongation

Enhancer RNA

Enhancers regulate transcription in a tissue-specific manner and are often regulated by specific stimuli. Recently described transcription of the enhancers is thought to be central to the enhancer function, however, the mechanism by which it elicits its effect is not understood

Chromatin immunoprecipitation

This technique allows a snapshot of a specific protein on DNA within a population of cells

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Highlights

- SERPINB2 is regulated by enhancer RNAs
- Enhancer RNAs interact with pause-release proteins CDK9 and NELF
- Manipulation of enhancer RNAs regulates expression of SERPINB2

Figure 1. The context of *SERPINB2*

(A) Histone marks and DNase hypersensitive sites (DS) in various human cells from ENCODE [63] data indicated that these ncRNAs are located within active enhancer regions, termed Enhancer 1 and Enhancer 2. The eRNAs described in the text are listed above the tracks. (B) The sequencing depth of H3K4me3 at the *SERPINB2* gene is displayed from a previously published dataset [33, 64]. The depth is the normalized average of 6 control or 6 SLE samples. The plotted data is in log2 scale. The magenta color indicates the overlap, with pink and blue representing the mean for SLE and control, respectively. H3K4me3 is higher in SLE patients at *SERPINB2* both upstream and downstream of the transcription start site (0). (C) Quantitative RT-PCR was used to measure the difference of *SERPINB2* mRNA level in 15 SLE patient monocyte samples and 13 healthy control monocyte samples. The RNA samples came from a previously published cohort [3]. *SERPINB2* mRNA levels are significantly higher (**** $p < 0.0001$). Results were normalized to 18S expression.

Figure 2. The characterization of *SERPINB2* ncRNAs

(A) The schematic diagram shows the luciferase reporter vector driven by a TK promoter. The enhancer DNA from 775 was subcloned downstream of luciferase in the antisense orientation relative to the *SERPINB2* gene.

(B) The firefly luciferase vectors were co-transfected with a constitutive Renilla control pRL-TK into HEK293 cell or K562 cells. Construct 172 is a negative control, which is an LPS-induced non-coding RNA but not an eRNA and not located in the *SERPIN* locus. PGL3-TK-A7 is a positive ncRNA enhancer control from near the *UBE2VI* gene (48). Inducible firefly luciferase was normalized to the Renilla signal. Results are the means + standard deviation (n=3, * p<0.05, ** p<0.01). The 775 construct induced expression in this transient transfection assay. (C) The *SERPINB2* eRNAs were mainly expressed in monocytes. The primary monocytes (M), T and B cells were purified from healthy people by Dynal bead purification and RNA was extracted and analyzed with qRT-PCR. Results were normalized to 18S expression (n= 3, error bars in panels denote SD). (D) The *SERPINB2* eRNAs were localized primarily to the chromatin fraction. RNA was extracted from

MonoMac6 cytoplasm, nucleoplasm, and chromatin fractions. PCR was performed after reverse transcription (n= 3, error bars in panels denote SD). Actin and HOTAIR are shown as positive controls for cytoplasmic and chromatin fractions, respectively. (E, F, G) Primary monocytes were purified by adherence and stimulated. RNA was harvested after 90 minutes of LPS treatment or LPS with the indicated inhibitors. *SERPINB2* eRNAs (E), *SERPINB2* mRNA (F), and *SERPINB10* mRNA (G) were quantitated. Error bars are standard deviations (n=3). The y-axis represents fold change over mock-treated cells (control). RNAs from the *SERPINB2* Enhancer 1 region and the *SERPINB2* mRNA were comparably regulated while *SERPINB10* behaved differently.

Figure 3. *SERPINB2* eRNAs regulate transcription

(A) LPS-induced *SERPINB2* RNA expression kinetic analysis. The eRNA and mRNA expression levels were measured using qRT-PCR at various time-points and normalized to 18S in MonoMac6 cells. *SERPINB10* is minimally LPS-inducible while *SERPINB2* responds to LPS. *GAPDH* is a non-inducible control. The eRNAs appear temporally before the mRNAs. (B) Knock-down of *SERPINB2* eRNAs decreased the LPS-induced *SERPINB2* mRNA. MonoMac6 cells were transfected with the indicated 20-bp phosphorothioate antisense oligonucleotides (ASO) designed to knock-down 774, 775 (two different ASO) or GFP for 3 hours and stimulated with or without 1 μ g/ml LPS for 60 minutes. The eRNAs and *SERPINB2* mRNAs were measured using qRT-PCR. The antisense knock-down was

specific and effective for each eRNA (left panel). *SERPINB2* expression was inhibited while that of *SERPINB10* (right panel) was not affected (n=4, * p<0.05, ** p<0.01). (C) Over-expression of *SERPINB2* eRNA led to increased *SERPINB2* mRNA. The constructs pcDNA3.1+(PC), pcDNA3.1-s774 (PC-774) and pcDNA3.1-775 (PC-775) were the eRNA over-expression vectors and pcDNA3.1-477 (PC-477) was the off-target control. Constructs were transfected into MonoMac6 cells and stimulated with 1 µg/ml LPS for 60 minutes. Total RNA was isolated and qRT-PCR performed. The left panel confirms over-expression by the constructs while the right panel demonstrates that 774 and 775 over-expression drives increased *SERPINB2* expression but not *SERPINB10* expression. Results are the means with standard deviation (n=3, * p<0.05, ** p<0.01, *** p<0.001).

