The Activating Transcription Factor 3 Protein Suppresses the Oncogenic Function of Mutant p53 Proteins*

Saisai Wei‡§, Hongbo Wang*,‡1, Chunwan Lu†, Sarah Malmut†, Jianqiao Zhang§, Shumei Ren‡, Guohua Yu**, Wei Wang**, Dale D. Tang³, and Chunhong Yan‡¶1

From the ‡Center for Cell Biology and Cancer Research and §Center for Cardiovascular Sciences, Albany Medical College, Albany, New York 12208, the GRU Cancer Center and ¶Department of Biochemistry and Molecular Biology, Georgia Regents University, Augusta, Georgia 30912, the †Key Laboratory of Molecular Pharmacology and Drug Evaluation, School of Pharmacy, Yantai University, Yantai 264005, China, and the **Department of Pathology, Affiliated Yantai Yuhuangding Hospital, Medical College of Qingdao University, Yantai 264000, China

Mutant p53 proteins (mutp53) often acquire oncogenic activities, conferring drug resistance and/or promoting cancer cell migration and invasion. Although it has been well established that such a gain of function is mainly achieved through interaction with transcriptional regulators, thereby modulating cancer-associated gene expression, how the mutp53 function is regulated remains elusive. Here we report that activating transcription factor 3 (ATF3) bound common mutp53 (e.g. R175H and R273H) and, subsequently, suppressed their oncogenic activities. ATF3 repressed mutp53-induced NFKB2 expression and sensitized R175H-expressing cancer cells to cisplatin and etoposide treatments. Moreover, ATF3 appeared to suppress R175H- and R273H-mediated cancer cell migration and invasion as a consequence of preventing the transcription factor p63 from inactivation by mutp53. Accordingly, ATF3 promoted the expression of the metastasis suppressor SHARP1 in mutp53-expressing cells. An ATF3 mutant devoid of the mutp53-binding domain failed to disrupt the mutp53-p63 binding and, thus, lost the activity to suppress mutp53-mediated migration, suggesting that ATF3 binds to mutp53 to suppress its oncogenic function. In line with these results, we found that down-regulation of ATF3 expression correlated with lymph node metastasis in TP53-mutated human lung cancer. We conclude that ATF3 can suppress mutp53 oncogenic function, thereby contributing to tumor suppression in TP53-mutated cancer.

The tumor suppressor p53 gene (TP53) is one of the most frequently mutated genes in human cancers. Found in about half of all human cancers, TP53 mutations frequently occur in residues residing in the central DNA-binding domain. These “hot spot” mutations (e.g. R175H and R273H) lose the ability to regulate expression of canonical p53 target genes and, thereby, are unable to suppress tumorigenesis caused by various oncogenic stimuli (1). In addition to the loss of tumor suppressor activity, mutant p53 proteins (mutp53)3 often acquire oncogenic activities, including promoting cell proliferation, conferring drug resistance, inducing angiogenesis, and, most importantly, promoting cancer cell invasion and metastasis (1). Indeed, although p53 mutation is often associated with a poor prognosis of cancer, mice carrying hot spot p53 mutations develop aggressive tumors characterized by a high frequency of metastasis (2, 3). Although mutp53 may directly regulate gene expression by binding to DNA elements distinct from the wild-type p53 (4), these mutated proteins more often achieve their gain of function through binding to transcriptional regulators (e.g. NF-Y and p63), thereby indirectly altering transcription (4, 5). p63 is a member of the p53 family of transcription factors and can regulate the expression of genes (e.g. SHARP1) involved in migration and invasion (6–8). p63 also controls cell migration and invasion by down-regulating integrin recycling (9). A subset of common p53 mutants, but not the wild-type p53, can efficiently bind and inactivate p63, thereby promoting cancer cells to invade and metastasize (9, 10). Relevant to our research interests, mutp53 was shown to suppress phorbol ester-induced expression of activating transcription factor 3 (ATF3) and prevent ATF3-mediated cell death (11).

ATF3 is a member of the ATF/cAMP-responsive element-binding protein family of transcription factors and can bind to the ATF/cAMP-responsive element-binding protein cis-regulatory element and regulate gene expression under various conditions. Although ATF3 is aberrantly expressed in a wide range of human cancers (12), its role in cancer remains unclear.

