Tumor-suppressive p53 Signaling Empowers Metastatic Inhibitor KLF17-dependent Transcription to Overcome Tumorigenesis in Non-small Cell Lung Cancer*

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Background: How Krüppel-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 the anti-EMT effect of KLF17 via p53-dependent pathway.

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

Metastasis, which is controlled by concerted action of multiple genes, is a complex process and is an important cause of cancer death. Krüppel-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 non-small cell lung cancer (NSCLC). p53 is recruited to the KLF17 promoter and results in the formation of p53-DNA complex. p53 enhances binding of p300 and favors histone acetylation on the KLF17 promoter. Mechanistically, we found that KLF17 increases the expression of tumor suppressor genes p53, p21, and pRB. Functionally, KLF17 required p53 to suppress cancer cell invasion and migration in NSCLC. In conclusion, our study highlights a novel insight into the anti-EMT effect of KLF17 via a p53-dependent pathway in NSCLC, and KLF17 may be a new therapeutic target in NSCLC with p53 status.

Non-small cell lung cancer (NSCLC)4 is an aggressive type of lung cancer, and about 80% lung cancers are NSCLC (1). Lung cancer is one of the leading causes of cancer-associated death and can be divided into squamous cell carcinoma, adenocarcinoma, and large cell lung carcinoma (2). Despite recent advances and improvements in the field of chemotherapy for lung cancer, still the prognosis of NSCLC is very poor, and about 30–55% of 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). Decreased 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

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4 The abbreviations used are: NSCLC, non-small cell lung cancer; EMT, epithelial-mesenchymal transition; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qRT-PCR, quantitative RT-PCR; p53RE, p53-responsive element.
cancer cell 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 a tumor suppressor transcription factor and binds to the CACCC sequence on target gene promoters (9, 10). Recent studies have shown 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 expression (4, 7).

Tumor suppressor p53 is known as a “guardian of the genome” and functions 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 have shown that p53 has anti-proliferative functions and inhibits cancer cell growth (17, 18). p53 functions as a transcription factor and has the ability to trigger multiple tumor-suppressive pathways by targeting key genes (19–21). Activation of p53 in response to DNA damage triggers induction of apoptosis and subsequently leading to cancer inhibition (22, 23).

The 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 multistep process, which finally leads to metastatic tumor development (29–31). EMT is considered a critical process for cancer progression to a metastatic stage (32).

Here, we report a novel molecular and functional link of KLF17 with p53. We show 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 KLF17-mediated EMT gene transcription and the tumor-suppressive function of KLF17. Intriguingly, chemotherapeutic agents that activate p53 show recruitment of KLF17 to EMT target gene promoters via a p53-dependent pathway. Moreover, our results show that novel cross-talk and a 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 the anti-EMT effect of KLF17 via a 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 the manufacturer’s protocol (Invitrogen).

Antibodies—The following antibodies were used in Western blot and ChIP experiments: anti-p53 (DO-1, Santa Cruz Biotechnology, Inc.), anti-KLF17 (Abcam), anti-GAPDH (Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology), anti-P300 (Santa Cruz Biotechnology), and anti-Ach4 (Millipore).

Cell Culture and Treatments—H1299 and A549 cells were described previously in our study (33). For cell treatments, we used Nutlin-3 (10 μM) (Sigma–Aldrich), Adriamycin (0.5 μM) (Sigma–Aldrich), and etoposide (10 μM) (Sigma–Aldrich).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed with 32P-radiolabeled probes. 2 μg of nuclear extract or different concentrations of purified proteins were incubated with 32P-radiolabeled probes in 20 μl of EMISA reaction buffer (2 μg of poly(dl-dC), 20 mM HEPES (pH 7.9), 1 mM MgCl2, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 12% glycerol). To perform the competition assay, an excess of unlabeled competitor oligonucleotide was added to the EMISA reaction mixture. Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5× TBE and exposed to a phosphor imager (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% agarose.

