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Mutant p53 promotes tumor cell malignancy by both positive and negative regulation of TGF-β pathway*

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*Running title: *Mutant p53 deregulates the TGF-β pathway*

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Keywords: Migration; wild type p53; mutant p53; TGF-β; Smad3

Background: The molecular mechanisms by which mutant p53 deregulates TGF-β pathway remain obscure.

Results: Mutant p53 disrupts the Smad3/Smad4 complex by occupying the MH2 domain in Smad3 upon ERK-activation.

Conclusion: Mutant p53 achieves gain of function by attenuating and cooperating with TGF-β pathway via targeting Smad3.

Significance: Discovery of a new mechanistic mode explaining the cross-talks among mutant p53, TGF-β and ERK pathways.

ABSTRACT

Specific p53 mutations abrogate the tumor suppressive functions by gaining new abilities to promote tumorigenesis. Inactivation of p53 is known to distort the TGF-β signaling, which paradoxically displays both tumor-suppressive and pro-oncogenic function. The molecular mechanisms how mutant p53 simultaneously antagonizes tumor-suppressive and synergizes tumor-promoting function of TGF-β pathway remain elusive. Here we demonstrate that mutant p53 differentially regulates subsets of TGF-β target genes, by an enhanced binding to the MH2 domain in Smad3 upon the integration of ERK signaling, therefore disrupting the Smad3/Smad4 complex formation. Silencing Smad2, inhibition of ERK or introducing a phosphorylation defective mutation at Ser392 in p53 abrogates the R175H mutant p53 dependent regulation of these TGF-β target genes. Our study provide a mechanism to reconcile the seemingly contradictory observations that mutant p53 can both attenuate and cooperate with the TGF-β pathway to promote cancer cell malignancy in a same cell type.

Wild type p53, a well-known tumor suppressor gene, is activated in response to oncogenic stress to prevent cancer development (1). The p53 gene is mutated in around 50% of all human cancers and more than 75% of p53 alterations are missense mutations that lead to the synthesis of a stable but functionally aberrant protein (2-4). Mainly clustered at 6 hot spots, p53 mutations occur with unusually high frequency and result in loss of DNA binding. Most p53 mutants fall into two subclasses, DNA-contact mutants and DNA-binding domain structural mutants exemplified by R273H and R175H respectively (5). It has been suggested that mutant p53 exert its influence by

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either a dominant-negative or a "gain of function" effect on cells. The dominant-negative effect is well accepted by the observation that mutant p53 oligomerizes with wild type p53 and inhibit its function (6). The "gain of function" hypothesis states that mutant p53 not only simply loses wild-type p53 function, but gains multiple new activities contributing to tumorigenesis (7,8). Recent years, compelling evidence has been accumulated in support of the mutant p53 "gain of function" theory (9-11). Expression of mutant p53 in human cancers has been linked with a poorer prognosis (12).

Many members of TGF-β superfamily play a role in tumorigenesis (13). TGF-β signaling is mediated through its binding to the type I and type II receptors, the activated ligand-receptor complex typically activates Smad-dependent signal transduction (14). The canonical Smad signaling cascade is initiated by phosphorylation of R-Smad (Smad2 and/or Smad3) by activated ALK5. This allows R-Smad binding to Smad4 and translocation of the complex to the nucleus where it can recruit transcriptional coactivators or corepressors to Smad binding elements (SBEs) in the promoters of TGF-β target genes (15).

It is known that Smad2 and Smad3 physically interact and are structurally similar with 90% homology in their amino acid sequences (16), yet, the distinct functions of these two genes in embryonic development have been noted (17). Experimental data indicates that TGF-β can induce different functions in the same cell lines, such as growth arrest and epithelial-to-mesenchymal transition (EMT). Even though both playing important roles in tumorigenesis, intriguingly, there is ample evidence to suggest that Smad2 and Smad3 have distinct and non-overlapping roles in TGF-β signaling (18). It is known that Smad2 and Smad3 require interactions with a number of common transcriptional regulators for their actions (19,20). Some of these "co-factors" can act to alter the balance of Smad2 versus Smad3 mediated TGF-β signaling in the nucleus (21,22). However, the mechanisms for selective activation of Smad2 versus Smad3 are still largely unknown.

A convergence of p53 and Smad signaling pathways has been established (23), yet fully understanding the cross-talk between TGF-β and mutant p53 is complicated by the fact that TGF-β can act both as a tumor suppressor at early stages in carcinogenesis and as a pro-metastatic signal transducers at advanced stages (24). The studies by Adorno and colleagues suggest that mutant p53 can bind to Smad2/p63 complex but not Smad2 itself and cooperate with TGF-β, leading to suppression of p63 growth-inhibitory signal and induction of pro-migratory events (25). Prior studies in a different laboratory found that mutant p53 can attenuate TGF-β induced migration through the suppression of a range of TGF-β-dependent genes including the receptor gene TGFBR2 (26). It is still unclear how to reconcile the seemingly contradictory observations.

In the present study, we show that mutant p53 breaks the balance between Smad2 and Smad3 mediated signal transduction by occupying the MH2 domain in Smad3 and disrupting the Smad3/Smad4 complex formation. This action of mutant p53 hijacks ERK signaling that is required for the formation of mutant p53/Smad3 complex. Our study provides insights how mutant p53 differentially regulates subsets of TGF-β target genes for tumor suppressors and tumor promoters in a same cell type.

EXPERIMENTAL PROCEDURES

Antibodies, reagents and plasmids-Rabbit anti-p53, HSP90, GFP and mouse anti-p53, GAPDH, Smad4 antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-Smad2, Smad3, p-Smad2, p-Smad3, p-ERK1/2, ERK1/2, p-p53 (Ser392), Slug antibodies were obtained from Cell Signaling Technology. Mouse anti-actin and rabbit anti-Flag were purchased from Sigma. Mouse anti-p21 antibody was obtained from BD Pharmingen. HRP-goat anti-rabbit IgG and HRP-goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories.

Recombinant human TGF-β1 was obtained from PeproTech and was reconstituted to a final concentration of 5 μg/ml. All TGF-β treatments were performed at a concentration of 5 ng/ml. The MEK/ERK inhibitors PD98059 and U0126 were purchased from Cell Signaling Technology and were dissolved in DMSO at 10mM. Wortmanin and SB203580 were kindly provided by Dr. Ping Wang (East China Normal University). TGF-β

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Receptor inhibitor SB431542 and M2-Flag beads were purchased from Sigma. Glutathione Sepharose™ 4B was obtained from GE Healthcare Life Sciences.

pRK5-Smad2, pRK5-Smad3, pRK5-Smad4, GST-Smad2, GST-Smad3 and SBE-Luc were kindly provided by Dr. Xin Hua Feng (Baylor College of Medicine). Different p53 point mutations and truncations were generated by PCR and cloned into pCDNA3.1 vector. The Smad2 and Smad3 truncations were amplified from pRK5-Smad2 and pRK5-Smad3 by PCR and constructed into pCDNA3.1 vector. caMEK1 and HA-ERK1 were kindly provided by Dr. Ping Wang (East China Normal University)

Cell cultures-Mouse oral cancer derived cell lines J4708 (p53-/-) and J4705 (R172H p53) were gifts from Dr. Carlous Caulin (MD Anderson Cancer Center). Detroit 562 Lenti and shp53 stable cell lines were kindly provided by Dr. Jeffery N Myers (MD Anderson Cancer Center). The Smad3 knock out MEF cells were provided by Dr. Xin Hua Feng. H1299 cells stably expressing empty vector and R175H p53 were previously generated (27).

Time-lapse imaging-Time-lapse phase-contrast microscopy was performed on a Nikon inverted microscope equipped with a phase contrast 10X objective, a sample heater (37°C) and a home-made CO_2 incubation chamber. Images were obtained every 10 min for the duration of 12 hr.

