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GALNT2-mediated O-GalNAcylation of METTL3 promotes radioresistance in esophageal squamous cell carcinoma by stabilizing GPX4 mRNA via m6A modification

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Abstract

Radiotherapy is a mainstay treatment for esophageal squamous cell carcinoma (ESCC), but radioresistance remains a major clinical challenge. Ferroptosis has been implicated in cancer therapy responses, yet how ESCC cells evade ferroptosis to acquire radioresistance is unclear. Here, ESCC cell lines (ECA109 and TE-1) and a subcutaneous xenograft mouse model were employed. GPX4 was modulated by shRNA or RSL3, and methyltransferase-like 3 (METTL3) was knocked down by shRNA or inhibited by DAA or STM2457. Glutathione peroxidase 4 (GPX4) was overexpressed using a plasmid. Radiosensitivity was assessed by CCK-8, colony formation, and tumor growth assays, while ferroptosis was evaluated by ROS and Fe^{2+} measurements. m6A modification of GPX4 mRNA was analyzed by MeRIP-qPCR, protein interactions by Co-IP and IF, O-GalNAcylation sites by site-directed mutagenesis and VVL pull-down, and protein stability by CHX chase assays. Irradiation (4 Gy) upregulated GPX4, and GPX4 knockdown or RSL3 significantly enhanced radiosensitivity. Mechanistically, irradiation induced METTL3, which installed m6A modifications on GPX4 mRNA, increasing its stability and expression. METTL3 knockdown reduced GPX4, promoted ferroptosis, and sensitized cells to radiation, effects rescued by GPX4 overexpression. The O-GalNAc transferase Glycosyltransferase 2 (GALNT2) interacted with METTL3 and mediated its O-GalNAcylation at S64. GALNT2 overexpression upregulated METTL3 and GPX4, suppressed ferroptosis, and promoted radioresistance, which was reversed by METTL3 knockdown or STM2457. Our study identifies a GALNT2-METTL3-GPX4 axis that promotes radioresistance in ESCC by suppressing ferroptosis. Targeting this axis via METTL3 or GALNT2 inhibition may represent a promising strategy to overcome radioresistance in ESCC.

Keywords

Esophageal squamous cell carcinoma; Radioresistance; ferroptosis; Glutathione peroxidase 4; methyltransferase-like 3; Glycosyltransferase 2

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common and aggressive malignancies worldwide, particularly prevalent in East Asia, with a high incidence and poor prognosis[1]. Despite advances in multimodal therapies, including surgery, chemotherapy, and radiotherapy, the overall 5-year survival rate for ESCC patients remains disappointingly low, largely due to local recurrence and distant metastasis[2-4]. Radiotherapy plays a pivotal role in the curative and palliative treatment of ESCC, either as a definitive treatment for inoperable patients or as an adjuvant approach following surgical resection[5]. However, the therapeutic efficacy of radiotherapy is frequently compromised by the development of acquired or intrinsic radioresistance, which enables tumor cells to survive genotoxic insults and repopulate[6]. Understanding the molecular mechanisms underlying radioresistance is therefore essential for developing novel strategies to sensitize ESCC cells to irradiation and improve clinical outcomes.

Accumulating evidence has implicated deregulated cell death pathways in the acquisition of radioresistance[7]. Among various forms of regulated cell death, ferroptosis has recently emerged as a critical determinant of cancer cell sensitivity to radiotherapy and chemotherapy[8, 9]. Ferroptosis is an iron-dependent, non-apoptotic form of cell death characterized by the accumulation of lipid peroxides and reactive oxygen species (ROS) to lethal levels[10]. Unlike apoptosis, ferroptosis is morphologically and biochemically distinct, involving mitochondrial shrinkage, increased membrane density, and loss of plasma membrane integrity[11]. The execution of ferroptosis is tightly controlled by a network of antioxidant systems, with glutathione peroxidase 4 (GPX4) serving as the central regulator[12]. GPX4 is a selenoenzyme that reduces toxic lipid hydroperoxides to non-toxic lipid alcohols, thereby protecting cells from oxidative membrane damage[13]. Depletion or inhibition of GPX4 leads to unchecked lipid peroxidation and ferroptotic cell death[14]. In the context of cancer, GPX4 has been shown to be upregulated in various tumor types,

where it promotes survival, chemoresistance, and radioresistance[15]. For example, elevated GPX4 expression has been reported in pancreatic, lung, and colorectal cancers, correlating with poor patient prognosis[16]. Conversely, pharmacological inhibition of GPX4 using agents such as RSL3 or ML162 sensitizes cancer cells to ionizing radiation (IR) by inducing ferroptosis[17]. However, the role of GPX4 in ESCC radioresistance and the upstream regulatory mechanisms governing its expression following irradiation remain incompletely understood.

Recent studies have highlighted the importance of epigenetic and epitranscriptomic modifications in regulating gene expression during stress responses, including those induced by radiotherapy[18]. N6-methyladenosine (m6A) is the most abundant internal modification of eukaryotic mRNA, and it influences nearly every aspect of RNA metabolism, including splicing, export, stability, and translation[19]. The m6A modification is dynamically installed by a multicomponent methyltransferase complex, with methyltransferase-like 3 (METTL3) serving as the catalytic core. METTL3 has been implicated in numerous cancer-related processes, including cell proliferation, invasion, and therapy resistance[20]. For instance, METTL3 promotes chemoresistance in gastric and breast cancer by stabilizing target mRNAs encoding anti-apoptotic proteins or drug efflux transporters[20]. With respect to radiotherapy, recent studies have shown that ionizing radiation can induce the expression of METTL3 and other m6A regulators, leading to altered m6A landscapes that contribute to DNA damage repair and cell survival[21]. However, whether METTL3 directly modulates ferroptosis-related genes such as GPX4 in response to irradiation remains unexplored, especially in ESCC.

In addition to RNA modifications, post-translational modifications (PTMs) of proteins, including phosphorylation, ubiquitination, and glycosylation, play crucial roles in regulating protein stability, activity, and interactions[22]. O-GlcNAcylation and O-GalNAcylation (mucin-type O-glycosylation) are two major forms of O-glycosylation[23]. O-GalNAcylation is catalyzed by a family of UDP-GalNAc

polypeptide N-acetylgalactosaminyltransferases (GALNTs)[24]. Among the 20 human GALNT family members, Glycosyltransferase 2(GALNT2) has been shown to be dysregulated in various cancers, influencing cell adhesion, migration, and signaling[25]. However, the potential role of GALNT2 in modulating radioresistance via glycosylation of epitranscriptomic regulators has not been reported. The observation that GALNT2 expression correlates with METTL3 in TCGA datasets and that GALNT2 is upregulated in ESCC prompted us to investigate whether GALNT2 might regulate METTL3 through O-GalNAcylation, thereby linking protein glycosylation to RNA methylation and ferroptosis in the context of radiotherapy.

In the present study, we sought to test these hypotheses by combining in vitro and in vivo approaches, including gene knockdown and overexpression, pharmacological inhibition, biochemical assays for m6A modification and protein glycosylation, and functional assessments of radiosensitivity and ferroptosis. Our results reveal a novel GALNT2-METTL3-GPX4 signaling axis that promotes radioresistance in ESCC by suppressing ferroptosis, and we identify METTL3 O-GalNAcylation at serine 64 as a critical post-translational event that stabilizes METTL3. These findings provide new insights into the molecular mechanisms of radioresistance and suggest potential therapeutic targets for overcoming treatment failure in ESCC.

2. Materials and methods

2.1 Cell lines and culture conditions

Human esophageal squamous cell carcinoma (ESCC) cell lines ECA109 and TE-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) or the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (HyClone, Logan, UT, USA). All cells were maintained in a humidified incubator at 37°C with 5% CO₂. Mycoplasma contamination was routinely tested using a PCR-based detection kit (Yeasen, Shanghai, China), and only mycoplasma-free cells were used for experiments.

2.2 Irradiation treatment

Cells or tumor-bearing mice were exposed to ionizing radiation (IR) using a Gammacell 40 Exactor irradiator (Best Theratronics, Ottawa, ON, Canada) with a ¹³⁷Cs source. A single dose of 4 Gy was administered at a dose rate of approximately 0.8 Gy/min. For in vitro experiments, adherent cells were irradiated at approximately 70–80% confluence. For in vivo experiments, mice were anesthetized with isoflurane and shielded with lead blocks except for the tumor-bearing area.

2.3 Generation of stable knockdown and overexpression cell lines

Short hairpin RNAs (shRNAs) targeting human GPX4 (shGPX4), METTL3 (shMETTL3), and a non-targeting control shRNA (shNC) were designed and cloned into the lentiviral vector pLKO.1 (Addgene, Watertown, MA, USA). The shRNA sequences were listed in the Supplementary Table 1. The full-length human GPX4 coding sequence was amplified and inserted into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) for overexpression. Lentiviral particles were produced by co-transfecting HEK293T cells with the shRNA-containing pLKO.1 vector together with packaging plasmids psPAX2 and pMD2.G using Lipofectamine 3000 (Invitrogen). After

48 h, the viral supernatant was collected, filtered, and used to infect ECA109 and TE-1 cells in the presence of 8 µg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Stably transduced cells were selected with 2 µg/mL puromycin (InvivoGen, San Diego, CA, USA) for 7 days. For transient overexpression, cells were transfected with pcDNA3.1-GPX4 or empty vector using Lipofectamine 3000 according to the manufacturer's instructions, and experiments were performed 48 h post-transfection.

2.4 Pharmacological inhibitors

The GPX4 inhibitor RSL3 (MedChemExpress, Monmouth Junction, NJ, USA) was dissolved in DMSO and used at a final concentration of 2 µM. The METTL3 inhibitor DAA (D-amino acid derivative, also known as STM2457 in some contexts; MedChemExpress) was dissolved in DMSO and used at 5 µM. The m6A inhibitor STM2457 (MedChemExpress) was dissolved in DMSO for in vitro use (5 µM) or in 0.9% saline containing 10% Cremophor EL and 10% ethanol for in vivo administration (50 mg/kg, intraperitoneal injection every 3 days). For all inhibitor treatments, cells were pre-incubated with the inhibitor or equal volume of DMSO for 2 h before irradiation and maintained throughout the experiment.

2.5 RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from cultured cells or frozen tumor tissues using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed with 1 µg of total RNA using the PrimeScript RT Master Mix (Takara, Kusatsu, Japan). Quantitative real-time PCR was carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green Premix Ex Taq II (Takara). The thermal cycling conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH as an internal control. The primer sequences

used were listed in the Supplementary Table 2.

2.6 Western blot analysis

Cells or tumor tissues were lysed in RIPA buffer (Beyotime, Shanghai, China) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (MedChemExpress). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (20–30 µg) were separated by 10% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies. The primary antibodies used were: anti-GPX4 (1:1000, ab125066, Abcam, Cambridge, UK), anti-METTL3 (1:1000, 96391, Cell Signaling Technology, Danvers, MA, USA), anti-GALNT2 (1:1000, sc-515166, Santa Cruz Biotechnology, Dallas, TX, USA), anti-β-actin (1:5000, 4970, Cell Signaling Technology), and anti-HA tag (1:2000, 3724, Cell Signaling Technology). After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Cell Signaling Technology) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) reagent (Millipore) and imaged with a ChemiDoc XRS+ system (Bio-Rad). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to β-actin.

2.7 Cell viability assay (CCK-8)

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). ECA109 and TE-1 cells were seeded into 96-well plates at a density of 5×10^3 cells per well and cultured overnight. After the indicated treatments (shRNA, inhibitor, irradiation, or combinations), 10 µL of CCK-8 solution was added to each well and incubated at 37°C for 2 h. The absorbance at 450 nm was measured using a microplate

reader (Bio-Tek, Winooski, VT, USA). Each condition was tested in five replicate wells, and experiments were repeated at least three times.

2.8 Colony formation assay

Cells were trypsinized, counted, and seeded into 6-well plates at densities appropriate to yield 50–200 colonies per well (typically 500–2000 cells per well depending on treatment). After allowing cells to adhere for 6 h, treatments (irradiation, inhibitors, etc.) were applied. Cells were then cultured for 10–14 days with medium changed every 3 days. At the end of the culture period, colonies were fixed with methanol for 15 min and stained with 0.5% crystal violet (Sigma-Aldrich) for 30 min. After washing with water and air drying, colonies containing ≥ 50 cells were counted manually under a light microscope. The plating efficiency and surviving fraction were calculated relative to untreated controls.

