IDENTIFICATION OF AN ERG-BOUND ENDOTHELIAL CELL RESTRICTED ENHANCER FOR IL1α AND IL1β.

By:

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Abstract:

The progression of atherosclerosis is characterized by plaque formation within the arterial walls that could manifest into coronary artery disease (CAD) leading to myocardial infarction or stroke. Our objective in this study is to identify regulatory elements in human aortic endothelial cells that are responsible for controlling the inflammatory environment. To do so we evaluated ERG's binding landscape, an important transcription factor that suppresses inflammation, and among the topmost ERG-bound enhancer-like elements was a putative enhancer located between IL1α/β, genes. After deleting this candidate with CRISPR we found a reduction in the magnitude of the IL1α/β RNA upon TNFa treatment ($p<0.05$). We, therefore, concluded that this region functions as an enhancer for the transcription of the IL1-α and IL1-β genes. Deleting this genomic region also reduced leukocyte adhesion to endothelial cells demonstrating the inflammatory functional consequence of the enhancer. Next, we evaluated ERG's function at the IL1αβ enhancer by knocking down and overexpressing ERG with siRNA transfections and lentiviral transduction, respectively. Findings suggested that ERG's inflammatory function in regulating IL1-α and IL1-β is in combination with the enhancer element and that greater transcriptional regulation of the genes comes from the enhancer versus ERG alone. Within this region, we also identified an ERG motif and NFkB motif as being the source of the majority of regulation surrounding enhancer activity. By identifying the role performed by this IL1αβ enhancer and its regulatory network new advanced strategies can be formulated to better target CAD such as those with therapeutic and preventative measures.

Introduction:

Coronary artery disease (CAD) is the leading cause of death in America[3] and is characterized by the process of atherosclerosis, the obstruction of blood vessels by the formation of plaque build-up causing arterial narrowing.

The most vulnerable area for plaque formation is at branching sites such as in the coronary arteries. This process entails both a lipid component, from LDL cholesterol accumulation, as well as the functional role of the immune system that leads to a pro-inflammatory response within the vessel wall [4]. Endothelial cells (ECs) line the blood

vessel walls and are frequently the first to respond to circulating stressors. This is why exploring their role in modulating the inflammatory process will be key in identifying specific genes and genomic areas that are involved in regulating atherosclerosis.

The formation of the atherosclerotic lesion is concurrent with the induction of pro-inflammatory cytokines which furthers the fatty streak formation through cytokine and inflammatory signaling. Specifically, the Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway is activated by proinflammatory cytokine-like tumor necrosis factor-alpha (TNFa) [5]. Cytokines act as the signaling chemical language between cells, while transcription factors initiate the transcription of DNA into RNA, thereby regulating gene expression. In the instance that this pathway is chronically upregulated in the lesion it could lead to increased inflammation and progression of atherosclerosis.

Although the pathology of atherosclerosis is well documented, many molecular mechanisms are not well characterized. New mechanistic insights have been described in the past years. One significant finding involves the transcription factor (TF), an ETS-related gene (ERG) that has been shown to maintain an anti-inflammatory EC state. Studying ways to suppress chronic inflammation could lead to preventative treatments for atherosclerosis and CAD. Randi et al., observed that ERG overexpression suppressed the TNFa-dependent NFκB pathway as well as inhibited TNFa and NFκB expression individually[6]. It is also reported that ERG expression is downregulated in atherosclerotic vessels, signifying ERG's role in the anti-inflammatory process[6].

Our study utilized a novel approach to examine ERG's role in inflammation by focusing on a putative enhancer located between two pro-inflammatory cytokines in ECs. The two cytokines belong to the Interleukin 1 family, IL1-α and IL1-β, that are part of the regulatory processes concerning acute inflammation involving upregulation of NFκB activity [7]. An enhancer is a region in the DNA that is bound by TFs and interacts with other proximal or distal transcriptional components to regulate the transcriptional activities of target genes. Using Chromatin Immunoprecipitation sequencing (ChIP-seq) we verified that in addition to ERG, this region was bound by NFκB in ECs. Motif analysis identified binding motifs for both factors, and we determined the presence of a ~200 bp stretch of DNA most likely functioning as an enhancer.

We aimed to characterize the regulatory role of the IL1α/β enhancer by studying its impacts on human aortic ECs (HAECs). We split our project into three major components. The first part involved studying the loss-of-function of the enhancer by utilizing CRISPR technology to delete the genomic region and compare the effect to wild-type ECs on the transcriptional level. Next, we wanted to focus on the interplay between this enhancer and ERG. As previously stated, ERG is involved in the suppression of inflammatory signals and the putative enhancer upregulates cytokine expression. We wanted to further understand what mechanism the inflammatory process is working through and if the control of the anti-inflammatory response stems from the regulatory roles of both ERG and the enhancer in combination, so we performed ERG gain- and loss-of-function experiments in the wild-type and enhancer-knockout ECs. In our third section, we explored which TF binding events are responsible for enhancer activity through ERG and/or NFκB motif(s) deletions within the relevant sequence. We aimed to differentiate the roles of both transcription factors and how their opposing functions impact enhancer activity.

Research conducted in this study is aimed to explore the unidentified molecular mechanisms behind ERG binding and identify points of intervention to ultimately inhibit chronic inflammation from arising.

Materials and Methods:

Cell Culture and Preparation:

The immortalized cell lines (TeloHAECs) purchased from ATCC were grown in full media containing M199, FPS, ECGS, Heparin, and Fungizone and incubated at 37 ℃ with 5% CO_2. HAECs were incorporated in the CRISPR transfections, Sanger Sequencing, CHIP-sequencing, qPCR analysis, adhesion assay, and Luciferase assay. Cells were treated depending on the experimentation with TNFα (1:5000 dilution), IL1β (1:1000), or control media.

CRISPR Transfections:

The Alt-RTM CRISPR-CAS9 System protocol [1] for genome editing was utilized in the creation of the enhancer KO variants in the HAECs. Boundaries of this deletion

were designed to span the minimum stretch of sequence encompassing ChIP-seq peaks and DNA motifs within the H3K27ac region. 50 uL of the transfections complexes were added to 100 uL of diluted HAECs and incubated at 37 ℃ at 5% CO_2 for four to six hours. The two guides were assembled separately and then combined to a 1:1 ratio in the transfection. Two CRISPR transfections were performed to obtain more deletions two days following the initial transfection. The expected wild-type(WT) band was 464 bp long while the knockout (KO) 329 bp long resulted in a 141 bp deletion for the enhancer. Following the transfection, we performed a limiting dilution to replicate the KO cell populations only. Cells were resuspended in 100 uL of PBS. DNA extraction [8] was carried out for verification purposes through Sanger sequencing. Two KO populations and 1 WT population were chosen for the study based on the alignment through ApE.

