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Qualitative Proteome-wide Lysine Crotonylation Profiling Reveals Protein Modification Alteration in the Leukocyte Extravasation Pathway in Systemic Lupus Erythematosus

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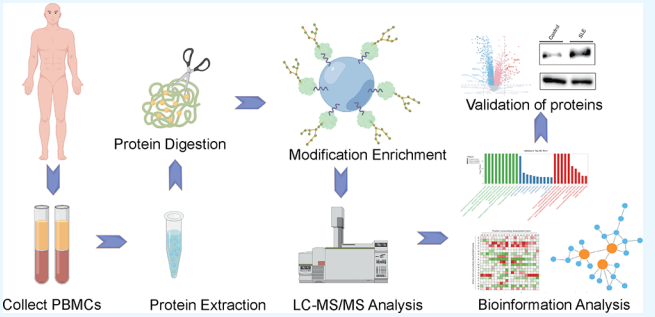
ABSTRACT: Background: Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease with multiple manifestations. Lysine crotonylation (Kcr) is a newly discovered posttranslational modification epigenetic pattern that may affect gene expression and is linked to diseases causally. Methods: We collected blood samples from 11 SLE individuals and 36 healthy subjects. Then, we used highly sensitive liquid chromatography−mass spectrometry technology to carry out proteomics and quantitative crotonylome analysis of SLE peripheral blood mononuclear cells in this investigation, which indicated the unique etiology of SLE. Finally, we verified the expression of critical protein in the leukocyte extravasation pathway by online database analysis and Western blot. Results: There were 618 differentially expressed proteins (DEPs), and 612 crotonylated lysine sites for 272 differentially modified proteins (DMPs) found. These DEPs and DMPs are primarily enriched in the leukocyte extravasation signaling pathway, such as MMP8, MMP9, and ITGAM. Conclusions: This is the first study of crotonylated modification proteomics in SLE. The leukocyte extravasation signaling pathway had a considerable concentration of DEPs and DMPs, indicating that this pathway may be involved in the pathogenic development of SLE.



INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the generation of nuclear autoantibodies and the formation of immune complexes, both of which can cause inflammation and organ damage. The overall incidence of SLE ranges from 0.3 to 31.5 cases per 100,000 individuals, and its widespread prevalence varies from 3.2 to 517.5 cases per 100,000 persons.[1] Women and nonwhite people are more prone to developing SLE.[2] The clinical presentation of SLE is heterogeneous, including the hematologic, renal, respiratory, neuropsychiatric, ocular, and gastrointestinal involvements.[3] The empirical treatments for SLE often caused medication toxicities, but the recent biological therapies, including belimumab and rituximab, have changed this situation.[4,5] Despite there has been a sizable amount of study on SLE, the pathogenic mechanism of SLE is still foggy because of its many symptoms and genetic heterogeneity.[6] Therefore, advanced research should be conducted to provide innovative orientations on the pathophysiology of SLE, and the development of novel therapeutic targets and pharmaceutical candidates.

By changing the stability and impact of histone and nonhistone proteins, protein posttranslational modifications (PTM) have been shown over the last several decades to play a crucial role in regulating pathological or physiological key processes. Protein PTM is a flexible regulatory process that maintains protein structure stability, promotes specific ligand−receptor interactions, and regulates signaling cascades.[7] Preliminary investigations have discovered more than 400 types of PTMs, including (but not limited to) phosphorylation, glycosylation, ubiquitination, methylation, N-acetylation, S-nitrosylation, and lipidation.[8] The role of PTM in the pathogenesis of SLE has become progressively acknowledged in the last 10 years. Until now, well-studied PTMs (e.g., phosphorylation, methylation, acetylation, and isoaspartylation) have been frequently found in the biological processes of SLE, with the majority of studies focusing on the regulatory role of PTMs in SLE pathogenesis. Recently, 1035 phosphorylation sites corresponding to 618 proteins in the peripheral blood mononuclear cells of SLE patients were identified by the highly sensitive liquid chromatography−mass spectrometry system

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(LC−MS/MS).[9] The U1-70k protein, which was phosphorylated susceptibly in SLE, has been demonstrated to play an essential part in apoptosis associated with cellular stress. In addition, the study reported that most phosphoproteins were located in the nucleus and the cell membrane. It also demonstrated that the major proteins of 50 metabolic pathways related to the pathogenesis of SLE were modified. Moreover, histone acetylation and methylation affect the gene expression of interleukin 17 (IL17) in SLE T cells, thereby exacerbating the proinflammatory phenotype of SLE.[10] Methylation may lessen the prevalence of infections in SLE patients by impairing cytolytic CD8+ T-cell function.[11] Abnormal carbamylation and citrullination can sustain autoimmunity and inflammation of SLE by modifying the epitopes of LL37, which acts as the T-cell/B-cell autoantigen.[12] Glycosylation may contribute to SLE renal complications by forming pathogenic IgG *n*-glycan, which damages podocytes.[13] These results implied that protein PTM assumed an enormous importance in SLE.

Lysine crotonylation (Kcr) is a newly discovered histone modification and one of the nonacetyl lysine acylations.[14,15] Chemically similar to the well-known acetylation, Kcr is composed of C4H5O elements and has a precise mass shift of +68.0230 Da. As an analogy to other PTMs, Kcr is regulated through "writers" (introducing modified marks), "erasers" (removing modified marks), and "readers" (recognizing epigenetic marks). It can be catalyzed by histone acetyltransferase p300 (the most promiscuous acyltransferase identified until now),[16] erased by class I histone deacetylases (HDACs),[17] and read by YEATS domain and double plant homeodomain finger proteins.[18,19] Chromodomain protein CDYL is a hydratase, which can cause crotonyl-CoA to transform into β-hydroxybutyryl-CoA. Since the intracellular relative concentration of crotonyl-CoA could directly influence the levels of crotonylation of histone, chromodomain Y-like transcription corepressor (CDYL) can negatively regulate Kcr.[20] Furthermore, there are innumerable connections between crotonylation and sickness. Crotonylation was involved in regulating spermatogenesis by chromodomain protein CDYL.[20] In 2021, scientists demonstrated that crotonylation played a critical part in cardiac homeostasis and embryonic development,[21,22] which has provided a fresh insight into the mechanisms about crotonylation in disease. However, the function of crotonylation in the development of SLE is currently unknown.

In our investigation, we used the LC−MS/MS approach to investigate the effect of Kcr on the pathogenic mechanism and potential new therapeutic targets in SLE. We assessed the protein crotonylation and proteome of PBMCs from 11 SLE patients and 36 healthy subjects, which identified unique features of SLE.

METHODS

Study Population. The American College of Rheumatology criteria for SLE categorization were met by 11 SLE patients.[23,24] Thirty-six healthy control subjects were not taking drugs and were able to rule out chronic diseases (hypertension, diabetes, hepatitis, etc.). Figure 2E shows the demographic differences between SLE individuals and healthy subjects. From earlier investigations conducted by our lab team, a more comprehensive list of the study population is available.[25] Every participant signed informed consent before being included in the study. The Key Laboratory of the Guangxi Metabolic Disease Research Ethics Committee accepted the study, which was carried out in accordance with the most recent edition of the Declaration of Helsinki. Gene expression profiles (GSE20864, GSE82221, and GSE112087) of SLE for further investigation were procured from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The material in Table of Contents is from https://www.figdraw.com/.

PBMCs Isolation and Protein Extraction. All subjects had their Peripheral blood mononuclear cells (PBMCs) separated using Ficoll density gradient centrifugation. The mononuclear cells were collected and washed after centrifugation and sonication, and the protein content was assessed using a BCA kit (Sigma Chemical Co., St. Louis, MO) in accordance with the manufacturer's recommendations. Please see the Supporting Information for detailed methods.

Trypsin Digestion and TMT Labeling. We used 5 mM dithiothreitol to reduce the protein and 11 mM iodoacetamide for alkylation. After that, the protein solution was diluted with 100 mM TEAB and then trypsinized. The peptides were then vacuum-dried and desalted using a Strata X C18 SPE column from Phenomenex in Torrance, CA. Finally, the resulting peptide mixtures were incubated with TMT mass tagging kits and reagents (Product No. 90068, Thermo) for labeling. Please see the Supporting Information for the detailed methods.

High-Performance Liquid Chromatography (HPLC) Fractionation. HPLC, using an Agilent 300Extend C18 column, was used to separate the tryptic peptides. The inner diameter of the column is 4.6 mm, the length of the column is 25 cm, and the interior is filled with 5 μm high-efficiency particle stationary phase. Simply said, peptides were divided into 60 fractions over the course of 1 h using a gradient of 8−32% acetonitrile, combined into 18 parts, and vacuum-dried.

