

## Therapeutic Targeting of FGL1-Driven Lymph Node Metastasis in Stage T1 Non-Small Cell Lung Cancer

Mikko Lahtinen<sup>1\*</sup>, Elina Salo<sup>1</sup>, Juhani Virtanen<sup>2</sup>

<sup>1</sup>Department of Early-Stage Lung Cancer and Metastasis Research, Faculty of Medicine, University of Helsinki, Helsinki, Finland.

<sup>2</sup>Department of Lymph Node Metastasis and Therapeutic Targeting, Faculty of Medicine, University of Eastern Finland, Kuopio, Finland.

\*E-mail ✉ mikko.lahtinen@helsinki.fi

### Abstract

Roughly 30% of individuals with stage T1 non-small cell lung cancer (NSCLC) exhibit mediastinal (N2) lymph node metastasis, yet the specific biological processes responsible for this phenomenon are still not well understood. Single-cell sequencing was used to identify the specific cell population most likely responsible for driving N2 lymph node metastasis in T1 NSCLC. Single-cell analysis further evaluated the expression patterns and functional significance of the primary gene of interest, fibrinogen-like protein 1 (FGL1), within this cell subset. Integrated multi-omics approaches—including transcriptome sequencing, metabolome profiling, and mass spectrometry—were combined with both in vitro and in vivo functional assays. Therapeutic potential was evaluated through targeted knockdown using shFGL1\_AAV9 and shFGL1\_AAV6. A previously unrecognized cell population, marked by FGL1 expression (CCNE1(+) cells), was discovered. The emergence of this subgroup closely paralleled FGL1 upregulation, indicating that FGL1-positive cells play a central role in mediating N2 lymph node metastasis in T1 NSCLC. Through mass spectrometry, transcriptional profiling, and metabolomic analyses, FGL1 was shown to modulate key glycolysis-related factors and drive epithelial-to-mesenchymal transition (EMT) in NSCLC cells primarily via activation of the PI3K/AKT/HIF-1 $\alpha$  signaling axis. Additional experiments demonstrated that FGL1 enhances tumor cell proliferation, migratory and invasive capabilities, and lymphatic vessel formation, thereby facilitating lymph node metastatic spread. These effects were consistently confirmed across in vitro and in vivo models. Conversely, FGL1 depletion effectively suppressed these malignant behaviors. In vivo validation confirmed that shFGL1\_AAV9 and shFGL1\_AAV6 serve as effective targeted delivery systems for FGL1 silencing, highlighting promising new therapeutic avenues to block NSCLC progression and metastasis. This study clarifies the critical contribution of FGL1 to NSCLC pathobiology, revealing its function as a “shield machine cutter” that promotes T1 NSCLC N2 lymph node metastasis by inducing lymph tube formation and establishing metastatic routes. These findings lay the groundwork for the development of innovative treatment strategies focused on targeting FGL1.

**Keywords:** N2 lymph node metastasis, T1 NSCLC, FGL1, CCNE1(+) cells, Single-cell sequencing, ShFGL1\_AAV6, ShFGL1\_AAV9

### Introduction

Lung cancer represents the leading cause of cancer-related incidence and mortality globally [1, 2].

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Individuals diagnosed with early non-small cell lung cancer (NSCLC), notably stage I cases where the primary tumor measures 3 cm or smaller (T1) and shows no evidence of spread (N0M0), typically experience excellent curative outcomes. Nonetheless, real-world clinical data indicate that nearly 30% of patients with T1 NSCLC still experience mediastinal (N2) lymph node involvement and distant metastases (M1) [3].

Contemporary research exploring the molecular drivers of tumor metastasis to lymph nodes has concentrated on several core themes: how cancer cells adapt their

metabolism to thrive in the lymph node environment [4], the crosstalk between chemokine receptors on malignant cells and their ligands present in nodal tissues, characteristic immunogenomic profiles tied to nodal dissemination [5, 6], and the ability of tumors to directly stimulate lymphangiogenesis [7]. Despite these insights, investigations specifically addressing the processes of lymph node metastasis or disease relapse in T1-stage neoplasms are relatively sparse. The existing literature has largely focused on identifying clinical risk factors and developing predictive models for nodal metastasis and recurrence in T1 tumors. As one example, pro-surfactant protein B expression strongly correlates with relapse in early NSCLC, and its suppression promotes recurrence via phosphoglycerate kinase 1-triggered AKT pathway activation [8]. Deep submucosal invasion has likewise been established as an independent predictor of lymph node metastasis in T1 colorectal cancer, and nomogram systems have been developed to predict it [9, 10]. Parallel modeling strategies have been utilized in T1 breast cancer research [11].

However, the detailed molecular pathways underlying lymph node metastasis in T1 tumors, particularly the underpinnings of N2 lymph node spread in stage T1 NSCLC, remain incompletely understood. Immune checkpoint proteins are recognized for advancing tumor malignancy by modulating the surrounding immune milieu and fostering aggressive traits such as uncontrolled proliferation and invasive spread [12]. Prominent examples encompass programmed cell death 1 (PD-1) [13-15], poliovirus receptor (PVR) [16, 17], SIRPA [18, 19], among others. Their specific contributions to N2 lymph node metastasis in stage T1 NSCLC have not been established. Thus, clarifying these mechanisms carries major importance for enhancing diagnostic accuracy, refining treatment options, and improving prognostic surveillance for affected patients. The current investigation used single-cell RNA sequencing (scRNA-seq) to examine tissues from T1N0M0 and T1N2M0 NSCLC cases, as well as matched N2 lymph node metastatic specimens. This approach uncovered a distinct, previously unidentified cell population designated as CCNE1(+) cells. These cells displayed prominent fibrinogen-like protein 1 (FGL1) expression, suggesting that FGL1 participates in N2 lymph node metastasis in stage T1 NSCLC, functioning like a “cutter head” within a shield machine. To test this concept, extensive laboratory (in vitro) and animal-based (in vivo) studies were executed. Results

showed that FGL1 drives enhanced glycolysis and epithelial-mesenchymal transition (EMT) by activating the phosphatidylinositol 3-kinase (PI3K)/AKT/hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) signaling pathway, thereby boosting metastatic capacity. Targeted interventions using shFGL1\_AAV6 and shFGL1\_AAV9 demonstrated substantial potential to restrain tumor expansion and dissemination. An overview of the experimental framework is presented in **Figure 1a**. In summary, the work outlines an FGL1-dependent pathway for N2 lymph node metastasis in stage T1 NSCLC and introduces a targeted therapeutic strategy against FGL1.

## Materials and Methods

### Mice

Four-week-old male BALB/c nude (BALB/cJGpt-Foxn1nu/Gpt) mice and NCG (NOD/ShiLtJGpt-Prkdcem26Il2rg em26/Gpt) mice were sourced from GemPharmatech Co., Ltd (Nanjing, China). Every in vivo procedure was reviewed and approved by the Ethics Committee of the Air Force Medical University (approval number 20220666).

### Cell lines

All cell lines were obtained from Wuhan Pricella Biotechnology Co., Ltd. (Wuhan, China). H226, H1299, and H1703 lines were propagated in Gibco RPMI-1640 medium (Cat# 11875119, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum. A549 cells were maintained in Ham's F-12K medium (Cat# PM150910B, Pricella, China) plus 10% fetal bovine serum (Cat# BS-1101, OPEL, China). Lewis lung carcinoma (LLC) cells were grown in high-glucose Dulbecco's modified Eagle medium (Cat# PM150210, Pricella, China) supplemented with 10% fetal bovine serum. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### NSCLC sample collection

The study assembled 316 NSCLC tissue specimens (comprising 158 adenocarcinomas, 133 squamous cell carcinomas, and 25 adenosquamous carcinomas) paired with adjacent normal tissues, supplemented by 15 T1N0M0 and 15 T1N2M0 NSCLC samples collected during surgeries at Tangdu Hospital (Xi'an, China). Eligible participants supplied written informed consent permitting the acquisition and analysis of their tissues and associated clinical information. All protocols

involving human materials were authorized by the Ethics Committee of the Air Force Medical University (No. 202203-040).

#### *Immunohistochemistry and immunofluorescence*

Protocols for immunohistochemistry (IHC) and immunofluorescence (IF) staining were conducted according to methods outlined in prior publications [20]. Antibodies deployed in the analyses included anti-FGL1 polyclonal antibody (Cat# 16000-1-AP, Proteintech, China), anti-Cyclin E1 polyclonal antibody (Cat# 40804, Signalway Antibody, China), GLUT1 polyclonal antibody (Cat# 40967, Signalway Antibody, China), GLUT3 Rabbit polyclonal antibody (Cat# 55439, Signalway Antibody, China), PGAM1 Rabbit monoclonal antibody (Cat# 49976, Signalway Antibody, China), LDHA antibody (Cat# 48492, Signalway Antibody, China), LDHB antibody (Cat# 48009, Signalway Antibody, China), PDK1 Rabbit mAb (Cat# 49573, Signalway Antibody, China), HK2 antibody (Cat# 32115, Signalway Antibody, China), TGF- $\beta$  (Cat# 21898-1-AP, Proteintech, China), Twist (Cat# 25465-1-AP, Proteintech, China), Slug (Cat# 53574, Signalway Antibody, China), Snail (Cat# 54899, Signalway Antibody, China), Vimentin (Cat# 10366-1-AP, Proteintech, China), and E-cadherin (Cat# 60335-1-Ig, Proteintech, China).

#### *Chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR)*

ChIP experiments utilized a dedicated kit (Cat# Bes5001, BersinBio, China) in conjunction with a specific anti-FGL1 antibody (Cat# 16000-1-AP, Proteintech, China). Enriched DNA fragments were quantified via qPCR. Primer sequences targeting the ETS1 and FGL1 promoter region primer 1 are listed below:

AATAATGTTACTCCAAGAAAATTTGGCTTTTGG  
AATTCCTCCACAAAGTATTGGCCCTAGAGTAGT  
CCTTACTGCCTGATTAACCTTTTGCAAGTTTCTCC  
TGAGGTAATGATGCTAGCCAAGTTTCCGAAAGA  
CAGCATAGTCTACCTTTAGTAGAAAAGAGGAC  
AGATTCTGAGCAGGATTGCCTTTGAGCCCAGCT  
ATACCTCTTGTTAGCTGTGTGACCTCGTGCAAG  
TCACTTAAACCTCTGTGTCTCAGTTTCGTCATTT  
GTGAAATGGAGATCATTAACAGTACCTACTTAT  
AG.

The sequence for the FGL1 promoter region primer 3 is: TATAGGGCTGTGTGGCTGAGTGAGTTGATAAAA  
TTACCATGTTTAGCACATGATAAGTATACCACA

TGTTAGCTGCTGTTAATGTTATGTCCCTCCAGTG  
AGGAAATGAACACAAGGCTAAATTATTGCTTG  
AATGGGGACAAAGCTAAAGGCATACAAGAAGG  
AAAGATAAGTTTGTATTTCAAATATGTCATTT  
TACCACAGAGATCATTATTGAATTAGCCGAATA  
ATCCCATATTTTGTCTTTGAAATTTAACATTAG  
CAGCTTGAATTCTCTACAATTATGAATGTATAA  
TTT.

#### *Luciferase reporter assay*

Mutations were introduced at three ETS1-binding sites in the FGL1 promoter region. FGL1-promoter-WT/Renilla and FGL1-promoter-MT/Renilla reporter plasmids were introduced into 293T cells via Invitrogen Lipofectamine 2000 (Cat# 11668019, Thermo Fisher Scientific, China). Promoter-driven luciferase activity was quantified using the Dual-Glo Luciferase Assay Kit (Cat# E1910, Promega, China).

#### *Immunoprecipitation and mass spectrometry*

FGL1-interacting proteins were isolated with a total protein extraction kit (Cat# SD-001/SN-002, Invent Biotechnologies, China) according to supplied instructions. Cells were lysed in SD-001 buffer on ice for 10 minutes, centrifuged at 12,000 rpm for 30 seconds, and the supernatant was incubated with anti-FGL1 antibody for 8 hours at 4 °C. Protein A/G agarose beads were then added for 2 hours. Precipitated material was subsequently submitted for mass spectrometric analysis performed by Beijing Tsingke Biotechnology Co., Ltd (Beijing, China).

#### *FGL1 knockdown*

Stable silencing of FGL1 in A549, H226, H1299, H1703, and LLC cell lines was achieved using specific short hairpin RNAs (shRNAs) obtained from HanBio Therapeutics (Shanghai, China). Transfection was facilitated with polybrene (2 mg/mL). Cells at approximately 70% confluence in 6-well plates were transfected for 24 hours, followed by puromycin-based selection of stable clones. The shRNA targeting sequence employed for FGL1 knockdown matches that detailed in our prior publication [21].