Sanger Sequencing:

Gel extraction[9] was carried out before sequence preparation to obtain cleaner samples and limit noise. Eurofins tube sequencing protocol[2] was used for verification of the enhancer WT and KO cell lines with a chosen template concentration of 30 ng/uL as well as in E.Coli transformations of the enhancer KO plasmid, WT, and relevant ERG and NFkB motif deletions. Alignments were analyzed based on the expected deletion sites of the enhancer and mutations of the motifs through ApE with a mismatch penalty of -2, gap penalty of -2, and gap extension penalty of -1.

qPCR:

RNA extraction was performed on the endothelial cells under different treatment conditions and the concentrations were measured by using a NanoDrop spectrophotometer. cDNA synthesis was followed and used in the preparation of the qPCR samples. Total volume resulted in 10uL and gene expression levels of the proinflammatory genes were determined. Samples were run in triplicates and mRNA levels were normalized to Gapdh. Paired t-tests (two-tailed) were performed for significance between treatment conditions.

Leukocyte Adhesion Assay

CRISPR KO cells were used for the generation of the leukocyte adhesion assay data as well as the WT counterpart with the enhancer intact. The three cells (KO 24, KO 25, and 1 WT) were all on passage 59. To measure adhesion THP-1 cells on passage 7 were used. The appropriate inflammatory stimulus was provided by TNFα with either .5, 1, 2, 4, or 6-hour time point treatment. Fifty to one hundred thousand cells were added to each plate (48 wells). After treatment with TNFα, the leukocytes and HAECs were incubated for 60 minutes together with leukocytes resuspended to 1 million cells/mL in serum-free media. A LeukoTracker was then added to a final concentration of 1X. Adherent molecules were counted using a fluorescence scope taking the average of three fields. Cells were read at 40/520 nm and samples were run in triplicates.

ChIP-sequencing:

A small-scale ChIP-sequencing protocol was performed for both ERG binding and H3K27ac marker presence. The core steps include immunoprecipitation, reverse crosslinking, dsDNA repair, dA-Tailing, adapter ligation, and qPCR for quantification. Increased site of ERG and H3K27ac markers used ERG/H3K27ac reduced sites as genomic backgrounds while those loci with decreased ERG and H3K27ac peaks utilized gained sites as points of comparison.

siRNA Transfection and Lenti Viral Transduction:

siRNA transfection and Lentiviral transduction were utilized to create ERG knockdown and overexpression respectively. Lentiviral transduction consisted of plating cells (WT, KO24, or KO 25) in complete media for 30-50% confluency a day before transduction occurs. Two vectors were used, GFP and ERG with HA tags. Incubation took place for 18-20 hours at 37℃ and 5% CO_2. Viral molecules were added and incubated overnight. Viral media was exchanged for complete media and incubated overnight. Cells expressing fluorescent genes were imaged after 24-48 hours. TNFα treatment for one hour was performed the next day. Selection for cells with antibiotic resistance was completed with puromycin at 1-2ug/mL concentration. Samples were in triplicates and were collected for RNA and qPCR was performed.

For siRNA transfection, OptiMem was mixed with siRNA for ERG and Lipofectamine. This mixture was then added to the cells and incubated for 3.5 to 4 hours. After the media was aspirated and exchanged with full growth media, cells were allowed to grow for 48 hours. TNFα treatment was added after siRNA treatment. RNA was collected and NanoDropped for the concentration. qPCR was performed on these samples.

PCR for Motif Mutation(s) Creation:

The initial step for the motif mutations was to create an IL1α/B enhancer plasmid that will be transformed into E. coli. We used a plasmid backbone in combination with two restriction enzymes BamH1 and Sal1 which would allow for the insertion of the enhancer that was already digested with the enzymes. After transformation, the plasmid was prepped and NanoDropped. Sanger sequencing was performed to verify the intact insert. The newly generated plasmid was then combined with various primers for each motif that contained base pair variations in the sequences. These mutations would then be integrated during the elongation phase of PCR. Samples were treated with DpnI to remove the parent plasmid and were transformed with 10 uL of E. coli and 2 uL of the sample. Colony PCR was performed, and half of the samples (10 ul) were run on an agarose gel while the other half were sent for sequencing verification. Samples were then grown up from the pipette tips that were saved from the colony PCR after the confirmation of the present mutations. Double motif mutations were performed with the same methods but now utilizing a different parent plasmid that contained one of the mutations we were seeking while the second change was added through primer annealing.

Luciferase Assay:

The luciferase assay was performed on the HAECs with their enhancer locus mutated at one of the six motifs or two in combination. The renilla plasmid concentration was 150 ng which allowed for normalization of the data and 300 ng was used for the luciferase plasmids. Cells were plated a day before in 24 well plates with about 75-81000 cells per well. Cells were washed two times with 500 uL of warmed PBS and

then placed into 500 uL of full media they were then placed in the incubator overnight and then treated the next day depending on the TNFα time point (0, 4, 6, or 24 hours).

Results:

Genome-Wide ERG Experiments.

ERG Mostly Functions as a Transcriptional Activator in Distal Enhancer Regions

To begin to characterize ERG's role in EC homeostasis we analyzed its binding profiles across the genome upon acute exposure of ECs to pro-inflammatory IL1-β. This is crucial to the study because ERG's anti-inflammatory mechanism of action is still unknown. It is unclear if it represses pro-inflammatory genes and/or activates anti-inflammatory genes. We used ERG CHIP-Seq data to characterize the binding profiles of 15 cultures HAECs upon no treatment (control media) and treatment (10 ng/mL of IL1β protein) for four hours. A total of 98,126 ERG-bound peaks were located for both treatments. A paired t-test revealed that ERG binding was unchanged at 79% of loci ($p < 0.05$), 18% of loci gained ERG binding, and 3% decreased binding upon IL1- β treatment compared to the control **(Fig, 1A)**. This signifies that most ERG binding sites are unaffected upon exposure to IL1-β. Differentiating the total number of ERG peaks into proximal (those of the gene promotors) and distal (those of the enhancer-like elements) regions we observed similar distributions. There was also a higher density of points closer to zero once again suggesting ERG's unaltered binding status upon IL1-β treatment. There were, however, three times as many ERG-bound sites at the gene-distal regions than that in proximal regions (**Fig. 1B)** and a greater density of overall peaks present (2803 v. 7144). These findings emphasize that ERG predominantly binds enhancer-like elements to regulate HAECs gene expression.

ERG binding alone does not describe its regulatory activity on distal target genes so we utilized the same 15 cultures but probed for acetylation marker, H3K27ac. Acetylation on lysine 27 on histone H3 (H3K27ac) is an epigenetic mark indicating genomic regions with activity. Focusing on the sites that gained ERG binding upon treatment, we discovered an enrichment of H3K27ac regions with more activity in IL1-β v. control (**Fig. 1C** $p<2e-16$). We also found enrichment of reduced H3K27ac markers

upon ERG binding reduction (**Fig. 1D** p<2e-16). This data supports the notion of ERG mostly being an activator in distal enhancer regions. Next, we wanted to establish a relationship between which transcription factors bind with ERG upon IL1β treatment and how they might play a collaborative role in modulating target gene expression. Motif enrichment analysis was performed on DNA sequences showing increased ERG and H3K27ac loci. Enriched motifs were found to be Ap-1, CEBP, and NFkB upon increased ERG biding and H3k27ac modification (**Fig. 1E**) while the reduced motifs were GATA, SOX, and NR under loss of binding (**Fig. 1F)**. These findings underscore ERG's collaborative role with other transcription factors in IL1β treated conditions in affecting

gene expression in ECs.