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2 To whom correspondence should be addressed: GRU Cancer Center, 1410 Laney Walker Blvd., CN-2134, Augusta, GA 30912. Tel.: 706-721-0099; E-mail: cyan@gru.edu.
3 The abbreviations used are: mutp53, mutant p53 protein; ATF, activating transcription factor; IP, immunoprecipitation; DMSO, dimethyl sulfoxide.
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Although it has been shown to promote tumorigenesis and metastasis in skin, breast, and prostate cancer (13–15), ATF3 has also been found to suppress oncogenic networks, induce cell death, and inhibit malignant dissemination in many cancer types, including glioblastoma, colon, prostate, bladder, and lung cancer (16–20). The context-dependent role that ATF3 plays in cancer is likely due to complex protein–protein interaction networks in which ATF3 is involved. Indeed, in addition to transcriptional regulation, ATF3 has been found to interact with many critical cellular proteins and regulate their functions. One of the well-characterized ATF3-binding proteins is the wild-type p53 (21–23). ATF3 binds the p53 C terminus via its leucine zipper domain, and this interaction prevents p53 from ubiquitin-mediated degradation, thereby activating p53 in response to DNA damage (23). Interestingly, ATF3 also binds the major p53 suppressor MDM2 and serves as a bona fide substrate for E3 ubiquitin ligase (24). Given that MDM2 is a p53 target gene, the ATF3–p53–MDM2 interplay likely fine-tunes p53 tumor suppressor activity in response to oncogenic challenges. ATF3 also binds human papillomavirus E6 protein and activates p53 in human papillomavirus-positive cancer cells by blocking p53 ubiquitination (25). Moreover, ATF3 represses androgen signaling by binding to the androgen receptor in prostate cancer (26). These findings support a notion that ATF3 can regulate cancer development and progression via protein–protein interaction. In line with this notion, we report here that ATF3 bound mutp53 to reverse drug resistance and suppress migration and invasion of p53-mutated cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfections—H1229 and MDA-MB-231 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, whereas SKBR3 cells were cultured in McCoy’s 5a medium. These cells are routinely maintained in our laboratory. The A431 cell line, cultured in DMEM, was a gift from Dr. Shi-Yong Sun. We obtained plasmids expressing the wild-type p53, R175H, R273H, R249S, and V143A from Dr. Bert Vogelstein. The coding sequences of these proteins were amplified by PCR and cloned into pGEX-3X to express GST fusion protein in bacteria. The construct expressing FLAG-p63 was obtained from Addgene (Cambridge, MA). Plasmids expressing ATF3 and ΔATF3 have been described previously (23, 26). These genes were cloned into pIRE2-GFP (BD Biosciences) expressing ATF3 and was obtained from Addgene (Cambridge, MA). Plasmids expressing the ATF3-p63-FLAG-p63 fusion protein in bacteria. The construct expressing FLAG-p63 was obtained from Dr. Shi-Yong Sun. We obtained plasmids expressing wild-type p53 (21–23). ATF3 binds the p53 C terminus via its leucine zipper domain, and this interaction prevents p53 from ubiquitin-mediated degradation, thereby activating p53 in response to DNA damage (23). Interestingly, ATF3 also binds the major p53 suppressor MDM2 and serves as a bona fide substrate for E3 ubiquitin ligase (24). Given that MDM2 is a p53 target gene, the ATF3–p53–MDM2 interplay likely fine-tunes p53 tumor suppressor activity in response to oncogenic challenges. ATF3 also binds human papillomavirus E6 protein and activates p53 in human papillomavirus-positive cancer cells by blocking p53 ubiquitination (25). Moreover, ATF3 represses androgen signaling by binding to the androgen receptor in prostate cancer (26). These findings support a notion that ATF3 can regulate cancer development and progression via protein–protein interaction. In line with this notion, we report here that ATF3 bound mutp53 to reverse drug resistance and suppress migration and invasion of p53-mutated cancer cells.

shRNA Knockdown and Retroviral Infections—p53 and ATF3 knockdown was performed using a lentivector-based shRNA system (pSIH-H1 shRNA cloning and lentivector expression system, System Biosciences). The p53-targeted sequence was 5′-GAC TCC AGT GGT AAT CTA C-3′, on the basis of a publication (27). The ATF3-targeted sequences have been reported previously (25). For negative controls, a luciferase-targeted sequence (5′-CTT ACG CTG AGT ACT TCG A-3′) was cloned into the lentivector. For retrovirally infected cells, the ATF3-coding sequence was cloned into pBabe and then transfected into Ampho293 (Clontech) to pack retrovirions for infections as described previously (23).

Immunoblotting and Coimmunoprecipitation Assays—Immunoblotting was carried out as described previously (28). For co-IP assays, transfected cells were lysed in radioimmune precipitation assay lysis buffer (10 mM Tris-HCl (pH 7.3), 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 0.2 mM DTT, 10% glycerol, and protease inhibitors). Cell lysates (1 mg) were incubated with 1 μg of the ATF3 antibody or IgG at 4 °C overnight. The immunocomplex was precipitated with 25 μl of protein A-agarose (Roche), washed with 1 ml of radioimmune precipitation assay buffer supplemented with 300 mM NaCl for 3 times, and detected by immunoblotting. For FLAG-p63 IP, cell lysates were incubated with 25 μl of anti–FLAG M2 affinity gel (Sigma). Antibodies for p53 (DO-1), ATF3 (C-19), and p63 (4A4) were purchased from Santa Cruz Biotechnology. The β-actin antibody was obtained from Sigma.

