Supplementary figures and figure legends

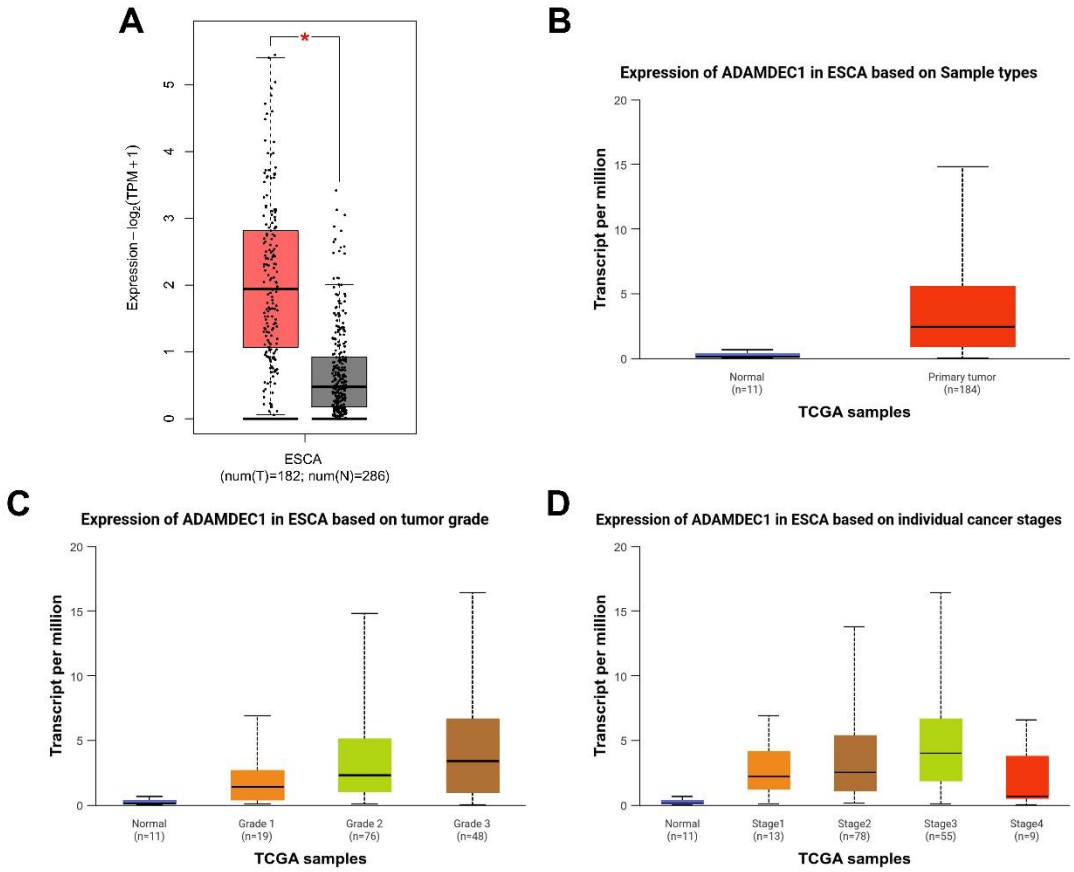

Figure S1: Related to Figure 1. (A) GEPIA2 database analysis of ADAMDEC1 mRNA expression in ESCA tissues (n=182 tumors) versus normal tissues (n=286 normals) using TCGA/GTEx RNA-seq data. (B) UALCAN database validation of ADAMDEC1 protein expression in ESCA (TCGA data, n=184 tumors vs. n=11 normals), with Kruskal-Wallis test for statistical analysis. (C) Expression of ADAMDEC1 in ESCA based on tumor grade. (D) Expression of ADAMDEC1 in ESCA based on individual cancer stages.

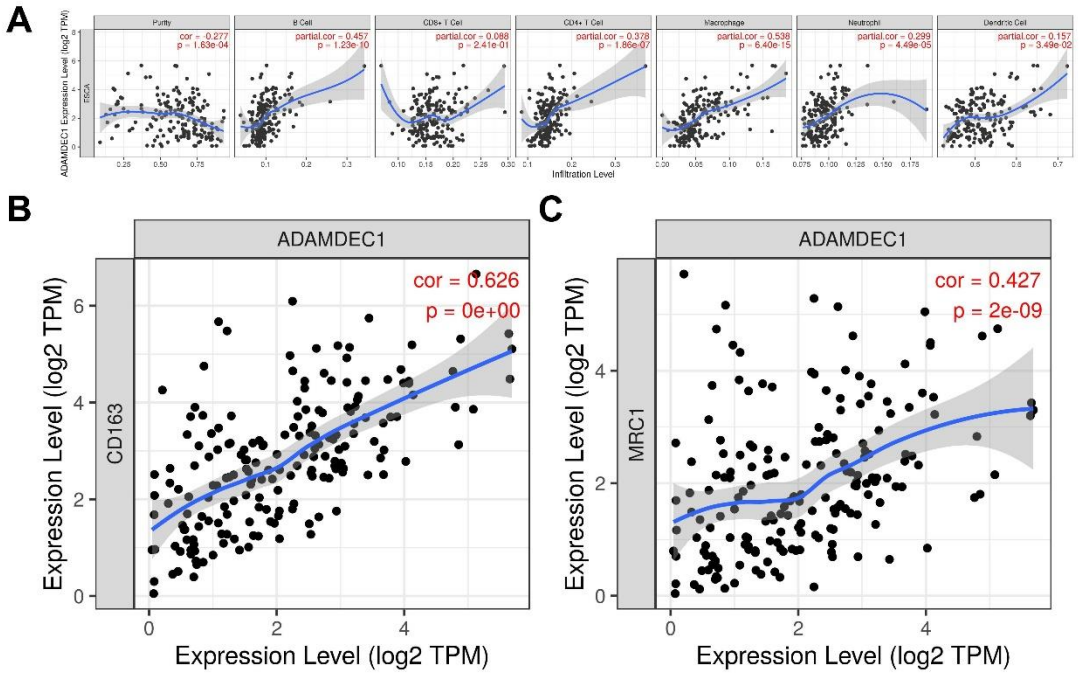

Figure S2: Related to Figure 3. (A) TIMER database analysis of the correlation between ADAMDEC1 expression and macrophage infiltration in ESCA (TCGA data), using partial correlation adjusted for tumor purity. (B) TIMER database analysis of the Pearson correlation between ADAMDEC1 and M2 macrophage marker CD206 in ESCA. (C) TIMER database analysis of the Spearman correlation between ADAMDEC1 and M2 macrophage marker MRC1 in ESCA.

