Xie et al. Cell Communication and Signaling (2025) 23:247
https://doi.org/10.1186/s12964-025-02259-z

Cell Communication and Signaling

RESEARCH Open Access

GLUT1 sensitizes tumor cells to EGFR-TKIs by binding with activated EGFR and regulating its downstream signaling pathways

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Abstract

Background We have previously demonstrated that GLUT1 can interact with phosphorylated EGFR and has an oncogenic role in lung cancer. Here, we aim to investigate their binding region and its signaling pathways.

Methods The AlphaFold 3 prediction, Co-immunoprecipitation, and Western blot were used to uncover the interaction conditions of GLUT1 and EGFR. The RNA-seq data was analyzed to evaluate the difference in signaling pathways between wild-type EGFR and activated mutated EGFR. The xenograft tumor model was established to determine the therapy effect of the combination of GLUT1 inhibitor BAY-876 and EGFR TKI Osimertinib.

Results We found that the interaction ability of GLUT1 and EGFR depended on the activation of EGFR. GLUT1 interacted with EGFRvIII (loss 2–7 exons) but not with EGFRvI (loss 1–16 exons), so GLUT1 interacts with EGFR in the EGFR extracellular transmembrane region. GLUT1 regulated EGFR downstream signaling pathways. GLUT1 inhibitor BAY-876 can sensitize tumor cells to EGFR TKI Osimertinib.

Conclusions GLUT1 participates in tumor progression by interacting with phosphor-EGFR, suggesting that inhibition of the GLUT1-EGFR axis may be a potential therapeutic strategy for lung cancer treatment.

Keywords LUAD, GLUT1, GLUT1 inhibitor, EGFRvIII, EGFRvI, EGFR TKIs

Introduction

Protein-protein interaction (PPI) is involved in various biological processes, and studying PPI helps design drug targets and intervention strategies [1]. Epidermal growth factor receptor (EGFR), a transmembrane protein, plays a key role in multiple biological processes such as signal transduction, cell proliferation, and survival [2, 3]. EGFR usually exists in monomeric form, and its extracellular PPI with ligands induces dimerization and activates phosphorylation of intracellular tyrosine kinase sites [4–6]; among these, phosphorylation at the Y1068 site is particularly critical for EGFR downstream signaling transduction [2, 7]. EGFR extracellular domain

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(ECD) mutations are relative to tumorigenic and drug-target cancer therapy [8]. Deletion of exons 2–7 in the extracellular domain generates the EGFRvIII variant, and complete removal of the extracellular structure, 1–16 exons, generates the EGFRvI variant. In both cases, EGFR loses its ligand-binding capacity, and its phosphorylation relies on the interaction with another wild-type EGFR protein [9–11]. However, the enormous differences in the percentage of extracellular domain between EGFRvIII and EGFRvI mutations may lead to changes in the PPI between EGFR and other proteins, which are likely caused by EGFR 8–16 exons, the extracellular transmembrane region (ETR).

Aberrant activation of the EGFR, in which the intracellular domain has the PPI with various downstream effector proteins, is an early event in lung adenocarcinoma (LUAD) pathogenesis [12], initiating several downstream signaling cascades [2, 13], such as the JAK-STAT, RAS-RAF, and AKT-mTOR pathways, and promoting tumor progression [14–16]. Gefitinib and Osimertinib are the EGFR tyrosine kinase inhibitors (TKIs) that are used in first-line clinical treatment in advanced non-small cell lung cancer (NSCLC) [17–19], which competitively bind to the ATP-binding site of EGFR, thereby blocking its abnormal phosphorylation and preventing activation of downstream signaling pathways [20, 21], ultimately suppressing LUAD progression [22, 23]. However, the effectiveness of EGFR TKI therapy is affected by drug resistance in patients [24, 25], highlighting the necessity for further treatment optimization.

GLUT1 (glucose transporter 1, also known as *SLC2A1*), known as a transmembrane protein, transports glucose in tumor cells [26]. In previous studies, GLUT1 has been primarily viewed as inducing a shift in glucose metabolism toward glycolysis involved in tumor cell metabolism [27–30]. Our team has further revealed that GLUT1 has the PPI with EGFR carrying activating mutations, therefore regulating EGFR expression independently of the metabolic pathway [31]. The GLUT1 inhibitors enhance the cell sensitivity of EGFR-TKIs and show tumor growth inhibitory effects in vitro, showing promising therapeutic potential [31–33]. However, the mechanisms of the interaction between GLUT1 and EGFR and their specific binding sites are unexplored.

This study reveals the essential role of EGFR activation in the GLUT1-EGFR interaction. Gradually mapping the specific interaction regions between these two proteins and exploring the impact of GLUT1 on protein expression within EGFR downstream signaling pathways. In addition, we conducted an in vivo experiment on the combination of EGFR TKIs and GLUT1 inhibitor, thereby providing new strategies for potential clinical applications in LUAD treatment.

Methods

Cell culture

Human non-small cell lung cancer cell lines (H838, H1299, H1650, H1975) and human embryonic kidney cell line 293T were obtained from ATCC. Cell culture media includes 10% fetal bovine serum (10270106, Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were cultured in RPMI-1640 medium (C3010-0500, Gibco) (for H838, H1299, H1650, H1975) or DMEM medium (C3113-0500, VivaCell) (for 293T), cell culture media were replaced every 2–3 days, and cells were maintained at approximately 80% confluence.

Reagents and drug treatment

6×10^6 lung cancer cells and 293T cells were seeded in 10-cm dishes following 24 h of culture. Gefitinib (HY-50895, MCE) and Osimertinib (HY-15772, MCE) were added to each dish for 48 h. The cells were collected for Co-immunoprecipitation (Co-IP). Gefitinib and Osimertinib were in a stock solution of 10μM, ensuring that the work solution contains less than 0.1% DMSO. 100U/ml EGF (AF-100-15, Peprotech) was added to each 10-cm dish or 6-well plate for 20 min before cells were collected. The solvent provided by this product created a stock solution of 1×10^4U/μl. The dosage selection was determined based on our research team's preliminary experimental findings.

Stable cell line Building

Lentivirus FLAG-vector (Ubi-MCS-3FLAG-CBh-gcGFP-IRES-puromycin), FLAG-EGFRvIII (delete 30–296 aa), or FLAG-EGFRvI (delete 25–645 aa), all purchased by Shanghai Genechem Co., Ltd. H1299 and 293T cells were transfected with lentiviral particles for 48 h, then incubated in fresh medium. 2 μg/mL Puromycin (REVG1001, Genechem) was used to build the transfected stable cell lines. Those stable cell lines were collected for Co-immunoprecipitation after 20 min of 100U/ml EGF treatment.