#### *Single-cell sequencing and analysis*

Ten NSCLC tissue specimens were processed for single-cell RNA sequencing, consisting of four T1N0M0 samples, three T1N2M0 samples, and three N2 lymph node metastatic samples derived from T1N2M0 patients.

All specimens were enzymatically dissociated to generate single-cell suspensions. Identification of major cell populations and their subpopulations, along with 10× Genomics library preparation and subsequent scRNA-seq data processing, was carried out by LC BioTechnology Co., Ltd (Hangzhou, China).

#### *RNA-sequencing and differential expression analysis*

H226 and A549 cells ( $1 \times 10^7$  cells each) were plated, followed by total RNA isolation with TRIzol reagent (Cat# MF035-01, Mei5bio, China). The extracted RNA underwent RNA-seq profiling. Downstream bioinformatics evaluation incorporated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping, Gene Ontology (GO) categorization, and Gene Set Enrichment Analysis (GSEA) to identify significantly altered genes. Sequencing and analysis for H226 cells were performed by LC BioTechnology Co., Ltd (Hangzhou, China), while those for A549 cells were handled by Beijing Tsingke Biotechnology Co., Ltd (Beijing, China).

#### *Metabonomic analysis*

A549 cells ( $1 \times 10^7$  cells) were harvested, and the resulting pellets were subjected to untargeted metabolomics profiling by Beijing Tsingke Biotechnology Co., Ltd. Comprehensive examination of differentially abundant metabolites was conducted through KEGG, GO, and GSEA frameworks.

#### *Western blotting*

Protein level alterations after FGL1 silencing were investigated by Western blot, with particular emphasis on epithelial-mesenchymal transition (EMT) indicators, glycolysis enzymes, and components of the PI3K/AKT signaling cascade. The primary antibodies applied were: TGF- $\beta$  (Cat# 21898-1-AP, Proteintech, China), Twist (Cat# 25465-1-AP, Proteintech, China), Slug (Cat# 53574, Signalway Antibody, China), Snail (Cat# 54899, Signalway Antibody, China), Vimentin (Cat# 10366-1-AP, Proteintech, China), E-cadherin (Cat# 60335-1-Ig, Proteintech, China), HIF-1 $\alpha$  (Cat# 41005, Signalway Antibody, China), PDK1 (Cat# 49573, Signalway Antibody, China), GLUT1 (Cat# 40967, Signalway Antibody, China), GLUT3 (Cat# 55439, Signalway Antibody, China), HK2 (Cat# 32115, Signalway Antibody, China), PGAM1 (Cat# 49976, Signalway Antibody, China), LDHA (Cat# 48492, Signalway Antibody, China), LDHB (Cat# 48009, Signalway

Antibody, China), dihydrolipoamide dehydrogenase (DLDH) (Cat# 56779, Signalway Antibody, China), AKT (Phospho-Ser473) (Cat# 11054, Signalway Antibody, China), AKT (Phospho-Thr308) (Cat# 11055, Signalway Antibody, China), AKT (Cat# 33748, Signalway Antibody, China), and PI3K (Cat# 41337, Signalway Antibody, China). The Western blotting procedure followed established methods described earlier [22].

#### *Glucose consumption, lactate production, and pyruvate production assays*

Cells were plated at  $3 \times 10^5$  cells per well in 24-well plates containing 300  $\mu$ L of growth medium and incubated for 24 hours. Culture supernatants were harvested and spun at 3000 rpm for 10 minutes. Levels of glucose, lactate, and pyruvate were quantified using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

#### *Lymph tube formation assay*

Twenty-four-well plates were pre-coated with poly-L-lysine solution (Cat# PB180522, Pricella, China). Matrigel matrix (Cat# 354234, Corning, China; 150  $\mu$ L per well) was added and allowed to solidify at 37 °C for 30 minutes. Lymphatic endothelial cells (Cat# CP-H026, Pricella, China; 50,000 cells in 600  $\mu$ L medium) were seeded in the lower compartment. In the upper chamber, 50,000 A549 or H1703 control (NC) cells, or their shFGL1 counterparts, were added in 200  $\mu$ L of medium to establish indirect co-culture conditions. Lymphatic vessel network formation was then monitored and documented.

#### *Tumor proliferation assays*

A549, H226, H1299, and H1703 cells were collected by centrifugation at 1,000 rpm for 5 minutes. For proliferation assessment, 1,000 cells per well were seeded in sextuplicate into 96-well plates with 100  $\mu$ L RPMI-1640 medium. After 48 hours, the medium was exchanged: the treatment arm received medium supplemented with 9 mM 2-Deoxy-D-glucose (2-DG; Cat# T6742, TargetMol, China), while controls continued with standard medium. Cell viability was evaluated using Cell Counting Kit-8 (CCK-8; Cat# C6005, New Cell & Molecular Biotech Co. Ltd, China) according to the supplier's protocol, with absorbance readings at 450 nm.

Separately, 1,000 cells were plated in 6-well dishes and grown for two weeks to evaluate colony-forming ability. In the 2-DG group, medium containing 9 mM 2-DG was introduced after the initial 4 days, whereas control wells received fresh regular medium. At endpoint, plates were rinsed twice with phosphate-buffered saline, fixed in paraformaldehyde for 20 minutes, and stained with 1% crystal violet for 30 minutes.

#### *Tumor migration assays*

A549, H226, H1299, and H1703 cells were grown to 80–90% confluence in 10 cm dishes, after which a scratch wound was created and images recorded over 48 hours. Following wounding, the 2-DG treatment group was switched to medium containing 9 mM 2-DG, while controls received standard medium.

For Transwell invasion/migration assays, 70,000 cells were loaded into the upper chamber. The upper compartment received 200  $\mu$ L serum-free RPMI-1640 (with 10  $\mu$ L of 180 mM 2-DG added for the treatment group), and the lower chamber was filled with 600  $\mu$ L RPMI-1640 containing 20% serum (plus 30  $\mu$ L of 180 mM 2-DG for the treatment group). After 24–48 hours, migrated cells were fixed with paraformaldehyde for 20 minutes and stained with 1% crystal violet for 30 minutes.

#### *Xenograft tumor experiments*

Male BALB/c nude mice (BALB/cJGpt-Foxn1nu/Gpt; 4 weeks old, 15–20 g) were acclimated for one week in a specific pathogen-free facility. Animals were randomly divided into groups and received subcutaneous injections into the forelimb with  $8 \times 10^6$  H226 or A549 cells, or  $8 \times 10^5$  LLC cells, either control (FGL1\_NC) or FGL1-knockdown (FGL1\_KD). Tumor dimensions were measured every 2–3 days, and volume ( $\text{mm}^3$ ) calculated as  $(\text{length [mm]} \times \text{width}^2 [\text{mm}^2]) / 2$ .

In a separate lymph node metastasis model,  $8 \times 10^5$  modified LLC cells (FGL1\_NC or FGL1\_KD) were inoculated into the footpad of similarly aged male BALB/c nude mice. After 14 days, tumor growth and metastatic spread were monitored via small-animal imaging. Inguinal and popliteal lymph nodes were subsequently excised and examined for metastatic deposits by hematoxylin and eosin (HE) staining.

#### *LLC cell in situ lung injection and shFGL1-AAV6 non-invasive tracheal injection*

Four-week-old male NCG mice (NOD/ShiLtJGpt-Prkdcem26Il2rgem26/Gpt) were anesthetized, and a small skin incision was made to expose the intercostal space. Luciferase-tagged LLC cells ( $5 \times 10^5$  cells in 100  $\mu$ L phosphate-buffered saline) were injected directly into the subpleural lung parenchyma using a 1-mL syringe.

Tumor establishment was confirmed by small-animal imaging, and shFGL1-AAV6 treatment was initiated 2 days later. Mice were anesthetized with isoflurane and positioned vertically by suspending them from the upper incisors with a fine thread. A suitable catheter was advanced into the trachea, and 50  $\mu$ L of HBAAV2/6-h-FGL1 shRNA1\_EGFP ( $1.2 \times 10^{12}$  vg/mL, produced by HanBio Therapeutics) was instilled and gently inhaled into the lungs.

#### *shFGL1-AAV9 intratumor injection targeted therapy*

Male BALB/c nude mice (4 weeks old, 15–20 g) were acclimated for one week in a specific pathogen-free environment. Mice were randomized and injected subcutaneously in the forelimb with  $8 \times 10^6$  H226 or A549 cells, or  $8 \times 10^5$  LLC cells. Once tumors reached 100–150  $\text{mm}^3$ , HBAAV2/9-h-FGL1 shRNA1\_EGFP ( $1.2 \times 10^{12}$  vg/mL, synthesized by HanBio Therapeutics) was administered via multi-point intratumoral injection (40  $\mu$ L total volume). Tumor growth kinetics were tracked until volumes approached 1,000  $\text{mm}^3$ . Upon reaching this size in control animals, tumors were excised for IHC analysis to assess the impact of targeted therapy on proliferation.

In parallel footpad experiments,  $8 \times 10^5$  LLC cells were injected, followed by intralesional shFGL1-AAV9 (20  $\mu$ L) every 4 days once tumors formed. 2-DG was given intraperitoneally at 1,800 mg/kg every other day. Combined treatment efficacy was evaluated by small-animal imaging. After 14 days, inguinal and popliteal lymph nodes were harvested, and metastatic involvement was evaluated by HE staining.

#### *Quantification and statistical analysis*

All quantitative results are expressed as mean  $\pm$  standard error of the mean. Statistical computations and figure preparation were performed using GraphPad Prism 8.0 (GraphPad Software, USA). Image quantification and cell counts were performed using ImageJ (National Institutes of Health). Differences between groups were analyzed by unpaired t-tests for biological triplicates in both in vitro and in vivo experiments. The Wilcoxon

signed-rank test was applied for datasets with sample sizes exceeding 60.

## Results and Discussion

### *Single-cell sequencing identifies a distinct CCNE1(+) cell population driving lymph node metastasis*

A previously unrecognized cell population, designated CCNE1(+) cells, emerged as a key contributor to lymphatic dissemination in stage T1 NSCLC. Seven NSCLC specimens underwent 10× Genomics single-cell RNA sequencing, comprising four T1N0M0 and three T1N2M0 cases. In total, 72,401 individual cells were resolved into nine major categories: B cells, endothelial cells, epithelial cells, fibroblasts, mast cells, myeloid cells, natural killer T (NKT) cells, plasma cells, and T cells (**Figure 1b**).

Further subdivision revealed 5,983 B cells grouped into memory, naïve, plasma, and plasmacytoid dendritic cell subsets. The 7,071 myeloid cells were resolved into cDC\_type 1, cDC\_type 2, cDC\_type 3, M1 and M2 macrophages, and neutrophils. Among 6,271 fibroblasts, seven subtypes were distinguished: antigen-presenting cancer-associated fibroblasts (CAFs), general CAFs, inflammation-associated fibroblasts, myofibroblasts, normal fibroblasts, proliferative fibroblasts, and pericytes. The 3,278 endothelial cells (ECs) were classified as alveolar, extra-alveolar, arterial, lymphatic, tumor-associated, and venous ECs. Finally, 29,131 T cells were partitioned into effector CD8+, follicular CD4+, naïve CD4+, regulatory (Treg) CD4+, tissue-resident memory CD4+, tumor Treg, and  $\gamma\delta$  T cell populations.

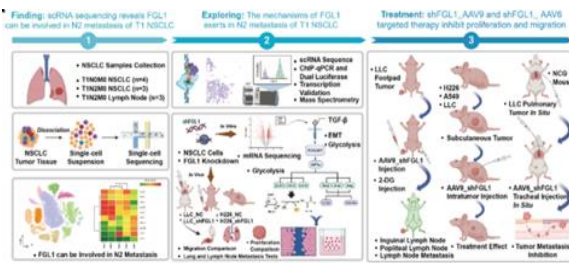
CCNE1(+) cells were markedly enriched in the more aggressive T1N2M0 tumors. Epithelial cells (14,232 total) segregated into eight distinct clusters: alveolar type 1 (AT1), AT2, basal, ciliated, club, cystatin-SN (CST1+), goblet, and CCNE1(+) cells (**Figure 1c**). Clusters 5, 6, and 16 displayed substantially higher abundance in T1N2M0 samples. Given their prominent and selective expression of CCNE1—a gene critically involved in driving tumor aggressiveness [23]—this population was termed CCNE1(+) cells. Characteristic marker genes for these cells included CENPF, CCNE1, STMN1, TOP2A, TPX2, and PBK, and they were confined exclusively to the epithelial compartment. Quantitative comparison confirmed a significantly elevated proportion of CCNE1(+) cells in T1N2M0 versus T1N0M0 specimens (**Figure 1d**).

