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Activation of TAK1 by Sef-S induces apoptosis in 293T cells

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ABSTRACT

Sef (similar expression to fgf genes, also named IL-17RD) was identified as a negative regulator of fibroblast growth factor signaling. Sef-S, an alternative splice isoform of Sef, inhibits FGF-induced NIH3T3 cell proliferation. Here we report that Sef-S physically interacts with TAK1, induces Lys63-linked TAK1 polyubiquitination on lysine 209 and TAK1-mediated JNK and p38 activation. Co-overexpression of TAK1 WT, K34R, K150R, K158R mutants with Sef-S induces Lys63-linked TAK1 polyubiquitination whereas TAK1 K63R and K209R mutants fail. Furthermore, co-overexpression of Sef-S and TAK1 induce 293T cells apoptosis. These results reveal Sef-S actives Lys63-linked TAK1 polyubiquitination on lysine 209, induces TAK1-mediated JNK and p38 activation and also results apoptosis in 293T cells.

1. Introduction

Mitogen-activated protein kinases (MAPKs) are signaling components that are important in converting extracellular stimuli into a wide range of cellular responses [1]. The Erk1 and Erk2 MAPKs are activated by mitogens and are found to be upregulated in human tumors [2], leading to the development of inhibitors for cancer therapeutics. Two other major MAPK pathways, the Jun N-terminal kinase (JNK) and p38 MAPK pathways, which are also called stress-activated protein kinase pathways, are also often deregulated in cancers [1]. JNK and p38 MAPKs are activated by environmental and genotoxic stresses and play key roles in inflammation, as well as in tissue homeostasis, as they control cell proliferation, differentiation, survival and the migration of specific cell types.

Growth factor signaling by receptor tyrosine kinases (RTKs) triggers a spectrum of responses in target cells, including proliferation, differentiation, migration and survival. Among the regulation mechanisms, the negative feedback loop is proposed as a classical model that RTK signaling can be attenuated effectively. Dysregulation of RTK signaling is associated with human diseases including cancer [3], skeletal dwarfism, and craniosynostosis [4], thus RTK signaling must be tightly regulated [1,5]. To date, several feedback inhibitors have recently been identified and members of the Sprouty [6] and SPRED families of polypeptides [7] are the most significant inhibitors for the RTK pathway.

Sef (similar expression to fgf genes), also named IL-17RD, is identified as a feedback inhibitor of fibroblast growth factor receptor signaling and encodes a transmembrane domain protein which is highly conserved in zebrafish, mouse and human [8–12]. Study demonstrate that Sef acts as a feedback inhibitor of FGF-mediated Ras–MAPK signaling and inhibits Erk1/2 activation [3,13–18]. In addition, an alternative splice isoform of human Sef, termed Sef-S [19,20], has been identified as a cytosolic protein that associates with FGFR and inhibits Erk1/2 activation [21]. However, it remains unclear whether Sef-S functions in other MAPK pathways besides Erk1/2.

The TAK1 kinase was discovered in 1995 by Matsumoto and colleagues in a complementation screen based on a MAPK pathway in yeast and characterized as a MAPK kinase kinase (MAP3K), which is activated by TGF-β and bone morphogenetic protein [5]. However, during the decade after the original report, TAK1 was characterized and widely accepted as a key player in pro-inflammatory cytokine signaling, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and Toll-like receptor (TLR) ligands [5,22].

In this report, we investigate the molecular mechanism of TAK1 activation induced by Sef-S. We find that TAK1 lysine 209 is required for Sef-S induced TAK1 polyubiquitination and phosphorylation [23]. We demonstrate that Sef-S induces JNK and p38 activation and 293T cells apoptosis.

2. Materials and methods

2.1. Reagents

Monoclonal antibodies to Flag and Myc were from Sigma. Antibodies against p38, p-p38, JNK, p-JNK, c-Jun, p-c-Jun and p-TAK1 were from

Abbreviations: Ub, ubiquitin; Ub, nubiquitination; wt, wild type; FBS, fetal bovine serum; IgG, immunoglobulin G; SPRED, Sprouty-related EVH1-domain containing; FGF, fibroblast growth factor.

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cell signaling and antibodies against His, HA, Erk1/2 and p-Erk1/2 were from Santa Cruz Biotechnology.

2.2. Constructs

Sef-S construct was in pcDNA3.1 and was described previously [21]. Flag-TAK1 and Flag-TAK1-K63R [24] were provided by Dr. Robert Friesel (Maine Medical Center Research Institute, Scarborough, ME). Flag-TAK1-K34R, Flag-TAK1-K150R and Flag-TAK1-K158R as well as Flag-TAK1-K209R [25–27] were provided by Dr. Jianhua Yang (Baylor College of Medicine, Houston, TX). The luciferase reporter plasmid pAP1 was provided by Dr. Huabing Qi (Third Military Medical University, Chongqing, China).

2.3. Cell transfection, immuno-precipitation and immuno-blotting

293T cells and MCF7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% of fetal bovine serum. Transient transfection experiments were carried out using Genejuice regent (VigoFect) according to the manufacturer's protocol. Cells were harvested 36 h after transfection by lysised in cell lysis buffer (80 mM KCl, 10 mM Na_2HPO_4, 1 mM EDTA (pH = 8.0), 0.5% NP-40, 10% glycerol) with protease inhibitors (1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM Na_3VO_4 (Sigma). Lysates were either subjected directly to immunoblot analyses or immunoprecipitated overnight with the indicated antibodies. Immune complexes were recovered with Protein G PLUS-Agrose (sc-2002, Santa Cruz Biotechnology) washed in cell lysis buffer, and subjected to immunoblotting.