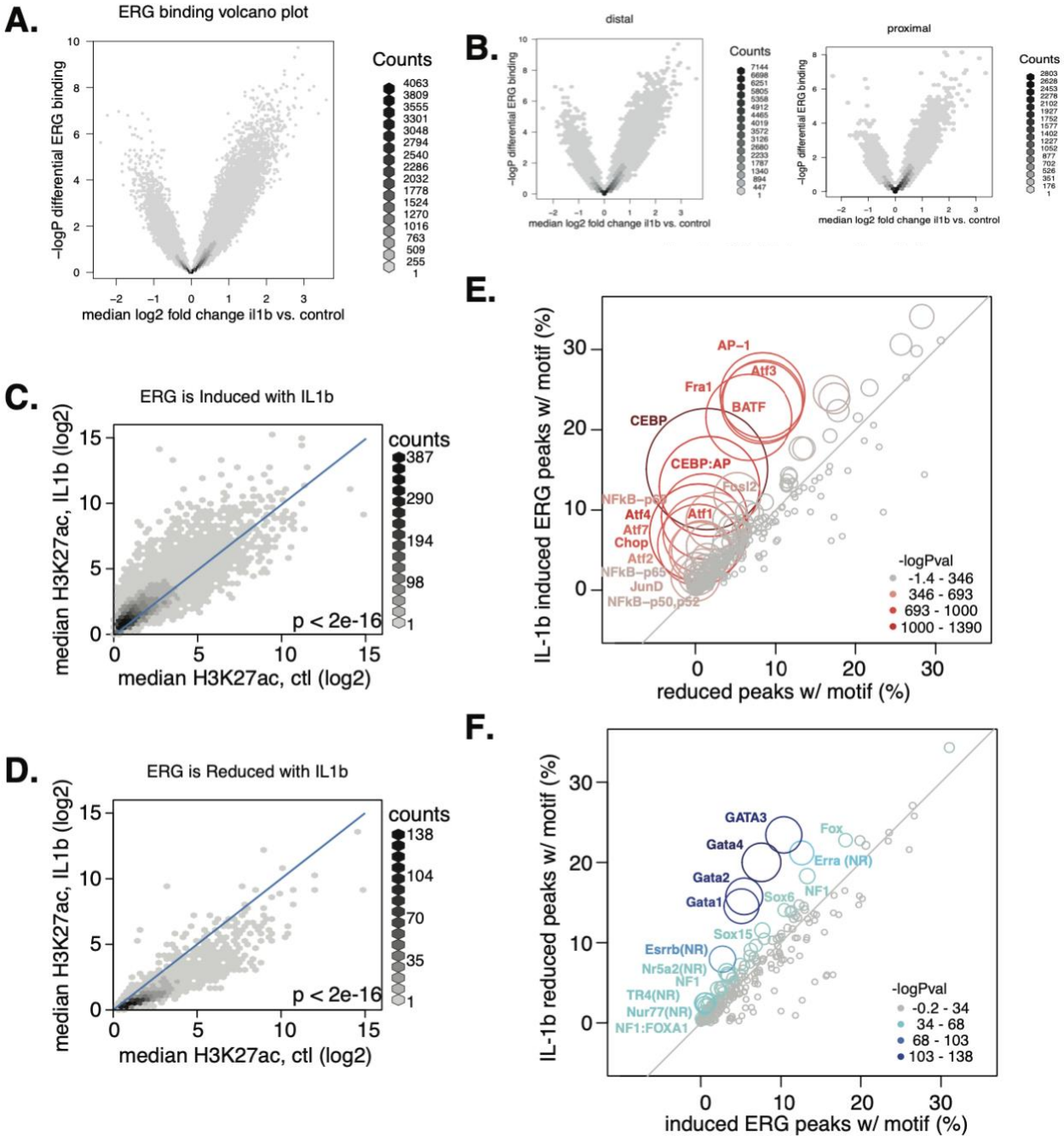

Figure 1: Genome-Wide ERG Experiments.
A. ChIP-seq data for 15 diverse cultures of HAECs with a total of 98,126 ERG-bound peaks. The movement towards the larger values of the x-axis signifies differential ERG binding upon treatment/no treatment conditions. **B.** Data from A. separated based on proximal or distal enriched binding events. **C**. Chip-seq was performed on H3K27ac in conjunction with sites that gained ERG binding under disease mimicking conditions. **D**. Similar methodology as with C. but now with ERG-reduced sites with IL1β treatment. **E.** De novo motif analysis for the loci with increased ERG and H3K27ac given treatment using reduced sites as genomic background and vice versa for **F**.

IL1A/B Enhancer Locus Experiments:

ERG Binds a Cytokine-inducible Putative Enhancer Element at the Human IL1α/β Locus.

To establish ERG's role in ECs we knocked down (KD) ERG levels through a siRNA transfection[10]. Three cells from different individuals (biological replicates) were used for the analysis. We were able to confirm the KD of ERG as well as its repressive role on the IL1α and IL1β cytokines **(Fig. 2A)**. IL1α p-values were found to be slightly above the 0.05 threshold and this result was replicated in past trials[10]. From these data, it can be inferred that the KD of ERG induces IL1α/β expression even without inflammatory stimulus. We also wanted to emulate a disease-like environment with the treatment with TNFα. Stress-inducing events on the ECs might cause differential gene expression which can be crucial in identifying the mechanistic pathway(s) involved in disease progression. The addition of TNFa and reduced ERG by siRNA augmented levels for both of the pro-inflammatory cytokines, IL1α and IL1β more than either condition alone **(Fig. 2B)**. This suggests that ERG downregulation works in addition to the inflammatory stimulus and activates the gene expression in endothelial cells.

