Supplemental Data

RNF144A sustains EGFR signaling to promote EGF-dependent cell proliferation

Shiuh-Rong Ho and Weei-Chin Lin

Content:

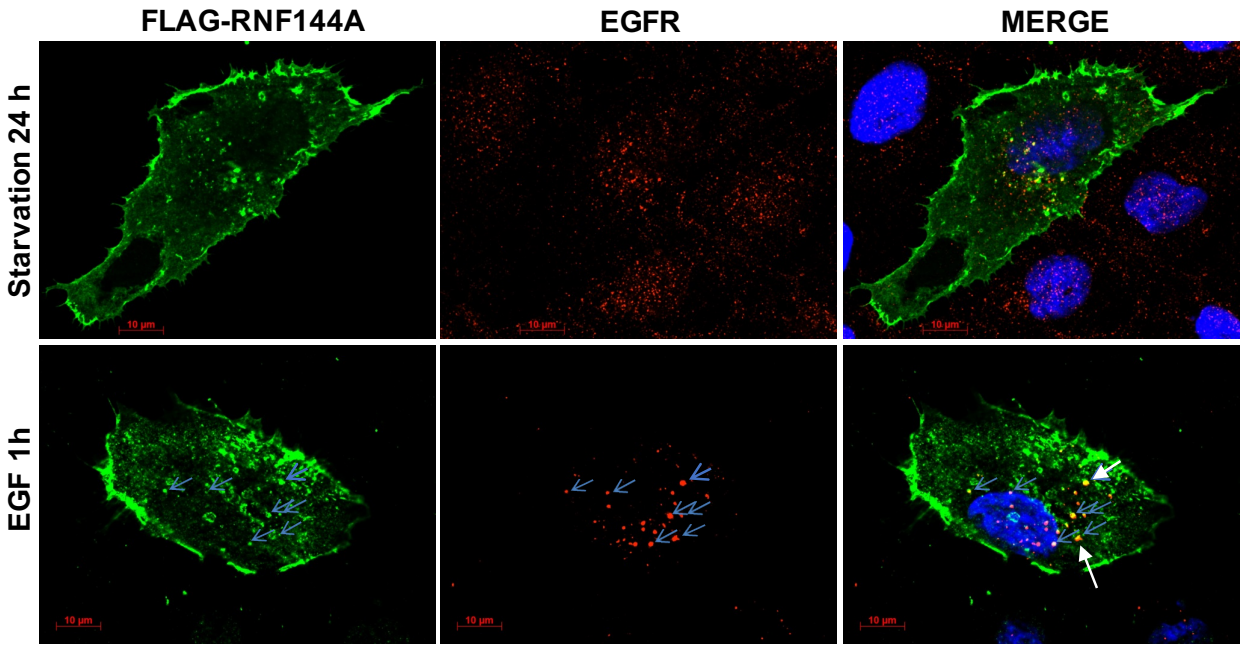

Figure S1. EGF induced colocalization of FLAG-tagged RNF144A (Green) and EGFR (Red) in the intracellular vesicles. Immunofluorescence analysis (X100) shows that EGF induced colocalization of RNF144A (Green) and EGFR (Red) in the intracellular vesicles (blue arrows; for clarity of the presentation, a few representative dots of colocalization are indicated by white arrows) upon EGF stimulation in U2OS cells. FLAG-tagged RNF144A was transfected into U2OS cells for 16 hours. The transfected cells were washed twice with 1X PBS and then incubated in 0.1% BSA for 24 hours. Then the cells were further treated with 50 ng/ml EGF for 1 hour. Cells were then fixed with 4% Paraformaldehyde for 10 minutes followed by permeabilization with 0.5% Triton-X 100 in 1X PBS for 10 minutes. The slides were then stained with anti-FLAG and anti-EGFR antibodies, followed by a fluorescein isothiocyanate- or Texas Red X-conjugated secondary antibody, respectively. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope equipped with ApoTome 2 (Axio Observer Inverted Microscope).