2.4. Immunofluorescent staining

MCF7 cells were cultured in cover glass chambers and transduced with the plasmids as indicated. Following a 36 h incubation period, cells were washed three times with cold PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. After another washing step with PBS, cells were permeabilized using PBS solution containing 10% FBS and 0.3% Triton X-100 for 50 min. Primary antibody was incubated at 4 °C overnight at a dilution of 1:100. All antibodies were diluted in PBS with 0.3% Triton X-100. The slides were washed three times in PBS. Cells were then incubated with the secondary antibodies, goat anti-mouse IgG/TRITC and goat anti-rabbit IgG/FITC (Jackson Research Laboratories) for 1 h and counterstained with Hoechst for 10 min. Finally, the immunostained cells were visualized with a confocal laser scanning microscope (OLYMPUS BX61).

2.5. Luciferase reporter assays

293T cells were seeded at 1×10^5 cells per well and cultured overnight in 24-well plates. The cells were transfected with indicated plasmids together with MAPK-dependent firefly luciferase constructs pAP1 and Renilla luciferase construct pRL-TK, which was used to

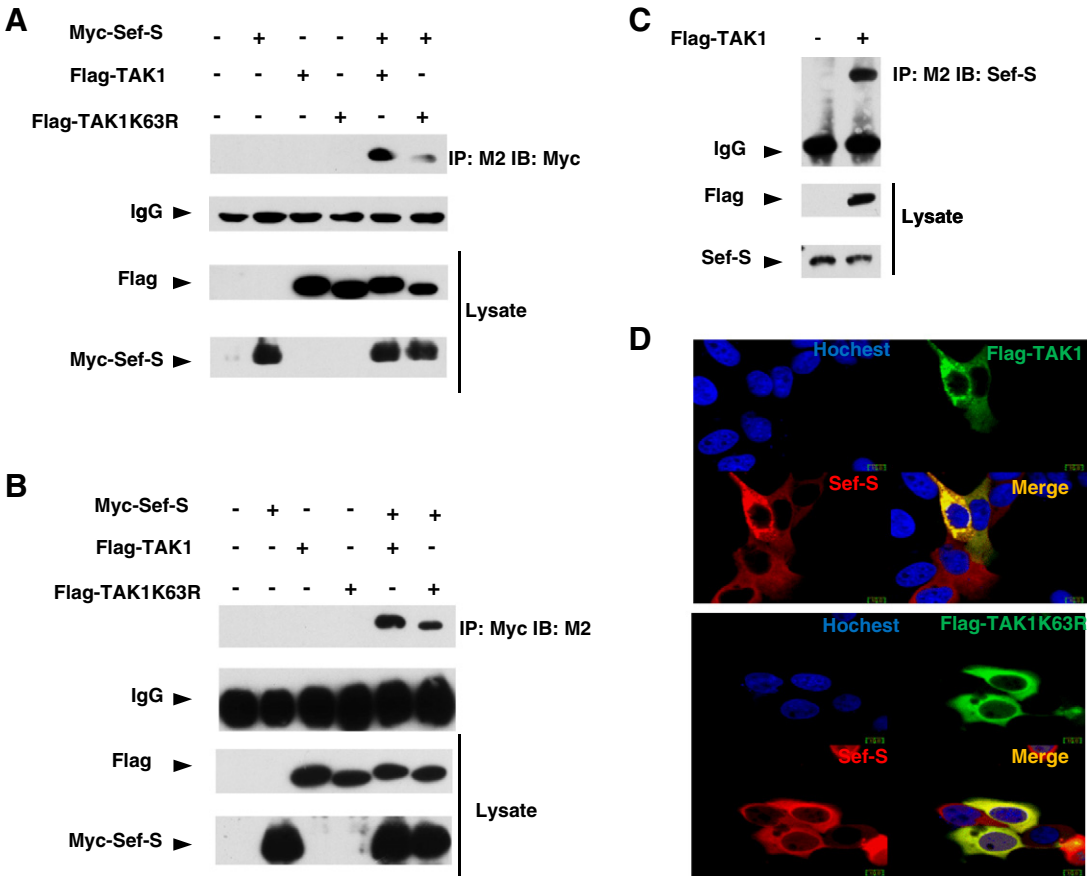


Fig. 1. Sef-S physically associates with TAK1. (A–B) 293T cells were co-transfected with Myc-Sef-S and Flag-TAK1 (or Flag-TAK1-K63R) constructs for 36 h. Cell lysates were immunoprecipitated with Flag (A) or Myc (B) antibodies overnight at 4 °C, and immune complexes were captured with Protein G PLUS-Agrose. Beads were washed extensively and eluted in lysis buffer and immunoblotted with Myc (A) or Flag (B) antibodies. (C) 293T cells were transfected with Flag-TAK1 for 36 h, and cell lysates were prepared and immunoprecipitated using Flag antibody and immunoblotted with Sef-S antibody. (D) MCF7 cells were grown on glass coverslips and transfected with 1.0 μg Sef-S-Myc and TAK1-Flag (or Flag-TAK1-K63R). After 36 h, cells were fixed and immunostained with mouse anti-Myc and rabbit anti-Flag antibody, followed by TRITC-conjugated mouse secondary antibody (red) and FITC-conjugated rabbit secondary antibody (green). Nuclei were counterstained with Hoechst. Scale bar: 10.0 μm.

