CELL CYCLE
2020, VOL. 19, NO. 24, 3458–3467
https://doi.org/10.1080/15384101.2020.1843811

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

FAIM-S functions as a negative regulator of NF-κB pathway and blocks cell cycle progression in NSCLC cells

Pengcheng Wang[a,b], Wenze Xun[a], Tianyu Han[b], and Zhujun Cheng[a]

[a]Department of Burn, The First Affiliated Hospital of Nanchang University, Nanchang, P.R.China; [b]Jiangxi Institute of Respiratory Disease, The First Affiliated Hospital of Nanchang University, Nanchang, P.R.China

ABSTRACT

Tumorigenesis is closely related to the disorder of the cell cycle. The cell cycle progression includes the interphase (G0/G1, S, and G2 phase) and mitosis (M phase). CCND1 is a key protein that regulates the entry of the G0/G1 phase into the S phase. In our study, we found that the short form of Fas Apoptosis Inhibitory Molecule 1 (FAIM-S) could regulate the expression of CCND1 and had a tumor-suppressing role in non-small cell lung cancer (NSCLC). Overexpressing FAIM-S significantly inhibited the proliferation and cell cycle progression in NSCLC cells. Further studies demonstrated that FAIM-S could interact with IKK-α, reducing its protein stability. This effect led to the suppression of the NF-κB pathway, resulting in the decreased expression of CCND1. Thus, our study demonstrated that FAIM-S functioned as a negative regulator of the NF-κB pathway and played a tumor-suppressing role through blocking cell cycle progression in NSCLC cells.

ARTICLE HISTORY
Received 3 November 2019
Revised 25 September 2020
Accepted 24 October 2020

KEYWORDS
FAIM-S; CCND1; cell cycle; NF-κB; non-small cell lung cancer

Introduction

The cell cycle is a ubiquitous and complex process involved in the regulation of DNA damage repair, cell proliferation, and tissue hyperplasia as a response to injury and diseases [1]. The cell cycle is primarily driven by Cyclins and Cyclin-dependent kinases and the dysregulation of these genes are closely related to cancer progression. CCND1, the regulatory subunit of CDK4 or CDK6, is required for the transition from the G1 phase to the S phase [1]. A recent study identified frequent genomic amplification of CCND1 in Squamous cell carcinomas [2]. In hepatocellular carcinoma, the mutations in the CCND1 gene were one of the most common molecular anomalies [3,4]. In non-small cell lung cancer, CCND1 was considered as a key driver of malignant transformation [5]. Thus, identifying the regulation mechanisms of CCND1 is crucial for understanding tumor initiation and progression.

The NF-κB pathway is a classical regulatory pathway that regulates the immune system, inflammation, cell proliferation, and bone development [6]. The canonical pathway is activated by stimuli such as the IL (interleukin)-1 or TNFα (tumor necrosis factor α). These cytokines led to the activation of IKK by a variety of pathways, resulting in the phosphorylation, ubiquitination, and degradation of IκBα by the proteasome [7]. The relationship between NF-κB and the cell cycle was first described in 1991, NF-κB was found to promote the transition of G0/G1 in mouse fibroblasts [8]. Since then, the NF-κB pathway has been shown to regulate the proliferation and cell cycle progression in a variety of cells, including osteosarcoma cells, mice Mammary epithelium, fibroblasts, or human mammary gland epithelial cells [9–11]. It has been reported that NF-κB could directly bind to the promoter region of CCND1 and control CCND1 transcription [12]. In addition, CDK2 was also reported to be a RelA target gene in laryngeal squamous cell cancer [13]. All these studies demonstrated that NF-κB was a key regulator of cell cycle.

The Fas Apoptosis Inhibitory Molecule 1 (FAIM1) is an evolutionarily highly conserved death-receptor antagonist [14]. The human FAIM1 gene is located in the long arm of chromosome 3 (3q22.3) and has four transcript splice variants encoding three isoforms. The shortest isoform of FAIM1, namely FAIM-S, was first identified from B-lymphocytes as a protein that counteracted to Fas-induced cell death [15]. Two years later, the long isoform was identified, named FAIM-

CONTACT Tianyu Han hantianyu87@163.com; Zhujun Cheng chengzj0613@163.com
Supplemental data for this article can be accessed here.

L [16]. FAIM-L is a 22 kDa protein expressed only in neurons, while FAIM-S is a 20 kDa highly conserved soluble protein that is ubiquitously expressed [16]. Recently, several studies have demonstrated that FAIM1 is closely related to cell proliferation. In neuronal models, FAIM-L protected neurons from DRs (death receptors)-induced cell death by binding to the Fas receptor and preventing the activation of caspase-8 [17]. In addition, FAIM-L could affect the stability of XIAP by binding to the BIR2 domain of XIAP [18,19]. Unlike FAIM-L, FAIM-S was dispensable for the death of neurons induced by DRs but promoted neurite outgrowth induced by growth factor in an ERK and NF-κB dependent manner in neuronal models [20]. However, little is known about the function of FAIM-S in cancer progression.

In this study, we discovered the opposite role of FAIM-S in non-small cell lung cancer, compared to that in neuronal models. We found that FAIM-S inhibited the proliferation and cell cycle progression by regulating the expression CCND1 in NSCLC cells. Further studies demonstrated that FAIM-S could block the activation of NF-κB by directly binding to IKK-α, accelerating its degradation, thereby, antagonizing the expression of CCND1. Thus, our study first demonstrated that FAIM-S blocked cell cycle progression and functioned as a tumor suppressor through suppressing the activation of the NF-κB pathway in NSCLC cells.

Materials and methods

Cell culture

Human non-small cell lung cancer (NSCLC) cell lines H1299, A549, H1650 from ATCC were cultured in RPMI 1640 (Invitrogen, C11875500BT), containing 10% fetal bovine serum (Excel, FCS100). The SPC-A1 cells from the National Infrastructure of Cell Line Resource were cultured in RPMI 1640 (Invitrogen, C11875500BT), containing 10% fetal bovine serum (Excel, FCS100). All the cell lines were cultured at 37°C with 5% CO_2.