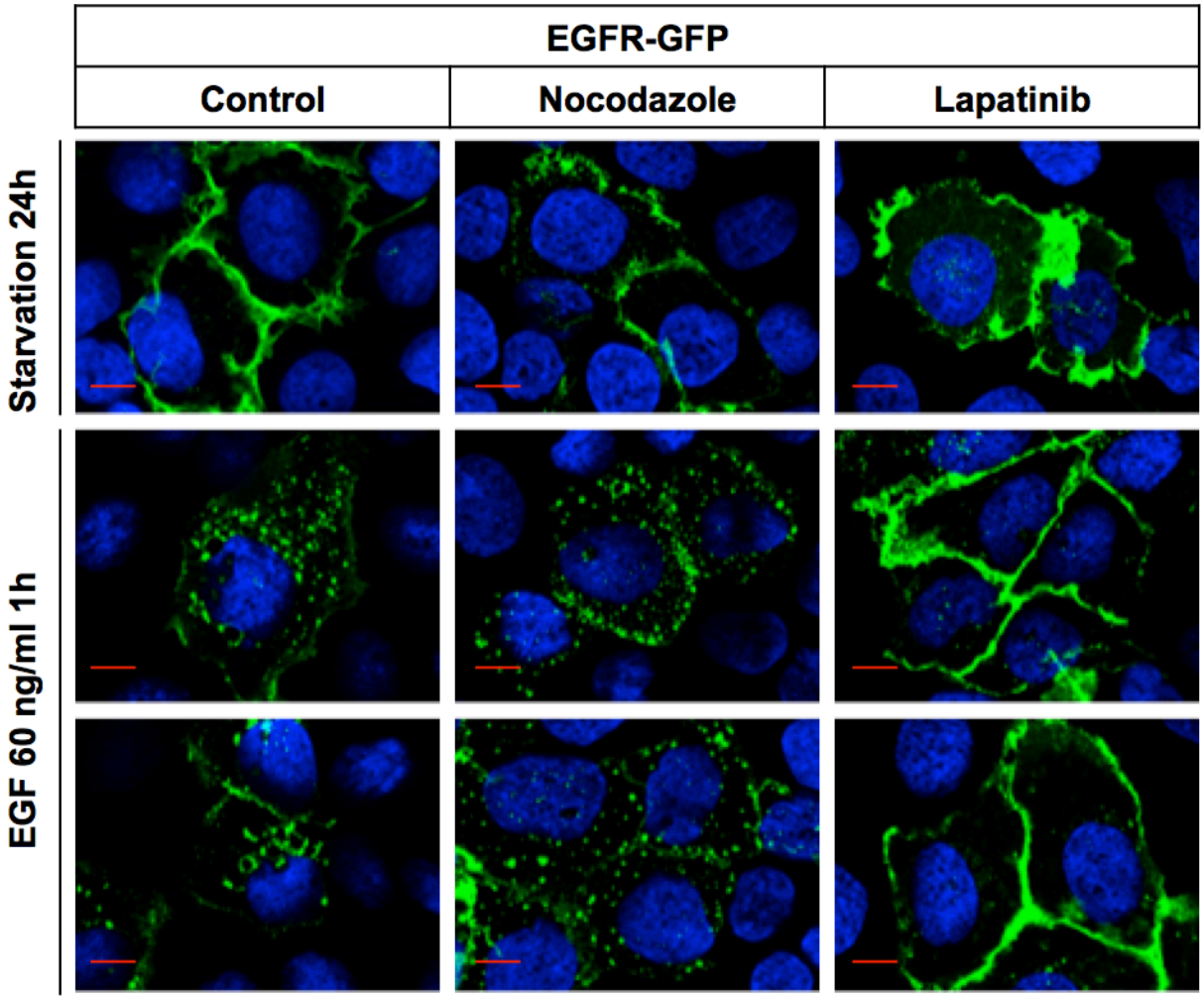

Figure S2. Lapatinib, but not Nocodazole, blocks endocytosis of EGFR upon EGF stimulation. Fluorescence analysis (X100) shows that Lapatinibi blocked EGF-mediated endocytosis of EGFR. U2OS cells were transiently transfected with EGFP-tagged EGFR for 24 hours. Transfected cells were starved for 24 hours and pretreated with Lapatinib (10 nM, 4 hours) or Nocodazole (1 μg/ml, 15 minutes). Then the cells were further treated with 60 ng/ml EGF for 1 hour. Cells were fixed with 4% paraformaldehyde for 10 min followed by permeabilization with 0.5% Triton-X 100 in 1X PBS for 10 min. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope (Axio Observer Inverted Microscope). Red bars: 10 μm. Green: EGFP-tagged EGFR; Blue: DNA.

