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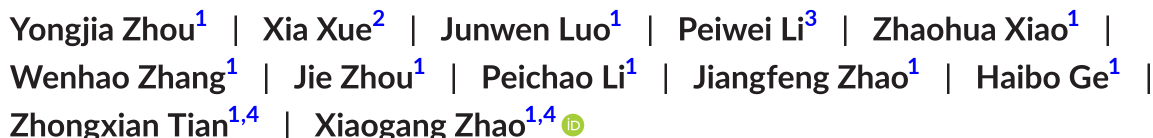
Circular RNA circ-FIRRE interacts with HNRNPC to promote esophageal squamous cell carcinoma progression by stabilizing *GLI2* mRNA



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Abstract

Increasing evidence has shown that circular RNAs (circRNAs) interact with RNA-binding proteins (RBPs) and promote cancer progression. However, the function and mechanism of the circRNA/RBP complex in esophageal squamous cell carcinoma (ESCC) are still largely unknown. Herein, we first characterized a novel oncogenic circRNA, circ-FIRRE, by RNA sequencing (Ribo-free) profiling of ESCC samples. Furthermore, we observed marked circ-FIRRE overexpression in ESCC patients with high TNM stage and poor overall survival. Mechanistic studies indicated that circ-FIRRE, as a platform, interacts with the heterogeneous nuclear ribonucleoprotein C (HNRNPC) protein to stabilize *GLI2* mRNA by directly binding to its 3'-UTR in the cytoplasm, thereby resulting in elevated GLI2 protein expression and subsequent transcription of its target genes *MYC*, *CCNE1*, and *CCNE2*, ultimately contributing to ESCC progression. Moreover, HNRNPC overexpression in circ-FIRRE knockdown cells notably abolished circ-FIRRE knockdown-mediated Hedgehog pathway inhibition and ESCC progression impairment in vitro and in vivo. Clinical specimen results showed that circ-FIRRE and HNRNPC expression was positively correlated with GLI2 expression, which reveals the clear significance of the circ-FIRRE/HNRNPC-GLI2 axis in ESCC. In summary, our results indicate that circ-FIRRE could serve as a valuable biomarker and potential therapeutic target for ESCC and highlight a novel mechanism of the circ-FIRRE/HNRNPC complex in ESCC progression regulation.

KEYWORDS
circRNA, esophageal squamous cell carcinoma, GLI2, hedgehog signaling, HNRNPC

Abbreviations: circRNA, circular RNA; ESCC, esophageal squamous cell carcinoma; GLI2, GLI family zinc finger 2; Hh, Hedgehog; HNRNPC, heterogeneous nuclear ribonucleoprotein C; IHC, immunohistochemistry; K-M, Kaplan–Meier; RBP, RNA-binding protein; RIP, RNA binding protein immunoprecipitation; ROC, receiver operating characteristic; RT-qPCR, quantitative real-time PCR.

Yongjia Zhou and Xia Xue contributed equally to this work.

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1 | INTRODUCTION

Globally, esophageal cancer is the sixth leading cause of cancer-related mortality.[1] Esophageal squamous cell carcinoma, the predominant histological subtype of esophageal carcinoma, accounts for approximately 90% of newly diagnosed esophageal cancer cases in China.[2] The first-line treatment for ESCC currently includes surgical resection, neoadjuvant chemotherapy, and radiotherapy; however, the prognosis of ESCC is poor, with a 5-year survival rate of 10%–15%.[3] Consequently, identification of applicable therapeutic targets and discovery of molecular biomarkers for early-stage diagnosis are urgently needed to improve the clinical outcomes of patients with ESCC.

Circular RNAs are noncoding RNAs with single-stranded loops with tissue-specific expression and tremendous stability and thus have potential application as therapeutic biomarkers and treatment targets.[4] Recent studies have revealed that circRNAs frequently show upregulated expression in tumors and play important roles in tumor development, including in ESCC.[5–7] Mechanistically, circRNAs can regulate the biological behaviors of cancer cells by serving as competitive endogenous RNAs, microRNA sponges, protein baits, or antagonists.[8] Numerous studies have revealed that circRNAs serve as microRNA sponges. However, the interactions and mechanisms of RBPs and circRNAs in ESCC remain unclear, limiting potential target identification. One crucial mechanism of circRNAs is their interactions with different RBPs to form circRNA-RBP complexes that influence the action of associated proteins.[9] For example, circ-CDKN2B-AS1 interacts with IGF2BP3 and thus promotes the malignant progression of cervical carcinoma by stabilizing hexokinase 2.[10] CircXPO1 promotes lung adenocarcinoma progression by binding to IGF2BP1 and stabilizing CTNNB1 mRNA.[11] Therefore, further investigations are needed to determine the exact mechanisms underlying the involvement of circRNAs in ESCC progression.

Heterogeneous nuclear ribonucleoprotein C, an RBP, is upregulated in multiple tumors and tumor cell lines.[12] Heterogeneous nuclear ribonucleoprotein C is well known for its regulatory roles in RNA splicing,[13] sequence-nonspecific RNA export,[14] and RNA expression,[15] stability,[16] and 3′-end processing.[17] For example, increased HNRNPC expression in metastatic-derived colon epithelial cells contributes to the upregulation of *MTHFD1L* mRNA by controlling poly(A) site selection.[18] However, the involvement of HNRNPC in the underlying mechanism of circRNAs remains largely unknown. GLI2 is a well-known oncogenic transcription factor targeting a series of downstream genes, such as *C-MYC*, *CCNE1*, and *CCNE2*,[19] as well as an indispensable mediator of Hh signaling activation.[20] The Hh signaling pathway plays an important role in embryonic development, cell proliferation, tissue polarity, and carcinogenesis.[21] In particular, Hh signaling is frequently activated in human cancers, including esophageal cancer,[22] osteosarcoma,[23] and retinoblastoma.[24] However, the association between GLI2 and circRNAs or HNRNPC in ESCC has not been reported.

In the present study, we identified a novel oncogenic circRNA, circ-FIRRE, whose expression was higher in ESCC tissues and was positively related to an advanced tumor stage and poor overall survival rate. In addition, circ-FIRRE could contribute to ESCC progression, including proliferation, migration, and invasion, by acting as a protein decoy binding with HNRNPC to promote Hh signaling activation. These findings suggest the potential application of circ-FIRRE as a therapeutic target in ESCC.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Human ESCC cell lines (KYSE-30, KYSE-150, KYSE-410, KYSE-510, and Eca-109) and a human esophageal epithelial cell line (HEEC) were purchased from Shanghai Fu Heng Biological. The HEEC cells were cultured in DMEM (Corning), and the other cells were cultured in RPMI-1640 medium (Corning). All cells were treated with 10% FBS (ExCell Bio) and 1% penicillin/streptomycin (NAM Biotech) and cultured at 37°C with 5% CO_2.

2.2 | Real-time PCR and RT-qPCR

RNA (1.0 μg) was used for first-strand cDNA synthesis with a lncRNA First-Strand cDNA Kit (Tiangen). Then RT-qPCR was carried out using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). The levels of β-actin were used to normalize the relative RNA expression level. The primer sequences are provided in Table S1.

FIGURE 1 Circular RNA circ-FIRRE is upregulated and correlated with malignant clinicopathologic characteristics in esophageal squamous cell carcinoma (ESCC). (A) Volcano plot of circRNA profiles. (B) Genomic loci of circ-FIRRE in the *FIRRE* gene are shown. Sanger sequencing results of the PCR product are shown. (C) Expression of circ-FIRRE and FIRRE was measured by real-time quantitative PCR (RT-qPCR) analysis after RNase R treatment in KYSE-410 and KYSE-510 cells. (D) Stabilities of circ-FIRRE and FIRRE in KYSE-410 and KYSE-510 cells after actinomycin D treatment are shown. (E) Expression of circ-FIRRE in the cytoplasm and nucleus of KYSE-410 and KYSE-510 cells. (F) Cy3 probes targeting circ-FIRRE showed their location in KYSE-410 and KYSE-510 cells. Scale bar, 10 μm. (G) Representative FISH fluorescence images of ESCC tissues and matched adjacent normal tissues are shown. Scale bar, 10 μm. (H) Expression of circ-FIRRE in ESCC tissues and normal tissues was analyzed by RT-qPCR. (I–K) RT-qPCR analysis of circ-FIRRE expression in tissues of ESCC patients; (I) clinical I + II stage vs. III + IV, (J) no lymphatic metastasis vs. lymphatic metastasis, (K) T1 + T2 stage vs. T3 + T4 stage are shown. (L) Area under the curve (AUC) values of circ-FIRRE for distinguishing ESCC patients from healthy controls are shown. (M) Kaplan–Meier analysis of circ-FIRRE in ESCC patients. LM, lymphatic metastasis; ns, no significance. $^{*}p < 0.05$, $^{***}p < 0.001$.

