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# Phosphorylation of REPS1 at Ser709 by RSK attenuates the recycling of Transferrin receptor

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**Abstract**

RalBP1 associated EPS domain containing 1 (REPS1) is conserved from *Drosophila* to humans and implicated in the endocytic system. However, an exact role of REPS1 remains largely unknown. Here, we demonstrated that mitogen activated protein kinase kinase (MEK)-p90 ribosomal S6 Kinase (RSK) signaling pathway directly phosphorylated REPS1 at Ser709 upon stimulation by epidermal growth factor (EGF) and amino acid. While REPS2 is known to be involved in the endocytosis of EGF receptor (EGFR), REPS1 knockout (KO) cells did not show any defect in the endocytosis of EGFR. However, in the REPS1 KO cells and the KO cells reconstituted with a non-phosphorylatable REPS1 (REPS1 S709A), the recycling of transferrin receptor (TfR) was attenuated compared to the cells reconstituted with wild type REPS1. Collectively, we suggested that the phosphorylation of REPS1 at S709 by RSK may have a role of the trafficking of TfR.

**Running Title:** REPS1-mediated recycling of transferrin receptor

**Keywords:** attenuation, phosphorylation, RSK, recycling, transferrin receptor

## Introduction

Receptor tyrosine kinases, such as EGF receptor (EGFR) undergo the rapid internalization and degradation after the binding of their cognate ligands (1-5). In contrast, the transferrin (Tf) receptor (TfR), which binds di-ferric Tf to deliver iron into the cell, internalizes constitutively and is recycled back to the plasma membrane (6-8). Both of the receptors utilize the clathrin coated pit for the internalization and subsequent endocytic machinery. The exact molecular mechanisms that confer the differences in the itinerary of the endocytosed EGFR and the TfR are, however, not entirely clear.

RalBP1 associated EPS domain containing 1 and 2 (REPS1 and REPS2) are first known to be associated with endocytosis and conserved from fly to human. REPS1 and REPS2 consist of 796 amino acids (87 kDa) and 660 amino acids (71 kDa), respectively. They all have two Eps homology (EH) domain, EF-hand domain, and RalBP1 interaction site with high homology (9). REPS2, a much more studied one among two paralogues, has been implicated in endocytosis (10-12). It has been demonstrated that Repts2 regulates internalization of receptors, such as the EGFR via AP-2 complex of clathrin-coated vesicles, attenuating the receptor activities (13-16). In case of REPS1, it has been shown that Intersectin 1, one of the components of AP-2 complex, binds to REPS1 (17). However, its role in the receptor trafficking remains unclear.

Recently, a company have generated an antibody against phospho-S709-REPS1. The sequence around S709 took our attention because it was RXRXXS/T motif that was the consensus phosphorylation motif of AGC kinases, such as Akt, S6K1, and p90 ribosomal S6 kinase (RSK) (18, 19). Using various inhibitors for those kinases, we found out that RSK was

a major kinase that phosphorylated REPS1 at S709. We showed that RSK directly binds and phosphorylates REPS1 via co-immunoprecipitation and *in vitro* kinase assay, and knockdown of RSK diminished the phosphorylation of REPS1 at S709. When REPS1 knockout (KO) cells were reconstituted by REPS1 wild type (WT) and Ser709Ala (SA), REPS1 KO and SA-expressing cells showed a delayed recycling of TfR compared to REPS1 WT-expressing cells, while the recycling of EGFR was not altered. Collectively, these results suggest that REPS1 may have a role in the recycling of TfR and the phosphorylation of REPS1 at S709 is required for the recycling.

## Results

### The phosphorylation of REPS1 S709 was downregulated by MEK and RSK inhibitors

Recently, Cell Signaling Technology has developed an anti-phospho S709 REPS1 (p-REPS1) antibody, which detects the phosphorylation of S709, a consensus phosphorylation site by AGC kinase family. To assess the selectivity of the antibody, 293T cells and REPS1-KO HEK293E cells (Supplementary Fig. 1A) were transfected with Myc-tagged WT and SA REPS1 constructs. Anti-p-REPS1 antibody detected endogenous and exogenous REPS1 WT, but not SA mutant (Fig. 1A). As mentioned earlier, S709 is a part of the conserved consensus phosphorylation motif for AGC kinase family. The kinase group AGC is one of the protein kinase family including protein kinase A (PKA), G (PKG), C (PKC), Akt, and ribosomal protein S6 kinase (RSK). Many of those, such as Akt and PKC, are phosphorylated and activated by mTORC2 (20) and p70 S6 kinase is phosphorylated by mTORC1 (21). Only RSK is phosphorylated by its C-terminal kinase (22). S709 is a part of RXRXXS motif, which may be recognized and phosphorylated by Akt, RSK, and p70S6K, so we tested which kinase is responsible for the phosphorylation of REPS1 at S709. GDC0349 (mTORC1 and mTORC2 inhibitor), rapamycin (mTORC1 inhibitor), BI-D1870 (RSK inhibitor), and U0126 (MEK inhibitor), were treated to the cells and the cell lysates were analyzed by western blotting with anti-p-REPS1 antibody and others. In HEK293E cells, BI-D1870 significantly inhibited the phosphorylation of REPS1 at S709 (Fig. 1B and C). In addition, U0126 have shown the similar effect on REPS1 (Fig. 1B and C), implicating that MEK-ERK-RSK signaling may be involved in the phosphorylation at this site.

