SLC6A14 facilitates epithelial cell ferroptosis via the C/EBPβ-PAK6 axis in ulcerative colitis

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Abstract
Emerging evidence suggests that ferroptosis is involved in the pathogenesis of ulcerative colitis (UC). However, the key regulator of this process remains uncertain. In this study, we aimed to explore the roles of solute carrier (SLC) family 6 member 14 (SLC6A14) in regulating ferroptosis in UC. The expression of SLC6A14 was significantly increased and positively associated with that of prostaglandin-endoperoxide synthase 2 (PTGS2) in tissue samples from patients with UC. Moreover, a series of in vitro and in vivo experiments showed that SLC6A14 knockdown markedly suppressed ferroptosis. RNA sequencing revealed that SLC6A14 inhibited the expression of P21 (RAC1)-activated kinase 6 (PAK6) and that PAK6 knockdown abolished the effects of SLC6A14 on RAS-selective lethal 3 (RSL3)-induced ferroptosis in Caco-2 cells. Furthermore, chromatin immunoprecipitation (ChIP) and Western blot analysis demonstrated that SLC6A14 negatively regulated PAK6 expression in a CCAAT enhancer binding protein beta (C/EBPβ)-dependent manner. Collectively, these findings indicate that SLC6A14 facilitates ferroptosis in UC by promoting C/EBPβ expression and binding activity to inhibit PAK6 expression, suggesting that targeting SLC6A14-C/EBPβ-PAK6 axis-mediated ferroptosis may be a promising therapeutic alternative for UC.

Keywords Ulcerative colitis · Ferroptosis · SLC6A14 · PAK6 · C/EBPβ

Introduction
Ulcerative colitis (UC) is a relapsing and remitting inflammatory bowel disease (IBD) and is characterized by colonic mucosal inflammation resulting in continuous ulceration and bloody diarrhea [1]. Although the complete pathogenesis of UC remains unclear, it is universally acknowledged that dysfunction of intestinal epithelial cells (IECs) and defects in the epithelial barrier play an essential role in chronic inflammation [2]. Excessive IEC death destroys host–microorganism homeostasis, mucosal immune regulation, nutrient circulation, and intestinal barrier integrity and eventually causes recurring protracted colitis. Accordingly, exploring the cause and mechanism of IEC death may facilitate the development of effective treatment strategies for UC patients.

Ferroptosis is a new form of regulated cell death (RCD) that is mainly based on uncontrollable iron-dependent accumulation of lethal lipid reactive oxygen species (ROS) and is usually manifested overtly as a shrunken cell morphology and cell detachment [3]. Generally, suppression of glutathione peroxides 4 (GPX4) expression, downregulation of cystine/glutamate antiporter system (System Xc−) activity, and accumulation of lipid ROS are the three main factors resulting in the induction of ferroptosis [4]. Emerging evidence indicates that ferroptosis is closely associated with the pathogenesis of multiple diseases, including IBD [5].
6]. For instance, impaired GPX4 activity and signs of lipid peroxidation were found in small IECs of Crohn’s disease (CD) patients. Moreover, dietary ω-6 polyunsaturated fatty acids (PUFAs), especially arachidonic acid (AA), trigger inflammatory responses, and cytokine production in GPX4-deficient IECs by ferroptotic mechanisms [5]. Ferroptosis was observed in IECs from UC patients and mice with colitis, and activation of the ferroptosis signalling pathway was found to be involved in NF-κB axis-mediated endoplasmic reticulum (ER) stress signaling [6]. In addition, numerous studies have shown that inhibition of ferroptosis can markedly alleviate murine experimental colitis [7–9]. Therefore, understanding the molecular basis of ferroptosis is crucial for improving targeted ferroptosis-based treatment options.

Solute carrier (SLC) transporter family proteins, such as SLC7A11, SLC3A2, and SLC25A28, have been reported to be important regulators of ferroptosis [10–12]. SLC7A11 and the chaperone SLC3A2, two basal subunits of System Xc−, efficiently protect cells against ferroptosis via biosynthesis of glutathione (GSH) [10, 11]. Moreover, SLC25A28 interacts with mitochondrial p53, leading to accumulation of redox-active iron and activation of the electron transfer chain (ETC) as well as ferroptosis induction [12]. In the present research, we focused on SLC6A14, a member of the SLC transporter family, which is upregulated in various colonic diseases, including UC [13]. Whether and how SLC6A14 modulates IEC ferroptosis in UC was investigated in both humans and mice. We demonstrated that SLC6A14 was highly expressed in UC patients, animal models of experimental colitis, and cell models of ferroptotic death. Furthermore, SLC6A14 upregulation contributed to iron overload and GSH consumption, which eventually resulted in lipid peroxidation and ferroptosis. Mechanistically, SLC6A14 positively regulated the multifunctional transcription factor CCAAT enhancer binding protein β (C/EBPβ) to inhibit P21 (RAC1)-activated kinase 6 (PAK6) transcription, which played a major role in ferroptosis by activating ERK signalling. In summary, our results indicate that SLC6A14 is a novel regulator of ferroptosis that promotes UC progression through the C/EBPβ-PAK6-ERK axis, thereby providing key insights into UC pathogenesis.