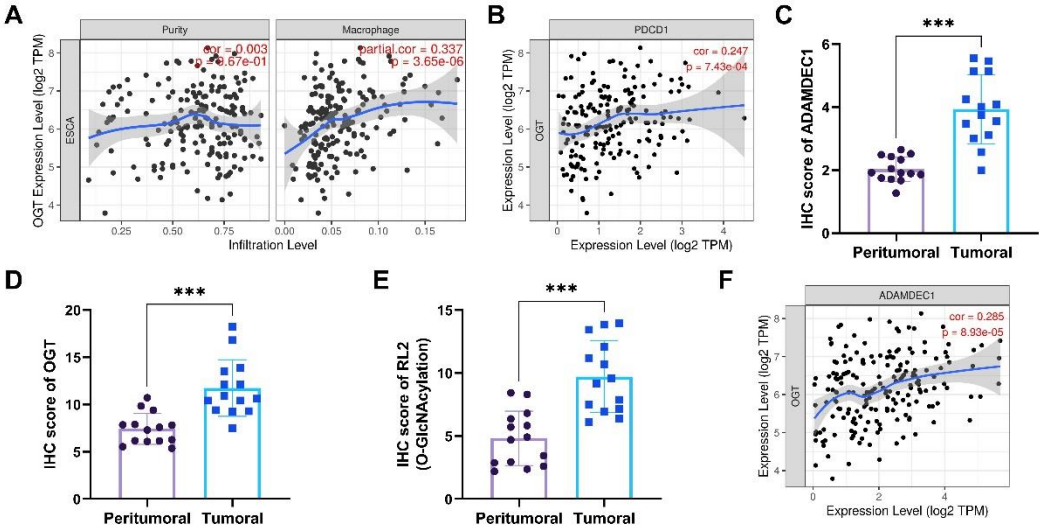

Figure S3: Related to Figure 4. (A) TIMER database analysis of the correlation between OGT expression and macrophage infiltration in ESCA (TCGA data), using

partial correlation adjusted for tumor purity. (B) TIMER database analysis of the Pearson correlation between OGT and PD-1 expression in ESCA. (C-E) Immunohistochemical staining of ADAMDEC1, OGT, and RL2 (O-GlcNAcylation marker) protein expression in clinical ESCA tissues and adjacent non-tumor tissues. (F) TIMER database analysis of the Pearson correlation between ADAMDEC1 and OGT expression in ESCA.

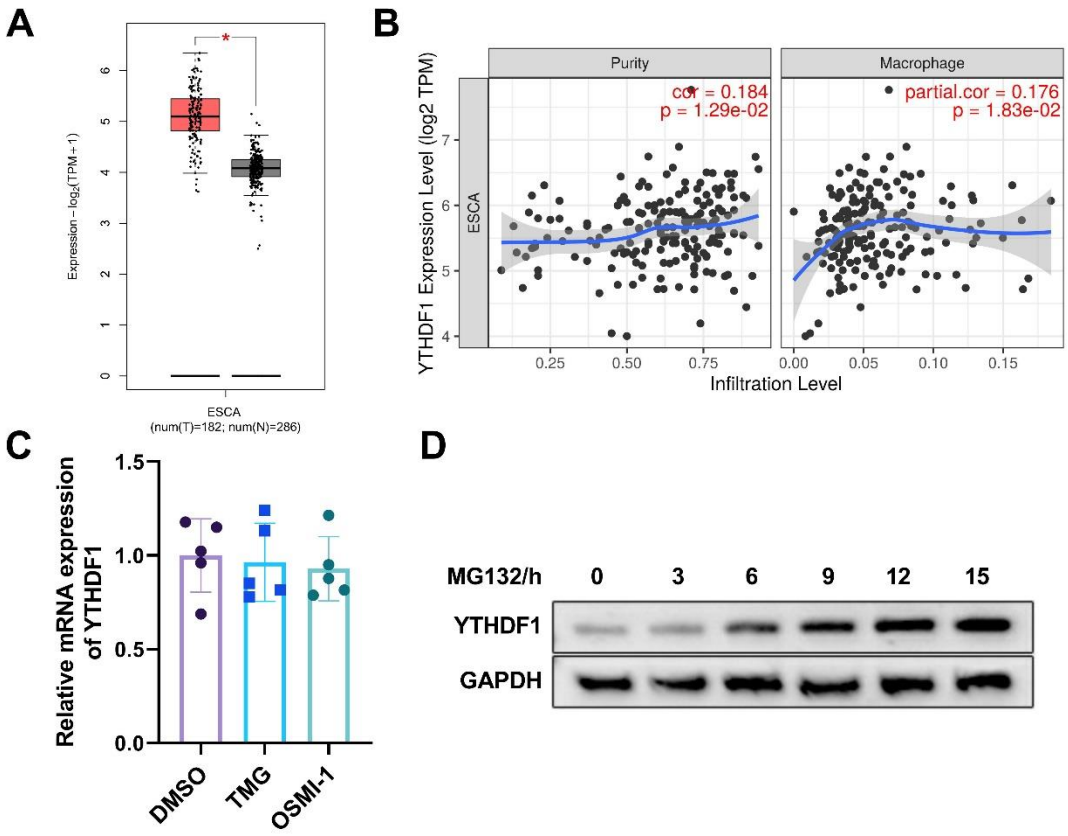

Figure S4: Related to Figure 5. (A) GEPIA2 database analysis of YTHDF1 mRNA expression in ESCA tissues (n=182 tumors) versus normal tissues (n=286 normals) using TCGA/GTEx RNA-seq data. (B) TIMER database analysis of the correlation between YTHDF1 expression and macrophage infiltration in ESCA (TCGA data), using partial correlation adjusted for tumor purity. (C) qRT-PCR analysis of YTHDF1 mRNA levels in ADAMDEC1-knockout (ADAMDEC1-KO) vs. control ESCA-PDOs, demonstrating no significant transcriptional change. (D) Western blot analysis of YTHDF1 protein levels in ESCA-PDOs treated with proteasome inhibitor MG132 for

indicated durations, assessing ubiquitin-proteasome pathway involvement in YTHDF1 degradation.

