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PAX5 and circ1857 affected DLBCL progression and B-cell proliferation through regulating GINS1

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Abstract

PAX5, a member of the paired box gene family of transcription factors, is a B-cell-specific activator protein that plays important roles during B lymphopoiesis. Two putative PAX5 binding sites in the human GINS1 promoter region were identified. EMSA, ChIP and luciferase assay showed that PAX5 functions as a positive transcription factor for GINS1 expression. Furthermore, coordinated expression of PAX5 and GINS1 was observed in mice B cells under physiological conditions and LPS stimulation situations. A similar pattern was also observed in human DLBCL cell lines under differentiation-inducing conditions. In addition, both PAX5 and GINS1 were highly expressed and significantly correlated in DLBCL specimens and cell lines. These findings suggested that dysregulation of PAX5 played an extremely important role in controlling the universal phenomenon of tumor progression through increased expression of GINS1 in DLBCL. In addition, circ1857 that was generated using back splicing of PAX5 pre-mRNA could further stabilize *GINS1* mRNA, modulate GINS1 expression and promote lymphoma progression. To the best of our knowledge, this report is the first to demonstrate the role of GINS1 in DLBCL progression, and the mechanism of GINS1 upregulation using both circ1857 and PAX5 in DLBCL was revealed. Our results suggested that GINS1 may be a possible therapeutic target for DLBCL.

KEYWORDS
DLBCL, GINS1, hsa_circ_0001857, PAX5, PSF1

Abbreviations: 3′UTR, 3′ untranslated regions; ALL, acute lymphoblastic leukemia; BSAP, B-cell-specific activator protein; ChIP-seq, ChIP followed by sequencing; DLBCL, diffuse large B-cell lymphoma; GEPIA, Gene Expression Profiling Interactive Analysis; GINS1, GINS (Go-Ichi-Ni-San) Complex Subunit 1; GINS2/3/4, GINS Complex Subunit 2/3/4; IPTG, isopropyl-β-D-thiogalactoside; MACS, magnetic-activated cell sorting; PAX5, paired box 5; PRDM1, PR domain zinc finger protein 1; PSF1, Partner of SLD five 1; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; TCGA, The Cancer Genome Atlas; TF, transcription factor.

1 | INTRODUCTION

DLBCL is the most common non-Hodgkin lymphoma, accounting for 30–40% of new diagnoses.[1] The current R-CHOP treatment is effective in 60–70% of patients. Approximately 30–40% of patients with DLBCL will relapse after first-line chemotherapy and the prognosis is poor.[2] Finding novel targets and effective therapies for these DLBCL patients are still urgently needed.

GINS1 (also known as PSF1) is a member of the heterotetrameric GINS complex, which contains GINS1, GINS2, GINS3, and GINS4.[3,4] In eukaryotic cells, the GINS complex regulates both the initiation and progression of DNA replication.[5,6] Recently, there has been an increasing number of studies on GINS1 in malignant tumors.[7–10] In addition, GINS1 was highly expressed in several types of leukemias, and knockdown of GINS1 reduced the growth of AML and CML cells, GINS1 was suggested as a possible therapeutic target to enhance the effect of chemotherapy.[11] However, it remains unknown whether GINS1 is involved in DLBCL progression.

PAX5 (also known as BSAP) plays a decisive role in B-cell proliferation and differentiation.[12,13] PAX5 represents a common deregulated molecule in multiple B-cell malignancies.[14–16] In a significant number of these cancers, PAX5 has been reported to be either upregulated or mutated due to aberrant hypermutation. In lymphoplasmacytic lymphoma and a few cases of DLBCL, elevated expression of PAX5 is the result of chromosomal translocation that juxtapositions PAX5 to the IgH gene promoter.[17] In addition, PAX5 overexpression without translocation was also reported.[18] Moreover, the *PAX5* gene has also been revealed to be a regulator in various non-hematologic tissues and cancers including malignant neuroblastoma and pediatric brain tumors, where PAX5 expression positively correlates with cell proliferation.[19] More interestingly, it has been reported that PAX5 haploinsufficiency could restrain cell proliferation and induce the G0/G1 arrest of lymphoma cells.[20] In addition, a mutation in PAX5 known as PAX5 P80R was reported to mediate a favorable outcome for a subtype of B-cell precursor ALL prognosis.[21,22] All these reports indicated that PAX5 expression might stimulate cells proliferation and contribute to enhanced cell survival.

In this study, a high expression of GINS1 in DLBCL was found and the mechanism of GINS1 upregulation was revealed. Both PAX5 and circ1857 (circBase ID: hsa_circ_0001857, back spliced from PAX5 pre-mRNA) could promote GINS1 expression in a coordinated way. Our finding indicated that dysregulation of PAX5, the key transcription factor in the B-cell lineage, plays an extremely important role in controlling the universal phenomenon of tumor progression through increased expression of GINS1 in DLBCL. Moreover, GINS1 regulated by PAX5 was also observed during B-cell proliferation and differentiation under physiological conditions.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

All the cell lines were confirmed using short tandem repeat (STR) profiling. IM-9 (EBV-transformed B lymphoblastoid cell line), Farage (a B lymphocyte cell line that was isolated in 1990 from a White, adult female patient with non-Hodgkin's B-cell lymphoma, DLBCL GCB subtype), DB (a B lymphoblast cell that was isolated from the ascites of a patient with large cell lymphoma, DLBCL GCB subtype) and SU-DHL-2 (a cell line that was isolated in 1974 from the lymph node of a White female with large cell lymphoma, DLBCL ABC subtype) were obtained from ATCC and cultured in RPMI1640 medium supplemented with 10% FBS. HEK293T was cultured in DMEM with 10% FBS.

2.2 | Patients and tissues

Fresh human biopsy tissues were obtained from 30 patients with DLBCL collected in Nanjing First Hospital. Lymph node biopsy samples from lymphadenitis individuals ($n=24$) were used as negative controls (Table S1).

2.3 | RT-qPCR

RNA isolation was performed using TRIzol reagent. cDNAs were synthesized using oligo(dT) and random hexamers. Real-time PCR was performed. Relative expression levels of circRNA or mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Table S2.

2.4 | Luciferase reporter assay

GINS1 promoter segments (−1500/+50; −550/+50; −400/+50) were obtained using PCR and inserted into pGL-4.17 vectors to construct luciferase reporter plasmids (Table S3). To prepare mutated promoter plasmids, a mutation was created from a wild-type promoter plasmid using PCR. Cells were transfected using electroporation and the AMAXA® Cell Line Nucleofector® Kit V (Lonza, Cologne, Germany). The used Nucleofector® programs were G-016. HEK293T cells were transfected with Lipofectamine 2000. After transfection for 48 h, the luciferase activity of the cells was evaluated using a luciferase assay kit and a β-galactosidase assay kit.

2.5 | Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed as previously described.[23] Cells were fixed in 11% formaldehyde and then re-suspended in cell lysis buffer. After sonication, the broken chromatin was subsequently incubated with an antibody at 4°C, then protein A+G agarose beads were added and incubated for 2 h. DNA fragments were purified and a PCR was performed. The primers used are shown in Table S4.

