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A. Colocalization of EGFR and EEA1 (%) Control vs CD44 KD

B. Colocalization of CD44 and EEA1 (%) Control vs CD44 KD

C. Colocalization of CD44 and LAMP1 (%) Control vs CD44 KD

D. Lipid Rafts Distribution (%)

E. BD blot

F. CD44 Rab7A LAMP1 Merge

G. CD44 LAMP1 EGFR Merge
A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Rab7A/T22N</th>
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<tr>
<td>EGFR</td>
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<tr>
<td>Rab7A/T22N</td>
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<tr>
<td>Actin</td>
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B

**GST-Pull down**

- GFP-HA-Rab7A
  - WT
  - Q67L
  - T22N

**Input**

- Actin
- Coomassie Blue
- GST-RILP-RBD

C

**Colocalization of EGFR and Rab7A (%)**

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<tr>
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<th>Ctrl (n=10)</th>
<th>CD44KD (n=10)</th>
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<tr>
<td>CD44 KD</td>
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<tr>
<td>Rab7A/T22N</td>
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D

**His-Rab7A**

- CD44s-FLAG
- His-Rab7A
- CD44s-FLAG

E

<table>
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<tr>
<td>Rab7A/T22N</td>
<td>-</td>
</tr>
<tr>
<td>p-AKT</td>
<td>-</td>
</tr>
<tr>
<td>AKT</td>
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<td>Actin</td>
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F

**LAMP1 EGFR Phalloidin Merge**

**CD44 KD**

- Rab7A/T22N
- CD44 KD
- Rab7A/T22N CD44 KD

Wang et. al._Figure S3
SUPPLEMENTAL INFORMATION

Supplemental Figure Legends

Figure S1. (A) Western blot analysis of CD44s and CD44v isoforms in HT1080 and HMLE cells (left panel), and control or CD44-knocked down (CD44 KD) U87MG cells (right panel). (B) EGFR degradation assay in HMLE cells. Cells were serum starved and then stimulated with EGF (100 ng/ml) for the indicated time points. CD44 knockdown efficiency was shown by western blot on the left panel. (C) CD44s overexpression (O/E) in 293FT cells attenuated EGF-induced EGFR degradation and sustained AKT signaling. pBrit-HA-EGFR-HA were cotransfected with a control vector or pBrit-CD44s-HA cDNA in 293FT cells. Cells were serum starved and stimulated with EGF (100 ng/ml) for the indicated times. (D) CD44v overexpression in 293FT cells did not attenuate EGF-induced EGFR degradation. pBrit-EGFR-HA were cotransfected with a control vector or pBrit-CD44v3-10-HA cDNA in 293FT cells. Cells were serum starved and stimulated with EGF (100 ng/ml) for the indicated times. (E) CD44 KD did not alter the mRNA levels of EGFR. HT1080 Control and CD44 KD cells were starved and stimulated with EGF (100 ng/ml) at the indicated time intervals. qRT-PCR analysis was performed using RNA extracted from the above cells. (F) Cyclohexymide (CHX) did not suppress EGFR degradation in HT1080 CD44 KD cells. EGFR degradation assay was performed by treating HT1080 control or CD44 KD cells with EGF (100 ng/ml) in the absence or presence of CHX (50 μg/ml). (G) CD44 KD did not affect the initial internalization of EGFR by FACS assay. HT1080 control or CD44 KD cells were serum starved and stimulated with EGF (100 ng/ml) for the indicated time. The mean fluorescence intensities were used to measure the cell surface EGFR level by FACS as described in the
Supplemental Materials and Methods section. The mean fluorescence intensity of control cells at starvation time point (0 min) was normalized to 100%. (H) CD44 KD did not affect the initial internalization of EGF. HT1080 control and CD44 KD cells were serum starved for 24 hours. Cells were replaced and incubated with serum starve media containing EGF conjugated to Alexa Fluor® 488 (green) at 4 °C for 1 hour. Then cells were incubated in pre-warmed starve media at 37 °C to allow EGF/EGFR internalization for the indicated time points. BF, Bright Field; Scale bars, 10 µm.