Scratch Wound Migration Assays and Time-lapse Imaging—These were performed according to a recent report (9). Briefly, cells were grown in monolayers, wounded with a pipette tip, and then grown in 1% FBS. The movement of individual cells into the wound was recorded with a phase contrast ×20 objective using an inverted microscope (Olympus IX70). Images were obtained every 10 min for a total of 16 h. 20–50 cells were then tracked and analyzed for differences in speed and net distance using ImageJ.

Transwell Migration and Invasion Assays—For Transwell migration assays, the lower surface of polycarbonate membranes (8-μm pore size, Corning Inc.) were coated with 100 μg of fibronectin. Cells (2–5 × 10^5) suspended in 0.1 ml of medium containing 0.1% BSA were then dispensed into the upper chambers of Transwells and incubated for 5 h. Cells migrated to the lower membrane surface were fixed, stained with hematoxylin/eosin (Fisher Scientific), and counted under a microscope. For invasion assays, 10^5 cells were plated in Transwells coated with Matrigel and cultured for 24 h. Invaded cells were stained and counted as described previously (29).

Colony Formation Assays—200, 1.5 × 10^4, or 2.5 × 10^4 cells plated in 6-well plates were treated with DMSO, 1.5 μM of etoposide for 48 h, or 2 μg/ml cisplatin for 24 h, respectively. After treatment, cells were washed and grown in normal culture medium for 9 days for colony formation. Colonies were stained with crystal violet and counted as described previously (25).

Real-time RT-PCR—Total RNA was prepared, reverse-transcribed, and subjected to real-time PCR assays as described previously (30). The primer sequences used for real-time PCR are available upon request.
Immunohistochemical Staining—Tumor samples were obtained from 36 lung cancer patients with radical resection in the Affiliated Yantai Yuhuangding Hospital, Medical College of Qingdao University, Yantai, China. All experimental procedures were approved by the Institutional Review Board of the Affiliated Yantai Yuhuangding Hospital, and written informed consent was obtained for all patient samples used in this study. Standard immunohistochemical procedures were carried out using anti-ATF3 antibody (catalog no. HPA001562, 1:200, Sigma) and anti-p53 antibody (catalog no. sc-6243, 1:600, Santa Cruz Biotechnology) as described previously (26). Briefly, 5-μm sections were incubated in hot citrate buffer to retrieve antigens and then blocked in 5% normal goat serum. After incubation with primary antibodies overnight, sections were stained using an ABC Elite kit and a DAB kit (Vector) according to the recommendation of the manufacturer. The specimens were scored independently by two pathologists on the basis of staining intensity and subcellular localization (0, no staining; 1, weak staining; 2, strong staining in both cytoplasm and nucleus; and 3, strong nuclear staining). Statistical analyses were carried out using Fisher’s exact test or Mann-Whitney test as indicated in the figure legends.

RESULTS

ATF3 Interacts with Mutp53—We showed previously that ATF3 binds the wild-type p53 at its C terminus (amino acids 363–393) (23) (Fig. 1A). Because most p53 mutations occur in the DNA-binding domain, we reasoned that ATF3 could bind mutp53 proteins and affect their oncogenic activities. To test this, we carried out GST pulldown experiments to examine the binding of ATF3 to GST-fused hot spot p53 mutants, including R175H, R273H, R249S, and V143A. The GST-mutp53 fusion proteins, but not GST, pulled down in vitro-translated ATF3 (Fig. 1B, lanes 4–7 versus lane 2), indicating that ATF3 indeed interacted with these mutp53 proteins. ATF3 bound mutp53 at an affinity similar to that for its binding to the wild-type p53 because the amount of ATF3 pulled down by mutp53 was comparable with that pulled down by the wild-type p53 (Fig. 1B, lanes 4–7 versus lane 3).

To determine whether ATF3 also interacts with mutp53 in vivo, we expressed ATF3 and mutp53 in p53-null H1299 cells and then precipitated ATF3 from cell lysates using an ATF3 antibody. The ATF3 antibody precipitated R175H, R273H, R249S, and V143A along with ATF3 (Fig. 1C), demonstrating that ATF3 could interact with these mutp53 proteins. ATF3 bound mutp53 at an affinity similar to that for its binding to the wild-type p53 because the amount of ATF3 pulled down by mutp53 was comparable with that pulled down by the wild-type p53 (Fig. 1B, lanes 4–7 versus lane 3).

To test whether endogenous ATF3 and mutp53 proteins interact, we carried out co-IP assays in R175H-harboring SKBR3 and R273H-expressing A431 cells to test whether endogenous ATF3 and mutp53 proteins interact. The ATF3 antibody or the p53 antibody could coimmunoprecipitate both ATF3 and mutp53 proteins (Fig. 1D, lane 3 versus lane 2 and lane 7 versus lane 6), demonstrating that ATF3 can bind mutp53 in cells.

