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In vitro study of FUZ as a novel potential therapeutic target in non-small-cell lung cancer

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ABSTRACT

FUZ is regarded as a planar cell polarity effector that controls multiple cellular processes during vertebrate development. However, the role of FUZ in tumor biology remains poorly studied. Our purpose of this study is to discover the physiological effects and mechanism of FUZ in non-small-cell lung cancer (NSCLC) *in vitro*. With the help of bioinformatics analysis, we noticed that the expression level of FUZ negatively correlates with prognosis of NSCLC patients. Exogenous FUZ expression markedly promoted cell proliferation of NSCLC cells. The phosphorylation of Erk1/2, STAT3 and related signaling molecules were induced activated after FUZ over-expression. FUZ also plays an important role in cell motility by regulating cell signaling pathways and inducing epithelial to mesenchymal transition (EMT). FUZ promotes EMT along with the up-regulation of N-cadherin, vimentin, Zeb1, Twist1 and decreased level of E-cadherin. Furthermore, we also carried out FUZ directed siRNA treatments to prove the above observations. Knockdown of FUZ resulted in delayed cell growth as well as impaired cell migration and reversed EMT phonotype. Importantly, we reported for the first time that FUZ is a BNIP3-interacting protein. Loss of FUZ resulted in decreased BNIP3 protein level, but no influence on BNIP3 mRNA level, suggesting weakened stability of BNIP3 protein. Overall, our results *in vitro* show that FUZ is responsible for NSCLC progression and metastasis, suggesting that FUZ can be a potential therapeutic target for NSCLC.

1. Introduction

Lung cancer has become the leading form of cancer in terms of both incidence and cancer related deaths [1]. Non-small-cell lung cancer (NSCLC) accounts for about 85% of all lung cancers [2], among all histological types of NSCLC, lung adenocarcinoma is the most common one that accounts for approximately 50% [3]. Chemotherapy was previously the conclusive recommendations, but it had a low cure rate and brought patients bad side effects and miserable experiences. Even worse, compared to small cell carcinoma, NSCLC relatively lacks sensitivity to chemotherapy. With the accumulation of our knowledge about tumor driver genes and promoter genes, targeted therapies against these genes have provided a better choice for advanced patients, which has much better treatment effects and lower side effects. Thus, the study about tumor driver or promoter genes has been a crucial breakthrough to solve these problems [4].

In the post-genome era, addressing gene function is an important and arduous task. While for most of the human encoding genes, their functions in specific cases are yet to know. In cancer diagnosis and treatment, many tumor-related genes are regarded as markers as they are involved in the selection of therapy as well as the prognosis of the patient. To our knowledge, highly activated oncogenes such as c-myc [5] and STAT3 [6] suggest poor prognosis of patient while an increased level of tumor suppressor genes such as p53 [7] and PTEN [8] indicated a better outcome. On the basis of this theory, it is vital for us to discover more potential markers to make clear of the mechanism of tumorigenesis and development as well as to assist diagnosis and treatment.

The *Drosophila melanogaster* homolog of FUZ is associated with planar cell polarity (PCP) and Hedgehog signaling [9,10]. In *Deosophila*, Fuz was reported to regulate hair polarity in a restricted set of fly tissues [11–13]. Fuz is a critical regulator of cilia structure and function in *Xenopus laevis* and mice. In *Xenopus laevis*, knockdown of Fuz (XFy) results in an open neural tube along the entire spinal axis, associated with body shortening, disruption of the cortical actin network and sparse rudimental cilia in skin cells [14]. A recently published study revealed that Fuz knockout mouse exhibits severe cranial neural tube defects (NTD) [15,16]. Actually, the functions of FUZ have been quite well elucidated, but limited to neural system development. Few reports

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of FUZ in tumor biology have been published yet, including in NSCLC. Planar polarity signaling is involved in the elongation of the body axis during development, a process that involves the coordination of multiple cell behaviors including cell rearrangement, cell division and cell-shape changes [17]. Interestingly, these are exactly what happen during tumorigenesis. As a PCP effector, dysregulation of FUZ could result in overactive PCP signaling pathway followed by subsequent uncontrollable cell division and cell-shape changes, which suggests FUZ as a potential tumor promoter.

BNIP3 is described as a pro-cell death protein, and its activity is dependent on the BH3 domain as well as on the transmembrane domain [18–20]. Unlike most BH3-only members that induce acute apoptotic cell death upon overexpression, transient expression of BNIP3 results in delayed cell death [21]. BNIP3 has also been implicated in autophagic cell survival possibly by facilitating removal of damaged mitochondrial [22,23]. High-level expression of BNIP3 is also reported in several human carcinomas compared with normal tissues [24]. Notably, recent studies suggest that BNIP3 plays an essential role in solid tumor development, including NSCLC [25].

In this study, we investigated the effects and mechanism of FUZ in NSCLC cells *in vitro*. Our results indicated that FUZ promotes cell proliferation along with the up-regulation of the phosphorylation of Erk1/2 and STAT3. Besides, FUZ accelerates the migration of NSCLC cells. From bio-informatics analysis, the expression of FUZ is found negatively correlated with the predicted overall survival of patients. FUZ is a BNIP3-interacting protein and the interaction enhances their stability. These results *in vitro* all support FUZ as an oncogene and a potential therapeutic target in NSCLC.

2. Materials and methods

2.1. Cell culture and reagents

A549 and H1299 cells were obtained from the American Type Culture Collection (ATCC) and routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 100 mg/ml penicillin-streptomycin in a humidified 5% CO_2 at 37 °C. Lipofectamine™ 3000 was purchased from Life Technologies (USA). All transfections were performed under the manufacturer's instructions. SKL2001 and KYA1797K reagents were purchased from Selleck. The MG-132 was purchased from EMD Millipore (Billerica, MA, USA). Antibodies against Flag-tag, Erk1/2, phosphor-Erk1/2 (Thr202/Tyr204), STAT3, phosphor-STAT3 (Tyr705), Ras, Raf1, p21, CyclinB, E-Cadherin, N-cadherin, Snail, TCF8/ZEB1, Twist1, Smad2/3, p-Smad3, Vimentin were purchased from Cell Signaling technology (USA). Anti-GAPDH and anti-β-actin (Proteintech group), anti-FUZ antibody (ab122742), anti-BNIP3 antibody was purchased from Abcam. Adenoviruses carrying the FUZ gene (AdFUZ) and empty adenovirus (AdNC) were packaged by Vigene Biosciences. The siRNAs were synthesized by GenePharma (Shanghai, China). The following sequences were used:

siFUZ-1:5′CCCUCAAUGGAGUCCACAUTT3′(sense), 5′AUGUGGACUCCAUUGAGGTT3′(antisense), siFUZ-2:5′GGUCCUUCUUGUGGGACUUTT3′(sense), 5′AAGUCCCACAAGAAGGACCTT3′(antisense); siBNIP3-1:5′AAGGAACACGAGCGUCAUGAATT3′(sense), 5′UUCAUGACGCUCGUGUGUUCCUUTT3′(antisense); siBNIP3-2:5′GUUCCAGCCUCGGUUUCUATT3′(sense), 5′UAGAAACCGAGGCUGGAACTT3′(antisense).