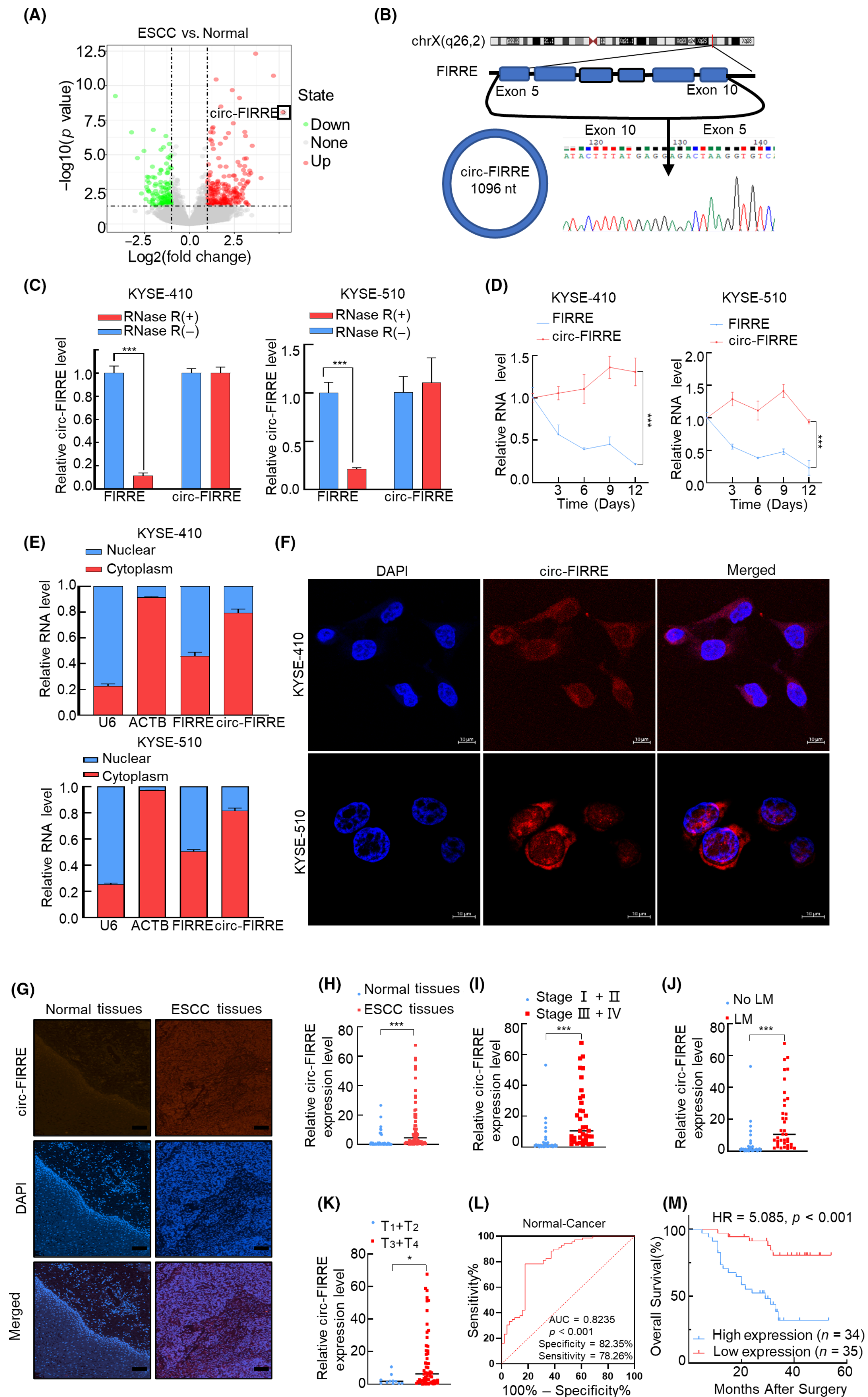

(A)
ESCC vs. Normal
circ-FIRRE
State
Down
None
Up
−log10(p value)
Log2(fold change)
(B)
chrX(q26,2)
FIRRE
Exon 5
Exon 10
circ-FIRRE
1096 nt
Exon 10
Exon 5
(C)
KYSE-410
RNase R(+)
RNase R(−)
Relative circ-FIRRE level
FIRRE
circ-FIRRE
KYSE-510
(D)
KYSE-410
FIRRE
circ-FIRRE
Relative RNA level
Time (Days)
KYSE-510
(E)
KYSE-410
Nuclear
Cytoplasm
Relative RNA level
U6
ACTB
FIRRE
circ-FIRRE
KYSE-510
(F)
DAPI
circ-FIRRE
Merged
KYSE-410
KYSE-510
(G)
Normal tissues
ESCC tissues
circ-FIRRE
DAPI
Merged
(H)
Normal tissues
ESCC tissues
Relative circ-FIRRE expression level
(I)
Stage Ⅰ + Ⅱ
Stage Ⅲ + Ⅳ
(J)
No LM
LM
(K)
T1+T2
T3+T4
(L)
Normal-Cancer
Sensitivity%
AUC = 0.8235
p < 0.001
Specificity = 82.35%
Sensitivity = 78.26%
100% – Specificity%
(M)
HR = 5.085, p < 0.001
Overall Survival(%)
High expression (n = 34)
Low expression (n = 35)
Months After Surgery

TABLE 1 Clinicopathologic characteristics of esophageal squamous cell carcinoma patients in low- and high-circ-FIRRE expression groups.

Clinicopathologic characteristics	Cases ($n=69$)	circ-FIRRE expression Low ($n=35$)	High ($n=34$)	χ^2-test	p value
Sex					
Male	55	27	28	0.289	0.591
Female	14	8	6	-	-
Age (years)					
<60	19	11	8	0.539	0.464
≥60	50	24	26	-	-
Smoking					
Yes	45	20	25	2.041	0.153
No	24	15	9	-	-
Alcohol					
Drinkers	47	24	23	0.007	0.934
Nondrinkers	22	11	11	-	-
Differentiation					
Well and medium	52	30	22	4.099	0.043*
Poor	17	5	12	-	-
Clinical stage					
I+II	31	25	6	20.16	0.001**
III+IV	38	10	28	-	-
T stage					
T_1+T_2	11	9	2	5.062	0.025*
T_3+T_4	58	26	32	-	-
LNM status					
No	32	25	7	17.93	0.001**
Yes	37	10	27	-	-

Abbreviation: LNM, lymph node metastasis.*$p<0.05$; **$p<0.01$.

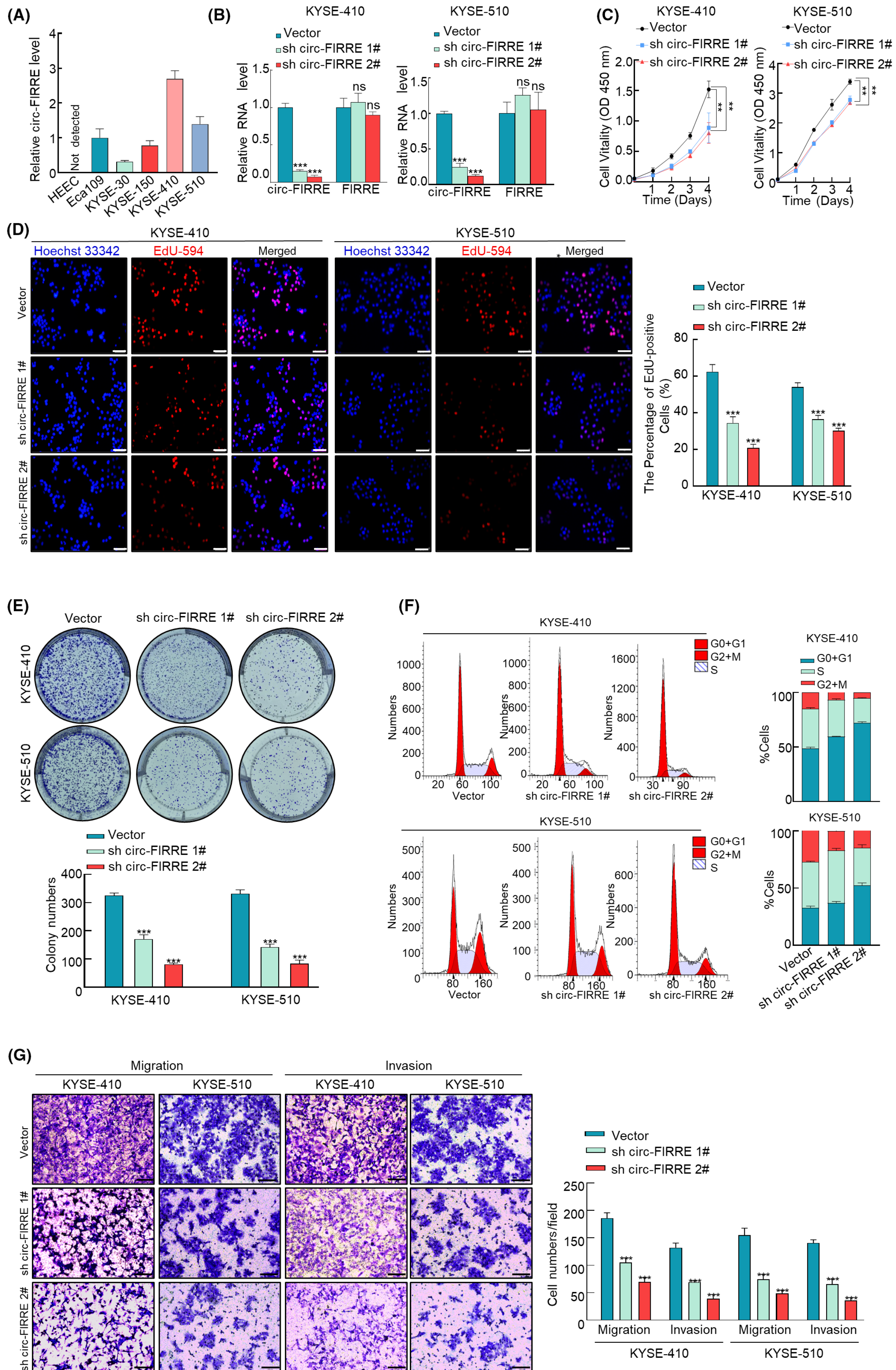
2.3 | Vector construction and cell transfection

