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Three-Dimensional Chromosomal Landscape Revealing miR-146a Dysfunctional Enhancer in Lupus and Establishing a CRISPR-Mediated Approach to Inhibit the Interferon Pathway

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Objective. The diminished expression of microRNA-146a (miR-146a) in systemic lupus erythematosus (SLE) contributes to the aberrant activation of the interferon pathway. Despite its significance, the underlying mechanism driving this reduced expression remains elusive. Considering the integral role of enhancers in steering gene expression, our study seeks to pinpoint the SLE-affected enhancers responsible for modulating miR-146a expression. Additionally, we aim to elucidate the mechanisms by which these enhancers influence the contribution of miR-146a to the activation of the interferon pathway.

Methods. Circular chromosome conformation capture sequencing and epigenomic profiles were applied to identify candidate enhancers of miR-146a. CRISPR activation was performed to screen functional enhancers. Differential analysis of chromatin accessibility was used to identify SLE-dysregulated enhancers, and the mechanism underlying enhancer dysfunction was investigated by analyzing transcription factor binding. The therapeutic value of a lupus-related enhancer was further evaluated by targeting it in the peripheral blood mononuclear cells (PBMCs) of patients with SLE through a CRISPR activation approach.

Results. We identified shared and cell-specific enhancers of miR-146a in distinct immune cells. An enhancer 32.5 kb downstream of miR-146a possesses less accessibility in SLE, and its chromatin openness was negatively correlated with SLE disease activity. Moreover, CCAAT/enhancer binding protein α, a down-regulated transcription factor in patients with SLE, binds to the 32.5-kb enhancer and induces the epigenomic change of this locus. Furthermore, CRISPR-based activation of this enhancer in SLE PBMCs could inhibit the activity of interferon pathway.

Conclusion. Our work defines a promising target for SLE intervention. We adopted integrative approaches to define cell-specific and functional enhancers of the SLE critical gene and investigated the mechanism underlying its dysregulation mediated by a lupus-related enhancer.

INTRODUCTION

Enhancers are the *cis*-acting DNA regulatory elements that determine the gene expression pattern.[1] The highly ordered spatial interactions between the enhancers and the promoter regions of genes are critical for the expression of cell type–specific genes.[2] In many diseases, enhancers are dysregulated and contribute to disease development by affecting the expression of disease-critical genes.[3,4] Identifying the functional disease-associated enhancers would help reveal the mechanisms that

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mediate the abnormal expression of genes in disease and enable us to better understand the disease pathogenesis.

Enhancers are usually cell-type specific and exclusively regulate gene expression[5]; these characteristics make it a promising therapeutic target for many diseases. For example, targeting an erythroid-specific enhancer of *BCL11A* by the CRISPR/Cas9 system has been applied to treat sickle cell disease and β-thalassemia, and the clinical outcomes are encouraging.[6] Therefore, further characterization of regulatory components can expand our toolset to target disease-critical genes. However, it is challenging to precisely locate active enhancers of a specific gene, especially for functional disease-associated enhancers.

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with the characteristics of autoantibody production and abnormal activation of the type I interferon (IFN) pathway.[7] Aberrations and dynamic changes of the epigenome have been identified in the immune cells of patients with SLE, some of which changed over time and might be associated with clinical phenotypes and medication use.[8,9] However, most of the epigenetic studies in SLE were observational, and the molecular basis of the epigenetic changes and their functional outcomes have not been deeply investigated. Dissecting the disease-associated enhancers that regulate SLE critical gene expression could provide important insight into SLE pathogenesis and provide an attractive target in disease therapy. However, few studies have experimentally mapped the functional disease-associated enhancers, let alone dissected the mechanism that mediates enhancer dysregulation.

To fill this gap, we chose gene *MIR3142HG* as an example to investigate its regulatory landscape in different cells and the epigenetic mechanism underlying its lower expression in SLE. Accumulated studies have shown that *MIR3142HG* is an SLE risk gene. *MIR3142HG* encodes microRNA 146-a (miR-146a), a critical microRNA that is abnormally expressed and negatively regulates the type I IFN pathway by targeting STAT1 and interferon regulatory factor 5 (IRF5) in SLE.[10] Previous studies by our group have found SLE risk variants located at the promoter and distal enhancer of miR-146a can lead to the aberrant expression of miR-146a by affecting the binding affinity with transcription factors.[11,12] Despite the well-recognized pathogenetic relevance of miR-146a in SLE, its regulatory network and epigenetic architecture remain largely uncharted. Here, we combined multiomics data, including chromatin accessibility, chromatin contacts, and histone modifications, with CRISPR activation (CRISPRa) screening to identify enhancers of miR-146a in distinct cell lineages. We discovered that the 32.5-kb enhancer was likely an SLE-associated enhancer because it was less accessible in monocytes through differential analysis of activation-induced, T cell–derived, and chemokine-related cytokine sequencing (assay for transposase-accessible chromatin sequencing [ATAC-seq]) data between patients with SLE and healthy controls. Additionally, this enhancer was enriched with CCAAT/enhancer binding protein α (C/EBPα) motifs. In particular, the reduced expression of *CEBPA* in SLE monocytes leads to the insufficient openness of the 32.5-kb enhancer and decreased expression of miR-146a, thus contributing to SLE pathogenesis. We further explored the therapeutic value of the SLE-associated enhancer. By adopting a CRISPRa system targeted to the 32.5-kb enhancer, the activity of the IFN signaling pathway was significantly inhibited. In conclusion, our research defines the epigenetic basis of miR-146a dysregulation in SLE and provides a therapeutic target for clinical intervention.

MATERIALS AND METHODS

Study design. The overall study design is presented in Figure 1. First, we performed chromatin conformation

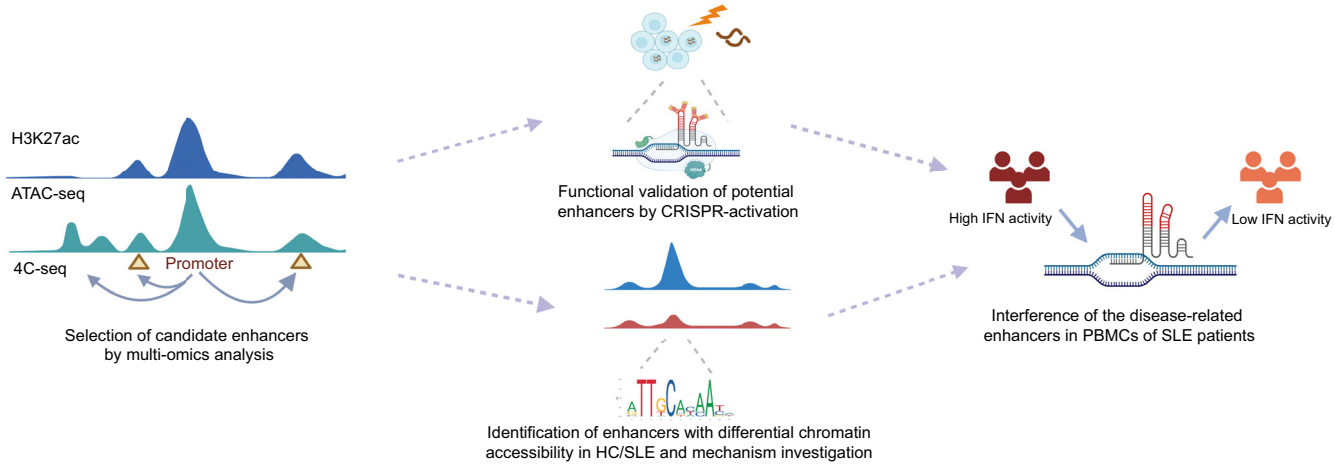

Figure 1. Overall study design and experiment steps. ATAC-seq, Assay for transposase-accessible chromatin sequencing, T cell–derived, and chemokine-related cytokine sequencing; HC, healthy control; H3K27ac, acetylation of histone H3 on lysine 27; IFN, interferon; PBMC, peripheral blood mononuclear cell; SLE, systemic lupus erythematosus; 4C-seq, circular chromosome conformation capture sequencing

