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LINC00629, a HOXB4-downregulated long noncoding RNA, inhibits glycolysis and ovarian cancer progression by destabilizing c-Myc

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Abstract

Ovarian cancer (OC) cells typically reprogram their metabolism to promote rapid proliferation. However, the role of long noncoding RNAs (lncRNAs) in the metabolic reprogramming of ovarian cancer, especially in glucose metabolic reprogramming, remains largely unknown. LINC00629 has been reported in our previous study to promote osteosarcoma progression. Upregulated LINC00629 was found to enhance the growth-suppressive effect of apigenin on oral squamous cell carcinoma. However, the precise function of LINC00629 in ovarian cancer development remains poorly understood. In this study, we found that LINC00629 was significantly downregulated in OC tissues and that low LINC00629 expression was associated with poor survival. Inhibition of LINC00629 was required for increased glycolysis activity and cell proliferation in ovarian cancer. In vivo, overexpression of LINC00629 dramatically inhibited tumor growth and lung metastasis. Mechanistically, LINC00629 interacted with and destabilized c-Myc, leading to its ubiquitination and proteasome degradation, further resulting in increased expression of downstream glycolysis-related genes and glucose metabolic reprogramming in OC. Interestingly, HOXB4 bound to the LINC00629 promoter and inhibited its transcription, indicating that LINC00629 is a transcriptional target of HOXB4. Collectively, these findings establish a direct role for LINC00629 in suppressing glucose metabolism, and HOXB4/LINC00629/c-Myc might serve as a potential biomarker and an effective therapeutic strategy for OC cancer treatment.

KEYWORDS
c-Myc, glycolysis, HOXB4, LINC00629, ovarian cancer

Abbreviations: OC, Ovarian cancer; TCGA, The Cancer Genome Atlas; GEPI, A Gene Expression Profiling Interactive Analysis; qRT–PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry; RIP, RNA immunoprecipitation; FISH, RNA fluorescence in situ hybridization; IF, immunofluorescence; LncRNA, Long noncoding RNA

Jia Liu, Yuan Zhu, and Huan Wang contributed equally to this article.

1 | INTRODUCTION

As a heterogeneous malignancy, ovarian cancer is the second leading cause of gynecological cancer-related death in China.[1] Ovarian cancer relapse and metastasis are reportedly associated with glycolysis and stemness properties.[2] It is of great significance to elucidate the basic mechanisms regulating tumor cell glycolysis and stem cell characteristics, which might open up new avenues for therapeutic development.

Myc (c-Myc), a well-established oncogenic transcription factor that is mainly localized in the nucleus, has been demonstrated to regulate many processes of cancer cells, including cell growth, glycolytic metabolism, proliferation, and cell stemness.[3–5] For example, c-Myc has been shown to excite many glycolytic enzymes, such as hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA).[6,7] Therefore, targeting c-Myc to treat ovarian cancer has attracted much attention. Due to its poor pharmacological properties, direct interaction with c-Myc has not shown good therapeutic effects. Therefore, inhibiting c-Myc at the posttranscriptional or translational level might represent an effective method for treating c-Myc-driven cancer.

Long noncoding RNAs (lncRNAs) are a class of noncoding transcripts longer than 200 nucleotides in length. Recent studies have highlighted the potential effects of lncRNAs on cancer glucose metabolism reprogramming. For example, AGPG regulates PFKFB3-mediated tumor glycolytic reprogramming,[8] GLCC1 promotes colorectal carcinogenesis and glucose metabolism by stabilizing c-Myc,[9] LINC01234 promotes hepatocellular carcinoma progression by orchestrating aspartate metabolic reprogramming,[10] LncRNA DIO3OS induces breast cancer glycolytic-dominant metabolic reprogramming to promote aromatase inhibitor resistance,[11] glycoLINC assembles a lower glycolytic metabolon to promote glycolysis,[12] LncRNA-AC020978 promotes proliferation and glycolytic metabolism of lung cancer cells by regulating the PKM2/HIF-1α axis,[13] and MALAT1 regulates cancer glucose metabolism by enhancing mTOR-mediated translation of TCF7L2.[14] LINC00629 has been reported in our previous study to promote osteosarcoma progression.[15] In our previous study, upregulated LINC00629 was also found to enhance the growth-suppressive effect of apigenin on oral squamous cell carcinoma.[16] Recently, it has also been reported to promote the proliferation and metastasis of osteosarcoma cells by elevating NUCKS1 expression and asparagine synthesis.[17] However, the function and mechanism of LINC00629 in ovarian cancer have not been reported.

Here, we observed that LINC00629 was significantly downregulated in ovarian cancer tissues, and the low expression of LINC00629 was correlated with poor prognosis in ovarian cancer patients. Elevated expression of LINC00629 suppressed ovarian cell proliferation and migration in vitro and in vivo. Subsequently, our data indicated that LINC00629 interacted with c-Myc and promoted its degradation, which led to a decrease in c-Myc in HeyA8 and SKOV3 cells. Furthermore, we demonstrated that HOXB4 binds to the LINC00629 promoter and represses its transcription. Collectively, our findings suggest that LINC00629 might be a biomarker and metabolism blocker target in ovarian cancer.

2 | OTHER MATERIALS AND METHODS

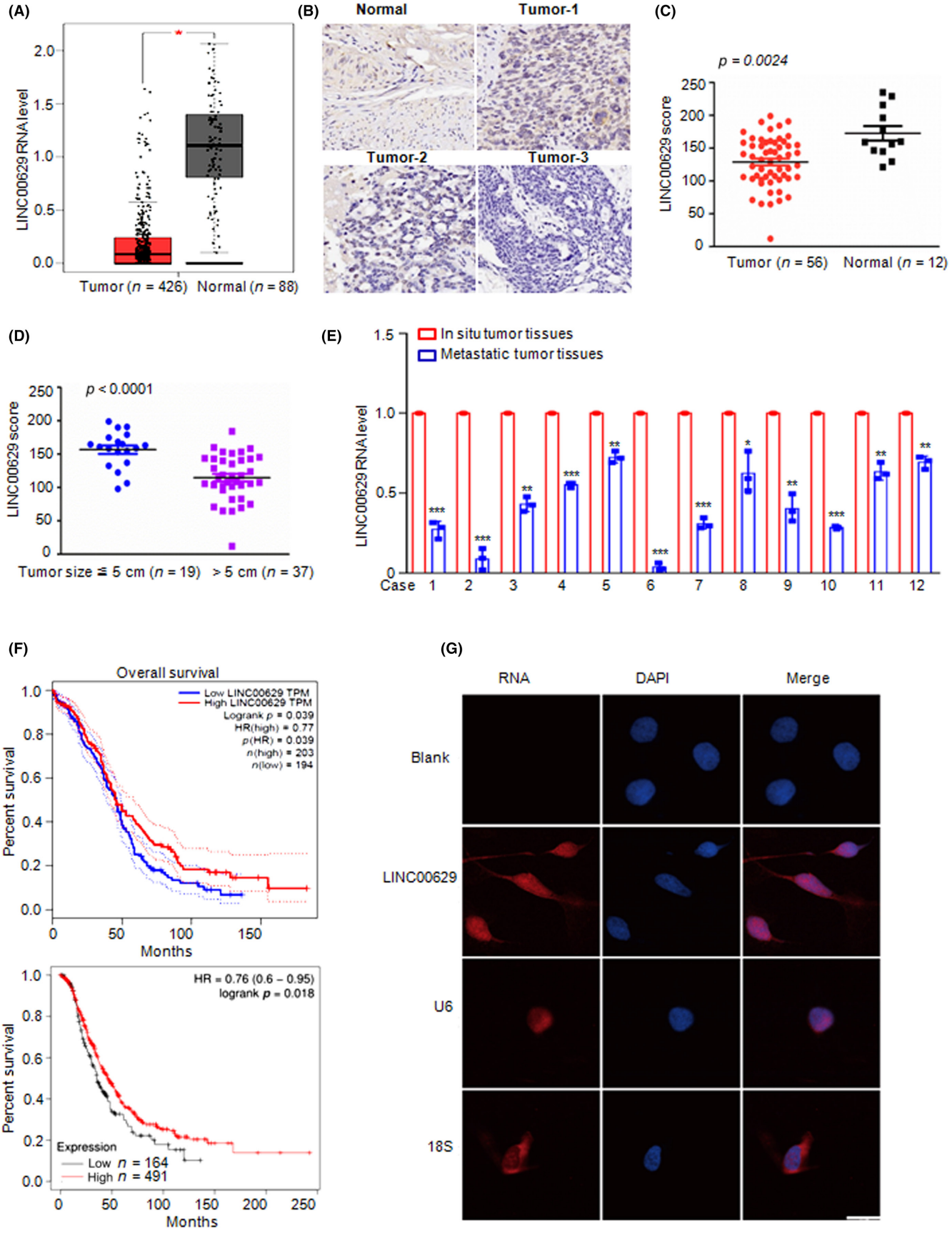
All materials and methods can be found in the supporting information 1.