When evaluating the HAEC regulome through the ChIP-seq data, we located an enhancer-like element bound by both ERG (IL1β treated and control) and NFκB. This region of the genome was also found to be flanked by H3k27ac marked histones, suggesting the presence of enhancer activity **(Fig. 2C** top**)**. When analyzing the normalized ChIP-seq. tags in both treated and non-treated conditions, we identified that this putative enhancer ranks in the top .04% peaks demonstrating that this IL1α/β locus harbors one of the most highly ERG-bound enhancer-like elements in the HAEC regulome **(Fig. 2D and 2E).** ENCODE's database also displayed that the epigenetic marker (H3K27ac) is most specific to the Human Umbilical Endothelial Cells (HUVECs) signifying the putative enhancers' ability to control expression specifically in ECs **(Fig. 2C** bottom**).** This result is presented in **Table 1** which shows that out of the 61 cell types analyzed in the ChIP-seq panel, HUVECs are the cells with the greatest H3K27ac normalized tags at this genomic locus. Next, we wanted to test if H3K27ac and ERG binding increase upon exposure to cytokine treatment to help us identify if this putative

enhancer is regulating gene expression. We identified that upon exposure to the IL1β recombinant protein both ERG binding and H3K27ac markers were significantly increased (p= 0.0113 and p= 0.0004 respectively) in HAECs **(Fig. 2F and 2G)**. This data suggest that this element does have some up-regulatory function within the HAECs compared to the control/untreated. To further verify this claim we cloned the IL1α/B enhancer sequence into a luciferase reporter plasmid and used Renilla to normalize both plasmid amounts **(Fig. 2H).** We performed these experiments in endothelial cells to evaluate the enhancer activity of the region. We determined that this enhancer does in fact increase luciferase activity in both IL1β treated and non-treated samples (p=1.95e-7 for no treatment control v 4.04e-5 for treated)**. (Fig. 2I).** These results validated our reasoning to study this putative enhancer further to characterize its functional role in endothelial cells.

The Enhancer Functions as an Activator of IL1α/β Gene Expression:

Our global question in this study is to evaluate the enhancer's impact on transcriptional activity of the IL1α and IL1β genes which are key modulators of the immune response. To study this, we created one WT and two KO cell lines with a 141 base pair deletion utilizing the CRISPR-Cas9 system and 2 guide RNAs in TeloHAECs **(Fig. 2H)**. Both control and TNFa treated media were analyzed for transcript levels of the two genes using qPCR. We did not observe a significant alteration in the control media samples between the WT and KO enhancer for transcript levels of IL1α and IL1β (**Fig. 3A)**. Upon TNFα treatment there was a significant decrease in IL1α and IL1β transcript levels in the enhancer KO cells at all the tested time points

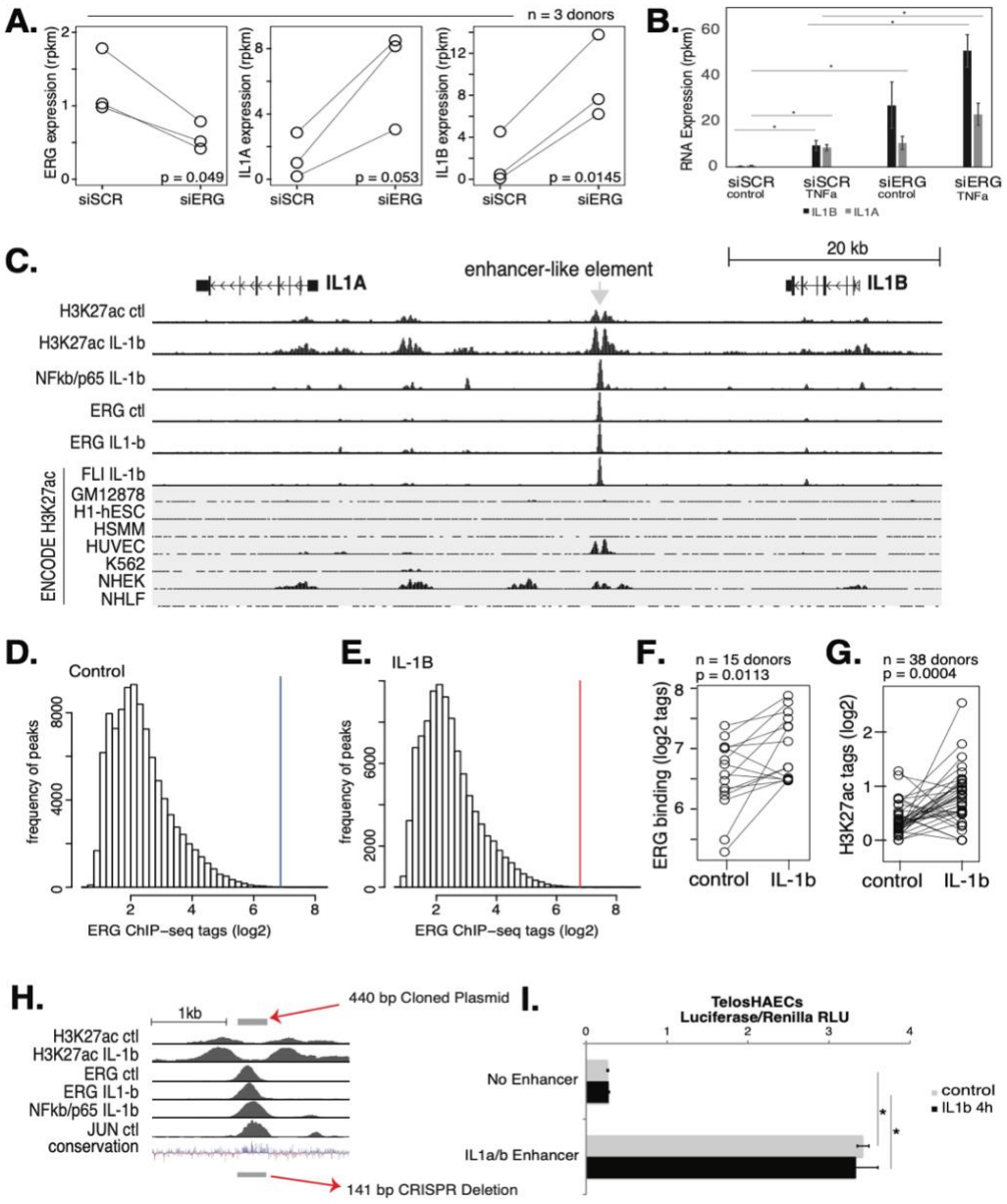

Figure 2: IL1A/B Enhancer Locus Experiments.
A. siRNA ERG knock-down and IL1α and IL1β expression levels in the absence of cytokine treatment. N=3 cells (non-biological replicates). **B**. qPCR data of ERG knock-down with TNFα treatment and its effect on the pro-inflammatory cytokine transcript levels **C.** Major transcription factor peaks and epigenetic markers in the HAEC regulome (top figure) with an enhancer-like element (arrow) located within their binding regions. ENCODE's panel of H3K27ac ChIP-seq data among various cell types with the majority coming from Human Umbilical Vein Endothelial Cells (HUVEC) of the bottom figure. **D.** Frequency of peak analysis among the ERG normalized ChIP-seq tags upon no treatment and **E.** treatment. **F.** ERG binding and **G.** H3K27ac marker enrichment upon HAEC exposure to recombinant IL1β protein. **H**. Luciferase assay data with a 440 bp cloned enhancer plasmid into HAECs. "No enhancer" variable consists of the plasmid backbone only (no insert). **I.** Relative representations of the size of the CRISPR deletion and luciferase plasmid insert within the HAEC regulome.