Functional annotation via KEGG pathway analysis, contrasting CCNE1(+) cells against AT2 cells, highlighted enrichment in cell adhesion processes such as tight junction and focal adhesion pathways, alongside key oncogenic cascades including PI3K/AKT signaling (**Figure 1e**). Assessment of malignant potential through inferCNV copy number variation analysis demonstrated that CCNE1(+) cells exhibited the highest CNV scores among epithelial subpopulations (**Figure 1f**).

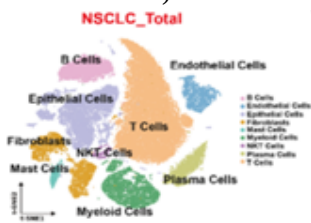
Importantly, CCNE1(+) cells present in primary lung tumors were found to be equivalent to those detected in metastatic lymph nodes. Three N2 lymph node metastases from T1N2M0 patients were profiled by scRNA-seq, yielding 23,950 cells that clustered into ten major groups: B cells, endothelial cells, epithelial cells, fibroblasts, neutrophils, myeloid cells, NK cells, NKT cells, plasma cells, and T cells (**Figure 1g**). Within the 982 epithelial cells identified, three main subsets emerged: CCNE1(+), cancer cells, and ciliated cells (**Figure 1h**). Copycat and inferCNV analyses confirmed that CCNE1(+) cells represented the dominant malignant epithelial subpopulation in the metastatic nodes, mirroring findings in primary NSCLC.

To confirm identity across sites, gene expression profiles were correlated. Most CCNE1(+) cell clusters showed correlation coefficients exceeding 0.9 between primary tumor and metastatic lymph node samples, establishing that the identical cell population is responsible for mediating N2 lymph node metastasis in stage T1 NSCLC (**Figure 1i**). Additional clustering details for B cells, myeloid cells, fibroblasts, endothelial cells, and T cells are provided.

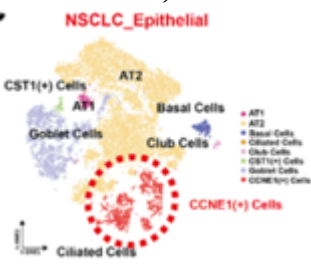
Publicly available datasets further corroborated these observations. Analysis of nine lung cancer-related samples from GSE131907 and dataset 10.24433CO.0121060.v1 [24]—including normal tissues (two stage IA and one stage IIIA lung adenocarcinomas) as well as tumor tissues (three stage IA and three stage IIIA)—validated an increased abundance of CCNE1(+) cells in more advanced stage IIIA disease. These cells were strongly linked to metastatic potential and glycolytic activity.



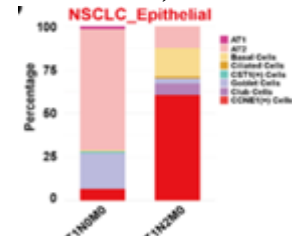
a)



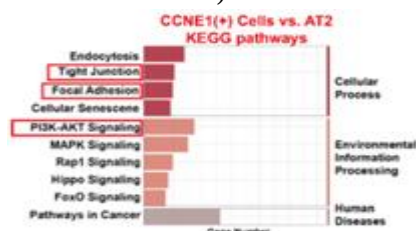
b)



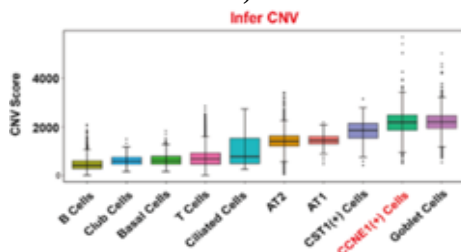
c)



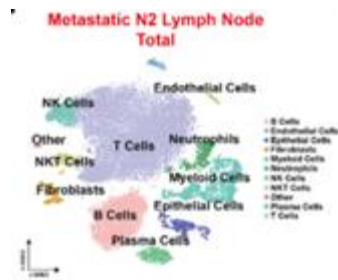
d)



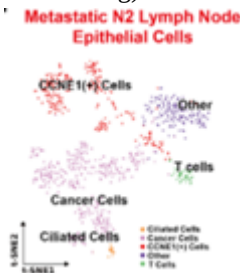
e)



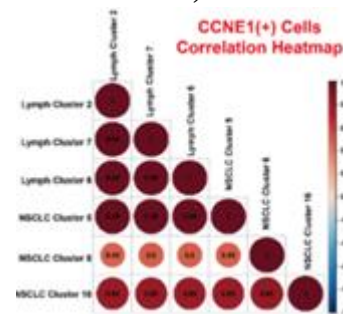
f)



g)



h)



i)

**Figure 1.** Single-cell sequencing uncovers a unique cell population that drives lymph node metastasis.

(a) Overview of the experimental workflow. (b) UMAP visualization showing the main cell clusters identified from scRNA-seq of seven NSCLC specimens. (c) Detailed subclustering of epithelial cells within the seven NSCLC samples. (d) Quantitative comparison of epithelial cell subpopulation proportions. (e) KEGG pathway enrichment comparing CCNE1(+) cells to AT2 cells, accompanied by differential gene expression analysis. (f) Copy number variation (CNV) profiling across epithelial subpopulations to assess malignancy levels. (g) UMAP plot of overall cell clusters derived from scRNA-seq of three metastatic N2 lymph node specimens. (h) Subclustering results for epithelial cells in the metastatic N2 lymph node samples. (i) Correlation matrix of gene expression profiles for CCNE1(+) cell clusters between primary NSCLC tumors and metastatic N2 lymph nodes.

*FGL1 shows strong expression in CCNE1(+) cells, emerges concurrently with them, and exerts a metastasis-promoting function independent of immune modulation*

FGL1 was identified as a critical gene contributing to N2 lymph node metastasis mediated by CCNE1(+) cells. Given that these cells appear capable of migrating from the lung to immune-rich lymph nodes without being eliminated, we hypothesized that they possess specialized immune-evasion capabilities. We therefore examined the expression of several immune checkpoint molecules (FGL1, LAG3, PD1, PD-L1, PVR, TIGIT, IGSF11, and VSIR) on CCNE1(+) cells and observed that FGL1 displayed highly specific and prominent expression in this population (**Figure 2a**).

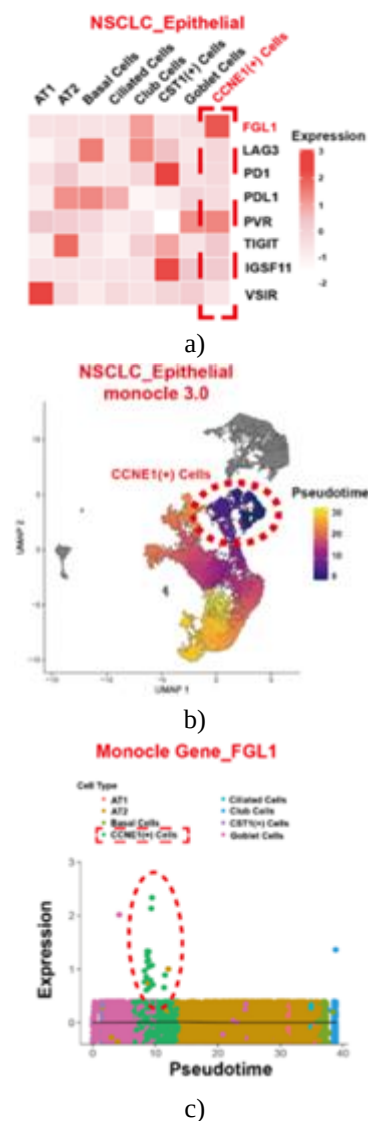
Monocle-based pseudotime trajectory analysis, together with differential gene expression profiling between FGL1(+) and FGL1(-) subsets within CCNE1(+) cells, indicated that FGL1 plays a substantial role in conferring metastatic properties to these cells. Expression profiling confirmed that FGL1 was predominantly found in epithelial cells, with the strongest signals in the CCNE1(+) subset. These observations suggest that, in addition to its established role in immunosuppression, FGL1 likely possesses an independent capacity to promote tumor spread.

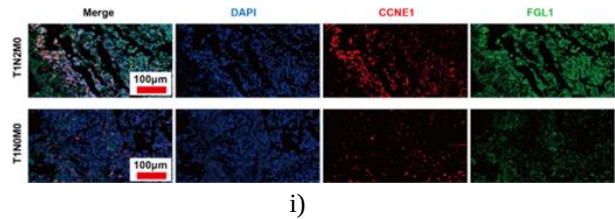
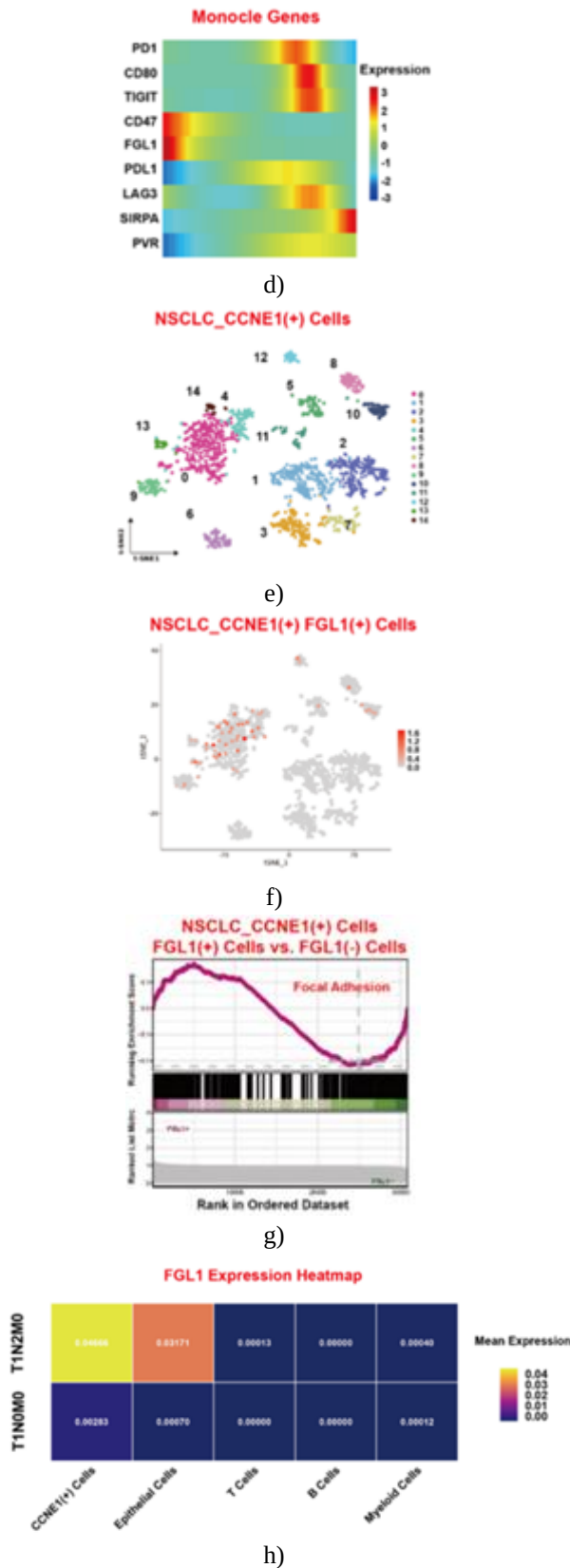
Pseudotime analysis further demonstrated that CCNE1(+) cells represent an early diverging population along the developmental trajectory (**Figure 2b**). FGL1 expression closely tracked the emergence of CCNE1(+) cells and was minimally detected in other cell types (**Figures 2c and 2d**). Within the CCNE1(+) population, cells were subdivided into FGL1(+) and FGL1(-) subsets according to FGL1 levels (**Figures 2e and 2f**). Gene Set Enrichment Analysis (GSEA) of differentially expressed genes between these subsets revealed significant enrichment in focal adhesion pathways, supporting the involvement of FGL1 in metastatic processes (**Figure 2g**).

Heatmap visualization confirmed markedly higher FGL1 expression in epithelial cells—especially CCNE1(+) cells—relative to immune lineages such as T cells, B cells, and myeloid cells. Moreover, FGL1 transcript levels were substantially elevated in T1N2M0 samples compared with T1N0M0 samples (0.047 versus 0.002), whereas no meaningful differences were detected in immune cell populations between N0 and N2 groups (**Figure 2h**). Taken together, these data emphasize that

the non-immune functions of CCNE1(+) epithelial cells are central to disease progression.