2.6 | Electrophoretic mobility shift assay

Nuclear extracts were obtained as described above.[23] Synthetic biotin-labeled oligonucleotides were synthesized and oligonucleotide

sequences are shown in Table S4. The probes of annealed oligonucleotides were subsequently incubated with nuclear extract or purified protein. The DNA–protein complex was separated using 6% non-denaturing polyacrylamide, and detected with HRP-conjugated streptavidin.

2.7 | PAX5 protein purification

Plasmids pET32a-PAX5 WT or pET32a-PAX5 P80R were transformed into *E. coli* BL21 and the expression of Re-PAX5 protein (recombinant wild-type PAX5) or Re-PAX5 P80R protein (recombinant PAX5 P80R) was induced using IPTG. Then, the cultures were centrifuged, and the cell pellet was collected and sonicated. The inclusion body in the precipitate was dissolved in 8 mol/L urea, then recombinant proteins were refolded and purified.

2.8 | Generation of stable cell lines

GINS1-expressing (NM_021067.5) and PAX5-expressing (NM_016734.3) plasmids were constructed based on pCDH-CMV-MCS-EF1-copGFP-T2A-puro. The circ1857 overexpression vector pLCDH-ciR-circ1857 was designed and exons 2–5 of PAX5 was inserted into pLCDH-ciR that contained an autocyclization sequence (Figure S1). The shRNA expressing vectors were generated by inserting short, double-stranded DNA oligos encoding a sense–loop–antisense sequence to the targeted gene in pLKO.1-puro. shRNA target sequences are shown in Table S2. To produce the recombinant viruses, the above recombinant lentiviral vectors were co-transfected with packaging plasmids into 293 T cells. The lentiviral particles were then used to infect cells in order to generate stable cell lines (Table S5). Briefly, cells were seeded at a density of 5×10^5 cells per well in a six-well plate, and then infected with virus. Following a 24 h incubation, cells were washed to remove free virus and selection was started using fresh medium containing puromycin; a mixed population of drug-resistant cells was obtained. To generate stable cell lines, monoclonal cell line screening with 1.5 μg/mL puromycin was performed.

2.9 | Western blot

Western blot was conducted with antibodies specific for PAX5, GINS1, PRDM1, a-tubulin and Flag (Table S6).

2.10 | CCK-8 assays

For the cell proliferation assay, cells were seeded into a 96-well plate. Cell viability was determined using the Cell Counting Kit-8 and according to the manufacturer's manual.

2.11 | Soft agar colony assay

Cells were seeded into six-well plates, with a 0.3% top agarose layer and a 0.5% bottom agarose layer in 2× RPMI-1640 medium containing 20% FBS. Cell colonies were formed following incubation for 10 days. Thereafter, the agarose was fixed with paraformaldehyde, and stained with crystal violet. The number of clones was counted.

2.12 | Xenograft lymphoma model

Six-week-old male BALB/c-nude mice were purchased from Gempharmatech Company, China. Each male nude mouse was injected subcutaneously with 8×10^6 cells. Tumor size was monitored. Finally, the mice were anesthetized, and the tumors were obtained and weighed.

2.13 | RNA stability assay

Cells were seeded into six-well plates and incubated with 4.5 μg/mL actinomycin D for the indicated time and then cells were collected.

2.14 | B-cell subpopulation sorting

C57BL/6, 6–8 weeks of age, were obtained from Gempharmatech Company, China. Spleen and bone marrow were isolated and homogenized. Freshly prepared single-cell suspensions were used for mononuclear cell isolation with Ficoll 400. Subsequent sorting of distinct B-cell subpopulations was performed using MACS. For spleen $CD138^+$ plasma cell preparation, BSA-immunized mice were used. Spleen $CD138^+$ plasma cells were isolated using the $CD138^+$ Plasma Cell Isolation Kit (Miltenyi Biotec).[24,25] For spleen IgD^+ B-cell isolation, cells were first stained with biotinylated IgD antibody. Subsequently the cells were magnetically labeled with Streptavidin MicroBeads and positively selected (Table S6). For bone marrow IgM^+ B cells isolation, anti-mouse IgM microBeads were used.[26] To increase the purity of each fraction, magnetic cell sorting was conducted twice. The purity of each fraction was determined as ~90% using flow cytometry.

2.15 | LPS-activated mice $B220^+$ splenocytes and IM-9 cells

Purified spleen $B220^+$ B cells were cultured in RPMI 1640 supplemented with 10% FBS. After 12 h, cells were stimulated with 10 μg/mL LPS. IM-9 cells were cultured and treated with LPS for the same concentration. After stimulation for the indicated time, cells were harvested and analyzed for western blot.

2.16 | CD40-activated B lymphocytes

SU-DHL-2 cells were seeded into six-well plates and induced to differentiate as previously described.[27,28] Briefly, cell culture was supplemented with IL-21 (50 ng/mL) and anti-CD40 antibodies (3 μg/mL) and cultured for the indicated time, then cells were collected for analysis.

2.17 | GINS1 3′UTR reporter assay

GINS1 3′UTR and GINS1 mutated 3′UTR sequences were amplified and cloned into pmirGLO dual luciferase report vectors. Then constructed pmirGLO GINS1 and pmirGLO GINS1 Mut were co-transfected with circ1857 overexpression plasmid or circ1857 knockdown plasmid. At 48 h after transfection, the Dual Luciferase Reporter Gene Assay Kit was used to detect luciferase activity.

2.18 | Statistical analysis

All experimental data were analyzed using Student's *t*-test or one-way ANOVA and GraphPrism7. All results were presented as the mean ± SD. A p-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | GINS1 was highly expressed in DLBCL and promoted tumor proliferation in vivo and in vitro

To explore the expression and significance of GINS1 in DLBCL, first, clinical data for DLBCL in TCGA database were used. GEPIA showed that GINS1 was elevated in DLBCL compared with normal. In addition, patients with high GINS1 expression had poorer disease-free survival than those with low GINS1 expression (Figure 1A1,A2). Next, GINS1 expression was examined in our collected tissue samples using RT-qPCR and western blotting, and the result was consistent with GEPIA. GINS1 was upregulated in biopsy specimens from DLBCL patients ($n = 30$) compared with from control lymphadenitis ($n = 24$) (Figure 1B1,B2). Furthermore, GINS1 expression in three DLBCL cell lines was also investigated, the results showed that GINS1 expression was higher in DLBCL cell lines than in the control IM-9 cells (Figure 1C1,C2).

To test the functional significance of GINS1 expression on DLBCL cell proliferation, GINS1 stable expression cell lines Farage-OE-GINS1 and GINS1 stable knockdown cell lines SU-DHL-2-shGINS1 were produced (Figure 1D–I). CCK-8 and clonogenic assays showed that overexpressing GINS1 promoted Farage-OE-GINS1 cell proliferation (Figure 1D–F). Knockdown of GINS1 suppressed SU-DHL-2-shGINS1 cell growth (Figure 1G–I). Then, an in vivo experiment further confirmed that the tumors formed using Farage-OE-GINS1 cells showed increased rates of proliferation when compared with the control group (Figure 1J–L). These results indicated that GINS1 was critical for DLBCL proliferation.

