

piR-164552 promotes malignant progression in head and neck squamous cell carcinoma via the RBM4/EIF4E2 signaling axis: Insights from multi-omics and functional analyses

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Received February 20, 2026; Accepted June 19, 2026

DOI: 10.3892/ijo.2026.5924

Abstract. Head and neck squamous cell carcinoma (HNSCC) is a highly aggressive malignancy with limited options for early diagnosis and poor clinical outcomes. In the present study, PIWI-interacting RNA (piR)-164552 was identified as a novel oncogenic regulator in HNSCC. piR-164552 was found to be markedly upregulated in tumor tissues and serum exosomes and its expression promoted the proliferation, migration, invasion and tumorigenicity of HNSCC cells both *in vitro* and *in vivo*. Mechanistic analyses revealed that

piR-164552 interacted with RNA-binding motif protein 4 (RBM4) and positively regulated RBM4 protein levels, which in turn enhanced the expression of eukaryotic initiation factor 4E-like 2 (EIF4E2), forming a piR-164552/RBM4/EIF4E2 axis. Integrated transcriptomic and translational profiling further demonstrated that this axis orchestrated extensive reprogramming of mRNA metabolism, ribosome biogenesis and cancer-associated signaling pathways, underscoring its multilayered role in tumor progression. The present findings highlighted the diagnostic potential of piR-164552 and uncovered its key contribution to the molecular network driving HNSCC, providing new insights into biomarker development and therapeutic strategies.

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; piRNA, PIWI-interacting RNA; RBP, RNA-binding protein; RBM4, RNA-binding motif protein 4; EIF4E2, eukaryotic initiation factor 4E-like 2; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; FISH, fluorescence *in situ* hybridization; IF, immunofluorescence; RIP, RNA immunoprecipitation; co-IP, co-immunoprecipitation; AUC, area under the curve; TCGA, The Cancer Genome Atlas; GEPIA2, Gene Expression Profiling Interactive Analysis 2; CPTAC, Clinical Proteomic Tumor Analysis Consortium; HPA, Human Protein Atlas; P-bodies, processing bodies; GSEA, Gene Set Enrichment Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; TE, translational efficiency

Key words: PIWI-interacting RNAs, head and neck squamous cell carcinoma, RNA-binding motif protein 4, eukaryotic initiation factor 4E-like 2, transcription, translation

Introduction

Head and neck squamous cell carcinoma (HNSCC), arising primarily from the mucosal epithelium of the oral cavity, pharynx and larynx, is the predominant histological subtype of head and neck cancer, which is ranked as the sixth most common cancer type worldwide, with ~946,000 new cases and 482,000 deaths according to the latest Global Cancer Observatory 2022 estimates (1,2). Clinically, HNSCC is characterized by a poor 5-year survival rate of ~35% due to its anatomically concealed tumor sites, challenges in early detection, high propensity for lymph node metastasis and frequent postoperative recurrence (3). In the absence of effective early screening methods, diagnosis still primarily relies on comprehensive physical examination, leading to ~60% of patients being diagnosed at advanced stages, when curative surgery is no longer feasible (4). Furthermore, the molecular mechanisms underlying HNSCC pathogenesis remain insufficiently understood, limiting therapeutic progress (5). Therefore, defining the genetic and epigenetic regulatory networks that drive HNSCC initiation and progression will not only further the current understanding of its molecular pathology, but will also provide a basis for the development of novel biomarkers and targeted treatment strategies.

PIWI-interacting RNAs (piRNAs) are a distinct class of small non-coding RNAs (ncRNAs) of 26-31 nucleotides in length, which were first identified in male germ cells in 2001 (6). piRNAs are characterized by 2'-O-methylation at the 3' end, a strong uridine bias at the 5' end and the enrichment of adenine at position 10 (7). Despite their nucleotide sequences being poorly conserved across species, piRNA genomic clusters display high evolutionary conservation. piRNAs exert their functions by forming complexes with PIWI proteins, serving key roles in both epigenetic and post-transcriptional regulation (8). Initially recognized for maintaining genomic stability in germ cells through transposon silencing, piRNAs have now been implicated in a wide range of physiological and pathological processes in somatic tissues, including tissue regeneration, metabolic disorders, neurological diseases and several cancer types, such as breast, colorectal and gastric cancer (9). Dysregulation of piRNA expression, including aberrant upregulation or downregulation, has been associated with tumor initiation, progression and prognosis (10). Furthermore, their intrinsic stability underscores the potential of piRNAs as biomarkers for the diagnosis, prognosis and therapeutic monitoring of immune-associated diseases, thus highlighting their clinical relevance in oncology (11-13).

RNA-binding proteins (RBPs) represent another key layer of post-transcriptional regulation (14). These highly conserved proteins are involved in nearly all aspects of RNA metabolism, including alternative splicing, polyadenylation, modification, stability and translation, as well as the processing of microRNAs (miRNAs) and circular RNAs (15). Accumulating evidence has indicated that RBPs are associated with cancer-related phenotypes such as proliferation, apoptosis, senescence, migration, invasion and angiogenesis (16,17). Aberrant expression or dysfunction of RBPs disrupts gene expression homeostasis and contributes to the development of human diseases, including cancer (18). Notably, recent research has demonstrated that piRNAs can interact with RBPs to form RNA-protein complexes that coordinate the regulation of downstream targets. For example, piR-1742 specifically binds to heterogeneous nuclear ribonucleoprotein U, facilitating mucin 12 deubiquitylation and thereby enhancing the invasive and metastatic potential of renal cell carcinoma (19). These findings suggest that piRNA-RBP interactions may constitute a novel regulatory axis in tumorigenesis. However, their molecular mechanisms and functional relevance in HNSCC remain poorly understood.

The present study, investigated the expression pattern, clinical relevance and biological function of piR-164552 in HNSCC. In addition, the molecular mechanism by which piR-164552 may contribute to HNSCC progression was explored, with a particular focus on its interaction with RNA-binding motif protein 4 (RBM4), regulation of eukaryotic initiation factor 4E-like 2 (EIF4E2), and its potential impact on transcriptional and translational regulatory programs.

Materials and methods

Patients and sample collection. A total of two independent patient cohorts were included in the present study. A tissue cohort consisting of 20 patients with HNSCC (17 men and

3 women; aged 51-72 years) who underwent primary surgical resection at the Department of Otorhinolaryngology Head and Neck Surgery, The Affiliated Hospital of Qingdao University (Qingdao, China) between March 2023 and April 2024 was used for RNA sequencing and reverse transcription-quantitative PCR (RT-qPCR validation). Tumor tissues and matched adjacent normal tissues (located ≥ 3 cm from the tumor margin) were collected during surgery and immediately snap-frozen in liquid nitrogen (-196°C).

In addition, a serum exosome cohort was established for exosomal piR-164552 expression, diagnostic evaluation and clinicopathological correlation analyses. Serum samples were obtained from 90 patients with HNSCC and 50 healthy volunteers at the aforementioned institution. A subset of samples was used for exosome characterization experiments, including transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and exosomal marker detection by western blotting. Ultimately, serum samples from 68 patients with HNSCC who had complete clinicopathological information and 38 healthy volunteers were included in the subsequent analyses.

For both the tissue cohort and the serum exosome cohort, all patients were newly diagnosed and treatment-naïve. The inclusion criteria were newly diagnosed HNSCC without prior antitumor treatment, and the exclusion criteria were receipt of chemotherapy, radiotherapy or targeted therapy prior to sample collection or surgery. Clinical data, including tumor site, tumor stage, lymph node status, clinical stage, smoking history and alcohol consumption, were collected from medical records. Human papillomavirus status was not available for all patients and was therefore not included in the present analysis. Clinical stage was determined according to the TNM staging system. The clinicopathological characteristics of the serum exosome cohort are summarized in Table I. Healthy controls were determined to be individuals undergoing routine health examinations with no history of malignancy. No significant differences in age or sex were observed between the healthy control group and the patient group.

The present study was approved by the Ethics Board of The Affiliated Hospital of Qingdao University (Qingdao, China; approval no. QYFY WZLL 29843) and written informed consent was obtained from all participants.

Cell culture. Human HNSCC cell lines CAL-27 and FaDu were supplied by the American Type Culture Collection. CAL-27 cells are derived from human tongue squamous cell carcinoma, whereas FaDu cells are derived from human hypopharyngeal squamous cell carcinoma. Normal human oral epithelial cells (HOK), used as non-malignant control cells, were purchased from Wuhan Procell Life Science & Technology Co., Ltd. CAL-27 and FaDu cells were cultured in DMEM (Dalian Meilun Biology Technology Co., Ltd.) supplemented with 10% FBS (Dalian Meilun Biology Technology Co., Ltd.) and 1% penicillin-streptomycin (Dalian Meilun Biology Technology Co., Ltd.). HOK cells were cultured in a commercially available HOK-specific culture medium purchased from Wuhan Procell Life Science & Technology Co., Ltd., according to the manufacturer's instructions. All cells were maintained at 37°C in a humidified incubator with 5% CO_2 .

Table I. Clinicopathological characteristics of patients with head and neck squamous cell carcinoma in the serum exosome cohort.

Variable	Total, n %
Sex	
Male	61 (89.7)
Female	7 (10.3)
Age, years	
≤50	5 (7.4)
>50 but ≤60	23 (33.8)
>60 but ≤70	28 (41.2)
>70	12 (17.6)
Tumor site	
Oropharynx	8 (11.8)
Hypopharynx	27 (39.7)
Larynx	33 (48.5)
Tumor stage	
T1-T2	40 (58.8)
T3-T4	28 (41.2)
Lymph node status	
N0	43 (63.2)
N+	25 (36.8)
Clinical stage	
I-II	37 (54.4)
III-IV	31 (45.6)
Histological differentiation	
Well/moderate	48 (70.6)
Poor	20 (29.4)
Smoking history	
Yes	52 (76.5)
No	16 (23.5)
Alcohol consumption	
Yes	55 (80.9)
No	13 (19.1)

Transient transfection. CAL-27 and FaDu cells were seeded into six-well plates and transfected when the cell confluence reached 50-60%. Transfection was performed using the riboFECT CP Transfection Kit (Guangzhou RiboBio Co., Ltd.) at 37°C in a humidified incubator with 5% CO₂. Chemically modified piR-164552 agomir and antagomir oligonucleotides (both from Shanghai GenePharma Co., Ltd.) were used to modulate piR-164552 levels. The agomir served as a synthetic mimic to increase intracellular piR-164552 levels, whereas the antagomir was an antisense oligonucleotide designed to inhibit endogenous piR-164552 activity. The following oligonucleotides were used: Agomir-negative control (NC) and antagomir-NC, both from Shanghai GenePharma Co., Ltd., agomir-piR-164552 and antagomir-piR-164552 at final concentrations of 50 nM for the agomirs and 100 nM for the antagomirs. Cells were harvested 24 h after transfection for expression validation and 48 h after transfection for subsequent

functional experiments. The sequences of the oligonucleotides are listed in Table SI.

Lentivirus production and construction of stable cells. Recombinant lentiviral vectors expressing piR-164552 or EIF4E2 were generated by cloning the precursor sequences of piR-164552 or EIF4E2 into the LV3-pGLVH1-Puro-GFP or LV5-pGLVEF1a-Puro-GFP lentiviral vector. Short hairpin RNAs targeting piR-164552, EIF4E2 and RBM4 were inserted into the LV3-pGLVH1-Puro-GFP lentiviral vector. The shRNA target sequences were as follows: shpiR-164552, 5'-TGGCGA ATGTGAGAACCACTACACTACGGAAAC-3'; shEIF4E2-1, 5'-CGCTTTCAGGAAGACATTATT-3'; shEIF4E2-2, 5'-CAC AGAGCTATGAACAGAATA-3'; shRBM4-1, 5'-CACCTG CACCAATAAGGAGCT-3'; and shRBM4-2, 5'-GCACCA ATAAGGAGCTTCGAG-3'. The corresponding lentiviral constructs and packaged lentiviral particles were prepared by Shanghai GenePharma Co., Ltd. An empty vector served as the control for all experiments. The lentiviral titer was 1x10⁸ TU/ml. FaDu cells were infected with lentiviruses at a multiplicity of infection (MOI) of 10, whereas CAL-27 cells were infected at an MOI of 15. CAL-27 and FaDu cells were infected with the lentiviruses for 48 h at 37°C in a humidified incubator with 5% CO₂. After infection, the cells were selected with 2 µg/ml puromycin (Dalian Meilun Biology Technology Co., Ltd.) for 72 h to establish stable cell lines, which were subsequently maintained in medium containing 1 µg/ml puromycin. Transduction efficiency was verified by RT-qPCR after puromycin selection, and stable cells were used for subsequent experiments after confirmation of successful transduction.

RNA extraction and quantification. Total RNA was extracted using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For RT, 1 µg total RNA was used. piRNA was reverse transcribed using the miRNA cDNA First Strand Synthesis Kit (stem-loop method), whereas mRNA was reverse transcribed using the *Evo M-MLV* Reverse Transcription Reagent Master Mix (Hunan Accurate Bio-Medical Technology Co., Ltd.). The RT conditions were 25°C for 5 min, 42°C for 15 min and 85°C for 5 sec for piRNA, and 37°C for 15 min followed by 85°C for 5 sec for mRNA. RT-qPCR was performed using a SYBR Green *Pro Taq* HS Premixed qPCR Kit (Hunan Accurate Bio-Medical Technology Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 55°C for 30 sec. U6 was used as the internal reference for piRNA quantification, whereas GAPDH was used as the internal reference for mRNA quantification. Relative RNA expression levels were calculated using the 2^{-ΔΔC_q} method (20). Table SI displays the sequences of the PCR primers used.