2.9 Measurement of reactive oxygen species (ROS)

Intracellular ROS levels were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich). After the indicated treatments, cells were harvested, washed twice with PBS, and incubated with 10 μ M DCFH-DA in serum-free medium at 37°C for 30 min in the dark. Cells were then washed with PBS and analyzed immediately using a flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and emission at 525 nm. For each sample, at least 10,000 events were acquired. ROS levels were expressed as mean fluorescence intensity (MFI) relative to control. For tumor tissues, fresh frozen sections (10 μ m thick) were incubated with 10 μ M DCFH-DA for 30 min at 37°C, washed, and imaged using a fluorescence microscope (Nikon Eclipse Ti-S, Tokyo, Japan). Quantification was performed using ImageJ.

2.10 Measurement of ferrous ion (Fe^{2+}) levels

Cellular ferrous iron levels were assessed using the fluorescent probe FerroOrange (Dojindo). Following treatments, cells were washed with Hank's balanced salt solution

(HBSS) and incubated with 1 μ M FerroOrange in HBSS at 37°C for 30 min in the dark. Cells were then washed twice with HBSS, and fluorescence was measured using a flow cytometer with excitation at 561 nm and emission at 579 nm. For tumor tissues, frozen sections were stained with 1 μ M FerroOrange for 30 min at 37°C, washed, and imaged by confocal microscopy (Leica TCS SP8, Wetzlar, Germany). Fe²⁺ levels were quantified as mean fluorescence intensity.

2.11 MeRIP-qPCR (methylated RNA immunoprecipitation coupled with qPCR)

m6A levels on GPX4 mRNA were analyzed using a MeRIP kit (Magna MeRIP m6A Kit, Millipore) according to the manufacturer's instructions. Briefly, total RNA (100 μ g) was extracted from cells under various treatments. RNA was fragmented to ~100–200 nucleotide fragments using RNA fragmentation reagent (Ambion, Austin, TX, USA) at 70°C for 5 min. Fragmented RNA was incubated with anti-m6A antibody (5 μ g, Millipore) or normal mouse IgG (control) in immunoprecipitation buffer at 4°C overnight with rotation. Protein A/G magnetic beads were added and incubated for another 2 h. The beads were washed, and the immunoprecipitated RNA was eluted with m6A competitor (N6-methyladenosine, Sigma-Aldrich). After phenol-chloroform extraction and ethanol precipitation, the enriched RNA was reverse transcribed and subjected to qPCR using primers specific for the GPX4 transcript region containing the predicted m6A site (forward: 5'-GCC TGA GTA ACA TCC TGA ATT-3', reverse: 5'-CCT GGG CTT CCT TAT CTA TAA-3'). The m6A enrichment level was calculated as the ratio of m6A-IP signal to input signal, normalized to IgG control.

2.12 Actinomycin D transcription inhibition assay

To assess mRNA stability, cells were treated with actinomycin D (5 μ g/mL, Sigma-Aldrich) to block new transcription. At various time points (0, 2, 4, 6, 8, 10 h) after actinomycin D addition, total RNA was extracted and GPX4 mRNA levels were determined by qPCR. The remaining fraction of GPX4 mRNA at each time point was normalized to the 0 h time point, and decay curves were fitted using a one-phase

exponential decay model. The half-life ($t_{1/2}$) was calculated using GraphPad Prism 9 software (GraphPad, San Diego, CA, USA).

2.13 Co-immunoprecipitation (Co-IP)

Cells were lysed in IP lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors. Lysates were pre-cleared by centrifugation and then incubated with anti-METTL3 antibody (5 μ g) or normal rabbit IgG (control) at 4°C overnight with gentle rotation. Protein A/G agarose beads (Santa Cruz) were added for another 3 h. Beads were washed three times with lysis buffer, and bound proteins were eluted by boiling in 2× SDS loading buffer. The eluates were analyzed by western blotting using anti-GALNT2 or anti-METTL3 antibodies. For HA-GALNT2 co-IP, HA-tagged GALNT2 was overexpressed, and pull-down was performed using anti-HA affinity beads (Thermo Fisher Scientific).

2.14 Immunofluorescence (IF) staining

ECA109 and TE-1 cells grown on glass coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 5% bovine serum albumin (BSA) in PBS for 1 h. Cells were then incubated with primary antibodies against GALNT2 (1:200, Santa Cruz) and METTL3 (1:200, Cell Signaling Technology) overnight at 4°C. After washing, Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 594-conjugated anti-rabbit secondary antibodies (1:500, Invitrogen) were applied for 1 h at room temperature. Nuclei were counterstained with DAPI (Sigma-Aldrich). Coverslips were mounted with antifade mounting medium and imaged under a confocal laser scanning microscope (Leica TCS SP8). Co-localization was assessed using the Pearson's correlation coefficient (PCC) calculated in ImageJ.

2.15 Identification of O-GalNAcylation sites and VVL pull-down assay

Potential O-GalNAcylation sites on METTL3 were predicted using the NetOGlyc-4.0 server (<https://services.healthtech.dtu.dk/service.php?NetOGlyc-4.0>) with a default

threshold score > 0.8. Site-directed mutagenesis was performed using a QuikChange II XL kit (Agilent, Santa Clara, CA, USA) to generate alanine substitution mutants (P48A, T50A, S54A, P57A, T58A, S64A, T65A, S67A, K203A) in the METTL3 expression construct. Wild-type or mutant METTL3 was co-expressed with GALNT2 in HEK293T cells. Cell lysates were incubated with biotinylated Vicia Villosa Lectin (VVL, 10 µg/mL, Vector Laboratories, Burlingame, CA, USA) at 4°C overnight, followed by streptavidin-agarose beads (Thermo Fisher Scientific) for 2 h. The precipitated proteins were washed and eluted for western blotting with anti-METTL3 antibody to detect O-GalNAcylated METTL3.

2.16 Cycloheximide (CHX) chase assay

To assess METTL3 protein stability, cells expressing wild-type or S64A mutant METTL3 were treated with cycloheximide (100 µg/mL, Sigma-Aldrich) to block de novo protein synthesis. At indicated time points (0, 2, 4, 6, 8, 10 h), cells were harvested and lysed for western blot analysis of METTL3. Protein band intensities were quantified and normalized to the 0 h time point. The degradation curve was plotted, and half-life was calculated by nonlinear regression using GraphPad Prism.

2.17 Bioinformatics analysis

TCGA (The Cancer Genome Atlas) ESCC data were analyzed using the GEPIA2 (Gene Expression Profiling Interactive Analysis 2) platform (<http://gepia2.cancer-pku.cn/>). Correlation analysis between GALNT family members (GALNT1-18) and METTL3 was performed using the Spearman correlation method. Differential expression of GALNT2 between normal esophageal tissues and ESCC tumor tissues was assessed using the GEPIA2 boxplot function with the threshold $|\log_2FC| > 1$ and adjusted $p < 0.05$. The m6A modification site on GPX4 mRNA was predicted using SRAMP (<http://www.cuilab.cn/sramp>) with the “high confidence” setting. Protein-protein interaction between GALNT2 and METTL3 was predicted using AlphaFold Server (<https://alphafold.ebi.ac.uk/>). The structures of GALNT2 and

METTL3 were downloaded from the AlphaFold Protein Structure Database, and docking was performed using the AlphaFold multimer prediction mode.

2.18 Animal studies and xenograft model

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the authors' institution and conformed to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Female BALB/c nude mice (4–6 weeks old, 18–20 g) were purchased from Vital River Laboratories (Beijing, China) and housed under specific pathogen-free (SPF) conditions with a 12 h light/dark cycle, controlled temperature (22–25°C), and humidity (50–60%). Food and water were provided ad libitum.

For subcutaneous xenograft models, ECA109 cells (5×10^6 cells in 100 μ L PBS) stably expressing shNC, shGPX4, shMETTL3, or shMETTL3 + GPX4 overexpression, as indicated, were injected subcutaneously into the right flank of each mouse. For GALNT2 overexpression experiments, ECA109 cells were transiently transfected with pcDNA3.1-GALNT2 or empty vector before injection. When tumors reached approximately 100 mm³ (at day 5 post-injection), mice were randomly assigned to treatment groups (n = 6 per group): (1) control (no irradiation), (2) 4 Gy irradiation alone, (3) 4 Gy irradiation + inhibitor (RSL3, DAA, or STM2457 as indicated). Irradiation (4 Gy) was administered once on day 5. Tumor dimensions were measured every 3 days using a digital caliper, and tumor volume was calculated using the formula: volume = (length $\times$ width²)/2. At the end of the experiment (day 26), mice were euthanized by cervical dislocation, and tumors were excised, weighed, photographed, and divided into three parts: one fixed in 4% paraformaldehyde for immunohistochemistry (IHC), one snap-frozen in liquid nitrogen for RNA and protein analysis, and one embedded in optimal cutting temperature (OCT) compound for ROS and Fe²⁺ staining.

2.19 Immunohistochemistry (IHC)

Paraffin-embedded tumor sections (4 μ m thick) were deparaffinized in xylene and

rehydrated through graded ethanol solutions. Antigen retrieval was performed by boiling sections in 10 mM sodium citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 min. Sections were then incubated with 5% normal goat serum for 1 h at room temperature to block non-specific binding, followed by overnight incubation at 4°C with anti-GPX4 antibody (1:200, Abcam). After washing, sections were incubated with HRP-conjugated secondary antibody (1:500) for 1 h at room temperature. Immunoreactivity was visualized using diaminobenzidine (DAB) substrate (Vector Laboratories), and sections were counterstained with hematoxylin. Stained slides were scanned using a digital slide scanner (Hamamatsu NanoZoomer, Hamamatsu, Japan). GPX4 staining intensity was scored by two independent pathologists blinded to the treatment groups using a semi-quantitative scoring system (0 = negative, 1 = weak, 2 = moderate, 3 = strong).

2.20 Statistical analysis

All experiments were performed at least three independent times, and data are presented as mean $\pm$ standard deviation (SD) or standard error of the mean (SEM) as indicated. Statistical analyses were conducted using GraphPad Prism 9 software. Comparisons between two groups were performed using the two-tailed Student's t-test (unpaired). For multiple group comparisons, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or two-way ANOVA followed by Šídák's multiple comparisons test was employed. Tumor growth curves were analyzed using two-way repeated measures ANOVA. A p-value < 0.05 was considered statistically significant. All statistical tests were two-sided, and no adjustments for multiple comparisons were made beyond those indicated. The sample sizes for animal studies were chosen based on power analysis to detect a 30% difference in tumor volume with 80% power at $\alpha = 0.05$. Investigators were not blinded to group allocation during data collection except for IHC scoring. No animals or data points were excluded from the analysis.

3. Results

3.1 GPX4 downregulation or pharmacological inhibition enhances radiosensitivity of ESCC cells in vitro and in vivo

To investigate the role of GPX4 in the response of esophageal squamous cell carcinoma (ESCC) cells to radiotherapy, we first examined the expression of GPX4 following irradiation. qPCR and western blot analyses revealed that exposure to 4 Gy radiation significantly upregulated both the mRNA (ECA-109: $P<0.001$; TE-1: $P<0.001$) and protein (ECA-109: $P<0.001$; TE-1: $P<0.001$) levels of GPX4 in ECA109 and TE-1 cells (Figs. 1A and 1B). This upregulation suggested a potential adaptive response that might contribute to radioresistance. To test this hypothesis, we knocked down GPX4 using specific shRNA (ECA-109: $P=0.02$; TE-1: $P=0.04$), which effectively abolished the radiation-induced increase in GPX4 expression (ECA-109: $P<0.001$; TE-1: $P<0.001$) (Figs. 1A and 1B).

Functional assays were then performed to evaluate the impact of GPX4 downregulation on ESCC cell radiosensitivity. CCK-8 assays demonstrated that GPX4 silencing alone did not significantly affect baseline cell viability in either ECA109 ($P=0.33$) or TE-1 ($P=0.86$) cells. However, when combined with 4 Gy irradiation, GPX4 knockdown markedly enhanced the reduction in cell viability induced by radiation (ECA-109: $P=0.02$; TE-1: $P=0.02$) (Fig. 1C). Similarly, colony formation assays showed that GPX4 shRNA had no effect on baseline clonogenic survival (ECA-109: $P=0.67$; TE-1: $P=0.99$) but significantly potentiated the suppressive effect of 4 Gy irradiation on colony formation (ECA-109: $P=0.03$; TE-1: $P=0.03$) (Fig. 1D). These results indicated that GPX4 downregulation sensitizes ESCC cells to radiation.