**Figure S2.** (A) CD44 KD did not alter EGFR distribution in the early endosomes. HT1080 control or CD44 KD cells were plated on cover slides, serum starved, and stimulated with EGF (100 ng/ml) for 15 min followed by fixation and staining of EGFR (red) and an early endosomal marker EEA1 (green). Confocal images and quantification of colocalization were shown. (B) CD44 preferentially localized in the late endosomes/lysosomes at 30 min after EGF (100 ng/ml) stimulation. The co-localization percentage (% intensities above threshold) in panel A & B was analyzed using the Fiji software. The colocalization values of the indicated numbers (n) of cells are presented as mean ± SEM.; ***: p < 0.001 (Student’s t-test). Scale bars: 10 µm. (C) Colocalization of CD44 and EGFR. HT1080 cells were starved (0 min) and stimulated with EGF (100 ng/ml) for 30 min. Cells were fixed and costained for CD44 (green) and EGFR (red). Scale bars indicate 10 µm. (D) CD44s was sedimented in lipid rafts. CD44s-overexpressing HT1080 cells were subjected to fractionation by ultracentrifugation. 12 fractions were collected from the top to the bottom and subjected to the detection of CD44 and Flotillin-1. The intensities of CD44 and Flotillin-1 in each fraction were
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quantified and plotted. (E) Western blot analysis depicts expression of GAL4-BD-CD44s and GAL4-AD-Rab7A in the yeast two-hybrid assay (corresponding to Fig. 2D) (F, G) Confocal imaging analysis of colocalization of CD44, Rab7A, LAMP1, and EGFR. GFP-HA-Rab7A plasmid were transfected in HT1080 cells, starved and stimulated with EGF (100 ng/ml) for 30 minutes. The cells were stained with indicated antibodies. Scale bars indicate 10 µm.

**Figure S3.** (A) The dominant negative mutant of Rab7A (Rab7A/T22N) inhibited EGF-induced EGFR degradation. HA-tagged Rab7A/T22N-overexpressing HT1080 cells were stimulated with EGF (100 ng/ml) for the indicated times and analyzed by western blots. (B) RILP proteins interacts with GTP bound Rab7A. 293FT cells were transfected with GFP-HA-Rab7A WT, or Q67L and T22N mutant constructs. Equal amount of GST-RILP-RBD protein was incubated with the above cells lysates for GST-pull down, followed by GFP-HA-Rab7A detection with an HA-HRP antibody. The recombinant GST-RILP-RBD protein was visualized by Coomassive Blue staining. The GFP-HA-Rab7A WT protein contains additional 20 amino acids between GFP and the HA tag, running slightly slower than the Rab7A mutants in SDS-PAGE. (C) CD44 KD promoted EGFR accumulation in Rab7A-containing endosomes. HT1080 control and CD44 KD cells were transfected with the GFP-HA-Rab7A plasmid and stimulated with EGF (100 ng/ml) for 15 min. Images and quantifications of EGFR (red) and Rab7A (green) were shown. (D) The recombinant His-Rab7A protein was detected with Coomassive Blue staining and CD44s-FLAG in the FLAG-immunoprecipitated fraction was detected with a FLAG antibody. (E) Ectopic expression of Rab7A/T22N restored p-AKT activity in
CD44 KD cells. Western blot images were shown in HT1080 control, CD44 KD, or CD44 KD overexpressing Rab7A/T22N cells after 1 h of EGF (100 ng/ml) stimulation. (F) Images showing LAMP1 (green) and EGFR (red) localization in cells stained with Phalloidin (F-actin). The cells were serum starved and stimulated with EGF (100 ng/ml) for 30 minutes. Scale bars, 10 µm.

**Figure S4.** (A) Western blot images of CD44 isoform detection in GBM cells. Locations of CD44s and CD44v were shown. GBM cells preferentially expressed CD44s with no detectable CD44v. (B) CD44s depletion inhibited GIC neurosphere formation and the effects were abrogated when silencing Rab7A. Representative images, and quantification number of neurospheres in control and CD44 KD GICs were shown. Data are mean ± SEM.; n=3. *: p < 0.05 (Student’s t-test). Scale bars, 10 µm. (C) c-Met protein level was decreased in CD44s-depleted GBM cells. Cell lysates were collected 48 h after plating. (D) CD44 KD did not affect mRNA levels of c-Met in GBM cells. (E) Enforced expression of Rab7A/T22N partially restores c-Met protein expression in CD44 KD cells. (F) CD44 KD diminished HGF-induced p-AKT signaling and showed additive inhibitory effect with SU11274. U87MG control and CD44 KD cells were starved and stimulated with HGF (30 nM) in the presence or absence of SU11274 (10 µM) for 1 h. Western blot images are shown. (G) CD44 KD in combination with SU11274 (10 µM) showed additive effect on inhibiting GBM cell viability. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test).
Figure S5. (A) TCGA analysis of 172 GBM patient specimens showed that CD44s was increased in recurrent tumors. (B) Gene Ontology analysis showed pathways that were significantly associated with the CD44s gene signature. (C) GSEA analysis demonstrates the positive association of the EGFR pathway signature and CD44s-correlated gene list in colon and rectum adenocarcinoma, liver hepatocellular carcinoma, pancreatic adenocarcinoma, and lung cancer.
Supplemental Materials and Methods