3 | RESULTS

3.1 | LINC00629 is downregulated in ovarian cancer

To investigate the role of LINC00629 in ovarian cancer, Gene Expression Profiling Interactive Analysis (GEPIA) was used to analyze the expression of LINC00629 in ovarian cancer tissues and normal ovarian tissues based on The Cancer Genome Atlas (TCGA) database. The results showed that LINC00629 was significantly downregulated in ovarian cancer tissues ($n=426$) compared with normal ovarian tissues ($n=88$) (Figure 1A; Figure S1A). To validate these findings, in situ hybridization (ISH) was used to evaluate LINC00629 expression in paraffin-embedded ovarian cancer and normal tissues. Consistently, LINC00629 expression was lower in ovarian cancer tissues than in normal tissues, and the expression of LINC00629 was negatively associated with tumor size (Figure 1B,D). In addition, the expression levels of LINC00629 in 12 cases of in situ ovarian cancer and their greater omentum metastatic ovarian cancer tissues were analyzed by qRT-PCR. The results indicated that greater omentum metastatic ovarian cancer tissues showed

FIGURE 1 LINC00629 is downregulated in ovarian cancer. (A) The Cancer Genome Atlas (TCGA) dataset results of LINC00629 in specimens of normal ovarian tissues ($n=88$) and ovarian cancer tissues ($n=426$). (B) The RNA Scope ISH of LINC00629 in one normal ovarian tissue and three representative ovarian cancer tissue samples (Tumor-1:tumor size less than or equal to 5 cm, Tumor-2 and 3 are greater than 5). (C) Statistical score analysis of LINC00629 expression between normal ovarian tissues ($n=12$) and ovarian tumor tissues ($n=56$). (D) Statistical score analysis of LINC00629 expression in ovarian tumor tissues between tumor size ≤5 cm ($n=19$) and tumor size >5 cm ($n=37$). (E) qRT–PCR analysis of LINC00629 expression between in situ ovarian cancer tissues ($n=12$) and greater omentum metastatic ovarian cancer tissues ($n=12$). (F) Kaplan–Meier survival curves showed poor overall survival with low expression of LINC00629 compared with high LINC00629 from GEPIA2 and the Kaplan–Meier Plotter network (top panel results comes from website of http://gepia2.cancer-pku.cn/#survival; bottom panel result comes from http://kmplot.com/analysis/index.php?p=service). (G) Localization of LINC00629 (red) in SKOV3 cells was detected by FISH, and 18S (red) and U6 (red) served as positive controls for cytoplasmic and nuclear fractions, respectively. The nuclei were stained with DAPI (blue). Scale bar = 50 μm.


(A)
LINC00629 RNA level
2.0
1.5
1.0
0.5
0.0
Tumor (n = 426)
Normal (n = 88)
(B)
Normal
Tumor-1
Tumor-2
Tumor-3
(C)
p = 0.0024
LINC00629 score
250
200
150
100
50
0
Tumor (n = 56)
Normal (n = 12)
(D)
p < 0.0001
LINC00629 score
250
200
150
100
50
0
Tumor size ≦ 5 cm (n = 19)
> 5 cm (n = 37)
(E)
In situ tumor tissues
Metastatic tumor tissues
LINC00629 RNA level
1.5
1.0
0.5
0
Case 1 2 3 4 5 6 7 8 9 10 11 12
(F)
Overall survival
Low LINC00629 TPM
High LINC00629 TPM
Logrank p = 0.039
HR(high) = 0.77
p(HR) = 0.039
n(high) = 203
n(low) = 194
Percent survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
Months
HR = 0.76 (0.6 – 0.95)
logrank p = 0.018
Percent survival
Expression
Low n = 164
High n = 491
0
50
100
150
200
250
Months
(G)
RNA
DAPI
Merge
Blank
LINC00629
U6
18S

lower LINC00629 expression than in situ ovarian cancer tissues (Figure 1E). Furthermore, Kaplan–Meier (K-M) survival analysis indicated that high expression of LINC00629 was significantly correlated with a longer survival time (Figure 1F; Figure S1B). These results suggest that LINC00629 was downregulated in ovarian cancer and that reduced LINC00629 was strongly associated with ovarian cancer development. RNA FISH was performed and revealed that LINC00629 was subcellularly located in both the cytoplasm and nucleus of SKOV3 cells (Figure 1G).

3.2 | LINC00629 suppresses ovarian cancer cell proliferation and migration in vitro

To explore the function of LINC00629 in ovarian cancer, we first knocked down or overexpressed LINC00629 in HeyA8 and SKOV3 cells, which was confirmed by qRT-PCR (Figure S2A,B). Subsequently, colony formation and Transwell assays were performed to measure the effects of LINC00629 on cell clonogenicity and migration. As shown in Figure 2A–F, the depletion of LINC00629 markedly promoted cell clonogenic potential and migration in HeyA8 and SKOV3 cells. In contrast, forced expression of LINC00629 inhibited cell clonogenicity and migration in HeyA8 and SKOV3 cells (Figure 2G–J). Subsequent sphere formation assays suggested similar results in the indicated cells (Figure 2K–N).

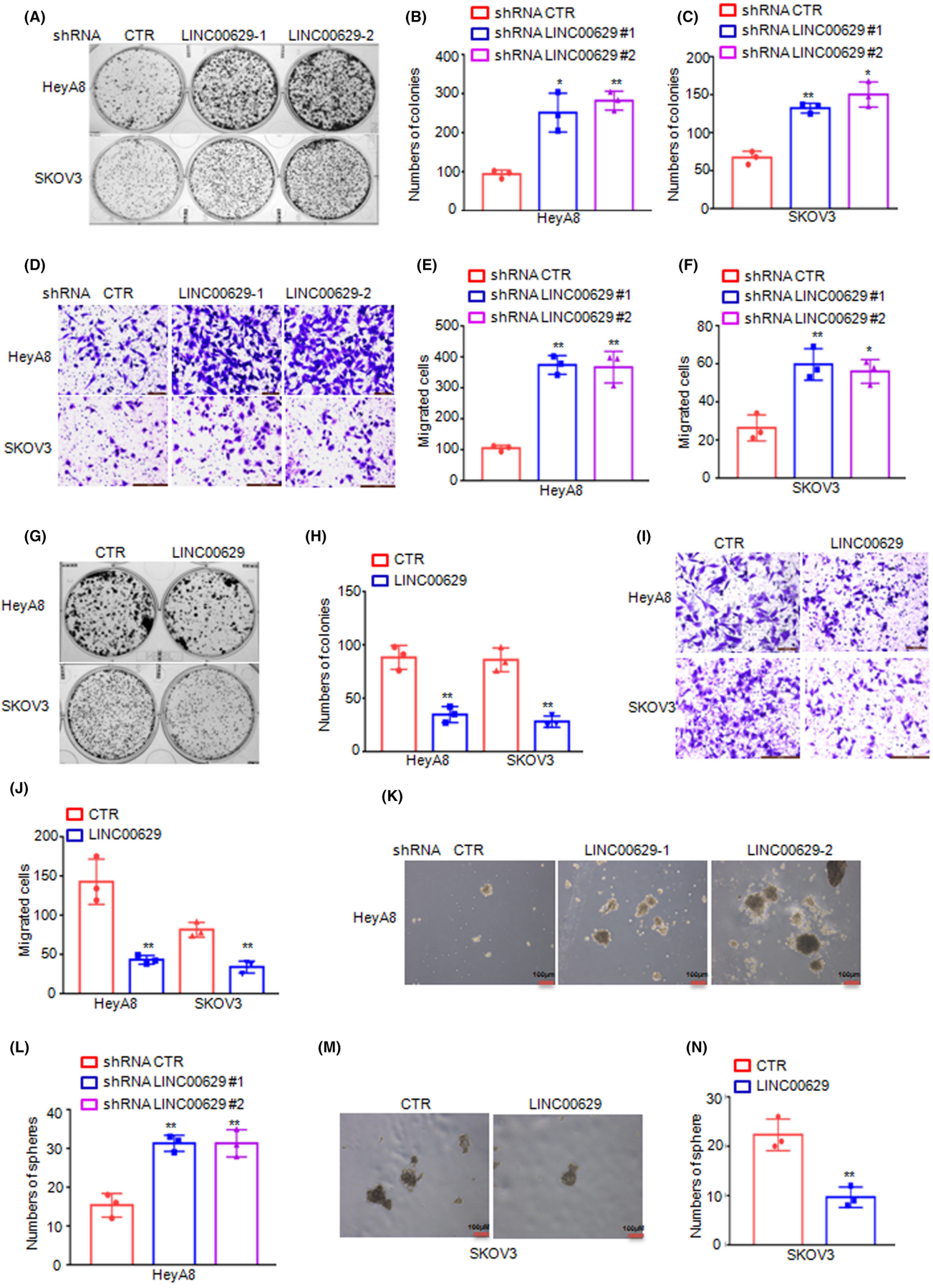
3.3 | LINC00629 inhibits tumorigenesis and lung metastasis of ovarian cancer cells in vivo

