UBE2S activates NF-κB signaling by binding with IκBα and promotes metastasis of lung adenocarcinoma cells

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Abstract
Purpose Nuclear factor (NF)-κB signaling in cancer cells has been reported to be involved in tumorigenesis. Phosphorylation and degradation of inhibitor of NF-κBα (IκBα) is a canonical pathway of NF-κB signaling. Here, we aimed to identify and characterize noncanonical activation of NF-κB signaling by ubiquitin-conjugating enzyme E2S (UBE2S) in lung adenocarcinoma cells.

Methods TCGA and the Human Atlas Protein Database were used to analyze the survival rate of lung adenocarcinoma patients in conjunction with UBE2S expression. In addition, PC9, H460, H441 and A549 lung adenocarcinoma cells were used in this study. PC9 and H460 cells were selected for further analysis because they expressed different UBE2S protein levels. Specific IKK inhibitors, PS1145 and SC514, were used to assess IκBα phosphorylation. Western blot analysis was used to assess protein levels in PC9 and H460 cells. A scratch wound-healing assay was used to analyze the migrative abilities of PC9 and H460 cells. Overexpression and knockdown of UBE2S in H460 and PC9 cells were used to analyze their effects on downstream protein levels. Immunoprecipitation, immunofluorescent staining, glutathione S transferase (GST) pull-down and in vitro binding assays were used to analyze the interaction between UBE2S and IκBα. A luciferase assay was used to analyze activation of NF-κB signaling regulated by UBE2S. An in vivo zebrafish xenograft model was used to assess metastasis of PC9 cells regulated by UBE2S.

Results We found that UBE2S expression in lung adenocarcinoma patients was negatively related to survival rate. The protein level of UBE2S was higher in PC9 cells than in H460 cells, which was opposite to that observed for IκBα. PC9 cells showed a higher UBE2S expression and migrative ability than H460 cells. Phosphorylation of IκBα was not changed by treatment with the IKK-specific inhibitors PS1145 and SC514 in PC9 and H460 cells. Overexpression and knockdown of UBE2S in H460 and PC9 cells were used to analyze their effects on downstream protein levels. Immunoprecipitation, immunofluorescent staining, GST pull-down and in vitro binding assays revealed direct binding of UBE2S with IκBα. Nuclear P65 protein levels and luciferase assays showed that NF-κB signaling was regulated by UBE2S. The expression of epithelial-to-mesenchymal (EMT) markers and the migrative ability of lung adenocarcinoma cells were also regulated by UBE2S. A zebrafish xenograft tumor model showed a reduction in the metastasis of PC9 cells that was induced by UBE2S knockdown.

Conclusions Higher UBE2S expression in lung adenocarcinomas may lead to increased binding with IκBα to activate NF-κB signaling and promote adenocarcinoma cell metastasis. UBE2S may serve as a potential therapeutic target for lung adenocarcinomas.

Keywords lung adenocarcinoma · UBE2S · IκBα · NF-κB · metastasis

1 Introduction

The ubiquitin-conjugating enzyme E2S (UBE2S) carrier protein is an E2 ubiquitination ligase that facilitates E1, E2 and E3 ligases to link ubiquitin with target proteins, which subsequently targets them for proteasome degradation [1–3]. The ubiquitin-proteasome pathway has been found to play critical roles in tumor formation and ...
progression [4]. The extent of UBE2S, which is highly expressed in several types of cancer, has been correlated with the progression of esophageal cancer [5], breast cancer [6], lung cancer [7], papillary renal cell carcinoma [8], endometrial cancer [9], glioblastoma multiforme [10] and colon cancer [11] development. UBE2S has been suggested to be a potential biomarker for diagnostic, prognostic and therapeutic purposes [12]. UBE2S is involved in the stabilization of hypoxia-inducible factor (HIF)-1α-induced tumorigenesis [13]. UBE2S has also been reported to be overexpressed in lung cancer [7, 14]. Nuclear factor (NF)-κB expression has been related to the tumor stage, lymph node metastasis and 5-year overall survival rate of non-small cell lung cancer patients [15].

NF-κB is constitutively activated in several types of cancer cells and is able to induce various aspects of tumorogenesis, including proliferation, anti-apoptosis, angiogenesis and metastasis [16]. Activation of NF-κB is generally involved in phosphorylation of inhibitor of NF-κBα (IκBα) by IκB kinase (IKK) for degradation. NF-κB is then translocated from the cytoplasm into nucleus where it binds to the promoter regions of target genes related to proliferation [17], anti-apoptosis [18, 19], drug resistance [20], angiogenesis and metastasis [21, 22]. Low IκBα and high constitutive NF-κB activities have been reported in adenocarcinoma cells, with only a weak NF-κB activity induced by tumor necrosis factor (TNF)-α treatment [23]. Stabilization of free IκBα is not regulated by IKK but, instead, is related to the presence of proline (P), glutamic acid (E), serine (S), threonine (T) (PEST) domains and proteasome degradation [24]. Degradation of IκBα to activate NF-κB signaling in cancer cells without IKK activation may play an important role in tumorogenesis.

Previously, we found that metastasis of tumor cells was activated by matrix metalloproteinase (MMP)-9 through epithelial-to-mesenchymal transition (EMT) signaling [25–30]. Snail and MMP-9 are downstream targets of NF-κB [31]. We also found that acceleration of nuclear entry of NF-κB activated the expression of EMT markers in A431-III cells [32]. Treatment with the dietary flavonoids luteolin and quercetin was able to inhibit stemness, reactive oxygen species (ROS), and the invasive capacity of A431-III cells by reducing EMT and inhibiting the phosphorylation of Akt and glycogen synthase kinase-3β (GSK3β) [32–34]. UBE2S is highly expressed and is known to promote the migratory and invasive abilities of cancer cells [30]. UBE2S is stabilized by Akt phosphorylation and is involved in DNA repair mechanisms by degradation of the Ku70 complex [10]. Akt in turn has been reported to activate NF-κB signaling for oncogenic transformation [35].