FIGURE 1. ATF3 interacts with mutp53. A, schematic representation of the regions responsible for ATF3 binding to p53. DBD, DNA-binding domain; ZIP, leucine zipper domain. B, the indicated GST-mutp53 fusion proteins were immobilized on glutathione-agarose and incubated with in vitro-translated ATF3 for GST pulldown assays. The bottom panel shows Ponceau S staining. C, H1299 cells were transfected with ATF3 and indicated mutp53 and lysed for co-IP assays using the ATF3 antibody or IgG. Precipitated mutp53 proteins were detected with the DO-1 antibody. D, SK-BR-3 and A431 cells were treated with 1.5 μM of camptothecin for 4 h to increase the ATF3 expression level and then subjected to co-IP assays using the ATF3 antibody or the p53 antibody, as indicated.
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A. Western blot analysis of p53 and β-actin levels in cell lines.

B. Western blot analysis of p53 and ATF3 levels in cell lines.

C. Graph showing cell numbers (x10^5) over days for different cell lines.

D. Bar graph showing relative mRNA level of V-pBabe, V-ATF3, R175H-pBabe, and R175H-ATF3.

E. Bar graph showing relative reporter activity for V-pBabe, V-ATF3, R175H-pBabe, and R175H-ATF3.

F. Bar graph showing relative reporter activity for AT3 in the presence or absence of V and R175H.

G. Images of colonies treated with DMSO, Cisplatin, and Etoposide for V-pBabe, V-ATF3, R175H-pBabe, and R175H-ATF3.

H. Bar graph showing colony number for different treatments.

I. Western blot analysis of p53, ATF3, and β-actin levels in cell lines treated with DMSO, Etoposide, and Cisplatin.

J. Bar graph showing colony numbers for H1299-R175H and SKBR3(R175H) treated with shLuc, shATF3, siLuc, and siATF3.
ATF3 Suppresses Mutant p53 Function

ATF3 Represses Mutp53-induced NFKB2 Expression and Reverse Drug Resistance—Given that ATF3 could bind mutp53, we tested whether ATF3 affects the gain of function of mutp53. Toward this end, we transfected H1299 cells and developed cell lines expressing the two most common mutp53 proteins, R175H and R273H. An empty vector cell line (V) was also developed and used as a control. The mutp53 expression level in these cells was comparable with that in A431 and MDA-MB-468 cells carrying native TP53 mutations (Fig. 2A, lanes 2 and 3 versus lanes 4 and 5). Because H1299 cells express a low level of ATF3, we overexpressed ATF3 in these mutp53 cell lines by retroviral infections, generating cells expressing mutp53 with or without ATF3 overexpression (Fig. 2B). Not surprisingly, neither mutp53 nor ATF3 expression altered cell growth (Fig. 2C).

It has been reported that mutp53 (e.g. R175H) induces NFKB2 expression to activate prosurvival NFκB signaling, thereby conferring drug resistance to cancer cells (31). Indeed, we found that the NFKB2 mRNA level and the NFκB transcriptional activity (represented by NFκB luciferase reporter activity) were increased in R175H-expressing cells (Fig. 2, D and E). Interestingly, ATF3 expression decreased the NFKB2 expression level and down-regulated the NFκB activity in R175H cells (Fig. 2, D and E). Transient expression of ATF3 in R175H cells was also sufficient to down-regulate NFκB transcriptional activity induced by the mutant protein (Fig. 2F). These results suggest that ATF3 might reverse mutp53-mediated drug resistance. To test this, we treated cells with cisplatin or etoposide and then determined cell survival using colony formation assays. Although the R175H cells survived much better than the empty vector cells, ATF3 expression significantly decreased the number of viable R175H cells (Fig. 2, G and H). ATF3 did not alter the mutp53 expression level under these conditions (Fig. 2J), suggesting that the ATF3-mediated inhibition of cell survival was a consequence of altered mutp53 activity. Of note, although it exerts proapoptotic activity under certain conditions (11, 17), ATF3 did not affect the survival of H1299 cells without R175H expression (Fig. 2, G and H). In line with these results, knockdown of ATF3 expression using shRNA increased the number of surviving R175H cells after etoposide treatment (Fig. 2J, left panel). Similarly, siRNA-mediated knockdown of ATF3 expression in SKBR3 cells carrying the endogenous R175H protein promoted cell resistance to etoposide treatment.

An early report demonstrated that resistance of SKBR3 cells to chemotherapeutic agents (including etoposide) was due to expression of the mutant p53 protein (32). Therefore, our results indicate that ATF3 can reverse drug resistance in p53-mutated cancer cells.