3.2 | PAX5 directly promotes transcription of GINS1

To further explore the mechanisms underlying the upregulation of GINS1 in DLBCL, the promoter region of the human *GINS1* gene was analyzed using JASPAR (an open-access database of curated, non-redundant TF binding profiles). The presence of two putative PAX5 binding sites was predicted (Figures 2A, S2). More importantly, target genes of PAX5 in public ENCODE ChIP-seq datasets were analyzed (http://amp.pharm.mssm.edu/Harmonizome/dataset/ENCODE+Transcription+Factor+Targets).[29] ChIP-seq datasets showed that *GINS1* was one of the target genes of PAX5. To further confirm whether PAX5 was involved in GINS1 activation, five promoter reporter plasmids were constructed (Figure 2B). Promoter reporter plasmids and pRSV-β-galactosidase control plasmids were co-transfected into SU-DHL-2 cells; the luciferase activity showed that mutation of any one of the predicted PAX5-binding sites decreased the GINS1 promotor activity. This result indicated that both PAX5-binding sites on the *GINS1* promoter were important.

Next, the EMSA was performed. Two sets of probes that contained a putative site1 or site2 were synthesized; DNA–protein complexes were detected when the probes were incubated with the nuclear extracts of SU-DHL-2 cells. The results showed that an increased number of unlabeled probes decreased the band for the complexes, and PAX5 antibody also super-shifted the band for the complexes (Figure 2C). Furthermore, ChIP assays also confirmed that PAX5 could be recruited onto the GINS1 promoters (Figure 2D). These results indicated that PAX5 could bind to the GINS1 promoter.

3.3 | PAX5 P80R mutant lacks the ability to bind to the *GINS1* promoter

The PAX5 P80R mutation was located in the paired domain for DNA binding (Figure S3A).

It has been reported that patients who have thePAX5 P80R mutation have a significantly higher probability of 5-year overall survival,[21] and it is suggested that the PAX5 P80R mutation disrupts DNA binding to its target genes.[30] To confirm that GINS1 was regulated by PAX5, recombinant re-PAX5 and re-PAX5 P80R protein were expressed in *E. coli* and purified. The EMSA assay showed that re-PAX5 P80R had lost its ability for DNA binding to probe1, while re-PAX5 could form the complex with the probe (Figure S3B). These results confirmed that the PAX5 P80R mutation created the loss of the ability for DNA binding. Consistent with these results, overexpression of PAX5 could activate the promoter activity of the reporter plasmids P-1500/+50-LUC significantly in 293T cells, whereas PAX5 P80R failed to stimulate the promoter activity of this reporter plasmid (Figure S3C). Moreover, western blotting demonstrated that PAX5 overexpression enhanced

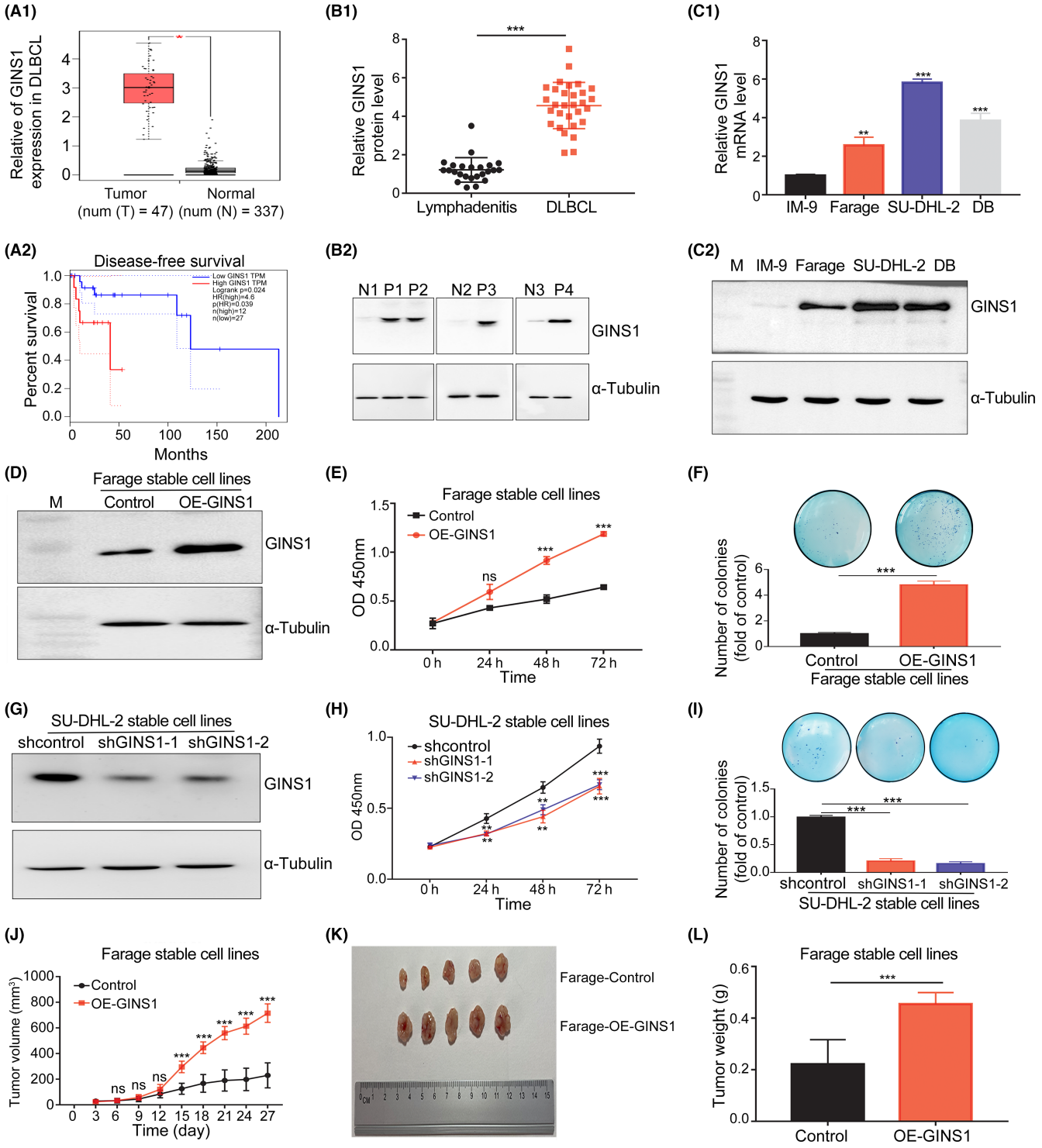

FIGURE 1 GINS1 was highly expressed and promoted tumor proliferation in DLBCL. (A) GEPIA analysis. A1, GINS1 expression was higher in the DLBCL tumor. A2, Disease-free survival curves of DLBCL patients. (B) GINS1 protein expression in our collected biopsy specimens from DLBCL ($n = 30$) and lymphadenitis patients ($n = 24$). B1, Western blot analysis. B2, Representative western blotting analysis of GINS1 protein expression (P, DLBCL sample; N, non-tumorous lymphadenitis control sample). (C) *GINS1* mRNA and protein expression levels in DLBCL cell lines. C1, RT-qPCR analysis. C2, Western analysis. (D–F) Overexpression of GINS1 in Farage cells promotes cell proliferation. D, Western blot. E, Cell viability assay. F, Soft agar assay for colony formation. (G–I) Knockdown of GINS1 inhibits the proliferation of SU-DHL-2 cells. G, Western blot. H, CCK-8 assay. I, soft agar colony. (J–L) Overexpression of GINS1 in Farage cells promotes cell proliferation in vivo. J, Tumor growth curve: subcutaneously injecting Farage-control cells, GINS1 overexpressing stable cells Farage-OE-GINS1 into the flank of nude mice, the tumor sizes of two groups were measured (five mice in each group). K, Tumor pictures. L, tumor weight (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