Cells transfection

The siRNAs were used at a concentration of 10nM. The transfection was conducted following the protocol provided for Lipofectamine RNAiMAX Reagent (13778-150, Invitrogen). The siRNA sequences are listed in Supplementary Table S1. Plasmids encoding FLAG-vector (Ubi-MCS-3FLAG-CBh-gcGFP-IRES-puromycin), FLAG-EGFR (full length), or FLAG-EGFR Y1068A (EGFR point mutation) were all purchased by Synbio Technologies, Suzhou, China. 293T cells were transiently transfected with these plasmids using Lipofectamine 3000 (13778150, Invitrogen), following the protocol. Cells were incubated for 48 h post-transfection and collected for subsequent analysis.

Co-Immunoprecipitation (Co-IP)

1×10^7 cells were washed twice with cold PBS and lysed in 1 ml NP40 Buffer (P0013F, Beyotime) with protease inhibitors and phosphatase inhibitors on ice for 30 min. The lysates were sonicated on ice and centrifuged at 15,000 × g for 20 min at 4℃. 100 µl supernatant was used as the input, and the remaining supernatants were aliquoted as the IgG and IP and respectively added primary antibodies overnight at 4℃. These samples were incubated with magnetic beads (36417ES08, Yeasen) for 4–5 h and high-temperature denatured with a metal bath at 90℃ for 10 min and retained for western blotting analysis.

Western blot analysis

20 µg protein samples were separated by the SDS-PAGE system (L00794 and L00747, GenScript) and transferred to PVDF membranes. Primary antibodies were incubated overnight at 4℃ after membranes were blocked with 5% BSA in TBST for 1 h at room temperature. Secondary antibodies were incubated for 1 h at room temperature after washing three times. The primary and secondary antibodies used in these experiments are listed in the Supplementary Table S1. Protein bands were visualized using ECL reagents and quantified with ImageJ software.

Colony formation assay

Cells were seeded at a density of 500 to 800 cells per well in 6-well plates and cultured for a period of 7 to 14 days, with the medium being renewed every 3 days. And the drugs were added during media renewal in all colony formation assays. After incubation, the cells were fixed with methanol and stained using a 0.1% crystal violet solution (C0121, Beyotime). The stained cells were subsequently scanned with an Epson Perfection V370 photo scanner.

Structural model prediction

Alphafold3 web server [34] was utilized for predictive modeling of the interaction between EGFR protein and GLUT1 protein, following the default parameters. The protein sequences of EGFR and GLUT1 proteins were obtained from UniProt [35] database. The ATP molecule required for the prediction was provided by Alphafold3. The ATP molecule's structure used in the prediction was derived from AlphaFold3's default small molecule template library. The tool automatically positions ATP in the binding pocket during complex prediction based on energy minimization and evolutionary constraints.

RNA-seq

LUAD cells with wild-type EGFR or activating mutated EGFR were transfected with siRNA and prepared for RNA-seq detection. HISAT-StringTie- DEseq2 [36, 37] was used to analyze the gene expression and differential expression.

Animal experiments

Male, 5-week-old, 20–25 g, BALB/c nude mice were obtained from Beijing Weitong Lihua Experimental Animal Technology Co., LTD. Animal experiments received ethical approval from the Institutional Animal Care and Use Committee of the Southern University of Science and Technology (SUSTech-JY202307038) (Ethics Code: 2017 – 0170). For the subcutaneous xenograft model, 5×10^5 H1975 cells were injected subcutaneously into nude mice. When the tumor volume reached 90 mm^3, mice were randomly assigned to one of four treatment groups, with five mice in each group. The treatments administered were as follows: (a) Saline (2–3 times per week, oral gavage, 100 µl per mouse); (b) Osimertinib (2–3 times per week, oral gavage, 5 mg/kg); (c) BAY-876 (2–3 times per week, oral gavage, 5 mg/kg); (d) Osimertinib combined with BAY-876 (Combination) (2–3 times per week, oral gavage, 5 mg/kg). The size of the tumor was measured with calipers every day during the treatment. The tumor volume was calculated using the following formula: (length × $width^2/2$). The mice were sacrificed on day 29, and the xenografts were excised and weighed. All tumor-bearing mice in both treatment (Osimertinib, BAY-876, and combination) and control groups survived until the predetermined endpoint (tumor volume = < 1500 mm^3). No treatment-related mortality occurred. At the end of the experiment, the mice were sacrificed by carbon dioxide anesthesia asphyxia.

Statistical analysis

All statistical analyses were conducted using the R V.4.2.0 (http://www.r-project.org). The Student's t-test was employed to assess the differences between the two variables, with statistical significance determined by a two-tailed p-value of less than 0.05. Differentially expressed genes were examined using clusterProfiler [38], an R package commonly utilized for comparing biological themes among gene clusters. The KEGG enrichment analysis assesses the enrichment of differentially expressed genes within the KEGG pathways compared to what would be expected by random chance. Meanwhile, GSEA illustrates changes in KEGG pathway activation based on the differentially expressed genes.

Results

EGFR phosphorylation level determines the interaction capacity of GLUT1 and EGFR

Our team has previously revealed that GLUT1 could interact with EGFR in lung cancer cells [31]. To determine whether GLUT1 interacts with EGFR depends on EGFR activation, we used Gefitinib and Osimertinib as