Serum exosome isolation. Serum samples were subjected to differential centrifugation at 4°C to remove cell debris and contaminating proteins, including centrifugation at 3,000 x g for 10 min followed by 12,000 x g for 10 min. Exosomes were then enriched using the Exosome Extraction and Purification Kit for Serum/Plasma (umibio®; Shanghai Yumei Biogi Biotechnology Co., Ltd.). Briefly, the supernatants were diluted with pre-cooled PBS, mixed with Blood PureExo Solution,

vortexed for 1 min and incubated at 4°C for ≥30 min. The mixtures were then centrifuged at 12,000 x g for 15 min at 4°C to precipitate exosomes, followed by an additional centrifugation at 12,000 x g for 2 min at 4°C to remove residual liquid. The pellets were resuspended in PBS and centrifuged again at 12,000 x g for 2 min at 4°C, and the resulting supernatants were further purified using an exosome purification filter at 3,000 x g for 10 min at 4°C to ensure high purity. Purified serum exosomes were stored at -80°C until further analysis.

Transmission electron microscopy (TEM) analysis of exosomes. Exosome suspensions were fixed with 4% paraformaldehyde at 4°C for 30 min, applied onto carbon-coated copper grids, negatively stained with 1% uranyl acetate at room temperature for 1 min, air-dried at room temperature and then observed under a transmission electron microscope to visualize their characteristic cup-shaped or spherical morphology.

Nanoparticle tracking analysis (NTA). Size distribution and concentration of exosomes were measured using a ZetaView nanoparticle tracking analyzer (software version 8.06.01 SP1; Particle Metrix GmbH). Exosome suspensions were diluted 1:200 in 1X PBS, yielding a measured particle concentration of 1.5×10^8 particles/ml. Measurements were performed using a 488 nm laser under identical instrument settings for all samples, with ZetaView software settings of sensitivity 80 and shutter 150, a frame rate of 30 fps, 1 cycle and 11 positions. Data were analyzed using constant analysis parameters, including a minimum brightness of 30 arbitrary units and a trace length of 15. Standard beads were used for system calibration before sample analysis.

Western blotting. Protein lysates from CAL-27 cells, FaDu cells and serum-derived exosomes were prepared using RIPA buffer (Dalian Meilun Biology Technology Co., Ltd.) supplemented with protease and phosphatase inhibitors. The lysates were centrifuged at 12,000 x g for 15 min at 4°C and the supernatants were collected as total protein. Protein concentrations were quantified using a BCA Protein Assay Kit (Dalian Meilun Biology Technology Co., Ltd.). Equal quantities of protein (20 µg/lane) were mixed with SDS loading buffer, denatured at 95°C for 5 min and separated by SDS-PAGE (Vazyme Biotech Co., Ltd.) on 10% gels. The proteins were then transferred onto methanol-activated 0.45-µm PVDF membranes (Roche Applied Science), which were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were then incubated with the corresponding primary antibodies against CD9 (1:1,000; cat. no. ab263019; Abcam), CD63 (1:1,000; cat. no. ab134045; Abcam), CD81 (1:1,000; cat. no. ab219209; Abcam), Tsg101 (1:1,000; cat. no. ab125011; Abcam), calnexin (1:5,000; cat. no. ab133615; Abcam), RBM4 (1:1,000; cat. no. 11614-1-AP; Proteintech Group, Inc.; and 1:500; cat. no. sc-373852; Santa Cruz Biotechnology, Inc.), EIF4E2 (1:1,000; cat. no. 12227-1-AP; Proteintech Group, Inc.; and 1:500; cat. no. sc-100731; Santa Cruz Biotechnology, Inc.), microtubule associated protein 1B (MAP1B) (1:1,000; cat. no. ab307286; Abcam). For β-actin detection, membranes were incubated with HRP-conjugated anti-β-actin (1:10,000; cat. no. ab20272; Abcam) overnight at 4°C on a shaker,

without the use of a secondary antibody. After washing with TBS-Tween 20, HRP-labeled goat anti-mouse IgG secondary antibody (1:10,000; cat. no. ab6789; Abcam) or goat anti-rabbit IgG secondary antibodies (1:10,000; cat. no. ab6721; Abcam) was added and incubated for 1 h at room temperature. Following additional washes, protein signals were detected using an ECL substrate (Vazyme Biotech Co., Ltd.) and visualized with an Amersham ImageQuant 800 chemiluminescence imaging system (Cytiva).

RNA fluorescence in situ hybridization (FISH). CAL-27 and FaDu cells were seeded in 24-well plates at a density of 1×10^4 cells per well and incubated overnight. After fixation and permeabilization, cells were blocked with 1X blocking solution provided in the RNA FISH Kit (cat. no. F40261; Shanghai GenePharma Co., Ltd.) at 37°C for 30 min. The piR-164552 probe (Shanghai GenePharma Co., Ltd.) was used at a working concentration of 1 µM, and hybridization was performed at 37°C in the dark overnight (12-16 h). After hybridization, cells were washed with kit-provided 0.1% Buffer F at 37°C, followed by washes with kit-provided 2X Buffer C at 60°C and 37°C, according to the manufacturer's protocol. Nuclei were counterstained with DAPI at room temperature for 20 min under light-protected conditions. Fluorescence signals were acquired and imaged using a laser confocal microscope.

Nuclear and cytoplasmic fractionation. Nuclear and cytoplasmic fractions were isolated using the PARIS™ kit (cat. no. AM1921; Thermo Fisher Scientific, Inc.), which provides reagents for both nuclear/cytoplasmic fractionation and RNA isolation, according to the manufacturer's instructions. Briefly, CAL-27 and FaDu cells in logarithmic growth phase were harvested, washed with cold PBS and resuspended in ice-cold Cell Fractionation Buffer, followed by incubation on ice for 5-10 min, and centrifugation at 4°C and 500 x g to separate cytoplasmic and nuclear fractions. RNA from each fraction was then isolated using the RNA isolation reagents provided in the PARIS™ Kit and treated with RNase-free DNase I to remove genomic DNA contamination.

Cell Counting Kit-8 (CCK-8) assay. CAL-27 and FaDu cells were seeded in 96-well plates at a density of 3×10^3 cells per well with five replicates per group and blank wells for background correction. At each time point (0, 24, 48 and 72 h), 1:10 (v/v) CCK-8 reagent (Dalian Meilun Biology Technology Co., Ltd.) was added and incubated at 37°C for 2 h. The absorbance at 450 nm was then measured using a microplate reader (SpectraMax i3x; Molecular Devices, LLC) and background-subtracted values were used to plot cell proliferation curves.

EdU assay. CAL-27 and FaDu cells were seeded in 24-well plates at a density of 1×10^5 cells per well. After 48 h, 10 µM EdU (Dalian Meilun Biology Technology Co., Ltd.) was added and incubated for 3 h at 37°C. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min, washed and permeabilized according to the manufacturer's instructions, and subjected to Click reaction staining at room temperature for 30 min in the dark. Nuclei were counterstained with 1X Hoechst 33342 at room temperature for 10 min under

light-protected conditions. Images were captured using an Olympus DP74 fluorescence microscope imaging system (Olympus Corporation).

Colony formation assay. A total of 1,000 cells in 2 ml complete medium were seeded per well in 6-well plates and cultured for 10-14 days, with the medium changed every 3-4 days. Colonies containing ≥ 50 cells were counted as positive colonies. Once colonies had formed, cells were fixed with 4% formaldehyde solution at room temperature for 30 min. After washing three times with PBS, cells were stained with crystal violet solution at room temperature for 15 min. After gently rinsing off excess liquid with running water, the plates were placed upside down on absorbent paper to air dry. Representative images were recorded, and colony numbers were quantified using ImageJ software (version 1.53k; National Institutes of Health).

Wound healing assay. CAL-27 and FaDu cells were seeded in 6-well plates and transfected with piR-164552 agomir or antagomir at 40-60% confluence. After 24 h, a scratch was made using a 200- μ l pipette tip. Floating cells were removed by washing three times with PBS and low-serum medium containing 1% FBS was added. Images of the wound area were captured at 0 and 24 h using an Olympus DP74 fluorescence microscope imaging system (Olympus Corporation). Wound closure was quantified using ImageJ software (version 1.53k), and the percentage of wound closure was calculated.

Transwell cell migration and invasion assays. After transfection, CAL-27 and FaDu cells were harvested and counted. A total of 2×10^5 cells in 200 μ l serum-free medium were added to the upper chambers of Transwell inserts (Corning, Inc.). For invasion assays, the upper chambers were pre-coated with Matrigel at 37°C for 2 h, whereas uncoated chambers were used for migration assays. The lower chambers were filled with 600 μ l complete medium containing 20% FBS as a chemoattractant. After incubation at 37°C for 36 h, non-migrated or non-invaded cells were removed. Cells on the lower surface were fixed with pre-cooled methanol at -20°C for 15 min, washed and stained with 0.5% crystal violet at room temperature for 20 min and images were captured using an Olympus DP74 fluorescence microscope imaging system (Olympus Corporation).

Xenograft tumor model. A total of 15 male BALB/c nude mice (4 weeks old; 16-18 g) were purchased from Beijing Huafukang Biotechnology Co., Ltd. Male mice were selected based on the higher clinical prevalence of HNSCC in males (21) and to maintain consistency within the animal cohort in the present initial xenograft validation experiment. As sex was not included as an independent biological variable for comparison, the present *in vivo* findings should be interpreted within the context of male BALB/c nude mice and potential sex-specific effects require further investigation in future studies including both male and female animals. All animals were housed under specific pathogen-free conditions at Qingdao University (Qingdao, China), with controlled temperature (22 $\pm$ 2°C), humidity (50-60%) and a 12-h light/dark cycle and were provided with sterilized food and water *ad libitum*. All animal procedures were approved by the Institutional Animal

Care and Use Committee of Qingdao University (approval no. 20241017BALB/c-nude20241219007) and performed in accordance with institutional guidelines and the Animal Research: Reporting of *In Vivo* Experiments guidelines (22).

For the xenograft tumor model, mice were randomly assigned to three groups (n=5 per group): i) Control; ii) piR-164552 overexpression; and iii) piR-164552 knock-down. A total of 2×10^6 FaDu cells suspended in 100 μ l PBS were subcutaneously injected into the left axilla of each mouse. Tumor size was measured every 3 days using calipers and tumor volume was calculated as the volume=(length x width²)/2.

Humane endpoints were predefined in accordance with institutional and internationally accepted guidelines (22,23). Mice were euthanized when tumor volume reached 1,200-1,500 mm³ or when the maximum tumor diameter exceeded 12-15 mm, whichever occurred first. Animals were also euthanized earlier if tumor ulceration or signs of distress were observed.

On day 28 after injection, mice were anesthetized with isoflurane (3-4% for induction and 1.5-2% for maintenance) and placed in supine position for imaging using the Living Image™ software (version 4.5.1.18294; Revvity, Inc.) with an excitation wavelength of 488 nm. GFP fluorescence was used as a surrogate indicator of tumor burden. Following imaging, mice were euthanized by cervical dislocation and mortality was determined by the absence of respiration, heartbeat and reflexes. No animals were found prematurely deceased during the experiment and no unexpected mortalities occurred. Therefore, the cause of mortality for all animals was protocol-specified euthanasia at the planned experimental endpoint. Tumors were subsequently excised, photographed, weighed and fixed in 4% paraformaldehyde at 4°C for 24 h for further analysis.

Immunofluorescence (IF). Tumor tissues were cryosectioned and fixed with 4% paraformaldehyde for 20 min at room temperature, followed by three washes with PBS (5 min each). Sections were then blocked with 5% BSA blocking solution (cat. no. AR0004; Boster Biological Technology) for 1 h at room temperature or overnight at 4°C, and then incubated in a humidified chamber at 4°C overnight in the dark with primary antibodies against RBM4 (1:50; cat. no. sc-373852; Santa Cruz Biotechnology, Inc.) and EIF4E2 (1:50; cat. no. GTX82524; GeneTex, Inc.). After washing with PBS to remove unbound antibodies, sections were incubated for 2 h at room temperature in the dark with FITC-conjugated goat anti-mouse IgG secondary antibody (1:100; cat. no. BA1101) and Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:100; cat. no. BA1032) (both from Boster Biological Technology). Nuclei were counterstained with DAPI at room temperature for 5 min. Multichannel fluorescence images were captured using a fluorescence microscope to assess protein expression and localization.