Table 1: H3K27ac ChIP-seq normalized tags at the IL1ab enhancer (chr2:113568367-113570991, hg19), ranked highest to lowest across cell and tissue types from 61 Roadmap Epigenomic samples

Cell Type or Tissue	H3K27ac normalized tag count (rpkm) at IL1ab enhancer; descending rank	Rank
HUVEC_GSM733691.tagDir	702.84	1
NHEK_GSM733674.tagDir	476.45	2
OSTEOBLAST_GSM733739.tagDir	425.5	3
NHA_GSM733763.tagDir	243.99	4
HMEC_GSM733660.tagDir	229.46	5
IMR90.GSM521887.tagDir	217.93	6
Bone_Marrow_Derived_Mesenchymal_Stem_Cell_Cultured_Cells.GSM1112792.tagDir	157.65	7
IMR90.GSM469966.tagDir	137.45	8
GM12878_GSM733771.tagDir	113.8	9
HELAS_GSM733684.tagDir	73.67	10
NHDFAD_GSM733662.tagDir	57.75	11
NHLF_GSM733646.tagDir	54.74	12
hESC_Derived_CD56+_Mesoderm_Cultured_Cells.GSM1112825.tagDir	54.09	13
HEPG2_GSM733743.tagDir	52.84	14
K562_GSM733656.tagDir	47.08	15
H1_Derived_Mesenchymal_Stem_Cells.GSM767341.tagDir	43.62	16
HUES48.GSM997250.tagDir	34.66	17
Right_Atrium.GSM910557.tagDir	30.81	18
CD4+_CD25-_IL17+_PMA-Ionomcyin_stimulated_Th17_Primary_Cells.GSM772987.tagDir	29.34	19
Chondrocytes_from_Bone_Marrow_Derived_Mesenchymal_Stem_Cell_Cultured_Cells.GSM1112786.tagDir	29.13	20
Left_Ventricle.GSM1127173.tagDir	25.62	21
HSMM_GSM733755.tagDir	24.23	22
Duodenum_Smooth_Muscle.GSM772908.tagDir	23.92	23
H1_BMP4_Derived_Mesendoderm_Cultured_Cells.GSM753425.tagDir	23.13	24
CD14_Primary_Cells.GSM1102782.tagDir	22.49	25
iPS-20b.GSM772848.tagDir	21.77	26
Breast_Fibroblast_Primary_Cells.GSM1127064.tagDir	20.28	27
Pancreatic_Islets.GSM1127061.tagDir	19.57	28
Penis_Foreskin_Fibroblast_Primary_Cells.GSM1127060.tagDir	19.28	29
HUES6.GSM1112774.tagDir	19.2	30
H1_BMP4_Derived_Trophoblast_Cultured_Cells.GSM753427.tagDir	18.76	31
Peripheral_Blood_Mononuclear_Primary_Cells.GSM1127137.tagDir	18.68	32
iPS_DF_19.11.GSM706065.tagDir	16.77	33
CD4_Memory_Primary_Cells.GSM772963.tagDir	16.59	34
iPS-18a.GSM773033.tagDir	15.96	35
iPS_DF_6.9.GSM752967.tagDir	15.46	36
Right_Ventricle.GSM1013124.tagDir	15.4	37
H1.GSM466732.tagDir	15.3	38
H9.GSM605307.tagDir	15.3	39
CD4+_CD25-_CD45RO+_Memory_Primary_Cells.GSM772859.tagDir	15.08	40
H1HESC_GSM733718.tagDir	14.97	41
Mobilized_CD34_Primary_Cells.GSM772870.tagDir	14.89	42
CD19_Primary_Cells.GSM1027287.tagDir	14.79	43
CD4+_CD25-_Th_Primary_Cells.GSM997239.tagDir	14.3	44
HSMMT_GSM733666.tagDir	13.58	45
Penis_Foreskin_Melanocyte_Primary_Cells.GSM1127072.tagDir	13.15	46
CD8_Memory_Primary_Cells.GSM772880.tagDir	12.9	47
HUES64.GSM1112775.tagDir	12.78	48
CD4+_CD25+_CD127-_Treg_Primary_Cells.GSM997233.tagDir	11.95	49
H1_Derived_Neuronal_Progenitor_Cultured_Cells.GSM753429.tagDir	11.75	50
CD56_Primary_Cells.GSM1027288.tagDir	11.7	51
Stomach_Smooth_Muscle.GSM916065.tagDir	11.7	52
CD3_Primary_Cells.GSM1058764.tagDir	10.87	53
hESC_Derived_CD184+_Endoderm_Cultured_Cells.GSM1112830.tagDir	10.71	54
Aorta.GSM906392.tagDir	10.37	55
CD4+_CD25-_CD45RA+_Naive_Primary_Cells.GSM773004.tagDir	9.96	56
hESC_Derived_CD56+_Ectoderm_Cultured_Cells.GSM1112824.tagDir	9.62	57
CD4+_CD25int_CD127+_Tmem_Primary_Cells.GSM916026.tagDir	8.7	58
CD8_Primary_Cells.GSM1102781.tagDir	8.26	59
CD4_Naive_Primary_Cells.GSM772835.tagDir	7.68	60
CD8_Naive_Primary_Cells.GSM772949.tagDir	6.57	61

except for the 0.5 TNFα treatment for IL1α. Taken together, this data validates this region's role as an enhancer in ECs for IL1α and IL1β pro-inflammatory cytokines. We also investigated this enhancer's impact on cellular function by performing a leukocyte adhesion assay of THP-1 monocytes for the WT and the KO enhancer ECs samples. This assay evaluates the extent of interactions between the endothelial cells and the monocytes that are recruited by the ECs after the release of cytokines and downstream adhesion molecules[12]. Upon inflammatory stimulus, we found a reduction in monocyte adhesion in the KO samples compared to the WT at all time points, but significantly at the 6-hour treatment **(Fig. 3B)**.