To further validate the effect of LINC00629 on tumor growth and metastasis in vivo, HeyA8 cells with or without LINC00629 knockdown were subcutaneously injected into BALB/c nude female mice ($n = 5$ in each group). Compared with the control group, LINC00629 depletion significantly promoted tumor formation, as indicated by increased tumor weights and sizes (Figure 3A–C). The subsequent immunohistochemistry analyses assay was performed to detect the resected HeyA8 xenograft tumor tissues from the control and LINC00629 knockdown groups using Ki-67 and cleaved caspase-3 antibodies. The results indicated that the LINC00629 knockdown group exhibited higher Ki67 and CD31 expression but lower cleaved caspase-3 expression (Figure 3D,E). Based on the experience of Zhang et al. (2023) in ovarian cancer with lung metastasis,[18] a lung metastasis model was established in our research to assess the effects of LINC00629 on metastasis. HeyA8 cells with or without LINC00629 knockdown were injected intravenously into nude mice via the tail vein. Three weeks later, the mice were killed to examine the lungs (Figure 3F). There was a significant increase in the number of lung metastatic nodules and lung weight in the LINC00629 knockdown group compared with the control group (Figure 3H,I). Correspondingly, there was the same result for H&E staining of the lungs (Figure 3G). Taken together, our data suggest that LINC00629 plays a tumor suppressive role in ovarian cancer.

3.4 | LINC00629 suppresses c-Myc expression and glycolytic reprogramming

To dissect the molecular mechanisms underlying LINC00629-mediated ovarian cancer growth, we used label-free quantitative proteomics to identify differentially expressed proteins in HeyA8 cells with or without LINC00629 overexpression. Thirty-six downregulated and 25 upregulated genes were observed (Figure S3A). Interestingly, among these altered genes, we found that c-Myc was significantly downregulated in LINC00629-elevated cells (Figure S3B). c-Myc is an important oncogenic transcription factor that plays a vital regulatory role in cancer cell glycolysis and ovarian cancer stem cell properties.[19,20] The expression levels of c-Myc were further detected in HeyA8 and SKOV3 cells with or without LINC00629 overexpression. Consistently, LINC00629 upregulation markedly decreased c-Myc protein levels but did not affect c-Myc mRNA levels (Figure 4A,B). Conversely, inhibition of LINC00629 increased c-Myc protein expression (Figure 4C). The above results proved the inhibitory role of LINC00629 on c-Myc. It is well known that c-Myc promotes glycolysis by transcriptionally regulating its target genes.[21–23] Given that LINC00629 was able to decrease c-Myc protein levels, we sought to detect the expression of the c-Myc downstream glycolysis genes HK2, GLUT1, and LDHA. We found that LINC00629 overexpression decreased the protein and mRNA expression of HK2, GLUT1, and LDHA (Figure 4D,E). However, the knockdown of endogenous LINC00629 in HeyA8 and SKOV3 cells elevated the expression of HK2, GLUT1, and LDHA (Figure 4F,G).To further confirm the role of LINC00629 in metabolic reprogramming, we detected the effects of LINC00629 on intracellular lactate levels and glucose uptake. As shown in Figure 4H,J, overexpression

FIGURE 2 LINC00629 suppresses ovarian cancer cell proliferation and migration in vitro. (A) HeyA8 and SKOV3 cells with or without LINC00629 knockdown were tested for cell growth in the colony formation assay. Viable colonies were counted and are pictured. (B–C) Numbers of colonies are depicted as bar graphs. (D–F) The migration of the indicated cells was detected by Transwell assays. Representative images of crystal violet-stained culture plates are shown. Data are depicted as bar graphs. (G–H) HeyA8 and SKOV3 cells with or without LINC00629 overexpression were tested for cell growth in the colony formation assay. Viable colonies were counted and are shown. Data are depicted as bar graphs. (I–J) The migration of the indicated cells was detected by Transwell assays. Representative images of crystal violet-stained culture plates are shown. Data are depicted as bar graphs. (K–L) Sphere formation abilities were detected in HeyA8 cells with or without LINC00629 knockdown. Data are depicted as bar graphs. (M–N) Sphere formation abilities were detected in SKOV3 cells with or without LINC00629 overexpression. Representative images of the spheres are shown. Data are depicted as bar graphs. Data in B, C, E, F, H, J, L, and N were analyzed by Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.


(A)
shRNA CTR LINC00629-1 LINC00629-2
HeyA8
SKOV3
(B)
shRNA CTR
shRNA LINC00629 #1
shRNA LINC00629 #2
Numbers of colonies
HeyA8
(C)
shRNA CTR
shRNA LINC00629 #1
shRNA LINC00629 #2
Numbers of colonies
SKOV3
(D)
shRNA CTR LINC00629-1 LINC00629-2
HeyA8
SKOV3
(E)
shRNA CTR
shRNA LINC00629 #1
shRNA LINC00629 #2
Migrated cells
HeyA8
(F)
shRNA CTR
shRNA LINC00629 #1
shRNA LINC00629 #2
Migrated cells
SKOV3
(G)
CTR LINC00629
HeyA8
SKOV3
(H)
CTR
LINC00629
Numbers of colonies
HeyA8 SKOV3
(I)
CTR LINC00629
HeyA8
SKOV3
(J)
CTR
LINC00629
Migrated cells
HeyA8 SKOV3
(K)
shRNA CTR LINC00629-1 LINC00629-2
HeyA8
(L)
shRNA CTR
shRNA LINC00629 #1
shRNA LINC00629 #2
Numbers of spheres
HeyA8
(M)
CTR LINC00629
SKOV3
(N)
CTR
LINC00629
Numbers of sphere
SKOV3