2.2. Cell proliferation assays

A549, H1299 cells infected with AdNC, AdFUZ or siNC, siFUZ were seeded in 96-well plates at a density of 2500 cells per well, cells were then incubated routinely. Cell proliferation was detected by using Cell Counting Kit-8 (CCK8) (Dojindo Molecular Technologies, Japan). 10 μl CCK-8 reagent was diluted in each well and incubated for 2 h at 37 °C. The absorbance as 450 nm was measured by a spectrophotometer. Results from three independent experiments were presented as the means ± standard deviation (SD).

2.3. Colony formation assay

1000 A549 cells that were equally treated in CCK-8 assay were plated in 6-well culture plates. 2 weeks later, cells were fixed with paraformaldehyde for 10 min and then stained with 0.5% crystal violet for 10 min at room temperature. Colonies were defined as a minimum of 50 cells in a group and counted with the image analysis software (IPP6). Results were obtained from two or three independent experiments.

2.4. Cell cycle analysis

A549 cells were harvested at 48 h after Ad-FUZ infection. Cells were washed with PBS for 3 times and then fixed in pre-cooled 70% ethanol overnight at −20 °C. Fixed cells were then pelleted through centrifugation, washed with PBS and incubated with 500 mg/ml RNase A (Sigma-Aldrich) in PBS at 37 °C for 30 min. Cells were then analyzed on BD FACS Calibur (BD bioscience, San Jose, CA, USA) after stained with 10 mg/ml PI (Sigma-Aldrich) in 0.1% Triton X-100. Cell cycle distribution was analyzed with the ModFit LT software (Verity Software House, Topsham, ME).

2.5. Western blotting analysis

Cells were lysed in RIPA (Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% protease inhibitor cocktail and 5% phosphatase inhibitor (Roche, Basel, Switzerland). Protein concentrations were determined using BCA protein assays (Pierce, Rockford, IL, USA). Cell lysates were then fractionated using 12.5% SDS-PAGE gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK), then probed with primary antibodies and subsequently HRP-labeled secondary antibodies. Signals were detected by LAS500 Quant Image (GE, New York City, NY, USA). GAPDH was used as a lysate loading control.

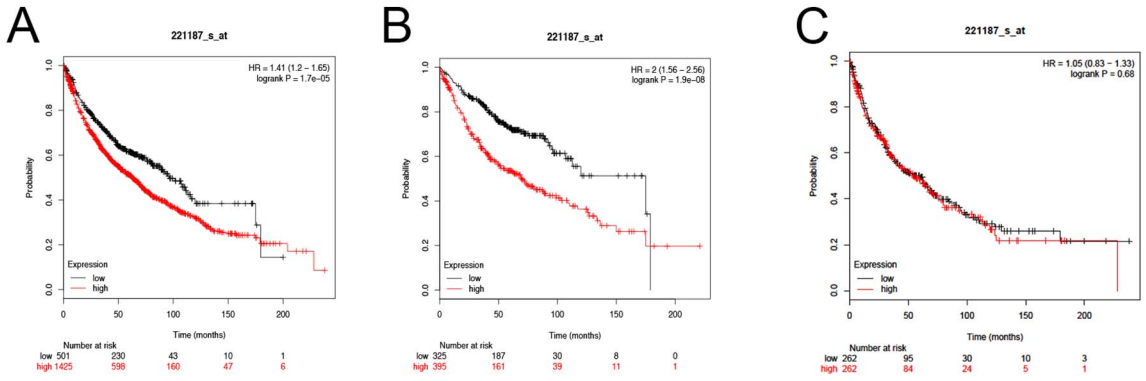
2.6. Cell migration assay

Transwell assay. 24 h after infection, A549 or H1299 cells were serum starved for 6 h, 3×10^4 cells (for A549 cells) or 5×10^4 cells (for H1299 cells) in 200 μl serum-free medium were seeded into the upper chamber of a Transwell chamber with a fibronectin-coated filter (8 mm pore size, Corning Life Sciences, NY, USA). Bottom chamber contained medium supplemented with 10% FBS. After a 24-hour incubation at 37 °C, non-migrated cells were scraped off the filter by using a wet cotton swab and migrated cells were stained with crystal violet following fixation with 4% paraformaldehyde. Number of cells was counted in 6 randomly chosen fields. Triplicate wells were performed in each assay and the assay was repeated for 3 times.

Wound healing assay. H1299 cells were infected with AdNC/AdFUZ or transfected with siNC/siFUZ for 48 h, then cells were moved into serum-free medium. At each indicated time point, photographs of wound closure were taken utilizing a Motic AE31E microscope (×40 magnification) and subsequently analyzed by Imagej.

2.7. Yeast two-hybrid

The FUZ and BNIP3 cDNA was fused to the GAL4 binding domain (BD) (pGBK-T7 vector) and activation domain (AD) (pGAD-T7 vector) respectively. The BD and AD plasmids were co-transformed into yeast AH109 strains using His (+) [SD (Leu-, Trp-)] plates. The transformants with the desired protein expression were plated on His (+) and His (−) [SD (Leu-, Trp-, His-)] plates. The strains were incubated at 30 °C till visible colonies appeared. Protein expression was analyzed by Western-



D

Normal vs cancer

Probe set	Tissue	Min	Q1	Median	Q3	Max	P value	Sample n
221187_s_at	Normal	35	82	109	159	409	NA	86
221187_s_at	Cancer	3	97	171	254	962	4.21e-07	2437

Fig. 1. Overall survival (OS) of NSCLC patients were analyzed using the Kaplan-Meier test.
(A) *Left*, NSCLC patients (including all subtypes) with high FUZ expression showed a significantly lower OS compared with patients with low FUZ expression ($P < 0.001$). *Right*, plot beeswarm graph of probe distribution.
(B) OS of patients with adenocarcinoma of lung. Patients with high FUZ expression showed a significantly lower OS compared with patients with low FUZ expression ($P < 0.001$).
(C) The expression level of FUZ in squamous cell lung carcinoma does not affect the OS ($P = 0.68$).
(D) Probe expression in NSCLC and normal tissue. The expression is higher in cancer than normal.

Blot using TCA precipitated cell lysates from the transformants grown on the His (+) [SD (Leu-, Trp-)] plates.

2.8. Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation assays were performed with control (flag-RIOK) or flag-FUZ transfected A549 cells. Cells were cultured for indicated hours and then harvested in a buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 5 μg/ml pepstatin. Cell lysates (1.5 mg) were incubated with indicated antibody or corresponding IgG for 4 h and further incubated for another 2 h with pre-equilibrated protein-G (GE, New York City, NY, USA), or cell lysates incubated with flag-affinity gel (Biotool, Switzerland) for 4 h. Beads were then washed thrice and analyzed using western blotting as previously described. Signals were detected by LAS500 (GE, New York City, NY, USA).

2.9. Confocal microscopy

Cells were washed with PBS, fixed and permeabilized in 3% paraformaldehyde containing 0.1% Triton X-100 for 30 min at 4 °C. The cells were blocked and incubated with primary antibodies and then Alexa Fluor 488 or 576-conjugated secondary antibodies. The cells were washed twice with PBS and stained with DAPI for 10 min before being imaged with a TCS-SP laser-scanning confocal microscope with a 63× oil immersion lens (Leica Microsystems, Mannheim, Germany).