Two short hairpin interfering RNAs specifically targeting circ-FIRRE (sh-circ-FIRRE #1 and sh-circ-FIRRE #2) and the PLENT-U6-GFP-Puro lentiviral vector (Vector) were purchased from Tsingke Bio. In addition, the lentiviral plasmid for circ-FIRRE overexpression was constructed using the pLC5-ciR vector by Geneseed Bio. Specific shRNAs against GLI2 (sh-GLI2#1 and sh-GLI2#2) along with the empty vector were constructed by Tsingke Bio. The GLI2 overexpression plasmid was purchased from Miaoling Bio. The HNRNPC overexpression lentivirus plasmid was synthesized by Tsingke Bio. Puromycin was used at 4 µg/mL for 1 week to select stably transfected cells. The shRNA sequences are shown in Table S2.

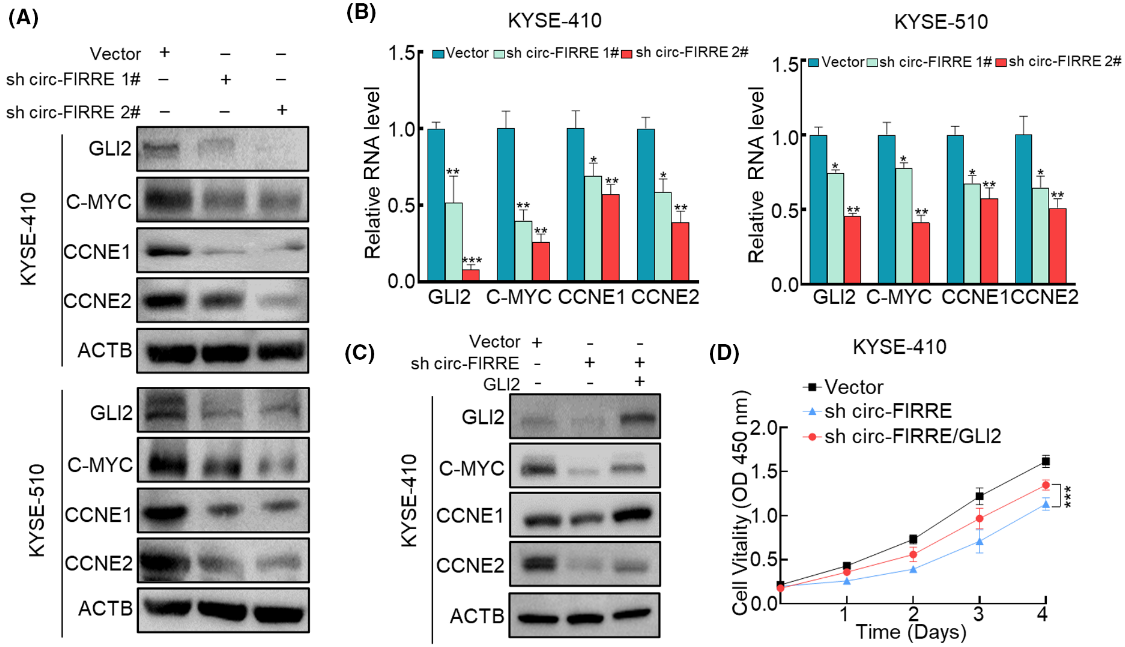
2.4 | Antibodies and western blot analysis

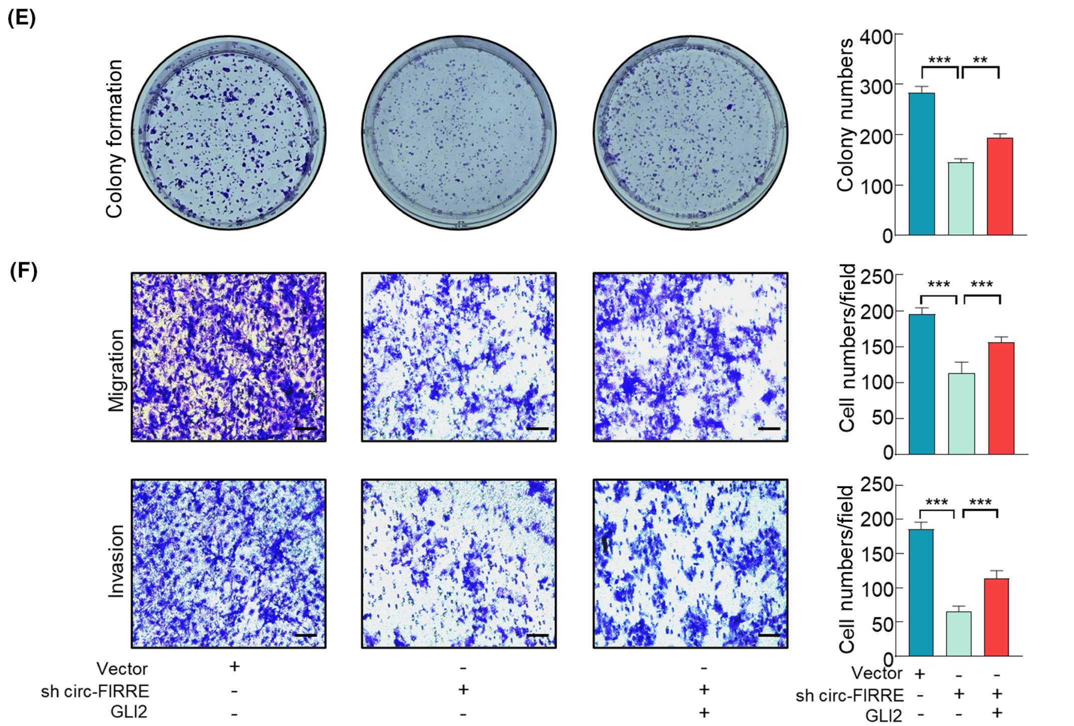
Equal amounts of total protein lysates (20 µg) collected from ESCC cells were separated by 6%–15% SDS-PAGE and transferred to a PVDF membrane (Millipore). After blocking with 5% skim milk, the membranes were incubated with primary Ab overnight at 4°C and then with HRP-labeled secondary Ab for 1 h at room temperature. The bands were then examined with an enhanced chemiluminescence

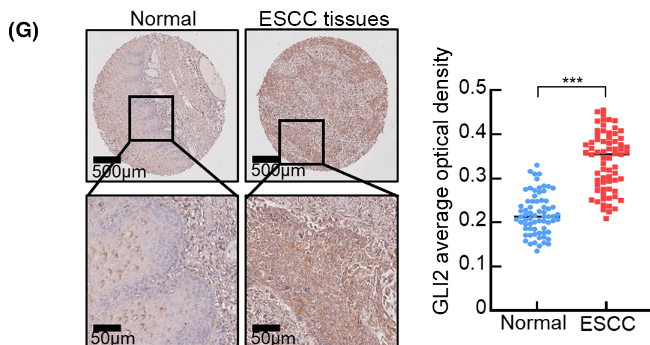
FIGURE 2 Circular RNA circ-FIRRE promotes proliferation, migration, and invasion of esophageal squamous cell carcinoma (ESCC) in vitro. (A) circ-FIRRE expression levels were detected in an esophageal epithelial cell line and five ESCC cell lines. (B) Efficacy of circ-FIRRE knockdown in KYSE-410 and KYSE-510 cells. (C–E) Proliferation abilities of KYSE-410 and KYSE-510 cells transfected with circ-FIRRE shRNAs were tested by CCK-8, EdU (scale bar, 50 µm), and colony formation assays (scale bar, 50 µm). (F) Flow cytometry shows the percentages of cells in the G_1, S, or G_2 phase in KYSE-410 and KYSE-510 cells. (G) Migration and invasion abilities of KYSE-410 and KYSE-510 cells transfected with circ-FIRRE shRNAs are shown by Transwell assays. Scale bar, 100 µm. **$p<0.01$, ***$p<0.001$, and all the above experiments were repeated three times. ns, no significance.