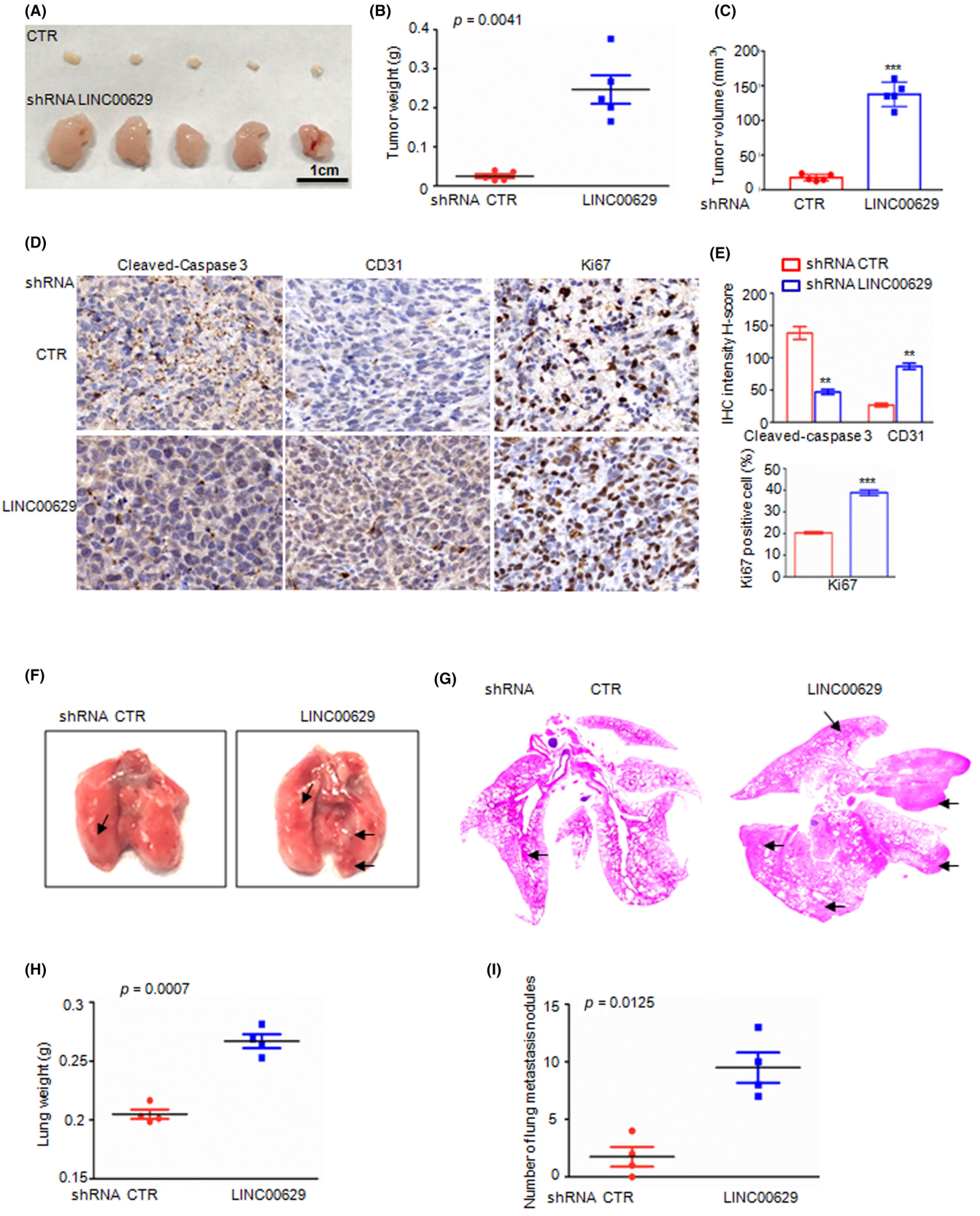


FIGURE 3 LINC00629 inhibits cell growth and lung metastasis of ovarian cancer cells in vivo. HeyA8 cells with or without LINC00629 knockdown were subcutaneously injected into female nude mice ($n = 5$). (A) Images of xenograft tumors of the two groups. (B) The tumor weights of the two groups. (C) The tumor volumes of the two groups. (D) Representative immunohistochemical results of cleaved caspase-3, Ki67, and CD31. (E) IHC intensity H-score of cleaved caspase-3, CD31, and Ki67 positive cell percentage. (F) Representative bright-field images of the lung and (G) lung metastasis HE staining are displayed. (H) Lung weight and metastatic nodules (I) were calculated for each group. Data were analyzed by Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

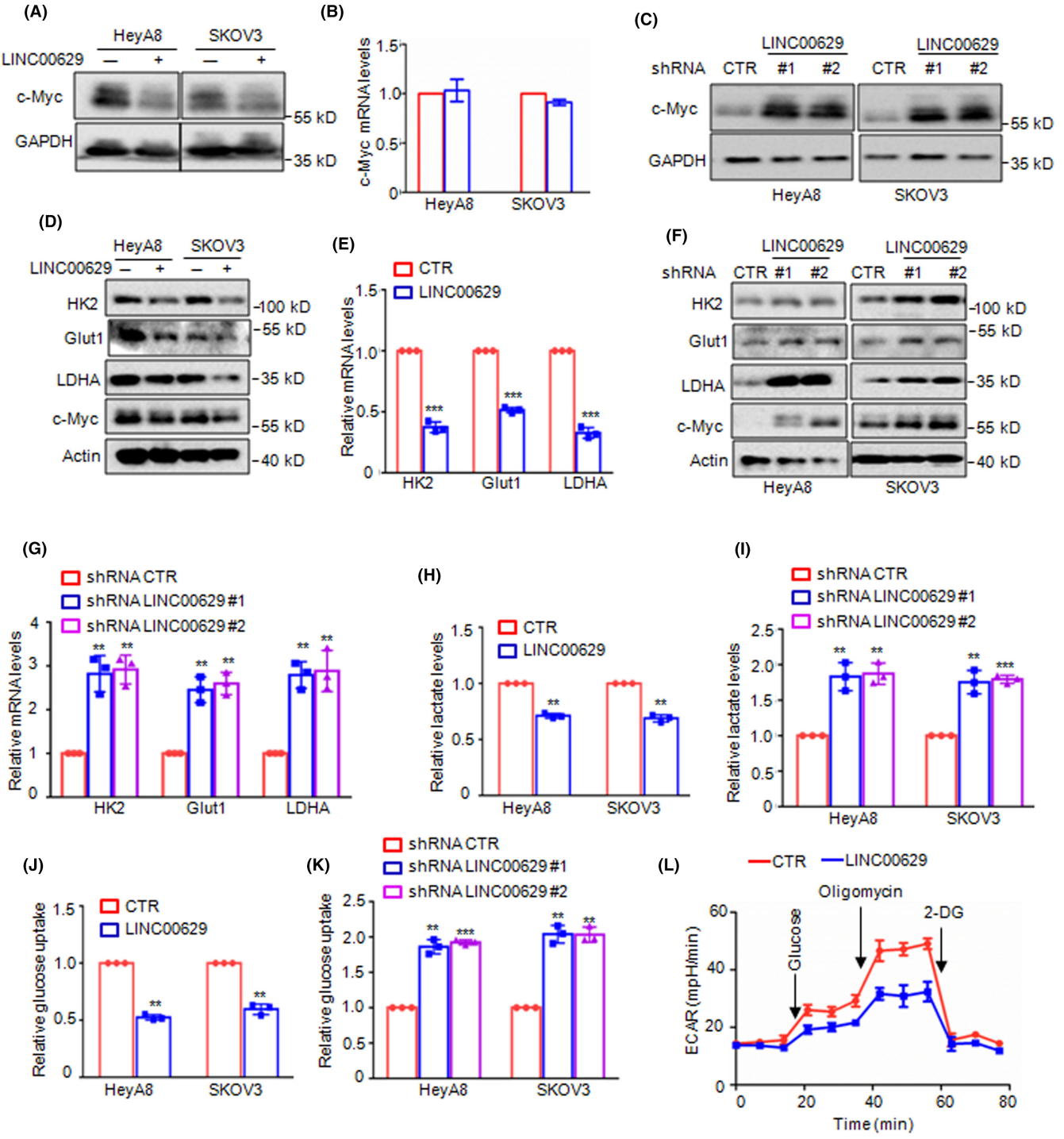


FIGURE 4 LINC00629 downregulates c-Myc expression and glycolytic reprogramming. (A) The expression levels of c-Myc were detected by western blotting in HeyA8 and SKOV3 cells with or without LINC00629 overexpression. (B) The mRNA levels of c-Myc were measured by qRT–PCR in HeyA8 and SKOV3 cells with or without LINC00629 overexpression. (C) The expression levels of c-Myc were detected by western blotting in HeyA8 and SKOV3 cells with or without LINC00629 knockdown. (D) The expression levels of HK2, Glut1, LDHA, and c-Myc were detected by western blotting in HeyA8 and SKOV3 cells with or without LINC00629 overexpression. (E) The mRNA levels of HK2, Glut1, and LDHA were measured by qRT–PCR in HeyA8 cells with or without LINC00629 overexpression. (F) The expression levels of HK2, Glut1, LDHA, and c-Myc were detected by western blotting in HeyA8 and SKOV3 cells with or without LINC00629 knockdown. (G) The mRNA levels of HK2, Glut1, and LDHA were measured by qRT–PCR in SKOV3 cells with or without LINC00629 knockdown. (H–I) The relative lactate levels were detected in HeyA8 and SKOV3 cells with or without LINC00629 overexpression or knockdown (H–I). (J–K) The relative glucose levels were detected in HeyA8 and SKOV3 cells with or without LINC00629 overexpression or knockdown. (L) The change in extracellular acidification rate levels was detected in HeyA8 cells with or without LINC00629 overexpression.