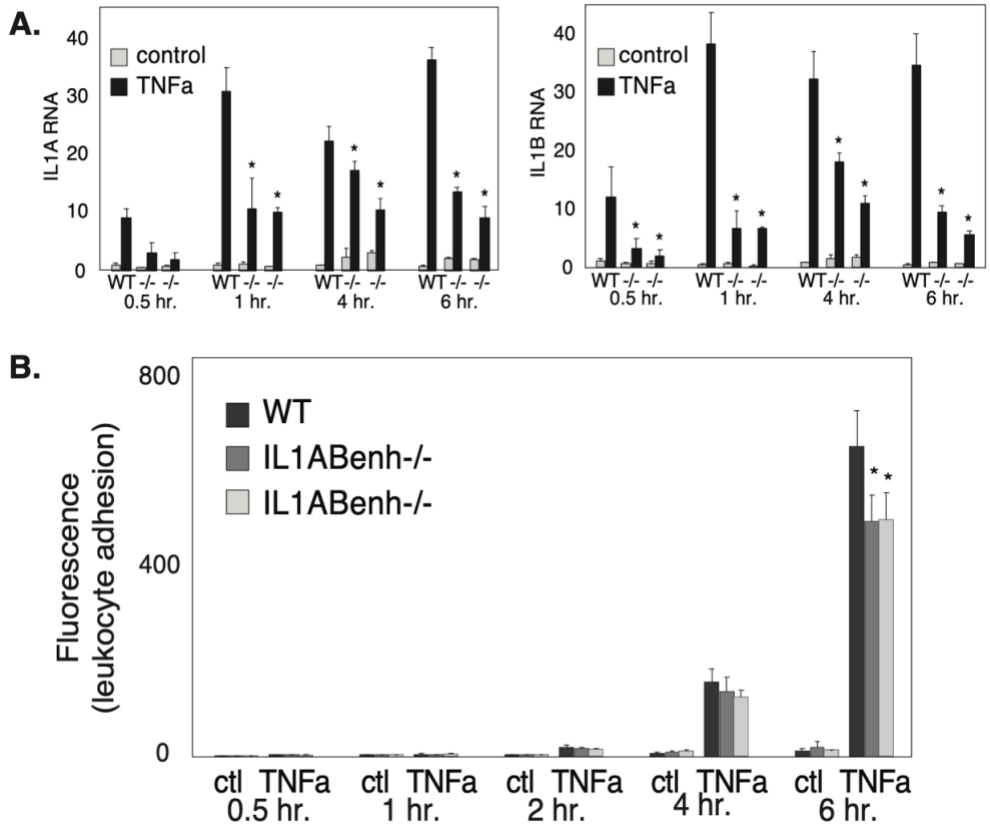

Figure 3: Identifying IL1A/B Enhancer Regulatory Function Through CRISPR-Cas9 Transfections and Leukocyte Adhesion Assays.
A. Transcript levels of IL1α and IL1β in untreated conditions over 0.5,1,4, and 6-hour period (control) compared to treated media conditions (TNFα) for the same samples. Time treatment of 2 hours did not show a significant difference between media types similar to WT 0.5 hours. **B.** Leukocyte adhesion assay was performed with the same samples and time frame. WT=wild type HAECs, IL1ABenh-/- KO clone. The asterisk symbolizes the significance of KO cells ($p<.05$) compared to their individual WT condition.

Combinatory Effects of ERG and theIL1α/B Enhancer on Regulating Pro-Inflammatory Cytokine Transcription:

Learning about the individual roles of ERG and the IL1α/B Enhancer we next wanted to evaluate the relative contributions of each of the components on inflammatory gene transcription using ERG gain and loss of function experiments in both WT and KO enhancer endothelial cells. Lentiviral transduction was performed with either ERG or GFP (control) particles in TeloHAECs and the transduction took place for three days. The cells that received the ERG lenti condition are those with overexpression of ERG with treatment. Our results showed that the effect of gene transcription is mediated more through the enhancer (WT v KO) than ERG alone (control v ERG lenti) as shown in **Fig. 4A** as significant change is present between KO and WT samples and only some of the WT v WT samples between ERG overexpression or control levels. This finding is suggestive of ERG's collaborative role with the Enhancer in modulatingIL1α/B transcription.

To test the impact of ERG's loss of function, we carried out a transfection with ERG validated siRNAs[10]. In the treated conditions, IL1α and IL1β transcript levels were three times higher in ERG KD cells than in controls (**Fig. 4B** dark bars) which signifies that reduced ERG overexpression and TNFa treatment are additive. There is also a reduction in IL1α and IL1β transcription when the enhancer is removed upon the treatment which suggests that regulation is prominently exerted through this element.

ERG Motif 5 and NFkB Motif are Points of Regulation Within the Enhancer:

In our last section, we wanted to further characterize the mechanism of action of ERG based on its binding landscape in the IL1α/β enhancer. We performed motif analysis in the enhancer region of our luciferase plasmid that we created (440 bp) and identified 4 ERG binding motifs and one NFkB motif **(Fig. 5A).**

Producing individual motif deletions through primer annealing and mutation creation, we sought to establish each motif's mutation effect on enhancer activity. The individual plasmids with their respective motif mutations and control samples were then

utilized for a luciferase assay with IL1β treated (4 hours) and under non-treated conditions. Compared to theIL1α/β enhancer sample ERG motif mutation 5 and NFkB motif mutation (p65) significantly decreased enhancer activity under both treated and non-treated conditions **(Fig. 5B)**. From this result, we can infer the possibility that both of these mutations might produce their effects in conjunction. ERG motif mutations 1, 2, and 3 saw no change, while ERG motif mutation 4 increased enhancer activity but was not replicable when various mutated versions were created (refer to future directions). We also went back to our CRISPR enhancer deletion (141 bp) and compared where the motifs would align in a program called ApE. We found that our CRISPR KO did not encompass motifs 1 and 2, which is consistent with our luciferase result finding these motif mutations to have no major effect. To test the hypothesis that both the P65 motif and motif 5 function independently to modulate enhancer activity upon ERG and NFkB binding, we repeated the luciferase assay (0, 6, and 24-hour treatments) with the incorporation of a double mutation. We also saw that the double mutation of motif 5 and NFkB creates a larger inhibitory response than the two mutations alone ($p<.05$ for both time treatments). Our results were replicable for motif 5 and p65 across all time treatments **(Fig. 5C).** This signifies that ERG and NFkB both collaborate in this enhancer sequence to modulate the inflammatory response.