Materials and methods

Microarray data and differential expression analysis

The microarray gene expression dataset GSE134025 from the Gene Expression Omnibus (GEO) database is based on the GPL20115 platform [6]. Differential expression analysis was performed with R software (package: ‘limma’). Differentially expressed genes (DEGs) were identified by the following criteria: (1) \( \log_2 \) (fold change) > 1 and (2) adjusted \( P \) value < 0.05. When DEGs were duplicated, the most significant genes were retained. The heatmap and volcano plot were generated with R software (packages: ‘pheatmap’, ‘ggplot2’, and ‘ggrepel’).

Human colon tissues

Paraffin-embedded colon specimens from 89 UC patients and 5 healthy individuals were obtained from the First Affiliated Hospital of Soochow University (Jiangsu, China). The clinical data of each patient are provided in Supplementary Table 1. The diagnosis of UC was confirmed histopathologically by haematoxylin and eosin (H&E) staining, and the Mayo score, Ulcerative Colitis Endoscopic Index of Severity (UCEIS), haemoglobin (Hb) level, C-reactive protein (CRP) level, and erythrocyte sedimentation rate (ESR) were available (Supplementary Tables 1–4) [14]. Ethical approval (reference number: 2021-325) was obtained from the Ethics Committee of the First Affiliated Hospital of Soochow University, and informed consent was obtained from all participants in this research.

Establishment of experimental colitis models

Six- to eight-week-old male C57/BL/6 mice (21.45 ± 1.12 g) and BALB/c mice (21.46 ± 1.25 g) were obtained from the Laboratory Animal Center of Soochow University. Animals were raised in compliance with regulations, and experiments were approved by the Ethics Committee of Soochow University (reference number: SUDA20210918A01).

Dextran sulphate sodium (DSS; MeilunBio, Dalian, China)-induced acute colitis was established as previously reported [15]. Briefly, control mice were provided sterile ddH2O, and others were provided sterile ddH2O containing 3% DSS for 8 days. A total of 20 C57/BL/6 mice were randomly divided into 3 groups: (1) Control \((n=6)\), (2) DSS + phosphate-buffered saline (PBS, \(n=7\)), and (3) DSS + α-methyl-DL-tryptophan (α-MT; Sigma-Aldrich, Bellefonte, USA; \(n=7\)). Mice in the DSS + PBS group were intraperitoneally injected with 100 μl of PBS, while mice in the DSS + α-MT group were intraperitoneally injected with 100 μl of α-MT (20 mg/ml, dissolved in sterile saline) once a day for 7 days. Weight loss, diarrhoea, and bleeding were monitored daily. The grade of DSS-induced intestinal inflammation was assessed using the disease activity index (DAI) as previously described (Supplementary Table 5) [16]. Eight days after induction of colitis, DSS-challenged mice were sacrificed.

To establish oxazolone (OXZ; Sigma–Aldrich)-induced colitis, BALB/c mice were randomly divided into 3 groups: (1) Control \((n=6)\), (2) OXZ + PBS \((n=8)\), and (3) OXZ + α-MT \((n=7)\). OXZ-induced acute colitis was induced according to previous protocols [15]. Briefly,
cutaneous sensitization to OXZ was induced in mice by application of 200 μl of 3% OXZ (dissolved in ethanol) for 2 consecutive days. Five days later, 150 μl 1% OXZ (dissolved in 50% ethanol) was carefully delivered into the colonic lumen by insertion of a catheter to a distance of 4 cm from the anus. Then, the mice were maintained in a vertical position for 30 s. Control mice were intrarectally administered 50% ethanol. Mice in the OXZ + PBS group and DSS + α-MT group were intraperitoneally injected with PBS and α-MT, respectively, once a day for 4 days. Weight loss, diarrhoea, bleeding, and DAI were recorded. Mice were finally sacrificed on Day 4 after colitis induction.

**Cell culture and treatment**

Caco-2 cells obtained from ATCC (Manassas, Virginia, USA) were cultured as an adherent epithelial monolayer in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% foetal bovine serum (FBS) and 1% penicillin–streptomycin–amphotericin solution in a 37 °C humidified incubator (Thermo Fisher Scientific, Waltham, USA) with 5% CO₂.

To establish a cell model of ferroptosis, Caco-2 cells were treated with RSL3 (MeilunBio, 15 μM) with or without ferrostatin-1 (Fer-1; Macklin, Shanghai, China; 4 μM) for 24 h. To explore the effect of SLC6A14 on Caco-2 cell ferroptosis, cells were treated with α-MT (2.5 mM) for 48 h and then treated with RSL3 (15 μM) for 24 h.

**Cell transfection**

SLC6A14-, PAK6-, C/EBPβ-specific small interfering RNAs (siRNAs; RiboBio, Guangzhou, China) and C/EBPβ-specific overexpression plasmid (Guangzhou iGene Biotechnology Co., Ltd., Guangzhou, China) were transfected into Caco-2 cells for 48–72 h using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions.

**RNA sequencing and bioinformatic analysis**

Total RNA was isolated from RSL3-induced Caco-2 cells transfected with si-SLC6A14-NC, si-SLC6A14-1, or si-SLC6A14-2 for poly(A) enrichment. mRNA transcripts with poly(A) tails were fragmented, reverse-transcribed into double-stranded cDNA, and inserted into a single-stranded circular DNA backbone. The qualified DNAs were sequenced on the DNBSEQ platform (Beijing Genomics Institute, Shenzhen, China). mRNA expression was obtained after quality control, filtering, and read alignment.

