iScience, Volume *23*

Supplemental Information

Rab25-Mediated EGFR Recycling Causes Tumor Acquired Radioresistance

Lu Zhang, Bowen Xie, Yanfang Qiu, Di Jing, Jing Zhang, Yumei Duan, Zhi Li, Ming Fan, Jiang He, Yuanzheng Qiu, Rong Tan, Jian Jian Li, and Lun-Quan Sun

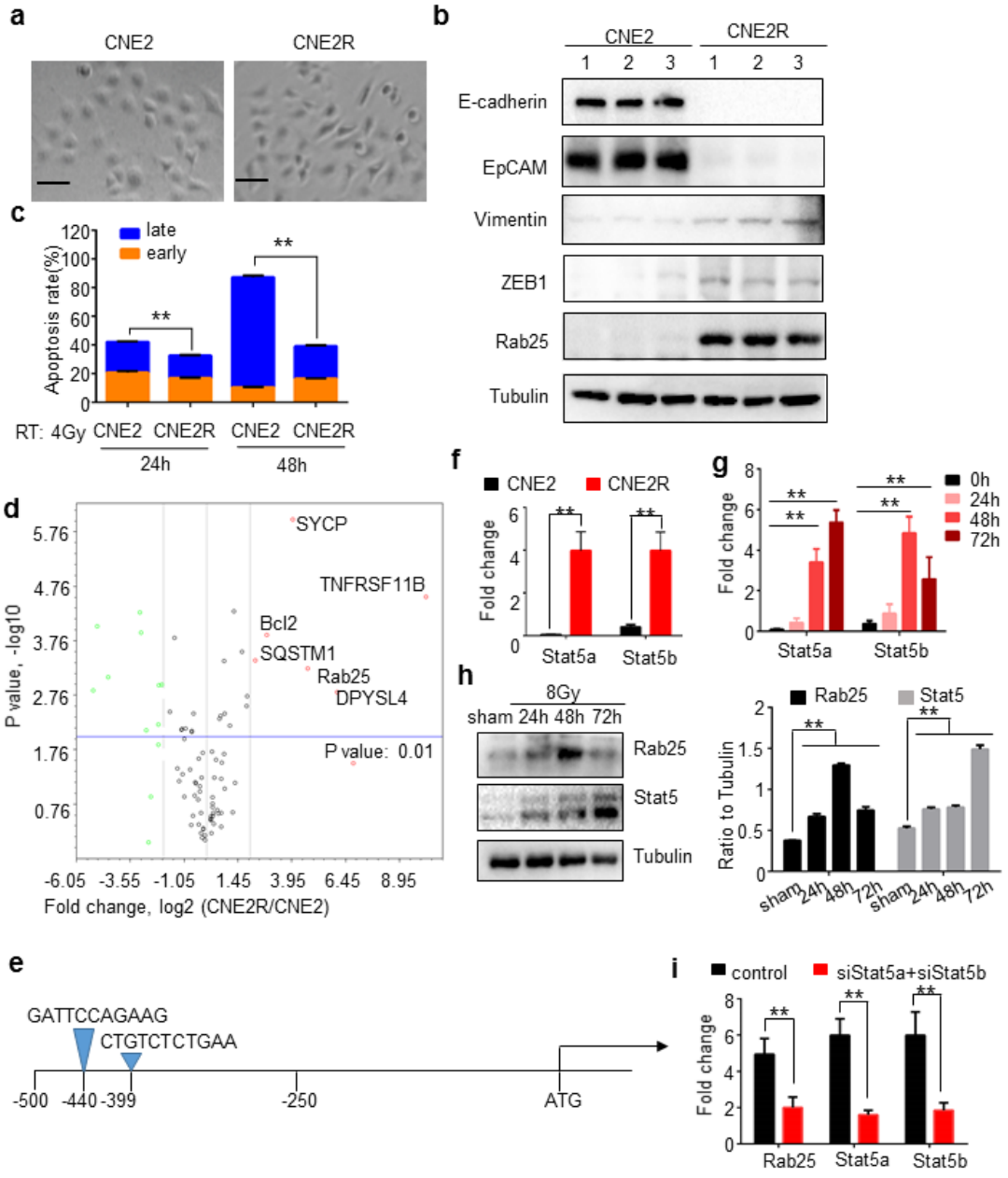

Figure S1 High expression of Rab25 in radioresistant cells was regulated by Stat5, Related to Figure 1.

a Morphological characterization of CNE2 and CNE2R cells (magnification: ×100). **b** Western blotting assay of EMT markers in CNE2 or CNE2R cells. α-Tubulin was used as a loading control. **c** Apoptosis rate of CNE2 or CNE2R cells after culturing in ultra-low attachment wells for indicated time. **d** Volcano plot of differentially genes between CNE2 and CNE2R cells. The total RNAs extracted from CNE2R or CNE2 cells were reverse-transcribed into single-stranded cDNA. Quantitative RT-PCR for cell death-

related genes was performed using the RT² Profiler™ PCR Array Human Cell Death Pathway Finder, and data analysis was performed using a website-based analysis tool from the Qiagene GeneGlobe Data Analysis Center. **e** Diagram to show the predicted binding sites of Stat5 in -500bp region of Rab25. **f** Transcriptional level of Stat5a and Stat5b in CNE2 and CNE2R cells, n=3, mean ± SD, **p < 0.01. **g** Transcriptional level of Stat5a and Stat5b in CNE2 cells irradiated at 8Gy with indicated time. n=3, mean ± SD, **p < 0.01. **h** Protein level of Rab25 or Stat5 in CNE2 cells irradiated at 8Gy with indicated time. **i** Transcriptional level of Rab25, Stat5a, and Stat5b in CNE2R cells or in CNE2R cells with Stat5 knockdown. n=3, mean ± SD, **p < 0.01.