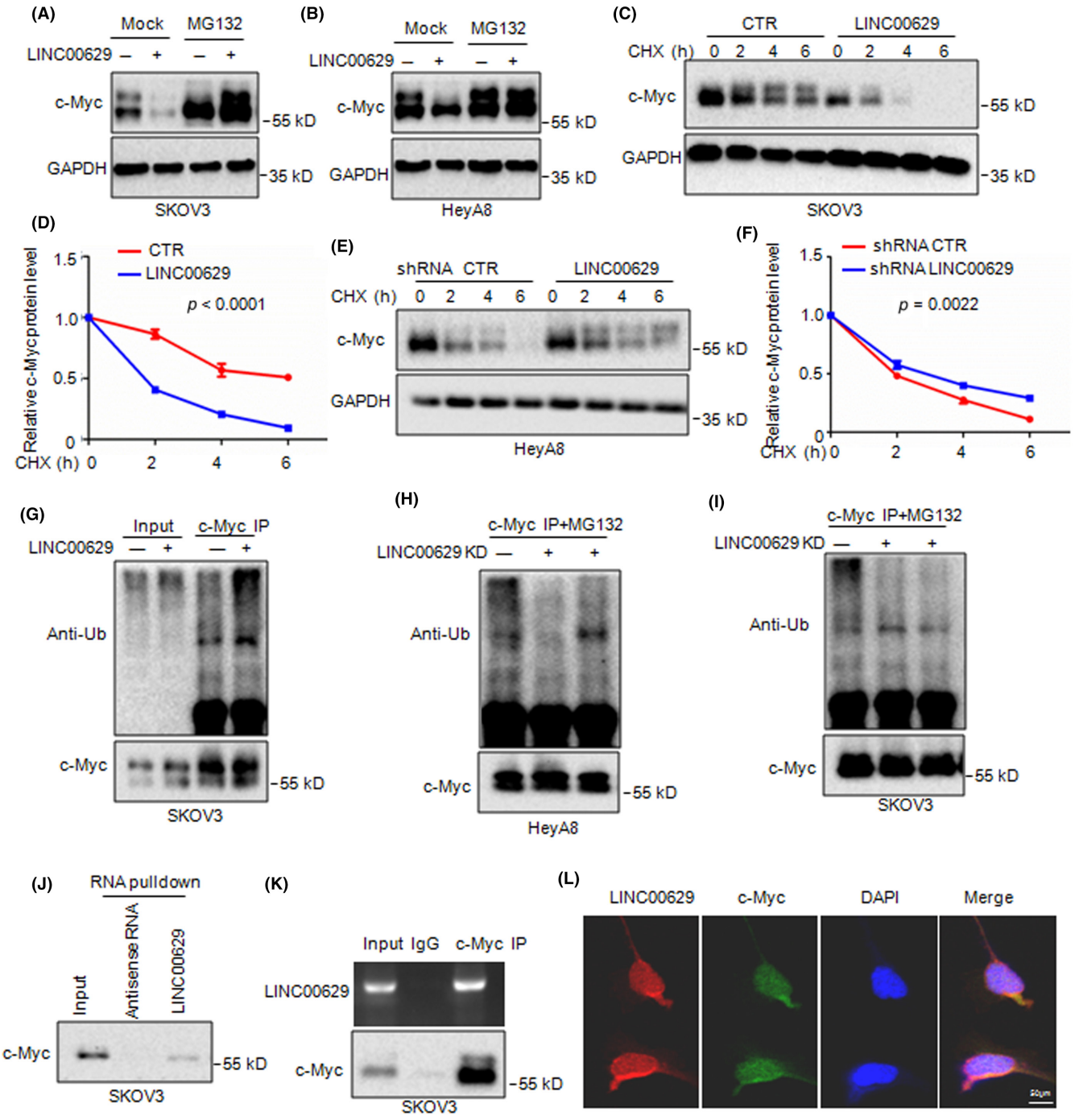
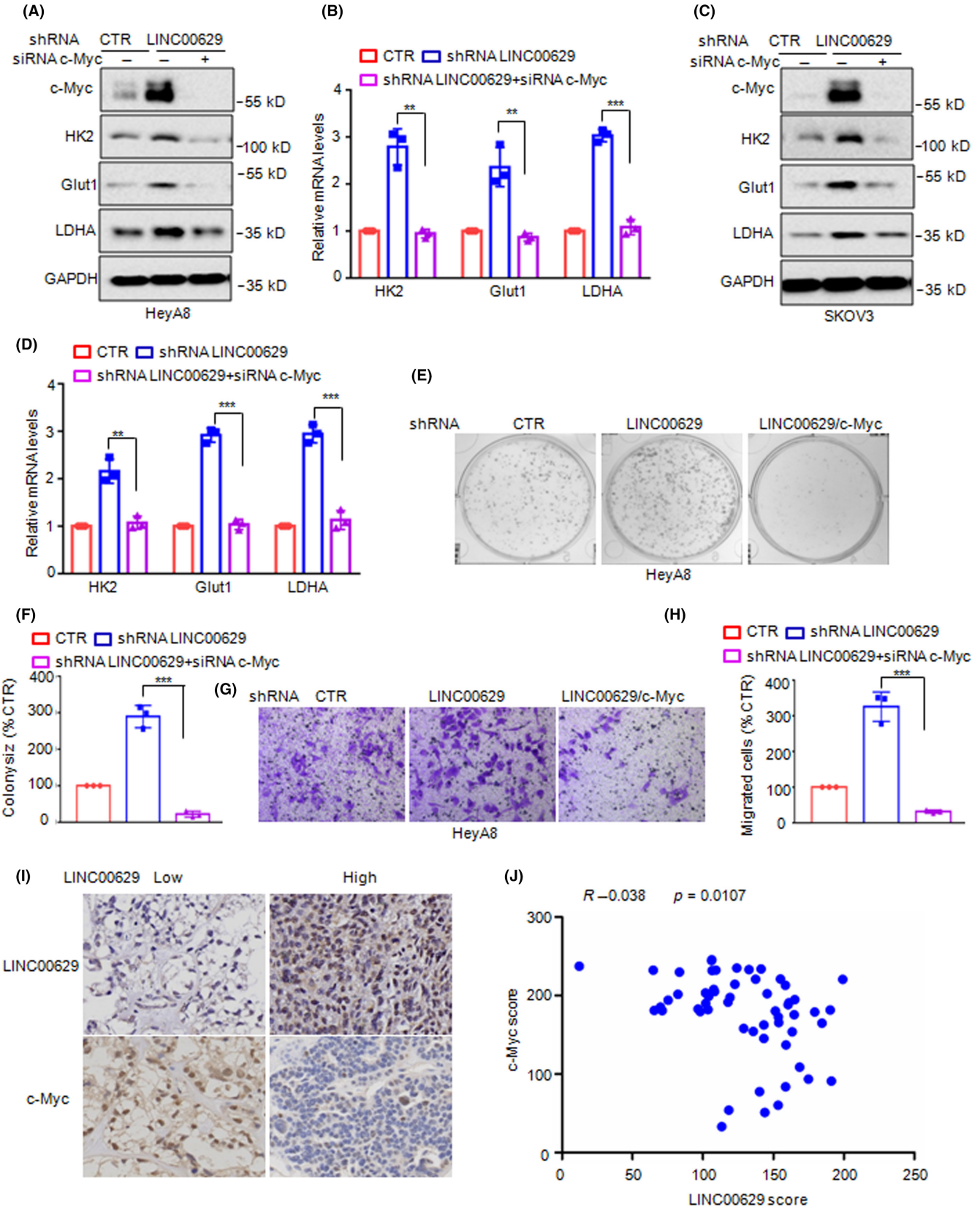


FIGURE 5 LINC00629 interacts with c-Myc and promotes its degradation. (A–B) SKOV3 and HeyA8 cells with or without LINC00629 overexpression were treated with 20 μM MG132 for 8 h. The expression levels of c-Myc were detected by western blotting. (C–D) SKOV3 cells with or without LINC00629 overexpression were treated with 10 mg/ mL cycloheximide (CHX) for the indicated times. The expression levels of c-Myc were detected by western blot (C), and the quantification of c-Myc levels relative to GAPDH is shown (D). (E–F) HeyA8 cells with or without LINC00629 knockdown were treated with 10 mg/mL cycloheximide (CHX) for the indicated times. The expression levels of c-Myc were detected by western blot, and the quantification of c-Myc levels relative to GAPDH is shown. (G) Whole-cell lysates were subjected to immunoprecipitation with a c-Myc antibody and western blotting with an anti-Ub antibody to detect ubiquitylated c-Myc. (H–I) HeyA8 and SKOV3 cells with or without LINC00629 knockdown were transfected with the indicated constructs and then treated with 20 μM MG132 for 8 h before collection. The whole-cell lysates were subjected to immunoprecipitation with a c-Myc antibody and western blotting with an anti-HA antibody to detect ubiquitylated c-Myc. (J) Biotin-labeled LINC00629 or antisense RNA was pulled down with c-Myc in whole-cell lysates of SKOV3 cells. c-Myc protein was detected by western blotting. (K) c-Myc antibody (2 μg) was used to coprecipitate with LINC00629 in whole-cell lysates of SKOV3 cells. The levels of LINC00629 were detected by RT–PCR, and c-Myc protein levels were analyzed by western blotting using a c-Myc antibody. (L) Immunofluorescence analysis showed that LINC00629 and c-Myc colocalized in SKOV3.



of LINC00629 inhibited intracellular lactate and glucose uptake. LINC00629 knockdown promoted intracellular lactate and glucose levels (Figure 4I,K). The extracellular acidification rate of OC cells was measured using a Seahorse XF24e Extracellular Flux to determine the glycolysis, glycolytic capacity, and glycolytic reserve of the indicated cells by measuring the extracellular acidification rate. The results showed that LINC00629 overexpression significantly impaired glycolysis capacity, further reducing the extracellular

FIGURE 6 LINC00629 suppressed the malignancy of ovarian cancer by downregulating c-Myc expression and negatively correcting c-Myc in tumor tissues. (A–D) LINC00629 was knocked down in SKOV3 and HeyA8 cells with or without c-Myc depletion. The expression levels of c-Myc, HK2, Glut1, and LDHA were detected by western blot and qRT–PCR. (E) LINC00629 was knocked down in HeyA8 cells with or without c-Myc depletion. These cells were then tested for cell growth in the colony formation assay. Viable colonies after 1 week were counted (shown here). (F) Data are depicted as bar graphs. (G) The migration of the indicated cells was detected by Transwell assays. Representative images of crystal violet-stained culture plates are shown. (H) Data are depicted as bar graphs. (I) Representative RNAScope ISH images for LINC00629 and immunohistochemical staining for c-Myc in ovarian cancer ($n = 56$). (J) Correlation analysis of LINC00629 with c-Myc in ovarian cancer tissues. Data in B, D, and H were analyzed by Student's *t*-test, $^{**}p < 0.01$, $^{***}p < 0.001$.

acidification rate (Figure 4L). Taken together, our data indicate that LINC00629 is functionally important in downregulating ovarian cancer metabolic reprogramming.