Plasmid constructs

The following plasmid constructs were purchased from addgene: GST-RILP-RBD (#79149), GFP-HA-Rab7A WT (Plasmid #12605), GFP-HA-Rab7A/T22N (Plasmid #28048), GFP-HA-Rab7A/Q67L (#28049). The cDNA coding for Rab7A WT and T22N were amplified with the above constructs as the templates and sub-cloned into the pBrit-HA/FLAG vector with a HA tag on its c-terminus. The Rab7A WT cDNA was also cloned into pET Duet vector, with an N-terminal His-tag. The mouse cDNA of CD44 was amplified from NIH3T3 cDNA and cloned into a pcDNA3.1 vector (pcDNA-mCD44s). The human EGFR, CD44s, and CD44v3-10 cDNA was cloned into pBrit-HA/FLAG vector with a HA tag on its c-terminus (pBrit-EGFR-HA, pBrit-CD44s-HA, pBrit-CD44v3-10-HA). Human CD44s was also cloned into the pcDNA3.1 vector with a FLAG tag on its c-terminus (pcDNA-CD44s-FLAG). All of the above constructs were sent for sequencing to confirm the correct insert. The control and CD44 shRNA plasmids were obtained from Dr. S Godar (1). The targeted shRNA sequence of CD44 is 5’- ATGCAATGTGCTACTGATTGT-3’, and were expressed in a pLKO.1 Lenti-viral plasmid. The control and CD44 shRNA-expressing pLKO.1 plasmids were co-transfected with the lenti-virus packaging plasmids psPAX2 and pMDG. 48 hours after transfection, viral supernatants were collected, filtered, and subjected to infection. Puromycin (1 µg/ml) was used for selecting cells that express control or CD44 shRNA. The Rab7A shRNA plasmid (clones V2LHS_41758 in the pGIPZ vector) was obtained from Open Biosystems (The targeted shRNA sequence of Rab7A is 5’ CGTAGGCTTCAACACAAAT 3’).
**Reagents**

The primary antibodies for western blot are as follows: EGFR (CST, #4267), p-AKT S473 (CST, #4051), AKT (CST, #9272), CD44 (R&D, BBA10), CD44 (Santa Cruz, sc-18849), Actin (Sigma, A5441), HA-HRP (Roche, No. 12013819001), Rab7A (Abcam, ab173250), Flotillin-1 (BD, #610820). The primary antibodies used for immunofluorescence staining are as follows: CD44 (Santa Cruz, sc-18849), EGFR (CST, #4267), EGFR (Santa Cruz, sc-120), EEA1 (Abcam, ab15846) and LAMP1 (CST, #9091). Antibodies for immunoprecipitation are as follows: EGFR (Santa Cruz, sc-120), CD44 (Santa Cruz, sc-18849), HA (Roche, No. 11666606001) and FLAG (Sigma, F1804). Alexa Fluor® 488 EGF (Invitrogen, #E13345), Phalloidin (Invitrogen, #A22281). Inhibitors are as follows: Chloroquine (Sigma, C6628), Cycloheximide (Sigma, C7698), Erlotinib (CST, #5083) and U11274 (Selleckchem, S1080).

**Cell culture**

HT1080, 293FT, U87MG, U373, U251, and U178 cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. GBM initiating cells (GICs) were cultured in the DMEM/F12 media (Gibco 11320-033) supplemented with N2 supplement (Gibco 17502-048), B27 Supplement (Gibco 17504-044), 20 ng/ml epidermal growth factor (EGF, PeproTech), and 20 ng/ml basic fibroblast growth factor (PeproTech) (2). The HT1080 and 293FT cells were purchased from the American Type Culture Collection (ATCC). All cells were incubated at 37°C in a humidified 5% (v/v) CO₂ incubator.
**Generation of stable cell lines**

The retrovirus and lentivirus systems were used to establish gene overexpressing and knockdown stable cell lines respectively as described (3). Briefly, the pBrit HA/FLAG plasmids were transfected together with the retrovirus packaging plasmids VSVG and CMVG/P. The pGIPZ or the pLKO.1 plasmids were transfected together with the lentivirus packaging plasmids psPAX2 and pMDG. The transfection reagents were FuGENE (Roche). After 48 hours of transfection, the virus supernatants were collected and filtered for cell infection. The cells were selected using puromycin (1 μg/ml) and the survived cell populations were subjected to gene overexpression or knockdown analysis.