2.10. $CoCl_2$ induced hypoxia

A549 cells were seeded in a 6-well plate. 12 h later, $CoCl_2$ (dilution = 500 μM) was applied into the medium and the treatment lasted for 12 h. Then cells were harvested for RT-PCR.

2.11. Reverse transcription PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, USA), and reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo, USA) following the manufacturer's instructions.

2.12. Statistical and survival analysis

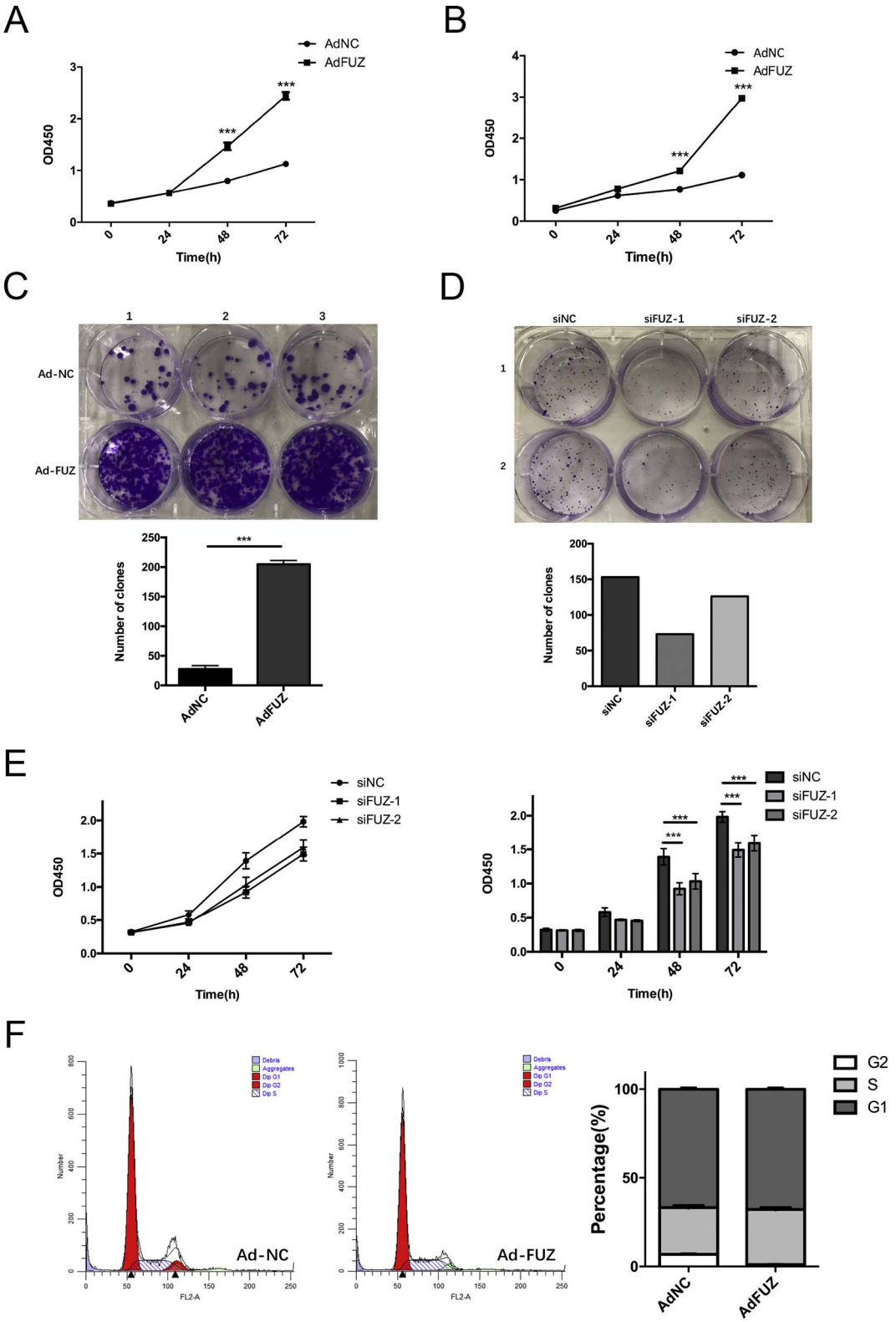
Differences between two independent groups were analyzed using Student's *t*-test. A P-value < 0.05 was considered to represent a statistically significant difference.

Survival curves were performed by the Kaplan-Meier method and compared by the log-rank test (www.kmplot.com/lung/). Gene expression data and overall survival information were downloaded from GEO (Affymetrix microarrays only), EGA and TCGA. To analyze the prognostic value of FUZ, the lung cancer samples (1926 samples) were split into two groups according to various quantile expressions of the proposed biomarker. For NSCLC histology subtypes analysis, 720 adenocarcinoma samples and 524 squamous cell carcinoma samples were analyzed. Only JetSet best probes were used. Univariate and multivariate Cox regression model was used for survival analysis and the number-at-risk was indicated below the main plot. Hazard ratio, 95% confidence intervals and logrank P were calculated and displayed.

3. Results

3.1. FUZ expression is associated with poor prognosis in NSCLC patients

We first explored the association between FUZ expression and prognosis of NSCLC patients. The Kaplan-Meier survival analysis showed that compared with the group with high expression of FUZ, FUZ low expression markedly had favorable overall survival in NSCLC patients (without restriction to tumor subtypes) (Fig. 1A). We then took NSCLC histology subtypes into consideration and found out that the influence of FUZ on overall survival is strikingly evident in lung



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adenocarcinoma (Fig. 1B), but no difference found in squamous cell lung carcinoma (Fig. 1C). As shown in Fig. 1D, according to the analysis (www.kmplot.com), the expression level of FUZ in cancer tissue is higher than that in normal tissue. Therefore, FUZ probably plays an important role in NSCLC.

3.2. FUZ promotes cell proliferation of NSCLC cells

According to survival analysis, lung adenocarcinoma cell lines were chosen to study the functions of FUZ. We looked into the effects of FUZ on cell phenotypes. By performing CCK-8 assay, we found that FUZ overexpression could markedly enhance A549 and H1299 cell growth

Fig. 2. Effects of FUZ on NSCLC cell lines.
(A) Overexpression of FUZ in A549 by infection with a FUZ-expressing adenovirus (AdFUZ) and then incubated for 48 h before harvest, MOI = 100, the empty adenovirus was used as a control. CCK-8 assay was performed to measure cell proliferation.
(B) Overexpression of FUZ in H1299 by infection with a FUZ-expressing adenovirus (AdFUZ), MOI = 100, cells were harvested 48 h later. The empty adenovirus was used as a control. CCK-8 assay was performed to measure cell proliferation.
(C) 1000 A549 cells infected by Ad-FUZ or Ad-NC were cultured routinely in a 6-well plate for 12 days, cells were fixed with paraformaldehyde (PFA) for 10 min and then stained with 0.5% crystal violet for 10 min at room temperature. Results are presented for triplicate wells.
(D) Knockdown of FUZ in A549 cells, 48 h after the transfection, cells were plated in a 6-well plate and cultured routinely in a 6-well plate for 14 days, 1200 cells per well.
(E) Knockdown of FUZ with two siRNAs (siFUZ-1 and 2) in A549 cells, cells were plated in a 96-well plate 48 h after the transfection, 3000 cells per well. CCK-8 assay was performed to measure cell proliferation.
(F) Cell cycle was analyzed 48 h after infection of AdNC/AdFUZ in A549 cells by flow cytometry. Representative histograms (*left*) and the percentage of cells at different phases (*right*) are shown. The data are expressed as the means ± SEM of three independent experiments.