Affinity Enrichment. First, 100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40 (NETN) buffer was used to break down tryptic peptides, and then prewashed antibody beads were added (PTM-503, Protein Translational Modifications Biolabs, Hangzhou, China) overnight with mild shaking. Peptides were extracted from the beads, vacuum-dried, and desalted with C18 ZipTips (Millipore, Sanit Louis, USA) for LC−MS/MS after washing with NETN buffer and ddH2O. Please see the Supporting Information for the detailed methods.

LC−MS/MS Analysis. Solvent A (0.1% formic acid) was used to dissolve the tryptic peptides before they were separated using an EASY-nLC 1000 UPLC machine. Then, it was loaded onto a reversed-phase analytical column, which was 15 cm in length and 75 μm i.d. The liquid phase gradient of solvent B (0.1% formic acid in 98% acetonitrile) increases from 6 to 23% over 26 min, 23 to 35% in 8 min, and climbs to 80% in 3 min and then holds at 80% for the last 3 min. The Nanospray Flex Ion (NSI) source and MS/MS in Q Exactive Plus (Thermo) were then applied to the peptides. Data are available via ProteomeXchange with the identifier PXD042217. Please see the Supporting Information for the detailed methods.

Data Analysis. The generated MS/MS data were processed using the Maxquant search engine (v.1.5.2.8). The detailed retrieve parameters, such as the database, enzyme cut method, fixed modification, variable modification, etc., were provided in the Supporting Information.

Bioinformatics Analysis. *Identification of the DEPs and the DMPs.* The differentially expressed proteins (DEPs) and the differentially modified proteins (DMPs) between SLE patients and controls were screened with cutoff conditions of fold change (FC) $\geq$ 1.2 or $\leq$0.83.

GO and KEGG. DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/), a predication tool for annotation

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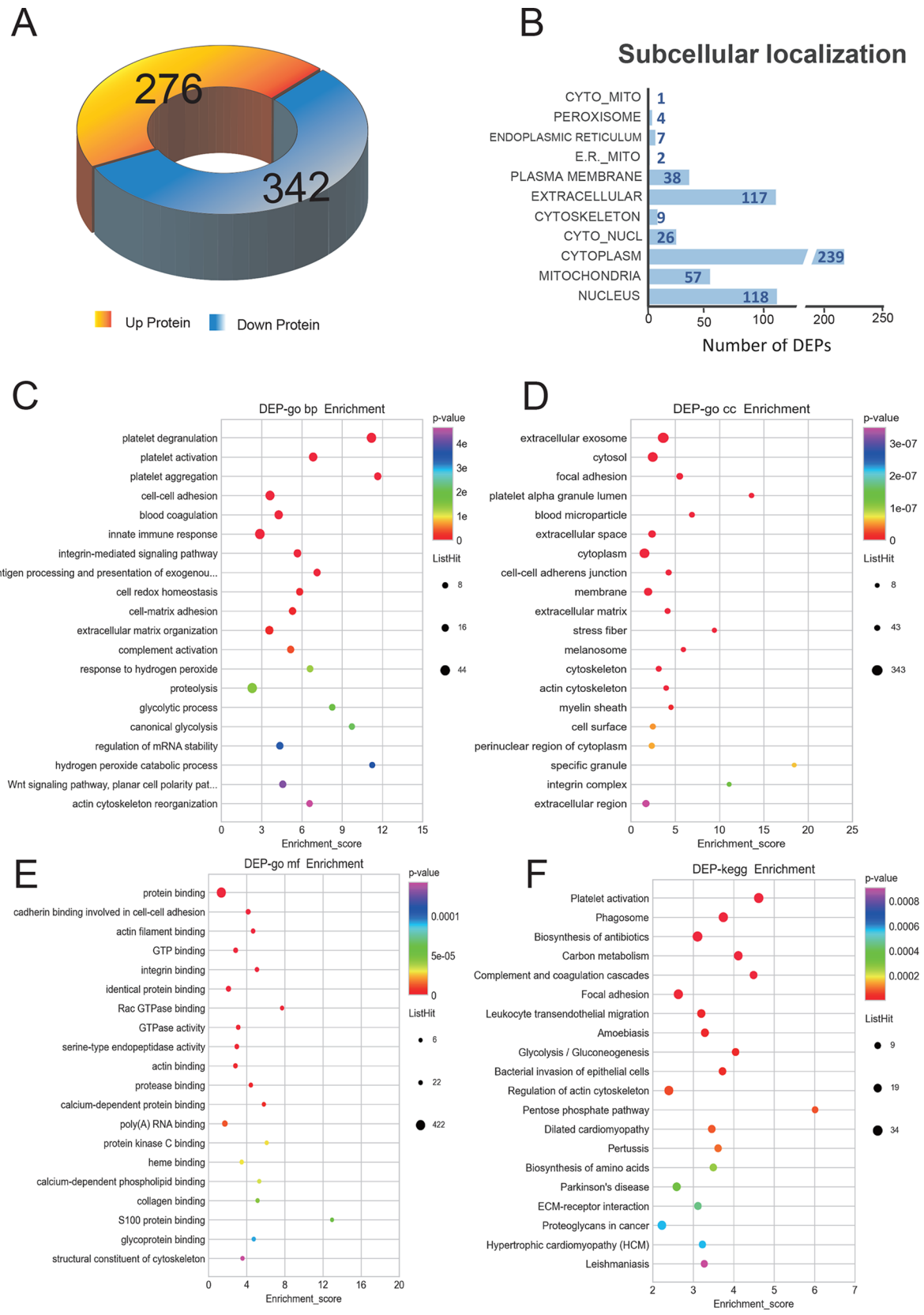

Figure 1. Qualitative analysis of DEPs between SLE individuals and healthy controls. (A) Pie chart representing the upregulated proteins and downregulated proteins. (B) Subcellular location of the DEPs. (C) Biological process in the GO-based enrichment analysis. (D) Cellular component in the GO-based enrichment analysis. (E) Molecular function in the GO-based enrichment analysis. (F) KEGG pathway enrichment analysis of the DEPs.

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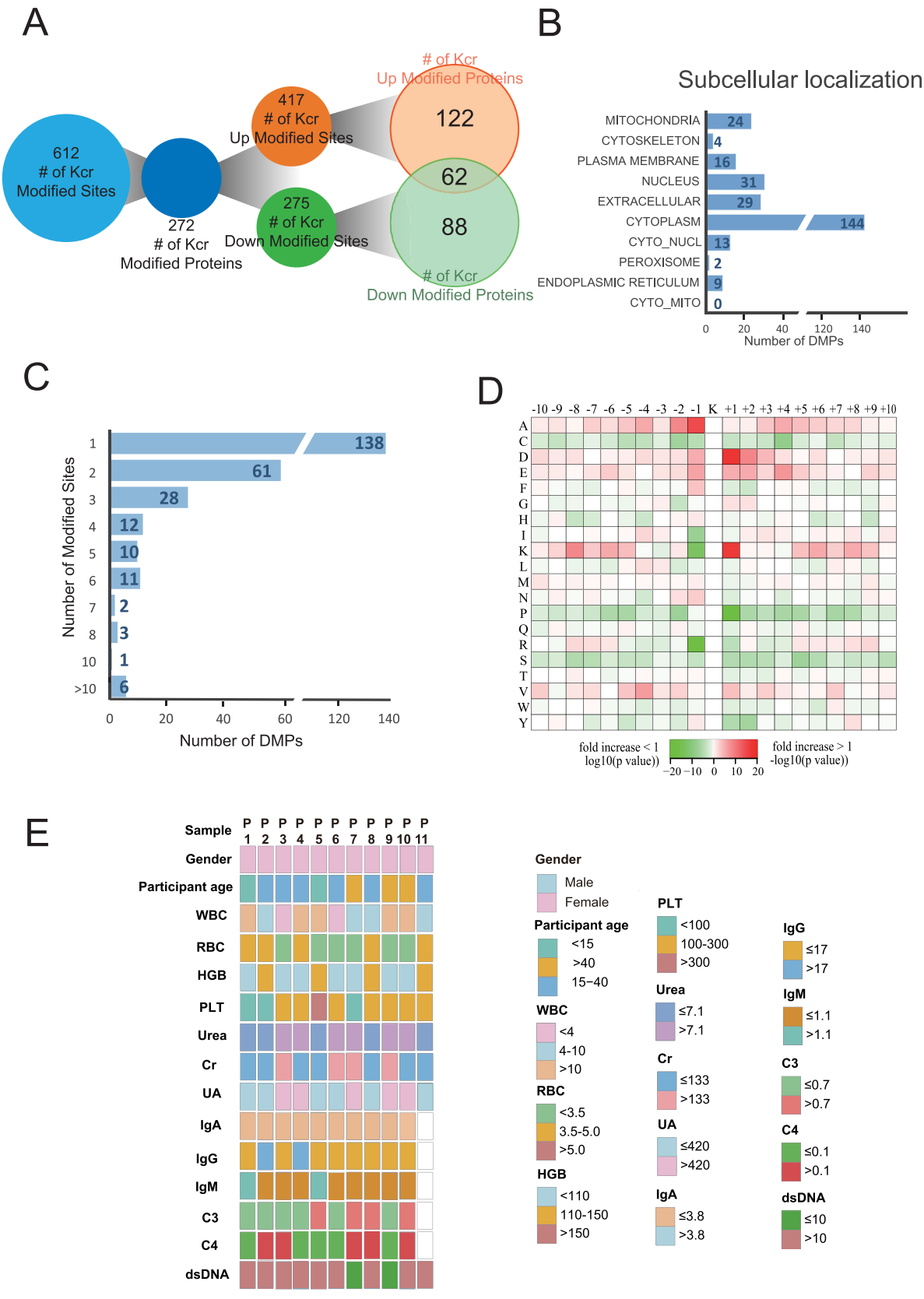

