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]

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.

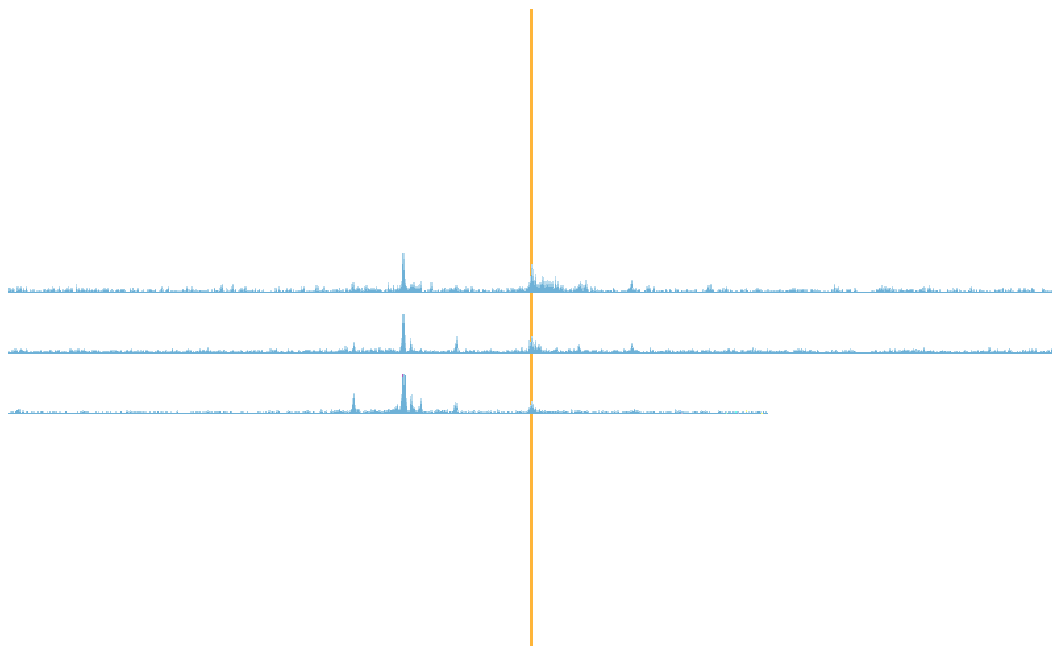
Statistical analysis. All statistical analyses were performed using GraphPad Prism 8 software and calculated using a paired or unpaired two-tailed Student's t-test as indicated in the figure legend unless otherwise mentioned. Where noted in the figures, asterisks define the significance level (*P < 0.05; **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-397[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).