(Fig. 2A, B). Besides, in a colony formation assay, cells infected with AdFUZ managed to establish lots more amount of clones than controls (Fig. 2C). These results were further supported as cell proliferation was restrained after siRNA induced endogenous FUZ knockdown (Fig. 2D, E). These results indicated that FUZ could promote the proliferation of NSCLC cells.

3.3. FUZ facilitates cell cycle process in A549 cells

Observed markedly enhanced cell proliferation after FUZ expression, we then studied the effect of FUZ on cell cycle progression by flow cytometry. AdFUZ infected cells had a significant increase population in the S phase compared with the Ad-NC infectants (Fig. 2F). We further examined several key cell cycle regulators by western blotting and found that the CDK inhibitor p21 expression was down-regulated in FUZ-expressing cells compared with controls, whereas cyclin B was markedly induced up-regulated (Fig. 3A, B). Consistently, FUZ knockdown led to increased level of p21 and downregulated cyclin B (Fig. 3C, D). These results suggested that FUZ stimulates cell mitosis.

3.4. FUZ expression activates Erk1/2 and STAT3 signal pathways

To further investigate the mechanism responsible for the effect of FUZ, signal pathways involved in cell survival and proliferation were analyzed by western blotting. Our results demonstrated that Erk1/2, STAT3 were induced activated after FUZ expression as well as elevated levels of Raf1 and Ras (Fig. 3A, B). Whereas phosphorylation of Erk1/2 and STAT3 were inhibited after FUZ knockdown, accompanied with decreased level of Raf1 and Ras (Fig. 3C, D). Erk1/2 activation is generally associated with enhanced cell survival and blockage of the Raf/Ras/Erk1/2 pathway is widely used as an antineoplastic method. While it seemed that p38 MAPK signals were not involved in this process. These results indicated that FUZ significantly enhances the Erk1/2, STAT3 signals and further promotes cell proliferation and survival.

3.5. FUZ accelerates A549 and H1299 cell migration

In the course of our study, we noticed that FUZ-overexpressed NSCLC cells transformed to an obvious spindle-like form (data not shown), which prompted us to consider the association of FUZ with the epithelial-mesenchymal transition (EMT) process. We chose to study the effects of FUZ on cell migration. Wound healing and Transwell assays were conducted to assess the motility of FUZ over-expressing or control cells. The results showed that over-expression of FUZ led to significantly increased migration and faster wound healing capacities (Fig. 4A, C, D), while FUZ knock-down resulted in decreased migration and slower wound healing capacities compared with control (Fig. 4B, E, F). Restoration of FUZ reversed the phonotype caused by siRNA-induced FUZ knockdown (Fig. 4G). These results indicated that FUZ could promote the mobility and facilitate the metastasis of NSCLC.

3.6. FUZ is associated with EMT process in NSCLC

After determined the effects of FUZ on NSCLC cell motility, we then aimed to investigate the effects of FUZ on EMT, which is important in the initiation and promotion of cell migratory and invasive properties [16]. First, the expression of EMT markers was analyzed in FUZ over-expressed A549 and H1299 cells. As shown in Fig. 5A and B, FUZ increased the expression of Twist1, Zeb1, Snail, Vimentin and N-cadherin but decreased the expression of E-cadherin. Among the EMT-related markers, Smad2/3 expression was not affected by FUZ, further analysis indicated that Smad3 was markedly induced phosphorylated after FUZ over-expression. We then confirmed the effects of FUZ on EMT in FUZ-knockdown A549 (Fig. 5C) and H1299 cells (Fig. 5D). The expression of E-cadherin was up-regulated whereas the expression of Twist1, Zeb1, Snail, Vimentin and N-cadherin was down-regulated. Notably, β-

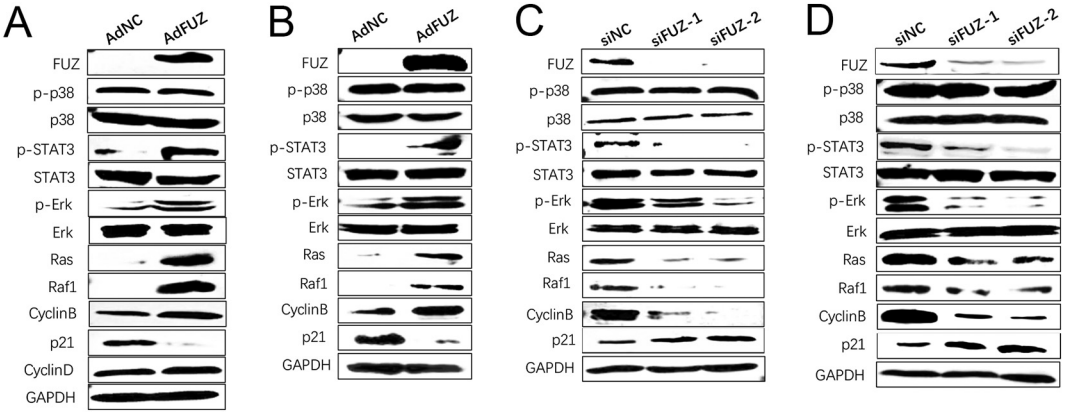

Fig. 3. FUZ promotes cell proliferation by upregulating phosphorylation of Erk and STAT3.
(A) Effects of FUZ overexpression on the phosphorylation of Erk1/2, STAT3 signaling pathways, A549 cells were infected by AdFUZ or AdNC (MOI = 100) for 48 h.
(B) Effects of FUZ overexpression on the phosphorylation of Erk1/2, STAT3 signaling pathways, H1299 cells were infected by AdFUZ or AdNC (MOI = 100) for 48 h.
(C) Effects of FUZ knockdown on the phosphorylation of ERK1/2, STAT3 signaling pathways, A549 cells were transfected with siFUZ-1 or 2 for 48 h.
(D) Effects of FUZ knockdown on the phosphorylation of ERK1/2, STAT3 signaling pathways, H1299 cells were transfected with siFUZ-1 or 2 for 48 h.