ATF3 Suppresses Mutp53-driven Cell Migration and Invasion—Mutp53 can promote cancer progression by driving cell migration and invasion. Therefore, we examined cell migration in scratch wound assays using time-lapse microscopy (Fig. 3A). Consistent with the migration-promoting activity of mutp53 (9), both R175H- and R273H-expressing H1299 cells moved significantly faster than the control cells, as evidenced by the significantly increased migration speed (Fig. 2B). The mutp53 cells appeared to move more erratically into the wound (Fig. 3A), and the net moving distance was significantly longer than that of the control cells (Fig. 2C). Interestingly, ATF3 appeared to counteract the mutp53 activity and, thus, ATF3 expression in both R175H and R273H cells significantly decreased the migration speed and the net distance (Fig. 3, B and C). ATF3 expression also increased the persistence of the mutp53 cells and changed the cell migration tracks to a pattern similar to that of the control empty vector cells (Fig. 3A). These results indicated that ATF3 suppressed mutp53-driven cell migration.

To corroborate this finding, we carried out Transwell assays to examine cell migration driven by fibronectin, a mutp53-mediated event (9). Although R175H and R273H increased the number of cells migrated through membranes, ATF3 significantly suppressed migration induced by mutp53 (Fig. 3, D and E). It is important to note that ATF3 had a minimal effect on the migration of cells without mutp53 expression (Fig. 3, A–E). Therefore, ATF3 appeared to counteract mutp53 and suppress cell migration mediated by the mutant proteins. Consistent with these results, ATF3 expression suppressed invasion of the R175H cells through Matrigel (Fig. 3F).

ATF3 Suppresses Migration in Cancer Cells Harboring TP53 Mutations—To test whether ATF3 affects the function of native mutp53, we determined whether ATF3 also suppresses migration in A431 cells harboring a native R273H mutation. The migration of A431 cells was largely driven by the native R273H protein because knockdown of its expression (Fig. 4A) dramatically decreased cell motility (Fig. 4B). ATF3 expression indeed significantly decreased the migration speed in A431 cells (Fig. 4B). In contrast, knockdown of ATF3 expression in these cells (Fig. 4C) increased cell motility (Fig. 4D). These results support a notion that ATF3 counteracts native mutant p53 proteins and suppresses their migration-promoting activity as well. The reason why ATF3 increased migration of the p53 knockdown cells (Fig. 4B) is unknown but might reflect the context-dependent nature of ATF3 function in cancer. We also determined the effects of ATF3 on migration of MDA-MB-231 cells carrying the native R280K mutation. ATF3 similarly suppressed migration in this cell line, although the inhibition was less effective compared with A431 cells (Fig. 4F). This was prob-
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Figure 3. ATF3 suppresses mutp53-induced cell migration and invasion. A, migration of indicated cells into scratch wounds was recorded with a time-lapse video microscope. The movement of individual cells are presented as track plots using ImageJ. B and C, migration speed (B) and net distance (C) of individual cells were extracted from the track plots using ImageJ. ns, no significant difference; **, p < 0.01; ***, p < 0.001; one-way analysis of variance and Bonferroni test. D and E, the indicated cells were plated in Transwells for migration assays. Three independent experiments were carried out, and representative fields are shown. The results are presented as mean ± S.E. *, p < 0.05; paired Student’s t test. F, the indicated cells were plated in Transwells coated with Matrigel for invasion assays. *, p < 0.05; Student’s t test.
ably because the mutp53 was not the major driving force for cell migration in MDA-MB-231 cells. Indeed, knockdown of p53 expression in MDA-MB-231 cells (Fig. 4E) only modestly decreased cell migration (Fig. 4F). Nevertheless, our results demonstrated that ATF3 can suppress the migration of cancer cells carrying native TP53 mutations.

**ATF3 Suppresses the Mutp53-p63 Interaction and Reactivates p63 in Mutp53 Cells**—A well characterized mechanism by which mutp53 promotes migration and invasion is related to the interaction between mutp53 and p63, which can inactivate p63 transcriptional activity by preventing p63 from binding to target genes (4). Indeed, it has been demonstrated recently that a mutp53-regulated p63-target gene, SHARP1, is a major migration and metastasis suppressor (7, 8). To explore the mechanism underlying the suppression of mutp53-driven migration by ATF3, we determined the effect of ATF3 on the mutp53-p63 interaction. We coexpressed FLAG-p63, R175H/R273H, and ATF3 in H1299 cells and carried out co-IP assays. The FLAG antibody efficiently coprecipitated R175H and p63 (Fig. 5A, lane 2) but precipitated only a small amount of R273H (Fig. 5A, lane 5), consistent with a previous report (9). These results confirmed the interaction between mutp53 and p63. ATF3 expression dramatically decreased the amounts of both R175H and R273H coprecipitated by FLAG-p63 (Fig. 5A, lane 3 versus lane 2 and lane 6 versus lane 5), indicating that ATF3 prevented mutant p53 from binding to p63. Interestingly, ATF3 was also found in the immunocomplexes, probably because of its interaction with R175H/R273H. Alternatively, ATF3 might directly bind p63. Indeed, although p63 was coprecipitated with ATF3 (Fig. 5B, lane 5), ATF3 appeared to directly bind p63, as revealed by GST pulldown assays (Fig. 5C, lane 3).