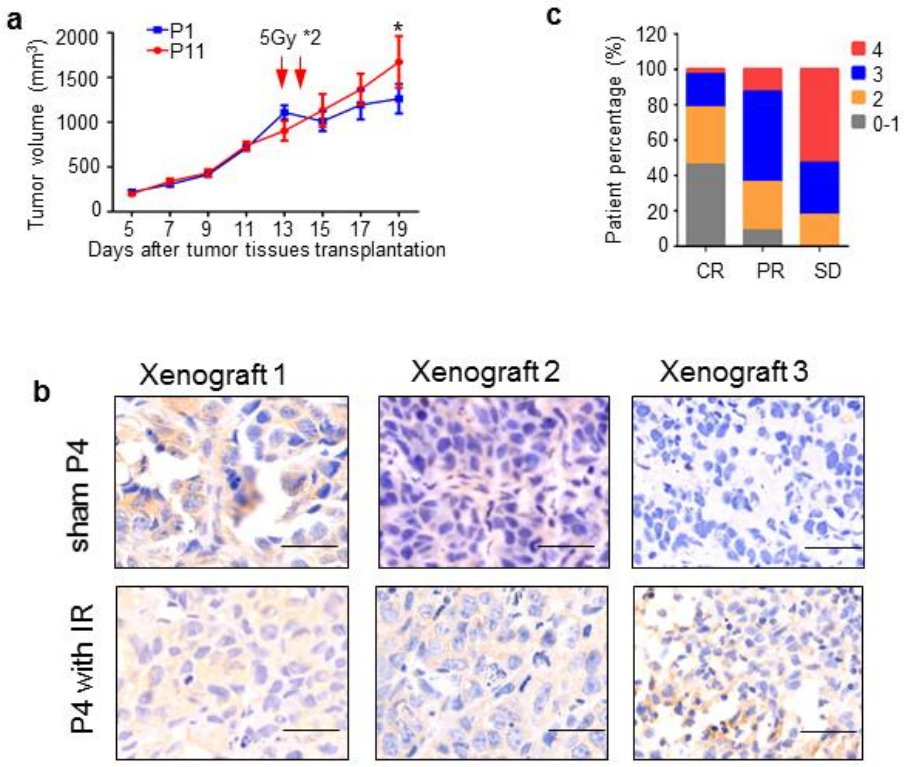

Figure S2 Rab25 was enhanced in radioresistant P4 xenografts, Related to Figure 2.

a Growth curve of tumors from transplanted P1 and P11 mice following radiation. Tumors were irradiated with 5 Gy x 2 when tumor reached 200 mm^3. Four mice were used in each group and data are represented as mean ± SD. The statistical significance between groups was determined with the two-tailed unpaired Student's t test. P values are presented as star marks in figures: *$p < 0.05$. **b** Representative IHC staining of Rab25 in sham P4 (passage alone without radiation) and P4 xenografts. scale bar, 50 μm. **c** Scores of negative, low, medium or high levels of Rab25 were shown as percentage for Rab25 IHC analysis.