Plasmid

Human FAIM-S expression plasmid was constructed by PCR with the following primers:

5′-GCGGTACCATGACAGATCTCGTAGCTGT −3′ (sense)

5′-GCCTCGAGCGACTTGCAATCTCTGGGAT TT −3′ (antisense).

The construct was cloned into pcDNA3.1-His vector using the restriction enzyme Xho1 and Kpn1, and verified by sequencing. The plasmid was transiently transfected in NSCLC cells using SuperFectin DNA Transfection Reagent (Pufei, Shanghai, 2103–100) and the overexpression efficiency was determined by western blot.

Cell proliferation assay

For cell proliferation assay, cells were seeded in 24-well plates at 5000 cells per well in 0.5 ml medium with 10% FBS. The medium was changed every 2 days. At the indicated time points, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid and the relative proliferation was determined by the absorbance at 595 nm.

Colony formation assay

Colony formation assay was carried out with NSCLC cells. After transfection, the cells were trypsinized, counted, and 500 cells were seeded in 6-well dishes. All cells were then grown for 10 days with medium changed every second day. Plates were fixed with 4% formaldehyde and stained with 2% crystal violet. The images were obtained by a digital camera.

Saturation density assay

The FAIM-S plasmid was transfected into NSCLC cells, and the cells were seeded at 10^5 cells per well in 12-well plates in 0.5 ml medium containing 10% FBS. The medium was changed every 2 days. After 6 days, the cells were digested and counted.

MTT assay

Cells were seeded in a 96-well plate at 5000 cells per well in 200 µl medium supplemented with 10% FBS. After that, the cells were treated with 20 µl

MTT (5 mg/ml) solution and incubated for 4 h at 37°C with 5% CO_2. Then, the culture medium was removed and 150 µl DMSO was added to each well and incubated on an end-over shaker for 20 min. Finally, the absorbance at 570 nm was measured. Measurements were done in triplicate.

Quantitative RT-PCR

Total RNA was extracted using trizol reagent (Invitrogen) and 1 µg total RNA was performed reverse transcription using a PrimeScript RT reagent kit with a gDNA eraser (TaKaRa), according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Green dye using (Applied Biosystems). The relative amount of cDNA was calculated by the comparative Ct method using GAPDH as a control. PCR reactions were performed in triplicate.

The following primers were used:
FAIM-S: 5′-GAGACGCTGGCCCTCCTT-3′ (sense)
5′-AAACTCACCCGCTGTCTC-3′ (antisense);
CDK2: 5′-GACACGCTGCTGGATGTCA-3′ (sense)
5′-CACTGGAGGAGAGGGTGAGA-3′ (antisense);
CDK4: 5′-GTGTATGGGGCCGTAGGAAC-3′ (sense)
5′-CCATAGGCACCGACACCAAT-3′ (antisense);
CDK6: 5′-CCGACTGACACTCGCAGC-3′ (sense)
5′-TCCTCGAAGCGAAGTCCTCA-3′ (antisense);
CCND1: 5′-TGAGGGACGCTTTGTCTGTC-3′ (sense)
5′-GCCTTTGGCCTCTCGATACA-3′ (antisense);
IKK-α: 5′-AACTCCTCAAGATGGGGAGACT-3′ (sense)
5′-TTTGGGGACAGTGAACAAGTGA-3′ (antisense);
p65: 5′-CTTCCAAGAAGAGCAGCGTG-3′ (sense)
5′-GATCTTGAGCTCGGCAGTGT-3′ (antisense);
GAPDH: 5′-AATGGGCAGCCGTTAGGAAA-3′ (sense)
5′-GCGCCCAATACGACCAAATC-3′ (antisense).

Cell cycle synchronization

The cells were seeded and attached by incubating 6-well plates with 2 ml medium supplemented with 10% FBS at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. For the thymidine block, prepared a 200 mM thymidine stock solution by dissolving 145.2 mg thymidine powder in 3 mL H2O (or equivalent amounts) and sterilized the solution by filtration through a 0.2 µm pore size filter. Slighted warming might help dissolve thymidine. Add 20 µl of the freshly prepared 40 mM stock to each 6-well plate (final concentration 2 mM). Incubated cells with thymidine in a humidified atmosphere with 5% CO_2 at 37°C for 24 h. To release from the thymidine block, removed thymidine containing growth medium in the afternoon of the following day; washed cells twice with prewarmed 1x PBS and add 2 ml of complete medium to each 6-well plate. Incubated cells for 5 h at 37°C in a humidified atmosphere with 5% CO_2. For mitotic cell arrest, added nocodazole to a final concentration of 50 ng/ml. Prepared a stock solution by dissolving nocodazole powder in DMSO (e.g.,5 mg/ml) and stored frozen at −20°C. Incubated cells with nocodazole for no longer than 10 h –11 h at 37°C in a humidified atmosphere with 5% CO_2. Released from nocodazole-mediated arrest in early M phase.

Cell cycle analysis

Freshly prepared cells were harvested and re-suspended in 0.5 ml PBS. Then, the cells were fixed with 70% alcohol on ice for at least 2 h. After centrifugation, the supernatant was discarded and sediment was washed with PBS twice. The cell pellet was resuspended in 5 ml PBS and then cells were counted. Resuspending 2×10^5 cells with 400 µl guava cell cycle reagent (Millipore, 4700–0160). After incubating in a water bath at 37°C for 10 min, then, filtering cells by the screen. The cell cycle was analyzed by the Millipore GuavaeasyCyte™ flow cytometer (Millipore).

Immunoprecipitation

NSCLC cells were lysed in NP-40 buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 20 mM β-glycerol phosphates, 1 mM Na orthovanadate, 20 mM NaF, 0.5% Nonidet P-40) supplemented with PMSF (Dingguo). Then, the cell lysates were centrifuged at 10,000 g for 20 min at 4°C. The supernatants

were precleared with protein G agarose beads (Roche) for 1 h at 4°C. Then the supernatants were added with indicated antibodies (1 μg/ml) supplemented with protein G agarose beads to incubate overnight at 4°C. On the second day, the immunocomplexes were washed with lysis buffer. The supernatants were suspended with 2× loading buffer and boiled for 10 min.