Quantitative RT-PCR-To assess mRNA levels, RNA was isolated from cells using a Trizol reagent (Takara), and cDNA synthesized using the MLV reserve transcriptase from Promega. For the quantitative RT–PCR analysis, the reverse-transcribed cDNA was subjected to RT-PCR using SYBR-green master mix (Toyobo) and the Mx3005P quantitative RT-PCR system (Stratagene). Each experiment was performed in duplicates and was repeated three times. Primers used for Q-PCR. For human version: p21 (5'-GGCAGACCAGCATGACAGATT-3' and 5'-GCGGATTAGGGCTTCCTCT-3'); p15 (5'-CGTTAAGTTTACGGCCAACG-3' and 5'-GGTGAGAGTGGCAGGGTCT-3'); TBC1D16 (5'-CCTGCGCTACATCACACCC-3' and 5'-CATGCGGTCTGGAACACTC-3'); DAB2IP (5'-GTACCGGGAGACCGACAAGA-3' and 5'-GATGCGGATCATGGGTCCAG-3'); MMP2 (5'-GATACCCCTTTGACGGTAAGGA-3' and 5'-CCTTCTCCCAAGGTCCATAGC-3'); MMP9 (5'-GGGACGCAGACATCGTCATC-3' and 5'-TCGTCATCGTCGAAATGGGC-3'); MMP10 (5'-TTTGGCTCATGCCTACCCAC-3' and 5'-TCTTGCGAAAGGCGGAACTG-3'); Slug (5'-TGTGACAAGGAATATGTGAGCC-3' and 5'-TGAGCCCTCAGATTTGACCTG-3'); Smad7 (5'-CTCCAGATACCCGATGGATTTTC-3' and 5'-GCATCTGGACAGTCTGCAGTTG-3'); ID1 (5'-CTGCTCTACGACATGAACGG-3' and 5'-GAAGGTCCCTGATGTAGTCGAT-3'); AKAP12 (5'-GAGATGGCTACTAAGTCAGCGG-3' and 5'-CAGTGGGTTGTGTTAGCTCTTC-3'); TGM2 (5'-GAGGAGCTGGTCTTAGAGAGG-3' and 5'-CGGTCACGACACTGAAGGTG-3'); ASTE1 (5'-ATGGGTATCCGAGGACTAATGAG-3' and 5'-GTCCCGCAACTTCAAATCAGT-3'); for mouse version: MMP2 (5'-CAAGTTCCCCGGCGATGTC-3' and 5'-TTCTGGTCAAGGTCACCTGTC-3'); MMP9 (5'-CTGGACAGCCAGACACTAAAG-3' and 5'-CTCGCGGCAAGTCTTCAGAG-3'); MMP10 (5'-GAGCCACTAGCCATCCTGG-3' and 5'-CTGAGCAAGATCCATGCTTGG-3'); Slug (5'-TGGTCAAGAAACATTTCAACGCC-3' and 5'-GGTGAGGATCTCTGGTTTTGGTA-3'); p21 (5'-CCTGGTGATGTCCGACCTG-3' and 5'-CCATGAGCGCATCGCAATC-3'); AKAP12 (5'-CTGAGTCCCAAGCTAATGACG-3' and 5'-GGACGGTATCTGACTTTTCGTT-3'); TGM2 (5'-GCTGGACCAACAGGACAATGT-3' and 5'-CTCTAGGCTGAGACGGTACAG-3').

Transfections and Lentiviral Infections-For transient overexpression and siRNA transfection, the plasmids or dsRNA oligos (synthesized by Shanghai GenePharma Co., Ltd.) were transfected by Hilymax (Dojindo Molecular Technologies) and Lipofectamine 2000 (Invitrogen) respectively. siSmad2 target sequence: GGATGAAGTATGTGTAAAC (28); siSmad3 target sequence:

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GGATTGAGCTGCACCTGAATG (29); siSlug target sequence: GGACCACAGTGGCTCAGAA (30); sip53 target sequence: CCGCGCCATGGCCATCTACA (25).

Lentiviral empty vectors, a vector coding for wild-type-p53 or mutant-p53 were transfected into 293T cells in combination with pMD2G and pSPAX2 to produce viral particles needed for H1299 cell infection.

Coimmunoprecipitation-H1299 or generated stable cells were transfected with constructs or treated as explained in the figures. Cells were then scraped into ice-cold PBS and lysed with lysis buffer containing 50 mM Tris-HCl, pH7.5, 1 mM EDTA, 1% NonidetP-40, 150 mM NaCl, 10% glycerol and protease inhibitors, 24hrs after transfection or after treatment with TGF-β or ERK inhibitor for indicated periods. Specific proteins were immunoprecipitated, followed by 3 washes with wash buffer (50 mM Tris-HCl, pH7.5, 1 mM EDTA, 0.1% Nonidet P-40, 150 mM NaCl, 10% glycerol and protease inhibitors). The pellet was then resuspended in SDS sample buffer and analyzed by indicated antibodies, as described in figure legends.

GST Pull-Down Assays-Plasmid DNA constructs encoding glutathione-S-transferase (GST) fusion proteins were transformed into BL21 *E. coli* and purified using a standard purification protocol with glutathione beads (GE Healthcare). Purified GST fusion proteins adsorbed to glutathione beads are eluted and stored at -80℃ in small aliquots. Different p53 derivatives were *in vitro* translated and labeled with ^{35}S Met using TNT Quick translation system (Promega). Translated protein or overexpression mutant p53 cell lysate was incubated with recombinant Smad2, Smad2ΔExson3, Smad3 or their truncation mutants at 4℃ for 2 hrs. Beads were then washed 4 times with Triton X-100 lysis buffer (50 mM Tris-HCl, pH7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol). Samples were subjected to SDS-PAGE, followed by Coomassie brilliant blue staining or specific antibodies incubation. Binding of labeled protein to GST-fusion proteins was analyzed by autoradiography or Western blotting.

Migration Assays-Transwell assays were performed in 24 well PET inserts (Millipore 8.0 µm pore size) for cell migration. Cells plated in 6 cm dishes with serum starved overnight were transfected with indicated siRNA for 48hr hours. 5×10^4 cells in serum free media were plated in the upper chamber of transwell inserts (2 replicas for each sample). The inserts were then put in 10% serum media for 6h migration. Cells in the upper chambers were removed with a cotton swab; migrated cells were fixed in PFA 4% and stained with Crystal Violet 0.5%. Filters were photographed and the total number of migrated cells was counted. Every experiment was independently repeated three times.

Electrophoretic mobility shift assay-DNA-binding assays for Smad3/Smad4 were performed following the protocols described previously (27,31,32). Briefly, ^{32}P-labeled double-stranded SBE oligonucleotides from the *p21waf1* gene promoter region were used as probes, and competition assays were performed with a 100-fold excess of unlabelled wild-type or mutant SBE oligonucleotides (31).

In vitro kinase assay-Purified GST-R175H p53 protein was incubated with HA-ERK enriched from ERK-transfected cell lysate in the kinase buffer with 50µM ATP at 30℃ for 30min (33). The reactions were terminated upon SDS sample buffer. The phosphorylation of R175H p53 was detected by site-specific antibody for Ser392.

RESULTS

Mutant p53 enhances the motility of lung cancer cells-To pursue mechanistic studies, we generated stable cells expressing mutant p53 (R175H) in non-small cell lung carcinoma cell line H1299, which lacks p53 expression and therefore precludes the interference of wild-type p53 (3). We analyzed both single clones and a pool of the cell lines for mutant p53 related features that the tumor-derived mutant p53 can interfere with cytostatic effects of TGF-β and promote cancer cell migration. By BrdU incorporation cell proliferation assays, H1299 cells expressing mutant p53 (mutp53-H1299) were less responsive than the control H1299 cells (expressing vector only) to TGF-β induced growth-inhibition (Fig. 1A). Western blotting showed that mutant p53 attenuated TGF-β induced expression of p21^{WAF1}

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without influencing the phosphorylation of R-Smads (Fig. 1B), indicating that the mutant p53 may attenuate TGF-β signal transduction by a previously unidentified mechanism.

Using time-lapse microscopy and a software to track single cell movement, we successfully recorded the migratory behaviors of the mutp53-H1299 and the control H1299 cells. In agreement with previous studies (3), the paths extracted from each single cell demonstrated that the mutp53-H1299 cells moved faster but with less orientation than the control cells (Fig. 1C). Then, the migration abilities of these cells in the presence or absence of TGF-β were examined by transwell assays. Interestingly, mutant p53 harboring cells migrated faster than control cells, meanwhile, the mutp53-H1299 cells displayed faster migration than the control cells upon TGF-β stimulation (Fig. 1D), indicating that mutant p53 could enhance cells' response to TGF-β dependent migration. Taken together, we speculate that mutant p53 attenuates TGF-β's anti-tumor functions, while cooperates with its tumorigenic functions.

Mutant p53 differentially modulates the transcription in subsets of TGF-β target genes-To investigate how mutant p53 deregulates TGF-β dependent transcription, pooled mutp53-H1299 and control H1299 cells were treated with TGF-β before harvested for quantitative RT-PCR. Expression of p21 and p15, two of the canonical tumor suppressor genes responsive to TGF-β, was significantly attenuated in R175H p53 cells, comparing with that in their p53 null counterpart controls. Given a recent finding that mutant p53 can inhibit the function of the tumor suppressor DAB2IP that encodes a GTPase-activating protein (GAP) (34), we speculated that TGF-β and mutant p53 might be involved in the regulation of a group of GAPs' expression. Expectedly, both DAB2IP and TBC1D16 GAP genes had reduced expression in mutant p53 cells. However, Some TGF-β responsive genes involved in regulation of cell migration and invasion, including MMPs and Slug, were further up-regulated after TGF-β stimulation in mutant p53 expressing cells (Fig. 2A).

To ensure that the expression of these genes was regulated upon activation of TGF-β signaling, SB431542, an inhibitor for TGF-β receptor, was applied to cells before TGF-β treatment (Fig. 2A). The inhibitor blunted expression of all these target genes in response to TGF-β in H1299 cells regardless of mutant p53 status. To preclude potential artifacts due to exogenous expression of R175H p53 in H1299 cells, a pharynx carcinoma cell line, Detroit562 which harbors an endogenous R175H p53 mutant, was employed for further analysis. A stable cell line silencing mutant p53 in Detroit562 was generated using a specific p53 shRNA established before (35). Consistent with our observation in mutp53-H1299 and control cells, genes controlling cell migration (MMPs and Slug) had better response to TGF-β in the mutant p53 containing Detroit562 cells (with integration of only a lenti-viral vector, therefore named "Detroit Lenti") than in the shp53 Detroit562 cells (Fig. 2B). However, the tumor suppressive genes (p21 and p15) had less response to TGF-β in the Detroit562 Lenti cells than in the shp53 Detroit562 cells. Similar expression pattern was observed in J4705 and J4708, two oral cancer cell lines derived from mutant p53 (R172H) knock-in and p53 knock out mouse respectively (Fig. 2C) To our surprise, we did not observe any difference in the phosphorylation of Smad2 and Smad3 either between control and mut-p53 H1299 cells, or Detroit Lenti and shp53 cells (Fig. 1B and data not shown). In addition, we checked changes in phosphorylation of R-Smads in J4705 and J4708 or in H1299 cells transfected viral vectors expressing wild type p53, R175H p53, or control vector. Consistent results were observed in these cells that mutant p53 did not induce differences in phosphorylation of R-Smads (Fig. 2D and 2E). As shown in Fig. 2F, TGF-β treatment for 30 min induced the translocation of Smad3 into the nucleus in H1299 cells expressing either wild type or mutant p53, suggesting that mutant p53 had no influences on Smad3's nuclear translocation.

