

Positive Feedback Loop via HIF-1 α Lactylation Driven by Kynurenine Promotes Glycolysis

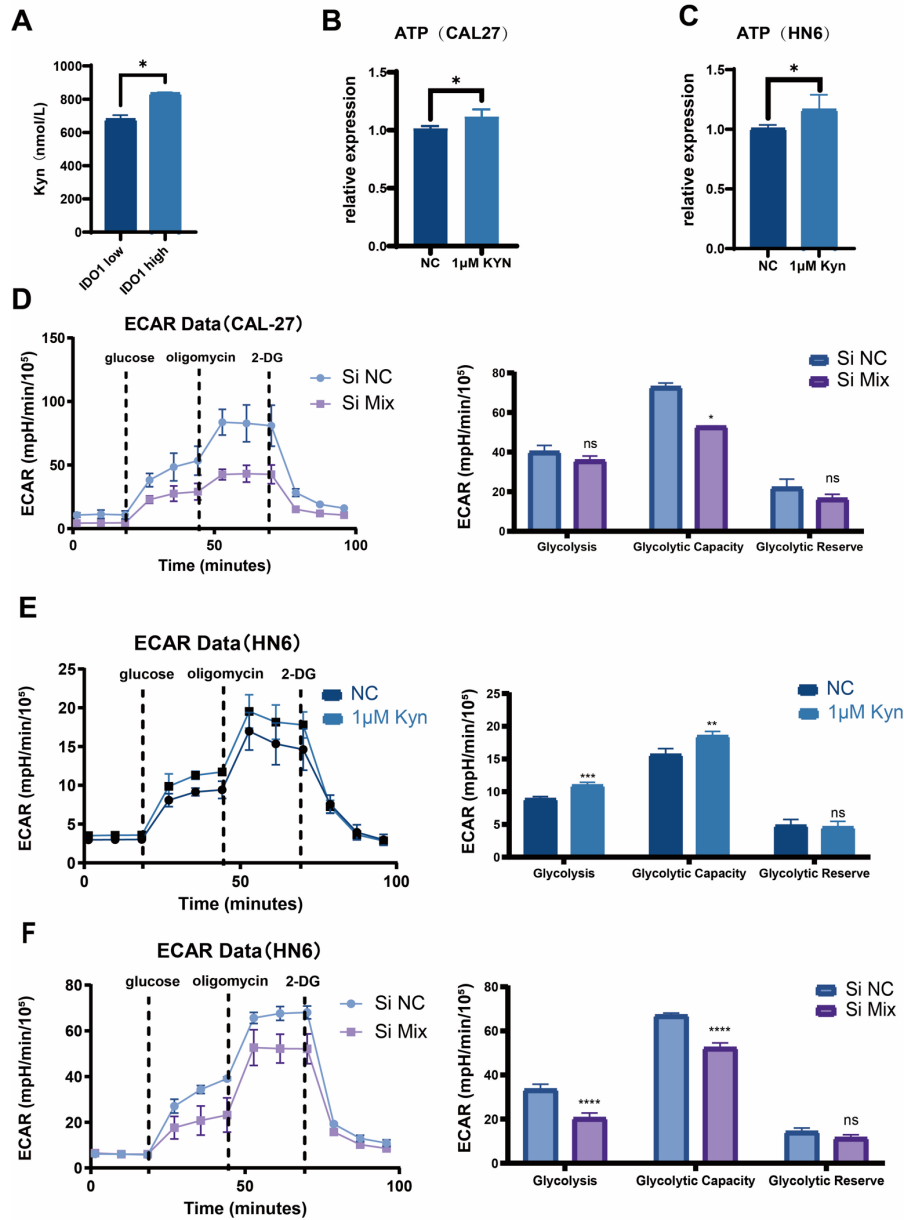


Figure S1. Kynurenine concentrations and effects on cellular energy metabolism. (A) LC-MS quantification of extracellular kynurenine in conditioned medium from IDO1-overexpressing OSCC cells. (A) LC-MS quantification of extracellular Kyn in conditioned medium from IDO1-overexpressing CAL27 OSCC cells. (B-C) Exogenous Kyn (1 μ M) increases ATP production in CAL27 (B) and HN6 (C) tumor cells. (D-F) Glycolytic stress tests showing ECAR in tumor cells, including representative curves and quantification of glycolysis, glycolytic capacity, and glycolytic reserve: IDO1-knockdown CAL27 cells (D), HN6 cells treated with 1 μ M Kyn (E), and IDO1-knockdown HN6 cells (F). ECAR values were normalized to cell number and expressed as mpH/min/ 10^5 cells. Data are presented as means $\pm$ SEMs from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

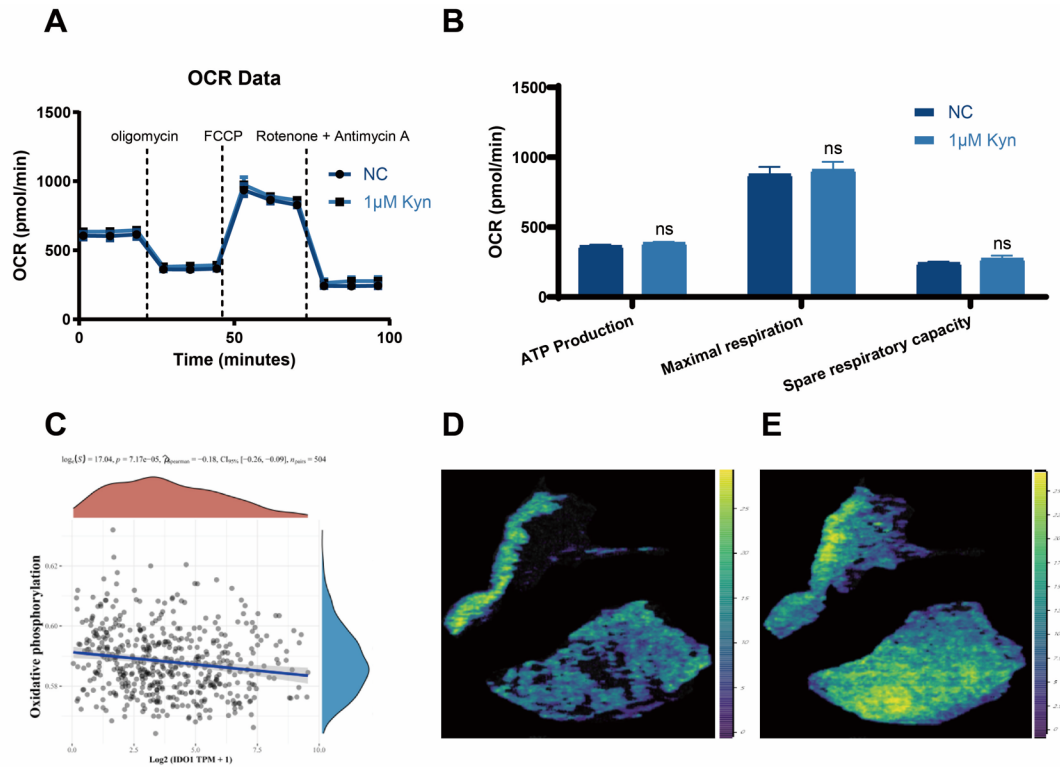


Figure S2. Kynurenine and oxidative phosphorylation in tumor cells. (A-B) Mitochondrial stress test in tumor cells, showing representative OCR curves (A) and quantification (B) of oxidative phosphorylation. OCR values were normalized to cell number and expressed as pmol/min/ 10^5 cells. (C) Correlation analysis between IDO1 expression and OXPHOS-related signatures in head and neck squamous cell carcinoma using the TCGA dataset. (D-E) Spatial metabolomics mapping the tissue distribution of OXPHOS-related metabolites, including cis-aconitate (D) and glutathione (E), in clinical samples. Data are presented as means $\pm$ SEMs from three independent experiments. ns indicates no significant difference.