### 2 Materials and methods

#### 2.1 Chemicals and reagents

Dulbecco’s modified Eagle medium (DMEM), RPMI-1640 medium, 0.25 % trypsin, Ly294002 and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Cleveland, OH, USA). MG132, S3I-201, DMSO, paraformaldehyde (PFA), lysogeny broth (LB), MS222, isopropyl β-d-thiogalactopyranoside (IPTG) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). Protein G Mag Sepharose, glutathione Sepharose 4B GST-tagged protein purification resin, Ni Sepharose high-performance histidine-tagged protein purification resin and Amicon ultracentrifugation filters were obtained from GE (Marlborough, MA, USA). Primers were purchased from Purigo Biotech (Taipei, Taiwan). KAPA HiFi polymerase chain reaction (PCR) kits were purchased from Roche (Wilmington, MA, USA). PolyJet was purchased from SigmaGen Laboratories (Rockville, MD, USA). Luciferase assay reagents were purchased from Promega (Madison, WI, USA). Anti-UBE2S, anti-lamin-A and anti-GAPDH antibodies were obtained from GeneTex (Irvine, TX, USA). Anti-p65 antibody was acquired from Cell Signaling Technology (Danvers, MA, USA), whereas anti-IκBα, anti-phosphorylated (p)-IκBα, anti-matrix metalloproteinase (MMP)-9, anti-Twist and anti-E-cadherin (E-Cad) antibodies were obtained from Abcam (Cambridge, UK). Anti-hemagglutinin (HA), anti-flag, anti-His and anti-β-glutathione S-transferase (GST) antibodies were purchased from Santa Cruz (Capitola, CA, USA).

#### 2.2 Database search

The survival rate of lung adenocarcinoma patients correlating with UBE2S mRNA expression was analyzed using the GEPIA2 website (http://gepia2.cancer-pku.cn) [36]. The protein level of UBE2S in lung adenocarcinoma patients was deduced form CAB015228 antibody staining data in the Human Atlas Protein Database (https://www.proteinatlas.org) [37].

#### 2.3 Cell culture

H441, A549, PC9, and H460 human lung adenocarcinoma cell lines were obtained and maintained as described previously [38]. The cells were incubated in RPMI-1640 medium containing 10 % FBS (Thermo Fisher Scientific) in a 5 % CO₂ atmosphere at 37 °C.
2.4 Plasmid construction

The pcDNA3-UBE2S-flag plasmid was obtained as described previously [30]. pCMV4-3 HA/IκBα was purchased from Addgene (Cambridge, MA, USA). The coding region of IκBα was amplified using a KAPA HiFi PCR Kit (Roche) and cloned into a pcDNA3-HA vector as pcDNA3-IκBa-HA plasmid. The pcDNA3-IκBa-flag plasmid was digested with BamHI and SalI and inserted into a PQE30 vector as PQE30-IκBa plasmid. The pcDNA3-UBE2S-flag plasmid was digested with BamHI and XhoI and inserted into a pGEX-4T-2 vector as pGEX-UBE2S plasmid. The following primer pairs were used for PCR: IκBα-forward (5'-ATG TTC CAG GCG GCC G-3') and IκBα-reverse (5'-TAA CGT CAG ACG CTG G-3').

2.5 Cell viability assay

H460 and PC9 cells (10^5 cells/well) were seeded into 12-well plates overnight. PC9 cells were treated with DMSO, SC514, PS1145 and Ly294002 for 24 h. Cells were refreshed with culture medium with 5 mg/ml MTT solution (Merck), after which incubation was continued at 37 °C for 3 h. Next, the culture medium was removed and 200 µl DMSO was added to resolve the precipitate. Absorbance was measured at 570 nm using a Spark multimode microplate reader (TECAN, Männedorf, Switzerland).

2.6 Preparation of cell lysates

Cultured cells were washed three times with phosphate-buffered saline (PBS). Next, Gold lysis buffer (20 mM Tris-HCl at pH 7.9, 1 mM EGTA, 0.8 % NaCl, 0.1 mM β-glycerolphosphate, 1 mM sodium pyrophosphate, 10 mM NaF, 1 mM Na₃VO₄, 10 mM Na₃VO₄, 10 % glycerol, 1 % Triton X-100, 1 mM PMSF, 10 µg/ml aprotonin, and 10 µg/ml leupeptin) was used to lyse cells. Total cell lysates were collected by centrifugation at 14,000 g for 20 min at 4 °C and quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Cultured cells in 10-cm culture plates were harvested, and nuclear extracts were collected using a mini-preparation method [39–42].

2.7 Western blotting

Protein samples were heated to 100 °C with sample buffer (250 mM Tris-HCl at pH 6.8, 10 % sodium dodecyl sulfate (SDS), 30 % glycerol, 5 % β-mercaptoethanol and 0.02 % bromophenol blue) for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) was used to separate proteins, which were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 3 % bovine serum albumin (BSA) for 30 min at room temperature, a specific primary antibody diluted in 3 % BSA was incubated with the membrane on a shaker at 4 °C overnight. Next, the membrane was washed with PBST (0.25 % Tween-20 in PBS) three times, and incubated with a secondary antibody conjugated with horseradish peroxide (Millipore) for 1 h at room temperature. After washing with PBST three times, proteins were detected using an enhanced chemiluminescence (ECL) reagent kit (Millipore) followed by exposure to Amersham Imager 600 imagers (GE). Protein images were quantified using ImageJ software (http://rsb.info.nih.gov/ij/index.html, National Institutes of Health, Bethesda, MA, USA).

2.8 Transfection and immunostaining

COS-1 cells (10^5 cells/well) were seeded into 12-well plates overnight. Next, pcDNA3-UBE2S-HA and pcDNA3-IkBα-flag plasmids were transfected into the cells by PolyJet (SignaGen) for 24 h. PFA at 4 % was used to fix the cells for 20 min, and PBST (0.1 % Triton X-100 in PBS) was used to permeabilize them for 10 min. After washing with PBS three times and blocking with 3 % BSA for 1 h at room temperature, the cells were incubated with anti-HA and anti-flag monoclonal antibodies at 4 °C overnight. Next, Cy3 AffiniPure goat anti-mouse immunoglobulin G (IgG) was added and incubated for 30 min, after which the cells were stained with 0.1 % DAPI for 5 min. Images were captured using an Olympus IX70-FLA inverted fluorescence microscope (Olympus, Tokyo, Japan) and a SPOT system (Diagnostic Instruments, Sterling Heights, MI, USA).

2.9 UBE2S knockdown by small interfering (si)RNA

UBE2S and control siRNAs were purchased from Thermo Fisher Scientific and prepared following the manufacturer’s instructions. In total, 10^6 cells were seeded into six-well plates overnight and then transfected with 40 nM siRNA using a GenMute siRNA transfection reagent (SignaGen Laboratories, Rockville, MD, USA) following the manufacturer’s instructions. All assays were performed 24 h after transfection.

2.10 Luciferase assay

H460 and PC9 cells (10^5) were seeded into 12-well plates overnight. pGL3-5xB-Luc and pcDNA3-UBE2S-flag plasmids were obtained as described before [30, 43]. The pGL3-Basic, pGL3-5xB-Luc, control siRNA, UBE2S siRNA and pcDNA3-UBE2S-flag plasmids were transfected into the cells using PolyJet transfection reagent (SignaGen Laboratories) according to the manufacturer’s instructions. Total cell lysates were collected at 48 h post transfection. Luciferase activity was monitored using Luciferase Assay
Reagent (Promega) and detected using a Spark multimode microplate reader (Tecan, Mannedorf, Switzerland).