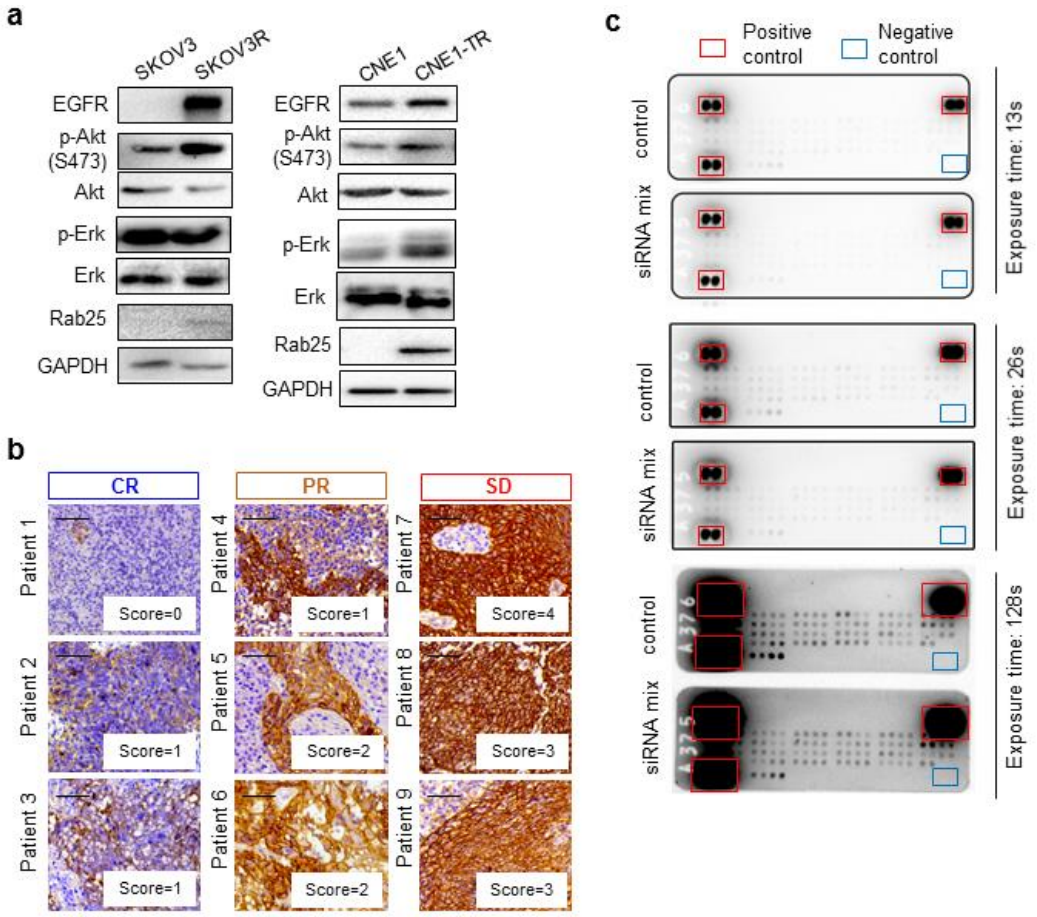

Figure S3 EGFR was hyperactive in therapeutic cells and patients samples, Related to Figure 3.

a Western blot analysis of EGFR and its downstream Erk and Akt signaling pathways in two taxol-resistant cell lines (NPC cell CNE1-TR and ovarian carcinoma cell SKOV3R). **b** Representative IHC staining of EGFR in tissues of NPC patients with different radiotherapy responses. The ASCO-CAP guideline 201336 was used for interpretation of EGFR IHC data. Expression levels of EGFR were classified into five grades (score 0-1: 0-25% , score 2: 25%-50%, score 3: 50%-75%, score 4: >75%). scale bar 50μm. **c** Protein array analysis with different exposure time.

Figure S4

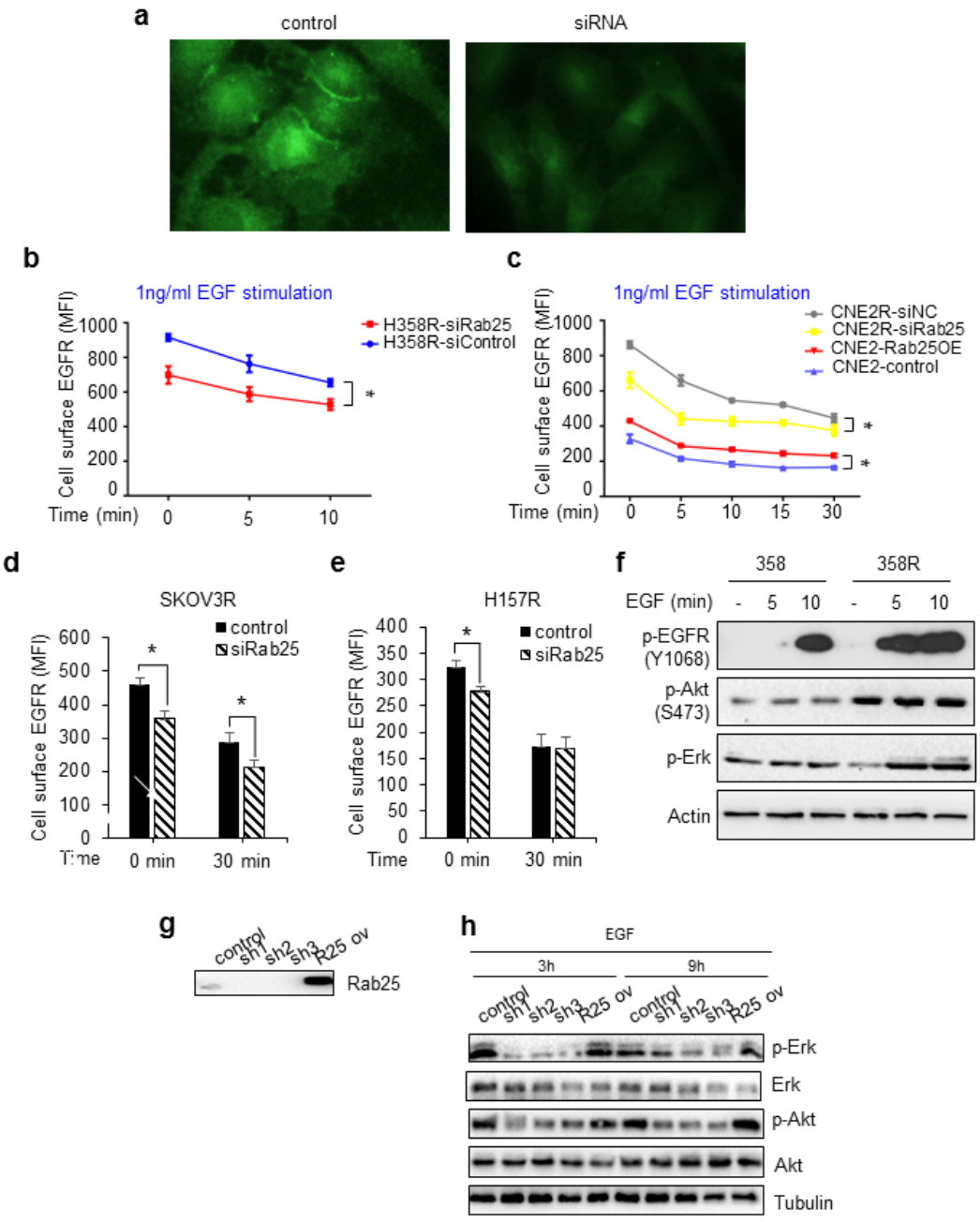

Figure S4 Surface content of EGFR was regulated by Rab25, Related to Figure 5.

