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Tumor suppressive p53 signaling empowers metastatic inhibitor KLF17-dependent transcription to overcome tumorigenesis in non-small cell lung cancer

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Running Title: KLF17 suppresses EMT via p53-dependent pathway in NSCLC.

Key words: Metastasis, KLF17, tumor suppressor p53, lung cancer, chemotherapy.

Capsule:

Background: How Kruppel-like-factor 17 (KLF17) controls metastasis and epithelial-mesenchymal-transition (EMT) during cancer progression remains unknown.

Results: Tumor-suppressive p53 signaling is critical for KLF17 to inhibit cancer metastasis in NSCLC.

Conclusion: These results indicate novel insights into anti-EMT affect of KLF17 via p53-dependent pathway.

Significance: Targeting KLF17 for cancer therapy may be applicable to NSCLC tumors with TP53 status, which may improve prognosis of NSCLC patients.

Abstract

Metastasis, which is controlled by concerted action of multiple genes, is a complex process, and is important cause of cancer death. Kruppel-like-factor 17 (KLF17) is a negative regulator of metastasis and epithelial-mesenchymal-transition (EMT) during cancer progression. However, the underlying molecular mechanism and biological relevance of KLF17 in cancer cells are poorly understood. Here, we show that tumor suppressor protein p53 plays an integral role to induce KLF17 expression in NSCLC. p53 is recruited to KLF17 promoter and results in the formation of p53-DNA complex. p53 enhances binding of p300, and favors histone acetylation on KLF17 promoter. Mechanistically, p53 physically interacts with KLF17 and thereby enhances anti-metastatic function of KLF17. p53 empowers KLF17 mediated EMT genes transcription via enhancing physical association of KLF17 to target gene promoters. Nutlin-3 recruits KLF17 to EMT target gene promoters and results in the formation of KLF17-DNA complex via p53-dependent pathway. p53 depletion abrogates DNA binding affinity of KLF17 to EMT target gene promoters. KLF17 is critical for p53 cellular activities in NSCLC. Importantly, KLF17 enhances p53 transcription to generate a novel positive feedback loop. KLF17 depletion accelerates lungs cancer cells growth in response to chemotherapy. Mechanistically, we found that KLF17 increases tumor suppressor genes p53, p21 and pRB expressions in NSCLC. Functionally, KLF17 required p53 to suppress cancer cell invasion and migration in NSCLC. In conclusion, our study highlights novel insight into anti-EMT affect of KLF17 via p53-dependent pathway in NSCLC, and KLF17 may be a new therapeutic target in NSCLC with p53 status.

Introduction

Non-small-cell lung cancer (NSCLC) is aggressive type of lung cancer and about 80% lung cancers are NSCLC [1]. Lung cancer is one of leading cause of cancer-associated death and can be divided into squamous cell carcinoma, adenocarcinoma, and large-cell lung carcinoma [2]. Despite of recent advances and improvements in the field of chemotherapy for lung cancer, still the prognosis of NSCLC is very poor and about 30–55% NSCLC patients show recurrence after chemotherapy [3]. Therefore, it is important to assess the important molecular mechanisms that are involved in inhibition of NSCLC for better treatment and prognosis of NSCLC patients.

KLF17 is a negative regulator of epithelial-mesenchymal-transition (EMT) and metastasis. Depletion of KLF17 promotes EMT and metastasis [4, 5]. Decrease expression of KLF17 has been detected in invasive breast cancer and adenocarcinoma cell lines [4-7]. In addition, KLF17 expression is an important predictor for lymph node metastasis in cancer [4, 6, 7]. Overexpression of KLF17 in cancer cell lines has been shown to inhibit cancer cells growth [7, 8]. Patients having low expression of KLF17 show greater tumor size, later pathological stage, and poor prognosis [4, 7, 8]. KLF17 is also tumor suppressor transcription factor and binds to CACCC sequence on target gene promoters [9, 10]. Recent studies showed that KLF17 inhibits EMT and metastasis by binding directly to the promoters of genes involved in EMT such as ID1, Vimentin, Fibronectin, ZO-1 and E-Cadherin and regulate their expressions [4, 7].

Tumor suppressor p53 known as “guardian of the genome”, and function as an important barrier against cancer [11-14]. Analysis of human cancers indicates a critical role for tumor suppressor p53 in cancer prevention. Tumor suppressor p53 functions are context-dependent and influenced by numerous factors [14-16]. Genetic studies of p53 showed that p53 have anti-proliferative functions and inhibits cancer cell growth [17, 18]. p53 functions as a transcription factor, and have ability to trigger multiple tumor suppressive pathways by targeting key genes [19-21]. Once, p53 activated in response to DNA damage, and then it leads to induction of apoptosis, suppression of cell cycle progression and subsequently leads to cancer inhibition [22, 23].

EMT program is often activated during cancer progression [24, 25]. During EMT, many transcription factors directly repress the adherent junction mediator E-Cadherin [26-28]. Metastasis is a complicated multi-step process, which finally leads to metastatic tumor development [29-31]. EMT is considering as a critical process for cancer progression to a metastatic stage [32].

Here, we report novel molecular and functional link of KLF17 with p53. We showed that p53 empowers tumor suppressive KLF17 signaling during cancer metastasis. KLF17 suppresses EMT and metastasis in a p53-dependent manner in NSCLC. Mechanistically, p53 forms a complex with KLF17 and hence potentiates KL17 mediated EMT genes transcription and tumor suppressive function of KLF17. Intriguingly, chemotherapeutic agents that activate p53 show recruitment of KLF17 to EMT target gene promoters via p53-dependent pathway. Moreover, our results show that novel crosstalk and positive feedback loop exist between p53 and KLF17 in NSCLC and play an important role in the inhibition of cancer

metastasis. Functionally, KLF17 required p53 to suppress cancer metastasis. Taken together, our results describe novel molecular and functional insights into anti-EMT affect of KLF17 via p53-dependent pathway in NSCLC. KLF17 may be a new therapeutic target with p53 status in NSCLC, which may improve treatment and prognosis of NSCLC patients.

Materials and Methods

Plasmids and Transfection

pcDNA3.1-p53 plasmid was described in our previous study[33]. H1299 and A549 lung cancer cell lines were transfected with Lipofectamine 2000 following manufactures protocol (Invitrogen).

Antibodies

Following antibodies were used in Western Blot and ChIP experiments. Anti-p53 (DO-1, Santa Cruz), anti-KLF17 (Abcam), anti-GAPDH (Santa Cruz), anti-β-actin (Santa Cruz), anti-P300 (Santa cruz), anti-AcH4 (Millipore).

Cell culture and Treatments

H1299 and A549 cells were described previously in our study [33]. For cells treatments we used Nutlin-3 (10 μM) (Sigma-Aldrich), Adriamycin (0.5 μM) (Sigma-Aldrich) and Etoposide (10μM) (Sigma-Aldrich).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed with ^{32}P-radiolabeled probes. 2 μg of nuclear extract or different Concentration of purified proteins was incubated with ^{32}P-radiolabeled-probes in 20μl of EMSA reaction buffer (2 μg of poly (dI-dC), 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 12% glycerol). To perform the competition assay, excess of unlabeled competitor's oligo was added to the EMSA reaction mixture. Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5x TBE and exposed to phoshoimager (Bio-Rad).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed as described previously [33]. PCR-amplification of the genomic-fragments was performed with specific primers flanking putative binding-sites on the KLF17 promoter. The PCR products were separated by electrophoresis through 2.0% agarose.

KLF17 luciferase reporter constructs

DNA-fragments containing KLF17genomic sequences were amplified from 293T cell genomic DNA using the polymerase chain reaction and primers derived from human genomic KLF17 and ligated into kpn1/xhol sites of the promoterless pGL3-Basic (Promega) vector and was named as pGL3-KLF17-luc. Different deletion constructs of KLF17 promoter were generated from 2kb KLF17 promoter and ligated into kpn1/xhol sites of pGL3-Basic vector.

Luciferase Assay

After transfection and/or treatment, the cells were washed with phosphate buffered saline (PBS) 3 times. The cells were then lysed in the luciferase cell culture lysis buffer provided with the luciferase Assay Kit (Promega,

Madison, USA). After a brief vortex, whole cell lysates were centrifuged in the cold (4°C) at 12000 rpm for 2 min. Supernatant was collected in a fresh-tube and 20-30 ml of that was added to luciferase assay substrate (60-80 ml). Luminescence was measured as relative-light-units (RLU), twice for each lysate, taking the reading of luciferase assay using a LUMIstar OPTIMA, BMG LABTECH. Each assay was repeated for three times. Fold repression values were represented as mean of the three experiments.

RNA Interference

Cells were cultured to 30% confluence. For each well in a 6-well culture dish, 20nM of KLF17/p53 siRNAs or appropriate negative controls siRNAs was transfected into cells using Lipofectamine 2000 (Invitrogen) following manufactures protocol. Cells were incubated at 37°C in a CO2 incubator, and 6-8 hr later, 10% serum growth medium was added to the transfection mixture. Cell-extracts were assayed by Western blot for KLF17/p53 protein expression at 72 hr post transfection, while for mRNA expression at 48 hr after transfection. Please see the supplementary information for primer sequences.

RT-PCR

RT-PCR was performed as described previously (33). Each experiment was performed in duplicates, and repeated thrice.

Preparation of total cell extract and western blot analysis

Cells were washed with PBS and treated with an extraction buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium-deoxycholate, 150mM NaCl, and 1 mM EDTA) supplemented with 1 mM phenylmethanesulfonyl-fluoride, 1 mM sodium-orthovanadate (Na3VO4), 0.1 mM dithiothreitol, 0.4 µg/ml leupeptin/ pepstatin). Cell extract was stored at -20°C until required. Protein samples were subject to electrophoresis in 10% SDS polyacrylamide-gel. Separated proteins were electroblotted to Nitrocellulose membranes (Bio-Rad), and blot was blocked for 1 hr at room temperature with blocking buffer 0.1% PBST with 5% fat-free dried milk powder. Blot was then incubated with primary antibodies, (1:1000 dilution) at 4°C overnight. Blot was washed with 0.1% TBST 3 times, and incubated with secondary antibodies (mouse, rabbit) (1:5000 dilution) for 1 hr. Blot was washed again 3 times and exposed to Odyssey LI-COR-scanner

MTT assay

Cell viability was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny ltetrazolium-bromide (MTT) assay in replicates. Cells were seeded in 96-well plate at 2.5×10^3 cells/well, and incubated in 10% FBS supplemented with DMEM for 24 hr. After that cells were treated with Nutlin-3/Adriamycin for indicated time points. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. After that drug-containing medium was replaced with 200µL of 10% FBS supplemented with DMEM containing 0.5 mg/mL MTT, and cells were incubated in the CO_2 incubator at 37°C for 2 hr, and absorbance (490 nm) was measured and analyzed.

Cell migration

Cell culture inserts with a pore size of 8 µm

were pre-coated by adding 10 μg/mL Collagen I in PBS to the upper chamber at room temperature for 2 hr. At the onset of each experiment, the cells were detached with Versene and resuspended as single cells in serum-free DMEM. For the migration experiments, we took 25,000 cells in 0.5 mL and diluted them in serum-low DMEM (supplemented with 2% FBS). Cells were added to the upper chamber and the lower chamber was filled with 0.70 mL DMEM supplemented with 10% FBS. The cells were allowed to migrate for 24 hr at 37°C in a humidified atmosphere of 5% CO_2. The experiment was terminated by discarding the medium and fixing the cells in the filter with 4% paraformaldehyde for 10 min. Non-invading cells on the upper side of the insert were removed by a cotton-tipped applicator. Staining of the cells on the bottom of the membrane was performed with DAPI (300 nM in PBS) for 5 min at room temperature and washed with PBS. Membranes were excised from the inserts and mounted on slides using Dako Fluorescent Mounting Medium. Cells were either counted manually or with the help of the Image J software.

Cell invasion

Cell invasion experiments were performed in BD Matrigel™ invasion chambers (BD Biosciences) with a membrane pore diameter of 8 μm. For the invasion experiments, we used 50,000 cells per well suspended in 0.5 mL serum-low DMEM (supplemented with 2% FBS). The rest of the protocol was identical to that described in the migration assay.