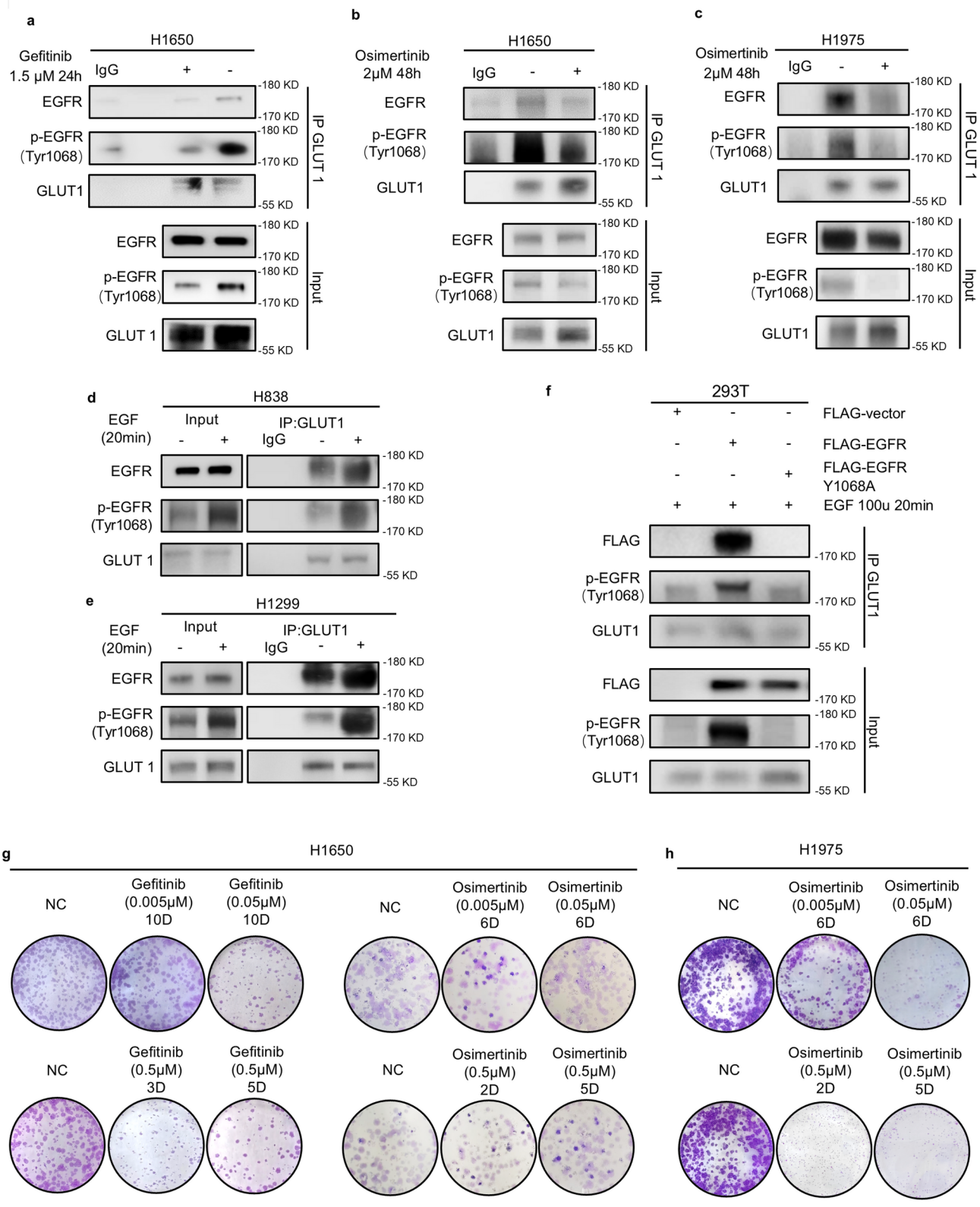

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Fig. 1 The Interaction of GLUT1 and EGFR depends on EGFR phosphorylation. **a-c**, Western blotting was used to verify the protein Co-IP results. GLUT1, EGFR, and p-EGFR (Y1068) were detected in protein samples pulled down by GLUT1 in H1650 and H1975 cells after being treated with EGFR TKIs. **a**, Western blot analysis of EGFR, p-EGFR (Tyr1068), and GLUT1 expressions in H1650 cells treated with Gefitinib (1.5 μM, 24 h); **b**, Western blot analysis of EGFR, p-EGFR (Tyr1068), and GLUT1 expressions in H1650 cells treated with Osimertinib (2 μM, 48 h); **c**, Western blot analysis of EGFR, p-EGFR (Tyr1068), and GLUT1 expressions in H1975 cells treated with Osimertinib (2 μM, 48 h); **d**, **e**, Western Blotting was used to verify the protein Co-IP results. GLUT1, EGFR, and p-EGFR (Y1068) were detected in protein samples that were pulled down by GLUT1 in H838 and H1299 cells after being treated with EGF (EGFR stimulator, 100 u/mL, 20 min). **f**, Western blot analysis of FLAG, p-EGFR (Tyr1068), and GLUT1 expressions in 293T cells transfected by Empty vector, FLAG-EGFR, and FLAG-EGFR Y1068A in the presence of EGF (100 u/mL, 20 min); **g**, **h**, Colony formation assays of H1650 and H1975 cells treated with Gefitinib or Osimertinib at various concentrations (0.005–0.5 μM) and durations, showed a time and concentration gradient dependence, demonstrating the inhibitory effects on cell proliferation

the EGFR activation inhibitors and found that the interaction of GLUT1 protein with both total EGFR protein and Y1068 p-EGFR protein decreased after being treated Gefitinib in H1650 cells (Fig. 1a), which is further confirmed with Osimertinib treatment in H1650 and H1975 cells (Fig. 1b, c).

We used EGF as an EGFR activation stimulator and found that the interaction between GLUT1 and both total EGFR and Y1068 p-EGFR enhanced in H838 and H1299 (Fig. 1d, e). In order to determine if Y1068 is the key phosphorylation region for EGFR binding to GLUT1, we performed GLUT1 Co-IP and Western blot analysis in 293T cells transfected by FLAG empty vector, FLAG-EGFR (wild type) and FLAG-EGFR Y1068A (Y1068 mutated with Alanine) in the presence of EGF. We found that GLUT1 protein could interact with EGFR protein but not with EGFR carrying Y1068A mutation (Fig. 1f) while the expressions of FLAG-EGFR and FLAG-EGFRY1068A plasmids were successfully detected (Supplementary Figure S1a). These results indicated that the interaction between GLUT1 and EGFR could depend on the phosphorylation of the EGFR Y1068 site, regardless of whether the phosphorylation of EGFR is due to EGFR activating mutations or EGF stimulation.

Cell colony formation ability was evaluated to verify the efficacy of Gefitinib and Osimertinib, and it decreased after being treated with Gefitinib and Osimertinib. This presented a time gradient dependence and concentration gradient dependence (Fig. 1g, h, Supplementary Figure S1b).

The binding of GLUT1 and EGFR depends on the presence of EGFR 8–16 exons

To verify if GLUT1 binds to EGFR depends on the dimeric change of EGFR, we predicted the binding of GLUT1 to monomeric EGFR and dimeric EGFR under the premise of introducing ATP. Whether we provide one or two EGFR sequences, we can only predict the signal of its interaction with GLUT1 on one EGFR sequence, and the binding in the intracellular region is highly probable (Fig. 2a). In the top figure, we provided one EGFR molecule, one GLUT1 molecule, and one ATP molecule in the protein-protein interaction prediction. The result showed the interaction signals of EGFR and GLUT1. In the bottom figure, we provided two EGFR molecules, one GLUT1 molecule, and two ATP molecules. The result showed that the extracellular structures of the two EGFR molecules interact with each other, which is consistent with the interaction form of the EGFR dimer in reality and does not change the mode in which GLUT1 interacts with monomeric EGFR. The AlphaFold3 signal prediction reflects a more macroscopic result, namely that the new spatial structure generated by the dimerization of EGFR does not affect the binding of GLUT1 to EGFR. In fact, GLUT1 interacts with only one EGFR monomer. Ligand-bond can activate EGFR, and then the extracellular dimerization arm is exposed while the affinity of the TK domain of EGFR to ATP is enhanced [39]. Therefore, in the AlphaFold3 prediction, we provided activated EGFR with ATP molecule added (blue structure in Fig. 2b) and wild-type EGFR without ATP molecule (blue structure in Fig. 2c) to explore the binding of EGFR and GLUT1 (purple structure) under different conditions. In the absence of an ATP molecule, most of the predicted sites were mainly concentrated in the intracellular region (Fig. 2b). However, in the presence of ATP molecules, more binding sites in the ETR were predicted (Fig. 2c). After ATP binding with the EGFR TK domain, the extracellular conformation changed, and more extracellular binding sites were predicted.