Table 1
Primers used for PCR.

Gene detection	Primers
c-Jun-F:	GCATGAGGAAACGCATCGCTGCCTCCAAGT
c-Jun-R:	GCGACCAAGTCCTTCCCACTCGTGCACACT
GAPDH-F:	AACGTGTCAGTGGTGGACCTG
GAPDH-R:	AGTGGGTGTCGCTGTTGAAGT
MYC-F:	GGAGGAACAAGAAGATGAGG
MYC-R:	GTTCGCCTCTTGACATTCTC
ELK1-F:	GGTGGTGAATTCAAGCTGGT
ELK1-R:	ATTTGGCATGGTGGAGGTAA
MEF2C-F:	GCCCTGAGTCTGAGGACAAG
MEF2C-R:	AGTGAGCTGACAGGGTTGCT
C/EBPβ-F:	ACAGCGACGAGTACAAGATCC
C/EBPβ-R:	GACAGTTGCTCCACCTTCTTCT
Bcl-2-F:	CAGTCTTCAGGCAAAACGTCGA
Bcl-2-R:	ACAGAAACCTTTAGGCTGGT
Cyclin D1-F:	CCGTCCATGCGGAAGATC
Cyclin D1-R:	GAAGACCTCCTCCTCGCACT
STAT1-F:	GATCTCTAACGTCTGTCAGCTG
STAT1-R:	GAGGTCCAGGATTCCTTCGATC
STAT3-F:	GAGGACTGAGCATCGAGCA
STAT3-R:	CATGTGATCTGACACCCTGAA

F: forward; R: reverse.

normalize the firefly luciferase activity. The control plasmid was added to sustain equal amount of total DNA. At 36 h after transfection, cell lysates were collected for dual luciferase reporter assays. Luciferase activity was measured according to the manufacturer's protocol. The relative luciferase activity was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity. Data represent three independent experiments performed in triplicate.

2.6. Real-time PCR

Total RNA was extracted from Vector or Sef-S 293T cell lines using TRIzol (Invitrogen). Gene expression was determined by Real-time PCR (RealMasterMix, Tiangen). Reactions were performed in triplicate in 20 μl containing 2.5 μl of 1:100-diluted cDNA. Reactions were run at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene expression was determined by the standard curve method and normalized to the level of GAPDH. Appropriate analyses were performed to determine that expression of control genes was unchanged under the experimental conditions described. RCR primer sequences for target genes are listed in Table 1. The endogenous GAPDH gene was used as an internal control.

2.7. Cell death assays

293T cells were transiently transfected with Sef-S or (and) TAK1 expression plasmids. 48 h after transfection, the cells were washed with PBS and stained with PI and FITC. Apoptosis was quantified by calculating the percentage of fluorescence-positive cells as determined by flow cytometry.