3.5 | LINC00629 interacts with c-Myc and promotes its degradation

Because depleted LINC00629 obviously reduced the protein level but not the mRNA level, we wanted to test whether LINC00629 decreased c-Myc expression in a proteasome-dependent manner. First, HeyA8 and SKOV3 cells were cultured with the proteasome inhibitor MG132, and the expression of c-Myc was examined by western blotting. As shown in Figure 5A,B, MG132 reversed the downregulation of c-Myc induced by LINC00629. Subsequently, to assess the alteration of the c-Myc half-life in LINC00629-overexpressing cells, ovarian cancer cells were treated with the protein synthesis inhibitor CHX, and we found that LINC00629 overexpression markedly decreased the stability of the endogenous c-Myc protein (Figure 5C,D). Inhibition of LINC00629 produced the opposite result (Figure 5E,F). Our further data indicated that elevated LINC000629 increased the ubiquitination of c-Myc (Figure 5G). However, the knockdown of LINC00629 decreased the ubiquitination of c-Myc in HeyA8 and SKOV3 cells (Figure 5H,I). Considering that LINC00629 impaired c-Myc protein expression, we aimed to determine whether LINC00629 interacts with c-Myc. To verify this hypothesis, an RNA pulldown assay proved that c-Myc could be precipitated by LINC00629 (Figure 5J). Conversely, an RNA immunoprecipitation (RIP) assay was performed to further demonstrate the interaction of LINC00629 with c-Myc (Figure 5K). Finally, an immunofluorescence colocalization assay revealed that LINC00629 and c-Myc colocalized mainly in the nucleus, with some colocalization in the cytoplasm, suggesting that the LINC00629/c-Myc combination might regulate cell proliferation and glycolysis in both the nucleus and cytoplasm (Figure 5L).

3.6 | LINC00629 suppressed the malignancy of ovarian cancer by downregulating c-Myc expression and negatively correcting c-Myc in tumor tissues

To determine whether LINC00629 depended on c-Myc in inhibiting tumorigenesis, we first inhibited c-Myc in LINC00629-depleted SKOV3 and HeyA8 cells. The expression levels of c-Myc and the downstream genes HK2, GLUT1, and LDHA were detected by western blotting and qRT-PCR (Figure 6A–D). The results indicated that inhibition of c-Myc eliminated the increase in HK2, GLUT1, and LDHA expression levels induced by LINC00629 knockdown. Similarly, depletion of c-Myc reversed the effects of LINC00629 knockdown on cell growth and migration (Figure 6E–H). Moreover, the expression of LINC00629 and c-Myc in OC tissues was examined by FISH and IHC. As expected, LINC00629 expression was negatively correlated with c-Myc expression in OC, further indicating the functional relationship between LINC00629 and c-Myc (Figure 6I,J).

3.7 | HOXB4 transcriptionally downregulates LINC00629 expression in ovarian cancer

Many transcription factors have been reported to exert a regulatory role in lncRNA biogenesis, including c-Myc.[24,25] To clarify the reason that LINC00629 was downregulated in OC, streptavidin/biotin pull-down assays and mass spectrometry assays were used to identify the LINC00629 promoter binding proteins. The transcription factor HOXB4 was selected as one possible candidate binding protein (Figure S4A,B). Research shows that HOXB4 is significantly overexpressed in ovarian cancer.[26] To verify the regulatory possibility, HOXB4 expression was first knocked down. Compared with the control cells, inhibition of HOXB4 notably promoted LINC00629 expression in both HeyA8 and SKOV3 cells (Figure 7A,B). Otherwise, overexpression of HOXB4 suppressed the expression of LINC00629 in the two ovarian cancer cell lines (Figure 7C,D). The binding site sequence of HOXB4 on the LINC00629 promoter was analyzed using JASPAR software (https://jaspar.genereg.net), and one positive binding site was identified. Two pGL3-based luciferase reporter plasmids containing the wild-type binding site (BS WT) and a mutant binding site (BS Mut) were constructed (Figure 7E,F). 293 T cells were individually transfected with these plasmids with or without HOXB4 overexpression. Subsequently, luciferase activity was measured. As shown in Figure 7G, the luciferase activities of WT were decreased in HOXB4-overexpressing 293 T cells but had no effect on Mut. Subsequently, we transfected the WT plasmid into HeyA8 and SKOV3 cells with or without HOXB4 overexpression or knockdown to further verify the previous results. As expected, as shown in Figure 7H,I, overexpression

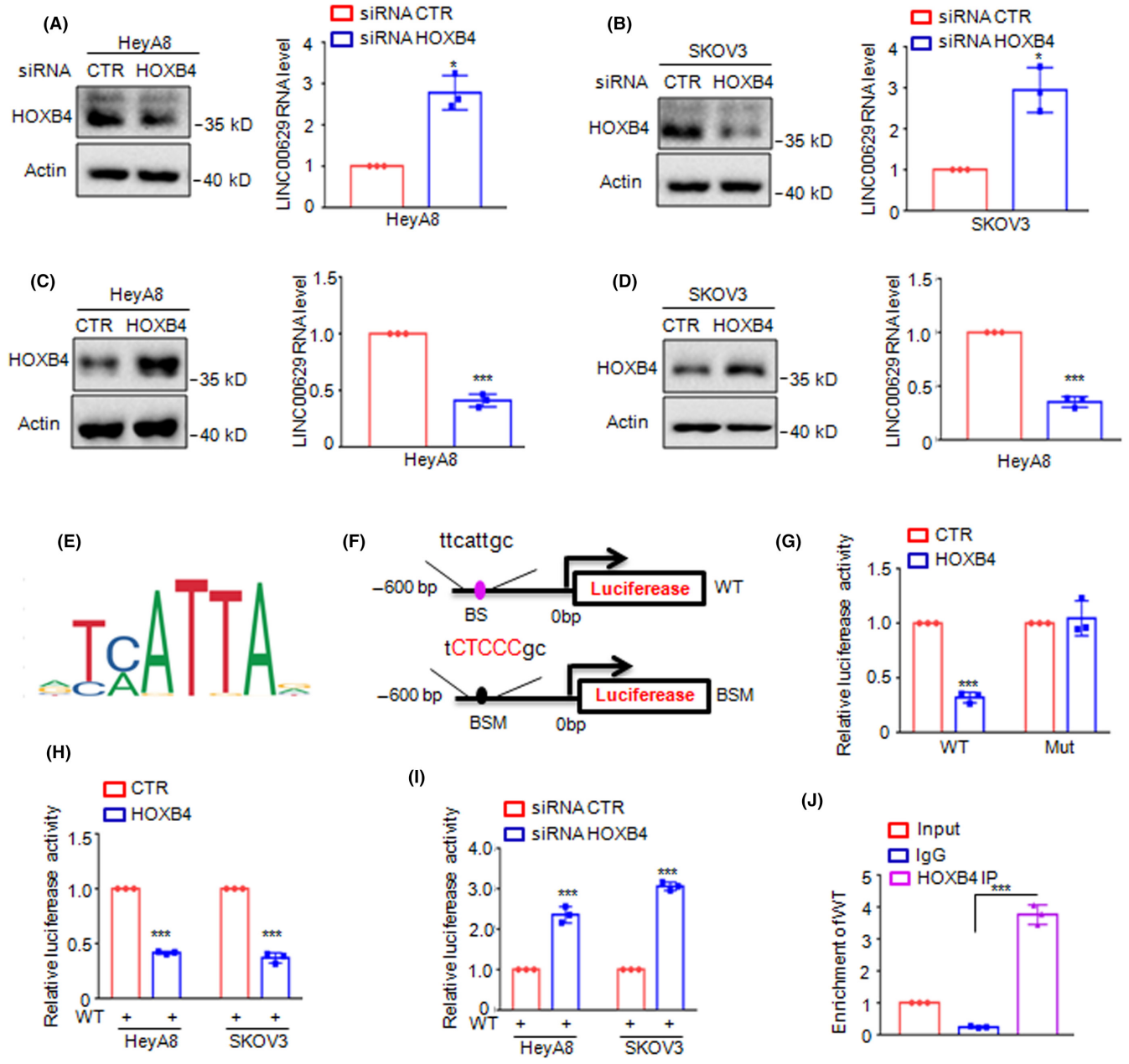


FIGURE 7 HOXB4 transcriptionally downregulates LINC00629 expression in ovarian cancer. (A–B) HOXB4 was knocked down in HeyA8 and SKOV3 cells, LINC00629 expression was measured by qRT–PCR, and the expression levels of HOXB4 were detected by western blotting. (C–D) HOXB4 was overexpressed in HeyA8 and SKOV3 cells, LINC00629 expression was measured by qRT–PCR, and the expression levels of HOXB4 were detected by western blotting. (E–F) Schematic illustration of the HOXB4 wild-type binding site (BS) and the matching mutant (BSM) that was used in luciferase assays. (G) The wild-type promoter (BS) or the matching mutant (BSM) together with the Renilla luciferase plasmid was individually transfected into 293T cells with or without HOXB4 overexpression. Dual luciferase activity was measured. (H) The wild-type promoter (BS) together with the Renilla luciferase plasmid was individually transfected into HeyA8 and SKOV3 cells with or without HOXB4 overexpression. Dual luciferase activity was measured. (I) The wild-type promoter (BS) together with the Renilla luciferase plasmid was individually transfected into HeyA8 and SKOV3 cells with or without HOXB4 knockdown. Dual luciferase activity was measured. (J) ChIP analysis showed the binding of HOXB4 to the promoter of LINC00629 in HeyA8 cells. Isotype-matched IgG was used as a negative control.