Immunohistochemistry. Paraffin-embedded tissue sections (4 μ m) were deparaffinized in xylene and rehydrated through a graded ethanol series (100-70%). Sections were equilibrated in PBS and subjected to microwave antigen retrieval at 95-100°C for 10 min. Endogenous peroxidase activity was blocked

with 3% hydrogen peroxide at room temperature for 10 min, followed by incubation with 5% BSA for 30 min at room temperature. Sections were then incubated overnight at 4°C in a humidified chamber with primary antibodies against Ki67 (1:200; cat. no. PB9026), α -SMA (1:1,000; cat. no. BM0002) and Vimentin (1:1,000; cat. no. BM0135) (all from Boster Biological Technology), followed by incubation with the corresponding HRP-labeled goat anti-mouse IgG secondary antibody (1:1,000; cat. no. ab6789) or HRP-labeled goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. ab6721) (both from Abcam) for 1 h at 37°C. Each incubation step was followed by three washes with PBS (5 min each). Protein signals were developed with a DAB chromogen kit (cat. no. AR1027; Boster Biological Technology) for 5–10 min and nuclei were counterstained with hematoxylin at room temperature for 1–3 min. Sections were dehydrated through graded ethanol, cleared in xylene, mounted with neutral resin and imaged under a bright-field microscope.

RNA-pulldown assay. An *in vivo* F2-RNA pull-down assay was performed using the F2-RNA Pull-Down Kit (cat. no. FI8701; Guangzhou FitGene Biotechnology Co., Ltd.) according to the manufacturer's instructions. piR-164552 containing an F2 tag sequence (5'-GGCGCUGACAAAGCGCC-3') were synthesized by Shanghai GenePharma Co., Ltd., and the corresponding sequence is listed in Table SI. The F2-piR-164552 construct was transfected into cells as described in the aforementioned transfection methods, with cells transfected with the corresponding empty vector serving as the control. After collection, cells were washed 2–3 times with pre-cooled RNase-free PBS. After each wash, the cells were centrifuged at 4°C and 500 x g for 5 min each time. Cell pellets were then lysed on ice in pre-cooled lysis buffer supplemented with protease inhibitor and RNase inhibitor, followed by centrifugation at 4°C and 12,000 x g for 15 min to collect the supernatant. Next, F2-ligand magnetic beads were pretreated according to the kit instructions and incubated with the experimental or control lysates at 4°C for 2–4 h to capture RNA-protein complexes. After washing three times with the kit-provided wash buffer, the bound complexes were eluted with the kit-provided elution buffer, stored at -80°C and subsequently analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and western blotting.

LC-MS/MS analysis. Eluted proteins from the RNA pull-down assay were subjected to shotgun protein identification by LC-MS/MS at Shanghai Bioprofile Technology Co., Ltd. Briefly, proteins were reduced, alkylated and digested with trypsin. The resulting peptides were desalted using C18 StageTips, reconstituted in 0.1% formic acid, separated using an Easy-nLC 1200 system coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Inc.) and analyzed using an untargeted data-dependent acquisition workflow. Peptides were separated using a C18 trap column and a C18 analytical column, and the MS analysis was performed in positive ion mode for 60 min. The MS/MS data were searched against the UniProt *Homo sapiens* reference proteome (<https://www.uniprot.org/proteomes/UP000005640>) using MaxQuant 2.4.14.0 (Max Planck Institute of Biochemistry; <https://www.maxquant.org/>), with the false discovery rate

set to 1% at both the peptide-spectrum match and protein levels.

RNA immunoprecipitation (RIP). RIP was performed using the PureBinding® RNA Immunoprecipitation Kit (cat. no. P0102; Guangzhou Genesee Biotech Co., Ltd.) according to the manufacturer's native RIP protocol. Briefly, cells were collected and lysed in Buffer A supplemented with protease inhibitor and RNase inhibitor. After centrifugation at 4°C and 10,000 x g for 10 min, the supernatant was collected. For each IP reaction, 400 μ l lysate supernatant was incubated with 5 μ g RBM4 antibody (cat. no. 11614-1-AP; Proteintech Group, Inc.) or 5 μ g rabbit IgG monoclonal isotype control (cat. no. ab172730; Abcam) and 100 μ l protein A/G magnetic beads at 4°C. After washing with Buffer B or Buffer C to remove non-specific interactions, RNA was purified according to the kit protocol, reverse transcribed into cDNA and analyzed by RT-qPCR. The RT-qPCR conditions were the same as those aforementioned.

Co-immunoprecipitation (co-IP). Co-IP was performed using the Co-IP Kit, Protein A/G Magnetic Beads (cat. no. FI8802; Guangzhou FitGene Biotechnology Co., Ltd.) according to the manufacturer's instructions. CAL-27 and FaDu cells were collected, washed 2–3 times with pre-cooled PBS. After each wash, the cells were centrifuged at 4°C and 500 x g for 5 min. Cell pellets were lysed on ice in the kit-provided lysis buffer supplemented with protease inhibitor, followed by centrifugation at 4°C and 12,000 x g for 15 min to remove cellular debris. A total of 400 μ l lysate supernatant was used for each IP reaction. For endogenous IP, EIF4E2 antibody (cat. no. sc-100731; Santa Cruz Biotechnology, Inc.) or normal mouse IgG control (cat. no. sc-2025; Santa Cruz Biotechnology, Inc.), and RBM4 antibody (cat. no. 11614-1-AP; Proteintech Group, Inc.) or rabbit IgG monoclonal isotype control (cat. no. ab172730; Abcam), were added to the respective lysates and incubated at 4°C overnight. Subsequently, 25 μ l protein A/G magnetic beads were added to each reaction and incubated at 4°C for 2 h. After washing three times with the kit-provided wash buffer, bound proteins were eluted with 1X SDS-PAGE loading buffer by heating at 95°C for 5 min. The eluted proteins were analyzed by western blotting to detect interacting proteins. The western blotting conditions were the same as those aforementioned.

Secondary structure prediction and molecular docking. For piR-164552-RBM4 docking analysis, the full-length RBM4 structure [RBM4_HUMAN, UniProt ID: Q9BWF3; <https://www.uniprot.org/uniprotkb/Q9BWF3/entry>] was retrieved from UniProt, and the three-dimensional model of piR-164552 was generated using AlphaFold Server, powered by AlphaFold3 (<https://alphafoldserver.com/>) (24). For RBM4-EIF4E2 docking analysis, the full-length RBM4 structure [RBM4_HUMAN, UniProt ID: Q9BWF3] and the EIF4E2 crystal structure [EIF4E2_HUMAN, UniProt ID: O60573; <https://www.uniprot.org/uniprotkb/O60573/entry>; Protein Data Bank ID: 2JGB; <https://www.rcsb.org/structure/2JGB>] were used. Molecular docking was performed using the HDOCK server (25), a hybrid docking platform for protein-protein and protein-DNA/RNA interactions, with default parameters. The

docking score and confidence score were used to evaluate the predicted binding models.

RNA sequencing (RNA-seq). Total RNA was extracted using TRIzol reagent, treated with DNase I to remove genomic DNA and assessed for quality (RNA integrity number ≥ 7.0). RNA-seq libraries were prepared using the Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit (cat. no. 12309ES; Shanghai Yeasen Biotechnology Co., Ltd.) according to the sequencing provider's standard protocol. Briefly, mRNA was enriched, fragmented and reverse-transcribed into cDNA, followed by second-strand synthesis, adaptor ligation and library amplification. Qualified libraries were sequenced on the Illumina NovaSeq platform (paired-end, 150 bp; Illumina, Inc.) by Guangzhou Genedenovo Biotechnology Co., Ltd. Raw data were quality-controlled and aligned to the reference genome. Gene expression was quantified using featureCounts from the Subread package (26). Differential expression analysis was performed using DESeq2 (27), with significance thresholds of false discovery rate < 0.05 and $\log_2$ fold-change (FC) ≥ 1 . Functional enrichment analysis through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted using clusterProfiler (28).

Ribosome profiling sequencing (Ribo-seq). To investigate translational regulation, ribosome profiling was performed according to the sequencing provider's standard protocol. Briefly, logarithmic-phase cells were treated with harringtonine (2 $\mu\text{g/ml}$) at room temperature for 2 min, followed by cycloheximide treatment (100 $\mu\text{g/ml}$) at room temperature for 1 min to stall ribosomes. Cells were then collected by centrifugation at 4°C and 500 x g for 5 min, lysed in cell lysis buffer (Guangzhou Genedenovo Biotechnology Co., Ltd.) on ice for 10 min, and clarified by centrifugation at 4°C and 17,000 x g for 10 min. Ribosome-polypeptide complexes were isolated using a MicroSpin S-400 Column (Cytiva), and ribosome-protected fragments were generated by RNase I digestion at room temperature for 45 min. Sequencing libraries were constructed using the NEBNext® Small RNA Library Prep Set for Illumina (cat. no. E7300; NEB) and sequenced on an Illumina platform by Guangzhou Genedenovo Biotechnology Co., Ltd. After quality control with fastp, rRNA reads were removed using Bowtie2 (v2.2.8; <http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>), and the remaining reads were further filtered to remove transfer RNA (tRNA) and other noncoding RNA reads. Reads of 20-40 nt were retained and mapped to the *Homo sapiens* reference genome (Ensembl release 113; <https://www.ensembl.org>) using STAR (v3.1.0; <https://github.com/alexdobin/STAR>). Downstream analyses were performed using riboWaltz (v1.1.0; <https://github.com/LabTranslationalArchitectomics/riboWaltz>) and RSEM (v1.2.19; <https://deweylab.github.io/RSEM/>).

Public database and enrichment analyses. Publicly available transcriptomic data were analyzed using Gene Expression Profiling Interactive Analysis 2 (GEPIA2; <http://gepia2.cancer-pku.cn/>), which integrates RNA-seq data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression projects. The mRNA expression levels of RBM4, EIF4E2 and MAP1B in HNSCC were analyzed using TCGA head and neck squamous cell carcinoma tumor data and normal tissue data

from healthy controls available through GEPIA2. The correlation between EIF4E2 and RBM4 expression was analyzed using Spearman's correlation analysis through GEPIA2, and pan-cancer EIF4E2 expression analysis was performed using GEPIA2. The protein expression levels of EIF4E2 and MAP1B in HNSCC were evaluated using Clinical Proteomic Tumor Analysis Consortium (CPTAC) mass spectrometry-based proteomic data available through the Human Protein Atlas (HPA; <https://www.proteinatlas.org/>). Single-cell transcriptomic data from the HNSC_GSE103322 dataset, comprising ~6,000 cells from 18 patients, were accessed through the Tumor Immune Single-cell Hub 2 (TISCH2; <http://tisch.comp-genomics.org/>) and used to examine RBM4 and EIF4E2 expression across cell populations in HNSCC using the visualization tools provided by TISCH2. Gene Set Enrichment Analysis (GSEA) was performed using the clusterProfiler package (28) in R software (version 4.4.2; Posit Software, PBC) to identify enriched biological processes associated with genes ranked by differential expression from the RNA-seq analyses of shpiR-164552 vs. Scramble and shEIF4E2 vs. Scramble cells using GO biological process gene sets.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 8.3.0; Dotmatics) and R software (version 4.4.2). Data are presented as the mean $\pm$ SD unless otherwise indicated. Paired comparisons were analyzed using paired t-tests, whereas comparisons between independent groups were performed using unpaired t-tests. Receiver operating characteristic curve analysis was performed to evaluate diagnostic and monitoring performance and area under the curve (AUC) values with 95% CIs were calculated. For agomir- and antagomir-based experiments with corresponding NCs, two-way ANOVA followed by Šidák's multiple comparisons test was used. For independent overexpression and knock-down validation experiments displayed in the same panel, piR-164552 overexpression and shpiR-164552 knockdown groups were analyzed separately against the Scramble control using unpaired t-tests. Rescue experiments were analyzed using one-way or two-way ANOVA followed by Šidák's multiple comparisons test according to the experimental design. Repeated tumor volume measurements over time were analyzed using two-way repeated-measures ANOVA. Differential RNA-seq and Ribo-seq analyses were performed using predefined thresholds; genes with an adjusted P-value < 0.05 and $\log_2$ fold change > 1 were considered significantly differentially expressed or translated. The corresponding number of biological replicates is detailed in each figure legend.

Results

Elevated expression of piR-164552 in tumor tissues and serum-derived exosomes of HNSCC. piRNAs are expressed in a tissue-specific manner and serve key roles in tumor initiation and progression (29). In the present study, tumor tissues and matched adjacent non-tumor tissues were collected from patients with clinically diagnosed HNSCC, alongside liquid biopsy samples to capture circulating tumor information. High-throughput RNA-seq was performed to systematically profile HNSCC-specific piRNA expression patterns. Candidate

piRNAs were subsequently validated in both tissue samples and serum-derived exosomes using RT-qPCR, thereby establishing a tissue-fluid integrated verification system to ensure the robustness of the results (Fig. 1A). Based on differential expression criteria ($FC \geq 2$ and $P < 0.05$), 10 piRNAs that were significantly dysregulated between tumor and adjacent tissues were identified, including seven upregulated and three downregulated piRNAs (Fig. 1B and C). To further define HNSCC-specific piRNAs, large-scale validation was conducted for the seven upregulated candidates. Among them, piR-164552 displayed significantly higher expression in tumor tissues compared with matched adjacent tissues (Figs. 1D and E and S1A-F).