capture–on-chip sequencing (circular chromosome conformation capture sequencing [4C-seq]) and ATAC-seq in different immune cell subsets and integrated the results with acetylation of histone H3 on lysine 27 (H3K27ac) data to select the candidate enhancers of miR-146a. Then, we employed CRISPRa to identify functional cell type–specific and shared enhancers of miR-146a. Meanwhile, we carried out the differential analysis of ATAC-seq data from the healthy controls and patients with SLE to identify SLE-associated enhancers of miR-146a. Finally, we dissected the mechanism that mediated the enhancer dysregulation, and the lupus-related enhancer was targeted by the CRISPRa system to intervene in pathogenic pathways.

Human study subject samples. The donors of healthy controls and patients with SLE were recruited and signed informed consent according to the internal review and ethics boards of Renji Hospital, Shanghai Jiao Tong University School of Medicine (SJTUSM). The experiments were approved by the internal review and ethics boards of Renji Hospital, SJTUSM. Patients with SLE were diagnosed based on the 1997 update of the American College of Rheumatology revised SLE criteria[13] in the rheumatology department at Shanghai Renji Hospital. Demographic and clinical information including age, sex, disease duration, clinical manifestations, and medications was collected through chart review. Laboratory test results with the closest time to sample collection for the study were collected from electronic patient records. The SLE Disease Activity Index 2000 (SLEDAI-2K)[14] were calculated based on chart review. Detailed demographic and clinical characteristics of the subjects are listed in Supplementary Tables 1 to 3. Blood samples of healthy controls or patients with SLE were collected with a 10-ml vacuum sterile tube, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8 software and calculated using a paired or unpaired two-tailed Student's *t*-test as indicated in the figure legend unless otherwise mentioned. Where noted in the figures, asterisks define the significance level (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Data are shown as mean ± SEM; "n" represents the number of biologically independent samples or experiments unless otherwise mentioned.

All data relevant to the study are included in the article or uploaded as supplementary information. The data from E-GEAD-39 7[15] were used to analyze miR-146a expression and CEBPA expression in healthy control patients and patients with SLE. The data from GSE131620[16] were used to analyze the chromatin accessibility induced by C/EBPα expression. The Cistrome database (http://cistrome.org/db/#/)[17] was used to analyze the chromatin immunoprecipitation sequencing (ChIP-seq) data of C/EBPα binding to the 32.5-kb enhancer. The 4C-seq data that support the findings of this study have been deposited in ArrayExpress database under accession codes E-MTAB-9581 and E-MTAB-13082. The ATAC-seq data of healthy control patients and patients with SLE in the current study are available from the corresponding author on reasonable request.

RESULTS

Integration of 3D genome and epigenomic analysis and identification of the candidate enhancers of miR-146a. Enhancers control the expression of genes by forming highly ordered chromatin structures. To define the enhancers that regulate miR-146a expression, we first performed a 4C-seq assay to construct the chromatin interaction landscape of the miR-146a promoter region in Jurkat T cells, Raji B cells, and U-937 monocytes (Supplementary Figure 1). Moreover, given that most functional regulatory elements are enriched of strong H3K27ac modification or possess high chromatin accessibility,[18,19] we further analyzed the epigenomic modification of genomic regions interacting with the promoter of miR-146a. The H3K27ac ChIP-seq from the ENCODE database and the ATAC-seq in our laboratory were used in this analysis. Then, we identified the candidate enhancers based on the following criteria (Supplementary Figure 2): 1) the genomic region should form a gene loop with the miR-146a promoter, 2) the genomic region should be enriched of H3K27ac modification and ATAC peak, and 3) the genomic region should be located in the −200-kb to 200-kb window of miR-146a transcription start site (TSS) because most gene loops are distributed in this distance (Supplementary Figure 1).[20] After analysis, we finally identified 10, 6, and 7 candidate enhancers in monocytes, B cells, and T cells, respectively (Figure 2A and B). Among these candidates, six enhancers are shared by all three cell types, four enhancers are monocyte specific, and one enhancer is T cell specific (Figure 2C). Notably, most candidates are located in the intergenic region, but some candidates are located in the promoter region and exonic region (Figure 2D).

Functional screen of miR-146a enhancers by CRISPRa system across different cell types. Although the combination of 3D genome data and epigenomic analysis could aid in the prediction of potential enhancers, experimental confirmation was indispensable in the investigation of functional regulatory elements. Thus, we used a dead Cas9 (dCas9)-VP64 CRISPRa system[21] to screen functional enhancers of miR-146a. First, we established U-937, Jurkat, and Raji cells with stable expression of the dCas9-VP64 system through lentiviral infection. Then, single-guide RNAs targeting candidate enhancers were designed, synthesized, and electroporated into dCas9-VP64–expressing cells. The expression levels of mature miR-146a were detected after electroporation for 12 hours (Figure 3A). Consistent with the epigenomic modifications, most putative enhancers could regulate miR-146a expression (Figure 3B).