(A)
Relative circ-FIRRE level
Not detected
HEEC
Eca109
KYSE-30
KYSE-150
KYSE-410
KYSE-510
(B)
KYSE-410
KYSE-510
Vector
sh circ-FIRRE 1#
sh circ-FIRRE 2#
Relative RNA level
circ-FIRRE
FIRRE
ns
(C)
Cell Vitality (OD 450 nm)
Time (Days)
(D)
Hoechst 33342
EdU-594
Merged
The Percentage of EdU-positive Cells (%)
(E)
Colony numbers
(F)
G0+G1
G2+M
S
Numbers
%Cells
(G)
Migration
Invasion
Cell numbers/field


(A)
Vector
sh circ-FIRRE 1#
sh circ-FIRRE 2#
GLI2
C-MYC
CCNE1
CCNE2
ACTB
KYSE-410
KYSE-510
(B)
KYSE-410
KYSE-510
Vector
sh circ-FIRRE 1#
sh circ-FIRRE 2#
Relative RNA level
GLI2
C-MYC
CCNE1
CCNE2
(C)
Vector
sh circ-FIRRE
GLI2
(D)
sh circ-FIRRE/GLI2
Cell Vitality (OD 450 nm)
Time (Days)


(E)
Colony formation
Colony numbers
(F)
Migration
Invasion
Cell numbers/field
Vector
sh circ-FIRRE
GLI2


(G)
Normal
ESCC tissues
500μm
50μm
GLI2 average optical density
ESCC

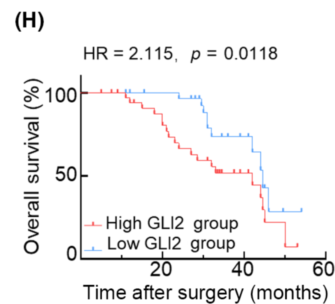

(H)
HR = 2.115, p = 0.0118
Overall survival (%)
High GLI2 group
Low GLI2 group
Time after surgery (months)

FIGURE 3 *GLI2* is a downstream target gene of circ-FIRRE. (A, B) Western blot analysis and real-time quantitative PCR showed the expression of *GLI2*, *C-MYC*, *CCNE1*, and *CCNE2* in vector and circ-FIRRE knockdown KYSE-410 cells. (C) Western blot showing the expression of *GLI2*, *C-MYC*, *CCNE1*, and *CCNE2* in cells transfected with vector or sh circ-FIRRE or cotransfected with sh circ-FIRRE and GLI2 overexpression plasmid. (D–F) CCK-8, colony formation, and Transwell assays (scale bar, 100 μm) were carried out in KYSE-410 cells transfected with vector or sh circ-FIRRE or cotransfected with sh circ-FIRRE and the GLI2 overexpression plasmid. (G) Representative images and quantitative analysis of GLI2 expression in esophageal squamous cell carcinoma (ESCC) patients. Scale bars: up, 500 μm; down, 50 μm. (H) Kaplan–Meier analysis of GLI2 in ESCC patients. $*p<0.05$, $**p<0.01$, $***p<0.001$.

detection system using a Tanon 5200 system (Tanon). The Ab information is listed in Table S3.

2.5 | Nude mouse xenograft experiments

BALB/c nude mice ($n=15$, female, 4 weeks) were obtained from Vital River and randomly divided into three groups for cell inoculation. The animal experimental procedures were approved by the Second Hospital of Shandong University Animal Care Commission, and the experiments were carried out following the Guide for the Care and Use of Laboratory Animals (GB/T35892-2018; Standardization Administration of the People's Republic of China). In total, 3×10^6 cells were resuspended in 100 μL PBS and injected s.c. under the armpits of nude mice. After treatment for 7 days, the length and width of the subcutaneous tumors were measured once every 5 days for 4 weeks, and the volume was calculated using the following formula: volume (mm^3) = (length × width^2)/2. The mice were killed by i.v. injection of pentobarbital (100 mg/kg), and the subcutaneous tumors were harvested.

2.6 | Statistical analysis

All statistical analyses were undertaken using GraphPad Prism 8.0 software (GraphPad Software). The differences between two groups were analyzed by Student's *t*-test. The χ^2-test was applied for categorical variables. The *r* values and *p* values for the correlations were obtained with Pearson's correlation analysis. The data are presented as the mean ± SD from three independent experiments. A *p* value less than 0.05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | Circ-FIRRE is upregulated and correlated with malignant clinicopathological characteristics in ESCC

To illustrate the circRNA expression levels in ESCC tissues, we analyzed the circRNA expression profile from 23 paired cancerous and noncancerous tissues of ESCC patients from the Gene Expression Omnibus database (GSE130078). A total of 160 upregulated and 31 downregulated circRNAs were identified, and heatmaps for the top 10 differentially expressed circRNAs were obtained in each segment ($\log_2$ |fold change| > 2 and adjusted *p* value <0.05; Figure S1A). We named the hsa_circ_0001944 circ-FIRRE, which is formed by the circularization of exons 5–10 of FIRRE, a long noncoding RNA region locus located at chromosome Xq26.2: 130883333–130928494 (GRCh37/hg19, Figure S1B). Circ-FIRRE was the most significantly upregulated circRNA in ESCC tumor tissues compared with adjacent tissues (Figure 1A). Specifically, Sanger sequencing of circ-FIRRE was carried out next to determine the head-to-tail junction points of circ-FIRRE, which was consistent with circBase database annotation (Figure 1B). In addition, circ-FIRRE and FIRRE expression was examined in cDNA reverse transcribed by oligo dT or random primer pairs as well as genomic DNA by 2% agarose gel electrophoresis. The results showed that circ-FIRRE was detected only by random primers reverse-transcribed in cDNA (Figure S1C). Moreover, we confirmed that circ-FIRRE was more stable than FIRRE after treatment with RNase R or actinomycin D in KYSE-410 and KYSE-510 cells (Figure 1C,D). These findings strongly indicate that circ-FIRRE truly exists in ESCC tissues and cell lines. The location of circRNAs indicates their biological function. Nuclear-cytoplasmic fractionation experiments and FISH assays showed that circ-FIRRE was mostly localized in the cytoplasm (Figure 1E,F). Furthermore, using the cy3-circ-FIRRE probe, we validated circ-FIRRE expression in ESCC tumorous tissues and adjacent nontumorous tissues (Figure 1G). In addition, we undertook RT-qPCR and found that circ-FIRRE was significantly overexpressed in 69 formalin-fixed paraffin-embedded primary ESCC tumorous tissues (T) compared with adjacent nontumorous tissues (N) (Figure 1H). To further investigate the correlation between circ-FIRRE expression and the clinicopathologic features of ESCC patients, we compared the clinicopathologic features between the low- and high-circ-FIRRE expression groups using the χ^2-test (Table 1). A significant correlation was found between circ-FIRRE expression levels and clinical stage ($p<0.001$), lymph node metastasis ($p<0.001$), and tumor stage ($p<0.05$) in ESCC tumor tissues (Figure 1I–K). The ROC curve analysis showed that circ-FIRRE had potential as an indicator to distinguish ESCC patients from healthy individuals, and its area under the ROC curve, sensitivity, and specificity values were 0.8235, 78.26%, and 82.35%, respectively (Figure 1L). Furthermore, Kaplan–Meier analysis revealed that ESCC patients with high expression of circ-FIRRE showed shorter overall survival than those with low expression (Figure 1M). In addition, we also collected 33 fresh normal esophageal tissue and 24 ESCC tumor tissue samples and carried out RT-qPCR. The results similarly showed that circ-FIRRE was significantly overexpressed in ESCC tumor tissues compared with normal esophageal tissues (Figure S1D). Collectively, these results suggest that circ-FIRRE is