### **Epidermal Growth Factor (EGF) or amino acid-induced phosphorylation of REPS1 was reduced by MEK and RSK inhibitors**

The previous study showed that REPS1 is tyrosine-phosphorylated in response to EGF stimulation (23). We wondered whether the phosphorylation at S709 is also regulated by EGF stimulation. EGF stimulation indeed elevated the phosphorylation of REPS1 at S709 significantly, but GDC0349 and rapamycin did not block this phosphorylation (Fig. 2A and B). However, BI-D1870 and U0126 almost abrogated the EGF-induced phosphorylation of REPS1 (Fig. 1A and B). The same phenomena were observed in HeLa cells and Caco-2 cells (Sup. Fig. 2). These data indicated that EGF-induced Ras-MAPK-RSK signaling may elicit the phosphorylation of REPS1.

Next, we tested whether amino acid stimulation, which is known to activate mTORC1-p70S6K signaling, regulates the REPS1 phosphorylation. It was intriguing that amino acid stimulation to HEK293E cells led to the increase in the phosphorylation of REPS1 at S709 (Fig. 2C and D). The mTOR inhibitors (GDC0349 and rapamycin) and U0126 restored the level of phospho-S709 to the control, while BI-D1870 strongly reduced it below the control level (Fig. 2C and D). This result demonstrated that mTOR-p70S6K signaling pathway can modulate the phosphorylation of REPS1 and, however, RSK is still the major kinase responsible for the phosphorylation. Taken together, we suggested that the phosphorylation of REPS1 at S709 is mediated by RSK.

### **RSK directly phosphorylates REPS1 at S709 *in vitro***

Judging from the above data, the phosphorylation of REPS1 at S709 could be inferred that it had a connection with RSK. We first wanted to see if there is an interaction between

REPS1 and RSK. We conducted the co-immunoprecipitation experiment with REPS1 variants (Fig. 3A) and RSK1. SA mutant showed a slightly less avidity toward RSK1, while REPS1 C had a stronger binding to RSK1 than REPS1 N (Fig. 3B).

RSK consists of four isoforms as RSK1, RSK2, RSK3, and RSK4. Because RSK4 has been known to be independent from growth factor stimulation (24), we targeted only RSK1~3 by shRNA. First, when we made shRNA stable cell lines for each RSK1, RSK2, and RSK3, the result showed that only RSK1 seemed to induce a slight decrease in p-REPS1 (Supplementary Fig. 3A). Therefore, we tried to generate the stable cell line expressing shRNAs for all RSK1/2/3. In that case, we were able to observe that the phosphorylation of REPS1 at S709 significantly decreased (Fig. 3C).

Next, to verify that RSK can directly phosphorylate REPS1 at S709, HEK293E cells were transfected with HA-chicken RSK wild type, and constitutively active (CA) and kinase dead (KD) mutants. Endogenous REPS1 S709 phosphorylation increased in RSK1 wild type and CA-transfected cells, but not in KD-expressed cells (Supplementary Fig. 3B-C). Confirming the direct phosphorylation further, we performed *in vitro* kinase assay using Myc-tagged chicken RSK and Flag-tagged REPS1. RSK wild type robustly phosphorylated REPS1 WT, but RSK KD did not (Fig. 3D). In addition, REPS1 SA was not phosphorylated by RSK wild type at all (Fig. 3D). Taken together, these data demonstrated that RSK directly phosphorylates REPS1 at S709 *in vitro* and *in vivo*.

**The phosphorylation of REPS1 at S709 was likely to be required for the internalization of transferrin receptor**



Given that REPS1 is tyrosine phosphorylated after EGF stimulation (23) and interacts with clathrin-coated pit complex (17, 23), we hypothesized that REPS1 and its phosphorylated form at S709 may be involved in the endocytosis of EGF receptor as its paralogue, REPS2, does (12, 13, 16, 25, 26). We made REPS1-KO HeLa cell line using CRISPR/Cas9 technology as described in Materials and Methods, and then, established stable cell lines expressing empty vector, REPS1 WT, and SA in KO cells (Supplementary Fig. 1). Those cells transfected with EGFR-GFP were treated with Alexa-488-conjugated EGF and subject to confocal microscopy. When EGF was bound to EGFR, they internalized and accumulated in the cytoplasmic speckles with or without REPS1 (Fig. 4A). Therefore, we concluded that REPS1 is not related to the endocytosis of EGFR complex.