KLF17 Luciferase Reporter Constructs—DNA fragments containing KLF17 genomic sequences were amplified from 293T cell genomic DNA using PCR and primers derived from human genomic KLF17 and ligated into KpnI/XhoI sites of the promoterless pGL3-Basic (Promega) vector to make this construct, pGL3-KLF17-luc. Different deletion constructs of the KLF17 promoter were generated from the 2-kb KLF17 promoter and ligated into KpnI/XhoI sites of pGL3-Basic vector.

Luciferase Assay—After transfection and/or treatment, the cells were washed with phosphate-buffered saline (PBS) three times. The cells were then lysed in the luciferase cell culture lysis buffer provided with the luciferase assay kit (Promega, Madison, WI). After a brief vortex, whole cell lysates were centrifuged in the cold (4 °C) at 12,000 rpm for 2 min. Supernatant was collected in a fresh tube, and 20–30 μl of that was added to luciferase assay substrate (60–80 μl). Luminescence was measured as relative light units, twice for each lysate, taking the reading of the luciferase assay using a LUMIstar OPTIMA (BMG LABTECH). Each assay was repeated three times. Fold repression values are represented as means of the three experiments.

RNA Interference—Cells were cultured to 30% confluence. For each well in a 6-well culture dish, 20 nm KLF17/p53 siRNAs or appropriate negative control siRNAs was transfected into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Cells were incubated at 37 °C in a CO2 incubator, and 6–8 h 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 h post-transfection and for mRNA expression at 48 h after transfection.

RT-PCR—RT-PCR was performed as described previously (33). Each experiment was performed in duplicates and repeated three times.

Preparation of Total Cell Extract and Western Blot Analysis—Cells were washed with PBS and treated with an extraction buffer (50 μM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1% Triton X-100) for 5 min on ice. Cell extracts were centrifuged at 20,000× g for 10 min at 4 °C, and supernatants were collected. Protein concentrations were measured by BCA assay (Pierce), and 10 μg of total protein was resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk in TBS-T for 1 h and incubated with primary antibodies overnight at 4 °C. After washing, membrane was incubated with the corresponding secondary antibody for 1 h at room temperature. Membranes were washed again, and primary antibody signals were detected using Pierce ECL kit (Thermo Scientific). Band intensity was quantified using ImageJ (NIH) software.
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sodium deoxycholate, 150 mM 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 subjected to electrophoresis in 10% SDS-polyacrylamide gel. Separated proteins were electroblotted to nitrocellulose membranes (Bio-Rad), and the blot was blocked for 1 h at room temperature with blocking buffer (0.1% BSA with 5% fat-free dried milk powder). The blot was then incubated with primary antibodies (1:1000 dilution) at 4 °C overnight. The blot was washed with 0.1% TBS three times and incubated with secondary antibodies (mouse and rabbit) (1:5000 dilution) for 1 h. The blot was washed again three times and exposed to an Odyssey LI-COR scanner.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay—Cell viability was assessed with an MTT assay in replicates. Cells were seeded in a 96-well plate at 2.5 × 10^3 cells/well and incubated in 10% FBS supplemented with DMEM for 24 h. After that, cells were treated with Nutlin-3/Arcimycin for the indicated times. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. After that, drug-containing medium was replaced with 200 μl of 10% FBS supplemented with DMEM containing 0.5 mg/ml MTT; cells were incubated in the CO2 incubator at 37 °C for 2 h; and absorbance (490 nm) was measured and analyzed.