DEGs were identified by the following criteria: (1) log₂ (fold change)> 0.585 and (2) adjusted P value < 0.05. The heatmap and volcano plot were generated with R software (packages: ‘heatmap’, ‘ggplot2’, and ‘ggrepel’). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with R software (packages: ‘clusterProfiler’, ‘org.Hs.eg.db’, ‘pathview’, and ‘topGO’).

**Haematoxylin–eosin staining and histological scoring**

For histopathological analysis, H&E staining (Beyotime, Shanghai, China) was performed according to the manufacturer’s protocols. Briefly, colon tissues of mice and humans were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. The paraffin-embedded colon tissues were sliced into 5 μm sections and stained sequentially with haematoxylin to visualize nuclei and eosin to visualize the cytoplasm. H&E staining was evaluated with the tissue damage index (TDI) as previously described (Supplementary Table 6) [17].

**Immunohistochemistry**

Immunohistochemical (IHC) staining was conducted as previously mentioned with a 3,3′-diaminobenzidine (DAB) Detection Kit (Gene Tech, Shanghai, China). Briefly, paraffin-embedded sections of colon tissue were incubated with the indicated primary antibodies overnight at 4 °C and then with the corresponding HRP-conjugated goat anti-mouse/rabbit IgG secondary antibody for 1 h at 37 °C. IHC reactions were visualized by DAB staining (brown) and haematoxylin counterstaining (purple). The IHC sections were scored blindly by two experienced pathologists and additionally analysed with ImageJ (IHC Profile) with the H-score semi-quantitative scoring system (Supplementary Table 7) [18]. The antibodies used for IHC analysis are shown in Supplementary Table 8.

**Cell viability assay**

Cell viability was evaluated with a Cell Counting Kit-8 (CCK-8; NCM, Suzhou, China) according to the manufacturer’s instructions. Briefly, (3–5) × 10⁵ Caco-2 cells were seeded in a 96-well plate with 100 μl of DMEM containing 20% FBS and incubated for 48 h. After the indicated treatments, 10 μl of CCK-8 reagent was added to each well and incubated at 37 °C for 3 h. The absorbance at 450 nm (A450) was measured using a microplate photometer (Biotek, Vermont, USA).

**Total iron content assay**

The total iron content in Caco-2 cells and mouse colon tissue was measured with an Iron Content Assay Kit (Solarbio, Beijing, China) according to the manufacturer’s protocols. Briefly, Caco-2 cells and the medium were centrifuged at 3000 × g for 15 min at room temperature. The precipitates
were resuspended in 170 μl of iron extraction reagent and homogenized by sonication (ice bath; 20% power; ultrasonication 3 s, interval 3 s, 10 repeats). For analysis of mouse colon tissue, 100 μg of colon tissue was homogenized. The supernatant of each sample was obtained by centrifugation at 4 °C and 4000×g for 10 min. The protein concentration (Cpr) in the supernatant was measured by normalization with a BCA protein assay (Beyotime). Next, the abovementioned supernatant (144 μl; ddH2O for the blank wells and 0.125 μmol/ml standard solution for the standard wells) was mixed with iron assay reagents (reagent one: 72 μl; reagent two: 144 μl) and then heated at 100 °C for 1 h. The inorganic phase was obtained after mixing with trichloromethane and centrifugation at 10,000 rpm for 10 min at room temperature. The absorbance at 520 nm (A520) was measured. Thus, total iron content (μg/mg prot) = 6.98 × (A_{est} − A_{blank}) / (A_{standard} − A_{blank})/Cpr. The relative iron content was determined by normalization to the controls.

**Reduced glutathione assay**

The content of reduced GSH in Caco-2 cells and mouse colon tissue was measured with a Reduced GSH Assay Kit (Njcbio, Nanjing, China) in accordance with the instructions. Briefly, the cell and colon samples were homogenized in 60 μl of RIPA lysis buffer and centrifuged at 4 °C and 8000×g for 10 min. The protein concentration (Cpr) in the supernatant (Cpr) in the supernatant was measured for normalization. Then, the supernatant (30 μl) was mixed with Reagent One Application Buffer (120 μl) and centrifuged at 4 °C and 4000 rpm for 10 min. The absorbance at 420 nm (A420) was measured, and the GSH concentration (CGSH) was calculated according to the standard concentration curve. Thus, GSH content (μmol/L prot) = CGSH/Cpr. The relative GSH content was determined by normalization to the controls.

**Malondialdehyde assay**

The malondialdehyde (MDA) content in Caco-2 cells and mouse colon tissues was assessed with an MDA Assay Kit (Beyotime) based on the manufacturer’s instructions. In brief, cell and colon samples were homogenized in 140 μl of RIPA lysis buffer and centrifuged at 4 °C and 12,000×g for 10 min. The protein concentration (Cpr) in the supernatant was measured for normalization. The supernatant (120 μl) was mixed with 240 μl of MDA assay buffer, incubated at 100 °C for 1 h, and then centrifuged at 25 °C and 10000×g for 10 min. The absorbance at 532 nm (A532) was measured. Then, the MDA concentration (μmol/mg prot) was calculated according to the standard concentration curve, and the relative MDA content was determined by normalization to the controls.