Given the established role of GPX4 as a key inhibitor of ferroptosis, we assessed markers of ferroptosis, namely reactive oxygen species (ROS) and ferrous ion (Fe^{2+}) levels. GPX4 knockdown did not alter baseline ROS (ECA-109: $P=0.97$; TE-1: $P=0.99$) or ferrous ion levels (ECA-109: $P=0.68$; TE-1: $P=0.99$) in untreated cells. Following 4

Gy irradiation, both ROS (ECA-109: $P<0.001$; TE-1: $P<0.001$) and Fe^{2+} (ECA-109: $P<0.001$; TE-1: $P<0.001$) levels were significantly elevated, and this elevation was further augmented by GPX4 silencing (ECA-109: $P<0.001$; TE-1: $P<0.001$) (Figs. 1E and 1F). These data suggest that irradiation induces ferroptotic stress, which is counteracted by GPX4 upregulation, and that GPX4 downregulation sensitizes ESCC cells by promoting ferroptosis.

To complement the genetic loss-of-function approach, we employed the pharmacological GPX4 inhibitor RSL3. As shown in Supplementary Figs. 1A and 1B, RSL3 treatment did not affect the radiation-induced upregulation of GPX4 mRNA (ECA-109: $P=0.88$; TE-1: $P=0.96$) or protein (ECA-109: $P=0.99$; TE-1: $P=0.96$), consistent with the fact that RSL3 inhibits GPX4 activity rather than its expression. Functional assays revealed that RSL3 alone had no effect on cell viability (ECA-109: $P=0.98$; TE-1: $P=0.56$) or colony formation (ECA-109: $P=0.38$; TE-1: $P=0.99$), but it significantly enhanced the growth-suppressive effects of 4 Gy irradiation on CCK-8 (ECA-109: $P=0.02$; TE-1: $P=0.02$) and colony formation (ECA-109: $P=0.002$; TE-1: $P=0.004$) (Supplementary Figs. 1C and 1D). Moreover, RSL3 treatment did not alter baseline ROS (ECA-109: $P=0.99$; TE-1: $P=0.97$) or Fe^{2+} (ECA-109: $P=0.94$; TE-1: $P=0.99$) levels but significantly amplified the radiation-induced accumulation of both markers (ECA-109: $P<0.001$; TE-1: $P<0.001$) (Supplementary Figs. 1E and 1F). These findings confirm that GPX4 inhibition, whether by genetic knockdown or pharmacological blockade, sensitizes ESCC cells to radiation by promoting ferroptosis.

We next evaluated the in vivo relevance of these observations using a subcutaneous xenograft model in nude mice. ECA109 cells with or without GPX4 shRNA were implanted, and mice were treated with 4 Gy irradiation or left untreated. As shown in Figs. 2A–2C, GPX4 knockdown ($P=0.97$) or RSL3 administration ($P=0.84$) alone did not significantly affect tumor growth under non-irradiated conditions. However, both interventions dramatically enhanced the anti-tumor effect of 4 Gy irradiation, leading to significantly smaller tumor volumes and weights compared to irradiation alone

(GPX4 knockdown: $P=0.02$; RSL3 administration: $P=0.03$). Immunohistochemical analysis confirmed that 4 Gy irradiation upregulated GPX4 protein expression in tumor tissues (GPX4 knockdown: $P<0.001$; RSL3 administration: $P=0.03$), an effect that was abrogated by GPX4 shRNA but not by RSL3 (GPX4 knockdown: $P<0.001$; RSL3 administration: $P=0.97$) (Fig. 2D). Consistent with the in vitro data, GPX4 downregulation or RSL3 treatment did not alter baseline ROS (GPX4 knockdown: $P=0.97$; RSL3 administration: $P=0.99$) or Fe^{2+} (GPX4 knockdown: $P=0.97$; RSL3 administration: $P=0.99$) levels in tumors but significantly exacerbated the radiation-induced elevation of these ferroptosis markers (GPX4 knockdown: $P<0.001$; RSL3 administration: $P<0.001$) (Figs. 2E and 2F). Collectively, these results establish that GPX4 plays a critical radioprotective role in ESCC both in vitro and in vivo, and that targeting GPX4 enhances radiosensitivity.

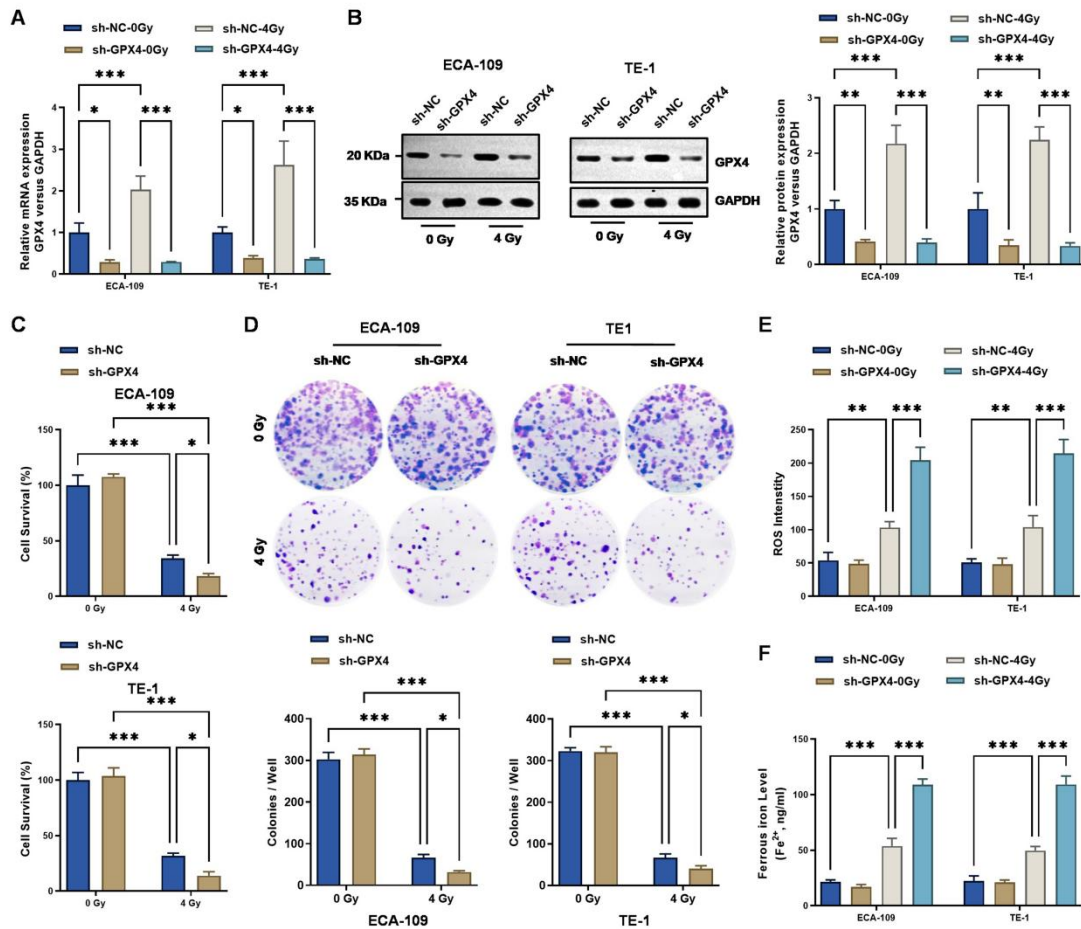


Fig. 1 GPX4 downregulation significantly enhances radiosensitivity in ESCC cells. (A)

qPCR analysis revealed that 4 Gy irradiation significantly upregulated *GPX4* mRNA expression, an effect that was effectively reversed by *GPX4* shRNA. (B) Western blot analysis demonstrated a marked increase in GPX4 protein levels following 4 Gy irradiation, which was successfully silenced by *GPX4* shRNA. (C) CCK-8 assays indicated that while GPX4 knockdown did not affect the baseline viability of ESCC cells (ECA109 and TE-1), it significantly exacerbated the reduction in cell viability induced by 4 Gy irradiation. (D) Colony formation assays showed that GPX4 knockdown did not alter baseline colony formation but significantly potentiated the inhibitory effect of 4 Gy irradiation. (E) GPX4 knockdown did not alter baseline ROS levels; however, it significantly augmented the 4 Gy-induced accumulation of ROS. (F) GPX4 knockdown had no impact on baseline ferrous ion levels but significantly amplified the 4 Gy-induced upregulation of ferrous ions. N=3; Data are presented as the mean $\pm$ SD; * P <0.05, ** P <0.01, *** P <0.001.

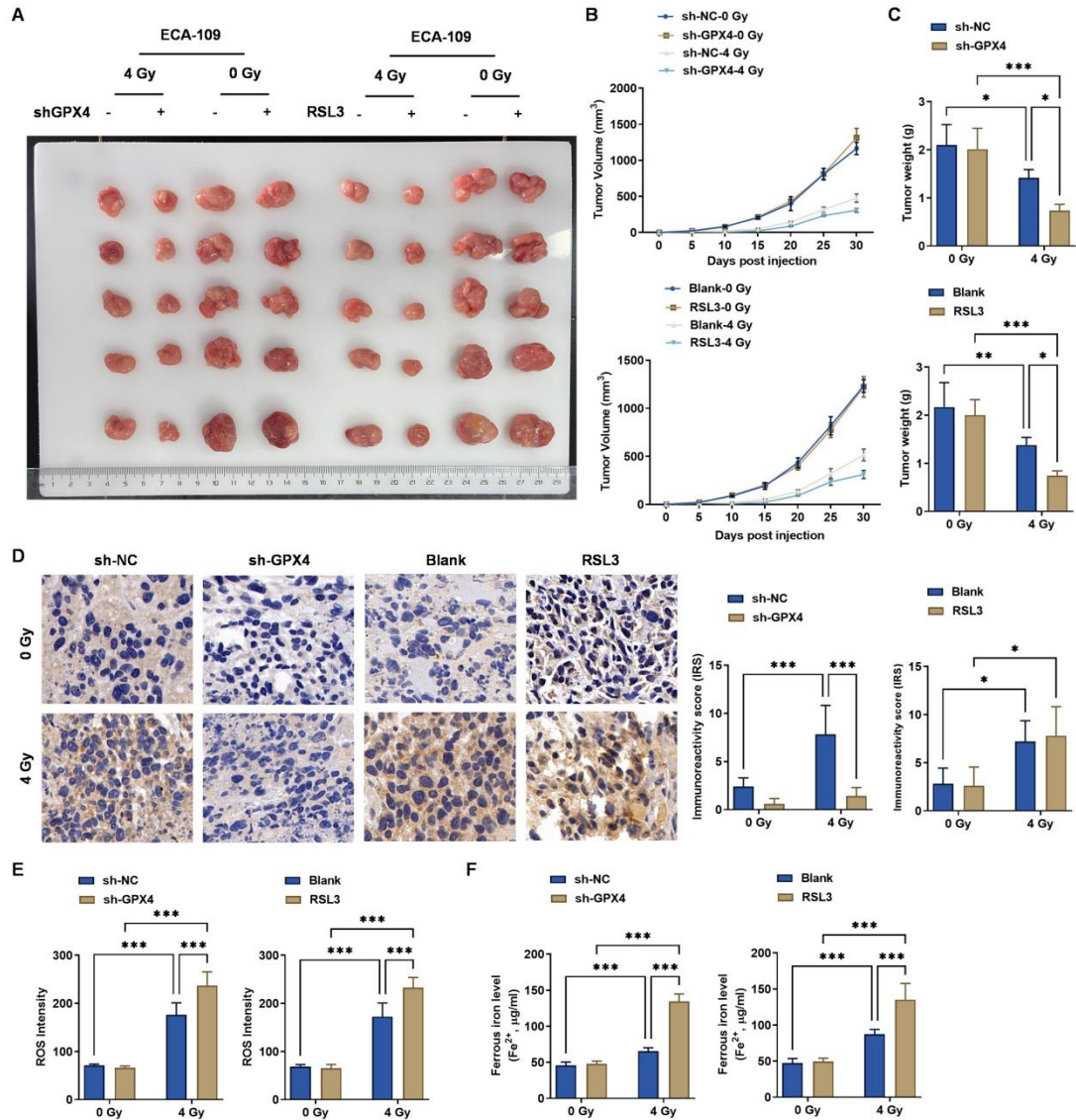


Fig. 2 In vivo validation demonstrates that GPX4 downregulation or pharmacological inhibition sensitizes ESCC tumors to radiotherapy. Nude mice bearing subcutaneous ECA109 xenografts were irradiated with 4 Gy on day 5 post-implantation, with non-irradiated mice serving as controls. The in vivo effects of GPX4 knockdown and the GPX4 inhibitor RSL3 were evaluated and compared. **(A)** Representative images of the resected tumors. **(B–C)** Tumor growth curves **(B)** and tumor weights **(C)** revealed that GPX4 silencing or RSL3 treatment alone did not significantly affect baseline tumor growth. However, both interventions significantly enhanced the tumor-suppressive effects of 4 Gy irradiation. **(D)** Immunohistochemical staining demonstrated that 4 Gy irradiation significantly upregulated GPX4 protein expression in tumor tissues. This upregulation was effectively abolished by GPX4

shRNA, whereas RSL3 treatment had no significant effect on GPX4 expression levels. **(E)** GPX4 knockdown or RSL3 treatment alone did not alter baseline ROS levels in tumor tissues, but both significantly amplified the 4 Gy-induced ROS accumulation. **(F)** Similarly, GPX4 knockdown or RSL3 treatment alone did not affect baseline ferrous ion levels, but both significantly augmented the 4 Gy-induced accumulation of ferrous ions. N=6; Data are presented as the mean $\pm$ SD; * P <0.05, ** P <0.01, *** P <0.001.