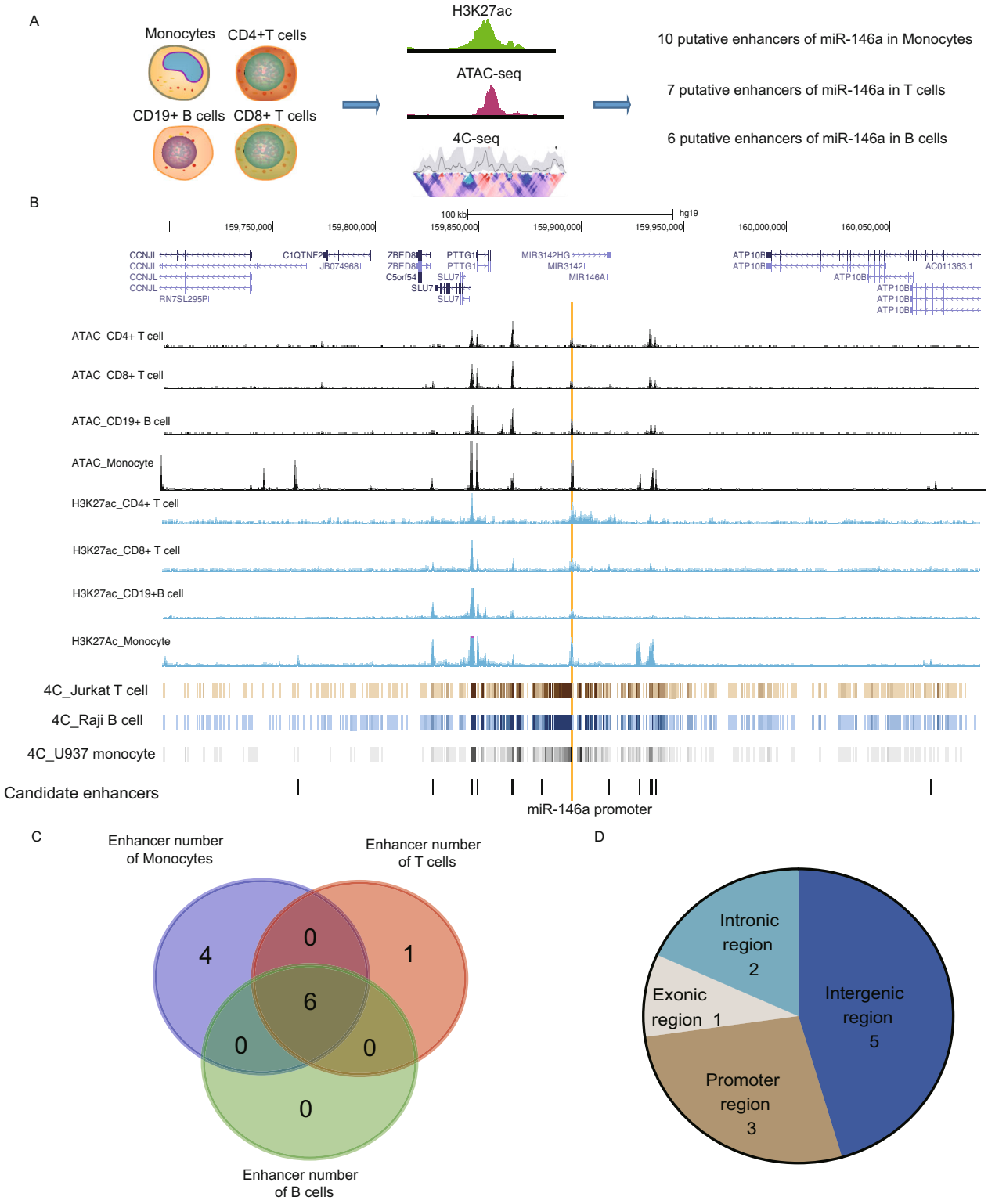

Figure 2. Identification of the putative enhancers of miR-146a in different immune subpopulations. (A) Flow scheme of identifying putative enhancers of miR-146a is shown. (B) Chromatin landscape of MIR3142HG locus based on ATAC-seq, H3K27ac-seq, and 4C-seq in monocytes, B cells, and T cells is shown. The 4C-seq reads were loaded into the University of California Santa Cruz (UCSC) (https://genome.ucsc.edu/cgi-bin/hgCustom) for 4C visualization. The intensity of colors indicates the interaction frequency. (C) Analysis of the shared and cell type–specific enhancers is shown. (D) Distribution of enhancers in the genomic region is shown. See also Supplementary Figures 1 and 2. ATAC-seq, activation-induced, T cell–derived, and chemokine-related cytokine sequencing; H3K27ac, acetylation of histone H3 on lysine 27; MIR, MicroRNA; miR-146a, microRNA-146a; 4C-seq, circular chromosome conformation capture sequencing.

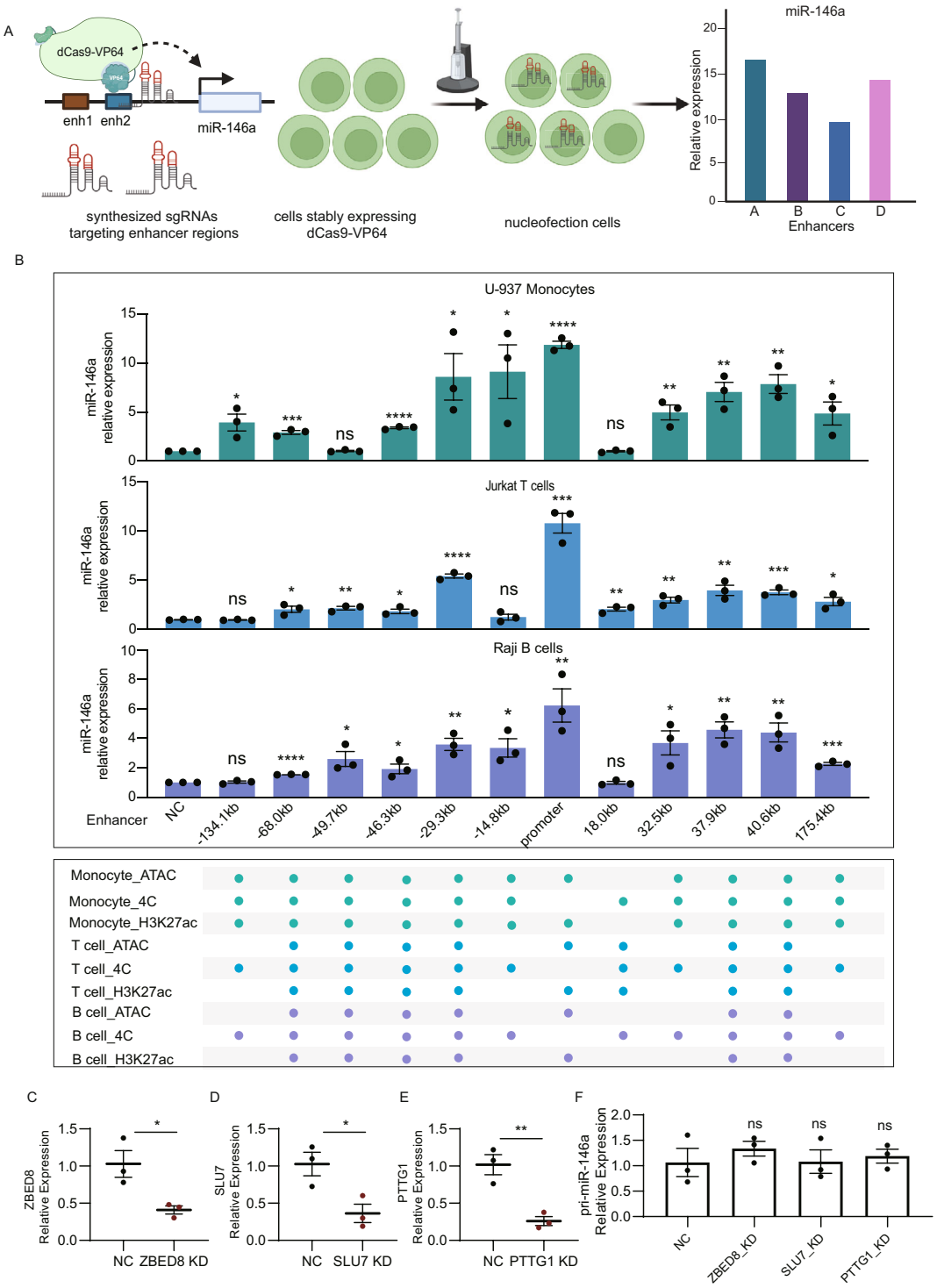

Figure 3. CRISPRa confirms the functional enhancers of miR-146a. (A) Flow scheme of confirming the functional enhancer of miR-146a is shown. (B) Top panel shows RT-qPCR analysis of the miR-146a expression by targeting different enhancer regions using CRISPRa system (n = 3, biologically independent experiments). Bottom panel shows a summary of characters of enhancers in different cell subsets; the dot indicates that the genomic regions possess H3K27ac modification, ATAC peak, or interaction with miR-146a promoter. Different colors of dot represent different cells. (C–E) RT-qPCR analysis of *ZBED8*, *SLU7*, and *PTTG1* expression with *ZBED8*, *SLU7*, and *PTTG1* knockdown, respectively, is given (n = 3, biologically independent experiments). (F) RT-qPCR analysis of pri-miR-146a expression with *ZBED8*, *SLU7*, and *PTTG1* knockdown, respectively, is shown (n = 3, biologically independent experiments). Data are represented as mean ± SEM, and *P* values are calculated using (B and C–F) unpaired two-tailed Student's *t*-test. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. See also Supplementary Figure 3. assay for transposase-accessible chromatin, activation-induced, T cell–derived, and chemokine-related cytokine; CRISPRa, CRISPR activation; dCas9, dead Cas9; enh, enhancer; H3K27ac, acetylation of histone H3 on lysine 27; KD, knockdown; miR-146a, microRNA-146a; NC, negative control; pri-, primary transcript; RT-qPCR, reverse transcriptase–quantitative polymerase chain reaction; sgRNA, single-guide RNA; 4C, circular chromosome conformation capture.