2.11 Expression and purification of GST-UBE2S and His-IkBα protein in Escherichia coli

PQE30-IkBα and pET4T2-UBE2S were transformed into E. coli DH5α cells after which expression was induced by 0.2 M IPTG. Cells grown overnight were regrown in 1/10 dilution of lysogeny broth (LB) for 1 h and induced by 0.2 M IPTG for 3 h. Next, the cells were collected by centrifugation and resuspended in cold GST buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA and a protease inhibitor cocktail) and His binding/wash buffer (0.5 M NaCl, 100 mM HEPES, 10 mM imidazole at pH 8.0, 0.5 % NP-40, 1 mM PMSF and a protease inhibitor cocktail). After processing the cells by an Untrasonic Processor (Chrom Tech, Apple Valley, MN, USA) on ice, the lysate was centrifuged at 13,000 g for 10 min at 4 °C. The supernatant was applied to glutathione Sepharose 4B GST-tagged protein purification resin (GE) and Ni Sepharose high-performance histidine-tagged protein purification resin (GE). After being washed with GST wash buffer (0.5% Triton X-100 and 1 mM EDTAU in PBS) and His binding/wash buffer, GST-UBE2S was resuspended in PBS. His-IkBα proteins were eluted with elution buffer (100 mM HEPES and 0.5 M imidazole at pH 8.0), followed by changing the buffer to PBS and filtering using Amicon Ultra Centrifugal Filters (GE).

2.12 Pull-down and in vitro binding assays

For the pull-down assay, GST-UBE2S proteins on beads were added to a total lysate of H460 cells and incubated at 4 °C overnight on a rolling shaker. After centrifugation at 500 g for 2 min and washing three times with PBS, the beads were resuspended in PBS and analyzed by Western blotting. The in vitro binding assay was performed by mixing equal volumes of GST-UBE2S and IkBα protein to interact at 4 °C overnight on a rolling shaker. After centrifugation at 500 g for 2 min and washing three times with PBS, the beads were resuspended in PBS and analyzed by Western blotting.

2.13 Immunoprecipitation (IP)

Cultured cells in 10-cm plates were collected and treated with IP lysis buffer (150 mM NaCl, 20 mM NaCl, 20 mM HEPES at pH 7.2, 10 mM NaF, 1 mM EDTA, 1 % NP-40, 1 mM Na3V04, 1 mM PMSF, 1 DTT and a protease inhibitor cocktail) for 10 min. Total cell lysates were collected by centrifugation at 13,000 rpm for 10 min. Pretreatment with protein G Mag Sepharose magnetic beads (GE) for 1 h was used to collect total proteins. An anti-UBE2S (GeneTex) or anti-mouse IgG (Abcam) monoclonal antibody was coated onto protein G Mag Sepharose magnetic beads (GE) for 1 h and washed with PBS three times. Coated protein G Mag Sepharose magnetic beads (GE) were added to total cell lysates, after which the complexes were pulled down overnight. After washing with PBS three times, the protein G Mag Sepharose complexes were immunoblotted using anti-UBE2S (GeneTex) or anti-IkBα (Abcam) monoclonal antibodies.

2.14 Scratch wound cell migration assay

PC9 cells (5 × 10⁵ cells/well) were seeded into six-well culture plates overnight in RPMI-1640 containing 10 % FBS. Next, monolayer cells were scratched with a pipette tip. After washing with PBS, the monolayers were incubated at 37 °C for 24 h. Images of the monolayers were captured at 0 and 24 h using a phase-contrast Zeiss Axio Vert.A1 inverted microscope (Zeiss, Jena, Germany) and a Leadview 2800AM-FL camera (Leadview, Taipei, Taiwan). Cell migration was calculated from triplicate determinations for each treatment group.

2.15 Zebrafish metastasis model

Zebrafish (Brachydanio rerio) embryos were supplied by the Zebrafish Core Facility of Taipei Medical University. The embryos were maintained in E3 buffer in a 28 °C incubator. All animal procedures were approved by the Institutional Animal Care and Use Committee or Panel (IACUC/IACUP) (protocol # LAC-2019-0355). The methods were carried out in accordance with the approved guidelines. The migration of cancer cells in zebrafish was analyzed as reported before [26, 44]. Briefly, GFP-expressing PC9 cells were transfected with control or UBE2S siRNA for 24 h. Next, the cells were detached with 0.25 % trypsin (Thermo Fisher) and stained with 1 μM CM-DiI (Thermo Fisher) for 20 min. After 3 washes with PBS, the cells were diluted to 2 × 10⁶ cells/ml with PBS and microinjected into the yolk of 3-days postfertilization (dpf) zebrafish larvae using an IM300 Microinjector (Narishige, Tokyo, Japan) at 30 psi. The zebrafish larvae were then incubated at 28 °C for 1 h and transferred to 32 °C for further incubation. Migrative tumor cells in zebrafish larvae were analyzed at 7 dpf using a phase-contrast Olympus IX70 microscope (Olympus, Tokyo, Japan) and a SPOT camera (Sterling Heights, MI, USA). The total number of zebrafish larvae containing migrative tumor cells was used to compare the migration of tumor cells between the knockdown and control groups.

2.16 Statistical analysis

Three independent experiments were used to analyze the mean ± standard deviation (SD). Statistically significant
differences were analyzed by one-way analysis of variation (ANOVA) followed by Tukey’s test. Statistical significance is indicated by * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).

3 Results

3.1 UBE2S expression affects the overall survival rate of lung adenocarcinoma patients

To investigate the role of UBE2S in lung adenocarcinoma patients, The Cancer Genome Atlas (TCGA) database [36] and Human Protein Atlas database [37] were used to analyze messenger (m)RNA and protein levels of UBE2S. In total 478 patients were separated into high and low groups at the median log2 value. We found that high levels of UBE2S mRNA were correlated with low survival rates in lung adenocarcinoma patients from the TCGA database (Fig. 1A). High and medium (HM) levels of UBE2S protein were noted in 81.8% of lung adenocarcinoma patients compared to low and not detectable (LN) levels by immunohistochemistry (IHC) in lung adenocarcinoma patients from the Human Protein Atlas database (Fig. 1B). These results indicate that UBE2S may play a role in the tumorigenesis of lung adenocarcinomas.

