SUPPLEMENTARY MATERIAL

Immunohistochemistry

Immunohistochemical analysis was conducted on tissue sections derived from patients and nude mice, following standard protocols. Two trained pathologists independently evaluated the stained slides using a light microscope. Staining intensity was semi-quantitatively classified into four categories: negative (-), weak (+), moderate (++), and strong (+++). Specificity was verified with goat anti-rabbit IgG (negative control) and human clear-cell renal-cell carcinoma tissue (positive control). Both controls are shown in Fig. S1. This study utilized the anti-ENO1 antibody (ab227978; Abcam) and the anti-Ki-67 antibody (Cell Signaling Technology).

Cell viability assay

Metabolic activity was assessed utilizing the Cell Counting Kit-8 (CCK-8) (Beyotime). Cells were distributed at a count of 4,000 cells per well in 96-well plates. At 24, 48, and 72 hours after seeding, the culture medium was supplemented with 10% solution of CCK-8 and thereafter placed in an incubator at a temperature of 37°C for a duration of 1 hour. After the incubation period, we measured the optical density (OD) at a wavelength of 450 nm utilizing a microplate reader. The experiment was repeated three times, with six technical replicates for each repetition.

Western blot

The proteins in cells were obtained by extracting them using a lysis solution (Beyotime) that included both protease and phosphatase inhibitors (Roche). The BCA Protein Assay Kit (Thermo Fisher Scientific) was used to assess protein concentrations. Equal amounts of total protein underwent electrophoresis and were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). These membranes were blocked using a 5% nonfat milk solution and left to incubate overnight at a temperature of 4°C with primary antibodies, followed by a one-hour incubation at room temperature with peroxidase-conjugated secondary antibodies. Blot bands were detected using an ECL reagent (Millipore), and imaging was performed on a chemiluminescence Imaging System (BioRad). The Western blot testing employed the subsequent antibodies: ENO1 (ab227978) and phospho-mTOR (ab109268) from Abcam; beta-actin (66009-1-Ig), CDK2 (101221-AP), CDK4 (11026-1-AP), cyclin D1 (60186-1-Ig), cyclin E1 (11554-1-AP), E-cadherin (20874-1-AP), N-cadherin (22018-1-AP), MMP1 (10371-2-AP) from Proteintech; phospho-AKT (4060), AKT (4691), and mTOR (2983) from Cell Signaling Technology.

Cell cycle analysis

The process of analyzing the cell cycle was carried out by employing Propidium Iodide (PI) staining, using the Cell Cycle Analysis Kit provided by KeyGEN BioTECH. Following the harvest, the cells were washed with phosphate-buffered saline (PBS) and subsequently immersed in 75% ethanol at a temperature of -20°C for the duration of the night. Subsequently, the cells were brought to room temperature, washed again with PBS, and stained with a PI and RNase buffer mixture for 30 minutes in a light-protected environment. Flow cytometry (CytoFLEX) was then employed to quantify DNA content. The analysis was performed three times, with three technical replicates for each analysis.

Cell apoptosis assay

The assessment of cellular apoptosis was carried out employing the FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Initially, cells were harvested, rinsed with cold PBS, and subsequently suspended in 100 μL of binding buffer. Next, 5 μL staining solution were added, followed by a 20-minute incubation in a light-protected environment. Following incubation, the cells underwent a double wash with PBS, were then sieved through a 200-mesh screen, and subsequently analyzed using flow cytometry. The assay was repeated three times, with three technical replicates for each run.

Caspase Activity Assay

Caspase activity was measured using the Caspase-9 Activity Assay Kit and Caspase-3 Activity Assay Kit (Beyotime), in accordance with the manufacturer's protocol. In brief, cSCC cells were collected, lysed, and centrifuged, followed by normalization of total protein concentrations. The lysates were then incubated with the respective substrates at a temperature of 37 degrees Celsius for a duration of 90 minutes. The samples were analyzed for absorbance employing a microplate reader (Thermo Scientific) at a wavelength of 405 nm. The experiment was repeated three times, with three technical replicates for each repetition.