Immunofluorescence staining on tissue microarrays verified the presence of CCNE1(+) cells in clinical specimens. It demonstrated significantly higher CCNE1 and FGL1 levels in T1N2M0 cases (n = 15) relative to T1N0M0 cases (n = 15) (**Figure 2i**). These results collectively establish that FGL1 functions as a key effector molecule within CCNE1(+) cells to regulate metastasis, operating beyond its conventional immune-regulatory activities. Analysis of the same nine lung cancer samples from public repositories further confirmed elevated FGL1 expression in CCNE1(+) cells, reinforcing the robustness of these findings.





**Figure 2.** FGL1 shows strong expression in CCNE1(+) cells, with its emergence paralleling the appearance of this population, underscoring its tight association with metastatic potential.

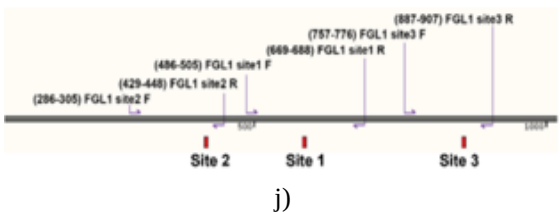
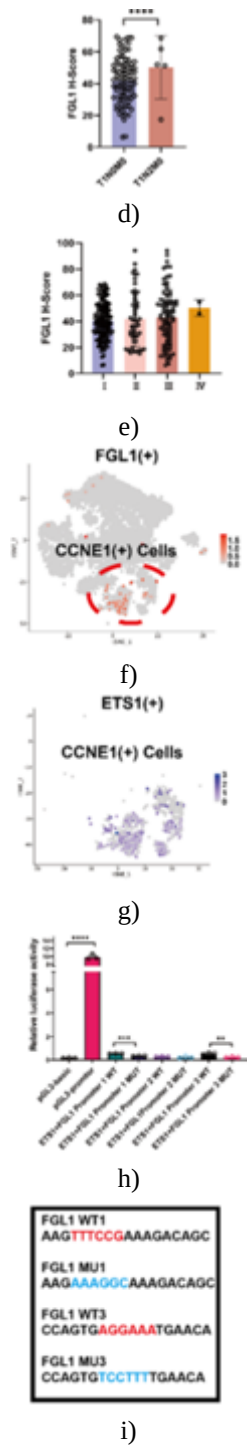
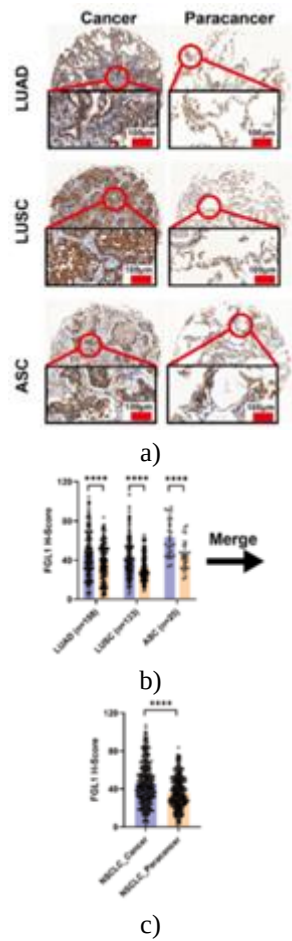
(a) Heatmap illustrating the expression patterns of selected immune checkpoint genes (FGL1, LAG3, PD1, PD-L1, PVR, TIGIT, IGSF11, and VSIR) across epithelial cells in NSCLC specimens. (b) Pseudotime trajectory analysis using Monocle demonstrating the developmental progression of CCNE1(+) cells. (c, d) Distribution of FGL1 expression across the full epithelial cell developmental trajectory. (e, f) Subdivision of CCNE1(+) cells into FGL1(+) and FGL1(-) subsets according to FGL1 expression status. (g) Gene Set Enrichment Analysis (GSEA) performed on differentially expressed genes (DEGs) between FGL1(+) and FGL1(-) cells to delineate the functional contribution of FGL1 within CCNE1(+) cells. (h) Comparative FGL1 expression levels in epithelial cells (particularly CCNE1(+) cells) versus immune cell lineages (T cells, B cells, and myeloid cells). (i) Dual immunofluorescence staining for CCNE1 (red) and FGL1 (green) confirming the presence of CCNE1(+) cells in T1N2M0 (n = 15) and T1N0M0 (n = 15) NSCLC tissues. Abbreviations: FGL1 = fibrinogen-like protein 1; NSCLC = non-small cell lung cancer; GSEA = Gene Set Enrichment Analysis.

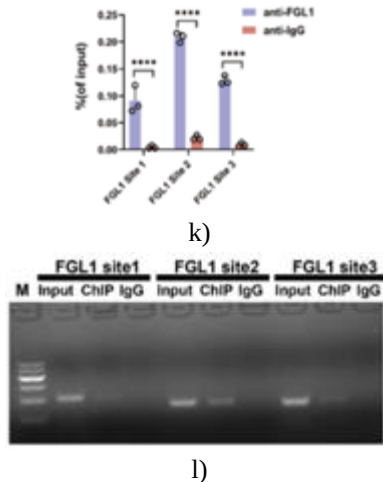
*FGL1 displays elevated expression in NSCLC and is transcriptionally controlled by ETS1*

Immunohistochemical analysis verified prominent FGL1 protein levels in malignant tissues. Complementary experiments using ChIP-qPCR and dual-luciferase reporter assays identified ETS1 as the key transcription factor driving this upregulation. Tissue microarrays were constructed from 316 NSCLC cases, encompassing 158 lung adenocarcinomas, 133 lung squamous cell carcinomas (LUSC), and 25 adenosquamous carcinomas.

Quantitative H-score assessment of FGL1 staining revealed significantly stronger expression in tumor tissues compared with matched adjacent non-cancerous

tissues ( $P < 0.0001$ ); (**Figures 3a–3c**). T1N2M0 specimens displayed markedly higher FGL1 levels than T1N0M0 samples ( $P < 0.0001$ ); (**Figure 3d**). A non-significant upward trend in FGL1 expression was noted with advancing disease stages (**Figure 3e**). Kaplan-Meier survival curves indicated no statistically significant correlation between FGL1 expression and overall patient survival ( $P = 0.492$ ). Prediction using the PROMO and hTFtarget databases indicated ETS1 as a potential upstream regulator. Transcription factor analysis of single-cell sequencing data was conducted to clarify the basis for elevated FGL1 in tumors. Both FGL1 and ETS1 showed high expression specifically in CCNE1(+) cells, suggesting that ETS1 is the primary transcriptional regulator of FGL1 (**Figures 3f and 3g**). Subsequent validation through ChIP-qPCR and dual-luciferase reporter assays confirmed direct binding and positive transcriptional regulation of FGL1 by ETS1 at two specific promoter sites (**Figures 3h–3l**).





**Figure 3.** FGL1 exhibits elevated levels in NSCLC and undergoes transcriptional control by ETS1.

(a–c). Tissue microarrays generated from 316 NSCLC cases (158 lung adenocarcinomas, 133 lung squamous cell carcinomas (LUSC), and 25 adenosquamous carcinomas) were subjected to FGL1 immunohistochemical staining. (d, e). Quantitative comparison of FGL1 expression across different stages of NSCLC. (f, g). Transcription factor analysis based on single-cell sequencing data to determine the mechanism underlying elevated FGL1 in tumor tissues. (h–k). Validation of ETS1 as a regulator of FGL1 and identification of binding sites using ChIP-qPCR and dual-luciferase reporter assays. In panels (b), (c), (d), and (e), data are shown as mean  $\pm$  SEM, with statistical significance determined by the Wilcoxon rank test. In panel H, data are presented as mean  $\pm$  SEM, and an unpaired t-test was applied. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . Abbreviations: FGL1= fibrinogen-like protein 1; NSCLC= non-small cell lung cancer; IHC= immunohistochemistry; ETS1= E26 transformation-specific transcription factor 1; ChIP-qPCR= chromatin immunoprecipitation quantitative PCR; SEM= standard error of the mean.

*FGL1 modulates glycolysis through the PI3K/AKT/HIF-1 $\alpha$  axis, thereby influencing tumor metastatic potential*  
FGL1 contributes to tumor malignant progression by regulating the PI3K/AKT signaling cascade. Stable lentiviral-mediated knockdown of FGL1 was successfully achieved in A549, H226, H1299, and H1703 cell lines, as verified by Western blot analysis ( $P < 0.01$ ); (Figures 4a–4c).

RNA sequencing of H226 cells following FGL1 silencing identified 322 upregulated and 1,176 downregulated genes, visualized in a volcano plot (Figure 4d). KEGG pathway enrichment analysis highlighted significant involvement in processes governing tumor growth and motility, such as Apoptosis, Tight Junction, and the PI3K/AKT Signaling Pathway, along with additional cancer-related routes (Figure 4f). In A549 cells, FGL1 knockdown resulted in 2,103 upregulated and 3,187 downregulated genes (Figure 4e). Corresponding KEGG analysis showed enrichment primarily in pathways associated with tumor proliferation and invasion, including Adherens junction, Gap junction, Focal adhesion, and Tight junction (Figure 4g).

Co-immunoprecipitation (Co-IP) coupled with mass spectrometry in A549 cells was employed to uncover FGL1-interacting partners (Figures 4h and 4i). The associated proteins were enriched in functions linked to tumor cell movement, including Focal Adhesion and Cell-Cell Junction. Notably, the data also implicated FGL1 in the regulation of glycolysis, lactate metabolism, and pyruvate metabolism by modulating key glycolytic enzymes, including ENOA, PGAM1, LDHA, and LDHB (Figures 4j–4l).

Further confirmation that FGL1 governs tumor glycolysis via the PI3K/AKT/HIF-1 $\alpha$  pathway came from integrated transcriptomic and metabolomic profiling. GSEA of RNA-seq data demonstrated enrichment of glycolysis-related gene sets in control versus shFGL1 A549 cells, with significant downregulation of critical glycolytic components (LDHB, DLDH, HIF-1 $\alpha$ , and GLUT3) upon FGL1 depletion (Figures 5a and 5b). Untargeted metabolomics revealed reduced concentrations of glycolytic intermediates including dihydroxyacetone phosphate, lactic acid, and glyceraldehyde, accompanied by elevated glucose levels. Additional alterations were noted in related metabolites such as 5'-deoxyadenosine, 6'-phosphogluconic acid, isocitric acid, and glucuronic acid (Figures 5c and 5d). Integrated multi-omics analysis (mass spectrometry, RNA-seq, and metabolomics) confirmed that FGL1 silencing perturbs glycolysis and HIF-1 $\alpha$  signaling (Figures 5e and 5f). Functional validation through glucose consumption, lactate production, and pyruvate production assays in the four NSCLC cell lines showed marked suppression of glycolytic activity following FGL1 knockdown ( $P < 0.05$ ); (Figure 5g). In clinical NSCLC tissue

microarrays, IHC and H-score quantification demonstrated positive correlations between FGL1 and multiple glycolysis markers: GLUT3 ( $r = 0.3891$ ,  $P < 0.0001$ ), GLUT1 ( $r = 0.1279$ ,  $P = 0.0195$ ), LDHB ( $r = 0.4097$ ,  $P < 0.0001$ ), PGAM1 ( $r = 0.2802$ ,  $P < 0.0001$ ), HK2 ( $r = 0.3396$ ,  $P < 0.0001$ ), and PDK1 ( $r = 0.2460$ ,  $P < 0.0001$ ) (**Figure 5h**).

Western blotting revealed that FGL1 depletion reduced activation of the PI3K/AKT pathway, evidenced by decreased levels of PI3K, pAKT-308, and pAKT-473 (**Figure 5i**). Additional protein analyses confirmed downregulation of the HIF-1 $\alpha$  pathway and glycolytic components (HIF-1 $\alpha$ , PDK1, GLUT1, LDHA, LDHB, and DLDH) after FGL1 knockdown (**Figure 5j**).

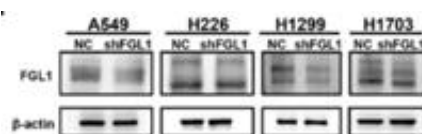
FGL1 silencing suppresses tumor progression, including proliferation and metastatic behaviors, by modulating glycolysis.

To further define the role of FGL1, a series of in vitro functional assays were conducted. A glycolytic blockade was induced using 9 mM 2-DG. Colony formation and CCK-8 assays showed reduced proliferative capacity in FGL1\_KD cells compared with NC controls across the A549, H226, H1299, and H1703 cell lines. This anti-proliferative effect was substantially attenuated under glycolytic inhibition ( $P < 0.05$ ); (**Figures 6a and 6b**).

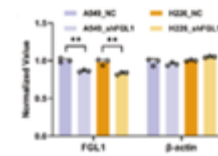
Transwell migration and wound-healing assays similarly showed decreased migratory ability in FGL1\_KD cells relative to controls, with the difference largely abolished in the presence of 2-DG ( $P < 0.05$ ); (**Figures 6c and 6d**).