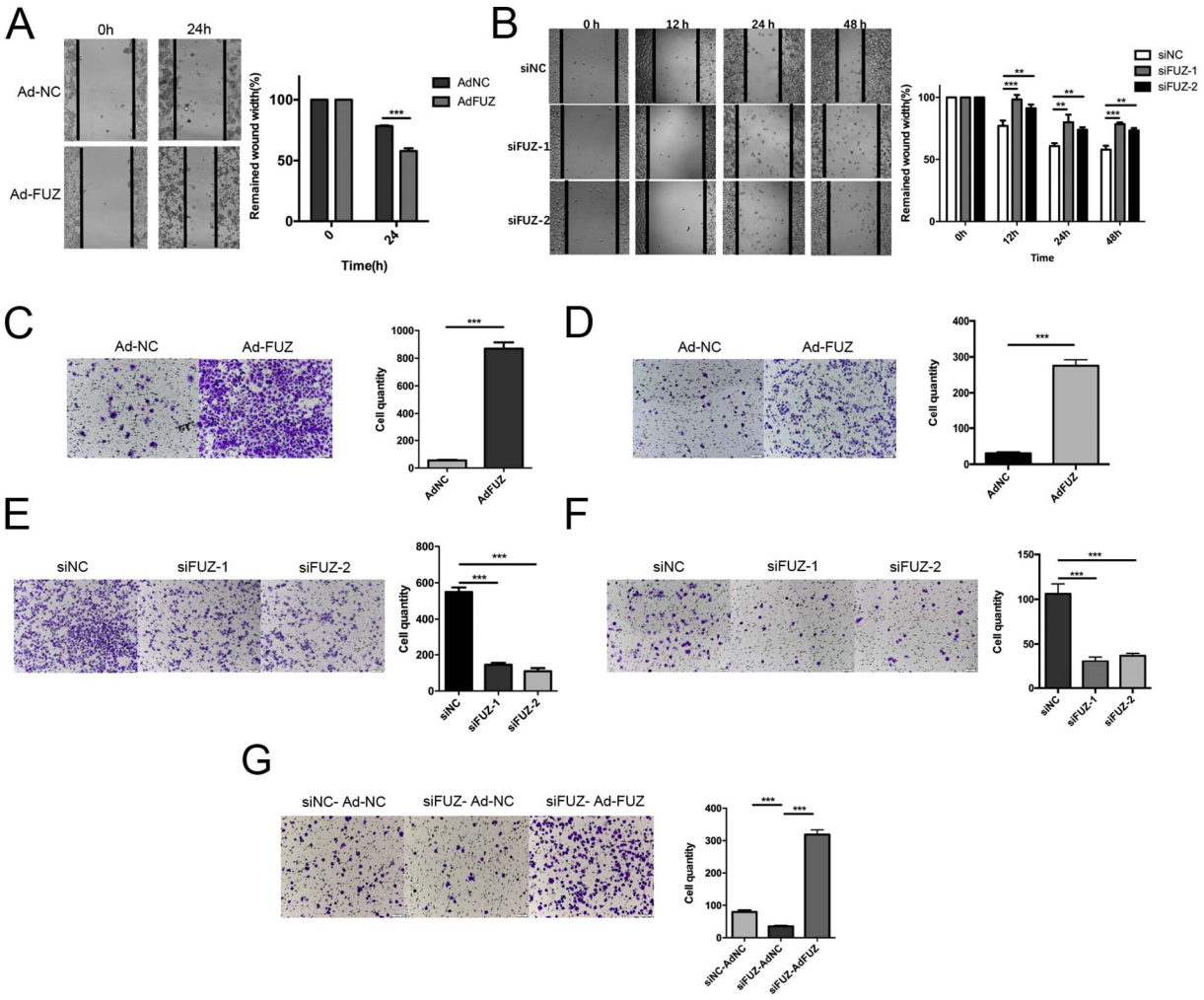

Fig. 4. FUZ expression promotes NSCLC migration.

(A) Cell migration activity was examined by wound healing assay. H1299 cells were infected with AdFUZ or AdNC (MOI = 100) for 48 h and cells were moved in medium without fetal bovine serum (FBS). Then cells were photographed. Representative images at indicated time point are shown.

(B) H1299 cells transfected with siNC, siFUZ-1 or siFUZ-2 were used in the wound healing assay. H1299 cells were transfected with siRNAs for 48 h and moved in medium without FBS. Then photographed. Representative images at indicated time point are shown.

(C) (D) Cell migration was examined by Transwell assay. A549 (C) or H1299 (D) cells were infected with AdFUZ or AdNC (MOI = 100) for 48 h, then seeded into a Transwell chamber ($4 * 10^4$/well) and incubated for 24 h before staining. Representative images are shown. The statistical graph indicated the mean ± SEM of the number of cells from 6 random fields from three independent experiments.

(E) (F) A549 (E) or H1299 (F) cells transfected with siNC, siFUZ-1 or siFUZ-2 were used in the Transwell assay ($4 * 10^4$/well), cells were treated identically to (C) (D). Representative images are shown. Each performed in triplicate. *P < 0.05.

(G) A549 cells transfected with siNC/siFUZ-1 for 48 h. Upon siRNAs transfection, AdNC/AdFUZ (MOI = 20) was applied to rescue the expression of FUZ and the incubation time was 48 h. Cells were then used to perform Transwell assay.

catenin, the transcriptional factor of FUZ [26] was induced markedly up-regulated after FUZ expression. As could be expected, in the context of FUZ-knockdown, exogenous expression of FUZ rescued the alterations of transductors involved in cell survival, proliferation as well as EMT signals (Fig. 5E). We then treated A549 cells with the agonist (SKL2001) or antagonist (KYA1797K) of β-catenin. As expected, the agonist SKL2001 restored the level of β-catenin, while simultaneously, endogenous level of FUZ was up-regulated in a SKL2001-dose dependent manner (Fig. 5F). Treatment of KYA1797K significantly reduced the level of β-catenin, and the level of FUZ was correspondingly down-regulated (Fig. 5G). These results indicated that β-catenin augmented the expression of FUZ gene and enhanced level of FUZ induced elevated level of β-catenin in return, which indicated a forward feedback loop to promote β-catenin downstream signals and tumorigenesis. Altogether, these changes suggested that FUZ significantly drove the EMT process.

3.7. FUZ is a BNIP3-interacting protein

In yeast two-hybrid screen, the interaction between FUZ and Bcl-2/E1b-19 kDa-interacting protein 3 (BNIP3) was identified (Supplementary Data). The interaction of FUZ and BNIP3 was further verified by co-immunoprecipitation (Co-IP) (Fig. 6A). Flag-FUZ and BNIP3-his plasmids were co-transfected in A549 cells and FUZ clearly colocalized with BNIP3 (Fig. 6B). Consistent with previous studies, exogenous BNIP3 expression did enhance the proliferation of A549 cells. While knockdown of FUZ could completely reverse the BNIP3-induced cell proliferation (Fig. 6C). Meanwhile, knockdown of BNIP3 significantly hampered FUZ-induced A549 migration (Fig. 6D). We then confirmed the above results by detecting the key signal mediators involved in cell proliferation and EMT. As shown in Fig. 6E, siRNA-induced BNIP3 knockdown partially (more or less) reversed FUZ-modified phenotype. All these results indicated that BNIP3 played a role in FUZ functioning.