Figure 4. *SERPINB2* eRNAs affect histone modifications

(A) ChIP-seq tracks from new data for LPS and control primary monocytes showed increased H3K4me2 and H3K27ac at the enhancer and promoter after LPS treatment. (B) EnhancerAtlas.org tracks (accessed December, 2016) display chromatin loops observed in CD14 monocytes at this locus, providing a three-dimensional framework for potential enhancer-promoter interactions [65]. Knock-down of 774 and 775 affected acquisition of H3K4me2 (C), H3K27ac (D), H3K4me3 (E), and H3K4me1 (F) at Enhancer 1 (left panels) and to a lesser degree extent at the promoter (middle panels) after LPS. The GAPDH

promoter was not affected by LPS or the knock-downs (right panels). These experiments were performed in MonoMac6 cells (n =4, * p<0.05, ** p<0.01).

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Figure 5. CDK9 and NELF-A binding is altered by LPS

MonoMac6 cells were treated with 1 μ g/ml LPS for the indicated time. ChIP assays were performed with antibodies to P-TEFb (CDK9) (A) or NELF-A (B). LPS led to increased CDK9 at the promoter and enhancer by 10 minutes and decreased NELF binding at the promoter and enhancer with slower kinetics (n = 4, * p<0.05, ** p<0.01). (C) Over-expression of 774 and 775 led to diminished NELF binding at the promoter even without LPS treatment. *GAPDH* is included as a non-LPS-inducible control and pc-DNA3.1 (PC) is a vector control. D) Knock-down of eRNAs did not alter the resting levels of NELF on the enhancer and promoter but there was a trend to preservation of NELF on DNA after LPS stimulation. *GAPDH*, in contrast, was unaffected by the knock-downs.

Figure 6. LPS induces CDK9 and NELF binding to the *SERPIN2* eRNA

MonoMac6 cells were treated with 1 μ g/ml LPS for 0, 10, 30 and 60 minutes. RNA-IPs were performed using NELF-A or CDK9 antibodies. Bound RNAs were detected by qRT-PCR. Input RNA was used for normalization. eRNAs were observed to significantly bind to CDK9 upon LPS stimulation with rapid kinetics. The effect was specific as neither the 896 nor 571 RNA species had significant induction of binding with LPS. The short mRNA, immediately downstream of the TSS (B2+80), exhibited a slight increase that did not reach significance. NELF, in contrast, bound eRNAs at baseline and increased binding after LPS, peaking at 30

minutes. The eRNAs remained associated with chromatin, as demonstrated by the H4ac RNA-IP. B2+80 is the first 80 nucleotides of the *SERPINB2* mRNA and 896 is an off-target control RNA from a regulatory region that is not LPS inducible. 571 is from an upstream region of *SERPINB2* that does not appear to represent an enhancer. Results are the means + standard deviation (n=3, * p<0.05).

Figure 7. The effect of chromatin inhibitors

Primary monocytes from healthy controls were pretreated with the 10 μ M iBET (A), 40 μ M DRB (B) or 20 μ M C646 (C) for 30 minutes before adding 1 μ g/ml LPS.

Total RNA was isolated and qRT-PCR was used to measure the ncRNA or mRNA level ($n=4$, error bars represent SD, * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$). The y-axis represents fold change over untreated cells. Each inhibitor treatment was associated with diminished *SERPINB2* mRNA as well as diminished levels of eRNAs. *SERPINB10* mRNA levels were enhanced by inhibitor treatment, a paradoxical response. To define effects on transcription factor binding, eRNAs 774 and 775 were over-expressed and c-JUN binding was determined by ChIP- assay (D). 775 over-expression was associated with increased c-

JUN at the enhancer. GST binding was unaffected (n=3, error bars represent SD, * indicates $p<0.05$).

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Figure 8. A model for *SERPINB2* eRNA regulation of *SERPINB2* transcription

In unstimulated cells, NELF binds the nascent mRNA and RNAPII. The *SERPINB2* enhancer transcribes eRNAs after LPS treatment. These eRNAs regulate the histone modifications in the enhancer and promoter regions. The eRNAs interact with NELF and CDK9 (P-TEFb). P-TEFb phosphorylates NELF and RNAPII serine 2. Once released from NELF-induced pausing, RNAPII is able to enter into productive elongation and *SERPINB2* mRNA is induced.