Recently a published article insisted that REPS1 mutation associated with neurodegeneration with brain iron accumulation may cause the defect in the recycling of TfR (27). To investigate a role of the phosphorylation of REPS1 at S709 in TfR recycling, we observed a kinetics of translocation of TfR using Alexa-555-conjugated transferrin (RTf) in the same HeLa cell lines we prepared above. After the addition of RTf, the internalization of RTf-TfR complexes occurred (Fig. 4B, 0 and 5 min panels) and they accumulated in the perinuclear region and disappeared (Fig. 4B, 15 and 30 min panels of REPS1 WT). However, in the cells expressing empty vector and REPS1 SA, the perinuclear accumulation of RTf was attenuated and significant amount of them remained until 30 min after transferrin treatment (Fig. 4B). These data suggested that REPS1 and its phosphorylation at S709 are required for the recycling of TfR.

## Discussion

In this study, we found out that RSK mediates the phosphorylation of REPS1 at S709 induced by EGF and amino acid stimulation, and showed an evidence that this phosphorylation is associated with the recycling TfR, not with the endocytosis of EGFR. To our knowledge, it was the first evidence that the phosphorylation of REPS1 has a role in its physiological function.

REPS1 has been demonstrated to be associated with several clathrin-coated pit complex proteins and signaling adaptor proteins via its two EH domains and proline-rich motif (13, 15, 16, 25, 28). However, its physiological roles were less studied compared to its closest isoform, REPS2. REPS2 has been demonstrated to be involved in the endocytosis of various receptors, such as EGFR (14) and Insulin receptor (26). It was intriguing that the endocytosis of transferrin receptor was not affected by REPS2 deletion mutant (26). We initially hypothesized that REPS1 may have a similar role with REPS2 because they share almost the same domain structure. However, we clearly demonstrated that the endocytosis of EGFR was unaffected in REPS1 KO and overexpressed cells (Fig. 4A). It seems that it also did not modulate the endocytosis of transferrin receptor like REPS2. We were surprised at the fact that REPS1 KO and SA mutant attenuated the recycling of the receptor (Fig. 4B). These results were consistent with the recent observations in which the mutations of REPS1 found in the patients with brain iron accumulation (NBIA), such as Val78Leu and Ala113Glu, may have caused the delayed recycling of transferrin receptor (27). Although the machinery that mediates the recycling of Tf/TfR complex is not well characterized, a couple of Rab family GTPases and dynamin have been known to be involved (29). There was also a report that Tf/TfR-containing endosomes generate buds in a clathrin-coat dependent manner (30, 31). Since it has been suggested that REPS1 is associated with clathrin-coat protein complex, REPS1 may be

involved in this clathrin-dependent budding process of endosomes to be recycled. In sum, we suggest that REPS1 and its phosphorylation by RSK have a distinct role in vesicle trafficking.

It is of note that the S709 phosphorylation by RSK was induced in response to the treatments of EGF and amino acids. It is well known that EGF activates Ras-ERK-RSK signaling pathway, so it is conceivable that EGF can elicit the S709 phosphorylation. However, there is no evidence that amino acid supplementation can induce RSK activity. Thus, amino acid-mediated RSK activation should be further investigated elsewhere.

In conclusion, the phosphorylation of REPS1 at S709 by growth factor-induced RSK may be required for the recycling of transferrin receptor. The differential role of REPS1 and REPS2 in the context of endocytosis and recycling of membrane receptors should be investigated in the future.

## Materials and Methods

### Cell culture and plasmid construction

HEK293E, HEK293T, HeLa, and Caco-2 cells were maintained in Dulbecco's Modified Eagle Medium (Welgene, Gyeongsan, South Korea) supplemented with 10% fetal bovine serum (FBS), 1X Pen strep (Gibco, Tulsa, USA), and 1X glutamax (Gibco). pcDNA3-3xFlag-REPS1, pcDNA3.1-REPS1-myc His(-), and pcDNA3.1-HA-RSK1 constructs were cloned. EGFR-EGFP plasmid was purchased from Addgene (32751, Watertown, MA, USA)

### Establishing REPS1 KO in 293E and HeLa cell lines.

Knockout target sequences are as follows: REPS1 KO: 5' - CCAAGAAGGTGGTGGTCAAC - 3' (for 293E cell lines), 5' - AGAAGGTGGTGGTCAACGGG - 3' (for HeLa cell lines). REPS1 knockout cell lines were established by pL-CRISPR.SFFV.tRFP vector (57826, Addgene). For REPS1-reconstituted cell lines, REPS1 (WT and SA)-Myc were cloned into pLVX-EF1 $\alpha$ -IRESpuro vector and the cells were infected with the lentivirus from the construct and selected with 1 $\mu$ g/ml puromycin.