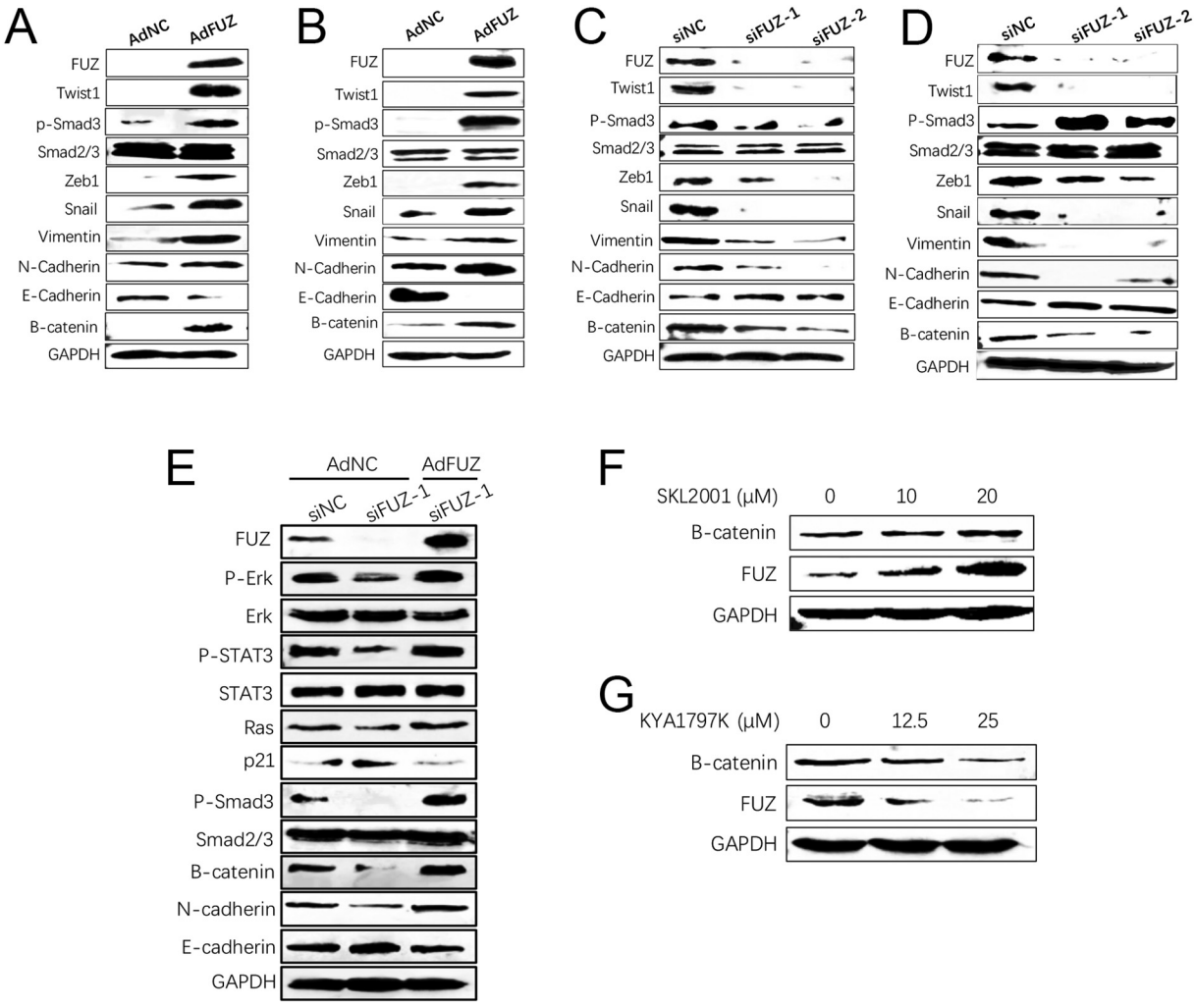

Fig. 5. FUZ drives the EMT of NSCLC cells.
(A) (B) A549 (A) or H1299 (B) cells were infected with AdFUZ or AdNC for 48 h, and the expression of Twist1, Smad2/3, Zeb1, Snail, Vimentin, N-cadherin, β-catenin and E-cadherin was examined.
(C) (D) A549 (C) or H1299 (D) cells were transfected with siNC, siFUZ-1 or siFUZ-2 for 48 h, and the expression of Twist1, Smad2/3, Zeb1, Snail, Vimentin, N-cadherin, β-catenin and E-cadherin was examined.
(E) A549 cells transfected with siNC/siFUZ-1 for 48 h. In the meantime of siRNAs transfection, AdNC/AdFUZ (MOI = 20) was applied. Cells were then harvested for western blotting analysis.
(F) A549 cells were treated with SKL2001 for 15 h, cell lysates were collected for western blotting, concentration gradient (1, 10 μM, 20 μM) was set for the analysis.
(G) A549 cells were treated with KYA1797K for 72 h, cells lysates were collected for western blotting, concentration gradient (1, 12.5 μM, 25 μM) was set for the analysis.

3.8. FUZ-BNIP3 interaction guarantees the stability of the two proteins

After determined that BNIP3 was functionally related to FUZ, we then discussed the significance of the FUZ-BNIP3 interaction. We exogenously expressed FUZ in A549 and H1299 cells. As shown in Fig. 7A, FUZ expression rendered enhanced level of BNIP3 in A549 and H1299 cells, but no influence on BNIP3 mRNA level. Likely, knockdown of FUZ resulted in significantly reduced level of BNIP3 (Fig. 7B). Interestingly, knockdown of endogenous BNIP3 also resulted in reduced FUZ level, these results suggested that the interaction of FUZ and BNIP3 is important for the stability of the two proteins. There were significant alterations in BNIP3 and FUZ protein levels upon FUZ and BNIP3 knockdown respectively, which indicated post-translational modifications. We applied MG-132 to see whether the level of BNIP3 or FUZ could be rescued. As expected, decreased level of BNIP3 under the circumstance of FUZ-knockdown was partially rescued after proteasome inhibition (Fig. 7C, *upper*). Similarly, FUZ level could be reverted after MG-132 treatment (Fig. 7C, *lower*).

Many researches on BNIP3 were based on the model of hypoxia. $CoCl_2$ was used to induce hypoxia and BNIP3 level was significantly up-regulated as previously reported. Meanwhile, exogenous expressed FUZ level was enhanced under hypoxia (Fig. 7D, F). Consistent with our previous results, the hypoxia itself did not alter the level of FUZ and BNIP3 mRNA (Fig. 7E). In summary, we demonstrated that the interaction of FUZ and BNIP3 is important for their stability.

4. Discussion

With the discovery of oncogenes and tumor suppressors, great improvement has been made in the understanding of tumorigenesis and tumor progression. Tumor-related genes are important because some of them can be tumor markers for therapy and outcome indication. Among all tumors, the number one with the increasing rapid incidence rate worldwide is lung cancer, which has the highest morbidity rate. In general case, chemotherapy is regarded as the conclusive recommendations. In the field of NSCLC, for instance, EGFR TKIs are considered as effective drugs. However, they had the low cure and brought patients severe side effects and miserable experiences in application. Worse yet, NSCLC relatively lacks sensitivity to chemotherapy. Thus, the exploration of alternative treatment is necessary.