3.2 METTL3 regulates GPX4 expression through m6A modification of GPX4 mRNA

Having established that GPX4 is upregulated by irradiation and promotes radioresistance, we next sought to elucidate the molecular mechanism underlying this upregulation. Using the SRAMP bioinformatics tool, we identified a high-confidence N6-methyladenosine (m6A) modification site at position A783 of the GPX4 mRNA (Fig. 3A). This finding suggested that RNA methylation might regulate GPX4 expression. We therefore examined the expression of METTL3, a key m6A methyltransferase, in irradiated ESCC cells. qPCR and western blot analyses showed that 4 Gy irradiation significantly upregulated both the mRNA and protein levels of METTL3 (ECA-109: P <0.001; TE-1: P <0.001), paralleling the increase in GPX4 expression (ECA-109: P <0.001; TE-1: P <0.001) (Figs. 3B and 3C). Importantly, knockdown of METTL3 using specific shRNA not only reduced METTL3 expression (ECA-109: P <0.001; TE-1: P <0.001) but also abrogated the radiation-induced upregulation of GPX4 at both the mRNA (ECA-109: P <0.001; TE-1: P <0.001) and protein levels (ECA-109: P <0.001; TE-1: P <0.001) (Figs. 3B and 3C).

To directly assess whether METTL3 modulates GPX4 via m6A modification, we performed MeRIP-qPCR (methylated RNA immunoprecipitation followed by qPCR) using an anti-m6A antibody. As shown in Fig. 3D, 4 Gy irradiation significantly increased the m6A level of GPX4 mRNA (ECA-109: P <0.001; TE-1: P <0.001), and this

increase was substantially suppressed by METTL3 knockdown (ECA-109: $P<0.001$; TE-1: $P<0.001$). Furthermore, actinomycin D transcription inhibition assays revealed that irradiation markedly enhanced the stability of GPX4 mRNA, as evidenced by a slower decay rate (ECA-109-3 hours: $P<0.001$; ECA-109-6 hours: $P<0.001$; TE-1-3 hours: $P=0.07$; TE-1-6 hours: $P=0.01$). METTL3 knockdown completely reversed this stabilizing effect (ECA-109-3 hours: $P<0.001$; ECA-109-6 hours: $P=0.09$; TE-1-3 hours: $P=0.04$; TE-1-6 hours: $P=0.004$) (Fig. 3E). These results indicate that METTL3 promotes GPX4 expression by installing m6A modifications on GPX4 mRNA, thereby enhancing its stability.

We confirmed these findings using the METTL3 inhibitor DAA. As shown in Supplementary Figs. 2A and 2B, DAA treatment did not affect the expression of METTL3 itself but completely abolished the radiation-induced upregulation of GPX4 at both the mRNA (ECA-109: $P<0.001$; TE-1: $P<0.001$) and protein (ECA-109: $P<0.001$; TE-1: $P<0.001$) levels. MeRIP-qPCR assays demonstrated that DAA treatment prevented the irradiation-induced increase (ECA-109: $P<0.001$; TE-1: $P<0.001$) in GPX4 mRNA m6A modification (ECA-109: $P<0.001$; TE-1: $P<0.001$) (Supplementary Fig. 2C), and actinomycin D assays confirmed that DAA reversed the stabilizing effect of irradiation on GPX4 mRNA (ECA-109-3 hours: $P<0.001$; ECA-109-6 hours: $P<0.001$; TE-1-3 hours: $P<0.001$; TE-1-6 hours: $P<0.001$) (Supplementary Fig. 2D). The efficiencies of METTL3 knockdown and GPX4 overexpression were validated by qPCR and western blot under both 0 Gy and 4 Gy conditions (ECA-109: $P<0.001$; TE-1: $P<0.001$) (Supplementary Figs. 3 and 4). Together, these data establish that METTL3-mediated m6A methylation is essential for the radiation-induced upregulation of GPX4.

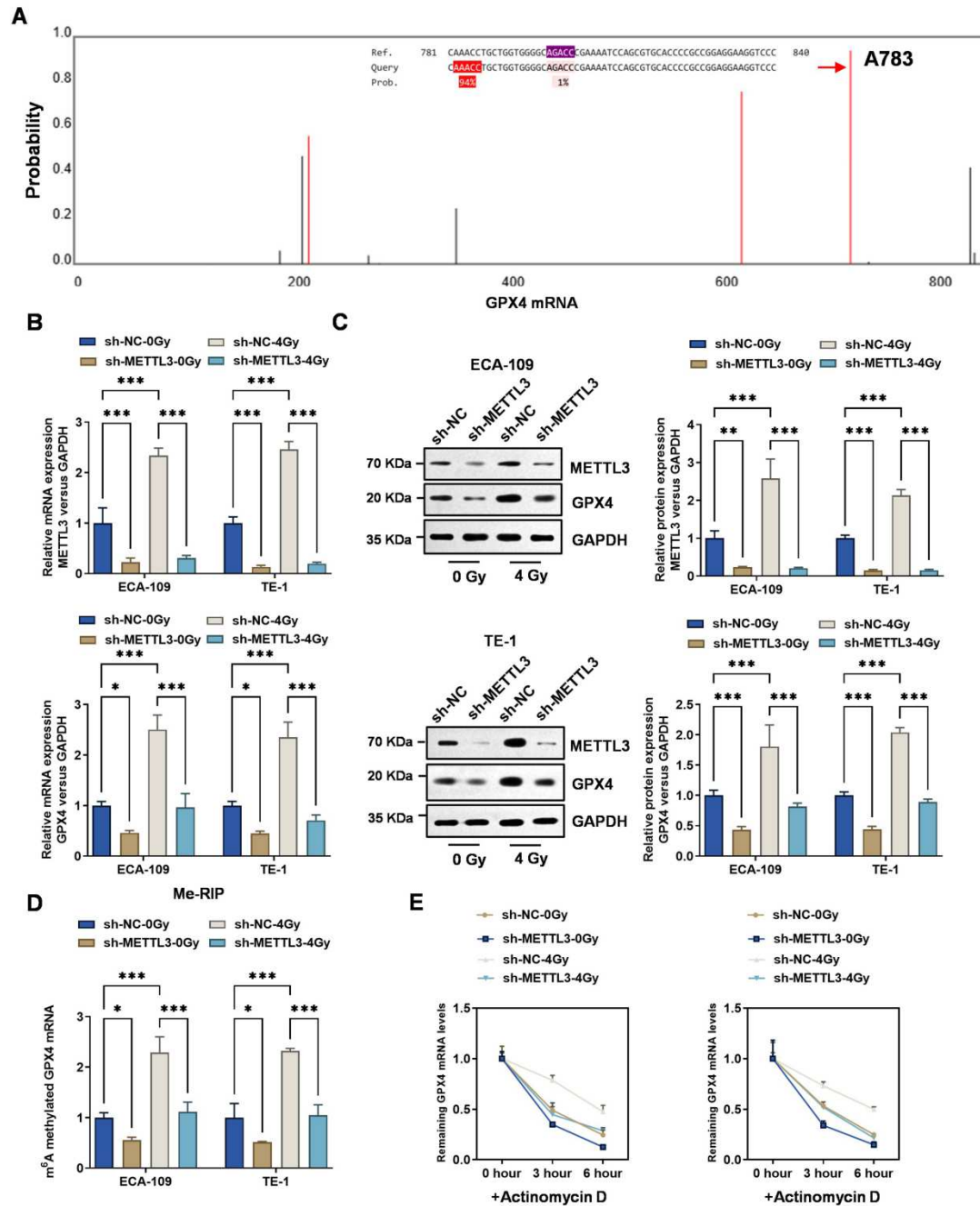


Fig. 3 METTL3 downregulation regulates the m6A modification and expression of *GPX4* mRNA. (A) The SRAMP (sequence-based RNA adenosine methylation site predictor) tool identified a high-confidence m6A modification site at position A783 of *GPX4* mRNA. qPCR analysis demonstrated that 4 Gy irradiation significantly upregulated the mRNA levels of both *METTL3* and *GPX4*, an effect markedly inhibited by *METTL3* shRNA. (C) Western blot analysis showed a significant irradiation-induced increase in *METTL3* and *GPX4* protein levels, which

was reversed following METTL3 knockdown. (D) MeRIP-qPCR assays revealed that 4 Gy irradiation significantly increased m6A methylation levels of *GPX4* mRNA, an increase that was suppressed by *METTL3* shRNA. (E) Actinomycin D transcription inhibition assays indicated that 4 Gy irradiation significantly enhanced *GPX4* mRNA stability, an effect abrogated by METTL3 knockdown. N=3; Data are presented as the mean $\pm$ SD; * P <0.05, ** P <0.01, *** P <0.001.

3.3 METTL3 promotes radioresistance by upregulating GPX4 and inhibiting ferroptosis

To determine whether the radioresistance conferred by METTL3 operates through GPX4, we performed rescue experiments in which METTL3 knockdown was combined with GPX4 overexpression. CCK-8 assays revealed that METTL3 knockdown alone had no effect on baseline cell viability (ECA-109: P =0.99; TE-1: P =0.63) under 0 Gy conditions but significantly reduced viability following 4 Gy irradiation (ECA-109: P <0.001; TE-1: P <0.001). Importantly, forced expression of GPX4, which alone did not affect baseline viability (ECA-109: P =0.99; TE-1: P =0.67), completely reversed the sensitizing effect of METTL3 knockdown, restoring cell viability to levels comparable to control irradiated cells (ECA-109: P <0.001; TE-1: P <0.001) (Fig. 4A). Similar results were obtained in colony formation assays: METTL3 knockdown inhibited radiation-induced colony formation (ECA-109: P <0.001; TE-1: P <0.001), and this inhibition was fully rescued by GPX4 overexpression (ECA-109: P <0.001; TE-1: P <0.001) (Fig. 4B).

We next examined ferroptosis markers. METTL3 knockdown or GPX4 overexpression alone did not alter baseline ROS or ferrous ion levels. Under 4 Gy irradiation, METTL3 knockdown significantly increased ROS (ECA-109: P =0.98; TE-1: P =0.99) and Fe^{2+} accumulation (ECA-109: P =0.99; TE-1: P =0.99), consistent with enhanced ferroptosis. GPX4 overexpression, on the other hand, reduced radiation-induced ROS (ECA-109: P <0.001; TE-1: P <0.001) and Fe^{2+} levels (ECA-109: P <0.001; TE-1: P <0.001) and

effectively reversed the pro-ferroptotic effect of METTL3 knockdown (Figs. 4C and 4D). These data demonstrate that METTL3 functions upstream of GPX4 to suppress ferroptosis and promote radioresistance.

The in vivo relevance of this axis was evaluated using the ECA109 xenograft model. Mice bearing tumors with METTL3 knockdown, GPX4 overexpression, or both were subjected to 4 Gy irradiation. As shown in Supplementary Figs. 5A–5C, neither METTL3 knockdown nor GPX4 overexpression alone affected baseline tumor growth in non-irradiated mice (METTL3 knockdown: $P=0.99$; GPX4 overexpression: $P=0.99$). Following irradiation, METTL3 knockdown significantly suppressed tumor growth, whereas GPX4 overexpression promoted tumor growth (METTL3 knockdown: $P<0.001$; GPX4 overexpression: $P<0.001$). Strikingly, GPX4 overexpression completely reversed the tumor-suppressive effect of METTL3 knockdown, restoring tumor growth to levels similar to control irradiated tumors ($P<0.001$). These results confirm that METTL3 promotes radioresistance in ESCC primarily through upregulation of GPX4.

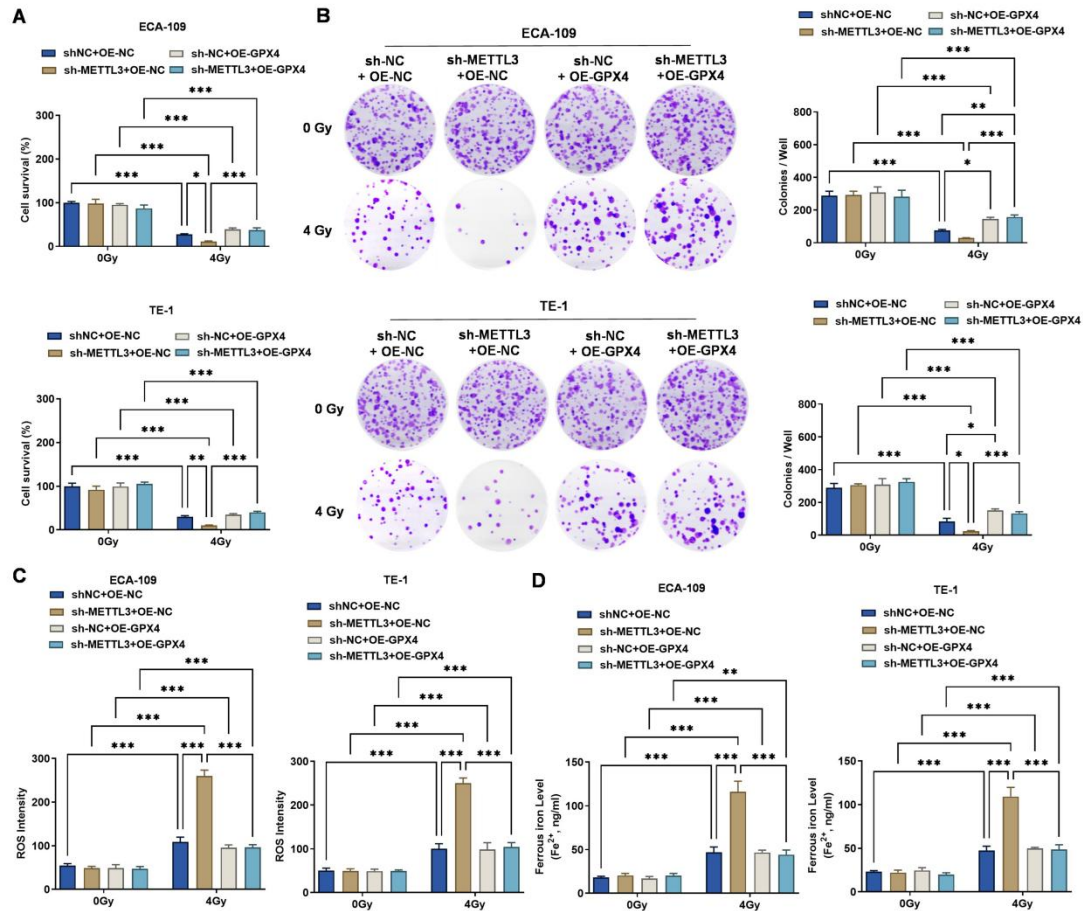


Fig. 4 METTL3 upregulates GPX4 expression via m6A modification, thereby inhibiting ferroptosis and promoting radioresistance in ESCC cells. (A) CCK-8 assays revealed that METTL3 knockdown or GPX4 overexpression alone did not significantly affect ESCC cell (ECA109 and TE-1) viability under control (0 Gy) conditions. Following 4 Gy irradiation, METTL3 knockdown significantly suppressed cell viability, whereas GPX4 overexpression restored viability, reversing the effects of METTL3 depletion. (B) Colony formation assays demonstrated that baseline colony formation (0 Gy) was unaffected by METTL3 knockdown or GPX4 overexpression. Under 4 Gy irradiation, METTL3 knockdown significantly inhibited colony formation, an effect effectively rescued by GPX4 overexpression. (C) Baseline ROS levels (0 Gy) remained unchanged following METTL3 knockdown or GPX4 overexpression. Under 4 Gy irradiation, METTL3 knockdown significantly increased ROS accumulation, which was mitigated by GPX4 overexpression. (D) Baseline ferrous ion levels (0 Gy) were not altered by METTL3 knockdown or GPX4 overexpression. Under 4 Gy irradiation, METTL3 knockdown

significantly elevated ferrous ion levels, an effect reversed by GPX4 overexpression. N=3; Data are presented as the mean $\pm$ SD; * P <0.05, ** P <0.01, *** P <0.001.

3.4 The O-GalNAc transferase GALNT2 interacts with METTL3 in ESCC cells

Having established the functional importance of METTL3, we next sought to understand how METTL3 itself is regulated. Given that protein glycosylation is a common post-translational modification that affects protein stability and function, we focused on O-GalNAc glycosylation (mucin-type O-glycosylation). Analysis of TCGA database revealed that among the GALNT family of O-GalNAc transferases, GALNT2 and GALNT11 showed the most significant positive correlation with METTL3 expression (Fig. 5A). Furthermore, GALNT2, but not GALNT11, was significantly upregulated in ESCC tissues compared to normal tissues (Fig. 5B, P =0.003), prompting us to focus on GALNT2 (P =0.018).

Using AlphaFold Server, we predicted a potential physical interaction between GALNT2 and METTL3, with favorable conformational complementarity (Fig. 5C). Co-immunoprecipitation (Co-IP) experiments in both ECA109 and TE-1 cells confirmed that endogenous METTL3 physically associates with GALNT2 (Fig. 5D). Moreover, immunofluorescence staining revealed prominent co-localization of GALNT2 and METTL3 in the cytoplasm of ESCC cells (Fig. 5E). These results indicate that GALNT2 and METTL3 form a protein complex in ESCC cells.

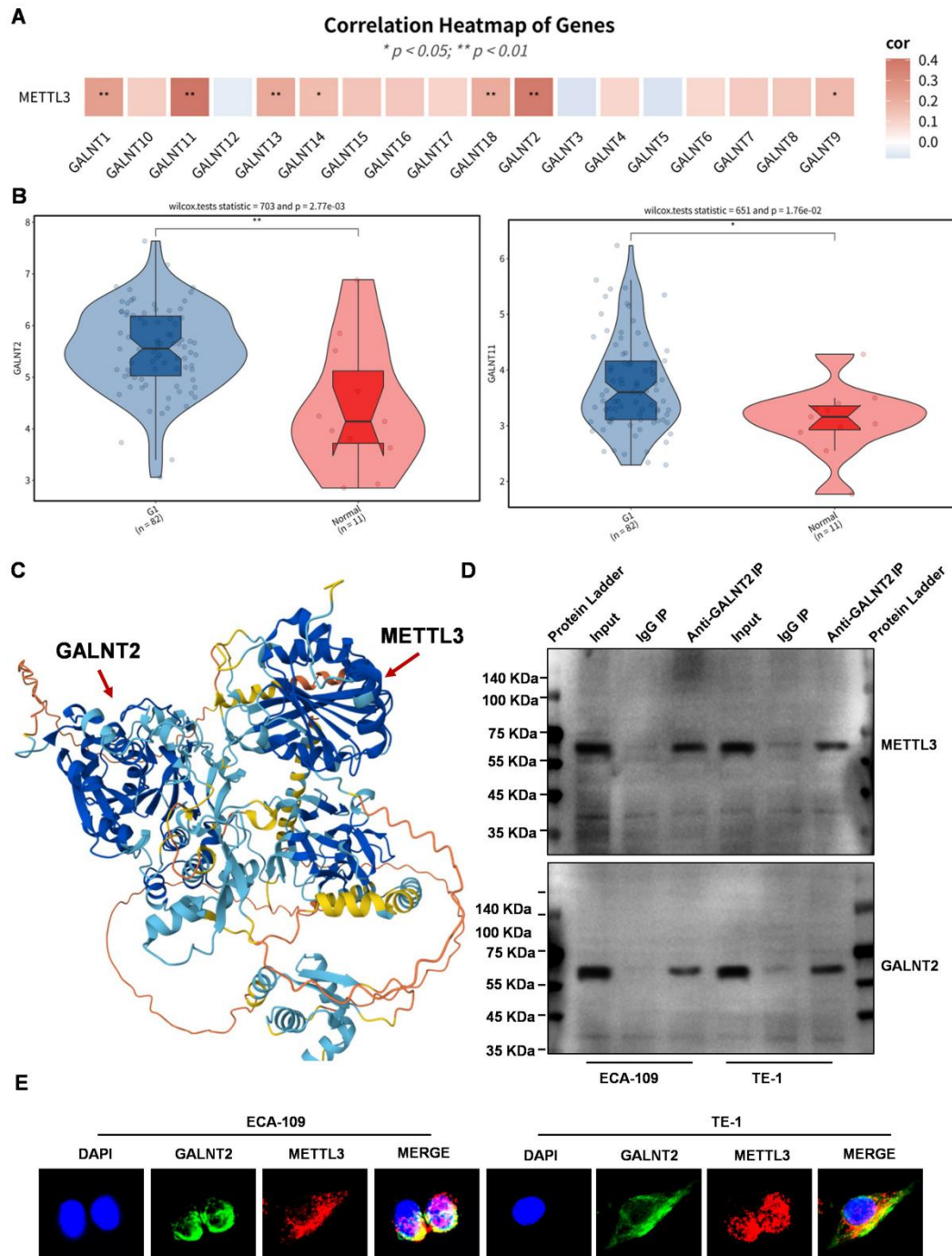


Fig. 5 The O-GalNAc transferase GALNT2 interacts with METTL3 in ESCC cells. (A) TCGA database analysis revealed a significant positive correlation between the expression of *METTL3* and multiple O-GalNAc transferases (*GALNT1-18*), with *GALNT2* and *GALNT11* exhibiting the strongest correlations. (B) Comparative analysis of normal and ESCC tissues from the TCGA database indicated a significant upregulation of *GALNT2* expression in ESCC. (C) AlphaFold Server predictions illustrated the simulated protein conformations and potential

interaction interfaces between GALNT2 and METTL3. (D) Co-immunoprecipitation (Co-IP) coupled with western blot analysis confirmed the physical binding of METTL3 to GALNT2 in ESCC cell lines (ECA109 and TE-1). (E) Immunofluorescence staining demonstrated the intracellular co-localization of GALNT2 and METTL3 in ESCC cells. N=3.

3.5 GALNT2 mediates O-GalNAcylation of METTL3 at Serine 64, enhancing METTL3 stability

To determine whether METTL3 is a substrate for GALNT2-mediated O-GalNAcylation, we first used NetOGlyc-4.0 to predict potential O-glycosylation sites on METTL3. The analysis identified nine high-confidence sites (score > 0.8), including P48, T50, S54, P57, T58, S64, T65, S67, and K203 (Fig. 6A). To identify the functionally relevant site(s), we generated a series of alanine substitution mutants and co-expressed them with GALNT2 in ESCC cells. O-glycosylated proteins were enriched using biotinylated Vicia Villosa Lectin (VVL), which specifically recognizes O-GalNAc glycans. Western blotting for METTL3 showed that GALNT2 overexpression markedly increased the O-GalNAcylation of wild-type METTL3. Among the mutants, only the S64A mutation completely abolished this enhancement, whereas mutations at other sites had minimal effects (Fig. 6B). These data identify serine 64 (S64) as the primary O-GalNAcylation site on METTL3.

We next examined whether the S64 glycosylation is required for the METTL3-GALNT2 interaction. Co-IP experiments demonstrated that the S64A mutation dramatically reduced the binding between METTL3 and GALNT2 compared to wild-type METTL3 (Fig. 6C). Furthermore, cycloheximide (CHX) chase assays were performed to assess the effect of S64 glycosylation on METTL3 protein stability. Wild-type METTL3 displayed a relatively long half-life, with gradual degradation over 10 hours. In contrast, the S64A mutant exhibited significantly accelerated degradation ($P<0.001$), with most of the protein lost within 6 hours (Fig. 6D, $P<0.001$). These results indicate that

GALNT2-mediated O-GalNAcylation at S64 stabilizes the METTL3 protein by promoting its interaction with GALNT2, thereby protecting it from degradation.

Figure 6

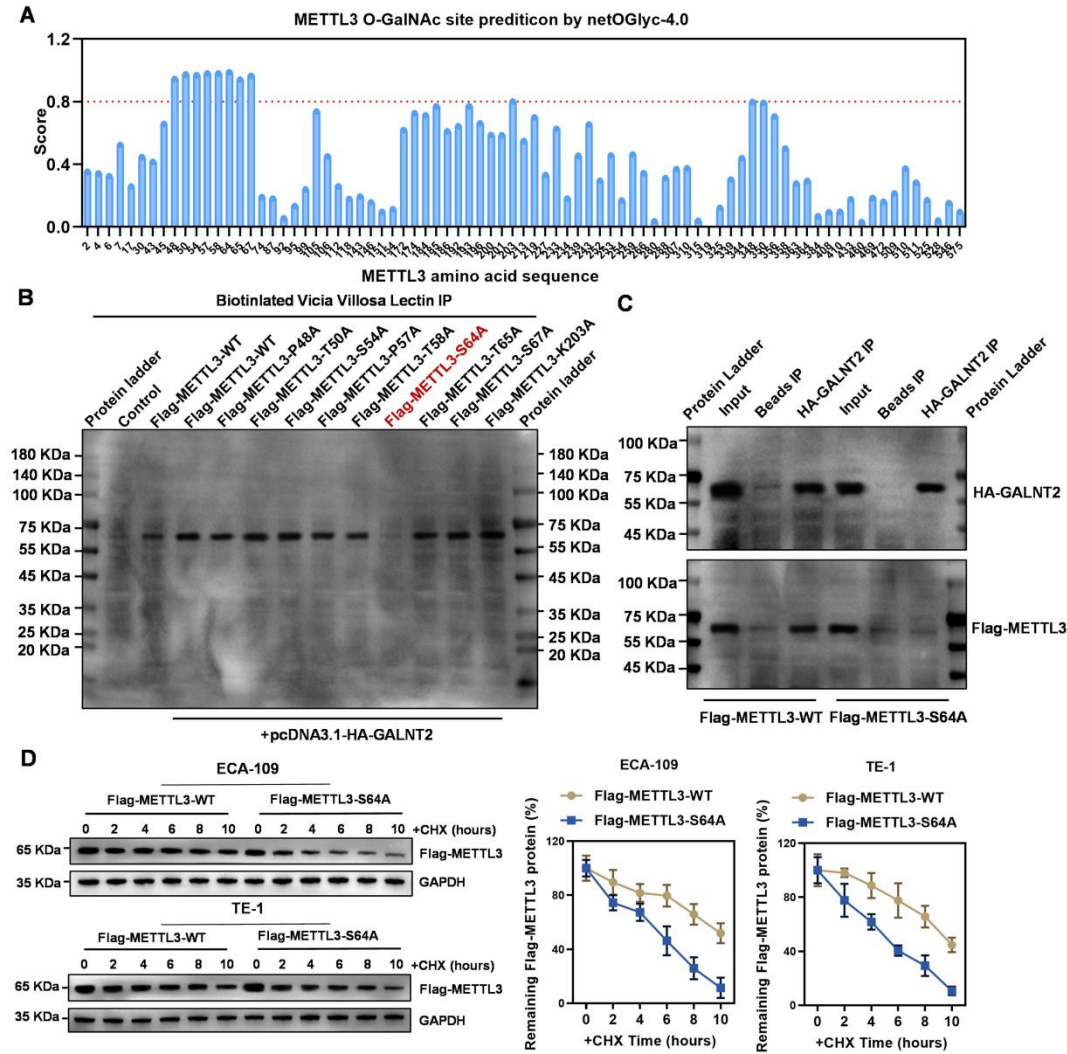


Fig. 6 GALNT2 mediates the O-GalNAcylation of METTL3 at Serine 64, enhancing METTL3 protein stability. (A) NetOGlyc-4.0 database predictions identified several high-confidence (score > 0.8) potential O-GalNAcylation sites on METTL3 (P48, T50, S54, P57, T58, S64, T65, S67, and K203). (B) Site-directed mutagenesis was employed to substitute these potential sites with alanine. Cells were co-transfected with wild-type or mutant METTL3 constructs alongside GALNT2. O-glycosylated proteins were enriched using biotinylated *Vicia villosa* lectin (VVL) and analyzed via western blotting. GALNT2 overexpression significantly enriched the O-GalNAcylation of wild-type METTL3, an effect abolished by the S64A mutation,

identifying S64 as the primary O-GalNAcylation site. (C) Co-IP of HA-GALNT2 followed by western blotting confirmed that the METTL3-GALNT2 interaction is dependent on the S64 residue; the S64A mutation significantly disrupted their physical binding. (D) Cycloheximide (CHX) chase assays (0, 2, 4, 6, 8, and 10 hours) revealed that the S64A mutation significantly accelerated the degradation of the METTL3 protein. N=3; Data are presented as the mean $\pm$ SD; * P <0.05, ** P <0.01, *** P <0.001.

3.6 GALNT2 promotes radioresistance by upregulating the METTL3/GPX4 axis and inhibiting ferroptosis

Finally, we investigated the functional consequences of GALNT2-mediated METTL3 regulation in the context of ESCC radiosensitivity. qPCR analysis revealed that GALNT2 overexpression significantly increased GPX4 mRNA levels (ECA-109: P <0.001; TE-1: P <0.001) but did not affect METTL3 mRNA levels (ECA-109: P =0.46; TE-1: P =0.81) (Fig. 7A), consistent with a post-translational regulation of METTL3 by GALNT2. Western blotting confirmed that GALNT2 overexpression elevated the protein levels of both METTL3 and GPX4 (ECA-109: P <0.001; TE-1: P <0.001), as well as the O-GalNAcylated form of METTL3 (ECA-109: P <0.001; TE-1: P <0.001) (Fig. 7B).

Functional assays showed that GALNT2 overexpression had no effect on baseline colony formation under 0 Gy conditions but significantly enhanced the clonogenic survival of ESCC cells following 4 Gy irradiation (ECA-109: P <0.001; TE-1: P <0.001) (Fig. 7C). Moreover, GALNT2 overexpression markedly reduced the radiation-induced accumulation of ROS (ECA-109: P <0.001; TE-1: P <0.001) and ferrous ions (ECA-109: P <0.001; TE-1: P <0.001), indicating suppression of ferroptosis (Figs. 7D and 7E). To confirm that these effects are mediated through the METTL3/GPX4 axis, we performed in vivo rescue experiments using the ECA109 xenograft model. Mice bearing tumors with GALNT2 overexpression were treated with 4 Gy irradiation alone or in combination with METTL3 knockdown or the m6A inhibitor STM2457. As shown in Figs. 7F–7I,

GALNT2 overexpression alone significantly promoted tumor growth under irradiated conditions ($P<0.001$). However, this pro-tumorigenic effect was completely reversed by either METTL3 knockdown ($P<0.001$) or treatment with STM2457 ($P<0.001$), demonstrating that GALNT2 acts upstream of the METTL3/m6A/GPX4 pathway. Collectively, these results define a novel signaling axis in which GALNT2 stabilizes METTL3 via O-GalNAcylation at S64, leading to increased METTL3-dependent m6A modification and stabilization of GPX4 mRNA, which in turn suppresses ferroptosis and promotes radioresistance in ESCC.

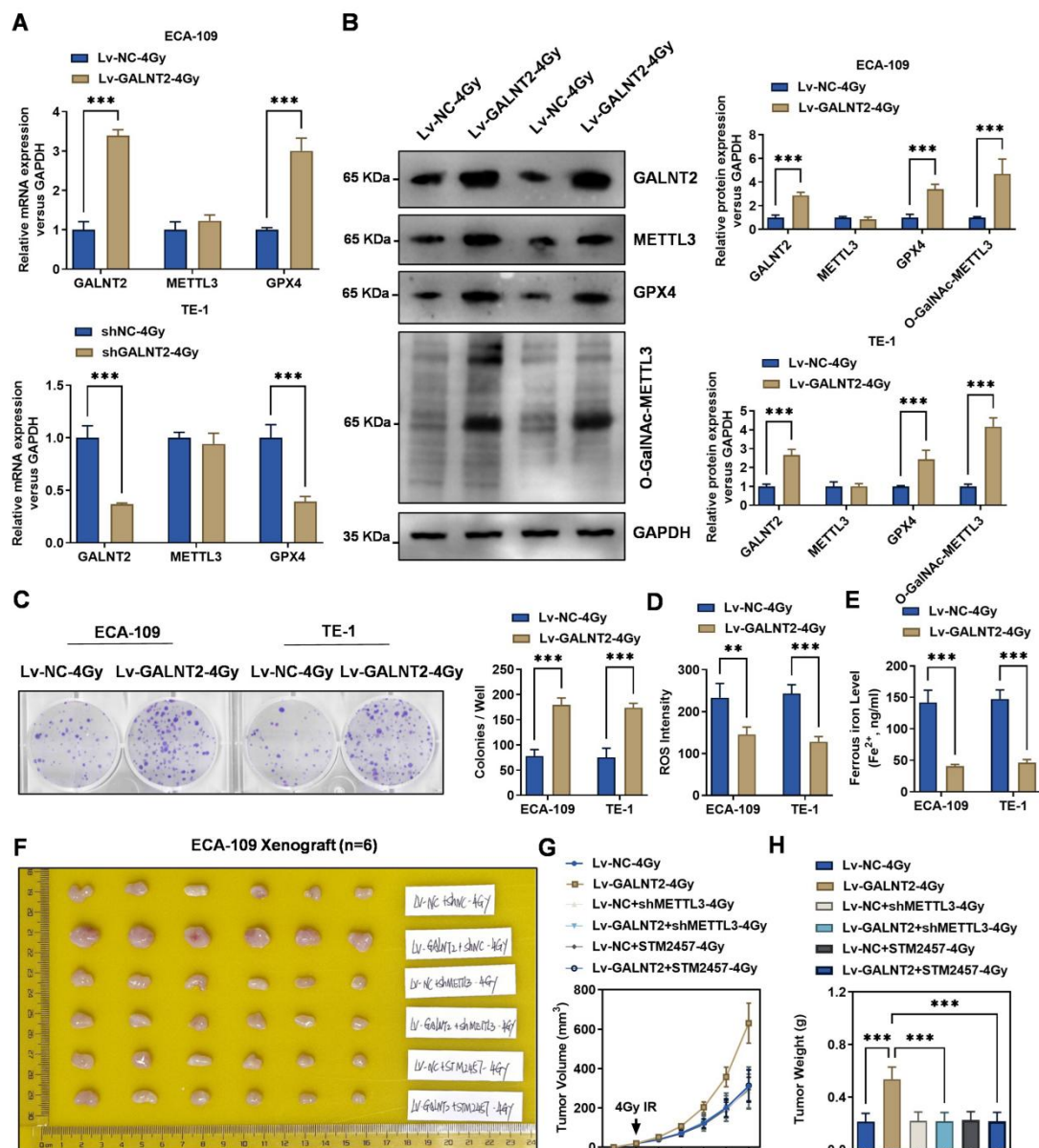


Fig. 7 GALNT2-mediated O-GalNAcylation of METTL3 at Serine 64 upregulates GPX4, thereby inhibiting ferroptosis and promoting radioresistance in ESCC cells. (A) qPCR analysis showed that GALNT2 overexpression significantly upregulated *GPX4* mRNA levels without significantly altering *METTL3* mRNA levels. (B) Western blot analysis indicated that GALNT2 overexpression elevated the protein levels of METTL3, GPX4, and O-GalNAcylated METTL3. (C) Colony formation assays demonstrated that GALNT2 overexpression significantly enhanced the clonogenic survival of ESCC cells following 4 Gy irradiation. (D) GALNT2 overexpression significantly attenuated ROS accumulation in ESCC cells under 4 Gy irradiation. (E) GALNT2 overexpression significantly reduced ferrous ion levels in ESCC cells following 4 Gy irradiation. (F–I) In vivo validation: Nude mice bearing subcutaneous ECA109 xenografts were irradiated with 4 Gy on day 5 post-implantation, alongside non-irradiated controls. The therapeutic impacts of GPX4 overexpression, METTL3 knockdown, and the m6A inhibitor STM2457 were compared. (G) Representative images of the resected tumors. (H) Tumor growth curves and (I) tumor weights demonstrated that GALNT2 overexpression significantly promoted subcutaneous tumor growth under 4 Gy irradiation. This effect was effectively reversed by METTL3 knockdown or treatment with STM2457. N=3 or 6; Data are presented as the mean $\pm$ SD; * P <0.05, ** P <0.01, *** P <0.001.

4. Discussion

The present study identifies a novel signaling axis—GALNT2-mediated O-GalNAcylation of METTL3, leading to m6A-dependent stabilization of GPX4 mRNA—that promotes radioresistance in esophageal squamous cell carcinoma (ESCC) by suppressing ferroptosis. Our findings provide multiple mechanistic insights into how ESCC cells adapt to ionizing radiation (IR) and reveal potential therapeutic targets to overcome treatment resistance.

First, we demonstrate that GPX4 is significantly upregulated by irradiation in ESCC cells and xenografts, and that this upregulation is functionally important for radioresistance. GPX4 is the central negative regulator of ferroptosis, reducing lipid hydroperoxides to lipid alcohols at the expense of glutathione (GSH)[12]. In various cancer models, GPX4 overexpression has been associated with resistance to chemotherapy, targeted therapy, and radiotherapy[26]. For instance, Lei et al. reported that IR induces GPX4 in pancreatic cancer cells, contributing to radioresistance [27]. Our results extend this observation to ESCC and show that both genetic knockdown and pharmacological inhibition of GPX4 (using RSL3) sensitize ESCC cells to IR, as evidenced by reduced viability, diminished colony formation, and increased tumor growth inhibition in vivo. Importantly, we also observed that GPX4 downregulation or RSL3 treatment further augmented IR-induced accumulation of ROS and ferrous iron, indicating that enhanced ferroptosis underlies the radiosensitizing effect. These data are consistent with the emerging concept that ferroptosis is a critical cell death pathway induced by IR[7], and that cancer cells upregulate GPX4 as a protective mechanism. Notably, RSL3 does not affect GPX4 expression levels, only its enzymatic activity; therefore, the combination of RSL3 with IR may represent a promising strategy to circumvent GPX4-mediated radioresistance without requiring complete transcriptional suppression.

Second, our study uncovers an epitranscriptomic mechanism by which GPX4 is upregulated in response to IR. We showed that IR induces METTL3 expression, which

in turn installs m6A modifications on GPX4 mRNA at position A783, thereby enhancing GPX4 mRNA stability and translation. Knockdown of METTL3 reduced GPX4 expression, promoted ferroptosis, and sensitized ESCC cells to IR, while forced GPX4 expression rescued these effects. This positions METTL3 as an upstream regulator of GPX4 in the radioresistance context. The role of METTL3 in cancer therapy resistance has been documented in several studies. For example, METTL3 promotes chemoresistance in gastric cancer by stabilizing zinc finger MYM-type containing 1 (ZMYM1) [28], and in pancreatic cancer, METTL3 enhances the stability of ID2 transcripts[29]. However, the connection between METTL3 and ferroptosis has only recently emerged. Sun et al. reported that TRIM21-mediated METTL3 degradation promotes PDAC ferroptosis and enhances the efficacy of Anti-PD-1 immunotherapy by m6A-modifying SLC7A11[30]. Our findings extend this paradigm by showing that METTL3 directly targets GPX4 mRNA, linking m6A methylation to the core ferroptosis execution machinery. The fact that the METTL3 inhibitor DAA (or STM2457) phenocopied METTL3 knockdown and reversed the effect of GALNT2 overexpression further supports the therapeutic potential of targeting METTL3 in combination with radiotherapy.

Third, we identified a novel post-translational regulation of METTL3 via O-GalNAcylation. O-GalNAc glycosylation, also known as mucin-type O-glycosylation, is catalyzed by the GALNT family of enzymes[23]. Although O-GalNAcylation is well-known for its roles in cell adhesion, signaling, and cancer metastasis, its involvement in regulating epitranscriptomic enzymes has not been previously reported[23]. Our bioinformatics analysis revealed a positive correlation between GALNT2 and METTL3 expression in TCGA ESCC datasets, and we experimentally confirmed that GALNT2 physically interacts with METTL3. Through site-directed mutagenesis and VVL pull-down assays, we identified serine 64 (S64) of METTL3 as the primary O-GalNAcylation site. The S64A mutation abolished the interaction between METTL3 and GALNT2 and dramatically reduced METTL3 protein stability, as

shown by CHX chase assays. These results indicate that GALNT2-mediated glycosylation stabilizes METTL3, likely by masking a degron or by preventing ubiquitin-proteasomal degradation. This is reminiscent of recent findings that O-GlcNAcylation or O-GalNAcylation can protect proteins from ubiquitination; for example, O-GlcNAcylation of c-Myc at T58 prevents its phosphorylation and subsequent degradation (Kamemura et al., 2002, *Journal of Biological Chemistry*). To our knowledge, our study is the first to report O-GalNAcylation of an m6A writer, thereby bridging protein glycosylation and RNA methylation in cancer biology.

The functional significance of the GALNT2-METTL3 axis in radioresistance was validated both in vitro and in vivo. GALNT2 overexpression upregulated METTL3 and GPX4, suppressed IR-induced ROS and ferrous ion accumulation, and enhanced colony formation and xenograft tumor growth following irradiation. Importantly, METTL3 knockdown or treatment with the m6A inhibitor STM2457 completely reversed the protective effect of GALNT2 overexpression, confirming that METTL3 acts as the immediate downstream effector. These data suggest that GALNT2 promotes radioresistance primarily through stabilizing METTL3 rather than through other potential substrates. However, we cannot exclude the possibility that GALNT2 may also modify other proteins involved in the DNA damage response or ferroptosis; future proteome-wide studies may identify additional targets.

From a clinical perspective, our findings have several implications. First, the expression levels of GPX4, METTL3, and GALNT2 might serve as predictive biomarkers for radiotherapy response in ESCC patients. Given that GPX4 and METTL3 are upregulated by irradiation, measuring their baseline or radiation-induced expression could help identify patients who are likely to develop radioresistance. Second, our study provides a strong rationale for combining GPX4 inhibitors (such as RSL3) or METTL3 inhibitors (such as STM2457) with radiotherapy to treat ESCC. The in vivo experiments showed that either GPX4 knockdown (which simulates inhibition) or METTL3 interference significantly enhanced the anti-tumor effect of 4 Gy irradiation

without causing obvious toxicity, but formal toxicology studies are needed before clinical translation. Third, targeting GALNT2, which is upregulated in ESCC, might provide an additional therapeutic avenue. However, because GALNT family members have overlapping substrate specificities, selective inhibitors are currently lacking, and systemic knockout of Galnt2 in mice leads to metabolic abnormalities (Kase et al., 2007, *Endocrinology*). Therefore, more specific strategies such as antibody-drug conjugates or proteolysis-targeting chimeras (PROTACs) targeting GALNT2 may need to be developed.

Limitations of this study should be acknowledged. First, we used a single radiation dose (4 Gy) in most experiments. Fractionated radiotherapy (e.g., 2 Gy per fraction for multiple days) is the clinical standard, and the mechanisms of adaptation may differ. Future studies should examine whether the GALNT2-METTL3-GPX4 axis is similarly engaged under clinically relevant fractionated regimens. Second, we focused on two ESCC cell lines (ECA109 and TE-1); verification in additional lines and in patient-derived organoids or xenografts (PDX) would strengthen the generalizability. Third, although we demonstrated that METTL3 knockdown reverses the effect of GALNT2 overexpression, we did not directly test whether non-glycosylatable METTL3 (S64A) fails to rescue radioresistance in GALNT2-depleted cells. Such rescue experiments would provide definitive evidence that S64 glycosylation is necessary for GALNT2 function. Fourth, the exact E3 ubiquitin ligase responsible for degrading non-glycosylated METTL3 remains unknown. Identification of this ligase could open additional therapeutic opportunities. Fifth, the in vivo studies used nude mice with subcutaneous tumors; the microenvironment of orthotopic esophageal tumors might influence the response, and future studies should consider orthotopic models.

In conclusion, our work reveals a previously unrecognized regulatory circuit linking O-GalNAc glycosylation, m6A RNA methylation, and ferroptosis in the context of ESCC radioresistance. We demonstrate that irradiation induces GALNT2 and METTL3 expression; GALNT2 glycosylates METTL3 at S64, enhancing its stability; METTL3

then methylates GPX4 mRNA, stabilizing it and increasing GPX4 protein levels; elevated GPX4 suppresses ferroptosis and thereby confers radioresistance. Interruption of any node of this axis—by GPX4 inhibition, METTL3 inhibition, or blocking GALNT2-METTL3 interaction—sensitizes ESCC cells to radiation. These findings not only advance the fundamental understanding of how cancer cells evade ferroptosis under genotoxic stress but also provide actionable targets for improving radiotherapy efficacy in ESCC.

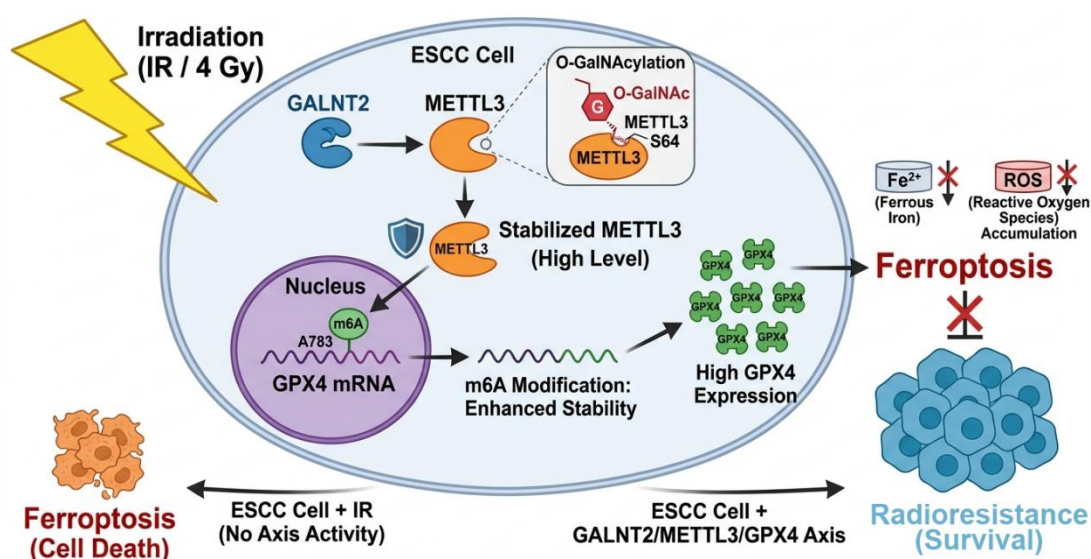


Fig. 8 A schematic model visually summarizes the GALNT2/METTL3/GPX4 signaling axis in esophageal squamous cell carcinoma, highlighting its role in promoting radioresistance. GALNT2 physically interacts with METTL3 and mediates its O-GalNAcylation at serine 64 (S64). This specific modification stabilizes the METTL3 protein by preventing its degradation. The stabilized METTL3 then installs m6A modifications on the GPX4 mRNA at position A783. This modification enhances the stability and expression of GPX4. Elevated levels of GPX4, a master antioxidant enzyme, effectively neutralize lipid peroxides and reduce the accumulation of reactive oxygen species (ROS) and ferrous ions (Fe^{2+}). By suppressing the execution of ferroptosis (an iron-dependent form of regulated cell death), this signaling axis allows ESCC cells to survive genotoxic stress, ultimately leading to radioresistance and tumor survival.

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Ethics approval and consent to participate

All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee/IRB (Institutional Animal Care and Use Committee of Shanghai Chengxi Biotechnology Co., Ltd.). The approval number is CX052511157. The welfare and handling of laboratory animals followed the National Institutes of Health guide for the care and use of Laboratory animals.

Author contributions

Chen Chen: Conceptualization, data curation, formal analysis, investigation, methodology, validation, writing – original draft preparation. Xiaoyan Shen: Methodology, Project Administration, Resources, Software. Jingting Jiang: Conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing – review & editing. Wendong Gu: Conceptualization, methodology, resources, supervision, writing – review & editing. Dan Xi, Yun Ding, Rui Sun:

Methodology, project administration, supervision. All authors have read and agreed to the published version of the manuscript.

Consent to publish

Not applicable.

Consent to participate

Not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Data Availability Statement

All data supporting the findings of this study are available within the article and its supplementary information files. Additional raw data and materials are available from the corresponding author upon reasonable request.

Publicly available databases and bioinformatics resources used in this study are explicitly listed below, with direct links to the specific analysis tools and records employed:

TCGA ESCC expression data: TCGA esophageal squamous cell carcinoma (ESCA) gene expression data were accessed and analyzed through the GEPIA2 web server, using the 'Expression DIY' and 'Correlation' modules (<http://gepia2.cancer-pku.cn/>).

m6A site prediction: Potential N6-methyladenosine (m6A) modification sites on GPX4 mRNA were predicted using the SRAMP tool (<http://www.cuilab.cn/sramp>) with the high-confidence setting.