Western blot

For western blot, the proteins were subjected to 10% SDS-PAGE and then transferred to the PVDF membrane (Milipore). After blocking with 5% skim milk (BD, 232,100) at room temperature for 1 h, the membranes were incubated with the indicated antibodies overnight at 4°C. On the following day, the membranes were washed 3 times at room temperature with 1× TBST for 10 min every time and incubated with secondary antibody (Thermo) at room temperature for 1 h. After washed 3 times at room temperature with 1× TBST, the membranes were stained with ECL western blot detection reagent (TIANGEN). Then, proteins were visualized by a digital gel image analysis system (TANON 5500). The expression of proteins was quantified using Tanon Gis software.

The following antibodies were used FAIM Antibody (CST, 6907), GAPDH Antibody (CST, 8884), Bcl-2 Antibody (CST, 15,071), NF-κB Pathway (CST, 9936), IKK-α Antibody (CST, 61,294), CDK2 Antibody (Invitrogen, MA5-32,017), CDK4 Antibody (Invitrogen, AHZ0202), CDK6 Antibody (Invitrogen, PA5-27,978), CCND1 Antibody (Invitrogen, MA5-16,356), RB1 Antibody (proteintech, 10,048-2-Ig), phospho-RB1-S780 Antibody (CST, 3590), TBP Antibody (Invitrogen, MA1-189), β-actin Antibody (proteintech, 60,008-1-Ig), Caspase3 Antibody (proteintech, 19,677-1-AP), HA-Tag Antibody (proteintech, 66,006-2-Ig), His-Tag Antibody (proteintech, 66,005-1-Ig).

Extraction of cytoplasmic and nuclear proteins

For the extraction of cytoplasmic and nuclear proteins, we used the Nuclear and Cytoplasmic Extraction Kit (CWBIO Cat.#CW0199S) according to the manufacturer's protocol.

Statistical analysis

For each experiment, three independent replicates were performed. All the data were expressed as mean ±SD. Statistical evaluation was conducted using the Student t-test. A p-value of less than 0.05 was considered statistically significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

FAIM-S inhibits the proliferation of non-small cell lung cancer cells

To determine the function of FAIM-S in non-small cell lung cancer cells, pcDNA3.1-His-FAIM-S was transfected into four NSCLC cell lines followed by cell proliferation assay. Figure 1(a) shows that overexpressing FAIM-S significantly reduced the proliferation rate of NSCLC cells. We also performed a saturation density assay to detect the effects of FAIM-S on the contact inhibition of NSCLC cells. Overexpressing FAIM-S sensitized NSCLC cells to contact inhibition (Supplementary Fig. 1A). Next detected the effects of FAIM-S on the colony-forming ability of NSCLC cells. The cells overexpressing FAIM-S showed reduced colony numbers and a smaller size compared with vector control (Figure 1 (b)). The MTT assay also showed that overexpressing FAIM-S reduced cell viabilities of NSCLC cells (Supplementary Fig. 1B). These data revealed that FAIM-S might function as a tumor suppressor in non-small cell lung cancer cells.

FAIM-S arrests cell cycle at G0/G1 phase and decreases the expression of CCND1

The inhibitory effects of FAIM-S on cell proliferation suggested that FAIM-S might perturb cell cycle-related events in non-small cell lung cancer cells. To examine the effects of FAIM-S on cell cycle progression, we transfected His-FAIM-S into NSCLC cells and the cell cycle progression was analyzed by flow cytometry after propidium iodide staining. Overexpression of FAIM-S significantly increased the proportion of the G0/G1 phase and decreased the proportion of and G2/M phase compared with that of control cells (Figure 2(a,b),