Accumulating evidence has indicated that tumor-derived exosomes are enriched in ncRNAs with diagnostic potential (30). Protected by the lipid bilayer, these ncRNAs are resistant to degradation by circulating ribonucleases, making them ideal biomarkers for liquid biopsy. Following the International Society for Extracellular Vesicles guidelines (31), exosomes were isolated from the serum of healthy controls and patients with HNSCC using a precipitation-based method, followed by multidimensional characterization (32). TEM revealed typical cup-shaped vesicles (Fig. 1F). NTA further determined that the majority of exosomes ranged from 100–150 nm in diameter (Fig. 1G). Western blotting further demonstrated the presence of specific exosomal markers, including CD9, CD63, CD81 and TSG101 (Fig. 1H). To evaluate the stability of exosomal piRNAs, a series of validation assays were conducted. piR-164552 levels remained stable in serum exosomes stored at room temperature for 0, 12, 24 and 48 h (Fig. 1I). RNase treatment alone did not significantly affect piR-164552 expression in comparison with the control, whereas RNase combined with Triton X-100-mediated membrane disruption caused a significant decrease in piR-164552 expression compared with the control group (Fig. 1J), demonstrating membrane-dependent protection.

Clinical validation showed that serum exosomal piR-164552 expression was significantly higher in patients with HNSCC compared with healthy controls (Fig. 1K). ROC curve analysis showed its diagnostic performance with an AUC of 0.7202 (95% CI: 0.6146–0.8258; Fig. 1L). Patients with advanced clinicopathological features, including higher tumor stage (T3–T4), lymph node metastasis (N+) and advanced clinical stage (III–IV), tended to exhibit higher serum exosomal piR-164552 expression compared with those with early-stage disease (T1–T2, N0 and stages I–II; Fig. S1G–I). Notably, serum exosomal piR-164552 levels declined significantly 7 days after surgical resection compared with preoperative levels (Fig. 1M), with a monitoring AUC of 0.7178 (95% CI: 0.5897–0.8458; Fig. 1N). Based on these analyses, the present findings suggested that serum exosomal piR-164552 may serve as a non-invasive biomarker associated with tumor progression and treatment response monitoring in HNSCC.

Expression characteristics and functional role of piR-164552 in HNSCC cells. To clarify the biological role of piR-164552, its expression profile and subcellular localization in HNSCC cells was examined. Quantitative analysis revealed that piR-164552 expression was significantly elevated in the HNSCC cell lines FaDu and CAL-27 compared with the normal oral epithelial

cell line HOK (Fig. 2A). FISH analysis using a Cy3-labeled piR-164552 probe revealed predominant cytoplasmic localization with weaker nuclear signals in both FaDu and CAL-27 cells (Fig. 2B). Consistently, subcellular fractionation analysis determined that piR-164552 was enriched in the cytoplasmic fraction, with slightly higher cytoplasmic than nuclear levels in both cell lines (Fig. 2C).

To explore the functional relevance of piR-164552, a piR-164552 agomir and antagomir were synthesized. RT-qPCR showed that agomir transfection significantly increased piR-164552 expression compared with the NC, whereas the antagomir effectively suppressed it compared with the NC (Fig. S2). CCK-8 assays revealed that overexpression of piR-164552 significantly promoted cell proliferation, whereas knockdown of piR-164552 significantly inhibited cell proliferation in both FaDu and CAL-27 cells, compared with the respective NCs (Fig. 2D). Consistently, EdU assays showed that piR-164552 overexpression increased the proportion of EdU-positive cells, whereas piR-164552 knockdown decreased this proportion (Fig. 2E). Colony formation assays further showed that piR-164552 overexpression enhanced the colony-forming ability of FaDu and CAL-27 cells *in vitro*, whereas piR-164552 knockdown reduced this ability (Fig. 2F). Wound healing assays demonstrated that overexpression of piR-164552 significantly enhanced the migratory capacity of FaDu and CAL-27 cells, compared with the NC, whereas knockdown of piR-164552 inhibited this ability, compared with the NC (Fig. 2G and I). Consistently, Transwell migration and invasion assays demonstrated increased cell traversal upon piR-164552 overexpression and a marked reduction following its knockdown, compared with the NC groups (Fig. 2H and J).

In vivo functional validation of the role of piR-164552 in promoting tumor growth. To assess the role of piR-164552 in tumor growth *in vivo*, a subcutaneous xenograft model was established by injecting FaDu cells into the axilla of nude mice. Palpable tumors were observed by day 7 post-injection, after which tumor volumes were measured every 3 days (Fig. 3A). Lentiviral vectors were used to establish stable HNSCC cell lines overexpressing and knocking down piR-164552 for further functional investigation (Fig. 3B).

In the early stages of tumor formation, overexpression of piR-164552 significantly accelerated tumor growth from day 10 onward, whereas knockdown of piR-164552 significantly reduced tumor volume from day 13 onward compared with the Scramble group (Fig. 3C). *In vivo* fluorescence imaging revealed stronger signals in the overexpression group and weaker signals in the knockdown group, indicating corresponding changes in tumor burden (Fig. 3D). Final analysis consistently showed that piR-164552 overexpression led to a significantly increased tumor volume and weight (compared with the scramble), while knockdown of piR-164552 produced the opposite effect (Fig. 3E and F). Immunohistochemical analysis further demonstrated significantly elevated expression levels of Ki67, α -smooth muscle actin and vimentin in piR-164552-overexpressing tumors, whereas knockdown groups exhibited significantly reduced levels of these markers, compared with the scrambles (Fig. 3G–J). Collectively, these findings indicated that piR-164552 promoted tumor growth *in vivo* and may have accelerated tumor progression through

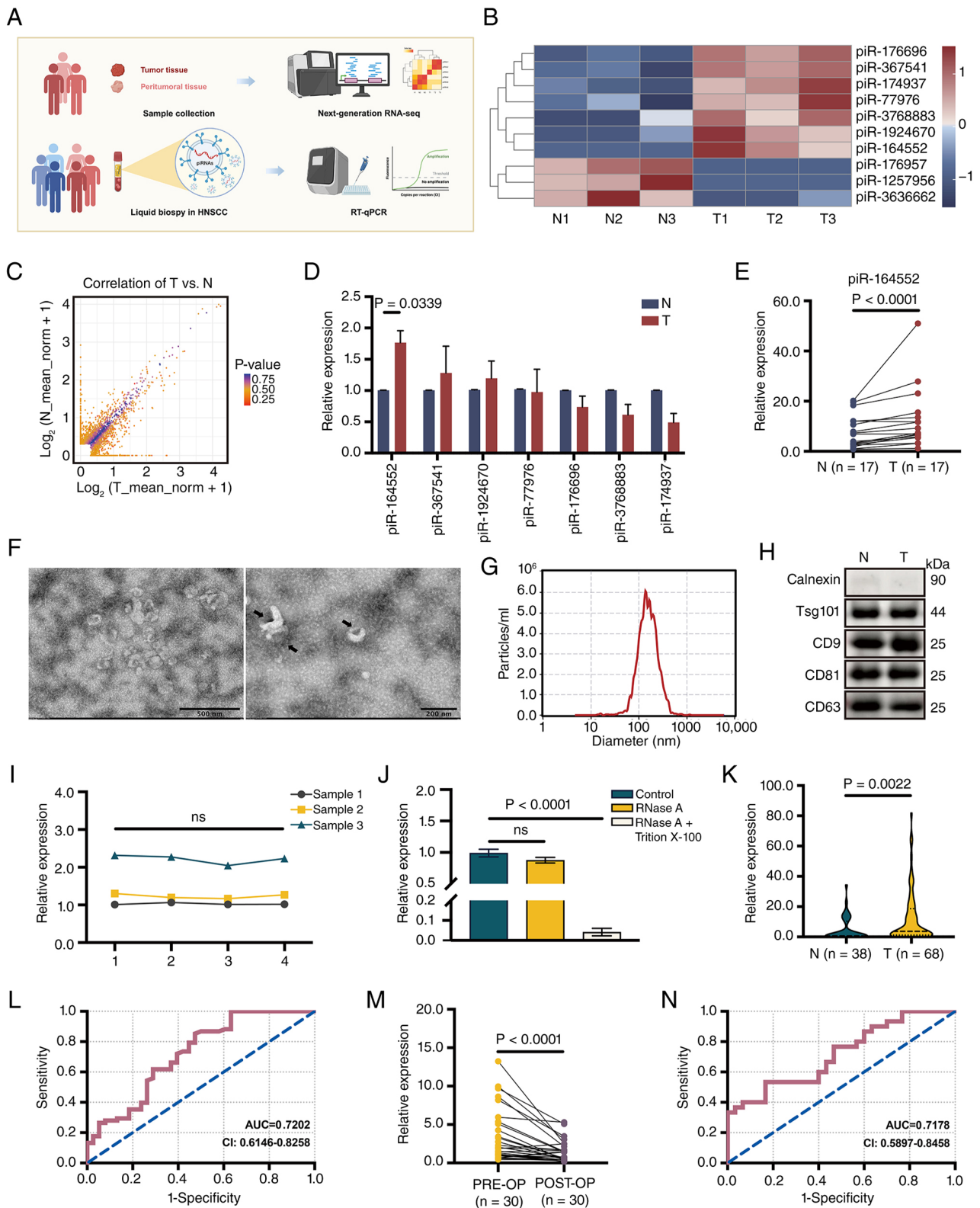


Figure 1. Elevated expression of piR-164552 in HNSCC tissues and serum-derived exosomes. (A) Workflow for collecting HNSCC tumor tissues, adjacent normal tissues and serum samples, followed by RNA-seq and RT-qPCR validation. Fig. 1A was created with BioRender.com. (B) Heatmap of significantly dysregulated piRNAs. (C) Correlation analysis of RNA-seq data. (D) Screening of candidate piRNAs in tissues revealed distinct expression patterns. (E) Paired analysis of piR-164552 expression in tissues (n=17). (F) Transmission electron microscopy exhibiting typical exosome morphology (scale bar, 500 nm in the left image and 200 nm in the right image). The data presented are from one representative experiment of three independent experimental replicates. (G) Nanoparticle tracking analysis of exosome size distribution. The data presented are from one representative experiment of three independent experiments. (H) Western blotting determined the presence of exosomal markers (Tsg101, CD9, CD81 and CD63) and the absence of calnexin. (I) Stability assay of exosomal piR-164552 at room temperature. (J) RNase A/Triton X-100 treatment of exosomes (n=3). (K) Serum exosomal piR-164552 levels in patients with HNSCC (n=38) and healthy controls (n=68). (L) ROC curve analysis for diagnostic potential. (M) Serum exosomal piR-164552 levels before and after surgery (n=30). (N) ROC curve analysis for monitoring surgical outcomes. piR/piRNA, PIWI-interacting RNA; HNSCC, head and neck squamous cell carcinoma; RNA-seq, RNA sequencing; ROC, receiver operating characteristic. Tsg101, tumor susceptibility 101; RT-qPCR, reverse transcription-quantitative PCR; N, normal; T, tumor; ns, not significant; AUC, area under the curve.