Cell Migration—Cell culture inserts with a pore size of 8 μm were precoated by adding 10 μg/ml collagen I in PBS to the upper chamber at room temperature for 2 h. At the onset of each experiment, the cells were detached with Versene solution 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 of DMEM supplemented with 10% FBS. The cells were allowed to migrate for 24 h at 37 °C in a humidified atmosphere of 5% CO2. 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 counted either manually or with the help of the ImageJ 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/well suspended in 0.5 ml of serum-low DMEM (supplemented with 2% FBS). The rest of the protocol was identical to that described for 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 increased 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 decreased expression of KLF17 in lung cancer cells (Fig. 1, B–D). We treated A549 cells with different p53-activating chemotherapeutic agents and observed that these drugs were able to induce KLF17 expression (Fig. 1E). To further verify our results, we performed protein analysis of KLF17 in response to Nutlin-3. We found that the KLF17 protein level enhanced in Nutlin-3-treated cell lines (Fig. 1F). 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 the KLF17-luc reporter in H1299 (p53-null) lung cancer cells (Fig. 1G). Next, we generated different deletion constructs of KLF17-luc to find the minimal responsive region required for KLF17 activation by p53 (Fig. 1H). Luciferase analysis indicated that the region between −2000 and −498 is responsive to p53 (Fig. 1I), whereas the region from −498 to +1 was unable to enhance the KLF17 transcription (Fig. 1I), suggesting that the p53-responsive region lies between −1015 and −498. Using the NCBI database, we found the p53 consensus-responsive element (p53RE) within the KLF17 promoter (Fig. 1J). Mutation analysis of this p53RE indicated that p53 was unable to activate the KLF17 transcription from this mutated region (Fig. 1J). These 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 a chromatin immunoprecipitation (ChIP) assay in A549 cells. ChIP analysis indicated that Nutlin-3-treated cells showed recruitment of p53 to the KLF17 promoter, which contains bona fide p53RE. In contrast, no recruitment of p53 was observed within the upstream region of the KLF17 promoter (Fig. 2, A and B). We obtained similar results using Adriamycin, an anti-cancer drug (Fig. 2C). To further verify our data, we performed a gel shift assay in A549 cells that were left untreated or treated with Nutlin-3 for 24 h. We observed a complex on this p53RE (Fig. 2D), whereas the region from −498 to +1 was unable to activate the KLF17 transcription (Fig. 2D). Using the NCBI database, we found the p53 consensus-responsive element (p53RE) within the KLF17 promoter (Fig. 1J). Mutation analysis of this p53RE indicated that p53 was unable to activate the KLF17 transcription from this mutated region (Fig. 1J). These data indicate that p53RE is critical for KLF17 regulation by p53 in lung cancer cells.

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

p53 Empowers KLF17-mediated EMT Gene 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 a CACCC DNA sequence (4, 6, 7). Therefore, we investigated the affect of p53 on KLF17-mediated gene transcription. KLF17 inhibits metastases of non-small cell lung cancer (4, 6). 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 a CACCC DNA sequence (4, 6, 7). Therefore, we investigated the affect of p53 on KLF17-mediated gene transcription. KLF17 inhibits metastases of non-small cell lung cancer (4, 6).
tasis and tumor growth by negatively regulating ID1, vimentin, and fibronectin expression while inducing the ZO-1 and E-cadherin transcripts (4, 7). We silenced p53 expression in lung cancer cells using siRNA targeting p53 (Fig. 3A, top). 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, bottom). Transcript analysis of these target genes revealed that the tumor-suppressive ability of KLF17 was higher in p53-containing A549 cells in comparison with p53-depleted cells (Fig. 3, B–F).

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 gene expression (Fig. 3G). In contrast, silencing of p53 sup-
pressed the 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 gene expression.

**p53 Enhances Binding of KLF17 to EMT Target Gene Promoters**—To further gain insight into how p53 potentiates KLF17-mediated EMT gene 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 (Fig. 4, A–D). Conversely, in p53-containing cells, KLF17 has significant physical association with its target gene promoters (Fig. 4, B–D).