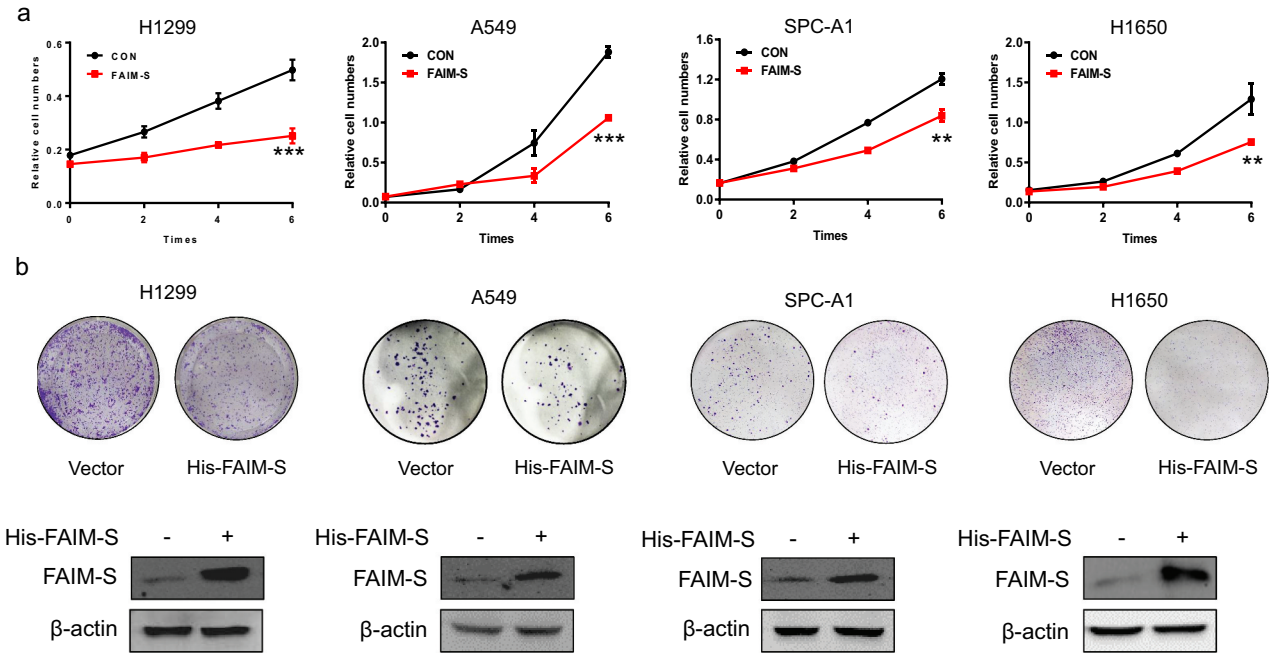


Figure 1. Overexpression of FAIM-S inhibits the proliferation of non-small cell lung cancer cells (a) The NSCLC cells (H1299, A549, SPC-A1, H1650) were cultured in RPMI 1640 with 10% FBS, cells were transfected with vector or pcDNA3.1-His-FAIM-S. At 2 days, 4 days and 6 days, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Data represent the average of three independent experiments (mean ± SD). **, $P < 0.01$; ***, $P < 0.001$. (b) The NSCLC cells were transfected with FAIM-S plasmid and 500 cells were seeded in 6-well plate. 10 days later, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet and the photographs were taken (upper panel). The expression of FAIM-S were detected by western blot using indicated antibodies (bottom panel).

supplementary Fig. 2A and 2B). These results showed that FAIM-S was indispensable for cell cycle progression in non-small cell lung cancer cells. To elucidate the mechanism of FAIM-S in cell cycle regulation, we examined the expression of cell cycle-related genes in the G0/G1 phase. Q-PCR was performed to detect the mRNA expression of CDK2, CDK4, CDK6, and CCND1. As shown in Figure 2(c), CCND1 showed a remarkable decrease. The western blot results showed that overexpressing FAIM-S decreased the expression of CCND1, while the CDKs functioned in the G0/G1 phase displayed slightly or no changes. The expression of tumor suppressor RB1 was increased and its phosphorylation at Ser780 was decreased significantly when overexpressing FAIM-S (Figure 2(d)). We also detected the effects of FAIM-S on the apoptosis of NSCLC cells. However, overexpressing FAIM-S did not significantly affect the apoptosis of NSCLC cells (Supplementary Fig. 3A and 3B). These findings suggested that the expression of FAIM-S was important for cell cycle progression.

FAIM-S inhibits NF-κB pathway by directly binding to IKK-α and promoting its degradation

Previous studies have demonstrated that the expression of CCND1 was regulated by the NF-κB pathway [12], so we first determined if NF-κB regulated the expression of CCND1. TNF-α was used to stimulate the activation of the NF-κB pathway and the expression of CCND1 was examined. Figure 3(a) shows that TNF-α treatment significantly increased the expression of CCND1. Also, overexpressing NF-κB/p65 increased the expression of CCND1, indicating that the expression of CCND1 was regulated by the NF-κB pathway in H1299 cells (Figure 3(b)). We next examined if FAIM-S regulated the NF-κB pathway. His-FAIM-S was transfected into H1299 cells and the changes of the NF-κB pathway were examined. The expression of phosphorylated-IKK, -IKB, and -p65

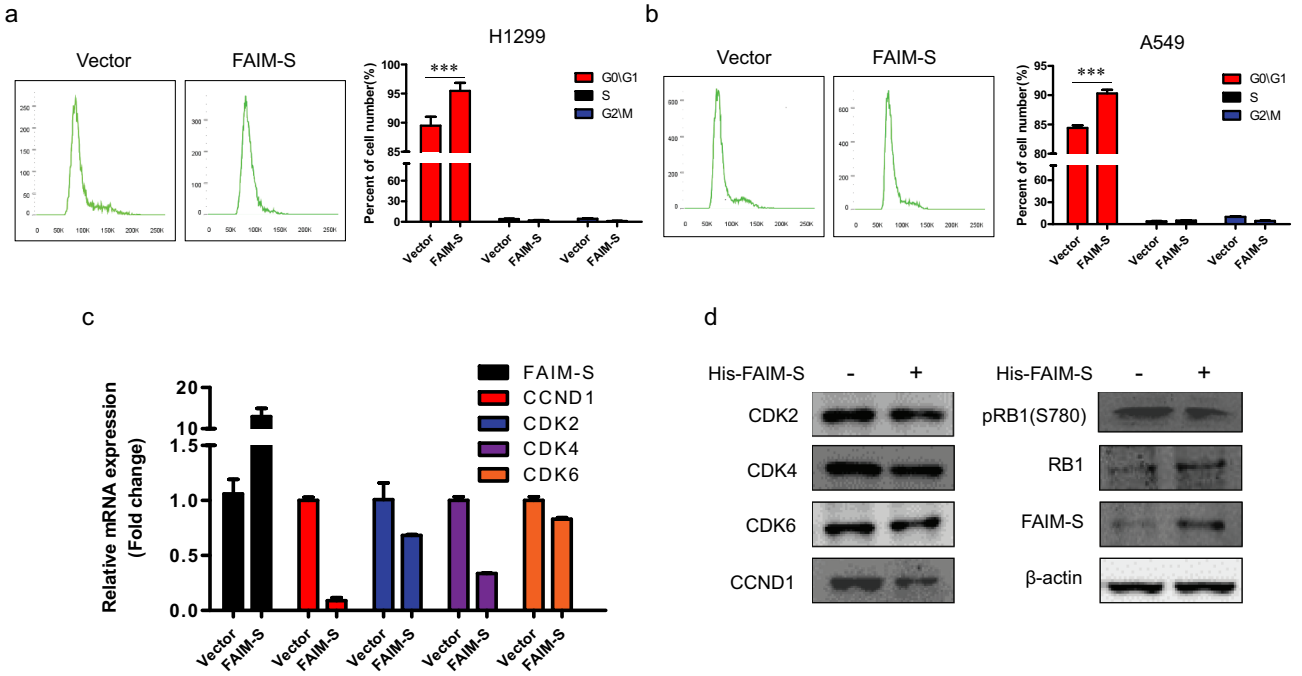
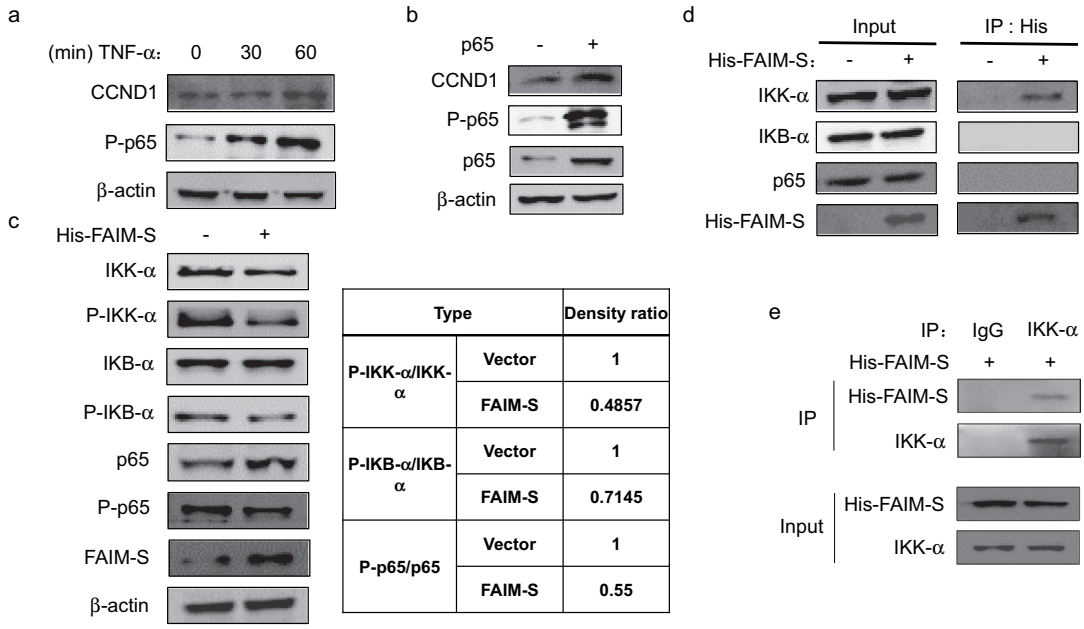