Figure 2. Lysine crotonylation sites identification in SLE. (A) Overview of the Kcr modified sites. (B) Subcellular location of the DMPs. (C) Number of modified sites in a protein. (D) Heat map of the amino acid compositions visualizing the frequency of amino acids nearby the Kcr sites. Red manifests enrichment, and green indicates depletion. (E) Sample trait heatmap. The colors represent different clinical traits, including gender, sex, and blood test results.

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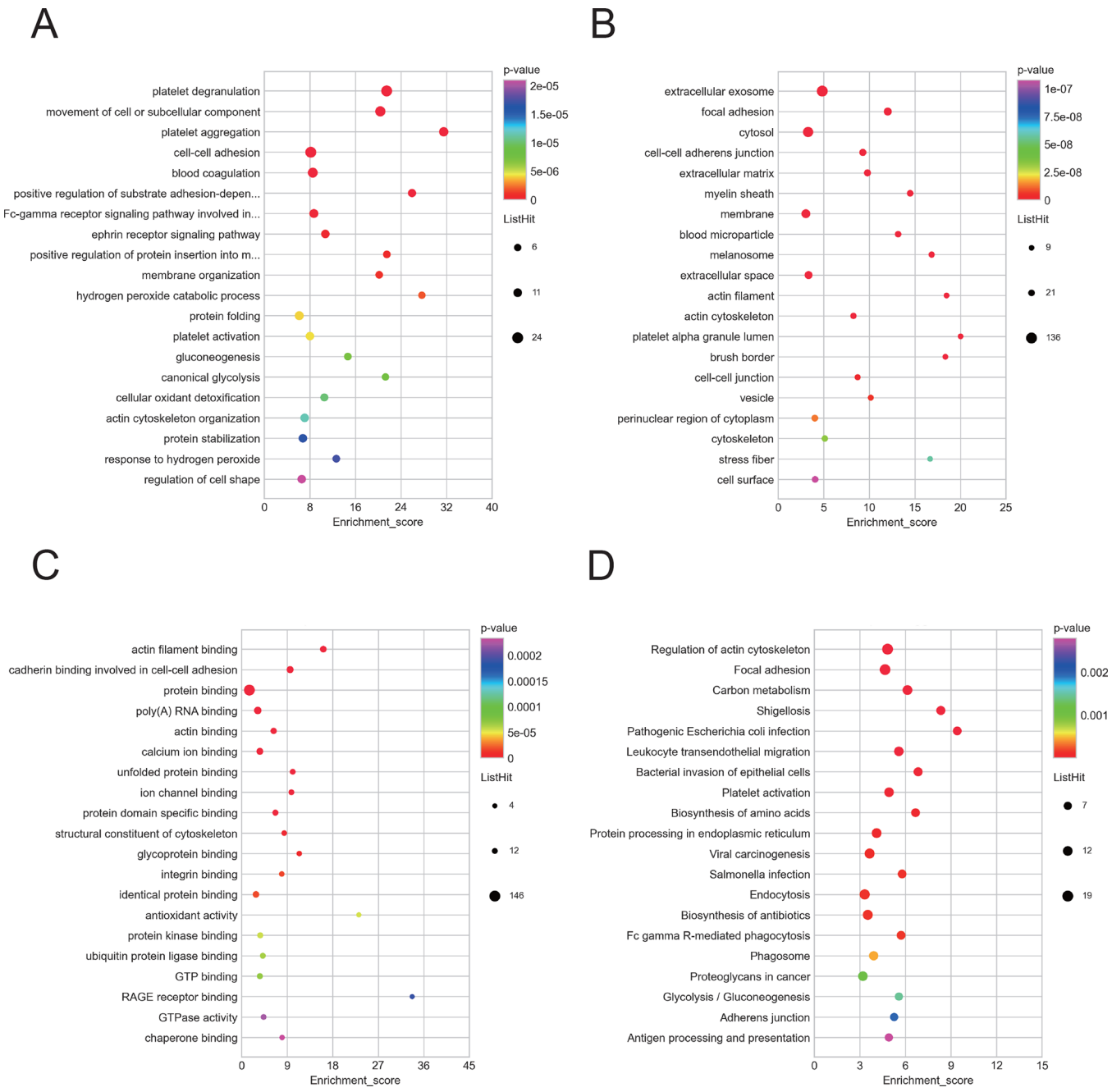

Figure 3. GO and KEGG enrichment analysis of the hyper-crotonylated proteins. (A) Biological process in the GO analysis. (B) Cellular component in the GO analysis. (C) Molecular function of GO enrichment analysis. (D) KEGG pathway enrichment analysis of the hyper-crotonylated proteins.

and visualization was used to analyze GO classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The whole human gene sequences were used as background gene sets, with all other parameters being set at default. The standard FDR control method was used to correct multiple hypothesis tests. All bioinformatics analysis with a corrected *p*-value <0.05 was considered significant.

Subcellular Localization. An online tool called the WoLF PSORT (http://www.genscript.com/wolf-psort.html) is used to forecast and describe the subcellular localization.

Motif Analysis. The motif-x algorithm was used to analyze the sequence models constituted with particular amino acids (10 amino acids up and downstream of the Kcr sites).

PPIs and Ingenuity Pathway Analysis. Identified proteins were examined thoroughly by the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING version 11.5; http://string-db.org) for protein−protein interaction analysis (PPIs). Then, we used the Cytoscape software (version 3.9.0) to picture the PPI network. To assess the densely connected regions, we employed Molecular complex detection (MCODE), using the following standards: degree cutoff = 2, MCODE scores >5, K-core = 2, and maximum depth = 100.

For further analysis, we submitted the DEPs and DMPs to an ingenuity pathway analysis, which was applied to map pathways and predict upstream regulators.

Western Blot Analysis. The PBMCs of six SLE patients were collected for protein extraction. After extraction by Radio