To further verify our results, we performed EMSA analysis using oligonucleotide probe from the ZO-1 promoter that contained the CACCC KLF17-responsive element. We transfected A549 cells with control siRNA or p53 siRNA and then transfected with expression plasmid encoding KLF17 for 24 h, qRT-PCR was performed to check different gene expressions. The average was calculated based on three independent experiments with mean ± S.D. (two-tailed Student’s t-test; **, p < 0.005).
Physically Interacts with KLF17—To further gain insight into how p53 enhances the tumor-suppressive ability of KLF17, we tested the interaction between KLF17 and tumor suppressor p53 by an 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 was further enhanced in Nutlin-3-treated cells (Fig. 5A). Nutlin-3 is an anti-cancer drug that 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). A similar co-precipitation of p53 and KLF17 was 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 (FL) and KLF17Δ1, KLF17Δ2, and KLF17Δ3, which contained deletions in the N terminus (Fig. 5D). The transfected cells were subjected to immunoprecipitation, carried out with anti-FLAG antibody. Positive interactions were obtained only between p53 and full-length KLF17, KLF17Δ1, or KLF17Δ2 but not with p53 and KLF17Δ3 (Fig. 5E). These results indicate that the region containing amino acids from 1 to 210 in the N-terminal region mediated the interaction between p53 and KLF17 and may have exerted an important effect on the tumor-suppressive function of KLF17 in lung cancer cells.

Activation of p53 Recruits KLF17 to EMT Gene Promoters in a p53-dependent Manner—Our results show that p53 enhances KLF17-mediated EMT gene 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. A 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 nutlin-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 (Fig. 6, B and C). In contrast, we did not observe any binding of KLF17 to EMT target gene promoters in p53-depleted cells treated with Nutlin-3 (Fig. 6, B and C). Furthermore, we did a detailed kinetics study of KLF17 recruitment on the ID1 promoter in response to Nutlin-3. A ChIP assay showed recruitment of KLF17 to the ID1 promoter in Nutlin-3 treated in a p53-dependent manner (Fig. 6 D). Conversely, we did not observe binding of KLF17 with the ID1 promoter in p53-knockdown cancer cells (Fig. 6 D).

Next we asked whether p53-dependent KLF17 recruitment to EMT target gene promoters is limited to the Nutlin-3-induced model or if other anti-cancer drugs that activate p53 can also show this phenomenon. We selected Adriamycin and etoposide chemotherapeutic agents that activate p53. Importantly, a ChIP assay revealed that both Adriamycin- and etoposide-treated cells have more recruitment of KLF17 to the ID1 promoter in a p53-dependent manner (Fig. 6, F and G). 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 cells. 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 cells treated with Nutlin-3, in comparison with KLF17-depleted cells (Fig. 7, A and B). Moreover, we observed decreased recruitment of p53 to PUMA and PIG3 target gene promoters in response to Nutlin-3 in KLF17-depleted cells (Fig. 7, C and D), 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 Nutlin-3 and more proliferative, even in the presence of p53 (Fig. 7E). Furthermore, knockdown of KLF17 decreased the apoptotic level of A549 cells in response to Nutlin-3 (10 μM) for 24 h, and chromatin immunoprecipitation was performed with the indicated antibodies. B–D, A549 cells were transfected with control siRNA or siRNA targeting p53 (20 nM) for 48 h and then left untreated or treated with Nutlin-3 (10 μM) for 24 h, and chromatin immunoprecipitation was performed with the indicated antibodies. B–D, the average was calculated based on three independent experiments with mean ± S.D. (error bars) (two-tailed Student’s t test; *p < 0.05). E, A549 cells were transfected with control siRNA or siRNA targeting p53 (20 nM) for 48 h and then left untreated or treated with Nutlin-3 (5 and/or 10 μM) for 24 h, and EMSA was performed with an oligonucleotide probe derived from the ZO-1 promoter. F, A549 cells were transfected with control siRNA or siRNA targeting p53 (20 nM) for 48 h and then left untreated or treated with Adriamycin for 24 h, and chromatin immunoprecipitation was performed with the 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 p53 (20 nM) for 48 h and then left untreated or treated with etoposide for 24 h, and chromatin immunoprecipitation was performed with the indicated antibodies. The average was calculated based on three independent experiments with mean ± S.D. (two-tailed Student’s t test; *p < 0.05).