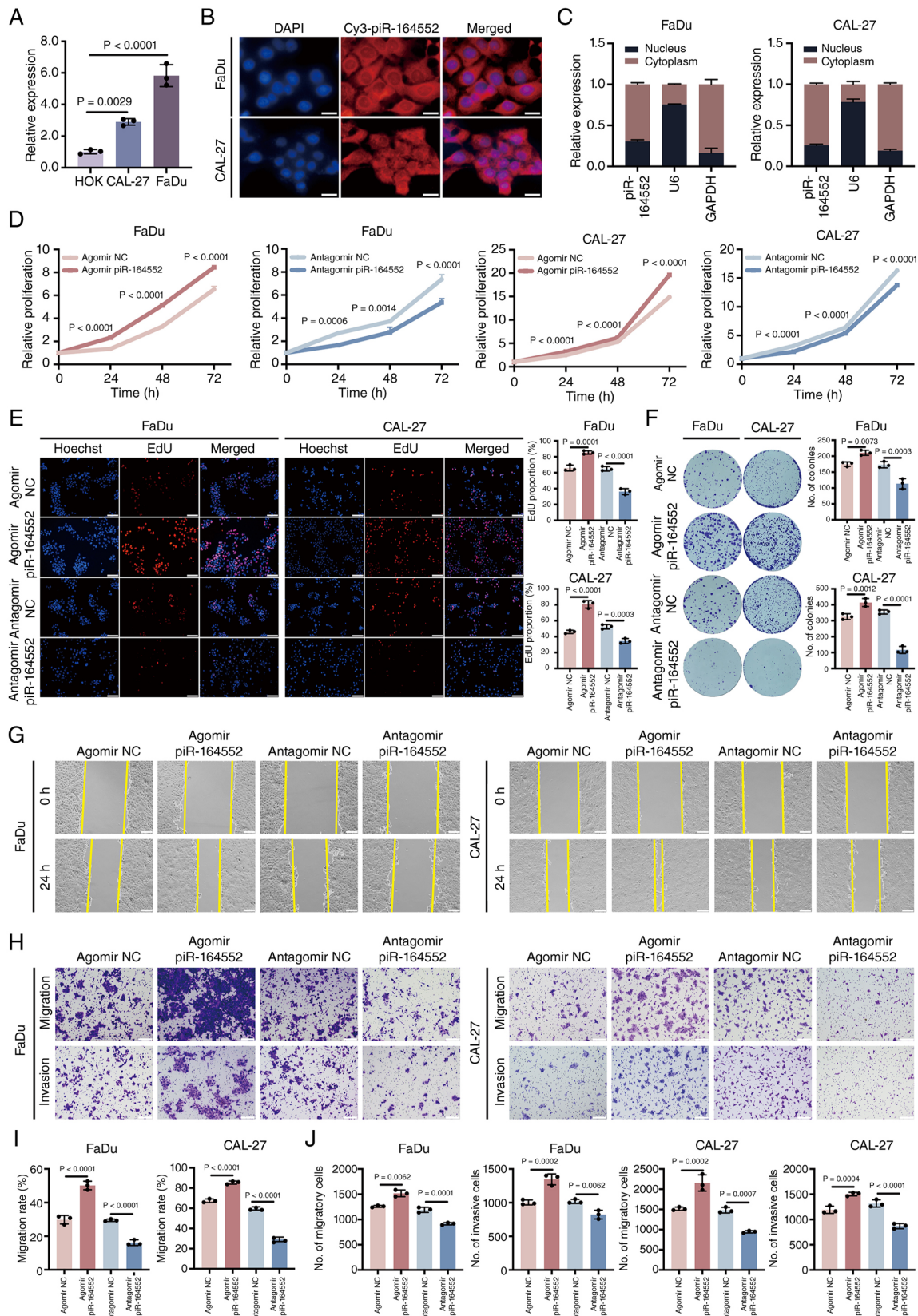


Figure 2. Expression pattern and functional role of piR-164552 in HNSCC cells. (A) Reverse transcription-quantitative PCR analysis of piR-164552 expression in FaDu, CAL-27 and HOK cells. (B) Fluorescence *in situ* hybridization analysis of subcellular localization of piR-164552 (scale bar, 25 μ m). The data presented are from one representative experiment of three independent experiments. (C) Nuclear and cytoplasmic RNA isolation was performed on FaDu and CAL-27 cells to detect piR-164552. (D) Cell Counting Kit-8 assay results of cell proliferation. (E) Representative results of EdU assay for HNSCC cells (FaDu and CAL-27). Dividing cells were labelled with EdU (red). DAPI staining is shown as blue in the nucleus (scale bar, 100 μ m). Histogram graph indicates normalized signaling rate of EdU cells. (F) Colony formation assay (representative images, n=3). (G) Wound healing assays of cell migration (scale bar, 100 μ m). (H) Transwell assays of cell migration and invasion (scale bar, 100 μ m). The data presented are from one representative experiment of three independent experiments. (I) Quantification of wound healing assays. (J) Quantification of Transwell assays. Data are presented as the mean $\pm$ SD of three independent experiments. HNSCC, head and neck squamous cell carcinoma; piR/piRNA, PIWI-interacting RNA; NC, negative control.

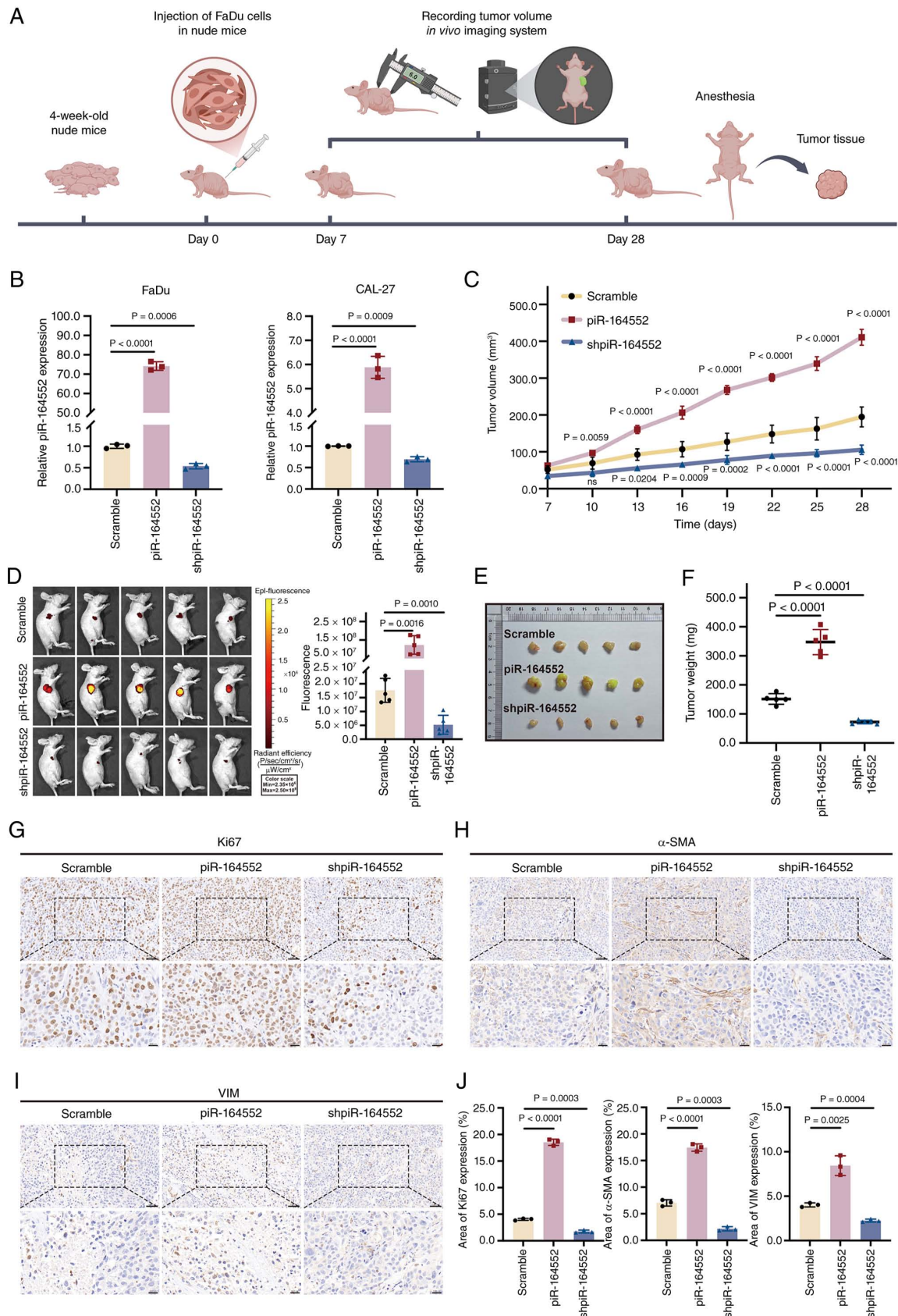


Figure 3. *In vivo* validation of the tumor-promoting role of piR-164552. (A) Schematic overview of the timeline for the *in vivo* study. Fig. 3A was created with BioRender.com. (B) Reverse transcription-quantitative PCR demonstrating the efficiency of lentivirus-mediated overexpression and knockdown of piR-164552 in stable HNSCC cell lines (n=3). (C) Tumor growth curves (n=5). (D) *In vivo* bioluminescence imaging of tumor progression (n=5). (E) Representative images of excised tumors (n=5). (F) Quantification of tumor weight (n=5). (G) Representative Ki67 staining images of tumors from different groups (scale bar, 200 μ m; enlarged view, 100 μ m). (H) Representative α -SMA staining images of tumors from different groups (scale bar, 200 μ m; enlarged view, 100 μ m). (I) Representative VIM staining images of tumors from different groups (scale bar, 200 μ m; enlarged view, 100 μ m). (J) Quantification of Ki67 (panel G), α -SMA (panel H) and vimentin (panel I) staining (n=3). piR/piRNA, PIWI-interacting RNA; HNSCC, head and neck squamous cell carcinoma; α -SMA, α -smooth muscle actin; VIM, vimentin; sh, short hairpin; ns, not significant.

mechanisms associated with the epithelial-mesenchymal transition.

piR-164552 directly interacts with RBM4 in HNSCC cells. To investigate the molecular mechanisms underlying the oncogenic role of piR-164552 in HNSCC, the present study first examined whether it interacts with protein partners. Since piRNAs often function through RNA-protein complexes, an F2-tagged piR-164552 was synthesized for RNA pull-down assays, followed by liquid chromatography-tandem MS (LC-MS/MS) analysis to identify binding proteins. Comparative analysis between piR-164552 and its antisense control identified 92 candidate proteins, including 44 RBPs (Figs. S3A and 4A) as RBPs were defined according to the RBPWorld database (<http://research.gzsys.org.cn/rbpworld/>). Among these, intensity-based absolute quantification values, FC and unique peptide count highlighted RBM4 as the most enriched candidate. RIP assays showed that piR-164552 was significantly enriched in RBM4 immunoprecipitates compared with IgG controls in both FaDu and CAL-27 cells, supporting an interaction between piR-164552 and RBM4 (Fig. 4B). Western blotting of RNA pull-down products further validated the presence of RBM4 protein (Fig. 4C).

The present study subsequently investigated the structural basis of this interaction. Secondary structure prediction and three-dimensional modeling of piR-164552, followed by molecular docking with RBM4, indicated a highly stable interaction (docking score: -298.22; confidence score: 0.9490; Fig. 4D). ImmunoFISH further demonstrated clear cytoplasmic co-localization of piR-164552 and RBM4 in HNSCC cells (Fig. 4E). Consistent with these findings, western blotting revealed a positive association between piR-164552 levels and RBM4 protein levels, namely piR-164552 overexpression significantly increased RBM4 protein levels, whereas piR-164552 knockdown significantly reduced them (Fig. 4F). By contrast, RT-qPCR exhibited no significant change in RBM4 mRNA levels (Fig. S3B), suggesting the involvement of post-transcriptional regulation.

RBM4 is a multifunctional RBP involved in alternative splicing and translational control, with context-dependent roles in tumorigenesis (33). However, its role in HNSCC remains poorly understood. Analysis using GEPIA2 (34) revealed that RBM4 mRNA expression was significantly upregulated in HNSCC tissues compared with normal head and neck epithelium (Fig. 4G). Single-cell transcriptomic profiling (HNSC_GSE103322, which comprises 6,000 cells from 18 patients) further showed that RBM4 was highly expressed in malignant epithelial cells (Fig. 4H), supporting its potential function as a tumor-promoting RBP in HNSCC (35). To validate this function, RBM4-knockdown HNSCC cell lines were generated and knockdown efficiency was determined by western blotting (Fig. 4I and K). Functional rescue experiments showed that RBM4 knockdown significantly reduced RBM4 and EIF4E2 protein levels compared with the corresponding scramble control group, whereas treatment with agomir piR-164552 significantly restored their expression in both FaDu and CAL-27 cells with RBM4-knockdown. The restored RBM4 level remained significantly lower than that in piR-164552-overexpressing cells without RBM4 knockdown in FaDu cells, whereas this difference was not significant in

CAL-27 cells (Fig. 5H). Consistently, Transwell assays demonstrated that RBM4 knockdown significantly suppressed cell migration and invasion in FaDu and CAL-27 cells, whereas piR-164552 overexpression partially rescued these effects, compared with the scramble + NC (Fig. 4J and L).

EIF4E2 is the target of the piR-164552/RBM4 complex in HNSCC cells. RBM4 is an RBP that interacts with numerous partners and regulates diverse processes, including alternative splicing and translational control (36). Protein-protein interaction network analysis identified EIF4E2 as a direct interactor of RBM4, which was also broadly associated with PER1, HNRNPK, RBM10, U2AF2, SRSF3, YTHDF2, AGO2 and EPAS1 (Fig. 5A). Molecular docking analysis further modeled the interaction between EIF4E2 and the piR-164552/RBM4 complex (Fig. 5B), showing a stable binding interface mediated by hydrogen bonds (including EIF4E2 residues Y253, Y255 and Y257 with RBM4 residues R350, R352 and R354) and hydrophobic contacts, with a docking score of -267.75 and a confidence score of 0.9133. Analysis of -HNSC dataset revealed a significant positive correlation between EIF4E2 and RBM4 expression (Fig. 5C). Co-IP assays demonstrated the direct interaction between RBM4 and EIF4E2 in HNSCC cells (Fig. 5D). In FaDu and CAL-27 cells, FISH combined with FISH-IF demonstrated prominent cytoplasmic co-localization between piR-164552 and EIF4E2 (Fig. 5E).

Next, the present study analyzed the association between piR-164552 and EIF4E2 protein expression. Western blotting showed that piR-164552 overexpression significantly increased EIF4E2 protein levels, whereas piR-164552 knockdown reduced them (Fig. 5F). In xenograft tumor tissues, IF staining revealed enhanced co-localization of RBM4 and EIF4E2 in the piR-164552 overexpression group compared with the knockdown group (Fig. 5G). Rescue experiments further showed that piR-164552 overexpression partially restored RBM4 and EIF4E2 protein levels in RBM4-silenced HNSCC cells (Fig. 5H).

EIF4E2 is an important downstream effector mediating the oncogenic role of piR-164552 in HNSCC. To compare EIF4E2 expression levels between tumor and normal tissues, a pan-cancer analysis using GEPIA2 was performed. The results revealed that EIF4E2 was highly expressed in the majority of tumors (Fig. S4A). Analysis using GEPIA2 showed that EIF4E2 mRNA expression was significantly higher in HNSCC tissues compared with normal tissue data available through GEPIA2, and CPTAC mass spectrometry-based proteomic data available through HPA further indicated elevated EIF4E2 protein expression in HNSCC (Figs. 6A and S4B). Single-cell RNA-seq data from the HNSC_GSE103322 dataset demonstrated that EIF4E2 was enriched in malignant cells (Fig. 6B), suggesting an oncogenic role in HNSCC progression (35). Kaplan-Meier survival analysis indicated that patients with low EIF4E2 expression exhibited significantly longer overall survival rates compared with that of patients with high EIF4E2 expression (Fig. 6C). These findings collectively suggested that EIF4E2 is involved in the initiation and progression of HNSCC.