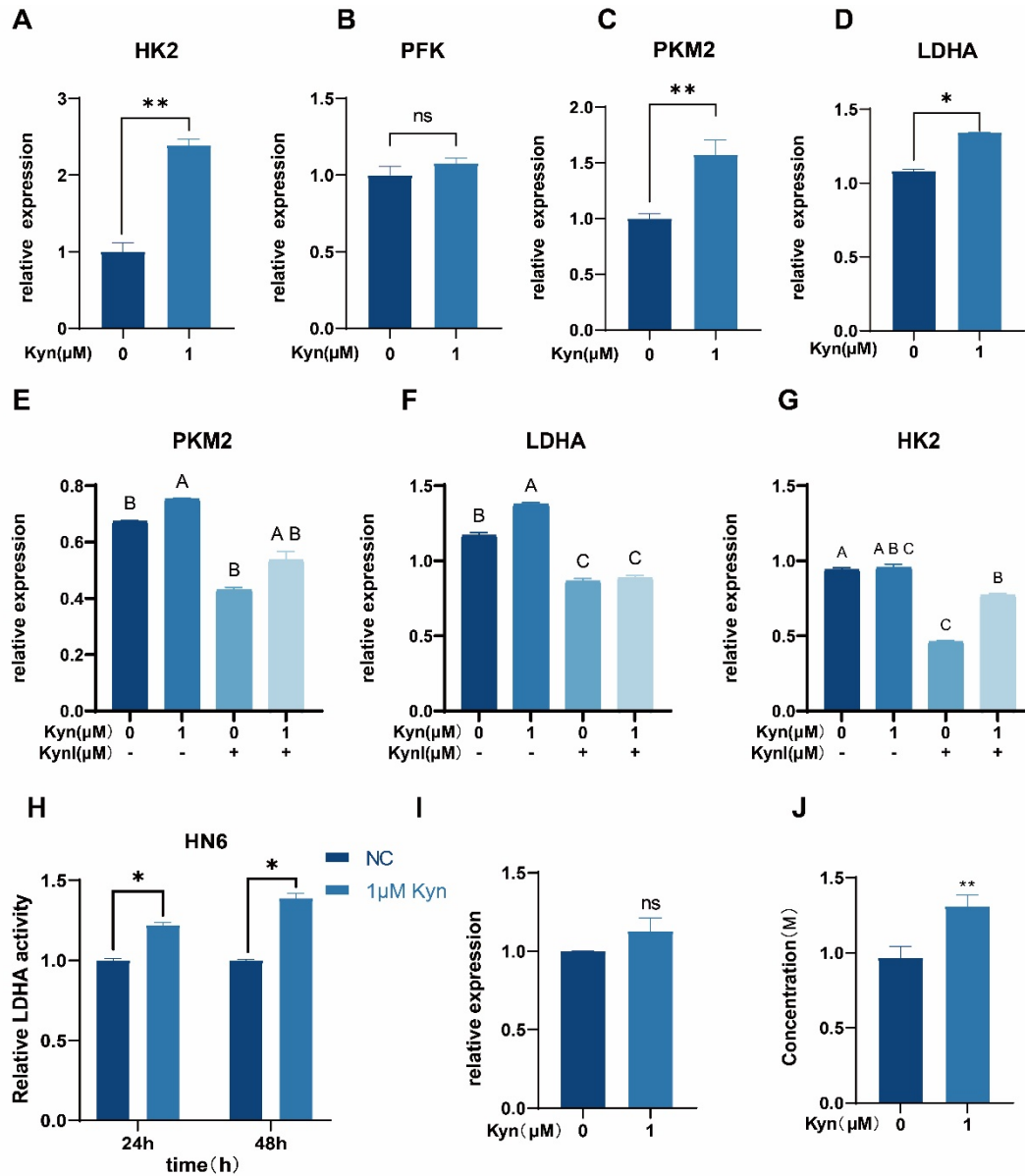


Figure S3. Effects of Kynurenine on glycolytic enzyme expression and metabolites.

(A-D) RT-qPCR analysis of HK2 (A), PFK (B), PKM2 (C), and LDHA (D) mRNA expression in HN6 cells treated with 1 μM Kyn. (E-G) Quantification of Western blot bands for PKM2 (E), LDHA (F), and HK2 (G) corresponding to Figure 1J. (H-J) Biochemical assays measuring LDH activity (H), pyruvate levels (I), and lactate production (J) in HN6 cells following 1 μM Kyn treatment. Data are presented as means ± SEMs from three independent experiments. * $p < 0.05$, ** $p < 0.01$, ns indicates no significant difference. Different letters (A, B, C) indicate statistically significant differences between groups