Figure 2. Overexpression of FAIM-S arrests cell cycle in G0/G1 phase. (a and b) H1299 (a) and A549 (b) cells were transiently transfected with pcDNA3.1-His-FAIM-S or vector. 24 h later, the cells were synchronized in early mitosis. The cells were then stained with propidium iodide. Adherent cells were collected and cell cycle progression was analyzed by flow cytometry (left panels). The percentage of the cells in each phase were counted and analyzed. Data represent the average of three independent experiments (mean ± SD). ***, $P < 0.001$. (right panels). (c) H1299 cells was transiently transfected with pcDNA3.1-His-FAIM-S or vector. 48 h later, total RNAs were extracted and the expression of cell cycle-related genes were analyzed by Q-PCR. (d) H1299 cells was transiently transfected with pcDNA3.1-His-FAIM-S or vector. 48 h later, The protein expression of cell cycle-related genes were checked by western blot using indicated antibodies.

were all decreased indicating the repression of the NF-κB pathway (Figure 3(c)). To better evaluate the activation of NF-κB, we performed nuclear and cytoplasmic extraction. Supplementary Figure 4A shows that overexpressing FAIM-S reduced the nuclear localization of p65 and increased its cytoplasmic localization, indicating the reduced activation of the NF-κB pathway. We also examined the effects of FAIM-L on the activation of the NF-κB pathway. Overexpressing FAIM-L showed no effects on the phosphorylation of p65, indicating that the regulation of the NF-κB pathway was FAIM-S specific (Supplementary Fig. 4B). IKK-α is the upstream kinase that phosphorylates IκB and activates NF-κB. We also observed that the total level of IKK-α was reduced, indicating that FAIM-S might suppress the NF-κB pathway by decreasing the expression of IKK-α (Figure 3(c)). Next detected the interactions of FAIM-S with the proteins in the NF-κB pathway. Figure 3(d,e) showed that FAIM-S could only interact with IKK-α and no bindings were observed between FAIM-S and the other proteins. Based on the observation that overexpressing FAIM-S did not affect the mRNA level of IKK-α (Supplementary Fig.5A), we next detected the effects of FAIM-S on the stability of IKK-α. Cycloheximide (CHX) was used to block protein translation and the protein degradation rate of IKK-α was detected by western blot. Figure 4(a) shows that overexpressing FAIM-S could accelerate the degradation of IKK-α. We also found that the increased degradation rate of IKK-α induced by FAIM-S overexpression could be recovered by adding MG132, a proteasome inhibitor, indicating that FAIM-S increased the degradation of IKK-α though the ubiquitin-proteasome pathway (Figure 4(b)). Next examined the ubiquitination of IKK-α under FAIM-S overexpressing conditions. Figure 4(c) shows that overexpressing FAIM-S increased the ubiquitination



Type		Density ratio
P-IKK-α/IKK-α	Vector	1
	FAIM-S	0.4857
P-IKB-α/IKB-α	Vector	1
	FAIM-S	0.7145
P-p65/p65	Vector	1
	FAIM-S	0.55

Figure 3. FAIM-S inhibits NF-κB pathway and directly binds to IKK-α. (a) The H1299 cells were treated with 100 μg/ml TNF-α for 0 min, 30 min, and 60 min. The expression of indicated proteins were detected by western blot. (b) H1299 cells was transfected with vector or pcDNA3.0-p65. 48 h later, western blot was performed. (c) H1299 cells was transfected with vector or pcDNA3.1-His-FAIM-S. After 48 h, the expression of proteins in NF-κB pathway were detected by western blot (left panel). The ratios of phosphorylated proteins to total proteins were calculated (right panel). (d) H1299 cells was transfected with vector or pcDNA3.1-His-FAIM-S. 48 h later, immunoprecipitation was performed using anti-His antibody followed by western blot using indicated antibodies. (e) H1299 cell was transiently transfected with His-FAIM-S. 48 h later, immunoprecipitation was performed using IKK-α antibody or normal rabbit IgG followed by western blot using indicated antibodies.

of IKK-α. These results demonstrated that FAIM-S inhibited the NF-κB pathway by directly binding to IKK-α and promoting its degradation.