Western blot analysis indicated that FGL1 downregulation significantly suppressed EMT-related proteins, including TGF- $\beta$ , Slug, and Snail, while upregulating E-cadherin ( $P < 0.05$ ); (**Figure 7a**), a key alteration that impacts metastatic potential. In LUSC tissue microarrays, IHC-based correlation analysis revealed positive associations between FGL1 and EMT markers: E-cadherin ( $r = 0.3903$ ,  $P < 0.0001$ ), Snail ( $r = 0.1960$ ,  $P = 0.0133$ ), Twist ( $r = 0.3769$ ,  $P < 0.0001$ ), Slug ( $r = 0.3882$ ,  $P < 0.0001$ ), TGF- $\beta$  ( $r = 0.3979$ ,  $P < 0.0001$ ), and Vimentin ( $r = 0.2136$ ,  $P = 0.0076$ ) (**Figure 7b**).

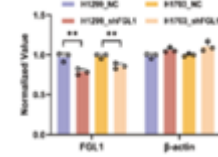
Collectively, these results demonstrate that FGL1 promotes tumor proliferation and metastasis, at least in part, by regulating cellular glycolysis.



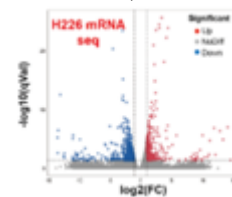
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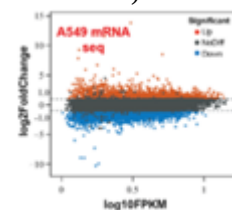
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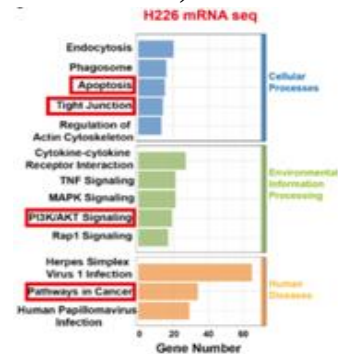
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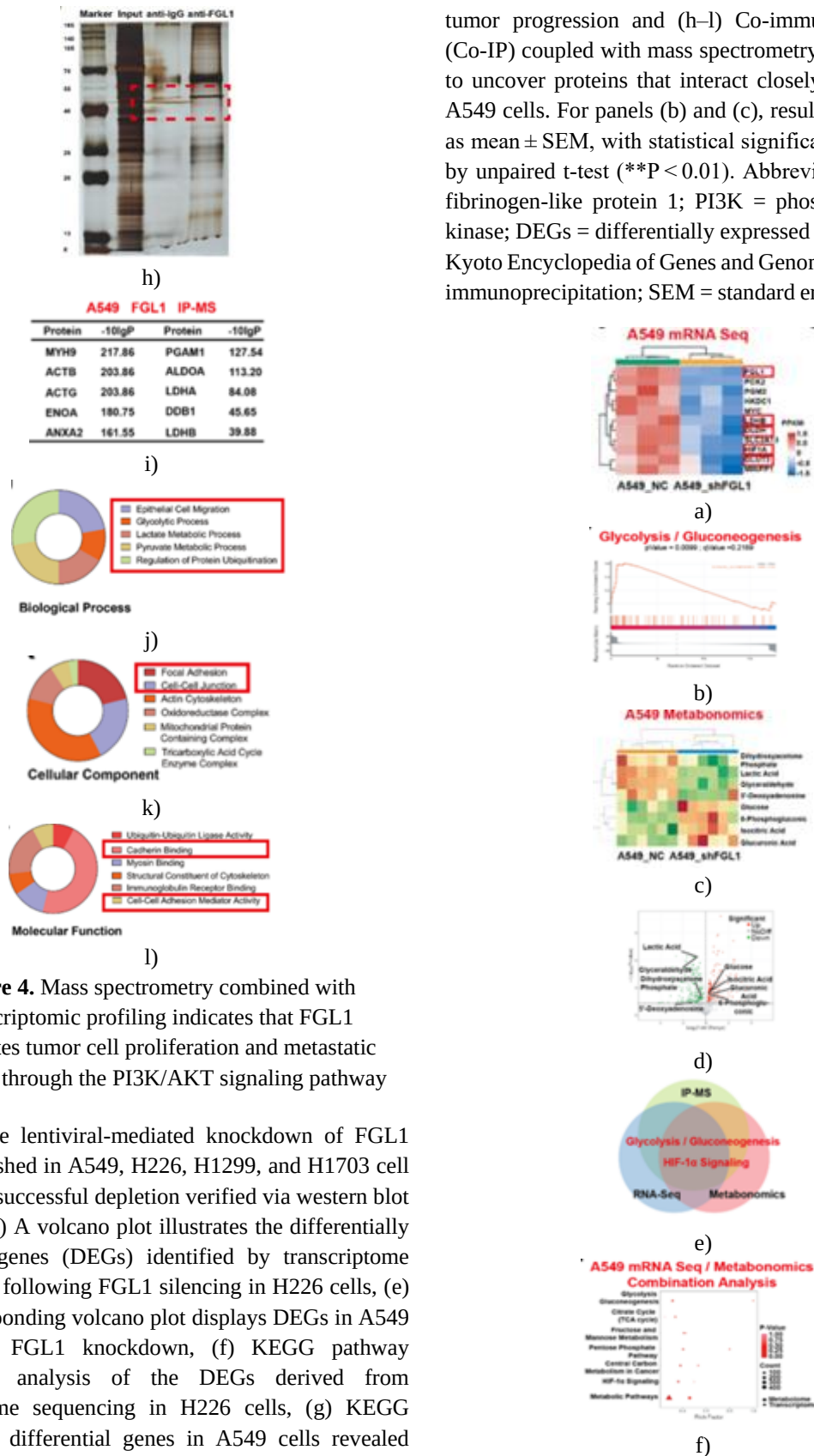
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f)



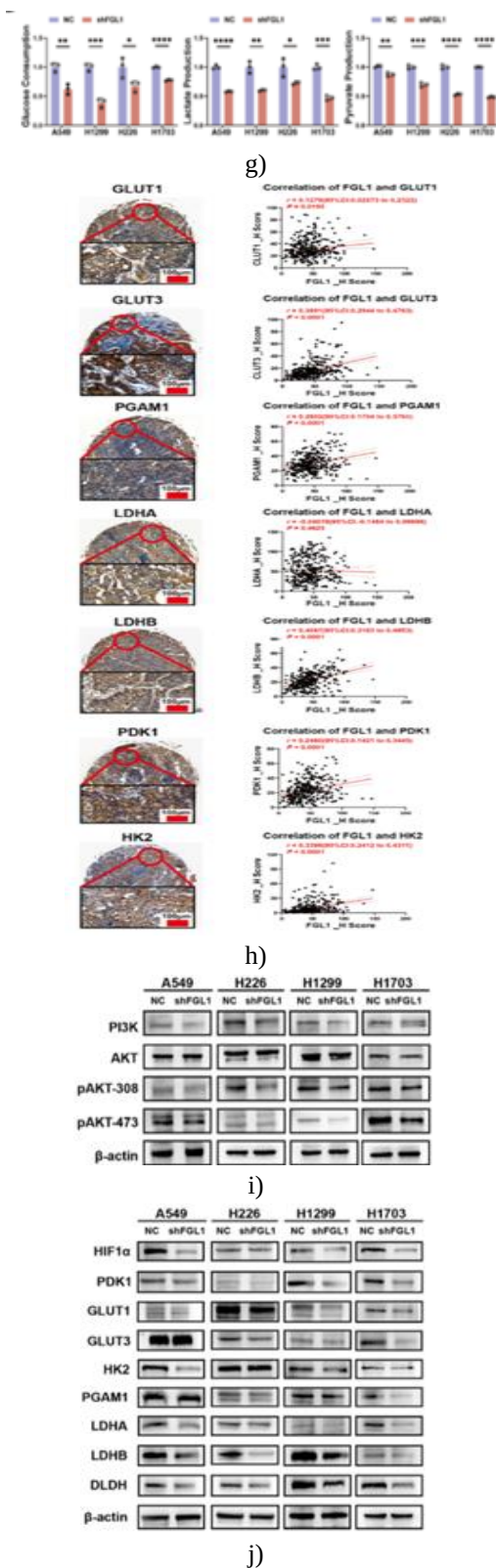
g)



**Figure 4.** Mass spectrometry combined with transcriptomic profiling indicates that FGL1 modulates tumor cell proliferation and metastatic potential through the PI3K/AKT signaling pathway

(a–c) Stable lentiviral-mediated knockdown of FGL1 was established in A549, H226, H1299, and H1703 cell lines, with successful depletion verified via western blot analysis, (d) A volcano plot illustrates the differentially expressed genes (DEGs) identified by transcriptome sequencing following FGL1 silencing in H226 cells, (e) The corresponding volcano plot displays DEGs in A549 cells after FGL1 knockdown, (f) KEGG pathway enrichment analysis of the DEGs derived from transcriptome sequencing in H226 cells, (g) KEGG analysis of differential genes in A549 cells revealed significant enrichment in pathways associated with

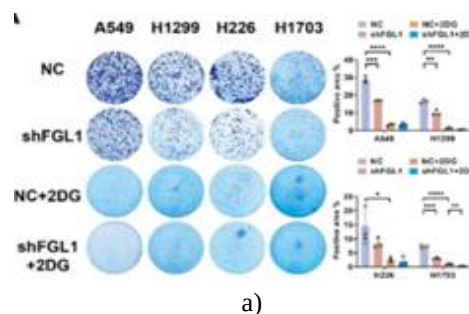
tumor progression and (h–l) Co-immunoprecipitation (Co-IP) coupled with mass spectrometry was conducted to uncover proteins that interact closely with FGL1 in A549 cells. For panels (b) and (c), results are expressed as mean  $\pm$  SEM, with statistical significance determined by unpaired t-test (\*\*P < 0.01). Abbreviations: FGL1 = fibrinogen-like protein 1; PI3K = phosphoinositide 3-kinase; DEGs = differentially expressed genes; KEGG = Kyoto Encyclopedia of Genes and Genomes; Co-IP = co-immunoprecipitation; SEM = standard error of the mean.

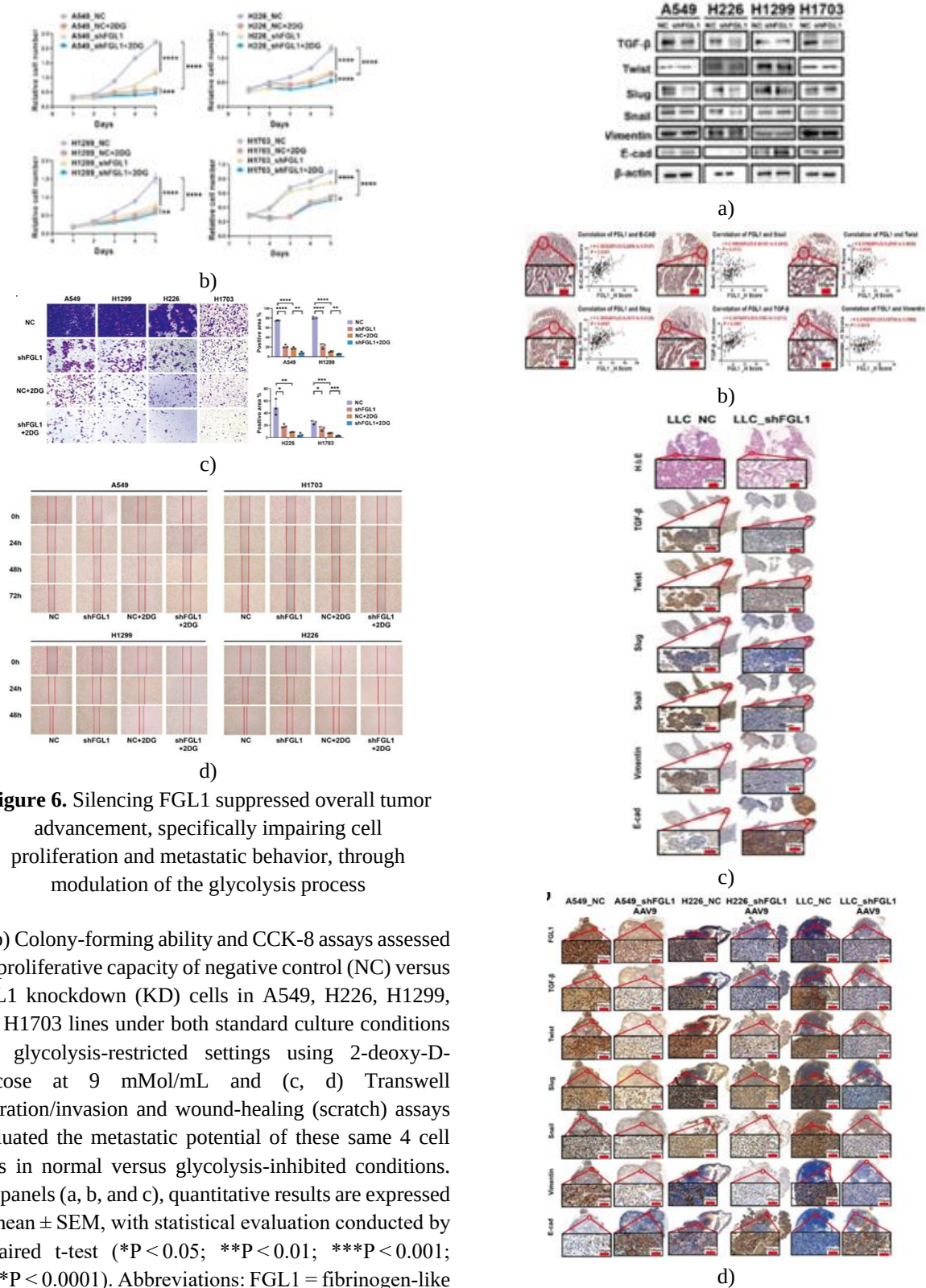


**Figure 5.** Multi-omics approaches incorporating mass spectrometry, transcriptomics, and metabolomics collectively point to FGL1 as a key

regulator of glycolytic metabolism in tumors, acting primarily through the HIF-1 $\alpha$  cascade