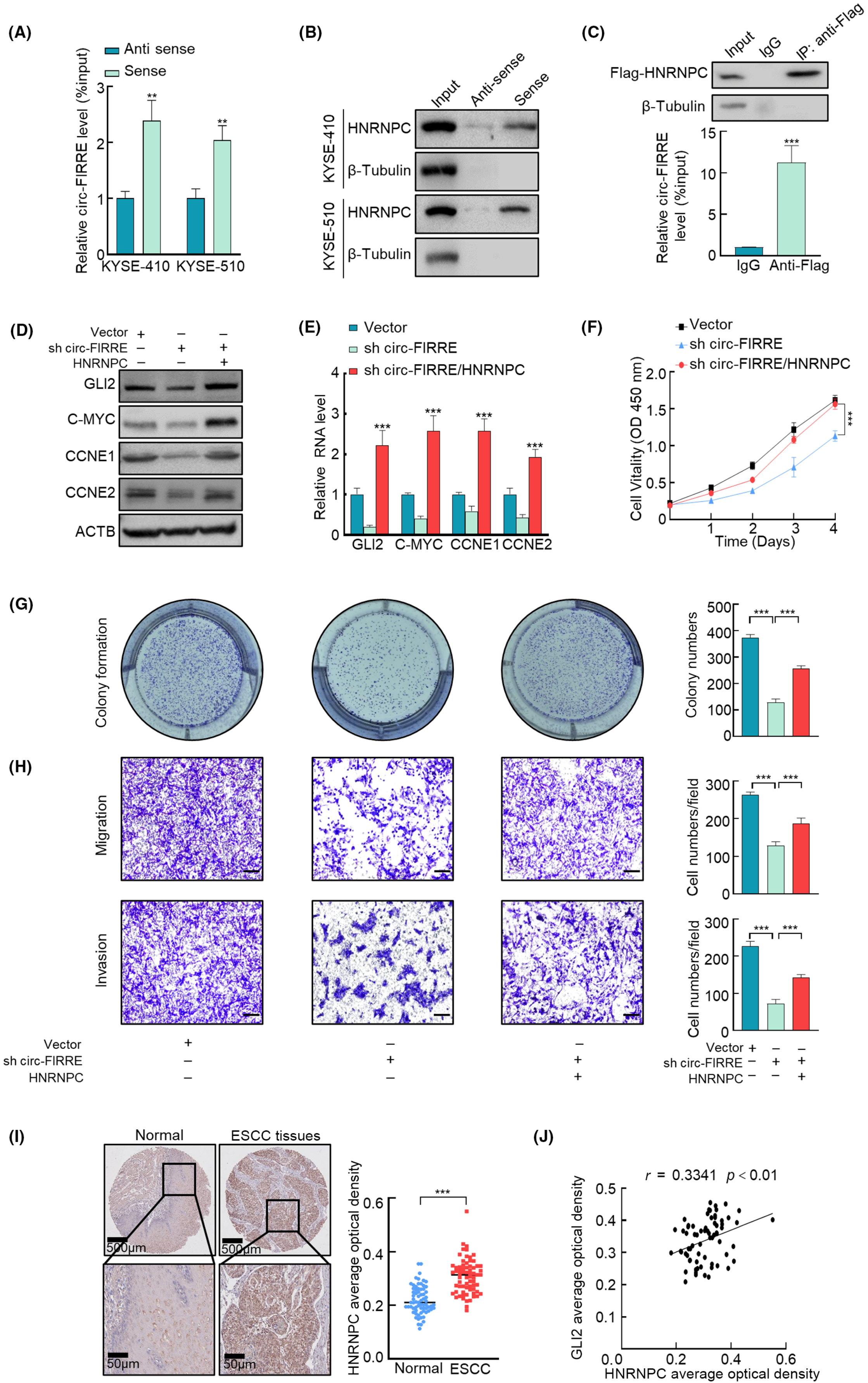

(A)
Anti sense
Sense
Relative circ-FIRRE level (%input)
KYSE-410 KYSE-510
(B)
Input
Anti-sense
Sense
KYSE-410
KYSE-510
HNRNPC
β-Tubulin
(C)
Input
IgG
IP: anti-Flag
Flag-HNRNPC
β-Tubulin
Relative circ-FIRRE level (%input)
IgG Anti-Flag
(D)
Vector
sh circ-FIRRE
HNRNPC
GLI2
C-MYC
CCNE1
CCNE2
ACTB
(E)
Vector
sh circ-FIRRE
sh circ-FIRRE/HNRNPC
Relative RNA level
GLI2 C-MYC CCNE1 CCNE2
(F)
Vector
sh circ-FIRRE
sh circ-FIRRE/HNRNPC
Cell Vitality (OD 450 nm)
Time (Days)
(G)
Colony formation
Colony numbers
(H)
Migration
Invasion
Cell numbers/field
Vector
sh circ-FIRRE
HNRNPC
(I)
Normal
ESCC tissues
500μm
50μm
HNRNPC average optical density
Normal ESCC
(J)
r = 0.3341 p < 0.01
GLI2 average optical density
HNRNPC average optical density

FIGURE 4 Circular RNA circ-FIRRE–heterogeneous nuclear ribonucleoprotein C (HNRNPC) complex facilitates the malignant phenotype of esophageal squamous cell carcinoma (ESCC) cells by regulating GLI2 expression. (A) RNA pull-down efficacy of sense and antisense probes in KYSE-410 cells. (B) Results of the RNA pull-down assay analyzed by western blotting. (C) Enrichment of circ-FIRRE pulled down by the HNRNPC-FLAG protein is shown. (D, E) Western blotting and quantitative PCR showed the expression of *GLI2*, *C-MYC*, *CCNE1*, and *CCNE2* in cells transfected with vector or sh circ-FIRRE or cotransfected with sh circ-FIRRE and the HNRNPC overexpression plasmid. (F–H) Proliferation, migration, and invasion abilities of KYSE-410 cells transfected with sh-circ-FIRRE or cotransfected with the HNRNPC overexpression plasmid by CCK-8, colony formation, and Transwell assays. Scale bar, 100 μm. (I) Representative images of immunohistochemistry (IHC) and quantitative analysis of HNRNPC expression in ESCC patients. Scale bars: up, 500 μm; down, 50 μm. (J) Correlation between HNRNPC and GLI2 expression in IHC data of ESCC patients. **$p < 0.01$, ***$p < 0.001$.

upregulated in ESCC and that its expression might be associated with malignant clinical features in ESCC patients.

3.2 | circ-FIRRE promotes proliferation, migration, and invasion of ESCC in vitro