### Antibodies and reagents

Primary antibodies used in this study were as follows: anti-phospho REPS1 S709 (12005), anti-REPS1 (6404), anti-phospho Akt S473 (4060), anti-Akt (4691), anti-phospho p70 S6K (9234), anti-p70 S6K (2708), anti-phospho threonine (9386), anti-phospho tyrosine (9416), anti-phospho ERK T202/T204 (4370), anti-ERK1/2 (4695), anti-phospho RSK S380 (11989), anti-RSK1/2/3 (9355), anti-RSK1 (8408), anti-RSK2 (5528), anti-Myc (2278) were

purchased from Cell Signaling Technology (Danvers, MA, USA). anti-phospho serine (61-8100, Invitrogen, Carlsbad, CA, USA), anti-RSK3 (sc-1431, Santa Cruz, Dallas, Texas, USA), anti-GAPDH (LF-PA0212, AB frontier, Seoul, South Korea), anti-Flag-M2 (F1804, Sigma Aldrich, Kenilworth, NJ, USA), and anti-HA (11 867 423 001, Roche, Basel, Switzerland). Flag magnetic beads (A36798) and Myc magnetic beads (88842) were purchased from Thermo Fisher (Waltham, MA, USA). GDC0349 (S8040, Selleckchem, Houston, TX, USA), rapamycin (37094, Sigma Aldrich, Kenilworth, NJ, USA), BI-D1870 (S2843, Selleckchem), and U0126 (1144, Selleckchem) were additionally used for experiments in this study. Alexa-555 EGF (E35350) and Alexa-555 Transferrin (T35352) were purchased from Thermo Fisher.

#### **Preparation of cell lysates and immunoprecipitation**

After cells were washed with cold PBS, they were lysed in lysis buffer A (20mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 2mM EGTA, 1% Triton X-100, 1X phosphatase inhibitor (B15001, Bimake, Houston, TX, USA), 1X protease inhibitor (P3100, GenDEPOT, Katy, TX, USA), 1mM PMSF, 1mM DTT. Each lysates for immunoprecipitation was mixed with the equal volume of lysis buffer and incubated with pre-washed magnetic beads for overnight at 4°C on a rotator. The beads were washed with lysis buffer A three times (Fig. 1A) or lysis buffer A twice, 500mM NaCl lysis buffer A twice, and then lysis buffer A once again (Fig. 3B).

#### ***In vitro kinase assay***

HEK293T cells transfected with Flag-REPS1 wild-type, SA mutant, Myc-chicken RSK wild-type and kinase-dead (KD) mutant (K112R), respectively, were lysed using lysis

buffer B (50mM HEPES KOH pH7.4, 40mM NaCl, 1mM EDTA, 1mM EGTA, 10mM Na pyrophosphate, 10mM Na  $\beta$ -glycerophosphate, 50mM NaF, 1mM NaVO<sub>4</sub>, 1% Triton X-100) containing protease inhibitor. The clarified lysates were immunoprecipitated with anti-Flag-magnetic beads (for Flag-REPS1) or anti-c-Myc-magnetic beads (for Myc-RSK) at 4°C for 3h. The beads were washed twice in lysis buffer B, twice in 500 mM NaCl lysis buffer B, once in lysis buffer B and once in kinase buffer (25mM HEPES-KOH pH7.4, 10mM MgCl<sub>2</sub>, 3mM  $\beta$ -mercaptoethanol, 0.1mg/ml BSA, 1mM DTT). In the last washing step, Flag-REPS1 and Myc-RSK binding beads were combined as indicated. The kinase assay was performed by the method previously described (32).

### **Immunocytochemistry**

HeLa/REPS1 KO cells infected with REPS1 (WT or SA)-Myc were seeded on poly-l-lysine-coated  $\mu$ -slide 8 well plates (80826, ibidi, Grafelfing, Germany). After serum starvation overnight, the plates were put on 4°C for 10 min. The cells were treated with 10 ng/ml Alexa-555 EGF or 25  $\mu$ g/ml Alexa-555 transferrin at 4°C for 50 min. Then, the cells were transferred to 37°C incubator for indicated times. The cells were fixed by 4% paraformaldehyde for 20 min at room temperature. Nucleus were stained by mounting solution with DAPI (ab104139, Abcam, Cambridge, UK) and Fluorescence was visualized using LSM 880 confocal microscope. Scale bar. 20 $\mu$ m.