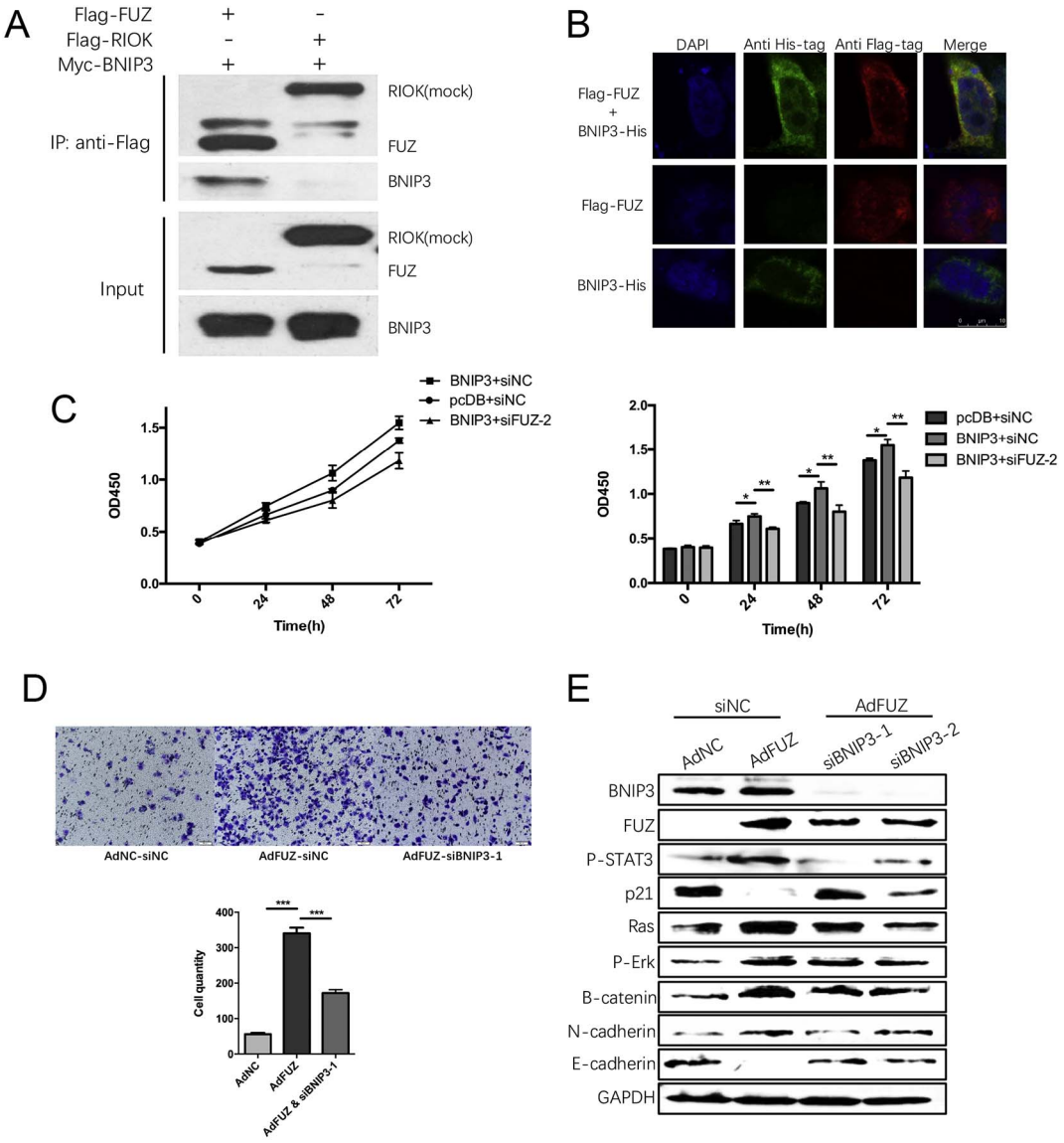

Fig. 6. FUZ is a BNIP3 interacting protein.

(A) Co-immunoprecipitation assay was performed to prove the interaction of FUZ and BNIP3. A549 cells were transfected with Flag-FUZ and BNIP3-His plasmids. Flag-RIOK plasmid was used as negative control.

(B) A549 cells were transfected with Flag-FUZ and BNIP3-His plasmids for 24 h prior to fixation in 3% PFA and immune-stained with antibodies against Flag-tag and His-tag. Images show colocalization of FUZ and BNIP3.

(C) A549 cells were transfected with siFUZ-2 to knock down the endogenous expression of FUZ. BNIP3-myc plasmid was then transfected. CCK8 assay was used to determine the proliferation.

(D) A549 cells infected with AdFUZ (or AdNC for control) and siBNIP3-1 (or siNC for control) simultaneously for 48 h. $4 * 10^4$ cells were seeded into Transwell chambers, incubated for 24 h and then fixed and stained. Representative images are shown. The statistical graph indicated the mean ± SEM of the number of cells from 6 random fields from three independent experiments.

(E) A549 cells infected with AdFUZ (or AdNC for control) and siBNIP3-1/siBNIP3-2 (or siNC for control) for 48 h. Cells were then harvested for western blotting.

Currently, with the accumulation of our knowledge about tumor drivers and promoters, targeted therapies against them have provided a better choice, which has better treatment efficacy and brings milder side effects. Hence, the discovery of potential markers is significative to clarify the mechanisms of tumorigenesis as well as to assist diagnosis and treatment.

FUZ was described as a planar cell polarity effector and its functions were rather well elucidated in the development of neuron system. Nonetheless, the role of FUZ in tumor initiation and progression has not been put forward. In this study, we demonstrated that FUZ promotes NSCLC cell proliferation as well as cell migration.

In order to study the biological function of FUZ, we firstly conducted a survival analysis to measure the possible association between the FUZ expression and NSCLC patient overall survival. FUZ is associated with poor prognosis of NSCLC patients, which indicates that FUZ is unfriendly in lung adenocarcinoma. Gene expression profiles in cancer and corresponding control (normal or non-tumor tissues) can provide clues to the function of it. The expression level of FUZ is up-regulated in cancer compared with normal tissues, indicating the involvement of FUZ in NSCLC.

According to our experiments, FUZ was found to promote cell proliferation, which was consistent with our speculation. Regular cell cycle progression is a key factor in cell proliferation, alterations of cell cycle may influence cell growth. Here, we observed that over-expression of FUZ induced elevated cell cycle distribution in S phase. Cyclin B was up-regulated in this process, while p21 was induced down-