The monocyte-specific –134.1-kb enhancer and T cell–specific 18.0-kb enhancer specially regulated miR-146a expression in monocytes and T cells, respectively (Figure 3B). Notably, the 32.5-kb and 175.4-kb enhancers, which were predicted to be monocyte-specific enhancers, could also regulate miR-146a expression in T cells and B cells. These two enhancers have interactions with the miR-146a promoter region in all three cell types, but enhancer markers are only in monocytes, suggesting that some unmodified regions also have regulatory potential. In addition, the –14.8-kb enhancer harbors an SLE risk variant rs2431697, which has been identified as the functional genetic variant that regulates the expression of miR-146a by affecting nuclear factor kappa-light-chain-enhancer of activated B cells p65 binding.[12] Interestingly, we also found that some enhancers, such as the –46.3-kb, –49.7-kb, and 68.0-kb ones located at the promoter regions of *PTTG1*, *SLU7*, and *ZBED8*, respectively (Supplementary Figure 3), can also regulate miR-146a expression. To explore whether miR-146a expression is regulated by these genes directly or by their promoters, which act as enhancers of miR-146a, we knocked down these genes and measured miR-146a expression. As shown in Figure 3C–F, the down-regulation of *PTTG1*, *SLU7*, and *ZBED8* expression could not affect the expression of miR-146a, suggesting that the promoter regions could function as enhancers to regulate the expression of nearby genes.

Differential analysis of chromatin accessibility discovers SLE-dysregulated enhancer of miR-146a. Immune cells from patients with SLE possess a distinct chromatin landscape compared with healthy controls.[9] It was found that variations in chromatin accessibility at gene regulatory elements were positively correlated with variations in the expression of the associated genes.[22] To find the epigenomic alterations driving the differential expression of miR-146a in SLE, we first performed ATAC-seq[23] in CD14+ monocytes, CD19+ B cells, CD4+ T cells, and CD8+ T cells collected from eight healthy controls and eight patients with SLE (discovery cohort; the demographic and clinical characteristics of the study subjects are listed in Supplementary Table 1). The difference of chromatin accessibility of ATAC-seq data was analyzed by an R package named ROTS (Figure 4A).[24] Focusing on the miR-146a locus, the enhancer with the significantly differentiated chromatin accessibility (false discovery rate <0.05) was 32.5 kb away from the miR-146a TSS in monocytes (Figure 4B). As demonstrated in Figure 4C and D, the chromatin accessibility at this enhancer is significantly reduced in SLE. Next, to confirm whether the chromatin accessibility of the 32.5-kb enhancer is dysfunctional in SLE, we further recruited 9 healthy controls and 20 patients with SLE (validation cohort; the demographic and clinical characteristics of the study subjects are listed in Supplementary Table 2) to sort the CD14+ monocytes and performed an ATAC-seq assay. Consistent with the results in the discovery cohort, we also observed that the chromatin accessibility of the 32.5-kb enhancer is obviously decreased in the validation cohort (Supplementary Figure 4). Moreover, the chromatin accessibility of the ATAC peak in this enhancer is positively correlated with the expression of miR-146a in patients with SLE (patients in the discovery cohort + validation cohort) (Figure 4E). To further explore the association between this enhancer and SLE, we performed a correlation analysis between the chromatin accessibility of this enhancer and the clinical phenotypes. As shown in Figure 4F, the SLEDAI scores, a widely adopted criteria evaluating the disease activity of lupus, of patients with SLE were negatively correlated with the chromatin accessibility of the ATAC-seq peaks in this region, suggesting that the chromatin accessibility at this locus might reflect the disease status.

Transcription factor C/EBPα drives the chromatin accessibility variation of the SLE-associated enhancer. The chromatin accessibility is significantly influenced by key transcription factors (TFs).[18,23] To explore the mechanism that drives the chromatin accessibility variation at the lupus-related enhancer, we first analyzed the transcription factor binding motifs enriched in this region using HOMER software (http://homer.ucsd.edu/homer/motif/). Supplementary Figure 5A lists the top 10 most significant transcription factors (TFs) predicted to bind at this enhancer. C/EBPα, a myeloid master regulator,[25] was predicted to bind to this enhancer, and ChIP-seq results in myeloid cell lines further confirmed the enrichment of C/EBPα in this enhancer (Figure 5A). Cell type–specific and stimulation-responsive regulation of gene expression are usually governed by the alteration of TF levels. Remarkably, we found that the expression of both miR-146a and C/EBPα were reduced in monocytes of patients with SLE (ImmuNexut database) (Figure 5B and C),[15] including CD16+ monocytes (healthy control [HC] = 78, SLE = 60), intermediate monocytes (HC = 63, SLE = 59), nonclassical monocytes (HC = 63, SLE = 59), and classical monocytes (HC = 77, SLE = 61), and were positively correlated in three subtypes of monocytes (Supplementary Figure 5B–D). Furthermore, the knockdown of CEBPA reduced the expression of miR-146a (Figure 5D and E). More importantly, this region is closed in BLaER1 cell (B cell precursor leukemia), but with the induction of C/EBPα expression, the chromatin accessibility of this enhancer was gradually increased as revealed by ATAC-seq (Figure 5F).[16] In addition, knockdown of *CEBPA* decreased the chromatin accessibility of this enhancer as detected by formaldehyde-assisted isolation of regulatory elements (FAIRE)-quantitative polymerase chain reaction (qPCR) (Figure 5G). These findings imply that C/EBPα plays a pivotal role in maintaining the chromatin architecture at this enhancer.

In an endeavor to delineate the mechanistic underpinnings of diminished CEBPA expression in SLE, we turned our focus to the dysregulated inflammatory cytokine milieu characteristic of this autoimmune condition.[7] Existing literature intimates a potential suppressive effect of specific cytokine combinations on *CEBPA*