To demonstrate the critical role of the ETR in the binding of GLUT1 and EGFR, we overexpressed the FLAG-EGFRvIII plasmid and FLAG-EGFRvI plasmid, which lack various EGFR extracellular regions: EGFRvIII missing 2–7 exons and EGFRvI missing the whole extracellular domain (Fig. 2d). The results of the Co-IP experiments showed the interaction of GLUT1 protein with FLAG-EGFRvIII. The overexpression of FLAG-EGFRvIII increased p-EGFR pulled down by GLUT1 in 293T and H1299 cells. As expected, we observed two bands of p-EGFR in the input samples, confirming the success of the expression and phosphorylation of the EGFRvIII protein (Fig. 2e, f). However, in the sample overexpressed FALG-EGFRvI, Co-IP results showed that GLUT1 cannot interact with FLAG-EGFRvI and phosphorylation FLAG-EGFRvI, while the expression and phosphorylation of EGFRvI can be seen in the input sample (Fig. 2g, h). The expressions of FLAG-EGFRvIII

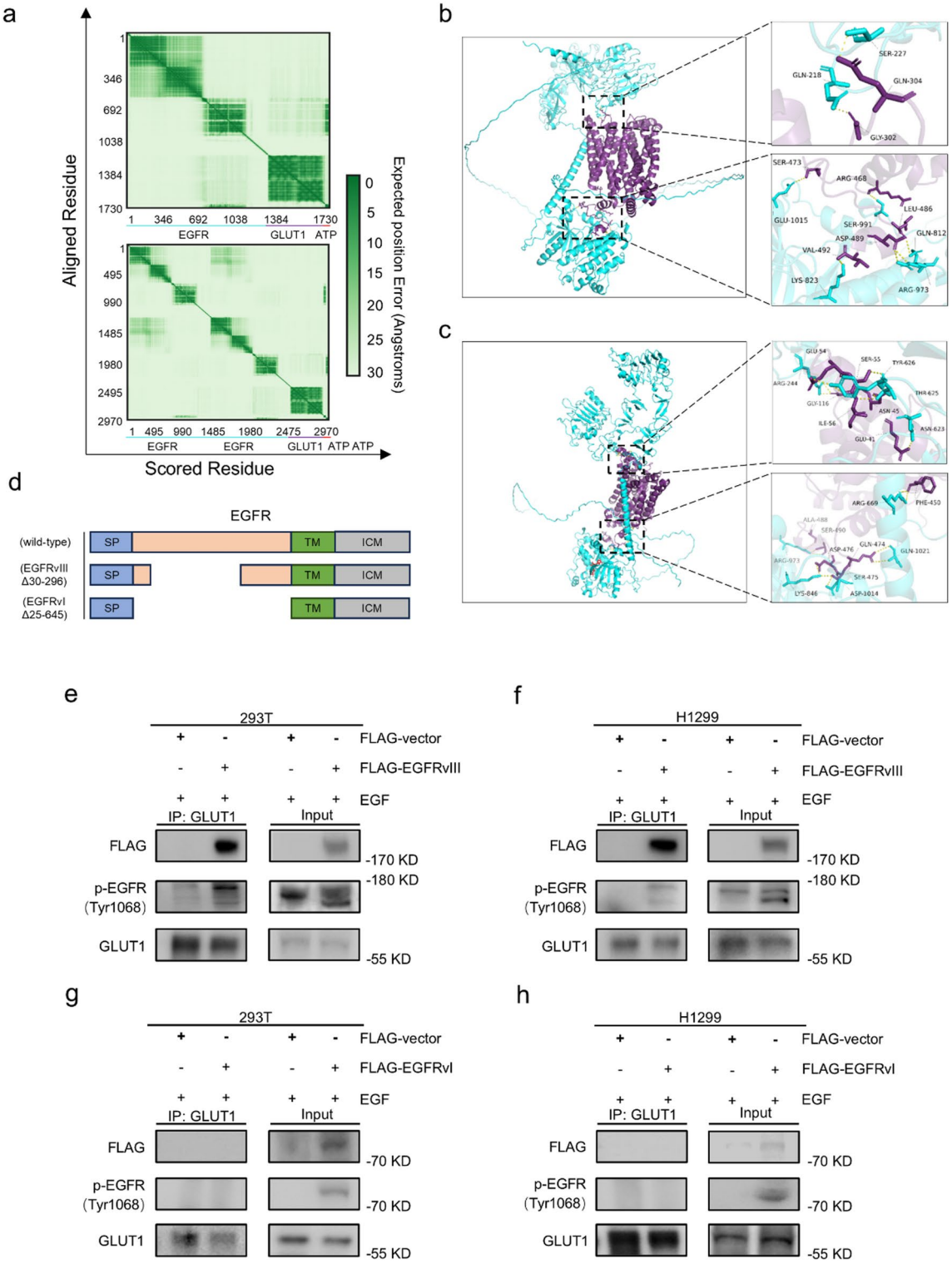

Fig. 2 (See legend on next page.)

and FLAG-EGFRvI plasmids were successfully detected in H1299 and 293T cells (Supplementary Figure S2a). At the same time, we unexpectedly found that the colony formation ability of EGFRvI is much weaker than that of EGFR and EGFRvIII (Supplementary Figure S2b, c). Taken together, in comparison to wild-type EGFR, when EGFR loses exons 8–16, GLUT1 is unable to bind to both wild-type EGFR and phosphorylated EGFR (p-EGFR).

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Fig. 2 GLUT1 binds to the extracellular region of EGFR. **a-c**, AlphaFold3 prediction of the binding sites between GLUT1 and EGFR. **a**, Prediction of GLUT1 binds to monomeric and dimeric EGFR in the presence of ATP; in the top figure, one EGFR molecular, one GLUT1 molecular, and one ATP molecular were provided; in the bottom figure, two EGFR molecules, one GLUT1 molecular, and two ATP molecules were provided; **b**, **c**, Prediction binding sites of GLUT1 and EGFR in the absence and presence of ATP. The purple structure represents the GLUT1 protein and its amino acids; blue structure indicates the EGFR protein and its amino acids; yellow dashed lines denote predicted hydrogen bonds at binding sites; numbered labels identify specific amino acid positions; **d**, EGFR truncations were constructed according to UniProt; **e**, **f**, Western blotting was used to verify the protein Co-IP results. FLAG, p-EGFR (Y1068), and heavy chain were detected in protein samples that were pulled down by GLUT1 in 293T and H1299 cells transfected with Empty vector and FLAG-EGFRvIII in the presence of EGF (100 u/mL, 20 min); **g**, **h**, Western blotting was used to verify the protein Co-IP results. FLAG, p-EGFR (Y1068), and heavy chain were detected in protein samples that were pulled down by GLUT1 in 293T and H1299 cells transfected with Empty vector and FLAG-EGFRvI in the presence of EGF (100 u/mL, 20 min)

Additionally, the cell colony formation ability was also affected by the absence of these exons.

