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The regulatory mechanism of oncogenic Ras-dependent RhoA/Rac1/Cdc42 activity

암유전자 **Ras**에 의한 **RhoA/Rac1/Cdc42** 활성조절 연구

조 선 대 학 교 대 학 원

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이 논문을 이학박사학위 신청 논문으로 제출함

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Abbreviations

KD	**Knock down**
DP	**Dominant positive**
DN	**Dominant negative**
WT	**Wild type**
EGF	**Epidermal growth factor**
FGF2	**Fibroblast growth factor 2 (Basic)**
PCR	**polymerase chain reaction**
Myr	**myristoylation**
CHX	**cyclohexamide**
PI3K	**phosphatidylinositol-3 kinase**
ERMC	**Ephexin1 and Ras Multi-Complex**

Abstract

The regulatory mechanism of oncogenic Ras-dependent RhoA / Rac1 / Cdc42 activity

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The Rho family GTPases RhoA / Rac1 / Cdc42 regulates cytoskeleton organization and membrane trafficking in physiological processes such as cell proliferation, motility and polarity. Also, Rho family GTPases plays downstream action in Ras / ERK pathway. Oncoprotein Ras act as molecular switches to control of cell growth, invasion, metastasis and differentiation. However, Rho family GTPases regulation mechanism by Ras is not fully understood. Here we demonstrate that, Ephexin1, a guanine nucleotide exchange factor for RhoA, Rac1 and Cdc42 GTPases, preferentially associates with activated GTP-bound Ras. Furthermore, interaction of Epheixn1 and Ras is increased by the Ephexin1 phosphorylation state. The phosphorylation of Epheixin1 includes Ser16, Ser18 residue by AKT. Additionally, Ephexin1 and activated Ras cooperate to cause synergistic formation of RhoA / Rac1 / Cdc42-GTP in AKT / PI(3) K-dependent manners. The active Ras or active AKT enhances Ephexin1,

EphA receptor, EGFR receptor and Ras multi-complex. Thus, Ephexin1 can function as master-effectors that directly mediate Ras activation of tumorigenesis and Rho family GTPases mediated cytoskeleton remodeling, and Ephexin1 itself or its downstream effectors may be valuable targets for the development of therapeutics.

국문초록

암유전자 Ras에 의한 RhoA / Rac1 / Cdc42 활성조절 연구

김지호

지도교수 : 유호진

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Rho family GTPase에 속하는 RhoA와 Rac1 그리고 Cdc42는 cell proliferation과 motility과 polarity과 같은 세포의 생리학적인 과정 안에서 cytoskeletal organization과 membrane trafficking을 조절한다. 또한, Rho family GTPase인 RhoA / Rac1 / Cdc42는 Ras-ERK pathway 아래에서 작e동한다. Oncoprotein인 Ras 단백질은 cell growth, invasion, metastasis 그리고 differentation을 조절하는 molecular switch로서 역할을 한다. 하지만, Ras 단백질에 의해서 Rho family GTPase가 조절되는 기전에 대해서는 완전히 이해되지 않고 있다. 이 논문에서는 Guanine nucleotide exchange factor (GEF)로써의 Ephexin1이 active form인 GTP-Ras와 interaction하고 있음을 밝혀냈다. 게다가 Ephexin1은 Ras와의 interaction에 Ephexin1의 phosphorylation 상태가 중요함을 알아냈으며, Ephexin1의 Ser16, Ser18에 AKT에 의해서 phosphoylation이 된다는 것을 밝혔다. 그리고

Ras와 Ephexin1이 synergistic formation을 이룰 수 있으며, Ras 단백질의 fully activation에 Ephexin1이 필요하다는 것을 밝혀냈다. 또한, Active Ras / AKT는 Ephexin1 / EphA receptor / EGFR receptor / Ras의 multi-complex를 형성함으로써 oncogenic signal을 더욱 증가 시킨다. 이와 같이 Ephexin1이 Ras에 의해서 이루어지는 tumorigenesis와 Rho family GTPases mediated cytoskeleton remodeling에서 Ephexin1이 매우 중요한 effector로서 작용 할 수 있음을 밝혔다. 그리고 Ephexin1 또는 Ephexin1 downstream effector들이 중요한 cancer therapeutics로 사용 될 수 있을 것으로 기대된다.

I. Introduction

Rho family GTPases are a family of proteins which control many different biological processes in the cell, including cell survival, proliferation, adhesion, migration, gene expression and apoptosis [1, 2, 3, 5]. Rho family GTPase contains at least 20 members, with RhoA, Rac1 and Cdc42 being among the best characterized [1]. These three proteins have both unique and overlapping functions. In terms of cell migration and cytoskeletal organization, they regulate distinct processes : RhoA controls the assembly of stress fibers [4, 8] and cell rounding, detachment, membrane blebbing [35] , Rac1 stimulates the formation of lamellipodia and membrane ruffles [4, 9], and Cdc42 regulates the assembly of filopodia [4, 7]. These proteins function as molecular switches, cycling between an active GTP-bound form by guanine nucleotide exchange factors (GEFs), and an inactive form that is bound to GDP by GTPase activating proteins (GAPs) [10, 11].

As well as contributions of the Rho family GTPases to the metastatic phenotype, several cell lines of evidence point to involvement of Ras oncoproteins [21, 22]. Three classes of Ras effectors are now well established: the Raf family of protein kinases, phosphatidylinositol-3 kinases (PI3Ks), and the Ral guanine nucleotide exchange factors (Ral-GEFs) [22, 24, 25]. The Raf kinases phosphorylate the dual specific kinase MEK, which in turn phosphorylates and activates the MAP kinases ERK 1 and 2 [22, 26]. However, the study on the control state of Rho family GTPases by Ras is not clear.

Therefore, there is a need to study the mechanism for control of Ras by Rho family GTPases.

Dbl family proteins are guanine nucleotide exchange factors (GEFs) for the Rho family of small GTPase [12]. All Dbl family proteins contain a tandem Dbl homology (DH) domain / pleckstrin homology (PH) domain structure [12]. The DH domain is the catalytic region of the protein, whereas the PH domain regulates the DH domain as well as the sub-cellular localization of the Dbl protein [12, 13]. Some DH domains act as GEFs for specific Rho GTPasaes. For example, Vav can act as a GEF for RhoA, RhoG, Rac1, and Cdc42 [14, 15, 16, 17], whereas Tiam1 is a specific activator of Rac1 [18], p115 RhoGEF / Lsc is an activator of RhoA [19], Fgd1 is a specific activator of Cdc42 [20], Ephexin4 is a specific activator of RhoG [33], and Ephexin1 can act for RhoA, Rac1, Cdc42 [27]. Stimulation of Eph receptors can either activate or repress Rho GTPases depending on the cell type and physiological context.

Ephexin is a subfamily of Dbl family GEFs that interacts directly with EphA receptors [27, 28]. At least five members of the Ephexin subfamily (Ephexin1–5) have been reported, and Ephexin1 (ARHGEF27/ NGEF), Ephexin2 (ARHGEF19 / WGEF), Ephexin3 (ARHGEF5 / TIM1), Ephexin4 (ARHGEF16 / nueroblastoma), and Ephexin5 (ARHGEF15 / Vsm–Rho GEF) activate Rho family GTPases [27, 29, 30, 31, 33]. The function of Ephexin1 is well characterized in neuronal cells, and it has been reported that Ephexin1 is many studies for the role of axon guidance and spine morphogenesis through the interaction with the cytoplasmic domain of EphA4, as the name of NGEF (Neuronal Guanine Nucleotide Exchange Factor). When over-expressed in cells,

Ephexin1 activates RhoA, Rac1, and Cdc42. The co-expression of activated EphA4 with Ephexin1 enhances the ability of Ephexin1 to activate RhoA relative to its ability to activate Rac1 and Cdc42, thereby promoting growth cone collapse [27, 28, 32]. Thus most previous researchers about Ephexin1 are focused in neuronal cell. Interestingly, however we show that Ephexin1 was high expression in several cancer cells. But, study of Ephexin1 have not been in cancer cells for invasion, metastasis and other tumorigenesis effects .

Eph receptors are structurally grouped into EphA receptors, preferentially binding glycophosphatidylinositol-liked A-type ephrins, and EphBs, binding transmembrane b-type ephrins [34]. Within these subtypes, ephrin-Eph interactions are largely promiscuous, but there is limited cross-reactivity between subtypes. Known exceptions include EphA4 ligating A and B type ephrins and ephrinA5 binding all EphA and some EphB receptors [35]. EphA receptors can directly activate RhoA through the exchange factors Ephexin or indirectly through the adaptor protein Crk. In neuronal cells, EphA4 receptor activation leads to increased Rho activity that is mediated by Ephexin1, resulting in growth cone collapse [27]. Whether EphA4 also activates RhoA under physiological conditions remains unclear, since ephrin-A1 does not appear to stimulate RhoA activity in primary VSMCs and siRNA inhibition of RhoA fails to alter cellular responses significantly [36]. Activation of EphA2 receptor increase Rac1 activity in microvascular endothelial cells [38].

Eph over-expression in multiple human malignancies pointed toward the Eph / Ephrin family as important players in tumorigenesis [41]. In breast cancer and brain tumor (glioblastoma multiforme (GBM)), Ephrin-A1 and EphA2 are

differentially expressed. Ephrin-A1 is down-regulated, a highly over-expressed EphA2 in several cell lines [42, 59], a pattern which correlates with more invasive and tumorigenesis in breast cancer cells [34]. In additionally, EphA2 is associated with higher tumor grade and decreased patient survival [44, 45]. Similarly, EphA2 is up-regulated in pancreatic cancer [46] and renal carcinoma [48], as well as in lung cancer, in which increased expression correlates with shorter patient survival and is a predictor of brain metastasis [48]. Moreover, EphA2 knockdown inhibits migration and proliferation in non-small cell lung cancer (NSCLC) cells [49]. Also, The EphA2 has been proposed as a therapeutic target in colorectal cancer and other cancers [50]. Additionally, there is a correlation between increased EphA2 mRNA levels in Her2 / neu (human epidermal growth factor receptor2)-positive breast cancer patients and a decrease in disease-free survival [51]. Eph receptors are no activated in a similar way as other RTKs but require pre-clustered ligands to induce efficient downstream signaling [40]. Before activation, the Eph receptors are loosely distributed on the cell surface and display minimal kinase activity, unless receptor expression levels are considerably elevated [52, 53]. Also, unlike other receptor tyrosine kinase, Ephs require oligomerization by cell surface-tethered or pre-clustered soluble ephrins for transphosphorylation, downstream signaling, and biological responses [39, 40]. Eph receptors and other receptor tyrosine kinases are forma hetero-dimer such as FGFRs, EGFR and VEGFR [54, 55, 56, 57, 58, 59]. However, hetero-dimerization of the Eph receptor and EGFR not detailed understanding of the downstream effects.

We propose that Ephexin1 was “core intermediate effector” in controlling

the Rho family GTPases activity by Ras, that its activity regulates AKT / PI3K dependent manner in cancer cells. We also show that EphA, Ephexin1, EGFR and Ras multi-complex (we called ERMC (Ephexin1 and Ras Multi-Complex)) is enhances by active Ras or active AKT. We suggest that Active ERMC leads to a fully MAPK activity in active Ras mutant cancer cells.

II. Materials and Methods

Cell culture and transfection

The human colorectal adnocarcinoma (SW480), human Colorectal Carcinoma (HCT116) and human embryonic kidney (HEK-293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 500 U/ml penicillin-streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 in air. SW480, HCT116 and HEK-293T cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 36 h, the cells were lysed by RIPA buffer (150 mM sodium cloride, 1% NP-40, 0.5% sodium deoxyclolate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) supplemented with protease inhibitor (1mM PMSF, 2.5ug/ml leupeptin, 5ug/ml aprotinin, 2ug/ml pepstain A) and phosphatase inhibitor (10mM NaF, 1mM Sodium orthovarate).

Construction of plasmid DNA

The HEK-293T total cDNA containing the Ephexin1 iso.1 (NM_019850) open reading frame was modified to include XhoI / NotI restriction sites at 5’ and 3’ ends by polymerase chain reaction (PCR). The modified cDNA was cloned into the XhoI / NotI site of modified pCI-neo-Flag or V5 vector (Promega) and sequenced completely. The Ephexin1 iso.2 (NM_001114090) open reading frame was modified to include XhoI / NotI restriction sites at 5’ and 3’ ends by PCR from pCMV6-entry-human-Ephexin1 iso.2 (Origene).

The HEK-293T total cDNA containing the EGFR (NM_005228) open reading frame was modified to include XhoI / NotI restriction sites at 5' and 3' ends by polymerase chain reaction (PCR). The modified cDNA was cloned into the XhoI / NotI site of modified pCI-neo-Flag or V5 vector (Promega) and sequenced completely. The EphA1 (NM_005232), EphA2 (NM_004431), EphA3 (NM_005233) and EphA4 (NM_004438) open reading frame were modified to include XhoI / NotI restriction sites at 5' and 3' ends by PCR from pDONR223-EPHA1 (Addgene 23929), pDONR223-EPHA2 (Addgene 23926), pDONR223-EPHA3 (Addgene 23911) and pDONR223-EphA4 (Addgene 23919) were purchased from Addgene. The pcDNA3-EGFP-RhoA (Addgene 12965), pcDNA3-EGFP-Rac1 (Addgene 12980), pcDNA3-EGFP-Cdc42 (Addgene 12975), pRK5-myc-RhoA (Addgene 15899), pRK5-myc-Rac1 (Addgene 12985) and pRK5-myc-Cdc42 (Addgene 15905) were purchased from Addgene. The pGEXTK-Pak1 70-117 (GST-Pak-PBD; Addgene 12217), pGEX-2T-RBD (GST-Rhotekin-RBD; Addgene 15247), pGEX-2T-Raf1-RBD 1-149 (GST-Raf1-RBD; Addgene 13338) were purchased from Addgene. The pCMV-Ras WT, Ras G12V and Ras S17N were purchased from Clontech. The pMEV-2HA-KRas WT, KRas G12V and S17N were purchased from Biomyx Technology (San Diego, CA, USA).

The sequences for various deletion mutants are as follows (refer to the maps in Fig) : Epehxin1 ΔSH3 (amino acids 1-612), Ephexin1 ΔPH/SH3 (amino acids 489), Ephexin1 ΔDH/PH/SH3(=RR) (amino acids 1-273), Ephexin1 ΔDH (amino acids 1-273 and 489-710), Ephexin1 ΔRR (amino

acids 273-710), Ephexin1 ΔPH (amino acids 1-457 and 601-710), Ephexin1 ΔDH/PH (amino acids 1-273 and 601-710) and Ephexin1 DH/PH (amino acids 273-612). Primer are listed in the **Supplementary Table 2**.

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Site Direct Mutagenesis of plasmids

K-Ras Q61L, Ephexin1 iso.1 S16A, S18A, S606A, S16D, S18D, S606D, S16/18A, S16/18D, S16A/18D and S16D/18A were prepared by applying the recombinant PCR method or site-directed mutagenesis using WT constructs as templates and a pair of appropriate mutation primer (**Supplementary Table 4**) All mutants were checked by DNA sequencing.

Reagents and antibodies

Cycloheximide (CHX.) was from Sigma-Aldrich (St Louis, MO, USA). Recombinant Human EphrinA1-Fc chimera was purchased from Sino Biological (Beijing, P.R.China). Recombinant Human FGF-basic was purchased from PEPROTECH (Rhocky Hill, NJ, USA). The Ephexin1 was purchased from ECM biosciences or abcam (Cambridge, MA, USA). β-actin (AC15) and HA (Chip grade) were purchased from abcam (USA). Phosphor-EphA4 (Y602) was purchased from ECM biosciences. Total ERK 1/2, phosphor-ERK 1/2 (T202/Y204), total AKT, phosphor-AKT (S473), phosphor-EphA2 (S897), phosphor-EphA2 (Y588), phosphor-AKT substrate (RXRXXS/T) and EGFR were purchased from Cell signaling (Danvers, MA, USA). Total RhoA, phospho-RhoA (S188), α-tubulin, GFP (FL), GFP (B-2),

Ub (P4D1), EphA2 and EphA4 were purchased from Santacruz (Dallas, TX, USA). Myc (4A6) was purchased from Millipore (Billerica, MA, USA). Rac1, Cdc42 and Ras were purchased from Pirece (Thermo Fisher Scientific. Waltham, MA USA). V5 was purchased from invitrogen (USA). The FLAG-M2 antibody was purchased from Sigma-Aldrich (USA).

Preparation of cell lysate and Western blot analysis

The SW480, HCT116 and HEK-293T cells were washed with PBS twice and lysed in RIPA buffer supplemented with protease and phosphatase inhibitor. After incubation on ice for 15 min, the supernatants were collected by centrifugation at 13,000 rpm for 15 min. Protein concentration was determined using Bradford protein assay (BioRad, Hercules, CA, USA). Aliquots containing 20~35 μg protein were separated on a 12% or 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The protein-bound membrane was incubated with antibody, followed by HRP-conjugaed secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), and visualized by chemiluminescence (ECL).

Immunofluorescence microscopic analysis

Cells were grown on glass coverslips, transfected, and incubated as indicated above, then washed twice in PBS-A (1× PBS with 0.02% sodium azide), immediately fixed with 4% (v/v) formaldehyde in PBS for 15 min at

4℃, and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min at RT. protein detection used primary antibody for overnight at 4℃, then washed twice in PBS-A, briefly stained with cy2 or cy3 conjugated immunoglobulin (Jackson ImmunoReserch) and Hoechst 33258 (Sigma) for 1 h at RT on dark condition, washed twice in PBS-A, and the coverslips were mounted on slide glasses. Fluorescence microscopic images were taken using Nicon fluorescence microscope (63×) and compiled using Adobe Photoshop software.

RT-PCR and quantitative RT-PCR

Total RNA was extrated from cell lysates with the TriZol (RBC), and 2 ㎍ was reverse-transcribed using oligo dT primer (Macrogen synthesis) and M-MLV Reverse Transcriptase (Invitrogen). Oligomers used for amplify PCR products and primer sequences are listed in **Supplementary Table 1.**

RNA interference

HEK-293T, HCT116 and SW480 cells were transfected with siRNAs (40 nM) using Lipofectamine 2000 or Lipofecatamin RNAi Max (Invitrogen). After 40 h, cells were trypsinized, reseeded and transfected again for another 40 h. For single transfection, cells were treated for 48 ~ 96h and processed for the next step. Knockdown efficiencies were verified by Western blot analysis. siRNAs were designed using published

recommendation and target mRNA coding region. siRNA synthesis was in bioneer (Daejeon, Republic of Korea). siRNA sequences used in this study are listed in the **Supplementary Table 3**.

Immunoprecipitation

Harvested cells were washed with ice-cold PBS, and lysed in IP-150 lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA(pH8.0), 0.5% NP-40) supplemented with protease inhibitor (1mM PMSF, 2.5ug/ml leupeptin, 5ug/ml aprotinin, 2ug/ml pepstain A) and phosphatase inhibitor (10mM NaF, 1mM Sodium orthovarate). After incubating for 20min on ice, samples were spin at 13,000 rpm for 15 min. The extracts were immunoprecipitated with antibody and Protein G-Sepharose 4 Fast Flow (GE Healthcare Life Sciences, Uppsala, Sweden) at 4°C for overnight. Beads were washed with IP-150 lysis buffer (without protease / phosphase inhibitor) for 5 times. The immunoprecipitates were resolved on a 12% or 10% SDS-PAGE and analyzed by western blot analysis for coimmunoprecipitation or proteins of interest.

GST-fusion protein purification

GST-PAK1-PBD, GST-Rhotekin-RBD and GST-Raf1-RBD were expressed in E.coli BL21(DE3) for 3 hours at 30°C, respectively, after induction with 0.1mM or 0.3mM isopropyl-β-D-thiogalaactopyranoside (IPTG). Bacteria (1L)

were harvested and lysed in 10ml ice-cold lysis buffer (GST and GST-PAK1-PBD: 1mg/ml Lysozyme, 50mM Tris-HCl (pH 7.6), 150mM NaCl, 0.5% NP-40, 5mM $MgCl_2$, 10ug.ml aprotinin, 10ug/ml Leupeptin, 1mM PMSF, 5% Glycerol ; GST-Rhotekin and GST-Raf1 : 1mg/ml Lysozyme, 50mM Tris-HCl (pH 7.6), 150mM NaCl, 0.5% NP-40, 5mM $MgCl_2$, 10ug.ml aprotinin, 10ug/ml Leupeptin, 1mM PMSF, 5% Glycerol, 0.18% or 0.2% Sarkosyl) by sonication (Input : 25% for 10sec five time). After incubating for 10min on ice, lysates were spin at 13,000 rpm for 15 min. Clarified lysates were mixed with 1ml of lysis buffer-equilibrated Glutathione Sepharose 4B (50% Slurry (GE Healthcare Life Sciences, Uppsala, Sweden) and incubated by end-over-end rotation for overnight at 4°C. The beads were collected by centrifugation at 4000rpm for 3minute at 4°C in a swing-buket centrifuge, washed four times with 10ml each for wash buffer (50mM Tris-HCl (pH 7.6), 150mM NaCl, 0.5% NP-40, 5mM $MgCl_2$, 10ug.ml aprotinin, 10ug/ml Leupeptin, 1mM PMSF, 5% Glycerol), and eluted tree times with 1ml each for elution buffer (50mM Tiri-HCl (pH8.0), 10mM glutathione reduced, 150mM NaCl), and finally resuspended in elution buffer containing 10%(v/v) glycerol. Aliquots were stored at -80°C.

Measurement of RhoA/Rac1/Cdc42/Ras activity

The GTP-bound from of Ras was determined by using as GST-fusion protein of the Ras-binding domain (RBD) of Raf-1 (amino acids 51-131) as

an activation-specific probe for endogenous Ras-GTP [60]. The recombinant GST-PAK1-PBD fusion protein, encompassing amino acids 56-141 of the CRIB-domain of PAK1B, was used as a probe for GTP-bound Rac1 and GTP-bound Cdc42 [3] and the recombinant Rhotekin-RBD fusion protein endoding the Rho-binding domain of Rhokein, amino acids 7-89, was used as an activation-specific probe for RhoA-GTP [61]. Twenty-four hours after tansfection, cells wre lysed in GST-pulldown lysis buffer (25mM Tirs-HCl (pH7.6), 150mM NaCl, 5mM $MgCl_2$, 1% NP-40, 1mM DTT, 5% Glycerol). Lysates were clarified by centrifugation at 13000rpm for 10min. GST-Pak1-PBD, GST-Rhotekin-RBD and GST-Raf1-RBD with Glutathione Sepharose 4B beads were incubated with 1mg of cell lysates in a final volume of 350ul for 1hr at 4°C. The beads were washed three with lysis buffer, and bound proteins were eluted in protein sample buffer and analyzed by 12% SDS-PAGE and Western blotting.

III. Results

III-1. Depletion of Ephexin1 causes defect at Ras/ERK pathway

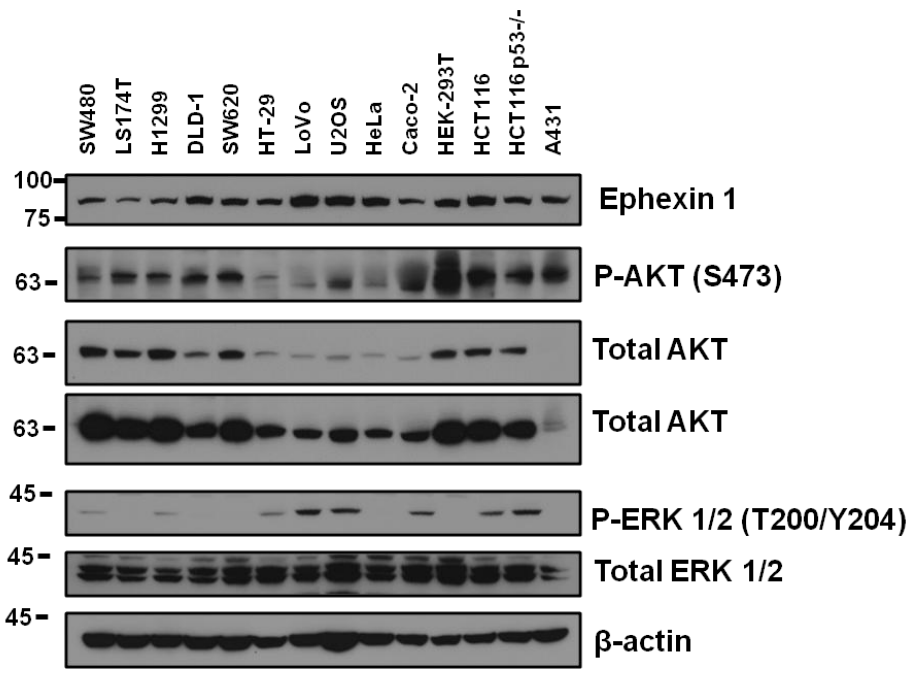
Many previous study reported that Ephexin1 was regulated the cell polarity and growth cone in neural cells [27, 28, 32]. However, little is known about the function of Ephexin1 in no-neuronal cells.

Therefore, we first determined to evaluate the correlation between Ephexin1 / p-AKT and Ephexin1 / p-ERK 1/2 in various cancer cells. Ephexin1 and active ERK 1/2 (p-ERK 1/2 (T202/Y204) per total ERK 1/2) were correlation in various cancer cells (R_2=0.3629). Also, Ephexin1 and active AKT (p-AKT (S473) per total AKT) were correlation in various cancer cells (R_2=0.4846) (Fig.1 A, B, C). And using Ephexin1 siRNA of six different sequences reduced the level of phospho-ERK and phospho-AKT in SW480, HEK-293T and HCT116 cells (Fig. 1 D, E).

Previous reported showed Ephexin1 may be involved in the FGFR receptor activation by the FGF_2 [62], we determined the effect of Ephexin1 on the growth factor-mediated downsteam signal. We observed that ERK and AKT activation in Ephexin1 knock-down was not observed when treated with EGF (100ng/ml) or FGF_2 (10ng/ml) (Fig. 1F, G). And ERK activation of Flag tagged-Ephexin1 over-expression cells was better than control cells (CONT). The amount of Ephexin1 protein did not change the Ras protein levels (Fig. 1F).

Taken together, these results suggest that Ephexin1 is oncogenic protein that can regulate the Ras / ERK / AKT pathway in the cancer cells.

A.



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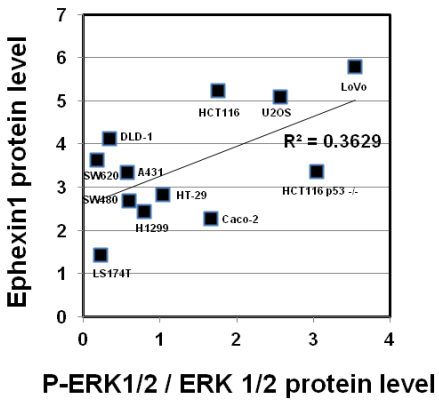

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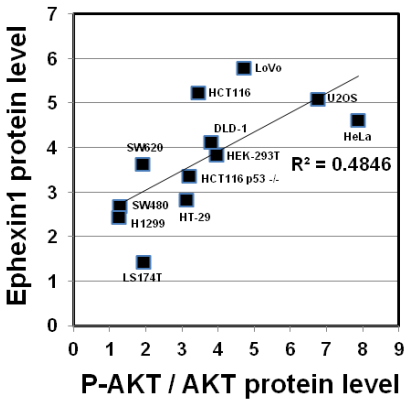

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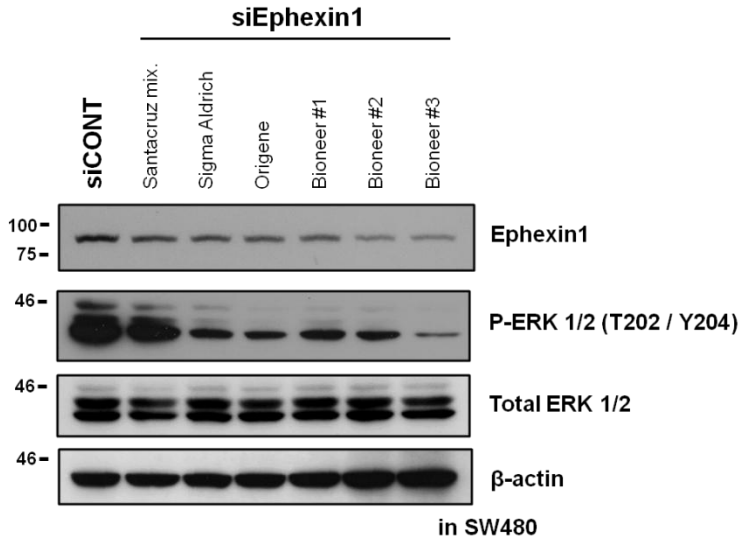

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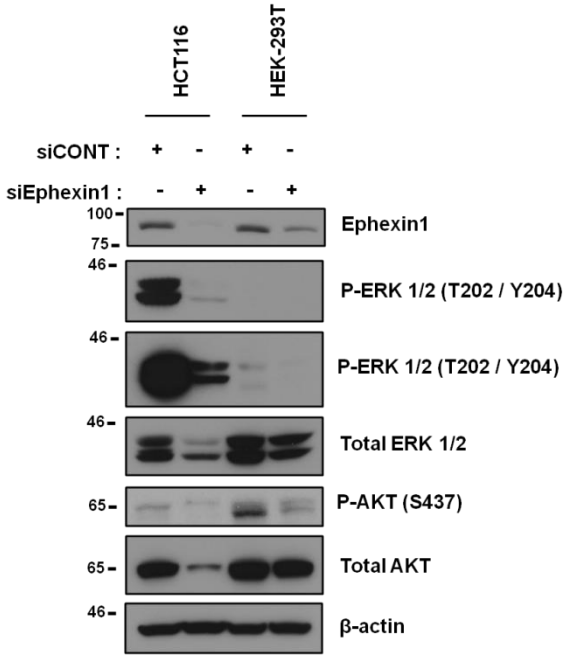

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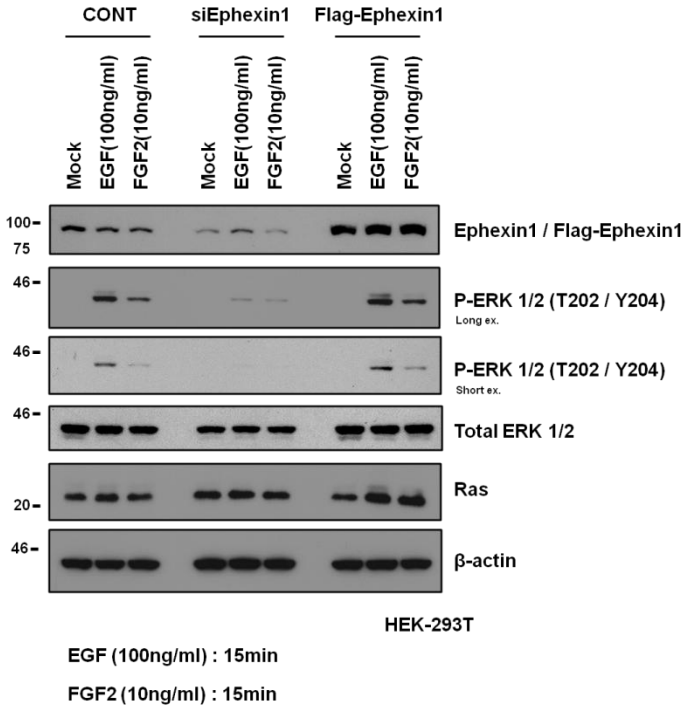

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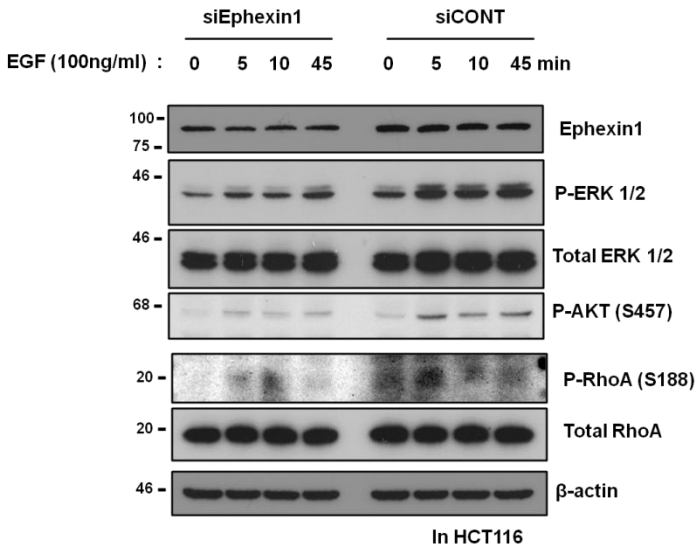


Figure 1. Ephexin1 knockdown leads to suppression of Ras/ERK signaling.