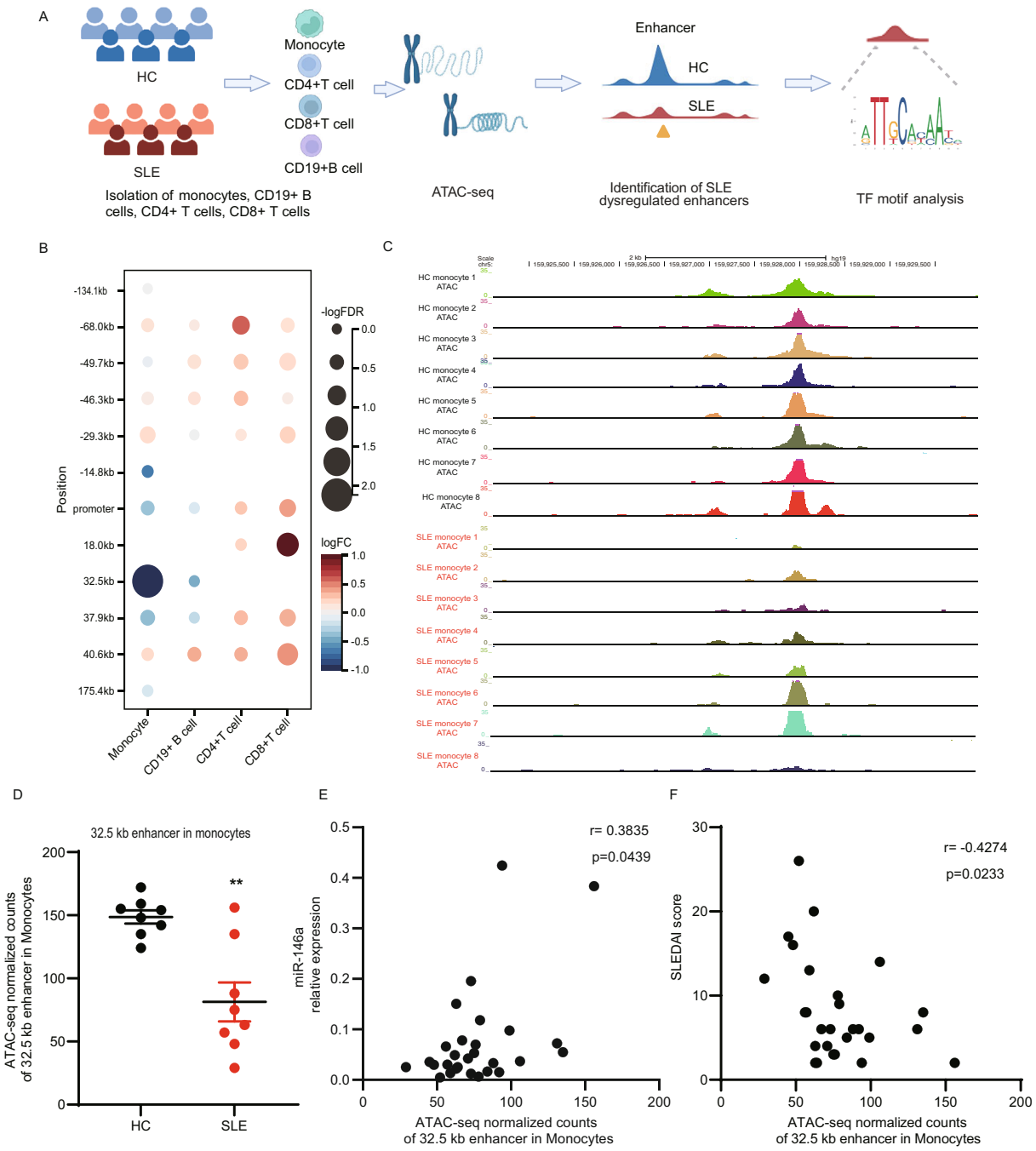

Figure 4. Chromatin accessibility analysis in SLE and healthy control reveals SLE-related enhancer of miR-146a. (A) Flow scheme of identifying SLE-dysregulated enhancers is shown. (B) Differential analysis of the chromatin accessibility of miR-146a functional enhancers in SLE and healthy control (n = 8, biologically independent individuals for SLE and healthy control) is shown. (C and D) Chromatin accessibility of the 32.5-kb enhancer is reduced in the monocytes of patients with SLE (n = 8, biologically independent individuals). (E) Chromatin accessibility of the 32.5-kb enhancer is positively correlated with the expression of miR-146a (n = 28, biologically independent individuals with SLE). (F) The chromatin accessibility of the 32.5-kb enhancer is negatively correlated with SLE SLEDAI scores (n = 28, biologically independent individuals with SLE). Data are represented as mean ± SEM, and *P* values are calculated using (D) unpaired two-tailed Student's *t*-test or (E and F) Spearman's test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. See also Supplementary Figure 4. ATAC-seq, assay for transposase-accessible chromatin sequencing, T cell–derived, and chemokine-related cytokine sequencing; chr, chromosome; FDR, false discovery rate; HC, healthy control; hg19, human genome build 19; miR-146a, microRNA-146a; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index; TF, transcription factor.

expression.[26] In this context, we postulated a potential nexus between SLE-associated cytokine perturbations and the attenuated expression of *CEBPA*, subsequently culminating in the down-regulation of miR-146a. Corroborating our hypothesis, our data revealed a marked diminution in the expression profiles of both *CEBPA* and miR-146a upon exposure to an IFNγ +