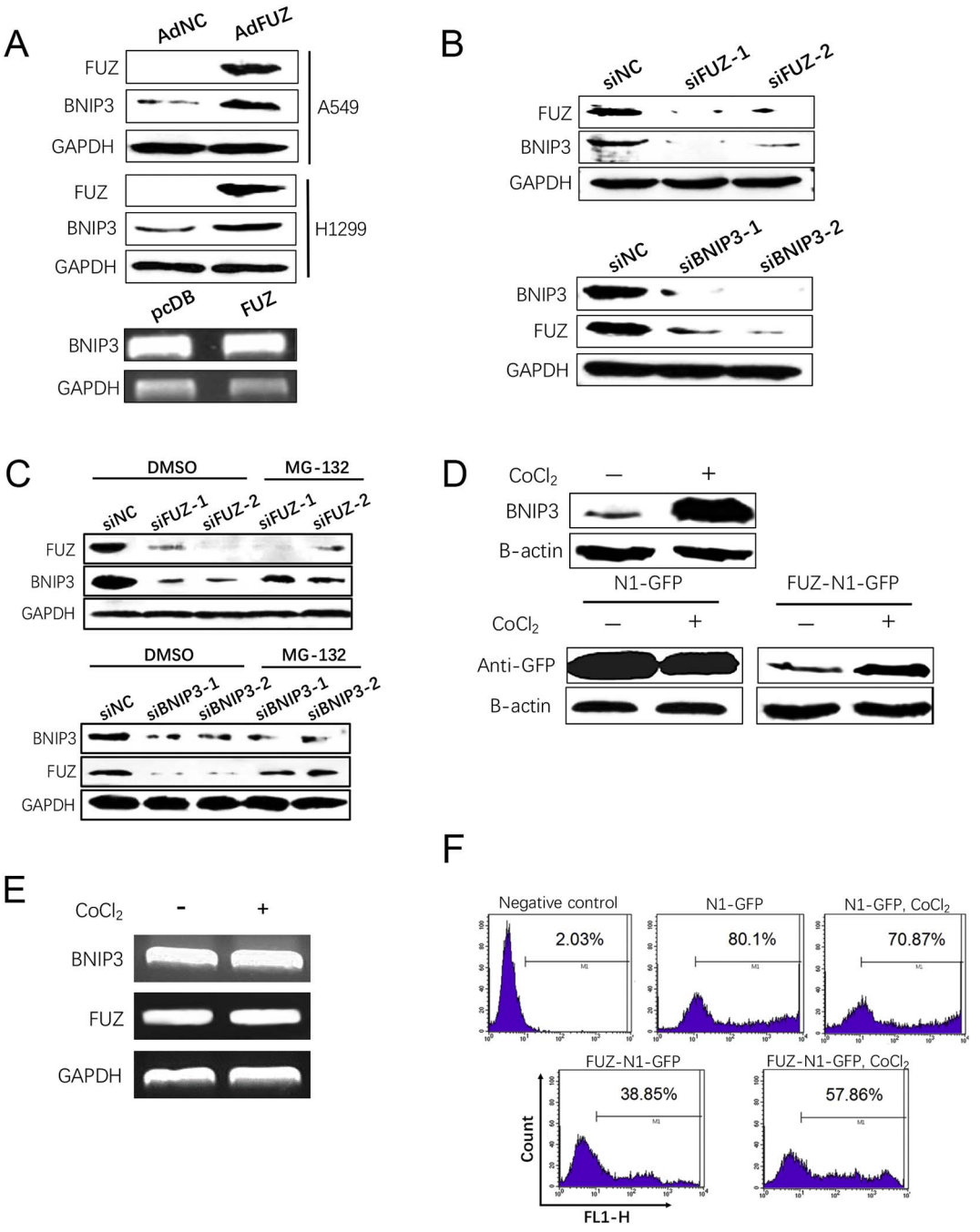

Fig. 7. The interaction of FUZ and BNIP3 is essential for their stability.
(A) A549 or H1299 cells were infected with AdFUZ or AdNC for 48 h and cell lysates were collected for western blotting (*upper*), and A549 cells transfected with FUZ or pcDB plasmid were collected for RT-PCR (*Lower*).
(B) A549 cells were transfected with siFUZ-1/2 (*upper*) or siBNIP3-1/2 (*lower*) for 48 h and cell lysates were collected for western blotting.
(C) A549 cells were transfected with siFUZ-1/2 (*upper*) or siBNIP3-1/2 (*lower*) for 48 h. 24 h after siRNA transfection, MG-132 (20 μM) was applied in the medium.
(D) $CoCl_2$ was applied to A549 cells at a concentration of 500 μM for 12 h. Cell lysates were collected for western blotting (*upper*). FUZ-N1-GFP or N1-GFP plasmids were transfected in A549 cells for 36 h, and at the time point of 24 h after the transfection, $CoCl_2$ was applied into the medium for 12 h, cells were then harvested for western blotting (*lower*).
(E) $CoCl_2$ was applied to A549 cells at a concentration of 500 μM for 12 h. The mRNA levels of FUZ and BNIP3 were tested by semi-quantitative RT-PCR.
(F) A549 cells were transfected with FUZ-N1-GFP or N1-GFP plasmids for 36 h, with or without $CoCl_2$-induced hypoxia (500 μM for 12 h), fluorescence intensity was measured by flow cytometry.

regulated. FUZ knockdown resulted in lowered p21 expression but elevated cyclin B. P21 can directly blocks cell cycle progression by inhibiting the activity of cyclin B/CDC2 complexes that promote G2/M phase progression [27,28]. Therefore, our results support the view that FUZ promotes cell cycle progression by regulating cyclin B and p21.

Investigation of signaling pathways in A549 and H1299 cells revealed that FUZ over-expression caused dramatic activation of Raf/Ras/Erk1/2 and STAT3 pathways but little effect on p38 signals, and FUZ knockdown resulted in restrained signals. MAPKs are fundamental survival pathways activated and regarded as vital targets of clinical chemotherapeutic drugs [29]. Activations of the key molecules, such as Erk1/2 and STAT3, are responsible for enhanced cell proliferation of A549 and H1299. Our results suggested that FUZ promotes cell proliferation *via* activating Raf/Ras/ERK1/2 and STAT3 pathways.

Tumor metastasis is also an important cause of cancer-related deaths, metastasis is not only a sign of deterioration but also a major cause of treatment failure in patients with NSCLC and blockade of metastasis is always a key part of an efficient therapy [16]. FUZ overexpression significantly enhanced the mobility of NSCLC cells. The level of E-cadherin was induced downregulated, while the level of N-cadherin, Twist1, Snail, Zeb1 are upregulated after FUZ expression. E-cadherin is the marker of epithelial cells and loss of this marker suggests mesenchymal transition of cells (EMT). Besides, importantly, FUZ expression significantly upregulated the level of β-catenin, a key down stream effector in the Wnt signaling pathway [30]. It has been previously reported that β-catenin can directly activate the FUZ promoter [26]. In embryonic development, FUZ negatively regulates the Wnt/β-catenin signal activity, which suggests that FUZ constitutes a negative feedback loop to maintain Wnt/β-catenin signals controllable. However, in NSCLC cells, the expression of FUZ does not inhibit the activity of β-catenin anymore but reversely up-regulates the level, this is a severe consequence as FUZ and β-catenin constitute a forward feedback loop and the signals become dramatically activated. Besides, interestingly, similar to FUZ, reports on β-catenin are implicated in two major biological process in vertebrates: early embryonic development [31] and tumorigenesis [32]. Hence, the FUZ-β-catenin forward feedback loop could be a target for clinic treatment.

By means of yeast-two-hybrid analysis, FUZ is identified as a BNIP3 interacting protein. According to our experiments, the interaction of FUZ and BNIP3 lead to enhanced stability of these two proteins. Loss of FUZ resulted in weakened BNIP3 stability and vice versa. BNIP3 level is up-regulated under the induction of hypoxia and FUZ level is correspondingly induced elevated. BNIP3 is described as a pro-cell death protein in several types of cells. However, it was previously reported high-level expression of BNIP3 in several human carcinomas compared with normal tissues [24]. A study involving non-small cell lung carcinoma samples suggested a strong correlation between BNIP3 expression and poor prognosis [33]. Analyses of human tumor tissues suggest a correlation between up-regulation of BNIP3 expression and tumorigenesis. Besides, overexpression of BNIP3 lead to enhanced A549 growth [25] and BNIP3-related-hypoxia induced autophagy can mediate chemotherapy resistance in lung cancer [34]. All these studies indicate an important role of BNIP3 in NSCLC. Therefore, the FUZ-BNIP3 interaction could be an important therapeutic target.

5. Conclusion

Our data demonstrate that FUZ promotes NSCLC proliferation and the EMT process *in vitro*. In addition, we show that FUZ and β-catenin constitute a forward feedback loop, which drives the activation of downstream signals. FUZ is a BNIP3-interacting protein and the interaction enhances the stability of these two proteins. Therefore, our research *in vitro* indicates that FUZ could be a potential tumor marker and target for diagnosis and treatment.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.02.007.

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