a Immonoflourence of EGFR in CNE2R cell or in CNE2R cells with Rab25 knockdown. **b** Quantification of cell surface EGFR content in H358R cells transfected with siControl or siRab25 mix over time following stimulation with 1 ng/ml EGF. **c** EGFR cell surface content by flow cytometry. Each time point for each cell line contained three replicates. Mean ± SD, *p < 0.05. **d,e** Taxol-resistant SKOVR3 or radioresistant

157R cells were firstly transfected with control or siRab25 mixture for 24h. Then, the transfected cells were stimulated with 1 ng/ml EGF for 30 mins. Cells were collected and analyzed for surface EGFR content with Alexa647-conjugated EGFR antibody by FACS. **f** Activity of EGFR downstream signaling Akt and Erk in H358 or H358R cells after short-time EGF stimulation. n=3, mean ± SD, *$p < 0.05$. **g** Rab25 expression in three CNE2R-shRab25 cells and in CNE2R-shRab25 cell transfected with Rab25. **h** Down-expression of Rab25 reduced the expression of EGFR and the activation of associated signaling pathways in radioresistant cells.

Figure S5

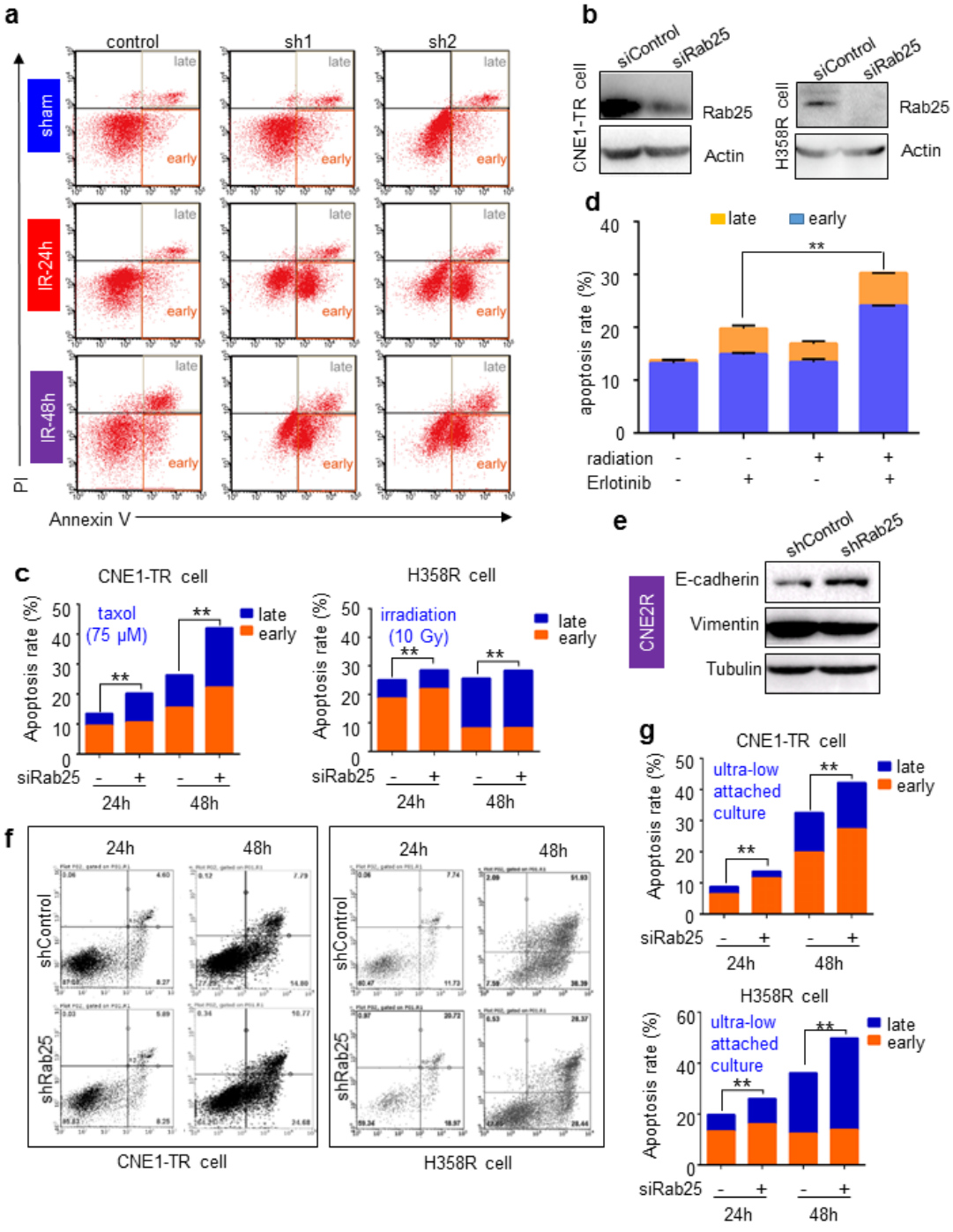

Figure S5 Rab25 knockdown by reverted cell EMT-like phenotype and sensitized cell to radiation, Related to Figure 6.