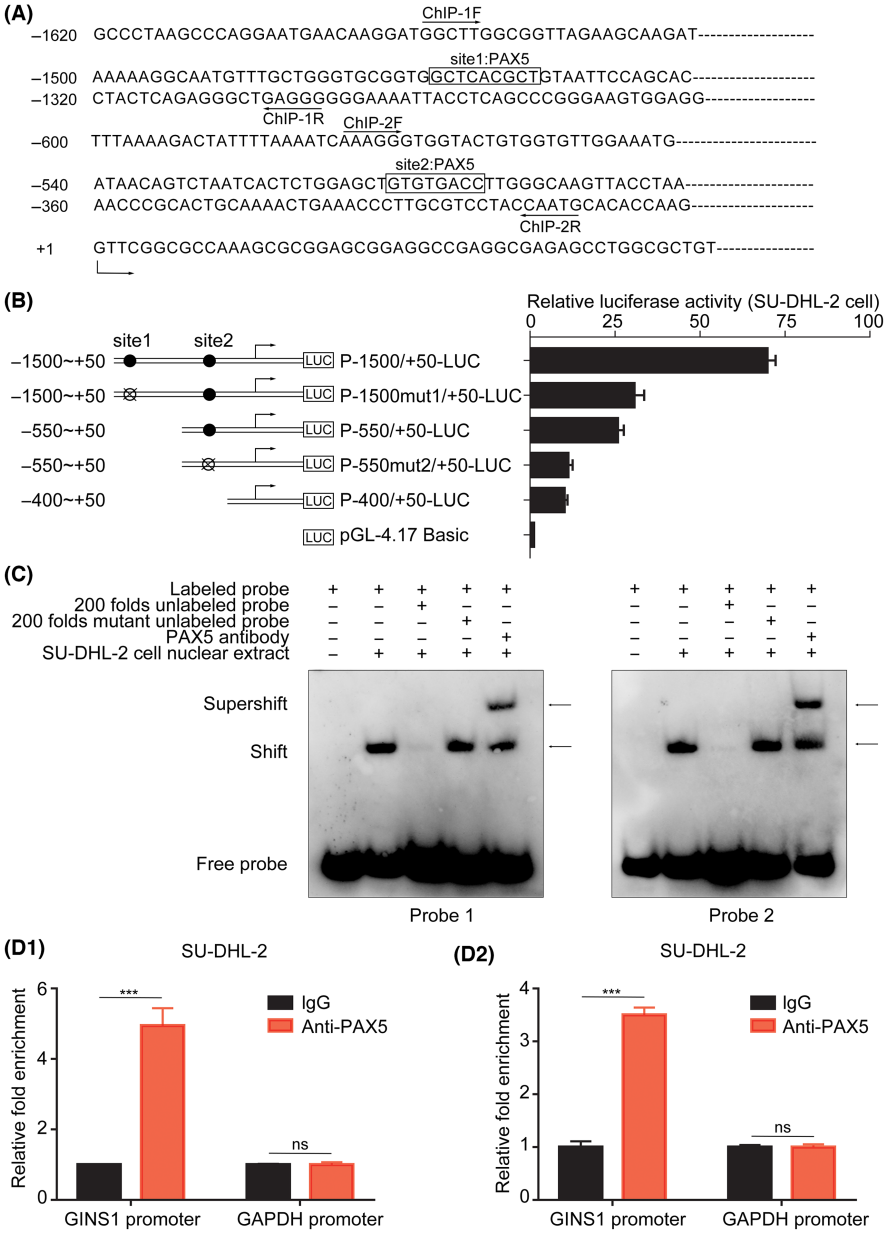

FIGURE 2 PAX5 is responsible for GINS1 expression. (A) Nucleotide sequence of the promoter region of the *GINS1* gene. Two predicted PAX5 binding sites (site1 and site2) are shown. The location of primers used for ChIP-1 and ChIP-2 are also shown here. +1 indicates the position of the transcription initiation site of the *GINS1* gene. (B) Left: the schematic diagram of the luciferase reporter constructs containing the indicated genomic fragments of the *GINS1* gene is shown. Right: the results of the luciferase reporter assay. (C) EMSA analysis. (D) ChIP assay. D1, ChIP-1. D2, ChIP-2 (***$p < 0.001$).

GINS1 expression in 293T cells, whereas PAX5 P80R overexpression did not increase the GINS1 level (Figure S3D). Altogether, these results suggested that *GINS1* was the target gene of PAX5.

3.4 | PAX5 promotes DLBCL progression through GINS1

Based on the above results, it was proposed that GINS1 upregulation might be caused by the PAX5 abnormality. Therefore, the expression levels of PAX5 and GINS1 in clinical specimens were analyzed, the results showed that both PAX5 and GINS1 were highly expressed and significantly correlated (Figures 1B, 3A1–A3). Moreover, TCGA dataset analysis using GEPIA also showed that the PAX5 level was higher in DLBCL samples than in the control (Figure 3B); a significantly positive correlation between GINS1 and PAX5 expression is shown in Figure S4A. Consistently, compared with the B lymphoblast cell line IM-9, a higher level of PAX5 expression in DLBCL cell lines was also observed (Figure 3C).

To further explore the functional significance of PAX5 on GINS1 in DLBCL, stable cell lines were established and evaluated. Overexpressing PAX5 in Farage cell and IM-9 cells strikingly enhanced the GINS1 level and facilitated cell proliferation (Figures 3D–F, S4B). Knockdown of PAX5 in SU-DHL-2 cells and DB cells significantly reduced GINS1 levels and inhibited cell proliferation (Figures 3G–I, S4C). Moreover, GINS1 overexpression reversed the decreased level of GINS1 in PAX5-knockdown cells and rescued the inhibited proliferation in SU-DHL-2 cell and DB cells (Figures 3J–L, S4D). In an in vivo xenograft lymphoma model, the decreased tumor growth in the PAX5-silencing group was rescued by overexpression of GINS1 in SU-DHL-2 stable cells (Figure 3M–O). Taken together, these data suggested that upregulated GINS1 participated in the tumorigenesis of DLBCL.