A) Protein level was measured between various cancer cell line by western blot analysis using antibodies against Ephexin1, phospho-AKT (S473), total AKT, phospho-ERK 1/2 (T202/Y204), total ERK 1/2 and loading control β-actin.

B, C) A Scatter plot was showed the positive correlation Ephexin1 and phospho-ERK (B) or phospho-AKT (C) expression. Quantification of protein level by AlphaImager 2000™ program.

D) Control siRNA (siCONT) and siRNAs specific for Ephexin1 (siEphexin1 Santacruz mix., Sigma Aldrich, Origene, and Bioneer #1, #2, #3) were transfected into SW480 cells for 96hr and anlyzed by western blot analysis to detect protein levels. β-actin was used loading control.

E) siCONT and siEphexin1 (bioneer #2) were transfected into HCT116 and HEK-293T cells for 96hr and anlyzed by western blot analysis to detect protein levels. β-actin was used loading control.

F) HEK-293T cells transfected with either siCONT, siEphexin1, Flag tagged-Ephexin1 were lysed. Cells were stimulated with EGF(100ng/ml) and FGF_2 (10ng/ml) for 15min and cell lysates were western blot analysis for indicated protein detected antibodies. β-actin was used loading control.

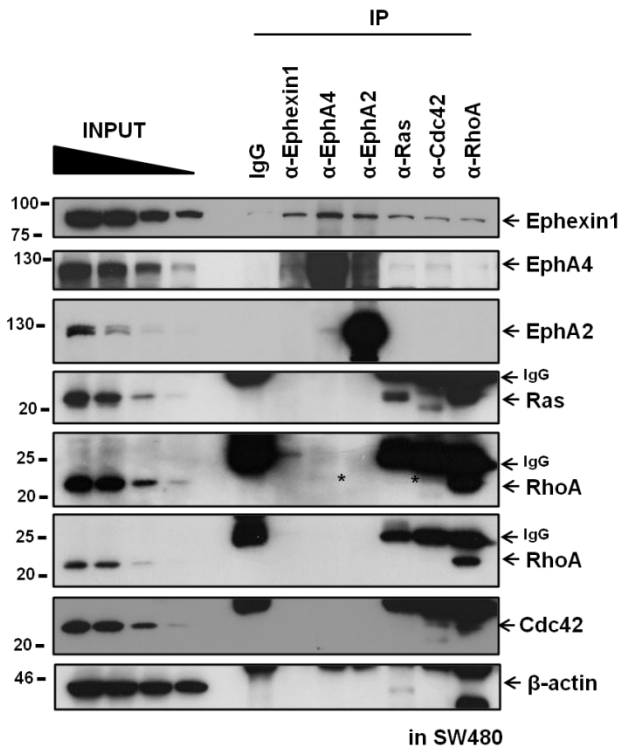
G) siCONT and siEphexin1 were transfected into HCT116 cells for 96hr and stimulated with EGF(100ng/ml) for 15min. cell lystes were anlyzed by western blot analysis to detect protein levels. β-actin was used loading control.

III-2. Ephexin1 interacts with Rho, Rac1 and Cdc42 in non-neuronal cells

We next investigated whether Ephexin1 interacts with Rho family GTPases. Co-immunoprecipitation experiments using endogenous proteins reveled that Ephexin1 interacts with Rho family proteins, such as Ras, RhoA, Rac1, and Cdc42 in SW480 cells (Fig. 2A). Also, We demonstrated that Flag tagged-Ephexin1 transfected HEK-293T, HCT116 and SW480 cells interacts with Rho family GTPases interaction using anti-Flag antibody (Fig. 2B). We then showed that this interaction take place in vivo by demonstrating that Flag-tagged Ephexin1 co-immunoprecipitated with GFP-tagged Rho family GTPases (Fig. 2C) or Myc-tagged Rho family GTPases (Fig. 2D) in HEK-293T cells.

Taken together, these data suggest that Ephexin1 can be controlled to Rho, Rac, Cdc42 in non-neuronal cells.

A.



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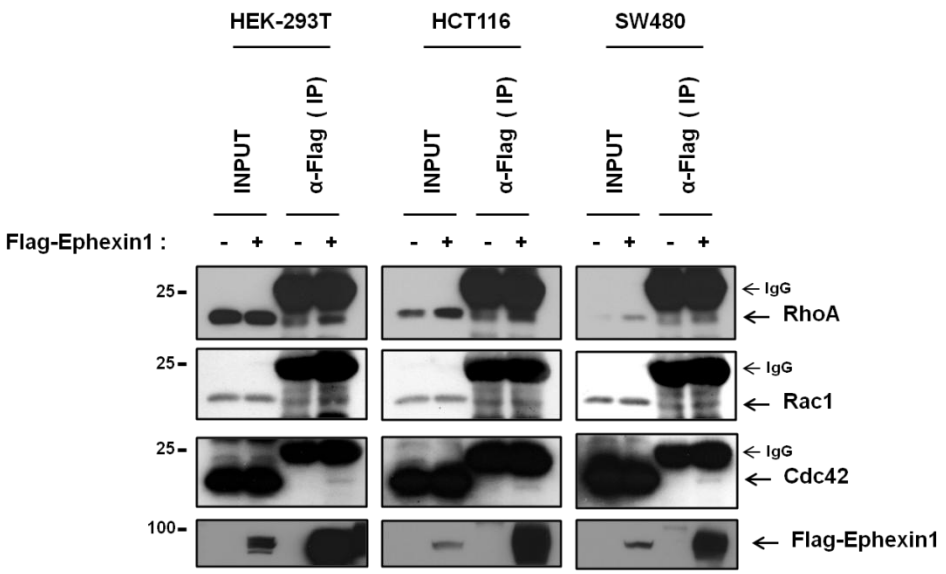

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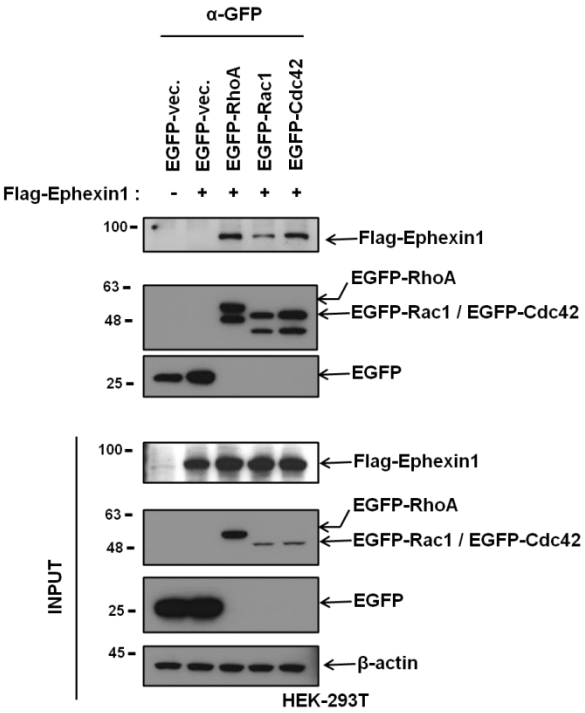

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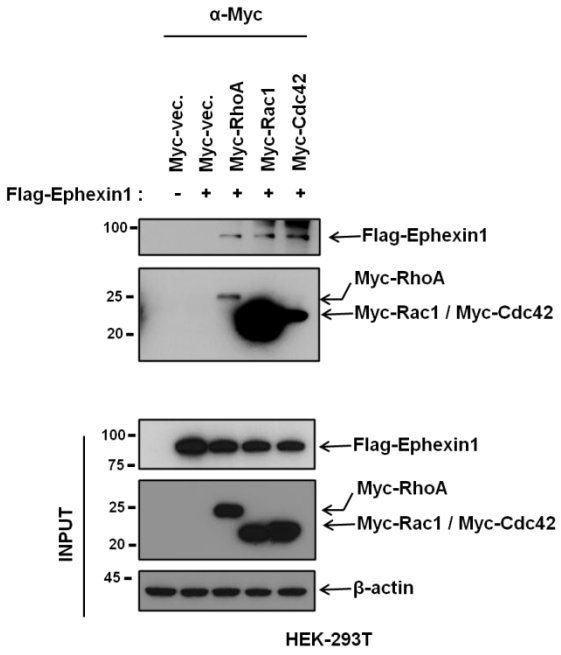

Figure2. Ephexin1 associates with Ras and Rho family proteins.

A) Whole-cell lysates from SW480 cells were subjected to immunoprecipitation for anti-Ephexin1, EphA2/A4, Ras, Cdc42 or RhoA followed by western blotting using the indicated antibodies. β-actin was used loading control.

B) Flag-tagged Ephexin1 was transfected with HEK-293T, HCT116 and SW480 cells. After 36hr, whole cell lystes were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies.

C,D)Flag tagged-Ephexin1 was co-expressed with EGFP tagged-RhoA/Rac1/Cdc42(c) or Myc tagged-RhoA/Rac1/Cdc42(d). Cell lysates were prepared and characterized by immunoprecipitation and western blot analysis. β-actin was used loading control.

III-3. Ephexin1 regulates activity of RhoA, Rac1 and Cdc42

Ephexin1 is well known that controlling the RhoA / Rac1 / Cdc42 as the Guanine nucleotide exchange factor (GEF) [27]. Therefore, we test control the RhoA, Rac1, Cdc42 in cancer cells as the neuronal cells. For these analyses, we used a glutathione S-trasnferase (GST) fusion protein that contains the Cdc42 / Rac-interactive binding region (CRIB) of the p21-activated serine / threonine kinase (PAK; designated GST-PAK-PBD), which specifically binds to the activated GTP-bound forms of Cdc42 and Rac1. For RhoA activity analyses, we used a GST-Rhokein-RBD, which specifically binds to the activated GTP-bound forms of RhoA. Only GST pull-down by Glutathione separose bead was not RhoA / Rac1 / Cdc42 / Ras pull-down (Fig. 3A). We observed that GTP-bound (active form) RhoA / Rac1 / Cdc42 levels were elevated in V5-Ephexin1 transfected HEK-293T, HCT116 and SW480 cells. Also phospho-AKT (S473) and phospho-ERK 1/2 (T202 / Y204) was up-regulation in V5 tagged-Ephexin1 over-expressing cells (Fig. 3A, B, C). On the contrary, depleted Ephexin1 using siEphexin1 in HEK-293T and HCT116 cells were reduced GTP-bound form of RhoA / Rac1 / Cdc42. and phospho-ERK 1/2 (T202 / Y204) was down-regulation in siEphexin1 cells (Fig. 3D, E).

Rho family GTPases are regulated by Ras [21, 22]. Therefore, we test that the regulation of RhoA / Rac1 / Cdc42 by overexpression / knockdown of Ephexin1 was due to changes the GTP-Ras. However, increasing the amount of Flag tagged-Ephexin1 in HEK-293T cells were did not affect active Ras (GTP-bound) (Fig. 3F).

Taken together, these data propose that Ephexin1 regulated activity of Rho, Rac, Cdc42 without affecting the activity on Ras in cancer cells.

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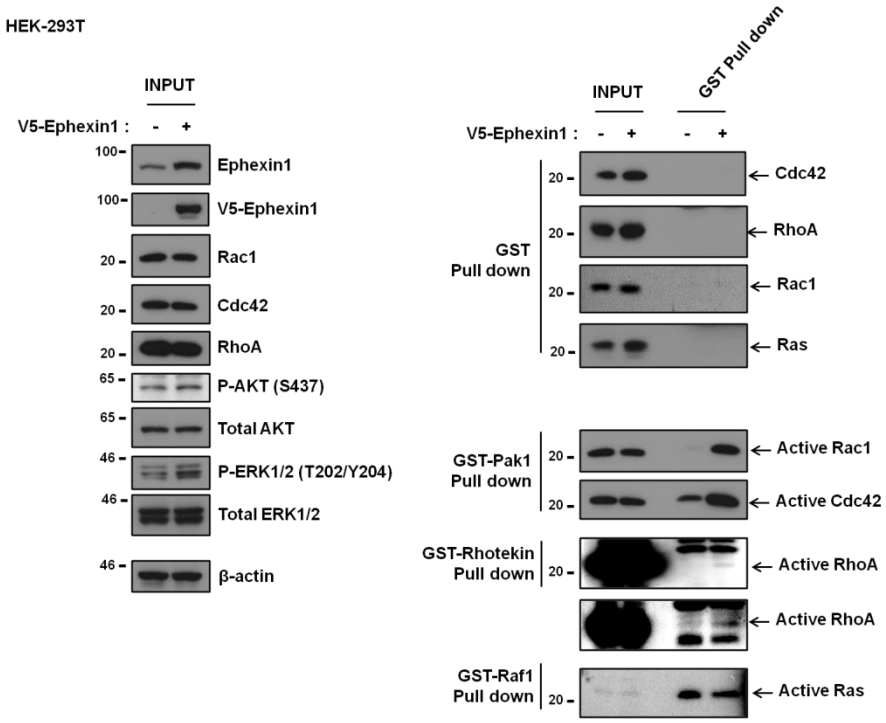

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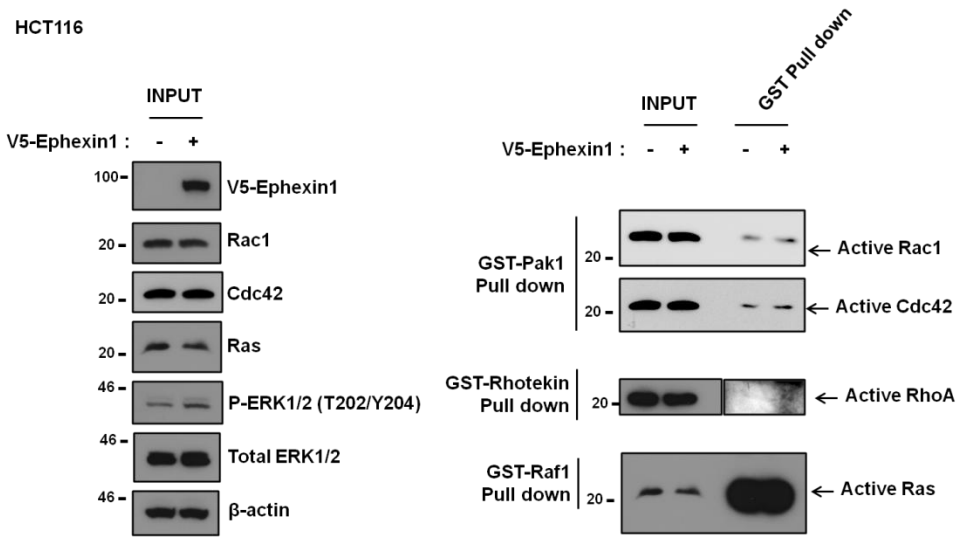

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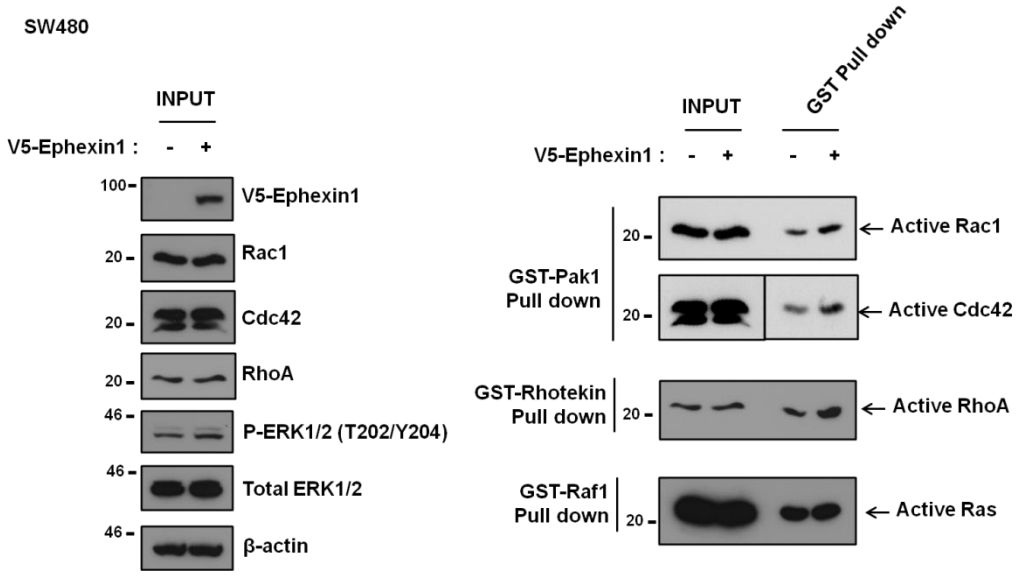

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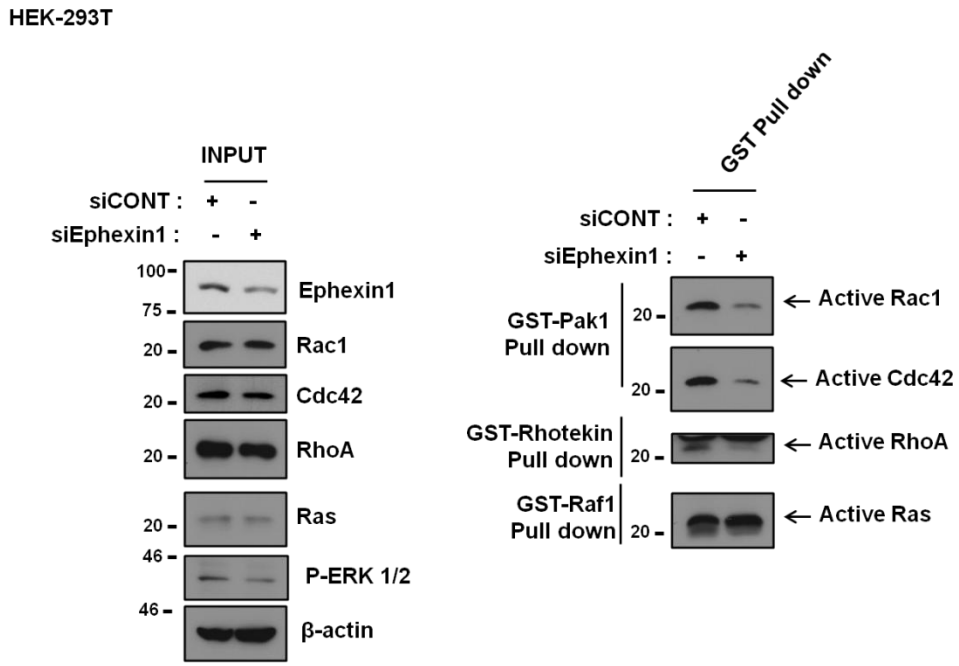

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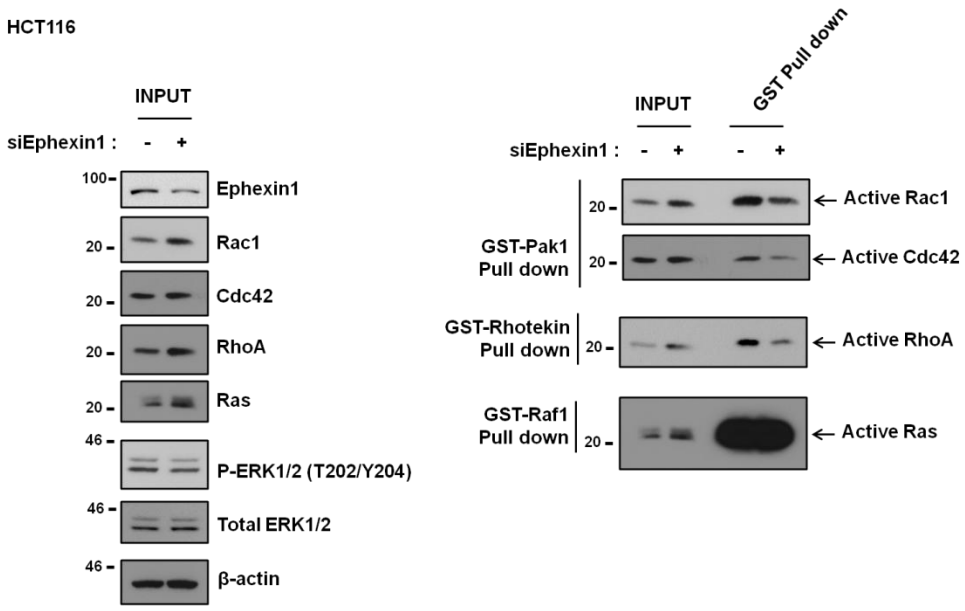

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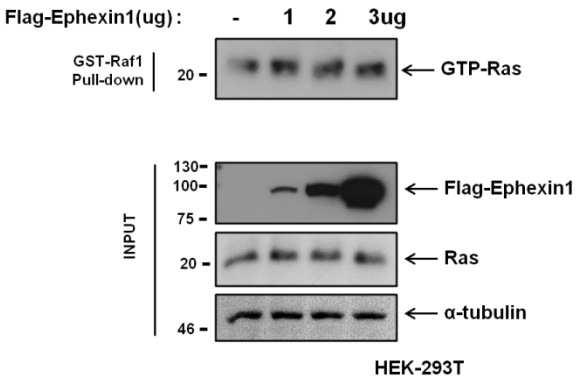

Figure3. Ephexin1 regulated RhoA / Rac1 / Cdc42 in non-neuronal cell .

A,B,C) HEK-293T, HCT116 and SW480 cells transfected with V5 tagged-Ephexin1 were measured by its ability to increase the levels of activated RhoA, as detected by GST-Rhotekin-RBD pull-down, or to increase the levels of activated Rac1 or Cdc42, as detected by GST-Pak1-PBD pull-down, or th increase the levels of activated Ras, as detected by GST-Raf1-RBD pull-down. Cell lystes and active Rho family GTPases levels were determined by western blotting using the indicated antibodies. β-actin was used loading control.

D,E) HEK-293T and HCT116 cells depleted Ephexin1 were measured by its ability to increase the levels of activated RhoA, as detected by GST-Rhotekin-RBD pull-down, or to increase the levels of activated Rac1 or Cdc42, as detected by GST-Pak1-PBD pull-down, or th increase the levels of activated Ras, as detected by GST-Raf1-RBD pull-down. Cell lystes and active Rho family GTPases levels were determined by western blotting using the indicated antibodies. β-actin was used loading control.

F) Flag tagged-Ephexin1 (0, 1, 2, 3ug) were transfected into HEK-293T cells for 36hr and measured by its ability to increase the levels of activated Ras, as detected by GST-Raf1-RBD pull-down. Cell lystes and active Ras level were determined by western blotting using the indicated antibodies. β-actin was used loading control.

III-4. Ephexin1 and Ras cooperate to activate Rho / Rac / Cdc42.

We test that Ephexin1 can activate RhoA / Rac1 / Cdc42 by over-expression of Ephexin1 independent of HA tagged-Kras WT, dominant positive (G12V), dominant negative (S17N). We showed that Ephexin1 can activate downstream effector of Ras (Fig. 4A). Next, we determined if Ras enhances the ability of Ephexin1 to activate RhoA, Rac1, and Cdc42 by using a pull-down assay and to assess whether activated K-Ras and Ephexin1 could cooperate to stimulate formation of GTP bound RhoA, Rac1, Cdc42 in HEK-293T cells. Expression of HA tagged-Kras G12V alone failed to stimulate significant formation of GTP bound RhoA, Rac1, Cdc42, whereas co-expression of HA tagged-KRas G12V with Flag tagged-Ephexin1 resulted in increased formation of GTP-bound RhoA, Rac1, Cdc42 (Fig. 4B). These results suggest that the association of Ras with Ephexin1 stimulates Ephexin1-mediate activation of Rho family GTPases.

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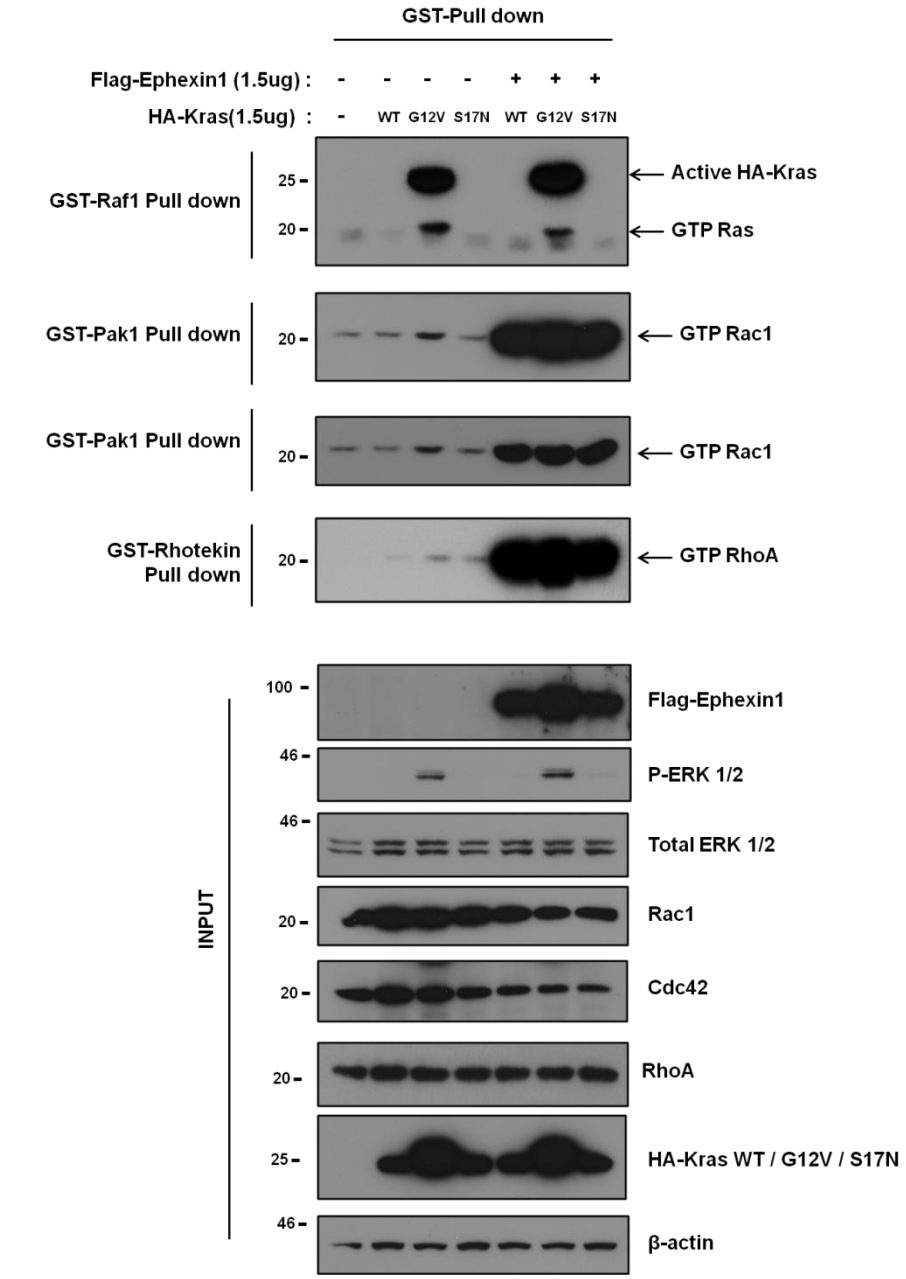

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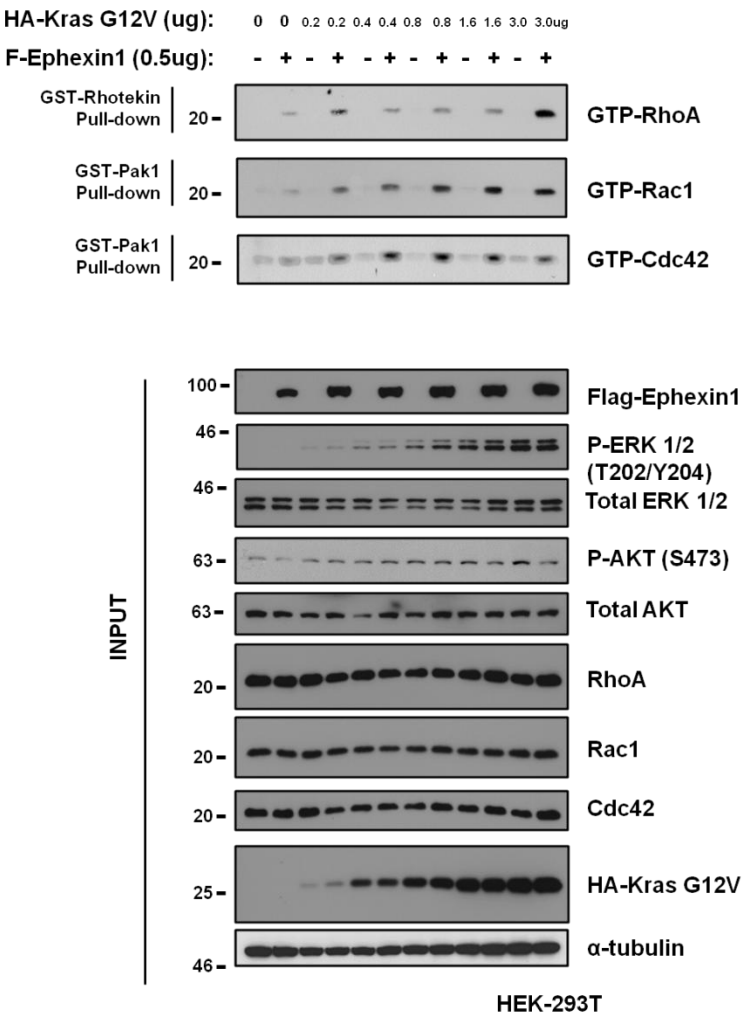

Figure4. Ephexin1 and Ras cooperate to activate Rho / Rac / Cdc42.

A) HEK-293T cells were co-transfected with Flag-Ephexin1 and either HA-Kras WT, G12V, S17N. Levels of RhoA-GTP / Rac1-GTP / Cdc42-GTP detected by GST-Rhotekin-RBD / GST-PAK1-PBD pull-down using Glutatione Separose. Protein expression and active Rho family GTPases levels were characterized by western blot analysis using the indicated antibodies. β-actin was used loading control.

B) Co-transfected with varying amounts of expression plasmids encoding HA tagged-KRas G12V and Flag tagged-Ephexin1, as indicated. Levels of RhoA-GTP / Rac1-GTP / Cdc42-GTP detected by GST-Rhotekin-RBD / GST-PAK1-PBD pull-down using Glutatione Separose. Protein expression and active Rho family GTPases levels were characterized by western blot analysis using the indicated antibodies. β-actin was used loading control.