Taken these results together, we conclude that mutant p53 may achieve its "gain of function" by attenuating and enhancing subsets of TGF-β responsive genes without affecting phosphorylation of Smad2 or Smad3, suggesting an effect downstream of R-Smad translocation to the nuclei.

Mutant p53 fulfills its gain of function by occupying the MH2 domain of Smad3-To understand the molecular details by which mutant

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p53 affects transcription of TGF-β responsive genes, we tested physical interactions between mutant p53 and Smad proteins by GST pull-down or immunoprecipitation assays. When we used GST-Smad2 or GST-Smad3 to pull down ^{35}S labeled wild type or mutant R175H p53 (Fig. 3A), both wild type and mutant p53 exhibited higher affinity to Smad3 than Smad2 whereas mutant p53 had no interaction with Smad4 (Data not shown). Interestingly, R175H p53 and several other p53 mutants all had higher affinity to Smad3 than smad2 (Fig. 3B), which was consistent with previous observation that mutant p53 barely interacted with Smad2 by itself (25). Differential affinity for Smad3 by wild type and mutant p53 encouraged us to analyze if the binding regions in Smad3 to p53 are the same or different. Consistent with previous report that wild type p53 binds to the MH1 domain in Smad3 (36), we found that wild type p53 interacted with Smad3 through MH1 domain only (Fig. 3C and D). However, the R175H p53 exclusively bound to MH2 rather than MH1 domain in Smad3 (Fig. 3D and 3E). To test if other mutant p53 may bind to Smad3 in similar fashion, constructs encoding R273H, R248W or R 282W p53 were transfected together with Flag-MH1 or MH2 from Smad3 to H1299 for the interaction assays. All tested mutant p53 were coimmunoprecipitated with Flag-MH2 but not MH1 domain in Smad3 (Fig. 3F). Given that Smad2 and Smad3 are 91% identical in full length amino acid sequence and 96% identical in their MH2 domains (37), we found no differences in the binding of mutant p53 to isolated MH2 domains from Smad2 or Smad3 (data not shown). This prompted us to explore the mechanism why Smad2 had reduced affinity to mutant p53. Previous research has shown that the MH1 domain can interfere with the function of MH2 domain by direct interactions (16). The Smad2 protein has a 30 amino acid region in the middle of MH1 domain coded by exson3, which does not exist in Smad3 or other Smads in mammals (38). It was suggested that Smad2Δexon3 may function as a Smad3-like molecule (16). By comparing the interactions between p53 and purified Smad2, Smad2Δexon3 or Smad3 proteins, we found that the extra 30 amino acid residues in Smad2 attenuates its interaction with both wild type and mutant p53 (Fig. 3G).

Given that MH2 domain of Smad3 is responsible for receptor interaction, formation of homomeric as well as heteromeric Smad complexes (39), we predicted that mutant p53 might disrupt the Smad3-Smad4 complex by occupying the MH2 domain in Smad3. We indeed observed attenuated interactions between endogenous Smad3 and Smad4 in cells expressing mutant p53 upon TGF-β treatment (Fig. 3H). Disruption of Smad3/Smad4 complex by mutant p53 resulted in reduced affinity to SBE probe as observed in EMSA assay (Fig. 3I). In addition, the SBE-Luc activity was attenuated by mutant p53 overexpression in H1299 cells (Fig. 3J). These results suggest that mutant p53 may disrupt Smad3 dependent TGF-β signal transduction. Expression profiles in additional cell lines, including naturally occurring mutant p53 cell ARO, were supportive of mutant p53 GOF (Fig. 2K and 2L). Despite that mutant p53 may directly influence transcription of some target genes, we found several classical and newly defined Smad3 dependent genes (40) that are not affected by mutant p53 (Fig. 2M). Taken together, the above results indicate that mutant p53 may achieve its gain of function by preventing the Smad3/Smad4 complex formation and alters TGF-β signal transduction.

ERK signal is required for the interaction between mutant p53 and Smad3-To determine the mechanisms for the mutant p53 and Smad3 interactions, we decided to define the domains required for this interaction on mutant p53. Using *in vitro* translated deletion mutants and purified GST-Smad3, we localized the Smad3-binding region to the C-terminus of mutant p53 since the mutant p53 lacking aa301-393 lost binding to Smad3 (Fig. 4A). To validate this, we tested if overexpression of MH2 or the mutant p53 C-terminal domain would block the interaction. By co-transfecting a GFP control vector, GFP tagged C-terminal domain of mutant p53 (GFP301-393), or GFP-MH2 domain of Smad3 together with Flag-Smad3 and R175H p53 to H1299 cells, we found that both GFP tagged MH2 and GFP301-393 fragments, but not GFP itself blunted the interaction between mutant p53 and Smad3 in a dominant-negative fashion (Fig. 4B).

ERK signal is known to synergize with mutant p53 in certain "gain of function" manifestation

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(25). Phosphorylation of Ser6 and Ser9 on the N-terminal of wild type p53 mediates the interactions between p53 and Smad3 (36). We speculated that kinase signals might affect mutant p53 interaction with Smad3. Kinase inhibitors for ERK, PI3K and p38 were tested for their influences on the interaction between mutant p53 and Smad3. Interestingly, both MEK inhibitor PD98059 and ERK inhibitor U0126, but not other kinase inhibitors, blocked the interaction between mutant p53 and Smad3 (Fig. 4C). Even in the presence of TGF-β, ERK inhibitors could prevent the interactions between the R175H p53 and Smad3 (Fig. 4E), indicating that MEK-ERK signal plays a vital role in mediating the interaction. As predicted, silencing ERK or the presence of U0126 in R175H expressing cells significantly attenuated the interaction between R175H p53 and Smad3 (Fig. 4D and 4E). To validate that protein modification is essential for R175H p53 and Smad3 interactions, we immunoprecipitated Flag R175H p53 or HA Smad3, followed by λ-phosphatase treatment (λ-pp) to erase phosphorylation and another round of HA cross-immunoprecipitation. Phosphatase efficiency was examined together with the utilization of a site specific phosphorylation antibody (Fig. 4F, lower panel). The results demonstrated that when phosphorylation on Flag-R175H p53 was removed, R175H p53 and Smad3 interactions was attenuated (Fig. 4F, upper left panel), suggesting the requirement of kinase signal in promoting mutant p53 actions. As controls, λ-pp treated or untreated HA Smad3 beads could equally precipitate Flag R175H p53 (Fig. 4F, upper right panel).

To further define the phosphorylation region/site in mutant p53, we performed GST pull-down assays using purified GST-Smad3 together with a series of *in vitro* translated C-terminal truncation mutants of R175H p53. GST-Smad3 failed to interact with mutant p53 truncations lacking aa320-342 or aa367-393 (Fig. 4G). Sequence analysis indicated that these mutant either missed the tetramerization domain or the C-terminal regulatory domain, consistent with previous finding that these domains might be required for the mutant p53 gain of function (41). Through a series of site directed mutagenesis and immunoprecipitation analysis, we found that phosphorylation at Ser392 was important for the interactions between R175H p53 and Smad3 (Fig. 4H). Although phosphorylation at N-terminus serines (S6/9A) had been known to mediate the interaction between wild type p53 and Smad3, mutations at these serines had no effect for mutant p53 actions (Fig. 4H). Furthermore, U0126 repressed the phosphorylation on Ser392 in R175H p53 (Fig. 4I). *In vitro* kinase assay defined the phosphorylation of mutant p53 at Ser392 by activated ERK1 (Fig. 4J). Together, these data suggest that MEK-ERK signal is important for the mutant p53 gain of function by promoting phosphorylation at Ser392 and formation of the mutant p53-Smad3 complex.

Mutant p53 deregulates TGF-β signaling by antagonizing Smad3 dependent transcription-Given that disrupting the Smad3 and Smad4 complex underlies the mechanism by which mutant p53 deregulates TGF-β signaling, we asked whether the gene expression patterns in mutant p53 expressing cells could be recapitulated by silencing endogenous Smad3 in control cells or tipping the balance between Smad2 and Smad3. The siRNA for Smad2 or Smad3 were previously demonstrated efficient and specific (28,29). Interestingly, silencing Smad3 resulted in a similar genes expression pattern to that in cells expressing mutant p53, showing attenuated p21 but increased MMPs and Slug expression in response to TGF-β induction (Fig. 5A and 5B). Similarly, mutant p53 had little effects on subsets of TGF-β responsive genes in Smad3 knockout MEFs (Fig. 5C and 5D). However, silencing Smad2 attenuated mutant p53 mediated up-regulation of MMPs and Slug (Fig. 5A and 5B), consistent with previous observation that the regulation of MMP2 by TGF-β was Smad2-dependent in epithelial cells (17,42). Our results suggest an inhibitory role for Smad3 in MMPs and Slug expression while Smad2 functions to counterbalance Smad3 effects. Taken together, we conclude that silencing Smad2 or inhibiting the ERK signal pathway in mutant p53 cells can repress the synergy between mutant p53 and TGF-β in their tumor promoting functions by affecting Smad3 dependent transcriptional regulation of MMPs and Slug.