a Gating strategies for apoptosis induced by radiation in CNE2R cells with or without transfection of shRab25. **b** Rab25 expression in therapeutic-resistant cells was transfected with Rab25 siRNA mixture. **c** Rab25 knockdown increased the apoptosis rate of therapeutic-resistant cells treated with taxol or radiation. The ratio of apoptotic

cells was measured by FACS using Annexin V and PI double staining method. **d** Erlotinib increased the radiation-induced apoptosis of EGFR mutant PC9 cells. Cellls were treated with 5uM Erlotinib alone or combined with 4Gy irradiation. The ratio of apoptotic cells was measured by FACS using Annexin V and PI double staining method. n=3, mean ± SD, **p < 0.01. **e** Decreased expression of EMT-markers in CNE2R cells knockdown with Rab25. **f** Gating strategies for apoptosis induced by detachment in CNE2R cells with or without transfection of shRab25. **g** Rab25 knockdown increased the apoptosis rate of therapeutic-resistant cells cultured in low-attachment culture dishes for the indicated time. n=3, mean ± SD, **p < 0.01.

Table S1. Transcription of Rab25 in different cancers, Related to Figure 2

Variables	n	Rab25 expression			p-value*
		negative/low	moderate	high	
Age					
>50 years	33	6	22	5	0.214
<=50 years	68	15	46	7	
Tumor size					
T1	1	0	1	0	0.0812
T2	28	6	19	3	
T3	30	8	18	4	
T4	42	8	29	5	
Stage					
II	5	0	5	0	0.073
III	30	8	20	2	
IVa	60	12	40	8	
IVb	6	1	3	2	
Lymph node metastasis					
N0	8	0	7	1	0.5116
N1/2	64	13	44	7	
N3	29	8	17	4	

*Statistical significance was determined by the χ^2 test.

Table S2. Patient samples for Rab25 transcript analysis*, Related to Figure 2.

Tumor abbreviation	Tumor name	Normal (number)	Tumor (number)
BLCA	Bladder urothelial carcinoma	28	404
BRCA	Breast invasive carcinoma	291	1085
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma	13	306
CHOL	Cholangio carcinoma	9	36
COAD	Colon adenocarcinoma	349	275
LUAD	Lung adenocarcinoma	347	483
LUSC	Lung squamous cell carcinoma	338	486
OV	Ovarian serous cystadenocarcinoma	88	426
PAAD	Pancreatic adenocarcinoma	171	179
PRAD	Prostate adenocarcinoma	152	492
READ	Rectum adenocarcinoma	318	92
STAD	Stomach adenocarcinoma	211	408
TGCT	Testicular germ cell tumors	165	137
THCA	Thyroid carcinoma	337	512
THYM	Thymoma	339	118
UCEC	Uterine corpus endometrial carcinoma	91	174
UCS	Uterline carcinosarcoma	78	57

* Information was from TCGA.

Transparent Methods

Cell lines

All of the radioresistant cancer cells were generated by saving the surviving residues of cells following the regimen of clinically mimic fractionated ionizing radiation (from Monday to Friday, 2Gy/day, total dose is 60 Gy) (Guo et al., 2003).Lung cancer cell lines A549, A549R, H157, H157R, H358, and H358R, were gifted from Xingming Deng (You et al., 2014). STR test was run for CNE2 and CNE2R cells. PC9 cell were bought from the cell bank of type culture collection of Chinese academy of sciences with STR authentication. Radioresistant nasopharyngeal

carcinoma cell line CNE2R (Li et al., 2013), the paclitaxel-resistant cell lines CNE1-TR and SKOV3R (Zhang et al., 2012, Hou et al., 2017, Zhou et al., 2015) were cultured following the published work. All of the cells except A549 and A549R were cultured in RPMI-1640 medium (Hyclone) with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher Scientific). A549 and A549R cells were cultured in DMEM/F12 medium (Hyclone) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher Scientific). All cell lines were cultured at 37 °C in a 5% CO2 incubator.

Reagents and antibodies

Reagents include EGF (RD, catalog # 236-EG, 10 ng/ml) and EGF-AlexaFluor 488 (Thermofisher, catalog # E13345, 10 ng/ml, Thermofisher). The R&D Systems™ Human Phospho-Receptor Tyrosine Kinase (RTK) Array Kit (catalog # ARY001B) was purchased from R&D Company.