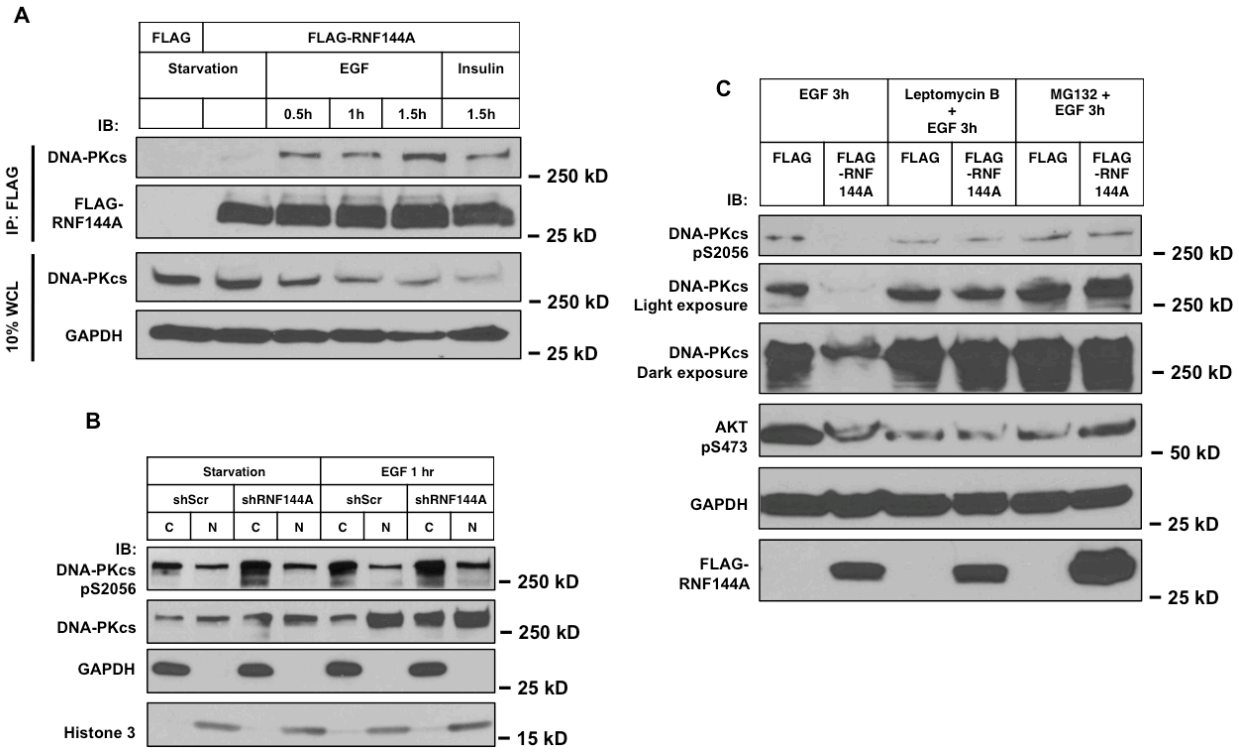

Figure S3. RNF144A regulates EGF-dependent proteasomal degradation of DNA-PKcs. (A) Western blot analysis shows that both EGF and insulin stimulation induced RNF144A-DNA-PKcs interaction and decreased DNA-PKcs protein. Cells were transfected with a FLAG empty vector or FLAG-tagged RNF144A for 24 hours, followed by incubation of 0.1% BSA without serum for additional 6 hours. Cell starvation was released by EGF or insulin for the indicated time. Cell lysates were harvested for FLAG IP and Western blot. **(B)** Western blot analysis shows depletion of RNF144A increased phospho-serine 2056 and total form of DNA-PKcs in both cytosol (labeled as "C") and nucleus (labeled as "N") after EGF treatment. **(C)** Western blot analysis shows nucleus export inhibitor Leptomycin B and proteasome inhibitor MG132 restored RNF144A-mediated decrease of phospho-serine 2056 and total form of DNA-PKcs.

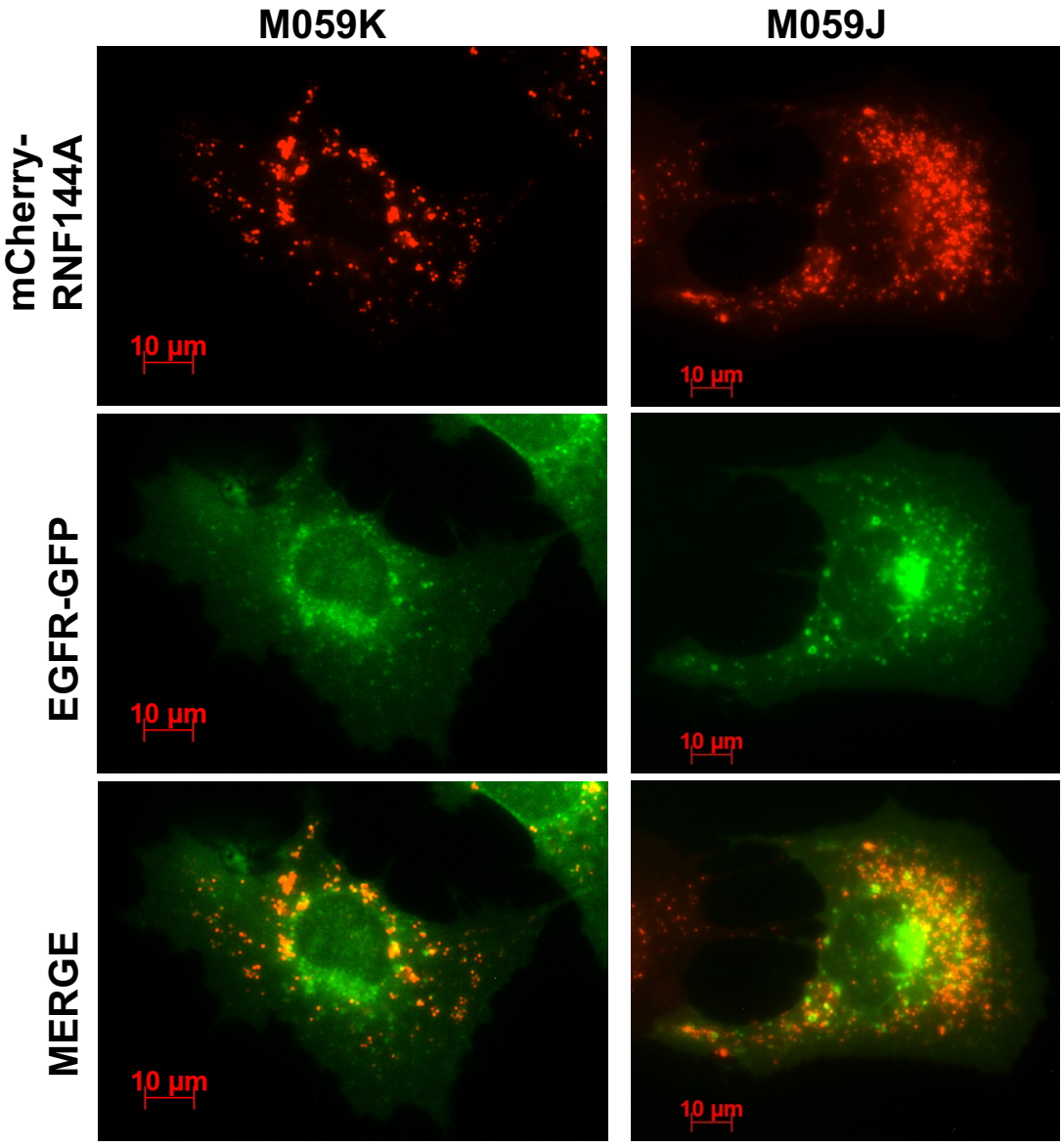

Figure S4. Colocalization RNF144A and EGFR in both DNA-PKcs-proficient cells (M059K) and -deficient cells (M059J). Live image analysis (X100) shows that EGF induced colocalization of RNF144A (Red) and EGFR (Green) in the intracellular vesicles upon EGF stimulation in both M059J and M059K cells. Cells were co-transfected with mCherry-tagged RNF144A and EGFP-tagged EGFR for 24 hours. Transfected cells were starved for 24 hours and the treated with 50 ng/ml EGF for 40 minutes. Red bars: 10 µm.