**Immunofluorescence staining**

A total of (3–5) × 10^3 Caco-2 cells were seeded on coverslips in a glass bottom dish (φ = 20 mm; NEST, Shenzhen, China) in 1 ml of DMEM containing 20% FBS and incubated for 48 h. After the indicated treatments, the cells were fixed with 4% PFA for 15 min at room temperature and then permeabilized by using 0.2% Triton X-100. The cells were incubated with a rabbit polyclonal anti-human/mouse prostaglandin-endoperoxide synthase 2 (PTGS2) antibody (Proteintech, Wuhan, China) overnight at 4 °C after blocking with 2% bovine serum albumin (BSA) for 1 h. Then, the cells were incubated with the corresponding Cy3-labelled goat anti-rabbit secondary antibody for 1 h at 25 °C and finally mounted in the confocal glass bottom dish with antifade medium containing 4′,6-diamidino-2-phenylindole (DAPI, Beyotime). The targeted cells were observed with a laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Relative PTGS2 expression was calculated by dividing the number of PTGS2+ cells (with red staining in the cytoplasm) by the number of DAPI+ cells (with blue staining in the nucleus) followed by normalization to the controls.

**C11 BODIPY581/591 fluorescence assay**

A total of (3–5) × 10^3 Caco-2 cells were seeded on coverslips in a glass bottom dish and incubated for 48 h. After the indicated treatments, the cells were incubated with C11 BODIPY581/591 dye (ABclonal, Wuhan, China; 10 μM) at 37 °C for 1 h. The targeted cells were observed with a laser scanning confocal microscope after excess dye was removed by wiping with PBS. Quantitative analysis of stained regions was performed with ImageJ (NIH, MD, USA). C11-BODIPY581/591 levels were calculated as the ratio of the area with lipid oxidation (green) to the area without lipid oxidation (red).

**Promoter prediction analysis**

The promoter sequences of PAK6 were obtained from the GRCh38/hg38 human assembly via the University of California Santa Cruz (UCSC) Genome Browser (https://genome.ucsc.edu/cgi-bin/hgGateway). Then, the potential transcription factors and their binding sites were predicted with the TRANSFAC, ChIP-Atlas, and CIS-BP databases.
The intersection of these three datasets was visualized with an interactive Venn diagram viewer, jvenn [19]. The predicted binding sites were visualized with the WebLogo program (http://weblogo.berkeley.edu/logo.cgi).

**Dual-luciferase reporter assay**

The relative luciferase activity of the C/EBPβ promoter was determined with a Dual-Luciferase Reporter Assay System (Promega, Wisconsin, USA) following the instruction manuals. In brief, Caco-2 cells were cotransfected with the C/EBPβ luciferase reporter plasmid (pGM C/EBPβ-Lu; Genomeditech, Shanghai, China) and SLC6A14-specific siRNAs with Lipofectamine 2000. After 48 h, the cells were lysed with passive lysis buffer (PLB), and firefly and Renilla luciferase activities were measured with a luminometer (Biotek). The relative luciferase activity was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity and was normalized to si-SLC6A14-NC.

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation (ChIP) assay was performed using an EZ-ChIP Kit (Sigma-Aldrich) according to the manuals, as previously reported. Briefly, RSL3-induced Caco-2 cells were cross-linked with 37% formaldehyde for 10 min. The cross-linked chromatin was sheared with the appropriate sonication protocol (ice bath; 10% power; ultrasonication 5 s, interval 10 s, 16–18 repeats) into fragments between 200 and 1000 bp in length, which were immunoprecipitated with a rabbit polyclonal anti-human/mouse C/EBPβ antibody (Proteintech). Normal mouse IgG was used for the negative control, while an anti-RNA polymerase antibody was used for the positive control. After the complexes were subjected to reverse cross-linking, the purified short hairpin DNA was detected by qPCR and DNA agarose gel electrophoresis (AGE) using primers specific for the PAK6 promoter region designed with Primer Premier 6 (Premier, Canada). The primers are listed in Supplementary Table 9.

**Protein extraction and Western blot analysis**

Caco-2 cells and mouse colon tissues (100 mg) were lysed with RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail. The total protein concentration was measured with a BCA protein assay kit. Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a 0.45 μm polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 1.5 h and incubated with the indicated primary antibodies overnight at 4 °C. The following day, the membrane was incubated with the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the membrane was visualized with ECL reagents in a ChemiDocTM MP Imaging System (Bio-Rad, CA, USA). The antibodies used in the Western blot analysis are listed in Supplementary Table 8.