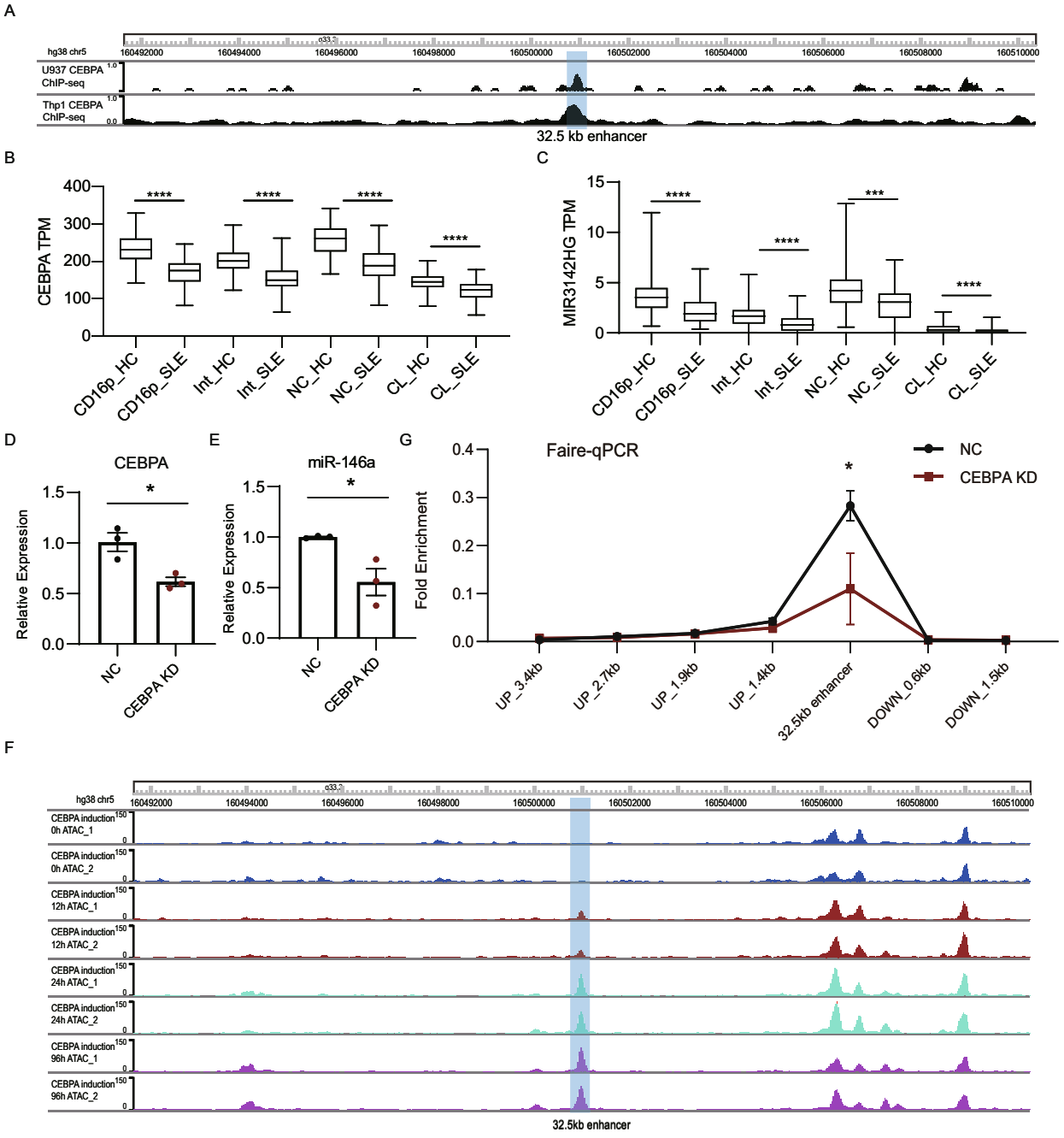

Figure 5. C/EBPα drives the chromatin accessibility of the 32.5-kb enhancer and affects miR-146a expression. (A) ChIP-seq demonstrates that C/EBPα binds to the 32.5-kb enhancer. (B and C) The expression of *CEBPA* and MIR3142HG (pri-miR-146a) is down-regulated in the monocytes of patients with SLE. CD16p, CD16+ monocytes (HC = 78, SLE = 60); Int, intermediate monocytes (HC = 63, SLE = 59); NC, non-classical monocytes (HC = 63, SLE = 59); CL, classical monocytes (HC = 77, SLE = 61). (D and E) RT-qPCR analysis of *CEBPA* and miR-146a expression after *CEBPA* knockdown in U-937 monocytes is shown (n = 3, biologically independent experiments). (F) C/EBPα induces the chromatin accessibility of 32.5-kb enhancer in BLaER1 cells as detected by ATAC-seq. (G) Knockdown of *CEBPA* decreased the chromatin accessibility of the 32.5-kb enhancer in U-937 monocytes as detected by FAIRE-qPCR (n = 3, biologically independent experiments). Fold enrichment was calculated as the ratio of the signal from the FAIRE sample to the signal from the input control DNA as measured by qPCR. Data are represented as mean ± SEM, and *P* values are calculated using (B–E and G) unpaired two-tailed Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. See also Supplementary Figure 5. ATAC-seq, Assay for transposase-accessible chromatin sequencing, T cell–derived, and chemokine-related cytokine sequencing; ChIP-seq, chromatin immunoprecipitation sequencing; chr, chromosome; C/EBPα, CCAAT/enhancer binding protein alpha; FAIRE, formaldehyde-assisted isolation of regulatory elements; hg38, human genome build 38; KD, Knockdown; MIR, MicroRNA; miR-146a, microRNA-146a; pri-, primary transcript; qPCR, quantitative polymerase chain reaction; RT-qPCR, reverse transcriptase–qPCR; SLE, systemic lupus erythematosus; TPM, transcripts per kilobase million.

interleukin-6 (IL-6) cocktail (Supplementary Figure 5E and F). It is noteworthy to highlight the aberrant elevation of IFNγ and IL-6 in the SLE landscape.[27,28] In essence, the *CEBPA* attenuation, presumably triggered by the cytokine disequilibrium, emerges as a potential determinant of the compromised chromatin accessibility at this enhancer locus in SLE monocytes, with plausible ramifications for miR-146a expression dynamics.

Perturbation of lupus-related enhancer possesses therapeutic potential. Disease-associated enhancers could function as a therapeutic target to intervene in disease progression, such as disruption of the erythroid-specific enhancer of *BCL11A* in the treatment of sickle cell disease and β-thalassemia.[6,26] As a critical negative regulator of the type I IFN pathway and the production of inflammatory cytokines, miR-146a has been demonstrated to be an attractive therapeutic target in SLE.[10,12] Our results imply that the 32.5-kb enhancer, as an SLE-dysregulated enhancer, controls miR-146a expression, so we hypothesized that targeting the 32.5-kb enhancer with CRISPR could restore miR-146a expression and thus restrain the abnormal activation of the type I IFN pathway and the aberrant production of inflammatory cytokines in patients with SLE. To test this hypothesis, we first used the CRISPR/Cas9 system to confirm the regulatory function of this region. As expected, the deletion of the 32.5-kb enhancer significantly decreased the expression of miR-146a (Figure 6A and Supplementary Figure 6A). More importantly, the decreased expression of miR-146a results in the high expression of interferon-stimulated genes (ISGs) such as ISG15 and 2′,5′-oligoadenylate synthetase 1 (Figure 6B–D). Next, we collected PBMCs from patients with SLE and delivered the plasmids of CRISPR-synergistic activation mediator (SAM) to the PBMCs using the Neon transfection system, thus activating the expression of miR-146a by targeting the 32.5-kb enhancer (Figure 6E). (The demographic and clinical characteristics of the study subjects are listed in Supplementary Table 3.) After transfection for 24 hours, the expression of miR-146a and the downstream effect genes were detected by reverse transcriptase–qPCR (RT-qPCR). As demonstrated in Figure 6F, targeting the 32.5 kb enhancer by CRISPR-SAM system could effectively increase the expression of miR-146a. More importantly, the downstream effects to correct the overactivation of the IFN signaling pathway and the overproduction of inflammatory cytokines can also be achieved by targeting this lupus-associated enhancer (Figure 6G and H; Supplementary Figure 6B and C). These results suggest that targeting this lupus-associated enhancer should be an effective method to regulate the activity of the IFN pathway and the production of inflammatory cytokines.

DISCUSSION

In this study, we integrated multiomics analysis with CRISPR screening to comprehensively decode the regulatory networks of a critical SLE gene in distinct cells and localize a lupus-related enhancer, which might act as a therapeutic target in the future. We also found that the reduced expression of *CEBPA* reshapes the epigenetic modification of the 32.5-kb enhancer, an SLE-related enhancer. Consequently, the decreased accessibility of the 32.5-kb enhancer leads to the reduced expression of miR-146a and contributes to SLE pathogenesis.

Enhancers are required to precisely regulate lineage- and stage-specific gene expression.[5] Currently, diverse strategies have been developed to predict enhancers but without functional confirmations, especially for enhancers of noncoding RNAs.[12,27–29] We designed an applicable procedure to comprehensively decode the regulatory networks of miR-146a by integration of epigenomic study, chromatin 3D structure study, and CRISPRa in distinct cell types. This established the functional enhancer landscape of miR-146a and enables us to better understand the regulatory network of gene expression. Notably, the expression of genes was regulated by a series of enhancers; enhancers in a cluster might work additively or synergistically to regulate their target genes.[30,31] We identified multiple enhancers regulating miR-146a expression individually, and how they coordinate to regulate miR-146a expression deserves to be studied in the future.

Enhancers are characterized by H3K27ac modification, monomethylation on lysine 4 of histone H3 modification, and high chromatin accessibility.[32] Consistent with these observations, we found that most functional enhancers possess specific epigenetic modifications.[33,34] However, we also found that some regions without enhancer-like modifications can also act as regulators. Besides, promoters of nearby genes also have enhancer activities to increase the transcription of miR-146a. This is consistent with the studies in coding genes.[35,36] These findings reveal the complexity of the gene regulation network.