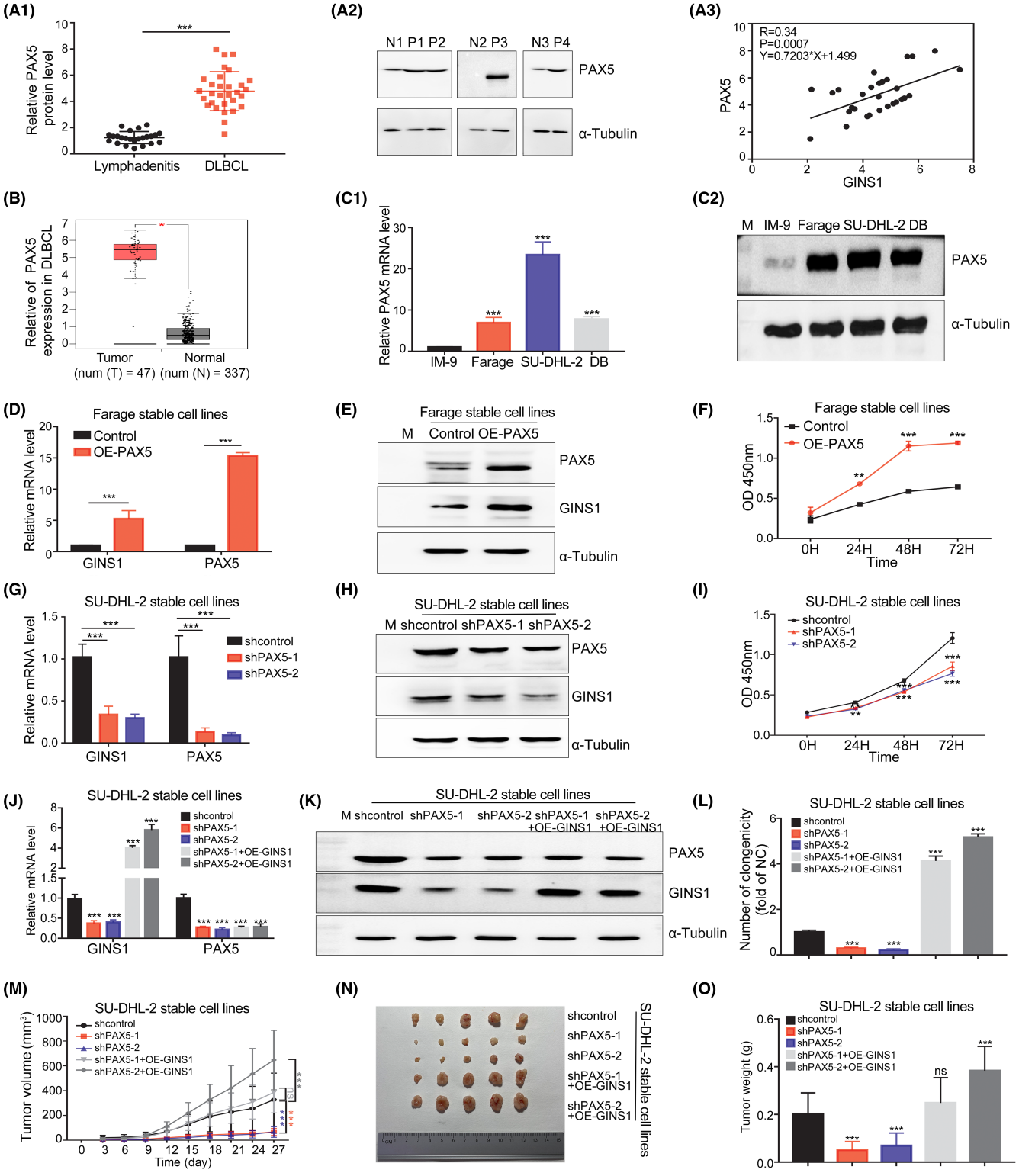

FIGURE 3 PAX5-mediated *GINS1* expression is critical for DLBCL proliferation. (A) PAX5 protein expression in DLBCL specimens. A1, Western blot analysis of DLBCL ($n=30$) and lymphadenitis ($n=24$) specimens. A2, Representative western blotting. P, DLBCL patients; N, lymphadenitis control. A3, Correlation analysis. (B) GEPIA analysis. (C) PAX5 level in DLBCL cell lines. C1, RT-qPCR. C2, Western blot. (D–F) Overexpression of PAX5 in Farage stable cell lines. D, RT-qPCR. E, Western blot. F, CCK8 assay. (G–I) Stable knockdown of PAX5 in SU-DHL-2 cells. G, RT-qPCR. H, Western blot. I, CCK8 assay. (J–L) GINS1 overexpression reverses the GINS1 expression level decrease caused by PAX5 knockdown in SU-DHL-2 stable cell lines. J, RT-qPCR. K, Western blot. L, Soft agar colony formation assy. (M–O) Xenograft tumor model. M, Tumor growth curve: indicates the groups of stable cell lines subcutaneously injected into the flanks of nude mice; tumor size was measured every 3 days. N, Tumor pictures. O, Tumor weight. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

3.5 | circ1857 upregulated *GINS1* expression in DLBCL

The above results showed that changes in PAX5 could promote GINS1 expression and enhance DLBCL cell proliferation. Accumulating evidence has shown that circRNAs generated by a noncanonical splicing event called back splicing, were involved in human cancer development and progression.[31] It was reported that circ1857 was markedly upregulated in ALL, and could be used for the diagnosis of ALL,[32] but the function of circ1857 was not investigated. Therefore, whether circ1857 was also involved in GINS1 regulation was explored. circ1857 was formed by back splicing exons 2–5 of the linear transcript of the *PAX5* gene. In Farage cells, DB cells and the SU-DHL-2 cells, the presence of circ1857 was confirmed (Figures 4A, S5A,B). Compared with linear PAX5, circ1857 was more resistant to RNase R digestion, more stable and primarily distributed in the cytoplasm (Figure 4B–D). Subsequently, circ1857 secondary structure was predicted using online RNAfold (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi; Figure S5C). More interestingly, the loop in circ1857 was predicted to bind in *trans* with the 3′UTRs of GINS1 mRNAs (Figure S5C). To determine whether circ1857 acted directly on *GINS1* mRNA, luciferase reporter assays were performed, the results showed that circ1857 overexpression promoted GINS1 3′UTR luciferase activity, but had no influence on the GINS1 mutated 3′UTR luciferase activity (Figure 4E). Similarly, circ1857 knockdown inhibited GINS1 3′UTR luciferase activity, but had no effect on the GINS1-mutated 3′UTR luciferase activity, suggesting that circ1857 directly bound to *GINS1* mRNA and promoted its stability. To explore the potential biological function of circ1857 in DLBCL, RNA levels of both *GINS1* and *circ1857* in biopsy specimens were detected using RT-qPCR. The results showed that circ1857 and GINS1 were upregulated (Figure 5A,B) and significant correlations between them were found (Figure 5C). Next, the expression of circ1857 in DLBCL cell lines was detected. As shown in Figure 5D, circ1857 levels were also high in DLBCL cell lines. Stable expression of circ1857 in Farage cells resulted in significantly upregulated *GINS1* mRNA and protein