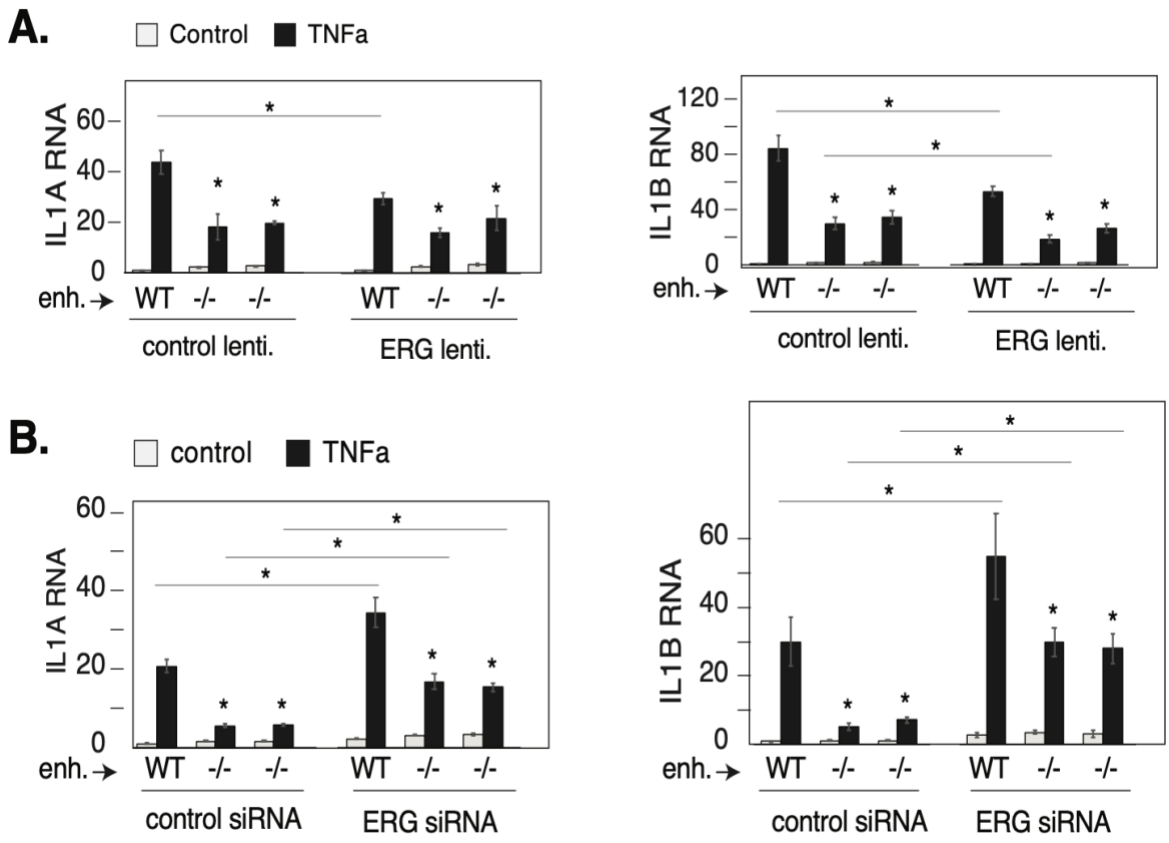

Figure 4: Inflammatory Pathway Contribution Analysis of ERG and the Enhancer through Loss-of-function and Gain-of-function Experimentations.
A. ERG Lentivirus transduction experiments with WT and KO enhancer cells. IL1α and IL1β transcript levels were measured through qPCR. **B.** ERG siRNA transfections with the same cell types and cytokines were measured as before. The asterisk symbol represents the significance of enhancer presence (WT and KO) in each respective group while the asterisk combined with a line compares the significance of ERG overexpression.

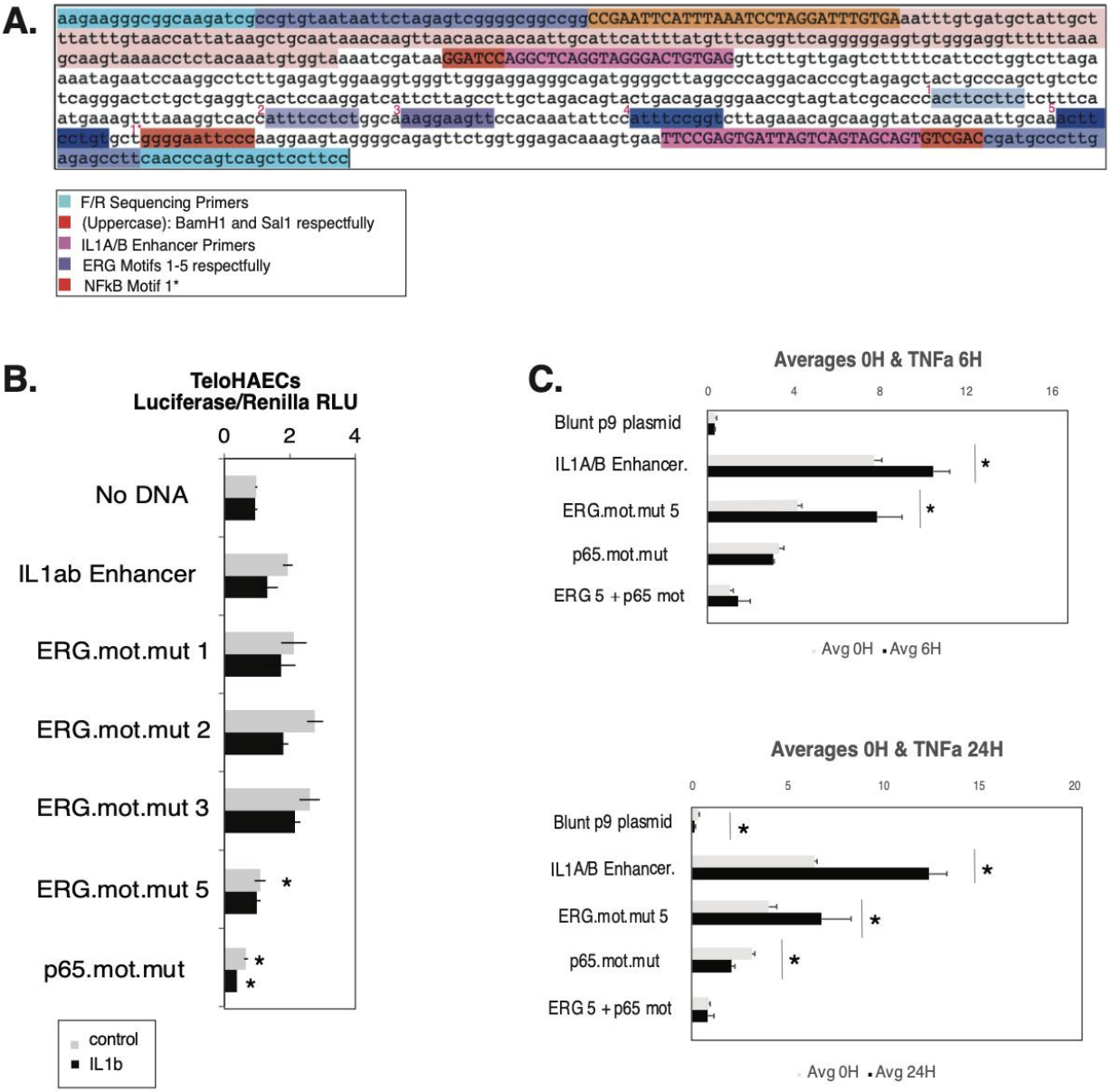

Figure 5: Transcription Binding Event Analysis Through Motif Deletions Inside the IL1A/B Enhancer Locus.

A. ERG and NFκB motif distribution within the luciferase plasmid. **B.** Luciferase data for single motif mutations with and without treatment. Significance is seen between no treatment conditions for IL1αβ Enhancer (control or treated) versus ERG motif 5 mutation control as well as p65 mutation control and treated (p=.003 and p=.0002, p=.029 respectively). **C.** Luciferase data with the addition of blunt plasmid (no enhancer) and ERG 5 + p65 motif mutation. Treatment period over 6 and 24 hours. A significant difference ($p<.05$) between the zero and six-hour TNFα treatment as well as zero and 24-hour treatment is displayed through the asterisk.