Cell line-derived xenograft model

Female BALB/c/nu nude mice, aged 4-5 weeks, were procured from the Xi'an Jiaotong University Animal Center. Each mouse received a subcutaneous injection on the right flank with 5×10^6 cells, either subjected to transfection using sh-control or sh-ENO1. Tumor volumes and body weights were recorded every three days, beginning one week after implantation. Tumor volume was determined using the formula: $\text{Volume} = \text{Length} \times (\text{Width}^2)/2$. Following a period of 22 days, the mice were humanely executed by means of cervical dislocation, and tumor tissues were then gathered for the purpose of weighing. The Institutional Animal Care and Use Committee at Xi'an Jiaotong University granted consent for all treatments.

Cell culture

Human epidermal keratinocytes, obtained from ATCC (Manassas, VA), and HaCat cells (catalogue number ZQ0044, from Shanghai Zhong Qiao Xin Zhou Biotechnology, with STR authentication), along with A431 (with STR authentication) and SCL-1 cell lines, procured from Xi'an Yobios Biotechnology, were cultured. The cells were grown in Dulbecco's Modified Eagle's Medium (Gibco), enriched with 10% fetal bovine serum (Zeta Life) and 1% penicillin-streptomycin. These were incubated in a humidified environment at 37°C and 5% carbon dioxide.

Wound healing assay

Cells were cultured in six-well plates until they reached approximately 95% confluence. The cell monolayers were then disrupted using the tip of a 200 μL pipette, followed by rinsing with PBS and a subsequent 24-hour incubation in serum-free growth medium. Photographs of the injured regions were taken at both 0 and 24 hours, with a minimum of three fields per time point documented. Analyzed wound closure via Image J, developed by the National Institutes of Health. The percentage of wound closure at time (x) was determined by using the formula: Wound Closure % = [1−Area at Time x/Area at Time 0)] x 100. The assay was repeated three times, with three technical replicates for each repeat.

Transwell migration and invasion assay

To conduct the migration test, a total of 7×10^4 cells were placed in each chamber, with 200 μL of serum-free media, on the top insert (8 μm pore size, Corning). For the lower compartment of a 24-well plate, we introduced 800 μL of medium enriched with 10% serum. Post a 20-hour incubation period, migrated cells on the underside of the insert membrane were fixed using 100% methanol for a duration of 10 minutes, followed by a 10-minute staining in Crystal Violet Staining Solution (Beyotime). In the invasion assay, we adopted a comparable procedure, utilizing Transwell inserts that had been pre-coated with Matrigel (356234; Corning) and rehydrated, with an extended incubation period of 34 hours. Migration and invasion assays were conducted three times, with three technical replicates for each measurement.

Seahorse Mito Stress Test

The XF Cell Mito Stress Test Kit was utilized on an XF96 Analyzer (Seahorse Biosciences) to assess the cellular oxygen consumption rate (OCR), which serves as an indication of mitochondrial respiration. A total of 10,000 cells were plated into each well of Seahorse 96-well microplates and allowed to adhere overnight. Prior to measurement, the cells were incubated for 1 hour in a CO2-free incubator. The assessment of OCR involved the stepwise introduction of various chemicals.

These included oligomycin (1 mM), an ATP synthase inhibitor, FCCP (0.5 μM), a protonophore, and a combination of rotenone and antimycin A (0.5 μM), inhibitors of Complex I and III, respectively. The test was conducted three times, with six technical replicates for each run.

ROS test

ROS levels were quantified using the dihydroethidium (DHE) Assay Kit (Abcam) in combination with flow cytometry. Cells were collected by centrifugation and then incubated with the diluted DHE fluorescent probe in a dark environment, shielded from light, for 1 hour. Fluorescence was detected at an excitation wavelength of 488 nm on the PE channel using a CytoFLEX instrument (Beckman Coulter Life Sciences). The experiment was repeated three times, with three technical replicates for each data point.

Fig. S1. Immunohistochemical controls for ENO1 staining. (**A**) Negative control: normal-skin section processed with goat anti-rabbit IgG isotype instead of the primary antibody, showing no specific signal. (**B**) Positive control: paraffin-embedded clear-cell renal-cell carcinoma, a tumor known to over-express ENO1, displaying strong cytoplasmic staining. Scale bar, 50 µm.