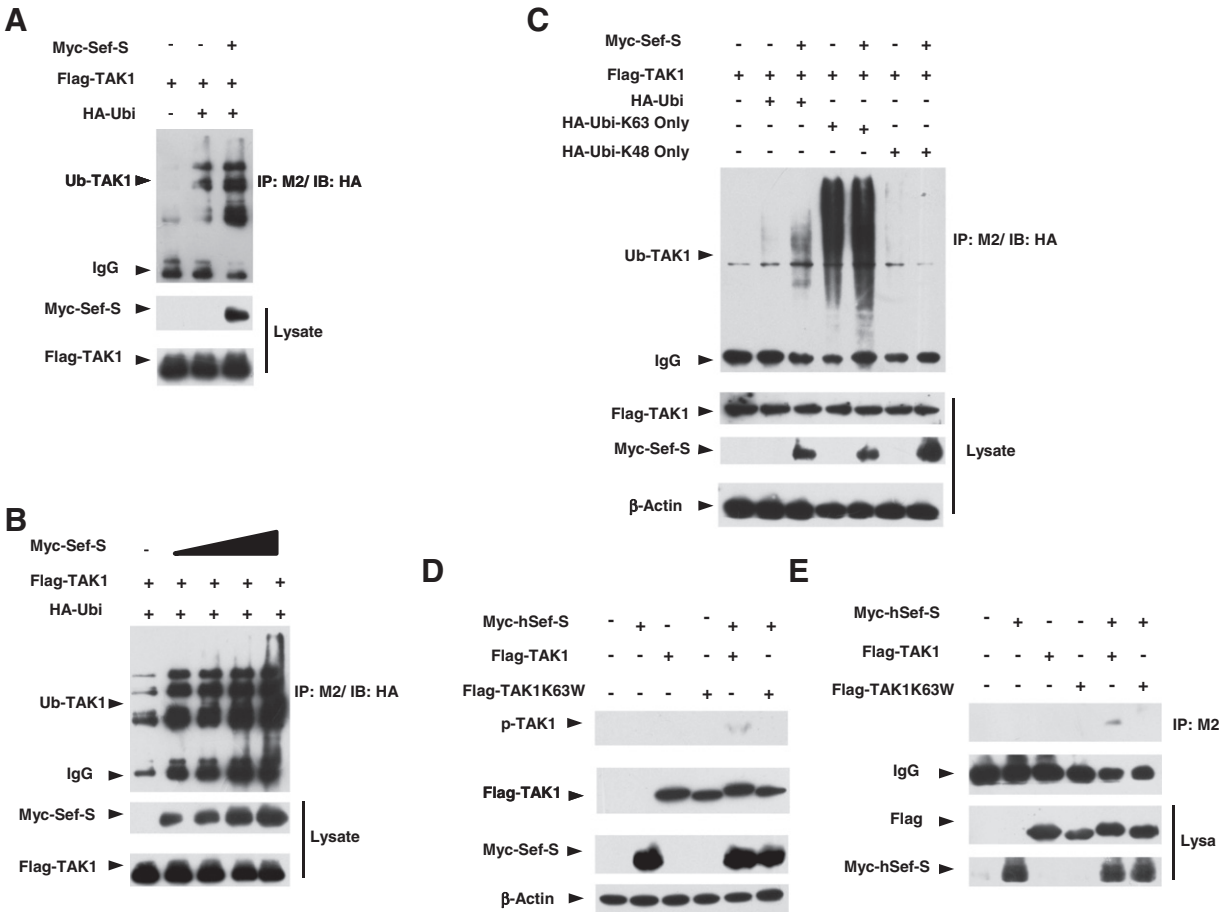


Fig. 2. Sef-S stimulates ubiquitination and activation of TAK1. (A) 293T cells were transfected with 1 μg TAK1 either in the absence (−) or presence (+) of Sef-S along with 400 ng HA-tagged ubiquitin. TAK1 was isolated by immunoprecipitation followed by immunoblot (with anti-hemagglutinin) to examine its ubiquitin conjugation. (B) TAK1 ubiquitination from 293T cells transfected as indicated was immunoprecipitated and immunoblotted by using a HA antibody. (C) 293T cells were transfected with Flag-TAK1 with or without Sef-S and with wild type, Lys63-only or Lys48-only HA-ubiquitin as indicated. Ubiquitination of TAK1 was detected by immunoblotting the TAK1 immunoprecipitants with HA antibody. (D–E) TAK1 immunoblot (D) or immunoprecipitas (E) from 293T cells transfected as indicated were immunoblotted with p-TAK1 (Thr184/187) antibody.

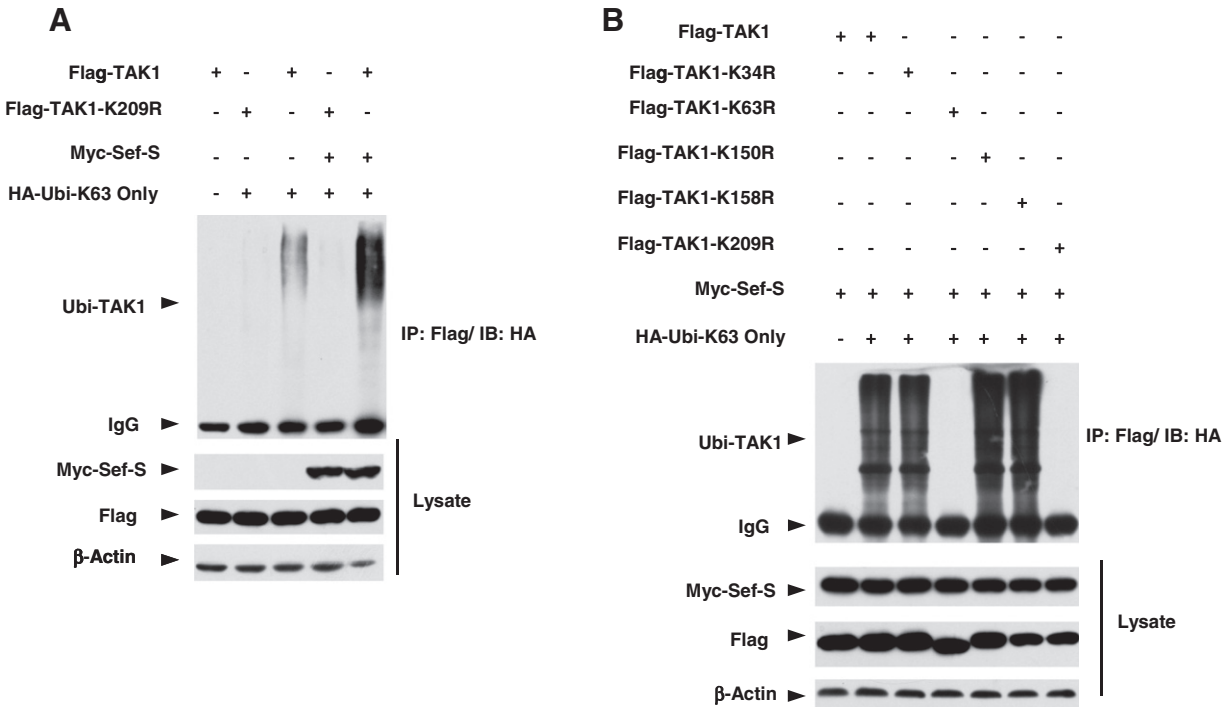


Fig. 3. The Lys63-linked TAK1 polyubiquitination acceptor site is at the Lys-209 residue. (A) Sef-S mediates Lys63-linked TAK1-WT polyubiquitination, but not TAK1-K209R. Expression vectors encoding HA-ubiquitin (K63-only) and Sef-S were co-transfected into 293T cells with control and expression vectors encoding TAK1-WT and -K209R respectively. TAK1 proteins in the transfected cells were precipitated with Nickel beads and immunoblotted with anti-HA antibodies to detect the presence of ubiquitinated TAK1-WT. Note that ubiquitination was enhanced when Sef-S was co-expressed. (B) Co-overexpression of TAK1 and Sef-S induces TAK1 polyubiquitination at Lys-209. Expression vectors encoding HA-ubiquitin (K63-only) and Sef-S were co-transfected into 293T cells with control vector and expression vectors encoding Flag-TAK1-WT, -K34R, -K63R, -K150R, -K158R and -K209R mutants, respectively. The cell lysates were heated in the presence of 1% SDS and diluted with lysis buffer in order to disrupt non-covalent protein–protein interactions. Flag-TAK1 proteins in the transfected cells were precipitated with Nickel beads and immunoblotted with an anti-HA antibody to detect the presence of ubiquitinated TAK1.