Results

p53 enhances metastatic suppressor KLF17 transcription

KLF17 negatively regulates metastasis [4-7]. Tumor suppressive p53 plays a key role in the inhibition of tumorigenesis by regulating the expression of several important genes [11-13]. However, the affect of p53 on KLF17 transcription remains unknown during cancer progression. Therefore, we investigated the role of p53 on KLF17 regulation in A549 cells. A549 cell lines express tumor suppressive p53 (TP53) [34, 35]. We depleted p53 expression in A549 cells using specific siRNA targeting p53. We found that KLF17 mRNA levels induced in a dose-dependent manner in response to Nutlin-3 (Fig. 1A). In contrast, we did not observed any induction in KLF17 transcript levels in p53 depleted cells treated with Nutlin-3 (Fig. 1A), suggesting that p53 is important for KLF17 induction. Nutlin-3 activates/stabilizes p53 by blocking the interaction between mdm2 and p53 [36, 37]. Furthermore, silencing of p53 by siRNA resulted in decrease expression of KLF17 in lung cancer cells (Figs. 1B-1E). We treated A549 cells with different p53 activating chemotherapeutic agents, and observed that these drugs were able to induce KLF17 expression (Fig. 1F). To further verify our results, we performed protein analysis of KLF17 in response to Nutlin-3. We found that KLF17 protein level induced in Nutlin-3 treated cell lines (Fig. 1G). Taken together, our data suggest that p53 enhances KLF17 mRNA and protein levels in lung cancer cells.

Next, we aimed to analyze the transcriptional regulation of KLF17 by p53. In a dose dependent manner, p53 was able to induce transcriptional activity of KLF17-luc reporter in H1299 (p53 null) lung cancer cells (Fig.

1H). Next, we generated different deletion constructs of KLF17-luc to find minimal responsive region required for KLF17 activation by p53 (Fig. 1I). Luciferase analysis indicated the region between (-2000/-498) is responsive to p53 (Fig. 1J). While, region beyond the (-498/+1) was unable to enhance the KLF17 transcription (Fig. 1J), suggesting that p53 responsive region lies between (-1015/-498) region. Using the NCBI database we found p53 consensus responsive element (p53RE) within KLF17 promoter (Fig. 1J). Mutation analysis of this p53RE indicated that p53 was unable to activate the KLF17 transcription from this mutated region (Fig. 1K). This data indicate that p53RE is critical for KLF17 regulation by p53 in lung cancer cells.

p53 interacts with KLF17 promoter via p53RE and recruits p300 in response to chemotherapy

To gain insight into the molecular details of KLF17 regulation by p53, we performed chromatin immunoprecipitation (ChIP) assay in A549 cells. ChIP analysis indicated that Nutlin-3 treated cells showed recruitment of p53 to KLF17 promoter, which contains bona fide p53RE. In contrast, no recruitment of p53 was observed within upstream region of KLF17 promoter (Figs. 2A and 2B). We obtained similar results using etoposide and adriamycin, anti-cancer drugs (Fig. 2C). To further verify our data, we performed gel shift assay in A549 cells that left untreated or treated with nultin-3 for 24 hr. We designed oligo-probe containing the putative p53RE from KLF17 promoter. Gel shift assay revealed formation of p53-DNA complex on this p53RE, which was further verified by competition assay (Fig. 2D). Next, we analyzed the kinetics of p53 binding to KLF17 promoter. ChIP analysis indicated that p53 recruitment to KLF17 promoter was increased in a time-dependent manner (Fig. 2E). Taken together, p53 binds directly to KLF17 promoter and enhances KLF17 transcription in lung cancer cells.

Next, we asked that how p53 induces KLF17 transcription in lung cancer cells. p53 is known to interacts with co-activators such as p300, and lead to histone acetylation [38, 39]. Therefore, we examined the physical association of p300 with KLF17 promoter via p53RE. We treated A549 cells with Nutlin-3 and observed binding of p300 and AcH4 (a marker of chromatin activation) [40, 41] to p53RE with in KLF17 promoter (Fig. 2F). Furthermore, we observed time dependent recruitment of p300 and AcH4 to KLF17 promoter in lung cancer cells treated with Nutlin-3. ChIP analysis showed that binding of p300 and AcH4 increased to KLF17 promoter in a time-dependent manner (Figs. 2G and 2H). In conclusion, p53 enhances binding of p300 to KLF17 promoter to favor histone acetyaltion, and ultimately induce KLF17 transcription in A549 lung cancer cells in response to chemotherapy.

p53 empowers KLF17 mediated EMT genes transcription in NSCLC

Our results indicate that KLF17 expression is induced by p53 in lung cancer cells. KLF17 is a transcription factor and binds to the promoters of its target genes via CACCC DNA sequence [4, 6, 7]. Therefore, we investigated the affect of p53 on KLF17 mediated genes transcription. KLF17 inhibits metastasis and tumor growth by negatively

regulating ID1, Vimentin and Fibronectin expression, while inducing the ZO-1 and E-Cadherin transcript [4, 7]. We silenced p53 expression in lung cancer cells using siRNA targeting p53 (Fig. 3A, upper panel). p53 depleted and control cells were left untransfected or transfected with KLF17 expression vector and qRT-PCR was performed to check the mRNA levels of the KLF17 target genes (Fig. 3A, lower panel). Transcript analysis of these target genes revealed that tumor suppressive ability of KLF17 was higher in p53 containing A549 cells in comparison with p53-depleted cells (Figs. 3B-F and Supplementary Figure S1A).

Furthermore, we performed immunoblot to detect protein levels of KLF17 downstream target genes in both control and p53-depleted cell lines. Importantly, we found that knockdown of p53 decreased both KLF17 and KLF17 positively regulated genes expression (Fig. 3G). In contrast, silencing of p53 suppressed KLF17 protein level and enhanced the expression of KLF17 negatively regulated genes (Fig. 3H). Taken together, these results suggest that p53 potentiates KLF17 to regulate EMT target genes expression.

p53 enhances binding of KLF17 to EMT target gene promoters

To further gain insight that how p53 potentiates KLF17 mediated EMT genes transcription, we aimed to detect the recruitment of KLF17 to EMT gene promoters in the presence or absence of p53. We selected Vimentin, Fibronectin and ZO-1 promoters, which are known targets of KLF17 [7]. ChIP analysis, indicated that knockdown of p53 in A549 cells reduced the recruitment of KLF17 to Vimentin, Fibronectin and ZO-1 promoters (Figs. 4A-D). Conversely, in p53 containing cells KLF17 have significant physical association with its target gene promoters (Figs. 4B-D and Supplementary Figure S2A).

To further verify our results, we performed EMSA analysis using oligo probe from ZO-1 promoter that contain CACCC KLF17 responsive element. We transfected A549 cells with control siRNA or siRNA targeting against p53 and then left untransfected or transfected with expression plasmid encoding KLF17. EMSA analysis showed decrease formation of KLF17-DNA complex in p53-depleted cells in comparison with control cells which have p53 (Fig. 4E, compare lane4 with lane5). Next, we co-transfected flag-KLF17 with flag-p53 in lung cancer cells and found that formation of KLF17-DNA complex was much stronger than the control (Fig. 4E, compare lane 4 with lane 6). Taken together, these results indicate that p53 is critical for binding ability of KLF17 to EMT target gene promoters and enhances formation of KLF17-DNA complex.

p53 physically interacts with KLF17

To further gain insight that how p53 enhances tumor suppressive ability of KLF17, we tested the interaction between KLF17 and tumor suppressor p53 by immunoprecipitation assay in A549 cells. We found that endogenous p53 co-immunoprecipitated with KLF17 and resulted in the formation of p53-KLF17 complex (Fig. 5A). Importantly, formation of this complex further enhanced in Nutlin-3 treated cells (Fig. 5A). Nutlin-3 is an anti-cancer drug, which activates tumor suppressor p53 [33, 34]. The endogenous interaction between p53 and KLF17 was further confirmed by immunoprecipitating

KLF17 in lung cancer cells. A positive interaction between p53 and KLF17 was observed in both control and Nutlin-3 treated cells (Fig. 5B). Similar co-precipitation of p53 and KLF17 were obtained when A549 cells were transfected with flag-KLF17 (Fig. 5C).

To elucidate the region of KLF17 that might mediate the interaction between KLF17 and p53, A549 cells were transfected with flag-tagged full-length KLF17 (KLF17 FL), KLF17 Δ1, KLF17 Δ2 and KLF17 Δ3, that contained deletions in N-terminal (Fig. 5D). The transfected cells were subjected to immunoprecipitation, carried out with anti-flag antibody. Positive interactions were obtained only between p53 and KLF17 FL, or KLF17 Δ1, or KLF17 Δ2, but not with p53 and KLF17 Δ3 (Fig. 5E). These results indicate that the region containing amino acids from (1 to 210) in N-terminal region mediated the interaction between p53 and KLF17, and may be exerted an important effect on tumor suppressive function of KLF17 in lung cancer cells.

Activation of p53 recruits KLF17 to EMT gene promoters via p53-dependent manner

Our results show that p53 enhances KLF17 mediated EMT genes transcription. These hints prompted us to investigate the affect of Nutlin-3 on recruitment of KLF17 to EMT target gene promoters. A549 cells were left untreated or treated with Nutlin-3 and subjected to ChIP analysis. ChIP assay showed recruitment of KLF17 to EMT target gene promoters in A549 cells treated with Nutlin-3, but not in the control cells (Fig. 6A).

Next we examined whether recruitment of KLF17 to EMT target gene promoters in response to nultin-3 is p53-dependent. We transfected A549 cells with control siRNA or siRNA targeting p53 and then left untreated or treated with Nutlin-3. ChIP analysis indicated recruitment of KLF17 to EMT target gene promoters in control cells (Figs. 6B and 6C). In contrast, we did not observed any binding of KLF17 to EMT target gene promoters in p53-depleted cells treated with Nutlin-3 (Figs. 6B and 6C). Furthermore, we did detailed kinetics study of KLF17 recruitment on ID1 promoter in response to Nutlin-3. ChIP assay showed recruitment of KLF17 to ID1 promoter in Nutlin-3 treated in a p53-dependent manner (Fig. 6D). Conversely, we did not observed binding of KLF17 with ID1 promoter in p53-knockdown cancer cells (Fig. 6D).

Furthermore, we did EMSA analysis to detect the formation of KLF17-DNA complex in response to Nutlin-3. We used oligo-probe from ZO-1 promoter and observed formation of KLF17-DNA complex in control cells treated with Nutlin-3 (Fig. 6E, lane1, 2 and 4). In contrast, we did not observed any formation of KLF17-DNA complex in p53-depleted cells treated with Nutlin-3 (Fig. 6E, lanes 5-8).

Next we asked whether p53-dependent KLF17 recruitment to EMT target genes promoters is limited to Nutlin-3 induced model or other anti-cancer drugs that activate p53 can also show this phenomenon. We selected adriamycin and etoposide chemotherapeutic agents that activate p53. Importantly, ChIP assay revealed that both adriamycin and etoposide treated cell have more recruitment of KLF17 to ID1 promoter in a p53-dependent manner (Figs. 6F and 6G).

To address whether endogenous p53 binds to KLF17 target gene promoters, we did ChIP analysis in A549 cells treated with Nutlin-3.

ChIP analysis showed no recruitment of p53 to KLF17 target gene promoters in both control and Nutlin-3 treated cells (Supplementary figures S3A-S3C).

Taken together, these results show that p53 is critical for KLF17 recruitment to EMT target gene promoters in lung cancer cells in response to different p53 induced chemotherapeutic agents.

Knockdown of KLF17 impairs tumor suppressive function of p53

Next, we aimed to address the impact of KLF17 regulation on p53 cellular activities. p53 suppresses cell growth, decreases drug resistance, enhances apoptosis and blocks cell-cycle progression in response to chemotherapy [12-16]. Therefore, we investigated the molecular and biological roles of p53 in control and KLF17-depleted lung cancer cell lines. We selected different p53 target genes such as PUMA and PIG3 that are up regulated by p53 [42, 43]. We carried out mRNA analysis of PUMA and PIG3 genes in control and KLF17-depleted lung cancer cells. qRT-PCR results indicated strong induction of PUMA and PIG3 expression in control cells treated with Nutlin-3, in comparison with KLF17-depleted cells (Figs. 7A and 7B). Moreover, we observed decrease recruitment of p53 to PUMA and PIG3 target gene promoters in response to Nutlin-3 in KLF17-depleted cells (Figs. 7C and 7D), suggesting that KLF17 may be important for full transcriptional activity of p53 in lung cancer cells.