**Western blot and immunoprecipitation**

Cell lysates were collected with the RIPA lysis buffer (50mM Tris–HCl, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktails (Roche). The lysates were centrifuged and protein concentrations of the supernatants were measured for the western blot analysis. For the co-immunoprecipitation assays, cells lysates were collected with lysis buffer (20mM Tris-HCl, 100 mM NaCl, 5mM EDTA, 0.2% NP40, 16% Glycerol, 20 mM NaF, 1mM Na3VO4, 20 mM, β-glycerophosphate) supplemented with protease inhibitor cocktails (Roche). The lysates were clarified by centrifugation at speed of 12,000 rpm for 5 minutes. 1 mg of cell lysates were incubated with the primary antibody and agitated overnight. Protein A or Protein G beads (depending on the species of the IP antibody) were added next day and agitated for 4 hrs. Beads were washed three times with the lysis buffer and boiled for 10 min in 40 ul 2xSDS sample buffer (100 mM Tris-HCl, 4% SDS,
20% glycerol, 2% β-mercaptoethanol and 0.005% Bromophenol Blue). For identification of CD44s-interacting proteins, Lysates from HT1080 cells that overexpressed CD44s-FLAG were subjected to ultracentrifugation. The CD44s-containing lipid rafts fractions were combined for immuno-precipitation (IP). The proteins associated with FLAG-protein A beads were eluted with FLAG peptides (150 ng/µl) and subjected to Mass Spectrometry analysis.

**Immunofluorescence**

Cells were plated on cover slides, starved and stimulated with EGF (100 ng/ml). Then cells were fixed with 4% Paraformaldehyde for 10 minutes followed by 10 minutes of 0.2% Triton X-100 permeabilization and 1 hr of blocking with 1% BSA in PBS at room temperature. The primary antibodies were incubated overnight and subjected to the corresponding fluorescent dye conjugated secondary antibody labeling. The localizations were observed and the pictures were taken using Nikon C2 confocal instrument and the co-localizations (the intensities above threshold) were analyzed using the Fiji software. The percent (%) co-localization was calculated by the thresholded Manders coefficients, which were expressed as percentages to show the fraction of intensities in one channel above threshold that was colocalized with intensities in the other channel above threshold. At least two independent experiments were performed.

**Fluorescence-activated cell sorting (FACS)**

EGFR internalization was assayed by flow cytometry as described (4). Briefly, the cells were starved and stimulated with or without EGF (100 ng/ml) for 5 min, 15 min and 30
min, respectively. The cells were placed on ice to stop internalization, Non-internalized EGF were washed with ice-cold acid stripping buffer (DMEM, 0.2% BSA, pH 3.5 adjusted with HCl). Cells were detached using TrypLE and were incubated with an EGFR antibody (Santa Cruz, sc-120) on ice for 1 h followed by FITC conjugated secondary antibody incubation on ice. The cells were then subjected to flow cytometry analysis. The mean surface EGFR fluorescence values of control cells in the absence of EGF were set as 100%. The percentage of EGFR in response to EGF stimulation at different timepoint was plotted.

**Rab7A activity assay**

The Rab7A activity was measured using the Rab7A Activation Assay Kit (Abcam, ab173250). Briefly, Rab7A-overexpressing cells were stimulated with EGF (100 ng/ml) for 15 minutes. 2 mg of protein lysates were incubated with the monoclonal antibody that specifically recognizes Rab7A-GTP, but not Rab7A-GDP, and protein A/G beads for 2 h in the cold room. The beads were centrifuged, washed with the lysis buffer, and boiled with 2xSDS sample buffer for western blot detection of the immune-precipitated active Rab7A by the total Rab7A antibody.

**Cell viability assay**

Cell viability assays were performed as described (5). Briefly, cells were plated in 96-well plates. The next day cells were incubated with RTK inhibitors (RTKI) or DMSO in the starved media for the following 3 days. Cell viability was measured using the CellTiter-Glo Assay Kit (Promega, #G7571) according to manufacturer’s instructions.
For measuring the cell viability of GIC, cells were treated with RTKI in GIC culture media the next day after plating. Each assay consisted of two or three replicate wells and was repeated at least three times. The luciferase value reflecting the cell viability was obtained. The cell viability was normalized to that of DMSO-treated control cells, which was set as 100%.