Down-regulated genes by si-GLUT1 only in EGFR-mutated cells are involved in the cell cycle and cancer-related signaling pathways

To explore the differences between EGFR wild-type and EGFR activating mutated cells in GLUT1 silent at the transcriptional level. Genes with absolute values of log-2FoldChange higher than 0.5 as differentially expressed genes. We classified these genes into upregulated and downregulated groups after knocking down GLUT1 in EGFR wild-type (H1299 and H838) and EGFR-activating mutated cells (H1650 and H1975). In EGFR-activating mutated cells, there are 1186 down and 1949 up-regulated genes. In EGFR wild-type cells, there are 1011 down and 580 up-regulated genes (Fig. 3a).

We divided these differentially expressed genes into six groups: genes only up-regulated in EGFR-activating mutated cells (G1), genes only down-regulated in EGFR-activating mutated cells (G2), genes only up-regulated in EGFR wild-type cells (G3), genes only down-regulated in EGFR wild-type cells (G4), genes up-regulated in both EGFR activating mutated and wild-type cells (G5), and genes downregulated in both EGFR activating mutated and wild-type cells (G6) (Fig. 3b). We found that 924 G2 genes were involved in the lysosome, cell cycle, and cancer-related signaling pathways, including the MAPK and Hippo signaling pathways, as well as Signaling pathways regulating the pluripotency of stem cells. In contrast, genes in G1, G3, and G4 did not correlate with cancer pathways (Fig. 3c).

To further investigate the effects of EGFR activating mutated cells on cellular biological functions and signaling pathways, we performed GSEA analysis to enrich KEGG signaling pathways. It can be observed that various cellular functions and signaling pathways were negatively regulated (Supplementary Figure S3a), which further revealed that GLUT1 participates in tumor progression through these KEGG pathways. Interestingly, EGFR downstream signaling pathways, mTOR signaling pathway, PI3K-Akt signaling pathway, JAK-STAT signaling pathway, and MAPK signaling pathway are negatively regulated (Fig. 3d). On the contrary, compared to EGFR activating mutated cells, EGFR wild-type cells have fewer cancer-regulated signaling pathways (Supplementary Figure S3b). To clarify the involvement of genes in different biological processes and molecular functions, we conducted a GO (Gene Ontology) analysis and found that biology processes about Ras protein signal transduction, regulation of protein localization to membrane, and small GTPase-mediated signal transduction can enrich more genes. Interestingly, genes in G2 are involved in some EGFR relatively molecular functions, such as protein tyrosine kinase activity and protein tyrosine/threonine phosphatase activity, by knocking down GLUT1 in EGFR activating mutated cells. (Fig. 3e, Supplementary Figure S3c). These analysis results indicate that GLUT1 has the potential to participate in the regulation of EGFR downstream signaling pathways by modulating tyrosine kinase activity. This means knocking down GLUT1 in EGFR activating mutated cells can regulate certain membrane localization processes, certain tyrosine kinase activity, and some relative signaling pathways.

GLUT1 regulates EGFR downstream signaling pathways

To further confirm that GLIUT1 regulates the RAS signaling pathway in H1650 cells but not in H1299 cells, we conducted a series of Western blot experiments. The results showed that the expressions of RAS and RAF, the phosphor-RAF, and phosphor-ERK were down-regulated by silencing GLUT1 in H1650 cells (EGFR-mutated cells). However, the expressions of RAF and RAF, the phosphor-RAF, and phosphor-ERK were not regulated by GLUT1 in H1299 cells (EGFR-wild type cells) despite the stimulation of the EGFR by EGF (Fig. 4a), indicating that GLUT1 might regulate the RAS signaling pathway only in EGFR-mutated cells.

Further exploration showed that the expressions of phosphor-AKT and mTOR were downregulated after knocking down GLUT1 in H1650 and H1975 cells, the expressions of phosphor-AKT and phosphor-mTOR were downregulated in H1299 with EGFR stimulation. The AKT- mTOR signaling pathway was regulated by GLUT1 in H1650 cells, but the regulation exclusively upon the EGF stimulation of EGFR in H1299, indicating that the high activation of EGFR may be inevitable involving in these regulations (Fig. 4b, Supplementary Figure S4a).

Fig. 3 (See legend on next page.)

In the JAK-STAT signaling pathway of H1650, the expressions of STAT1, phosphor-STAT1 (Tyr701) and phosphor-STAT3 (Tyr705) were downregulated after silencing GLUT1. The expressions of JAK1, phosphor-JAK1, and STAT3 were not regulated by GLUT1. And we found expressions of phosphor-JAK3 (Tyr980/981) in H1650, JAK1, phosphor-JAK1 (Tyr1034/1035), and phosphor-STAT1 (Ser727) in

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Fig. 3 Down-regulated genes by siGLUT1 only in EGFR-mutated cells were involved in the cell cycle and cancer-related signaling pathways. **a**, The proportion of down-regulated genes and up-regulated genes after knockdown GLUT1 in the RNA-seq datasets of EGFR activating mutated cells and EGFR wild-type cells. **b**, The Venn diagram of six differentially expressed gene groups in the RNA-seq datasets of EGFR activating mutated cells and EGFR wild-type cells. Group 1 included differentially expressed genes only up-regulated in EGFR-activating mutated cells (G1). Group 2 included differentially expressed genes only down-regulated in EGFR-activating mutated cells (G2). Group 3 included differentially expressed genes that are only up-regulated in EGFR wild-type cells (G3). Group 4 included differentially expressed genes only down-regulated in EGFR wild-type cells (G4). Group 5 included differentially expressed genes that up-regulated both in EGFR-activating mutated cells and EGFR wild-type cells (G5). Group 6 included differentially expressed genes that down-regulated both in EGFR activating mutated cells and EGFR wild-type cells (G6). **c**, The KEGG pathways that are enriched in the four differentially expressed gene groups (G1-G4). Genes (G2) down-regulated only in EGFR-activating mutated cells were involved in the cell cycle and cancer-related signaling pathways. **d**, The GSEA results of EGFR downstream signaling pathways in EGFR activating mutated cells. **e**, The GO-Biological Process (BP) enrichment analysis results of differentially expressed genes that down-regulated in EGFR activating mutated cells after knocking down GLUT1

H1975 were regulated by GLUT1 (Supplementary Figure S4b, c). Differently, the expressions of JAK1, phosphor-JAK1, phosphor-STAT1 (Tyr701), and phosphor-STAT3 (Tyr705) were down-regulated depending on the stimulation of EGFR by EGF in H1299 while GLUT1 protein was knocked down (Fig. 4c). The results showed that the regulations of GLUT1 mainly focus on the STAT family proteins in the EGFR-mutated cells, while GLUT1 regulates one JAK family protein, and the phosphorylation of STAT family proteins depends on the EGF stimulation in the EGFR wild-type cells.