To further evaluate the functional role of EIF4E2 as a downstream effector of piR-164552, stable EIF4E2-overexpressing and EIF4E2-knockdown HNSCC cell models were established

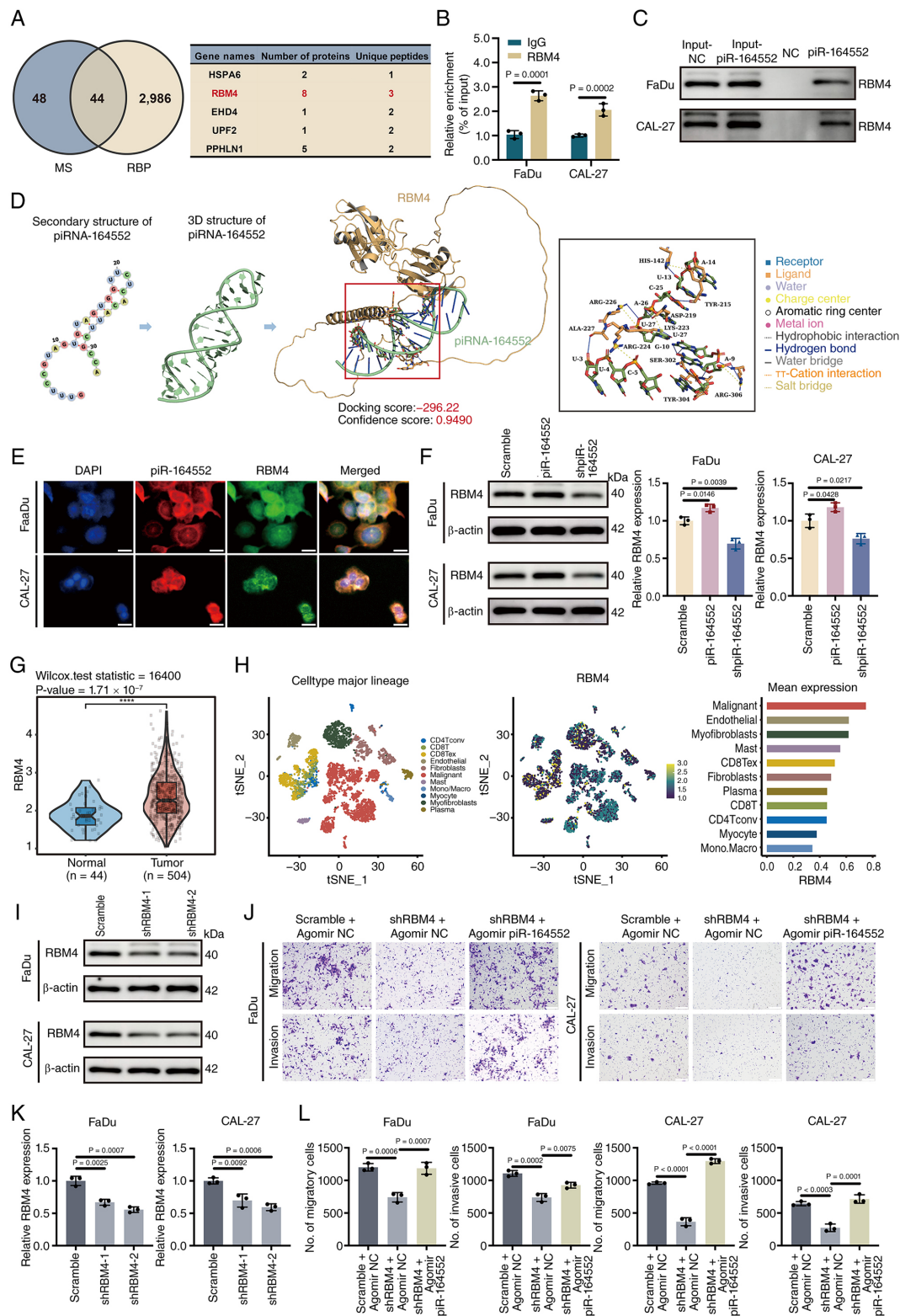


Figure 4. piR-164552 directly interacts with the RNA-binding protein RBM4 and regulates HNSCC cell functions. (A) Venn diagram showing the overlap between MS results and the RBP database. (B) RNA immunoprecipitation assay validating the interaction between piR-164552 and RBM4. (C) RNA pulldown assay determining direct binding of RBM4 to piR-164552. The data presented are from one representative experiment of three independent experiments. (D) Molecular docking analysis. (E) Fluorescence *in situ* hybridization-immunofluorescence double staining showing partial co-localization of piR-164552 and RBM4 (scale bar, 25 μ m). The data presented are from one representative experiment of three independent experiments. (F) Western blotting analysis of RBM4 expression regulated by piR-164552. (G) Differential expression of RBM4 in tumor and paired normal tissues in The Cancer Genome Atlas database. (H) Single-cell transcriptomic analysis revealing RBM4 distribution across different cell subpopulations. (I) Western blotting analysis of RBM4 knockdown efficiency. (J) Transwell migration and invasion assays (scale bar, 100 μ m). The data presented are from one representative experiment of three independent experiments. (K) Quantitative analysis demonstrating the knockdown efficiency of RBM4. (L) Quantification of Transwell assays. Data are presented as the mean $\pm$ SD of three independent experiments. ****P<0.0001. piR/piRNA, PIWI-interacting RNA; HNSCC, head and neck squamous cell carcinoma; RBP, RNA-binding protein; RBM4, RNA-binding motif protein 4; NC, negative control; sh, short hairpin; HSPA6, heat shock protein family A member 6; EHD4, EH-domain containing 4; UPF2, up-frameshift suppressor 2 homolog; PPHLN1, periphilin-1; t-SNE, t-distributed stochastic neighbor embedding; MS, mass spectrometry.

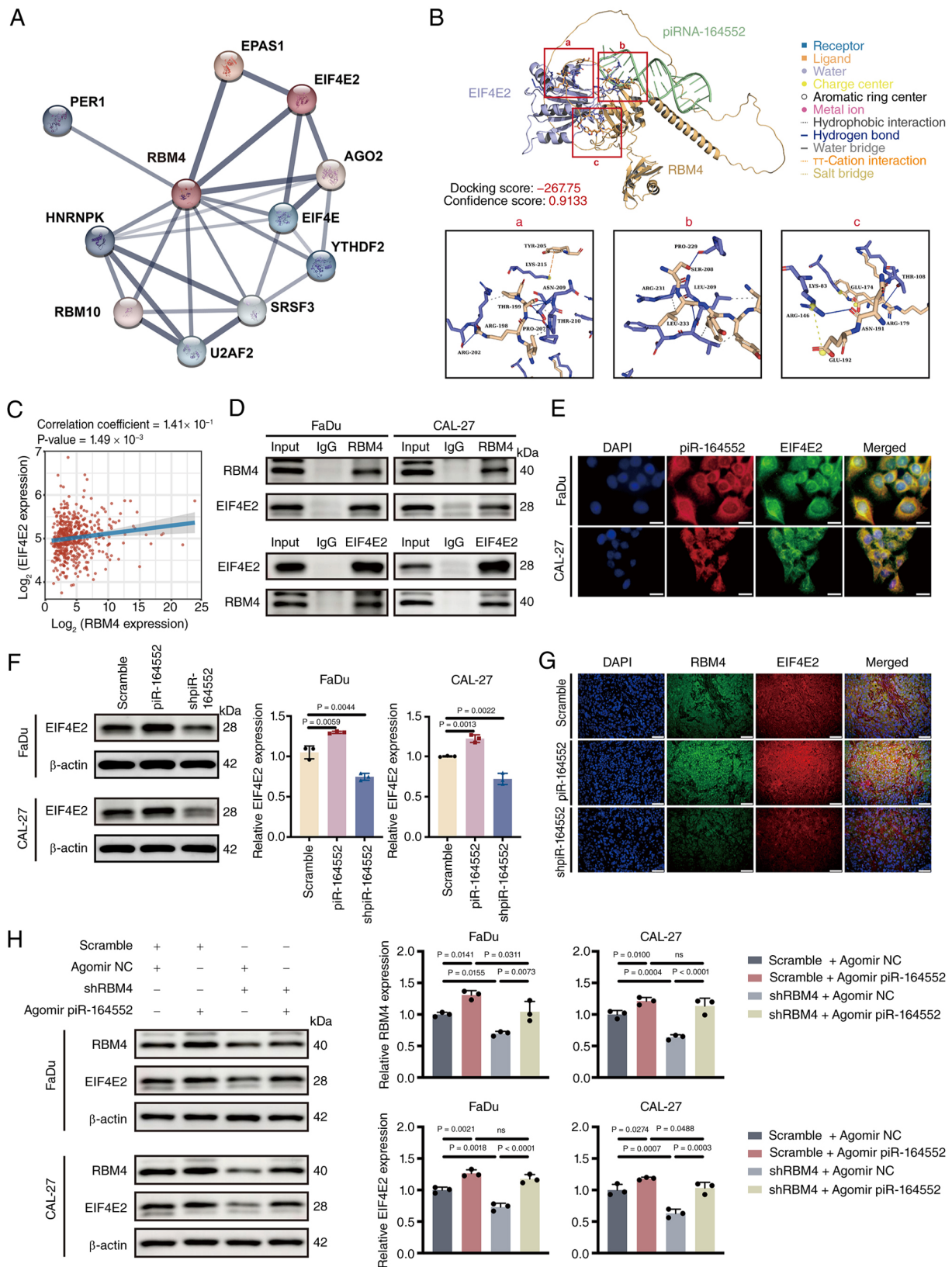


Figure 5. EIF4E2 is the target of the piR-164552/RBM4 complex in HNSCC cells. (A) Protein-protein interaction network showing interactions of RBM4 with EIF4E2 and other proteins. (B) Molecular docking analysis of EIF4E2 with the piR-164552/RBM4 complex. (C) Correlation analysis of RBM4 and EIF4E2 expression in The Cancer Genome Atlas HNSCC dataset. (D) Co-immunoprecipitation assay demonstrating the interaction between RBM4 and EIF4E2. The data presented are from one representative experiment of three independent experiments. (E) Fluorescence *in situ* hybridization-IF co-localization assay of piR-164552 and EIF4E2 in FaDu and CAL-27 cells (scale bar, 25 μ m). The data presented are from one representative experiment of three independent experiments. (F) Western blotting analysis of EIF4E2 expression regulated by piR-164552. (G) IF staining analysis of protein expression levels of RBM4 and EIF4E2 in tumor tissues (scale bar, 50 μ m). The data presented derive from one representative experiment of three independent experiments. (H) Rescue assay showing that piR-164552 overexpression partially restores RBM4 and EIF4E2 expression in RBM4-silenced HNSCC cells (FaDu and CAL-27). Data are presented as the mean $\pm$ SD of three independent experiments. EIF4E2, eukaryotic initiation factor 4E-like 2; piR/piRNA, PIWI-interacting RNAs; RBM4, RNA-binding motif protein 4; HNSCC, head and neck squamous cell carcinoma; IF, immunofluorescence; NC, negative control; sh, short hairpin; ns, not significant.