Next, we treated p53 containing A549 lung cancer cell lines with Nutlin-3 and observed that KLF17 depleted cells were less sensitive to nultin-3 and more proliferative, even in the presence of p53 (Fig. 7E). Furthermore, knockdown of KLF17 decreased apoptotic level of A549 cells in response to Nutlin-3 (Fig. 7F). Next, we assessed proliferative role of KLF17 and p53 in A549 cells. Importantly, knockdown of KLF17 and p53 independently enhanced cell growth (Fig. 7G). Strikingly, co-silencing of KLF17 and p53 further induced cell proliferation in A549 cells (Fig. 7G). These data indicate that KLF17 plays important role in the inhibition of A549 lung cancer cell growth in response to chemotherapy and is important for full tumor suppressive function of p53.

Overexpression of KLF17 enhances cyostatic ability of p53

Next, we sought whether enforce expression of KLF17 suppress lung cancer cells growth in response to chemotherapy. MMT analysis showed that both Nutlin-3 (which activates p53) and KLF17 independently decreased the cell viability of lung cancer cells. While, the combination of both nultlin-3 and KLF17 further inhibited the lung cancer cell growth (Fig. 8A). Apoptotic analysis of lung cancer cells showed the same results (Fig. 8B).

Next, we aimed to address the mechanism that how KLF17 inhibits lung cancer cells growth. Intriguingly, we found that KLF17 increased tumor suppressor p21 and pRB expressions in lung cancer cells (Fig. 8C and Supplementary Figures S4A-S4C). p21 and pRB are the inhibitor of cell growth and enhance apoptosis [12, 13, 44-47]. Importantly, depletion of KLF17 decreased mRNA and protein levels of p21 and pRB (Figs. 8D and 8E). Taken together, these results show that KLF17 is critical for inhibition of cell growth and induction of apoptosis in response to chemotherapy in lung cancer cells.

KLF17 enhances p53 transcription to generate a positive feedback loop

Next, we asked whether a positive feedback loop exists between KLF17 and p53, we depleted KLF17 expression in A549 cells. qRT-PCR analysis showed that depletion of KLF17 suppressed mRNA level of p53 (Figs. 9A and 9B). We obtained similar results at protein level (Fig. 9C).

To further assess the effect of KLF17 on p53 expression, we established A549 transfectants stably expressing KLF17 using lenti-viral infection. A qRT-PCR analysis demonstrated that KLF17 expression level in lenti-KLF17 A549 cell lines were significantly higher than in the control stable cell lines (Fig. 9D). Consistently, we observed higher expression of p53 both at mRNA and protein levels in A549 cells stably expressing KLF17 (Figs. 9E and 9F).

Next, we investigated whether KLF17 enhance p53 transcription, we co-transfected p53-Luc reporter with KLF17 encoding plasmid into A549 cells. Induction was observed when reporter construct was co-trasnfected with different doses of KLF17 (Fig. 9G). Importantly, we found higher luciferase activity of p53-Luc construct in lenti-KLF17 A549 cells (Fig. 9H). Bioinformatics analysis, using NCBI database, revealed that human p53 promoter contains three putative KLF17 responsive elements termed as KLF17RE (Fig. 9I, upper panel).

We attempted to indentify the regulatory region conferring KLF17 responsiveness within the p53 promoter. We constructed different p53-Luc constructs that contain KLF17RE(s) (Fig.9I, lower panel). Deletion of KLF17RE-2 construct abolished its response to KLF17 induction, validating that KLF17 binds to KLF17RE-2 to induce p53 transcription in A549 cells stably expressing KLF17 (Fig. 9J and supplementary fig.S5).

To determine whether KLF17 directly binds to KLF17RE-2 within p53 promoter, a chromatin immunoprecipitation assay was performed in A549 expressing lenti-KLF17. The anti-KLF17 antibody specifically pull down DNA fragments corresponding to KLF17RE-2, which suggest direct binding of KLF17 to p53 promoter (Fig. 9K). Moreover, we observed recruitment of p300, a co-activator, to KLF17RE-2 region within p53 promoter in A549 cells stably expressing KLF17 (Fig. 9L).

Taken together, these results suggest that KLF17 enhances p53 transcription and generate a novel positive feedback with p53 in lung cancer cells to jointly control cancer progression.

KLF17 suppresses EMT and metastasis in a p53-dependent manner

Mechanistically, our results indicate that p53 enhance KLF17 mediated EMT genes transcription. These hints prompted us to investigate the functional effect of KLF17 on tumor cells migration and invasion in both control and p53 depleted cells. Overexpression of KLF17 inhibited cell migration in A549 cells (Fig. 10A, upper panel). Importantly, depletion of p53 lost the ability of KLF17 to suppress cell migration (Fig. 10A, lower panel, and 10B).

Next, we performed invasion assay in A549 cells. Enforce expression of KLF17 decreased the invasion of A549 cells (Fig. 10C, upper panel). In contrast, silencing of p53 abolished KLF17 ability to significantly suppress invasion of lung cancer cells (Figs. 10C,

lower panel, and 10D). Taken together, these results suggest that KLF17 inhibits metastasis and invasion of lung cancer cells in a p53-dependent manner.

Discussion

Activation of tumor suppressive signaling is linked with inhibition of cancer progression and metastasis. Metastasis is a complex multistep process, which is control by joint regulation of several signaling cascade and is one of main cause of cancer-associated death. NSCLC is aggressive type of lung cancer and prognosis of NSCLC patients is very poor and about 30-55% NSCLC patients after chemotherapy show recurrence. The inhibitory effect of KLF17 on tumor cell migration and metastasis has been reported; however, the underlying molecular mechanism that how KLF17 control cancer metastasis remains elusive. Only a limited number of KLF17 target genes that regulate cancer cell migration and metastasis have been identified. Several studies showed that KLF17 suppresses cancer cell migration through targeting EMT-inducing transcription factors such as ID1 and ZO-1. We previously showed that mutant-p53 proteins exerts gain-of-function (GOF) ability to inhibit KLF17 expression [48]. Similarly, recent study showed that microRNA-9 represses KLF17 expression [6]. However, the signaling that positively control KLF17 pathway to suppress cancer metastasis remains unknown. Here, we showed novel molecular and functional link of KLF17 with p53. Our data reveals for the first time that KLF17 suppresses EMT and metastasis in a p53-dependent manner (Fig.11). Our study provides new insight into KLF17 pathway during cancer metastasis and for the first time, links KLF17 signaling with p53. Our results indicate that tumor suppressor p53 plays an integral role to potentiate KLF17 tumor suppressive function to suppress EMT and metastasis. Depletion of p53 abrogates KLF17 mediated inhibition of EMT and metastasis in NSCLC. KLF17 is one of the key inhibitor of EMT and metastasis [4, 48,49]. Knockdown of KLF17 promotes EMT and metastasis [4-8, 48]. Mechanistically, we found that endogenous p53 interacts with KLF17 and thereby enhances anti-EMT and tumor suppressive function of KLF17. Our results highlight novel crosstalk between p53 and KLF17 tumor suppressive signaling in lung cancer cells (Fig.11). Moreover, we show that novel positive feedback loop exists between p53 and KLF17, which suggest that these two tumor suppressor proteins amplify each other signaling (Fig.11). We found that endogenous p53 and KLF17 proteins form a complex in lung cancer cells. Importantly, formation of p53-KLF17 complex further enhanced in response to chemotherapeutic agent that activates p53 such as Nutlin-3.

p53 is a tumor suppressor transcription factor and plays a key role in the inhibition of tumor development and metastasis [12,13, 15]. Our study indicates that KLF17 is a novel transcriptional target of p53 signaling in lung cancer cells. We show that p53 enhances KLF17 transcription in lung cancer cells via p53RE. Depletion of p53 is associated with decrease expression of KLF17 in lung cancer cells. EMSA analyses showed formation of p53-DNA complex on KLF17 promoter, which suggest that KLF17 is a direct target of p53. Our study highlights that induction of KLF17 by p53 may reduce risk of metastasis,

EMT and cancer development in lung cancer cells.

KLF17 suppresses EMT by regulating the expression of EMT markers [4-6]. In this study, we examined whether KLF17 regulates its target gene expression in a p53-dependent manner. Indeed, we found that p53 potentiates KLF17 mediated EMT target genes expression during cancer metastasis. Our results show that knockdown of p53 decreased both KLF17 and KLF17 downstream target genes expression. With respect to the mechanism of KLF17 target genes regulation by p53, we found that depletion of p53 reduced recruitment of KLF17 to EMT target gene promoters. Moreover, p53 enhanced formation of KLF17-DNA complex on EMT target gene prompters. Our results indicate that p53 is critical for binding/recruitment of KLF17 to EMT target gene promoters and p53 plays a key role to empower tumor suppressive function of KLF17.

It is interesting to note that different chemotherapeutic agents that activate p53 such as Nutlin-3, Adriamycin and Etoposide promote recruitment of KLF17 to EMT target gene promoters in a p53-dependent manner. EMSA analysis showed that Nutlin-3 treatment results in the formation of KLF17-DNA complex on EMT target gene promoters, which was p53-dependent. These results pointing towards that p53 activating anti-cancer drugs may induce KLF17 expression and recruits KLF17 to EMT promoters via p53-dependent pathway. Our data show that p53 is crucial for KLF17 recruitment to EMT target gene promoters in response to chemotherapy. These results highlight the importance of p53 in triggering KLF17 signaling to overcome cancer progression.

p53 has been shown to suppress cancer progression by inhibiting cancer cell growth and proliferation [12,13, 15]. In this study, we showed that KLF17 is important for full tumor suppressive function of p53. Depletion of KLF17 impairs p53 signaling to inhibit cancer progression significantly. KLF17 is a tumor suppressive transcription factor and regulates the expression of its target gene promoters. We showed that p53 is a novel direct target of KLF17 in lung cancer cells. KLF17 induces p53 expression to generate a novel feedback loop. Our results show that KLF17 is important to maintain high level of p53. Depletion of KLF17 is associated with decrease expression of p53 in lung cancer cells. These results highlight interplay between p53 and KLF17 during cancer metastasis and show that these two proteins are engaged in a positive feedback loop.

Functionally, we examined whether KLF17 suppresses the tumor cell migration and invasion via p53-dependent pathway. Both KLF17 and p53 inhibit EMT and metastasis. Our study defines a novel functional link between these two tumor suppressor proteins during cancer metastasis. Importantly, our data show that KLF17 suppresses cancer cell migration and invasion in a p53-dependent manner. Depletion of p53 abrogates KLF17 function to inhibit tumor cell migration and invasion significantly in lung cancer cells. These results indicate for the first time that inhibition of tumor cell migration and invasion by KLF17 is p53-dependent in NSCLC.

Functional and molecular crosstalk of KLF17 with other tumor suppressive signaling remains unknown. Our study revealed novel molecular and functional link between KLF17 and p53 signaling. p53 and KLF17 form a novel positive feedback loop to amplify each other signaling. p53 is important to trigger KLF17 pathway in lung cancer cells to overcome EMT and cancer metastasis. Endogenous p53 and KLF17 form a complex and hence enhances the KLF17 tumor suppressive function. This finding extends the repertoire of roles for both p53 and KLF17 signaling during cancer progression. Our finding contributes to comprehensive understanding of KLF17 function in metastatic process, including its role as a transcriptional activator for inducing adherent protein expression and as a transcriptional repressor for suppressing oncogenic protein expression in tumor microenvironment.

These findings define novel mechanism by which p53-KLF17 pathway mutually affect each other during EMT and cancer metastasis, provide a new model of regulation of KLF17 tumor suppressive pathway by p53 and defines novel insights into anti-metastatic function of KLF17 via p53-dependent pathway in NSCLC. Thus, p53-KLF17 novel positive feedback play important role in inhibition of cancer metastasis. In addition, these results indicate that KLF17 may be a new therapeutic target in NSCLC tumors with p53 status, which may improve prognosis and treatment of NSCLC patients.

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Competing financial interests:
The authors declare no competing financial interests.

Author contributions:
Amjad Ali and Muhammad Zeeshan Bhatti, designed project, performed major experiments and contributed to writing; Abdus Saboor Shah, Hong-Quan Duong, Huda Mohammad Alkreathy, Shah Faisal Mohammad and Rahmat Ali performed experiments and analyzed data; Ayaz Ahmad, designed project and wrote the manuscript.