FAIM-S regulates the expression of CCND1 through affecting NF-κB pathway

As NF-κB regulates the transcription of CCND1, we next want to figure out if FAIM-S regulates the expression of CCND1 through the NF-κB pathway. TNF-α was used to activate the NF-κB pathway. Figure 5(a) shows that TNF-α treatment increased both the mRNA and protein level of CCND1 while overexpressing FAIM-S alleviated this effect. To further confirm this result, we transfected pcDNA3.0-p65 with or without pcDNA3.1-His-FAIM-S into H1299 cells. Overexpressing p65 significantly increased the expression of CCND1; however, FAIM-S overexpression abolished the upregulation of CCND1 caused by p65 overexpression (Figure 5 (b)). These results demonstrated that FAIM-S regulated the expression of CCND1 through the NF-κB pathway.

Discussion

FAIM proteins were first known as a death-receptor antagonist and mainly functioned in the apoptosis pathway [14]. It is reported that FAIM-L could directly bind to the BIR2 domain of XIAP and inhibit its self-ubiquitination, thus enhanced the stability of XIAP [18,21]. Another study found that FAIM-L bound to the DISC (death-inducing signaling complex) and prevented the recruitment of caspase-8 precursor, thereby inhibiting the activation of caspase-8 [17]. In addition, A recent study showed that FAIM-L could protect cells

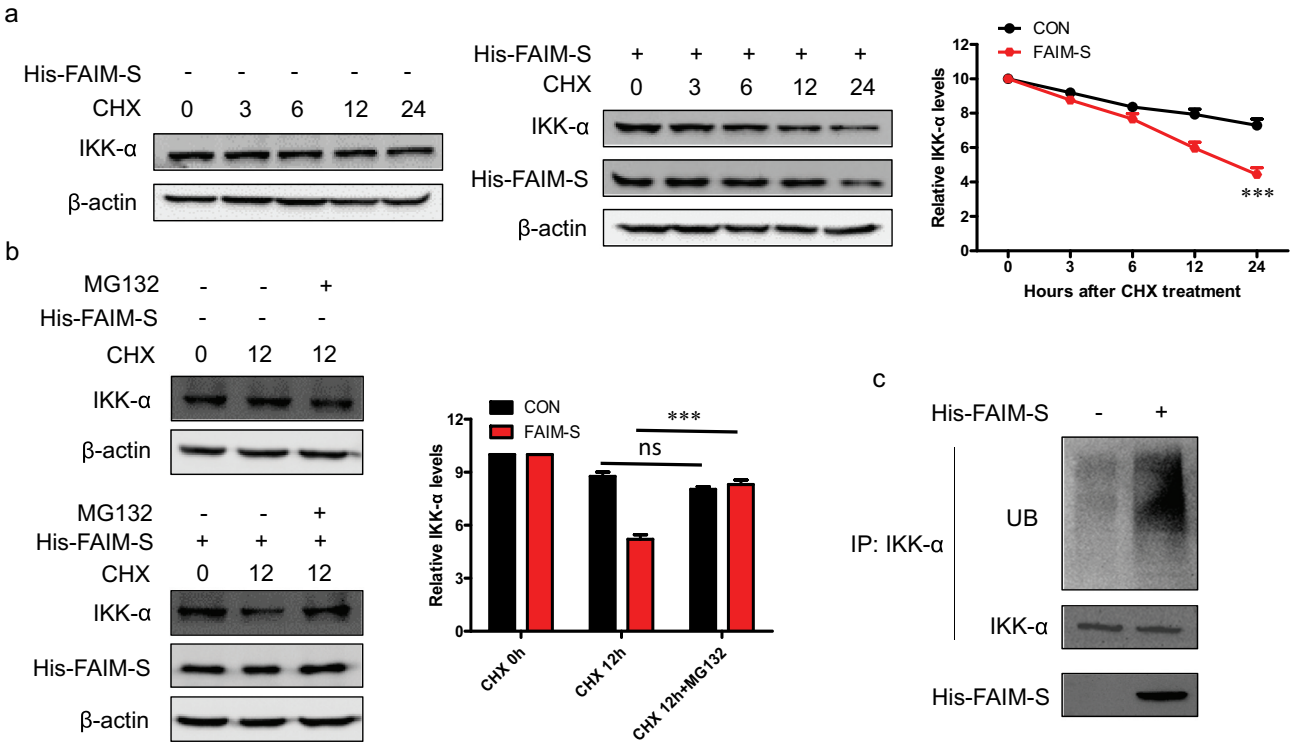


Figure 4. FAIM-S promotes the degradation IKK-α through increasing its ubiquitination. (a) H1299 cell was transiently transfected with His-FAIM-S or vector. After 24 h, the cells were treated with 50 μg/ml CHX for indicated times followed by western blot (left and middle panel). The expression of IKK-α were quantified using Tanon Gis software. Data represent the average of three independent experiments (mean ± SD). ***, $P < 0.001$. (right panel). (b) H1299 cell was transiently transfected with His-FAIM-S or vector. After 24 h, the cells were treated with 50 μg/ml CHX with or without MG132 for 12 h followed by western blot (left panel). The expression of IKK-α were quantified using Tanon Gis software. Data represent the average of three independent experiments (mean ± SD). ns, $P > 0.05$; ***, $P < 0.001$. (right panel). (c) H1299 cell was transiently transfected with His-FAIM-S or vector. 48 h later, immunoprecipitation was performed using IKK-α antibody followed by western blot. The ubiquitination level was detected using ubiquitin antibody.