Mutant p53 promotes cell motility by enhancing TGF-β induced Slug expression-One of the most heavily investigated EMT regulators in lung

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cancers is Slug, whose expression is associated with lung cancer metastasis and resistance to target therapy (43). The observation that both mutant p53 and lack of Smad3 can enhance TGF-β induced Slug expression in human lung cancer cells (Fig. 6A and Fig. 5A and 5B) prompted us to test Slug expression in additional cancer cells. Interestingly, we found up-regulation of Slug in the oral cancer cell line J4705 (derived from p53 R172H-knock-in mouse) and Detroit562 cells compared to control cells with p53 depletion (data not shown and Fig. 6B). To understand expression of R-Smad and Slug in the regulation of cell motility, we silenced Smad3 alone or together with Slug in H1299. Smad3 knockdown clearly enhanced cells motility in a transwell analysis, however, cell movement was slowed down by silencing Smad3 and Slug together (Fig. 6C). Next, we compared migration ability in R175H p53 cells with Smad2 or Smad3 knockdown. Silencing Smad3 in R175H p53 cells enhanced cell migration. However, depleting Smad2 reduced TGF-β induced chemokinesis (Fig. 6D), consistent with previous observation in pancreatic ductal adenocarcinoma cell line (44). By single cell migration analysis, we demonstrated that the ability of mutant p53 to drive enhanced migration in H1299 cells was dependent upon Slug expression since depletion of Slug in the mutant p53 expressing cells reversed its migratory behaviors (Fig. 6E). In conclusion, mutant p53 can enhance cancer cell motility by antagonizing Smad3-dependent inhibition of Slug expression.

DISCUSSION

TGF-β and p53 signaling network play a vital role in regulating cell growth and migration. Wild type p53 is known to be required for full activity of TGF-β mediated regulation by cooperating with Smads (36). Deregulation in either of these signal pathways may disturb cell's ability to maintain normal behaviors, eventually leading to development of cancer.

Despite that mutant p53 is reported to mediate repression of TGF-β-signaling by interference with TGFβ receptors (26), a recent study has shown that mutp53 activates TGF-β-induced migration, invasion, and metastasis in breast cancer cells (25). Our study suggests that these seemingly contradictory observations can be partially reconciled. We demonstrate that in mutant p53 expressing cells, expression of some TGF-β responsive tumor suppressor genes is attenuated; in contrast, expression of several tumor promoter genes is enhanced. Clearly, mutant p53 can achieve its "gain of function" activity by hijacking the Smad3 mediated TGF-β signaling transduction rather than blocking the entire TGF-β regulated gene network. Identification of preferential interactions between mutant p53 and R-Smads, for example, stronger interactions with Smad3 than Smad2 in our study, provides an alternative mechanism how mutant p53 suppresses TGF-β signaling. Mutant p53 mainly interacts with MH2 domain on Smad3 through its C-terminus, this explains why mutant p53 attenuated the interaction between Smad3 and Smad4 without influencing the phosphorylation of Smad3. Different from the previously discovered Smad2/p63/mutant p53 complex formation in breast cancer cells (25), our finding of Smad3-mutant p53 complex in lung cancer cells may reflect a cell specific and signal specific mechanism.

The C-terminus of mutant p53 contains a tetramerization domain (TD) and a regulatory region. The TD of p53 has long been ignored because it is not a hot-spot region often mutated in cancers (41). However, a number of structural and functional analyses have revealed the importance of TD for p53 function and TD is also found required for some mutant p53 "gain of function" (45,46). In this study, we found that mutant p53 deleted the TD or regulatory domain is unable to interact with Smad3. Intriguingly, when we mutate the Serine at 392 whose phosphorylation will stimulate the wild type p53 tetramerization (47), the interactions between mutant p53 and Smad3 are disrupted. The data presented so far indicate that the Ser392 phosphorylation may influence mutant p53 gain of function in twisting the Smad3 dependent signal transduction, consistent with previous finding that post-translational modification at C-terminus regulatory domain may affect p53 function (47).

In addition, selective actions of Smad2 and Smad3 on specific target genes may further empower mutant p53 gain of function. In the TGF-β signaling network, how cells selectively

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activate Smad2 versus Smad3 remains poorly understood. Based on large scale analyses of Smad2 and Smad3 dependent TGF-β target genes in knockout MEFs, Smad2 and Smad3 have distinct roles in TGF-β signaling despite of their functional redundancy (17). Mutant p53 may achieve its "gain of function" by tipping the balance between the Smad2 and Smad3 actions on specific target genes. Previous research indicated that regulators, such as Hic-5 and EID-2, can alter the balance of the Smad3-specific versus the Smad2-specific arm of TGF-β signaling by blocking the Smad3 signal transduction through occupying its MH2 domain (22, 48, 49) . Here, we provide an example that mutant p53 can function as a molecular switch to alter the balance between Smad2 and Smad3 dependent transcriptional output of TGF-β signaling.

A recently research has disclosed that knocking down Smad3 could increase migratory response while silencing of Smad2 decrease the chemokinesis in PDC cells (44). Mechanistically, we found that expression of MMPs and Slug is reduced by silencing Smad2 but enhanced by depletion of Smad3, whereas knocking down Smad2 or Slug in mutant p53 expressing cells attenuates motility in lung cancer cells. These findings endorse our hypothesis that mutant p53 enhances the TGF-β induced MMPs and Slug expression and cell migration by attenuating Smad3-mediated signal transduction. Consistently, clinical research has found high p-Smad2 expression in stromal fibroblasts predicted poor survival in patients with clinical stage I to IIIA non-small cell lung cancer (50), indicating that Smad2 may play an important role in mediating the lung cancer metastasis. The association between high level of p-Smad2 and malignant phenotype and poor prognosis in patients with advanced carcinoma (51) suggests that targeting mutant p53 may amend the balance between Smad2 and Smad3 signaling for late stage cancers.

In conclusion, our present studies provide evidence for a new mechanism by which mutant p53 protein hijacks Smad3 mediated TGF-β signal transduction, in turn unleashing the Smad2 specific pathway. Thus, mutant p53 may be employed by cancer cells as a molecular switch to alter TGF-β signaling at the R-Smad levels from a tumor-suppressive towards a tumor-promoting pathway. Our findings that physical interactions between mutant p53 and Smad3 are required for the gain of function of mutant p53 propose a potential to develop anticancer drugs able to suppress specific tumors.

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References

1. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Surfing the p53 network. *Nature* **408**, 307-310
2. Soussi, T., and Beroud, C. (2001) Assessing TP53 status in human tumours to evaluate clinical outcome. *Nature reviews. Cancer* **1**, 233-240
3. Muller, P. A., Caswell, P. T., Doyle, B., Iwanicki, M. P., Tan, E. H., Karim, S., Lukashchuk, N., Gillespie, D. A., Ludwig, R. L., Gosselin, P., Cromer, A., Brugge, J. S., Sansom, O. J., Norman, J. C., and Vousden, K. H. (2009) Mutant p53 drives invasion by promoting integrin recycling. *Cell* **139**, 1327-1341
4. Muller, P. A., and Vousden, K. H. (2013) p53 mutations in cancer. *Nature cell biology* **15**, 2-8
5. Bullock, A. N., Henckel, J., DeDecker, B. S., Johnson, C. M., Nikolova, P. V., Proctor, M. R., Lane, D. P., and Fersht, A. R. (1997) Thermodynamic stability of wild-type and mutant p53 core domain. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 14338-14342
6. Willis, A., Jung, E. J., Wakefield, T., and Chen, X. (2004) Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes. *Oncogene* **23**, 2330-2338
7. Sigal, A., and Rotter, V. (2000) Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer research* **60**, 6788-6793
8. Kim, E., and Deppert, W. (2004) Transcriptional activities of mutant p53: when mutations are more than a loss. *Journal of cellular biochemistry* **93**, 878-886
9. Olive, K. P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., and Jacks, T. (2004) Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**, 847-860
10. Lang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., El-Naggar, A. K., and Lozano, G. (2004) Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* **119**, 861-872
11. Hanel, W., Marchenko, N., Xu, S., Yu, S. X., Weng, W., and Moll, U. (2013) Two hot spot mutant p53 mouse models display differential gain of function in tumorigenesis. *Cell death and differentiation* **20**, 898-909
12. Petitjean, A., Achatz, M. I., Borresen-Dale, A. L., Hainaut, P., and Olivier, M. (2007) TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene* **26**, 2157-2165
13. Derynck, R., Akhurst, R. J., and Balmain, A. (2001) TGF-beta signaling in tumor suppression and cancer progression. *Nature genetics* **29**, 117-129
14. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Mechanism of activation of the TGF-beta receptor. *Nature* **370**, 341-347
15. Massague, J., and Chen, Y. G. (2000) Controlling TGF-beta signaling. *Genes & development* **14**, 627-644
16. Yagi, K., Goto, D., Hamamoto, T., Takenoshita, S., Kato, M., and Miyazono, K. (1999) Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3. *The Journal of biological chemistry* **274**, 703-709
17. Meng, X. M., Huang, X. R., Chung, A. C., Qin, W., Shao, X., Igarashi, P., Ju, W., Bottinger, E. P., and Lan, H. Y. (2010) Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis. *Journal of the American Society of Nephrology : JASN* **21**, 1477-1487
18. Giampieri, S., Manning, C., Hooper, S., Jones, L., Hill, C. S., and Sahai, E. (2009) Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nature cell biology* **11**, 1287-1296
19. Feng, X. H., and Derynck, R. (2005) Specificity and versatility in tgf-beta signaling through Smads. *Annual review of cell and developmental biology* **21**, 659-693