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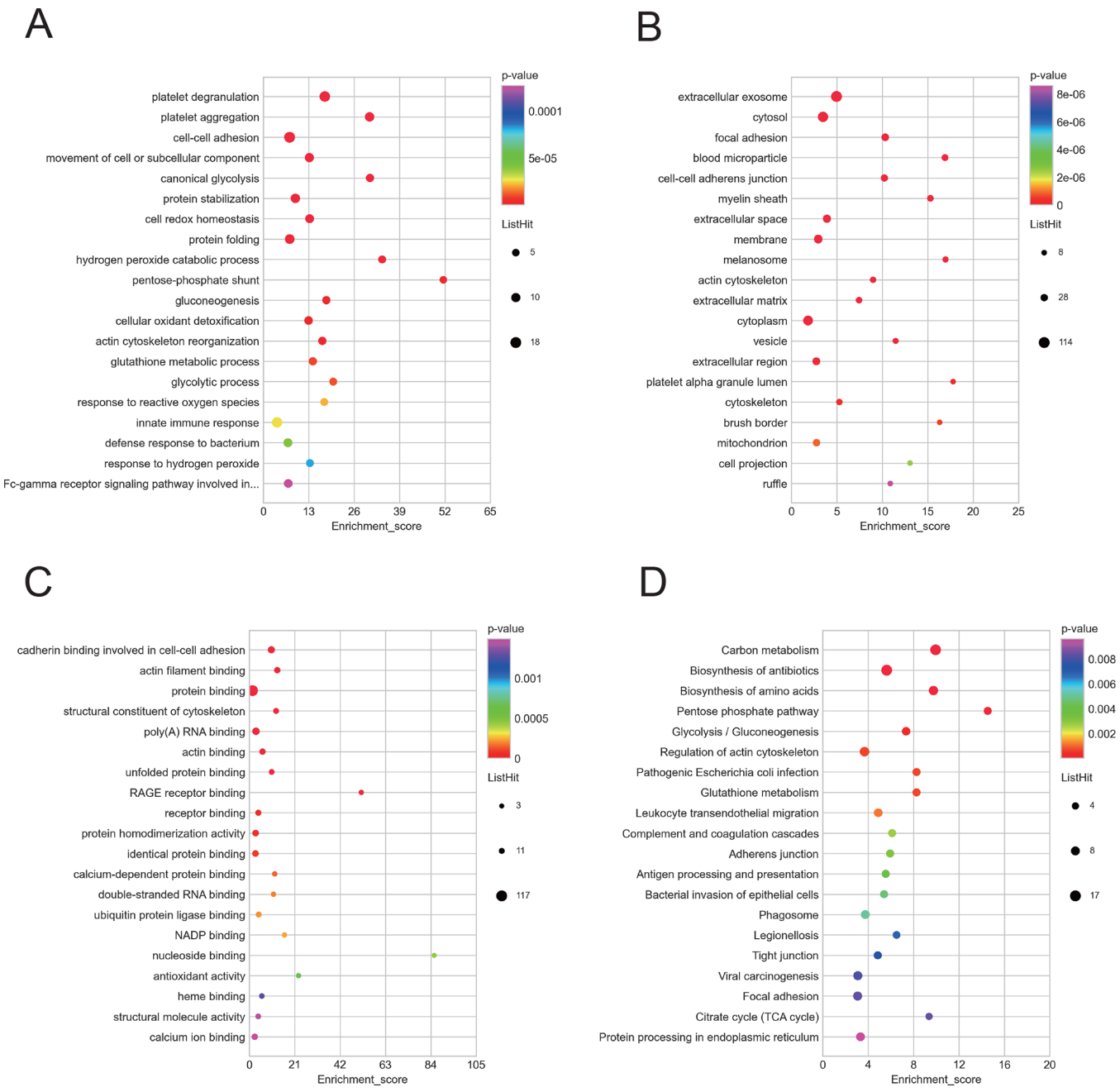

Figure 4. GO analysis and KEGG pathway analysis of the hypo-crotonylated DMPs. (A) Biological process in the GO enrichment. (B) Cellular component in the GO enrichment. (C) Molecular function of GO enrichment. (D) KEGG pathway enrichment analysis of the downregulated DEPs.

Immunoprecipitation Assay (RIPA) buffer and Phenylmethanesulfonyl fluoride (PMSF), the protein was detected by the BCA kit for concentration measured. sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 10 or 12% was used to separate 20 μg of protein samples, which were subsequently transferred to a poly(vinylidene difluoride) membrane (Immobilon, Germany). The membranes were treated with MMP9 (1:1000; Servicebio, A23535) overnight at 4 °C after being blocked in 5% defatted milk at room temperature for 2 h. After being cleaned with TBS-T, the membranes were incubated with secondary antibodies from Servicebio for 2 h at room temperature before being detected using ThermoFisher's enhanced chemiluminescence solution.

RESULT

We collected blood samples and isolated PBMCs from 11 SLE individuals and 36 healthy subjects. Then, we extracted the protein from PBMCs and digested it with trypsin. After digestion, the peptides were taken part in proteomics and quantitative crotonylome analysis by LC−MS/MS. The conclusion was drawn after the bioinformatics analysis, which indicated the unique etiology of SLE. A total of 11 SLE individuals (aged from 9 to 53 years) were diagnosed with SLE, and their clinical data are shown in Figure 2E. Most of them have leucopenia, anemia, thrombocytopenia, and high titers of dsDNA.

Analysis of DEPs. In our investigation, 3179 proteins were identified, of which 2580 proteins could be quantifiable in the

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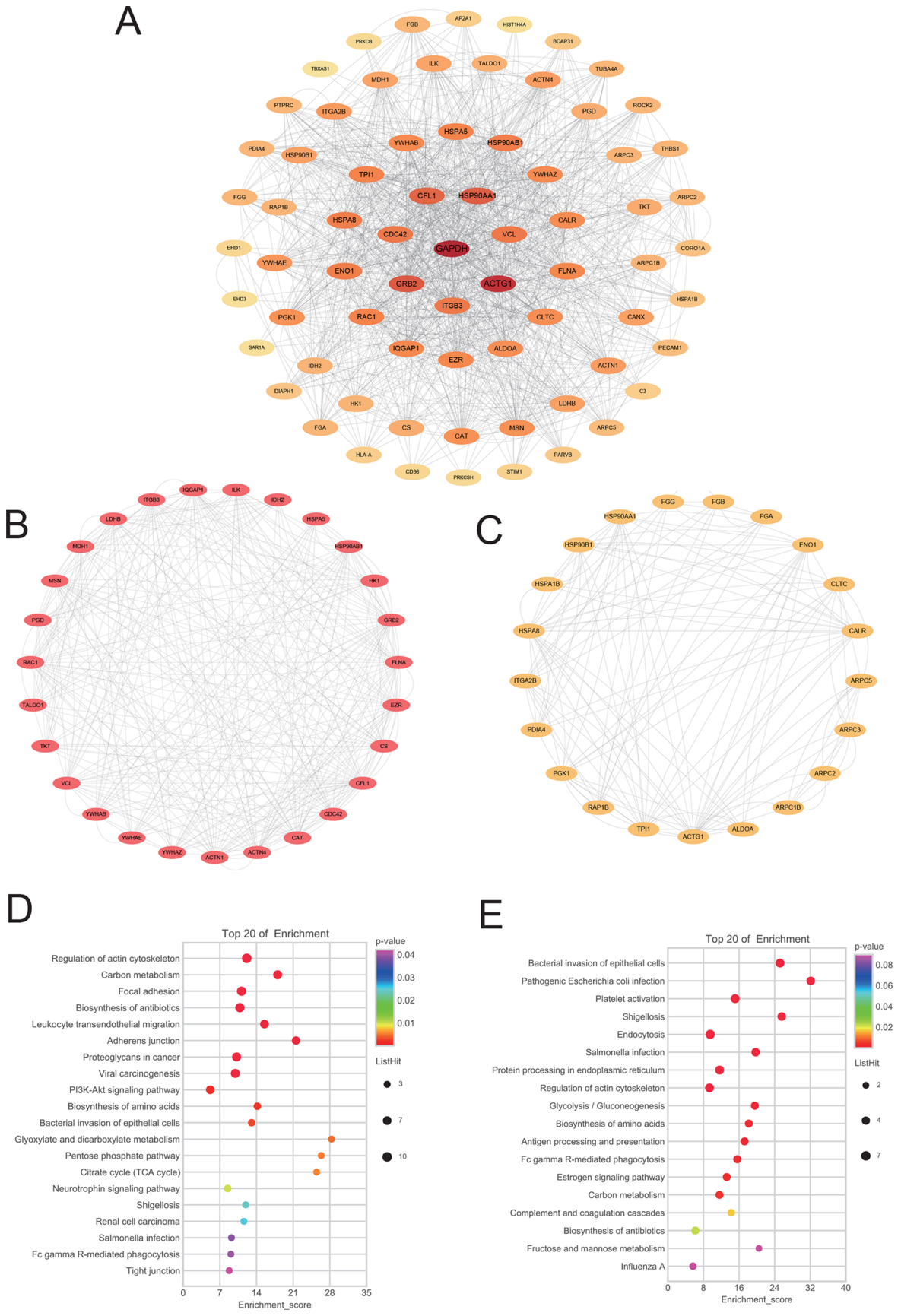

Figure 5. PPI network of hyper-crotonylated proteins in the SLE. (A) Overview of the hyper-crotonylated PPI network. (B) Cluster 1 was built of 27 nodes and 294 edges. (C) Cluster 2 was built of 21 nodes and 132 edges. (D) KEGG pathway enrichment analysis of the cluster 1. (E) KEGG pathway enrichment analysis of the cluster 2.