3. Results

Studies in several previous reports indicate that Sef plays an essential role in cell apoptosis [24]. But the role of Sef-S, a short form of Sef, in cell apoptosis remains unknown. To identify the function of Sef-S, we screened the components of MAPK pathway by using a luciferase reporter assay. We noted that when co-transfection of Sef-S with TAK1, JNK–p38 MAPK pathway was activated (data not shown). To determine whether JNK and p38 activations are involved in Sef-S and TAK1 induced apoptosis of cells [28], we performed cell death assays in Sef-S and TAK1 transfected 293T cells. We observed that 293T cells with Sef-S plus TAK1 coexpression underwent apoptosis significantly.

3.1. Sef-S physically associates with TAK1

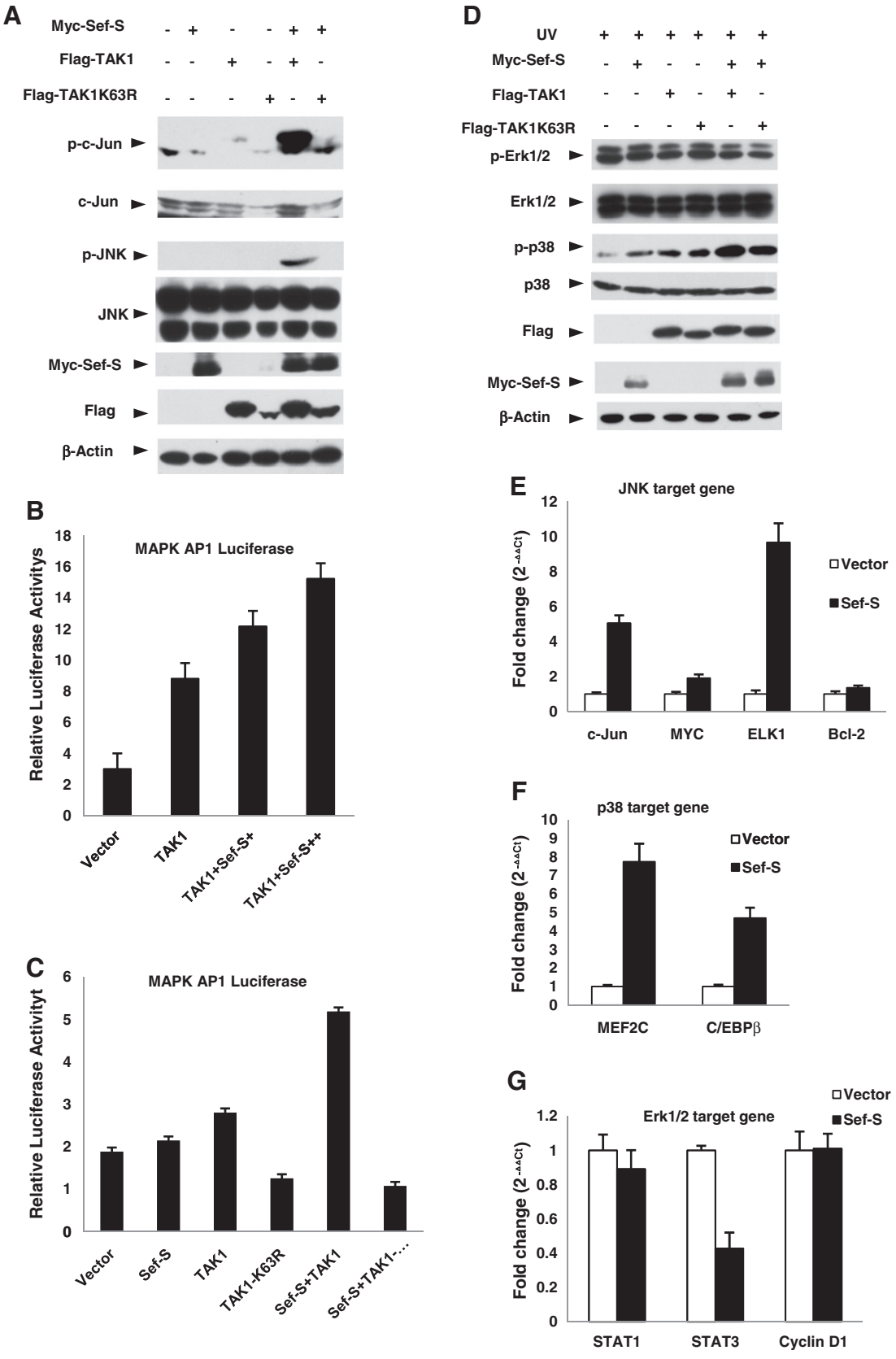
To test our hypothesis that Sef-S induces apoptosis probably through activating TAK1 and its downstream JNK-p38 pathway, we first examined whether Sef-S interacts with TAK1 in cultured cells. When Sef-S and TAK1 were co-expressed in 293T cells, Sef-S was immunoprecipitated with TAK1 and TAK1-K63R (Fig. 1A and 1B). This experiment revealed that TAK1 associates with Sef-S. To confirm the interaction, immunoprecipitation of cell lysates from Vector and Flag-TAK1 transfected cells with an anti-Sef-S antibody was performed and the result revealed that Flag-TAK1 also interacts with endogenous Sef-S (Fig. 1C). Furthermore, we examined whether these two molecules colocalize within cell. For this, we co-expressed Myc-Sef-S and Flag-TAK1 or (Flag-TAK1-K63R) in MCF7 cells and performed immunostaining assays. The results show that when co-transfected, Myc-Sef-S and Flag-TAK1 or (Flag-TAK1-K63R) are colocalized and associates with each other within cells (Fig. 1D).