**Statistical analysis**

All statistical data were analysed with GraphPad Prism 8 (La Jolla, CA, USA). The results with normal distribution are presented as the means ± standard error of means (SEMs) and the non-normal results are presented as medians ± interquartile ranges (IQRs). An unpaired/Welch’s t test was used to analyse normal data and Mann–Whitney U test was used to analyse non-normal data in two groups. Analysis of variance (ANOVA)/Welch’s ANOVA analysis was performed to analyse normal data and Kruskal–Wallis test was used to analyse non-normal data in multiple groups. Pearson correlation analysis was performed to analyse correlations between numerical normal data, while Spearman correlation analysis was performed in non-normal data in two groups. R² represents the coefficient of determination, and r represents the Pearson/ Spearman correlation coefficient. P < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001). All experiments in this study were performed in triplicate.
Results

SLC6A14 was overexpressed in UC tissue specimens and associated with ferroptosis in mice with experimental colitis

To identify the possible critical factors mediating ferroptosis in UC, we retrieved the microarray expression dataset GSE134025 from the GEO database and screened the DEGs (adjusted \( P \) value < 0.05 and \( \log_{2} \) (fold change) > 1). In total, 26 genes were upregulated and 8 genes were downregulated in the tissues of UC patients compared with healthy individuals (Figs. 1A and S1A; Supplementary Table 10). Given that SLC proteins, such as SLC7A11 and SLC25A28, are critical to ferroptosis regulation [10, 12], SLC6A14, among the 26 upregulated genes, attracted our attention. Then, IHC analysis was conducted to validate SLC6A14 expression. SLC6A14 protein expression was markedly elevated in 89 tissues of patients with UC compared with 5 tissues of healthy individuals (Figs. 1B and S1B; Supplementary Table 1). In addition, in UC patients, SLC6A14 expression had a positive relationship with the Mayo score and UCEIS (Fig. 1C, D), two objective evaluative instruments of clinical and endoscopic activity in UC.

To analyse the correlation between SLC6A14 and ferroptosis, we next examined the expression of PTGS2, a well-known biomarker of lipid peroxidation [20], in the tissues of UC patients. The PTGS2 protein level was increased in UC tissues and positively correlated with the Mayo score and UCEIS in UC patients (Figs. 1B and S1C, D). Importantly, there was a positive correlation between the expression of SLC6A14 and PTGS2 (Fig. 1E).

To further evaluate the influence of SLC6A14 on ferroptosis in UC, DSS-induced and OXZ-induced colitis were established in mice. The results of the Western blot revealed that the SLC6A14 level was apparently higher in colon tissues of colitic mice than in control mice (Figs. 1F and S1E). Moreover, both SLC6A14 and PTGS2 expressions were significantly increased in the colons of mice with DSS-induced or OXZ-induced colitis (Fig. 1G). In addition, the protein level of SLC6A14 was markedly increased in Caco-2 cells after treatment with RSL3, which was reversed by Fer-1 (Figs. 1H and S1F).

SLC6A14 inhibition inhibits ferroptosis in colonic epithelial cells

To investigate the function of SLC6A14 in ferroptosis, three non-overlapping siRNAs targeting SLC6A14 (si-SLC6A14-1, si-SLC6A14-2, and si-SLC6A14-3) were used to knock down the mRNA and protein expression of SLC6A14 in Caco-2 cells. Among the three siRNAs, si-SLC6A14-1 and si-SLC6A14-2 had the greatest inhibitory effect on SLC6A14 expression (Figs. 2A and S2A, B). Therefore, we chose si-SLC6A14-1 and si-SLC6A14-2 for further experiments. As shown in Fig. S2C, D, depletion of SLC6A14 increased the viability and inhibited the ferroptotic death of Caco-2 cells treated with RSL3. In addition, knockdown of SLC6A14 reduced the expression of PTGS2, FTL, and FTH (ferroptosis-related genes; Figs. 2F and S2E, F) and decreased the total cellular iron content and GSH consumption as well as the MDA level in RSL3-treated Caco-2 cells (Fig. 2B–D). Furthermore, we observed decreased lipid peroxidation, as determined by C11-BODIPY581/591 staining (Fig. 2E), in Caco-2 cells after cotreatment with SLC6A14 siRNA and RSL3. These results were further confirmed by treatment with the chemical SLC6A14 inhibitor α-MT (Figs. 2G–K and S2G-J).

SLC6A14 suppression ameliorates inflammation and ferroptosis in experimental colitis

To further investigate whether SLC6A14 modulates ferroptosis in vivo, α-MT was administered to mice with DSS-induced and OXZ-induced colitis (Figs. S3A, B). As shown in Fig. 3, compared with those in the DSS group, mice in the DSS + α-MT group exhibited higher body weights, lower DAI scores, and longer colon lengths (Figs. 3A–C and S3C). Additionally, α-MT treatment markedly alleviated macroscopic colon inflammation and histological damage (Figs. 3G and S3D). A similar result was found in mice with OXZ-induced colitis (Figs. 3H–J and S3E, F).

Next, we explored the effect of ferroptosis in colitic mice by analysing ferroptosis-related indices. DSS-challenged mice administered α-MT showed a lower iron load and MDA level and exhibited a higher level of GSH (Fig. 3D–F). The results of IHC analysis of the mouse colon also indicated that treatment with α-MT reduced the protein expression of PTGS2 in DSS-challenged mice (Fig. 3G). Furthermore, a decreased iron load, reduced MDA and PTGS2 levels, and an increased GSH level were observed in OXZ-challenged mice treated with α-MT (Fig. 3K–N).