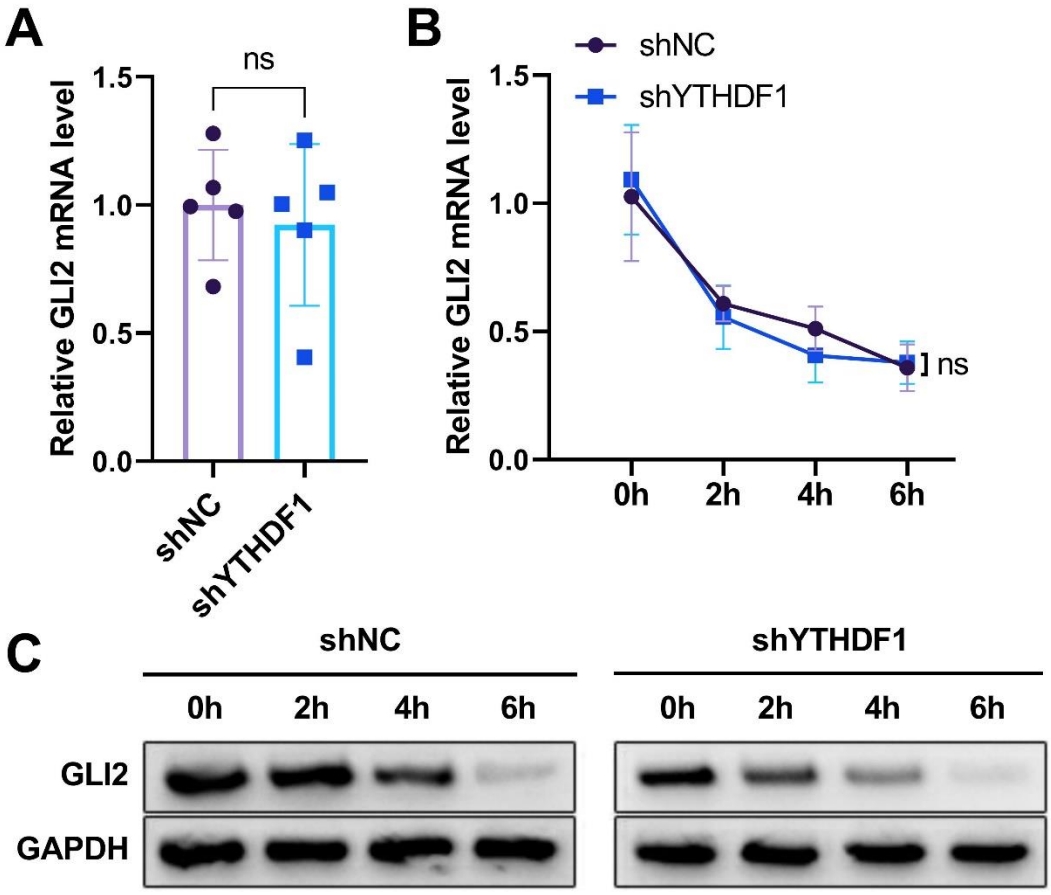

Figure S5: Related to Figure 6. (A) qRT-PCR analysis of GLI2 mRNA levels in YTHDF1-knockdown (sh-YTHDF1) vs. control ESCA-PDOs, assessing transcriptional changes. (B) Actinomycin D chase assay to measure GLI2 mRNA half-life in YTHDF1-knockdown vs. control ESCA-PDOs, with RNA isolated at indicated time points and quantified by qRT-PCR. (C) Western blot analysis of GLI2 protein levels in YTHDF1-knockdown ESCA-PDOs treated with cycloheximide (CHX, protein synthesis inhibitor) and proteasome inhibitor (MG132), evaluating effects on GLI2 degradation rates.