Discussion:

In this study, we aimed to characterize the molecular mechanisms behind atherosclerosis in endothelial cells by exploring a region of the genome that is located between IL1-α and IL1-β and is bound by an important anti-inflammatory transcription factor, ERG. We demonstrated that this putative enhancer controls inflammation by promoting upregulation of the pro-inflammatory cytokines which is crucial in cell homeostasis, as well as located regions of the enhancer that are specific to its regulatory function in the presence of ERG and NFkB. Our study design consisted of emulating disease-like conditions with TNFa or IL1β to perturb the EC environment towards downstream coronary artery disease. Both in vitro (luciferase assay) and in vivo studies (CRISPR deletion) were conducted to establish the enhancer's regulatory role while RNA transcript levels were measured for gene expression analysis.

Our hypothesis stemmed from the relationship between ERG's binding in the EC genome and its high affinity toward the IL1-α/β enhancer. As this TF is an important regulator of inflammation, knowing its binding affinity and location could lead to possible interventional therapies to control chronic damage. We identified that iERG's binding landscape is enriched with H3K27ac, a marker for enhancer activity specifically between IL1-α and IL1-β genes and that it is most saturated in this genomic area compared to its total regulome. We also uncovered that ERG's control over expression occurs in conjunction with the binding of other TF such as NFkB through de novo motif analysis under disease-causing stimuli. This implies that ERG could have possible downstream partners in controlling inflammation which is still a major topic of study today. What is important is that we showcased that ERG's influence on gene expression is limited without a stimulus like TNFa in which cytokine transcript levels are significantly upregulated in its presence **(Fig. 2B).** This phenomenon could be possibly explained by the close ties TNFa has to the NFkB activation pathway. It translocates this TF into the nucleus and increases its pro-inflammatory activity. ERG with TNFa treatment results in less RNA expression than with siERG and TNFa and this could be due to the notion that ERG initially inhibits the NFkB pathway but with its depression, NFkB could act upon the genes with its full potential[6]. These results indirectly imply this relationship and will be explored more in the future directions.

From this information, we hypothesized that ERG binding in the putative enhancer region causes an increase in IL1-α and IL-β transcripts (and protein levels) and that this region's deletion causes dysregulation of both of these cytokines. To test our claim's first part, we transformed E.coli with a plasmid backbone that lacked the enhancer insertion site and compared that to the fully intact plasmid through a luciferase assay in ECs. We found that in the presence of this region there was an increase in enhancer activity in both cytokine treated and nontreated conditions ($p=4e-5$ and $p=1.9e-7$ respectively) implying the differential gene expression it can provide. We also verified this finding in CRISPR KO endothelial cells where there was a significant decrease ($p<.05$) of transcript levels in the absence of this genomic region. This was further supported by the leukocyte adhesion assay results showing a lack of monocyte binding due to the absence of a pro-inflammatory cytokine signal implying that this enhancer functions upstream of the regulatory network involved in leukocyte adhesion a crucial aspect of the inflammatory process.

With the characterization of this area as a true enhancer, we hypothesized that there could be differential gene regulation coming from ERG and the enhancer in combination. We specifically wanted to quantify the amount of control that is coming from the TF versus the enhancer. Performing lentiviral transduction assays for overexpression of ERG and siRNA transfection for KD of the TF in both WT and KO enhancer cell lines we identify the ERG and the enhancer function in conjunction as can be seen by the significance of the paired t-tests between WT v KO samples (enhancer impact) as well as WT v WT (ERG impact within control and ERG lenti/ siRNA). What is important to consider is the fact that most of the significance is located between cells with variable enhancer samples (KO and WT) and only some of the samples compare the ERG overexpression or knockdown. This could suggest that regulation is primarily controlled through the enhancer element and that only in the KD of ERG do we see significant changes between samples. There is something that needs to be considered when interpreting this portion of the data which is the relative difference in the TNFa treatment between the two methods where the lentiviral transduction had a 1 hour treatment time point while the siRNA included a 1 hour and 48-hour time point after the inclusion of the siERG. This difference could relate to the greater change between

enhancer samples in the transfection portion compared to the transduction and will need to be controlled for in the future.

In the last section of our project, we wanted to identify motifs within the enhancer that control its activity. Through de novo motif analysis we located five potential ERG motifs and one NFkB motif **(Fig. 5A).** We showed multiple times that ERG motif mutation 5 and NfkB motif mutations are major points of regulation within the IL1-α/β enhancer region with and without treatment. This mechanism of action is likely due to ERG binding and activation of the enhancer as seen by the decrease in enhancer activity without the motif presence. Comparatively, ERG motifs 1, 2, and 3 did not show any significant difference implying the lower frequency of binding and control coming from these motif regions. What is interesting to note is that we obtained a significant difference in ERG motif 4 but in the opposite direction where without its presence, enhancer activity would increase signifying that binding upon motif 4 produces a repressive response onto the enhancer. Data surrounding motif four was inconsistent even after various mutation versions of the same area and so this section of the experiment will be explored more in the future directions. We also showed that when combining the two motif mutations (ERG 5 and p65) onto one plasmid we created an even greater depressed response than compared each mutation alone in both treatment and non-treatment ($p<.05$) implying their cumulative nature and the possibility of ERG and NFkB combinatory binding that creates the greatest stimulation when the motifs are present.

In conclusion, we identified an HAEC-specific enhancer that controls IL1-α and IL1-β gene expression. This region works in combination with ERG to regulate cytokine expression and utilizes ERG motif 5 and p65 of NFkB as the major contributors to enhancer control. Impact on enhancer activity can be modified through double motif mutations signifying the possibility of ERG and NFkB acting together to modulate the inflammatory environment in the ECs through this enhancer. We hope this knowledge can bring forth various genetic therapies and treatments in preventative medicine to reduce the devastating impacts of prolonged inflammation and atherosclerosis.

Future Directions:

Although we were able to uncover important mechanistic processes regarding inflammation regulation within the endothelial cells, there is still much we would like to focus on. In this study, gene expression was represented through RNA transcript levels which we then deduced to suggest the relative protein concentration of the genes. Currently, we are working on replicating our ELISA assays to add in combination with **Figure. 3** and **Figure 4.** This could reinforce our present data and verify the trend on both a transcriptomic and protein level.

As described in the discussion, our ERG 4 motif mutation resulted in an unexpected trend in **Figure. 5B** suggesting that ERG acts both as a repressor through motif 4 and an activator through motif 5 and p65. We compared, using the program Homer's, the motif list with the mutation we synthesized for motif 4 in the program R. HIF1b was found to have the greatest motif match score supporting the idea that an accidental production of its motif site led to the inaccurate interpretation of the data, but when creating two other versions of motif 4, we were not able to replicate the trend of depression seen in ERG motif 5 and p65 levels. This discontinuous trend between the various versions compelled us to look at the possibility of FII1, a transcription factor with a similar motif sequence to ERG[11], as a possibility for these results. We hope to conduct ERG and FLI1 KD experimentation to distinguish the relative contributions of both TFs within the enhancer.

Lastly, we want to establish the relationship NFkB has with ERG in this IL1α/β enhancer. We are in the process of performing NFkB inhibitor trials and hypothesized that with an inclusion of an inhibitor the enhancer activity would decrease throughout all the motif mutations as ERG is likely inhibited from binding without a downstream partner that controls target gene expression.

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