SLC6A14 regulates PAK6 expression

To clarify the possible mechanisms underlying the role of SLC6A14 in intestinal epithelial cell ferroptosis, RNA sequencing (RNA-seq) was performed to profile the transcriptome changes in SLC6A14-knockdown Caco-2 cells after RSL3 treatment. In summary, 14 upregulated and 3 downregulated genes (adjusted \( P \) value < 0.05 and \( \log_{2} \) (fold change) > 0.585) were identified in SLC6A14-knockdown Caco-2 cells compared to control cells (Figs. 4A and S4A, B; Supplementary Table 11). To validate the results of the RNA-seq analysis, several genes identified as dysregulated
SLC6A14 facilitates epithelial cell ferroptosis via the C/EBPβ-PAK6 axis in ulcerative colitis

Fig. 1 SLC6A14 expression increased in UC tissue specimens and correlated with ferroptosis. A Heatmap of 34 differentially expressed genes between UC patients (n=3) and healthy individuals (n=3) in colonic biopsy samples from the GSE134025 dataset. Red represents upregulated genes and blue represents downregulated genes. The adjusted P value <0.05 and |log2 (fold change)|>1. SLC6A14 is labelled with a red star. B Representative images of IHC staining of SLC6A14 and PTGS2 in colon tissues from UC patients (n=89) and healthy individuals (n=5) (upper). Scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score and is shown by the rose plot. The blue part represents UC patients and the orange part represents healthy individuals (lower). The correlations between SLC6A14 expression and the Mayo score (C), the UCEIS (D), and PTGS2 expression (E) in human colonic tissues. F Western blot analysis of SLC6A14 in colon tissues from mice with DSS-induced and OXZ-induced colitis. β-actin was used as the control. G Representative images of IHC staining of SLC6A14 and PTGS2 in colon tissues from mice with DSS-induced and OXZ-induced colitis. Scale bar, 50 μm. H Western blot analysis of SLC6A14 in the Caco-2 cell model of RSL3-induced ferroptosis. β-actin was used as the control. The results are presented as the means ± SEMs; ***P<0.001 vs. normal.
in the RNA-seq data were analysed by qRT-PCR. Consistent with the RNA-seq results, the expression of PAK6, HABP4, CDKN1A, and ACAD10 was increased but the expression of DACT3 was decreased in SLC6A14-knockdown Caco-2 cells (Fig. S4C). Among these DEGs, PAK6, a member of the type II PAK family, was obviously upregulated in ferroptotic SLC6A14-knockdown cells and is involved in the regulation of mitochondrial activity and ROS production [21, 22]. Hence, we hypothesized that PAK6 may participate in SLC6A14-mediated ferroptosis. IHC analysis showed that the expression of PAK6 in UC tissue samples was slightly upregulated compared with that in the samples of healthy individuals and was negatively correlated with the Mayo score and UCEIS in UC patients (Fig. 4B–D). Although
the expression levels of both SLC6A14 and PAK6 were increased in UC tissue specimens, we observed an inverse correlation between SLC6A14 and PAK6 protein levels in our cohort (Fig. 4E). Additionally, α-MT administration evidently elevated the protein expression of PAK6 in Caco-2 cells after treatment with SLC6A14 siRNA or α-MT (Figs. 4H and S4H, I). Collectively, our results suggested that SLC6A14 facilitates epithelial cell ferroptosis via the C/EBPβ-PAK6 axis in ulcerative colitis. 

Fig. 3 Inhibition of SLC6A14 ameliorating inflammation and ferroptosis in mice with experimental colitis. The body weight (A), disease activity index (DAI) score (B), and photograph of the colon (C) in DSS-challenged mice treated with α-MT. Iron content (D), GSH content (E), and MDA content (F) in DSS-challenged mice treated with α-MT. G Representative images of H&E staining (upper) and IHC staining (lower) of PTGS2 in colon tissues from DSS-challenged mice treated with α-MT. Scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score. The body weight (H), DAI score (I), and photograph of the colon (J) in OXZ-challenged mice treated with α-MT. Iron content (K), GSH content (L), and MDA content (M) in OXZ-challenged mice treated with α-MT. N Representative images of H&E staining (upper) and IHC staining (lower) of PTGS2 in colon tissues from OXZ-challenged mice treated with α-MT. Scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score. The results are presented as the means ± SEMs or medians ± IQRs; *P < 0.05, **P < 0.01, ***P < 0.001 vs. DSS-Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. DSS + PBS; &P < 0.05, &&P < 0.01, &&&P < 0.001 vs. OXZ-Control; @P < 0.05, @@P < 0.01, @@@P < 0.001 vs. OXZ + PBS.
that SLC6A14 negatively modulates PAK6 expression in UC.

**PAK6 is involved in SLC6A14-mediated ferroptosis**

To assess whether SLC6A14-mediated ferroptosis is PAK6 dependent, we synthesized PAK6 siRNA-2 and PAK6 siRNA-3, which markedly decreased the protein level of PAK6 in Caco-2 cells after RSL3 treatment (Figs. 5A and S5A). Upon siRNA-mediated silencing of PAK6 in Caco-2 cells, RSL3-induced ferroptosis was significantly increased (Fig. S5B), and cell viability was apparently decreased (Fig. 5B). In addition, knockdown of PAK6 obviously elevated the iron content, GSH consumption, MDA level, and
SLC6A14 facilitates epithelial cell ferroptosis via the C/EBPβ-PAK6 axis in ulcerative colitis