Taken together, we determined that GLUT1 can bind to EGFR and regulate the expressions and activations of certain proteins in EGFR downstream signaling pathways including JAK-STAT, AKT-mTOR and RAS-RAF-ERK in both the EGFR-mutated cells and the EGFR wild-type lung cancer cells (Fig. 4d).

GLUT1 inhibitor BAY-876 sensitized tumor cells to EGFR TKI osimertinib

To investigate the role of the combination of GLUT1 inhibitor and EGFR TKIs, we conducted experiments in vitro and *in vivo*. Cell colony formation ability significantly decreased after combining BAY-876 and Osimertinib, while separately using these two drugs had a worse effect (Fig. 5a, b). Western blot was used to detect the effect of the drugs combination, and we found that phosphor-AKT, p-STAT1 and p-JAK3 were decreased significantly after combining these two drugs (Supplementary Figure S5a, b). In Vivo, experiments were conducted using subcutaneous injection of H1975 cells in nude mice to establish the xenograft tumor model. Different treatments were carried out after palpable tumors had developed in the mice. Drugs (5 mg/kg) (BAY-876 or Osimertinib) were given to the drug treatment groups by oral gavage 2–3 times per week, and saline was given to the NC group on the same day (Fig. 5c). While drugs remarkably retarded tumor growth as expected, it is notable that all treatment groups, including the Combination group, did not appear to have apparent animal body weight loss (Fig. 5d). At the end of the experiment day (day 25), the NC group showed a larger tumor size and heavier tumor weight than the Combination group (Fig. 5e, f). Additionally, the combination of BAY-876 and Osimertinib significantly inhibited the tumor volume in mice (Fig. 5g). Collectively, these results demonstrate that GLUT1 inhibitor BAY-876 could induce tumor cells sensitizing to EGFR TKI Osimertinib and decrease tumor progression.

Discussion

In the current study, we predicted a groundbreaking discovery of protein interactions between GLUT1 and EGFR, emphasizing the innovative aspect of our work linking glucose metabolism regulation with EGFR signaling in LUAD. Specifically, we found that GLUT1, traditionally known as glucose transporter, exerts a dual function by interacting with EGFR in a phosphorylation-dependent manner, especially at the Y1068 phosphorylation site. At the same time, it provided a detailed understanding of how the combination of EGFR-TKIs such as Gefitinib and Osimertinib and GLUT1 inhibitors made tumor cells highly sensitive. Collectively, our results suggested that targeting the EGFR-GLUT1 axis may provide a basis for novel therapeutic strategies against lung cancer proliferation.

GLUT1, as a well-known glucose transporter, is instrumental in the metabolic reprogramming of cancer cells [40], which is a vital mechanism for tumor cells to adapt to their proliferation and migration [41, 42]. In the combined analysis of GSEA and GO, GLUT1 was found to be closely related to cell cycle and signal transduction. This could be a hint that GLUT1 is involved in tumor regulation with non-metabolic dependent functions. In our Co-IP experiments, GLUT1 bound with EGFR, and in the western blot experiments, GLUT1 regulated EGFR downstream signaling pathways. Our results solidified the regulation of GLUT1 in LAUD cells. This demonstrated the crosstalk of metabolic reprogramming on tumor signaling pathways.

Previous research shows that EGFR phosphorylation plays a role in downstream signaling transduction, which affects the proliferation and survival of tumor cells and serves as a diagnostic criterion for LAUD under certain conditions [2, 43]. Gefitinib and Osimertinib are two commonly used EGFR TKIs that can prevent the