### **Statistical analysis**

Statistical significance of differences was tested using Student's t test in Microsoft Excel (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.005$ ). Western blots were quantified using ImageJ. The error bars represent standard deviation.

**Figure Legends****Figure 1. Phosphorylation of REPS1 at S709 was controlled by MEK-RSK signaling.**

A. 293T and 293E/REPS1 KO cell lysates transfected with REPS1-Myc constructs were subjected to immunoprecipitation with anti-Myc magnetic beads. Immunoblots were completed with the indicated antibodies. B-E. 293E (B-C) cells were treated with inhibitors, 1 $\mu$ M GDC0349, 1 $\mu$ M rapamycin, 10 $\mu$ M BI-D1870, 10 $\mu$ M U0126, for 30min. The intensity of the p-REPS1 bands was quantified by ImageJ program and divided by total REPS1. All the data were analyzed from two biological experiments to calculate a significance score using Student's t-test. \*,  $p < 0.05$ .

**Figure 2. MEK-RSK mediated the phosphorylation of REPS1 at S709 in response to EGF and amino acid stimulation.**

After 293E cells were serum-starved overnight, these cells were treated with the inhibitors for 30min, and then 10ng/ml human EGF for 1hr (A-B). Or the serum-starved cells were cultured in D-PBS for 1hr and treated with inhibitors for 30min and changed to DMEM including 25mM HEPES, 1X Glutamax for 1hr (C-D). All of the lysates were subject to immunoblots with the indicated antibodies. The intensity of the p-REPS1 bands was quantified by ImageJ program and divided by total REPS1. All of the data were obtained from two independent experiments.

\*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.005$ .

**Figure 3. RSK interacts with REPS1 and phosphorylates p-REPS1 S709.**



A. The domain structure of REPS1 and its mutants. B. 293T cells transfected with Flag-REPS1 and HA-RSK1 plasmids were subjected to co-immunoprecipitation with anti-Flag magnetic beads and the western blot were completed with anti-HA and anti-Flag M2 antibodies. C. HEK239E cells stably expressing RSK1/2/3 shRNA were lysed and the lysates were subjected to immunoblots with the indicated antibodies. D. *in vitro* kinase assay was carried out as described in Materials and Methods. The data presented here was a representative of two independent experiments.

**Figure 4. The phosphorylation of REPS1 at S709 may be required for the recycling of TfR.**

A. REPS1-KO HeLa cells expressing REPS1 wild type or SA mutant were transfected with EGFR-EGFP construct (green). 10 ng/ml Alexa-555 EGF (red) were added to the cells and they were visualized by confocal microscopy as described in Materials and Methods. B. The same cell used in A were treated with 25  $\mu$ g/ml Alexa-555 transferrin (red) and the samples were subjected to a confocal microscopy as described in Materials and Methods.

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**Conflicts of interest**

The authors declare no conflicting interests.

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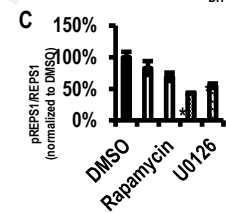
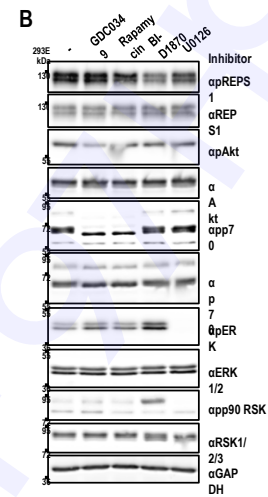
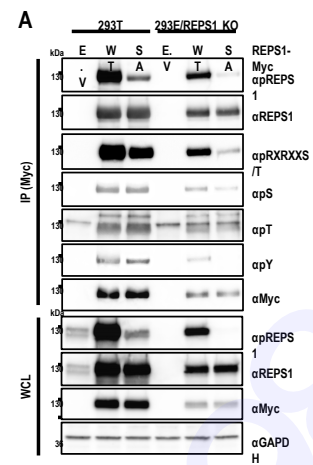