3.2. Sef-S stimulates ubiquitination and phosphorylation of TAK1

As polyubiquitination is important for the activation of TAK1 and TAK1 is essential for activation of JNK and p38, we next examined whether Sef-S stimulates polyubiquitination of TAK1. Our results showed that TAK1 is modestly ubiquitinated in the presence of ubiquitin, and this ubiquitination is markedly enhanced by co-transfection of Sef-S (Fig. 2A and 2B). Polyubiquitination of TAK1 by Sef-S indicates that Sef-S is crucial for polyubiquitination of TAK1 [29,30]. Importantly, the enhanced ubiquitination of TAK1 is Lys63-linked rather than Lys48-linked, as enhanced ubiquitination of

Fig. 4. Sef-S and TAK1 induce activation of JNK and p38 but not Erk1/2.(A), 293T cells were transfected with indicated plasmids and then incubated for 48 h. Endogenous p-c-Jun, c-Jun, p-JNK and JNK were subjected to Western blot analyses. (B) Sef-S and TAK1 were co-transfected with a pAP1-luciferase reporter plasmid into 293T cells, as indicated. At 36 h after transfection, luciferase activity was measured. (C) 293T cells were transfected with 50 ng of TAK1 or 100 ng of TAK1-K63R mutant, 10 ng of pAP1-Luc and 5 ng of pRL-TK. After 36 h, luciferase activity was analyzed using the Dual Luciferase Reporter Assay System. Data was normalized by co-transfection with a Renilla reniformis luciferase reporter vector. Results presented were from one experiment assayed in triplicate. (D) Activation of p38 but not Erk1/2 by Sef-S and TAK1. 293T cells were transfected with indicated plasmids and incubated for 24 h and exposed to UV (200 J per m) for 5 min. Total cell protein was extracted at indicated times, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with anti Erk1/2, p-Erk1/2, p38 and phospho-p38 antibodies as described in Materials and methods (Section 2). (E–G)Presences of JNK (E), p38 (F) and Erk1/2 (G) target genes mRNA in Vector or Sef-S expressed 293T cell lines. Real-time PCR was performed. GAPDH was used as the endogenous control gene. Standard errors of means, were presented.

TAK1 could only be detected in the presence of wild type or Lys63-only ubiquitin, but not in the presence of Lys48-only ubiquitin (Fig. 2C), indicating that ubiquitination of TAK1 enhanced by Sef-S probably promotes the activity of TAK1 rather than its proteasomal degradation [31,32]. We next examined whether Sef-S can activate TAK1. A Western blot result showed that co-expression of Sef-S enhances the kinase



activity of TAK1, as is demonstrated by the auto-phosphorylation of TAK1 at Thr184/187 (Fig. 2D and 2E).

3.3. Sef-S induces K63-linked TAK1 polyubiquitination at Lys-209

Overexpression of TAK1 with Sef-S leads to TAK1 K63-linked polyubiquitination and activation in the cells. Our above results suggest that Sef-S is required for TAK1 polyubiquitination. To reveal which lysine residue is required for K63-linked TAK1 polyubiquitination and activation, the expression vectors encoding the C-terminal Flag tagged TAK1 WT, K34R, K63R, K150R, K158R, and K209R mutants are co-transfected with or without Sef-S into 293T cells along with a HA-Ub mutant containing one lysine only at position 63 (K63-only) [33]. Then the cell lysates from the transfected cells were boiled in the presence of 1% SDS and immunoprecipitated with Nickel beads, following by SDS-PAGE and immunoblotting with anti-HA antibody. In this assay, we find that TAK1 polyubiquitination is induced in the presence of TAK1-WT and other mutants but not K209R mutant (Fig. 3).