(a, b) Application of Gene Set Enrichment Analysis (GSEA) to the transcriptome sequencing results highlighted strong enrichment of glycolysis pathways among the differentially expressed genes (DEGs) when comparing A549\_NC cells with A549\_shFGL1 cells, (c, d) Metabonomic profiling demonstrated substantial reductions in critical metabolites involved in glycolysis following the knockdown of FGL1, (e, f) Integration of findings from mass spectrometry, RNA-seq, and metabolomics datasets revealed that loss of FGL1 markedly disrupted both glycolytic flux and HIF-1 $\alpha$  signaling activity, (g) Functional assays measuring glucose utilization, lactate output, and pyruvate levels were conducted across four non-small cell lung cancer (NSCLC) cell lines to determine how FGL1 influences tumor glycolytic capacity, (h) Examination of NSCLC tissue arrays by immunohistochemistry (IHC) and subsequent H-score analysis uncovered a significant association between FGL1 levels and the expression of various glycolysis-related factors, (i) Immunoblotting experiments established that silencing FGL1 led to suppression of the PI3K/AKT pathway, and (j) Additional western blot assays confirmed that FGL1 reduction downregulated the HIF-1 $\alpha$  axis and associated glycolytic components. Data in panel G are shown as mean  $\pm$  SEM, with group comparisons performed via unpaired t-test (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ). Abbreviations: FGL1 = fibrinogen-like protein 1; HIF-1 $\alpha$  = hypoxia-inducible factor 1-alpha; GSEA = Gene Set Enrichment Analysis; DEGs = differentially expressed genes; NC = negative control; scRNA-seq = single-cell RNA sequencing; IHC = immunohistochemistry; SEM = standard error of the mean; PI3K = phosphoinositide 3-kinase.





**Figure 7.** Depletion of FGL1 resulted in the suppression of important epithelial-mesenchymal transition (EMT) factors

(a) Immunoblotting experiments showed that decreasing FGL1 substantially lowered the levels of EMT-linked proteins, (b) Tissue microarray analysis of lung squamous cell carcinoma (LUSC) samples via immunohistochemistry (IHC) and H-score calculation revealed a clear relationship between FGL1 and EMT indicators ( $n = 133$ ), (c) FGL1 knockdown in LLC cells markedly reduced metastatic dissemination in an orthotopic pulmonary tumor model established in nude mice (**Figure 8c**). IHC staining further validated the alterations in major EMT components due to FGL1 reduction, and (d) An shFGL1\_AAV9 construct was developed and administered intratumorally to knock down FGL1 in LLC, A549, and H226 lines. Subsequent IHC assessment confirmed modifications in EMT molecules following shFGL1\_AAV9 delivery. Abbreviations: FGL1 = fibrinogen-like protein 1; EMT = epithelial-mesenchymal transition; IHC = immunohistochemistry; LUSC = lung squamous cell carcinoma; LLC = Lewis lung carcinoma; AAV9 = Adeno-Associated Virus serotype 9.

*FGL1 silencing effectively curbed tumor metastasis, with a pronounced impact on lymph node involvement by blocking the development of new lymphatic vessels.*

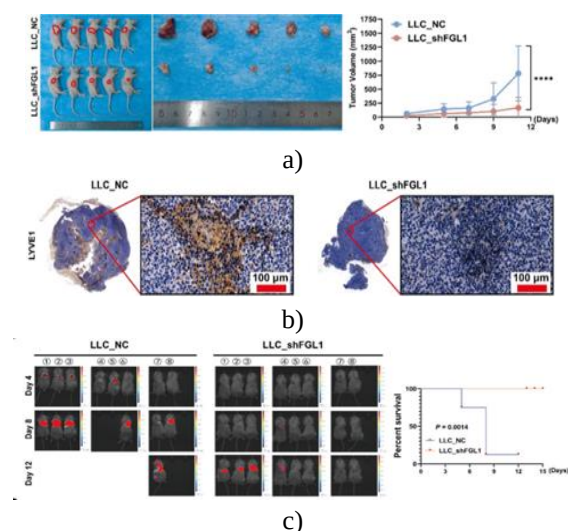
Consistent with earlier observations, FGL1 serves as an immune checkpoint regulator within non-small cell lung cancer (NSCLC). Experiments used the Node and NCG mouse strains as relevant in vivo systems [25–27] to dissect the direct, independent influence of the FGL1 protein on both proliferative growth and metastatic behavior.

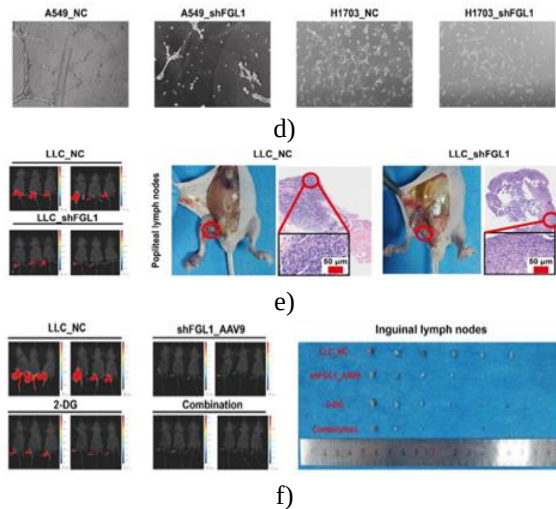
Lowering FGL1 expression attenuated in vivo tumor expansion. Stable LLC and H226 lines featuring lentivirus-driven FGL1 knockdown were established. Subcutaneous implantation into nude mice demonstrated that FGL1 depletion in these lines led to prominent restraint of tumor volume increase ( $P < 0.05$ ); (**Figure 8a**). Analysis of glycolytic markers in tumors from LLC\_NC compared to LLC\_shFGL1 cohorts indicated pronounced downregulation of central glycolysis regulators—HK2, GLUT1, GLUT3, LDHA, LDHB, PDK1, and PGAM1—upon FGL1 loss.

FGL1 reduction also hindered metastatic progression in living models. Orthotopic implantation of LLC\_NC or LLC\_shFGL1 cells into the left lung of NCG mice was employed to recapitulate lung tumor dissemination. In vivo bioluminescence imaging tracked primary tumor establishment and secondary spread at days 4, 8, and 12

post-injection. Imaging data indicated that by day 8, 80% of control-group mice had developed pulmonary metastases, which rapidly progressed and led to lethality. Distal metastases appeared in one control animal by day 12 (**Figure 8c**). Histological examination (HE staining) of lungs harvested from moribund control mice confirmed heavy tumor cell infiltration along with alveolar structural disruption (**Figure 7c**), directly accounting for metastasis-associated death. By comparison, animals with FGL1\_KD displayed only sparse metastatic deposits and achieved considerably longer survival times ( $P = 0.0014$ ). Concurrently, EMT protein profiles shifted, showing diminished mesenchymal markers and elevated E-cadherin following FGL1 knockdown (**Figure 7c**).

Attenuated FGL1 activity additionally prevented de novo lymph vessel assembly, thereby blocking subsequent lymph node colonization by tumor cells. Human lymphatic endothelial cells were co-incubated with control or FGL1-deficient A549 cells to evaluate lymphatic network formation, which was strongly impaired in the knockdown condition (**Figure 8d**). Assessment of lymphatic vessel endothelial receptor-1 (LYVE-1) in subcutaneous LLC tumors revealed markedly lower LYVE-1 signals in the LLC\_shFGL1 group relative to LLC\_NC, directly supporting the conclusion that FGL1 loss disrupts lymphangiogenesis (**Figure 8b**). In a separate footpad injection model, LLC\_NC or LLC\_shFGL1 cells were introduced into the footpad, followed by monitoring of inguinal and popliteal lymph node colonization. Lymph node metastatic burden was notably diminished in the FGL1 knockdown cohort ( $P < 0.05$ ); (**Figure 8e**).





**Figure 8.** FGL1 silencing simultaneously restrained tumor cell proliferation, metastatic dissemination, and lymphatic vessel assembly, thereby blocking lymph node colonization

(a) In subcutaneous xenograft models using nude mice, FGL1 knockdown in LLC cells produced a substantial reduction in tumor growth ( $n = 10$ ), (B) LYVE-1 staining was conducted on subcutaneous tumors originating from LLC\_NC and LLC\_shFGL1 cells, (C) LLC\_NC and LLC\_shFGL1 cells were orthotopically implanted into the left lung of NCG mice, with tumor development and metastatic progression monitored through small animal imaging on days 4, 8, and 12. Corresponding survival curves were generated ( $n = 16$ ), (D) Control and FGL1-knockdown A549 and H1703 cells were co-incubated with lymphatic endothelial cells to assess lymph tube assembly, € A footpad-based lymph node metastasis assay was executed by injecting LLC\_NC or LLC\_shFGL1 cells into the footpad, followed by evaluation of metastatic spread to inguinal and popliteal lymph nodes, and (F) Following footpad tumor establishment, combined treatment with shFGL1\_AAV9 and 2-DG was administered, and therapeutic outcomes were assessed. For panels A and C, results are presented as mean  $\pm$  SEM, with comparisons performed using an unpaired t-test (\*\*\*\* $P < 0.0001$ ). Abbreviations: FGL1 = fibrinogen-like protein 1; LLC = Lewis lung carcinoma; NC, negative control; LYVE-1 = lymphatic vessel endothelial receptor 1; 2-DG = 2-deoxy-D-glucose; SEM = standard error of the mean.

*Targeting FGL1 represents a viable therapeutic approach to suppress metastatic progression in NSCLC.*

To evaluate translational potential, adeno-associated viral vectors were used to assess the impact of FGL1 knockdown on tumor growth and metastasis, with emphasis on lymph node involvement. LLC\_NC cells were introduced into the footpad for lymph node metastasis modeling. Once tumors formed, shFGL1\_AAV9 was locally injected into the footpad, while 2-DG was given systemically via the intraperitoneal route for combination treatment. Data demonstrated that shFGL1\_AAV9 delivery potently suppressed lymph node metastasis (**Figure 8f**).

shFGL1\_AAV9 constructs were engineered to deplete FGL1 and assess its effect on in vivo tumor growth. Subcutaneous tumor models were generated in nude mice using A549, H226, and LLC cell lines. When tumor volumes reached 150–200 mm<sup>3</sup>, shFGL1\_AAV9 was administered intratumorally. Across all three lines, local FGL1 inhibition led to significant attenuation of tumor progression ( $P < 0.05$ ). Immunohistochemical evaluation of EMT markers in tumors from these groups showed that FGL1 knockdown upregulated E-cadherin while downregulating TGF- $\beta$ , Twist, Slug, Snail, and Vimentin (**Figure 7d**). Collectively, these findings indicate that in vivo FGL1 depletion curbs tumor proliferation primarily by impairing glycolytic metabolism.

Additionally, shFGL1\_AAV6 was developed to examine the effects of FGL1 knockdown on pulmonary tumor dissemination in vivo. LLC cells were first implanted intrapulmonarily, followed two days later by non-invasive tracheal delivery of shFGL1\_AAV6. Treatment efficacy was assessed by small-animal imaging at days 4, 8, and 12. FGL1 knockdown at the primary site effectively limited metastatic outgrowth and enhanced overall survival ( $P = 0.0751$ ).