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20. Attisano, L., Silvestri, C., Izzi, L., and Labbe, E. (2001) The transcriptional role of Smads and FAST (FoxH1) in TGFbeta and activin signalling. *Molecular and cellular endocrinology* **180**, 3-11
21. Long, J., Matsuura, I., He, D., Wang, G., Shuai, K., and Liu, F. (2003) Repression of Smad transcriptional activity by PIASy, an inhibitor of activated STAT. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 9791-9796
22. Lee, H. J., Lee, J. K., Miyake, S., and Kim, S. J. (2004) A novel E1A-like inhibitor of differentiation (EID) family member, EID-2, suppresses transforming growth factor (TGF)-beta signaling by blocking TGF-beta-induced formation of Smad3-Smad4 complexes. *The Journal of biological chemistry* **279**, 2666-2672
23. Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C., and Piccolo, S. (2003) Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* **113**, 301-314
24. Massague, J. (2008) TGFbeta in Cancer. *Cell* **134**, 215-230
25. Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M. B., Guzzardo, V., Parenti, A. R., Rosato, A., Bicciato, S., Balmain, A., and Piccolo, S. (2009) A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* **137**, 87-98
26. Kalo, E., Buganim, Y., Shapira, K. E., Besserglick, H., Goldfinger, N., Weisz, L., Stambolsky, P., Henis, Y. I., and Rotter, V. (2007) Mutant p53 attenuates the SMAD-dependent transforming growth factor beta1 (TGF-beta1) signaling pathway by repressing the expression of TGF-beta receptor type II. *Molecular and cellular biology* **27**, 8228-8242
27. Ali, A., Wang, Z., Fu, J., Ji, L., Liu, J., Li, L., Wang, H., Chen, J., Caulin, C., Myers, J. N., Zhang, P., Xiao, J., Zhang, B., and Li, X. (2013) Differential regulation of the REGgamma-proteasome pathway by p53/TGF-beta signalling and mutant p53 in cancer cells. *Nature communications* **4**, 2667
28. Lin, X., and Feng, X. H. (2005) Design and application of a versatile expression vector for RNAi in mammalian cells. *Journal of RNAi and gene silencing : an international journal of RNA and gene targeting research* **1**, 38-43
29. Jazag, A., Ijichi, H., Kanai, F., Imamura, T., Guleng, B., Ohta, M., Imamura, J., Tanaka, Y., Tateishi, K., Ikenoue, T., Kawakami, T., Arakawa, Y., Miyagishi, M., Taira, K., Kawabe, T., and Omata, M. (2005) Smad4 silencing in pancreatic cancer cell lines using stable RNA interference and gene expression profiles induced by transforming growth factor-beta. *Oncogene* **24**, 662-671
30. Park, S. H., Cheung, L. W., Wong, A. S., and Leung, P. C. (2008) Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. *Molecular endocrinology* **22**, 2085-2098
31. Duda, D. G., Sunamura, M., Lefter, L. P., Furukawa, T., Yokoyama, T., Yatsuoka, T., Abe, T., Inoue, H., Motoi, F., Egawa, S., Matsuno, S., and Horii, A. (2003) Restoration of SMAD4 by gene therapy reverses the invasive phenotype in pancreatic adenocarcinoma cells. *Oncogene* **22**, 6857-6864
32. Peng, B., Fleming, J. B., Breslin, T., Grau, A. M., Fojioka, S., Abbruzzese, J. L., Evans, D. B., Ayers, D., Wathen, K., Wu, T., Robertson, K. D., and Chiao, P. J. (2002) Suppression of tumorigenesis and induction of p15(ink4b) by Smad4/DPC4 in human pancreatic cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **8**, 3628-3638
33. Powell, D. W., Rane, M. J., Chen, Q., Singh, S., and McLeish, K. R. (2002) Identification of 14-3-3zeta as a protein kinase B/Akt substrate. *The Journal of biological chemistry* **277**, 21639-21642
34. Di Minin, G., Bellazzo, A., Dal Ferro, M., Chiaruttini, G., Nuzzo, S., Bicciato, S., Piazza, S., Rami, D., Bulla, R., Sommaggio, R., Rosato, A., Del Sal, G., and Collavin, L. (2014) Mutant p53

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Reprograms TNF Signaling in Cancer Cells through Interaction with the Tumor Suppressor DAB2IP. *Molecular cell* **56**, 617-629
35. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553
36. Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S., and Piccolo, S. (2007) Integration of TGF-beta and Ras/MAPK signaling through p53 phosphorylation. *Science* **315**, 840-843
37. Brown, K. A., Pietenpol, J. A., and Moses, H. L. (2007) A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. *Journal of cellular biochemistry* **101**, 9-33
38. Takenoshita, S., Mogi, A., Nagashima, M., Yang, K., Yagi, K., Hanyu, A., Nagamachi, Y., Miyazono, K., and Hagiwara, K. (1998) Characterization of the MADH2/Smad2 gene, a human Mad homolog responsible for the transforming growth factor-beta and activin signal transduction pathway. *Genomics* **48**, 1-11
39. Shi, Y., and Massague, J. (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700
40. Kretschmer, A., Moepert, K., Dames, S., Sternberger, M., Kaufmann, J., and Klippel, A. (2003) Differential regulation of TGF-beta signaling through Smad2, Smad3 and Smad4. *Oncogene* **22**, 6748-6763
41. Chene, P. (2001) The role of tetramerization in p53 function. *Oncogene* **20**, 2611-2617
42. Zhang, L., Liu, C., Meng, X. M., Huang, C., Xu, F., and Li, J. (2014) Smad2 protects against TGF-beta1/Smad3-mediated collagen synthesis in human hepatic stellate cells during hepatic fibrosis. *Molecular and cellular biochemistry*
43. Shih, J. Y., and Yang, P. C. (2011) The EMT regulator slug and lung carcinogenesis. *Carcinogenesis* **32**, 1299-1304
44. Ungefroren, H., Groth, S., Sebens, S., Lehnert, H., Gieseler, F., and Fandrich, F. (2011) Differential roles of Smad2 and Smad3 in the regulation of TGF-beta1-mediated growth inhibition and cell migration in pancreatic ductal adenocarcinoma cells: control by Rac1. *Molecular cancer* **10**, 67
45. Lanyi, A., Deb, D., Seymour, R. C., Ludes-Meyers, J. H., Subler, M. A., and Deb, S. (1998) 'Gain of function' phenotype of tumor-derived mutant p53 requires the oligomerization/nonsequence-specific nucleic acid-binding domain. *Oncogene* **16**, 3169-3176
46. Deb, D., Scian, M., Roth, K. E., Li, W., Keiger, J., Chakraborti, A. S., Deb, S. P., and Deb, S. (2002) Hetero-oligomerization does not compromise 'gain of function' of tumor-derived p53 mutants. *Oncogene* **21**, 176-189
47. Sakaguchi, K., Sakamoto, H., Lewis, M. S., Anderson, C. W., Erickson, J. W., Appella, E., and Xie, D. (1997) Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53. *Biochemistry* **36**, 10117-10124
48. Wang, H., Song, K., Sponseller, T. L., and Danielpour, D. (2005) Novel function of androgen receptor-associated protein 55/Hic-5 as a negative regulator of Smad3 signaling. *The Journal of biological chemistry* **280**, 5154-5162
49. Wang, H., Song, K., Krebs, T. L., Yang, J., and Danielpour, D. (2008) Smad7 is inactivated through a direct physical interaction with the LIM protein Hic-5/ARA55. *Oncogene* **27**, 6791-6805
50. Chen, Y., Xing, P., Chen, Y., Zou, L., Zhang, Y., Li, F., and Lu, X. (2014) High p-Smad2 expression in stromal fibroblasts predicts poor survival in patients with clinical stage I to IIIA non-small cell lung cancer. *World journal of surgical oncology* **12**, 328
51. Shinto, O., Yashiro, M., Toyokawa, T., Nishii, T., Kaizaki, R., Matsuzaki, T., Noda, S., Kubo, N., Tanaka, H., Doi, Y., Ohira, M., Muguruma, K., Sawada, T., and Hirakawa, K. (2010) Phosphorylated smad2 in advanced stage gastric carcinoma. *BMC cancer* **10**, 652

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Mutant p53 enhances lung cancer cell malignancy. A. Mutant p53 attenuates cytostatic effect by TGF-β. H1299 EV and R175H cells were cultured with TGF-β at the concentration of 5ng/ml for indicated days. Cell growth was measured by BrdU incorporation cell proliferation assays. Results represent the mean ± SD of three experiments performed in triplicate. * indicates $p < 0.05$ as determined by a Student's t test. B. Mutant p53 attenuates p21 expression. H1299 EV and R175H cells were starved for 24hrs before treated with TGF-β for various amount of time. Indicated proteins were detected by Western blotting (Left), and quantification of p21 levels from 3 independent experiments was shown (Right). C. Mutant p53 promotes cell migration. H1299 cells stably expressing mutant p53 or a control vector were monitored by time-lapse video microscopy. The movement of individual cells was recorded using Image J cell-tracking software. The speed and persistence of migration were extracted from the track-plots. Directionality (Persistence) is defined as the ratio of the vectorial distance to the total path length traveled by the cells. Values are means ± SEM of 90 track-plots from three independent experiments. D. H1299 cells were seeded on transwell membranes where the cells were treated with or without TGF-β. The migrated cells were fixed and stained with crystal viole (Left). The percentage of migrated cell was quantified (right).