III-5. Ras-mediated activtion of Rho / Rac / Cdc42 is impaired in Ephexin1-depletion cells.

We found previously in figure.4 that Ephexin1 is downstream effector of Ras. Therefore, we examined that activity of Rho family GTPases measured in depleted of Ephexin1 with active Ras. The RhoA, Rac1, and Cdc42 were successfully activated by Epidermal growth factor (EGF) (100ng/ml) for 30min treatment. However, RhoA, Rac1, and Cdc42 activation in depleted Ephexin1 cells were not working on EGF for 30min treatment (Fig. 5A). In addition, when transfected with HA tagged-Kras G12V showed Rho family GTPases, RhoA, Rac1, and Cdc42, successfully activation of GTP-RhoA (2.15 fold increase), GTP-Rac1 (1.44 fold increase) and GTP-Cdc42 (2.13 fold increase). However in siEphexin1 and HA tagged-Kras G12V transfected cells, the level of GTP-bound RhoA, Rac1, and Cdc42 were back to normal state. And siEphexin1 transfection did not change Ras activity (Fig. 5 B).

These findings (Fig.4 and Fig.5) provide evidence that Ephexin1 is a novel, functionally relevant downstream effector of Ras. Additionally, activated Ras and Ephexin1 can cooperate to cause synergistic formation of Rho family GTPases such as RhoA, Rac1, Cdc42.

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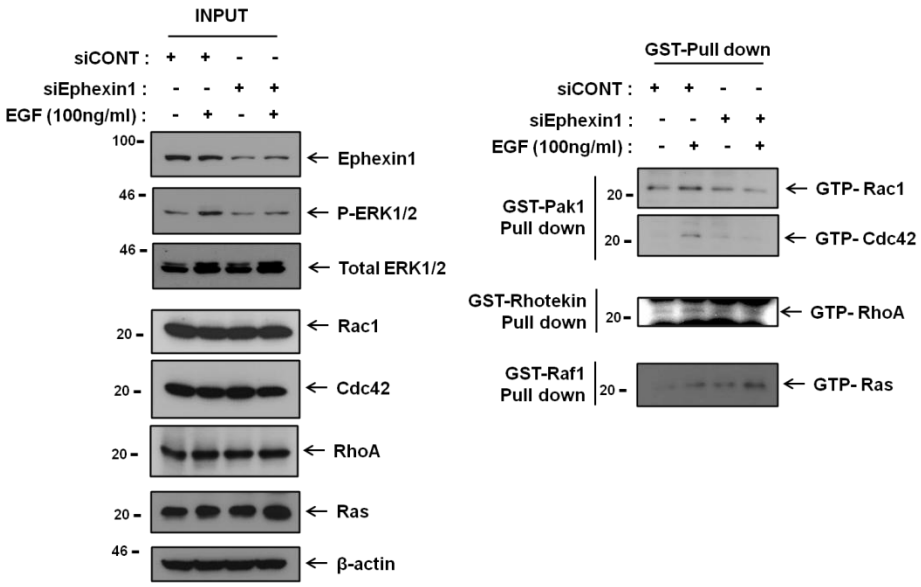

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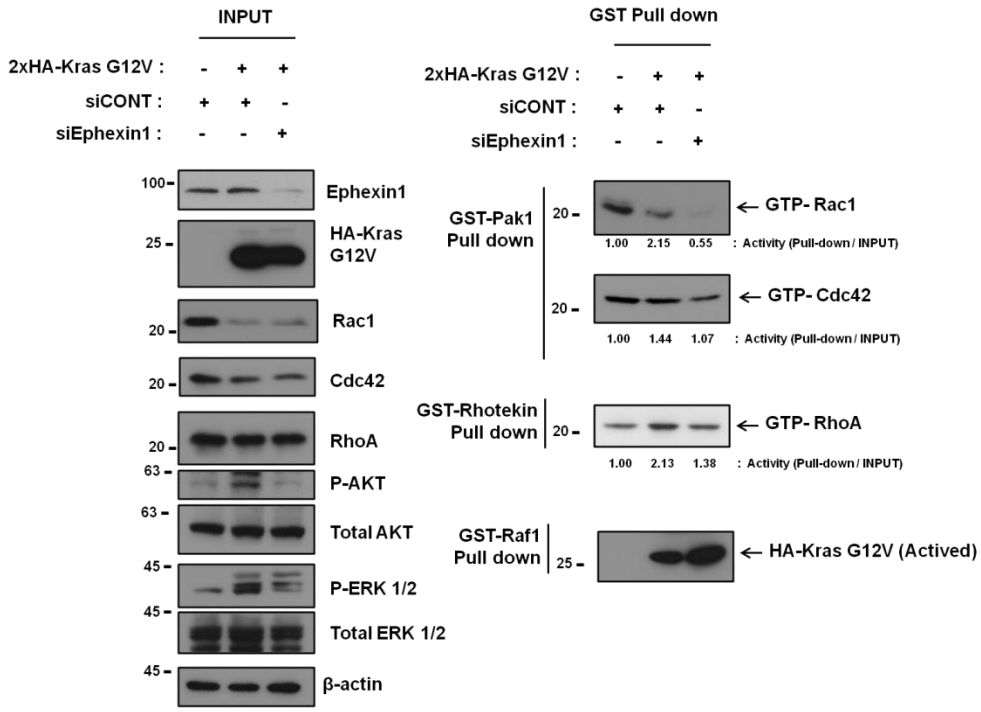

Figure5. Ras-mediated activtion of Rho / Rac / Cdc42 is impaired in Ephexin1-depleletion cells.

A) siCONT and/or siEphexin1 were transfected into HEK-293T cells and stimulated the EGF(100ng/ml) for 15min, as indicated. In this state was measured by GST-Rhotekin-RBD / GST-PAK1-PBD / GST-Raf1-RBD pull-down using the Glutatione Separose. Protein expression, active Ras and active Rho family GTPases levels were characterized by western blot analysis using the indicated antibodies. β-actin was used loading control.

B) siCONT and/or siEphexin1 and co-expression with HA tagged-Kras G12V were transfected into HEK-293T cells, as indicated. Rho family GTPase activity measured by GST-Rhotekin-RBD, GST-PAK1-PBD / GST-Raf1-RBD pull-down using the Glutatione Separose. Protein expression, active Ras andactive Rho family GTPases levels were characterized by western blot analysis using the indicated antibodies. β-actin was used loading control. Quantification of protein level used AlphaImager 2000™ program.

III-6. Ephexin1 binds activated Ras form. Also, Ephexin1-Ras binding increased Ras-Rho/Rac/Cdc42 interaction.

To determine whether Ephexin1 and Ras complex formation occurs in a Ras activity dependent manner, we compared the ability of Ephexin1 to co-precipitate with wild-type (WT) and activaved (G12V and Q61L) K-Ras. Consistent with an effector function of Ephexin1, we found that Flag tagged-Ephexin1 immunoprecipitated preferentially with activated HA tagged-Kras G12V or Q61L, but not bound to inactivated HA-KRas S17N (Fig. 6A, B). And the interaction between active Ras and Ephexin1 confirmed that both dominant positive form (G12V) of H and K-Ras strongly with Ephexin1 (Fig. 6C). In addition, we confirmed once again that in Ras mutated several cancer cells, there is a strong interaction between Ras and Ephexin1. However, Ras Wild type cell (HEK-293T cells) showed week binding. Interaction of Ephexin1 and Ras were high in activated Ras against mutated state. However, there are exceptions. HT-29 cells were Ras WT type and low GTP-Ras. But, Unlike other cells, HT-29 cells showed strong Ephexin1 and Ras interaction. This is probably due to unknown mechanisms in HT-29 cells (Fig. 6D). Next experiment, we experimented that Ephexin1 effect on the interaction between Ras and Rho family GTPases. Overexpression of Ephexin1 cells increases between Ras (WT and G12V) and RhoA, Rac1, Cdc42, than non-overexpression of Ephexin1. Interestingly, strength of the binding between Ras and Rho family GTPases are similar to between Ras and Ephexin1 / EphA receptor / β-actin, but not EGFR (Fig. 6E). In addition, between Ephexin1 and Ras interaction were important DH

/ PH domain of Ephexin1 (Fig. 6F).

Taken together, these result suggest that activated Ras show strong interaction with Ephexin1 that strong Ras-Ephexin1 complex can regulate Rho family GTPases.

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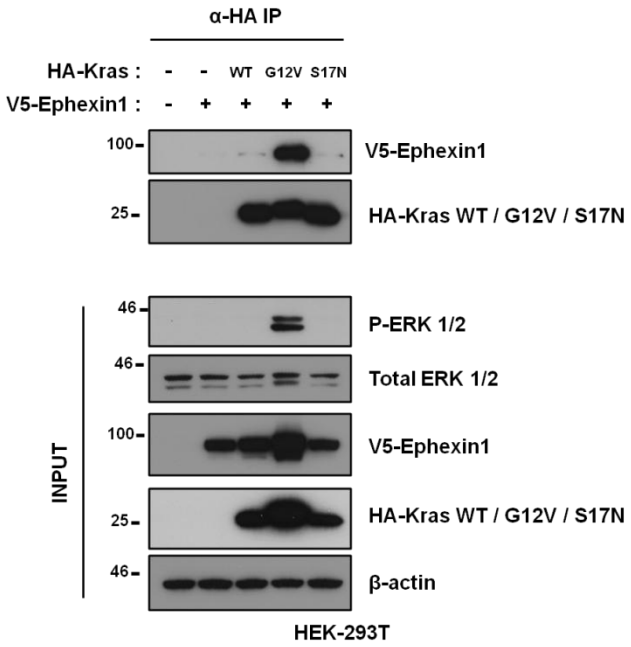

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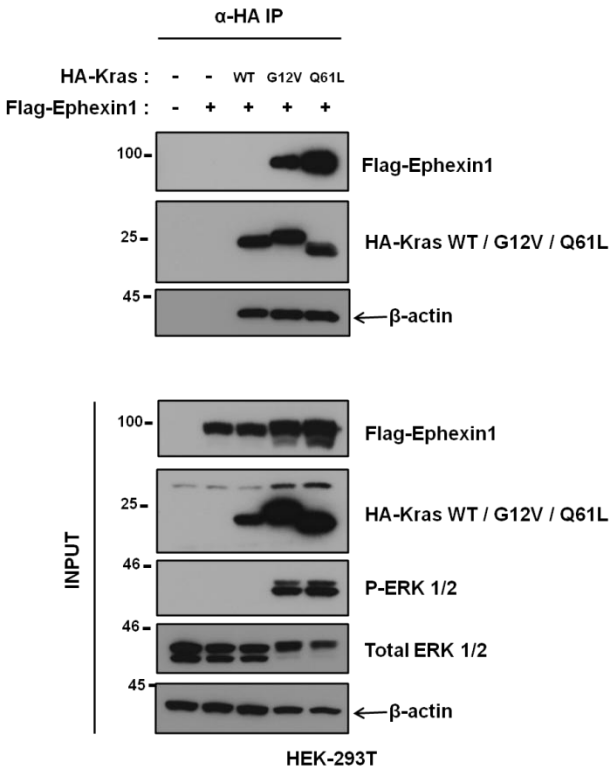

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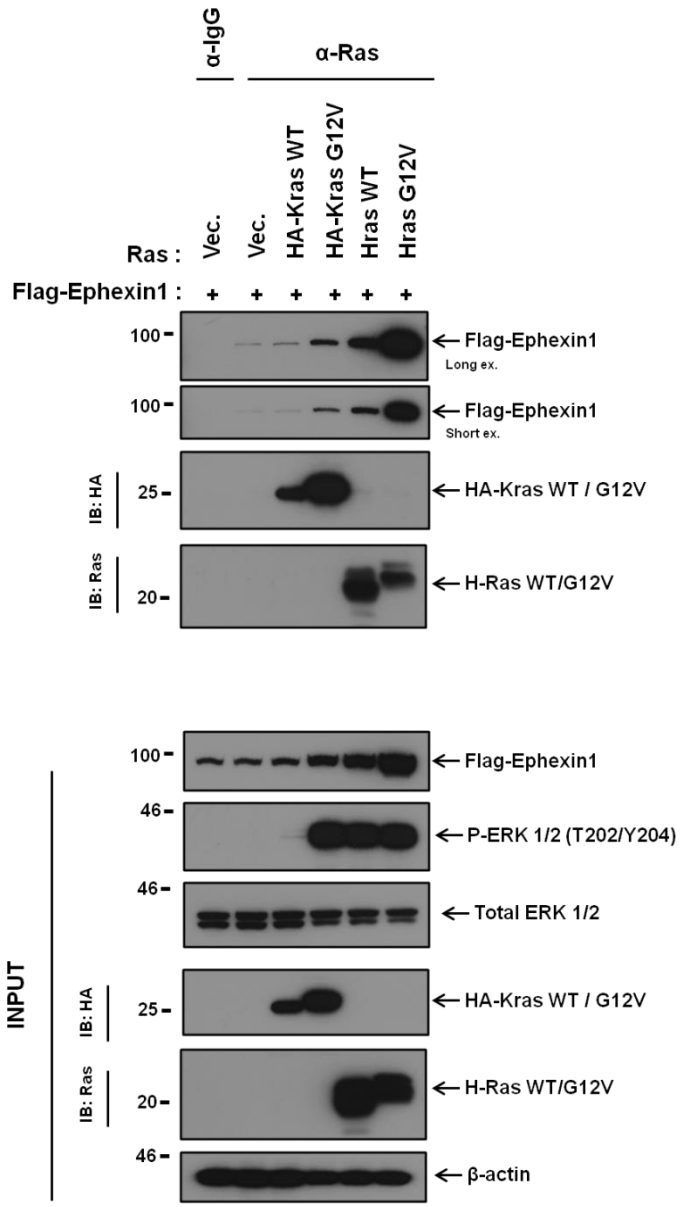

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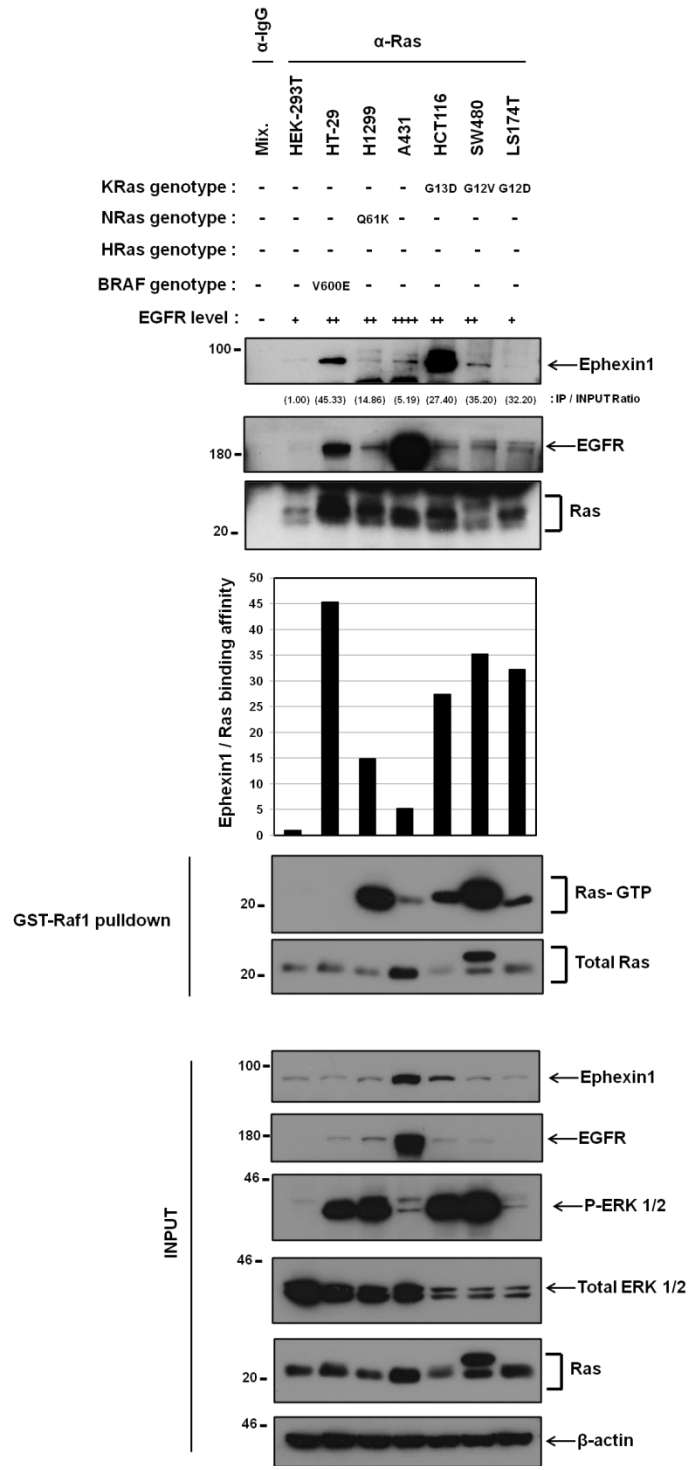

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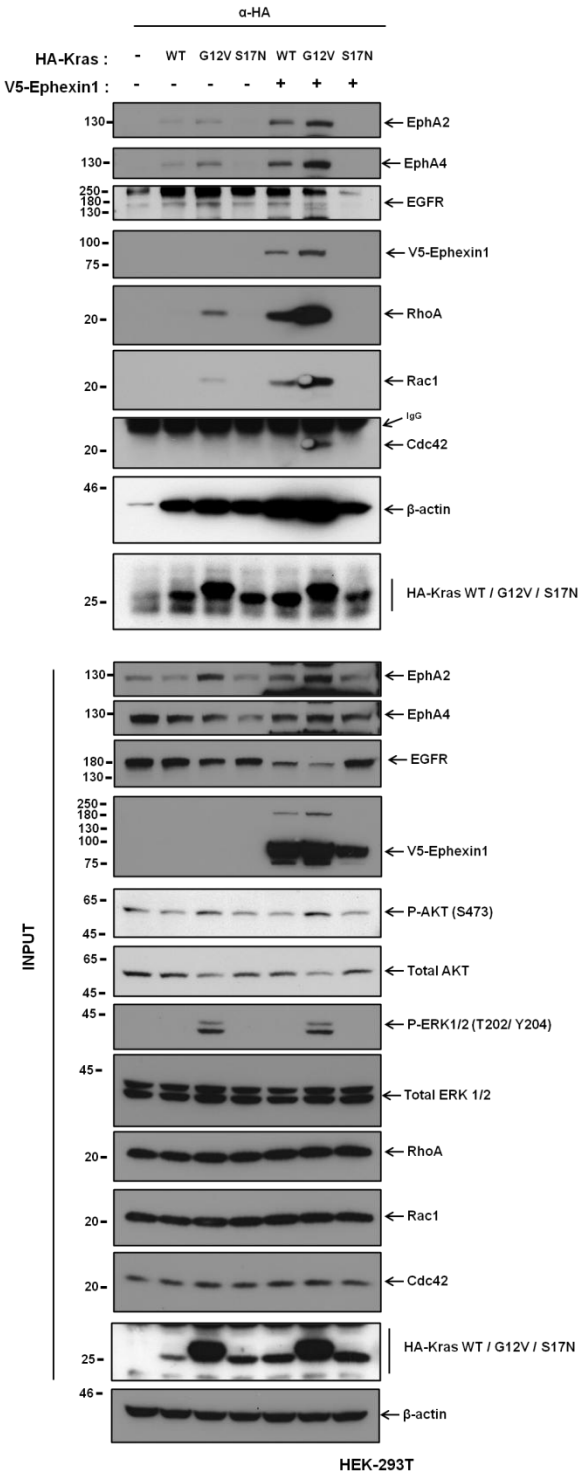

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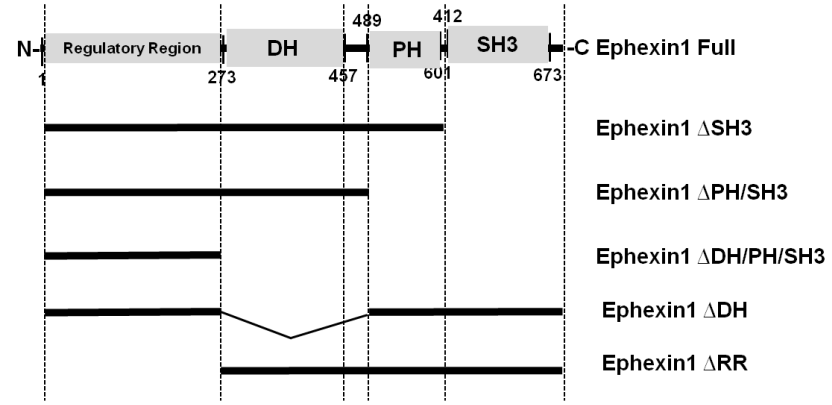

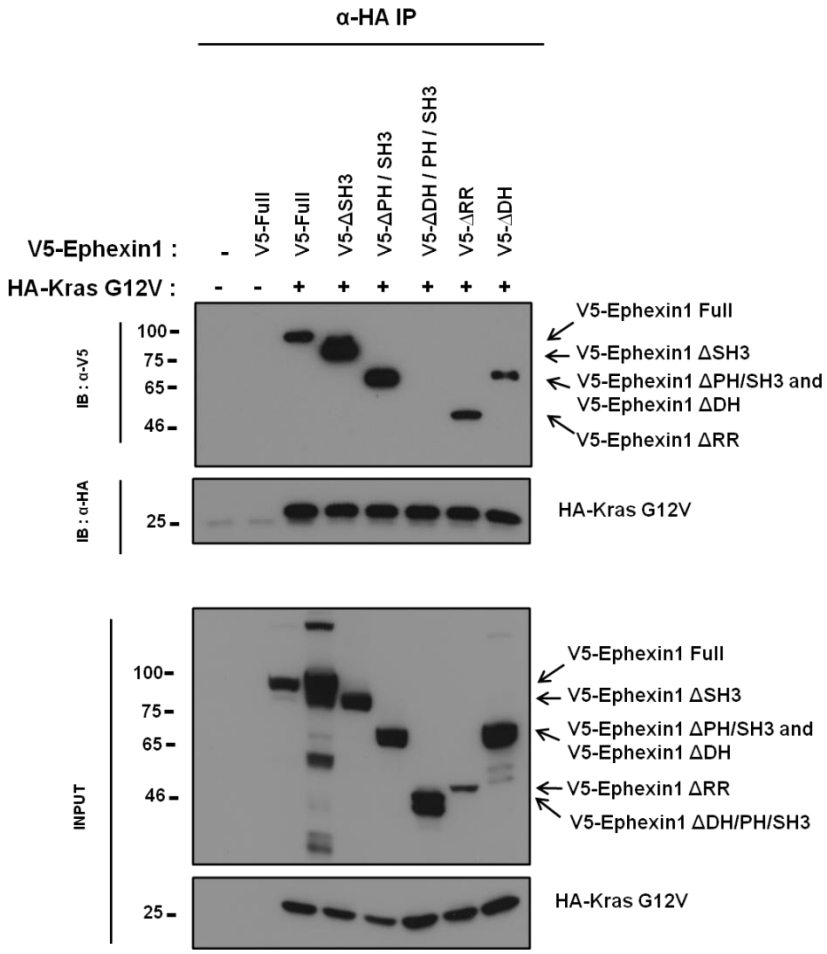

Figure6. Ephexin1 binds activated Ras form. Also, Ephexin1-Ras binding increased Ras-Rho/Rac/Cdc42 interaction.

A,B) Co-expression withFlag or V5 tagged-Ephexin1 and either HA tagged-Kras WT, G12V, Q61L, S17N were transfected into HEK-293T cells. After 36hr, whole cell lysates were subjected to immunoprecipitation for anti-HA antibody. Immunoprecipitates and cell lysates were characterized by western blot analysis. β-actin was used as a loading control.

C) HEK-293T cells transfected with either vector (vec.), HA tagged-K-ras WT, G12V and non-tagged H-ras WT, G12V were co-expressed with either Flag-Ephexin1. After 36hr, whole cell lysates were subjected to immunoprecipitation for anti-Ras antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

D) Whole cell lysates from HEK-293T cells and various cancer cell lines (HT-29, H1299, A431, HCT116, SW480 and LS1747 cells) were subjected to immunoprecipitation and GST pulldown assay for either anti-Ras antibody, GST-Raf-RBD. Immunoprecipitates and GST-Raf-RBD pulldown were characterized by western blot analysis with indicated antibodies. β-actin was used as a loading control. Quantification of protein level used AlphaImager 2000™ program.

E) Co-expression V5 tagged-Ephexin1 with either HA tagged-Kras WT, G12V, S17N were transfected into HEK-293T cells. After 36hr, immunoprecipitation for anti-HA antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

F) HEK-293T transfected with HA tagged-Kras G12V were co-transfected with different V5 tagged-Ephexin1 deletion mutants (or an empty vector (vec.)) At 36 h after transfection, cell lysates were immunoprecipitated with anti-HA followed by western blotting with indicated antibodies.

III-7. Ephexin1 / Ras interaction promoted is by AKT and regulation of Rho family GTPases by Ephexin1 is a AKT / PI3K dependent mechanism.

We showed a strong interaction with Ephexin1 and active Ras in the previous experiment (Fig.6). Therefore, we did an experiment to find the regulation factor between Ephexin1 and Ras protein. We use the inhibitors of kinase in various signaling pathway to determine the regulatory factors. We identified an interesting reduction in LY294002 (PI3K inhibitor) and SP600125 (SAPK/JNK inhibitor) (Fig. 7A). We have performed a more detailed test for AKT. AKT or CK1ε or GSK3β were transfected into HEK-293T cells. We showed that over-expression of Myc tagged-AKT-myr (kinase activation form) increased interaction of Ephexin1 and Ras (Fig. 7B, C). Also, Myc tagged-AKT-KD (kinase death form) did not show binding (Fig. 7C). Ephexin1 and Myc tagged-AKT myr exhibited strong interaction, where as Kinase death AKT (Myc tagged-AKT-KD) did not interact (Fig. 7D). Therefore, we tested the possibility that Ephexin1 can be phosphorylated by AKT. The Myc tagged-AKT and Flag tagged-Ephexin1 after co-transfection were subjected to immunoprecipitation with Flag antibody and it was detected using phosphor-AKT substrate (RXRXXS/Tp) antibody. Interestingly, we showed that Ephexin1 was phosphorylated by AKT (Fig. 7E).

Then we found the site by using the program (PhosphoSitePlus® : http://www.phosphosite.org/staticUsingPhosphosite.do) that may occur AKT phosphorylation in Ephexin1. The program predicted serine 16 and serine 606 phosphorylation site in Ephexin1 isoform1, but serine 18 was not predicted. However, RXKXXS/Tp motif was same to Serine 16 residue. AKT substrate

motif in S16, S18, S606 residues is conserved in four species (Fig. 7F). We therefore tested that Ephexin1 S16A mutant in the presence or absence of phosphorylation by AKT. The Ephexin1 S16A phosphorylation did not accur, by Myc tagged-AKT-myr. And Ephexin1 S18A has a little phosphorylation reduction by Myc tagged-AKT myr. In addition, Ephexin1 S606A was possible phosphorylation site by AKT. But, this site was not actually (Fig. 7G).

The Phosphorylation of Ephexin1 by AKT is critical for interaction with Ras and Ephexin1 (Fig. 7A, B, C, D). So, we demonstrate that If Flag tagged-Ephexin1 and HA tagged-KRas G12V along with Myc-AKT-KD (Kinase death, dominant negative) were co-expressed, it was confirmed that the interaction between HA tagged-Kras G12V and Flag tagged-Ephexin1 was reduced. As expected, co-expression of Myc tagged-AKT-KD and HA tagged-Kras G12V/Q61L reduced interaction between the Flag-Ephexin1 and HA-KRas G12V/Q61L. And interacton of HA tagged-KRas G12V/Q61L, and β-actin was also reduced (Fig. 7H). We found previously in figure.6 that The Ephexin1 and K-Ras S17N (dominant negative form) showed no interaction (Fig.6 B, E). Interestingly, the Myc tagged-AKT-myr or WT and HA tagged-Kras S17N (DN) co-expression increased the interaction between the Flag tagged-Ehexin1 and HA tagged-Kras S17N (Fig. 7I).

Next we investigated the role of AKT / PI3K in the activation of Rho family GTPases by Ephexin1 and Ras. First, If Ras functions through AKT / PI3K to stimulate Ephexin1-mediated activation of RhoA, Rac1 and Cdc42, then treatment with the LY294002 (PI3K inhibitor, 50mM) should prevent the activation of RhoA, Rac1 and Cdc42 caused by the co-expression of HA

tagged-Kras G12V and Flag tagged-Ephexin1. We found that RhoA and Rac1 were to inhibit the ability of Ephexin1 and KRas G12V to cooperate and stimulate formation of GTP-bound RhoA and Rac1. However Cdc42 was not affected. Second, then co-expression of actived PI3K subunit (in the form of the membrane-targeted and constitutively activated chimeric protein : PI3K p110-CAAX) with Ephexin1 should result in a level of RhoA, Rac1 and Cdc42 activation which cooperate and stimulate formation of GTP-bound RhoA, Rac1, Cdc42. The results suggest that RhoA and Rac1 was AKT/PI3K and Ephexin1 dependent mechanism, but Cdc42 was AKT/ PI3K and Ephexin1 plus other factor mechanism (Fig. 7J).

Taken together, these results suggest that S16, 18 phosphorylation sites of Ephexin1 by AKT is an essential factor between the Ras and Ephexin1 interaction. Even though dominant negative Ras form. Additionally, this data suggest that the ability of Ras to activate Rho family GTPases through Ephexin1 is mediated by PI3K / AKT activation.