from apoptosis induced by TNFα in neurodegenerative diseases, especially in Alzheimer's disease (AD) [22,23]. Recently, it was reported that FAIM protein is also involved in metabolism. Compared with wild-type mice, FAIM-KO mice had increased fatty acid synthesis, activated SREBP-1 and SREBP-2, and enhanced lipid synthesis, displaying a phenotype of spontaneous non-feeding obesity, adipocyte hypertrophy, dyslipidemia, hyperglycemia, and hyperinsulinemia [24]. These studies suggested that the functions of FAIM protein did not restrict to apoptosis and more functions of the FAIM gene need to be further clarified. Until now, several studies reported that FAIM1 had important functions in cancer cells. In multiple myeloma cells (MM), insulin-like growth factor 1 (IGF-1) could up-regulate the expression of FAIM1. High expression of FAIM1 promotes the activation of AKT followed by the up-regulation of IRF4, which is important for MM survival [25]. In prostate cancer, miR-133b could directly bind to the 3′UTR of FAIM mRNA and regulate the expression of FAIM protein, inducing proliferation arrest in PC3 cells [26]. However, most cancer-related studies of the FAIM gene have focussed on FAIM-L, little is known about the function and molecular mechanisms of FAIM-S in cancer progression.

In the neural model, it was reported that both FAIM-S and FAIM-L expressed in neurons but had different physiological effects. FAIM-S is primarily responsible for promoting growth factor-induced

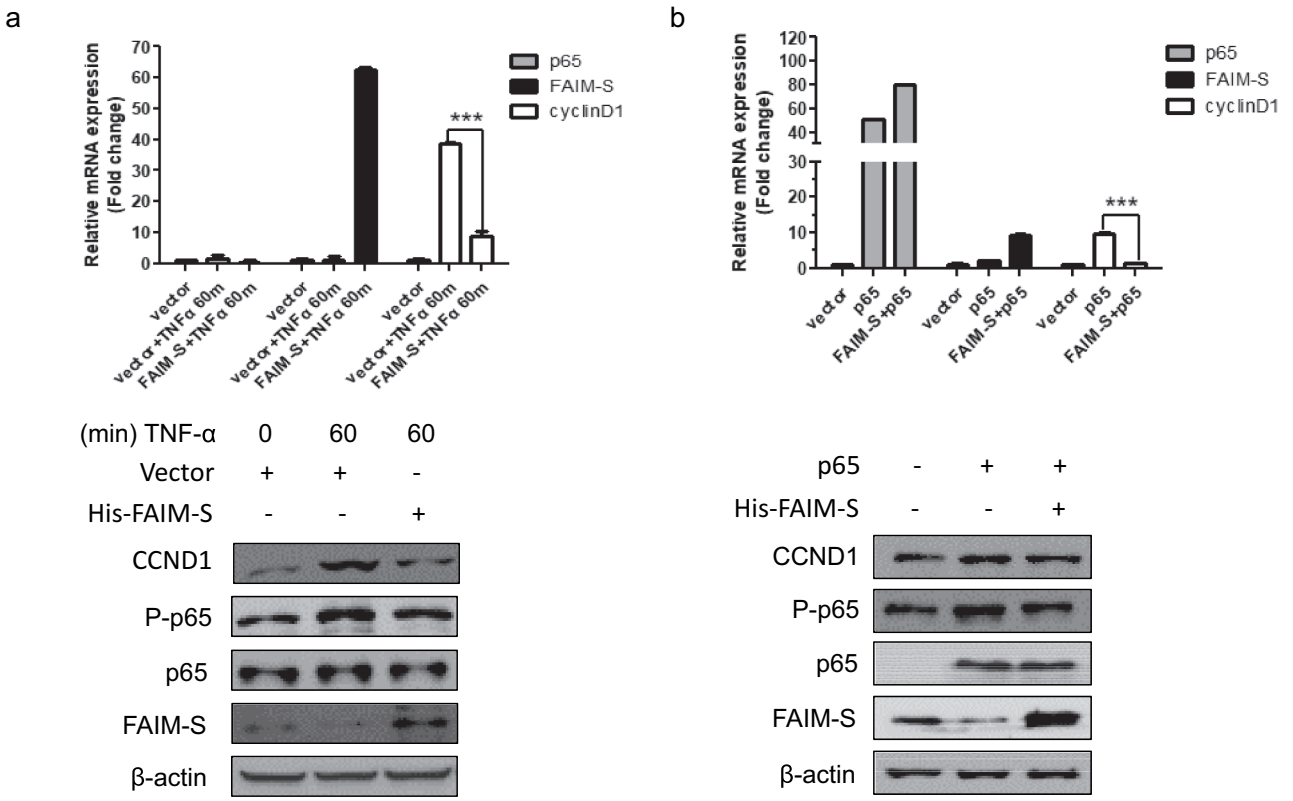


Figure 5. FAIM-S regulates the expression of CCND1 through affecting NF-κB pathway. (a) H1299 cell was transiently transfected with or without FAIM-S. Then, the cells were treated with TNF-α for indicated times. Q-PCR was performed to detected the mRNA expression of indicated genes. Data represent the average of three independent experiments (mean ± SD). ***P < 0.001 (upper panel). Western blot was used to detect the protein expression of indicated genes (bottom panel). (b) H1299 cells were transfected with pcDNA3.0-p65 or co-transfected with pcDNA3.0-p65 and pcDNA3.1-His-FAIM-S. Q-PCR was performed to detected the mRNA expression of indicated genes. Data represent the average of three independent experiments (mean ± SD). ***P < 0.001 (upper panel). Western blot was used to detect the protein expression of indicated genes (bottom panel).