SLC6A14 inhibits PAK6 expression via C/EBPβ

We next sought to investigate how SLC6A14 controls the expression of PAK6. Predictions by the UCSC Genome Browser, TRANSFAC, ChIP-Atlas, and CIS-BP databases identified three possible binding sites of C/EBPβ in the
PAK6 promoter (Fig. 6A, B). To confirm this prediction, we performed a ChIP assay in Caco-2 cells induced by RSL3. The ChIP results indicated that compared with the DNA purified with control IgG, the DNA purified with the anti-C/EBPβ antibody was significantly enriched in the three predicted sequences in the PAK6 promoter (Fig. 6C). Furthermore, transfection with C/EBPβ siRNA-1 and C/EBPβ siRNA-2 obviously reduced C/EBPβ protein expression in Caco-2 cells (Figs. 6D and S6A) and significantly elevated in PAK6 expression in Caco-2 cells induced by RSL3 (Fig. 6E). These results indicated that as a transcription factor, C/EBPβ can directly bind to the promoter of PAK6. To elucidate the importance of SLC6A14 in relation to C/EBPβ, we first detected by a luciferase reporter assay whether the activity of C/EBPβ (as determined by the C/EBPβ DNA-binding activity) is enhanced by SLC6A14. SLC6A14 silencing significantly decreased C/EBPβ activity in Caco-2 cells. Western blot analysis of SLC6A14, PAK6, and C/EBPβ expression in RSL3-induced Caco-2 cells transfected with SLC6A14-siRNA and C/EBPβ overexpression plasmid. GAPDH was used as the control. The Western blot band densities were quantified using ImageJ software. The results are presented as the means ± SEMs. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. IgG; φφP < 0.01, φφφP < 0.001 vs. si-C/EBPβ-NC + RSL3; ###P < 0.01 vs. si-SLC6A14-NC; ***P < 0.001 vs. si-SLC6A14-NC + RSL3; @@@P < 0.001 vs. si-SLC6A14-2 + RSL3.
The results of Western blot analysis showed that SLC6A14 knockdown greatly decreased C/EBPβ expression and increased PAK6 expression in Caco-2 cells after RSL3 induction (Fig. 6G). Moreover, treatment with C/EBPβ overexpression plasmid, which significantly upregulated the C/EBPβ protein levels in Caco-2 cells (Fig. S6B), reversed the effect of SLC6A14 knockdown on PAK6 expression (Figs. 6H and S6C).

Discussion

Recently, ferroptosis has been recognized as a key factor in the occurrence and development of UC [6]. Hence, accumulating evidence has indicated the roles and underlying molecular mechanisms of ferroptosis in UC. Inhibition of ferroptosis by ferrostatin-1, liproxstatin-1, or deferiprone was found to effectively ameliorate DSS-induced UC by negatively regulating the Nrf2/HO-1 signalling pathway [9]. Natural active ingredients from plants, such as curcuriligoside and astragalus polysaccharide, were found to obviously ameliorate DSS-induced UC through suppression of IEC ferroptosis [7, 23]. However, the key protein involved in this process remains obscure. Herein, we analysed the GSE134025 dataset to identify DEGs between UC patients and healthy individuals and found that SLC6A14, which shares analogous structures with other SLC transport proteins and is functionally coupled to SLC1A5, SLC7A5, and SLC7A11, was one of the upregulated DEGs in UC [24, 25]. Thus, in the current study, we concentrated on the potential impact and molecular mechanisms of SLC6A14 in mediating IEC ferroptosis in UC.

Multiple studies have demonstrated that SLC6A14 is upregulated in various colonic diseases, including UC [13]. Notably, microarray analysis of colon tissues from UC patients and control individuals revealed that SLC6A14 mRNA expression was noticeably increased in UC [26], in line with our analysis of recent microarray expression data [6]. Moreover, SLC6A14 expression was upregulated in human UC specimens in our cohort and was positively correlated with disease activity and endoscopic mucosal injury. DSS exerts toxicity toward intestinal epithelial cells of the basal crypts resulting in epithelial damage and infiltration of neutrophils, macrophages, and lymphocytes in mucosa and submucosa. OXZ triggers type-2- and type-9-related immune responses and causes ulcer formation, loss of epithelial cells, and infiltrations with neutrophils. Both DSS and OXZ can chemically induce colitis models to mimic some key immunological and histopathological features of UC in humans [15]. Hence, we further demonstrated that SLC6A14 was elevated in mice with DSS- and OXZ-induced colitis. Upregulation of SLC6A14 is closely associated with the progression of UC. More importantly, our study revealed that SLC6A14 was positively correlated with lipid peroxidation in UC patients, and this finding was validated in mice with DSS- and OXZ-induced inflammation and in ferroptotic cells. To evaluate the role of SLC6A14 in the regulation of ferroptosis, SLC6A14 siRNAs, decreased the mRNA and protein expression of SLC6A14, and α-MT, blocked SLC6A14 transport function, were used. Our data showed that SLC6A14 knockdown by siRNA or inhibition by α-MT dramatically alleviated ferroptosis in colonic epithelial cells and experimental colitis, as evidenced by the regulatory effects on the expression of ferroptosis-related genes, the iron content, GSH consumption, the MDA level, and lipid peroxidation. These results indicate that SLC6A14 plays a crucial role in mediating IEC ferroptosis in UC.