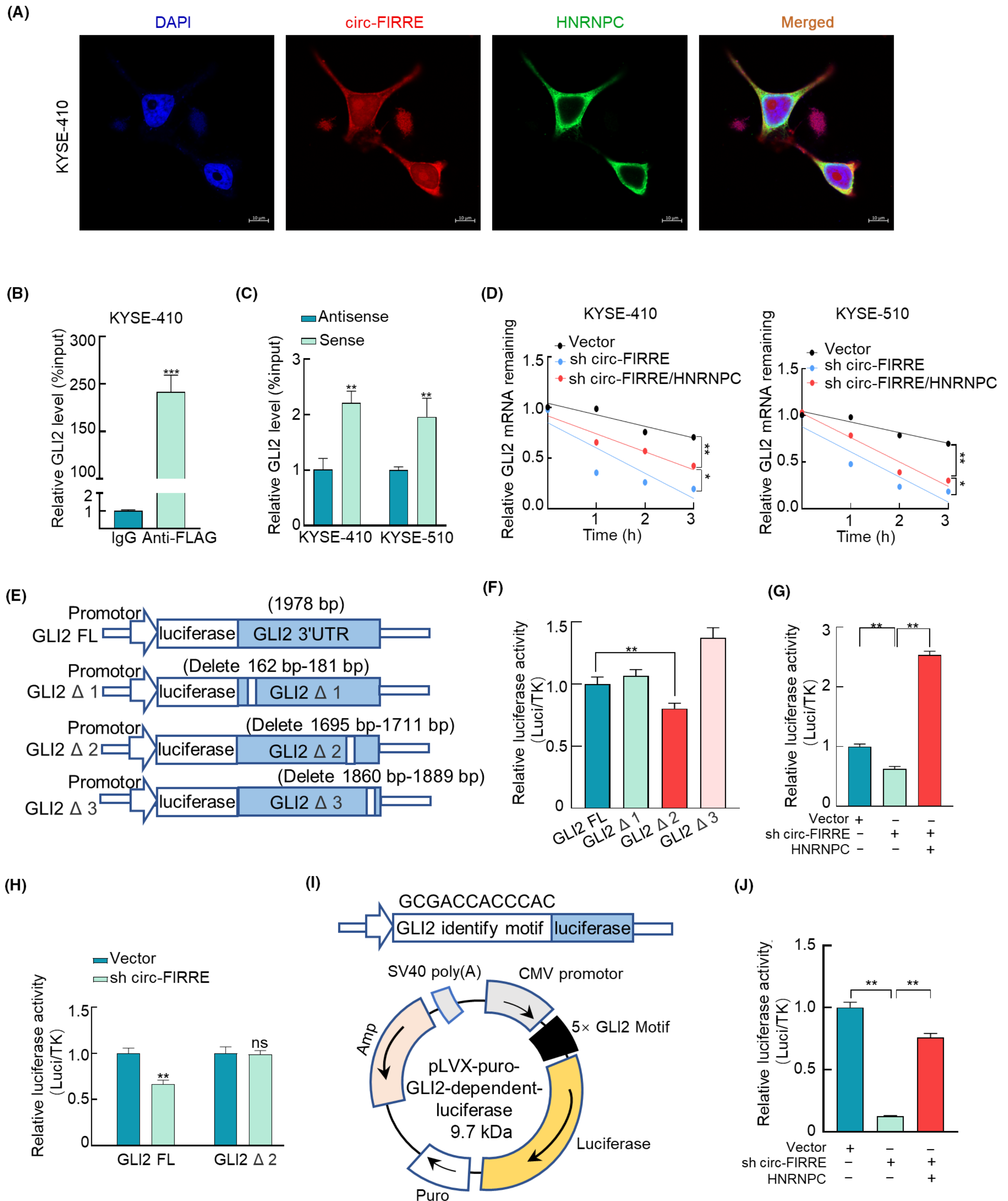
Expression of circ-FIRRE was upregulated in five ESCC cell lines compared with normal esophageal epithelial HEEC cells. Among these, KYSE-410 and KYSE-510 cells showed relatively high levels of circ-FIRRE, whereas KYSE-30 and KYSE-150 cells showed relatively low levels (Figure 2A). To study the functions of circ-FIRRE, two sh RNAs (sh-circ-FIRRE#1 and sh-circ-FIRRE#2) that specifically targeted the junction sites of circ-FIRRE were designed, and their efficiency was tested to confirm the exact knockdown of circ-FIRRE but not FIRRE (Figure 2B). Similarly, we constructed circ-FIRRE-overexpressing KYSE-410 cells and tested the efficiency (Figure S2A). We then carried out CCK-8, colony formation, EdU, and soft agar colony formation assays. The results showed that the proliferation and anchorage-independent colony formation abilities were notably suppressed after knockdown of circ-FIRRE and meaningfully increased after overexpression of circ-FIRRE in ESCC cells (Figures 2C–E and S2B–D). Flow cytometry analysis of the cell cycle showed that knockdown of circ-FIRRE caused G_0/G_1 blockade, and opposite results were observed in KYSE-410 and KYSE-510 cells overexpressing circ-FIRRE (Figures 2F and S2E). Moreover, as shown by wound healing and Transwell assays, the migration and invasion abilities were restrained by circ-FIRRE knockdown and improved by overexpression of circ-FIRRE in ESCC cells (Figures 2G and S2F,G). The abovementioned results suggest that circ-FIRRE is an oncogenic circRNA in ESCC and promotes the malignant behavior of ESCC cells.

3.3 | *GLI2* is a downstream target gene of circ-FIRRE

Figure 2 reveals that circ-FIRRE promotes the proliferation, migration, and invasion abilities of ESCC cells. The transcription factor GLI2 controls the transcriptional regulation of *C-MYC*, *CCNE1*, and *CCNE2* to promote cancer progression,[25,26] and we surprisingly discovered that the protein and RNA expression levels of C-MYC, CCNE1, CCNE2, and GLI2 were decreased after knockdown of circ-FIRRE in KYSE-410 and KYSE-510 cells (Figure 3A,B). To confirm the function of circ-FIRRE induced by GLI2, we undertook western blotting, qPCR, and cell function assays. The results showed that C-MYC, CCNE1, and CCNE2 expression could be decreased by knockdown of GLI2 in KYSE-410 cells (Figure S3A,B). In addition, knockdown of GLI2 decreased KYSE-410 cell proliferation, migration, and invasion abilities (Figure S3C–E). Furthermore, overexpression of GLI2 in circ-FIRRE knockdown KYSE-410 cells rescued the protein expression levels of C-MYC, CCNE1, and CCNE2 (Figure 3C). The cell function of circ-FIRRE knockdown could be partially reversed by GLI2 overexpression in KYSE-410 cells (Figures 3D–F and S3F). These results strongly indicated that GLI2 is a downstream target gene of circ-FIRRE. Furthermore, IHC of the ESCC patients and The Cancer Genome Atlas database analysis also showed that the GLI2 protein and mRNA expression levels were clearly increased in ESCC (Figures 3G and S3G), and its high expression level was also related to poor overall survival in ESCC patients, as shown by K-M analysis (Figure 3H). All these findings strongly suggest that circ-FIRRE promotes ESCC cell proliferation, migration, and invasion through GLI2.

3.4 | circ-FIRRE/HNRNPC complex facilitates the malignant phenotype of ESCC cells

To explore potential RBPs binding to circ-FIRRE, we used RNA pulldown combined with silver staining to identify potential circ-FIRRE-binding proteins (Figure S4A). Based on the overlap of circ-FIRRE-binding proteins predicted by CircInteractome, CDSC, RBPsuite, and catRAPID, we identified six potential circ-FIRRE-interacting proteins: HNRNPC, DGCR8, IGF2BP2, MOV10, ZC3H7B, and AGO2 (Figure S4B). The RNA pulldown results clearly indicated enrichment in the 25–35 kDa range; only one of the predicted proteins was identified as HNRNPC (34 kDa). Accordingly, we then validated the interaction between circ-FIRRE and HNRNPC by RNA pulldown and RIP assays (Figure 4A–C). To explore whether HNRNPC promotes circ-FIRRE biogenesis or circ-FIRRE regulates HNRNPC protein expression levels, we first examined the efficiency of HNRNPC overexpression in KYSE-410 cells (Figure S4C). Then, we measured HNRNPC protein levels in circ-FIRRE-knockdown cells and circ-FIRRE levels after HNRNPC overexpression in KYSE-410 cells. The results showed that circ-FIRRE did not directly regulate HNRNPC expression and that HNRNPC did not play a role in circ-FIRRE biogenesis (Figure S4D,E). In addition, to investigate whether GLI2 is a direct downstream target of the circ-FIRRE/HNRNPC complex, we utilized siRNA to knockdown HNRNPC and



evaluated its effect on the expression of GLI2 and its downstream target genes. The results showed that knockdown of HNRNPC significantly decreased the mRNA and protein levels of GLI2 and its downstream targets (Figure S4F,G). We also examined the effects of HNRNPC knockdown on the proliferation, migration, and invasion of KYSE-410 cells. The results showed that knockdown of HNRNPC resulted in a significant decrease in cell proliferation, migration, and invasion (Figure S4H–J). Conversely, overexpression of HNRNPC in circ-FIRRE-knockdown KYSE-410 cells led to increased expression of GLI2, C-MYC, CCNE1, and CCNE2 (Figure 4D,E). Functionally,

FIGURE 5 Circular RNA circ-FIRRE–heterogeneous nuclear ribonucleoprotein C (HNRNPC) complex promotes esophageal squamous cell carcinoma (ESCC) tumor growth in vivo. (A) FISH and immunofluorescence analyses showed circ-FIRRE and HNRNPC colocalization in KYSE-410 cells. Scale bar, 10 μm. (B) RNA binding protein immunoprecipitation showed the combination of HNRNPC and *GLI2* mRNA in KYSE-410 cells. (C) Enrichment of *GLI2* mRNA pulled down by the circ-FIRRE probe is shown. (D) mRNA stability of *GLI2* under treatment with actinomycin D in KYSE-410 and KYSE-510 cells with circ-FIRRE knockdown or co-overexpression of HNRNPC measured by real-time quantitative PCR. (E) Schematic illustration of the *GLI2* 3′-UTR full-length or mutant luciferase reporter. (F, G) The relationship between the circ-FIRRE/HNRNPC complex and the 3′-UTR of *GLI2* mRNA was verified by the dual-luciferase reporter assay. (H) GLI2 Δ 2 is the binding site of circ-FIRRE/HNRNPC and GLI2 verified by the dual-luciferase reporter assay. (I) Schematic illustration of the GLI2-dependent luciferase reporter. (J) Hedgehog signaling activity in circ-FIRRE knockdown or co-overexpressing HNRNPC KYSE-410 cells tested by the dual-luciferase reporter assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

we observed that overexpression of HNRNPC partially restored cell functions in circ-FIRRE-knockdown KYSE-410 cells (Figures 4F–H and S4K). Furthermore, IHC analysis showed that HNRNPC was obviously upregulated in ESCC, and its high expression was also related to poor overall survival in ESCC patients, as shown by K-M analysis (Figures 4I and S4L). In addition, IHC of ESCC patients and GSE130078 database analysis revealed that the HNRNPC expression level was positively correlated with the expression levels of GLI2 (Figures 4J and S4M). UCSC Xena and K-M Plotter showed that HNRNPC is highly expressed in ESCC and related to overall survival (Figure S4N,O). These results show that circ-FIRRE binds to HNRNPC to form a complex regulating GLI2 expression to achieve their biological functions together.