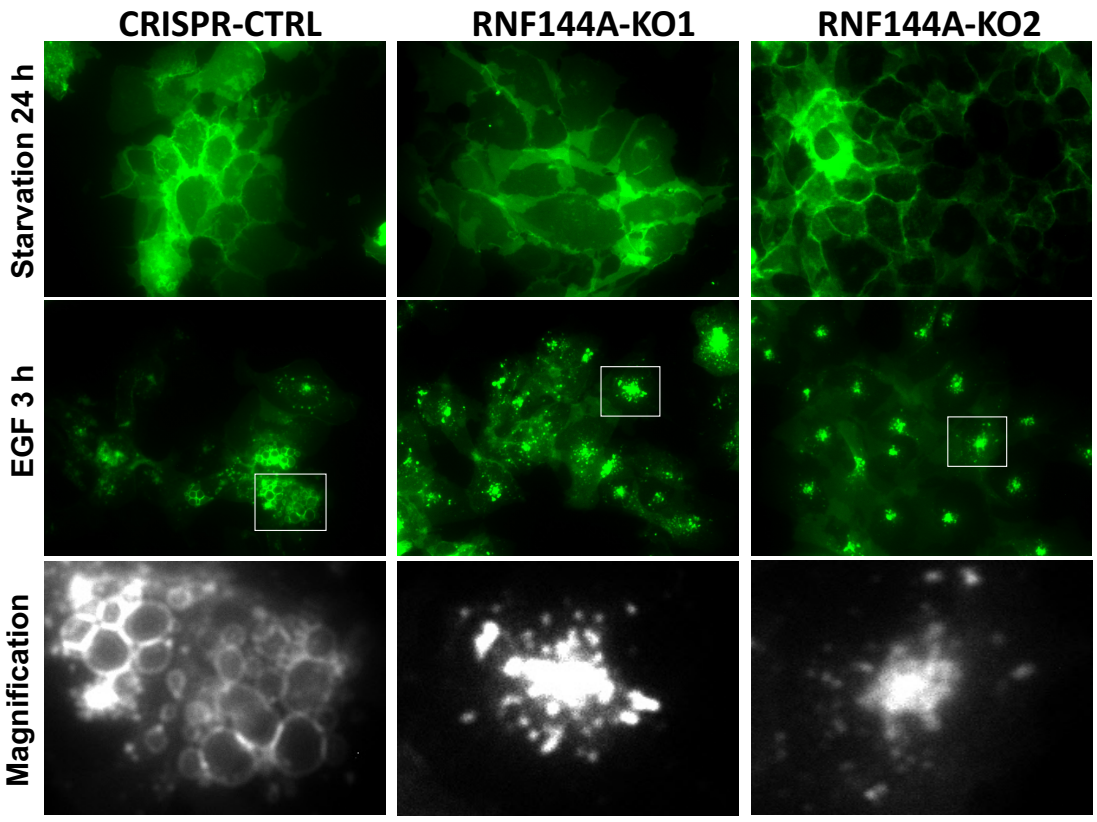

Figure S5. Depletion of RNF144A reduces EGF-induced formation of the intracellular big ring-shaped EGFR vesicles after 3 hours of EGF stimulation. Immunofluorescence analysis (X40) shows EGF treatment significantly induced big ring-shaped EGFR-GFP positive vesicles in the CRISPR-CTRL cells, but not in two RNF144A-depleted cell lines. Bottom panels show high magnification of images from the corresponding white open boxes in the middle panels. EGFP-tagged EGFR was transfected into CRISPR-CTRL and RNF144A KO U2OS cells. Next day, cells were starved in BSA for 24 h. Some cells were then treated with EGF (50 ng/ml) as indicated.