FIGURE 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 h, and chromatin immunoprecipitation was performed with the indicated antibodies. B–D, A549 cells were transfected with control siRNA or siRNA targeting p53 (20 nM) for 48 h and then left untreated or treated with Nutlin-3 (10 μM) for 24 h, and chromatin immunoprecipitation was performed with the indicated antibodies. B–D, the average was calculated based on three independent experiments with mean ± S.D. (error bars) (two-tailed Student’s t test; *p < 0.05). E, A549 cells were transfected with control siRNA or siRNA targeting p53 (20 nM) for 48 h and then left untreated or treated with Nutlin-3 (5 and/or 10 μM) for 24 h, and EMSA was performed with an oligonucleotide probe derived from the ZO-1 promoter. F, A549 cells were transfected with control siRNA or siRNA targeting p53 (20 nM) for 48 h and then left untreated or treated with Adriamycin for 24 h, and chromatin immunoprecipitation was performed with the 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 p53 (20 nM) for 48 h and then left untreated or treated with etoposide for 24 h, and chromatin immunoprecipitation was performed with the indicated antibodies. The average was calculated based on three independent experiments with mean ± S.D. (two-tailed Student’s t test; *p < 0.05).

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Overexpression of KLF17 Enhances Cytostatic Ability of p53—Next, we sought to determine whether enforced expression of KLF17 suppresses lung cancer cells growth in response to chemotherapy. MTT analysis showed that both Nutlin-3 (which activates p53) and KLF17 independently decreased the cell viability of lung cancer cells, whereas the combination of both Nutlin-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 of how KLF17 inhibits lung cancer cell growth. Intriguingly, we found that KLF17 increased tumor suppressor p21 and pRB expression in lung cancer cells (Fig. 8C). p21 and pRB are inhibitors of cell growth and enhance apoptosis (12, 13, 44–47). Importantly, depletion of KLF17 decreased mRNA and protein levels of p21 and pRB (Fig. 8, D and E). 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, in order to determine 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 the mRNA level of p53 (Fig. 9, A and B). We obtained similar results at the protein level (Fig. 9 C).

To further assess the effect of KLF17 on p53 expression, we established A549 transfectants stably expressing KLF17 using lentiviral infection. A qRT-PCR analysis demonstrated that the KLF17 expression level in lenti-KLF17 A549 cell lines was significantly higher than in the control stable cell lines (Fig. 9 D).

Consistently, we observed higher expression of p53 both at mRNA and protein levels in A549 cells stably expressing KLF17 (Fig. 9, E and F).

Next, to investigate whether KLF17 enhances p53 transcription, we co-transfected p53-Luc reporter with KLF17-encoding plasmid into A549 cells. Induction was observed when the reporter construct was co-transfected with different doses of KLF17 (Fig. 9 G). Importantly, we found higher luciferase activity of the p53-Luc construct in lenti-KLF17 A549 cells (Fig. 9 H).

Bioinformatics analysis, using the NCBI database, revealed that the human p53 promoter contains three putative KLF17-responsive elements termed KLF17RE (Fig. 9 I, top).

We attempted to identify the regulatory region conferring KLF17 responsiveness within the p53 promoter. We constructed different p53-Luc constructs that contain KLF17RE(s) (Fig. 9 I, bottom). Deletion of the 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. 9 J).

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

Taken together, these results suggest that KLF17 enhances p53 transcription and generates 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 enhances KLF17-mediated EMT gene transcription. These hints prompted us to investigate the functional effect of KLF17 on tumor cell migration and invasion in both control and p53-depleted cells. Overexpression of KLF17 inhibited cell migration in A549 cells (Fig. 10A, top). Importantly, depletion of p53 decreased KLF17 ability to suppress cell migration (Fig. 10, A (lower panel) and B).