3.5 | circ-FIRRE/HNRNPC complex stabilizes *GLI2* mRNA to enhance biological functions of GLI2 protein

Through confocal microscopy and immunofluorescence, we found that circ-FIRRE and HNRNPC were colocalized in the cytoplasm (Figure 5A). Accordingly, we hypothesized that the circ-FIRRE/HNRNPC complex might increase the expression of GLI2 by enhancing *GLI2* mRNA stability. To verify this hypothesis, we then undertook RNA pulldown and RIP assays validating the interaction between *GLI2* mRNA and HNRNPC or circ-FIRRE (Figure 5B,C). In addition, we carried out RT-qPCR to determine the mRNA stability of *GLI2* at different time points following actinomycin D treatment. The results showed that *GLI2* mRNA stability was decreased in circ-FIRRE knockdown cells, and this reduction could be partly rescued by HNRNPC overexpression in KYSE-410 and KYSE-510 cells (Figure 5D). It has been reported that HNRNPC binds specifically to the 3′-UTR of mRNA to increase protein production.[27] Therefore, we constructed luciferase reporter vectors that contained the full-length *GLI2* 3′-UTR or deletion mutants that bound to HNRNPC predicted by POSTAR3 (Figures 5E and S5A). A dual-luciferase reporter assay showed that luciferase activity decreased after deletion of the HNRNPC-binding site on the *GLI2* 3′-UTR (Figure 5F). Similarly, reduced activity was observed after knockdown of circ-FIRRE, and it was rescued by overexpression of HNRNPC after transfection with the full-length *GLI2* 3′-UTR reporter in KYSE-410 cells (Figure 5G). To determine whether GLI2 del2 luciferase activity is dependent on circ-FIRRE, we carried out cotransfection of sh-circ-FIRRE and the GLI2 del2 luciferase construct in KYSE-410 cells. Our findings showed that the transfection of GLI2 del2-luciferase plasmids in sh circ-FIRRE cells did not have any effect on luciferase activity, suggesting that GLI2 del2 is indeed the binding site of circ-FIRRE/HNRNPC and GLI2 (Figure 5H). To summarize, GLI2 is a direct target of the circ-FIRRE/HNRNPC complex. Because GLI2 is an essential transcription factor in the Hh signaling pathway,[28] we speculated that the circ-FIRRE/HNRNPC complex influences Hh signaling activity by regulating *GLI2* mRNA and protein levels. Consequently, a GLI2-dependent luciferase reporter was constructed to characterize Hh signaling activity (Figure 5I). A dual-luciferase reporter assay showed that knockdown of circ-FIRRE decreased Hh signaling activity, but the activity could be partly rescued by overexpression of HNRNPC (Figure 5J). In addition, after circ-FIRRE knockdown, the mRNA and protein levels of PTCH1 and SHH, which are critical components of the Hh pathway, were decreased in both KYSE-410 cells and KYSE-510 cells (Figure S5B,C). All results fully indicated that GLI2 is the downstream target of the circ-FIRRE/HNRNPC complex.

3.6 | circ-FIRRE/HNRNPC complex promotes ESCC tumor growth in vivo

To further evaluate the oncogenic role of circ-FIRRE in vivo, a xenograft tumor model was established in BALB/c nude mice. KYSE-410 cells stably transfected with vector, sh-circ-FIRRE, or sh-circ-FIRRE/HNRNPC were s.c. injected into mice. The results showed that the volume and weight of tumors from the sh-circ-FIRRE group were significantly lower than those of tumors from the vector group or sh-circ-FIRRE/HNRNPC group (Figure 6A–C). In addition, IHC confirmed that the sh-circ-FIRRE group showed a lower average optical density for Ki-67 and GLI2 than the vector group, but the average optical density of the sh-circ-FIRRE/HNRNPC group was higher than that of the sh-circ-FIRRE group (Figure 6D,E). Similarly, a positive correlation between the average optical density for HNRNPC and GLI2 was also found in mouse tumor tissues (Figure 6F). The RT-qPCR and western blot analyses also revealed that the circ-FIRRE/HNRNPC-GLI2 axis promoted ESCC tumor growth in vivo (Figure 6G,H). All of the abovementioned results support the oncogenic role of circ-FIRRE in promoting ESCC progression in vivo. Overall, in this study, we showed that circ-FIRRE enhances GLI2 mRNA stability and expression