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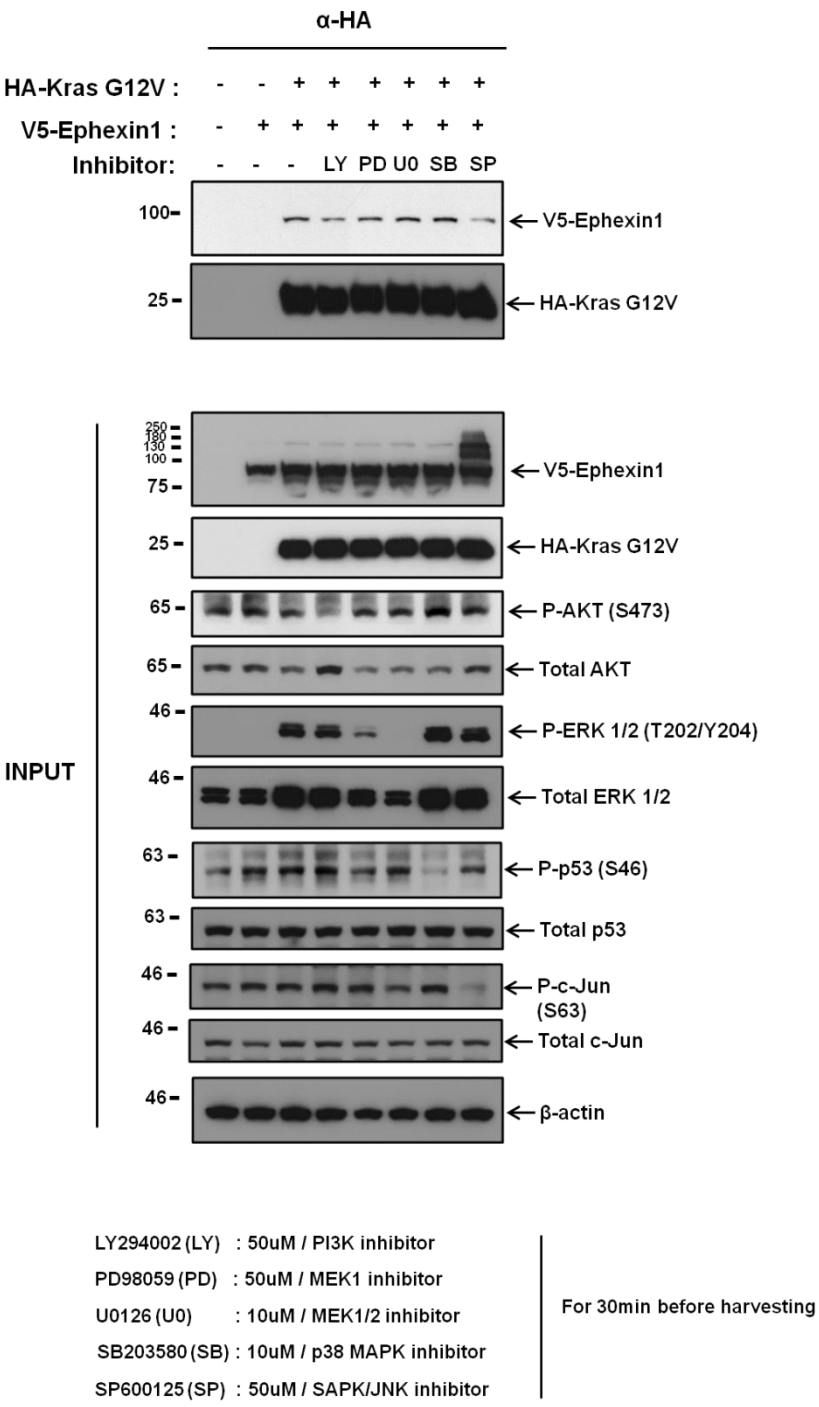
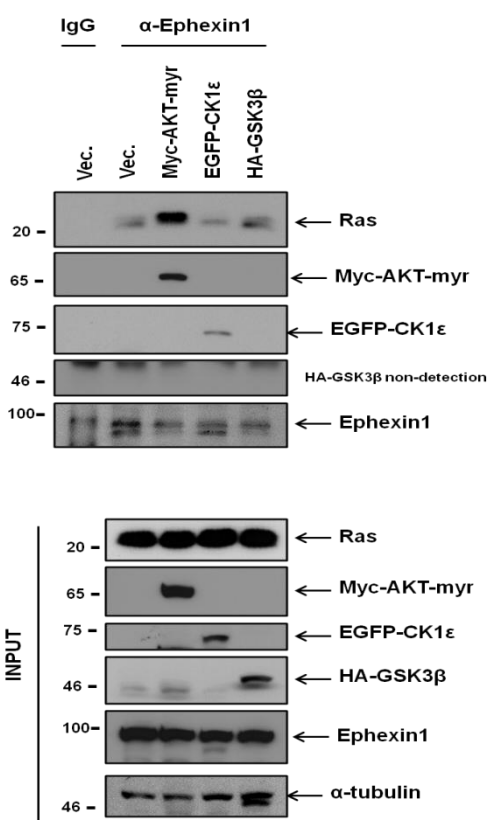


LY294002 (LY) : 50uM / PI3K inhibitor
PD98059 (PD) : 50uM / MEK1 inhibitor
U0126 (U0) : 10uM / MEK1/2 inhibitor
SB203580 (SB) : 10uM / p38 MAPK inhibitor
SP600125 (SP) : 50uM / SAPK/JNK inhibitor

For 30min before harvesting

B.



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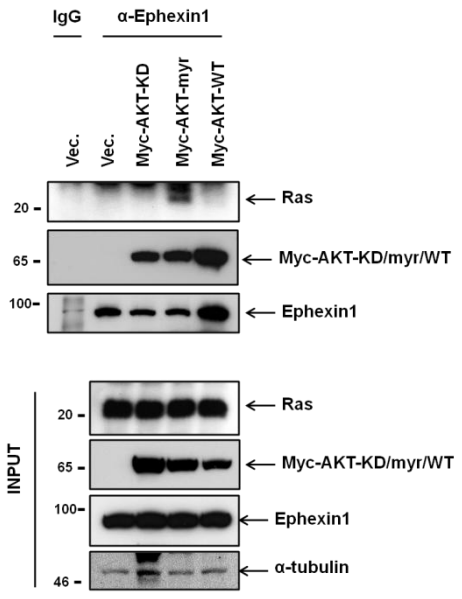

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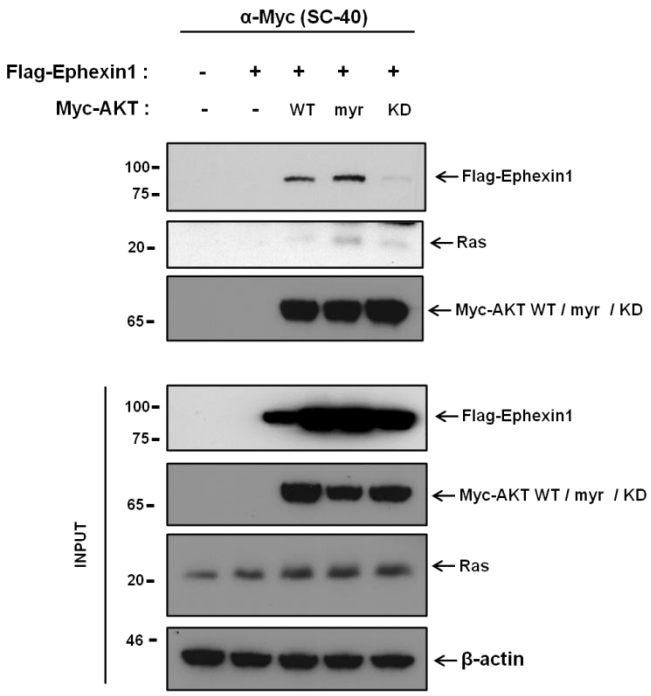

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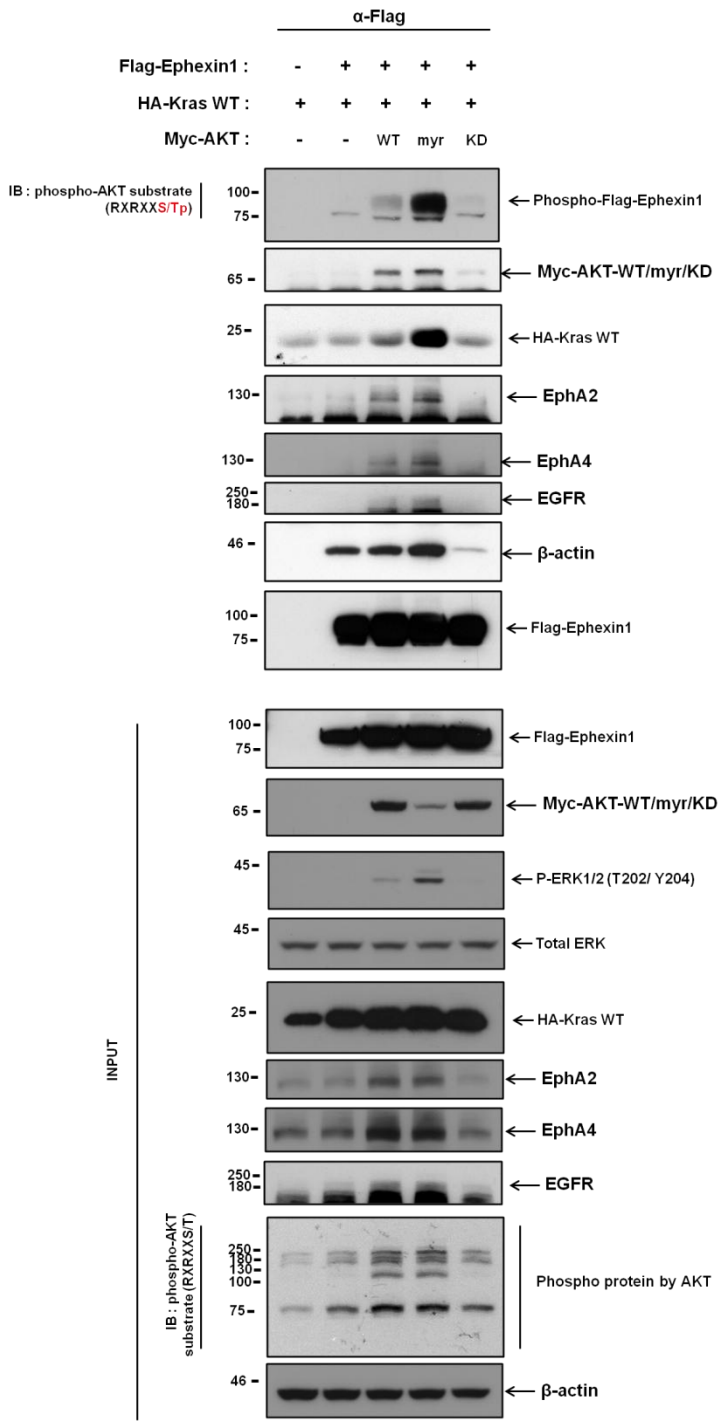

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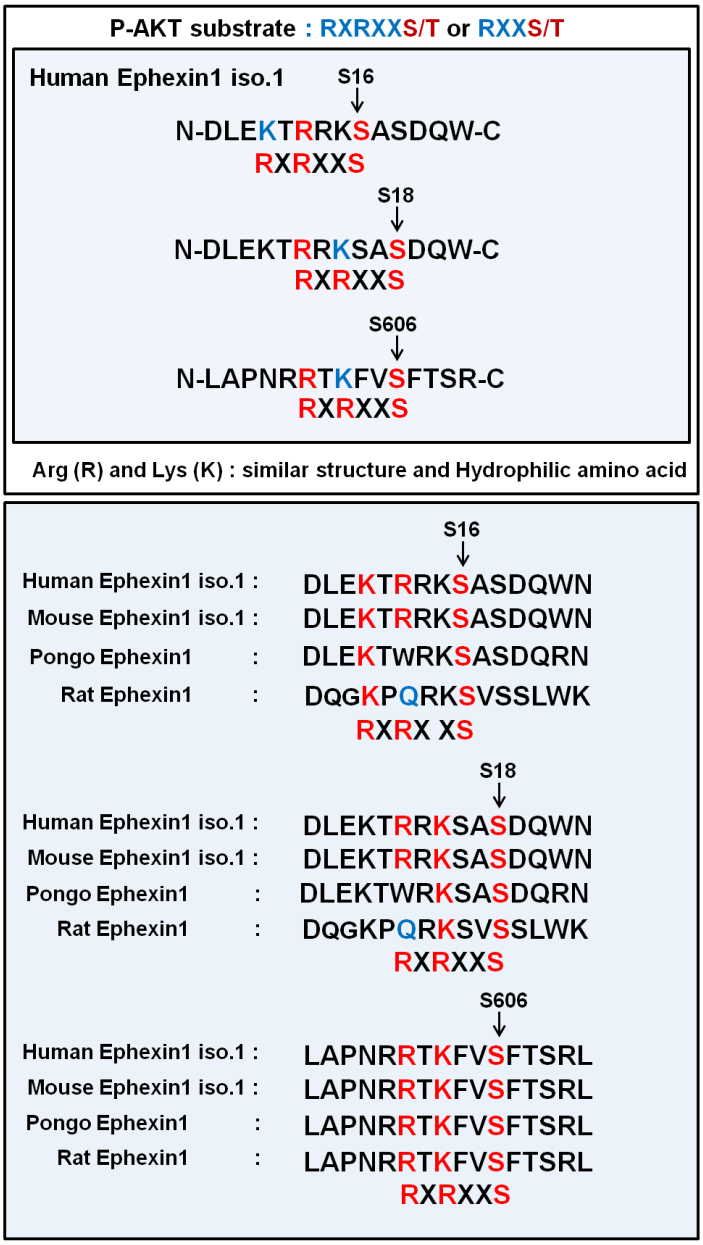

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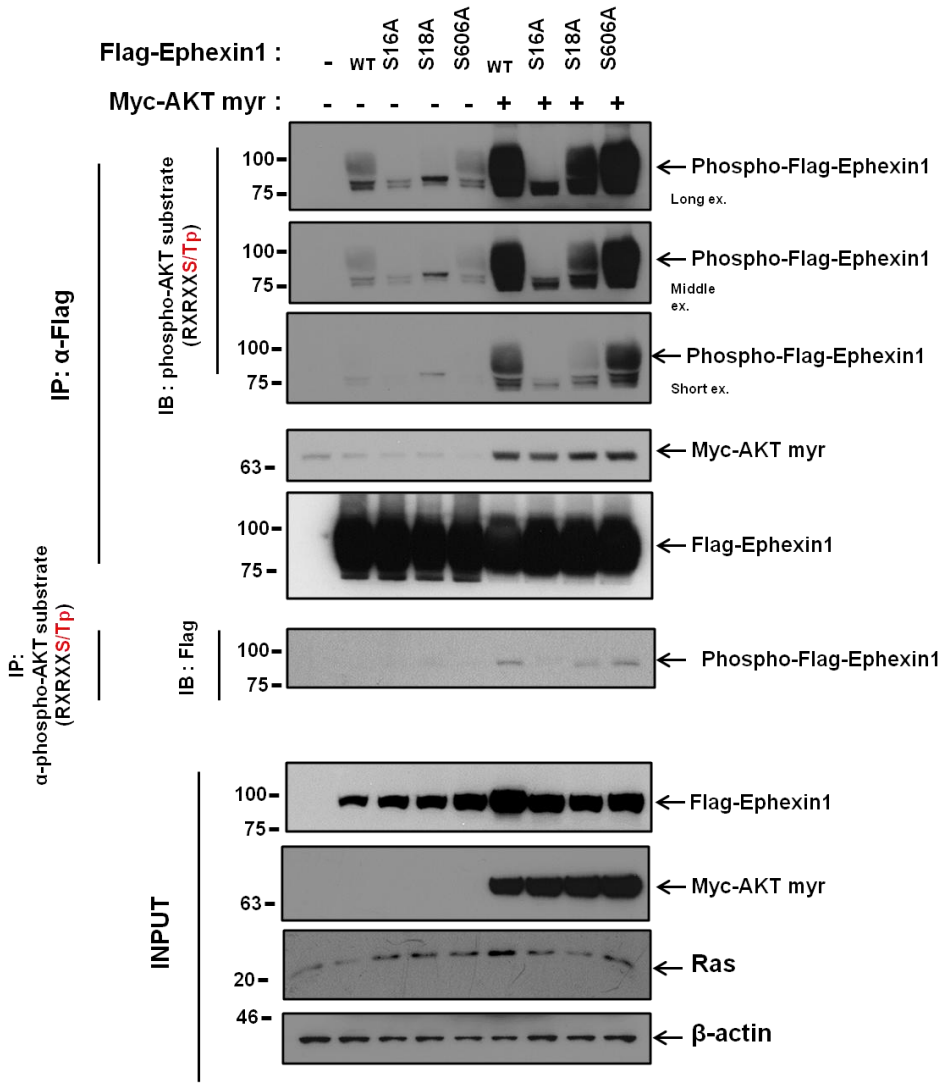

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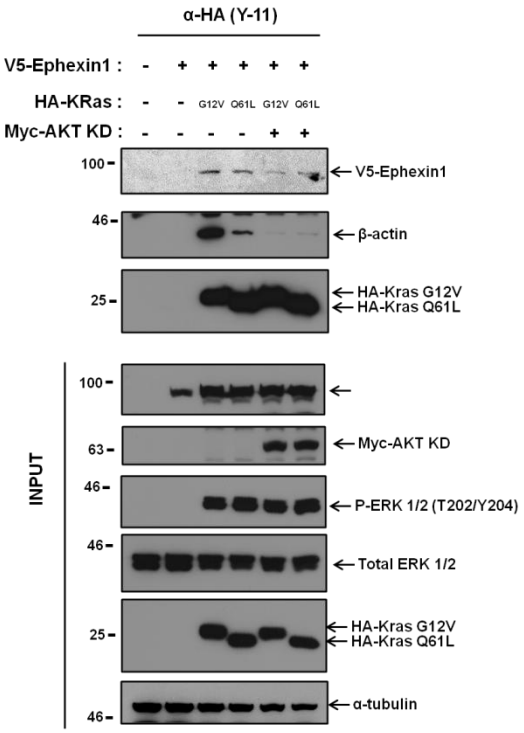

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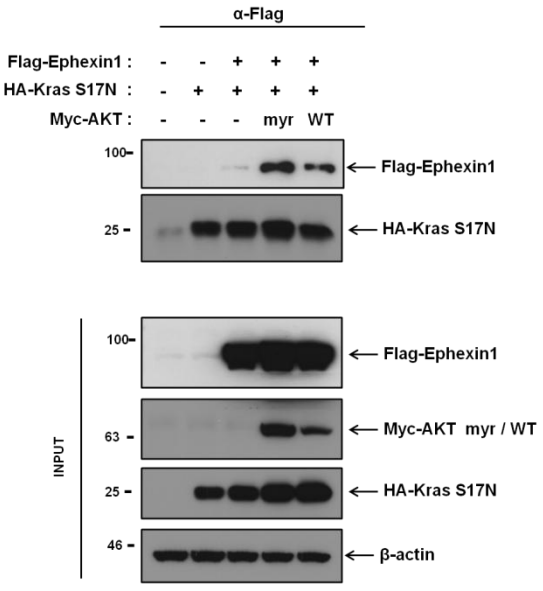

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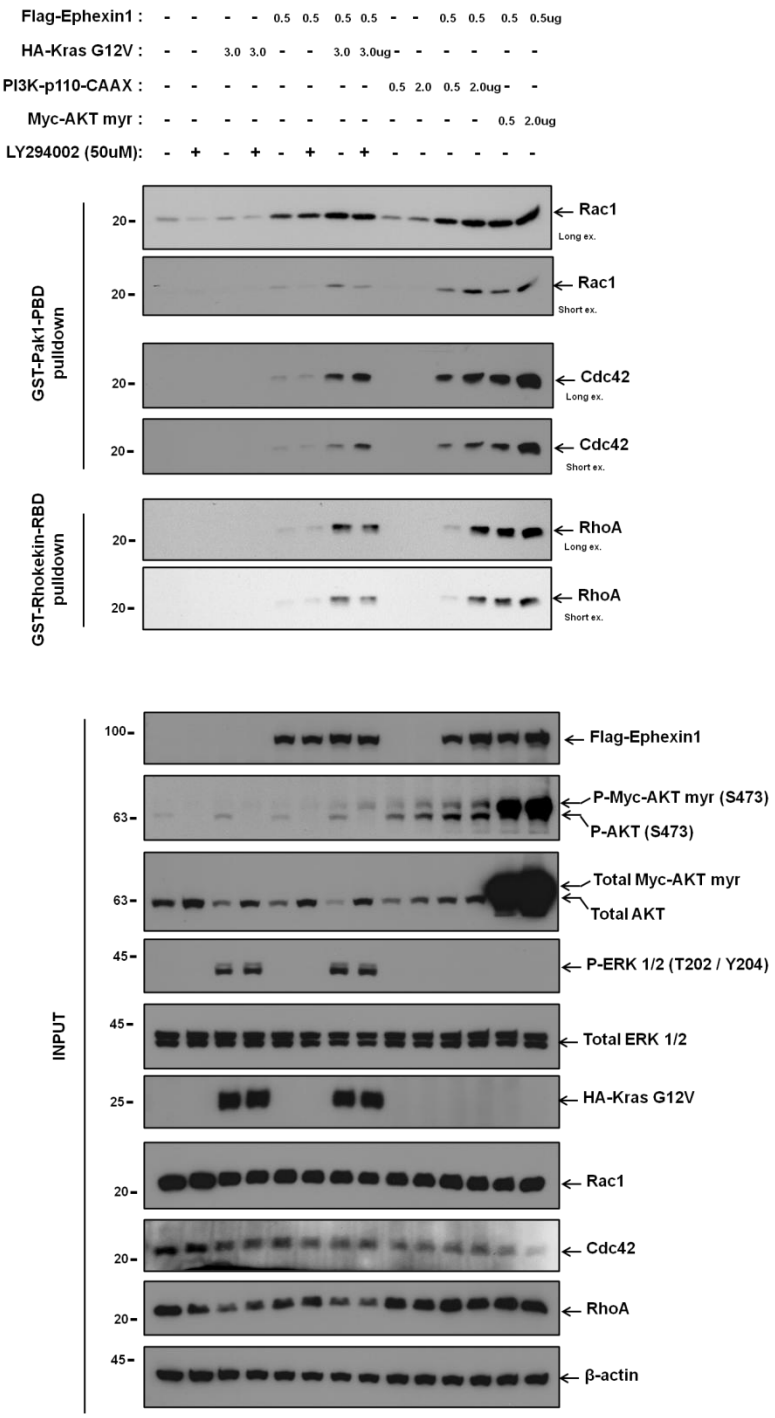

Figure7. Ephexin1 / Ras interaction promoted is by AKT and regulation of Rho family GTPases by Ephexin1 is a AKT / PI3K dependent mechanism.

A) HA tagged-Kras G12V and V5 tagged-Ephexin1 were transfected into HEK-293T cells for 36hr and various inhibitor treatment for 30min before harvesting. Whole cells lysates were immunoprecipitated with the anti-HA antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

B) Myc tagged-AKT myr, EGFP tagged-CK1ε and HA tagged-GSK3β either transfected HEK-293T cells were immunoprecipitated with anti-Ephexin1 (ECM biosiences) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

C) Myc tagged-AKT-KD, Myc tagged-AKT-myr, Myc tagged-AKT-WT either transfected HEK-293T cells were immunoprecipitated with anti-Ephexin1 (ECM biosiences) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

D) Co-expression with Flag tagged-Ephexin1 and either Myc tagged-AKT-WT, Myc tagged-AKT-myr, Myc tagged-AKT-KD were transfected into HEK-293T cells. After 36hr, whole cell lysates were subjected to immunoprecipitation for anti-HA antibody. Immunoprecipitates and cell lysates were characterized by western blot analysis. β-actin was used as a loading control.

E) Myc tagged-AKT WT, myr, KD were co-transfected with either Flag tagged-Ephexin1 and HA tagged-Kras WT into HEK-293T cells. After 36hr,

immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

F) phohospho-AKT substrate search used the program (PhosphoSitePlus® : http://www.phosphosite.org/staticUsingPhosphosite.do) in Ephexin1 protein amino acid sequences. And homology search in several species.

G) Co-expression with WT or site-specific mutant series (S16A, S18A, S606A) of Flag tagged-Ephexin1 and Myc tagged-AKT myr or empty vector were transfected into HEK-293T cells. At 36hr after transfection, immunoprecipitation for anti-Flag (M2) antibody or anti-phospho-AKT substrate (RXRXXS/Tp) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

H) HEK-293T cells transfected with V5 tagged-Ephexin1 and Myc tagged-AKT KD or empty vector were co-transfected with either HA tagged-Kras G12V, Q61L, as indicated. At 36hr after transfection, Immunoprecipitation for anti-HA antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

I) Flag tagged-Ephexin1, Myc tagged-AKT myr, WT, and HA tagged-Kras S17N were transfected into HEK-293T cells, as indicated. At 36hr after transfection, Immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

J) HEK-293T cells were co-transfected with varying amounts of expression plasmids encoding HA tagged-Kras G12V, Flag tagged-Ephexin1 or PI3K-p110 CAAX or Myc tagged-AKT-myr, as indicated. Cells were treated with

PI3K inhibitor, LY294002 (50uM) for 30min before harvesting. Cell lysates were measured by its ability to increase the levels of GTP-RhoA, as detected by GST-Rhotekin-RBD pull-down, or to increase the levels of GTP-Rac1 or Cdc42, as detected by GST-Pak1-PBD pull-down. Cell lysates and GTP bound Rho family GTPases were determined by western blot analysis with indicated antibodies. β-actin was used as a loading control.

III-8. EGF/ active Ras increased Ephexin1 / Eph A2/ A4 / EGFR / Ras complex.

The interaction between EphA receptor and Ephexin1 is well known [27, 28, 32]. Therefore, we have investigated the correlation between Ras and Ephexin1 and the impact from the upstream level. First, we test whether Ephexin1 will interact with EphA1, EphA2, EphA3 and EphA4. Interestingly, Ephexin1 interaction was most strong with EphA2 and EphA4 (Fig. 8A). EphA2 and EphA4 is known that there are many associated with a cancer [3, 41, 42, 43, 44, 45 46, 47, 49, 50].

We found that Ephexin1 was interaction EphA2/A4 receptor and EGFR in HCT116 cells after EGF (100ng/ml) for 30min treatment. Therefore, we suggest that Ephexin1, EphA receptor, Ras, EGFR, and Rho family GTPase was possible to form the "Ephexin1 and Ras Multi-Complex (ERMC)" (Fig. 8B). We showed that Flag tagged-Ephexin1 and HA tagged-Kras G12V co-transfection was promoted ERMC (Ephexin1 and Ras Multi-Complex) than Flag tagged-Ephexin1. In addition, Ephexin1 in ERMC showed increased interaction with RhoA / Rac1 / Cdc42. Also, we showed that immunoprecipitation of V5 tagged-EphA2 using anti-V5 antibody confirmed ERMC (Ephexin1 and Ras Multi-Complex) as Flag tagged-Ephexin1 immunoprecipitation (Fig. 8C, D). In addition, EphA2 and EGFR interaction was Ephexin1 and Ras dependent manner (Fig. 8F, G). These data suggest that Ephexin1 was multi-complex with Ras pathway component. In additionally, this complex, ERMC promoted the activity state of Ras, or the level of Ephexin1. (Fig. 8E, F, G).

We had shown that change in the form of a multi-complex could be due to Growth factor. The EGF is a major growth factor, and thus activated Ras-Raf-ERK pathway by binding to the EGFR. We showed that ERMC formation was increased on EGF (100ng/ml) for 5min, 15min and 30min treatment. Also, serum (10% FBS) was almost similarly ERMC being promoted as EGF (Fig. 8H, I). However, EphrinA1-Fc (1ug/ml) for 5min, 15min and 30min treatment caused dissociation of ERMC followed by inactivation of ERK 1/2 (Fig. 8J).

Taken together, these results propose that active Ras or large amounts of Ephexin1 formed ERMC (Ephexin1 and Ras Multi-Complex). And this complex is important for ERK activation.

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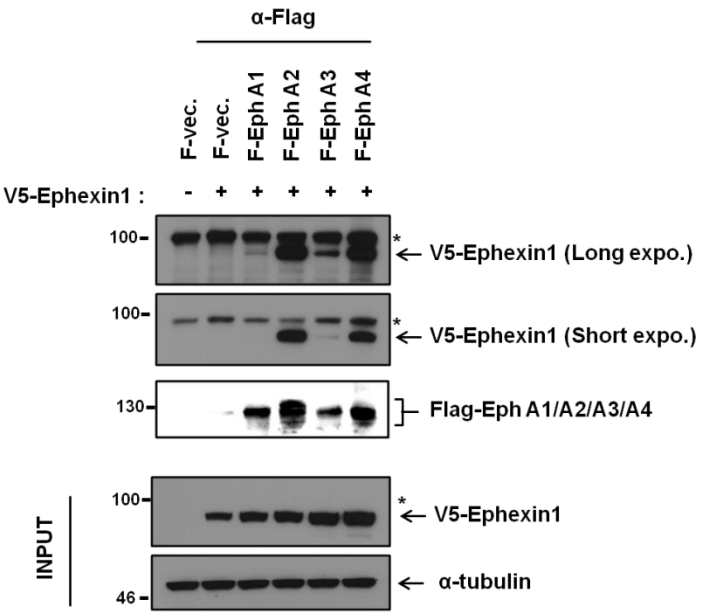

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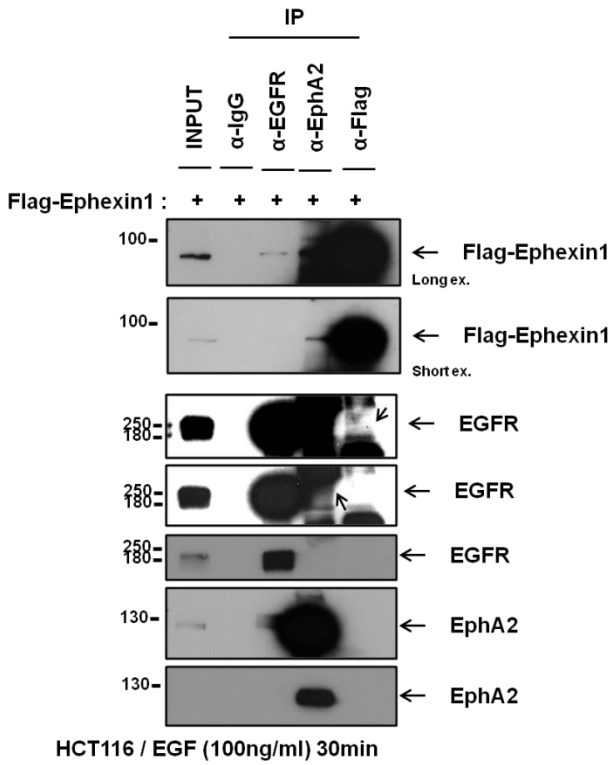

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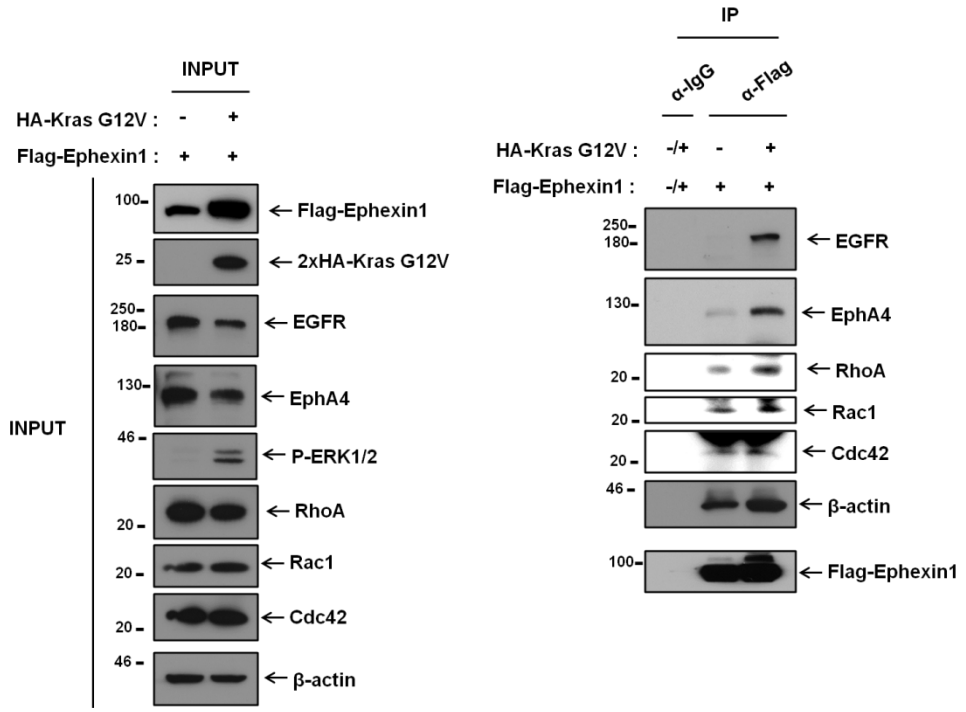