Figure 1

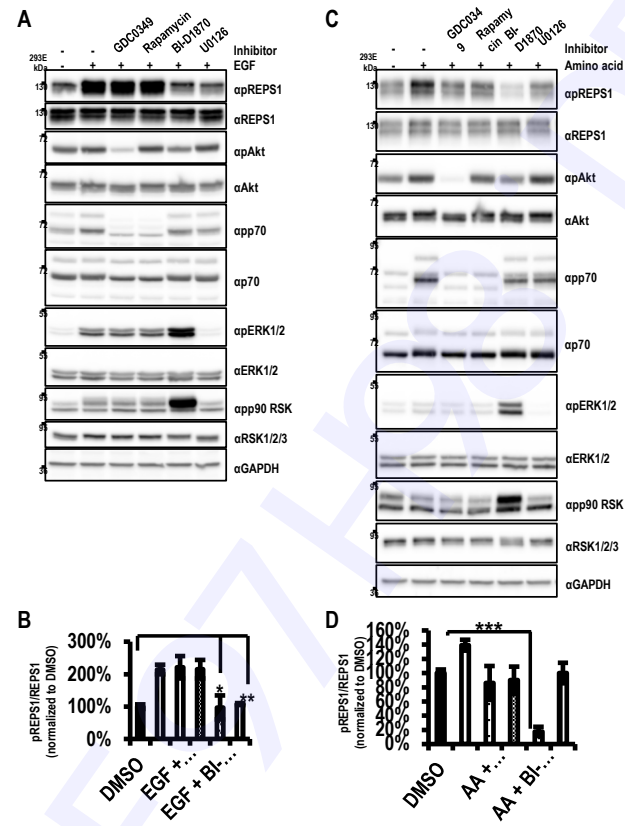


Figure 2





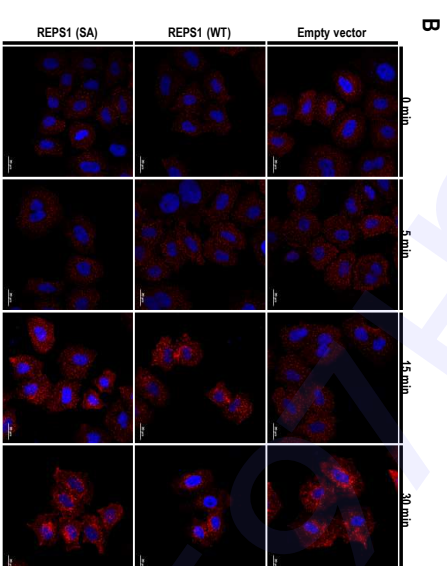
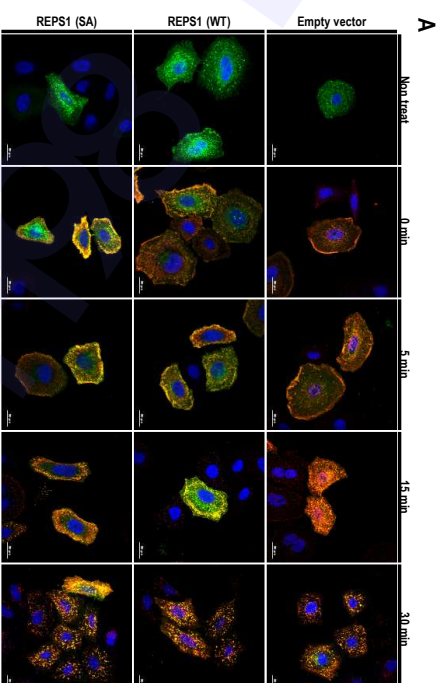
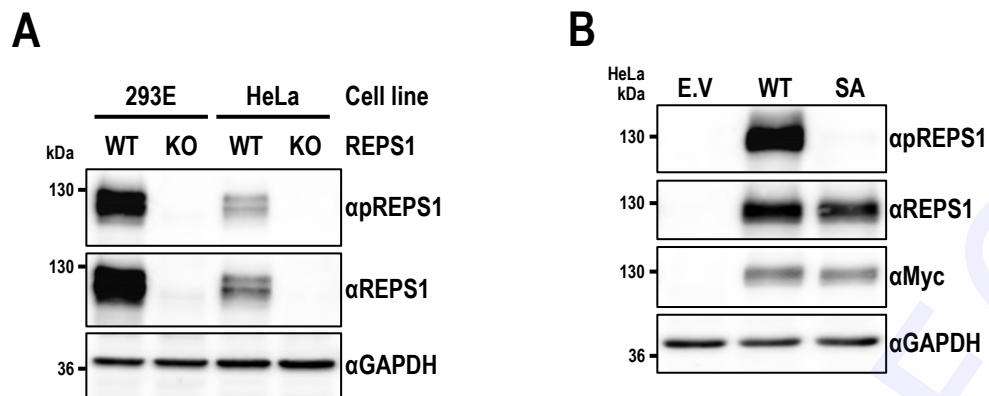