axon hyperplasia that dependent on ERK and NF-κB channels [20]. Unlike FAIM-S, FAIM-L protects neurons from DRs(death receptors)-induced cell apoptosis [17]. This indicated that FAIM-S might have different roles and molecular mechanisms in cancer cells. In this study, we focus on the shortest isoform of FAIM1 – FAIM-S. We discovered that overexpression of FAIM-S significantly inhibited the proliferation of non-small cell lung cancer cells and arrested cell cycle at the G0/G1 phase. Interestingly, overexpressing FAIM-S did not affect the apoptosis of NSCLC cells. This indicated that the functions of FAIM-S in NSCLC cells were independent of the anti-apoptosis effect and we will further investigate its effects on senescent or autophagy. We also demonstrated that FAIM-S could decrease the expression of CCND1, which functioned as a regulatory subunit of CDK4 or CDK6, and is required for the transition from the G1 phase to the S phase. In particular, we found that FAIM-S affected the transcription of CCND1. As NF-κB was proved to be a key transcription factor for CCND1, we detected the effects of FAIM-S on the NF-κB pathway [9]. We discovered that FAIM-S could interact with IKK-α, increasing its ubiquitination and accelerating its degradation, thus blocking the activation of the NF-κB pathway. However, the E3 ligase in this process is still not clear and we will further study the detailed molecular mechanisms. We finally demonstrated that FAIM-S regulated the expression of CCND1 through the NF-κB pathway. Taken together, our study demonstrates for the first time that FAIM-S regulates the expression of CCND1 through the NF-κB pathway and functions as a tumor suppressor to inhibit the proliferation of NSCLC cells.

Disclosure statement

The authors declare that there are no conflicts of interest.

Funding

This work is supported by the National Natural Science Foundation of China (81902346) and the Natural Science Foundation of Jiangxi Province (20192BAB215038).

References

[1] Schafer KA. The cell cycle: a review. Vet Pathol. 1998;35(6):461–78.
[2] Cancer Genome Atlas Research, N., et als. Integrated genomic characterization of oesophageal carcinoma. Nature. 2017;541(7636):169–175.
[3] Llovet JM, Villanueva A, Lachenmayer A, et al. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. Nat Rev Clin Oncol. 2015;12(8):436.
[4] Schulze K, Imbeaud S, Letouzé E, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nat Genet. 2015;47(5):505–511.
[5] Gautschi O, Ratschiller D, Gugger M, et al. Cyclin D1 in non-small cell lung cancer: a key driver of malignant transformation. Lung Cancer. 2007;55(1):1–14. .
[6] Hayden MS, Ghosh S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev. 2012;26(3):203–34.
[7] Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol. 2007;8(1):49–62.
[8] Baldwin AS Jr., Azizkhan JC, Jensen DE, et al. Induction of NF-kappa B DNA-binding activity during the G0-to-G1 transition in mouse fibroblasts. Mol Cell Biol. 1991;11(10):4943–4951.
[9] Hinz M, Krappmann D, Eichten A, et al. NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol. 1999;19(4):2690–8.
[10] Brantley DM, Chen C-L, Muraoka RS, et al. Nuclear factor-kappaB (NF-kappaB) regulates proliferation and branching in mouse mammary epithelium. Mol Biol Cell. 2001;12(5):1445–55.
[11] Westerheide SD, Mayo MW, Anest V, et al. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. Mol Cell Biol. 2001;21(24):8428–36.
[12] Guttridge DC, Albanese C, Reuther JY, et al. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol. 1999;19(8):5785–99.
[13] Liu JL, Ma HP, Lu XL, et al. NF-kappaB induces abnormal centrosome amplification by upregulation of CDK2 in laryngeal squamous cell cancer. Int J Oncol. 2011;39(4):915–24.
[14] Planells-Ferrer L, Urresti J, Coccia E, et al. Fas apoptosis inhibitory molecules: more than death-receptor antagonists in the nervous system. J Neurochem. 2016;139(1):11–21.
[15] Schneider TJ, Fischer GM, Donohoe TJ, et al. A novel gene coding for a Fas apoptosis inhibitory molecule (FAIM) isolated from inducibly Fas-resistant B lymphocytes. J Exp Med. 1999;189(6):949–56.
[16] Zhong X, Schneider TJ, Cabral DS, et al. An alternatively spliced long form of Fas apoptosis inhibitory molecule (FAIM) with tissue-specific expression in the brain. Mol Immunol. 2001;38(1):65–72.
[17] Segura MF, Sole C, Pascual M, et al. The long form of Fas apoptotic inhibitory molecule is expressed specifically in neurons and protects them against death receptor-triggered apoptosis. J Neurosci. 2007;27(42):11228–41. .
[18] Moubarak RS, Planells-Ferrer L, Urresti J, et al. FAIM-L is an IAP-binding protein that inhibits XIAP ubiquitinylation and protects from Fas-induced apoptosis. J Neurosci. 2013;33(49):19262–75. .
[19] Yu LY, Saarma M, Arumae U. Death receptors and caspases but not mitochondria are activated in the GDNF- or BDNF-deprived dopaminergic neurons. J Neurosci. 2008;28(30):7467–75.
[20] Sole C, Dolcet X, Segura M, et al. The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-kapp B signaling. J Cell Biol. 2004;167(3):479–92.
[21] Martinez-Marmol R, Barneda-Zahonero B, Soto D, et al. FAIM-L regulation of XIAP degradation modulates synaptic long-term depression and axon degeneration. Sci Rep. 2016;6:35775.
[22] Carriba P, Comella JX. Amyloid beta, TNFalpha and FAIM-L; approaching new therapeutic strategies for AD. Front Neurol. 2014;5:276.
[23] Carriba P, Jimenez S, Navarro V, et al. Amyloid-beta reduces the expression of neuronal FAIM-L, thereby shifting the inflammatory response mediated by TNFalpha from neuronal protection to death. Cell Death Dis. 2015;6:e1639.
[24] Huo J, Ma Y, Liu -J-J, et al. Loss of Fas apoptosis inhibitory molecule leads to spontaneous obesity and hepatosteatosis. Cell Death Dis. 2016;7(p):e2091.
[25] Huo J, Xu S, Lin B, et al. Fas apoptosis inhibitory molecule is upregulated by IGF-1 signaling and modulates Akt activation and IRF4 expression in multiple myeloma. Leukemia. 2013;27(5):1165–71.
[26] Patron JP, Fendler A, Bild M, et al. MiR-133b targets antiapoptotic genes and enhances death receptor-induced apoptosis. PLoS One. 2012;7(4):e35345.