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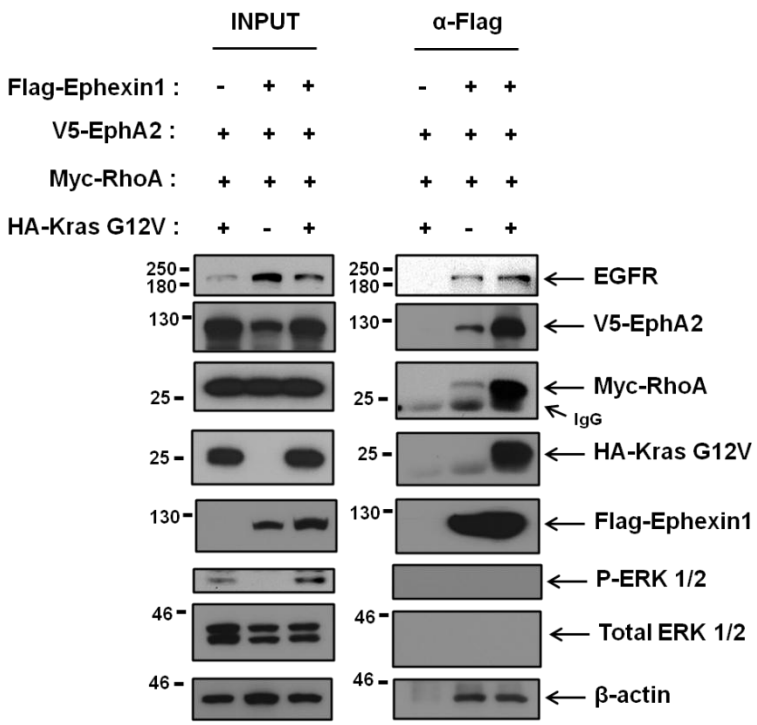

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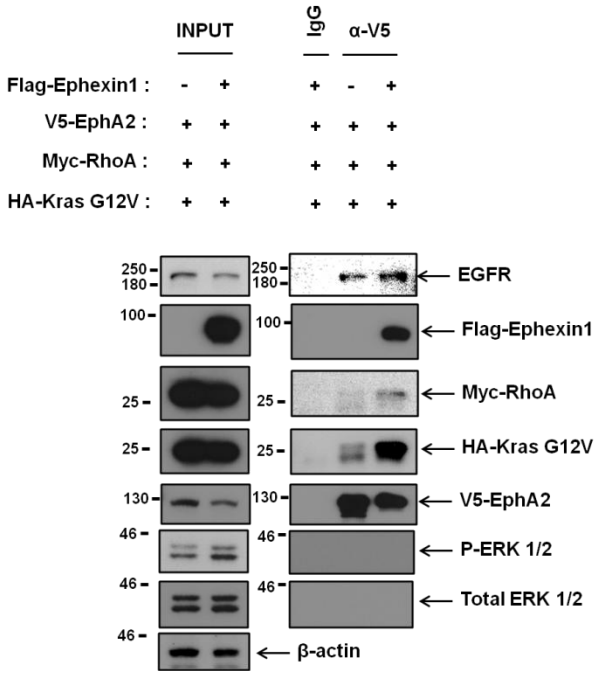

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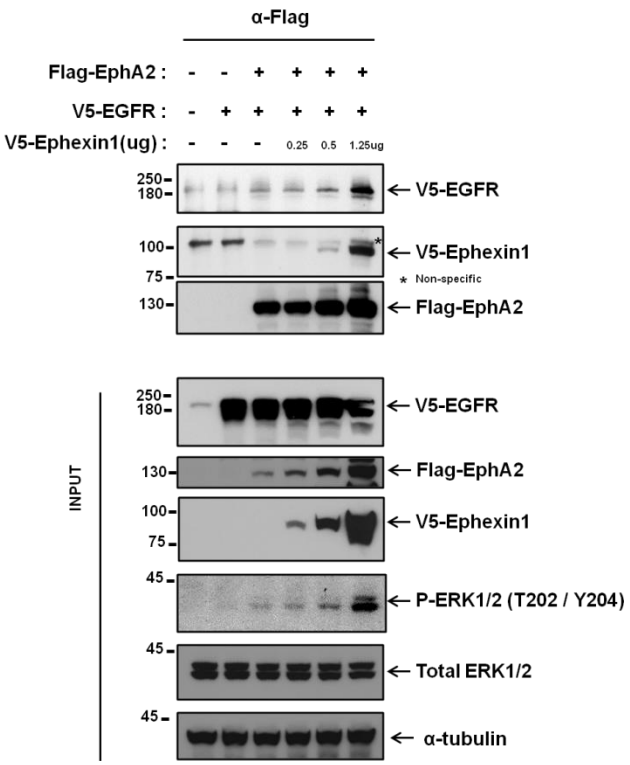

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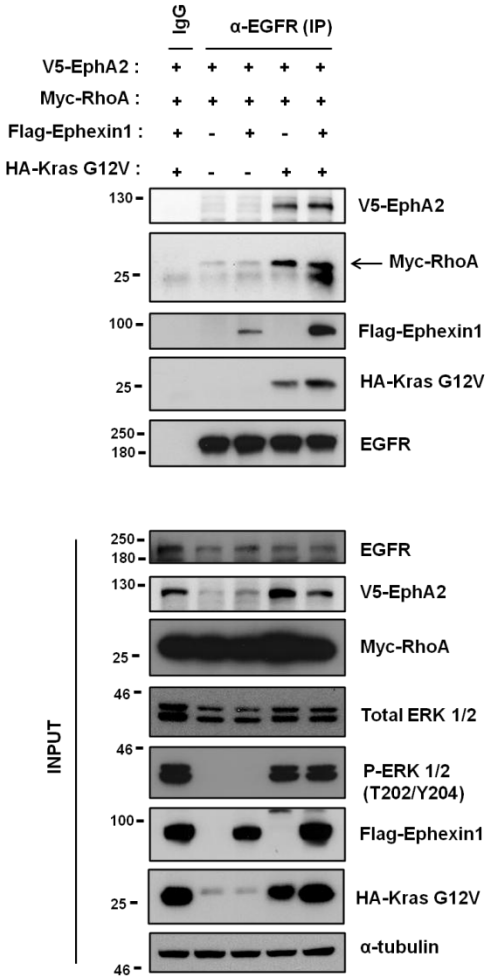

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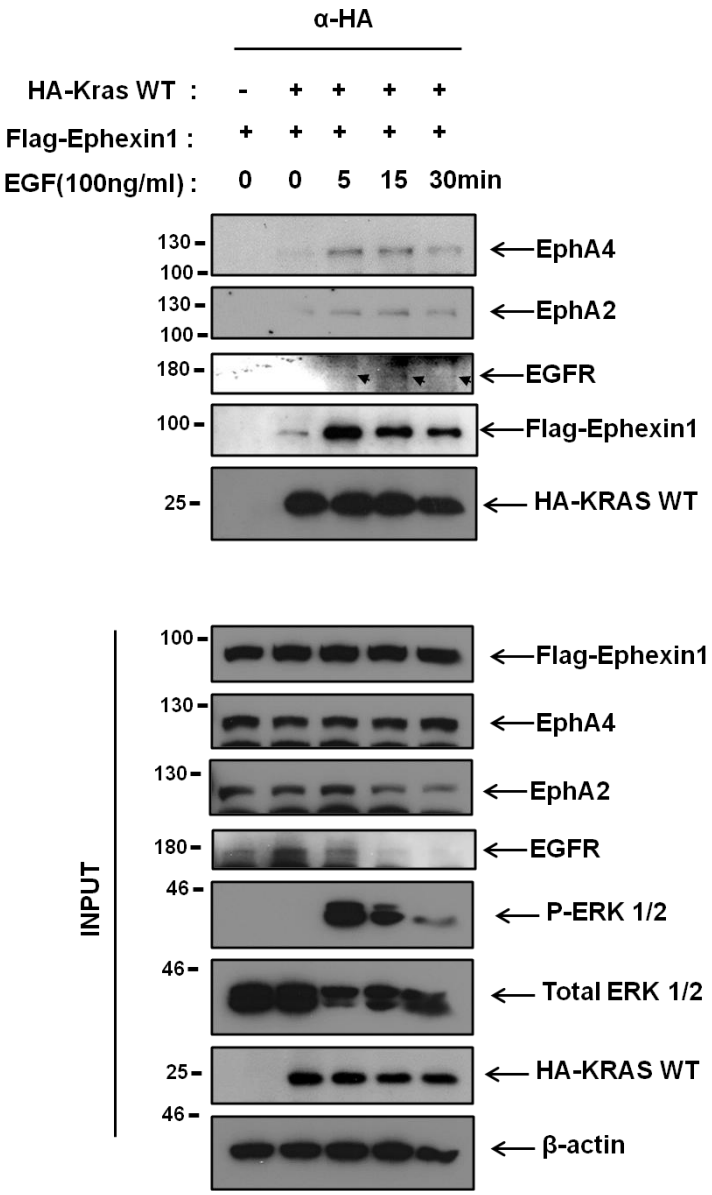

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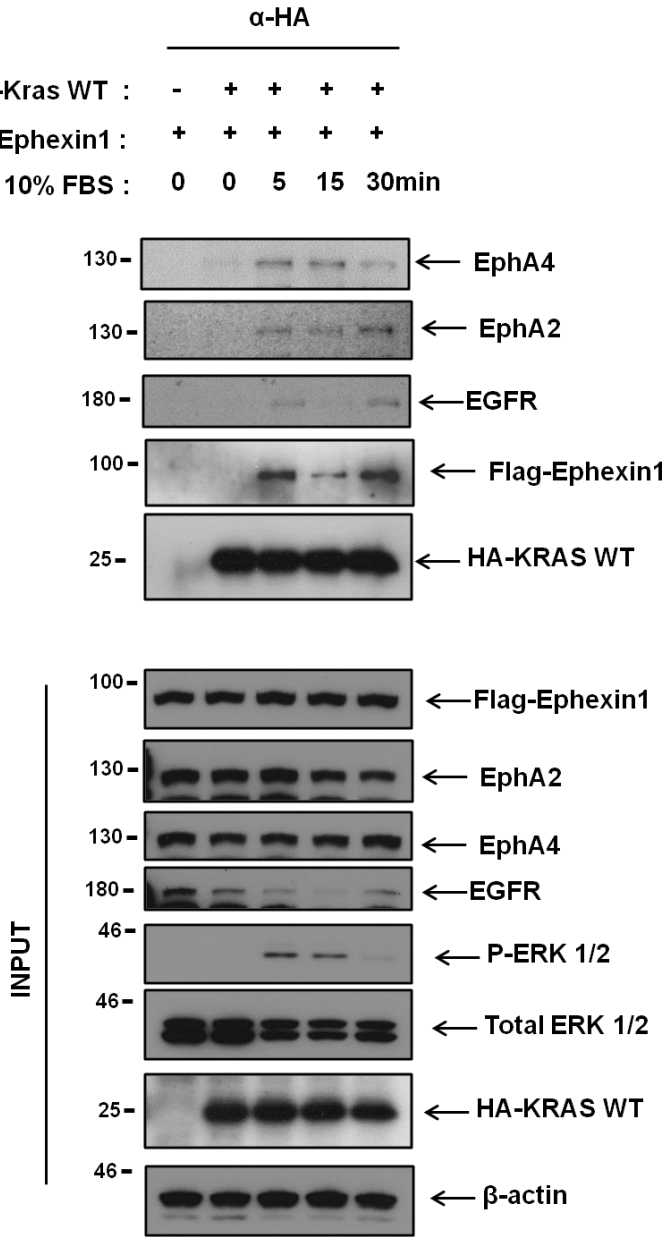

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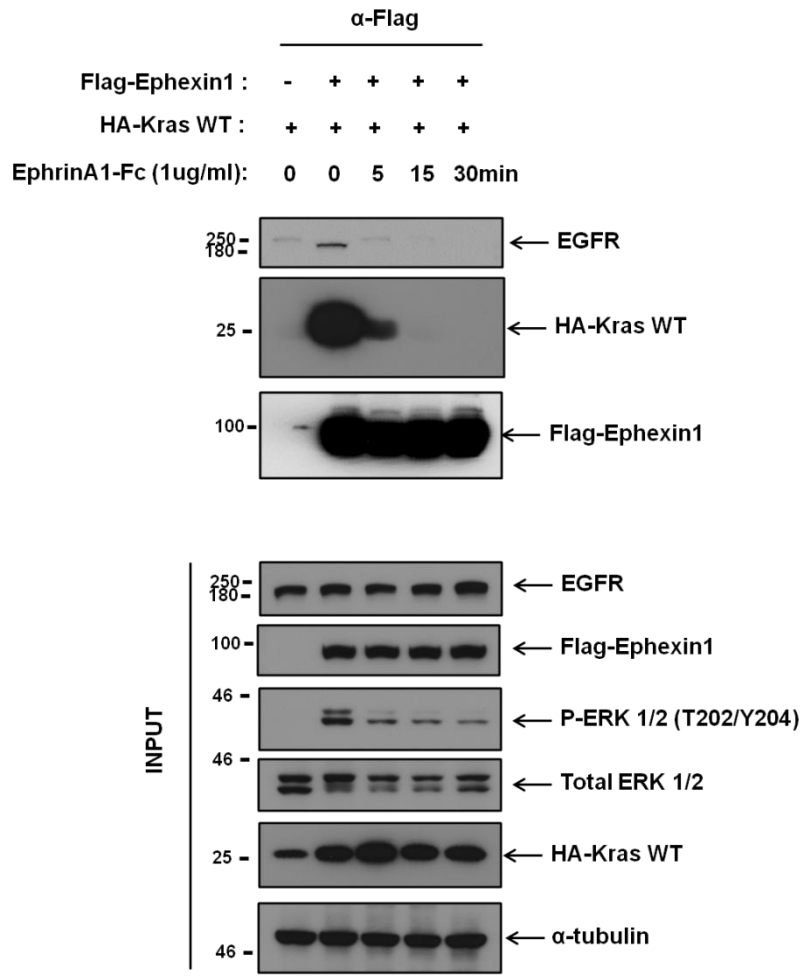

Figure8. EGF/ active Ras increased Ephexin1 / Eph A2/ A4 / EGFR / Ras complex.

A) Flag tagged-EphA1 / A2/ A3 /A4 were co-transfected with either V5 tagged-Ephexin1 into HEK-293T cells. At 36hr after transfection, immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

B) Flag tagged-Ephexin1 transfected and EGF(100ng/ml) for 30min treated before harvesting in HCT116 cells were subjected to immunoprecipitation for anti-EGFR, EphA2, Flag antibody and anti-IgG followed by western blotting with indicated antibodies.

C) Flag tagged-Ephexin1 and either vector or HA tagged- Kras G12V were transfected into HEK-293T, as indicated. After 36hr, lysates were subjected to immunoprecipitation for anti-IgG and anti-Flag antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control

D) Flag tagged-Ephexin1, V5 tagged-EphA2, Myc tagged-RhoA and either empty vector or HA tagged- Kras G12V were co-transfected into HEK-293T, as indicated. After 36hr, lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control

E) V5 tagged-EphA2, Myc tagged-RhoA, HA tagged-Kras G12V and either empty vector or Flag tagged-Ephexin1 were co-transfected into HEK-293T, as indicated. After 36hr, lysates were subjected to immunoprecipitation for anti-IgG and anti-V5 antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control β-actin

F) HEK-293T cells were co-transfected with Flag tagged-EphA2, V5-EGFR and varying amounts of V5 tagged-Ephexin1, as indicated. At 36hr after transfection, immunoprecipitation using anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control

G) V5 tagged-EphA2, Myc tagged-RhoA with either Flag tagged-Ephexin1 and/or HA tagged-Kras G12V were co-transfected into HEK-293T, as indicated. After 36hr, lysates were subjected to immunoprecipitation for anti-IgG and anti-EGFR antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

H) Co-expression with Flag tagged-Ephexin1 and HA tagged-Kras WT were as indicated. After 36hr, this cells were stimulated using EGF(100ng/ml) for 0, 5, 15, 30min before cell harvesting. Whole-cell lysates were subjected to immunoprecipitation for anti-HA antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

I) Co-expression with Flag tagged-Ephexin1 and HA tagged-Kras WT were as indicated. After 36hr, this cells were stimulated using 10% FBS for 0, 5, 15, 30min before cell harvesting. Whole-cell lysates were subjected to immunoprecipitation for anti-HA antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

J) Co-expression with Flag tagged-Ephexin1 and HA tagged-Kras WT were as indicated. After 36hr, this cells were stimulated using EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting. Whole-cell lysates were subjected

to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

III-9. Ephexin1 / EGF was controlled according to phospho state of EphA2/A4.

Previous reports stated that, upon EphrinA1-Fc treatment there is phosphorylation in EphA receptor such as phospho-EphA2 Y588, 594 and phospho-EphA4 Y596, 602 in juxtamembrane domain of cytoplasmic region [63, 64, 65, 66]. The phosphorylation of EphA-receptor is caused activation of downstream effectors (ex. c-Src, Vav2/3 etc.). And that EphrinA1-Fc causes MAPK inactivation followed suppression of tumorigenesis [67, 68, 69, 70, 71, 72]. Also, phosphorylation of EphA2 Serine 897 by AKT is promotes tumor progression as oncogenic effect [73, 74]. EphrinA1-Fc stimulation causes Serine897 de-phosphorylation and simultaneous inactivation of AKT [76]. We observed that EphrinA1-Fc (1ug/ml) treatment increased phospho-EphA2 (Y588) / A4 (Y602) and decreased phosphor-EphA2 (S897) / A4 (S887). Alternatively, EGF (100ng/ml) treatment decreased phospho-EphA2 (Y588) / A4 (Y602) and increased phosphor-EphA2 (S897) / A4 (S887) (Fig. 9A, B). Therefore, we demonstrated that Ephexin1 and phospho-EphA receptor that what kind of interaction with phospho-EphA receptor state on EphrinA1-Fc or EGF treatment. Also, we together test that changed test the interaction between Rho family GTPases and Ephexin1. We confirmed that when treated with EphinA1-Fc (1ug/ml) it decreased phospho-EphA2 (S897) and increased phospho-EphA2 (Y588, 594). And, treatment of EGF (100ng/ml) increased phosph-EphA2 (S897) and decreased phospho-EphA2 (Y588, 594) (Fig. 9A). We checked with the Ephexin1 and EphA receptor interaction. When treatment with a EphinA1-Fc

(1ug/ml) were increased between the Flag tagged-Ephxin1 and V5 tagged-EphA2 (total) or EphA4 (total) receptor interaction. Interestingly, Flag tagged-Ephexin1 and phospho-EphA2 (Y594) / A4 (Y602) increased interaction. However Flag tagged-Ephexin1 and phosphor-EphA2 (S897) / A4 (S887) decreased interaction (Fig. 9C, D, E top panels). At this state, between Flag tagged-Ephexin1 and Rac1 / RhoA the interaction was decreased, but not changed interaction of Cdc42 and Ephexin1. And interaction of cytoskeleton such as β-actin was decreased (Fig. 9C, D, E bottom panels). In addition, EGF (100ng/ml) treatments increased between the Flag tagged-Ephxin1 and V5 tagged-EphA2 (total) or EphA4 (total) receptor interaction as same EphrinA1-Fc treatment. However, Flag tagged-Ephexin1 in EGF condition was decreased with phospho-EphA2 (Y594) / A4 (Y602) interaction. Interestingly, Flag tagged-Ephexin1 and phosphor-EphA2 (S897) / A4 (S887) increased interaction (Fig. 9F, G, H top panels). At this state, between Flag tagged-Ephexin1 and RhoA interaction were decreased, and increased interaction of Rac1, but not changed interaction of Cdc42 and Flag tagged-Ephexin1. In addition, interaction of cytoskeleton such as β-actin was increased (Fig. 9F, G, H bottom panels).

Taken together, these results suggest that Ehexin1 interacts with the EphA receptors in different states by EphrinA1-Fc and growth signal (ex. EGF and other growth factors), and this represents a very different effect such as tumor suppression and tumor progression.

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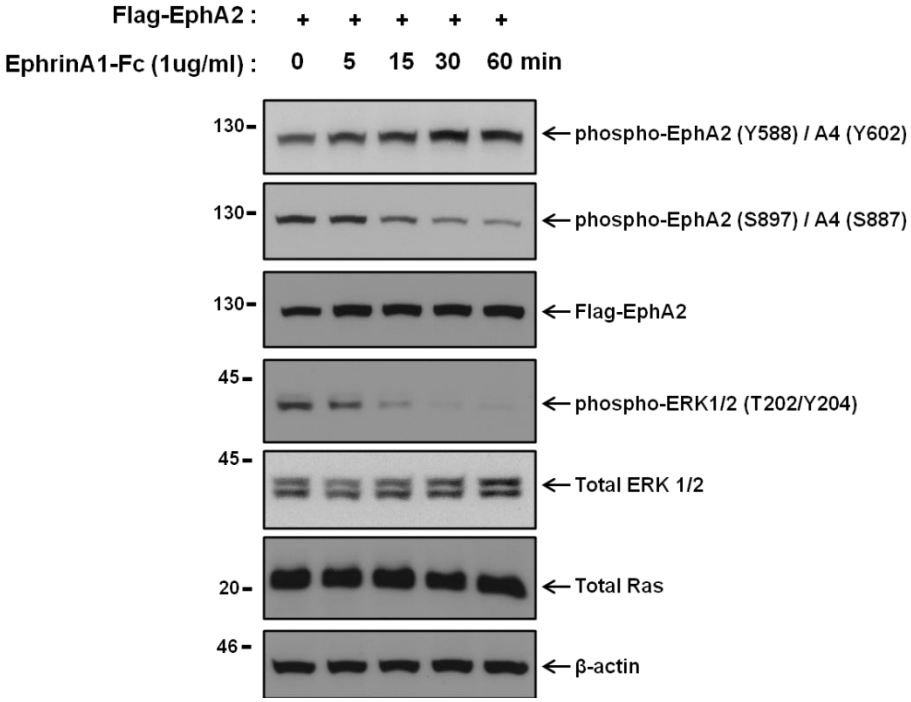

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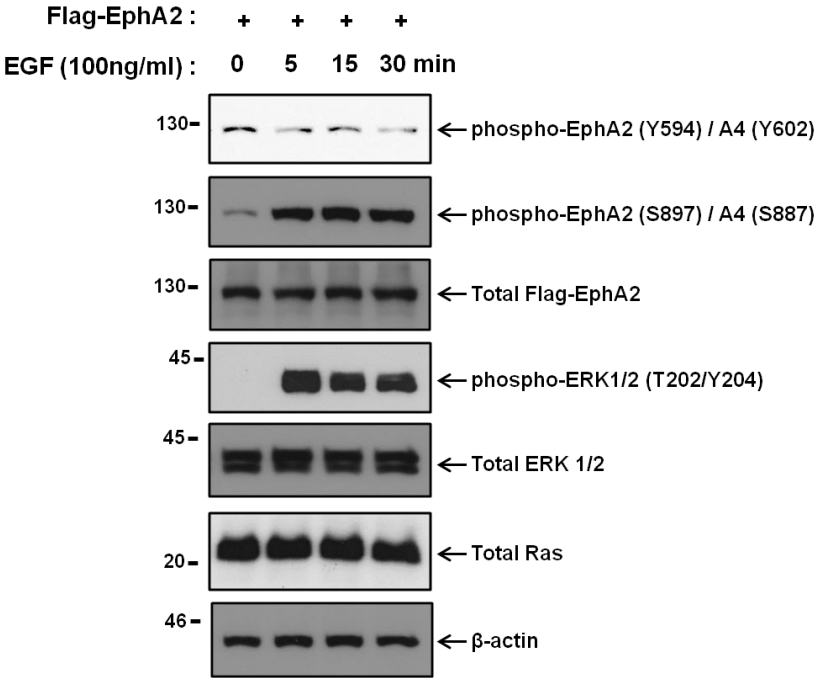

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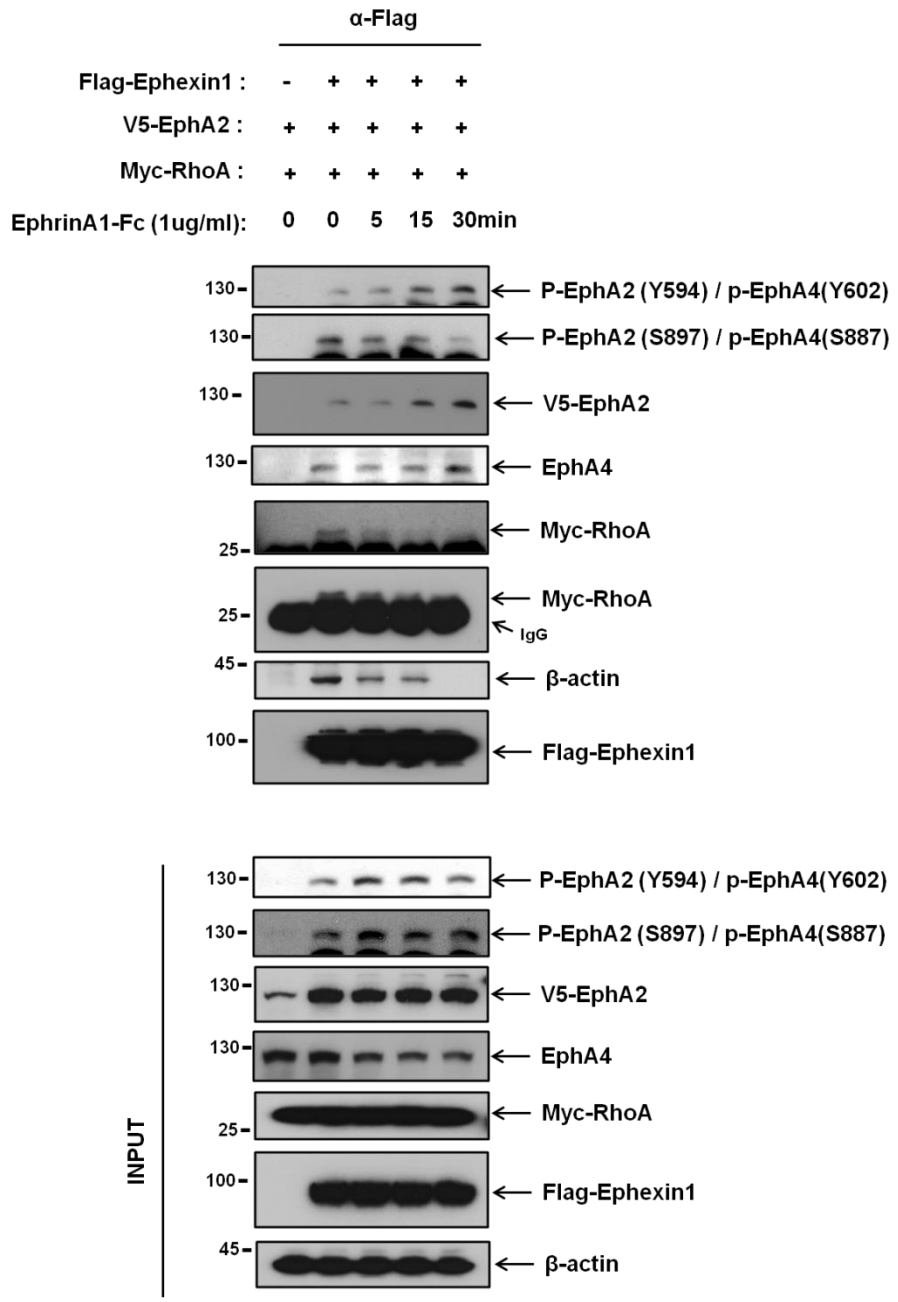

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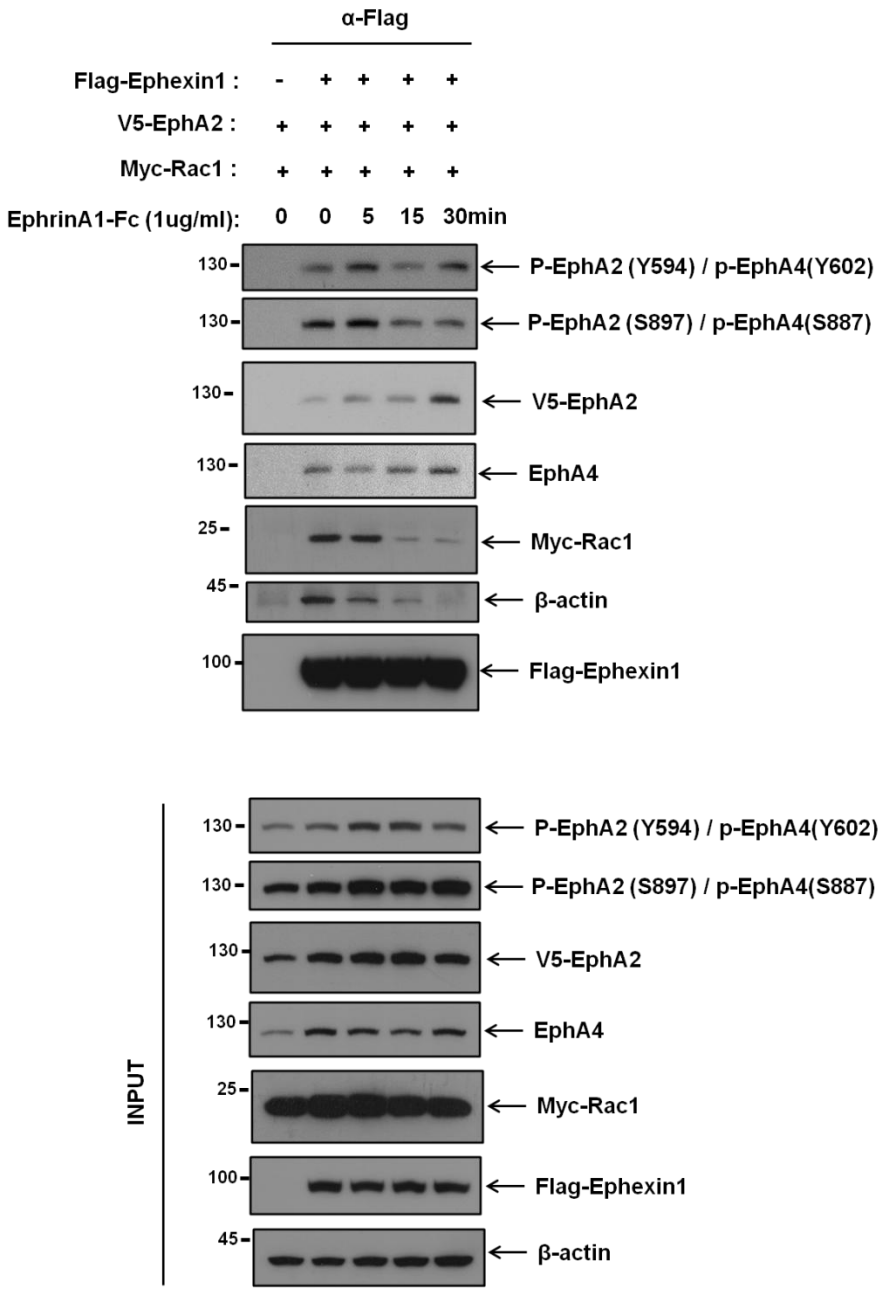