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Figure legends:

Fig.1. Tumor suppressor p53 induces KLF17 expression

(A) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then left untreated or treated with Nutlin-3 (10 μM/L) for indicated time points. Total RNA was isolated and subjected to qRT-PCR. Data are representative of three independent experiments (mean ± s.d.). (Two-tailed Student's *t*-test, P**<0.005).

(B, C and D) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48

hr. Total RNA was extracted and subjected to RT-PCR analysis. **(B, C)** Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(E) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 72 hr and subjected to western blot analysis with indicated antibodies.

(F) A549 cells were left untreated or treated with Nutlin-3 (10 µM), Etoposide (10 µM) and Adriamycin (0.5 µM) for 12 hr and analyzed by qRT-PCR. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(G) A549 cells were treated with Nutlin-3 (10µM) for 24 hr and subjected to western blot analysis with indicated antibodies.

(H) H1299 cells were co-transfected with KLF17 reporter construct (2 µg) in combination with different concentrations of p53 for 24 hr prior to lysis, and analyzed for luciferase-activity**.** Data are representative of three independent experiments (mean ± s.d.). (Two-tailed Student's *t*-test, P**<0.005).

(I) Schematic representation of different deletion luciferase constructs of KLF17.

(J) Schematic representation of p53 responsive element (p53RE) within KLF17 promoter. Different luciferase constructs of KLF17 (each 2 µg) were co-transfected with p53 (50 ng) for 24 hr and analyzed by luciferase assay. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P**<0.005).

(K) H1299 cells were co-transfected with wild-type (2 µg) or mutated (2 µg) p53RE KLF17 luciferase reporter constructs in combination with p53 (50 ng) for 24 hr, and luciferase activity was measured. Data are representative of three independent experiments (mean ± s.d.). (Two-tailed Student's *t*-test, P**<0.005).

Fig.2. p53 interacts and recruits p300 to KLF17 promoter in response to chemotherapy

(A) Schematic representation of ChIP primers from KLF17 promoter.

(B and C) A549 cells were left untreated or treated with Nutlin-3, Adriamycin and Etoposide for 24 hr, and chromatin immunoprecipitation were performed with indicated antibodies. **(B)** Data are representative of three independent experiments (mean ± s.d.). (Two-tailed Student's *t*-test, P*<0.05).

(D) EMSA was performed using nuclear extract from A549 cells treated with Nutlin-3 (10 µM) for 24 hr. 2 µg of nuclear extract protein were incubated with ^{32}p-radiolabeled probe containing p53RE from KLF17 promoter.

(E) A549 cells were left untreated or treated with Nutlin-3 (10 μM) for indicated time points and chromatin immunoprecipitation were performed with indicated antibodies. Data are representative of three independent experiments (mean ± s.d.). (Two-tailed Student's *t*-test, P*<0.05).

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(F-H) A549 cells were left untreated or treated with Nutlin-3 (10 μM) for 24 hr, and chromatin immunoprecipitation were performed with indicated antibodies. **(G, H)** Data are representative of three independent experiments (mean ± s.d.). (Two-tailed Student's *t*-test, P*<0.05).

Fig.3. p53 potentiates KLF17 mediated EMT target genes expression

(A) (Upper panel**)** A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and subjected to western blot analysis with indicated antibodies. **(**Lower panel**)** A549 cells were transfected with expression plasmid enconding KLF17 for 24 hr and subjected to western blot analysis with indicated antibodies.

(B-F) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then transfected with expression plasmid enconding KLF17 for 24 hr. qRT-PCR was performed to check different genes expressions. The average was calculated based on three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P**<0.005).

(G and H) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 72 hr, and subjected to western blot analysis with indicated antibodies.

Fig.4. Depletion of p53 decreases binding of KLF17 to EMT gene promoters

(A) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and subjected to western blot analysis with indicated antibodies.

(B-D) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then transfected with expression plasmid enconding KLF17 for 24 hr. ChIP was performed with antibody specific for KLF17. The average was calculated based on three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

(E) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then transfected with control vector or expression plasmid enconding KLF17 for 24 hr. EMSA was performed with oligo-pbrobe derived from ZO-1 promoter that conatin corresponding KLF17 responsive element.

Fig.5. KLF17 physically interacts with KLF17

(A) A549 cells were immunoprecipitated with anti-p53 antibody and then subjected to western blot with indicated antibodies.

(B)A549 cells were immunoprecipitated with anti-KLF17 antibody and then subjected to western blot with indicated antibodies.

(C) A549 cells were transfected with flag-vector or flag-KLF17 for 24 hr and then immunoprecipitated with anti-Flag antibody and analyzed by western blot with indicated antibodies.

(D) Mapping the interaction domain between KLF17 and p53. Different deletion constructs of KLF17 were generated to find out the region required for p53 and KLF17 interaction.

(E) A549 cells were transfected with full length flag-KLF17 or with different deletion constructs of KL17 for 24 hr and then immunoprecipitated with flag antibody and analyzed by western blot with indicated antibodies.

Fig.6. Nutlin-3 recruits KLF17 to EMT gene promoters in a p53-dependent manner.

(A) A549 cells were left untreated or treated with Nutlin-3 (10 μM) for 24 hr, and chromatin immunoprecipitation were performed with indicated antibodies.

(B-D) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then left untreated or treated with Nutlin-3 (10 μM) for 24 hr, and chromatin immunoprecipitation were performed with indicated antibodies. **(B-D)** The average was calculated based on three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

(E) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then left untreated or treated with Nutlin-3 (5 and/or 10 μM) for 24 hr, and EMSA was performed with oilgo-probe derived from ZO-1 promoter.

(F) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then left untreated or treated with Adriamycin for 24 hr, and chromatin immunoprecipitation were performed with indicated antibodies. The average was calculated based on three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

(G) A549 cells were transfected with control siRNA or siRNA targeting against p53 (20 nM) for 48 hr, and then left untreated or treated with Etoposide for 24 hr, and chromatin immunoprecipitation were performed with indicated antibodies. The average was calculated based on three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

Fig.7. Depletion of KLF17 affects the cytostatic function of p53

(A and B) A549 cells was transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 48 hr and then treated with Nutlin-3 (10 μM) for 24 hr. Total RNA was extracted and subjected to qRT-PCR analysis for indicated genes. Lower panel shows knockdown efficiency of KLF17 in A549 cells.

(C and D) A549 cells was transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 48 hr and then treated with Nutlin-3 (10 μM) for 24 hr. ChIP was performed with indicated target gene promoters with anti p53 antibody.

(E) A549 cells was transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 48 hr and then left untreated or treated with Nutlin-3 (10 μM/L) for 24 hr and MTT assay was performed. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(F) A549 cells were transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 48 hr and then left untreated or treated with Nutlin-3 (10 μM/L) for 24 hr and FACS analysis was performed to detect the apoptotic level. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(G) A549 cells were transfected with siRNAs targeting against p53 (20 nM) and KLF17 (20 nM) either independently or in combination for 72 hr and then subjected to MTT analysis. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

Fig.8. Overexpression of KLF17 enhances p53 tumor suppressive function.

(A) A549 cells were transfected with flag-vector or vector encoding KLF17 plasmid for 24 hr and then left untreated or treated with Nutlin-3 (10 μM/L) for 24 hr and MTT assay was performed to detect cell growth. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

(B) A549 cells were transfected with flag-vector or vector encoding KLF17 plasmid for 24 hr and then left untreated or treated with Nutlin-3 (10 μM/L) for 24 hr and FACS analysis was performed to detect apoptotic level. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

(C) A549 cells were transfected with flag-vector or vector encoding KLF17 plasmid for 24 hr and subjected to

western blot analysis with indicated antibodies.

(D) A549 cells were transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 48 hr and subjected to qRT-PCR analysis. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(E) A549 cells were transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 72 hr and subjected to western blot analysis with indicated antibodies.

Fig.9. KLF17 enhances p53 transcription to generate a feedback loop

(A and B) A549 cells were transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 48 hr and subjected to qRT-PCR analysis. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(C) A549 cells were transfected with control siRNA or siRNA targeting against KLF17 (20 nM) for 72 hr and subjected to western blot analysis with indicated antibodies.

(D) Lenti-KLF17 transfection significantly increased KLF17 expression compared to lenti-control transfection in A459 cells and subjected to qRT-PCR analysis. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P***<0.0005).

(E) A549 cells stably expressing lenti control vector or lenti KLF17 were subjected to qRT-PCR analysis for p53 expression. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P**<0.005).

(F) A549 cells stably expressing lenti control vector or lenti KLF17 were subjected to western blot analysis with indicated antibodies.

(G) A549 cells were transfected with p53-Luciferase construct in combination with 10, 25 and/or 50 ng of Flag-KLF17 for 24 hr prior to lysis and subjected to luciferase assay. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(H) A549 cells expressing lenti control vector or lenti KLF17 were transfected with p53-Luciferase construct for 24 hr prior to lysis and subjected to luciferase assay. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P**<0.005). Lower panel represent lenti-KLF17 expression in A459 cells.

(I) Schematic representation of different luciferase constructs for KLF17 responsive elements in p53 promoter.

(J) A549 cells stably expressing lenti control vector or lenti KLF17 vector were transfected with different p53-Luciferase construct for 24 hr prior to lysis and subjected to luciferase assay. Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05).

(K) A549 cells expressing lenti control vector or lenti KLF17 vector were subjected to ChIP analysis with anti-KLF17 antibody. IgG serve as a negative control.

(L) A549 cells expressing lenti control vector or lenti KLF17 vector were subjected to ChIP analysis with anti-p300 antibody. IgG serve as a negative control.

Fig.10. KLF17 suppresses EMT in a p53-dependent manner

(A) A549 cells were transfected with Flag-vector encoding KLF17 or transfected with siRNA targeting against p53 and cell migration assay was performed.

(B) Statistical analysis of **(A).** Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

(C) A549 cells were transfected with Flag-vector encoding KLF17 or transfected with siRNA targeting against p53 and cell invasion assay was performed.

(D) Statistical analysis of **(C).** Data are representative of three independent experiments with mean ± s.d. (Two-tailed Student's *t*-test, P*<0.05 and P**<0.005).

Fig.11. Model showing that (a) KLF17 suppresses cancer metastasis via p53-dependent pathway and (b) showing novel positive feedback loop exist between p53 and KLF17.