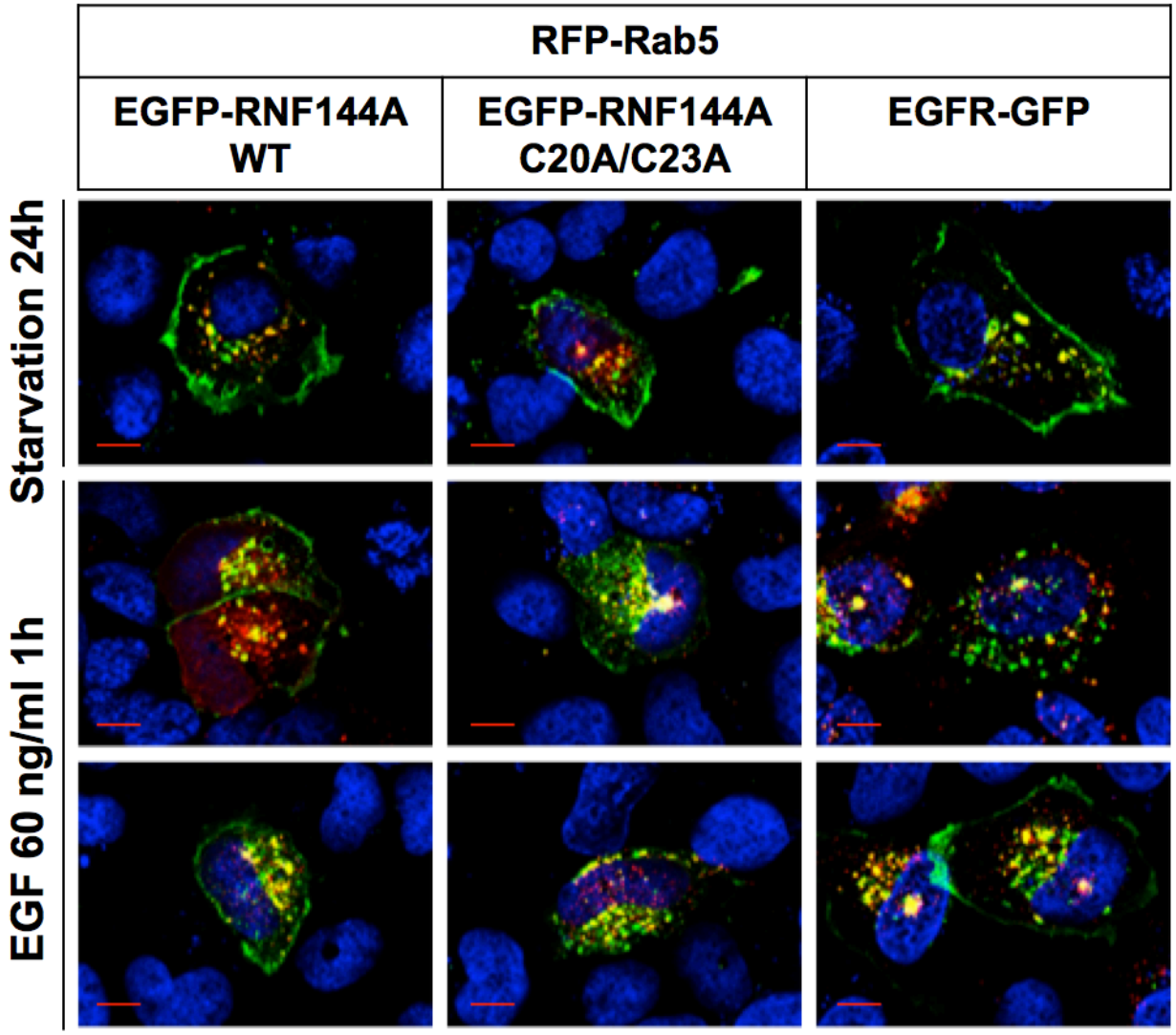

Figure S6. Colocalization between Rab5 with wild-type RNF144A, ligase-dead mutant RNF144A(C20A/C23A), and EGFR upon EGF stimulation. Fluorescence analysis (X100) shows that colocalization between Rab5 with RNF144A, RNF144A(C20A/C23A) and EGFR. U2OS cells were transiently co-transfected with RFP-Rab5 and EGFP-tagged EGFR or RNF144A wild-type or RNF144A(C20A/C23A) for 24 hours. Transfected cells were starved for 24 hours prior a treatment of 60 ng/ml EGF for 1 hour. Cells were fixed with 4% paraformaldehyde for 10 min followed by permeabilization with 0.5% Triton-X 100 in 1X PBS for 10 min. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope (Axio Observer Inverted Microscope). Red bars: 10 μm. Red: RFP-Rab5; Green: EGFP-tagged RNF144A wild-type, C20A/C23A, or EGFR; Blue: DNA.