The following antibodies were used for western blot: anti-Rab25 from Sigma (catalog # R8532, 1:500 dilution); anti- PMCA1 (catalog # ab76020, 1: 5000 dilution), from abcam, anti-Akt, (catalog #9272, 1:1000 dilution), anti-phospho-Akt S473 (catalog#4060, 1:1000 dilution), anti-ERK1/2 (catalog#4695, 1:1000 dilution), anti-E-cadherin (catlog#14472, 1:1000 dilution), anti-N-cadherin (catlog#13116, 1:1000 dilution), anti-Vimentin (catlog#5741, 1:1000 dilution), anti-p-Tyr (catalog#8954, 1:2000 dilution), anti-phospho-EGFR (Y1068) (catalog#2234, 1:1000 dilution) and anti-phospho-ERK1/2 (catalog#4370, 1:1000 dilution), HSP90 (catalog#4877, 1:1000) from Cell Signaling; and anti-EGFR (catalog#sc-03, 1:1000

dilution), anti-GAPDH (catalog#sc-47724, 1:1000 dilution), anti-actin (catalog#sc-58673, 1:1000 dilution), and anti-Tubulin (catalog#sc-166729, 1:1000 dilution) from Santa Cruz.

The following antibodies were used for immunofluorescence and Duolink assays: anti-EGFR (catalog#ab-30, Abcam, 1:200 dilution); anti-Rab25 (catalog#HPA010872, Sigma, 1:100 dilution); and anti-EEA1 (catalog#3288, Cell Signaling, 1:100 dilution). The following antibody was used for flow cytometry: Alexa Fluor® 647 mouse anti-human EGFR (catalog#563577, BD Pharmingen, 5ul/test). The following antibody was used for IHC: anti-Rab25 from Sigma (catalog#R8532, sigma,1:100 dilution), anti-EGFR (catalog#ab-30, Abcam,1:200 dilution).

Rab25 transfection

Cells were transfected with DharmaFECT1 siRNA transfection reagent (Dharmacon) as described by the manufacturer's guidelines for 48 h. The siRNAs were generated on the basis of the following sequences: Rab25: 1# 5'-GGAAGACCAATCTACTCTC-3', and 2# 5'-TTGAGCTAGCCTTTGAGAC-3', and 3# 5'-GAACTCATTGCGCGTGAATC-3', Stat5a: 1# 5'- : GCGCTTTAGTGACTCAGAA-3', 2# 5'- ACAGAACCCTGACCATGTA-3', Stat5b: 1# 5'-ATGGGACTCAGTAGATCTT-3', 5'-GCATCACCATTGCTTGGAA-3' (Fu et al., 2019), and the control siRNA sequence was 5'-TTCTCCGAACGTGTCACGT-3'.

Construction of lentiviruses and cell transduction

Full-length Rab25 was cloned into the pLV-EF1α-MCS-IRES-Bsd lentiviral vector with a blasticidin-selectable marker (cDNA-pLV03, Biosettia). The Rab25 short hairpin RNA (shRNA) sequence (sh1# GATCCGGGAAGACCAATCTACTCTCTCAAGAGAGAGAGTAGATTGGTCTT CC TTTTTTACGCGTG, sh2#GATCCG TTGAGCTAGCCTTTGAGACTCAAGAGAGTCTCAAAGGCTAGCTCAA TTTTTTACGCGTG, sh3#GATCCGGAACTCATTGCGCGTGAATCTCAAGAGA GATTCACGCGCAATGAGTTC TTTTTTACGCGTG) and the control shRNA sequence (GATCCG TTCTCCGAACGTGTCACGTTCAAGAGAACGTGACACGTTCGGAGAA TTTTTTACGCGTG) were cloned into Lenti-X™ shRNA Expression Systems (Clontech) with a puromycin-selectable marker. The Gag-Pol + Rev expression vector and VSV-G expression vector packaging plasmids were co-transfected with the pLV-EF1α-MCS-IRES-Bsd-Rab25 construct or the shRab25 construct in HEK293T cells for 48 h according to the manufacturer's instructions. The virus was collected 48 h after transfection, and cells were selected with blasticidin (8 μg/ml) or puromycin (1 μg/ml), respectively.

Immunoblotting

Cells were harvested and lysed in RIPA buffer plus protease inhibitor cocktail and phosphatase inhibitor cocktail (Selleck). The protein samples were separated by SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membranes (0.22 and 0.45 μm, Millipore, Bedford, MA, USA).

Subsequently, the membrane was blocked with 5% skim milk in TBST buffer (TBS containing 0.1% Tween-20) for 1 h at room temperature and then hybridized with primary antibody with gentle agitation overnight at 4°C. After being washed with TBST three times, the membrane was incubated with HRP-conjugated secondary antibody (Santa Cruz) for 1 h at room temperature. Bands were detected with a Pierce ECL Western Blotting Substrate detection system (Thermo Scientific) and visualized using a Gel Doc™ XR+ System (Bio-Rad). Three independent experiments were performed.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from cells according to the manufacturer's protocol using TRIzol reagent (Invitrogen). The dissolved RNA sample was measured on a spectrophotometer to determine the concentration and quality before being converted to complementary DNA (cDNA). RNA samples were reverse transcribed using a SuperScript III First-Strand Synthesis SuperMix for qPCR Kit (Life Technologies, California, USA), and quantitative real-time PCR (qRT-PCR) analysis was performed using a CFX-96 Real-time PCR System (Bio-Rad, Hercules, CA, USA). The values were expressed as fold changes compared with the corresponding values for the control using the $2^{-\Delta\Delta Ct}$ method.