FIGURE 2. Mutant p53 modulates the transcription of TGF-β target genes without alterations in the phosphorylation of R-Smads. A-C. Mutant p53 inhibits tumor suppressor genes while enhances tumor promoter genes. H1299 EV and R175H and Detroit Lenti and shp53 were pretreated with TGF-β receptor inhibitor SB431542 for 1h, followed by TGF-β stimulating for another 6hrs, and J4708 and J4705 cells were directly treated with TGF-β for 6hrs after 24hrs serum starvation. Total RNA was extracted and specific gene expression was analyzed (H1299 EV: H1299 cells with a control vector, R175H: H1299 stably expressing R175H p53, Detroit lenti: Detroit562 cells with a control shRNA integration, shp53 cells: Detroit562 stably expressing an shRNA for p53, J4708: mouse oral cancer cells derived from p53 null background, J4705: mouse oral cancer cells derived from p53 R172H background). D and E. J4708

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and J4705 mouse cancer cells (D) or H1299 infected lenti viruses expressing a control vector, wild type p53 or R175H p53 (E), were treated with TGF-β for indicated time course, followed by Western blotting analysis of Smad2 and Smad3 phosphorylation levels. F. H1299 cells transfected with vector control, wild type p53 or R175H p53 were serum starved 24hrs before stimulation with or without (control vector cells) TGF-β for 30 min at 37°C. Cells were then fixed and labeled for Smad3 and p53. The Quantitative measurements of the relative Smad3 levels (nuclear/cytoplasmic ratio) represent results from 3 independent experiments.

FIGURE 3. Mutant p53 fulfill its gain of function by occupying the MH2 domain of Smad3. A. Preferential interaction with Smad3 by mutant p53. Pull down assays were performed with GST, GST-Smad2 or GST-Smad3 together with S^{35} labeled wild type and mutant p53. B. Mutant p53 showed higher affinity for Smad3. The GST-Smad2 or GST-Smad3 was incubated with lysates from H1299 overexpressed different mutant p53 for pull down assays. C and D. Distinct domains in Smad3 bind to WT p53 and mutant p53. C. A diagram displays Flag-tagged human Smad3 and its truncation constructs expressing MH1 domain, Linker region, or MH2 domain. Fragments a-e refers to constructs expressing distinct domains and v means control vector. D. H1299 cells were transfected with Flag tagged full length Smad3, MH1, MH2 together with WT p53 or R175H p53, and the cells were harvested for co-immunoprecipitation assays 24hrs later. E. R175H p53 binds to MH2 domain in Smad3. Co-immunoprecipitation assays were performed to determine the binding of R175H p53 to specific domain(s) in Flag-Smad3. F. Different p53 mutants bind to MH2 domain in Smad3. Flag-MH1, Flag-MH2 and mutant p53s were transiently transfected to H1299 for co-immunoprecipitation assays. G. 30 amino acid residues coded by exson3 of Smad2 gene attenuates its interaction with p53. GST, GST-Smad2ΔExson3 (short for GST-S2ΔE3) and GST-Smad3 were incubated with *in vitro* translated WT p53 or R175H p53 respectively followed by pull down assays. H. Mutant p53 attenuates endogenous Smad3-Smad4 interactions. Cell lysates from H1299 EV and H1299 R175H were immunoprecipitated with a mouse anti-Smad4 antibody before and after TGF-β treatment, and the precipitates were analyzed with rabbit anti-Smad3 antibody. I. Mutant p53 attenuates the Smad3/Smad4 complex affinity to SBE probe. H1299 were transfected with indicated constructs and treated with or without TGF-β and EMSA assays were performed with the double-stranded oligonucleotides containing the SBE box from the p21 promoter. J. H1299 cells were transfected with SBE-Luc together with R175H p53 or wild-type p53 expression plasmids, and luciferase activity was measured following TGF-β treatment. K-M. Mutant p53 attenuates the Smad3 dependent signal transduction., J4708 and J4705, ARO cells with transient depletion of R273H p53 and H1299 EV and R175H were treated with TGF-β for 6hrs. The RNA was extracted and specific gene expression was analyzed.

FIGURE 4. ERK signal promotes the interactions between mutant p53 and Smad3. A. C-terminus of mutant p53 binds to Smad3. Pull down assays with GST-Smad3 and Full length, N-terminal or C-terminal truncations of R175H p53. B. Dominant-negative effect of mutant p53 C terminal fragment and MH2 domain. GFP 301-393 of R175H p53 or GFP-MH2 of Smad3 was co-transfected to H1299 together with Flag Smad3 and R175H p53 to test interactions. C. Inhibition of MEK-ERK signaling prevents mutant p53 interactions with Smad3. Following co-transfection of Flag Smad3 and R175H p53 to H1299 for 24hrs, different kinase inhibitors were applied to the cells for an additional 6hrs before harvesting for co-immunoprecipitation assays. D. Silencing endogenous ERK1/2 in H1299 significantly attenuated mutant p53 and Flag Smad3 interactions in co-immunoprecipitation assays. E. ERK inhibitor antagonizes TGF-β induced R175H-Smad3 interaction. R175H expressing H1299 cells treated with TGF-β, U0126, or in combination were lysed for co-immunoprecipitation of Smad3 by an anti-p53 antibody. F. Phosphorylation on mutant p53 is required for R175H-Smad3 interaction. H1299 cells transfected with Flag R175H p53 or HA Smad3 were immunoprecipitated with anti-Flag or anti-HA beads, followed by treatment of λ-phosphatase (λPP) or a vehicle. Then λPP-treated or untreated Flag proteins were eluted by Flag peptide for another round of immunoprecipitation by HA-beads bound Smad3. a: HA-Smad3; b: λPP treated HA-Smad3; c: Flag-R175H p53 and d: λPP treated Flag-R175H p53. The efficiency of λ-

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phosphataes treatment was evaluated for loss of Ser423/425 phosphorylation in Smad3 or Ser392 phosphorylation in mutant p53 by IP-λPP-Western blot analysis with specific phospho-antibodies. G. Diagram of R175H p53 deletion mutant constructs (A-F). Following *in vitro* translation ^{35}S labeled R175H p53 derivatives were incubated with purified GST-Smad3 for pull down assays. H. Phosphorylation at S392 on mutant p53 is important for R175H-Smad3 interaction. H1299 cells were co-transfected with Flag Smad3 and a series of site-directed R175H p53 mutants followed by co-immunoprecipitation assays (C-2A: Ser315/392A R175H p53, C-3A: Ser303/315/392A R175H p53). I. U0126 inhibited phosphorylation of mutant p53 at Ser392. H1299 cells were transfected with R175H p53 and treated with U0126 (10μM for 6h). Changes in phosphorylation at Ser392 were detected by a specific phospho-antibody. J. The Ser392 in mutant p53 is a directly target of ERK1. Following co-transfection of caMEK1 and HA-ERK1 to H1299 cells for 24hrs, activated HA-ERK1 was purified and incubated with purified GST-R175H p53 *in vitro* at 30℃ for 30min. The phosphorylation levels at Ser392 were detected by site-specific phosphorylation antibody.

FIGURE 5. Mutant p53 distorts TGF-β signaling by antagonizing Smad3 function. A and B. Mutant p53 attenuates p21 but promotes MMPs and Slug expression in a Smad3 dependent manner. Specific siRNA targeting Smad2 or Smad3 were transfected into H1299 EV and R175H cells for 60hrs, followed by treatment with TGF-β or vehicle for 6hrs. The RNA level was monitored by Q-PCR (A), and the related protein levels were confirmed by Western blotting (B). C and D. Related protein and genes expression in WT MEFs, Smad3KO MEFs and Smad3KO with R175H p53 overexpression MEFs were monitored by Western blotting (C) and Q-PCR (D) respectively.

FIGURE 6. Mutant p53 promotes cell motility by enhancing TGF-β induced Slug expression. A and B. Mutant p53 enhances TGF-β induced Slug expression. Following different time course of TGF-β treatment, indicated proteins from H1299 EV and R175H cells (A) and Detroit Lenti and shp53 cells (B) were detected by Western blotting. C. Positive and negative regulation of cell migration by Slug and Smad3. The migration ability of H1299 cells were tested by transwell assays following siRNA depletion of Smad3 alone or in combination with Slug (Left). The expression of Smad3 and Slug protein levels was confirmed by Western (Middle) and the percentage of migrated cell were quantified (Right). D. Silencing Smad3 but not Smad2 enhances cell migration. Following siRNA depletion of Smad2 or Smad3 for 36hrs, H1299 R175H cells were serum starved overnight before transwell assays, with the H1299 EV cells as a control. E. Microscopic analysis of cell migration by silencing the Smad3 or Slug. The movement of individual EV and R175H cells was monitored by time-lapse video microscopy following siRNA knockdown of Smad3 or Slug. The speed of migration was extracted from the track-plots. Values are means ± SEM of 90 track-plots from three independent experiments. * indicates $p < 0.05$ as determined by Student's t test.