The dysfunction of regulatory elements in the genome plays a vital role in disease pathogenesis.[4] Several studies revealed that the epigenetic modifications of regulatory elements in patients with SLE were altered, presenting as an SLE-specific signature.[9,37] However, the functional consequences, the target genes, and the mechanisms leading to these changes remain unclarified. By integration of the CRISPRa screen and chromatin accessibility differential analysis, we identified an SLE-associated enhancer, 32.5 kb away from the miR-146a, that regulates the expression of miR-146a. The accessibility of this enhancer was reduced in patients with SLE, positively correlated with the expression of miR-146a, and negatively correlated with the SLEDAI scores, indicating that the dysfunction of this enhancer may contribute to SLE pathogenesis.

Targeting disease-associated enhancers is a novel potential strategy for disease therapy.[6,38] Here, we found that targeting the SLE-associated 32.5-kb enhancer with the CRISPRa system effectively down-regulated the activity of the IFN pathway and the production of inflammatory cytokines, suggesting a promising target for therapy in the future.

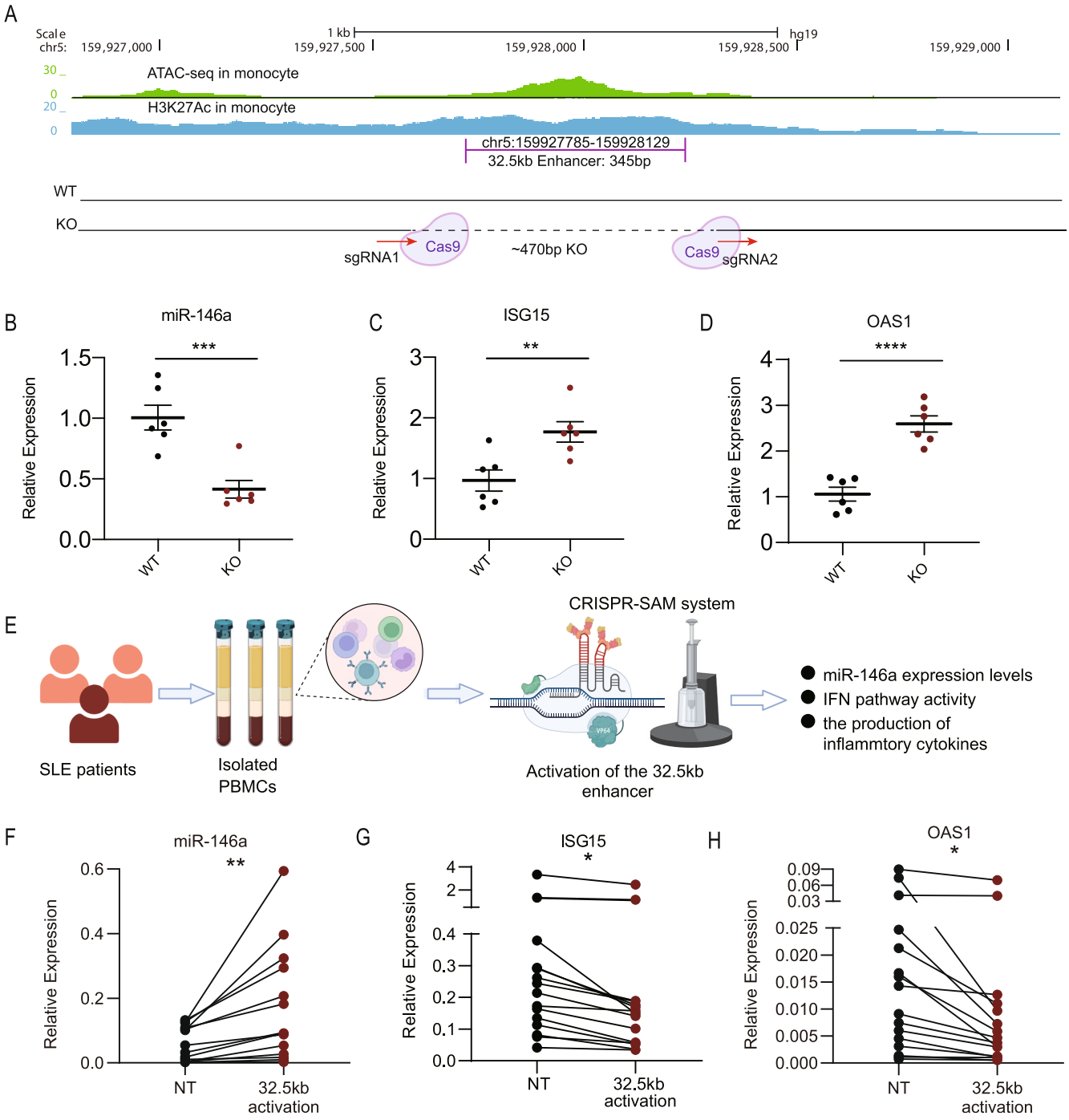

Figure 6. Targeting the 32.5-kb enhancer attenuates the activity of the type I IFN pathway by regulating miR-146a expression. (A) Deletion of the 32.5-kb enhancer in U-937 monocytes using the CRISPR-Cas9 tool is shown. (B) Deletion of the 32.5-kb enhancer strongly reduced miR-146a expression in U-937 monocytes (n = 6, biologically independent samples). (C and D) WT clones and 32.5-kb enhancer–deleted clones were treated with 100 U/ml IFN-α for 6 hours, and the expression of ISGs was detected by RT-qPCR (n = 6, biologically independent samples). (E) Experimental design to inhibit the expression of ISGs and inflammatory cytokines by CRISPRa system in PBMCs of patients with SLE is shown. (F–H) CRISPRa therapy decreases the expression of ISGs in the PBMCs of patients with SLE by up-regulating miR-146a expression through targeting the 32.5-kb enhancer (n = 16, replicates represent biological samples from unique individuals). Data are represented as mean ± SEM, and *P* values are calculated using (B–D) unpaired two-tailed Student's *t*-test and (F–H) paired two-tailed Student's test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. See also Supplementary Figure 6. ATAC-seq, Assay for transposase-accessible chromatin sequencing, T cell–derived, and chemokine-related cytokine sequencing; chr, chromosome; CRISPRa, CRISPR activation; H3K27ac, acetylation of histone H3 on lysine 27; IFN, interferon; ISG, interferon-stimulated gene; KO, knockout; miR-146a, microRNA-146a; NT, negative; PBMC, peripheral blood mononuclear cell; RT-qPCR, reverse transcriptase–quantitative polymerase chain reaction; SAM, synergistic activation mediator; sgRNA, single-guide RNA; SLE, systemic lupus erythematosus; WT, wild type.

The chromatin accessibility was mediated by transcription factor binding.[25,39] By analysis of binding motif and ChIP-seq data, we identified that C/EBPα, as the predominant transcription factor, binds to this SLE-associated enhancer, and the down-regulated *CEBPA* in patients with SLE contributes to the decreased expression of miR-146a. More importantly, we provided direct evidence that C/EBPα determines the chromatin accessibility difference of this enhancer in patients with SLE by ATAC-seq and FAIRE-qPCR. Our study demonstrates the mechanisms that mediate enhancer dysfunction in lupus research for the first time.