3.2 UBE2S expression levels affect IκBα, p-IκBα and p65 in lung adenocarcinoma cells

To assess the role of UBE2S in the tumorigenesis of lung adenocarcinomas in further detail, we analyzed UBE2S expression levels in H441, A549, PC9 and H460 cells. We found that UBE2S was higher expressed in PC9 cells than in H441, A549 and H460 cells (Fig. 2A). To investigate the role of UBE2S in NF-κB signaling, we analyzed the protein levels of UBE2S, IκBα and phosphorylated (p)-IκBα in H460 and PC9 cells that expressed different levels of UBE2S (Fig. 2A). The protein levels of IκBα were higher in H460 cells than in PC9 cells, which was opposite to the results obtained with UBE2S. The p-IκBα protein level was similar to that of UBE2S in these two cell lines (Fig. 2B). In a further analysis of p65 protein levels in the cytosol and nucleus, we found that PC9 cells expressed a higher level of p65 than H460 cells in both the cytosol and nucleus (Fig. 2C, S1). A scratch wound-healing assay revealed that PC9 cells exhibited greater migratory abilities than H460 cells (Fig. 2D, E). Collectively, these results suggest that UBE2S expression may be correlated with IκBα expression in lung adenocarcinoma cells.

3.3 UBE2S regulates IκBα stability without IKK activation

We found that the expression levels of UBE2S and IκBα, but not p-IκBα, were opposite in H460 and PC9 cells. UBE2S is an E2 ligase that regulates the degradation of proteins through polyubiquitination. To elucidate whether UBE2S regulates the degradation of IκBα and activates NF-κB signaling to promote tumorigenesis, we treated PC9 cells with the IKK inhibitors SC514 and PS1145 to reduce IκBα phosphorylation. We found that the protein levels of p-IκBα did not significantly differ between the control and treatment groups (Fig. 3A, B). In an earlier report, UBE2S was found to be activated by Akt and to be inhibited by Ly294002 [10]. We found that the protein levels of p-Akt and UBE2S were decreased and that of IκBα was increased after treatment with 5 and 10 µM Ly294002 (Fig. 3C). These data suggest that the reduction in IκBα in PC9 cells may not be induced...
by IKK phosphorylation, but instead correlates with UBE2S expression. To further analyze the relevance of UBE2S and IκBα expression in lung adenocarcinoma, UBE2S knockdown and overexpression was performed in PC9 and H460 cells to assess the effect on IκBα expression. We found that the protein levels of IκBα were increased after UBE2S knockdown with two small interfering (si)RNAs compared to control siRNA in PC9 cells (Fig. 3D). UBE2S overexpression in H460 cells showed that the IκBα protein levels decreased in a dose-dependent manner compared to the control group (vector) (Fig. 3E). Overexpression of UBE2S and treatment with MG132 in H460 cells led to retained IκBα protein levels compared to the control group (vector) (Fig. 3F). Collectively, these results indicate that UBE2S may regulate the stability of IκBα in PC9 and H460 cells.

3.4 UBE2S directly binds to IκBα

To assess whether UBE2S interacts with IκBα, COS-1 cells overexpressing UBE2S and IκBα were prepared. Subsequent immunostaining showed that UBE2S and IκBα colocalized in the cytosol in these cells (Fig. 4A, B). Next, we found that UBE2S precipitated with IκBα in PC9 cells using IP (Fig. 4C). To investigate whether UBE2S directly binds IκBα, GST-tagged UBE2S (GST-UBE2S) and His-tagged IκBα (His-IκBα) were individually expressed in PC9 cells (Fig. 4D, E). An in vitro pull-down assay showed that GST-UBE2S bound to IκBα in the cell lysates (Fig. 4F). An in vitro binding assay confirmed the binding of GST-UBE2S with His-IκBα (Fig. 4G). These data indicate a direct binding of UBE2S with IκBα.

3.5 UBE2S activates NF-κB signaling in lung adenocarcinoma cells

UBE2S directly bound to IκBα may degrade and promote activation of NF-κB downstream signaling. To assess NF-κB signaling activation by UBE2S, we conducted overexpression and knockdown experiments and subsequently analyzed p65 expression in PC9 and H460 cells. We found that UBE2S overexpression in H460 cells increased p65 protein levels in the nucleus and decreased its protein levels in the cytosol (Fig. 5A, S2). UBE2S knockdown in PC9 cells decreased p65 protein levels in the nucleus and increased its protein levels in the cytosol (Fig. 5B, S3). We additionally performed a luciferase reporter assay to analyze activation of NF-κB signaling by UBE2S. The pGL3-5×κB-Luc plasmid contains five repeats of κB-binding sites that are activated by NF-κB signaling. We found that the transactivation activity increased 13-, 23- and 35-fold after transfection with 0.25, 0.5 and 1 μg pGL3-5×κB-Luc compared to the control group (vector) (Fig. 5C, S4).
to pGL3-basic in H460 cells (Fig. 5C). After combined transfection with 0.5, 1 and 2 µg pcDNA3-UBE2S-flag and 0.5 µg pGL3-5xκB-Luc plasmid DNA, the transactivation activity of pGL3-5xκB-Luc increased 1.4-, 1.8- and 2.9-fold compared to the control group (pGL3-5xκB-Luc only) in H460 cells (Fig. 5D). In PC9 cells, the transactivation activity of pGL3-5xκB-Luc increased 40-, 72.2- and 81-fold after transfection with 0.25, 0.5 and 1 µg pGL3-5xκB-Luc compared to pGL3-basic (Fig. 5E). UBE2S expression knockdown with siRNA (siUBE2S#1 and -#2) decreased the transactivation activity to 42% and 53% of that of the control group (control siRNA) (Fig. 5F). These results indicate that UBE2S can activate NF-κB and EMT signaling in lung adenocarcinoma cells. Previously, we found that UBE2S can promote migration and invasion through EMT signaling in A431-III cells [30]. Collectively, these data indicate that UBE2S may activate NF-κB and EMT signaling in lung adenocarcinoma cells.

### 3.6 UBE2S promotes EMT signaling and metastasis of lung adenocarcinoma cells in a xenograft model

To investigate the function of UBE2S in the metastasis of lung adenocarcinoma cells, we first carried out a wound-healing experiment to analyze the migratory abilities of PC9 cells. We found that UBE2S knockdown by siRNA (siUBE2S#1 and -#2) decreased the migratory abilities to 42% and 23%, respectively, of control siRNA (Con) in PC9 cells (Fig. 6A, B). To investigate the function of UBE2S in activating EMT signaling, we prepared H460 and PC9 cells with UBE2S overexpression and knockdown. We found that UBE2S overexpression in H460 cells increased MMP-9 and TWIST protein levels while the E-Cadherin (E-Cad) protein levels decreased (Fig. 6C). Contrary, UBE2S knockdown by siRNA (siUBE2S#1 and -#2) decreased MMP-9 and TWIST protein levels while
the E-Cad protein levels increased (Fig. 6D). These data suggest that UBE2S may promote migration and EMT signaling in lung adenocarcinoma cells.