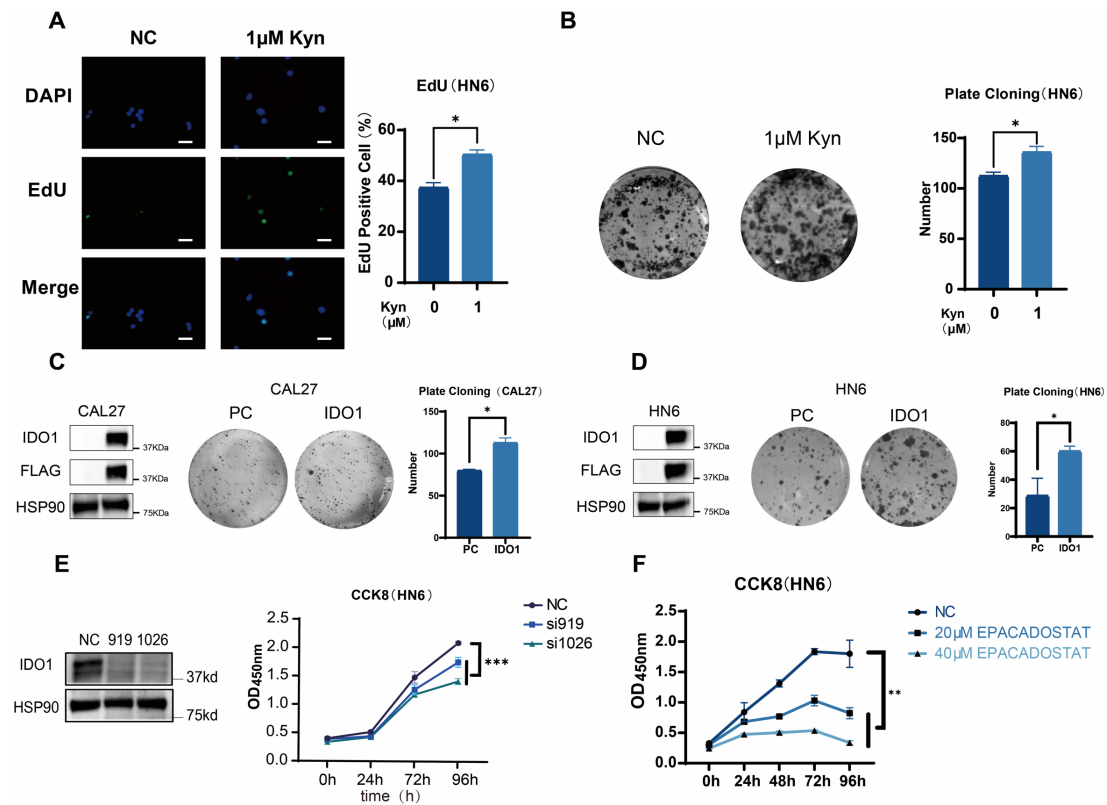


Figure S4. Effects of Kyn and IDO1 manipulation on OSCC cell proliferation. (A) EdU assay measuring DNA replication in HN6 cells treated with 1 μM Kyn. (B) CCK-8 assay evaluating proliferation of HN6 cells following 1 μM Kyn treatment. (C-D) Colony formation assays assessing the effects of IDO1 overexpression in CAL27 (C) and HN6 (D) cells. (E-F) CCK-8 assays showing reduced proliferation in HN6 cells upon IDO1 knockdown (E) or treatment with an IDO1 inhibitor (F). Data are presented as means ± SEMs from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant ($p > 0.05$). Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

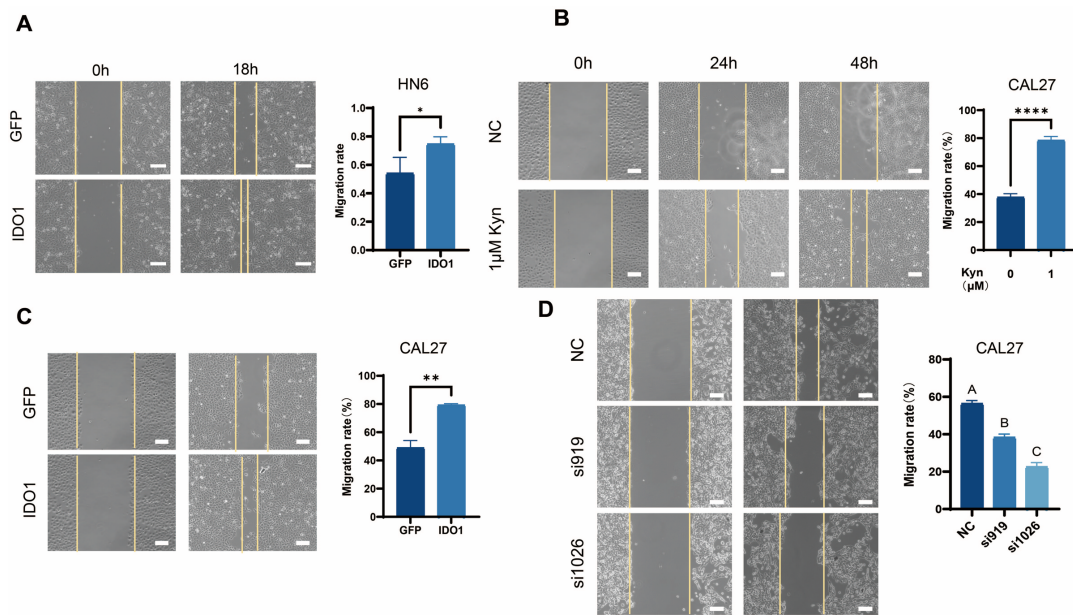


Figure S5. Effects of IDO1 and Kyn on OSCC cell migration. (A) Wound-healing assay assessing migration of HN6 cells overexpressing IDO1. (B) Wound-healing assay in CAL27 cells treated with 1 μ M Kyn. (C) Wound-healing assay in HN6 cells overexpressing IDO1. (D) Wound-healing assay in CAL27 cells following IDO1 knockdown. Data are presented as means $\pm$ SEMs from three independent experiments. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; ns, not significant ($p > 0.05$). Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