3.4. Sef-S and TAK1 induce activation of JNK and p38 but not Erk1/2

Since Sef-S stimulates ubiquitination and phosphorylation of TAK1, we therefore speculated that Sef-S may also mediate TAK1 downstream signaling pathway. To examine the role of Sef-S on the TAK1

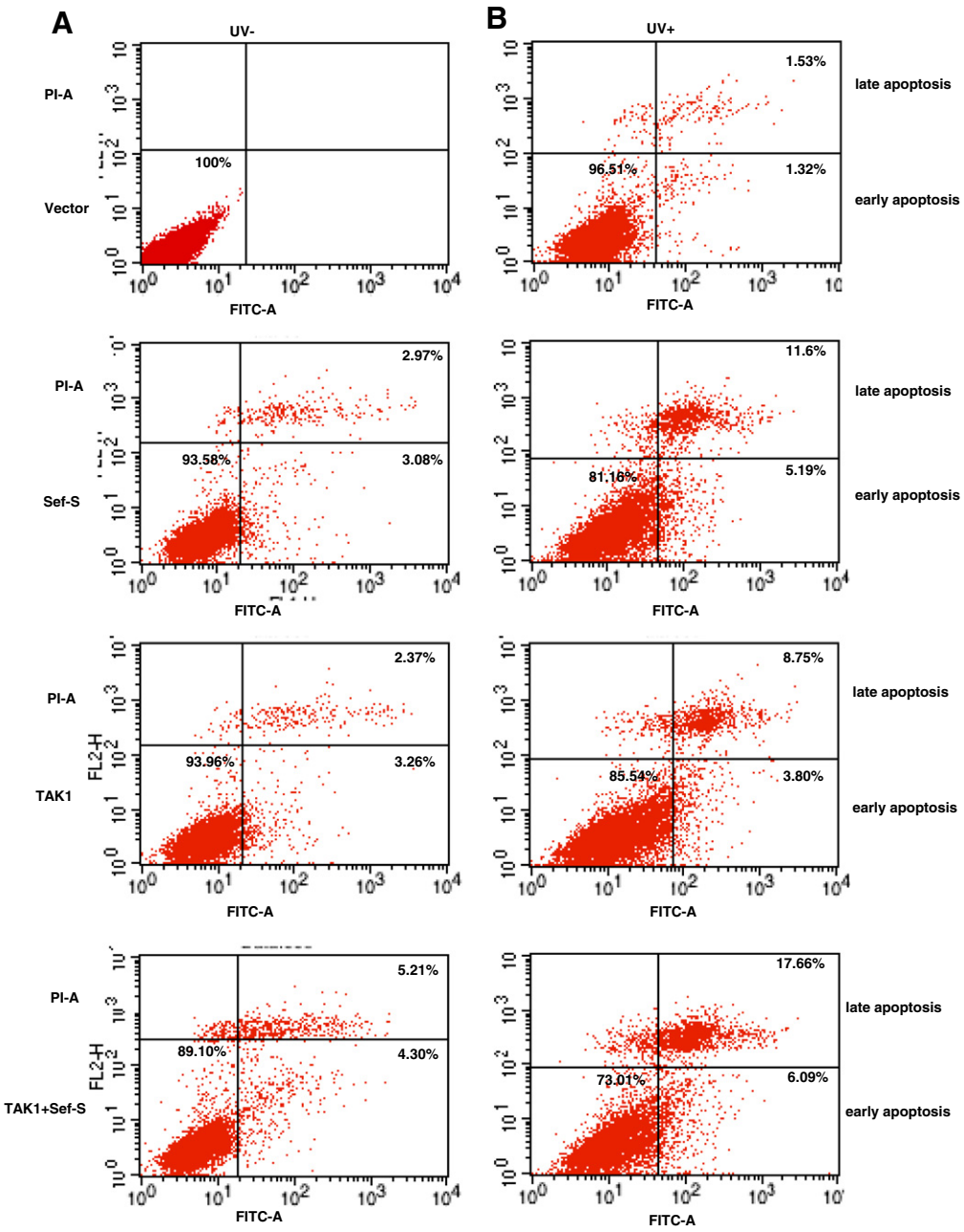


Fig. 5. Sef-S and TAK1 promote cell apoptosis. (A) Effect of Sef-S and TAK1 overexpression on apoptosis, as measured by annexin V/PI staining and flow cytometry. 293T cells were stained with annexin V-FITC and PI and analyzed by flow cytometry after transfection with the empty vector, Sef-S or (and) TAK1 for 48 h. Cells undergoing early apoptosis (annexin positive and PI negative) are located in the right lower box. Cells in the late stage of apoptosis and dead cells (annexin V-FITC and PI positive) are located in the right upper box. (B) Overexpression of Sef-S and TAK1 enhanced UV-induced apoptosis. 293T cells were transfected with the empty vector, Sef-S or (and) TAK1 for 36 h. The cells were treated with UV irradiation at 120 mJ/cm^2. 12 h after UV irradiation, cells were stained with annexin V–FITC and PI and analyzed by flow cytometry. The cells were treated the same way as in (A).

downstream events, we first measured the phosphorylation of c-Jun and JNK. 293T cells were transfected and the western blot results indicated that co-expression of Sef-S and TAK1 induced activation of c-Jun and JNK (Fig. 4A). Consistently, luciferase reporter experiments indicated that Sef-S stimulates the MAPK AP1 reporter in a dose dependent manner (Fig. 4B). Interestingly, Sef-S failed to activate the reporter in the presence of TAK1-K63R mutant (Fig. 4C). While the results suggest that Sef-S activates JNK (Fig. 4A), we also observed that Sef-S promotes phosphorylation of p38 (Fig. 4D, third panel) but not phosphorylation of Erk1/2 (Fig. 4D, top panel). Furthermore, the downstream genes of Erk1/2 and JNK–p38 were detected by Real-time PCR. Clearly, over-expression of Sef-S promoted the expression of c-Jun and ELK1, two downstrean genes of JNK pathway (Fig. 4E), and expression of MEF2C and C/EBPb, two downstream genes of p38 pathway (Fig. 4F), but had no effect on the expression of STAT1 and Cyclin D1, two downstream genes of Erk1/2 (Fig. 4G). These results were consistent with those data from luciferase assay or western blot. Together, those data indicate that the association of Sef-S with TAK1 enhances the ability of TAK1 to activate JNK–p38, and Sef-S plays a crucial role for TAK1 activation.