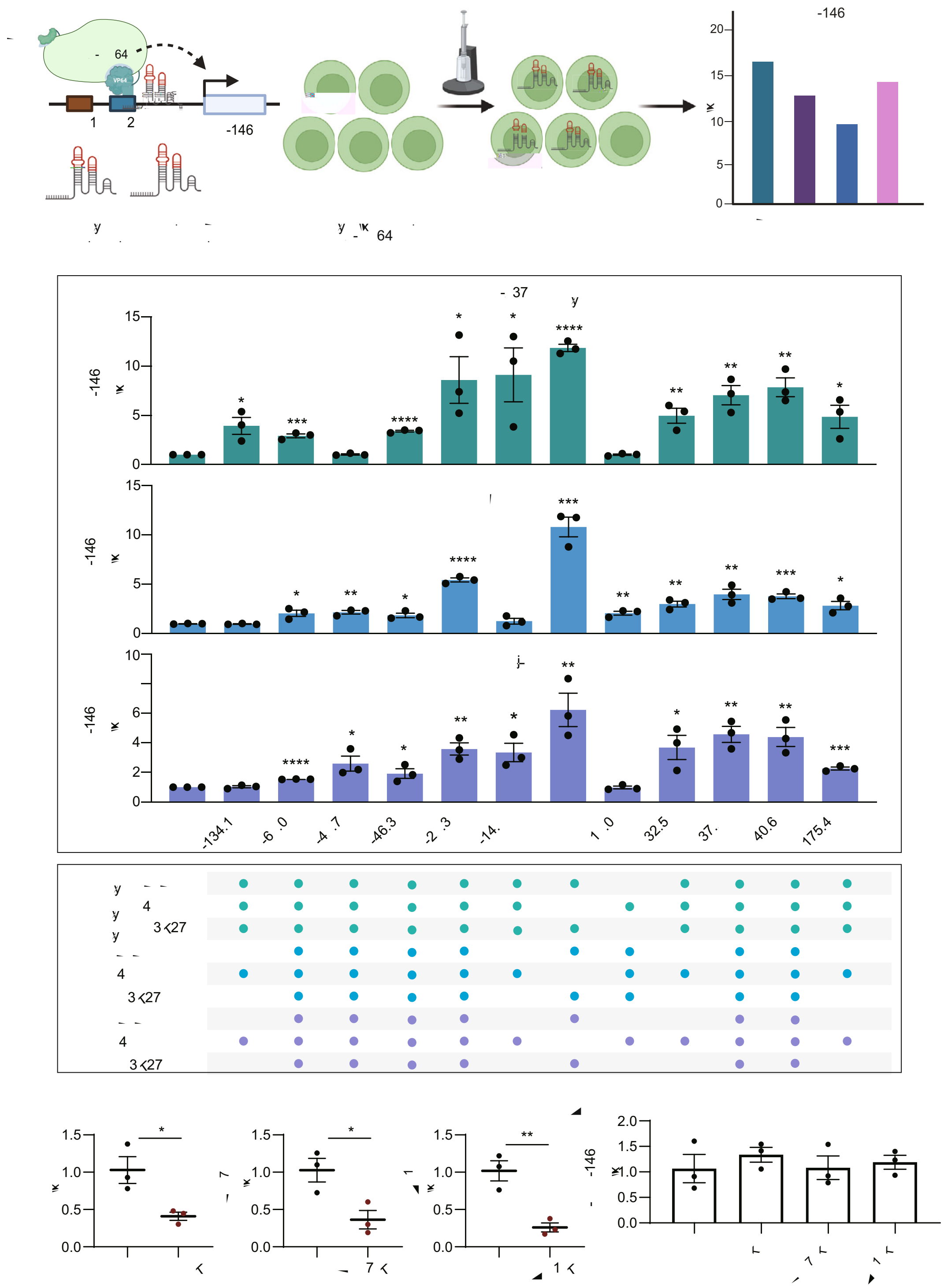


Figure 3. CRISPRa conrms the functional enhancers of miR-146a. (A) Flow scheme of coning the functional enhancer of miR-146a is shown. (B) Top panel shows RT-qPCR analysis of the miR-146a expression by targetingdifferent enhancer regions using CRISPRa system (n = 3, biologically independent experiments). Bottom panel shows a summary of characters ofenhancers in different cell subsets; the dot indicates that the genomic regions possess H3K27ac modication, 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 signicant; *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-speci c −134.1-kb enhancer and T cell–speci c 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-speci c 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 unmodi ed regions also have regulatory potential. In addition, the −14.8-kb enhancer harbors an SLE risk variant rs2431697, which has been identied 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 nd the epigenomic alterations driving the differential expression of miR-146a in SLE, we rst 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 signi