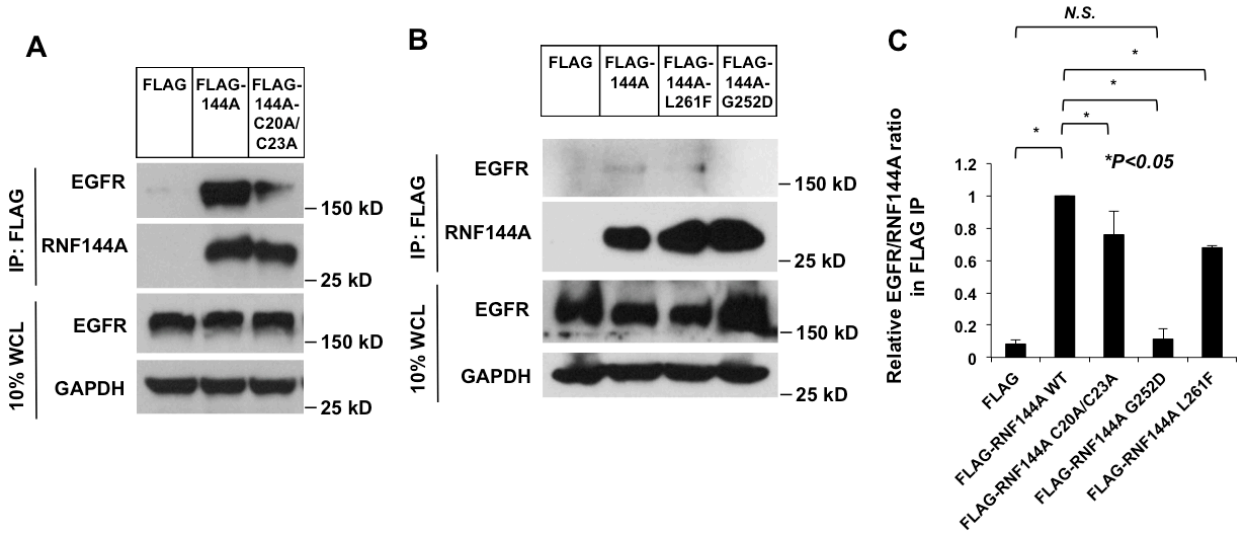

Figure S7. Reduced interaction between EGFR and RNF144A mutants. Western blot analysis shows a significant reduction in the interaction between the RNF144A mutants and EGFR in 293T cells. Cells were transfected with FLAG-tagged empty control vector or FLAG-tagged RNF144A wild-type and mutants for 24 hours. The transfected cells were starved for 24 hours and then were pretreated 30 minutes of proteasome inhibitor MG132 and lysosome inhibitor CQ prior to EGF treatment for 1 hour (50 ng/ml). **(A and B)** EGF stimulated the interaction between EGFR and RNF144A wild type, RNF144A ligase-dead mutant (RNF144A-C20A/C23A) and RNF144A TM domain somatic mutants (RNF144A-G252D and RNF144A-L261F). **(C)** Quantification of EGFR/RNF144A ratios from three independent experiments of FLAG IPs from (A) and (B). Data represent means ± S.D. *N.S.*=Not significant.

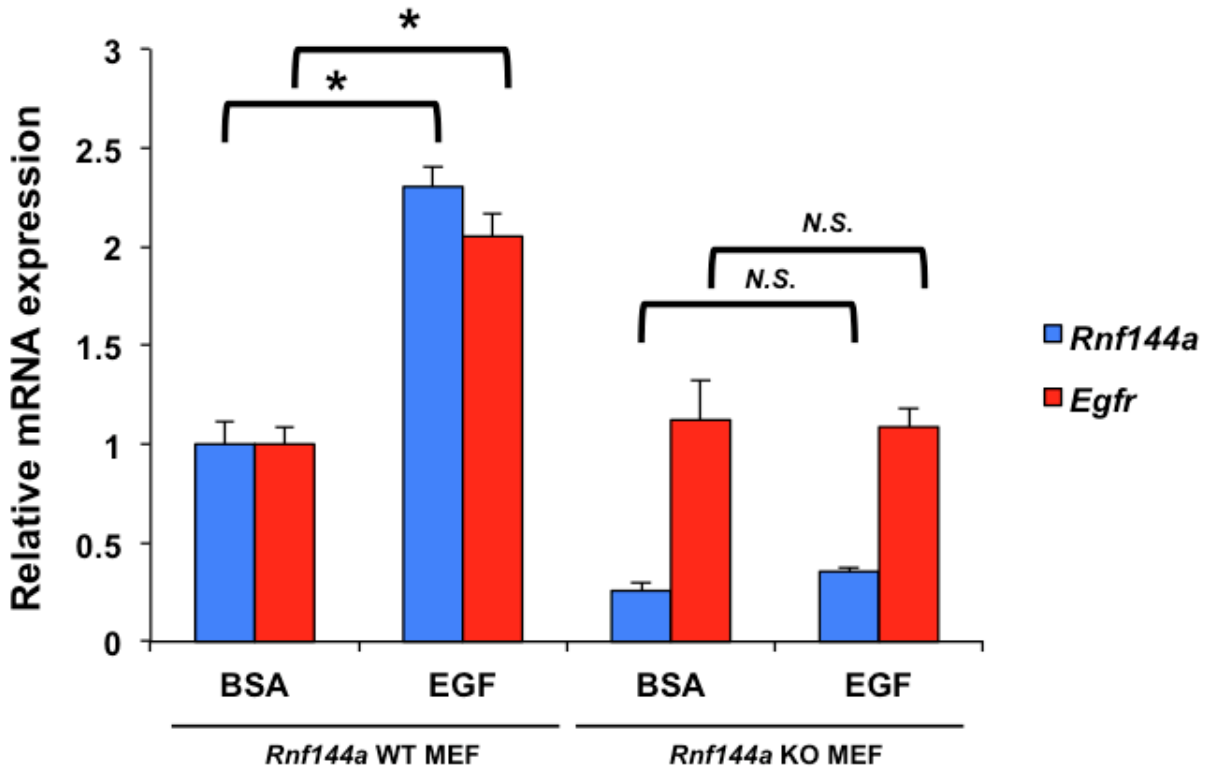

Figure S8. Knockout of Rnf144a in mouse embryonic fibroblasts (MEFs) inhibits EGF-dependent up-regulation of Rnf144a and Egfr expression. Real-time quantitative RT-PCR analysis shows that EGF induced the expression of Rnf144a and Egfr in the Rnf144a wild type (WT) but not in the Rnf144a knockout (KO) transformed MEFs. Rnf144a WT and KO MEFs were generated as described in Materials and methods, and real-time RT-PCR was performed. The cells were starved in 0.1% BSA for 36 hours, and then released by EGF (40 ng/ml) treatment for 24 hours. Results were normalized to GAPDH and the means ± S.D. (n = 3) were expressed relative to the expression of genes in starved Rnf144a WT MEFs. * $p< 0.05$; *N.S.*=Not significant.