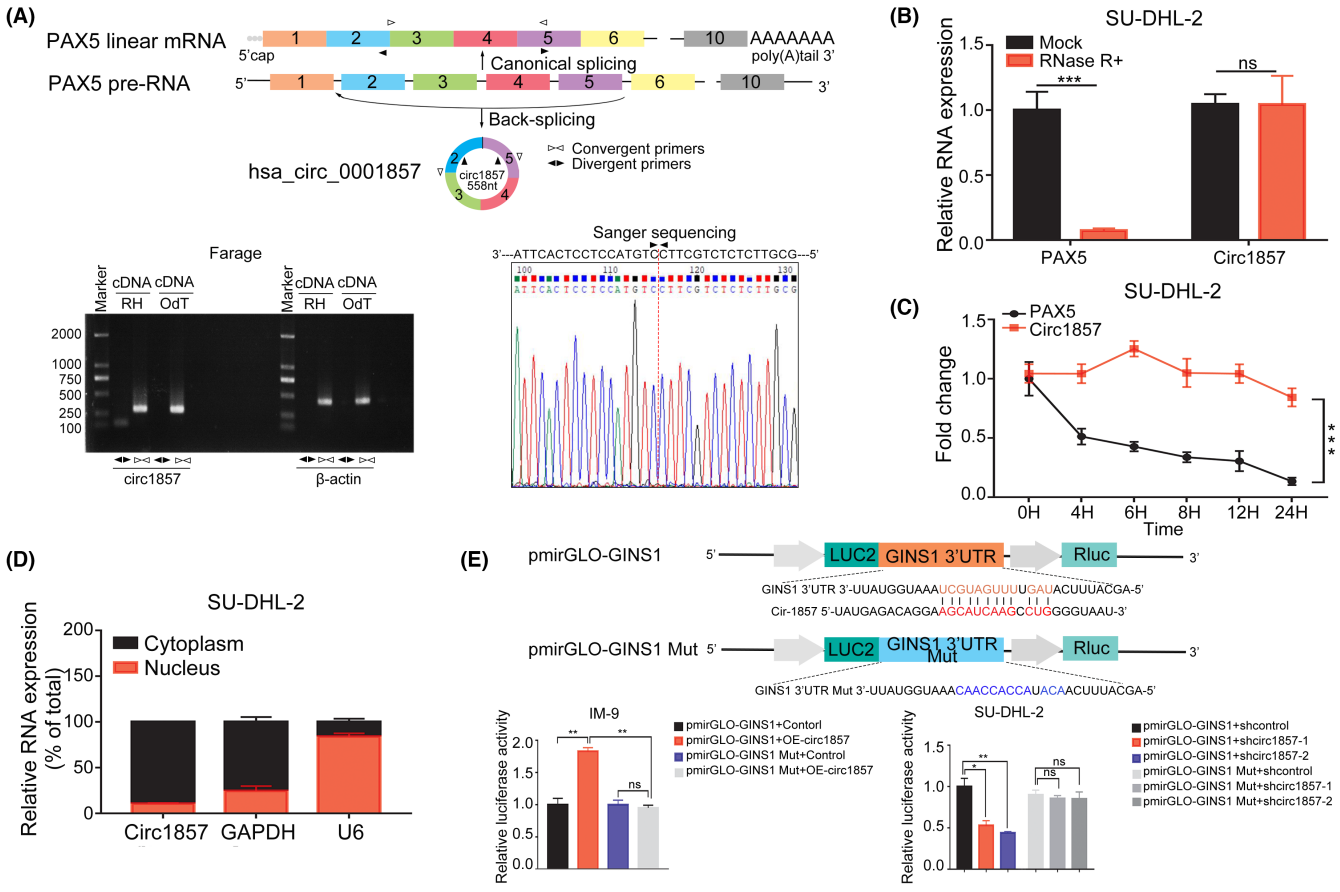

FIGURE 4 circ1857 in DLBCL. (A) Diagram shows the PAX5 pre-mRNA canonical splicing and back splicing that produces circ1857. circ1857 in Farage cells was validated using RT-PCR with convergent and divergent primers and confirmed using Sanger sequencing. PCR analysis for circ1857 and β-actin in cDNA. RH, random hexamers, OdT, oligo(dT)18 primers. Two independent experiments were carried out with similar results. (B) RT-qPCR analysis of linear *PAX5* mRNA and circ1857 after treatment with RNase R in SU-DHL-2. (C) RT-qPCR analysis of circ1857 and PAX5 mRNA in SU-DHL-2 cells after treatment with actinomycin D for the indicated time. (D) Relative expression of circ1857 in the nucleus and cytoplasm, respectively. GAPDH and U6 were used as controls for the cytoplasm and nucleus. (E) Luciferase activity was measured using dual luciferase assays when cells were co-transfected with a GINS1 3′UTR- or a GINS1 mutated 3′UTR-expressing plasmid with circ1857-overexpression or -knockdown plasmids. The construction of the GINS1 3′UTR or GINS1-mutated 3′UTR luciferase reporter plasmid is shown. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