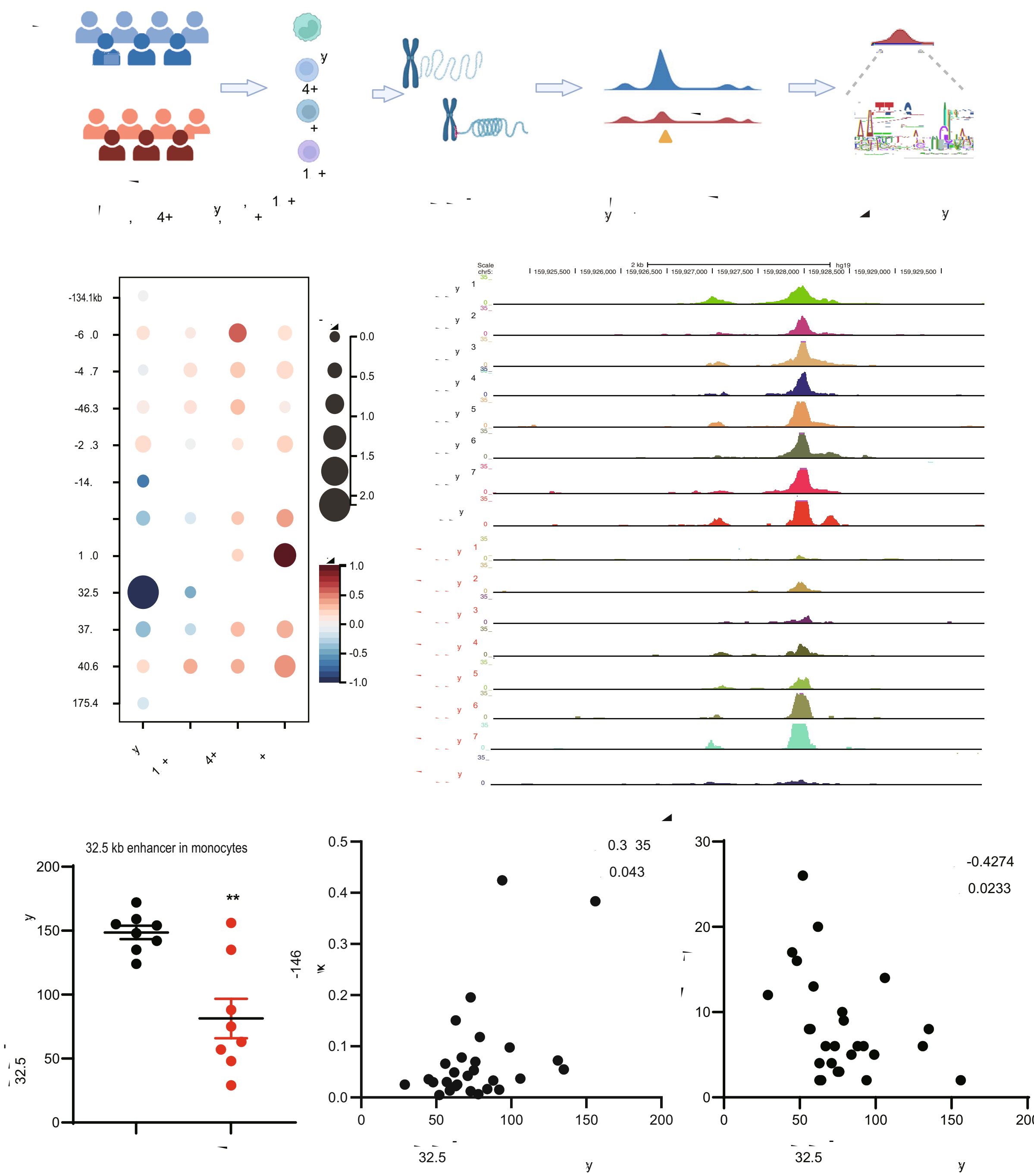
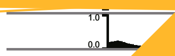


Figure 4. Chromatin accessibility analysis in SLE and healthy control reveals SLE-related enhancer of miR-146a. (A) Flow scheme of identifying SLE-dysregulated enhancers is shown. (B) Differential analysis of the chromatin accessibility of miR-146a functional enhancers in SLE and healthy control (n = 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 +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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.

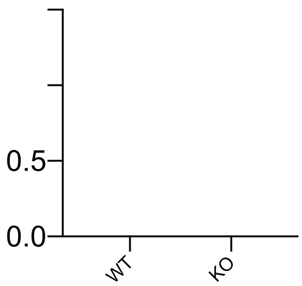
Enhancers are required to precisely regulate lineage- and stage-specific gene expression.[5] Currently, diverse strategies have been developed to predict enhancers but without functional confirmations, especially for enhancers of noncoding RNAs.[12,27–29] We designed an applicable procedure to comprehensively 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.

B



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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

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