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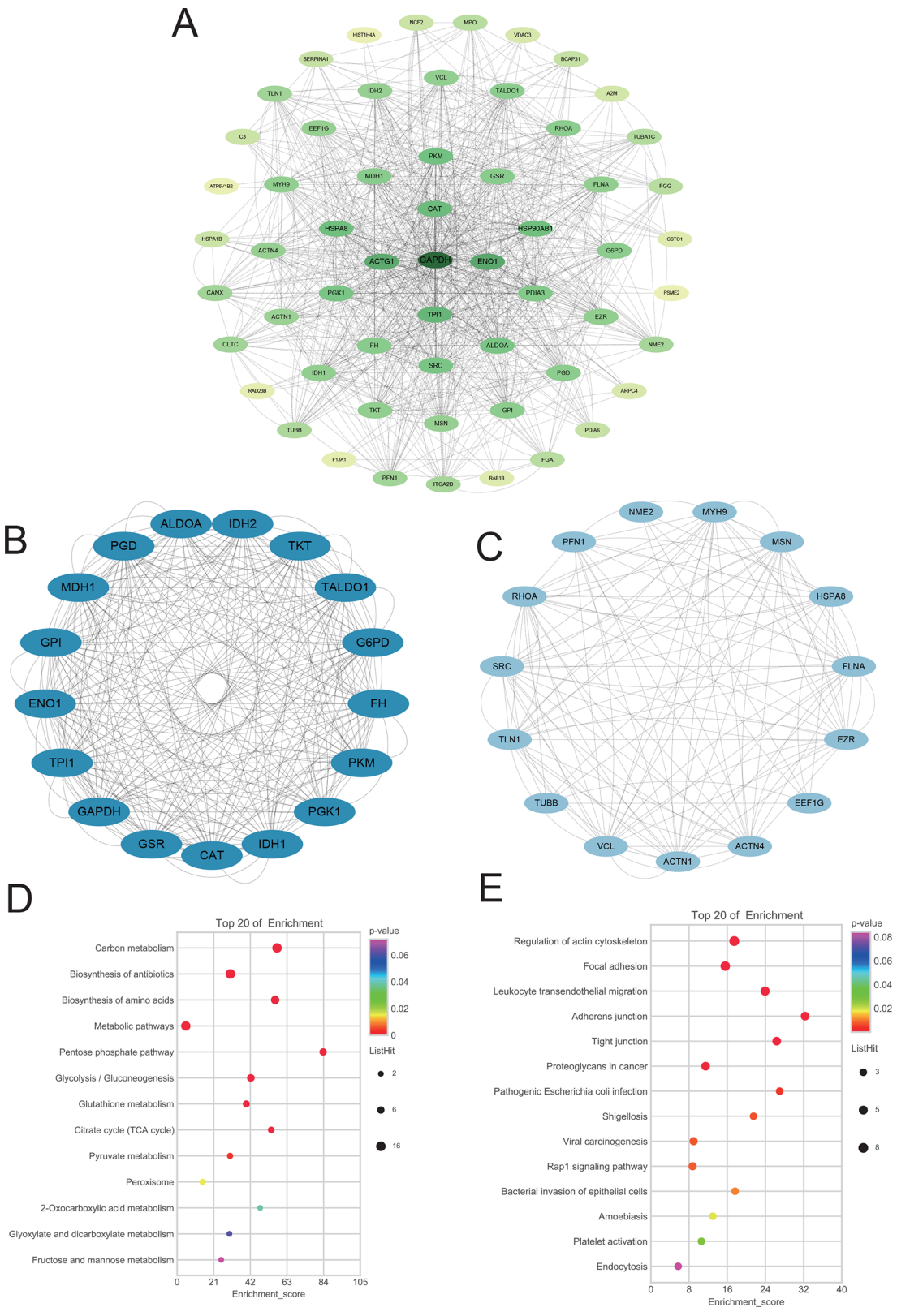

Figure 6. PPI network of hypo-crotonylated proteins in the SLE. (A) Overview of PPI network in the hypo-crotonylated proteins. (B) Cluster 1 (score = 16.3) was constructed with 17 nodes and 260 edges. (C) Cluster 2 (score = 8.7) was constructed with 15 nodes and 122 edges. (D) KEGG pathway enrichment analysis of cluster 1. (E) KEGG pathway enrichment analysis of cluster 2.

PBMCs of SLE individuals and healthy subjects. Based on the criteria of "FC ≥ 1.2 or FC ≤ 0.83" between SLE patients and NCs, 618 DEPs were determined (Table S1), of which 276 proteins were upregulated (FC ≥ 1.2) and 342 proteins (FC ≤ 1/1.2) were downregulated (Figure 1A). The subcellular localization analysis of DEPs showed that the DEPs between SLE patients and NCs were mainly located in cytoplasm (239), nucleus (118), extracellular (117), mitochondria (57) or plasma

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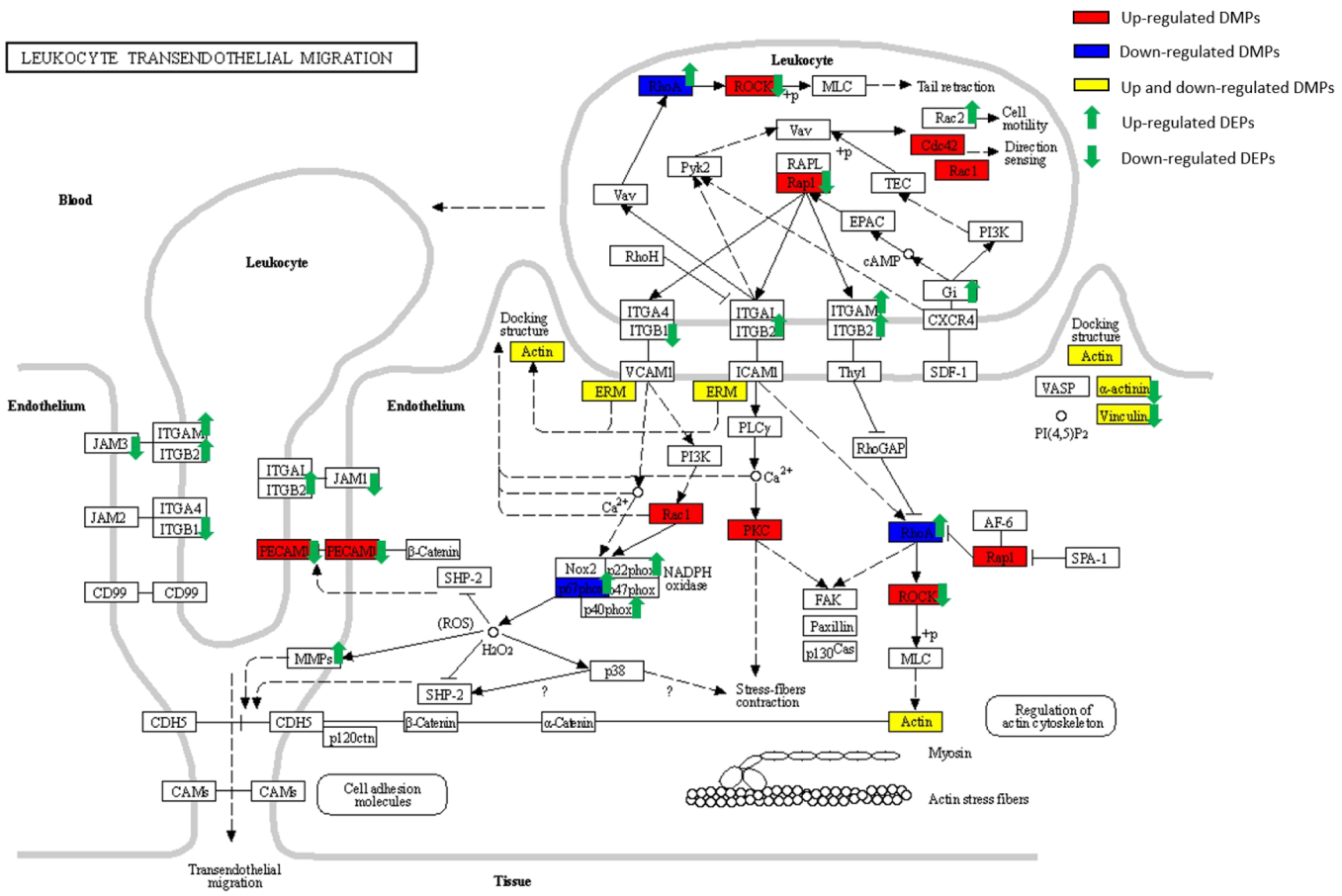

Figure 7. Expression condition of DEPs and DMPs in the leukocyte transendothelial migration pathway. Red indicates that the proteins are hyper-crotonylated, blue indicates hypo-crotonylated, and yellow indicates both modification change. The protein with a green arrow means expression change of the protein, and the up arrow indicates upregulated expression, while the down arrow means downregulated.

membrane (38) (Figure 1B). In the biological process category, most proteins were associated with platelet degranulation, platelet activation, platelet aggregation, cell−cell adhesion, and blood coagulation (Figure 1C). With respect to the cellular component category, most proteins were related to extracellular exosome, cytosol, focal adhesion, platelet alpha granule lumen, and blood microparticle (Figure 1D). With respect to the molecular function category, protein binding, cadherin binding involved in cell−cell adhesion, actin filament binding, GTP binding, and integrin binding were most significantly enriched (Figure 1E). Additionally, KEGG-based functional enrichment analysis showed that most proteins were highly enriched in platelet activation phagosome, leukocyte transendothelial migration, etc. (Figure 1F), all of which had a meaningful relationship with SLE (Table S3).