Taken together, this work uncovered a highly malignant CCNE1(+) cell population characterized by elevated FGL1 expression, which is more abundant in T1N2M0 than in T1N0M0 specimens. The enhanced migratory activity of FGL1(+) cells within this cluster appears to drive lymph node metastasis in early-stage (T1) NSCLC, especially N2 involvement, with FGL1 upregulation coinciding with the emergence of this subpopulation. Transcriptomic profiling suggested that beyond its established role as an immune checkpoint, FGL1 itself may actively facilitate metastatic behavior. We therefore propose a “shield machine cutter” model in which FGL1 creates a metastatic route for N2 lymph node spread in T1 NSCLC, analogous to establishing a dedicated transport corridor. ETS1 was further identified as the

upstream transcription factor driving high FGL1 levels in NSCLC. Integrated mass spectrometry and RNA sequencing data, supported by functional validation, positioned FGL1 as a participant in the EMT program of NSCLC cells via the PI3K/AKT/HIF-1 $\alpha$  axis, modulating key glycolytic components such as HIF-1 $\alpha$ , PDK1, GLUT1, LDHA, LDHB, and DLDH. In vitro and in vivo experiments confirmed that FGL1 promotes tumor proliferation, metastatic capacity, and lymphangiogenesis, culminating in lymph node metastasis, while its depletion reverses these effects. Finally, the therapeutic potential of shFGL1-AAV6 and shFGL1-AAV9 was validated in animal systems, with IHC confirming corresponding changes in glycolysis- and EMT-related molecules upon FGL1 knockdown. Overall, the study delineates a mechanistic framework for FGL1-driven N2 lymph node metastasis in stage T1 NSCLC and highlights FGL1-directed intervention as a potential treatment strategy.

The incidence of N2 lymph node metastasis in T1 NSCLC is notably high despite limited understanding of its drivers. Lung cancer remains the leading cause of cancer-related death globally, with NSCLC comprising roughly 80% of cases. Tumors classified as T1 ( $\leq 3$  cm) are generally viewed as suitable for curative surgical resection [28]. Nevertheless, clinical data indicate that approximately 30% of T1 NSCLC patients harbor lymph node metastases, with risk increasing alongside solid tumor components [3]. Even among radiologically node-negative (cT1N0M0) cases, 20–30% reveal occult nodal metastasis (N1–N2) upon pathological examination after surgery. Moreover, around 30% of stage IA patients experience postoperative recurrence or distant spread [29], adversely impacting long-term outcomes. Given the unclear biology underlying lymph node dissemination in T1 disease, identifying relevant cellular subsets and molecular mediators is of high clinical importance. The present study characterizes the CCNE1(+) cell population and elucidates its FGL1-dependent metastatic mechanism.

Although FGL1 is implicated in immune suppression, direct supporting evidence remains incomplete. As a ligand for lymphocyte-activation gene 3 (LAG3) [30], FGL1 (also designated hepassocin, HRFREP-1, or FREP1 [31–34]) is mainly detected on the membrane and in the cytoplasm of lung and breast carcinoma cells. It exerts immunosuppressive effects by binding LAG3 and dampening T cell responses [31–34]. The prevailing model holds that LAG3 itself mediates immune

inhibition, with its overexpression potentially amplifying FGL1-driven T cell dysfunction [30]. Prior work by Liu *et al.* [35] linked LAG3-associated genes to PD-1/PD-L1 signaling, cytotoxicity, and antigen presentation pathways, noting that elevated LAG3 correlates with reduced T and B lymphocyte counts. In breast cancer, co-expression of LAG3 and PD-1 has been tied to impaired T cell cytokine production [36, 37], whereas other reports associate high LAG3 with increased NK and dendritic cell infiltration in breast cancer, lung adenocarcinoma, and LUSC [38]. In LAG3-overexpressing 3A9 T cells, FGL1 strongly suppresses proliferation, an effect reversible by anti-FGL1 antibodies that restore TNF- $\alpha$  and IFN- $\gamma$  secretion [30]. Our earlier findings showed that FGL1 downregulation in lung adenocarcinoma protects Jurkat T cells from apoptosis and enhances TNF- $\alpha$  release [22], supporting an immune-evasion mechanism in which FGL1 enables CCNE1(+) cells to evade immune surveillance and promote N2 metastasis in T1 NSCLC. FGL1 may also exert direct pro-tumorigenic effects on growth, invasion, and motility, though its precise contribution in lung cancer warrants additional clarification. In colorectal cancer models, FGL1 appears oncogenic; MC38 colon carcinoma cells grew more slowly in FGL1-knockout hosts than in wild-type controls, and anti-FGL1 monoclonal antibodies displayed robust antitumor activity [30]. In contrast, FGL1 may function as a tumor suppressor in certain contexts, including LKB1-mutant lung adenocarcinoma, SGC-7901 gastric cancer cells, and hepatocellular carcinoma. For instance, in A549 cells overexpressing LKB1, FGL1 knockdown accelerated proliferation, migration, angiogenesis, and EMT, potentially facilitating metastasis [39]. In SGC-7901 gastric cancer cells, FGL1 depletion enhanced invasion, growth, and motility [40]. Similarly, in liver cancer, FGL1 levels decline during progression, and FGL1-knockout mice exhibit faster tumor growth, possibly mediated by AKT/mTOR signaling [41]. Thus, FGL1 exhibits context-dependent roles across tumor types, sometimes acting as a suppressor (e.g., in LKB1-mutant lung adenocarcinoma). However, data on its function in broader lung adenocarcinoma subtypes are scarce and require further validation. Our prior work established FGL1's roles in proliferation and immune modulation, demonstrating that its downregulation curbs lung adenocarcinoma growth by interfering with the MYC pathway [21]. The current study also uncovers a

non-immune, metastasis-regulating function of FGL1 that operates via the PI3K/AKT/HIF-1 $\alpha$  cascade.

This study is the first to identify a regulatory function of FGL1 in cellular glycolysis. Several other immune checkpoint molecules are known to influence glycolytic processes as part of their immune-modulatory activities. For example, the PD-1/PD-L1 axis participates in immune control of glucose metabolism. Earlier research established that PD-1 engagement with PD-L1 triggers signaling downstream of the T cell receptor (TCR). Src family kinases, including Lck and Fyn, phosphorylate tyrosine residues within the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) of the PD-1 intracellular domain, recruiting the phosphatase SHP-2. This leads to attenuation of the Ras/AKT pathway linked to TCR signaling and interference with the PI3K/AKT/mTOR cascade associated with CD28 co-stimulation—both central to T cell metabolic reprogramming. Coordinated input from TCR, CD28, and cytokine receptors normally drives glycolysis, but elevated PD-1 activity counteracts these signals, thereby diminishing T cell glycolytic rate, expansion, and effector functions [42-44]. CTLA-4 ligation to B7 molecules blocks CD28 signaling and activates protein phosphatase 2A, which in turn inhibits AKT/mTORC1 activity within the PI3K/AKT/mTORC1 pathway. Consequently, expression of key metabolic enzymes, such as GLUT1, hexokinase 2, and pyruvate kinase M2, is reduced, limiting activation-induced glycolysis and impairing the differentiation of T cells into effector subsets [45, 46]. He *et al.* [47] co-cultured CD8<sup>+</sup> T cells with gastric cancer cells to enhance TIGIT-CD155 interactions, observing that tumor cells competitively deprive CD8<sup>+</sup> T cells of glucose, compromising their function. TIGIT blockade on CD8<sup>+</sup> T cells enhanced AKT/mTOR phosphorylation, boosting metabolic activity and cytokine output. Similarly, CD155 blockade in gastric cancer cells inhibited tumor growth, reduced tumor glucose consumption, and restored the metabolic fitness and immune responsiveness of nutrient-competing CD8<sup>+</sup> T cells. Parallel findings in triple-negative breast cancer showed that the TIGIT/CD155 axis suppresses PI3K, p-AKT, and p-mTOR signaling, leading to decreased glucose uptake and lactate production in CD8<sup>+</sup> T cells and consequent impairment of their activation and effector functions within the tumor microenvironment. Disruption of TIGIT/CD155

signaling reversed these metabolic and functional deficits [48].

Our integrated mass spectrometry, transcriptomic, and metabolomic investigations indicate that FGL1 modulates glycolysis via the PI3K/AKT/HIF-1 $\alpha$  signaling route, thereby driving malignant behaviors including tumor cell proliferation and metastatic dissemination. Functional assays confirmed that FGL1 influences multiple glycolytic intermediates both in vitro and in vivo, notably affecting glucose uptake, lactate output, and pyruvate generation.

At present, no clinically approved monoclonal antibodies directed against FGL1 exist, underscoring the need for continued basic mechanistic studies. Several LAG3-targeted monoclonal agents, such as IMP321 and relatlimab, are advancing through clinical evaluation [49, 50], encompassing both monotherapy and combination regimens worldwide [50]. These trials span breast cancer [51], hepatocellular carcinoma [52], prostate cancer [53], colorectal cancer [54], and lymphoma [55]. Most combination approaches remain relatively straightforward, predominantly pairing LAG3 blockade with anti-PD-1 or anti-PD-L1 antibodies [54, 56, 57]. Additionally, epigenetic dysregulation has been implicated in modulating immunotherapy responses in lung cancer, with ongoing clinical investigations in this area summarized elsewhere [58]. Given that FGL1 functions both as an oncogene and an immune checkpoint, exploring its epigenetic regulation in lung cancer could yield deeper insights into its multifaceted roles and inform the rational design of targeted therapeutics. Epigenetic modifications of FGL1 may emerge as promising targets for future clinical translation. Because clinically usable anti-FGL1 monoclonal antibodies are currently unavailable—likely due to gaps in foundational and in vivo data—we developed AAV6-shFGL1 and AAV9-shFGL1 vectors. Their antitumor efficacy was validated through non-invasive intratracheal and direct intratumoral delivery routes in animal models, thereby providing additional preclinical support for FGL1 as a novel therapeutic target.

## Conclusion

Nevertheless, several limitations should be acknowledged. First, technical and sampling restrictions prevented isolation and direct functional study of the CCNE1(+) cell population. Second, other immune

checkpoint molecules exhibited elevated expression in CCNE1(+) cells within lymph nodes, which could contribute to subsequent tumor progression and immune evasion. Third, additional marker genes highly expressed by these cells may independently drive metastatic potential and require further experimental confirmation. In summary, the present work examined the involvement of FGL1 in NSCLC pathobiology, with particular emphasis on its contribution to lymph node metastasis in stage T1 disease. The goal was to establish a viable FGL1-directed therapeutic approach, backed by initial preclinical evidence that may help expand the repertoire of immunotherapy targets.