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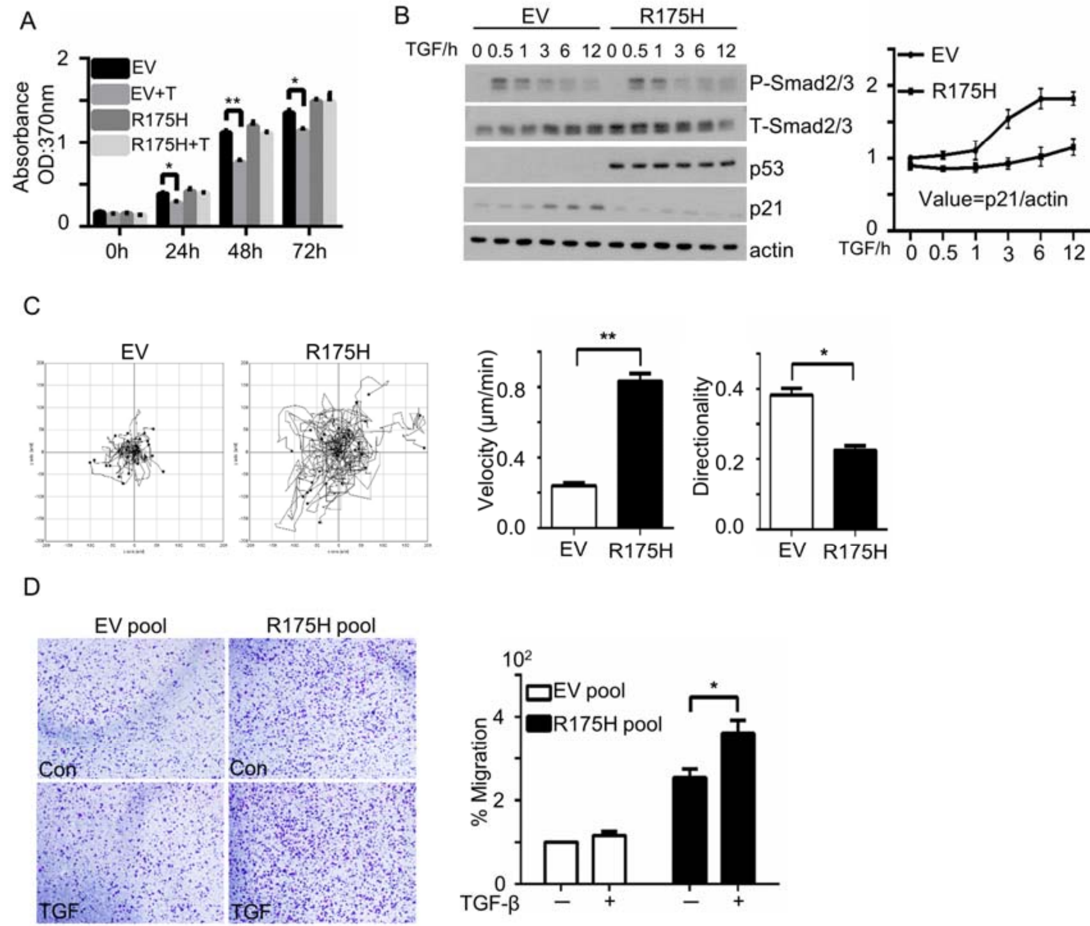
Figure 1



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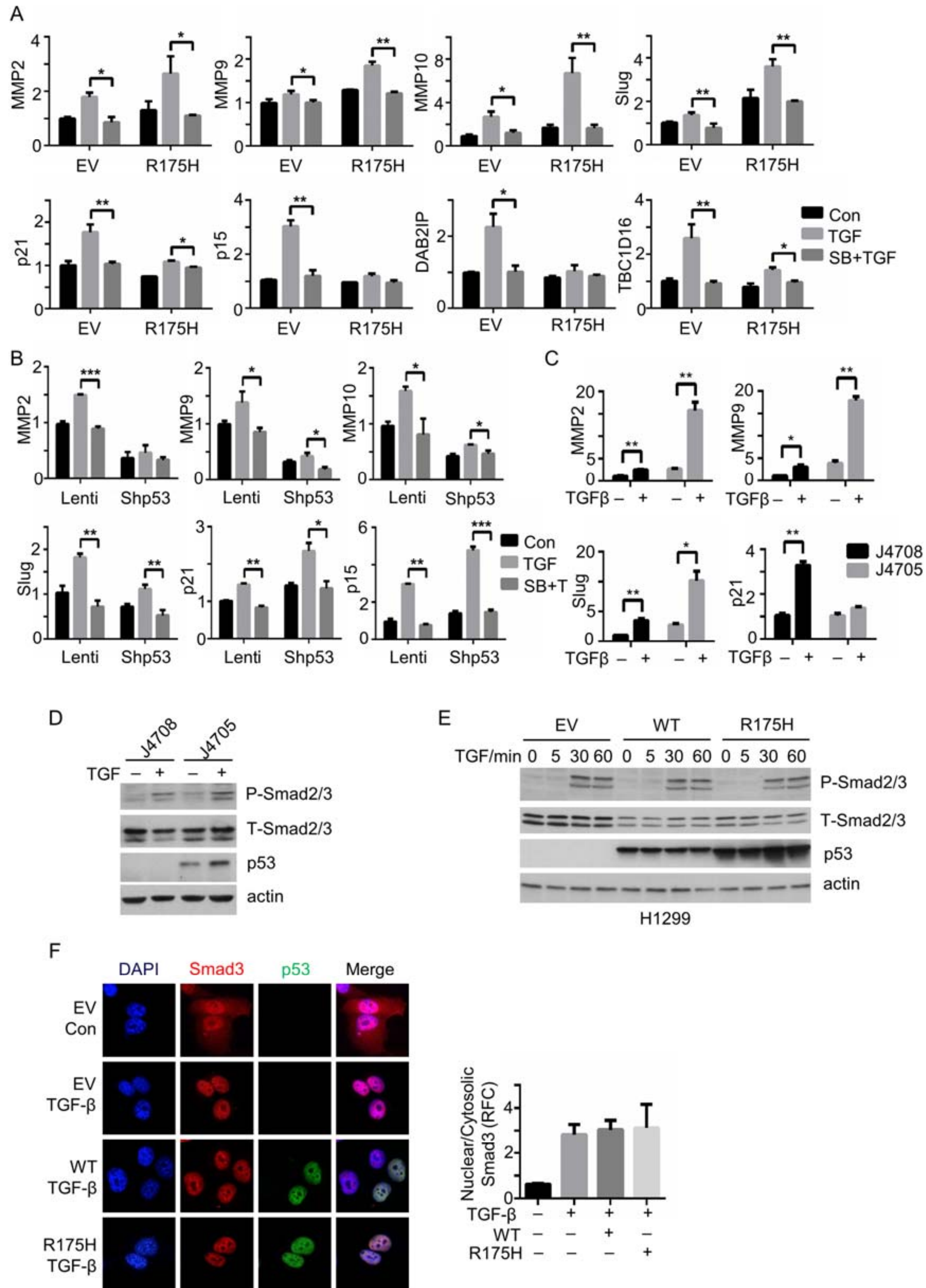