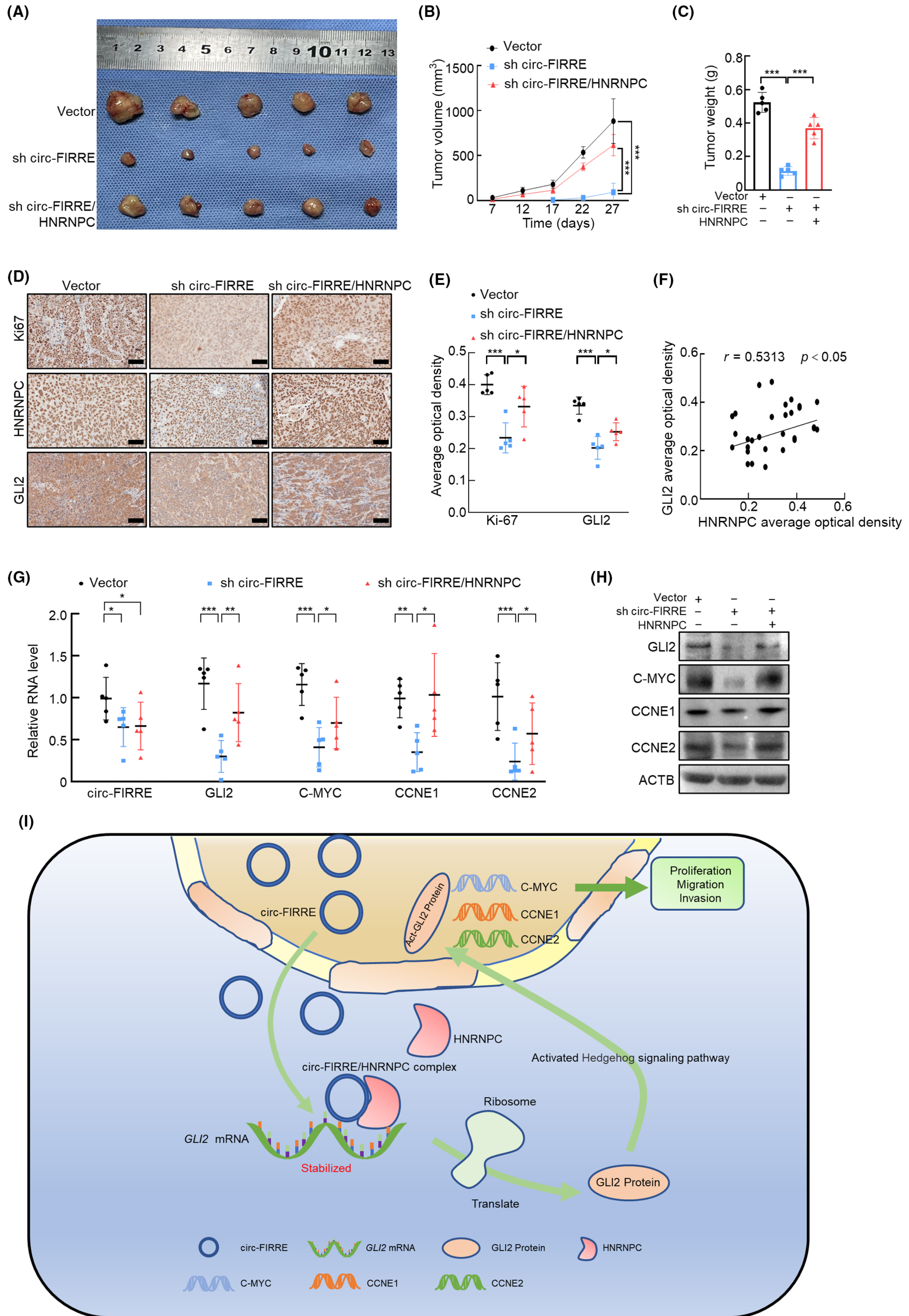

(A)
Vector
sh circ-FIRRE
sh circ-FIRRE/
HNRNPC
(B)
Vector
sh circ-FIRRE
sh circ-FIRRE/HNRNPC
Tumor volume (mm³)
Time (days)
(C)
Tumor weight (g)
Vector
sh circ-FIRRE
HNRNPC
(D)
Vector
sh circ-FIRRE
sh circ-FIRRE/HNRNPC
Ki67
HNRNPC
GLI2
(E)
Vector
sh circ-FIRRE
sh circ-FIRRE/HNRNPC
Average optical density
Ki-67
GLI2
(F)
r = 0.5313 p < 0.05
GLI2 average optical density
HNRNPC average optical density
(G)
Vector
sh circ-FIRRE
sh circ-FIRRE/HNRNPC
Relative RNA level
circ-FIRRE
GLI2
C-MYC
CCNE1
CCNE2
(H)
Vector
sh circ-FIRRE
HNRNPC
GLI2
C-MYC
CCNE1
CCNE2
ACTB
(I)
circ-FIRRE
Act-GLI2 Protein
C-MYC
CCNE1
CCNE2
Proliferation
Migration
Invasion
HNRNPC
circ-FIRRE/HNRNPC complex
Activated Hedgehog signaling pathway
Ribosome
GLI2 mRNA
Stabilized
GLI2 Protein
Translate
circ-FIRRE
GLI2 mRNA
GLI2 Protein
HNRNPC
C-MYC
CCNE1
CCNE2

FIGURE 6 Circular RNA circ-FIRRE–heterogeneous nuclear ribonucleoprotein C (HNRNPC) complex promotes esophageal squamous cell carcinoma (ESCC) tumor growth in vivo. (A) Representative images of the inoculated tumor tissues of each group. (B) Time-course evaluation of the tumor volumes in the indicated groups. (C) Tumor weight in each group. (D) Representative immunohistochemical staining images of Ki-67, HNRNPC, and GLI2 in different groups. Scale bar, 50 μm. (E) Average optical density of GLI2 and Ki-67 in the indicated groups. (F) Correlation between the average optical density of HNRNPC and GLI2 in tumor tissues. (G) Relative RNA levels extracted from tumor tissues of each group are shown. (H) Protein expression levels of tumor tissues from each group with five mixed samples. (I) Schematic diagram elucidating the mechanism of the circ-FIRRE/HNRNPC-GLI2 axis, which mediates the promotion of ESCC. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

levels by directly binding with HNRNPC, which results in activation of the Hh pathway and thus in the promotion of ESCC progression (Figure 6I).

4 | DISCUSSION

Esophageal squamous cell carcinoma is the leading cause of cancer-related mortality worldwide.[29] Esophageal cancer treatment methods include neoadjuvant radiochemotherapy, surgery, definitive chemoradiation, and systemic chemotherapy.[30] After several landmark trials indicated the benefits of immunotherapy for esophageal carcinoma,[31–33] immune checkpoint inhibitors in combination with chemotherapy became the standard treatment for late-stage esophageal cancer.[34,35] However, immune checkpoint therapy still has many drawbacks, including low response rates, resistance, and hyperprogression.[36] The discovery of effective therapeutic targets for ESCC remains an urgent issue. In the last few years, circRNAs, which have unique molecular structures and stage-specific expression, have presented remarkable potential as precise therapeutic targets in ESCC.[37–39] In this work, we first characterized a novel circRNA, circ-FIRRE, from the RNA sequencing (Ribo-free) profile of ESCC and proved its existence in ESCC. Real-time qPCR indicated that circ-FIRRE expression was higher in ESCC tissues than in normal tissues. Importantly, the expression level of circ-FIRRE was positively correlated with an advanced tumor stage and shorter overall survival time in ESCC patients. Subsequently, we found that knockdown of circ-FIRRE could meaningfully inhibit cell proliferation, migration, and invasion in vitro and suppress oncogenesis in vivo, while overexpression of circ-FIRRE showed the opposite effects. This evidence highlights the imperative role and clinical significance of circ-FIRRE in ESCC progression.

Heterogeneous nuclear ribonucleoprotein C belongs to a class of proteins associated with heterogeneous nuclear RNAs.[40] It has been reported that HNRNPC can bind pre-mRNAs and facilitate nuclear assembly,[41] interact with the 3′-UTR or 5′-UTR of mRNA to modulate their stability, and interact with long noncoding RNAs to enhance mRNA stability in the cytoplasm.[42] Little is known about how HNRNPC interacts with circRNAs. Here, we thoroughly investigated circ-FIRRE as a platform that interacts with HNRNPC to promote malignant behavior in vitro and in vivo, elucidating a new mechanism by which HNRNPC contributes to the development of ESCC.

Hedgehog signaling undoubtedly plays a pivotal role in ESCC progression. Notably, high Hh signaling activity was observed in approximately 60% of 104 ESCC tumors.[43] A canonical Hh signal is initiated in vertebrates when Sonic (Shh), Indian (Ihh), or Desert (Dhh) Hh ligands are engaged by Patched (Ptch) receptors on target cells, resulting in smoothened (Smo) being dissociated and activated. Active Smo accumulates in primary cilia, leading to the glioma-associated oncogene family zinc finger 2 transcription factor (Gli) being processed, relocalized, and activated.[44] GLI2 is a well-known oncogenic transcription factor targeting a series of downstream genes,[45] such as *C-MYC*, *CCNE1*, and *CCNE2*, and is also an indispensable mediator of Hh signaling activation in ESCC. For example, Zhang et al. proved the critical role of GLI2 in ESCC progression.[22] Accordingly, we surprisingly discovered that overexpression of GLI2 in circ-FIRRE knockdown cells attenuated the downregulation of circ-FIRRE inhibition in ESCC cells; we also confirmed the interaction between circ-FIRRE/HNRNPC complex components and their direct downstream target GLI2 by RIP and RNA pulldown assays. Moreover, we found that the circ-FIRRE/HNRNPC complex activated the Hh signaling pathway by elevating GLI2 protein expression, thereby initiating C-MYC, CCNE1, and CCNE2 transcript expression and eventually facilitating malignant behavior in ESCC. In addition, the GLI2-dependent dual-luciferase reporter assay showed the same result for the circ-FIRRE/HNRNPC-GLI2 axis. Our study provides clear evidence that circ-FIRRE worsens ESCC prognosis through Hh signaling activation, strongly supporting the potential of circ-FIRRE as a therapeutic target in ESCC. Based on previous reports, RBPs can directly bind to RNA and play a pivotal role in posttranscriptional processes.[46] In the present study, we also confirmed through actinomycin D and dual-luciferase reporter assays that *GLI2* mRNA stability was regulated by the circ-FIRRE/HNRNPC complex, which binds to its 3′-UTR. Promisingly, the confirmation that *GLI2* mRNA is regulated by the circ-FIRRE/HNRNPC complex is consistent with the characteristics of the circRNA-RBP complex in transcriptional regulation, expanding the knowledge of these RBPs beyond their conventional functions in mRNA processing.

In conclusion, we provide the first evidence that circ-FIRRE is upregulated and positively correlated with an advanced tumor stage and shorter overall survival time in ESCC patients. Mechanistically, circ-FIRRE binds to the HNRNPC protein as a circ-FIRRE/HNRNPC complex, promoting the progression of ESCC in vitro and in vivo. We showed that the circ-FIRRE/HNRNPC complex enhanced *GLI2* mRNA stability, which in turn increased the expression of *MYC*, *CCNE1*, and *CCNE2* and facilitated the malignant behavior of ESCC. The present study extends our knowledge of circ-FIRRE-HNRNPC interactions and their regulatory role in ESCC progression and suggests that circ-FIRRE could serve as a diagnostic and therapeutic target in ESCC in the future.

AUTHOR CONTRIBUTIONS

XGZ and ZXT conceived and designed the experiments. YJZ and XX performed most of the experiments, collected the data, and drafted the manuscript. JWL, PWL, and ZHX conducted the animal experiments. WHZ, JZ, and PCL revised the manuscript. JFZ and HBG assisted in the experiments. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was approved by the Ethics Committee of The Second Hospital of Shandong University.

Informed consent: Patients provided informed consent for obtaining samples, and the study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of the Second Hospital of Shandong University.

Registry and registration no. of the study/trial: KYLL-2020(LW)-081.

Animal studies: All animal experiments were approved by the Ethics Committee of The Second.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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