Figure 1

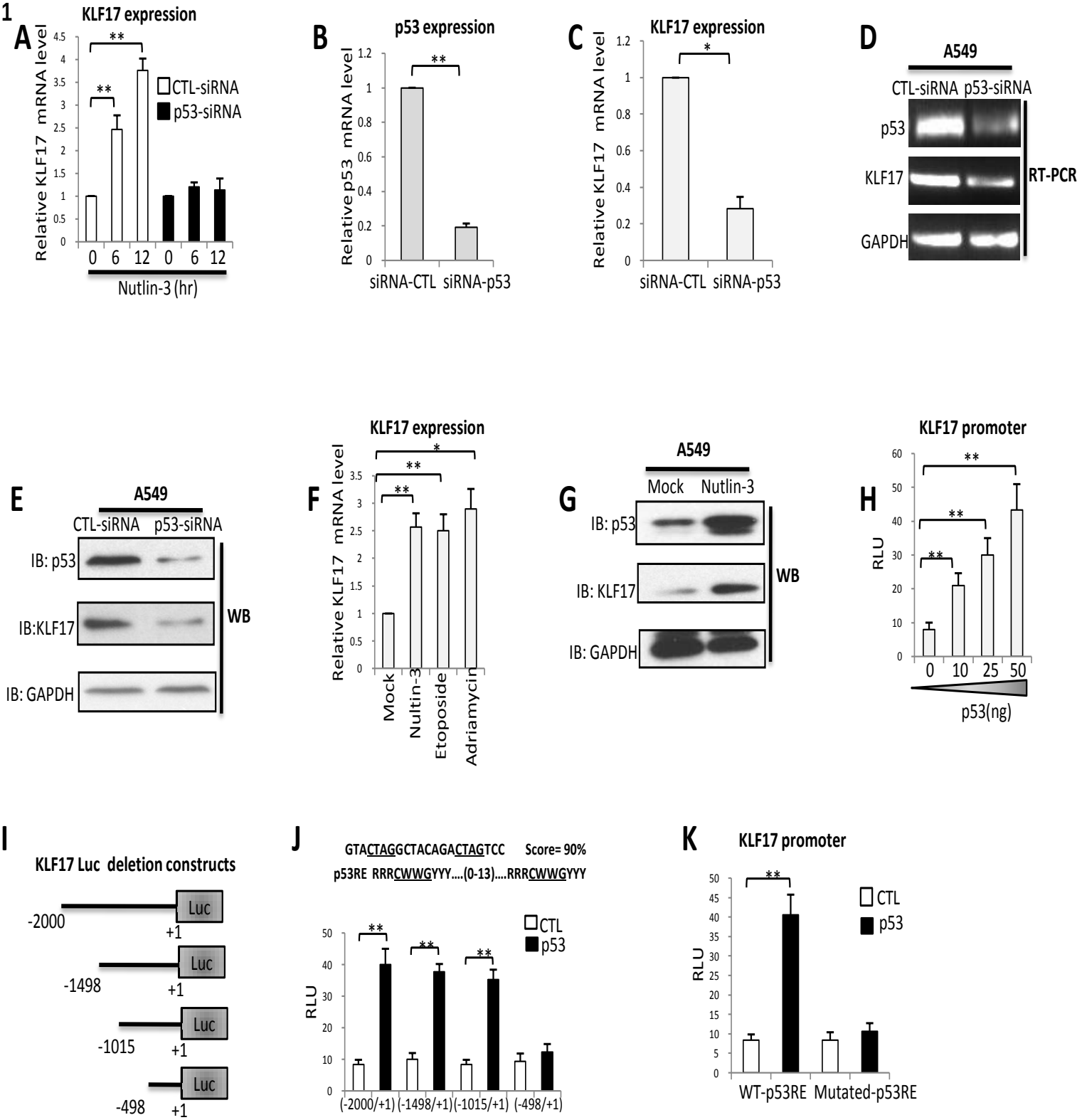

A
KLF17 expression
Relative KLF17 mRNA level
CTL-siRNA
p53-siRNA
0 6 12 0 6 12
Nutlin-3 (hr)
B
p53 expression
Relative p53 mRNA level
siRNA-CTL siRNA-p53
C
KLF17 expression
Relative KLF17 mRNA level
siRNA-CTL siRNA-p53
D
A549
CTL-siRNA p53-siRNA
p53
KLF17
GAPDH
RT-PCR
E
A549
CTL-siRNA p53-siRNA
IB: p53
IB:KLF17
IB: GAPDH
WB
F
KLF17 expression
Relative KLF17 mRNA level
Mock
Nultin-3
Etoposide
Adriamycin
G
A549
Mock Nutlin-3
IB: p53
IB: KLF17
IB: GAPDH
WB
H
KLF17 promoter
RLU
0 10 25 50
p53(ng)
I
KLF17 Luc deletion constructs
-2000
-1498
-1015
-498
Luc
+1
J
GTACTAGGCTACAGACTAGTCC Score= 90%
p53RE RRRCWWGYYY....(0-13)....RRRCWWGYYY
CTL
p53
RLU
(-2000/+1) (-1498/+1) (-1015/+1) (-498/+1)
K
KLF17 promoter
CTL
p53
RLU
WT-p53RE Mutated-p53RE

Figure 2

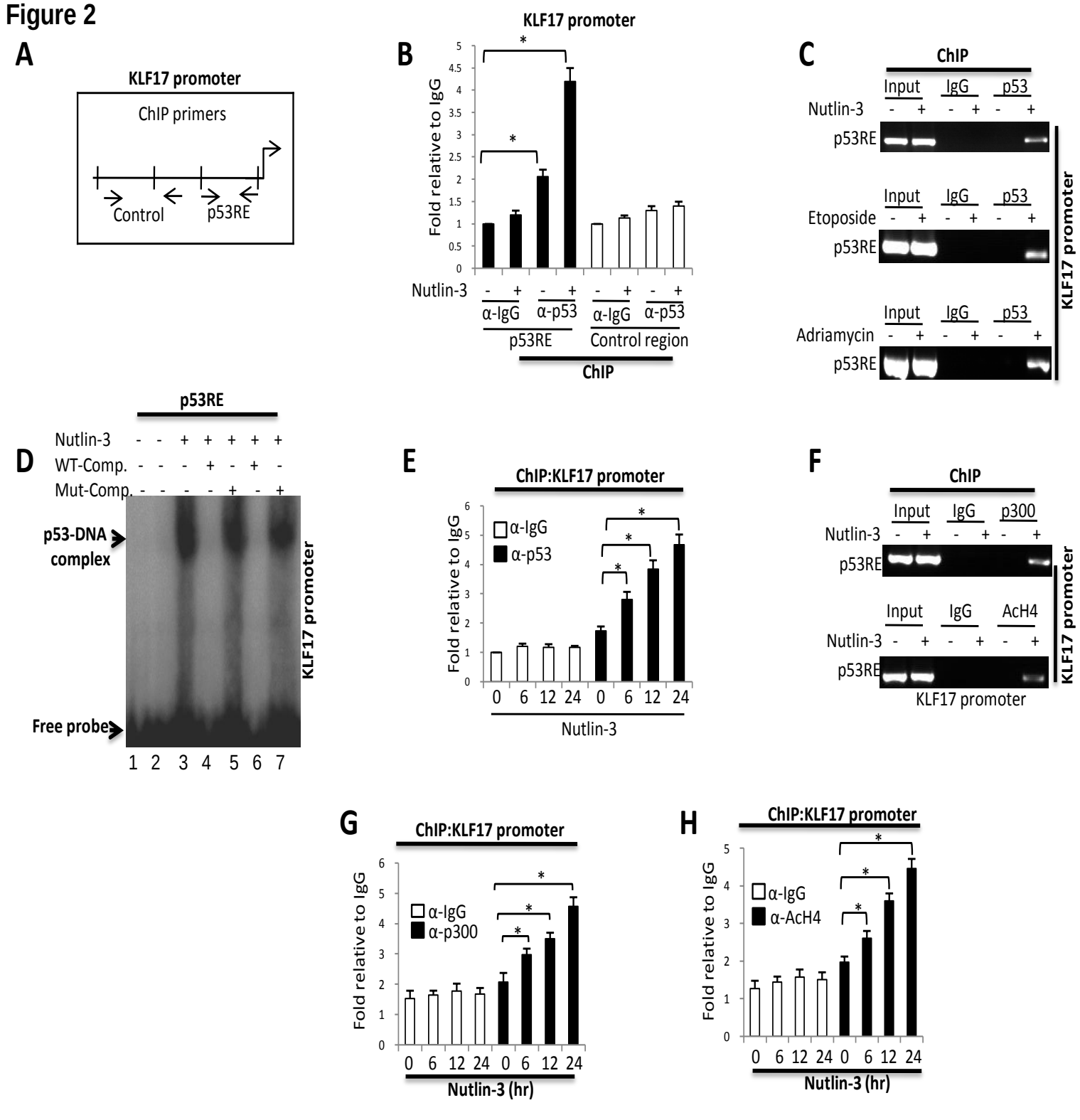

A
KLF17 promoter
ChIP primers
Control
p53RE
B
KLF17 promoter
Fold relative to IgG
Nutlin-3
α-IgG
α-p53
p53RE
Control region
ChIP
C
ChIP
Input
IgG
p53
Nutlin-3
p53RE
Etoposide
Adriamycin
KLF17 promoter
D
p53RE
Nutlin-3
WT-Comp.
Mut-Comp.
p53-DNA complex
Free probe
KLF17 promoter
1 2 3 4 5 6 7
E
ChIP:KLF17 promoter
α-IgG
α-p53
Fold relative to IgG
Nutlin-3
F
ChIP
Input
IgG
p300
AcH4
Nutlin-3
p53RE
KLF17 promoter
G
ChIP:KLF17 promoter
α-IgG
α-p300
Fold relative to IgG
Nutlin-3 (hr)
H
ChIP:KLF17 promoter
α-IgG
α-AcH4
Fold relative to IgG
Nutlin-3 (hr)

Figure 3

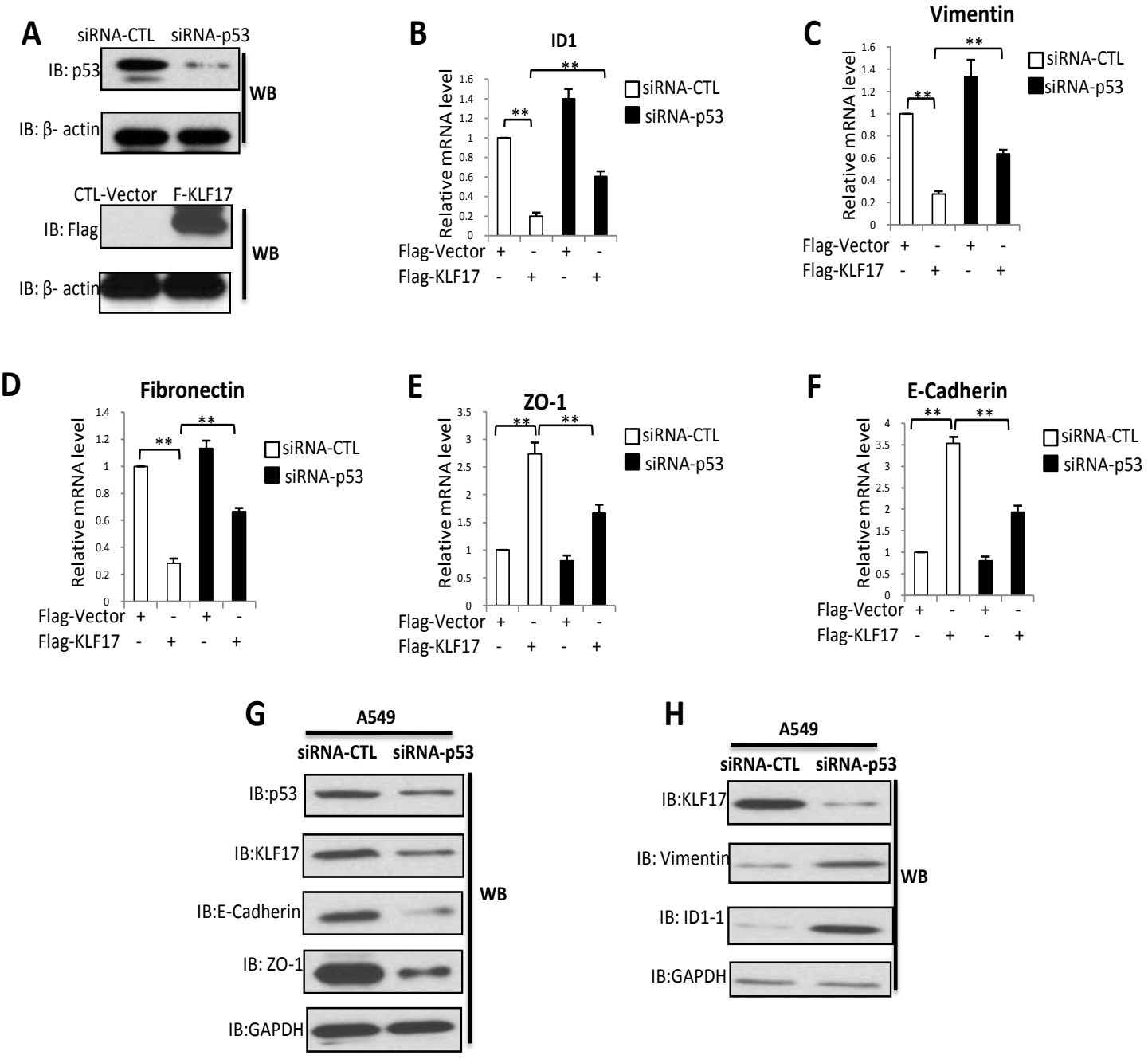

A
siRNA-CTL
siRNA-p53
IB: p53
IB: β- actin
WB
CTL-Vector
F-KLF17
IB: Flag
IB: β- actin
WB
B
ID1
Relative mRNA level
siRNA-CTL
siRNA-p53
Flag-Vector + - + -
Flag-KLF17 - + - +
C
Vimentin
Relative mRNA level
siRNA-CTL
siRNA-p53
Flag-Vector + - + -
Flag-KLF17 - + - +
D
Fibronectin
Relative mRNA level
siRNA-CTL
siRNA-p53
Flag-Vector + - + -
Flag-KLF17 - + - +
E
ZO-1
Relative mRNA level
siRNA-CTL
siRNA-p53
Flag-Vector + - + -
Flag-KLF17 - + - +
F
E-Cadherin
Relative mRNA level
siRNA-CTL
siRNA-p53
Flag-Vector + - + -
Flag-KLF17 - + - +
G
A549
siRNA-CTL siRNA-p53
IB:p53
IB:KLF17
IB:E-Cadherin
IB: ZO-1
IB:GAPDH
WB
H
A549
siRNA-CTL siRNA-p53
IB:KLF17
IB: Vimentin
IB: ID1-1
IB:GAPDH
WB

Figure 4

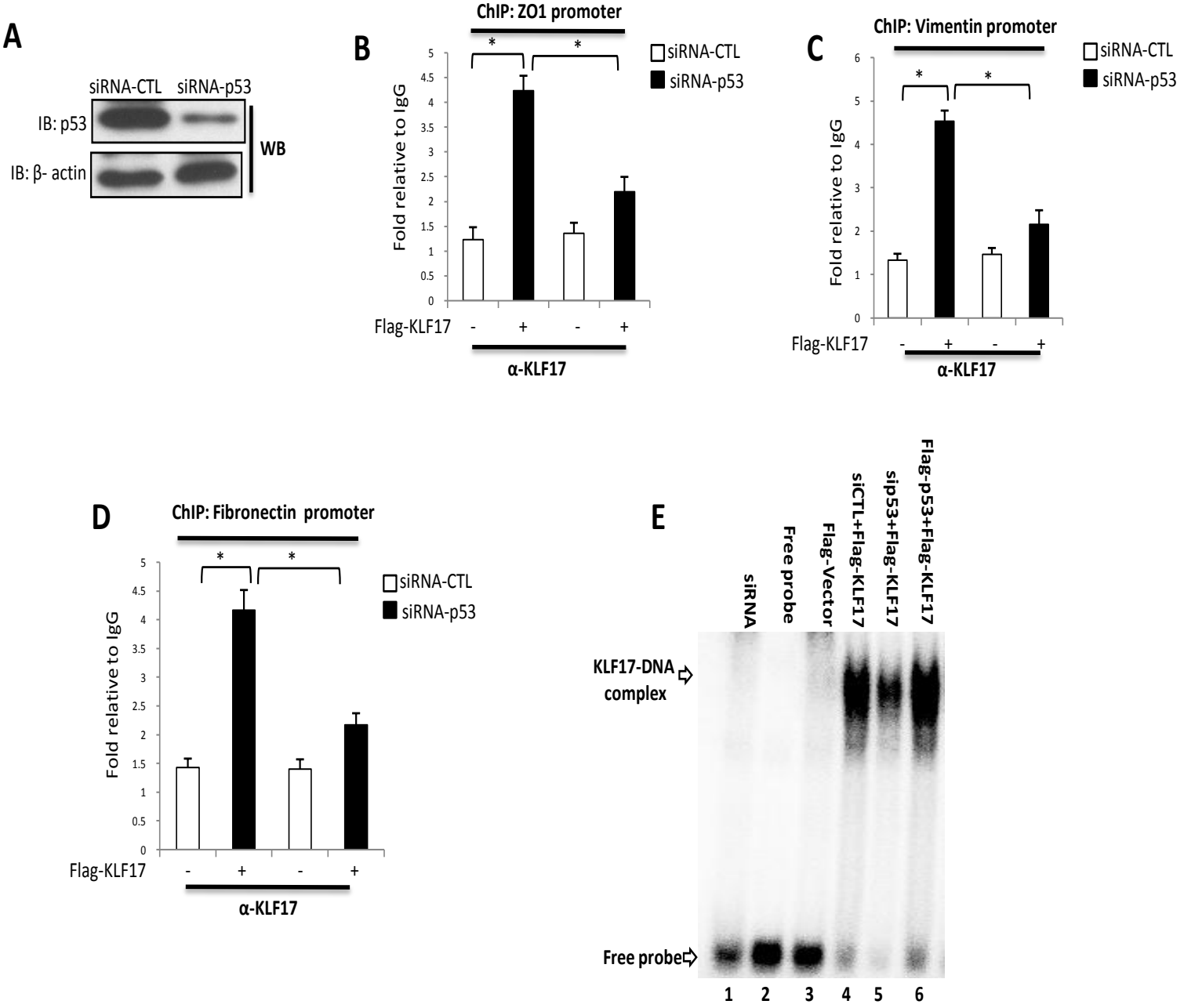

A
siRNA-CTL
siRNA-p53
IB: p53
IB: β- actin
WB
B
ChIP: ZO1 promoter
siRNA-CTL
siRNA-p53
Fold relative to IgG
Flag-KLF17
α-KLF17
C
ChIP: Vimentin promoter
siRNA-CTL
siRNA-p53
Fold relative to IgG
Flag-KLF17
α-KLF17
D
ChIP: Fibronectin promoter
siRNA-CTL
siRNA-p53
Fold relative to IgG
Flag-KLF17
α-KLF17
E
siRNA
Free probe
Flag-Vector
siCTL+Flag-KLF17
sip53+Flag-KLF17
Flag-p53+Flag-KLF17
KLF17-DNA complex
Free probe
1 2 3 4 5 6

Figure 5

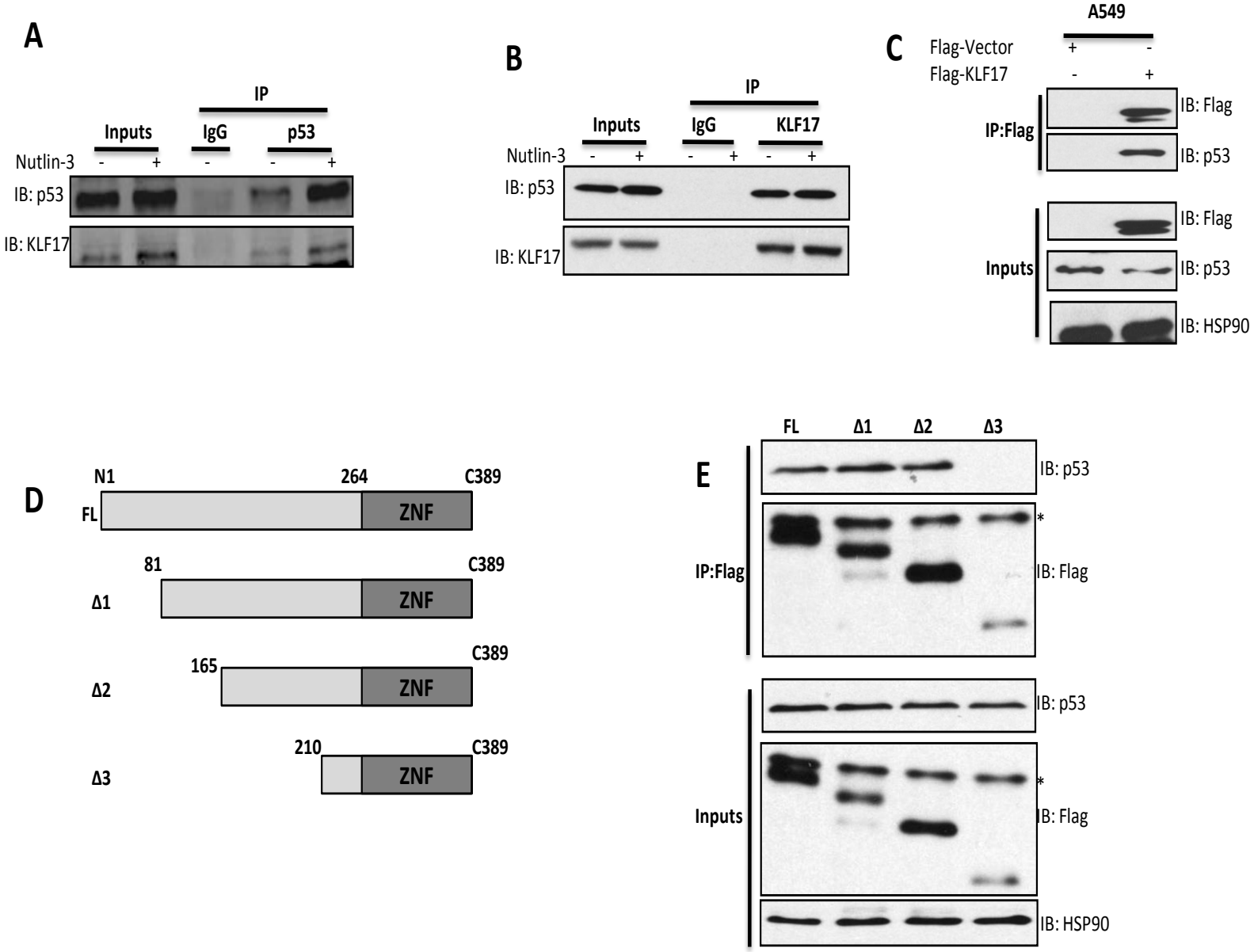

A
IP
Inputs
IgG
p53
Nutlin-3
- + - - +
IB: p53
IB: KLF17
B
IP
Inputs
IgG
KLF17
Nutlin-3
- + - + - +
IB: p53
IB: KLF17
C
A549
Flag-Vector
Flag-KLF17
+ -
- +
IP:Flag
IB: Flag
IB: p53
Inputs
IB: Flag
IB: p53
IB: HSP90
D
N1
264
C389
FL
ZNF
81
C389
Δ1
ZNF
165
C389
Δ2
ZNF
210
C389
Δ3
ZNF
E
FL
Δ1
Δ2
Δ3
IB: p53
IP:Flag
*
IB: Flag
IB: p53
Inputs
*
IB: Flag
IB: HSP90

Figure 6

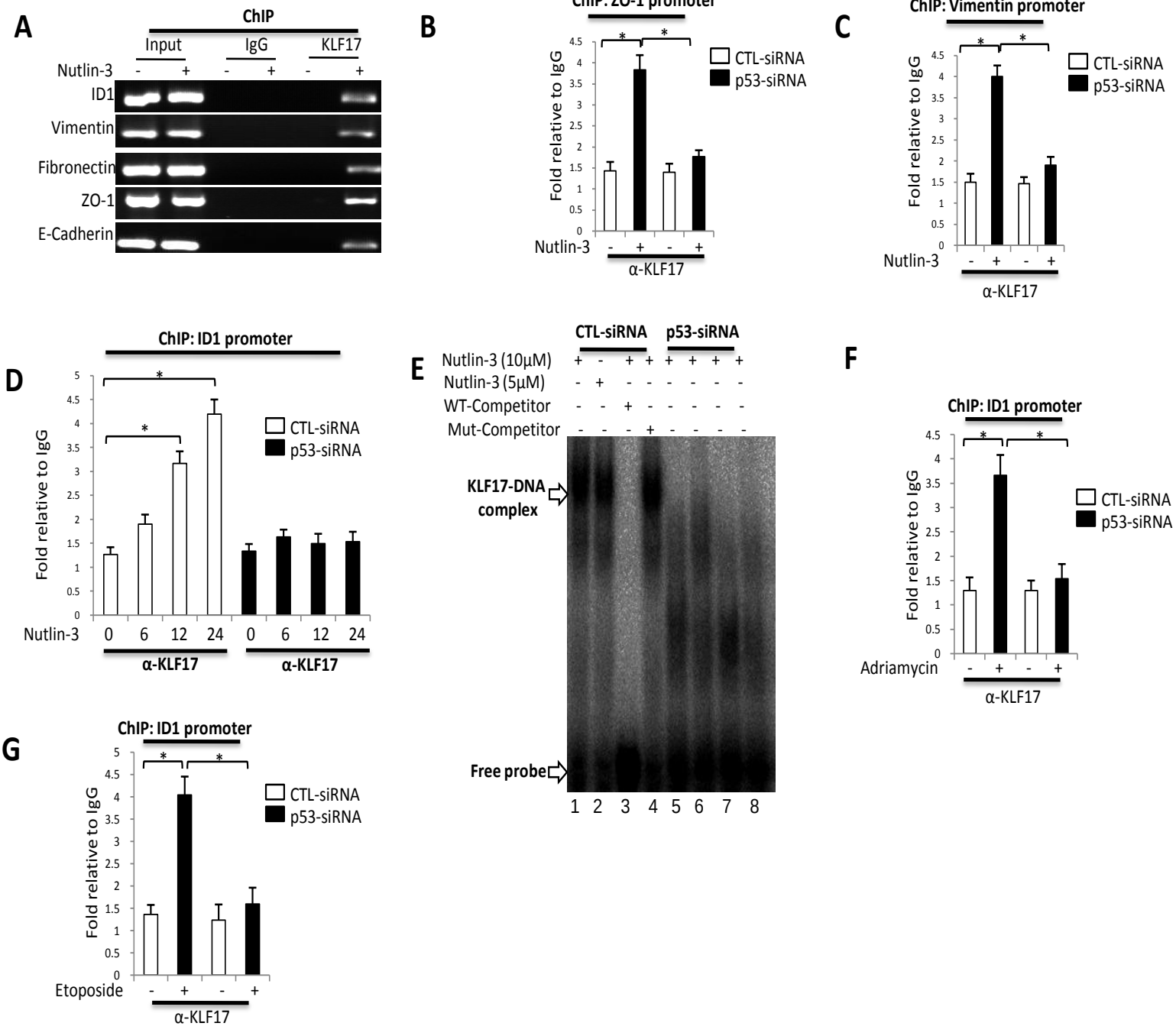

A
ChIP
Input
IgG
KLF17
Nutlin-3
ID1
Vimentin
Fibronectin
ZO-1
E-Cadherin
B
ChIP: ZO-1 promoter
Fold relative to IgG
CTL-siRNA
p53-siRNA
Nutlin-3
α-KLF17
C
ChIP: Vimentin promoter
Fold relative to IgG
CTL-siRNA
p53-siRNA
Nutlin-3
α-KLF17
D
ChIP: ID1 promoter
Fold relative to IgG
CTL-siRNA
p53-siRNA
Nutlin-3
0 6 12 24 0 6 12 24
α-KLF17
α-KLF17
E
CTL-siRNA
p53-siRNA
Nutlin-3 (10μM)
Nutlin-3 (5μM)
WT-Competitor
Mut-Competitor
KLF17-DNA complex
Free probe
1 2 3 4 5 6 7 8
F
ChIP: ID1 promoter
Fold relative to IgG
CTL-siRNA
p53-siRNA
Adriamycin
α-KLF17
G
ChIP: ID1 promoter
Fold relative to IgG
CTL-siRNA
p53-siRNA
Etoposide
α-KLF17

Figure 7

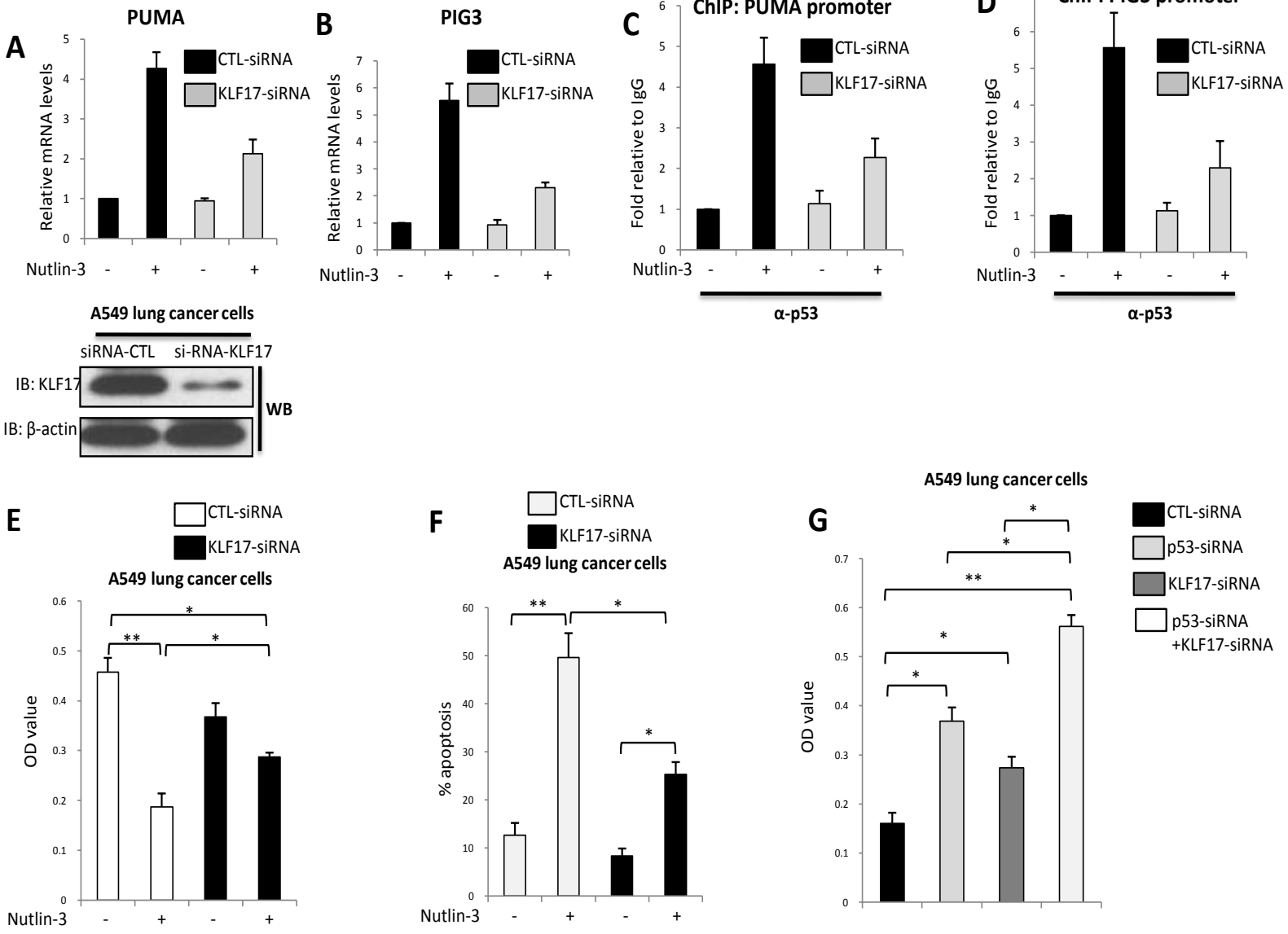

A
PUMA
Relative mRNA levels
CTL-siRNA
KLF17-siRNA
Nutlin-3 - + - +
B
PIG3
Relative mRNA levels
CTL-siRNA
KLF17-siRNA
Nutlin-3 - + - +
C
ChIP: PUMA promoter
Fold relative to IgG
CTL-siRNA
KLF17-siRNA
Nutlin-3 - + - +
α-p53
D
ChIP: PIG3 promoter
Fold relative to IgG
CTL-siRNA
KLF17-siRNA
Nutlin-3 - + - +
α-p53
A549 lung cancer cells
siRNA-CTL si-RNA-KLF17
IB: KLF17
IB: β-actin
WB
E
CTL-siRNA
KLF17-siRNA
A549 lung cancer cells
OD value
Nutlin-3 - + - +
F
CTL-siRNA
KLF17-siRNA
A549 lung cancer cells
% apoptosis
Nutlin-3 - + - +
G
A549 lung cancer cells
OD value
CTL-siRNA
p53-siRNA
KLF17-siRNA
p53-siRNA +KLF17-siRNA

Figure 8

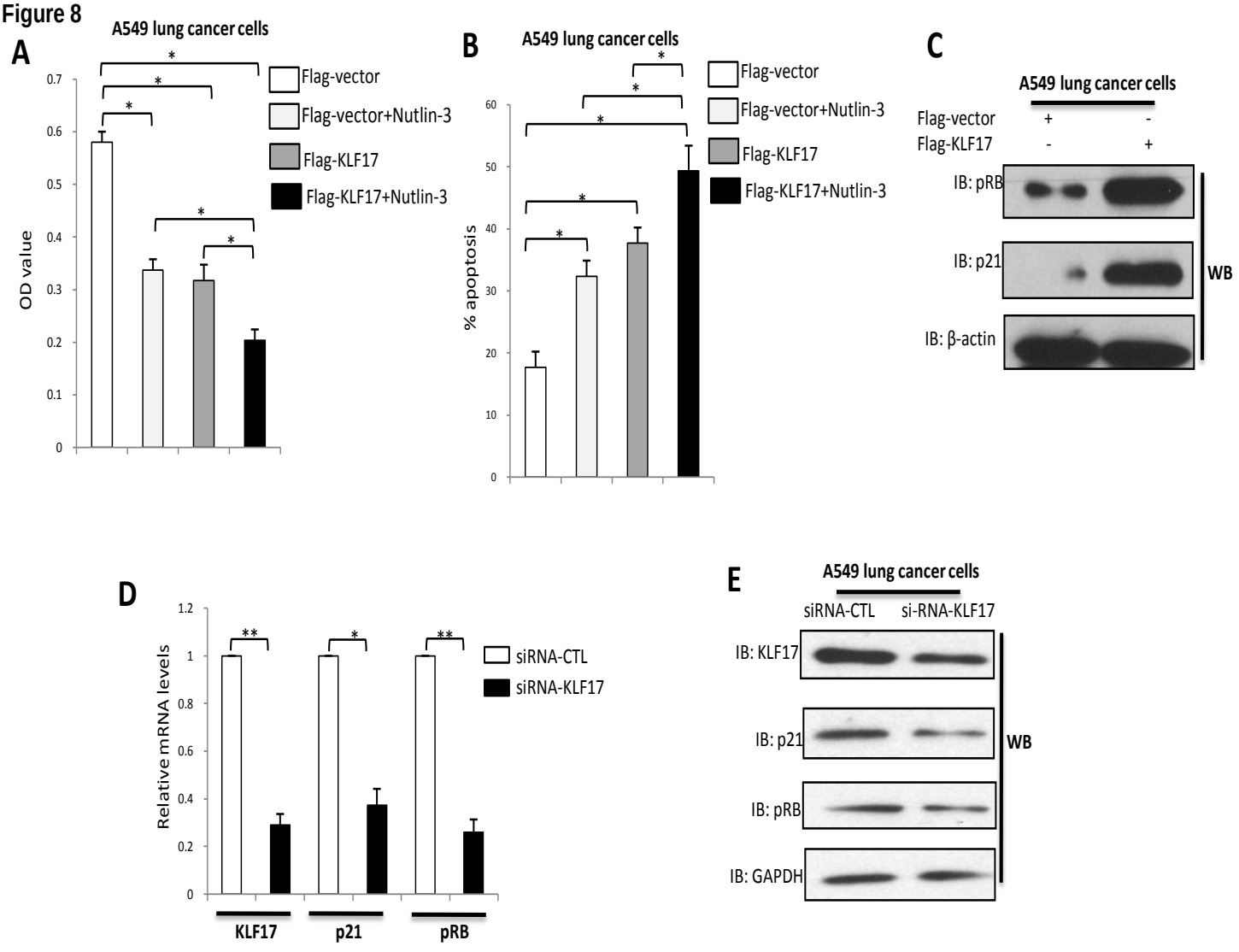

A
A549 lung cancer cells
OD value
Flag-vector
Flag-vector+Nutlin-3
Flag-KLF17
Flag-KLF17+Nutlin-3
B
A549 lung cancer cells
% apoptosis
Flag-vector
Flag-vector+Nutlin-3
Flag-KLF17
Flag-KLF17+Nutlin-3
C
A549 lung cancer cells
Flag-vector
Flag-KLF17
IB: pRB
IB: p21
IB: β-actin
WB
D
Relative mRNA levels
siRNA-CTL
siRNA-KLF17
KLF17
p21
pRB
E
A549 lung cancer cells
siRNA-CTL
si-RNA-KLF17
IB: KLF17
IB: p21
IB: pRB
IB: GAPDH
WB

Figure 9

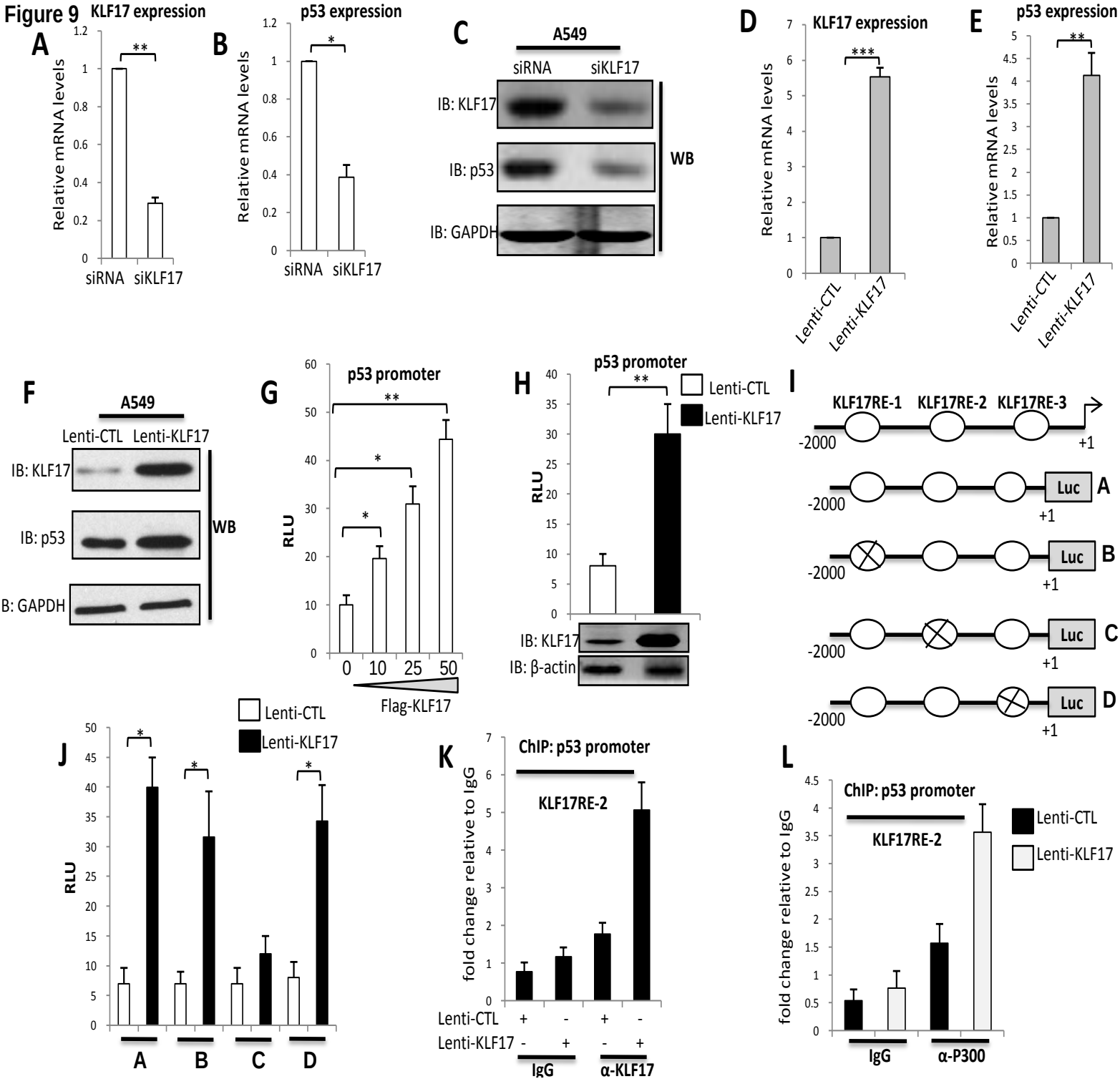

A
KLF17 expression
Relative mRNA levels
siRNA
siKLF17
B
p53 expression
C
A549
IB: KLF17
IB: p53
IB: GAPDH
WB
D
KLF17 expression
Lenti-CTL
Lenti-KLF17
E
p53 expression
F
A549
G
p53 promoter
RLU
Flag-KLF17
H
p53 promoter
IB: β-actin
I
KLF17RE-1
KLF17RE-2
KLF17RE-3
Luc
J
K
ChIP: p53 promoter
fold change relative to IgG
IgG
α-KLF17
L
α-P300

Figure 10

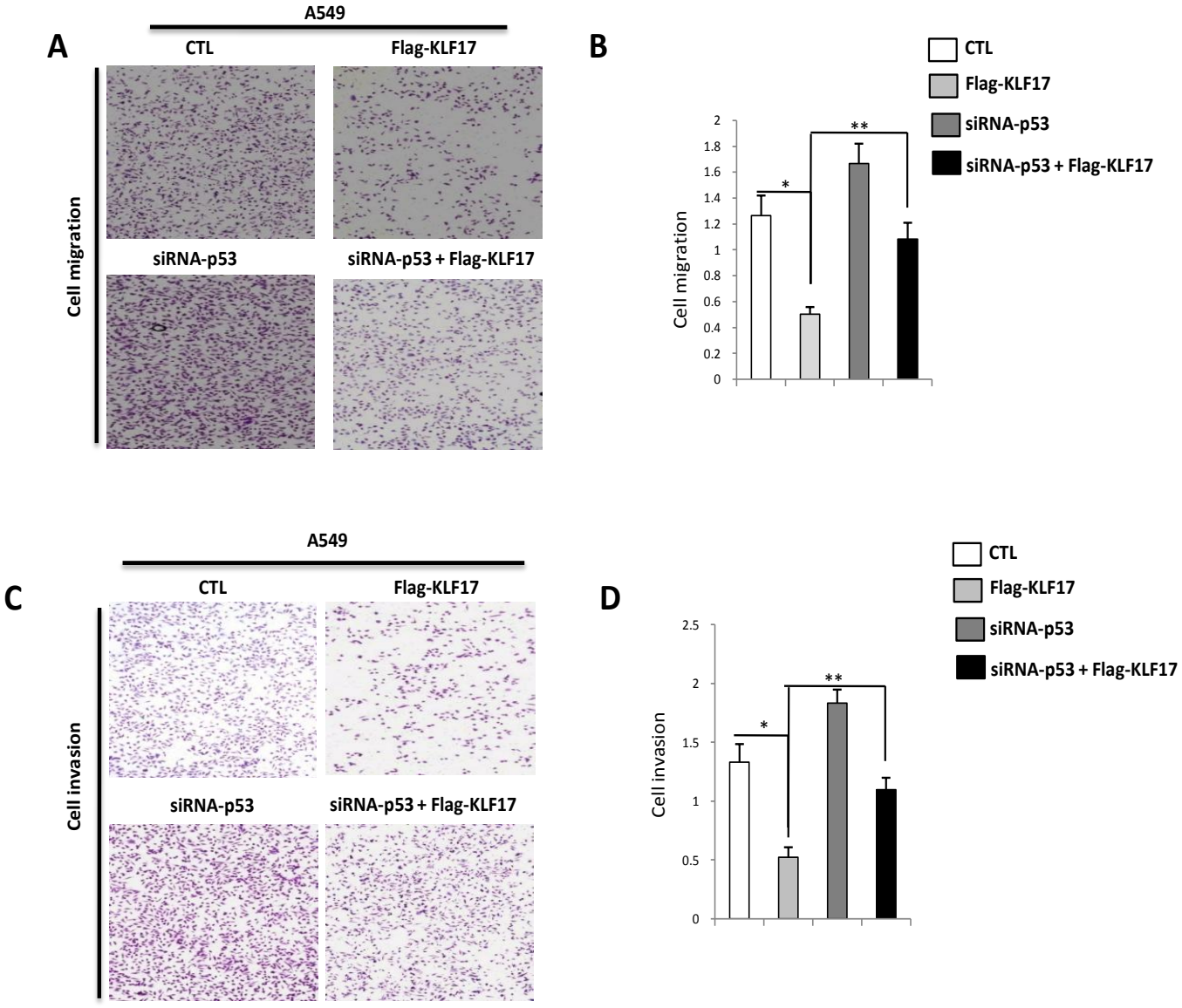
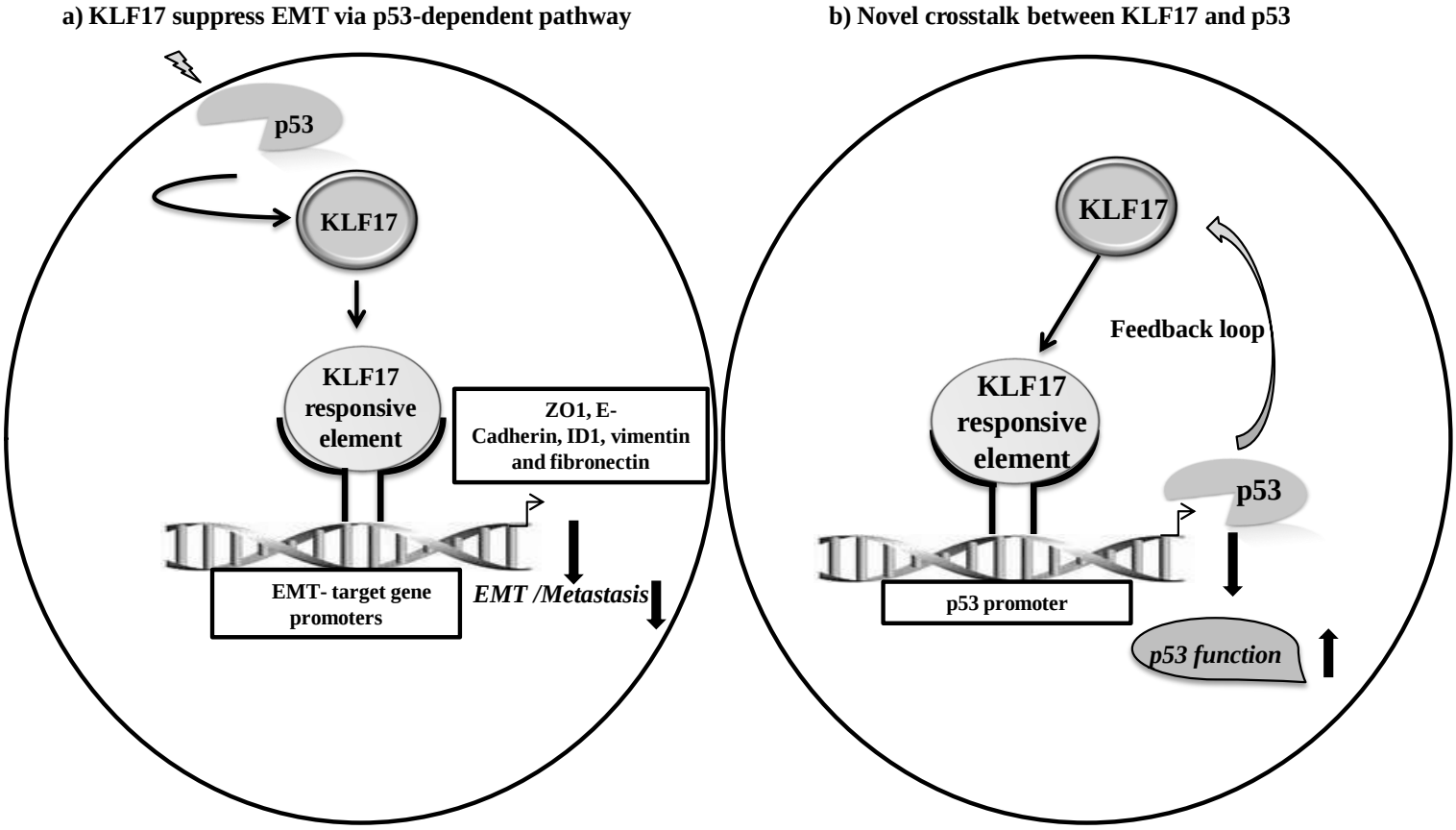

A
A549
CTL
Flag-KLF17
Cell migration
siRNA-p53
siRNA-p53 + Flag-KLF17
B
CTL
Flag-KLF17
siRNA-p53
siRNA-p53 + Flag-KLF17
Cell migration
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
*
**
C
A549
CTL
Flag-KLF17
Cell invasion
siRNA-p53
siRNA-p53 + Flag-KLF17
D
CTL
Flag-KLF17
siRNA-p53
siRNA-p53 + Flag-KLF17
Cell invasion
0
0.5
1
1.5
2
2.5
*
**

Figure 11


a) KLF17 suppress EMT via p53-dependent pathway
p53
KLF17
KLF17 responsive element
ZO1, E-Cadherin, ID1, vimentin and fibronectin
EMT- target gene promoters
EMT /Metastasis
b) Novel crosstalk between KLF17 and p53
KLF17
Feedback loop
KLF17 responsive element
p53
p53 promoter
p53 function