To extensively explore the mechanisms underlying SLC6A14-mediated ferroptosis, RNA-seq analysis was performed using Caco-2 cells cotreated with SLC6A14 siRNAs and RSL3. The results of this RNA-seq analysis indicated that PAK6 was obviously upregulated in ferroptotic SLC6A14-knockdown cells. As a member of the type II PAK family, PAK6 has been demonstrated to be associated with mitochondrial function and ROS production [21, 22]. Given that dysregulation of mitochondrial function and ROS elevation contribute to ferroptosis [27–29], we speculated that PAK6 is involved in regulating IEC ferroptosis in UC. Herein, we observed that silencing PAK6 significantly enhanced ferroptosis in Caco-2 cells after treatment with RSL3. Although PAK6 expression levels were increased in UC tissue specimens, an inverse correlation between SLC6A14 and PAK6 protein levels in our cohort was observed. Additionally, suppression of SLC6A14 by α-MT evidently upregulated the protein expression of PAK6 in the colons of mice with either DSS-induced or OXZ-induced colitis. Importantly, PAK6 knockdown abolished the effects of SLC6A14 on RSL3-induced ferroptosis in Caco-2 cells. These results suggested that SLC6A14 promotes IEC ferroptosis in UC via negative regulation of PAK6.

Previous studies have indicated that PAK6 can activate multiple signalling pathways and perform its biological functions. For instance, overexpression of PAK6 was found to promote the proliferation, migration, and invasion of cervical cancer cells by activating the Wnt/β-catenin signalling pathway [30]. PAK6 is involved in the modulation of androgen receptor signalling in various types of prostate cancer [31]. Moreover, pharmacological inhibition of PAK6 was found to sensitize therapy-resistant cells to tyrosine kinase inhibitors in chronic myeloid leukaemia by disrupting the RAS/MAPK pathway and mitochondrial activity [22]. In the current study, we identified that the RAS signalling pathway was upregulated in ferroptotic SLC6A14-knockdown cells (Fig. S6D). Moreover, the
results of KEGG pathway enrichment analysis (https://www.genome.jp/kegg/) showed the association between PAK6 and the RAS signalling pathway (Fig. S6E). Additionally, PAK6 knockdown significantly increased the protein level of phosphorylated ERK1/2, the key protein in the RAS signalling pathway (Fig. S6F). Given that the RAS signalling pathway plays an important role in the regulation of ferroptosis [32], we concluded that the RAS-ERK signalling pathway is required for SLC6A14-PAK6 axis-mediated ferroptosis. Since numerous factors and intersecting pathways were related to RAS signalling, we did not verify all involved elements, and there may be other potential pathways involved in this process.

C/EBPβ, an important transcription factor belonging to the leucine zipper family, plays a critical role in several cellular processes, including the inflammatory response [33–36]. C/EBPβ has been demonstrated to modulate inflammation in intestinal epithelial cells [37]. Moreover, upregulation of C/EBPβ induces inflammatory responses, resulting in mitochondrial dysfunction and ROS accumulation [38]. Herein, we predicted possible transcription factor binding sites in the PAK6 promoter region and fortunately found binding sites for the most compelling factor, C/EBPβ. Our ChIP assay showed that the DNA precipitated with the anti-C/EBPβ antibody was significantly enriched in PAK6 promoter sequences. Importantly, the results of the luciferase reporter assay indicated that SLC6A14 knockdown significantly decreased C/EBPβ activity. In addition, SLC6A14 silencing greatly decreased C/EBPβ expression and increased PAK6 expression in Caco-2 cells after treatment with RSL3. Therefore, our results suggested that SLC6A14 controls the expression of PAK6 via C/EBPβ. However, we have yet to fully understand the specific mechanism by which SLC6A14 activates C/EBPβ. Further exploration is required in future research.

In conclusion, we investigated the function and molecular mechanism of SLC6A14 in mediating IEC ferroptosis in UC. Our findings indicated that SLC6A14 overexpression promotes IEC ferroptosis in UC via the C/EBPβ-PAK6 pathway. Moreover, high SLC6A14 expression was positively associated with ferroptosis in UC tissue samples. Therefore, our results revealed that SLC6A14 promotes ferroptosis in UC via control of the C/EBPβ-PAK6 pathway. And targeting SLC6A14-C/EBPβ-PAK6 axis-mediated ferroptosis may be a promising therapeutic alternative for UC.

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Author contributions YJC, TGS, and WCC contributed to the study conception and design. Material preparation, data collection, and analysis were performed by YJC, WYY, JYW, YQC, JHZ, HYJ, HYW, GBZ, TGS, QHX, and SHZ. The first draft of the manuscript was written by YJC and TGS, and all authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

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Data availability The microarray datasets analysed during the current study are available in the GEO (GSE134025) repository, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134025.

Declarations Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval All animal procedures were performed in line with the principles of the Ethics Committee of Soochow University (reference number: SUDA20210918A01). All studies for human tissue samples compiled the Ethics Committee of the First Affiliated Hospital of Soochow University (reference number: 2021-325).

Consent to participate Written informed consent was obtained from the parents.

Consent for publication The authors affirm that human research participants provided informed consent for publication of the images in Figs. 1B, 4B, and S1B.

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