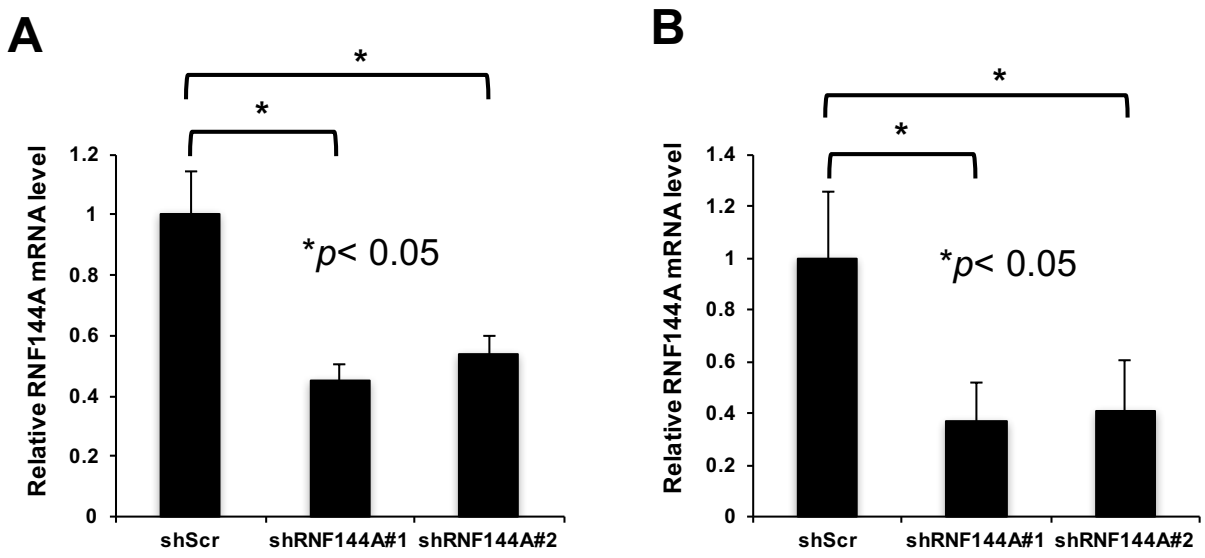

Figure S9. Establishment of RNF144A knockdown stable cells. HepG2 cells (A) and M059J cells (B) were transfected with two different validated RNF144A shRNA #1 and #2 constructs containing a puromycin resistant gene, and then selected by puromycin for three weeks. Stable cells were used in the subsequent experiments. Real-time quantitative PCR were performed to confirm the establishment of RNF144A knockdown HepG2 (A) and M059J (B) stable cells. Results were normalized to GAPDH levels and the means ± S.D. (n = 3) are expressed relative to the expression of RNF144A in scramble control cells. *= $p< 0.05$. RNF144A primer set: Human RNF144A-qPCR-1F: 5′-CTGTTTGATCCCTGTCGGACT-3′; Human RNF144A-qPCR-1R: 5′-GATGGGCGCGTCATCTTCTT-3′.