**EGFR degradation**

HT1080 or U87MG cells were seeded on 6 well plates. After 24 h of serum starvation, cells were stimulated with EGF (100 ng/ml) for the indicated time periods. Cells were collected in 1xSDS sample buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.0025% Bromophenol Blue) and lysates were used for western blot analysis.

**Yeast two-hybrid**

The CD44s and Rab7A cDNA was fused to the GAL4 binding domain (BD) (pGBK-T7 vector) and activation domain (AD) (pGAD-T7 vector) respectively. The BD and AD plasmids were co-transformed into yeast AH109 strains using His (+) [SD (Leu-, Trp-)] plates. The transformants with the desired protein expression were plated on His (+) and His (-) [SD (Leu-, Trp-, His-)] plates. The strains were incubated at 30 °C till visible colonies appeared. Protein expression was analyzed by Western-Blot using TCA precipitated cell lysates from the transformants grown on the His (+) [SD (Leu-, Trp-)] plates.
GST- RILP-RBD pull down Assay

The GST-RILP-RBD plasmid was obtained from addgene (#79149) (6) and transformed into the *Escherichia coli* strain BL21. The GST-RILP-RBD protein expression was induced by IPTG (0.2 mM) for 4 hours at room temperature. IPTG induced bacteria were collected and resuspended in lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, 10 mM pyrophosphate, 10 mM glycerophosphate, and protease inhibitor cocktails). After sonication, cleared bacterial lysates were incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 3 hours at 4°C, washed with lysis buffer, and resuspended in lysis buffer as a 50% slurry. The GST-RILP-RBD conjugated beads were incubated with mammalian cell lysates expressing either endogenous or exogenous Rab7A proteins overnight. Beads were washed three times with lysis buffer, followed by boiling in 2× Sample buffer (+DTT) for 5 minutes.

EGF internalization

The experiment was performed as described (7). Briefly, HT1080 control and CD44 KD cells were serum starved for 24 hours. Cells were replaced and incubated with serum starve media containing Alexa Fluor® 488 conjugated EGF (Invitrogen, # E13345) at 4 °C for 1 hour. This step allows EGF binding to EGFR but does not allow EGF/EGFR internalization. Cells were then incubated in pre-warmed starve media at 37 °C to allow EGF/EGFR internalization. Cells were washed with an acidic washing buffer (0.2M acetic acid pH 2.8, 0.5 M NaCl) to cleave any residual EGF/EGFR bound to the plasma membrane at different time intervals and fixed with 4% PFA. EGF/EGFR internalizations were observed under a fluorescence microscope, and the pictures were acquired and
processed using the AxioVision software (Carl Zeiss).

**Rab7A GTP hydrolysis assay**

The Rab7A cDNA was cloned into a pET Duet vector, with a His-tag at the N terminal. The resulting His-Rab7A plasmid was transformed into BL21 for IPTG induction for 4 hours at room temperature. The Rab7A protein was purified with Ni-NTA Agarose (QIAGEN, #30230). Control and CD44s-FLAG overexpressing plasmids were transfected into 293FT cells. The CD44s-FLAG protein was enriched with anti-FLAG antibody beads (Sigma, #F2426). Intrinsic Rab7A GTPase activity was measured according to the manufacture’s instruction (Promega, #V7681). Briefly, the GTP hydrolysis reaction was initiated by incubating 1μg Rab7A proteins, FLAG beads (Control) or CD44s-FLAG beads, 5μM GTP and 0.5mM DTT in GTPase/GAP Buffer for 2 hours at room temperature. This step allows for GTP hydrolysis of Rab7A. The reconstituted GTPase-Glo™ Reagent was added to the completed reactions, and incubated for 30 minutes at room temperature. This step allows for the conversion of the remaining GTP to ATP. Detection Reagent was added and incubated for 10 minutes at room temperature. This allows for detection of ATP using a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and luciferin substrate to produce bioluminescence. The signal was obtained with a luminescence microplate reader (BMG LABTECH). The GTP hydrolysis activity of the Rab7A protein was inversely correlated to the amount of light produced.
Statistics

P values were calculated using 2-tailed unpaired Student’s t test. P values of less than 0.05 were considered statistically significant. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

References: