

## hSef potentiates EGF-mediated MAPK signaling through affecting EGFR trafficking and degradation

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### Abstract

Sef (similar expression to *fgf* genes) was identified as an effective antagonist of fibroblast growth factor (FGF) in vertebrates. Previous reports have demonstrated that Sef interacts with FGF receptors (FGFRs) and inhibits FGF signaling, however, its role in regulating epidermal growth factor receptor (EGFR) signaling remains unclear. In this report, we found that hSef localizes to the plasma membrane (PM) and is subjected to rapid internalization and well localizes in early/recycling endosomes while poorly in late endosomes/lysosomes. We observed that hSef interacts and functionally colocalizes with EGFR in early endosomes in response to EGF stimulation. Importantly, we demonstrated that overexpression of hSef attenuates EGFR degradation and potentiates EGF-mediated mitogen-activated protein kinase (MAPK) signaling by interfering EGFR trafficking. Finally, our data showed that, with overexpression of hSef, elevated levels of Erk phosphorylation and differentiation of rat pheochromocytoma (PC12) cells occur in response to EGF stimulation. Taken together, these data suggest that hSef plays a positive role in the EGFR-mediated MAPK signaling pathway. This report, for the first time, reveals opposite roles for Sef in EGF and FGF signalings.

### Keywords

Sef; EGFR; Ras/MAPK; Endocytosis; PC12 cells; Differentiation

## 1. Introduction

Sef, similar expression to *fgf* genes, was identified as a novel negative regulator of FGF signaling during zebrafish development [1,2]. To date, Sef has been reported to be an antagonist of FGF signaling and to regulate Ras/MAPK signaling at different levels in

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vertebrates [2–8]. Evidence has been provided that heterozygous expression of Sef inhibits FGF signaling, but that underexpression of Sef leads to too much signaling, causing characteristic malformations in zebrafish embryos [1,2]. Chicken Sef demonstrated a spatiotemporal expression pattern during the limb development, suggesting a role in regulating limb outgrowth [3]. Studies in cultured cells also revealed that Sef inhibits FGF-induced Ras/MAPK signaling pathway [4,7–9]. In addition, Sef has been observed to synergistically regulate the expression of *Gbx2* (a downstream target gene of Fgf8) together with Sprouty2, an inhibitor of FGFR [10]. All these observations strongly indicated that Sef downregulates FGF signaling.

Interestingly, Sef has also been implicated as a regulator of other receptor tyrosine kinases (RTKs)-mediated signal pathways. Our previous studies have suggested that Sef exerts a negative effect not only on FGF2-induced but also on nerve growth factor (NGF) induced PC12 differentiation through inhibition of Ras/MAPK signaling [7]. We reasoned that Sef inhibits Ras/MAPK through interaction with FGFRs. More recently, Sef was reported to be distributed on the Golgi apparatus surface, blocking the dissociation of the MEK-Erk complex [9]. This observation suggested that Sef inhibits the nuclear translocation of activated Erk, and consequently inhibits the activation of nuclear but not cytoplasmic Erk substrates. Therefore, Sef was predicated to inhibit both FGF- and EGF-mediated nuclear signaling at the Erk level. Besides Ras-Erk signaling, overexpression of Sef has also been shown to induce apoptosis through the activation of c-jun amino-terminal kinase (JNK). Sef was demonstrated to activate JNK through a TAK-MKK4-JNK pathway, and to associate with TAK1 in a coimmunoprecipitated complex [11].

Recent pathological investigation into the role of Sef in tumorigenesis revealed that hSef is expressed in both androgen-dependent and independent cells but is reduced in highly metastatic derivative clones of prostate cancer cells [12]. Moreover, hSef expression correlates well with Her-2/ErbB2 oncogene expression in the breast carcinoma cell lines, although the hSef mRNA levels in breast cancer tissues vary between different breast tumor types [8]. These observations implied that hSef is expressed in some tumors *in vivo*, and may be playing a critical role in the pathologic process of certain tumors.

In this study, we present evidence that Sef interacts and colocalizes with epidermal growth factor receptor (EGFR). We found that Sef affects EGFR trafficking and attenuates EGFR degradation and thereby potentiates EGF-mediated Ras/MAPK signaling. Intriguingly, we demonstrated that the EGF-induced Erk activation sustained by Sef leads to the neurite outgrowth in rat pheochromocytoma (PC12) cells.

## 2. Materials and methods

### 2.1. Antibodies and other reagents

Monoclonal anti-Myc (9E10), anti-phospho-Erk1/2, anti- $\beta$ -Actin, anti-hemagglutinin (anti-HA) and anti-LAMP-1 antibodies, rabbit polyclonal anti-Erk and anti-green fluorescent protein (anti-GFP) antibodies, and goat and rabbit anti-EGFR antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-early endosome autoantigen 1 (anti-EEA1) antibody was from BD Biosciences Pharmingen. Fluorescent secondary antibodies

(goat anti-rabbit IgG, goat anti-mouse IgG, rabbit anti-goat IgG, and donkey anti-mouse IgG) were purchased from Jackson ImmunoResearch Laboratories. The HRP-conjugated secondary antibodies for ECL were from Pierce. Human EGF, bFGF were purchased from R&D System. The Texas Red conjugated EGF and Alexa594-conjugated transferrin were purchased from Molecular Probes, Invitrogen.

## 2.2. Plasmid construction and reverse transcription-PCR analysis

The construct pcDNA3.1-hSef-Myc and pEGFP-N1-Sef were described previously [13]. pEGFP-N1/EGFP-Rab5a WT, and S34N plasmids were gifts from Dr. Brian J. Knoll, University of Houston, Texas, USA. Dynamin T65A was kindly provided by Ye-Guang Chen, Tsinghua University, Beijing, China. The EGFR-GFP construct was a gift from Dr. Xuejun Jiang, Institute of Microbiology, Chinese Academe of Science, Beijing, China. The Elk-1 luciferase reporter plasmid and the GFP-Erk2 construct were provided by Dr. Akihiko Yoshimura, Kyushu University, Fukuoka, Japan. Reverse Transcription-PCR (RT-PCR) analysis was carried out as previously described [14]. The sequences of EGFR primers were described in a previous report [15]. The sequences of  $\beta$ -actin primers are as follow: '5CACACTGTGCCCATCTACGA3' (forward) and '5CTGCTTG-CTGATCCAC ATCT3' (reversed).

## 2.3. Cell culture and transfection

Cos7, HEK293T, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Rat pheochromocytoma (PC12) cells were maintained in DMEM supplemented with 10% FBS and 5% horse serum (Invitrogen). Cos7, HEK293T, and HeLa cells were transfected with the different plasmids using VigoFect (Vigorous). In the luciferase assay, HEK293T cells were transfected using Tfx20 (Promega). The PC12 cells were transfected using the Transfast transfection reagent (Promega).

## 2.4. Generation of stably transfected cell clones

The PC12 cell lines stably expressing hSef were generated previously [7]. For establishing stable cell lines in HeLa cells, the cells were transfected with pcDNA3.1/hSef-Myc or pEGFP-N1-hSef or the control vectors using VigoFect (Vigorous). After transfection for 24 h, the cells were grown in the medium supplemented with the antibiotic Genectin (G418, Life Technologies, GIBCO, Invitrogen) at 600  $\mu$ g/ml. Individual G418-resistant clones were expanded and maintained in 300  $\mu$ g/ml of G418. Clones with overexpression of hSef-Myc or hSef-GFP were confirmed by Western blotting.

## 2.5. Western blotting

Cells were washed twice with cold PBS and were lysed in lysis buffer. The appropriate volume of the protein was resolved by 8–10% SDS-PAGE and subjected to Western blotting as described previously [6]. The ECF detection system (Amersham Pharmacia Biotech) was used to visualize the protein bands. Alternatively, the ECL detection system was also used for chemiluminescence of proteins, and the blots were then exposed to photographic films (Kodak, USA).

## 2.6. Co-immunoprecipitation and Immunoblotting

Coimmunoprecipitation assays were performed using a protocol as described previously [6]. Essentially, HEK293T cells in 60-mm dishes were transfected and lysed in 800  $\mu$ l cell lysis buffer. 500  $\mu$ l whole cell lysates were incubated with 2  $\mu$ g of the indicated monoclonal antibodies and 30  $\mu$ l protein G Sepharose beads at 4 °C for 4 h. The beads were washed 4 times with the lysis buffer and the precipitates were eluted with 2 $\times$ SDS-PAGE sample buffer and analyzed by Western blotting.

## 2.7. Immunostaining and confocal microscopy

Immunostaining was performed using a protocol as previously described [6]. ACMA-, FITC-, TRITC-conjugated goat anti-rabbit or anti-mouse IgG purchased from Jackson ImmunoResearch Laboratories. The cover slips were mounted in glycerol and analyzed using a laser scanning confocal microscopy with a 60 $\times$ oil-immersion objective.

## 2.8. Texas red-conjugated EGF and Alexa594-conjugated transferrin uptake

The Texas Red-EGF assay was performed according to published protocols with minimal modifications [16,17]. HeLa cells stably expressing hSef or GFP were plated onto glass cover slips in 6-well plates and cultured for 24 h. The cells were starved for 24 h without feeding with serum, then followed by incubation with 100 ng/ml Texas Red conjugated EGF (Molecular Probes, Invitrogen) in the binding buffer (20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, and 0.1% bovine serum albumin) at 4 °C for 60 min. The cells were then either fixed with 4% paraformaldehyde (PFA) or allowed to internalize EGF at 37 °C for 30 min. After internalization, the cells were placed on ice and subjected to an acid wash (0.5 M NaCl, 0.2 M acetic acid, pH 2.8) for 5 min to remove residual cell-surface-bound EGF and then fixed. The coverslips were immunostained with the anti-EEA1 antibody or directly mounted on slides using glycerol. The confocal images were obtained using a laser scanning confocal microscope as described above. For examination of Alexa594-conjugated transferrin uptake, HeLa cells were rinsed briefly with serum-free medium and incubated with Alexa594-conjugated transferrin at 4 °C for 30 min at a concentration of 20  $\mu$ g/ml. Internalization was initiated by replacing the medium with fresh medium pre-warmed at 37 °C. At the end of internalization, the cells were rinsed briefly in ice-cold PBS, fixed with 4% PFA and observed with a confocal microscope.

## 2.9. Luciferase assay

The Elk-1 luciferase activity assay was performed using trans-reporter constructs including PFA-Elk-1 and PFR-luciferase plasmids (PathDetect in vivo signal transduction pathway trans-reporter system, Stratagene) according to the manufacture's instructions. The Elk-1 luciferase activity was measured using Luciferase Assay System (Promega). The results were expressed as mean $\pm$ S.D. from three independent experiments. The data were normalized with an internal control.

## 2.10. Differentiation of PC12 cells

PC12 cells stably expressing hSef and the mock cells were maintained in medium as described previously [7]. The cells were plated at a subconfluent density on 12-well culture

plates coated with poly-L-lysine and cultured overnight. The next day, the cell were stimulated with or without bFGF (50 ng/ml), or EGF (50 ng/ml) in DMEM supplemented with 1% fetal bovine serum (FBS) and 0.5% horse serum for 24–48 h. The cell morphology was examined by microscopy. Cells with processes longer than 1.5 times the diameter of the cell body were considered to be positive for neurite outgrowth. The numbers of undifferentiated and differentiated cells were counted in three randomly selected fields containing about 150 cells each. Data were expressed as means±S.D.

### 2.11. Statistical analysis

Data were analyzed with the Excel program (Microsoft), and presented as means±SD. Student's *t*-test was used to evaluate the significance of differences between groups of experiments. A value of  $P<0.05$  was considered statistically significant.

## 3. Results

### 3.1. Ectopically expressed hSef protein is localized into the endocytic vesicles

In our previous study [6], we found that hSef is translocated to the plasma membrane (PM) after FGF stimulation but has a typical punctuate localization in the quiescent condition (without FGF stimulation). This observation indicated that Sef might be involved in the endocytic process under an FGF quiescent condition. To test this hypothesis, we co-stained ectopically expressed hSef with endogenous EEA1, an early endosome marker protein [18], in Cos7 cells. Our result shows that hSef and EEA1 are colocalized in the early endosomes in the cytoplasm (Fig. 1A, top panels). To confirm the endosome localization, we also stained the cells with an antibody against Rab5, another early endosome marker [19]. The data demonstrated that Sef is also colocalized with Rab5 (Fig. 1A, bottom panels). Based on these observations, we concluded that Sef could be localized to the early endosomes.

We predicted that an endocytic process would affect Sef localization based on the above observations. Indeed, when we ectopically expressed hSef with GFP-Rab5a in Cos7 cells, we observed that the hSef targeted endocytic vesicles became enlarged (Fig. 1B, a, b). On the other hand, when Rab5 S34N, a dominant negative form of Rab5, which reduces the size of endosomes and inhibits internalization of transferrin and transferrin receptors [20], was overexpressed, the ectopically expressed hSef was increasingly distributed on PM with diminished punctuate structures in the cytoplasm (Fig. 1B, c). Furthermore, when DynaminT65A, a dominant negative form of Dynamin, which inhibits both clathrin- and caveolae-dependent endocytosis [21], was overexpressed, the Sef positive endocytic vesicles were decreased and the PM localization of Sef was increased significantly (Fig. 1B, d). These data indicate that hSef is a PM protein and may be internalized into early endosomes through an endocytic process.

To further investigate the itinerary of Sef trafficking, we compared the distribution of Sef with that of internalized transferrin, because transferrin is known to reach the early endosomes at earlier stage of internalization and to enter into recycling compartments at later stage before returning to the cell surface, but not to be sorted into late endosomes/lysosomes [22,23]. For convenient observation by immunostaining assay, we stably

expressed hSef-GFP and used Alexa594-conjugated transferrin in HeLa cells. The data showed that Alexa594-conjugated transferrin bound to the cell surface at 4 °C and demonstrated little colocalization with Sef in the cytoplasm (Fig. 1C, a). After 10 min of internalization, Alexa594-conjugated transferrin was found in the punctuate endosomal structures distributed through the cytoplasm. At this point, significant co-localization of transferrin and Sef was observed (Fig. 1C, b). After 20 min of internalization, the Alexa594-conjugated transferrin was significantly superimposed on the perinuclear structures together with Sef (Fig. 1C, c). Because the perinuclear accumulation of transferrin-containing endosomes is regarded as recycling endosome compartment [23], we can clearly draw a conclusion that Sef is also localized into the recycling endosomes as well as the early endosomes.

We also investigated whether the cytoplasmic Sef protein could localize to the late endosomes/lysosomes. By comparison of the localization of hSef-GFP with LAMP-1 (the lysosome-associated membrane glycoprotein-1), a marker of the late endosomes/lysosomes, our immunostaining result showed that cytoplasmic hSef-GFP demonstrates barely co-localization with endogenous LAMP-1 in the hSef-GFP expressing stable cell line (Fig. 1D), suggesting that Sef hardly enters into the late endosomes/lysosomes. Together with our above data, we concluded that Sef mainly localizes at PM and enters into the early/recycling endosomes but not into the late endosomes/lysosomes. These observations indicate the dynamic localization of Sef during the endocytic process.

### 3.2. hSef colocalizes and interacts with EGFR in response to EGF stimulation

The feature of Sef localization on PM and the early/recycling endosomes is reminiscent of the distribution of EGFR under stimulation. We hypothesized that Sef might undergo a similar endocytic process to that of EGFR. To test our hypothesis, we used HeLa cells expressing hSef-GFP, as the cells have a relatively high level of endogenous EGFR. Confocal microscopy observation showed that, when the cells were incubated at 4 °C, the EGFR distribution (demonstrated by using Texas Red conjugated EGF, referred to as EGF-EGFR hereafter) on the cell surface can be easily seen (Fig. 2A, panel c), but, when the cells were warmed to 37 °C for 30 min, EGF-EGFR was dramatically internalized into the Sef-GFP positive vesicles (Fig. 2A, panel d). As a control, GFP did not colocalize with EGFR (Fig. 2A, panel a,b). Moreover, Sef-GFP was colocalized very well with EGF-EGFR at both 4 °C (on PM) and 37 °C (in the vesicles), suggesting that Sef is closely colocalized with EGFR and might interact with the EGF-EGFR complexes.

To further confirm the above results, we also examined the endogenous EGFR protein in the same cell lines with anti-EGFR antibodies. In the rested cells (at 4 °C), Sef was mainly localized to the cell surface (Fig. 2B, panel c). In contrast, when the cells were stimulated with EGF, Sef was clearly localized to the cytosolic vesicles together with EGFR (Fig. 2B, panel d, see merged picture). Comparing to the GFP control (Fig. 2B, panel a, b), the internalized EGFR vesicles under EGF stimulation were significantly increased (Fig. 2B, comparing panel b with d). These data confirm that Sef is colocalized in the endocytic vesicles with EGFR in response to EGF stimulation.

To demonstrate whether the internalized hSef and EGFR proteins are colocalized in the early endosome, we immunostained the cells with an antibody against EEA1. We examined the colocalization of Texas Red conjugated EGF (red) and Sef-GFP protein (green) with EEA1 (blue). Our results show that, in most of the stimulated cells, internalized EGFR and hSef were colocalized with EEA1. However, the PM-localized Sef and EGF-EGFR demonstrated little colocalization with EEA1 in the rested cells (at 4 °C) (Fig. 2C, top panel). Colocalization of Sef, EGF-EGFR and EEA1 was easily observed in cytoplasmic vesicles when the cells were warmed to 37 °C (Fig. 2C, bottom panel) suggesting that the colocalization of Sef and EGFR occurs in the early endosomes.

The close colocalization of Sef and EGFR implies a physical interaction between the two proteins. To test this hypothesis, we coexpressed hSef and EGFR in HEK293T cells followed by an immunoprecipitation assay. The results showed that the EGFR-GFP protein can be detected in the complexes by immunoprecipitation using anti-Myc antibody when Sef-Myc and EGFR-GFP were coexpressed, suggesting that hSef interacts with EGFR physically in the cells (Fig. 2D). Further immunoprecipitation experiments using an anti-GFP antibody demonstrated that hSef interacts with endogenous EGFR in the stable cells expressing hSef-GFP (Fig. 2E). Interestingly, the interaction of hSef with endogenous EGFR can only be observed in the presence of EGF stimulation (Fig. 2E). Taken together, our data suggest that Sef interacts with EGFR as it is colocalized with EGFR on PM or in the early endosomes upon EGF stimulation.

### **3.3. Overexpression of hSef attenuates the down-regulation of EGFR by blocking EGFR trafficking into the late endosomes/ lysosomes**

The internalization of EGFR is a critical step for its activation and subsequently degradation or recycling [24]. As hSef colocalizes and interacts with EGFR in the early endosomes in response to EGF stimulation (see Fig. 2), we speculated whether the Sef-EGFR association could affect the stability of EGFR. To examine this, we measured the levels of the endogenous EGFR protein in HeLa cells stably expressing Sef-GFP and Sef-Myc respectively. Compared with the mock cells, the EGFR protein levels in the two Sef expressing cell lines are significantly elevated (Fig. 3A). In contrast, the levels of EGFR mRNA in these cell lines show no significant change (Fig. 3B), suggesting that Sef might affect the stability of the EGFR protein. As EGFR undergoes degradation after EGF stimulation, we further measured the protein levels of EGFR in the Sef expressing cells and the mock cells after different periods of time of EGF stimulation. Our results indicated that the total EGFR protein level was decreased at 1 h after EGF stimulation and was almost undetectable at 3 h after EGF stimulation in the mock cells (Fig. 3C, left 4 lanes). However, in hSef overexpressing cells, the EGFR levels were elevated (consistent with the results in Fig. 3A) and the EGF-induced decrease of the receptor was obviously delayed (Fig. 3C, right 4 lanes). Together with the fact that Sef is colocalized with internalized EGFR in the early and recycling endosomal vesicles (Fig. 2), our data suggest that Sef might interfere with the sorting process of EGFR from the early endosome to late endosomes/lysosomes and thereafter inhibits lysosomal EGFR degradation.

To confirm the above results, the effect of Sef overexpression on the translocation of EGFRs into late endosomes/lysosomes upon EGF stimulation was examined. HeLa cells stably expressing Sef-Myc or mock cells were treated with or without EGF. Costaining the endogenous EGFR (green) and LAMP-1 (red) proteins demonstrated that EGFR is obviously localized in PM, and EGFR/LAMP-1 colocalization is hardly observed in both quiescent HeLa/Sef-Myc cells and the mock cells (Fig. 3D, panel a,c). However, when the cells were treated with EGF for 30 min, the mock cells demonstrated a strong colocalization between EGFR and LAMP-1, implying that endocytic EGFR has entered into the late endosomes/lysosomes (Fig. 3D, panel b). In contrast, only small portions of endocytic EGFR have been shown colocalization with LAMP-1 in the HeLa cells stably expressing Sef-Myc (Fig. 3D, panel d), suggesting that Sef expression blocks EGFR trafficking from early endosomes to late endosomes/lysosomes. Taken together, these observations suggest that Sef enhances EGFR internalization but inhibits EGFR entering into the late endosomes/lysosomes for degradation.

We further investigated whether Sef has any effect on EGFR recycling. To this end, we compared the recycling endosome localization of EGFR using Alexa594-conjugated transferrin as a marker. In the rested cells (at 4 °C), EGFR and Alexa594-conjugated transferrin were observed to be localized in PM in both mock cells and Sef-Myc expressing stable cells (Fig. 3E, a, c). In contrast, after the cells were treated with EGF for 20 min at 37 °C, EGFR was observed to be internalized into transferrin positive endosomes, which represent recycling endosomes, with enlarged vesicles in Sef-Myc expressing stable cells but smaller ones in the mock cells (Fig. 3E, b, d). This result implies that Sef may play a role in increasing EGFR entering into the recycling endosomes, in concomitance with the role of blocking EGFR entering into the late endosomes/lysosomes.

#### 3.4. hSef facilitates EGF signaling while inhibits FGF signaling

Previous studies have demonstrated that Sef interacts with FGFR and inhibits FGF mediated signal transduction [1,4,7,8]. Based on the observations that Sef associates and co-localizes with EGFR and attenuates the degradation of the receptor, we sought to determine whether Sef could affect the downstream signaling pathway mediated by EGFR. To this end, we ectopically expressed hSef together with GFP-Erk2 in HEK293T cells and tested the ligand-induced activation of the MAPK pathway using an antibody specifically against the phosphorylated Erk proteins. In line with previous reports [4,7,8], hSef significantly reduced the activation of Erk in response to FGF stimulation at different time points (Fig. 4A). In contrast, the activation of Erk by EGF stimulation was significantly elevated with overexpression of hSef in the cells (Fig. 4B). To further confirm the effect of Sef on EGF signaling, we measured the levels of activated (phosphorylated) Erk in HeLa cells stably expressing hSef upon EGF stimulation at different periods of time. Our results show that the phosphorylation of the Erk protein was rapidly elevated by EGF stimulation at 7.5 min in both the mock and the Sef expression cells. However, the phosphorylation of Erk remained at high levels at 15 and 30 min in the Sef overexpression cells but decreased in the mock cells (Fig. 4C). These data suggest that Sef maintains the duration of EGFR activation indicated by Erk phosphorylation.



Activation of EGF signaling can be demonstrated by Elk-1 activity [25]. To measure the affect of Sef on EGFR signaling, we used an Elk-1-Gal4 luciferase reporter system, which responds to both EGF and FGF stimulation in activation of Elk-1 [26]. Our data show that both FGF and EGF stimulated the activation of the reporter in 293T cells with transfection of control vector (Fig. 4D, left group of columns). However, in the presence of overexpressed hSef, FGF almost totally lost activity while EGF showed an increased activity on the luciferase reporter (Fig. 4D, right group of columns). Similar results were obtained from the HeLa cells (Fig. 4E). These data, consistent with the Erk phosphorylation results, confirm that hSef has opposite effects on FGF and EGF mediated Erk signaling.

### 3.5. EGF-induced Erk activation sustained by hSef leads to neurite outgrowth in rat pheochromocytoma (PC12) cells

Under ordinary culture conditions, nerve growth factor (NGF) or FGF stimulation promotes sustained MAPK activation and neuronal differentiation, whereas transient MAPK activation triggered by EGF increases proliferation of PC12 cells [27,28]. Also, enhancement of the strength and duration of EGFR mediated signaling can result in outgrowth of neurites in PC12 cells [29–31]. As Sef enhanced EGFR mediated Erk activation, we hypothesized that overexpression of Sef might affect the EGF induced PC12 cell differentiation. To test our hypothesis, we stably expressed hSef in PC12 cells treated with EGF and FGF respectively. Our data show that overexpression of hSef inhibited FGF induced PC12 cell differentiation, which is consistent with our previous report [7]. In contrast, EGF stimulation elicited significant differentiation of the PC12 cells stably overexpressing hSef, greater than that observed in mock cells (Fig. 5). These results suggest that EGFR induced Erk activation is sustained in hSef overexpression cells, causing enhanced PC12 cell differentiation.

## 4. Discussion

EGF and FGF function through their cognate receptors and mainly activate Ras/MAPK signaling, which plays critical roles in both invertebrates and vertebrates to control organ morphogenesis, patterning, cellular proliferation, and differentiation [32]. In most cases, both EGF and FGF are regulated by similar mechanisms and positive and negative regulators have been identified for their activity modulation. However, it remains unclear how EGF and FGF can differentially regulate the activation of Ras/ MAPK. In this paper, we demonstrate that Sef enhances EGF- but inhibits FGF-induced Ras/MAPK signaling. In this way, Sef plays a distinct role in regulating EGF and FGF signalings.

Sef was previously reported to function mainly as a negative regulator to antagonize FGF signaling. Herein, we found that Sef interacts with EGFR and sustains EGF signaling. We first observed that Sef undergoes an internalization process, which is similar to the behavior of EGFR. Using immunostaining experiments we found that Sef is localized well into early/recycling endosomes while barely into late endosomes/lysosomes. Interestingly, Sef localization pattern can be changed by dominant negative Rab5 (Rab5 S34N) and dominant negative Dynamin (Dynamin T65A), the inhibitory molecules for endosome formation. These observations indicated that Sef is subjected to endocytosis. On the other hand, we also observed that Sef and EGFR are colocalized in early endosomes in response to EGF

stimulation. Based on these observations, we predicted that Sef might interact with EGFR to affect EGF signaling. From our immunoprecipitation results we found that Sef interacts strongly with EGFR and the interaction is regulated by EGF stimulation. Intriguingly, we found that overexpression of hSef attenuates EGFR degradation mediated by EGF stimulation. Furthermore, overexpression of hSef facilitates EGF signaling while inhibits FGF signaling as measured by Erk phosphorylation. Therefore, we proposed that Sef has opposite roles in the activation of Erk signaling mediated by FGF and EGF. Consistent with the Erk activation experiment by Western blotting, we showed that, Sef inhibits FGF-, but promotes EGF-mediated Elk-1 activation by luciferase assay. This opposing effect was also demonstrated by PC12 differentiation experiments. All the observations were in concordance to indicate that Sef functions in an opposite way on FGF and EGF signaling.

It is interesting to address how Sef upregulates EGF — but downregulates FGF-induced signaling. In the previous reports, we and others presented evidence that Sef could interact with FGFR [1,4,7] or the downstream mediators such as RAS [6] to inhibit the signaling. In the presence of FGF stimulation, Sef seems to localize onto PM [see Fig. 1 in [6]]. It was also proposed that Sef might sequester Erk in the Golgi membrane to prevent the phosphorylated Erk from translocation into nucleus [9]. These different observations suggested that Sef might function at different levels to inhibit FGF signaling. On the other hand, in this report, we observed that Sef interacts with EGFR and undergoes the internalization process with EGFR. We proposed that the internalized Sef protein might inhibit the process of EGFR trafficking from early endosomes to late endosomes/ lysosomes. Indeed, our experiments indicated that overexpression of hSef enhances the stability of EGFR (see Fig. 3A and B) and inhibits the translocation of EGFRs into late endosomes/ lysosomes and that Sef is localized well into early/recycling endosomes while barely to late endosomes/lysosomes. As internalized EGFR (but not FGFRs) has also been demonstrated to mediate the EGF downstream signaling [33,34], we propose that Sef facilitates EGF signaling by maintaining EGFR in the early or recycling endosomes and blocking EGFR trafficking into the late endosomes/lysosomes for degradation (Fig. 6). Therefore, although Sef interacts with both FGFR and EGFR, the distinct roles of Sef on the two receptors make it function both positively and negatively in MAPK signaling pathway.

How Sef stabilizes EGFR in the endosomes remains to be determined. In general, EGFR has been known to be translocated into the early endosomal vesicles by clathrin-mediated endocytosis upon EGF stimulation, and subsequently, EGFR is degraded in lysosomes [35]. Based on the function of Sprouty2, a negative regulator for FGF but a positive regulator of EGF [29,36], we speculated that Sef might inhibit an adaptor protein (which may mediate EGFR degradation) for the stabilization of EGFR. Several reports proposed that the recruitment of Cbl to the activated EGFR leads to ubiquitination of the cytoplasmic tail of the receptor, thereby promoting endocytosis and lysosomal degradation [35,37]. Furthermore, Cbl has also been found to be required for EGFR exit from the early endosomes but not for the internalization of FGFR [38]. In contrast, studies in mammalian cultured cells have suggested that in addition to mediating lysosomal/proteasomal degradation, Cbl-containing complexes promote the assembly of active intracellular EGFR signaling modules, either by traffic to particular endosomal structures or through receptor

recycling [39]. It is of interest to study whether Sef interacts with such a kind of adaptor to stabilize EGFR in the endosomes.

The proliferation and differentiation of cells in response to extracellular signals is influenced by the differential regulation of MAPK [28]. PC12 cells have been widely used as a cell model for the study of growth factor-stimulated cell function, since evidence has been well documented that the amplitude and longevity of the MAPK signal governs the proliferation and differentiation of PC12 cells under different stimulation [27]. Our experiments with PC12 cells expressing hSef demonstrate that incubation with EGF results in neurite outgrowth. This supports the proposed threshold theory that differentiation is determined by the duration of Erk activation [40]. Since EGF is also proved to be involved in determining the threshold level of Erk activation required for directional migration of PC12 cells [41,42], it would be worthy of investigating whether hSef plays a role in this process through regulating the extent and duration of Erk activation in response to EGF stimulation. Together with our previous results that hSef inhibits FGF and NGF induced PC12 differentiation, our data indicate that hSef plays a critical role in determination of cell fate by controlling the balance of FGF and EGF signalings.

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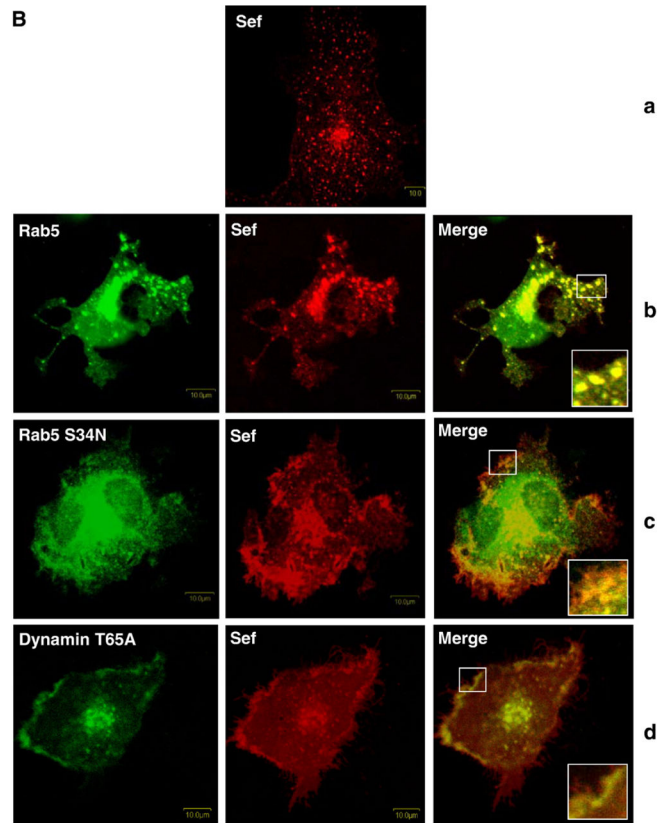
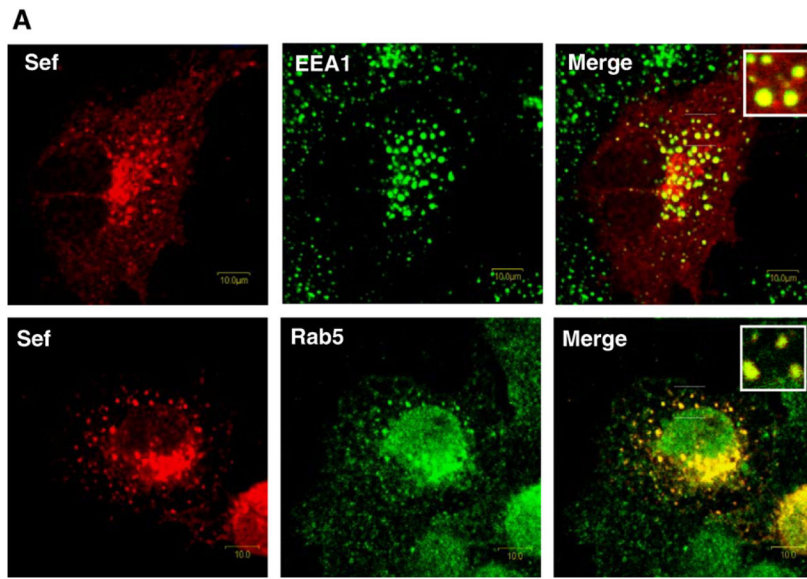
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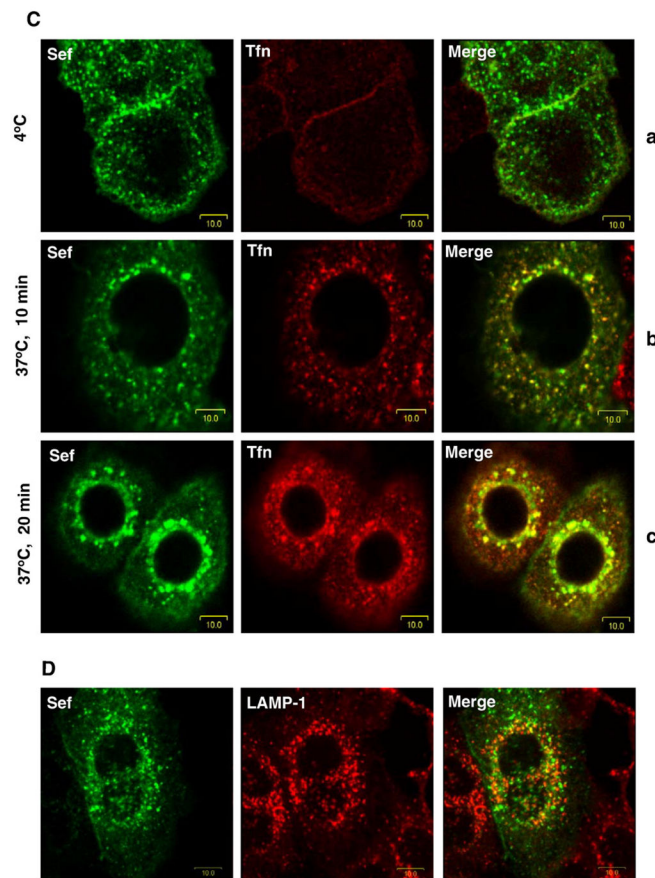
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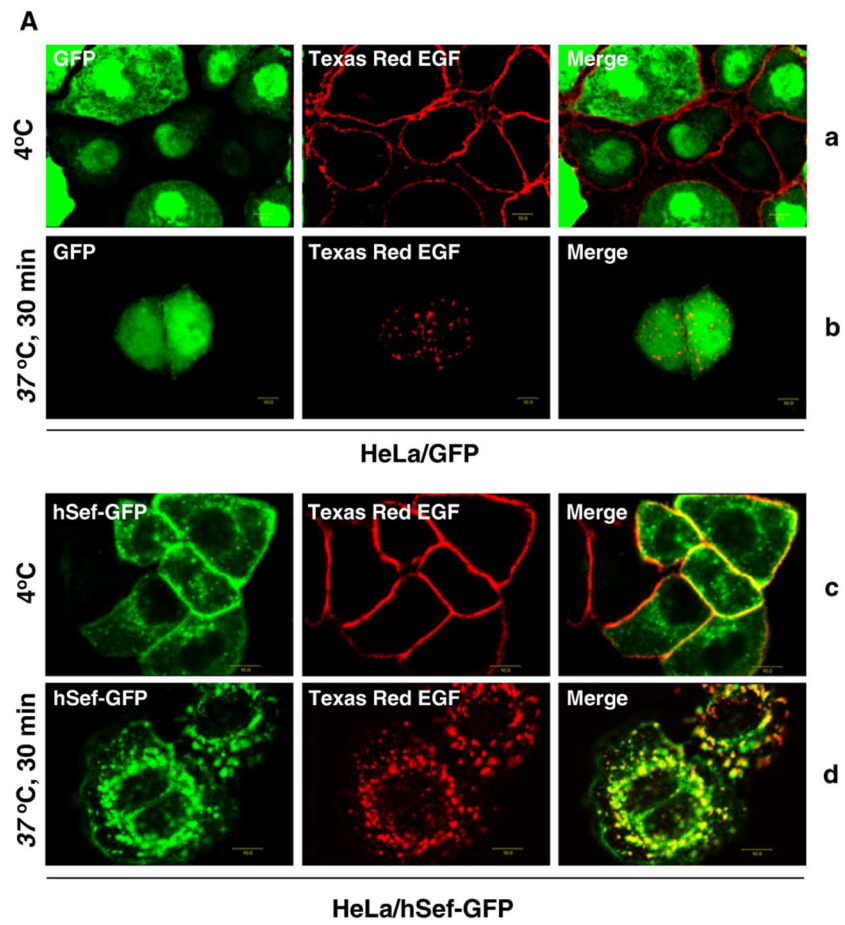
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**Fig. 1.** Characterization of the trafficking route of ectopically expressed Sef. (A) Sef is localized into the early endosomes. Cos7 cells transfected with hSef-Myc were double labeled with a rabbit polyclonal anti-Myc antibody (red) and either a mouse anti-EEA1 (upper panel, green) or anti-Rab5 antibody (lower panel, green). Enlarged area indicates overlap of hSef-Myc and endogenous EEA1 or Rab5a. (B) Molecules affecting the early endosome trafficking influence Sef localization. Cos7 cells co-transfected with hSef-Myc expression plasmid, and the plasmid encoding either GFP-Rab5a or GFP-Rab5S34N (a dominant negative form of Rab5) or HA-DynaminT65A (a dominant negative form of Dynamin) were immunostained with anti-Myc antibody (panel a, b, c) (red) or with anti-HA antibody (green) and anti-Myc antibody (red) (panel d). Enlarged area indicates overlap of hSef-Myc and ectopically expressed GFP-Rab5a or GFP-Rab5S34N or HA-DynaminT65A. Scale bars, 10 μm. (C) Sef undergoes into the recycling endosomes. HeLa-Sef-GFP cells were incubated with Alexa-conjugated transferrin (Tfn) at 4 °C for 30 min, followed by warming to 37 °C for 10 or 20 min. Confocal images show Tfn surface labeling at 4 °C or uptake at 37 °C. Scale bars, 10 μm. (D) Sef does not enter into the late endosomes/lysosomes. HeLa-Sef-GFP stable cells were labeled with mouse monoclonal anti-LAMP-1 antibody (red). Scale bars, 10 μm.





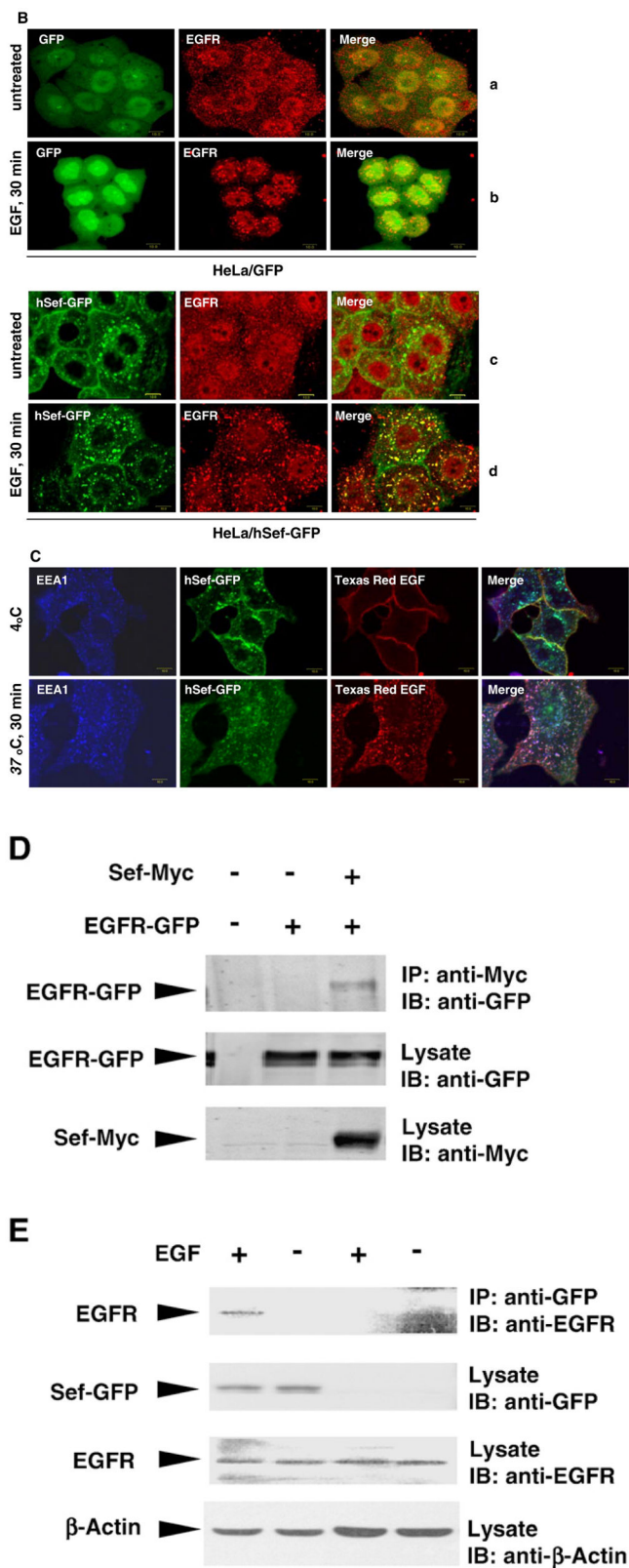
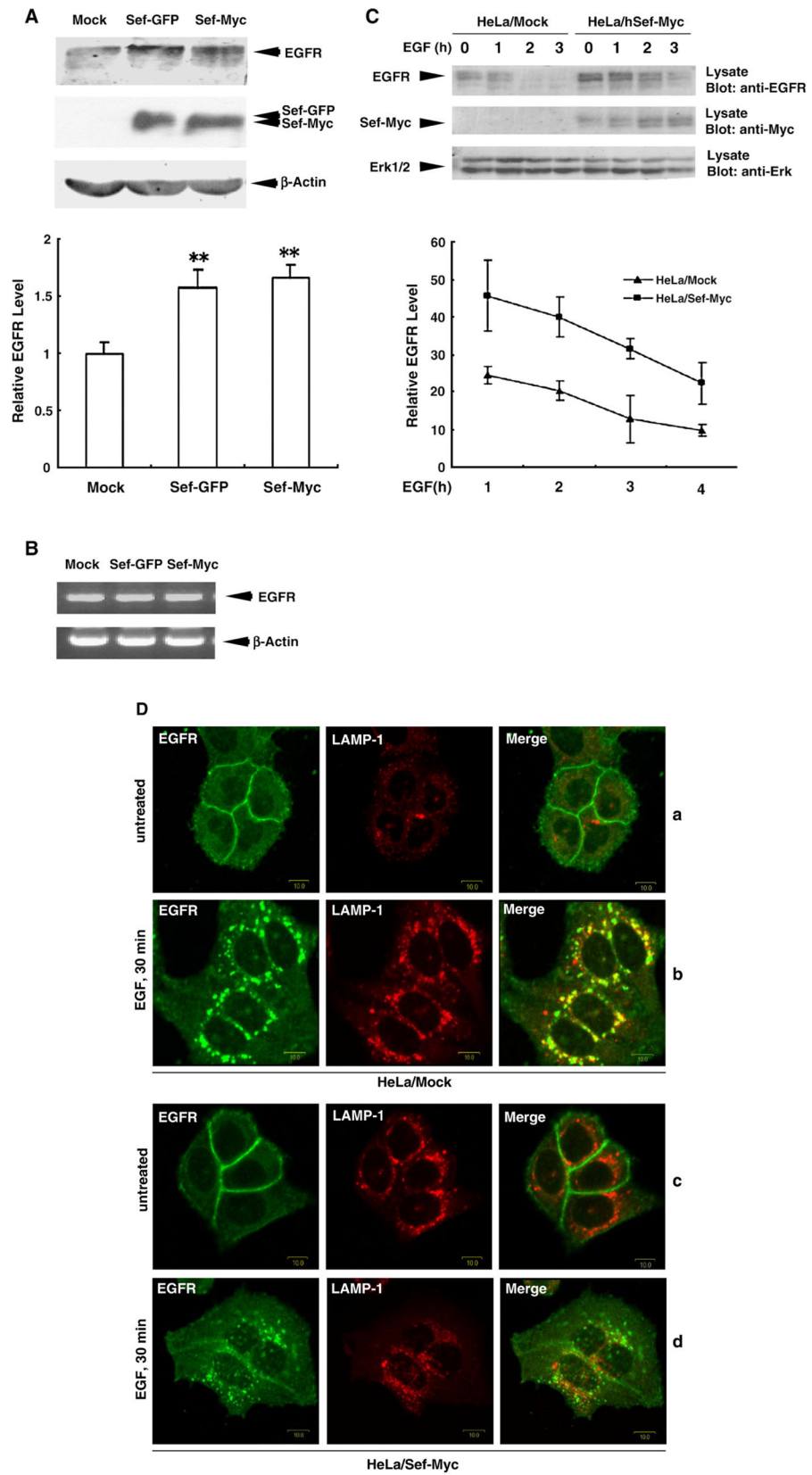
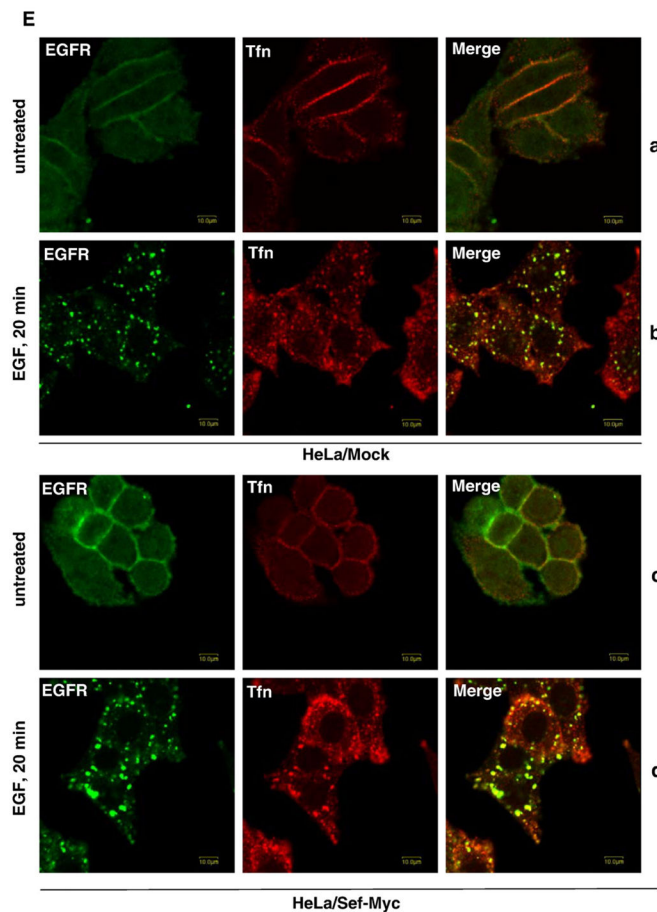


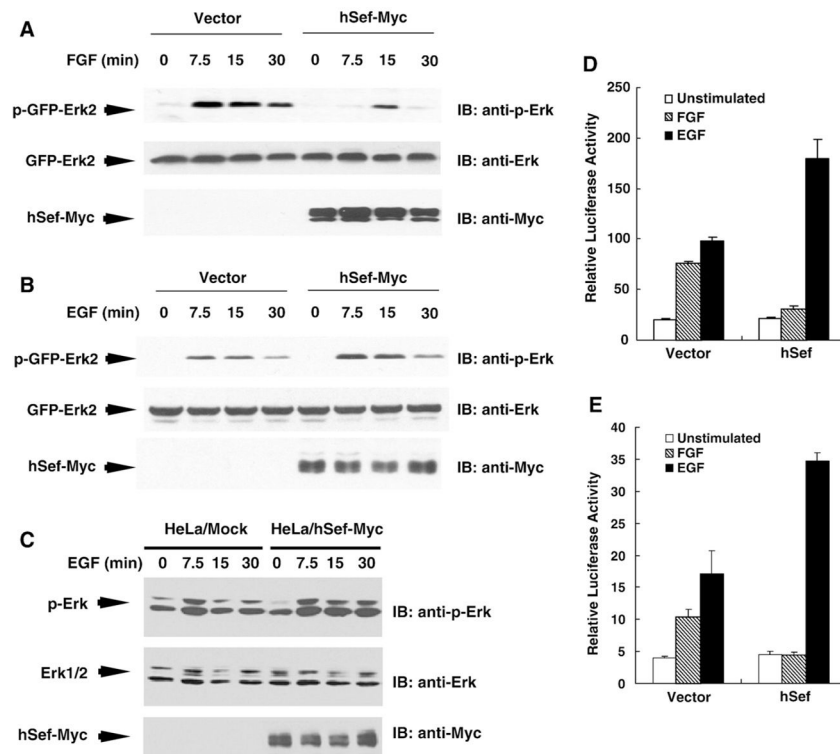
Fig. 2.

hSef colocalizes and interacts with EGFR. (A) Sef colocalizes with EGFR. HeLa cells stably expressing hSef-GFP or control GFP were incubated with Texas Red EGF (100 ng/ml) at 4 °C followed by warming to 37 °C (b,d) or directly fixed (a,c). Confocal images show Texas Red EGF surface labeling at 4 °C or uptake at 37 °C. Scale bars, 10 μm. (B) Colocalization of Sef with EGFR in response to EGF stimulation. hSef-GFP or GFP expressing HeLa stable cells were labeled with a goat polyclonal anti-EGFR antibody (red) after treatment of EGF (100 ng/ml) at 37 °C. Scale bars, 10 μm. (C) The vesicle colocalization of Sef with EGFR is in the early endosomes. hSef-GFP expressing HeLa stable cells were incubated with Texas Red EGF (100 ng/ml) at 4 °C, followed by warming to 37 °C. Confocal images show Texas Red EGF surface uptake at 37 °C and anti-EEA1 immunostaining (blue) of the corresponding coverslips. Scale bars, 10 μm. (D) Sef interacts with EGFR in the 293T cells. HEK293T cells co-transfected with Myc-tagged Sef and GFP tagged EGFR or the vector control were immunoblotted with anti-Myc and anti-GFP antibodies or immunoprecipitated with anti-Myc antibody and immunoblotted with anti-GFP antibodies. (E) Sef interacts with EGFR in Sef expressing HeLa stable cells in response to EGF stimulation. hSef-GFP or GFP expressing HeLa stable cells were used for the experiment.

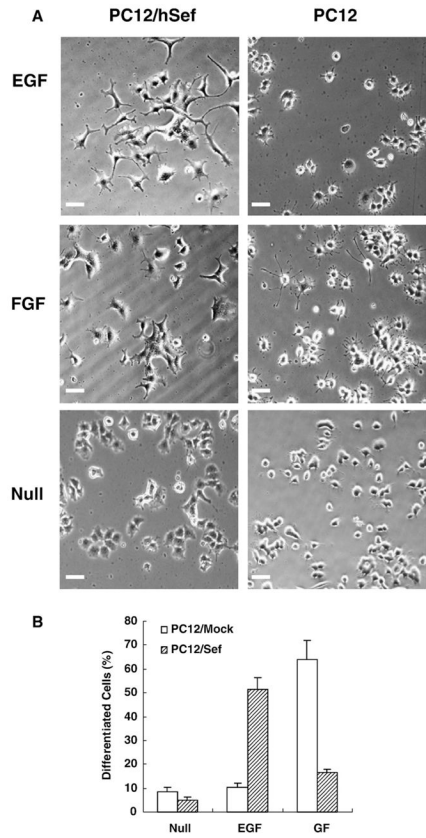




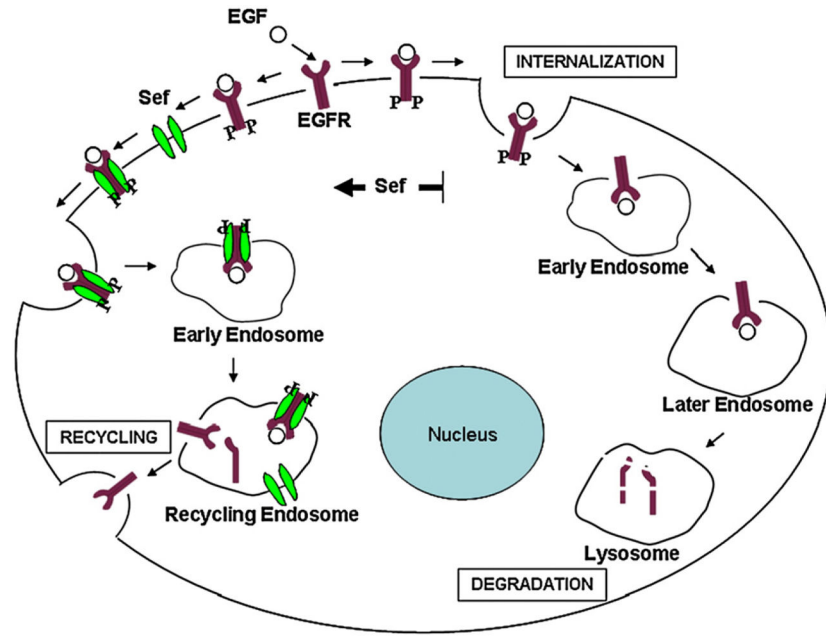
**Fig. 3.** hSef attenuates EGFR degradation and trafficking into the late endosomes/lysosomes. (A) Sef enhances the EGFR protein levels. The whole cell lysates from the indicated cells were immunoblotted with anti-EGFR antibody. The histograms represent the average of three independent experiments. Asterisks,  $p < 0.01$  (compared with control). (B) Sef does not affect EGFR mRNA levels. The mRNA level of EGFR in the indicated cell lines were analyzed by RT-PCR. (C) Sef increases the basal levels of EGFR. The whole cell lysates from the indicated cells treated with EGF (50 ng/ml) were immunoblotted with anti-EGFR and anti-Myc antibodies. The endogenous Erk1/2 proteins are shown as loading controls. The graph depicts the intensity of the EGFR bands from the average of three independent experiments. (D) Sef blocks EGFR trafficking into the late endosomes/lysosomes. The indicated cells stimulated with EGF (100 ng/ml) were double labeled with a rabbit polyclonal anti-EGFR antibody (green) and mouse anti-LAMP-1 antibody (red). Scale bars, 10  $\mu$ m. (E) Sef increases the recycling endosome localization of EGFR. The cells were incubated with Alexa-conjugated transferrin (Tfn) at 4  $^{\circ}$ C followed by warming to 37  $^{\circ}$ C for 20 min in the presence or absence of 100 ng/ml EGF, and labeled with a rabbit polyclonal anti-EGFR antibody (green). Scale bars, 10  $\mu$ m.

**Fig. 4.**

Overexpression of hSef demonstrates different effects on FGF/EGF-mediated signal transductions. (A) Sef inhibits FGF-activated Erk phosphorylation. Western blotting was performed for the cell lysates from HEK293T cells transfected with GFP-Erk2 (0.8  $\mu$ g) and hSef or a control vector in the presence of 25 ng/ml of bFGF for different times. (B) Sef enhances EGF activated Erk phosphorylation. HEK293T cells were treated with 25 ng/ml of EGF for the indicated times. (C) Sef increases the duration of EGF induced Erk phosphorylation in HeLa cells. Western blot shows the levels of the endogenous proteins from the cells over-expressing hSef in the presence or absence of EGF. (D, E) Sef inhibits FGF-induced but enhances EGF-induced Elk transcriptional activity. The luciferase activities were measured for the HEK293T (D) or HeLa (E) cells transfected with Elk-1 responsive reporter plasmids PFA-Elk-1, PFR-Luciferase and internal control pRL-TK plasmids together with or without hSef plasmids in the presence of 25 ng/ml of EGF or bFGF. Results presented are from one experiment assayed in triplicate.



**Fig. 5.** Ectopic expression of hSef leads to neurite outgrowth in rat pheochromocytoma (PC12) cells. Sef increases EGF-induced but inhibits FGF-induced neurite outgrowth of PC12 cells. PC12 cells with or without over-expression of hSef were stimulated with or without bFGF or EGF and then examined for the morphology changes by microscopy. Scale bars, 50  $\mu$ m. (B) Quantification of PC12 cell differentiation. Cells with processes longer than 1.5 times the diameter of the cell body were considered to be positive for neurite outgrowth. The graph shows the average percentage of cells with neurite outgrowth from three experiments. The numbers of undifferentiated and differentiated cells were counted in three randomly selected fields containing about 150 cells each.



**Fig. 6.** A schematic model of Sef in regulation of trafficking and degradation of EGFR. The activation of EGF receptors by EGF on the PM induces their phosphorylation and followed by rapid internalization of activated receptors to early endosomes. Sef also undergoes endocytosis to the early endosomes together with EGFR. The EGFR-Sef positive endosomes traffic to recycling endosome while the EGFR endosomes without Sef undergo trafficking to late endosomes and lysosomes. In such a way, Sef blocks the degradation of EGFR and potentiates the EGFR-mediated signaling.