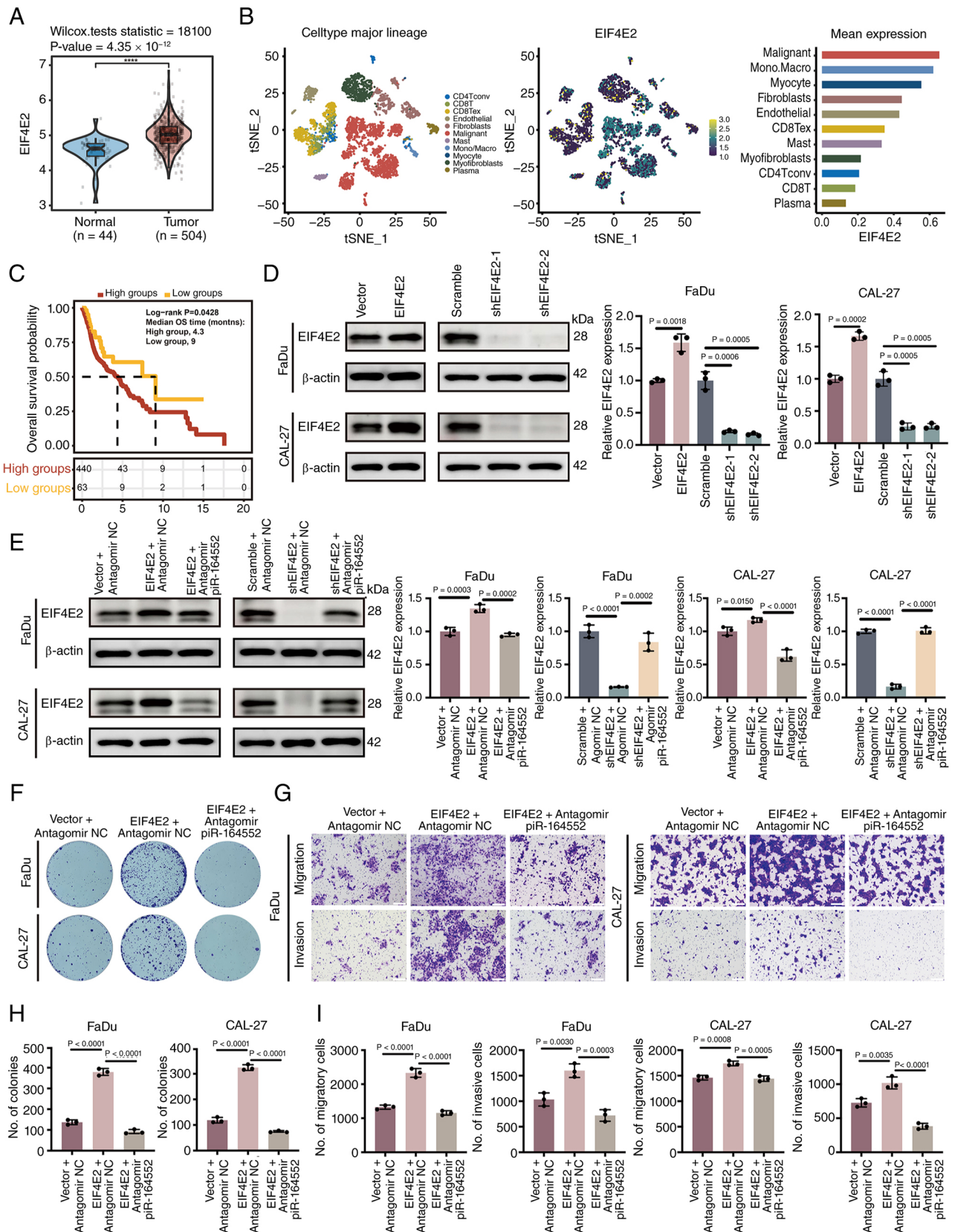


Figure 6. piR-164552 partially reverses the effects of EIF4E2 on HNSCC cell proliferation, migration and invasion. (A) Differential expression of EIF4E2 in tumor tissues and paired normal tissues in The Cancer Genome Atlas database. (B) Single-cell transcriptomic analysis revealing EIF4E2 distribution across different cell subpopulations. (C) Kaplan-Meier overall survival analysis of patients with high vs. low EIF4E2 expression. (D) Western blotting validation of EIF4E2 overexpression and knockdown efficiency in HNSCC cells (FaDu and CAL-27). (E) Rescue experiments showing that piR-164552 regulates EIF4E2 protein expression. (F) Colony formation assays (representative images, n=3). (G) Transwell migration and invasion assays (scale bar, 100 μ m). The data presented are from one representative experiment of three independent experiments. (H) Quantification of colony formation assays. (I) Quantification of Transwell migration and invasion assays. Data are presented as the mean $\pm$ SD of three independent experiments. ****P<0.0001. HNSCC, head and neck squamous cell carcinoma; piR/piRNA, PIWI-interacting RNA; EIF4E2, eukaryotic initiation factor 4E-like 2; sh, short hairpin; NC, negative control; t-SNE, t-distributed stochastic neighbor embedding.

and the efficiency of overexpression and knockdown were demonstrated by western blotting (Fig. 6D). Rescue experiments demonstrated that knockdown of piR-164552 partially attenuated EIF4E2 upregulation in EIF4E2-overexpressing cells, whereas overexpression of piR-164552 partially restored EIF4E2 expression in EIF4E2-silenced cells (Fig. 6E). Colony formation assays revealed that EIF4E2-overexpressing cells treated with antagomir NC formed significantly more colonies than the corresponding vector control group. By contrast, treatment with antagomir piR-164552 significantly reduced colony formation compared with EIF4E2-overexpressing cells treated with antagomir NC in both FaDu and CAL-27 cells (Fig. 6F and H). Similarly, Transwell assays demonstrated that EIF4E2 overexpression significantly enhanced migration and invasion, results which were then partially inhibited by piR-164552 knockdown (Fig. 6G and I). Furthermore, compared with the control group, overexpression of piR-164552 significantly rescued the inhibitory effects of EIF4E2 knockdown on cell proliferation (Fig. S4C and E), migration and invasion (Fig. S4D and F).

Effects of piR-164552 on the transcriptome and translome of HNSCC. To elucidate the functional significance of the piR-164552/RBM4/EIF4E2 axis in HNSCC, GO enrichment analysis was first performed. RBM4 was found to be predominantly enriched in 'Regulation of mRNA metabolic process', 'Regulation of mRNA splicing, via spliceosome' and 'miRNA-mediated gene silencing by inhibition of translation', whereas EIF4E2 was mainly associated with 'Translational initiation', 'Regulation of translational initiation' and 'Post-transcriptional gene silencing' (Fig. S5A and B). Processing bodies (P-bodies) are cytoplasmic granules enriched with decapping complexes (DCP1A/DCP2), RNA helicases (DDX6) and miRNA effectors (AGO2 and GW182), which serve key roles in mRNA degradation, storage and translational repression (37). Previous studies have reported that RBM4 and EIF4E2 partially co-localize with P-bodies in the cytoplasm (38,39). IF analysis revealed that both RBM4 and EIF4E2 showed marked co-localization with P-body markers in HNSCC cells (Fig. S5C), suggesting their involvement in post-transcriptional gene regulatory networks.

Based on the broad regulatory effects of piR-164552 on RBM4 and EIF4E2, RNA-seq and Ribo-seq were subsequently performed in piR-164552-depleted FaDu cells and corresponding control cells to systematically evaluate the impact of piR-164552 on the transcriptome and translome of HNSCC. At the transcriptional level, 1,745 upregulated and 994 downregulated genes upon piR-164552 knockdown were identified (Fig. 7A). GSEA revealed that genes were primarily enriched in processes associated with mRNA metabolism and protein synthesis, including 'tRNA aminoacylation', 'Amino acid activation', 'Preribosome', 'Small-subunit processome' and 'Cellular response to topologically incorrect protein' (Fig. 7B). KEGG analysis further indicated significant enrichment of multiple cancer-associated pathways, including 'pathways in cancer', 'PI3K-Akt signaling pathway', 'transcriptional misregulation in cancer' and 'complement and coagulation cascades' (Fig. 7C). These results suggested that piR-164552 promotes HNSCC progression by reprogramming transcriptional profiles toward mRNA metabolism and translation-associated pathways.

At the translational level, piR-164552 knockdown induced widespread changes, with 1,189 genes showing increased and 1,069 showing decreased translation (Fig. 7D). Analysis of the top differentially affected genes revealed selective alterations in translational efficiency (TE), including reduced TE of MAT1A and NICN1 and increased TE of ARHGAP31 and ZNF804A (Fig. 7E). KEGG analysis of differentially translated genes enriched 'ribosome', 'mTOR signaling pathway', 'Wnt signaling pathway' and cancer-associated pathways (Fig. 7F). Integrated analysis of RNA-seq and Ribo-seq data demonstrated both concordant and discordant regulation across transcriptional and translational levels (Fig. 7G), indicating multilayered gene regulation. Collectively, these results demonstrated that piR-164552 functions as an upstream regulator that orchestrates multilayered transcriptomic and translational reprogramming to drive malignant progression.

piR-164552 shapes the landscape of transcriptome and translome via the piR-164552/RBM4/EIF4E2 axis in cancer. To further delineate the role of the piR-164552/RBM4/EIF4E2 axis in transcriptional and translational regulation, RNA-Seq and Ribo-Seq analyses were performed in EIF4E2 knockdown cells. At the transcriptional level, depletion of EIF4E2 resulted in 1,418 upregulated and 1,224 downregulated genes, indicating extensive transcriptomic remodeling (Fig. 8A). GSEA showed significant enrichment of post-transcriptional and translational processes, including 'tRNA aminoacylation for protein translation', 'amino acid activation' and translational elongation, as well as activation of the 'endoplasmic reticulum unfolded protein response' (Fig. 8B). EIF4E2 silencing was also associated with enrichment of the unfolded protein response, underscoring its key role in sustaining translational homeostasis and protein quality control. KEGG analysis further revealed enrichment of numerous tumor- and survival-associated pathways, including the 'PI3K-Akt signaling pathway', 'MAPK signaling pathway', 'TNF signaling pathway', 'mTOR signaling pathway' and 'AMPK signaling pathway' (Fig. 8C). These findings demonstrated that EIF4E2 broadly modulates transcriptomic programs associated with translational control and cancer-related signaling.

At the translational level, differential expression analysis revealed widespread alterations in protein synthesis following EIF4E2 knockdown (Fig. 8D), highlighting its broad impact on mRNA metabolism. TE analysis identified a distinct subset of EIF4E2-dependent genes, including extracellular matrix components (COL3A1, COL4A3 and COL5A2), adhesion molecules (CXADR and CTAGE15) and developmental regulators (HOXB8 and KLF15; Fig. 8E). KEGG analysis showed that these genes were enriched in pathways associated with mRNA metabolism, translational control and oncogenic signaling, including 'MAPK signaling pathway', 'Wnt signaling pathway', 'VEGF signaling pathway', 'FoxO signaling pathway' and 'sphingolipid signaling pathway', as well as a number of cancer-associated pathways (Fig. 8F). These results suggest a selective role for EIF4E2 in modulating cellular architecture, intercellular communication and lineage specification at the translational level. Notably, integrative analysis of transcriptomic and translational profiles revealed that, while certain genes were concordantly regulated at both levels, a large number of them exhibited translational changes independent

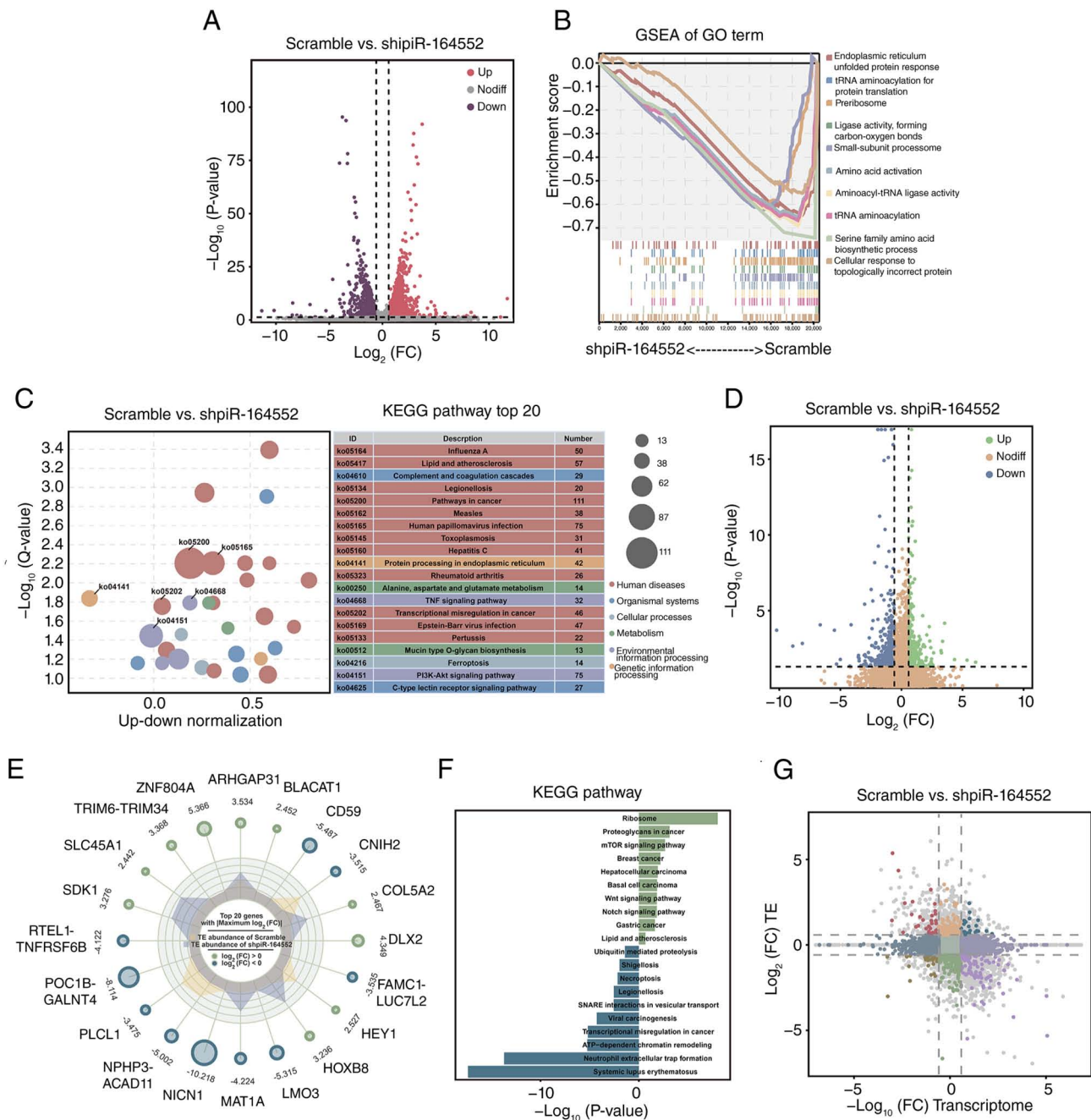


Figure 7. Regulatory effects of piR-164552 on the transcriptome and translate of HNSCC. (A) Volcano plot of RNA-seq data. (B) GSEA of RNA-seq data. (C) KEGG pathway analysis of RNA-seq data. (D) Volcano plot of Ribo-seq data. (E) Top 20 differentially translated genes identified by Ribo-seq. (F) KEGG pathway analysis of Ribo-seq data. (G) Scatter plots showing the association between gene expression fold-changes in RNA-seq and Ribo-seq data. The data presented derive from one representative experiment of three independent experiments. HNSCC, head and neck squamous cell carcinoma; piR/piRNA, PIWI-interacting RNA; RNA-seq, RNA sequencing; Ribo-seq, ribosome profiling sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; Nodif, no difference; Up, upregulated; Down, downregulated; sh, short hairpin; FC, fold change; GO, Gene Ontology; tRNA, transfer RNA; TE, translational efficiency.

of mRNA abundance, underscoring the post-transcriptional regulatory function of EIF4E2 (Fig. 8G). Collectively, EIF4E2 emerged as a central regulator of post-transcriptional gene expression, coordinating mRNA metabolism and protein synthesis to modulate cancer-associated pathways and drive tumor progression.

Subsequently, the Ribo-Seq datasets from piR-164552- and EIF4E2-knockdown cells were integrated (Fig. 8H). As exemplified by MAP1B, transcriptional efficiency and TE exhibited opposite trends (Fig. 8I). Specifically, depletion of piR-164552

or EIF4E2 reduced MAP1B mRNA levels, while paradoxically increasing its TE. Analysis using GEPIA2 showed that MAP1B mRNA expression was elevated in HNSCC tissues compared with normal tissue data available through GEPIA2, whereas CPTAC mass spectrometry-based proteomic data available through HPA indicated reduced MAP1B protein expression in HNSCC (Fig. S6A and B). Correlation analysis indicated that MAP1B expression was positively correlated with apoptosis (Fig. S6C) but negatively correlated with proliferative signatures (Fig. S6D), suggesting a potential tumor-suppressive

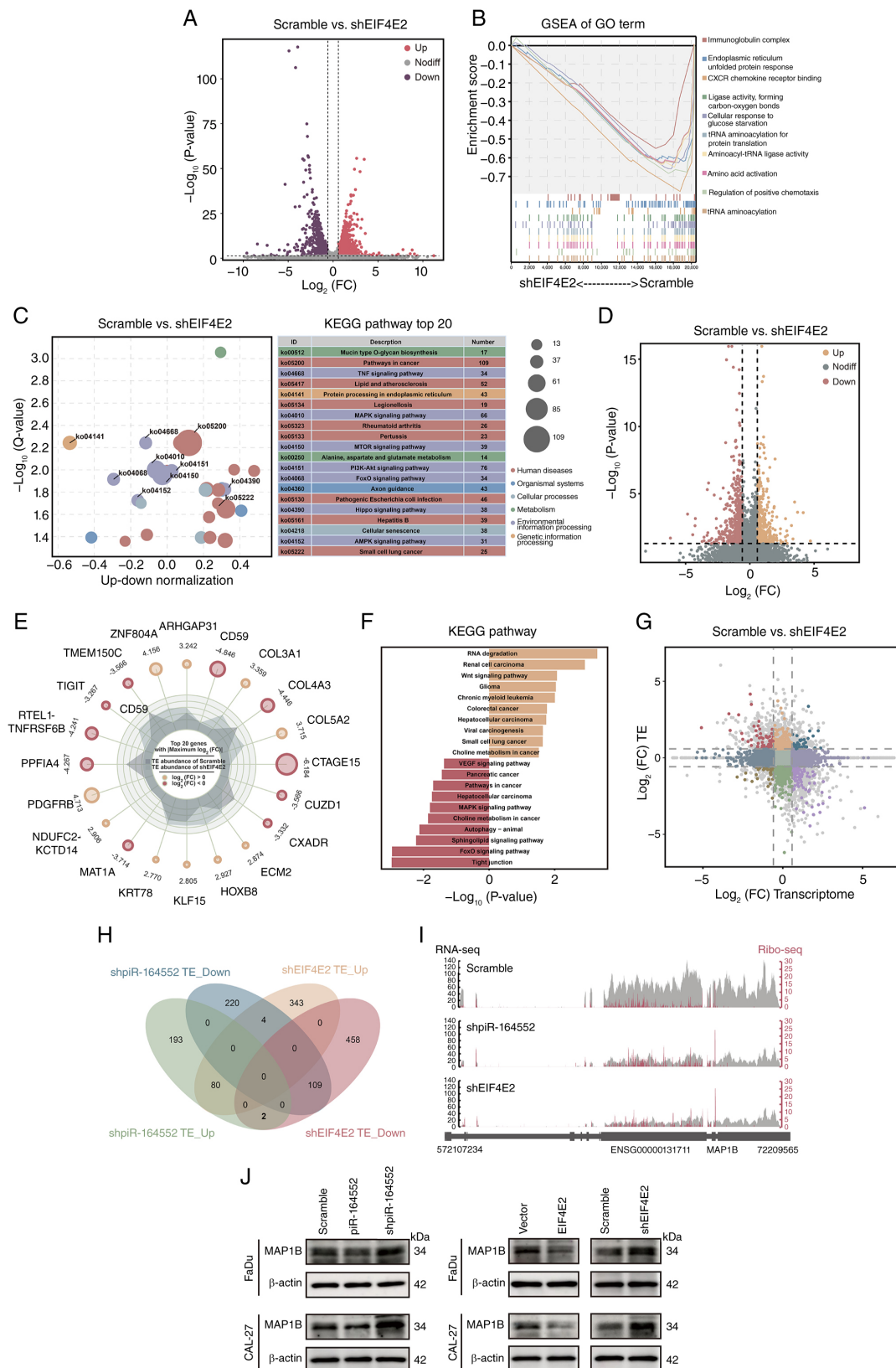


Figure 8. Role of the piR-164552/RBM4/EIF4E2 axis in transcriptional and translational regulation. (A) Volcano plot of RNA-seq data. (B) GSEA of RNA-seq data. (C) KEGG pathway analysis of RNA-seq data. (D) Volcano plot of Ribo-seq data. (E) Top 20 differentially expressed genes identified by Ribo-seq. (F) KEGG pathway analysis of Ribo-seq data. (G) Scatter plots showing the association between gene expression FC in RNA-seq and Ribo-seq data. (H) Venn diagram of TE changes in piR-164552-KD and EIF4E2-KD cells. (I) A representative example (MAP1B) of genes showing inverse changes in TE in response to piR-164552-KD or EIF4E2-KD. Ribosome density and the MAP1B transcript from Ribo-seq are marked in red. The input for Ribo-seq presented as mRNA levels (RNA) is marked in gray. (J) Western blotting validation of MAP1B protein expression. Data are presented as the mean $\pm$ SD of three independent experiments. piR/piRNA, PIWI-interacting RNA; RBM4, RNA-binding motif protein 4; EIF4E2, eukaryotic initiation factor 4E-like 2; RNA-seq, RNA sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes; Ribo-seq, ribosome profiling sequencing; TE, translational efficiency; KD, knockdown; MAP1B, microtubule associated protein 1B; FC, fold change; sh, short hairpin; tRNA, transfer RNA; TE, translational efficiency; GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; Up, upregulation; Down, downregulation; Nodiff, No difference.

role. Consistent with the RNA-seq data, RT-qPCR validation demonstrated that overexpression of piR-164552 and EIF4E2 significantly upregulated MAP1B mRNA, whereas their depletion resulted in the opposite effect (Fig. S6E). In line with the Ribo-seq findings, western blotting demonstrated that overexpression of piR-164552 and EIF4E2 reduced MAP1B protein abundance, while their knockdown significantly increased MAP1B protein levels (Figs. 8J and S6F).

Discussion

Within the present study, a previously unrecognized piRNA-mediated regulatory axis that promotes malignant progression in HNSCC was systematically investigated. It was found that piR-164552 is abnormally upregulated in HNSCC, where it interacts with the RBP RBM4 to enhance EIF4E2-dependent translational regulation, thereby exerting oncogenic piRNA functions. This piR-164552-RBM4-EIF4E2 signaling axis drives synergistic reprogramming of the transcriptome and translome, supporting an invasive tumor phenotype.

Despite prior studies having predominantly explored the contributions of miRNAs and long ncRNAs to HNSCC pathogenesis, the role of piRNAs has remained largely unexplored (40-44). piRNAs are classically associated with the preservation of genome integrity through suppression of transposable elements in germline cells (45). However, increasing evidence has suggested their involvement in somatic gene regulation and cancer development (46-48). The present data provide notable experimental support for piR-164552 as a functional oncogenic piRNA in HNSCC.

A key contribution of the present study is the identification of a previously unrecognized piRNA-protein-translation regulatory axis. Through LC-MS/MS, RNA pull-down, RIP and molecular docking analyses, it was demonstrated that piR-164552 directly binds to RBM4. RBM4 is a multifunctional RBP implicated in alternative splicing and translational control (49). In addition, despite RBM4 having been described as a tumor suppressor in certain cancer types, the present data reveal that RBM4 is highly expressed in HNSCC malignant cells and promotes tumor progression (50,51). Mechanistically, piR-164552 may promote tumor progression by interacting with RBM4, upregulating RBM4 protein levels and facilitating the interaction between RBM4 and the non-canonical cap-binding protein EIF4E2.

Integrating RNA-seq with ribosomal analysis further demonstrated that the piR-164552/RBM4/EIF4E2 axis exerts multi-level control at both the transcriptional and translational levels. The frequent decoupling of mRNA abundance and translation efficiency highlights the importance of post-transcriptional regulation in HNSCC. In this context, MAP1B, as a key downstream target, exhibits transcription-translation mismatch, suggesting that piR-164552-mediated translational regulation may influence the balance between tumor cell proliferation and apoptosis. The partial localization of RBM4 and EIF4E2 to P-bodies further corroborates the role of this axis in regulating mRNA storage and transport processes.

Beyond its mechanistic relevance, piR-164552 also demonstrates potential clinical importance. In the present study, piR-164552 was detectable in serum-derived exosomes, and its

association with advanced clinical and pathological features as well as postoperative changes suggests its potential utility as an adjunctive biomarker for disease monitoring. Furthermore, targeting components of the piR-164552/RBM4/EIF4E2 axis may represent a potential therapeutic strategy. As RNA-based therapeutics and oncogenic non-coding RNAs are being increasingly explored in cancer research, oncogenic piRNAs may also represent potential targets for therapeutic intervention (52,53).

Despite this, a number of limitations should be acknowledged. The clinical cohort size of the present study was limited, necessitating larger-scale studies to validate the translational potential of exosomal piR-164552. In addition, the specific mechanism by which piR-164552 regulates RBM4 protein levels remains to be fully elucidated. Although the present study supports an interaction between piR-164552 and RBM4, the precise mechanism underlying the regulation of RBM4 protein levels remains unclear and warrants further investigation using protein stability assays, such as cycloheximide chase and ubiquitination analyses. Furthermore, beyond EIF4E2, other downstream effectors may participate in piR-164552-mediated carcinogenic effects. Future research should therefore aim to further explore the therapeutic feasibility of targeting this pathway and its potential impact on treatment response in HNSCC.

In conclusion, the present study identified piR-164552 as a previously unrecognized oncogenic piRNA that drives HNSCC progression through the piR-164552/RBM4/EIF4E2 signaling axis, thereby reshaping transcriptional and translational regulatory programs. These findings expand the current understanding of piRNA-mediated cancer regulation mechanisms and suggest potential applications in diagnostic and therapeutic fields.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Shandong Provincial Natural Science Foundation (grant no. ZR2025MS1494) and the National Key Research and Development Program of China (grant no. 2022YFC2401500).

Availability of data and materials

The RNA-sequencing dataset used for preliminary piRNA screening in HNSCC in the present study may be found in the National Center for Biotechnology Information under BioProject accession number PRJNA1367908 or at the following URL: (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1367908>). The RNA-sequencing and ribosome profiling sequencing datasets related to piR-164552- and EIF4E2-modulated HNSCC cells in the present study may be found in the National Center for Biotechnology Information under BioProject accession number PRJNA1473757 or at the following URLs: (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1473757> and <https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1473757>). The other data generated in

the present study may be requested from the corresponding author.

Authors' contributions

SY conceived the present study, designed the experimental protocol, conducted the experiments and drafted the manuscript. ZS assisted with animal experiments, transcriptomic and proteomic sequencing and manuscript writing. SX and HL collected clinical samples and assisted with experimental procedures. SY, SX and XW performed data analysis, organized the results and created visualizations. HL, WX and YD contributed to the conceptualization of the experimental design and ensured quality control throughout the present study. HL and WX supervised all aspects of the project and wrote the final approval of the version, contributing equally to the present study. SY and HL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Board of The Affiliated Hospital of Qingdao University (Qingdao, China; approval no. QYFY WZLL 29843). The animal experiments were approved by the Institutional Animal Care and Use Committee of Qingdao University (Qingdao, China; approval no. 20241017BALB/c-nude20241219007). All human tissue and serum samples were collected after obtaining written informed consent from patients, in compliance with the provisions of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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