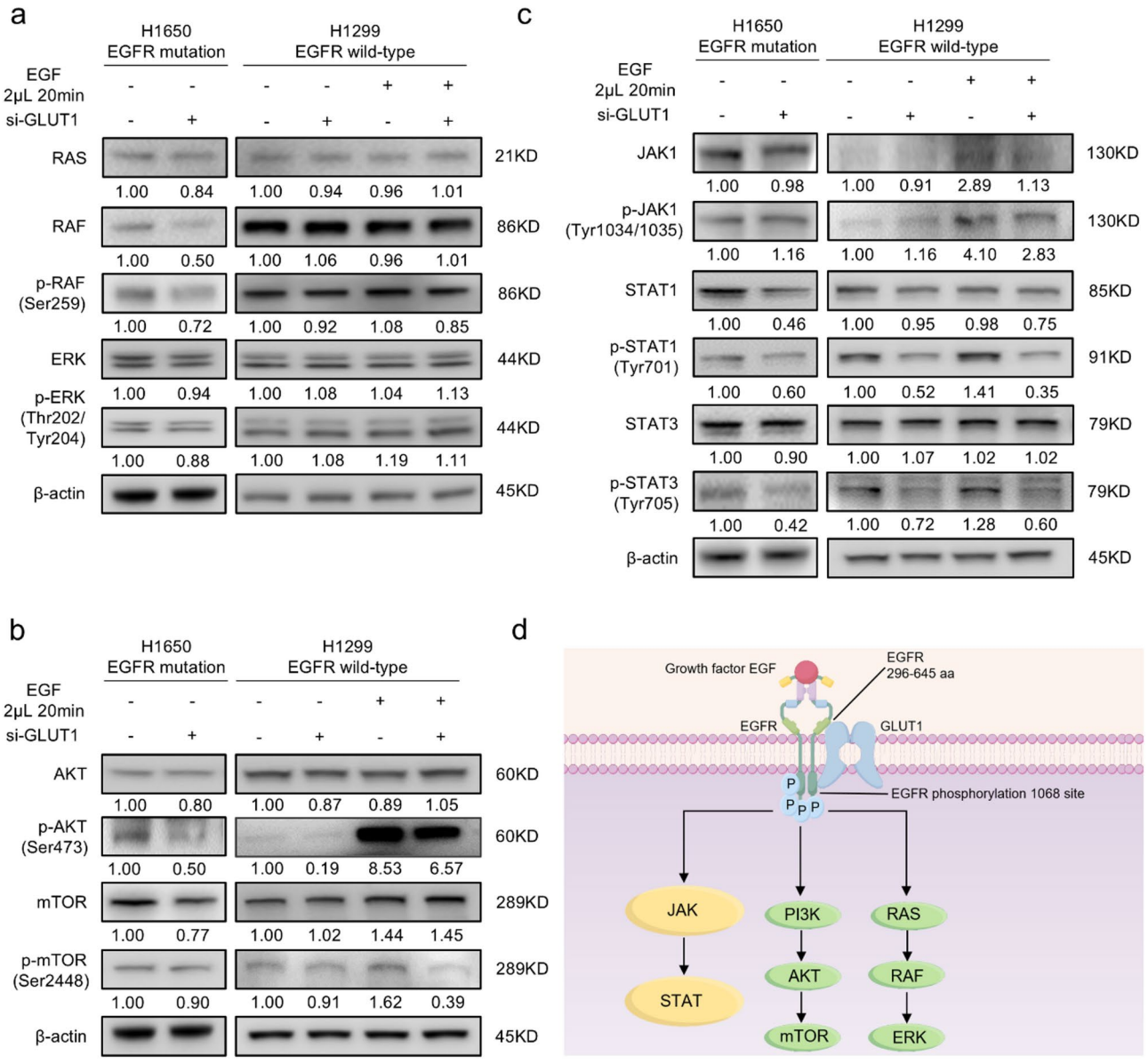

Fig. 4 GLUT1 regulates EGFR downstream signaling pathways depending on the high phosphorylation level of EGFR. **a-c**, Western blotting was used to verify the protein expressions and phosphorylation of different EGFR downstream signaling pathways. **a**, Western blot was used to detect the protein expressions of RAS, RAF, p-RAF (Ser259), ERK, p-ERK (Thr202/Tyr204) in H1650 and H1299 cells treated with siRNA knockdown of GLUT1 in the absence and presence of EGF (100 u/mL, 20 min); **b**, Western blot was used to detect the expressions of ATK, p-AKT (Ser473), mTOR, and p-mTOR (Ser2448) in H1650 and H1299 cells treated with siRNA knockdown of GLUT1 in the absence and presence of EGF (100 u/mL, 20 min); **c**, Western blot was used to detect the protein expressions of JAK1, p-JAK1 (Tyr1034/1035), STAT1, p-STAT1 (Tyr701), STAT3, p-STAT3 (Tyr705) in H1650 and H1299 cells treated with siRNA knockdown of GLUT1 in the absence and presence of EGF (100 u/mL, 20 min); **d**, Schematic diagram of the interaction between GLUT1 and EGFR and regulates downstream signaling pathways of EGFR

phosphorylation of EGFR, decreasing the activation of downstream signaling pathways [19, 22, 44]. We currently found that when EGFR TKIs inhibited the phosphorylation of EGFR, the binding to GLUT1 decreased, but the expression of GLUT1 was increased, suggesting that lung cancer cells can counteract the negative effects of the inability to phosphorylate by increasing the expression of glucose transporters. Among EGFR tyrosine kinase sites, Y1068 is an essential site for EGFR phosphorylation [45–47]. After mutating this tyrosine site to alanine (Y1068A), EGFR lost its phosphorylation and the ability to bind with GLUT1. Combining the results of these experiments, we demonstrated the interaction between EGFR and GLUT1 is dependent on EGFR phosphorylation.

The extracellular domain is necessary for the interaction of EGFR ligands [48, 49], and its conformation

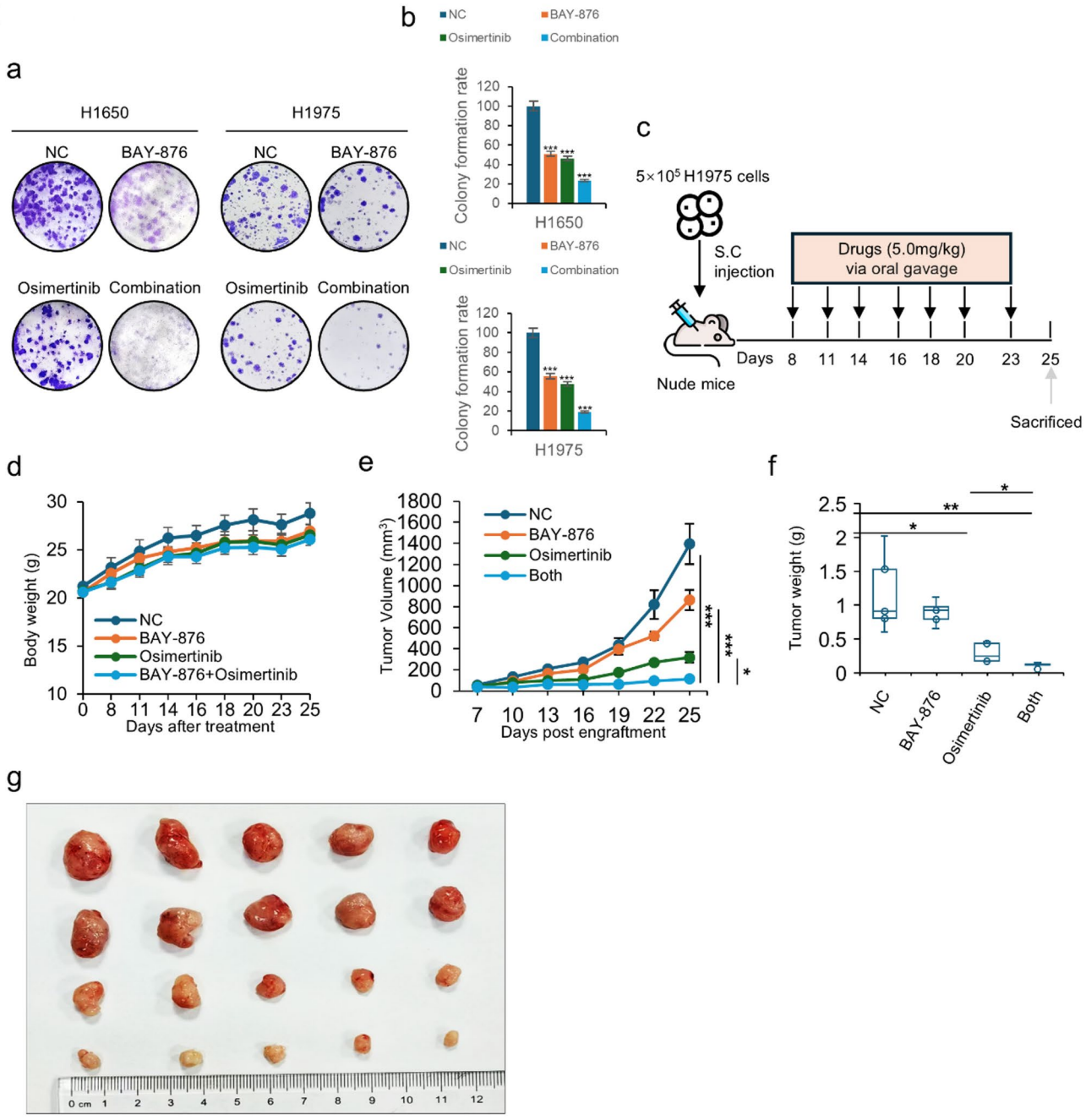

Fig. 5 BAY-876 sensitized lung cancer cells to Osimertinib in vitro and *in vivo*.**a**, Colony formation assay of H1975 and H1650 cells treated by BAY-876 (0.05μM/ml), Osimertinib (0.05μM/ml) and Combination of two drugs for 10 days, **b** is the quantitative value of a. **c**, Schematic diagram of the in vivo experimental procedure. **d**, Line graph displaying the weight changes of mice during the experimental period ($n=5$). **e**, Tumor growth curve of four treatment groups during this experiment ($n=5$). **f**, Comparison of the tumor weight of four treatment groups at end point ($n=5$). **g**, Tumor growth curve at the indicated days with different treatments in BALB/c-nu/nu mice ($n=5$). f Macroscopic view of tumor harvested at termination of four treatment groups ($n=5$). All data are presented as means ± SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

changes when EGFR is activated [50], which facilitates more possible interaction for EGFR, regulating EGFR downstream signaling pathways. Unlike wild-type EGFR, which possesses an entire extracellular domain allowing typical dimerization and ligand binding, EGFRvI and EGFRvIII lack all or parts of this domain, leading to distinct binding properties. EGFRvIII has been detected in over 50% of both high-grade and low-grade gliomas [51]. It has also been found in 5 out of 32 lung carcinomas [52], 21 out of 27 breast carcinomas [53, 54], 4 out of 6 pediatric gliomas, 6 out of 7 medulloblastomas, and 24 out of 32 ovarian carcinomas [54]. In contrast, EGFRvI

has only been observed in a single tumor cell line, specifically a xenograft derived from a malignant human glioma [55]. The differences in EGFRvIII and EGFRvI caused by the varying degrees of extracellular structural lack may suggest that EGFRvI mutations are an evolutionary disadvantage for tumor cells, while also highlighting the significance of the extracellular region of EGFR.

Our findings revealed that GLUT1 fails to interact with EGFRvI, which lacks the complete extracellular domain, but does interact with EGFRvIII. This distinction highlighted that the presence of an intact or partially intact extracellular domain is necessary for GLUT1 to bind EGFR, suggesting that GLUT1-EGFR binding may require specific extracellular conformations (269–645 bp). Interestingly, our Co-IP results showed that more phosphorylation signals can be observed when using GLUT1 pull-down in EGFRvIII, demonstrating that intact EGFR can dimerize with EGFRvIII. This dimer only binds one EGF ligand, producing two phosphorylation molecules. The insights gained from EGFRvIII and EGFRvI studies also underscore the broader functional role of the EGFR extracellular domain in non-ligand binding contexts. Our data suggested that even in the absence of typical ligand interactions, the extracellular domain facilitates essential interactions, such as with GLUT1, which may influence receptor stability, localization, or dimerization potential. This unexpected role of the extracellular domain highlights the structural requirements of non-ligand-dependent interactions that have not been fully understood before. It suggests that other transmembrane proteins might similarly rely on their extracellular domain to exert binding and regulatory effects.

Extracellular protein-protein interactions (exPPIs) are necessary for researching the biological function of receptor proteins [56]. These interactions play a pivotal role in transducing extracellular signals into the cell, thereby initiating signaling cascades and influencing protein dynamics [57, 58]. Additionally, they facilitate intercellular communication and molecular recognition [59]. In this study, we identified a novel form of transmembrane PPIs: The binding of two membrane proteins in the extracellular region depends on the activation of one of them, usually EGFR, and this activation site, such as Y1068, is also their binding site inside the cell.

EGFR activates multiple downstream signaling pathways, each regulating distinct cellular processes such as proliferation, differentiation, migration, and survival [57, 60, 61]. RAS/RAF/MEK/ERK pathway regulates cell proliferation, differentiation, and growth [62]. PI3K/AKT/mTOR pathway governs cell survival and metabolism [63]. Notably, while investigating the AKT-mTOR pathway in H1650 cells, we observed that GLUT1 exhibited limited regulatory effects on mTOR activity. This attenuated regulation may be attributed to the PTEN-deficient status of H1650 cells, which is known to profoundly alter PI3K/AKT signaling dynamics [64]. However, our experiments clearly demonstrated that GLUT1 remains capable of modulating AKT phosphorylation in H1650 cells, suggesting the existence of PTEN-independent regulatory mechanisms (Supplementary Figure S5a, b). JAK/STAT pathway regulates cell differentiation, immune modulation, and anti-apoptotic signaling [65]. Our exploration results on these EGFR downstream signaling pathways showed the regulation of GLUT1 to these signaling pathways, indicating that metabolism-related protein is involved in the regulation of tumor signaling pathways. This relativity between tumor metabolism and signaling pathways suggests that tumor cells might regulate these two biological progressions through the same system.

While our study provides mechanistic insights into GLUT1-mediated regulation of oncogenic signaling pathways, we acknowledge several limitations. Most notably, the current findings are derived entirely from cell line models and murine xenografts, which may not fully recapitulate the heterogeneity of human tumors. The absence of clinical specimen validation or patient-derived organoid models represents an important constraint in translating these findings into clinical settings. More clinical studies should be provided to verify the therapeutic potential of targeting GLUT1 in combination with EGFR inhibition.

Conclusion

Our study uncovered a novel, phosphorylation-dependent interaction between GLUT1 and EGFR, which regulated tumor downstream signaling pathways. We found the importance of phosphorylation in the structure and function of EGFR. This discovery provides profound implications for both basic cancer biology and clinical therapy. We demonstrated the pivotal role of the extracellular domain of EGFR, which can facilitate EGFR with more possibilities for interaction with other proteins or ligands. We further explored the possibility that GLUT1 inhibitors could transform EGFR-TKI therapy by enhancing drug sensitivity. Therefore, the GLUT1-EGFR axis may be a promising therapeutic target for LUAD and possibly other EGFR-driven cancer treatments. This study provides a significant contribution to cancer research by presenting a strategic framework that integrates the targeting of metabolic regulators with conventional targeted therapies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-025-02259-z.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Acknowledgements
The authors thank Huijie Zhao, Yue Liu and YUhui Du's helpful technical assistance and discussion on this project.

Author contributions
ZX and GC conceived and designed the experiments and wrote the original draft; ZX, ZZ, SC, YL, and XH performed the experiments and analyzed data; GC conceptualization, supervision, and funding acquisition. All authors read and approved the final manuscript.

Funding
This work was partly supported by the Shenzhen Municipal Science and Technology Innovation Commission Foundation in China (JCYJ20220530114415036, JCYJ20210324104800001 to G.C.).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Institutional Animal Care and Use Committee of the Southern University of Science and Technology.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 11 March 2025 / Accepted: 20 May 2025
Published online: 28 May 2025

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