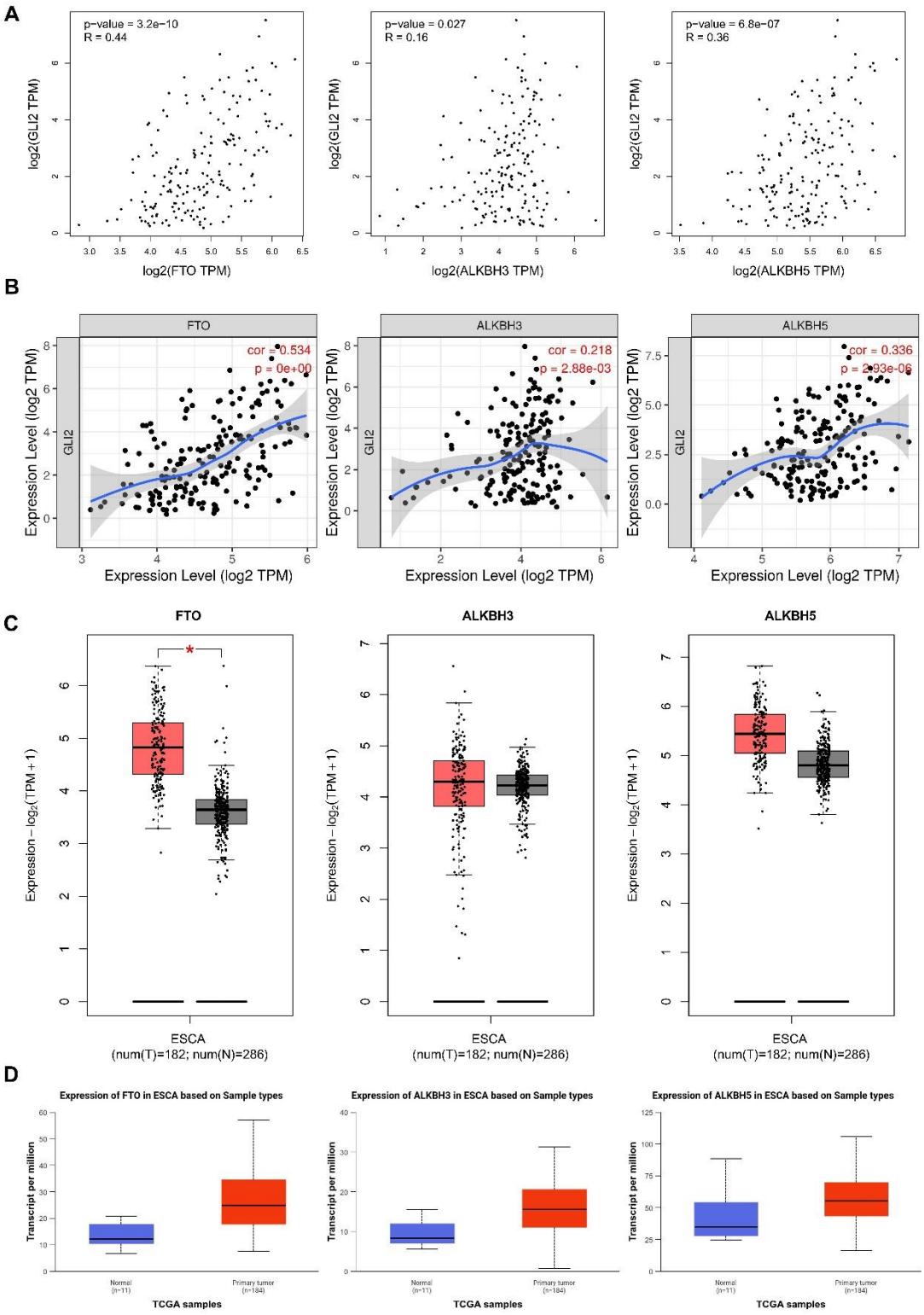

Figure S6: Related to Figure 7. (A) GEPIA2 database analysis of Pearson correlations between GLI2 and m^6 A demethylases (FTO, ALKBH3, ALKBH5) mRNA expression in ESCA. (B) TIMER database analysis of partial correlations between GLI2 and demethylases (adjusted for tumor purity), with Spearman

correlation coefficients shown. (C) GEPIA2 database comparison of FTO, ALKBH5, and ALKBH3 mRNA expression in ESCA tumors (n=182) vs. normal tissues (n=286). (D) UALCAN database analysis of FTO, ALKBH5, and ALKBH3 protein expression in ESCA (TCGA data, tumor n=184 vs. normal n=11).

Supplementary detailed methods

Clinical ESCA Tissue Sample Collection and Groups

Fresh esophageal cancer tissues and adjacent non-tumor tissues (⩾5 cm from tumor margins) were obtained from 14 patients with ESCA undergoing surgical resection at Henan Cancer Hospital between March 2022 and January 2024. All patients provided written informed consent, and the study was approved by the Institutional Ethics Committee of Henan Cancer Hospital.

Inclusion criteria: (1) histopathologically confirmed ESCA diagnosis; (2) age ⩾ 18 years; (3) scheduled for curative-intent surgical resection; (4) patients in the Cancer_Treated and Control_Treated groups received neoadjuvant paclitaxel plus camrelizumab according to the study protocol (dose and cycles specified in the treatment schedule); (5) availability of paired tumor and adjacent non-tumor tissues.

Exclusion criteria: (1) tumour invasion of the aorta or trachea; (2) hepatic metastasis involving >50% of total liver volume; (3) history of other malignant tumours (except non-melanoma skin cancer or cured carcinoma in situ); (4) concurrent severe cardiovascular, hepatic, or renal dysfunction; (5) active autoimmune disease or history of autoimmune disease requiring systemic immunosuppressive therapy; (6) neoadjuvant treatment with chemotherapy, immunotherapy, or radiotherapy other than paclitaxel plus camrelizumab; (7) insufficient tissue sample quality or quantity as assessed by pathologists; (8) interstitial lung disease requiring corticosteroids; (9) inability to provide informed consent.

Tissues were categorized into four groups: the Cancer_Untreated group (n=5) comprising untreated esophageal cancer tissues from patients without neoadjuvant

therapy, the Cancer_Treated group (n=9) consisting of esophageal cancer tissues from patients who received neoadjuvant paclitaxel plus camrelizumab, the Control_Untreated group (n=5) containing untreated adjacent non-tumor tissues, and the Control_Treated group (n=9) including adjacent non-tumor tissues from treated patients.

RNA Sequencing (RNA-Seq) Analysis

Sample Preparation and Total RNA Isolation

Total RNA was extracted from frozen esophageal cancer tissues (n=14) and adjacent non-tumor tissues (n=14) using an RNeasy mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. Briefly, 50–100 mg of tissue was homogenized in 1 mL TRIzol, followed by chloroform extraction and isopropanol precipitation. RNA pellets were washed with 75% ethanol, air-dried, and resuspended in RNase-free water. RNA purity and concentration were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was evaluated via the RNA Integrity Number (RIN $\geqslant$8.0) using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) with the RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA).

cDNA Library Construction and RNA Sequencing

mRNA was purified from 1 μg of total RNA using magnetic oligo (dT) beads (NEBNext Poly(A) mRNA Magnetic Isolation Module, New England Biolabs, USA). Purified mRNA was fragmented into ~200 bp segments using divalent cations at 94 ℃ for 5 min. First-strand cDNA was synthesized using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen), followed by second-strand cDNA synthesis with DNA Polymerase I and RNase H. cDNA fragments were end-repaired, adenylated at the 3' ends, and ligated to NEBNext adaptors (New England Biolabs) containing a unique index for multiplexing. Adaptor-ligated cDNA was amplified via PCR (98 ℃ for 30 s, 15 cycles of 98 ℃ for 10 s, 60 ℃ for 30 s, 72 ℃ for 30 s, followed by 72 ℃ for 5 min) using NEBNext Ultra II Q5 Master Mix, and PCR products were size-selected (150–200 bp) on a 2% agarose gel. Library quality and quantity were

validated using the Agilent Bioanalyzer and Qubit 4.0 Fluorometer (Thermo Fisher Scientific). Sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, USA) using a paired-end (PE) 150 bp configuration, generating ⩾6 Gb of clean data per sample. Raw reads were demultiplexed and processed to remove adapter sequences and low-quality reads (Q-score <20, ambiguous nucleotides "N" >5%) using Trimmomatic v0.39.