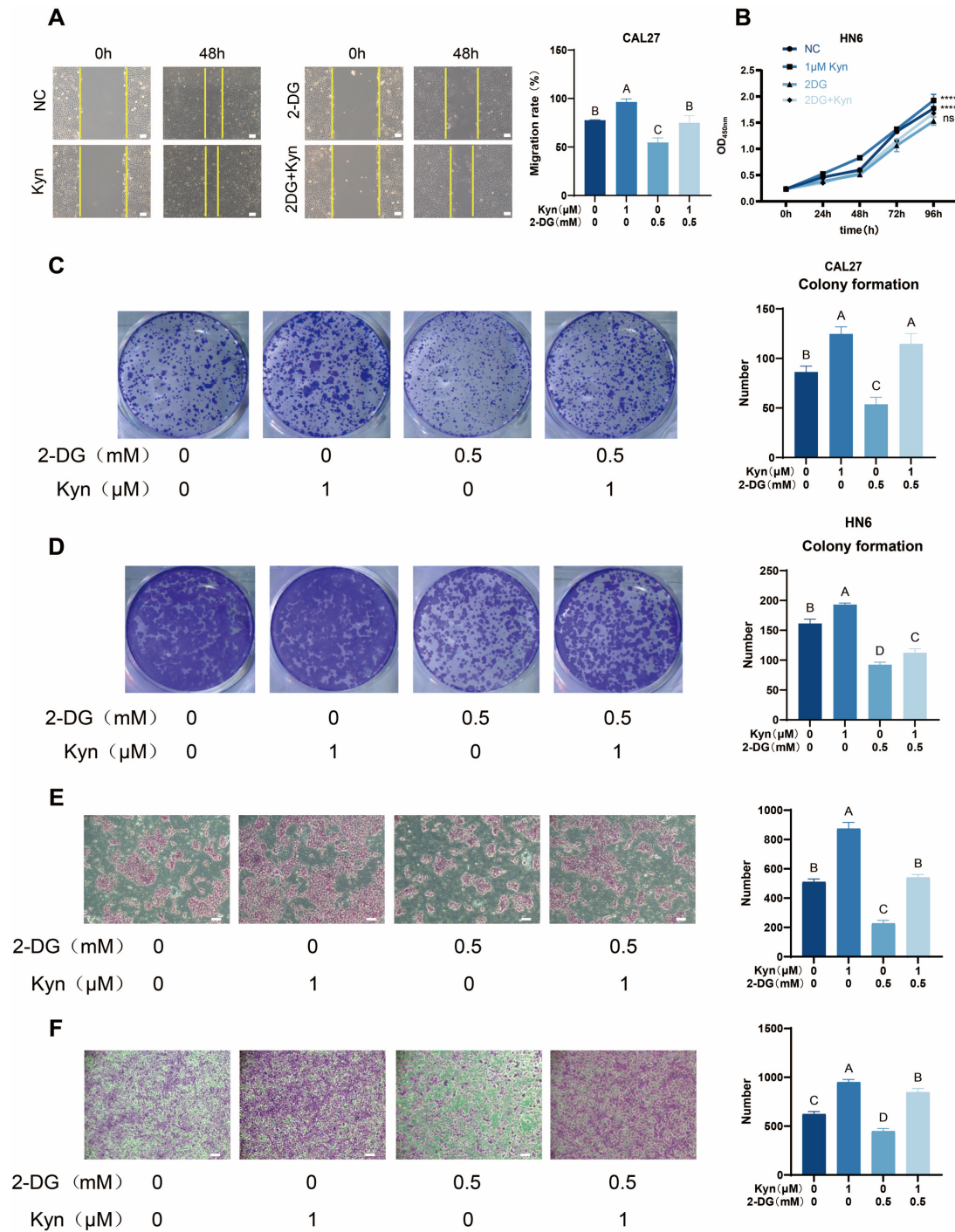


Figure S6. Effects of 2-DG and Kyn on OSCC cell proliferation, colony formation, and migration. (A) Wound-healing assay in CAL27 cells treated with 2-DG alone or in combination with 1 μM Kyn. (B) CCK-8 assay evaluating proliferation of HN6 cells 2-DG and/or Kyn treatment. (C-D) Colony formation assays in CAL27 (C) and HN6 (D) cells following 2-DG and/or Kyn treatment. (E-F) Transwell migration assays in CAL27 (E) and HN6 (F) cells under 2-DG and/or Kyn treatment. **** $p < 0.0001$; ns, not significant ($p > 0.05$). Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

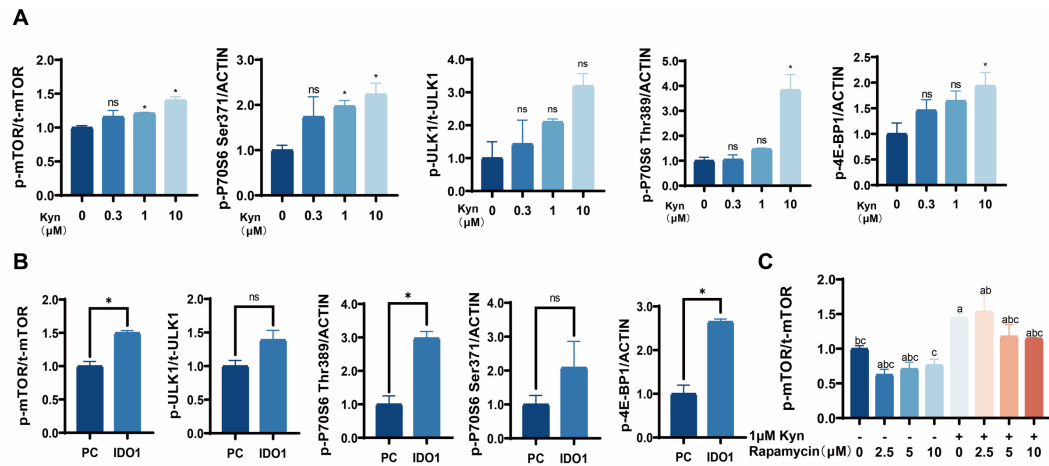


Figure S7. Western blot quantification of glycolytic enzymes corresponding to Figure 3. (A) Quantification of Western blot bands corresponding to Figure 3B. (B) Quantification of Western blot bands corresponding to Figure 3C. (C) Quantification of Western blot bands corresponding to Figure 3D. * $p < 0.05$, ns, not significant ($p > 0.05$). Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

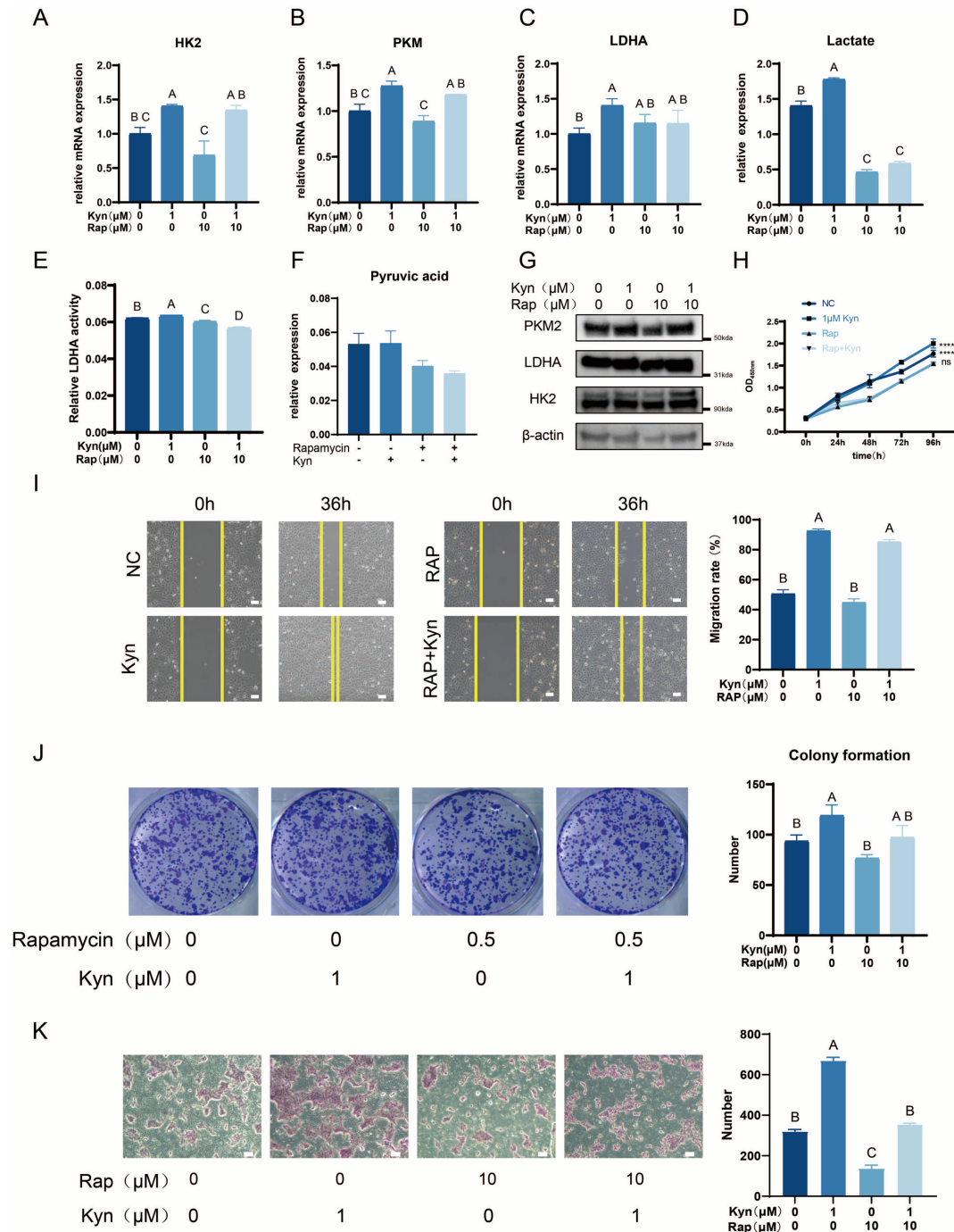


Figure S8. Pharmacological inhibition of mTOR attenuates Kyn-induced glycolytic reprogramming and malignant phenotypes in OSCC cells. (A-C) RT-qPCR analysis of HK2 (A), PKM2 (B), and LDHA (C) mRNA expression in OSCC cells treated with 1 μ M Kyn in the presence or absence of rapamycin. (D-F) Biochemical assays showing the effects of rapamycin on Kyn-induced lactate production (D), LDH activity (E), and pyruvate levels (F). (G) Western blot analysis showing the effect of rapamycin on Kyn-induced expression of glycolysis-related enzymes. (H-K) Functional assays showing that rapamycin attenuates the pro-tumor effects of Kyn in OSCC cells, as assessed by CCK-8 assay (H), wound-healing assay (I), colony formation assay (J), and transwell migration assay (K). **** $p < 0.0001$; ns, not significant ($p > 0.05$). Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