Given that ATF3 disrupted the mutp53-p63 interaction, we determined whether ATF3 affects the p63 transcriptional activity in mutp53-expressing cells. Using a luciferase reporter driven by a synthetic p63-responsive promoter, we found that p63 transcriptional activity was repressed by R175H or R273H, as expected (Fig. 5D). ATF3 expression in these mutp53-expressing cells significantly increased p63 transcriptional activity (Fig. 5D), arguing for the notion that ATF3 can counteract mutp53 and reactivate p63 in cells harboring mutp53. In line with these results, although R175H or R273H repressed SHARP1 expression, ATF3 significantly increased the mRNA level of this p63-target gene in mutp53-expressing cells (Fig. 5E). The increase in p63 reporter activity by ATF3 in the absence of R175H (Fig. 5D) might be due to direct binding of ATF3 to p63. However, ATF3 did not appear to affect SHARP1 expression in control cells (Fig. 5E).

**An ATF3 Mutant Deficient in Mutp53 Binding Fails to Suppress Mutp53-mediated Migration and p63 Inactivation**—Because ATF3 can bind mutp53, we reasoned that this interaction could account for the suppression rendered by ATF3 on mutp53 function. To test this, we used an ATF3 mutant (ΔATF3) devoid of the leucine zipper domain (amino acids 102–139) known to mediate ATF3 binding to wild-type p53 (23). This ATF3 mutant failed to bind R175H or R273H (Fig. 6A, lanes 8 and 9) but retained the capability of binding p63 (Fig. 6B, lane 3). We expressed ATF3 and ΔATF3 bicstronically with GFP in R175H cells (Fig. 6C) and examined the migration of ΔATF3-expressing (GFP-positive) cells and cells not expressing ΔATF3 (GFP-negative). Consistent with the previous results (Fig. 3), ATF3 expression dramatically suppressed the migration of R175H cells (Fig. 6, D and E, compare
In contrast, \( R175H \) ATF3 did not significantly alter the net migration distance of the mutp53 cells while decreasing the migration speed to an extent significantly smaller than wild-type ATF3 (Fig. 6, D and E, compare \( R175H \) \( \rightarrow \) and \( R175H \) ). Because \( \Delta \text{ATF3} \) failed to bind mutp53 but still bound p63, these results suggest that ATF3-mtp53 binding is required for the suppression of mutp53-mediated migration by ATF3. The residual migration-inhibitory activity of \( R175H \) ATF3 might be due to dimerization of \( R175H \) ATF3 with the endogenous wild-type ATF3 (23), which might result in binding of a small amount of \( R175H \) to R175H in cells. Of note, \( \Delta \text{ATF3} \) failed to counteract R175H-driven resistance to etoposide treatment as well (Fig. 6F), supporting the notion that ATF3 suppresses mutp53 function through interaction with mutp53.

We next carried out co-IP assays to determine whether \( \Delta \text{ATF3} \) lost the ability to disrupt the mutp53-p63 interaction. Indeed, the mutant ATF3 failed to prevent mutp53 from binding to p63 (Fig. 6G, lane 4 versus lane 3). Consistent with these results, \( \Delta \text{ATF3} \) failed to increase the p63 transcriptional activity repressed by R175H (Fig. 6H). We also sorted \( \Delta \text{ATF3} \)-expressing (GFP-positive) cells for quantitative PCR to quantify the \( \text{SHARP1} \) mRNA level. In contrast to the effects of ATF3, \( \Delta \text{ATF3} \) failed to induce \( \text{SHARP1} \) expression in R175H cells (Fig. 6I). These results suggest that the ATF3 mutant deficient in mutp53 binding lost the ability to reactivate p63 transcriptional activity in mutp53-expressing cells. Taken together, our results indicate that the binding of ATF3 to mutp53 reactivates p63 and accounts for the suppression of mutp53 oncogenic activities by ATF3.

**ATF3 Expression Negatively Correlates with Metastasis of TP53 Mutated Lung Cancer**—Given that ATF3 suppressed mutp53-mediated migration and invasion, we carried out immunohistochemical staining in human lung cancer samples to determine whether ATF3 expression correlates with metastasis in human cancer. We also stained the tissues with an anti-p53 antibody and used p53 immunopositivity as a surrogate marker for \( \text{TP53} \) mutations (33). Both the ATF3 and p53 anti-
FIGURE 6. An ATF3 mutant deficient in mutp53 binding fails to suppress mutp53 function. A, the indicated GST-mutp53 fusion proteins were incubated with in vitro-translated ATF3 or ΔATF3 for GST pulldown assays. B, immobilized GST-p63 or GST was incubated with purified recombinant ΔATF3 for a GST pulldown assay. C, R175H or control cells (V) were transfected with ATF3-IRES-GFP or ΔATF3-IRES-GFP. Expression of GFP and ATF3 was determined by immunoblotting and fluorescence microscopy. D and E, R175H-expressing cells (R175H) or control cells transfected with ATF3-IRES-GFP or ΔATF3-IRES-GFP were subjected to scratch wound migration assays as described in Fig. 3A, except that a fluorescence microscope was used to record cell movements. GFP-positive (ATF3 +) and GFP-negative (ATF3 −) cells were tracked and used to calculate migration speed (C) and net distance (D). ns, no significant difference; *, p < 0.05; **, p < 0.01; ***, p < 0.001; analysis of variance and Bonferroni test. F, R175H cells transiently transfected with GFP, ATF3-IRES-GFP, or ΔATF3-IRES-GFP were treated with DMSO or etoposide and subjected to colony formation assays as described in Fig. 2J. *, p < 0.05; Student’s t test. G, R175H cells were transfected with FLAG-p63, ATF3, or ΔATF3 as indicated and subjected to co-IP assays using anti-FLAG M2 affinity gel. H, the indicated cells were transfected with p63, ATF3, a p63-responsive luciferase reporter, or pRL-TK, as indicated, for Dual-Luciferase activity assays. Bottom panel, immunoblotting results of cell lysates. ***, p < 0.001; Student’s t test. I, R175 or control cells were transfected with GFP, ATF3-IRES-GFP, or ΔATF3-IRES-GFP for 2 days. GFP-positive cells were sorted and used for RNA preparation and quantitative PCR assays to determine SHARP1 expression. ***, p < 0.001; Student’s t test.
body generated clear staining on tumor sections (Fig. 7, A and B). Interestingly, neither ATF3 expression nor p53 mutation correlated with lymph node metastasis in these lung tumors (Fig. 7C). However, when we examined ATF3 expression in p53-positive samples, we found a significant inverse correlation between ATF3 expression and metastasis (Fig. 7C). Although ATF3-positive staining was frequently found in p53-positive tumors free of lymph node metastasis, fewer metastatic, p53-positive tumors expressed ATF3 (Fig. 7C). Such a correlation was not found in p53-negative tumors (Fig. 7C). Further analysis by scoring ATF3 staining intensity (Fig. 7D) (20) revealed that high ATF3 staining scores were often found in p53-positive tumors lacking lymph node metastasis (Fig. 7E). These results are consistent with the notion that ATF3 counteracts mutp53 and suppresses metastasis in TP53-mutated lung cancer.

DISCUSSION

The fact that TP53 mutations often correlate with a poor prognosis of cancer underscores the importance of unveiling the mechanisms by which the mutp53 oncogenic function is regulated. In this study, we found that ATF3 bound and counteracted common mutp53 proteins, thereby reversing drug resistance and suppressing migration of TP53-mutated cancer cells. Because ATF3 binds to the p53 C terminus (23), often unaffected by TP53 mutations, it is likely that ATF3 could bind a majority of mutp53 proteins and regulate their oncogenic function. Thus, our findings argue that ATF3 is a novel suppressor of mutp53. To our knowledge, ATF3 is the only protein, other than ANKRD1 (34), that has been demonstrated to suppress the oncogenic function of mutp53. Several other mutp53-binding proteins (e.g., PLK2 and TopBP1) promote the mutp53 gain of function (35, 36). This mutp53-suppressing activity is supported by our observations that ATF3 expression was lost or down-regulated in metastatic lung cancer harboring TP53 mutations (Fig. 7). Although the mechanism(s) underlying ATF3 loss/down-regulation in these tumors is currently unknown, our results suggest that increasing ATF3 expression and/or enhancing the ATF3-mutp53 interaction through therapeutic approaches would benefit patients with TP53-mutated metastatic cancer. Indeed, enforced expression of ATF3 in the TP53-mutated cancer cell lines A431 and MDA-MB-231 suppressed malignant cell migration (Fig. 3). Interestingly, ATF3 expression appeared to be down-regulated by R175H and R273H in response to etoposide and cisplatin treatment (Fig. 2I, lane 8 versus lane 6 and lane 12 versus lane 10), reminiscent of a previous finding that mutp53 repressed ATF3 expression upon phorbol ester treatment (11). Given that ATF3 is a mutp53 suppressor, mutp53-mediated down-regulation of ATF3 expression might serve as a mechanism to ensure maxi-

FIGURE 7. ATF3 expression negatively correlates with metastasis in TP53 mutated lung cancer. A, representative p53 staining in lung tumor samples. B, representative low/no or high ATF3 staining in p53-positive tumor samples (#6193 and #2523). C, summary of the staining results. The p values were calculated with Fisher’s exact test. D, scoring of ATF3 staining on the basis of intensity/nuclear localization in representative lung tumor sections. E, ATF3 staining scores were used to analyze the correlation between ATF3 expression and metastasis (Met). ns, no significant difference. The p value was calculated with a Mann-Whitney test.
ATF3 Suppresses Mutant p53 Function