of HOXB4 decreased LINC00629 promoter luciferase activity, while HOXB4 knockdown had the opposite result. Chromatin immunoprecipitation (ChIP) assays further demonstrated that the binding potential of HOXB4 to the LINC00629 promoter was specific (Figure 7J). Taken together, these results indicate that HOXB4 inhibits the transcription of LINC00629.

3.8 | HOXB4 enhances ovarian cancer malignancy and c-Myc-mediated glycolytic reprogramming by suppressing LINC00629 expression

To determine whether HOXB4 downregulates c-Myc expression by regulating LINC00629, we elevated HOXB4 in HeyA8 cells with

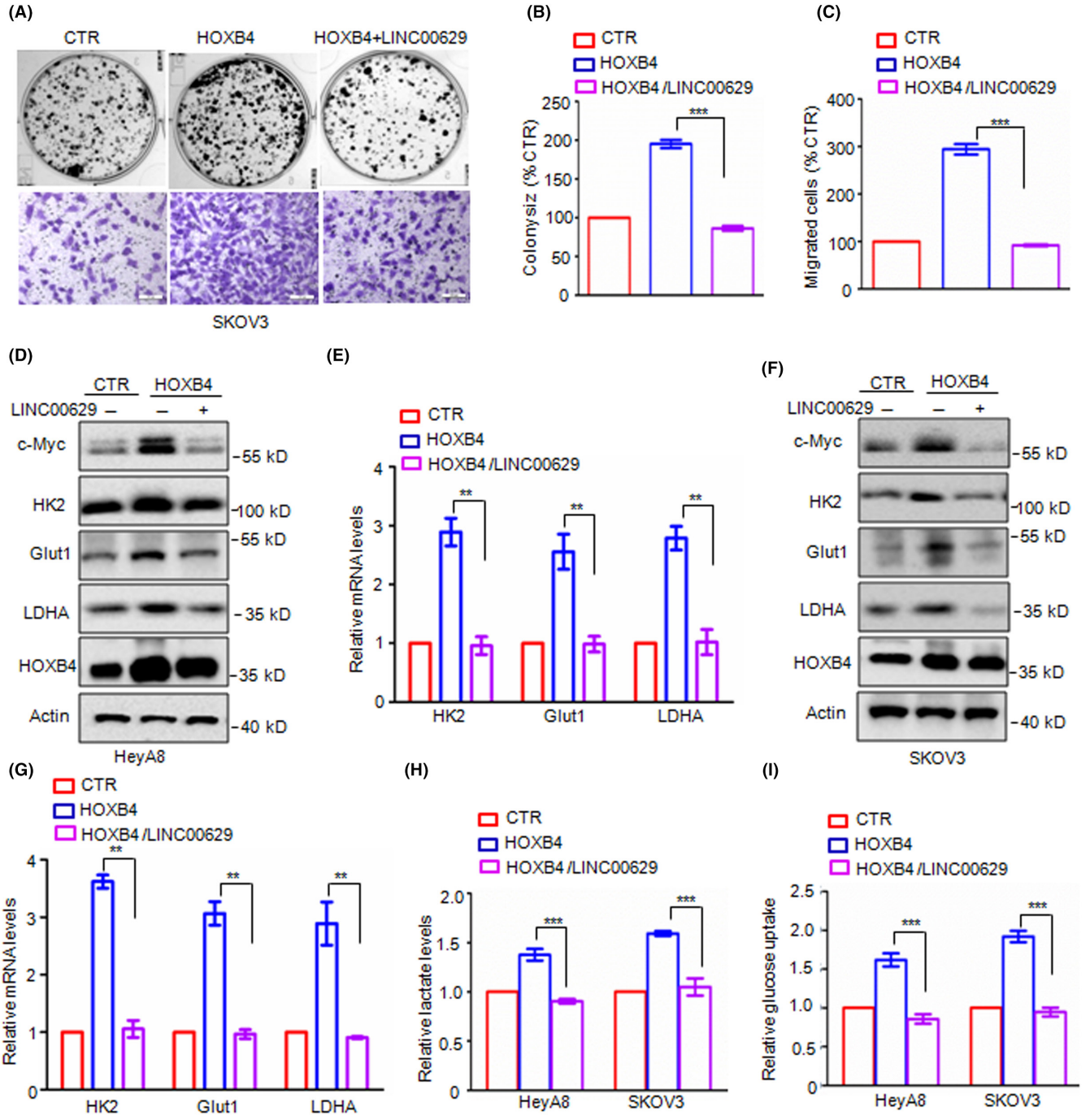


FIGURE 8 HOXB4 enhances ovarian cancer malignancy and c-Myc-mediated glycolytic reprogramming by suppressing LINC00629 expression. (A–C) LINC00629 was overexpressed in SKOV3 cells with or without HOXB4 overexpression. These cells were then tested for cell growth in the colony formation assay. Viable colonies after 1 week were counted and are shown. The migration of the indicated cells was detected by Transwell assays. Representative images of crystal violet-stained culture plates are shown. Data are depicted as bar graphs. (D–G) HOXB4, c-Myc, HK2, Glut1, and LDHA expression in SKOV3 and HeyA8 cells was determined by western blotting (D, F). HK2, Glut1, and LDHA mRNA levels in SKOV3 and HeyA8 cells were determined by qRT-PCR. (E,G). (H) The relative lactate levels were detected in HeyA8 and SKOV3 cells. (I) The relative glucose uptake levels were detected in HeyA8 and SKOV3 cells.

or without LINC00629 overexpression and found that HOXB4 increased the clonogenicity and migration of HeyA8 cells. However, the effects of HOXB4 on cell growth and migration were abolished by LINC00629 overexpression (Figure 8A–C). Subsequently, western blotting and qRT–PCR were performed to detect c-Myc and glycolysis-related genes, such as HK2, Glut1, and LDHA. The results showed that elevated HOXB4 promoted c-Myc, HK2, Glut1, and LDHA expression, which was then reversed by LINC00629 overexpression (Figure 8D–G). Consistently, the elevation of relative lactate levels and relative glucose uptake caused by HOXB4 were also