Data Analysis

Clean reads were aligned to the human reference genome (GRCh38/hg38) using STAR v2.7.10 with default parameters, and gene expression levels were quantified as transcripts per million (TPM) using Salmon v1.4.0.

Differentially expressed gene analysis

Differential gene expression analysis between the two groups tested for each pairwise comparison was performed using DESeq2 v1.34.0, with genes considered significantly differentially expressed at |log2(fold change)| ⩾2 and adjusted p <0.05 (Benjamini-Hochberg correction for multiple testing).

Genes with log2fold change > 2 were selected as upregulated genes in both C_Untreated_vs_N_Untreated (untreated cancer tissues vs. untreated normal tissues) and C_vs_N (cancer vs. Normal) comparisons, while genes with $\log_2$ fold change < -2 were identified as downregulated genes in the C_Treated_vs_C_Untreated (treated cancer tissues vs. untreated cancer tissues) comparison. Intersection analysis was performed using the Venny 2.1 webtool to obtain genes that were upregulated in esophageal cancer tissues and downregulated following combination treatment with paclitaxel and camrelizumab.

Bioinformatics Analysis

GEPIA2 Database

The GEPIA2 web server (http://gepia2.cancer-pku.cn/#index) was employed to analyze ADAMDEC1, YTHDF1, GLI2, FTO, ALKBH3, and ALKBH5 expression in ESCA using TCGA/GTEx RNA-seq data (n=182 tumors, n=286 normals). Pearson's correlation evaluated associations between GLI2-FTO, GLI2-ALKBH5,

GLI2-ALKBH3, and YTHDF1-OGT. For prognosis, Kaplan-Meier DFS curves were generated by median OGT expression, with log-rank tests and HR calculation using the survival module. Results were visualized via box plots, scatter plots, and GraphPad Prism-generated survival curves.

UALCAN Database

The UALCAN web tool (http://ualcan.path.uab.edu/) was used to analyze ADAMDEC1, GLI2, FTO, ALKBH5, and ALKBH3 expression in ESCA using TCGA data, comparing tumor (n=185) vs. normal (n=16) tissues and assessing grade-specific differences via Kruskal-Wallis tests. GLI2 promoter CpG methylation in ESCA was assessed using TCGA Illumina 450K data, comparing tumor (n=184) and normal (n=11) tissues.

TIMER Database

The TIMER database analyzed ADAMDEC1 associations with the immune microenvironment in ESCA (TCGA, n=184), using partial correlation (adjusted for tumor purity) to assess links with macrophage/$CD8^+$ T cell/neutrophil infiltration and M2 markers (CD206, CD163). Pearson's correlations evaluated ADAMDEC1 with PDCD1, CCL2, CCR2, OGT, and GLI2 with CCL2, FTO, ALKBH5, ALKBH3, visualized as scatter plots using default parameters.

Prediction of Transcription Factors

The National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) was used to retrieve the CCL2 promoter sequence (-2000 to +1 bp relative to TSS, Gene ID: 6356). JASPAR (http://jaspar.genereg.net/) predicted GLI2 binding sites in the promoter using the MA0133.2 matrix (relative score $\geqslant$ 85%, p-value <0.05), identifying potential transcriptional regulatory interactions.

2.13.5 Prediction of m6A Modification Site

The SRAMP server (http://www.cuilab.cn/sramp) predicted m6A sites in GLI2 mRNA (NM_001164343.2 CDS) using default parameters (threshold $\geqslant$ 0.5), classifying sites as Very high ($\geqslant$0.8), High (0.6 – 0.8), Moderate (0.5-0.6), or Low (<0.5).

qRT-PCR

Total RNA extraction was carried out with TRIzol reagent (Invitrogen, 15596026), followed by cDNA synthesis through RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622) as per standard protocols. For RT-qPCR analysis, the UltraSYBR One Step Kit (CWBIO, CW2624) was employed on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, 4329001). Primer sequences (Table S1) were custom-synthesized by Sangon Biotech (Shanghai, CHN). Gene expression quantification utilized the $2^{-\Delta\Delta CT}$ method, with GAPDH serving as the endogenous reference gene.

Table S1. Primers for qRT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
human		
ADAMDEC1	AACTCAAGCCTCAAACTGTG	CAGGTGTTTGCTTTATGGCT
Arg-1	ACTGACAACCACAAGTGGA	GCACATCGGGAATCTTTCC
YTHDF1	GTCAAATCAGAGTAACAGTTACCC	GTAAGGAAATCCAATGGACGG
CCL2	CCAGATGCAATCAATGCCC	TGGTCTTGAAGATCACAGCT
CCR2	ACAAGCTGAACAGAGAAAGTG	AACCGAGAACGAGATGTGG
β-actin	AAGTGTGACGTTGACATCCG	GATCCACATCTGCTGGAAGG

Western blot

The cellular or tissue samples underwent sequential processing starting with three washes in PBS pre-chilled to 4℃. Subsequently, mechanical disruption was performed using RIPA lysis buffer (Beyotime Biotechnology, P0013B) supplemented with phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology, ST506) as a protease inhibitor. Protein concentration quantification was carried out via the Bradford method (Bradford Protein Assay Kit, Beyotime Biotechnology, P0006). For immunoblotting, equal protein aliquots were electrophoresed and transferred to membranes. Following sequential incubation with species-matched primary and

secondary antibodies (adjusted to optimal concentrations), chemiluminescent signals were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095). Image acquisition was performed on a Tanon 5200 system (Shanghai, China). All antibodies used in this study, including anti-ADAMDEC1 (0.2-1 μg/mL, PA5-44267, Thermo Fisher Scientific), anti-CCL2 (1:100000, ab214819, Abcam), anti-CCR2 (10 μg/mL, ab313463, Abcam), anti-PD-1 (1:1000, ab234444, Abcam), anti-Arg-1 (1:20000, ab133543, Abcam), anti-OGT (1:1000, ab177941, Abcam), anti-RL2 (1:3000, ab2739, Abcam), anti-YTHDF1 (1:100000, ab220162, Abcam), anti-GLI2 (2 μg/mlab277800, Abcam), and anti-GAPDH (1:5000, ab8245).