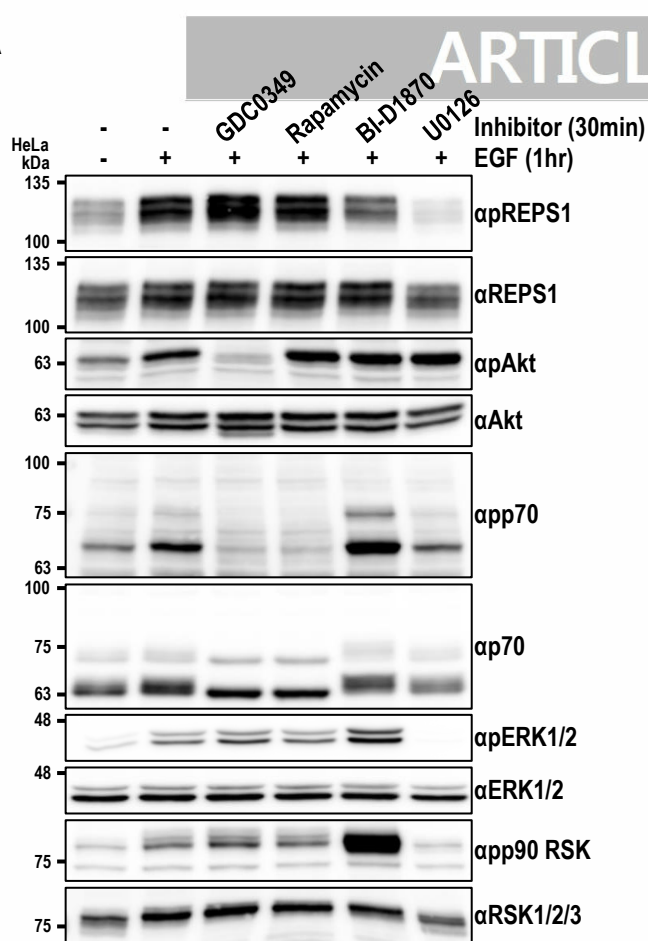
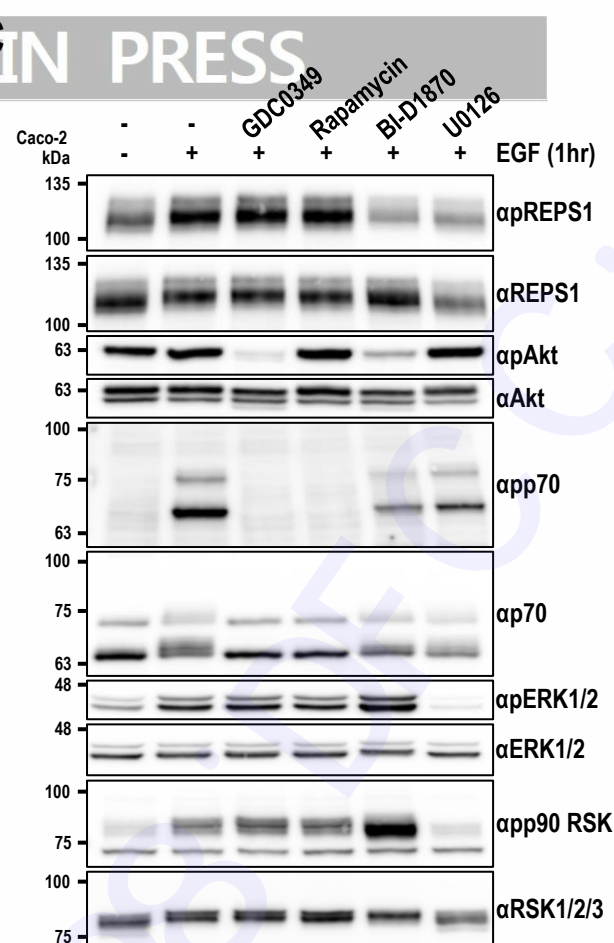
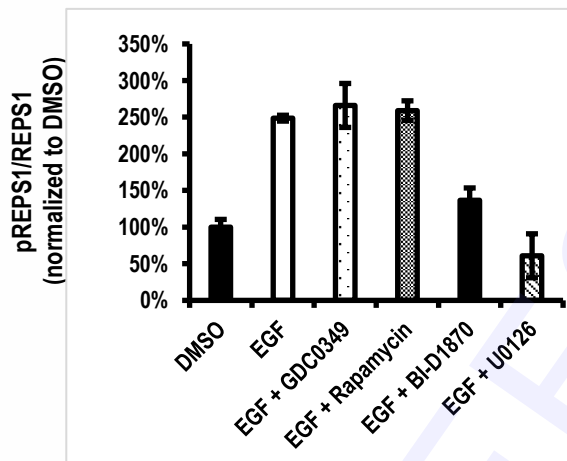
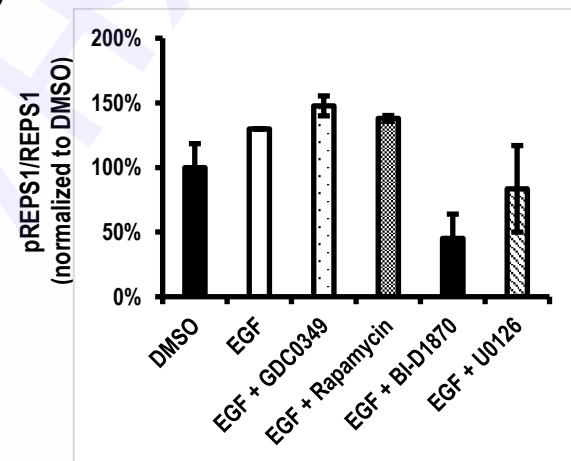