To next investigate the metastasis of lung adenocarcinoma cells in vivo, we prepared a xenograft zebrafish model. To this end, PC9 cells were transfected with control siRNA and UBE2S siRNA (siUBE2S#1, #2) for 24 h. Next, the cells were collected, labeled with CM-DiI (Fig. 6Ea-b) and microinjected into the yolk of zebrafish larvae at 2 days post-fertilization (dpf). Migrative tumor cells were evaluated in 7-dpf zebrafish larvae (Fig. 6Ec-e). We found that the migrative behavior of tumor cells with siUBE2S-mediated knockdown (Fig. 6Ef-k) was significantly decreased to 32 % and 33 % (Fig. 6Ei) compared to that of cells with control siRNA (Fig. 6Ec-e). These combined results suggest that UBE2S may activate the metastatic capacity of lung adenocarcinoma cells both in vitro and in vivo.

4 Discussion

We revealed novel UBE2S-mediated noncanonical activation of NF-κB signaling in lung adenocarcinoma cells. NF-κB signaling was activated by UBE2S in lung adenocarcinoma cells without activation of IKK. The expression of downstream EMT markers was also activated. We found that high mRNA expression levels of UBE2S in lung carcinoma patients were correlated with a poor survival rate in the TCGA database. Higher protein levels of UBE2S in lung carcinoma patients from the Human Protein Atlas were also observed. These data underscore an important role of UBE2S in lung adenocarcinoma. UBE2S has been reported to promote the proliferation and survival of adenocarcinoma cells by activating multiple genes, but detailed regulatory signaling mechanisms have remained unknown [14]. We analyzed the protein...
levels of UBE2S in various adenocarcinoma cells. The expression in PC9 was higher than that in H460, A549 and H441 cells. The stability of UBE2S is regulated by PI3K/Akt signaling [10]. NF-κB signaling is activated by Akt and correlates with oncogenic transformation [35]. This information raised the possibility that UBE2S may be involved in the activation of NF-κB signaling. Analysis of the protein levels of UBE2S, IκBα and p-IκBα in PC9 and H460 cells revealed simultaneous expression of IκBα, but not p-IκBα. The nuclear accumulation of p65 was higher in PC9 cells than in H460 cells. These data indicated activation of NF-κB signaling in PC9 cells by reducing IκBα, but not p-IκBα, which may be regulated by UBE2S. In a previous report it was shown that degradation of IκBα, but not p-IκBα, may induced continuous nuclear entry of p65 to activate NF-κB signaling [24]. In our present data, IκBα and p-IκBα protein levels were not affected by treatment with the specific IκB kinase (IKK) inhibitors SC514 and PS1145 in PC9 cells, but they were reduced by treatment with Ly294002, which has been reported to inhibit phosphorylation of UBE2S by Akt [10]. Overexpression of UBE2S in H460 cells increased protein levels of IκBα, accumulation of p65 in the nucleus, and transactivation activity of the 5x κB promoter. siRNA-mediated knockdown of UBE2S reversed these results, indicating a role of UBE2S in activating NF-κB signaling in these cells. Further analysis of the interaction between UBE2S and IκBα by immunoprecipitation, immunostaining, in vitro pull-down and binding assays revealed a direct binding of UBE2S with IκBα. Further analysis of downstream EMT markers, which are activated by NF-κB signaling, showed promotion of EMT by overexpression of UBE2S in H460 cells and reversal of EMT by knockdown of UBE2S in PC9 cells. Previously, we found that reversed

Fig. 5 Ubiquitin-conjugating enzyme E2S (UBE2S) activates nuclear factor (NF)-κB signaling in PC9 and H460 cells. A Overexpression of UBE2S (UBE2S/oe) in H460 cells increased nuclear localization of p65. B Knockdown of UBE2S by siRNAs (siUBE2S#1 and -#2) decreased nuclear localization of p65 in PC9 cells. Transactivation activity of the 5xκB promoter was induced by increasing the pGL3-5xxB-Luc (5xxB-Luc) C and pcDNA3-UBE2S-flag (UBE2S-flag) D plasmid DNA concentrations in H460 cells. In PC9 cells, transactivation activity of the 5xκB promoter was induced by increasing the pGL3-5xxB-Luc (5xxB-Luc) plasmid DNA concentration E and knockdown of UBE2S with siRNA (#1, and #2) compared to control siRNA F.
EMT reduced the migratory and invasive abilities of cancer cells. This raised the possibility of reducing the metastasis of lung cancer cells. We also reported that zebrafish may serve as an efficient in vivo tumor cell migration model [26, 44]. Here we found that UBE2S knockdown in PC9 cells reduced their metastatic ability in a zebrafish xenograft model. Collectively, we propose a novel noncanonical activation mechanism of NF-κB signaling by UBE2S in lung adenocarcinoma cells (Fig. 7).

UBE2S is an E2 ubiquitination ligase and is involved in different signaling pathways during tumorigenesis, and is involved in K11-linked polyubiquitination of component proteins to promote NF-κB signaling in tumor cells [1, 45, 46]. UBE2S promotes the proliferation and survival of lung adenocarcinoma cells [14]. In addition, UBE2S has been reported to mediate tumor progression through Sox6/β-catenin signaling in endometrial cancer [47], and has been associated with malignant properties of breast cancer [48], enhanced ubiquitination of p53 to exert oncogenic activities in hepatocellular carcinoma [49], mediated VHL/Hif1α/Stat3 signaling to promote EMT [50], and promoted the proliferation and survival of lung adenocarcinoma cells by activating the expression of multiple genes [14]. We and others have also shown that UBE2S can activate EMT signaling to promote migration and invasion of squamous cell carcinoma cells [30, 50]. Although UBE2S is involved in the tumorigenesis of multiple cancer types, its detailed mechanism of action has remained unclear. Here, we found using an in vitro binding assay that UBE2S directly binds with IκBα without adding other ubiquitination components. This finding indicates that the UBE2S-regulated stability of IκBα may not be brought about by ubiquitination, but potentially through other mechanisms. This result is similar to a previous report showing that UBE2S regulated the stability of Ku70 complexes during DNA repair [10]. The PEST domain of the C-terminal region of IκBα, which has been suggested to be a binding region of free IκBα for degradation [24],

Fig. 6 Metastasis of PC9 lung cancer cells is inhibited by siRNA-mediated knockdown of UBE2S. A UBE2S knockdown with siRNAs (siUBE2S#1 and #2) reduced the migratory activity of PC9 cells. B Statistical results of the migratory ability analyzed from A. Overexpression of UBE2S (UBE2S o/e) in H460 cells increased the protein levels of EMT markers. C UBE2S knockdown using siRNAs (siUBE2S#1 and #2) in PC9 cells D decreased the protein levels of EMT markers. E Metastasis analysis of PC9 cells in zebrafish larvae. The PC9 cells were labeled with CM-DiI (a-b). PC9 cells treated with siUBE2S (f-k) or control siRNA (c-e) and labeled with CM-DiI were microinjected into 3 dpf zebrafish larvae after which migrative PC9 cells were analyzed at 7 dpf zebrafish larvae. (l) Statistical analysis of metastatic PC9 cells in zebrafish larvae after knockdown with UBE2S (siUBE2S#1, #2) and control (control) siRNA.