Next, we performed an invasion assay in A549 cells. Enforced expression of KLF17 decreased the invasion of A549 cells (Fig. 10C, top). In contrast, silencing of p53 abolished the ability of KLF17 to significantly suppress invasion of lung cancer cells (Fig. 10, C (bottom) and D). 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 that is controlled by joint regulation of several signaling cascades and is one of the main causes of cancer-associated death. NSCLC is an aggressive type of lung cancer; 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 of how KLF17 controls 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 exert gain-of-function ability to inhibit KLF17 expression (48). Similarly, a recent study (6) showed that microRNA-9 represses KLF17 expression. However, the signaling that positively controls the KLF17 pathway to suppress cancer metastasis remains unknown. Here, we showed a novel molecular and functional link of KLF17 with p53. Our data reveal for the first time that KLF17 suppresses EMT and metastasis in a p53-dependent manner.
tasis in a p53-dependent manner (Fig. 11). Our study provides new insight into the 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 inhibitors 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 the tumor-suppressive function of KLF17. Our results highlight novel cross-talk between p53 and KLF17 tumor-suppressive signaling in lung cancer cells (Fig. 11). Moreover, we show that a novel...
positive feedback loop exists between p53 and KLF17, which suggests that these proteins enhance tumor suppressive function of each other (Fig. 11). We found that endogenous p53 and KLF17 proteins form a complex in lung cancer cells. Importantly, formation of the p53-KLF17 complex was further enhanced in response to chemotherapeutic agents that activate 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 decreased expression of KLF17 in lung cancer cells. EMSA analyses showed formation of a p53-DNA complex on the KLF17 promoter, which suggests that KLF17 is a direct target of p53. Our study highlights that induction of KLF17 by p53 may reduce the 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 gene expression during cancer metastasis. Our results show that knockdown of p53 decreased both KLF17 and KLF17 downstream target gene expression. Moreover, p53 enhanced formation of the KLF17-DNA complex on EMT target gene promoters. 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 the 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

FIGURE 10. KLF17 suppresses EMT in a p53-dependent manner. A, A549 cells were transfected with FLAG-vector encoding KLF17 or transfected with siRNA targeting p53, and a cell migration assay was performed. B, statistical analysis of A. Data are representative of three independent experiments with mean ± S.D. (error bars) (two-tailed Student’s t test; *, p < 0.05; **, p < 0.005). C, A549 cells were transfected with FLAG-vector encoding KLF17 or transfected with siRNA targeting p53, and a 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; **, p < 0.005).
that Nutlin-3 treatment results in the formation of a KLF17-DNA complex on EMT target gene promoters, which was p53-dependent. Importantly, p53-activating anti-cancer drugs induce KLF17 expression and recruit KLF17 to EMT promoters via a 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 a high level of p53. Depletion of KLF17 is associated with decrease expression of p53 in lung cancer cells. These results highlight the 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 a 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 cross-talk of KLF17 with other tumor-suppressive signaling remains unknown. Our study revealed a novel molecular and functional link between KLF17 and p53 signaling. p53 and KLF17 form a novel positive feedback loop to enhance tumor suppressive function of each other. p53 is important to trigger the KLF17 pathway in lung cancer cells to overcome EMT and cancer metastasis. Endogenous p53 and KLF17 form a complex and hence enhance 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 during metastasis, including its role as a transcriptional activator for inducing adherent protein expression and as a transcriptional repressor for suppressing oncogenic protein expression in the tumor microenvironment.

These findings define a novel mechanism by which p53 and KLF17 signaling affect each other during EMT and cancer metastasis, provide a new model of regulation of the KLF17 tumor-suppressive pathway by p53, and define novel insights into the anti-metastatic function of KLF17 via a p53-dependent pathway in NSCLC. Thus, p53-KLF17 novel positive feedback plays an 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 the prognosis and treatment of NSCLC patients.
KLF17 Suppresses EMT via p53-dependent Pathway in NSCLC

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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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