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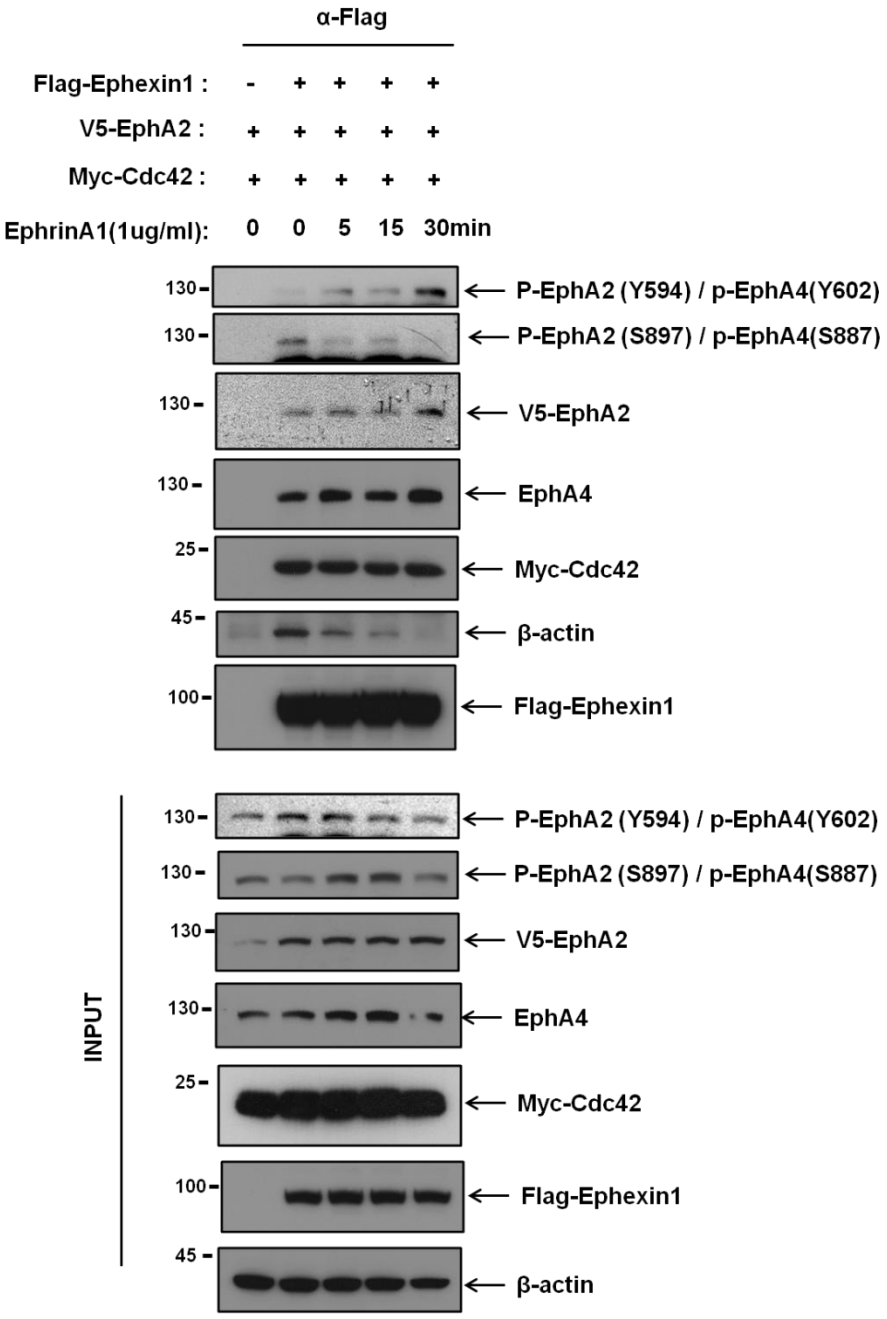

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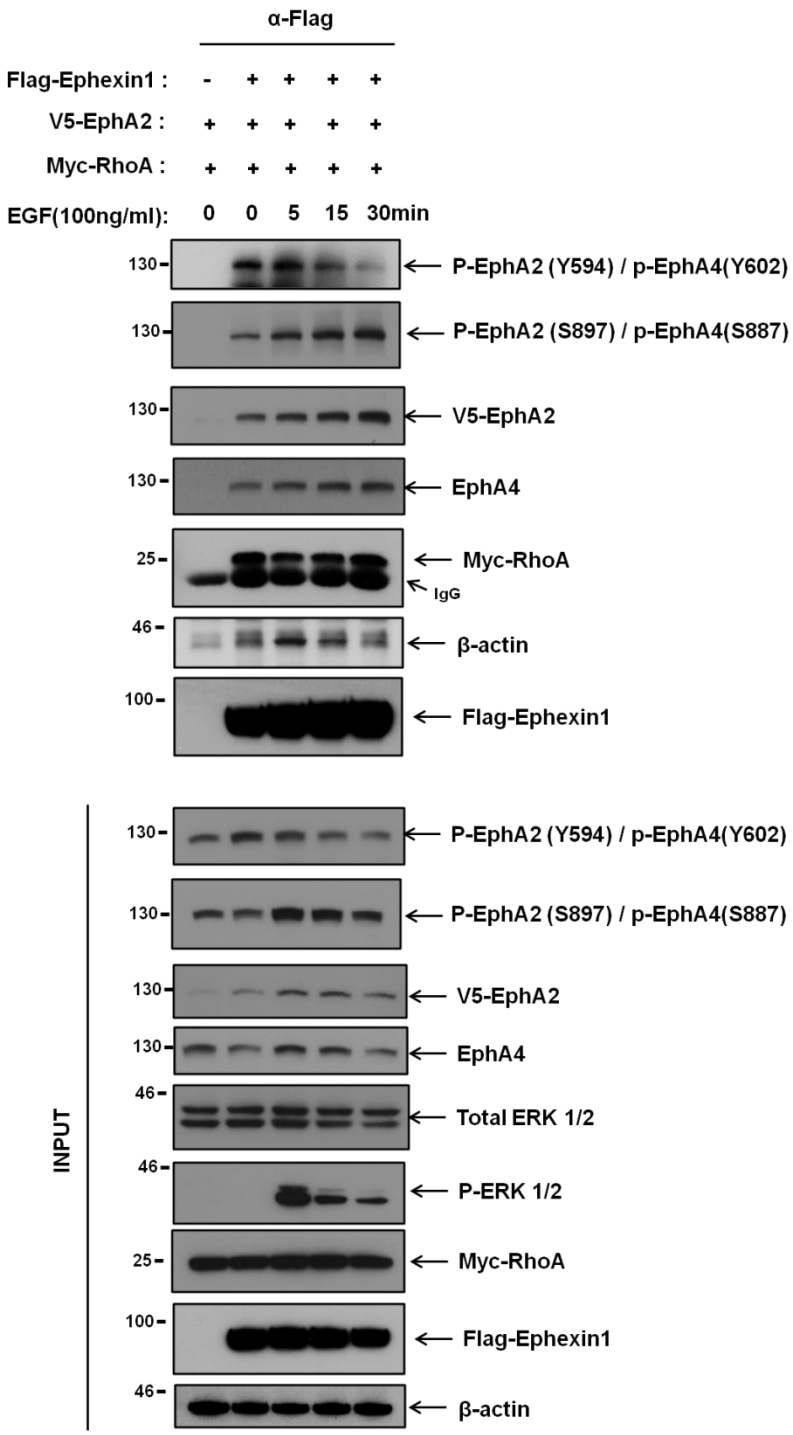

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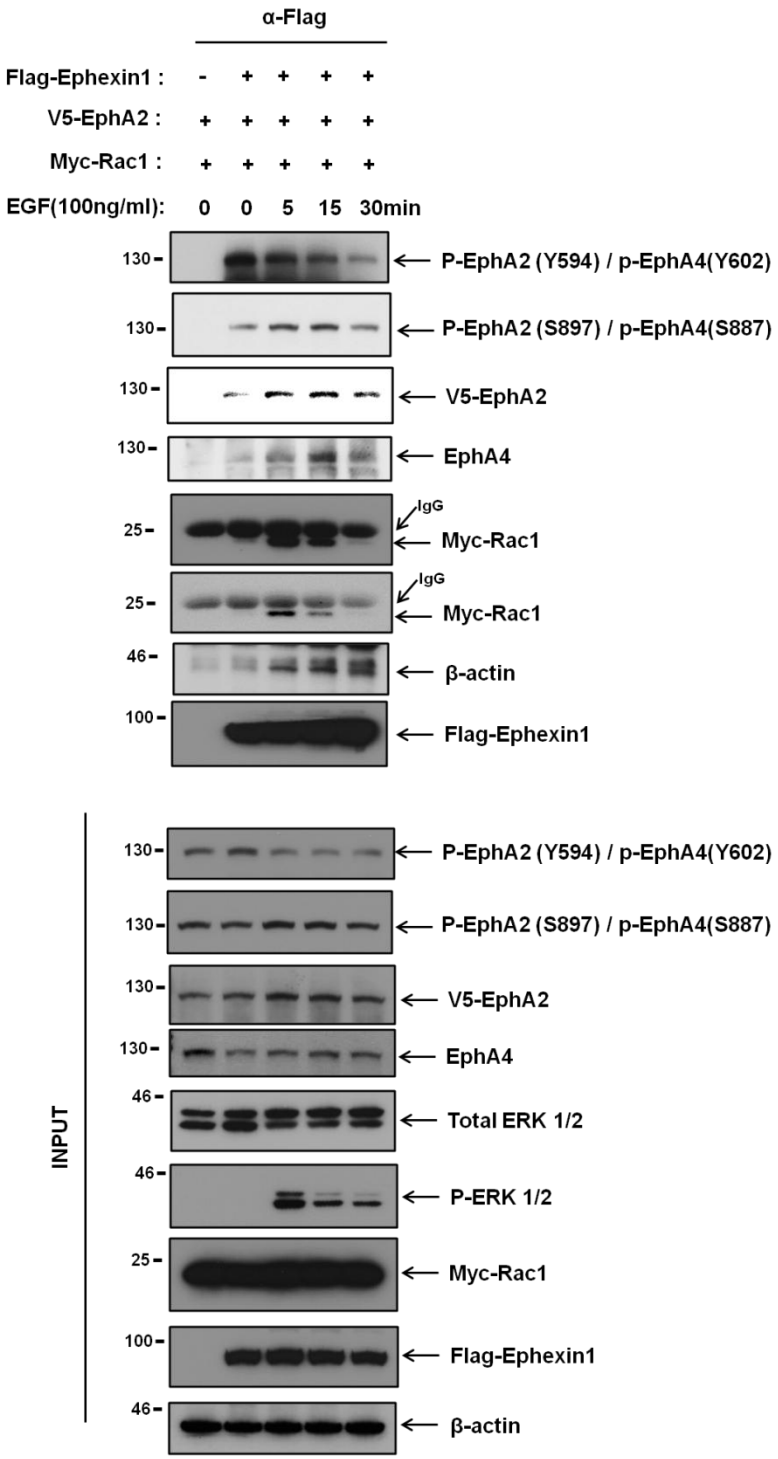

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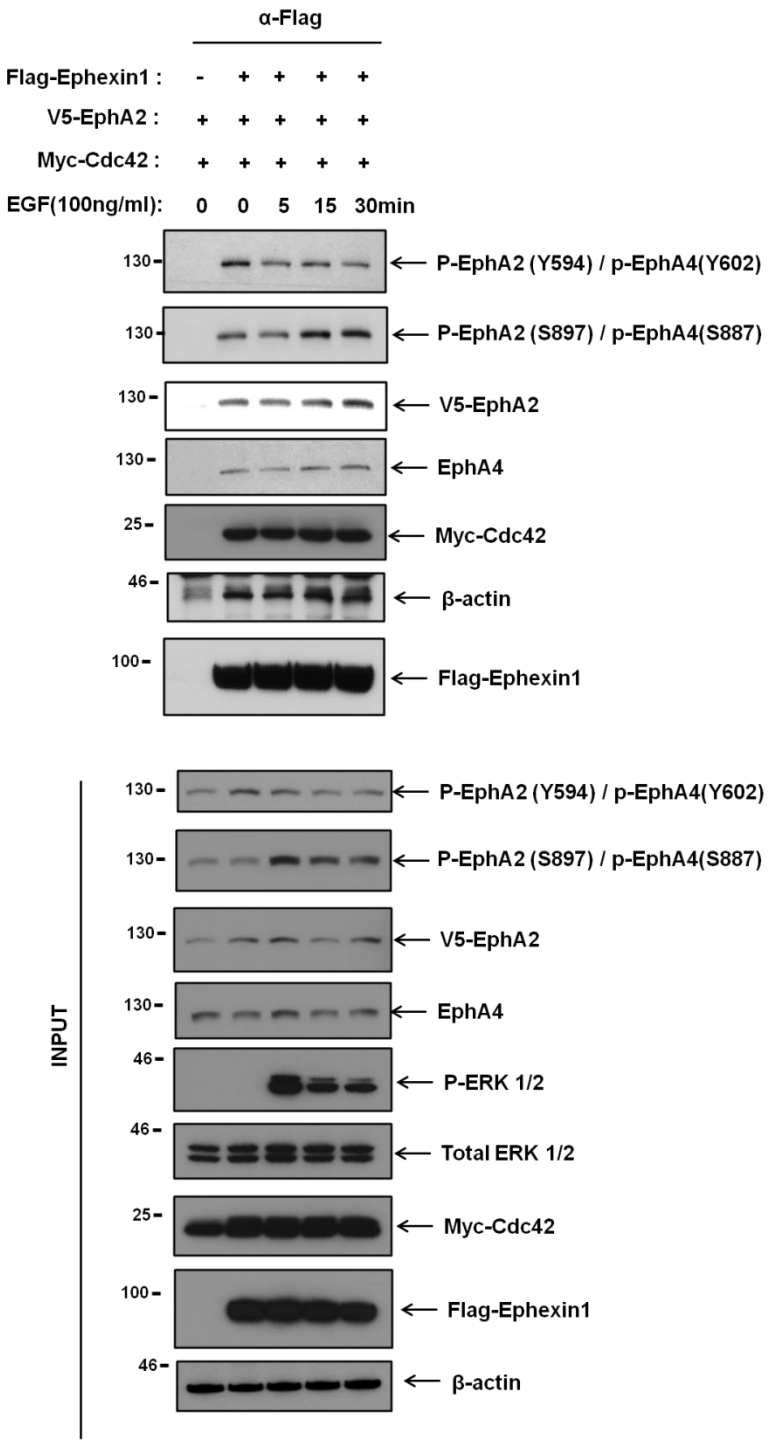

Figure9. Ephexin1 / EGF was controlled according to phospho state of EphA2/A4.

A) Flag tagged-EphA2 was transfected into HEK-293T cells for 36hr and stimulated with EphrinA1 (1ug/ml) for 0, 5, 15, 30min times. Whole cell lysates were determined used western blot analysis with indicated antibodies. β-actin was used as a loading control.

B) Flag tagged-EphA2 was transfected into HEK-293T cells for 36hr and stimulated with EGF (100ng/ml) for 0, 5, 15, 30min times. Whole cell lysates were determined used western blot analysis with indicated antibodies. β-actin was used as a loading control.

C) Flag tagged-Ephexin1 was co-transfected with V5 tagged-EphA2 and Myc tagged-RhoA into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

D) Flag tagged-Ephexin1 was co-transfected with V5 tagged-EphA2 and Myc tagged-Rac1 into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

E) Flag tagged-Ephexin1 was co-transfected with V5 tagged-EphA2 and Myc tagged-Cdc42 into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml)

for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control..

F) Flag tagged-Ephexin1 was co-transfected with V5 tagged-EphA2 and Myc tagged-RhoA into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

G) Flag tagged-Ephexin1 was co-transfected with V5 tagged-EphA2 and Myc tagged-Rac1 into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

H) Flag tagged-Ephexin1 was co-transfected with V5 tagged-EphA2 and Myc tagged-Cdc42 into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

III-10. EphrinA1 / Ras regulated EphA2/A4 -EGFR homo/hetero-dimer.

The Eph receptors form a homo or hetero-dimer/oligomer on EphrinA/B ligands treatment [40, 41, 78, 79]. And EGFR forms hetero-dimer with various RTKs (Receptor tyrosine kinases) [57, 58, 59]. We demonstrate EGF and EphrinA1-Fc treatment was for the dimerization of EphA receptor and EGFR. Like published, each Flag/V5 tagged-EphA2 and Flag/V5 tagged-EphA4 increased homo-dimer on the EphrinA1-Fc (1ug/ml) treat time dependent. And increase of EphA2/A2 or EphA4/A4 dimerization showed ERK inactivation (Fig. 10A, B). In contrary, each Flag/V5 tagged-EphA2 and Flag/V5 tagged-EphA4 decreased homo-dimer on the EGF (100ng/ml) treatment. And decrease of EphA2/A2 or EphA4/A4 dimerization observed ERK activation (Fig. 10C, D). Interestingly, hetero-dimerization of EphA2 receptor and EGFR was reduced by EphrinA1-Fc (1ug/ml) treatment with decreased between Flag tagged-EphA2 and cytoskeleton such as α-tubulin (Fig. 10E). Also, Flag tagged-EphA4 and V5 tagged-EGFR were same result as Flag tagged-EphA2 and V5 tagged-EGFR (Fig. 10F). However, EGF (100ng/ml) treatments were hetero-dimerization of Flag tagged-EphA2 and V5 tagged-EGFR was promoted (Fig. 10G). And Flag tagged-EphA4 and V5 tagged-EGFR in EGF treatment condition were same result as Flag tagged-EphA2 and V5 tagged-EGFR (Fig. 10H).

We demonstrated that dimerization of EphA receptors and EGFR changed on state of Ras. The interaction of Flag tagged-EphA2/A4 receptor and V5 tagged-EGFR were dramatic increased by HA tagged-Kras G12V or Q61L

(active form) co-expression (Fig. 10I, J, K, L). However, overexpression of inactivated Ras (HA tagged-KRas S17N) did not improve interaction of Flag tagged-EphA2/A4 receptor and V5 tagged-EGFR (Fig. 10K, L). In addition, Myc tagged-AKT WT, myr, KD controlled formation of hetero-dimerization of Flag tagged-EphA2 and V5 tagged-EGFR that Myc tagged-AKT WT and myr could increase hetero-dimerization, and Myc tagged-AKT KD could decrease hetro-dimeriation (Fig. 10N) Taken together, our results suggest that activated Ras or activated AKT formed EGFR-EphA complex that this complex caused strong ERK activation effect.

In Summary, our findings suggest that Ephexin1 is a strong oncoprotein and that Ephexin1 with active Ras can cooperate to cause synergistic effect of Rho family GTPases via phosphorylation of Ephexin1 by PI3K / AKT dependent mechanism. Also, Ephexin1 is formed together with Ras-EGFR-EphA receptor complex and this complex promoted tumorigenesis signal.

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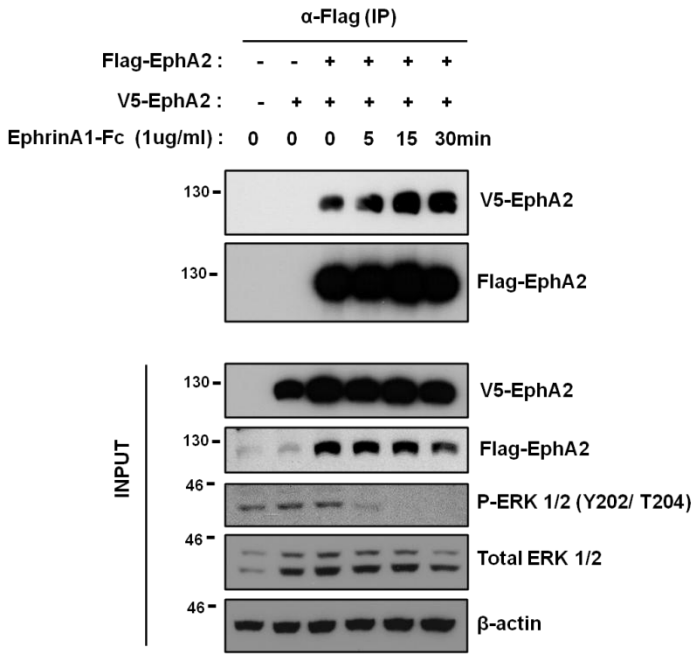

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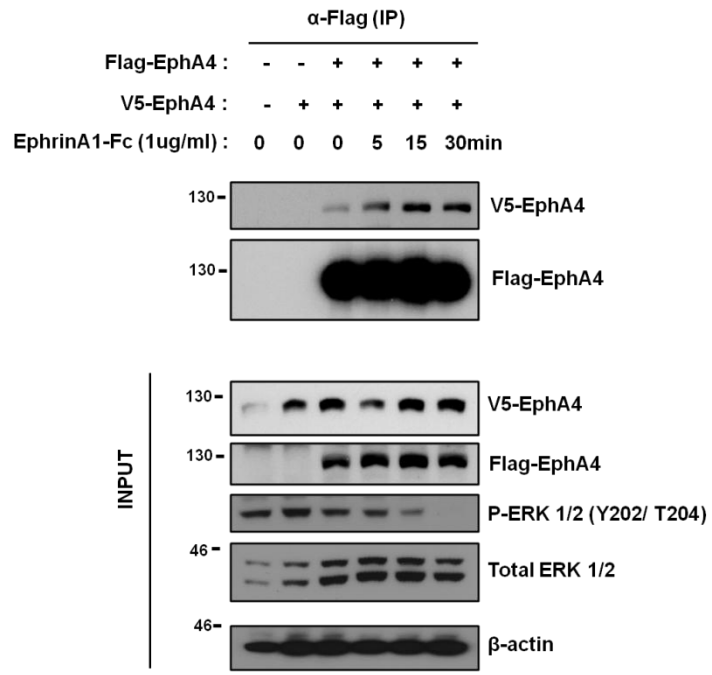

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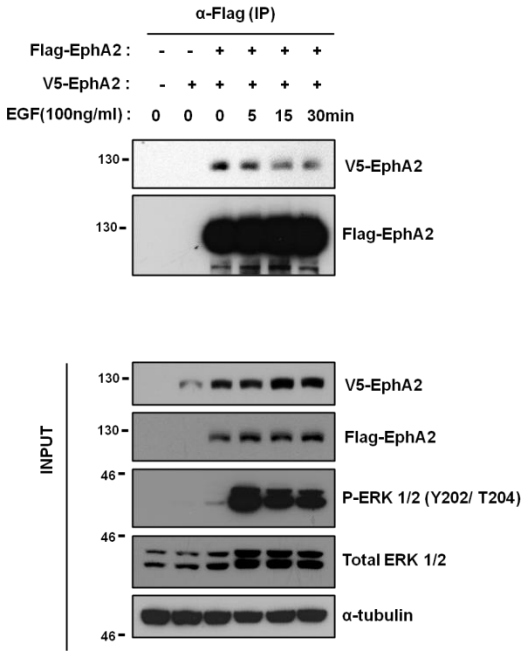

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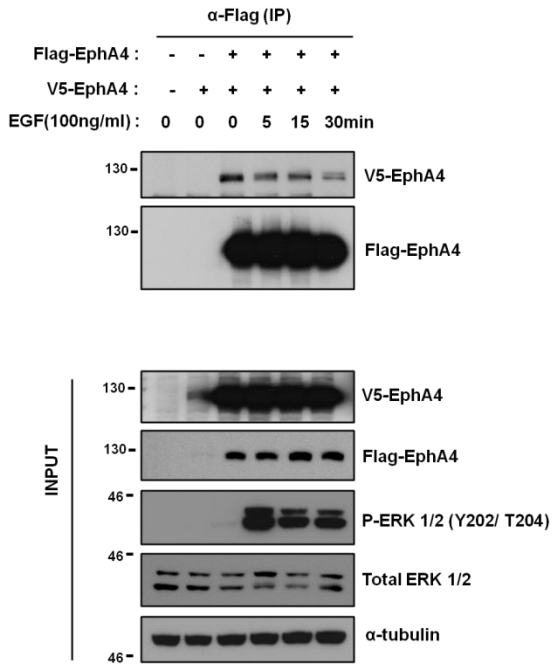

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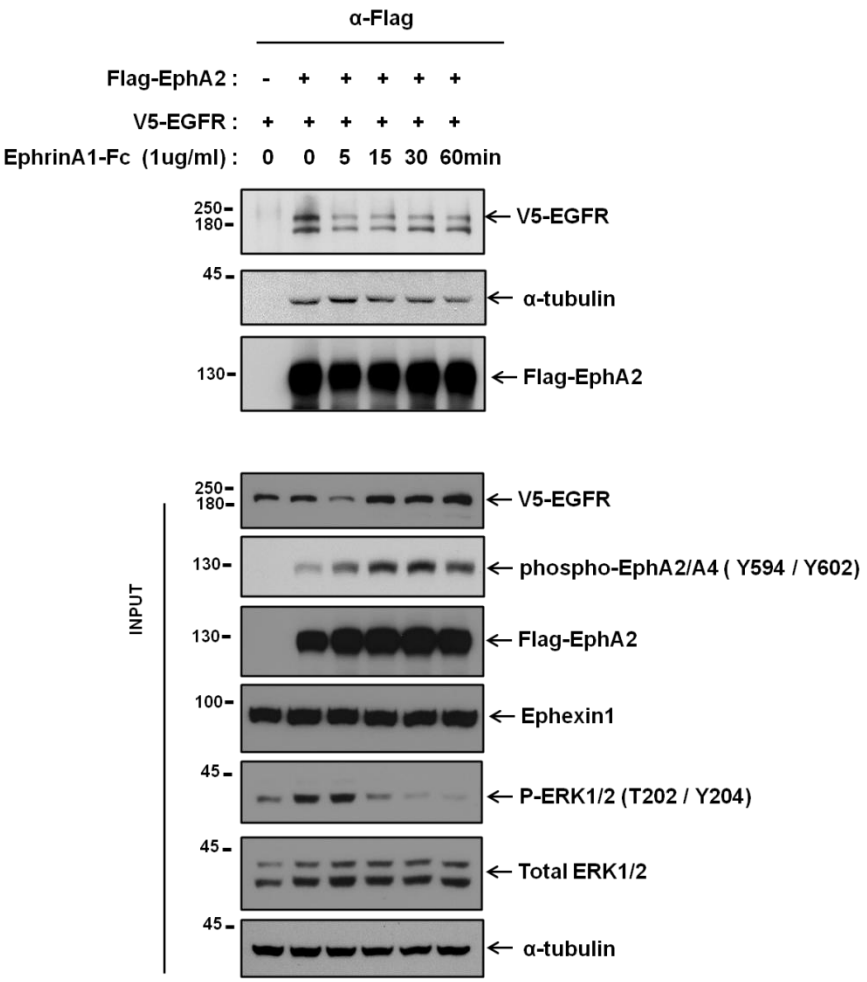

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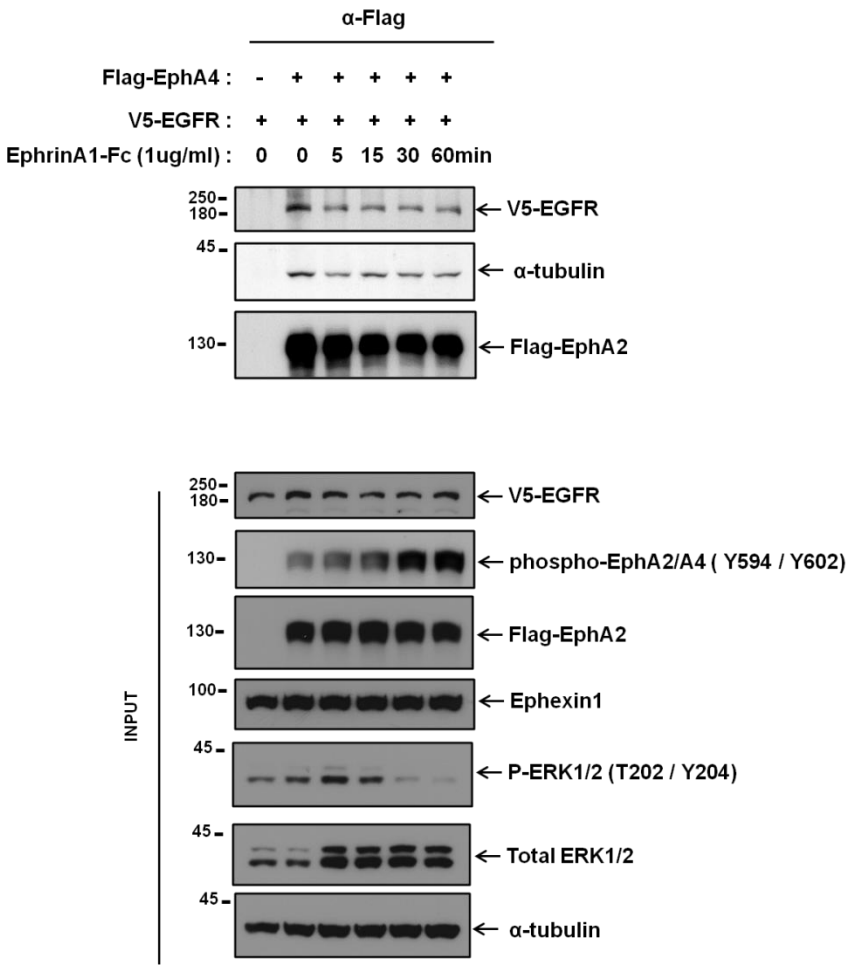

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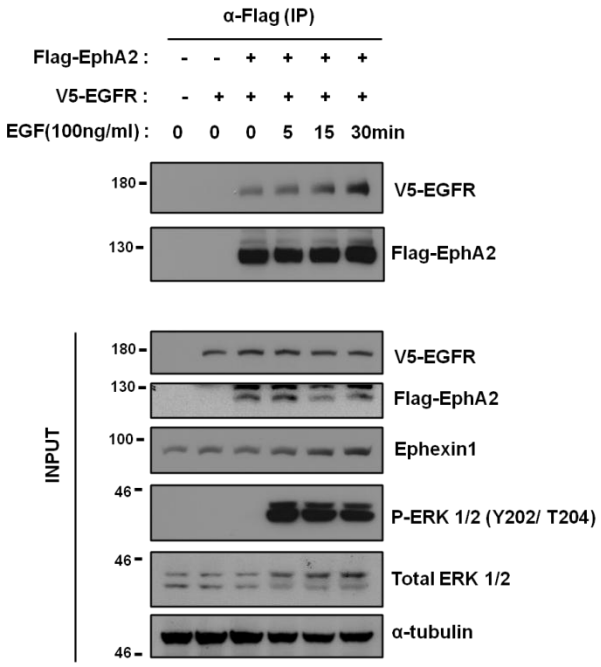

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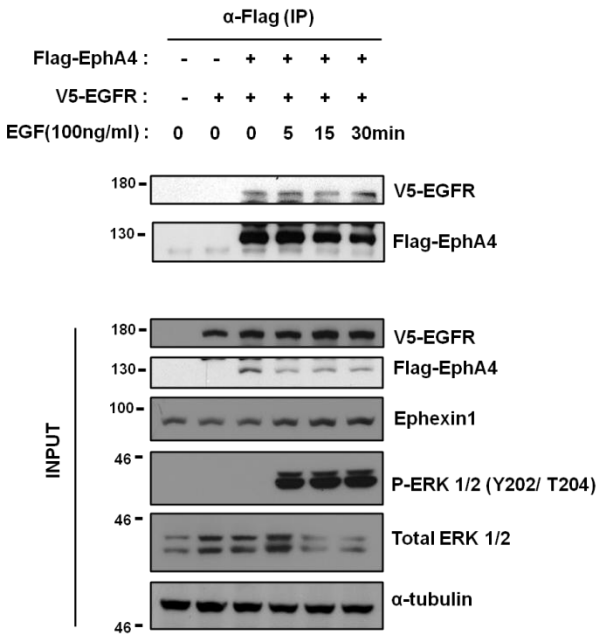