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may be a potential site for UBE2S binding for degradation. This suggestion requires further investigation.

In earlier reports, NF-κB signaling has been suggested to represent an important tumorigenesis and drug-resistance pathway in lung cancer [51–53]. EMT signaling is activated by NF-κB signaling [54] and high constitutive NF-κB activity and low IκBα expression have been found in some cancer cell lines [55, 56]. Phosphorylation and degradation of IκBα by IKK to activate NF-κB signaling constitute the canonical pathway. The noncanonical NF-κB pathway may not involve degradation of IκBα and may instead be regulated by p100 [57]. However, it has been shown that NF-κB signaling was activated in I KK1/2-devoid mouse embryo fibroblasts. Degradation of IκBα was enhanced by doxorubicin-induced DNA damage and was inhibited by the phosphatidylinositol 3-kinase inhibitor LY294002 [58]. UBE2S is regulated by Akt and is associated with the DNA damage response brought about by the Ku70 complex [10]. In our present data, Ly294002 reduced the protein level of UBE2S and increased the protein level of IκBα in lung adenocarcinoma cells. Knockdown and overexpression of UBE2S in adenocarcinoma cells increased the nuclear entry of p65 and activated the transactivation activity of the NF-κB reporter. This activation may further induce EMT signaling, which is involved in migration and invasion in cancer cells [25–27, 30, 59]. The zebrafish xenograft model has recently been reported as a useful model for analyzing the migration and metastasis of tumor cells by us and others [26, 44, 60–63]. Here, in a zebrafish xenograft model, knockdown of UBE2S reduced the metastasis of PC9 cells. These results indicate that UBE2S may serve as a new therapeutic target in lung adenocarcinoma.

In conclusion, we found that UBE2S expression in primary lung adenocarcinomas was negatively related to survival. In addition, we found that UBE2S in lung adenocarcinomas directly binds IκBα to activate NF-κB signaling. As a result, downstream EMT signaling was activated to promote the metastasis of adenocarcinoma cells. UBE2S may be a potential therapeutic target for lung adenocarcinomas.

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**Data availability** All data generated or analyzed during this study are available.