O-GalNAcylation site prediction: Candidate O-GalNAcylation sites on METTL3 were predicted by NetOGlyc 4.0 (<https://services.healthtech.dtu.dk/service.php?NetOGlyc-4.0>) using the default score threshold (>0.8).

Protein structure retrieval: The predicted structures of GALNT2 (UniProt Q10471) and METTL3 (UniProt Q86U44) were downloaded from the AlphaFold Protein Structure Database

(<https://alphafold.ebi.ac.uk/entry/Q10471> and <https://alphafold.ebi.ac.uk/entry/Q86U44>, respectively).

Protein-protein interaction prediction: Docking and interaction prediction between GALNT2 and METTL3 were performed using the AlphaFold Server (<https://alphafold.ebi.ac.uk/>) in multimer prediction mode.

References

1. Siegel RL, Kratzer TB, Wagle NS, Sung H, Jemal A. Cancer statistics, 2026. *CA Cancer J Clin.* 2026;76(1):e70043. doi: 10.3322/caac.70043. PubMed PMID: 41528114; PubMed Central PMCID: PMC12798275.
2. Chen X, Zhao Y, Wang Y, Wang X, Liu Y, Liu Z, et al. Single-cell atlas of the esophageal squamous cell carcinoma immune ecosystem to predict immunotherapy response. *Signal Transduct Target Ther.* 2025;10(1):348. doi: 10.1038/s41392-025-02446-x. PubMed PMID: 41111072; PubMed Central PMCID: PMC12536040.
3. Deng Z, Pu L, Deng K, Liu W, Zhang J, Zhang L, et al. Targeting TFAP2beta condensation suppresses the development of esophageal squamous cell carcinoma. *Cell.* 2026;189(3):887–905 e25. doi: 10.1016/j.cell.2025.11.019. PubMed PMID: 41406964.
4. Jiao H, Lin S, Gu J, Jiang D, Cui P, Huang Z, et al. Perioperative nivolumab and chemotherapy in locally advanced squamous cell carcinoma of the oesophagus: a randomized multicentre phase 2 study with circulating tumor DNA dynamics monitoring. *Mol Cancer.* 2025;24(1):143. doi: 10.1186/s12943-025-02332-8. PubMed PMID: 40375301; PubMed Central PMCID: PMC12079821.
5. Liu X, Lv M, Feng B, Gong Y, Min Q, Wang Y, et al. SQLE amplification accelerates esophageal squamous cell carcinoma tumorigenesis and metastasis through oncometabolite 2,3-oxidosqualene repressing Hippo pathway. *Cancer Lett.* 2025;621:217528. doi: 10.1016/j.canlet.2025.217528. PubMed PMID: 39924077.
6. Song J, Song X, Xie Y, Guo H, Cun Y. MIF-expressing tumor cells mediate immunotherapeutic resistance in esophageal squamous cell carcinoma. *Theranostics.*

- 2026;16(3):1613–29. doi: 10.7150/thno.118269. PubMed PMID: 41355948; PubMed Central PMCID: PMC12679699.
7. Zheng Y, Liu F, Huang Y, Feng Y, Yang X, Wu J, et al. SNORA58 Facilitates Radioresistance via Suppressing JNK1-Mediated Ferroptosis in Esophageal Squamous Cell Carcinoma. *Adv Sci (Weinh)*. 2025;12(40):e08515. doi: 10.1002/advs.202508515. PubMed PMID: 40714828; PubMed Central PMCID: PMC12561220.
 8. Chen B, Chen J, Wang S, Bai K, Li Z, Chen B, et al. Serum cytokines predict response and survival in esophageal squamous cell carcinoma receiving chemoradiotherapy combined with anti-PD-1 antibody: analyses of two phase II clinical trials. *J Immunother Cancer*. 2026;14(1). doi: 10.1136/jitc-2025-013065. PubMed PMID: 41526165; PubMed Central PMCID: PMC12815036.
 9. Gao J, Wang Q, Fu F, Zhao Y, Yang T, Li X, et al. Comprehensive genomic and transcriptomic analyses reveal prognostic stratification for esophageal squamous cell carcinoma. *Signal Transduct Target Ther*. 2025;10(1):223. doi: 10.1038/s41392-025-02306-8. PubMed PMID: 40670349; PubMed Central PMCID: PMC12267757.
 10. Wu Y, Li H, Yue K, Jing C, Duan Y. Ferroptosis in cancer: metabolism, mechanisms and therapeutic prospects. *Mol Cancer*. 2025;24(1):303. doi: 10.1186/s12943-025-02520-6. PubMed PMID: 41327191; PubMed Central PMCID: PMC12667084.
 11. Toyokuni S, Kong Y, Maeda Y, Lyu Q, Ohara Y, Sato K, et al. Ferroptosis and cancer: when iron turns against tumors. *Cell Mol Life Sci*. 2025;83(1):16. doi: 10.1007/s00018-025-05982-5. PubMed PMID: 41288738; PubMed Central PMCID: PMC12748493.
 12. Xue X, Wang M, Cui J, Yang M, Ma L, Kang R, et al. Glutathione metabolism in ferroptosis and cancer therapy. *Cancer Lett*. 2025;621:217697. doi: 10.1016/j.canlet.2025.217697. PubMed PMID: 40189013.
 13. Yu D, Hu H, Zhang Q, Wang C, Xu M, Xu H, et al. Acevaltrate as a novel ferroptosis inducer with dual targets of PCBP1/2 and GPX4 in colorectal cancer. *Signal Transduct Target Ther*. 2025;10(1):211. doi: 10.1038/s41392-025-02296-7. PubMed PMID: 40619432; PubMed Central PMCID: PMC12230175.
 14. Wahida A, Conrad M. Decoding ferroptosis for cancer therapy. *Nat Rev Cancer*. 2025;25(12):910–24. doi: 10.1038/s41568-025-00864-1. PubMed PMID: 41073537.
 15. Zhao H, Ao L, Sorina, Wei Y, Yin HZ, Zhang N, et al. Ferroptosis and gastric cancer: from molecular mechanisms to clinical implications. *Front Immunol*. 2025;16:1581928. doi: 10.3389/fimmu.2025.1581928. PubMed PMID: 40861444; PubMed Central PMCID: PMC12375570.
 16. Ma T, Guo X, Zhao J, Wang C, Yu X, Mo R, et al. HPGD induces ferroptosis and autophagy to suppress esophageal squamous cell carcinoma through the LXA4-ERK1/2-U2AF2-TFRC axis. *Mol Cancer*. 2025;24(1):241. doi: 10.1186/s12943-025-02464-x. PubMed PMID: 41039452; PubMed Central PMCID: PMC12492633.
 17. Lin J, Lai Y, Lu F, Wang W. Targeting ACSLs to modulate ferroptosis and cancer immunity. *Trends Endocrinol Metab*. 2025;36(7):677–90. doi: 10.1016/j.tem.2024.09.003. PubMed PMID: 39424456.
 18. Zhang L, Wei J, Zou Z, He C. RNA modification systems as therapeutic targets. *Nat Rev Drug Discov*. 2026;25(1):59–78. doi: 10.1038/s41573-025-01280-8. PubMed PMID:

40962853.

19. Huang T, Chen S, Ding K, Yuan L, Lv W, Chen K, et al. METTL3/ALKBH5-Mediated N6-Methyladenosine Modification Drives Macrophage M1 Polarization via the SLC15A3-TASL-IRF5 Signaling Axis in Psoriasis. *Adv Sci (Weinh)*. 2025;12(36):e01408. doi: 10.1002/advs.202501408. PubMed PMID: 40679079; PubMed Central PMCID: PMC12462922.
20. Li S, Zhou J, Wang S, Yang Q, Nie S, Ji C, et al. N(6)-methyladenosine-regulated exosome biogenesis orchestrates an immunosuppressive pre-metastatic niche in gastric cancer peritoneal metastasis. *Cancer Commun (Lond)*. 2025;45(8):941 – 65. doi: 10.1002/cac2.70034. PubMed PMID: 40370322; PubMed Central PMCID: PMC12365546.
21. Xie Y, Li H, Pan J, Li Y, Zhang D. Inhibition of Mettl3 alleviates low-dose cisplatin-induced renal fibrosis and enhances the chemotherapeutic efficacy in mouse models of cancer. *Int J Biol Sci*. 2025;21(10):4293–311. doi: 10.7150/ijbs.117443. PubMed PMID: 40765834; PubMed Central PMCID: PMC12320029.
22. Wu T, Zhao Y, Zhang X, Wang Y, Chen Q, Zhang M, et al. Short-chain acyl post-translational modifications in cancers: Mechanisms, roles, and therapeutic implications. *Cancer Commun (Lond)*. 2025;45(10):1247–84. doi: 10.1002/cac2.70048. PubMed PMID: 40703012; PubMed Central PMCID: PMC12531430.
23. Iyer SC, Srinivasan DK, Parameswaran R. GalNAc-Transferases in Cancer. *Biomedicines*. 2025;14(1). doi: 10.3390/biomedicines14010005. PubMed PMID: 41595542; PubMed Central PMCID: PMC12838155.
24. Liu J, Wu S, Zhang G, Li J, Yin Y, Ullah A, et al. The Critical Role of GALNTs-Regulated O-GalNAc Glycosylation in Cancer Malignancy. *FASEB J*. 2026;40(8):e71790. doi: 10.1096/fj.202504815R. PubMed PMID: 42012463; PubMed Central PMCID: PMC13098630.
25. Wang C, Chen J, Ding X, Wang X, Liang X, Wen X, et al. Establishing and validating a new metabolic marker-driven prognosis signature for cutaneous melanoma. *Sci Rep*. 2025;15(1):43045. doi: 10.1038/s41598-025-26955-5. PubMed PMID: 41330957; PubMed Central PMCID: PMC12675589.
26. Wang S, Zhang C, Zhou S, Liu S, Li Q, Cheng X, et al. RNF217-KEAP1-NRF2 feedback loop confers therapeutic resistance by inhibiting ferroptosis in esophageal squamous cell carcinoma. *Drug Resist Updat*. 2025;83:101296. doi: 10.1016/j.drug.2025.101296. PubMed PMID: 40911942.
27. Lei G, Zhang Y, Koppula P, Liu X, Zhang J, Lin SH, et al. The role of ferroptosis in ionizing radiation-induced cell death and tumor suppression. *Cell Res*. 2020;30(2):146–62. doi: 10.1038/s41422-019-0263-3. PubMed PMID: 31949285; PubMed Central PMCID: PMC7015061 Beyond Spring Pharmaceuticals, New River Labs; honorarium from Varian Medical Systems; and serves in Advisory Board for AstraZeneca and Beyond Spring Pharmaceuticals. Other authors declare no competing interests.
28. Yue B, Song C, Yang L, Cui R, Cheng X, Zhang Z, et al. METTL3-mediated N6-methyladenosine modification is critical for epithelial-mesenchymal transition and metastasis of gastric cancer. *Mol Cancer*. 2019;18(1):142. doi: 10.1186/s12943-019-1065-4. PubMed PMID: 31607270; PubMed Central PMCID: PMC6790244.

29. Chen J, Zhang H, Xiu C, Gao C, Wu S, Bai J, et al. METTL3 promotes pancreatic cancer proliferation and stemness by increasing stability of ID2 mRNA in a m6A-dependent manner. *Cancer Lett.* 2023;565:216222. doi: 10.1016/j.canlet.2023.216222. PubMed PMID: 37196908.
30. Mao W, Jiang Q, Feng Y, Peng C, Peng H, Li X, et al. TRIM21-mediated METTL3 degradation promotes PDAC ferroptosis and enhances the efficacy of Anti-PD-1 immunotherapy. *Cell Death Dis.* 2025;16(1):240. doi: 10.1038/s41419-025-07550-y. PubMed PMID: 40175350; PubMed Central PMCID: PMC11965403 and consent to participate: Ethical approval of this study was obtained from the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (2024-KY-0327-002). The animal experiments were approved by the Animal Protection Committee of Zhengzhou University (No. ZZU-LAC20230331 [04]). Pancreatic tissues were procured from Shanghai Outdo Biotech Co., Ltd. and this experiment was ethically approved by the Ethics Committee in accordance with the principles of the Helsinki Declaration. All participating patients provided written informed consent (No. YB M-05-02).

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