Establishment and Cultivation of Patient-Derived Organoids (PDOs)

Fresh ESCA tissues were sectioned into 1 mm^3fragments and subjected to three cold PBS washes containing 10% penicillin-streptomycin. The specimens were enzymatically dissociated using a digestion solution composed of 1.5 mg/mL collagenase II, 500 U/mL collagenase IV, 0.1 mg/mL dispase II, 10 mM Y-27632 (Selleck), and 1% fetal bovine serum in PBS with 10% antibiotics, incubated at 37℃ for 45 minutes under continuous agitation. Post-digestion pellets were washed thrice with chilled PBS, followed by centrifugation at 200 ×g for 5 minutes. Isolated tumor cells were suspended in Matrigel (BD Biosciences) and seeded into 96-well plates. Following matrix polymerization, organoids were maintained in Advanced DMEM/F12 medium (Thermo Fisher) enriched with: (1) Noggin (0.1 mg/mL) and R-spondin (1 μg/mL) from Peprotech/Nuvelo, (2) EGF (50 ng/mL), Glutamax, HEPES, N2/B27 supplements (Invitrogen), (3) N-acetyl-L-cysteine (1 mM), Gastrin (10 nM), Nicotinamide (10 mM) from Sigma, (4) A83-01 (0.5 μM, Tocris) and FGF10 (100 ng/mL, Preprotech). Organoid cultures underwent medium replacement every 48 hours, with routine subculturing performed between days 7-10 of expansion. Experimental procedures utilized early-passage organoids (P3-P6) to maintain cellular integrity and functional consistency.

To evaluate clonogenic potential, enzymatically dissociated single-cell

suspensions (TrypLE + Y-27632) were filtered through 40-μm meshes and embedded in 70% Matrigel/DMEM-F12 composite supplemented with Y-27632, seeded at 1,000 cells/well in 96-well plates. Organoid quantification was performed during days 10-15 post-seeding through systematic microscopic analysis. Representative 10× magnification fields per well were selected for both organoid enumeration and cross-sectional area measurement using standardized morphometric protocols.

Lentiviral Transduction of Organoids

Following two washes with chilled DPBS, organoids underwent enzymatic dissociation using 500 μL TrypLE (Thermo Fisher Scientific, 12604013) at 37°C for 10 minutes, with mechanical dissociation through repetitive pipetting to disrupt Matrigel. The enzymatic reaction was neutralized by adding 10 mL ice-cold serum-free DMEM/F12, followed by centrifugation (200 × g, 5 minutes) to obtain single cells/small clusters. The pellet was reconstituted in lentivirus-containing infection medium supplemented with polybrene and ROCK inhibitor. This suspension underwent spinoculation (2000 × g, 1 hour) in 6-well plates before 5-6 hour incubation at 37℃. Cells were subsequently washed twice with serum-free DMEM/F12 and plated in 24-well plates pre-coated with 70% Matrigel. After adding 500 μL organoid medium, cultures were stabilized at 37℃/5% CO2 for 20 minutes prior to routine medium replacement every 48 hours. A 3-4 day puromycin selection regimen (1 μg/mL) established stable transductants, with successful infection confirmed through quantitative RT-PCR or western blot analysis.

CellTiter-Glo-3D assay

To assess the viability of 3D organoid cultures, Promega's CellTiter-Glo® 3D Luminescent Cell Viability Assay (cat: G9681) was employed. Following medium aspiration, organoid cultures were washed to remove residual medium and treated with 50 μL of pre-equilibrated ATP detection reagent as per standardized protocols. After 10-minute equilibration under ambient conditions, bioluminescent signals reflecting intracellular ATP levels were quantified using a BioTek microplate reader

(λem = 560 nm). This homogeneous detection system demonstrates enhanced penetration capacity for 3D microtissues while maintaining compatibility with high-throughput screening formats.

Humanised mouse models

NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) aged 4-5 weeks were subjected to total-body γ-irradiation (150-170 cGy per animal) followed by intravenous administration of 5×10^4 umbilical cord blood-sourced $CD34^+$ hematopoietic progenitor cells (STEMCELL) within a 24-hour post-irradiation window. Engraftment efficiency was evaluated through serial flow cytometric quantification of human $CD45^+$ cell populations in peripheral blood at 8- and 16-week intervals post-transplantation. Mice demonstrating >20% human $CD45^+$ leukocyte reconstitution were selected for subsequent xenograft establishment.

Establishment of esophageal cancer PDOX mouse models

NOD/SCID/IL2Rγ$^-$ /$^-$ (NSG) mice (6–8 weeks old, female, Jackson Laboratory), a highly immunodeficient strain, were used to minimize host immune rejection. Esophageal cancer organoids (passage 3–5) were dissociated into single cells using Gentle Cell Dissociation Reagent, resuspended in Matrigel at 1×10^6 cells/100 μL, and subcutaneously injected into the right flank of each mouse (n=5/group). Tumor growth was monitored twice weekly by caliper measurements, and mice were euthanized at 12 weeks or when tumors reached ~1000 mm^3.

All experimental procedures were conducted in compliance with institutional animal care protocols approved by the Animal Experimentation Ethics Committee at the Affiliated Cancer Hospital of Zhengzhou University.