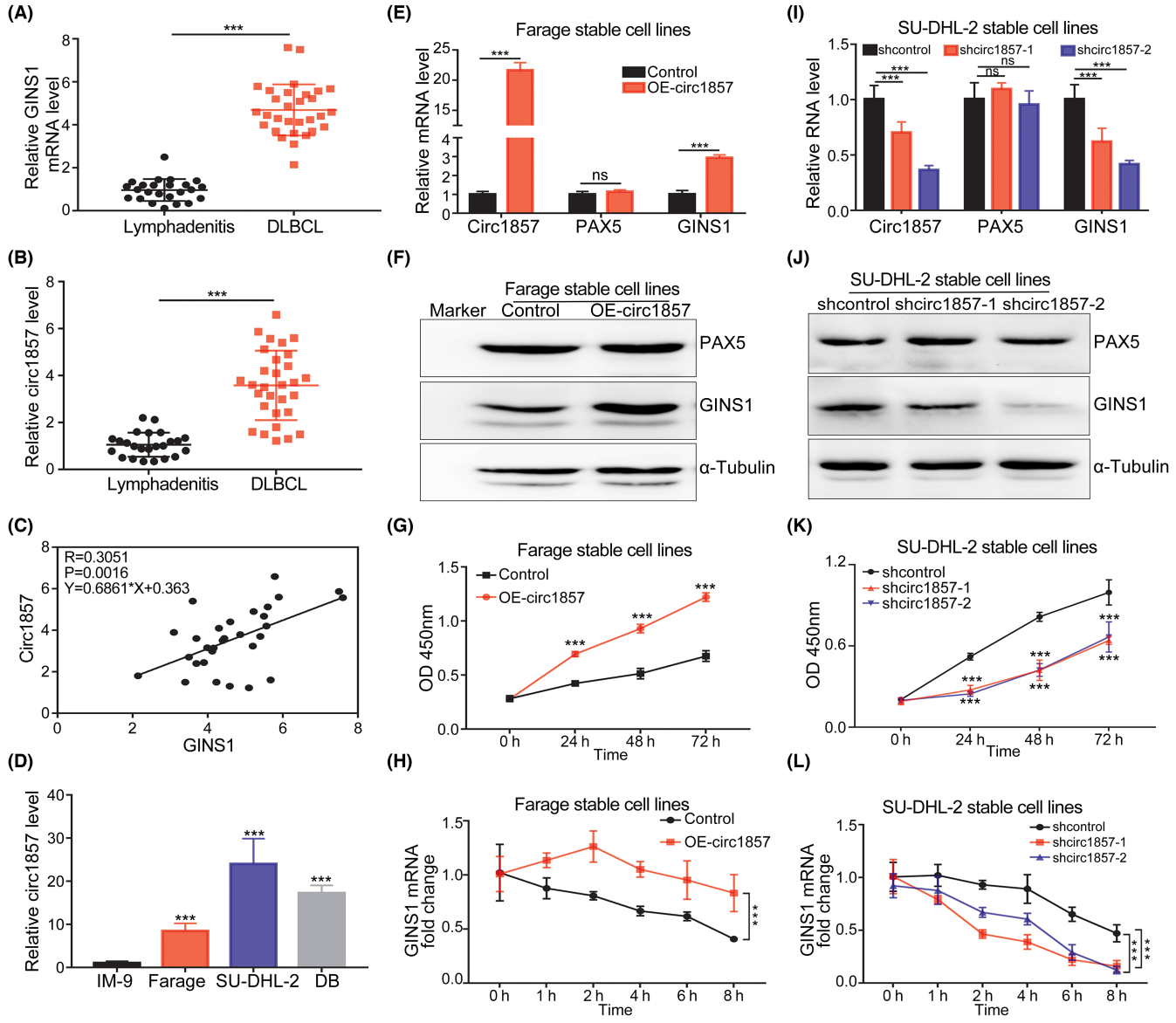

FIGURE 5 circ1857 promotes GINS1 expression in DLBCL cells. (A, B) GINS1 and circ1857 expression in DLBCL biopsy specimens detected by RT-qPCR. (C) Correlation between GINS1 and circ1857 in DLBCL specimens. (D) circ1857 levels in DLBCL cell lines analyzed by RT-qPCR. (E–H) Farage stable cells overexpressing circ1857. E, RT-qPCR. F, Western blot. G, CCK8. H, RT-qPCR analysis of GINS1 mRNA in Farage stable cells after treatment with actinomycin D for the indicated time. (I–L) SU-DHL-2 stable knockdown of circ1857 cells. I, RT-qPCR. J, Western blot. K, CCK8. L, After treatment with actinomycin D, RT-qPCR analysis of expression of *GINS1* mRNA in SU-DHL-2 stable knockdown circ1857 cells. $^{***}p < 0.001$.

levels (Figure 5E,F). Furthermore, CCK-8 assays showed that cell proliferation was significantly increased by overexpressing circ1857 (Figure 5G). Moreover, circ1857 overexpression significantly stabilized the *GINS1* mRNA (Figure 5H). These results suggested that circ1857 plays a role in fine tuning the expression of GINS1 at the posttranscriptional level. To further ascertain the role of circ1857 on GINS1, stable knockdown of circ1857 in SU-DHL-2 cell lines was established (Figure 5I,J). mRNA and protein levels for GINS1 were markedly decreased in circ1857 stable knockdown cells. There was no significant change for the mRNA and protein levels of PAX5 when circ1857 was overexpressed or knocked down. Lower cell viability was also observed when circ1857 was reduced (Figure 5K). These results suggested that the regulation of circ1857 on the target gene *GINS1* was independent of the PAX5 protein. Moreover, the stability of GINS1 decreased in circ1857 knockdown cells (Figure 5L). Altogether, these results indicated that circ1857 could stabilize *GINS1* mRNA.

3.6 | Coordinated expression of PAX5 and GINS1 in B cells

PAX5 was physiologically expressed in normal B cells and silenced in plasma cells. Next PAX5 and GINS1 protein expression patterns at different B-cell stages were evaluated. For PAX5 and the plasma cell regulator PRDM1 were mutually exclusive and cross-antagonized

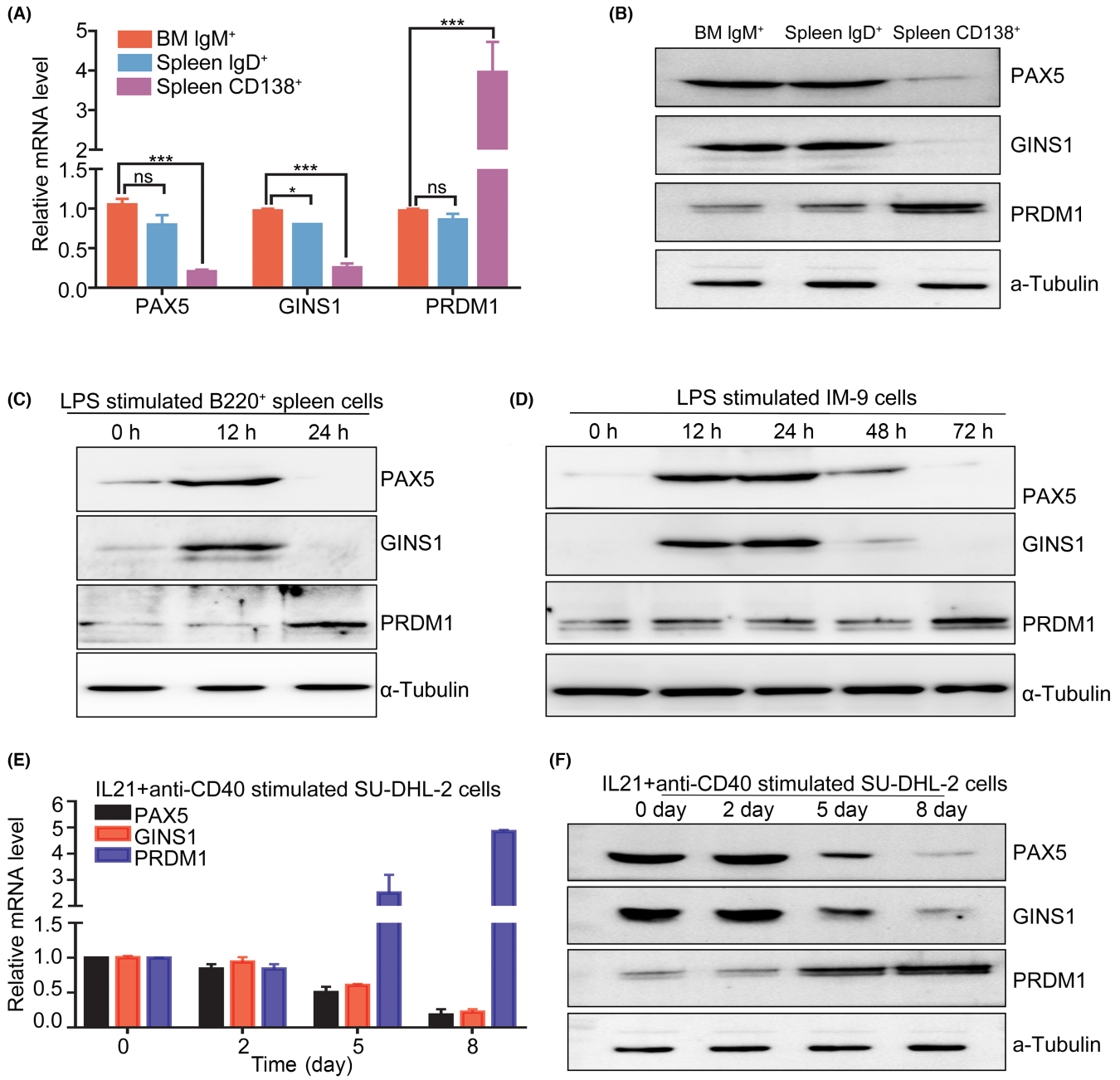

FIGURE 6 GINS1 is regulated by PAX5 in B-cell proliferation and differentiation. (A, B) Expression of PAX5 and GINS1 during the mouse B-cell proliferation and differentiation. (C) After LPS stimulation, the expression of GINS1, PAX5 and PRDM1 in mouse spleen cells was analyzed. Cells were incubated with 10 mg/mL LPS for the indicated time and harvested for western blot analysis. (D) After LPS stimulation, the expression of GINS1, PAX5 and PRDM1 in IM-9 cells was analyzed using western blot. (E, F) After anti-CD40 and IL21 stimulation for the indicated time, cells were collected, the expression levels of PAX5, GINS1 and PRDM1 were analyzed using RT-qPCR and western blot. ***$p < 0.001$.