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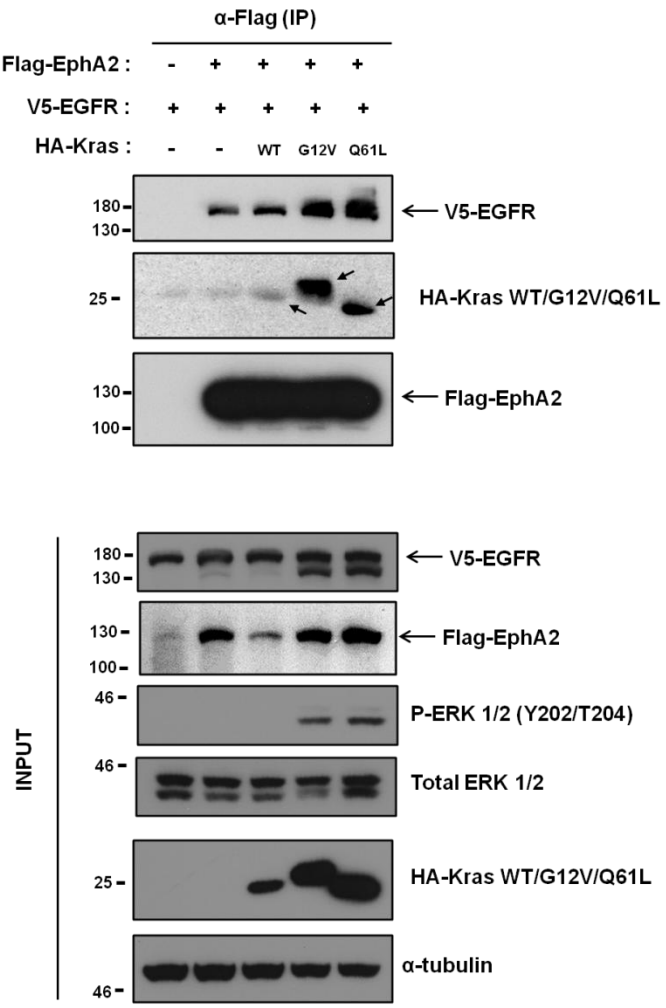

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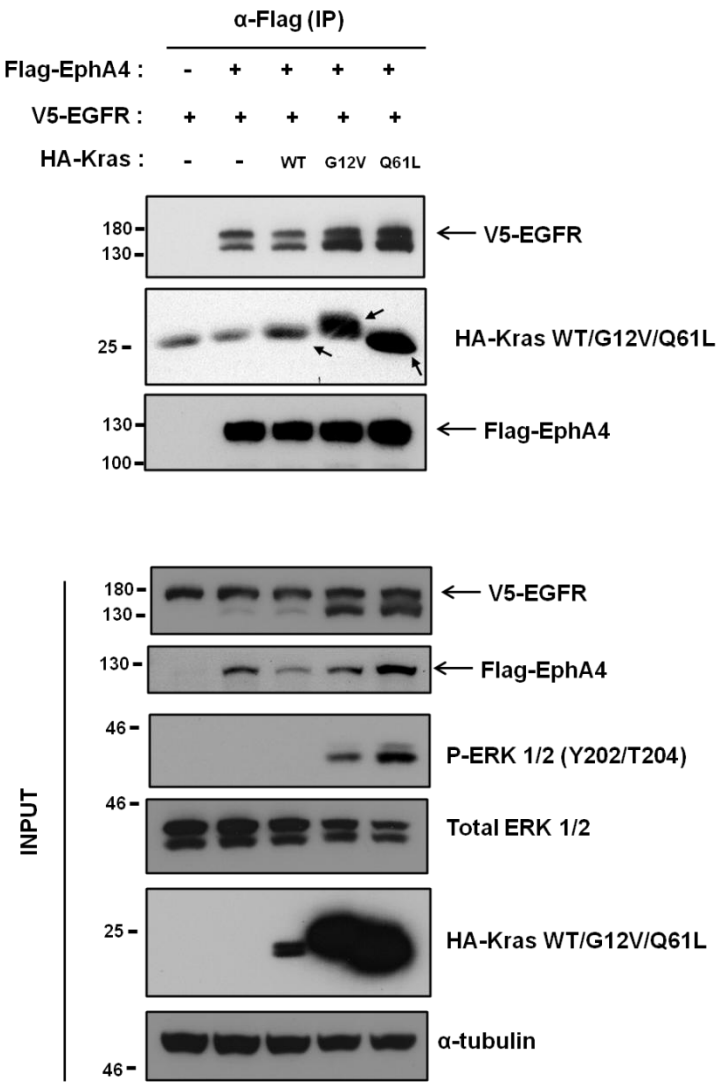

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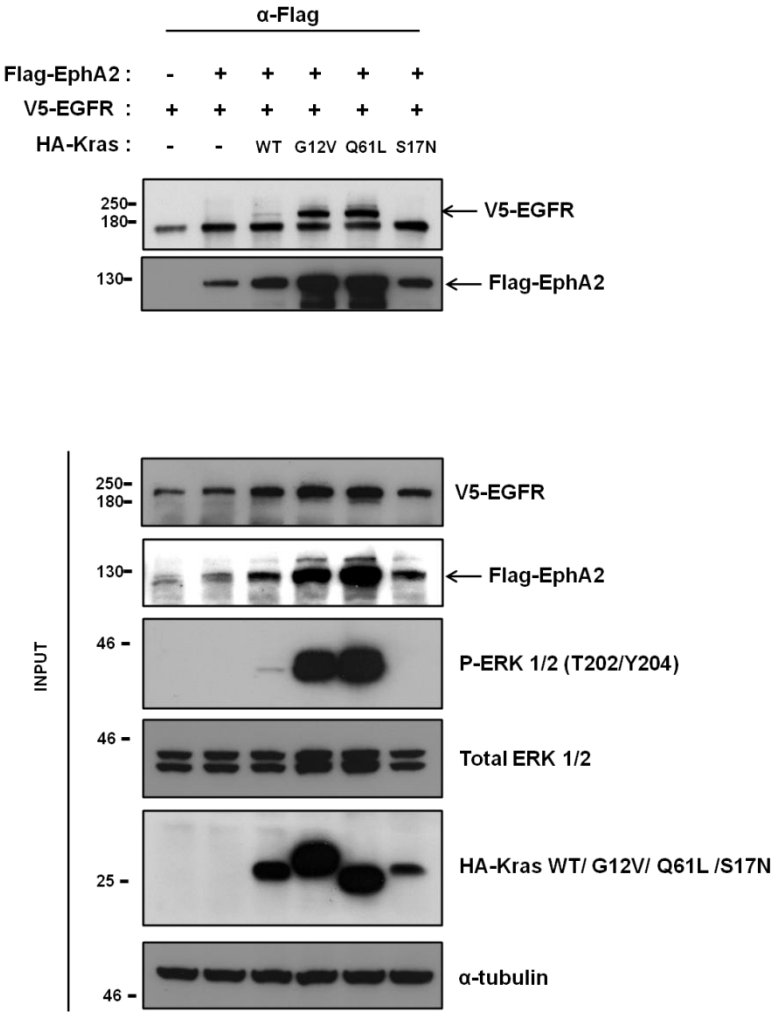

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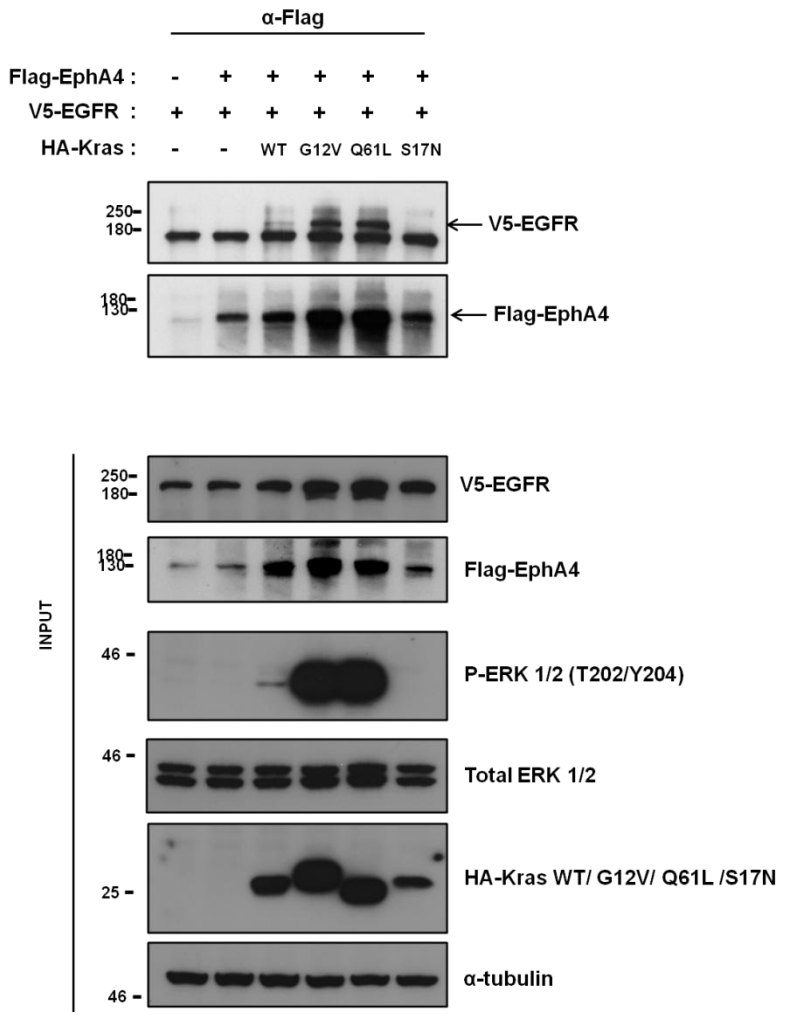

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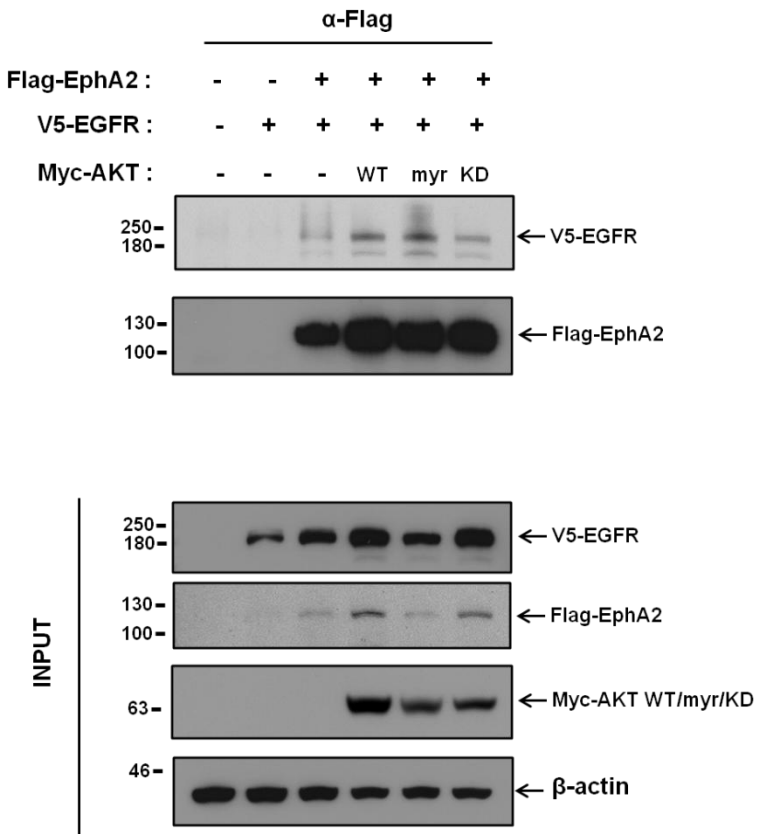


Figure10. EphrinA1 / Ras regulated EphA2/A4 -EGFR homo/heterodimer.

A) Flag / V5 tagged-EphA2 were co-transfected into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

B) Flag / V5 tagged-EphA4 were co-transfected into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

C) Flag / V5 tagged-EphA2 were co-transfected into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

D) Flag / V5 tagged-EphA4 were co-transfected into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

E) Flag tagged-EphA2 and V5 tagged-EGFR were co-transfected into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to

immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

F) Flag tagged-EphA4 and V5 tagged-EGFR were co-transfected into HEK-293T cells and treatment with EphrinA1-Fc (1ug/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

G) Flag tagged-EphA2 and V5 tagged-EGFR were co-transfected into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

H) Flag tagged-EphA4 and V5 tagged-EGFR were co-transfected into HEK-293T cells and treatment with EGF (100ng/ml) for 0, 5, 15, 30min before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

I) Flag tagged-EphA2, V5 tagged-EGFR and either HA tagged-Kras WT. G12V, Q61L were co-transfected into HEK-293T cells, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

J) Flag tagged-EphA4, V5 tagged-EGFR and either HA tagged-Kras WT. G12V, Q61L were co-transfected into HEK-293T cells, as indicated. Whole-cell

lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

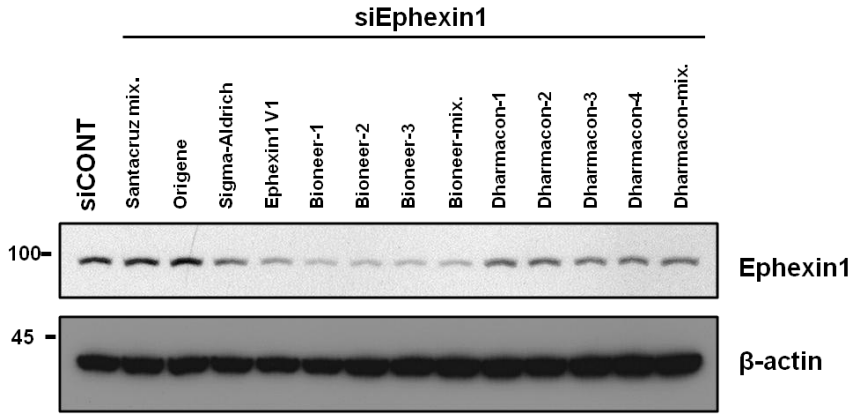
I) Flag tagged-EphA2, V5 tagged-EGFR and either HA tagged-Kras WT. G12V, Q61L, S17N were co-transfected into HEK-293T cells, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

J) Flag tagged-EphA4, V5 tagged-EGFR and either HA tagged-Kras WT. G12V, Q61L, S17N were co-transfected into HEK-293T cells, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

N) Flag tagged-EphA2, V5 tagged-EGFR and either Myc tagged-AKT WT, myr, KD were co-transfected into HEK-293T cells, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

III-Supplementary 1. Ephexin1 siRNA selection

We screened siEphexin1 using with thirteen different Ephexin1 siRNA. In this paper, we use the siEphexin1 Bioneer # 2 or siEphexin1 Bioneer mix. (Supplementary Fig. 1).



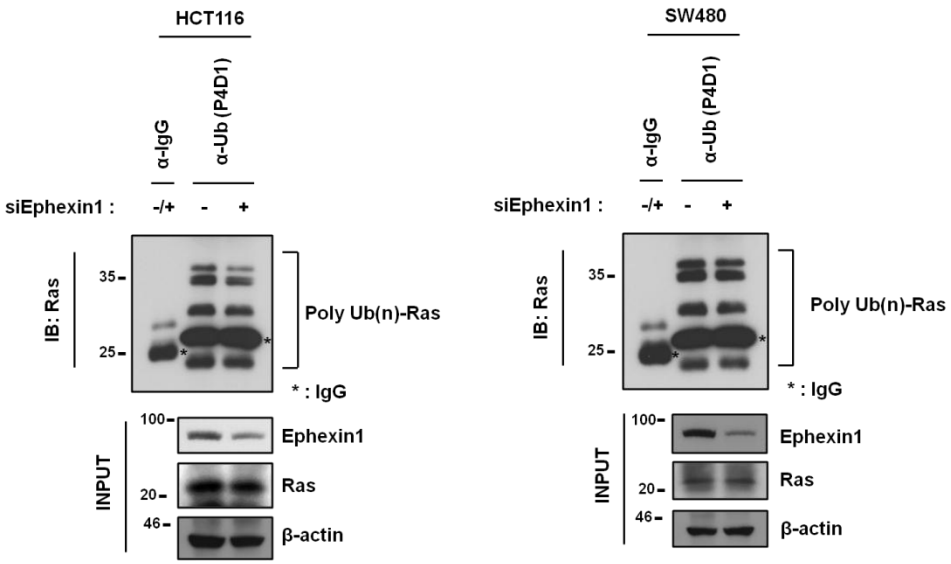
Supplementary Figure1 . Ephexin1 siRNA selection

HEK-293T cells were transfected with control (siCONT) and various siEphexin1 for 96hr and western blot analysis was performed to measure protein levels.

III-Supplementary.2. Ephexin1 did not regulated Ras ubiqutination and protein level.

The previous reports stated that the protein level of Ras is regulated by GSK3β, and proteosomal degradation by βTrcP E3 liagase [77, 78]. Therefore, we showed for the regulation of Ras / ERK pathway by Ephexin1 does not affect the protein level of Ras (Supplementary Fig. 2).

These results suggest that Ephexin1 does not regulate the protein level of Ras.



Supplementary Figure2. Ephexin1 not regulated Ras ubqutination and protein level.

Transfected with control (siCONT) and siEphexin1 for 96hr, followed by Immunoprecipitation using an anti-Ub(P4D1) antibody. Immunoprecipitation and cell lysates were determined by western blot analysis with indicated antibodies.

III-Supplementary 3. Ras regulated Ephexin1 protein stability by ubiquitin pathway.

EGF / EGFR / RAS pathway increased protein level of Ephexin1. Thus, we have shown that using the K-Ras over-expression and EGF treatment increased Ephexin1 protein level. HA tagged-Kras (WT, G12V and Q61L) overexpression increased endogenous (Supplementary Fig. 3A, C, D) and exogenous (Supplementary Fig. 3B) Ephexin1 protein levels. And EGF (100ng/ml) induced Ephexin1 protein level increase (Supplementary Fig. 3E). Therefore, we tested Ephexin1 ubiquitin level change by Ras signaling such as EGF, FBS. poly-ubiquitin of Ephexin1 decreased by EGF (100ng/ml) or Serum (10% FBS) (Supplementary Fig. 3F, G). And the reduction of Ephexin1 of poly-ubiquitin by EGF or Serum together HA-Kras S17N was not reduced (Supplementary Fig.3 H, I). In addition, we tested Ephexin1 protein stability test used Cyclohexamide (CHX.) that HA-Kras G12V over-expression were increased the protein half-life than empty vector transfected condition (Supplementary Fig. 3J).

Taken together, these data suggest that Ras and growth signal is Ephexin1 protein stabilization via proteosomal degradation block, ubiquitin pathway.

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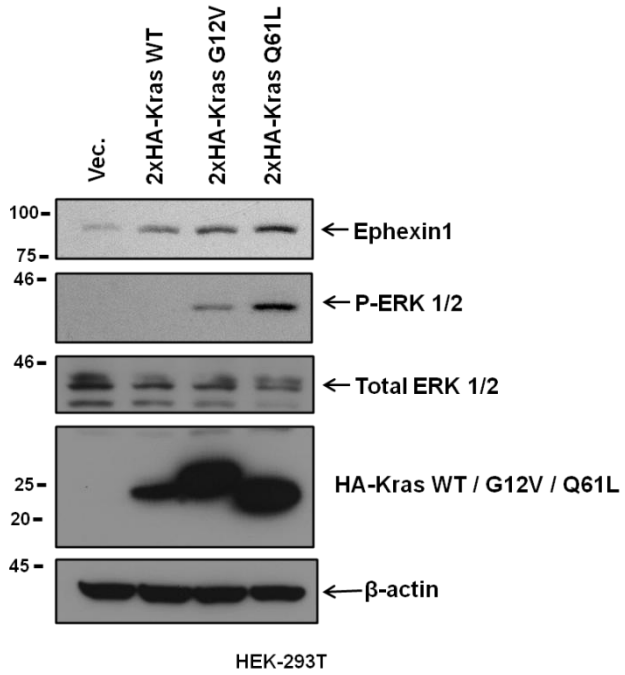

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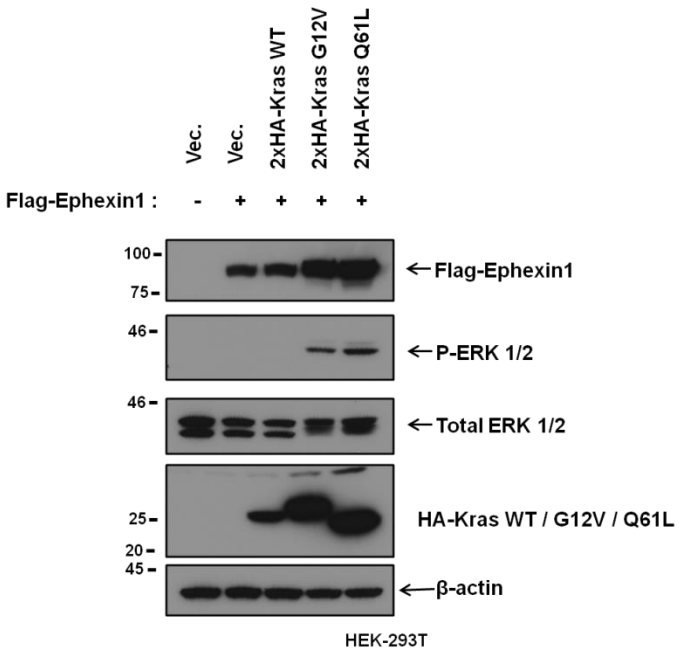

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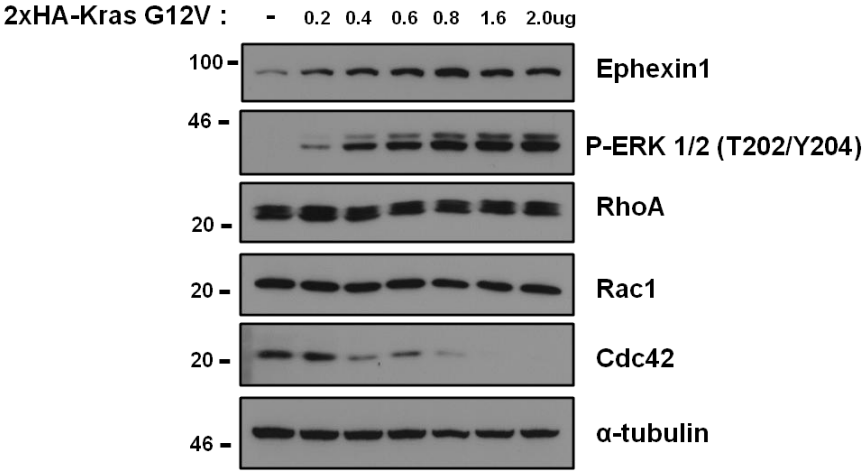

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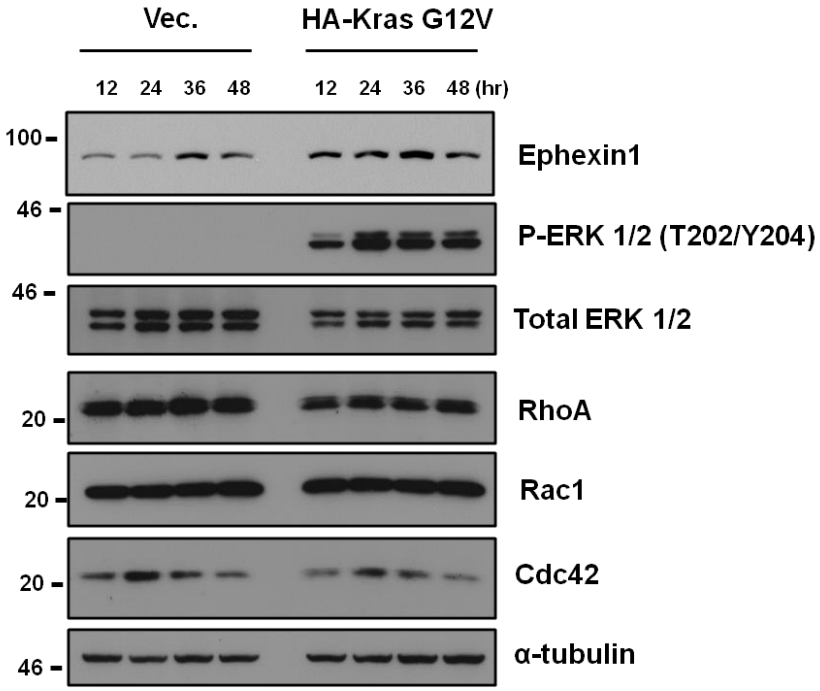

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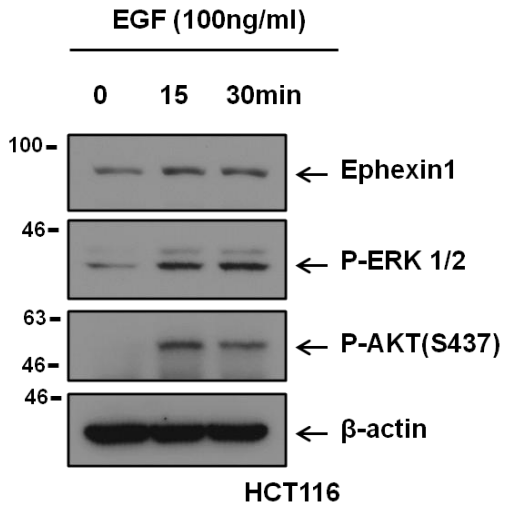

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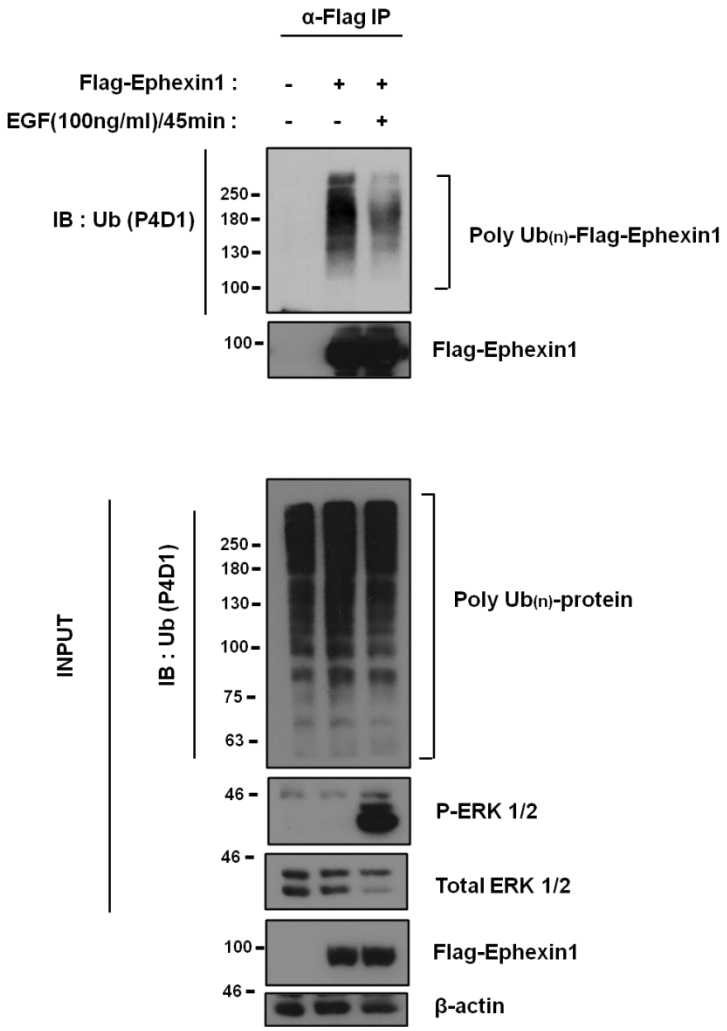

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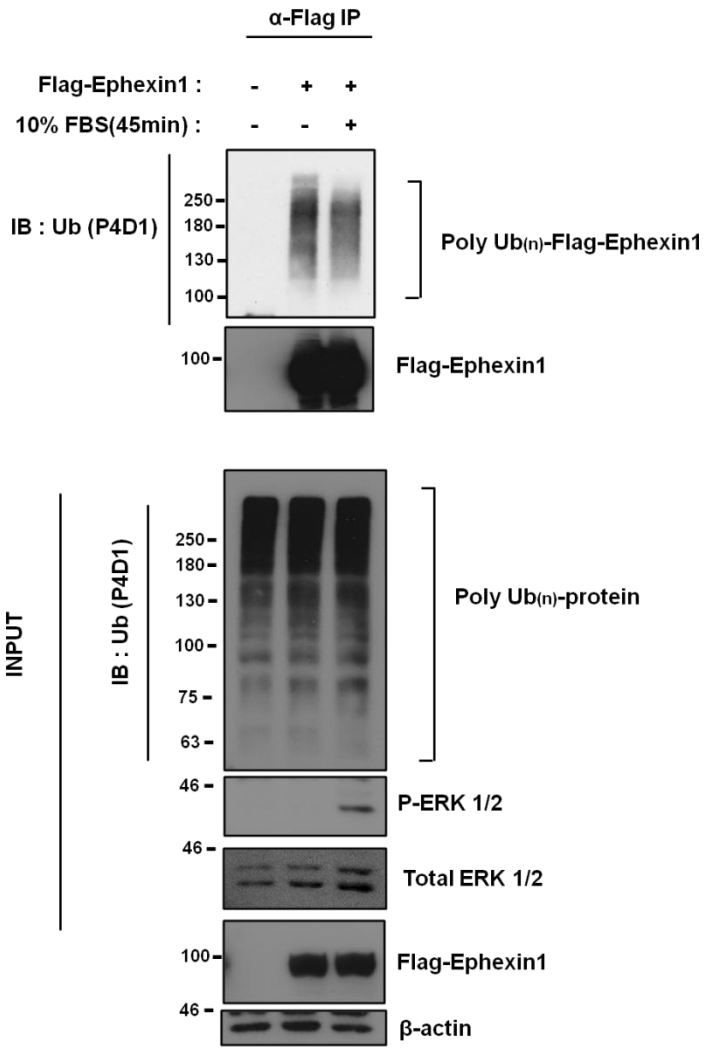

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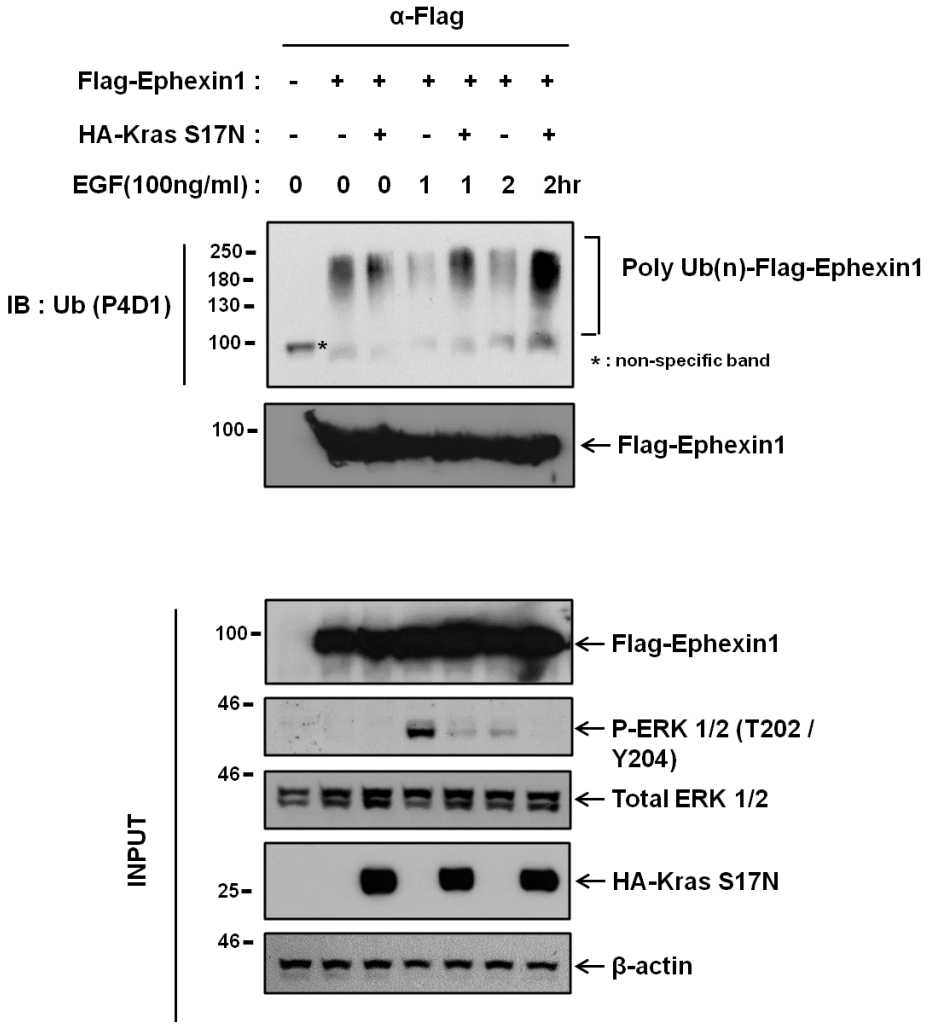