The down-regulation of *CEBPA* in SLE results in the dysfunction of SLE-associated enhancer. However, the mechanism that mediates the reduction of *CEBPA* is unclear. Here, we found that the cytokine combination IFNγ + IL6, the cytokines aberrantly produced in SLE,[40,41] could decrease the expression of both *CEBPA* and miR-146a, suggesting that the cytokine imbalance in SLE might influence the expression of critical transcription factors and thus affect the epigenomic aberration to regulate the downstream gene expression. This suggests that the cytokine imbalance of SLE may affect key transcription factors, subsequently influencing epigenomic aberrations and downstream gene expression. These findings hint at a potential therapeutic strategy: Targeting dysregulated cytokines with specific inhibitors or antibodies might restore miR-146a expression by stabilizing the epigenetic balance, thereby regulating the IFN pathway. Exploring this treatment avenue further is warranted.

Altogether, our study illustrates the landscape of functional enhancers of miR-146a in distinct immune cells and discovers a lupus-related enhancer in monocyte that could serve as a target for treatment. Looking forward, the dissection of the regulatory network of the lupus risk gene could shed light on our understanding of the pathogenesis of SLE and the discovery of novel therapeutic targets for lupus.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Authors Guo, Hou, and N. Shen had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Hnisz D, Abraham BJ, Lee TI, et al. Super-enhancers in the control of cell identity and disease. Cell 2013;155:934–947.
2. Schoenfelder S, Fraser P. Long-range enhancer-promoter contacts in gene expression control [review]. Nat Rev Genet 2019;20:437–455.
3. Mansour MR, Abraham BJ, Anders L, et al. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. Science 2014;346:1373–1377.
4. Yanchus C, Drucker KL, Kollmeyer TM, et al. A noncoding single-nucleotide polymorphism at 8q24 drives IDH1-mutant glioma formation. Science 2022;378:68–78.
5. Heinz S, Romanoski CE, Benner C, et al. The selection and function of cell type-specific enhancers [review]. Nat Rev Mol Cell Biol 2015;16:144–154.
6. Frangoul H, Altshuler D, Cappellini MD, et al. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. N Engl J Med 2021;384:252–260.
7. Tsokos GC, Lo MS, Reis PC, et al. New insights into the immunopathogenesis of systemic lupus erythematosus [review]. Nat Rev Rheumatol 2016;12:716–730.
8. Lanata CM, Nititham J, Taylor KE, et al. Dynamics of methylation of CpG sites associated with systemic lupus erythematosus subtypes in a longitudinal cohort. Arthritis Rheumatol 2022;74:1676–1686.
9. Scharer CD, Blalock EL, Mi T, et al. Epigenetic programming underpins B cell dysfunction in human SLE. Nat Immunol 2019;20:1071–1082.
10. Tang Y, Luo X, Cui H, et al. MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. Arthritis Rheum 2009;60:1065–1075.
11. Luo XB, Yang WL, Ye DQ, et al. A functional variant in microRNA-146a promoter modulates its expression and confers disease risk for systemic lupus erythematosus. PLoS Genet 2011;7:e1002128.
12. Hou G, Harley IT, Lu X, et al. SLE non-coding genetic risk variant determines the epigenetic dysfunction of an immune cell specific enhancer that controls disease-critical microRNA expression. Nat Commun 2021;12:135.
13. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997;40:1725.
14. Gladman DD, Ibañez D, Urowitz MB. Systemic lupus erythematosus disease activity index 2000. J Rheumatol 2002;29:288–291.
15. Ota M, Nagafuchi Y, Hatano H, et al. Dynamic landscape of immune cell-specific gene regulation in immune-mediated diseases. Cell 2021;184:3006–3021.e3017.
16. Choi J, Lysakovskaia K, Stik G, et al. Evidence for additive and synergistic action of mammalian enhancers during cell fate determination. Elife 2021;10:e65381.
17. Liu T, Ortiz JA, Taing L, et al. Cistrome: an integrative platform for transcriptional regulation studies. Genome Biol 2011;12:R83.
18. Klemm SL, Shipony Z, Greenleaf WJ. Chromatin accessibility and the regulatory epigenome [review]. Nat Rev Genet 2019;20:207–220.
19. Creyghton MP, Cheng AW, Welstead GG, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A 2010;107:21931–21936.
20. Zeng W, Liu Q, Yin Q, et al. HiChIPdb: a comprehensive database of HiChIP regulatory interactions. Nucleic Acids Res 2022;51(D1):D159–D166.
21. Konermann S, Brigham MD, Trevino AE, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 2015;517:583–588.

22. Buenrostro JD, Wu B, Litzenburger UM, et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 2015; 523:486–490.
23. Buenrostro JD, Giresi PG, Zaba LC, et al. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods 2013;10:1213–1218.
24. Faux T, Rytkönen KT, Mahmoudian M, et al. Differential ATAC-seq and ChIP-seq peak detection using ROTS. NAR Genom Bioinform 2021;3(3):lqab059.
25. Jin F, Li Y, Ren B, et al. PU.1 and C/EBPα synergistically program distinct response to NF-κB activation through establishing monocyte specific enhancers. Proc Natl Acad Sci U S A 2011;108:5290–5295.
26. Fu B, Liao J, Chen S, et al. CRISPR-Cas9-mediated gene editing of the BCL11A enhancer for pediatric β(0)/β(0) transfusion-dependent β-thalassemia. Nat Med 2022;28:1573–1580.
27. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 2012;489:57–74.
28. Moore JE, Purcaro MJ, Pratt HE, et al; ENCODE Project Consortium. Expanded encyclopaedias of DNA elements in the human and mouse genomes. Nature 2020;583:699–710.
29. Kundaje A, Meuleman W, Ernst J, et al; Roadmap Epigenomics Consortium. Integrative analysis of 111 reference human epigenomes. Nature 2015;518:317–330.
30. Huang J, Liu X, Li D, et al. Dynamic control of enhancer repertoires drives lineage and stage-specific transcription during hematopoiesis. Dev Cell 2016;36:9–23.
31. Bahr C, von Paleske L, Uslu VV, et al. A Myc enhancer cluster regulates normal and leukaemic haematopoietic stem cell hierarchies. Nature 2018;553:515–520.
32. Heintzman ND, Hon GC, Hawkins RD, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature 2009;459:108.
33. Fulco CP, Munschauer M, Anyoha R, et al. Systematic mapping of functional enhancer-promoter connections with CRISPR interference. Science 2016;354:769–773.
34. Simeonov DR, Gowen BG, Boontanrart M, et al. Discovery of stimulation-responsive immune enhancers with CRISPR activation. Nature 2017;549:111–115.
35. Dao LT, Galindo-Albarrán AO, Castro-Mondragon JA, et al. Genome-wide characterization of mammalian promoters with distal enhancer functions. Nat Genet 2017;49:1073–1081.
36. Ren X, Wang M, Li B, et al. Parallel characterization of cis-regulatory elements for multiple genes using CRISPRpath. Sci Adv 2021;7: eabi4360.
37. Ballestar E, Esteller M, Richardson BC. The epigenetic face of systemic lupus erythematosus [review]. J Immunol 2006;176: 7143–7147.
38. Hou G, Zhou T, Xu N, et al. Integrative functional genomics identifies SLE causal genetic variant in the IRF5 risk locus. Arthritis Rheumatol 2023;75:574–585.
39. Whyte WA, Orlando DA, Hnisz D, et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 2013;153:307–319.
40. Ripley BJ, Goncalves B, Isenberg DA, et al. Raised levels of interleukin 6 in systemic lupus erythematosus correlate with anaemia. Ann Rheum Dis 2005;64:849–853.
41. Lu R, Munroe ME, Guthridge JM, et al. Dysregulation of innate and adaptive serum mediators precedes systemic lupus erythematosus classification and improves prognostic accuracy of autoantibodies. J Autoimmun 2016;74:182–193.