Apoptosis detection by TUNEL assay

Tumor specimens fixed in 10% neutral buffered formalin were processed through paraffin embedding and sectioned into 5-μm slices. Apoptotic cells were identified via TUNEL staining following the supplier's instructions (Roche, 11684795910).

Fluorescent microscopic examination was performed to detect cells exhibiting green fluorescence signals indicative of apoptosis. Nuclear counterstaining was achieved using 4',6-diamidino-2-phenylindole (DAPI). Digital image acquisition was conducted with a Zeiss fluorescence microscope under 400× magnification.

H&E staining

PDOX tissue samples underwent fixation in 4% paraformaldehyde at 4℃ overnight, followed by paraffin embedding. Sequential 5 mm sections were hydrated, deparaffinized, and subjected to hematoxylin and eosin (H&E) staining. Histopathological evaluation was independently conducted by two blinded pathologists.

Immunohistochemistry (IHC) staining

To assess Ki67 expression levels in pancreatic adenocarcinoma specimens, immunohistochemical staining procedures were implemented. On the initial day, formalin-fixed paraffin-embedded esophageal carcinoma tissue sections underwent dewaxing through xylene immersion followed by heat-induced epitope recovery using pressurized thermal treatment (2.5 minutes). Primary antibodies were applied and maintained overnight at 4° C for specific antigen binding. During subsequent processing, secondary antibodies were introduced for 30 minutes under controlled 37° C incubation conditions to amplify detection signals. Chromogenic visualization was achieved using a commercial 3,3'-diaminobenzidine detection system (ORIGENE, ZLI-9019). Staining intensity was graded using a four-tiered scale: 0 (absent), 1 (mild), 2 (moderate), and 3 (strong). The proportion of positively stained cells was categorized as: 0 (0%), 1 (1-25%), 2 (26-50%), or 3 (51-100%). Quantitative analysis involved examination of five randomly selected microscopic fields under 100× objective magnification.

Flow Cytometry Analysis

Esophageal cancer organoids were minced and digested in HBSS (Sigma-Aldrich) containing 0.2% collagenase type 2 (Worthington) at 37 ℃ for 60 minutes with shaking, followed by inactivation of collagenase with DMEM containing 10% FBS. The cell suspension was filtered through a 40-μm nylon mesh (BD Biosciences), centrifuged at 350 ×g for 5 minutes, and resuspended in PBS. Single-cell viability was assessed by trypan blue exclusion, and 1×10^6 viable cells were pre-incubated with Fc receptor blocking solution (BD Biosciences) for 15 minutes at room temperature to reduce non-specific antibody binding.

For TAMs and M2 macrophage analysis, cells were stained with fluorophore-conjugated antibodies: PerCP/Cy5.5-conjugated anti-CD11b (Biolegend, 101228), PE Rat Anti-F4/80 (BD Pharmingen, 565410), APC anti-CD206 (Biolegend, 141708), for 30 minutes at 4 ℃. For anti-tumor effector T cell exhaustion ($CD8^+$ CTLs), cells were stained with PerCP-Cy5.5-conjugated anti-CD3 (BD Pharmingen, 560527) and PE-conjugated anti-CD8α (BioLegend, 100708). Dead cells were excluded using 7-AAD staining (BD Biosciences, 559925).

Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo v10 software. Gating strategies first excluded doublets and dead cells, followed by sequential gating on $CD45^+$ leukocytes. For TAMs, cells were gated as $CD11b^+$ $F4/80^+$; M2 macrophages were defined as $F4/80^+$ $CD206^+$. $CD8^+$ CTLs were identified as $CD3^+$ $CD8\alpha^+$ cells. Isotype-matched control antibodies were used to set background fluorescence thresholds. Data are presented as mean ± SEM from five independent experiments.

Molecular docking

The protein amino acid sequences were retrieved from the UniProt database (https://www.uniprot.org/) and processed through the AlphaFold3 platform (https://deepmind.google/technologies/alphafold/alphafold-server/) for predicting interaction patterns. From the five generated structural models, the top-ranked prediction (designated as Model 0) was selected for subsequent structural analysis of potential binding regions. Molecular visualization was performed using PyMOL's

open-source toolkit (v2.6) to elucidate critical residue interactions at the predicted binding interface.

Co-Immunoprecipitation (Co-IP) Assay

Esophageal cancer organoid lysates (wild-type or ADAMDEC1-KO) were prepared in ice-cold RIPA buffer, clarified by centrifugation, and pre-cleared with protein A/G agarose beads conjugated to normal mouse IgG. Pre-cleared lysates were incubated overnight at 4℃ with rabbit anti-ADAMDEC1 antibody (1 mg/mL, PA5-103574, Thermo Fisher Scientific) or normal rabbit IgG (negative control), followed by 2-hour incubation with protein A/G agarose beads (Santa Cruz Biotechnology, sc-2003). After washing, bound proteins were eluted by boiling in SDS loading buffer and analyzed via Western blot using mouse anti-OGT antibody (1:1000, ab177941, Abcam) and rabbit anti-ADAMDEC1 antibody (0.2-1 μg/mL, PA5-44267, Thermo Fisher Scientific), detected with HRP-conjugated secondary antibodies and ECL chemiluminescence.

Protein stability assay

To assess the stability of OGT protein in organoids, a cycloheximide (CHX, Sigma-Aldrich, catalog number: C7698) chase assay was performed. Organoids were divided into shADAMDEC1 and control groups. Each group was treated with CHX at concentrations of 5 μM and 10 μM, with samples collected at 0, 6, 12, and 24 hours post - treatment. Organoids were lysed in RIPA buffer containing protease inhibitors to extract total protein. Protein concentrations were normalized, and equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non - fat dry milk in TBST, then incubated overnight at 4℃ with anti-OGT antibody (to detect OGT) and anti-β-Actin antibody (as a loading control). After washing, membranes were incubated with HRP-conjugated secondary antibodies, and protein bands were visualized using an ECL detection system. This approach enabled evaluation of the effect of ADAMDEC1 silencing (via shADAMDEC1) on OGT protein half-life by monitoring

OGT level changes over time following CHX-induced protein synthesis inhibition.