Identification and Pattern Analysis of Kcr. Performing PBMCs' proteome-wide lysine crotonylation identification among donors with or without SLE, we identified 1209 crotonylated lysine sites across 377 crotonylated proteins, in which 1109 sites on 347 proteins were quantifiable. On the basis of FC $\geq$ 1.20 or $\leq$1/1.2, 417 hyper-crotonylated lysine sites for 184 hyper-crotonylated proteins and 275 hypo-crotonylated sites for 150 proteins were identified between SLE patients and normal control individuals (Figure 2A). Mostly DMPs are located in the cytoplasm (114) according to subcellular localization analysis and distributed at the nucleus (31), extracellular (29), mitochondria (24), or plasma membrane (16) (Figure 2B). Among 272 dyscrotonylated proteins, most proteins (199) contained 1 or 2 dyscrotonylation sites, and 12.1% of DMPs (33) had more than 5 dyscrotonylation sites (Figure 2C). For more details, see Table S2.

Analyzing the frequency of each kind of amino acid around the crotonylated lysine from −10 to +10 to study the context of crotonylated lysine, hydrophobic amino acids (A) and acidic amino acids (D, E) are located near the crotonylated lysine. According to our observation, D or E residues were enriched in the +1, + 2, or +3 positions, with R or K at +5 or +6 (Figure 2D).

Functional Enrichment Analysis of Hyper-Crotonylated Proteins. As shown in Figure 3B, the hyper-crotonylated proteins were observably enriched among extracellular exosome, focal adhesion, cytosol, cell−cell adherens junction, and extracellular matrix. Those proteins are significantly involved in biological processes, including endocytic recycling, cortical actin cytoskeleton organization, cellular response to IL-4, cell adhesion, and leukocyte-cell adhesion (Figure 3A). With respect to the molecular function category, most proteins were related to actin filament binding, cadherin binding involved in cell−cell adhesion, protein binding, poly(A) RNA binding, and actin binding (Figure 3C). Additionally, most proteins were highly enriched in the regulation of actin cytoskeleton, leukocyte transendothelial migration, and platelet activation, based on the KEGG-based functional enrichment analysis (Figure 3D). More details are represented in Table S4.

Functional Enrichment Analysis of Hypo-Crotonylated Proteins. Within the biological process grade, heavily hypo-crotonylated proteins were related to platelet degranula-

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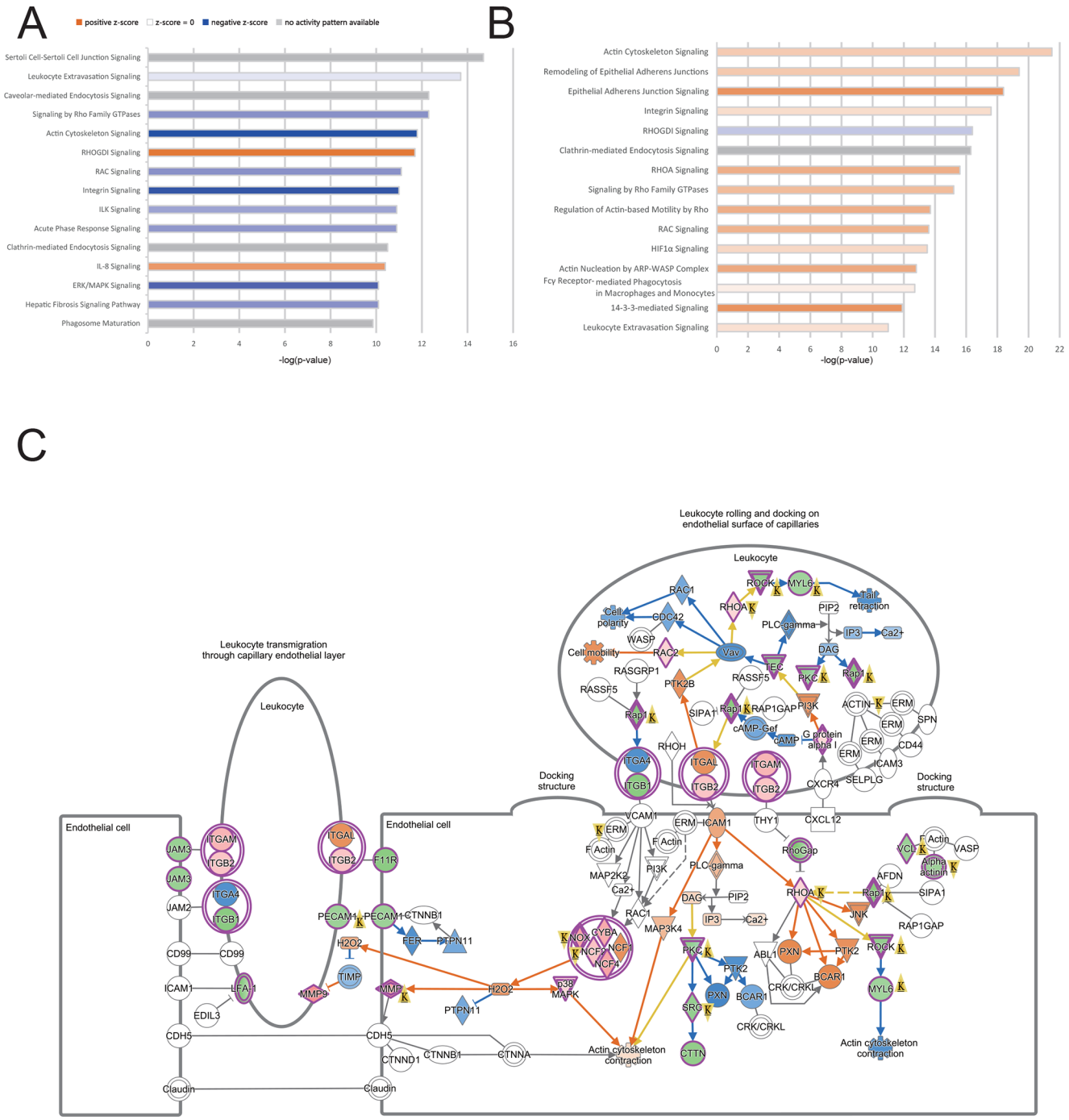

Figure 8. IPA canonical pathway analysis of DEPs and DMPs. (A) Top five enriched canonical pathways of DEPs; (B) top five enriched DMP canonical pathways. IPA's ingenuity knowledge database of DEPs and DMPs, along with the z-score, were used to predict the leukocyte extravasation signaling pathway; (C) protein that is enriched in this pathway is represented by the fuchsia outer ring. Green denotes downregulation, whereas red denotes upregulation. Proteins, pathways, and biological processes that are predicted to be activated and inhibited are, respectively, shown in orange and blue. The protein marked with a yellow arrow indicates that the protein has undergone modification; the up arrow denotes hypercrotonylation and the down arrow denotes hypocrotonylation. Yellow arrows that link two nodes indicate that the effect of changes in expression levels on this function is not consisting of literature reports.

tion, platelet aggregation, cell−cell adhesion, movement of cell or subcellular components, and canonical glycolysis (Figure 4A). In terms of cellular components, most hypo-crotonylated proteins were significantly enriched among extracellular exosome, cytosol, focal adhesion, blood microparticle, and cell−cell adherens junction (Figure 4B). These proteins were associated with many molecular function items, including the cadherin binding involved in cell−cell adhesion, actin filament binding, protein binding, structural constituent of cytoskeleton, and poly(A) RNA binding, among others (Figure 4C). KEGG-based functional enrichment analysis showed that most hypo-crotonylated proteins were significantly enriched in three processes, including carbon metabolism, biosynthesis of amino acids, leukocyte transendothelial migration, etc. (Figure 4D).