PCR array analysis

Quantitative RT-PCR for cell death-associated genes was performed using a Cell Death Pathway Finder PCR Array (PAHS-212Z, QIAGEN, MD, USA) according to the manufacturer's protocol. For data analysis, a web-based software from data

analysis center of QIAGEN was used. The fold-changes were then calculated and expressed as log-normalized ratios of the values from the CNE2R cells to those of the CNE2 cells.

Tumor xenografts and radiotherapy

All of the animal experiments were approved by the Animal Ethics Committee of Xiangya Hospital Central South University and followed the Guidelines of Animal Handling and Care of Central South University. The BALB/c nude mice used in our animal model were 4- to 6-week-old female mice. Cells ($5x10^5$ cells/mouse) stably transduced with or without the Rab25 shRNA lentivirus were suspended in 100 μl of RPMI-1640 medium mixed with Matrigel (1:1) and injected subcutaneously into the left flank of the mice for NPC or lung cancer tumor formation. Tumor growth was recorded every other day with a caliper according to the following formula: tumor volume = the shortest diameter^2 × the largest diameter × 0.5. After the tumors reached a volume of approximately 200 mm^3, the irradiation treatment groups were irradiated with 2 Gy locally at the tumor every two days, for a total dose of 4 Gy.

For establishment of radio-insensitive NPC xenografts, CNE2 cells ($5x10^5$ cells/mouse) were injected subcutaneously into the left flank of the mice for NPC tumor formation (named P0 mice). When the tumors reached a volume of approximately 200 mm^3, tumor tissues were harvested and divided into equally sized pieces (volume of 1-2 mm^3) immediately after removal from the mouse. Approximately 5-6 tumor pieces were then inoculated subcutaneously into the left flank of recipient mice for tumor formation (named P1 mice); the remaining tissue

was fixed in 10% formalin for IHC analysis. An X-ray treatment of 5 Gy was administered locally to the tumor for two days when the tumor volume of the P1 mice reached 200 mm^3. After 5 days of treatment, the mice were sacrificed, and the tumors were immediately cut into small pieces for inoculation into the left subcutaneous flank of recipient mice for tumor formation (named P2 mice). When the tumor pieces had been transferred to the P11 mice, we compared the growth rate of tumors between P2 and P11 mice. Data are presented as the tumor volume (n=4, Mean ± SD, *$p<0.05$). Statistical analysis was performed using the Student's t test and the software Graphpad Prism.

Immunohistochemistry

Paraffin-embedded slides were incubated at 70°C for 90 min and then deparaffinized with xylene and rehydrated. Endogenous peroxidase activity was quenched with 3% H_2O_2 solution for 15 min at 37°C, and antigen retrieval was performed in a pressure cooker using sodium citrate buffer (10 mM, pH 6.0). Sections were immuno-stained with antibodies against Rab25 and EGFR. All immunostainings were performed with the avidin-biotin-peroxidase complex technique in combination with diaminobenzidine (DAB), and the slides were counterstained with hematoxylin and eosin (H&E) for surgical pathology specimens. The use of NPC patient tumor samples in this study was approved by the Human Ethical Committee of Xiangya Hospital of Central South University.

The immunoreactive proteins in the tissue were evaluated with scores based on the proportion of positive tumor cells. Random 3-5 sections of each sample and 5-6

high-power fields (HPF) of each section were used for analysis and quantification. Fields with different staining intensity were counted separately when the staining was not homogeneous. According to the IHC staining intensity, the following criteria were used for interpretation of IHC data. High expression was defined as strong staining intensity in more than 75% of tumor cells (score=4); medium expression was moderate staining intensity in more than 50% of tumor cells (score=3); low expression was weak staining intensity in more than 25% of tumor cells (score=2); negative expression was weak staining in less than 25% of tumor cells (score=0-1).

Immunofluorescence

Cells were quickly rinsed with pre-warmed PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at 37°C, permeabilized in 0.5% Triton X-100 for 20 min and blocked with BlockAid^{TM} blocking solution (Thermo Fisher) for 1 h. After labeling with primary antibodies overnight at 4°C, cells were washed in PBS and incubated with Alexa Fluor-conjugated secondary antibodies for 45 min at room temperature. All antibody incubations were performed in BlockAid^{TM} blocking solution. The coverslips were mounted with DABCO anti-fade agent on glass slides and imaged using DeltaVision OMX SR (GE) equipped with a 63x/1.4 numerical aperture oil-immersion objective. For quantification of the EGFR internalization in early and late endosomes or to evaluate the co-localization of EGFR and Rab25, the software MetaMorph was used to analyze the co-localization of signals from the Rab25 and EEA1 channels.

Immunoprecipitation

Cells were fixed with 4% PFA for 15 min and lysed in RIPA buffer. Protein A/G magnetic beads were first suspended in binding buffer (50 mM Tris, 150 mM NaCl, 0.1%-0.5% Triton 100 or Tween 20, pH 7.5) and then incubated with 5 μg Rab25 antibody or normal rabbit IgG antibody for 1 h at room temperature with end-over-end rotation. After the supernatant was removed, 200 mg of cell lysate was added to each tube, which was incubated with rotation for 10 min at room temperature. The immunoprecipitated proteins were released by boiling for 5 min at 95°C in SDS–PAGE sample buffer. The magnetic beads were removed with a magnetic separator before the samples were loaded onto a 12% SDS–PAGE gel.

Duolink proximity ligation assay

The in situ proximity ligation assay was performed using a Duolink® In Situ Red Starter Kit for Mouse/Rabbit (DUO92101, Sigma) according to the manufacturer's instructions. Briefly, cells were seeded onto coverslips and circled with a hydrophobic pen the day before the experiment. After treatment, the cells were fixed, permeabilized, blocked, and then incubated with primary antibodies at 4°C overnight. After washing, the oligonucleotide (Minus and Plus)-conjugated secondary antibodies were added and incubated for another hour at 37°C. Subsequently, cells were washed and incubated with ligation solution for 30 min at 37°C. The ligated nucleotide circles were amplified using polymerase via the addition of amplification solution and incubation for 100 min at 37°C. The slides were washed briefly, and Duolink® In Situ Mounting Medium with DAPI (DUO82040, Sigma) was added to each sample to

stain cell nuclei for fluorescence microscopy. The visualized fluorescence spots represented the clusters of protein-protein interactions.

EGF uptake and EGFR internalization assay

Cells were serum-starved for 6 hours and used for either EGF uptake or EGFR internalization assays. For the quantitative EGF uptake assay, cells were treated with 1 ng/ml or 100 ng/ml EGF-Alexa488 (Invitrogen) for the indicated time at 37°C. For the EGFR internalization assay, cells were treated with 1 ng/ml EGF (RD) for the indicated time at 37°C. A plate incubated in RPMI-1640 medium without ligand for 30 min or 180 min was used as a control. EGF uptake was halted by placing the cells on ice followed by two washes in ice-cold phosphate-buffered saline (PBS). To remove surface-bound EGF, cells were washed three times for 5 min each with cold acid buffer (RPMI-1640/0.2% BSA, pH 3.5 adjusted with HCl). The 'pulse-chase' assay was conducted as previously described (Kondapalli et al., 2015). Cells were treated 1 ng/ml EGF for 30 min and then transferred to ice to halt EGFR internalization. After three washes with cold acid buffer, the plates used for observing the EGFR recycling process were cultured in RPMI 1640 with glutamine (Hyclone) in an incubator at 37°C and 5% CO_2 for the indicated time. The EGFR recycling process was halted by transferred the plates to ice. For the quantitative EGF uptake assay, cells were detached from the culture dishes, centrifuged at 300 ×g for 5 min at 4°C and suspended in 0.2% BSA in PBS. For the EGFR internalization assay, cells were detached and incubated with Alexa 647-EGFR antibody in PBS for 20 min. After washing once with 0.2% BSA in PBS, cells were fixed in 4% PFA in PBS. Flow

cytometry was performed using a Millipore Guava® easyCyte HT Sampling Flow Cytometer.

Flow cytometry analysis of anoikis and apoptosis

An anoikis resistance assay was performed by seeding cells in a 6-well ultra-low attachment surface polystyrene culture dish (Corning 3473) using regular culture medium. For cell apoptosis analysis, the cells were collected at different time points and resuspended in Annexin V binding buffer supplemented with Annexin V and propidium iodide (Roche). Anoikis was quantitated by flow cytometry, and the degree of apoptotic cell death (%) was determined by flow cytometry (Millipore) using Annexin V-FITC/PI staining.

Colony-forming assay

Cells were seeded at a density of approximately 200 cells per well in six-well plates and irradiated with various doses 24 h after plating. The cells were then cultured for 12 days to allow colony formation. Only the single clones that contained more than 50 cells were counted. During colony growth, the culture medium was replaced every 3 days. Each treatment was performed in triplicate.

Tumor sphere formation assay

Single-cell suspensions were seeded into 6-well ultra-low attachment surface polystyrene culture dish (Corning 3473) at a density of 500 cells/ml. Cells were grown in serum-free medium, supplemented with B27 (Life Technology), 20 ng/ml EGF (Biovision), 20 ng/ml basic-FGF, and 4 μg/ml heparin (VWR). Cells were cultured for 10 days and tumor spheres were counted, sphere size were measured and

calculated under light microscopy and collected for further experiments. Three independent experiments were done in triplicate.

Kaplan-Meier survival analyses

TCGA RNA-seq data for LUAD samples were obtained from UCSC Xena browser (http://xena.ucsc.edu/). The overall survival or disease-free survival of LUAD patients were evaluated using the Kaplan–Meier method, and statistical differences in survival times were determined using the log-rank test as described elsewhere. The cohort include 720 lung adenocarcinoma samples obtained from Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=service&cancer=lung) were used to generate the overall survival analyses of LUAD patient based on Rab25 (Affy ID, 218186_at) status. Patients were split by median expression of Rab25. The cohort include 478 LUAD samples were obtained and analysis by GEPIA website for disease free survival analysis. A log rank test was used to test for differences of more than one survival curve.

Quantification and statistical analysis

Data was presents as mean ± standard deviation (SD). GraphPad Prism was used for statistical analysis. ANOVA analysis was used for comparison study of more than two data groups and student's t-test was used to compare two groups of independent samples. Pearson correlation test was utilized to evaluate the association of staining intensities of EGFR and Rab25 in NPC patients who received radiotherapy. Image J was used to quantified and normalized data. Significance was indicated as follow: NS, not significant; * $p<0.05$; ** $p<0.01$.