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## References

1. Zheng RS, Chen R, Han BF, Wang SM, Li L, Sun KX, et al. Cancer incidence and mortality in China, 2022. *Zhonghua Zhong Liu Za Zhi*. 2024;46(3):221–31.
2. Filho AM, Laversanne M, Ferlay J, Colombet M, Piñeros M, Znaor A, et al. The GLOBOCAN 2022 cancer estimates: data sources, methods, and a snapshot of the cancer burden worldwide. *Int J Cancer*. 2025;156(7):1336–46.
3. Zhang Y, Fu F, Wen Z, Deng L, Wang S, Li Y, et al. Segment location and ground glass opacity ratio reliably predict node-negative status in lung cancer. *Ann Thorac Surg*. 2020;109(4):1061–8.
4. Lee CK, Jeong SH, Jang C, Bae H, Kim YH, Park I, et al. Tumor metastasis to lymph nodes requires YAP-dependent metabolic adaptation. *Science*. 2019;363(6427):644–9.
5. Kawada K, Taketo MM. Significance and mechanism of lymph node metastasis in cancer progression. *Cancer Res*. 2011;71(4):1214–8.
6. Meng F, Li H, Jin R, Yang A, Luo H, Li X, et al. Spatial immunogenomic patterns associated with lymph node metastasis in lung adenocarcinoma. *Exp Hematol Oncol*. 2024;13(1):106.
7. Su JL, Yen CJ, Chen PS, Chuang SE, Hong CC, Kuo IH, et al. The role of the VEGF-C/VEGFR-3 axis in cancer progression. *Br J Cancer*. 2007;96(4):541–5.
8. Luo H, Li Q, Wang RT, Zhang L, Zhang W, Deng MS, et al. Downregulation of pro-surfactant protein B contributes to the recurrence of early-stage non-small cell lung cancer by activating PGK1-mediated Akt signaling. *Exp Hematol Oncol*. 2023;12(1):94.
9. Kajiwara Y, Oka S, Tanaka S, Nakamura T, Saito S, Fukunaga Y, et al. Nomogram as a novel predictive tool for lymph node metastasis in T1 colorectal cancer treated with endoscopic resection: a nationwide, multicenter study. *Gastrointest Endosc*. 2023;97(6):1119–28.e5.
10. Zwager LW, Bastiaansen BAJ, Montazeri NSM, Hompes R, Barresi V, Ichimasa K, et al. Deep submucosal invasion is not an independent risk factor for lymph node metastasis in T1 colorectal cancer: a meta-analysis. *Gastroenterology*. 2022;163(1):174–89.
11. Takada K, Kashiwagi S, Asano Y, Goto W, Kouhashi R, Yabumoto A, et al. Prediction of lymph node metastasis by tumor-infiltrating lymphocytes in T1 breast cancer. *BMC Cancer*. 2020;20(1):598.
12. Zheng S, Wang W, Shen L, Yao Y, Xia W, Ni C. Tumor battlefield within inflamed, excluded or desert immune phenotypes: the mechanisms and strategies. *Exp Hematol Oncol*. 2024;13(1):80.
13. Zheng H, Ning Y, Zhan Y, Liu S, Wen Q, Fan S. New insights into the important roles of tumor cell-intrinsic PD-1. *Int J Biol Sci*. 2021;17(10):2537–47.
14. Kamada T, Togashi Y, Tay C, Ha D, Sasaki A, Nakamura Y, et al. PD-1(+) regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. *Proc Natl Acad Sci U S A*. 2019;116(20):9999–10008.
15. Wang R, He S, Long J, Wang Y, Jiang X, Chen M, et al. Emerging therapeutic frontiers in cancer: insights into posttranslational modifications of PD-1/PD-L1 and regulatory pathways. *Exp Hematol Oncol*. 2024;13(1):46.
16. Jin AL, Zhang CY, Zheng WJ, Xian JR, Yang WJ, Liu T, et al. CD155/SRC complex promotes hepatocellular carcinoma progression via inhibiting the p38 MAPK signalling pathway and correlates with poor prognosis. *Clin Transl Med*. 2022;12(4):e794.
17. Li M, Xia P, Du Y, Liu S, Huang G, Chen J, et al. T-cell immunoglobulin and ITIM domain (TIGIT)

- receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon- $\gamma$  production of natural killer cells via  $\beta$ -arrestin 2-mediated negative signaling. *J Biol Chem*. 2014;289(25):17647–57.
18. Li Z, Li Y, Gao J, Fu Y, Hua P, Jing Y, et al. The role of CD47-SIRP $\alpha$  immune checkpoint in tumor immune evasion and innate immunotherapy. *Life Sci*. 2021;273:119150.
  19. Zhou Z, Chen MM, Luo Y, Mojumdar K, Peng X, Chen H, et al. Tumor-intrinsic SIRPA promotes sensitivity to checkpoint Inhibition immunotherapy in melanoma. *Cancer Cell*. 2022;40(11):1324–e8.
  20. Tang X, Yang J, Shi A, Xiong Y, Wen M, Luo Z, et al. CD155 cooperates with PD-1/PD-L1 to promote proliferation of esophageal squamous cancer cells via PI3K/Akt and MAPK signaling pathways. *Cancers (Basel)*. 2022;14(22):5610.
  21. Tang XY, Xiong YL, Shi AP, Sun Y, Han Q, Lv Y, et al. The downregulation of fibrinogen-like protein 1 inhibits the proliferation of lung adenocarcinoma via regulating MYC-target genes. *Transl Lung Cancer Res*. 2022;11(3):404–19.
  22. Tang XY, Xiong YL, Zhao YB, Yang J, Shi AP, Zheng KF, et al. Dual immunological and proliferative regulation of immune checkpoint FGL1 in lung adenocarcinoma: the pivotal role of the YY1-FGL1-MYH9 axis. *Front Immunol*. 2022;13:1014053.
  23. Watkins TBK, Lim EL, Petkovic M, Elizalde S, Birkbak NJ, Wilson GA, et al. Pervasive chromosomal instability and karyotype order in tumour evolution. *Nature*. 2020;587(7832):126–32.
  24. Bischoff P, Trinks A, Obermayer B, Pett JP, Wiederspahn J, Uhlitz F, et al. Single-cell RNA sequencing reveals distinct tumor microenvironmental patterns in lung adenocarcinoma. *Oncogene*. 2021;40(50):6748–58.
  25. Xu A, Zhang J, Zuo L, Yan H, Chen L, Zhao F, et al. FTO promotes multiple myeloma progression by posttranscriptional activation of HSF1 in an m(6)A-YTHDF2-dependent manner. *Mol Ther*. 2022;30(3):1104–18.
  26. Jiang C, Wang X, Teng B, Wang Z, Li F, Zhao Y, et al. Peptide-targeted high-density lipoprotein nanoparticles for combinatorial treatment against metastatic breast cancer. *ACS Appl Mater Interfaces*. 2021;13(30):35248–65.
  27. Ning Z, Liu K, Zhang H, Dong G, Wang X, Xiong H. Platelets induce CD39 expression in tumor cells to facilitate tumor metastasis. *Br J Cancer*. 2024;130(9):1542–51.
  28. Lin A, Wei T, Meng H, Luo P, Zhang J. Role of the dynamic tumor microenvironment in controversies regarding immune checkpoint inhibitors for the treatment of non-small cell lung cancer (NSCLC) with EGFR mutations. *Mol Cancer*. 2019;18(1):139.
  29. Altorki N, Wang X, Kozono D, Watt C, Landrenau R, Wigle D, et al. Lobar or sublobar resection for peripheral stage IA non-small-cell lung cancer. *N Engl J Med*. 2023;388(6):489–98.
  30. Wang J, Sanmamed MF, Datar I, Su TT, Ji L, Sun J, et al. Fibrinogen-like protein 1 is a major immune inhibitory ligand of LAG-3. *Cell*. 2019;176(1-2):334–47.e12.
  31. Visan I. New ligand for LAG-3. *Nat Immunol*. 2019;20(2):111.
  32. Xu F, Liu J, Liu D, Liu B, Wang M, Hu Z, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. *Cancer Res*. 2014;74(13):3418–28.
  33. Kouo T, Huang L, Pucsek AB, Cao M, Solt S, Armstrong T, et al. Galectin-3 shapes antitumor immune responses by suppressing CD8<sup>+</sup> T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. *Cancer Immunol Res*. 2015;3(4):412–23.
  34. Graydon CG, Mohideen S, Fowke KR. LAG3's enigmatic mechanism of action. *Front Immunol*. 2020;11:615317.
  35. Liu Q, Qi Y, Zhai J, Kong X, Wang X, Wang Z, et al. Molecular and clinical characterization of LAG3 in breast cancer through 2994 samples. *Front Immunol*. 2021;12:599207.
  36. Du H, Yi Z, Wang L, Li Z, Niu B, Ren G. The co-expression characteristics of LAG3 and PD-1 on the T cells of patients with breast cancer reveal a new therapeutic strategy. *Int Immunopharmacol*. 2020;78:106113.
  37. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov*. 2018;8(9):1069–86.
  38. Tu L, Guan R, Yang H, Zhou Y, Hong W, Ma L, et al. Assessment of the expression of the immune checkpoint molecules PD-1, CTLA4, TIM-3 and LAG-3 across different cancers in relation to treatment response, tumor-infiltrating immune cells and survival. *Int J Cancer*. 2020;147(2):423–39.

39. Bie F, Wang G, Qu X, Wang Y, Huang C, Wang Y, et al. Loss of FGL1 induces epithelial–mesenchymal transition and angiogenesis in LKB1 mutant lung adenocarcinoma. *Int J Oncol*. 2019;55(3):697–707.
40. Zhang Y, Qiao HX, Zhou YT, Hong L, Chen JH. Fibrinogen–like–protein 1 promotes the invasion and metastasis of gastric cancer and is associated with poor prognosis. *Mol Med Rep*. 2018;18(2):1465–72.
41. Nayeb-Hashemi H, Desai A, Demchev V, Bronson RT, Hornick JL, Cohen DE, et al. Targeted disruption of fibrinogen like protein-1 accelerates hepatocellular carcinoma development. *Biochem Biophys Res Commun*. 2015;465(2):167–73.
42. Boussiotis VA, Patsoukis N. Effects of PD-1 signaling on immunometabolic reprogramming. *Immunometabolism*. 2022;4(2):e220007.
43. Wang S, Liu C, Yang C, Jin Y, Cui Q, Wang D, et al. PI3K/AKT/mTOR and PD–1/CTLA–4/CD28 pathways as key targets of cancer immunotherapy (review). *Oncol Lett*. 2024;28(6):567.
44. Baldanzi G. Immune checkpoint receptors signaling in T cells. *Int J Mol Sci*. 2022;23(7):3529.
45. Oyewole-Said D, Konduri V, Vazquez-Perez J, Weldon SA, Levitt JM, Decker WK. Beyond T-cells: functional characterization of CTLA-4 expression in immune and non-immune cell types. *Front Immunol*. 2020;11:608024.
46. DePeaux K, Delgoffe GM. Metabolic barriers to cancer immunotherapy. *Nat Rev Immunol*. 2021;21(12):785–97.
47. He W, Zhang H, Han F, Chen X, Lin R, Wang W, et al. CD155T/tigit signaling regulates CD8(+) T-cell metabolism and promotes tumor progression in human gastric cancer. *Cancer Res*. 2017;77(22):6375–88.
48. Huang M, Yu X, Wang Q, Jiang Z, Li X, Chen W, et al. The immune checkpoint TIGIT/CD155 promotes the exhaustion of CD8+ T cells in TNBC through glucose metabolic reprogramming mediated by PI3K/AKT/mTOR signaling. *Cell Commun Signal*. 2024;22(1):35.
49. Legat A, Maby-El Hajjami H, Baumgaertner P, Cagnon L, Abed Maillard S, Geldhof C, et al. Vaccination with LAG-3Ig (IMP321) and peptides induces specific CD4 and CD8 T-Cell responses in metastatic melanoma Patients–Report of a phase i/IIa clinical trial. *Clin Cancer Res*. 2016;22(6):1330–40.
50. Qi Y, Chen L, Liu Q, Kong X, Fang Y, Wang J. Research progress concerning dual blockade of Lymphocyte-Activation gene 3 and programmed Death-1/Programmed Death-1 Ligand-1 blockade in cancer immunotherapy: preclinical and clinical evidence of this potentially more effective immunotherapy strategy. *Front Immunol*. 2020;11:563258.
51. Stovgaard ES, Kümmler I, List-Jensen K, Roslind A, Christensen IJ, Høgdall E, et al. Prognostic and clinicopathologic associations of LAG-3 expression in triple-negative breast cancer. *Appl Immunohistochem Mol Morphol*. 2022;30(1):62–71.
52. Wang J, Wei W, Tang Q, Lu L, Luo Z, Li W, et al. Oxysophocarpine suppresses hepatocellular carcinoma growth and sensitizes the therapeutic blockade of anti-Lag-3 via reducing FGL1 expression. *Cancer Med*. 2020;9(19):7125–36.
53. Zahm CD, Moseman JE, Delmastro LE. PD-1 and LAG-3 blockade improve anti-tumor vaccine efficacy. *Oncoimmunology*. 2021;10(1):1912892.
54. Kraman M, Faroudi M, Allen NL, Kmiecik K, Gliddon D, Seal C, et al. FS118, a bispecific antibody targeting LAG-3 and PD-L1, enhances T-cell activation resulting in potent antitumor activity. *Clin Cancer Res*. 2020;26(13):3333–44.
55. Nagasaki J, Togashi Y, Sugawara T, Itami M, Yamauchi N, Yuda J, et al. The critical role of CD4+ T cells in PD-1 blockade against MHC-II-expressing tumors such as classic Hodgkin lymphoma. *Blood Adv*. 2020;4(17):4069–82.
56. Jiang H, Ni H, Zhang P, Guo X, Wu M, Shen H, et al. PD-L1/LAG-3 bispecific antibody enhances tumor-specific immunity. *Oncoimmunology*. 2021;10(1):1943180.
57. Bhagwat B, Cherwinski H, Sathe M, Seghezzi W, McClanahan TK, de Waal Malefyt R, et al. Establishment of engineered cell-based assays mediating LAG3 and PD1 immune suppression enables potency measurement of blocking antibodies and assessment of signal transduction. *J Immunol Methods*. 2018;456:7–14.
58. Luo H, Shan J, Zhang H, Song G, Li Q, Xu CX. Targeting the epigenetic processes to enhance antitumor immunity in small cell lung cancer. *Semin Cancer Biol*. 2022;86(Pt 3):960–70.