PPI Network Analysis of Dyscrotonylated Proteins. To broaden the landscape of the functional and physical

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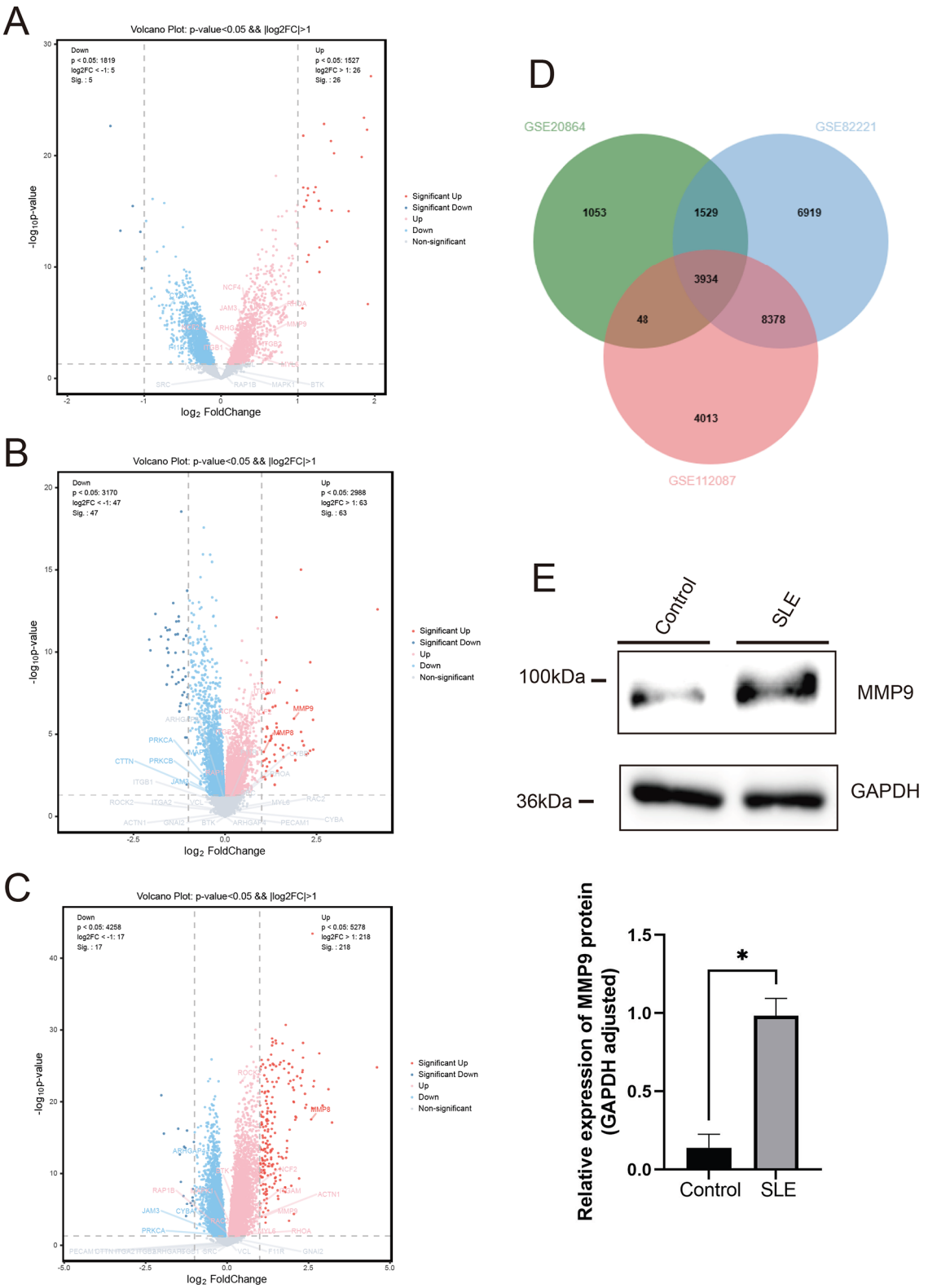

Figure 9. Expression of DEGs in the leukocyte transendothelial migration pathway of different experimental data set. (A−C) Volcano plot of the DEGs from GSE20864, GSE112087, and GSE82221. Red dots indicate upregulated DEGs, and blue dots express downregulated DEGs. (D) DEGs intersection of GSE20864, GSE112087, and GSE82221. (E) Western blot analysis examined the protein expression of MMP9 ($p < 0.05$).

interactions of DMPs, the PPI networks of DMPs were constructed using the STRING database and plotted with Cytoscape v3.9.0. The PPI among hyper-crotonylated proteins is shown in Figure 5A, containing 71 nodes and 1140 edges (Table S5). Additionally, we used MCODE, a theoretical clustering algorithm, to further analyze densely connected regions. The clusters of the top 2 MCODE scores are shown in Figure 5B,C: 27 nodes and 294 edges were included in the PPI network in cluster 1, and 21 nodes and 132 edges were included in the PPI network in cluster 2. KEGG-based functional

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enrichment analysis showed that most hyper-crotonylated proteins in cluster 1 were related to the following processes, including regulation of actin cytoskeleton, focal adhesion, leukocyte transendothelial migration, etc. (Figure 5D), and the proteins in cluster 2 were highly enriched in bacterial invasion of epithelial cells, pathogenic *Escherichia coli* infection, platelet activation, etc. (Figure 5E). As shown in Figure 6A, there were 58 hypo-crotonylated proteins mapped in the PPI network model, including containing 58 nodes and 798 edges (Table S6). In the network, proteins with a higher degree of affinity were closer to the center. Based on the aforementioned criteria, two major modules were chosen by clustering the PPI network with MCODE (Figure 6B,C). Cluster 1 scored 11.3, while Cluster 2 got 6.6. The hypo-crotonylated proteins in the two modules were then subjected to functional enrichment analysis using the KEGG database. Hypo-crotonylated proteins were highly enriched in carbon metabolism, the regulation of actin cytoskeleton, and leukocyte transendothelial migration. The results are shown in Figure 6D,E.

Leukocyte Transendothelial Migration Participates in the Occurrence and Progress of SLE. In our study, the leukocyte transendothelial migration was one of the top pathways enriched by both DEPs and DMPs (Figure 7), which had been previously demonstrated to be related to the pathogenicity of SLE. In our results, 19 DEPs were involved in this pathway, of which 9 DEPs were upregulated and 10 DEPs expressions were downregulated. Besides, 14 DMPs were significantly enriched in this pathway, while 47 sites in 14 DMPs were all hyper-crotonylated.

Functional analysis using IPA was performed further to explore the proteomics and Kcr profiling in SLE. Most of DEPs were enriched in the Sertoli cell−Sertoli cell junction signaling, the leukocyte extravasation signaling, the caveolar-mediated endocytosis signaling, the signaling by Rho family GTPases, and the actin cytoskeleton signaling (Figure 8A). Most of the DMPs were related to the actin cytoskeleton signaling, the remodeling of epithelial adherens junctions, the epithelial adherens junction signaling, integrin signaling, and RHOGDI signaling (Figure 8B). The expression of MMP9(FC = 5.04), NCF4 (FC = 3.81), MMP8(FC = 3.80), CYBA (FC = 3.51), ITGAM (FC = 3.02), NCF2(FC = 2.28), ITGB2 (FC = 2.17) were up-expressed. At the same time, RAP1B (FC = 0.58), ITGB1 (FC = 0.59), F11R (FC = 0.60), and PRKCA (FC = 0.67) were decreased in SLE (Table S7). Upon reviewing the KEGG results and the top pathways of IPA, the leukocyte extravasation signaling pathway (−log(*p*-value) = 13.7 in DEPs) was found particularly intriguing in the enrichment analysis and it had a high rank of expression changes and crotonylated modification. Based on the *z*-score, IPA anticipated that the DEPs correlated to actin cytoskeleton contraction, which then mediated the dysfunctional leukocyte extravasation in SLE (Figure 8C).

To further the conclusions of our study and investigate the regulatory mechanism of the leukocyte extravasation signal pathway in SLE, we intersected the differentially expressed mRNA of GSE20864, GSE112087, and GSE82221, uncovering 3934 common mRNA (Figure 9D). The volcano plots in Figure 9A−C represent the differential gene analysis performed on the SLE data sets from GEO. After that, we annotated the volcano plots with the DEGs of our study's leukocyte extravasation signaling pathway. As anticipated, the differential expression trends of DEGs in our study were identical to those in the GEO database. In addition, we collected PBMCs of six SLE patients to detect the expression of MMP9 by Western blotting. According to Figure 9E, we found that SLE patients had MMP9 expression considerably higher than that of healthy controls. All of these results suggested that the MMP family regulated the signaling pathway for leukocyte extravasation, which initiated the local SLE inflammation.