ATF3 can increase the stability of wild-type p53 by preventing it from MDM2-mediated ubiquitination and degradation (23). However, ATF3 did not appear to affect mutp53 expression in the tested cells (Figs. 2B and 4, A and E). This result is consistent with the previous reports that MDM2 promotes mutp53 degradation only in specific contexts (e.g. precancerous situations prior to oncogene activation) (37, 38). Rather than altering protein stability, our results argue that ATF3 directly regulates the mutp53 oncogenic activity by binding to mutp53. The ATF3 binding prevented mutp53 from interacting with p63, resulting in reactivation of p63 and expression of anti-invasive p63 target genes (e.g. SHARP1) in mutp53-expressing cells. Because ATF3 binds to the p53 C terminus distinct from the region where p63 binds (10), it is unlikely that the disruption of the mutp53-p63 interaction by ATF3 is due to steric hindrance. More likely, ATF3 binding alters the mutp53 conformation required for p63 binding (10). Interestingly, although it only weakly interacted with p63 (Fig. 5A), R273H inactivated p63 and repressed SHARP1 expression as efficiently as R175H (Fig. 2D). These results are consistent with the notion that the mutp53 C terminus can inactivate p63 independently of its binding to p63 (9), suggesting that the interaction of ATF3 with the mutp53 C terminus might directly reactivate p63 as well. It is important to note that ATF3 also appeared to bind p63 (Fig. 5, B and C). However, it was the ATF3-mutp53 interaction rather than the ATF3-p63 interaction that reactivated p63 because the ATF3 mutant (ΔATF3) that can bind p63, but not mutp53, failed to increase p63 activity (Fig. 6H). Consistent with an early report (39), we also found that ATF3 interacted with p73 (data now shown), the other p53 family member bound by mutp53 (10). Whether ATF3 interferes with the mutp53-p73 interaction to regulate mutp53 function remains elusive. Unlike p63, however, the role of p73 in cancer development and progression has not been well established.

In addition to p63 targets, we found that ATF3 also regulated expression of other mutp53 targets, including NFKB2 (Fig. 2D). However, ATF3 did not appear to regulate NFKB2 expression in cells that did not carry mutp53 (Fig. 2D), arguing against the possibility that ATF3 transcriptional activity caused NFKB2 down-regulation in mutp53-expressing cells. Rather, the binding of ATF3 to mutp53 might alter the interactions of mutp53 with other transcriptional regulators (e.g. vitamin D receptor and ETS2) (40, 41) or DNA elements (5), thereby regulating gene expression. These could be achieved through conformational changes or steric hindrance. Given that mutp53 interacts with proteins or DNA through varying mechanisms (5), ATF3 might affect a subset of mutp53 target genes but leave other mutp53 targets unaffected. Indeed, we found that ATF3 also repressed hTERT (human telomerase reverse transcriptase) expression, but not ITAG6 or E2F expression, in R175H cells (data not shown). Expression of other reported mutp53 target genes, including EBAG9, survivin, cdc25A, and CXCL1 (5), was not altered by R175H in our H1299 cells (data not shown), suggesting that the cell context might contribute to gene expression regulated by mutp53.

ATF3 has been identified as a metastasis promoter in an early study investigating the metastatic potentials of B16 murine melanoma cell lines (42). ATF3 has recently been shown to up-regulate Twist/Snail/Slug expression and promote TGF-β-induced epithelial-to-mesenchymal transition in MCF10CA1a breast cancer cells (14), a finding consistent with the role of ATF3 in promoting cancer progression. However, emerging evidence supports that ATF3 can also serve as a metastasis suppressor. Whereas we and others demonstrated that ATF3 represses proinvasive matrix metalloproteinase 2 expression (21, 43, 44), ATF3 has been shown to suppress migration, invasion, and hepatic metastasis in colon cancer (19, 45). A recent report demonstrated that ATF3 suppresses the invasion of lung cancer cells and that ATF3 expression correlates with a better survival of lung cancer patients (20). More recently, ATF3 was found to suppress invasion and metastasis in bladder cancer by regulating cytoskeleton remodeling (18). These findings are in line with our results, supporting the metastasis-suppressing role of ATF3. Therefore, it appears that ATF3 contributes to cancer progression in a context-dependent manner. Such context-dependent regulation of cancer progression is not without precedent. Zbtb7a, a transcription factor shown previously to promote cellular transformation (46), has recently been found to suppress invasion in prostate cancer (47). Interestingly, the bladder cancer cell lines used by Yuan et al. (18) carry TP53 mutations. Whether the effects of ATF3 on cancer progression are dependent on TP53 mutation status is an interesting question and remains to be answered.

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