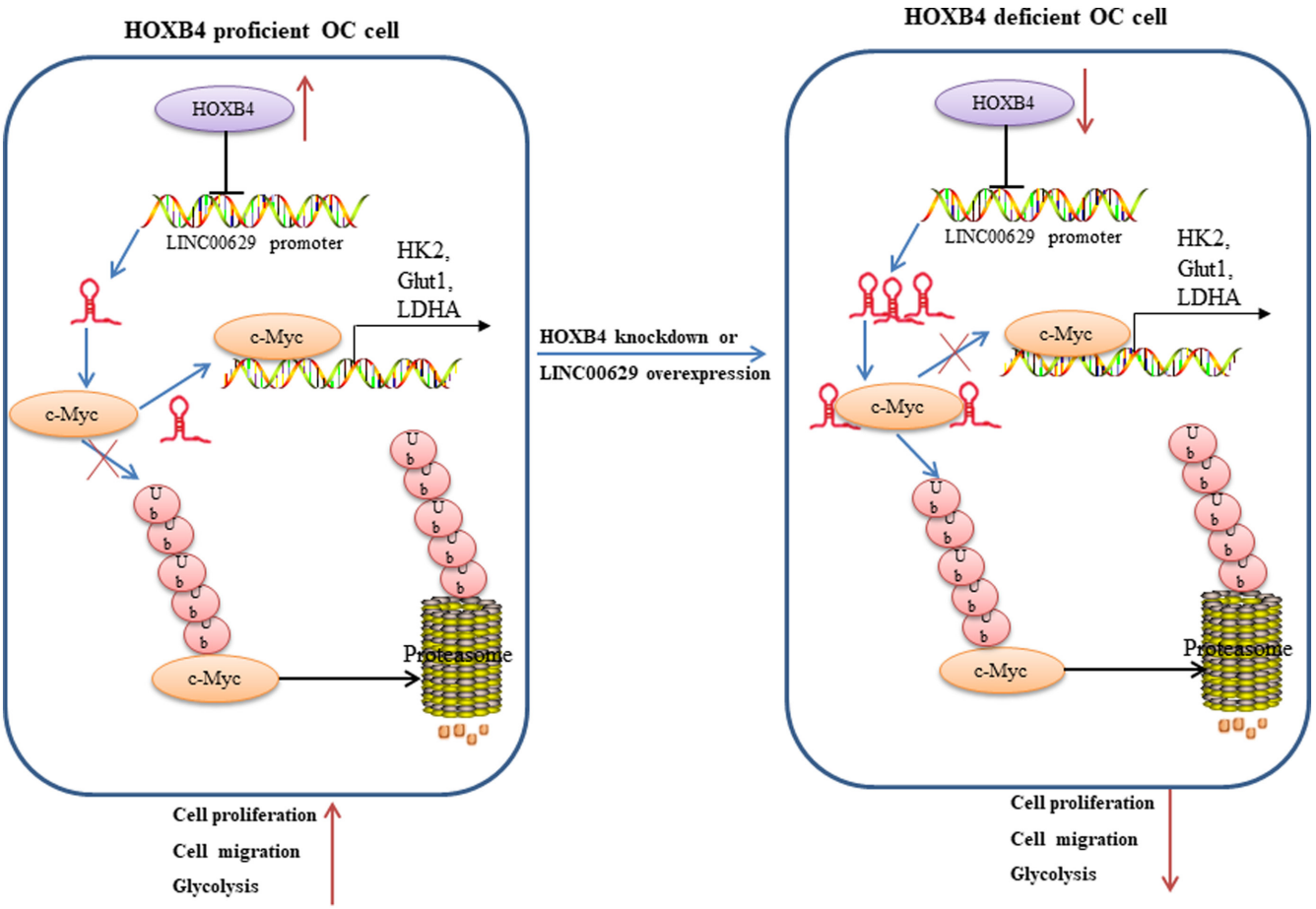


FIGURE 9 Proposed schematic diagram describing how LINC00629, a HOXB4-downregulated long noncoding RNA, inhibits glycolysis and ovarian cancer progression by destabilizing c-Myc.

abolished by LINC00629 overexpression (Figure 8H, I). Collectively, our data suggest that HOXB4 possibly enhances the malignancy of ovarian cancer and c-Myc-mediated glycolytic reprogramming by suppressing LINC00629 expression.

4 | DISCUSSION

Reprogramming glucose metabolism toward aerobic glycolysis endows tumor cells with unique metabolic pathways, which increase their biomass and sustain uncontrolled proliferation, contributing to cancer carcinogenesis.[27] However, the potential involvement of glycolytic metabolism in human ovarian cancer is poorly studied. Here, we found that the upregulation of LINC00629 mediated by HOXB4 inhibits cell proliferation by directly binding to and destabilizing the oncogene c-Myc, which regulates both glucose metabolism and cell proliferation (Figure 9).

LINC00629 is a long intergenic noncoding RNA mapped to chromosome X (Xq26). One study indicated that LINC00629 protects osteosarcoma cells from apoptosis and facilitates tumor progression by elevating the stability of KLF4.[15] LINC00629 also enhances asparagine synthesis by upregulating NUCKS1 expression.[17] Another study reported that LINC00629 exerts an anticancer function by upregulating AQP4 in gastric cancer.[28] Additionally, LINC00629 could promote sapigenin-induced cell apoptosis by decreasing Mcl1 expression.[16] Similarly, in our study, we found that LINC00629 plays a tumor-suppressive role in HeyA8 and SKOV3 cells based on the following evidence. First, LINC00629 is downregulated in ovarian cancer, and knockdown of LINC00629 promotes ovarian cancer cell proliferation, migration, and stemness in vitro, whereas overexpression of LINC00629 inhibits cell growth and stemness. Second, knockdown of LINC00629 promoted tumorigenesis and metastasis in vivo.

c-Myc expression is deregulated in multiple cancers, including ovarian cancer.[3,29] c-Myc plays a crucial role as a carcinogenic transcription factor in multiple processes of ovarian cancer, including glycolysis and stemness properties. lncRNAs have been reported to participate in regulating protein stability and modification.[30,31] Our label-free quantitative proteomics data first demonstrated that c-Myc is regulated by LINC00629. RNA pull-down data demonstrated that LINC00629 directly interacts with c-Myc and further destabilizes the c-Myc protein, thus decreasing the transcriptional and translational levels of HK2, Glut1, and LDHA. These genes promote glycolysis and are regulated by

c-Myc,[25,32] finally inactivating glycolytic metabolism. Three lines of experimental evidence supported this notion: (i) the expression level of c-Myc was reversed by the proteasome inhibitor MG132; (ii) overexpression of LINC00629 decreased the stability of endogenous c-Myc protein; and (iii) elevated LINC00629 increased the ubiquitination of c-Myc, while LINC00629 knockdown decreased the ubiquitination of c-Myc. RIP and RNA pulldown data support the binding of LINC00629 with c-Myc. Consistent with our observation, lncRNAs, OCC-1, and SDCBP-AS1 assist in destabilizing HuR and β-catenin, downregulating their protein and weakening their functions.[33,34] Briefly, LINC00629 is an lncRNA capable of binding to and increasing the ubiquitination level of c-Myc. Notably, LINC00629 can regulate downstream targets in many complicated ways due to its expression in both the cytoplasm and nucleus. For instance, LINC00629 can regulate AQP4 expression through sponge adsorption of miR-196b-5p, which might be involved in the progression of gastric cancer.[28] Therefore, there might be other signaling pathways mediating the function of LINC00629 in ovarian cancer, which remains to be investigated.

Another interesting discovery of ours was the transcriptional regulation of LINC00629 by HOXB4. Many important transcription factors have been indicated to regulate lncRNA expression in cancer, such as c-Myc and p53.[25,35,36] To elucidate the mechanism by which this factor increases the level of LINC00629 in OC cells, we screened the altered transcription factors by streptavidin/biotin pull-down assay and mass spectrometry. HOXB4 was found to have a binding site in the promoter region of LINC00629. HOXB4 is a transcription factor encoded by HOX family genes, which play a dual role in tumorigenesis as either a defensive or carcinogenic role in a number of cancers.[37] For example, in breast cancer and cervical cancer, HOXB4 was reported to inhibit cell proliferation.[38,39] However, research has shown that the expression of HOXB4 is upregulated in ovarian cancer tissue, and HOXB4-mediated upregulation of DHDDS promotes the malignancy of ovarian cancer.[40] HOXB4 knockdown also enhances the cytotoxic effect of paclitaxel and cisplatin by downregulating ABC transporters in ovarian cancer cells.[41] These studies demonstrate the cancer-promoting properties of HOXB4. In this study, we found that LINC00629 is a transcriptional target negatively regulated by HOXB4. LINC00629 overexpression reversed the tumor-promoting function of HOXB4 in upregulating cell proliferation and metabolism. Taken together, the imbalance of the HOXB4-LINC00629-c-Myc axis leads to metabolic remodeling and OC cell proliferation. HOXB4-LINC00629 could very possibly serve as a key candidate target for ovarian cancer diagnosis and treatment and further emphasizes the critical role of targeting of HOXB4 and LINC00629, sequentially or simultaneously, in ovarian cancer treatment.

However, our research has certain limitations. Ubiquitination or deubiquitination enzyme modification regulation is one of the main regulatory forms of c-Myc.[42–44] Although our study found that LINC00629 might promote c-Myc ubiquitination, a more detailed elaboration of the mechanism by which LINC00629 regulates the ubiquitination or deubiquitination of c-Myc needs further research.

AUTHOR CONTRIBUTIONS

Jia Liu: Conceptualization; data curation; formal analysis; investigation; software; writing – original draft. **Yuan Zhu:** Data curation; methodology; software; writing – original draft. **Huan Wang:** Data curation. **Chuanchun Han:** Data curation; investigation. **Yongpeng Wang:** Resources; supervision; writing – original draft. **Ranran Tang:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional reviewer board: N/A.

Informed Consent: Written informed consent was signed by each patient.

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

Human ovarian cancer sample studies: This sample collection was reviewed and ethically approved by the Ethics Committee of the Cancer Hospital of China Medical University (approval number: 20201145 K).

Animal Studies: This animal study was approved by the Medical Ethics Committee of the Dalian Medical University with procedure number AEE 21090.

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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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