Figure 4



**Supplementary Figure 1. Knockout of REPS1 in HEK293E and HeLa cells**

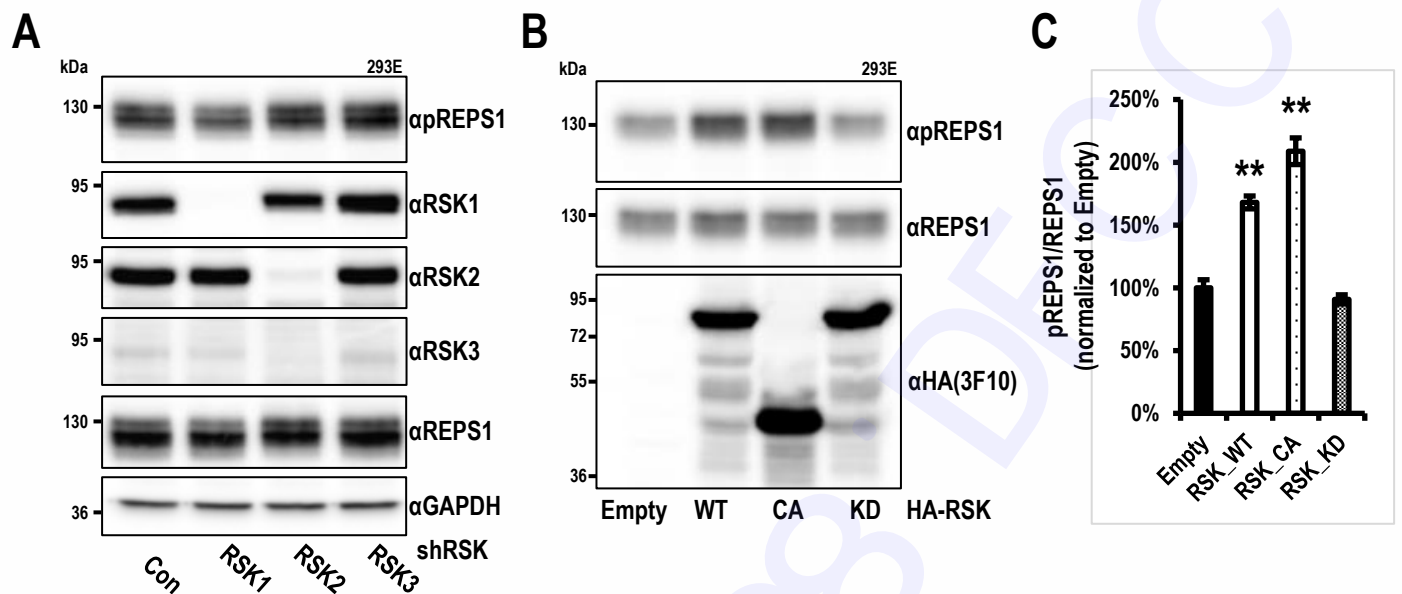
A. REPS1 knockout in HEK293E and HeLa cells was confirmed by immunoblots. B. REPS1-KO HeLa cells were reconstituted with wild-type and SA mutant REPS1 via lentivirus-mediated stable transfection.

**A****C****B****D**

**Supplementary Figure 2. REPS1 phosphorylation by the EGF-induced MEK-RSK pathway is conserved in HeLa and Caco-2 cells**

Lysates of HeLa (A-B) and Caco-2 (C-D) cells treated with the indicated inhibitors and EGF were subjected to SDS-PAGE and immunoblotting conducted with the indicated antibodies.

All data are representative of two independent experiments. \*,  $p < 0.05$ .



### Supplementary Figure 3. RSK phosphorylates REPS1 at S709

A. HEK293E cell lysates expressing shRNA of RSK1, RSK2, and RSK3 were subjected to western blot to assess phosphorylation of REPS1 at S709. B-C. 293E cells transfected with HA-RSK wild-type, CA, and KD were lysed and subjected to immunoblot. All experiments were conducted twice. The representative results are presented. \*\*,  $p < 0.01$ .