To assess the stability of O-GlcNAc-modified YTHDF1 protein in esophageal cancer organoids, the organoids were treated with 20 μM Thiamet G (TMG), 20 μg/mL OSMI-1, or DMSO (control) for 12 h. Subsequently, 50 μM cycloheximide (CHX, MCE), an inhibitor of protein synthesis, was added. Organoids were harvested at 0, 3, 6, and 9 h after CHX treatment. Western blot analysis was conducted using an anti-YTHDF1 antibody to monitor YTHDF1 protein levels, with GAPDH serving as a loading control.

Immunoprecipitation (IP)

Esophageal cancer organoids were treated with DMSO, 20 μM Thiamet G (TMG, MCE), or 20 μg/mL OSMI - 1 for 48 h. Cells were lysed in lysis buffer (50 mM Tris - HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100) supplemented with Protease Inhibitor Cocktail (Roche, 4693159001) and Phosphatase Inhibitor (NCM Biotech). After centrifugation, the supernatant was incubated with anti-YTHDF1 antibody at 4 ℃ overnight. Protein A/G magnetic beads were then added to capture the antibody - protein complexes. Following washing, the bound proteins were eluted. The eluates were subjected to Western blot analysis, probed with anti - O - GlcNAc antibody and anti-YTHDF1 antibody to detect O-GlcNAcylation on YTHDF1, with IgG as a control to ensure specificity. This method confirmed the presence of O-GlcNAcylation on YTHDF1 in esophageal cancer organoids, as shown in the experimental results.

ChIP-qPCR analysis

For ChIP-PCR to validate GLI2 as a transcription factor for CCL2, organoids were pelleted in cold PBS and resuspended in fixing buffer (50 mmol/L Hepes-KOH, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mol/L EGTA), cross - linked in 1% formaldehyde for 5 min, then quenched with glycine on ice. After centrifugation, pellets were resuspended in lysis buffer (50 mmol/L HEPES pH 8.0, 140 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X100), washed with

washing buffer (10 mmol/L Tris pH 8.0, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 200 mmol/L NaCl), and resuspended in shearing buffer (0.1% SDS, 1 mmol/L EDTA, pH 8, 10 mmol/L Tris HCl, pH 8). Sonication was performed using Covaris E220. Chromatin fragments were immunoprecipitated with anti-GLI2 antibody (or IgG as control) and protein G beads. After washing, samples were treated with proteinase K and RNase A. Purified DNA was used for PCR with primers targeting the CCL2 promoter: forward "ACTGCTGCCTGCTATGCTAG" and reverse "AGCCTAGCAGAACTGTAGGA". Experiments were performed in triplicate for each condition (sh-NC and sh-GLI2), and each experiment was repeated five times.

Luciferase reporter assay

For the dual-luciferase assay to confirm GLI2 as a transcription factor of CCL2 in esophageal cancer organoids, esophageal cancer organoids (3×10^4 cells/well) were seeded into 24-well plates in triplicate and incubated for 24 h. Using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Cat. No. L3000008), the wild-type (WT) or mutant (MUT) CCL2 promoter-luciferase reporter plasmids were transfected, along with 1.5 ng of pRL-TK Renilla plasmid. Two groups (sh-NC and sh-GLI2) were set. At 48 h post-transfection, a Dual Luciferase Reporter Assay Kit (Promega, Cat. No. E1980) measured luciferase and Renilla signals per the manufacturer's instructions. Relative luciferase activity was normalized to Renilla activity, with each transfection in triplicate and the experiment repeated five times.

Methylated RNA immunoprecipitation (MeRIP)

The MeRIP assay was performed according to standard protocols using an N6-methylated RNA Immunoprecipitation (MeRIP) Kit (BersinBio, Guangdong, China). Total RNA was first fragmented to ~300 nucleotides by incubation in a fragmentation buffer at 94 °C for 2 minutes, followed by EDTA treatment to halt the reaction. A fraction (~1/9) of the fragmented RNA was saved as an input control. The remaining RNA was incubated overnight at 4 °C with 5 μg of anti-m6A antibody for immunoprecipitation. Protein A/G Magnetic Beads were pre-treated with IP buffer at

4 °C for 3 hours prior to the addition of the RNA-antibody complex. After immunoprecipitation, the beads were digested with Proteinase K at 55 °C for 45 minutes to release the bound RNA. The enriched RNA fragments were then purified via Phenol-Chloroform-Isoamyl alcohol extraction and analyzed by RT-qPCR. Enrichment levels were quantified relative to the input control.

mRNA stability assay

Esophageal cancer organoids were incubated with actinomycin D (S8964, Selleck, USA) at 5 μg/mL for 0, 2, 4, and 6 hours. Total RNA was isolated using TRIzol reagent, and mRNA expression levels were quantified via qRT-PCR. The mRNA degradation constant (k) was derived from the exponential decay curve, and the half-life (t1/2) was determined using the equation: $t1/2= \ln 2/k_{decay}$.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 9.0 and R software, with data expressed as mean ± standard error of the mean (SEM). Experimental replicates represented independent biological repeats. Categorical variables in clinical samples were analyzed using the chi-squared test or Fisher's exact test. Differential gene expression analysis of RNA-Seq data was performed using DESeq2, with significant genes defined as those with $|\log_2$ fold change (FC)$| \geqslant 2$ and adjusted $p < 0.05$ (Benjamini-Hochberg correction). For qRT-PCR and western blot data, comparisons were performed using Student's t-test or analysis of variance (ANOVA). Flow cytometry and two-way repeated measures ANOVA were used to assess immune cell populations and xenograft tumor growth kinetics, respectively. Survival analysis was performed using Kaplan-Meier curves with log-rank tests. Pearson's correlation coefficient was used to evaluate gene-gene correlations, and transcription factor binding sites were predicted using JASPAR (relative score ⩾85%, $p < 0.05$). Statistical significance was defined as $p < 0.05$, with p values denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All experiments were repeated at least five times to ensure reproducibility.