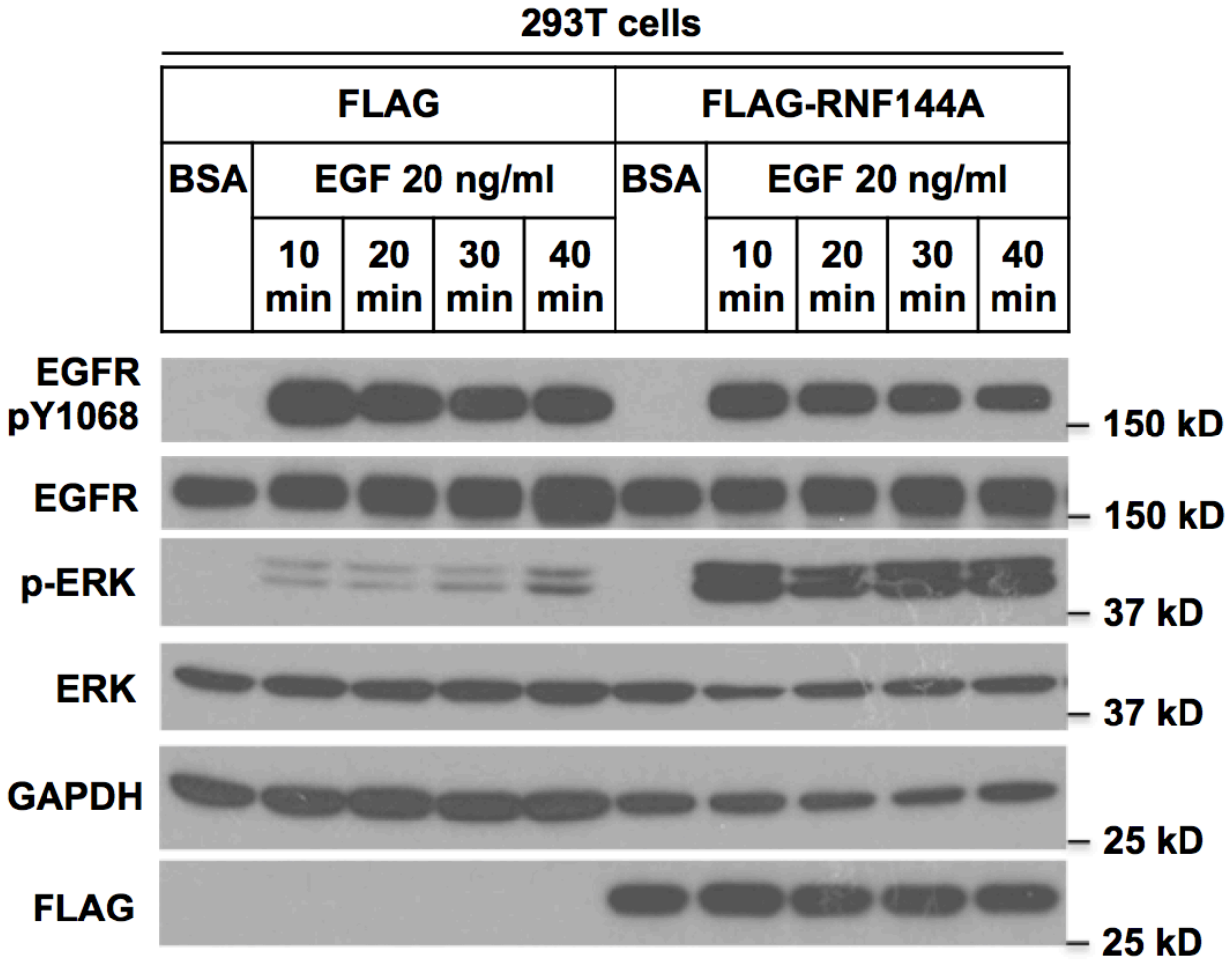

Figure S10. Overexpression of RNF144A increases EGF/EGFR signaling. Western blot analysis shows that overexpression of wild-type RNF144A induced the EGF-dependent phosphorylation of ERK T202/Y204. Cells were transfected with a FLAG empty vector or FLAG-tagged RNF144A for 24 hours, followed by incubation of 0.1% BSA without serum for additional 24 hours. Cell starvation was released by EGF stimulation for the indicated time. Cell lysates were harvested for Western blot analysis.

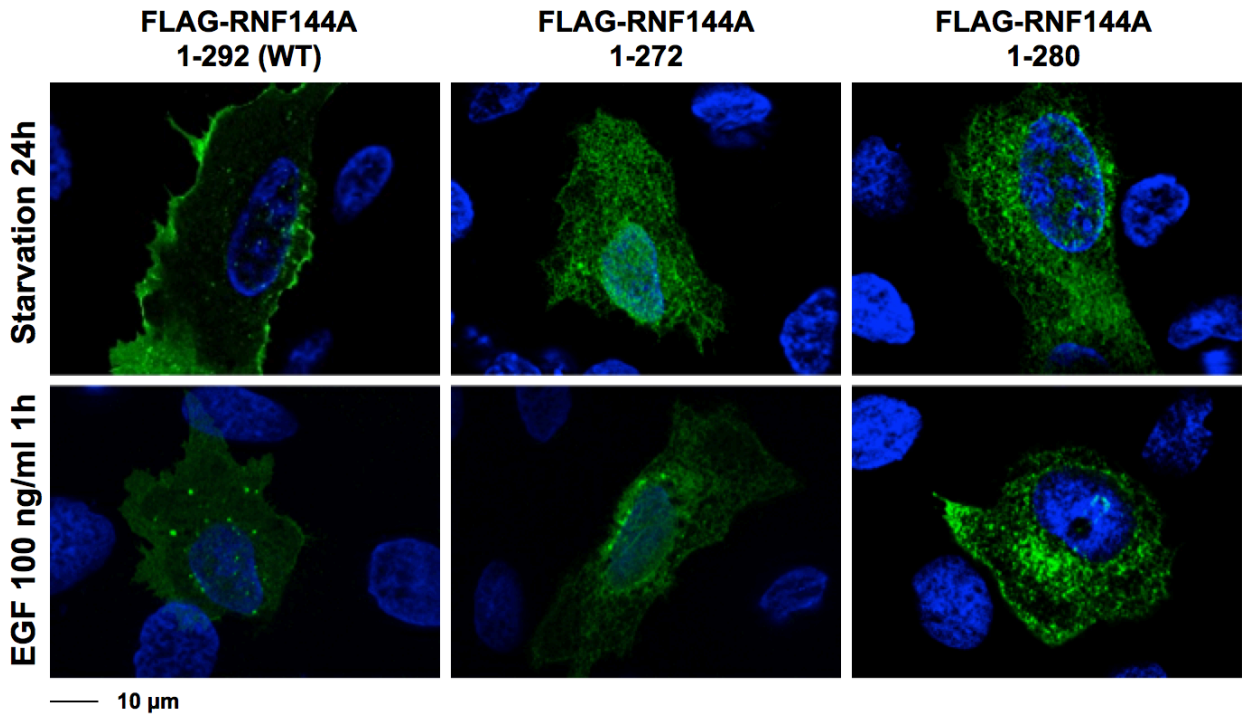

Figure S11. RNF144A(1-272) and RNF144A(1-280) have different subcellular localization pattern. Fluorescence analysis (X100) shows that FLAG-RNF144A(1-272) and RNF144A(1-280) truncate mutants have an ER-like net pattern distribution. U2OS cells were transiently transfected with indicated plasmids for 24 hours. Transfected cells were starved for 24 hours prior a treatment of 100 ng/ml EGF for 1 hour. Cells were fixed with 4% paraformaldehyde for 10 min followed by permeabilization with 0.5% Triton-X 100 in 1X PBS for 10 min. The nuclei were stained with Hoechst 33258. Images were captured on a Zeiss fluorescence microscope (Axio Observer Inverted Microscope). Green: FLAG-tagged RNF144A wild-type, a.a.1-272, and a.a. 1-280; Blue: DNA.

Movie S1. RNF144A transports with EGFR in the cytoplasm after EGF stimulation. Time-lapse movie of U2OS cells transiently expressing EGFR-GFP and mCherry-RNF144A. The movie was filmed using Zeiss fluorescence microscope for 5 minutes (5 seconds/frame) after 20 ng/ml EGF treatment (X100). Green signal: EGFR-GFP; red signal: mCherry-RNF144A. Cells were seeded on glass bottom dishes (MatTek corporation) and transfected with EGFR-GFP and mCherry-RNF144A for 24 hours. The cells were starved for 24 hours before 20 ng/ml EGF stimulation. After EGF treatment, the movie was taken between 20 and 25 minutes after EGF treatment.