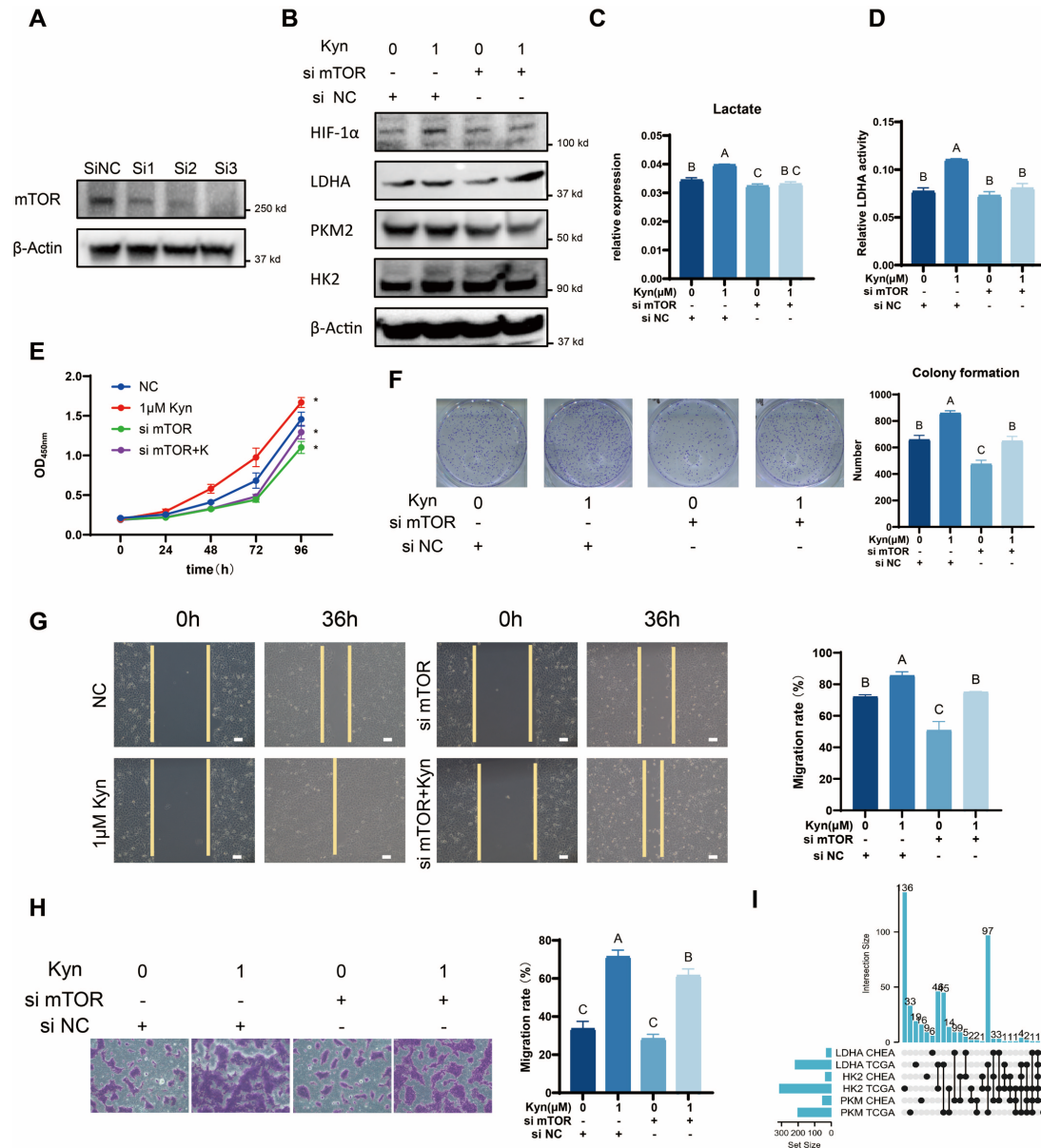


Figure S9. Genetic knockdown of mTOR attenuates Kyn-induced glycolytic reprogramming and malignant phenotypes in OSCC cells. (A) Western blot validation of siRNA-mediated mTOR knockdown efficiency. (B) Western blot analysis showing that mTOR knockdown attenuates Kyn-stimulated expression of glycolysis-related enzymes. (C-D) Biochemical assays showing that mTOR knockdown reduces Kyn-induced lactate production (C) and LDHA enzymatic activity (D). (E-H) Functional assays showing that mTOR knockdown weakens the pro-tumor effects of Kyn, as assessed by CCK-8 assay (E), colony formation assay (F), wound-healing assay (G), and transwell migration assay (H). (I) UpSet plot showing transcription factors predicted to regulate HK2, PKM, and LDHA based on the TCGA and ChEA databases. * $p < 0.05$, Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

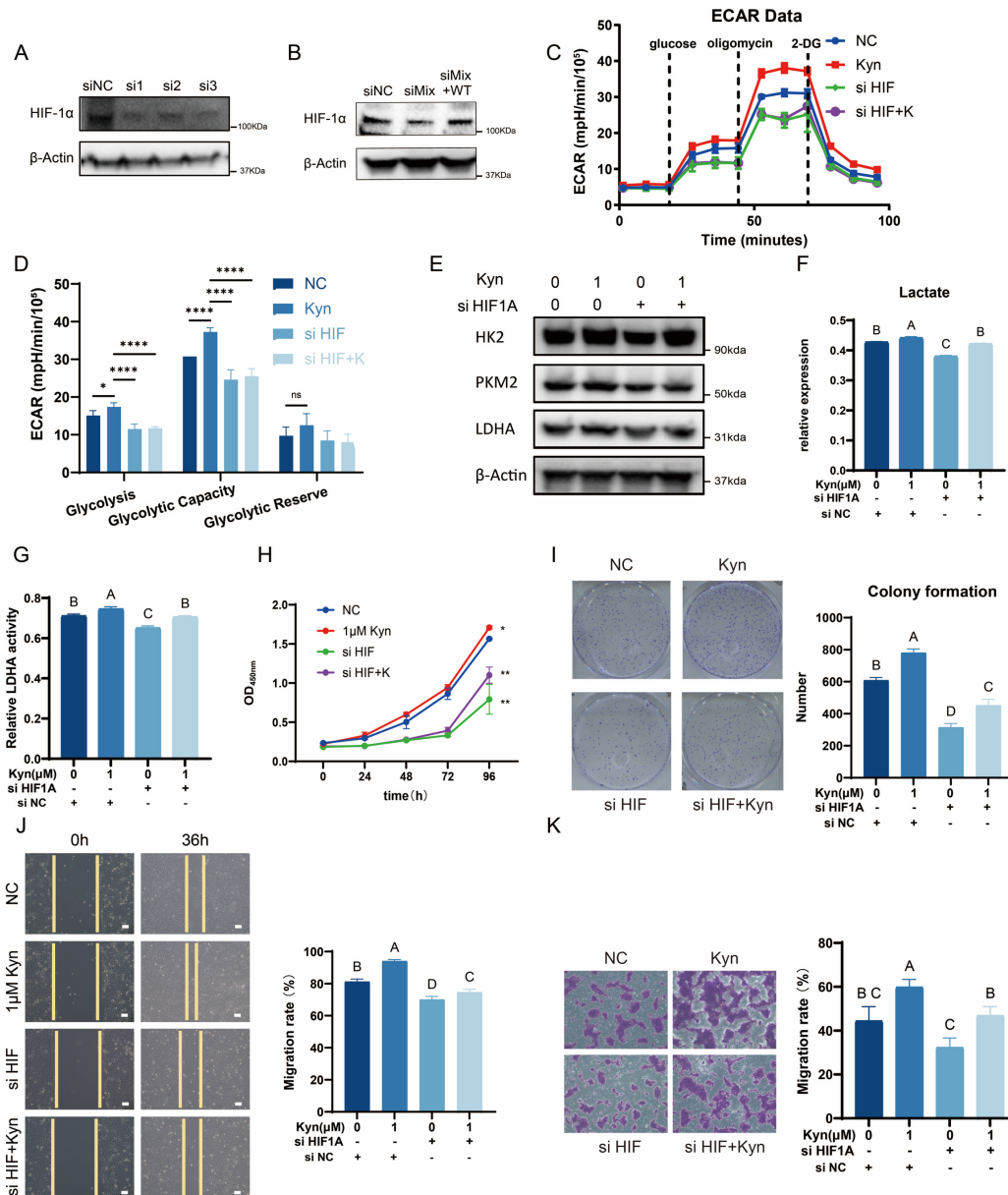


Figure S10. HIF1A knockdown attenuates Kyn-induced glycolysis and malignant phenotypes in OSCC cells. (A) Western blot confirming siRNA-mediated HIF1A knockdown in OSCC cells. (B) Western blot showing restoration of HIF-1α expression by HIF1A overexpression in HIF1A-knockdown cells. (C-D) Glycolysis stress assay measuring extracellular acidification rate (C) and corresponding quantification of glycolytic parameters (glycolysis, glycolytic capacity, and glycolytic reserve) (D) in OSCC cells following HIF1A knockdown and Kyn treatment. ECAR values were normalized to cell number and expressed as mpH/min/10⁵ cells. (E) Western blot analysis of glycolysis-related enzyme expression in HIF1A-knockdown OSCC cells following Kyn treatment. (F-G) Biochemical assays showing the effects of HIF1A knockdown on Kyn-induced lactate production (F) and LDHA enzymatic activity (G). (H-K) Functional assays showing that HIF1A knockdown weakens the pro-proliferative and pro-migratory effects of Kyn, as assessed by CCK-8 assay (H), colony formation assay (I), wound-healing assay (J), and transwell migration assay (K). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

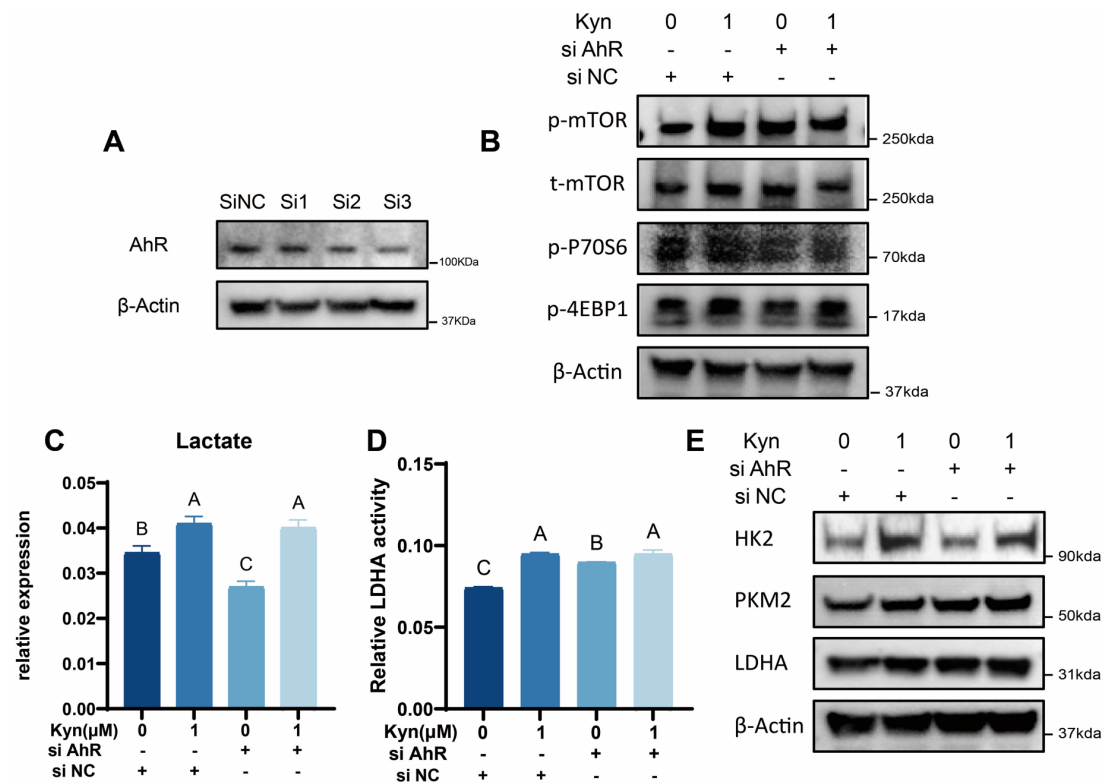


Figure S11. Effects of AhR knockdown on mTOR signaling and glycolytic parameters in OSCC cells. (A) Western blot showing siRNA-mediated AhR knockdown efficiency in OSCC cells. (B) Western blot analysis of mTOR signaling in AhR-knockdown cells following exposure to 1 μ M Kyn. (C-D) Biochemical assays measuring lactate production (C) and LDHA enzymatic activity (D) in AhR-knockdown cells cultured with 1 μ M Kyn. (E) Western blot analysis of glycolysis-related enzyme expression in AhR-knockdown cells after incubation with 1 μ M Kyn. Different letters (A, B, C) indicate statistically significant differences between groups ($p < 0.05$).

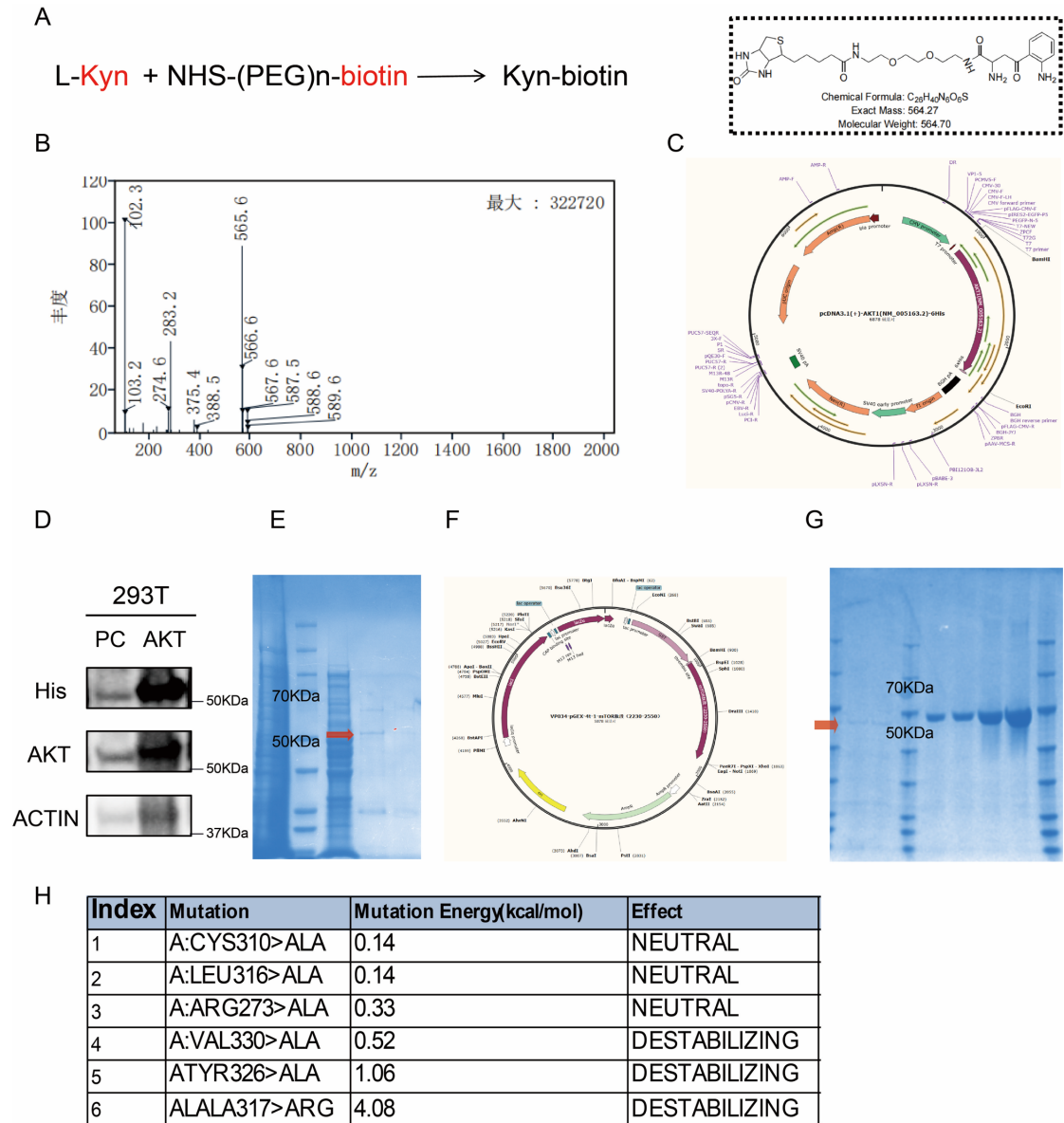


Figure S12. Validation of experimental tools for Kyn-AKT/mTOR interaction studies. (A) Synthetic route of Kyn-biotin. (B) Mass spectrometry analysis confirming the identity of Kyn-biotin. (C) Plasmid map of the AKT overexpression vector. (D) Western blot analysis verifying AKT overexpression in 293T cells. (E) Coomassie Brilliant Blue staining of purified AKT protein from 293T cells. (F) Plasmid map of the mTOR fragment expression vector. (G) Coomassie Brilliant Blue staining of purified mTOR fragment protein expressed in BL21 cells. (H) Molecular dynamics simulation of AKT mutant binding sites to evaluate changes in binding stability with Kyn.

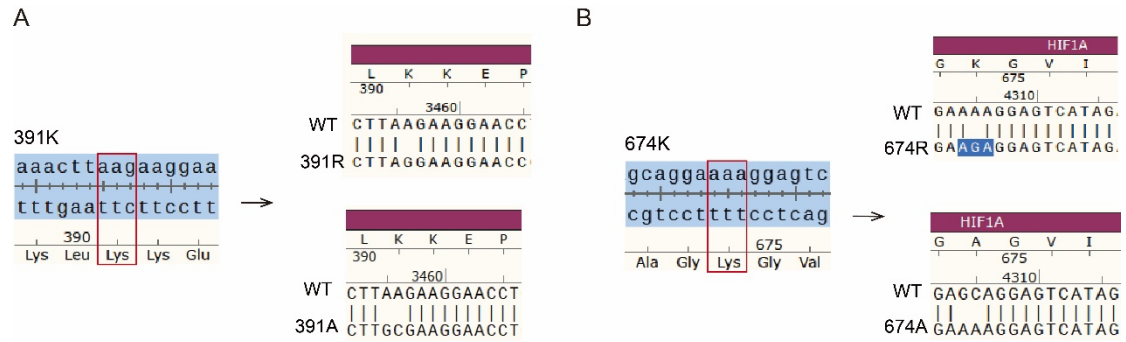


Figure S13. Sequencing validation of HIF-1 α lysine mutant constructs. (A) Sanger sequencing chromatograms confirming successful mutation of HIF-1 α K391 to arginine (K391R) or alanine (K391A). (B) Sanger sequencing chromatograms confirming successful mutation of HIF-1 α K674 to alanine (K674A) or arginine (K674R).

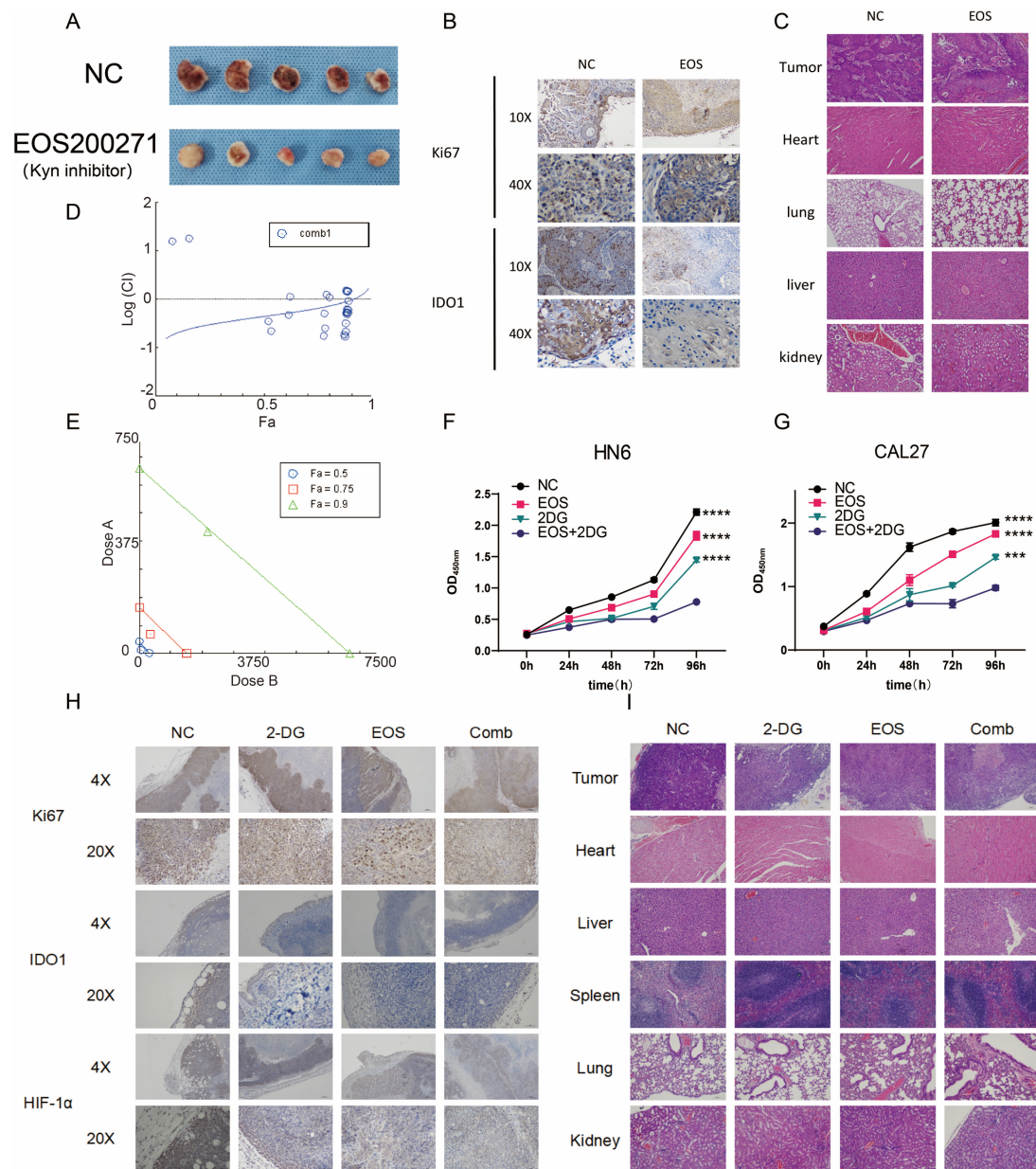


Figure S14. Antitumor efficacy of Kyn inhibition in oral squamous cell carcinoma models. (A) Tumor growth curves of OSCC-bearing mice treated with the Kyn inhibitor EOS200271. (B) Immunohistochemical staining for tumor-associated markers in EOS200271-treated mice. (C) H&E staining of major organs from mice treated with EOS200271 alone to evaluate systemic toxicity. (D-E) Combination index and drug interaction effect analysis evaluating the potential synergy between EOS200271 and 2-DG. (F) In vitro inhibitory effect of combined EOS200271 and 2-DG treatment on HN6 cells. (G) Tumor growth curves. (H) Immunohistochemical analysis of tumor-associated markers following combination treatment. (I) Histological evaluation of major organs from mice receiving combination therapy.

Table S1 IDO1 siRNA Sequence List

Gene	Sequence
siRNA-IDO1-homo-NC	5'-UUCUCCGAACGUGUCACGUTT-3'
	5'-ACGUGACACGUUCGGAGAATT-3'
siRNA-IDO1-homo-919	5'-GUGCUCAUUAGAGUCAAUUTT-3'
	5'-AUUUGACUCUAAUGAGCACTT-3'
siRNA-IDO1-homo-1026	5'-GGAGCUACCAUCUGCAAAUUTT-3'
	5'-AUUUGCAGAUGGUAGCUCCTT-3'

Table S2 AhR siRNA Sequence List

Gene	Sequence
siRNA-AhR-homo-NC	5'-UUCUCCGAACGUGUCACGUTT-3'
	5'-ACGUGACACGUUCGGAGAATT-3'
siRNA-AhR-homo-1	5'-GCUGAAGGAAUCAAGUCAATT-3'
	5'-UUGACUUGAUUCCUUCAGCTT-3'
siRNA-AhR-homo-2	5'-GCAUAGAGACCGACUUAUUTT-3'
	5'-AUUAAGUCGGUCUCUAUGCTT-3'
siRNA-AhR-homo-3	5'-CGGCAUAGAGACCGACUUATT-3'
	5'-UAAGUCGGUCUCUAUGCCGTT-3'

Table S3 HIF1A siRNA Sequence List

Gene	Sequence
siRNA-HIF1A-homo-NC	5'-UUCUCCGAACGUGUCACGUTT-3'
	5'-ACGUGACACGUUCGGAGAATT-3'
siRNA-HIF1A-homo-1	5'-GCCACAUUCACGUUAUAUGATT-3'
	5'-UCAUAUACGUGAAUGUGGCTT-3'
siRNA-HIF1A-homo-2	5'-UGAUGAAAGAAUACCGAATT-3'
	5'-UUCGGUAAUUCUUUCAUCATT-3'
siRNA-HIF1A-homo-3	5'-GGAGCUACCAUCUGCAAAUUTT-3'
	5'-AUUUGCAGAUGGUAGCUCCTT-3'

Table S4 mTOR siRNA Sequence List

Gene	Sequence
siRNA-mTOR-homo-NC	5'-UUCUCCGAACGUGUCACGUTT-3' 5'-ACGUGACACGUUCGGAGAATT-3'
siRNA-mTOR-homo-1	5'-CGACAAGUACUGCAAAGAATT-3' 5'-AUCUUUGCAGUACUUGUCGTT-3'
siRNA-mTOR-homo-2	5'-GCACUAUGUCACCAUGGAATT-3' 5'-UUCCAUGGUGACAUAGUGCTT-3'
siRNA-mTOR-homo-3	5'-GAGCCUUGUUGAUCCUUAATT-3' 5'-UUAAGGAUCAACAAGGCUCTT-3'

Table S5 Primer sequences

Primer	Sequence
β -Actin-F (Homo)	AAGGAGCCCCACGAGAAAAAT
β -Actin-R (Homo)	ACCGAACTTGCATTGATTCCAG
HK-F (Homo)	CGACAGACTGGTGGACGAATA
HK-R (Homo)	CGATTAAGATGTTGCGGACGATT
PFK-F (Homo)	CTGACACAGCACTCAATACTATCT
PFK-R (Homo)	TAGCCAGGTAGCCACAGTAG
PKM-F (Homo)	TAGCCAGGTAGCCACAGTAG
PKM-R (Homo)	G TTCAGACGAGCCACATTCATT
HIF1A-F (Homo)	GAACGTCGAAAAGAAAAGTCTC
HIF1A-R (Homo)	CCTTATCAAGATGCGAACTCACA
LDHA-F (Homo)	GATTCAGCCCGATTCCGTTAC
LDHA-R (Homo)	AGAGACACCAGCAACATTCATTC

Table S6 ChIP Primers

Primer	Sequence
HIF-HK2 FZ1 F	GCCTTGCCTCAATTTCTCATC
HIF-HK2 FZ1 R	TCTTACCTATTGTAGCACGGCC
HIF-PKM1/2 FZ1 F	CGGAAGGACACAGATTCAGAGT
HIF-PKM1/2 FZ1 R	AAGAAAAGACACCCCATCTGGC
HIF-LDHA FZ1 F	AACAGATCCAAGCCTTCCTGAG
HIF-LDHA FZ1 R	CGCTGGGAGTCTAAGTAAGGTG