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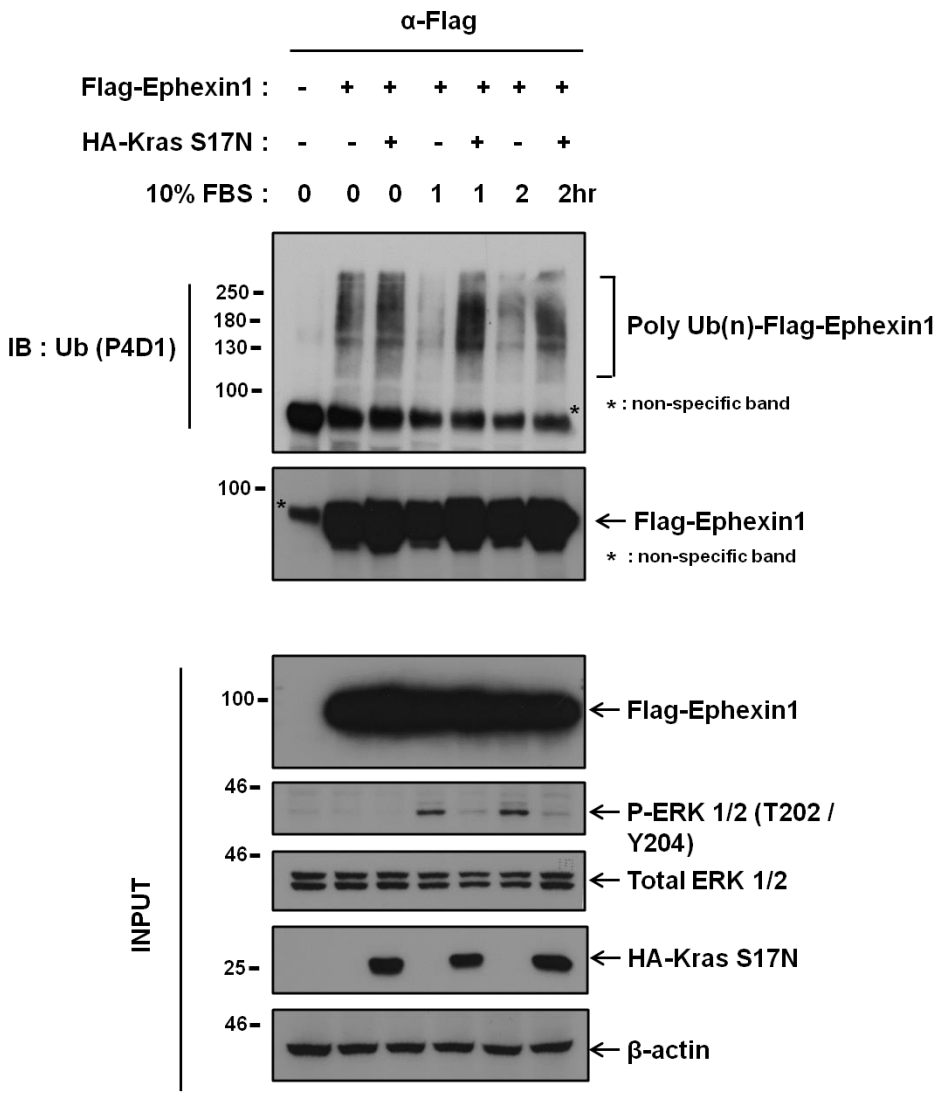

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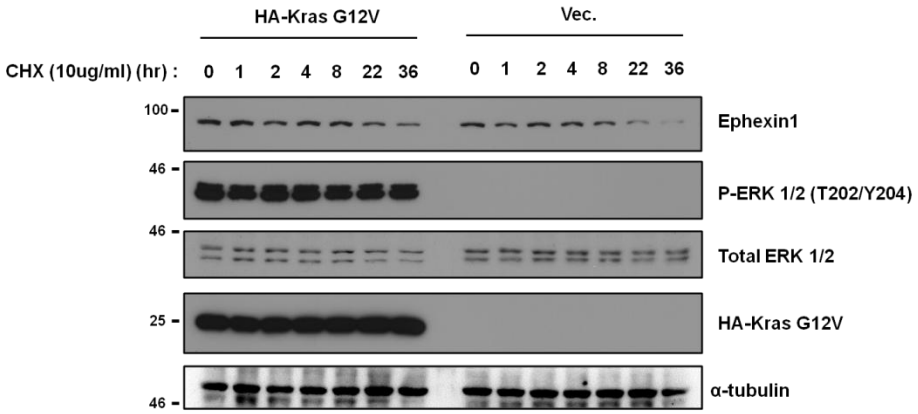


Supplementary Figure3. Ras regulated Ephexin1 protein stability by ubiquitin pathway.

A) HEK-293T cells were transfected with either HA tagged-Kras WT, G12V, Q61L for 36hr followed by western blotting with indicated antibodies. β-actin was used as a loading control.

B) Flag tagged-Ephexin1 transfected HEK-293T cells were co-transfected with either HA tagged-Kras WT, G12V, Q61L for 36hr followed by western blotting with indicated antibodies. β-actin was used as a loading control.

C) Transfected with various amount of HA-Kras G12V (0.2ug to 2.0ug) measured Endogenous Ephexin1 protein levels in HEK-293T cells. Western blot analysis was performed to measure protein levels with indicated antibodies. α-tubulin was used as a loading control.

D) Transfected with HA-Kras G12V for 12, 24, 36, 48hr measured Endogenous Ephexin1 protein levels in HEK-293T cells. Western blot analysis was performed to measure protein levels with indicated antibodies. α-tubulin was used as a loading control.

E) Treated EGF(100ng/ml) for 0, 15, 30min in HEK-293T cells. western blot analysis was performed to measure protein levels with indicated antibodies. β-actin was used as a loading control.

F) Transfected with Flag-Ephexin1 for 36hr and serum starvation for over-night followed EGF(100ng/ml) for 45min treated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

G) Transfected with Flag-Ephexin1 for 36hr and serum starvation for over-night followed 10% FBS for 45min treated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

H) Flag tagged-Ephexin1 was co-transfected with empty vector or HA tagged-Kras S17N into HEK-293T cells and treatment with EGF (100ng/ml) for 1, 2hr before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

I) Flag tagged-Ephexin1 was co-transfected with empty vector or HA tagged-Kras S17N into HEK-293T cells and treatment with 10% FBS for 1, 2hr before cell harvesting, as indicated. Whole-cell lysates were subjected to immunoprecipitation for anti-Flag (M2) antibody followed by western blotting with indicated antibodies. β-actin was used as a loading control.

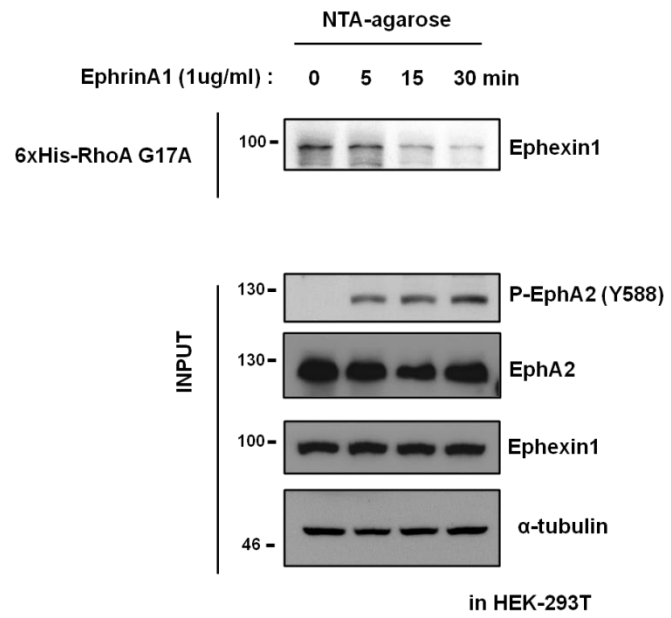
J) Endogenous Ephexin1 protein levels in HEK-293T cells transfected with HA-Kras G12V were monitored at the indicated time points after cyclohexaminde (CHX) (10 μg/ml) treatment followed by western blotting with indicated antibodies. α-tubulin was used as a loading control.

III-Supplementary.4. EphrinA1 / Ras regulated GEF activity of Ephexin1.

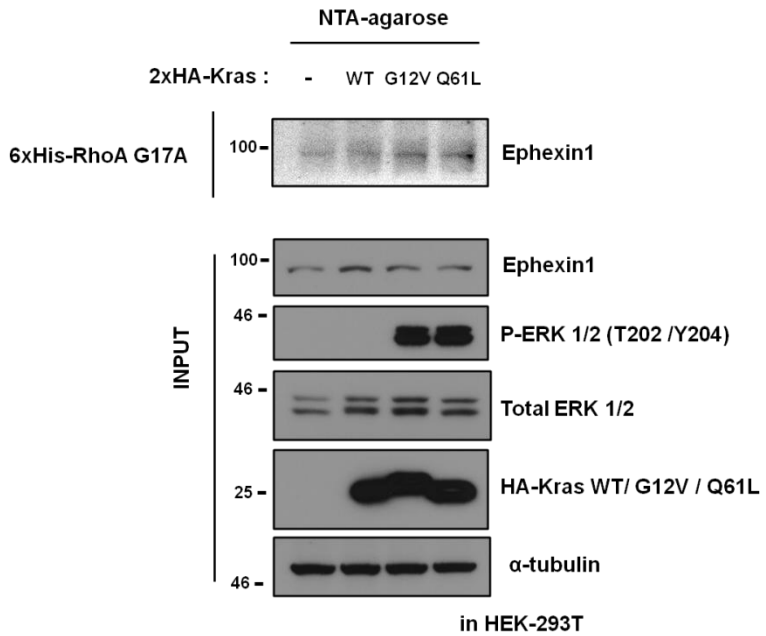
GEF activity of Ephexin1 on RhoA were reduced by EphrinA1-Fc (1ug/ml) (Supplementary Fig. 4A). EGF (100ng/ml) treatment increased Ephexin1 GEF activity to RhoA (Supplementary. Fig. 4B).

These data propose that EphrinA1-Fc decreased Ephexin1 GEF activity to RhoA, but, active Ras is increased Ephexin1 GEF activity to RhoA.

A.



B.



Supplementary Figure4 . EphrinA1 / Ras regulated GEF activity of Ephexin1.

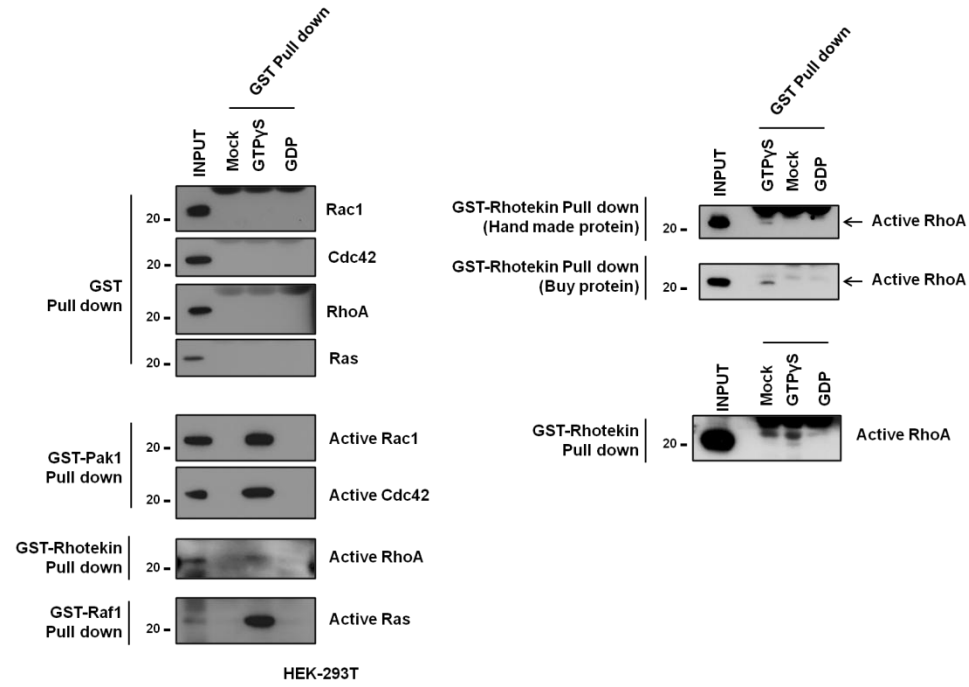
A) HEK-293T cells were treated EphreinA1-Fc (1ug/ml) for 5, 15, 30 min. Following treatment the cells were lysed and active Ephexin1 were captured using His-RhoA G17A bound NI-NTA agarose beads. Cell lysates and His-RhoA G17A pulldown assay were determined by western blot analysis with indicated antibodies. α-tubulin was used as a loading control.

B) HA tagged-Kras WT, G12V and Q61L were either transfected into HEK-293T cells. Following cells were lysed and active Ephexin1 were captured using His-RhoA G17A bound NI-NTA agarose beads. Cell lysates and His-RhoA G17A pulldown assay were determined by western blot analysis with indicated antibodies. α-tubulin was used as a loading control.

III-Supplementary 5. Active Rho / Rac / Cdc42 pulldown assay test

Before performing the GTPase activity, test of the Rho family GTPase confirmed the normal action of the GST-fusion protein. GST-pulldown using GTPγS increased activity. However, GST-pulldown using GDP did not pulldown. Therefore, any GST-fusion protein was confirmed that the normal working (Supplementary Fig. 5).

These data show that this is a GST-fusion protein functioning normally.

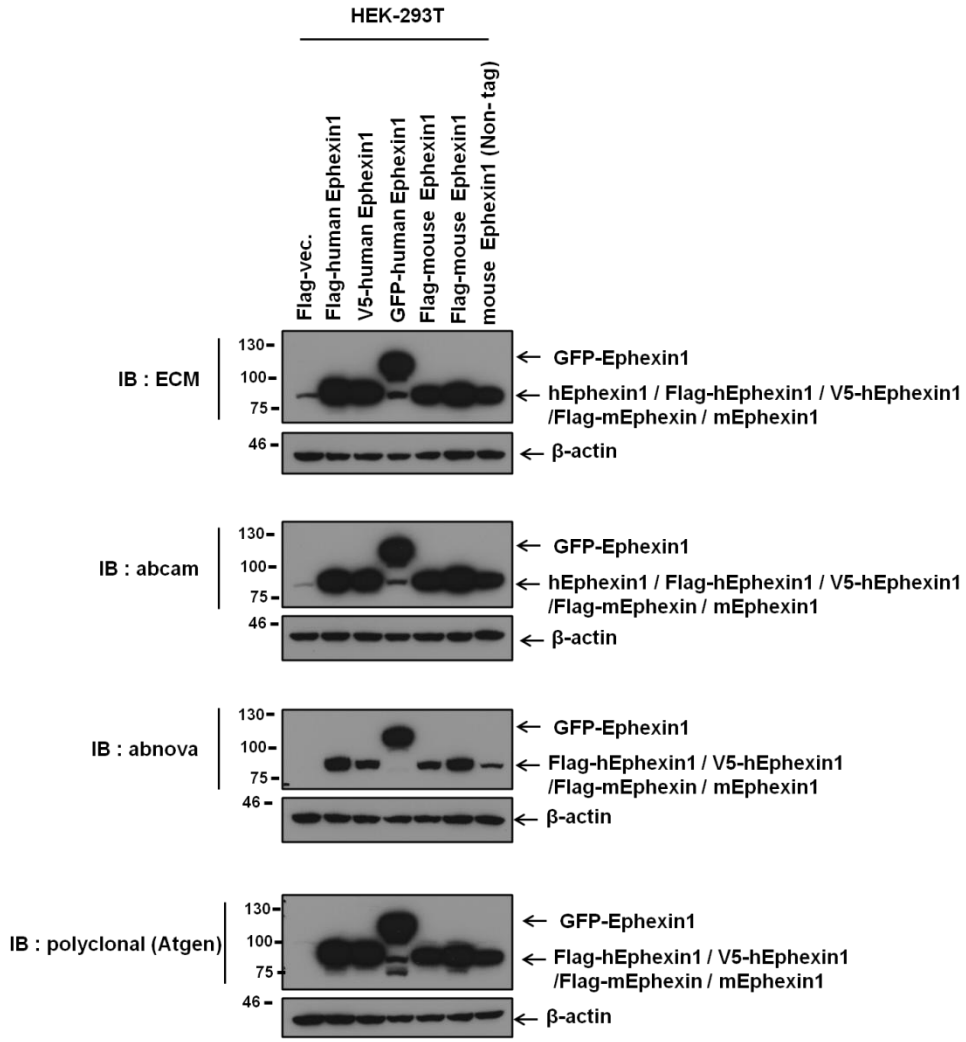


Supplementary Figure5 . Active Rho / Rac / Cdc42 pulldown assay test

HEK-293T cell lysates were treated *in vitro* with GTPγS or GDP to activate or inactivated Rac1, Cdc42, RhoA and Ras . GTPγS or GDP treated lysate was also incubated with GST alone (negative control), measured the levels of activated Rac1,Cdc42, RhoA and Ras as detected by GST-Rhotekin-RBD, GST-Pak1-PBD and GST-Raf1-RBD pulldown by Glutatione separose bead. The levels of Rac1, Cdc42, RhoA, Ras in the cell lysates were determined by western blot analysis.

III-Supplementary 6. Ephexin1 antibody test

We confirmed the specificity of the antibody that can be purchased from a company (ECM, abcam and abnova) and produced antibodies from our laboratory (Atgen). All antibody detected over-expressed mouse and human Ephexin1. The abcam and ECM bioscience is same epitope, and can endogenous Ephexin1 detection (Supplementary Fig. 6). In this paper, we detected of endogenous Ephexin1 used abcam or ECM bioscience products.



Supplementary Figure6. Ephexin1 antibody test

Cell lysates was transfected with Flag-human Ephexin1, V5-human Ephexin1, GFP-human Ephexin1, Flag-mouse Ephexin1 and non-tagged mouse Ephexin1 in HEK-293T cells. The cell lysates were determined by western blot analysis with anti-Ephexin1 (ECM, abcam, abnova, Atgen) antibody.

III-Sup.6. Supplementary Table1

Sequences of primers for RT-PCR / qRT-PCR

primer	Forward	Reverse
Ephexin1	5'GAGATGAAGCGTTGGATGACC-3'	5'-GCCTCTCTCCTGGTCGTGC-3'
EphA2	5'-GCAGCAGTATACGGAGCACTTC-3'	5'-AAGTATTCTTGGCCGATGGG-3'
EphA4	5'-CAACCCCAACAGCTTGAAGAG-3'	5'-TGTGATACCAATTCTTGCCAGG-3'
EGFR	5'-CAGCGCTACCTTGTCATTCA-3'	5'-AGCTTTGCAGCCCATTTCTA-3'
GAPDH mRNA	5'-TGACATCAAGAAGGTGGTGA-3'	5'-TCCACCACCCTGTTGCTGTA-3'
β-2-miocroglobulin (B2M)	5'-TTTCATCCATCCGACATTGA-3'	5'-CCTCCATGATGCTGCTTACA-3'

III-Sup.6. Supplementary Table2

Sequences of primers for cloning

primer	Forward	Reverse
Flag or V5-Ephexin1 full -XhoI/NotI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGCGGCCGCTCATTGCCGATTCCGGC-3'
Flag or V5-Ephexin1 ΔSH3 -XhoI/NotI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGCGGCCGCTCACAGCAGCCGGGATGTGAA-3'
Flag or V5-Ephexin1 ΔPH/SH3 -XhoI/NotI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGCGGCCGCTCACCAGCGGGAGTGGGAGAT-3'
Flag or V5-Ephexin1 ΔDH/PH/SH3 -XhoI/NotI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGCGGCCGCTCACTTAATCTCCTCGGGCTGTAGG-3'
Flag or V5-Ephexin1 ΔDH -Fragment 1-XhoI / EcoRI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGAATTCCTTAATCTCCTCGGGCTGTAGG-3'
Flag or V5-Ephexin1 ΔDH -Fragment 1-EcoRI / NotI	5'-AAAAGAATTCCTGCTGAAGCAGGGTGAGCT-3'	5'-AAAAGCGGCCGCTCATTGCCGATTCCGGC-3'
Flag or V5-Ephexin1 ΔRR-XhoI / EcoRI	5'-AAAACTCGAGATGCTGCAGGAGGCCATGTTCG-3'	5'-AAAAGCGGCCGCTCATTGCCGATTCCGGC-3'
Flag or V5-Ephexin1 ΔPH-Fragment 1-XhoI / EcoRI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGAATTCGCCCTCGTTGCATGCCTT-3'
Flag or V5-Ephexin1 ΔPH-Fragment 2-EcoRI / NotI	5'-AAAAGAATTCACCAAGTTTGTTTCGTTCACATC-3'	5'-AAAAGCGGCCGCTCATTGCCGATTCCGGC-3'
Flag or V5-Ephexin1 ΔDH/PH-Fragment 1-XhoI / EcoRI	5'-AAAACTCGAGATGGAGACCAGGGAATCTGAAG-3'	5'-AAAAGAATTCCTTAATCTCCTCGGGCTGTAGG-3'
Flag or V5-Ephexin1 ΔDH/PH-Fragment 2-EcoRI / NotI	5'-AAAAGAATTCACCAAGTTTGTTTCGTTCACATC-3'	5'-AAAAGCGGCCGCTCATTGCCGATTCCGGC-3'
Flag or V5-Ephexin1 DH/PH-XhoI / NotI	5'-AAAACTCGAGCTGCAGGAGGCCATGTTCG-3'	5'-AAAAGCGGCCGCTCACAGCAGCCGGGATGTGAA-3'
Flag or V5-EphA1-EcoR / NotI	5'-AAAAGAATTCAATGGAGCGGCGCTGGC-3'	5'-AAAAGCGGCCGCTCAGTCCTTGAATCCCTGAATACTG-3'
Flag or V5-EphA2-XhoI / SmaI	5'AAAAGCGGCCGCTCAGTCCTTGAATCCCTGAATACTG-3'	5'-AAAACCCGGGTCAGATGGGGATCCCCACA-3'
Flag or V5-EphA3-XhoI / NotI	5'-AAAACTCGAGATGGATTGTCAGCTCTCCATCC-3'	5'-AAAAGCGGCCGCTTACACGGGAACTGGGCC-3'
Flag or V5-EphA4-XhoI / NotI	5'-AAAACTCGAGATGGCTGGGATTTTCTATTTCG-3'	5'-AAAAGCGGCCGCTCAGACGGGAACCATTCTGC-3'
Flag or V5-EGFR-XhoI / NotI	5'-AAAACTCGAGATGCGACCCTCCGGGAC-3'	5'-AAAAGCGGCCGCTCATGCTCCAATAAATTCACTGC-3'

III-Sup.6. Supplementary Table3

Sequences of siRNA

siRNA	Sequence (Sense strand)
siCONT (si-GFP)	5'-GGCUACGUCCAGGAGCGCACC(dTdT)-3'
siEphexin1 (santacruz mix.)	SC-94358
siEphexin1 (Origene)	5'-AGGCUGUUGGUCCAGAACAUCCUG(dTdT)-3'
siEphexin1 (v1)	5'-UGGCAGAUUCACAGGACAA(dTdT)-3'
siEphexin1 (Bioneer #1)	5'-GAGAUGAAGCGUUGGAUGA(dTdT)-3'
siEphexin1 (Bioneer #2)	5'-GAGAUCUUGAAUCCCAAGA(dTdT)-3'
siEphexin1 (Bioneer #3)	5'-CUGCUUUGGAUGCUCACAA(dTdT)-3'
siEphexin1 (Dharmacon #1)	5'-GGAGAAACCUCAUUGAACA(dTdT)-3'
siEphexin1 (Dharmacon #2)	5'-GGACCAAGUUUGUUUCGUU(dTdT)-3'
siEphexin1 (Dharmacon #3)	5'-GAAAUUCCAUCUUCAAUCG(dTdT)-3'
siEphexin1 (Dharmacon #4)	5'-UAAAUCGACUCUCCAAGAA(dTdT)-3'

III-Sup.6. Supplementary Table4

Sequences of primers for site-direct mutagenesis

primer	Forward	Reverse
Ephexin1 S16A	5'-attgatcacttgctgctttcctccgggtcttttcc-3'	5'-ggaaaagacccggaggaaagcagcaagtgatcaat-3'
Ephexin1 S16D	5'-agtgttccattgatcacttgcatctttcctccgggtcttttccaaatc-3'	5'-gatttggaaaagacccggaggaaagatgcaagtgatcaatggaacact-3'
Ephexin1 S18A	5'-cagtgttccattgatcagctgctgatttcctccgggtct-3'	5'-agacccggaggaaatcagcagctgatcaatggaacactg-3'
Ephexin1 S18D	5'-tcagtgttccattgatcatctgctgatttcctccgggtctt-3'	5'-aagacccggaggaaatcagcagatgatcaatggaacactga-3'
Ephexin1 S606A	5'-ccgggatgtgaacgcaacaaacttggtcctcc-3'	5'-ggaggaccaagtttgttgcgttcacatcccgg-3'
Ephexin1 S606D	5'-agccgggatgtgaaatcaacaaacttggtcctcctgttgggggg-3'	5'-cccccaacaggaggaccaagtttgttgatttcacatcccggct-3'
Ephexin1 S16,18A	5'-tcagtgttccattgatcagctgctgctttcctccgggtctttttccaa-3'	5'-ttggaaaagacccggaggaaagcagcagctgatcaatggaacactga-3'
Ephexin1 S16,18D	5'-ggttcattatcagtgttccattgatcatctgcatctttcctccgggtcttttccaaatcttcaga-3'	5'-tctgaagatttggaaaagacccggaggaaagatgcagatgatcaatggaacactgataatgaacc-3'
Ephexin1 S16A,S18D	5'-ttatcagtgttccattgatcatctgctgctttcctccgggtcttttccaaatc-3'	5'-gatttggaaaagacccggaggaaagcagcagatgatcaatggaacactgataa-3'
Ephexin1 S16D,S18A	5'-gttcattatcagtgttccattgatcagctgcatctttcctccggggtcttttccaaatcttcag-3'	5'-ctgaagatttggaaaagacccggaggaaagatgcagctgatcaatggaacactgataatgaac-3'
RhoA G17A	5'-ggtgatggagcctgtgcaaagacatgcttgctc-3'	5'-gagcaagcatgtctttgcacaggctccatcacc-3'
Kras Q61L	5'-tcgacacagcaggtctagaggagtacagtgc-3'	5'-gcactgtactcctctagacctgctgtgtcga-3'

IV. Discussion

Most researchers were interested in the functions of the Ephexin1 on neuronal cells. However, we are focused on role of the Ephexin1 in cancer cells. Interestingly, Ephexin1 was high level expression in several cancer cells. And we show that Ephexin1 and AKT / ERK pathway were significantly correlation. Thus, we suggest that Ephexin1 can be controlled as an intermediate mediator of Rho family GTPases (RhoA / Rac1 / Cdc42) by Ras in cancer cell in this role as well as the neuronal cell.

The typical oncogenic protein, Ras act as molecular switches to control of cell growth, invasion, metastasis and differentiation. And this function of Ras is known to regulate cell mobility, migration, invasion and cytoskeleton organization through the Rho family GTPases [1, 2, 3, 4, 5, 7, 8, 9, 35]. So far it is the most well-known activation of Rac1 by Tiam1 activation by Ras [3, 18, 79]. Tiam1 is a powerful oncogenic protein. However, it is not enough to explain the regulation, such as Rho, Cdc42 of the Rho family GTPase by Ras. Therefore, we try to find the regulation factors of Rho family GTPases (RhoA, Rac1 and Cdc42) such as Rac1 activator Tiam1.

The first name of the Ephexin1 was NGEF (Neuronal Guanine Nucleotide Exchange Factor). Most researchers, Ephexin1 are thought to be expressed only in neurons [27, 28, 32]. Also, function of Ephexin1 in Eph receptor pathway has been thought the formation of neuronal cell functions such as growth cone of a neuronal cell. The study of the role of Ephexin1 did not research in cancer cells. We showed that Ephexin1 is strong expressed in cancer cells (figure 1).

Therefore, Ephexin1 was possible to intermediate regulator of Rho family GTPases by Ras, because Ephexin1 known as the regulation of RhoA, Rac1, and Cdc42 in nerve cells. Also, we have shown that AKT / PI3K can control function of Ephexin1 such as Ras binding and regulation of Rho family GTPases activity (figure 4, 7). The Serine 16, 18 residues in Ephexin1 is phosphorylated by AKT. This phosphorylation site is a very important residue in interaction with Ras and Ephexin1. Also its phosphorylation can play a role just like “Lock & Unlock switch”.

In additionally, Ephexin1 iso.2 is mainly expression in neuronal cells. And this form is not controlled by AKT (not shown). Interestingly, Ephexin1 iso.1 is mainly expression in cancer cells, and tightly controlled by AKT (Figure 1, 7). So possibly, Ephexin1 iso.2 in nerves cells is to act as a GEF to neuronal differentiation and functional growth. Ephexin1 iso.1 is tightly and more complex regulated by AKT / PI3K, which is controlled turmorigenesis in cancer cells.

In addition to the studies, EphA receptor is the tumor suppression by EphrinA1-Fc [67, 68, 69, 70, 71, 72]. And EphA2 / A4 were the tumor progression by over-expression [34, 44, 45, 46, 48]. The EphrinA1-Fc treatment reduced Ras activity [68]. However this paper was mechanism unknown. These reports were phenomenon. However, we showed that EGF growth factor and EphrinA1-Fc occur different phosphorylation state in EphA receptors (figure 9). At this time, Ephexin1 was different complexes formation with phospho-EphA (figure 8, 9, 10). Thus, EphrinA1 ligand or Growth factor was determined tumor suppression or progression due to the different components of complex such as Ephexin1, EphA receptor, EGFR, Ras and Rho family GTPases.

In summary, Ephexin1 is regulatory intermediator of Rho family GTPases by the Ras, Growth factor or active Ras has been induced phosphorylation to Ephexin1 S16, 18 by AKT. Also, phospho-Ephexin1 (S16, 18) increased activity of the Rho family GTPases. At the same time, The Ephexin1 / EGFR / EphA receptor / Ras / Rho family GTPases-multicomplex (ERMC) are increased eventually to tumorigenesis. Therefore, we propose that Ephexin1 could be used as important cancer therapeutics.

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