**Declarations**
Not applicable.

**Consent for publication** Not applicable.

**Conflict of interests** The authors declare that they have no conflicts of interest.
References

1. A Bremm, D Komander, Emerging roles for Lys11-linked polyubiquitin in cellular regulation. Trends Biochem. Sci. 36, 355–363 (2011)
2. A Bremm, D Komander, Synthesis and analysis of K11-linked ubiquitin chains. Methods Mol. Biol. 832, 219–228 (2012)
3. KE Wickliffe, S Lorenz, DE Wemmer, J Kuriyan, M Rape, The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. Cell 144, 769–781 (2011)
4. AL Schwartz, A Ciechanover, The ubiquitin-proteasome pathway and pathogenesis of human diseases. Annu. Rev. Med. 50, 57–74 (1999)
5. MF Chen, KD Lee, MS Lu, CC Chen, MJ Hsieh, YH Liu, PY Lin, WC Chen, The predictive role of E2-EPF ubiquitin carrier protein in esophageal squamous cell carcinoma. J. Mol. Med. (Berlin) 87, 307–320 (2009)
6. D Tedesco, J Zhang, L Trinh, G Lalehzadeh, R Meisner, KD Yamaguchi, DL Ruderman, H Dinter, DA Zajchowski, The ubiquitin-conjugating enzyme E2-EPF is overexpressed in primary breast cancer and modulates sensitivity to topoisomerase II inhibition. Neoplasia 9, 601–613 (2007)
7. J Liang, H Nishi, ML Bian, C Higuma, T Sasaki, H Ito, K Isaka, The ubiquitin-conjugating enzyme E2-EPF is overexpressed in cervical cancer and associates with tumor growth. Oncol. Rep. 28, 1519–1525 (2012)
8. FC Roos, AJ Evans, W Brenner, B Wondergem, J Klomp, P Heir, O Roche, C Thomas, H Schimmel, KA Furge, BT Teh, JW Thuroff, C Hampel, M Ohh, Deregulation of E2-EPF ubiquitin carrier protein in papillary renal cell carcinoma. Am. J. Pathol. 178, 853–860 (2011)
9. M Lin, T Lei, J Zheng, S Chen, L Du, H Xie, UBE2S mediates tumor progression via SOX6/beta-Catenin signaling in endometrial cancer. Int. J. Biochem. Cell Biol. 109, 17–22 (2019)
10. L Hu, X Li, Q Liu, J Xu, H Ge, Z Wang, H Wang, Z Wang, C Shi, X Xu, J Huang, Z Lin, RO Pieper, C Weng, UBE2S, a novel substrate of Akt1, associates with Ku70 and regulates DNA repair and glioblastoma multiforme resistance to chemotherapy. Oncogene 36, 1145–1156 (2017)
11. Z Li, Y Wang, Y Li, W Yin, L Mo, X Qian, Y Zhang, G Wang, F Bu, Z Zhang, X Ren, B Zhu, C Niu, W Xiao, W Zhang, Ube2s stabilizes beta-Catenin through K11-linked polyubiquitination to promote mesendoderm specification and colorectal cancer development. Cell Death Dis. 9, 456 (2018)
12. SM Hosseini, I Okoye, MG Chaleshtari, B Hazhirkarzar, J Mohamadnejad, G Azizi, M Hojat-Farsangi, H Mohammadi, SS Shotorbani, F Jaddi-Niaraghi, E2 ubiquitin-conjugating enzymes in cancer: Implications for immunotherapeutic interventions. Clin. Chim. Acta. 498, 126–134 (2019)
13. CR Jung, KS Hwang, J Yoo, WK Cho, JM Kim, WH Kim, DS Im, E2-EPF UCP targets pVHL for degradation and associates with tumor growth and metastasis. Nat. Med. 12, 809–816 (2006)
14. Z Lin, L Xu, UBE2S promotes the proliferation and survival of human lung adenocarcinoma cells. BMB Rep. 51, 642–647 (2018)
15. L. Gu, Z. Wang, J. Zuo, H. Li, L. Zha, Prognostic significance of NF-kappaB expression in non-small cell lung cancer: A meta-analysis. PLoS One 13, e0198823 (2018)
16. P Viator, MP Merville, Y Bours, A Chariot, Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem. Sci. 30, 43–52 (2005)
17. Y. Cao, G. Bonizzi, T.N. Seagroves, F.R. Greten, R. Johnson, E.V. Schmidt, M. Karin, IKKalpha provides an essential link between RANK signaling and cyclophilin D1 expression during mammary gland development. Cell 107, 763–775 (2001)
18. M Karin, A Lin, NF-kappaB at the crossroads of life and death. Nat. Immunol. 3, 221–227 (2002)
19. GA Webster, ND Perkins, Transcriptional cross talk between NF-kappaB and p53. Mol. Cell. Biol. 19, 3485–3495 (1999)
20. CY Wang, JC Cusack Jr., R Liu, AS Baldwin Jr., Control of inducible chemo-resistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat. Med. 5, 412–417 (1999)
21. H. Takeshita, T. Yoshizaki, W.E. Miller, H. Sato, M. Furukawa, JS. Pagano, N. Raab-Traub, Matrix metalloproteinase 9 expression is induced by Epstein-Barr virus latent membrane protein 1 C-terminal activation regions 1 and 2. J. Virol. 73, 5548–5555 (1999)
22. M Bond, RP Fabunmi, AH Baker, AC Newby, Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappaB. F. EBFS Lett. 35, 29–34 (1998)
23. E Dejardin, V Deregowski, M Chapelier, N Jacobs, J Gielen, MP Merville, V Bours, Regulation of NF-kappaB activity by I kappaB-related proteins in adenocarcinoma cells. Oncogene 18, 2567–2577 (1999)
24. E Mathes, EL O’Dea, A Hoffmann, G Ghosh, NF-kappaB dictates the degradation pathway of IkappaBalpha. EMBO J. 27, 1357–1367 (2008)
25. KC Chen, WH Hsu, JY Ho, CW Lin, CY Chu, CC Kandaswami, MT Lee, CH Cheng, Flavonoids Luteolin and Quercetin Inhibit RPM19 and contributes to metastasis of cancer cells through c-Myc reduction. J. Food Drug Anal. 26, 1180–1191 (2018)
26. JI Fan, WH Hsu, K.C. Lee, K.C. Chen, CW Lin, Y.A. Lee, T.P. Ko, L.T. Lee, M.T. Lee, M.S. Chang, C.H. Cheng, Dietary flavonoids luteolin and quercetin inhibit migration and invasion of squamous carcinoma through reduction of Src/Stata/S100A7 signaling. Antioxidants (Basel) 8, 557–573 (2019)
27. WT Kao, CY Lin, LT Lee, PP Lee, CC Hung, YS Lin, SH Chen, FC Ke, JI Hwang, MT Lee, Investigation of MMP-2 and – 9 in a highly invasive A431 tumor cell sub-line selected from a Boyden chamber assay. Anticancer Res. 28, 2109–2120 (2008)
28. CW Lin, GM Lai, KC Chen, TH Lin, JI Fan, RL Hsu, CM Chou, CM Lin, CC Kandaswami, MT Lee, CH Cheng, RPS12 increases the invasiveness in cervical cancer activated by c-Myc and inhibited by the dietary flavonoids luteolin and quercetin. J. Funct. Foods 19, 236–247 (2015)
29. CY Lin, PH Tsai, CC Kandaswami, PP Lee, CJ Huang, JI Hwang, MT Lee, Matrix metalloprotease-9 cooperates with transcription factor Snail to induce epithelial-mesenchymal transition. Cancer Sci. 102, 815–827 (2011)
30. TH Lin, WH Hsu, PH Tsai, YT Huang, CW Lin, KC Chen, IH Tsai, CC Kandaswami, CJ Huang, GD Chang, MT Lee, CH Cheng, Dietary flavonoids, luteolin and quercetin, inhibit invasion of cervical cancer by reduction of UBE2S through epithelial-mesenchymal transition signaling. Food Funct. 8, 1558–1568 (2017)
31. R Malaguarnera, A Belliore, The emerging role of insulin and insulin-like growth factor signaling in cancer stem cells. Front Endocrinol. (Lausanne) 5, 10 (2014)
32. CY Lin, PH Tsai, CC Kandaswami, GD Chang, CH Cheng, CJ Huang, PP Lee, JI Hwang, MT Lee, Role of tissue transglutaminase 2 in the acquisition of a mesenchymal-like phenotype in highly invasive A431 tumor cells. Mol. Cancer 10, 87 (2011)
33. PH. Tsai, C.H. Cheng, C.Y. Lin, Y.T. Huang, L.T. Lee, C.C. Kandaswami, Y.C. Lin, K.P. Lee, C.C. Hung, J.I. Hwang, F.C. Ke, G.D. Chang, M.T. Lee, Dietary flavonoids luteolin and quercetin suppressed cancer stem cell properties and metastatic potential of isolated prostate cancer cells. Anticancer Res. 36, 6367–6380 (2016)
34. JJ Fan, WH Hsu, HH Hung, WJ Zhang, YA Lee, KC Chen, CY Chu, TP Ko, MT Lee, CW Lin, CH Cheng, Reduction in MnSOD
promotes the migration and invasion of squamous carcinoma cells. Int. J. Oncol. 54, 1639–1650 (2019)

35. D Bai, L Ueno, PK Vogt, Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of P13K and Akt. Int. J. Cancer. 125, 2863–2870 (2009)

36. Z Tang, B Kang, C Li, T Chen, Z Zhang. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. Nucleic Acids Res. 47, W556–W560 (2019)

37. M Uhlen, L Fagerberg, BM Hallstrom, C Lindskog, P Oksvold, A Mardinoglu, A Sivertsson, C Kampf, E Sjostedt, A Asplund, I Olsson, K Edlund, E Lundberg, S Navani, CA Szgyarto, J Odeberg, D Djureinovic, JO Takanen, S Hober, T Alm, PH Edqvist, H Berling, H Tegel, J Mulder, J Rockberg, P Nilsson, JM Schwenk, M Hamsten, K von Felitzen, M Forsberg, L Persson, F Johansson, S Mzwalhen, G von Heijne, J Nielsen, F Ponten. Proteomics. Tissue-based map of the human proteome. Science 347, 1260419 (2015)