each other, therefore the PRDM1 level was also analyzed here. PAX5 and GINS1 were coordinately expressed in sorted bone marrow IgM$^+$ B cells and splenic IgD$^+$ B cells but both declined in spleen CD138$^+$ plasma cells (Figure 6A,B). LPS is a mitogen that stimulates B cells, causing proliferation and then differentiation into plasma cells.[33] The expression of PAX5 and the *GINS1* expression pattern in LPS-treated splenocytes were analyzed next. In sorted spleen B220$^+$ cells, expression of both PAX5 and GINS1 in splenocytes increased after LPS treatment for 12 h, during which time cells entered an active proliferation stage. Following this increase period, the expression of both proteins began to decline at 24 h, which could be explained using terminal cell differentiation (Figure 6C). In IM-9 cells after LPS stimulation, PAX5 and GINS1 levels also increased at 12 h, and then both decreased at 48 h (Figure 6D). More interestingly, circ1857 expression showed a similar trend as *GINS1* mRNA in LPS-stimulated IM-9 cells (Figure S6A).

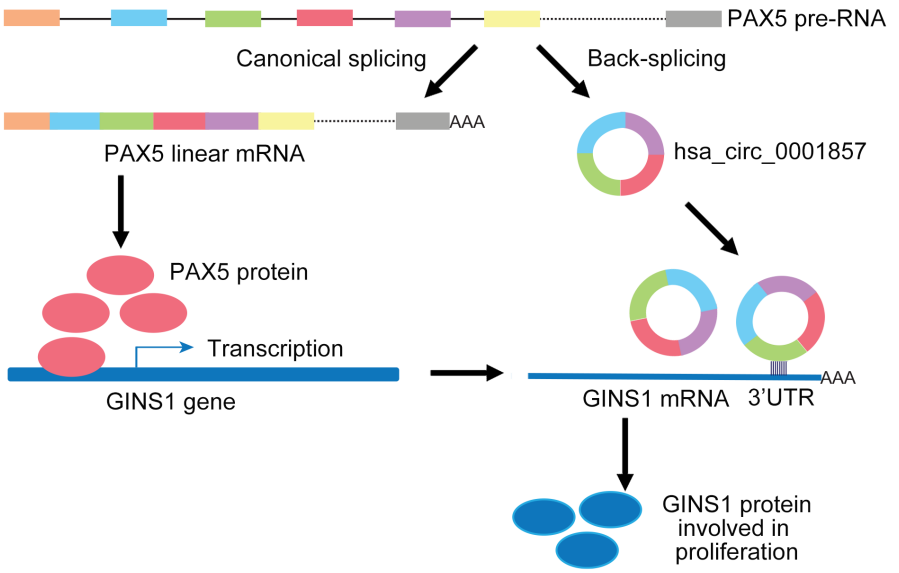

FIGURE 7 Schematic diagram of the regulation mechanism of GINS1 expression using PAX5 and circ1857 in normal B cells and DLBCL.

It was noted that PAX5 was dysregulated in B-cell tumors ranging from ALL to many mature B-cell lymphoma subtypes.[14,17,19,27] At the normal physical late stage of B-cell differentiation, PAX5 expression was downregulated. High-level PAX5 at this stage of differentiation would perturb the plasma cell differentiation program initiated by PAX5 repression, thereby contributing to the development of a fraction of the DLBCL. Therefore, DLBCL cells were induced to differentiate into plasma cells. SU-DHL-2 was induced to differentiate under differentiation-inducing conditions. Through stimulation with interleukin 21 and anti-CD40 (CD40L), when PAX5 was observed to decrease at day 5, GINS1 was also decreased simultaneously. In addition the plasma cell-specific gene *PRDM1* was induced (Figure 6E,F). The correlation of circ1857 and GINS1 expression in SU-DHL-2 cells under induced differentiation conditions was also evaluated (Figure S6B). All these results indicated that *GINS1* was one of the PAX5 targeted genes. Tight control of PAX5 is crucial not only for normal B lymphopoiesis, but also for preventing tumor formation.

4 | DISCUSSION

In this work, aberrant expression levels of PAX5, circ1857 and GINS1 in DLBCL were observed. We further demonstrated that knocking down one of them suppressed cell proliferation. Mechanistically, PAX5 is a transactivator for *GINS1* gene transcription, and circ1857 stabilized *GINS1* mRNA. circ1857 and PAX5 coordinately promoted DLBCL progression by upregulating GINS1 expression (Figure 7).

GINS1 is essential for the initiation and elongation of DNA replication by recruiting DNA polymerase.[6,34] Initially, GINS1 was found to be predominantly expressed in highly proliferating cells such as stem cells and progenitor cells instead of mature cells.[35,36] GINS1 homozygous null mutations in mice were embryonic lethal and heterozygous mutations of GINS1 in humans displayed intrauterine growth retardation, chronic neutropenia, and NK cell deficiency.[37,38] Later, it was found that GINS1 was involved in the tumorigenesis of various cancers.[11,39,40] However, GINS1 in DLBCL was never investigated.

In DLBCL, frequent alterations of the *PAX5* gene were reported.[17] IgH-PAX5 translocation resulted in the intact coding sequences for PAX5 under the control of strong enhancers or promoters of the IgH.[41] Consequently, the expression of translocated PAX5 was strongly increased.[42,43] Other mechanisms also contributed to the increased PAX5.[18,44]

circRNAs are a group of covalently closed loop-like RNAs.[45] Growing numbers of studies have shown that circRNAs are important regulator molecules in cancer progression, but they have been poorly studied in DLBCL. circ1857 was previously found in ALL.[32] Here, circ1857 and its host gene product PAX5 protein were found both upregulated in DLBCL. Although some antisense circular RNA was found to suppress parental gene splicing and translation,[46] actually more circular RNA was found positively correlated with that of linear parental mRNA.[47] Here both circ1857 and PAX5 upregulated the same target gene *GINS1* in DLBCL in a coordinated manner. To the best of our knowledge, this kind of coordinated function pattern of circular RNA and its parental mRNA product has not been reported previously.

In conclusion, we showed that proper control of GINS1 expression was critical for B-cell proliferation and differentiation. From an oncological view, aberrant high expression of PAX5 and GINS1 blocked B-cell differentiation. In addition, GINS1 recently was reported to have anti-apoptotic functions,[48,49] all these findings suggested that deregulated PAX5 and *GINS1* are an oncogene for a fraction of DLBCL. Furthermore more cell lines, more tissue samples and further studies are needed to establish the therapeutic potential of GINS1 in DLBCL.

AUTHOR CONTRIBUTIONS

Ting Wang performed experiments and analyzed the data. Zhenfa Chen, Cui Li, Wei Zhang and Wenbin Huang provided suggestions and performed the analysis. Jun Xue and Jundong Wang provided

patient samples. Shufeng Li designed experiments, reviewed the data, and wrote the manuscript. All authors read and approved the final submitted manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Review Board: This study was approved by the ethics committee of the Nanjing First Hospital Medical University.

Informed Consent: Written informed consent was obtained from each individual.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: Research protocols were approved by the Animal Experimentation Ethics Committee of Southeast University.

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SUPPORTING INFORMATION

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