DISCUSSIONS

As one of the most heterogeneous illnesses treated by physicians, there still exists a substantial medical need because of the increasing morbidity and damage accrual in SLE.[26] Earlier diagnosis and better treatment of SLE remain challenging. Crotonylation is a newly emerged PTM that plays critical roles in mammalian cells.[14,17] Modified nonhistone proteins of different human cell lines have recently been found to participate in metabolic and cellular processes, which has increased the range of modified proteins.[27] PTM of nonhistone proteins opened up a new regulatory mechanism to control protein function.

However, the investigation of Kcr is woefully underdeveloped and has not yet been investigated in SLE. Herein, we performed proteome and Kcr analyses in PBMCs from SLE individuals and healthy subjects to determine the molecular characteristics of SLE and bridge this knowledge gap in autoimmune diseases. Our study identified 272 DMPs and 618 DEPs in SLE, and we observed that most of the proteins with high-rank expression changes were modified by Kcr. The KEGG enrichment results of those proteins all pointed to the immune system and inflammation pathways, particularly the leukocyte extravasation signaling pathway. For further exploration, we combined the results of KEGG and IPA, and then came to the conclusion that leukocyte extravasation was mediated by Kcr in SLE.

SLE has long been regarded as a complicated illness because of its tangled etiology and multiple symptoms. Although genetic, environmental, hormonal, epigenetic, and immunoregulatory variables all largely influence the process of SLE, their effects are similar: initiating local inflammation and inducing damage to different organs.[28] Leukocytes, which circulate in the blood, trigger inflammatory reactions by migrating from the bloodstream to injured tissues. The function of leukocytes in SLE has previously been established, and its dysfunction may lead to inflammatory cytokine response enhancement and autoimmune disorders.[29] The inflamed tissue releases chemokines, which promotes leukocytes adhering and transmigrating across vascular endothelial cells (VECs) and then reaching the extravascular space.[30,31] In the inflammatory site, endothelial cells express IL-8, switching the leukocyte integrins LFA-1 and VLA-4 to affinitive states, then encouraging leukocytes to move from rolling to adhesion and migration.[32] As recruited to sites of infection, they release reactive oxygen species, activating the antimicrobial peptides and proteins.[33] Furthermore, the stimulated neutrophils from SLE can release NETs and promote plasmacytoid DCs to activate type I interferon, which can trigger the response of other immune cells, stimulate inflammatory cytokine expression, and promote vascular inflammation.[34] Abnormal leukocyte function contributes to pathogenicity in the SLE via vascular inflammation. In contrast to the general population, SLE patients had a greater incidence of cardiovascular illness, which, to a significant extent, is related to dysregulated leukocyte functions.

Other different PTMs among the leukocyte extravasation have been identified, including citrullination,[35] acetylation,[36] N-glycosylation,[37] and so forth. The function of crotonylation in leukocyte extravasation remains unknown. Our study first

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detected 18 DMPs with 51 Kcr sites enriched in the leukocyte extravasation signaling pathway (Table S8). We discovered that crotonylation could covalently modify proteins at the same sites as acetylation. ACTN1(K195) was found to be acetylated in a previous study, which can impact protein phosphorylation susceptibility and regulate gene expression.[38] Acetylation of ACTN1 reduced binding to two phosphopeptides, the receptor of the phosphorylation signaling pathway, thus affecting the downstream signaling network. Mutation of ACTG1(K50) could form an iso-glutamyl lysine iso-peptide bond and lock actin protomers into an orientation, inhibiting actin polymerization significantly.[39] Basing their same targeted sites (ε-amino side chain of lysine), we considered that crotonylation affected protein functions similar to the well-known acetylation. Crotonylation has a regulatory scope that is equivalent to those of other major PTMs. We look forward to witnessing how further research efforts on crotonylation can explore the underlying pathogenic progression and potential novel treatment targets of SLE.

In the results of IPA, 29 DEPs and 18 DMPs were enriched in the leukocyte extravasation signaling pathway including several members of the MMP family. As modulators of cell−cell and cell-extracellular matrix communication, matrix metalloproteinases (MMPs) have a crucial influence on the behavior of cells and stroma homeostasis. MMP9, also known as gelatinase B, is the largest molecular weighted protease of the MMP family, exerting a critical role in the differentiation of early endothelial progenitor cells.[40] MMP9, which controls chemokines to regulate leukocyte exudation, can especially degrade type IV collagen in the extracellular matrix.[41] MMP9 is involved in physiological processes such as neutrophil function, angiogenesis, endochondral ossification, and cell migration.[42] MMP9 also regulates immune reactions via the pathways of immune response and cytokine production, according to Xu et al.[43] Contained in NETs, MMP9 impairs murine endothelium-dependent vasorelaxation and plays an essential part in endothelial dysfunction.[44] Our study's results were consistent with earlier studies' findings that SLE patients' PBMCs emitted considerably more MMP9 than the healthy control group.[45,46] Previous studies have revealed that PTMs affect MMPs, potentiating their action and causing an increase in leukocyte migration during inflammation.[47] MMP8 is a neutrophil-produced endopeptidase that plays a significant role in neutrophil infiltration and function.[48] Once neutrophils are activated, MMP8 cleaves collagens and cell adhesion proteins, thereby promoting neutrophil migration.[49] In this study, we observed that MMP8 is one of the upregulated proteins (SLE/NC ratio: 3.80) that has undergone considerable Kcr modification. MMP8 was associated with the leukocyte pathway, one of the top processes enriched in DEPs and DMPs. Crotonylation modifications have been shown to stimulate transcription to a greater degree. After modification, lysine positive charge will be neutralized, rendering electronegative DNA more receptive to being bound.[16] In nonhistone proteins, lysine is frequently involved in protein function, and its positively charged residues can be easily neutralized by PTM. Thus, PTM could regulate enzymes, protein degradation, PPI, and DNA binding relevant to physiology and disease.[50] After modification, the loop motif of the enzyme would be activated to make the protein function. It was upregulated in neutrophil extracellular traps of SLE, and this similar finding has been reported in previous proteomic analysis studies.[51] We hypothesized that the modification of MMP8 might be responsible for its relevance to physiology and disease.

We predicted that Kcr modifications may cause local inflammatory damage in SLE patients by affecting the essential molecule MMPs of leukocyte migration based on the biological annotation enrichment data and the identification of proteins in the enriched immune system pathways. It could be a potential therapeutic direction for SLE with multiple organ damage in the future.

Despite the power of LC−MS/MS methods and the utilization of bioinformatics analyses, our research still has some limitations. First, the number of SLE patients in our study was relatively modest. Therefore, our results require further confirmation in a larger panel of patients. Second, since the healthy control samples were from health examination centers, the dearth of test data connected to SLE made it difficult to depict the clinical characteristics map of the healthy group. Finally, this is a cross-sectional study with a lack of confirmation of the selected candidate proteins. Further experimental verification of the presented hypothesis is therefore necessary.

CONCLUSIONS

Collectively, we characterized the proteome and crotonylation of PBMCs from SLE individuals and healthy subjects to identify the molecular characteristics. We managed to identify hundreds of altered and modified proteins that are important in the immune system by combining proteomics with Kcr modifications. In particular, the identification of more crotonylated proteins in PBMCs of SLE individuals compared to healthy control individuals, most of them pointed to the inflammation pathways, particularly the leukocyte extravasation signaling pathway, which highlighted a potential role of crotonylation in organ damage of SLE. Our results will help generate a new perspective to focus future studies on elucidating the functions of these altered proteins and crotonylated proteins in SLE.

ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange (http://proteomecentral.proteomexchange.org) with identifier PXD042217.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06293.

PBMCs isolation and protein extraction; trypsin digestion and TMT labeling; HPLC fractionation; affinity enrichment; LC−MS/MS analysis; and data analysis (PDF)

Detailed information on protein and peptide quantification of the 618 DEPs and 612 DMPs; KEGG-based functional enrichment of the DEPs and DMPs; PPI network for the DMPs with significant differences between healthy and SLE patients; 29 DEPs enriched in regulation of actin cytoskeleton pathway; and 18 DMPs with 51 Kcr sites enriched in leukocyte extravasation signaling pathway (XLSX)

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Notes

The authors declare no competing financial interest.

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