38. W.Y. Lee, P.C. Chen, W.S. Wu, H.C. Wu, C.H. Lan, Y.H. Huang, C.H. Cheng, K.C. Chen, C.W. Lin. Panobinostat sensitizes KRAS-mutant non-small-cell lung cancer to gefitinib by targeting TAZ. Int. J. Cancer. 141, 1921–1931 (2017)

39. CH Cheng, CM Chou, CY Chu, GD Chen, HW Lien, PP Hwang, MS Chang, CJ Huang. Differential regulation of Tetradon nigroviroidi Mi gene promoter activity by constitutively-active forms of STAT1, STAT2, and IRF9. Fish Shellfish Immunol. 38, 230–243 (2014)

40. F Derckere, F Gannon. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. Biotechniques 16, 405 (1994)

41. C.J. Huang, C.M. Chou, H.W. Lien, C.Y. Chu, J.Y. Ho, Y. Wu, C.H. Cheng. IRF9-Stat2 fusion protein as an innate immune inducer to activate mx and interferon-stimulated gene expression in zebrafish larvae. Mar. Biotechnol. (NY). 19, 310–319 (2017)

42. HW Lien, RY Yuan, CM Chou, YC Chen, CC Hung, CH Hu, SP Hwang, PP Hwang, CN Shen, CL Chen, CH Cheng, CJ Huang. Zebrafish cyclin Dx is required for development of motor neuron progenitors, and its expression is regulated by hypoxia-inducible factor 2alpha. Sci. Rep. 6, 28297 (2016)

43. WT Lee, TH Lee, CH Cheng, KC Chen, YC Chen, CW Lin, Antroquinol from Antrodia Camphorata suppresses breast tumor migration/invasion through inhibiting ERK-AP-1- and AKT-NF-kappaB-dependent MMP-9 and epithelial-mesenchymal transition expressions. Food Chem. Toxicol. 78, 33–41 (2015)

44. WJ Hsu, MH Lin, TC Kuo, CM Chou, FL Mi, CH Cheng, CW Lin. Fucoidan from Laminaria japonica exerts antitumor effects on angiogenesis and micrometastasis in triple-negative breast cancer cells. Int. J. Biol. Macromol. 149, 600–608 (2020)

45. ZH Wu, Y Shi. When ubiquitin meets NF-kappaB: a trove for anti-cancer drug development. Curr. Pharm. Des. 19, 3263–3275 (2013)

46. JN Dynek, T Goncharov, EC Dueber, AV Fedorova, A Izraelovic, L Phu, E Helgason, WJ Fairbrother, K Deshayes, DS Kirkpatrick, D Vucic, c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J. 29, 4198–4209 (2010)

47. K.C. Hung, T.C. Huang, C.H. Cheng, Y.W. Cheng, D.Y. Lin, J.J. Fan, K.H. Lee. The expression profile and prognostic significance of metallothionein genes in colorectal cancer. Int. J. Mol. Sci. 20, 3849–3860 (2019)

48. AK Ayesha, T Hyodo, E Asano, N Sato, MA Mansour, S Ito, M Hamaguchi, T Senga. UBE2S is associated with malignant characteristics of breast cancer cells. Tumour Biol. 37, 763–772 (2016)

49. YH Pan, M Yang. LP Liu, DC Wu, MY Li, SG Su. UBE2S enhances the ubiquitination of p53 and exerts oncogenic activities in hepatocellular carcinoma. Biochem. Biophys. Res. Commun. 503, 895–902 (2018)

50. L. Wang, Y. Liang, P. Li, Q. Liang, H. Sun, D. Xu, W. Hu. Oncogenic activities of UBE2S mediated by VHL/HEF-1alpha/STAT3 signal via the ubiquitin-proteasome system in PDAC. Onco Targets Ther. 12, 9767–9781 (2019)

51. TG Bivona, H Hieronymus, J Parker, K Chang, M Taron, R Rosell, P Moonsamsy, K Dahlan, VA Miller. C Costa, G Hannon, CL Sawyers. FAS and NF-kappaB signalling modulate dependence of lung cancers on mutant EGFR. Nature 471, 523–526 (2011)

52. W. Chen, Z. Li, L. Bai, Y. Lin. NF-kappaB in lung cancer, a carcinogenesis mediator and a prevention and therapy target. Front. Biosci. (Landmark Ed.). 16, 1172–1185 (2011)

53. Z Cai, KM Tchou-Wong, WN Rom, NF-kappaB in lung tumorigenesis. Cancers (Basel) 3, 4258–4268 (2011)

54. MA Huber, A Azoitei, B Baumann, S Grunert, A Sommer, H Pehamberger, N Kraut, H Beug, T Wirth, NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. J. Clin. Invest. 114, 569–581 (2004)

55. K Taniguchi, M Karin. NF-kappaB, inflammation, immunity and cancer: coming of age. Nat. Rev. Immunol. 18, 309–324 (2018)

56. M Karin. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene 18, 6867–6874 (1999)

57. C.H. Shin, D.S. Choi. Essential roles for the non-canonical IkappaB kinases in linking inflammation to cancer, obesity, and diabetes. Cells 8, 178 (2019)

58. V Tergaonkar, V Bottero, M Ikawa, Q Li, IM Verma. IkappaB kinase-independent IkappaBalpha degradation pathway: functional NF-kappaB activity and implications for cancer therapy. Mol. Cell. Biol. 23, 8070–8083 (2003)

59. YS Lin, PH Tsai, CC Kandaswami, CH Cheng, FC Ke, PP Lee, JJ Hwang, MT Lee. Effects of dietary flavonoids, luteolin, and quercetin on the reversal of epithelial-mesenchymal transition in A431 epidermal cancer cells. Cancer Sci. 102, 1829–1839 (2011)

60. HK Brown, K Schiavone, S Tazzyman, D Heymann, TJ Chico, A431 epidermal cancer cells. Cancer Sci. 102, 379–389 (2017)

61. J.T. Gamble, DJ. Elson, J.A. Greenwood, R.L. Tanguay, S.K. Kolluri, The zebrafish xenograft models for investigating cancer and cancer therapeutics. Biology (Basel) 10, 252 (2021)

62. W Shen, J Pu, J Sun, B Tan, W Wang, L Wang, J Cheng, Y Zuo. Zebrafish xenograft model of human lung cancer for studying the function of LINC00152 in cell proliferation and invasion. Cancer Cell Int. 20, 376 (2020)

63. ME Avci, AG Keskus, S Targen, ME Isilak, M Ozturk, RC Atalay, MM Adams, O Konu. Development of a novel zebrafish xenograft model in acah mutants using liver cancer cell lines. Sci. Rep. 8, 1570 (2018)

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