3.5. Sef-S and TAK1 promote apoptosis in 293 T cells

The above data demonstrate that Sef-S and TAK1 promote activation of JNK–p38, which is a critical process in apoptosis. To address whether Sef-S and TAK1 are involved in apoptosis, we used an annexin staining and flow cytometry method as a sensitive, quantitative assay to examine cell apoptosis in both early and late stage. We observed that more Sef-S plus TAK1 overexpression cells undergo apoptosis than the mock cells (Fig. 5A). Consistent with this result, UV induced cell apoptosis was also enhanced in Sef-S plus TAK1 overexpression group (Fig. 5B). Collectively, these results suggested that Sef-S and TAK1 induce apoptosis in 293T cells (Fig. 5).

4. Discussion

One of our previous studies suggested that Sef-S inhibited FGF-induced cell proliferation, similar to the function of Sef in inhibition of FGF signaling [21]. In screening the targets of Sef-S, we found that Sef-S induces JNK–p38 activation and apoptosis (data not shown). In this study we revealed the mechanism by which Sef-S mediates JNK–p38 activation and apoptosis Our data showed that Sef-S mediated apoptosis, at least in part, via a Sef-S–TAK1-JNK–p38 pathway. We have demonstrated that Sef-S activates TAK1 by enhancing TAK1 ubiquitylation and then auto-phosphorylation, consistently with previous reports [4,25,26,29]. Importantly, we revealed that activation of TAK1 by Sef-S resulted in further activation of both JNK and p-38 but not Erk1/2. Although the Erk1/2, JNK and p38 MAP kinases are related structurally, they are activated by different extracellular stimulins. The Erks are activated in response to growth factor stimulation, whereas the JNK–p38 MAP kinase are activated by various forms of environmental stress. In this study, we found that Sef-S activates JNK and p38, suggesting a specific role of Sef-S in regulation of environmental stress. Indeed, we observed that Sef-S induced stronger apoptosis when the cells were stressed by UV irradiation. The curerent study explains the observations that Sef-S inhibited cell proliferation independent of Erk1/2. Rather it turned out that Sef-S induced activation of JNK and p38, which induced apoptosis. Therefore, we speculate that Sef-S mainly activates JNK and p38 to initiate apoptosis under stress (e.g. UV) while Sef inhibits FGF-induced Erk1/2-MAPK. These differences in regulation of MAP kinase family members provide a diverse way for cells to response to different stimulins. Our findings raise questions regarding how Sef and Sef-S function coordinatively since Sef-S is an alternative splice form of Sef. Our studies provided a scenario that Sef inhibits Erk1/2, which results inhibition of cell proliferation, while Sef-S activates JNK and p38 to induce apoptosis. However, it is unclear that how Sef and Sef-S differentiate their substrates since Sef was also reported to interact with TAK1 [24] as we demonstrated that Sef-S associated with TAK1 in this study. Nevertheless, inhibition of Erk1/2 and activation of JNK-p38 result in apoptosis as reported in other cells [34]. Interestingly, our results indicate that the apoptosis in 293T cells is regulated by TAK1 polyubiquitination and JNK–p38 MAP kinase activation mediated by Sef-S. However, it remains unclear how Sef-S mediates the TAK1 K63-linked polyubiquitiantion. Specific E2 or E3 ligase should be identified for this process.

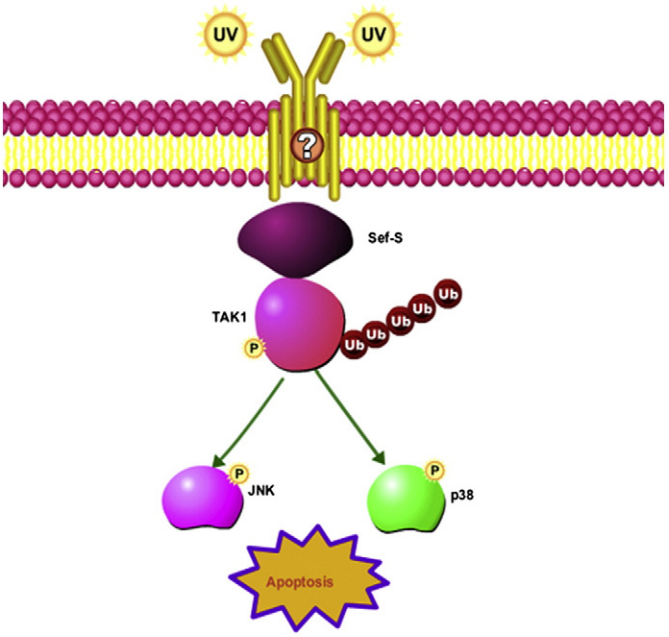


Fig. 6. Working model: Sef-S mediates TAK1 polyubiquitination on the Lys-209 within the kinase domain which in turn triggers the activation of TAK1-mediated p38–JNK activation and induces cell apoptosis.

In view of the data presented here and in previous reports, we propose a working model in which, Sef-S induces TAK1 polyubiquitination on lysine 209 within the kinase domain which in turn triggers the activation of TAK1-mediated JNK and p38 and then cell apoptosis (Fig. 6).

Conflict of interest statement

The authors have no financial conflicts of interest.

Acknowledgments

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