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Figure 3

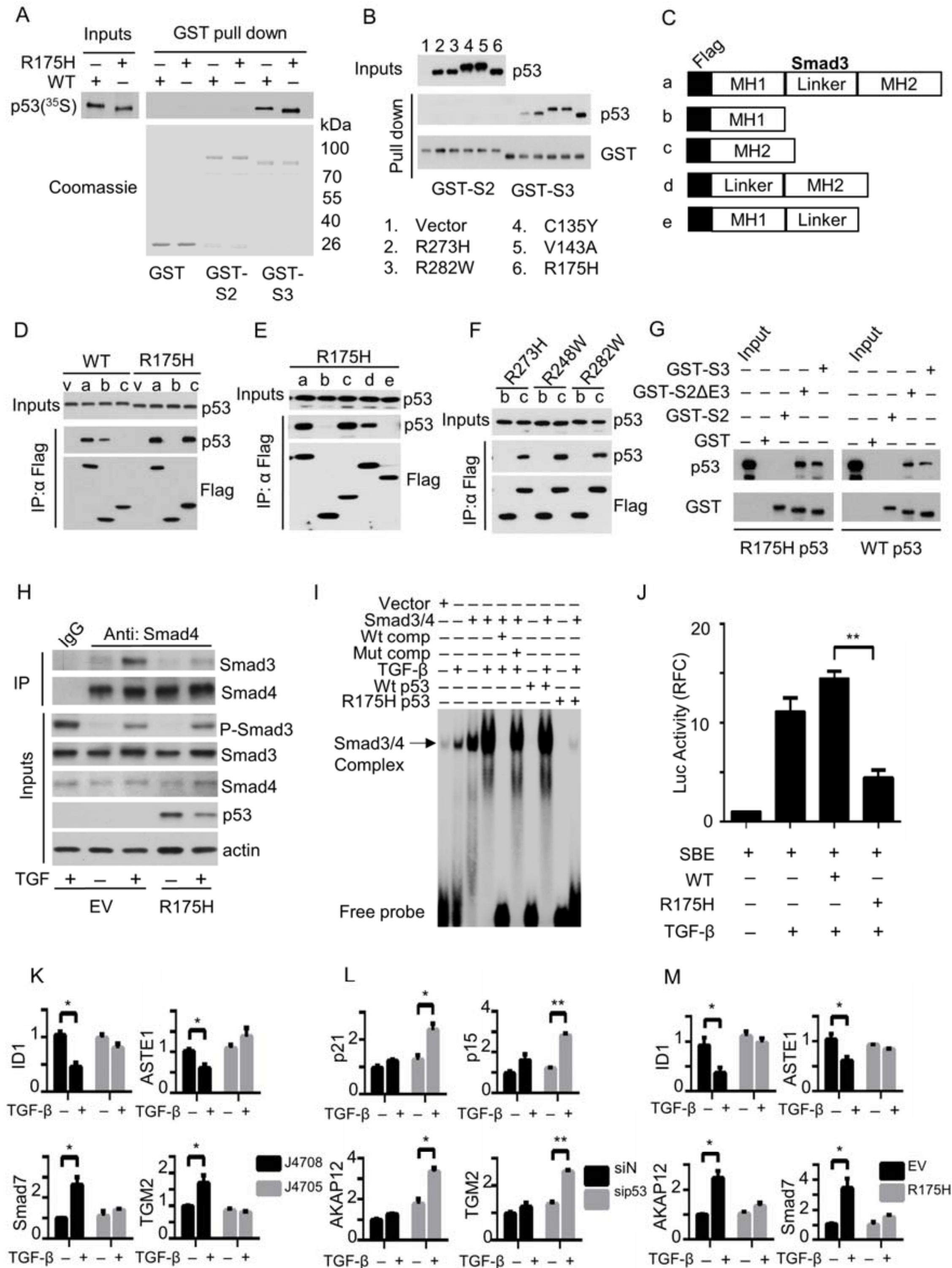

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Figure 4

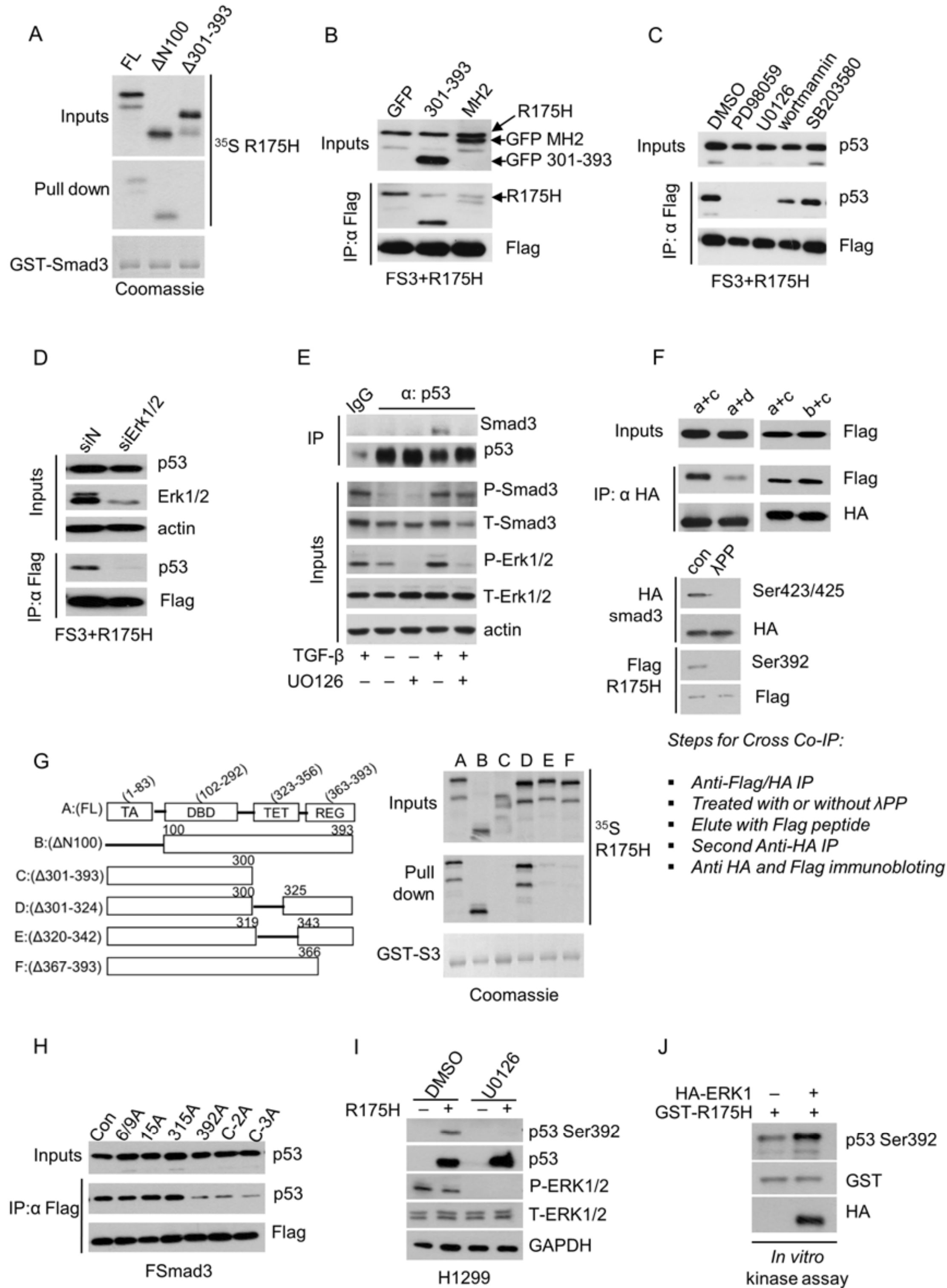

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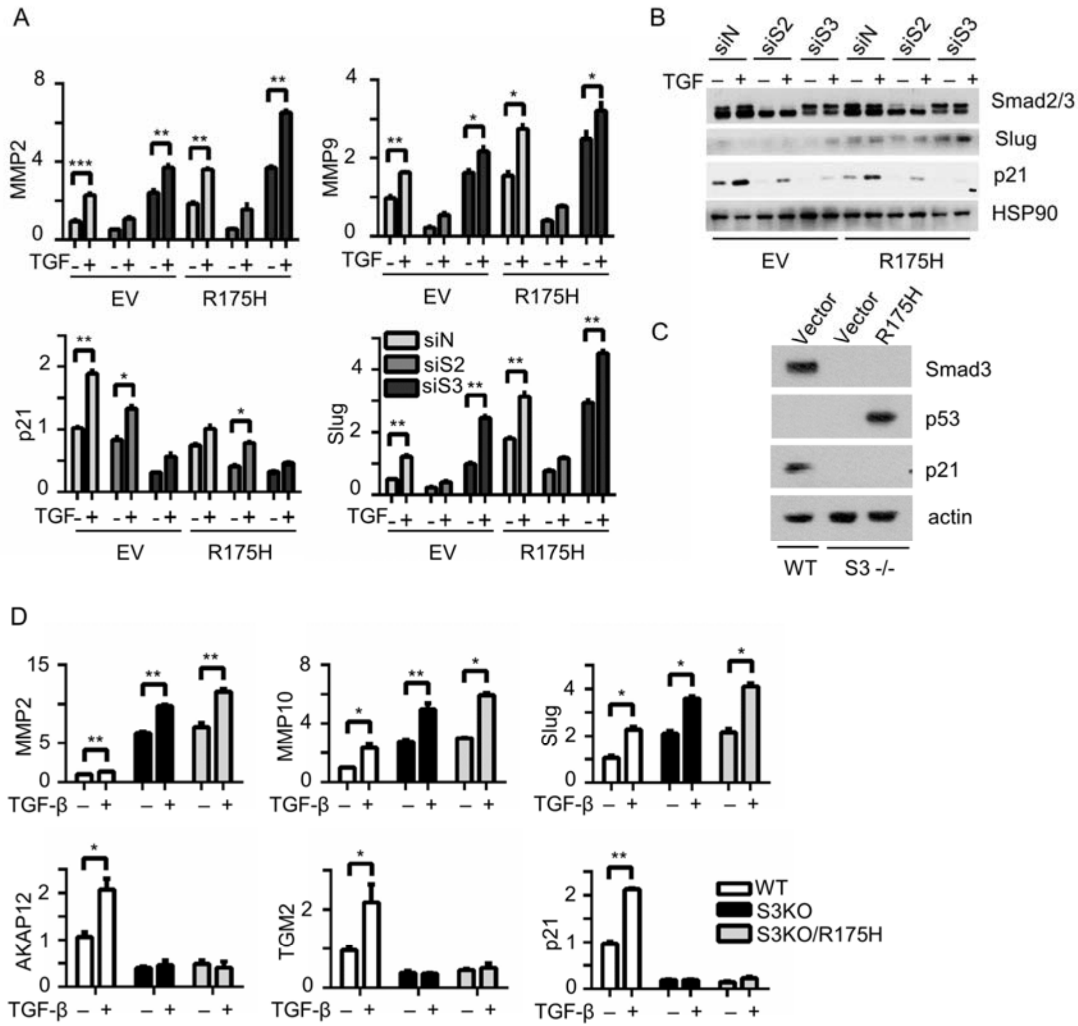
Figure 5



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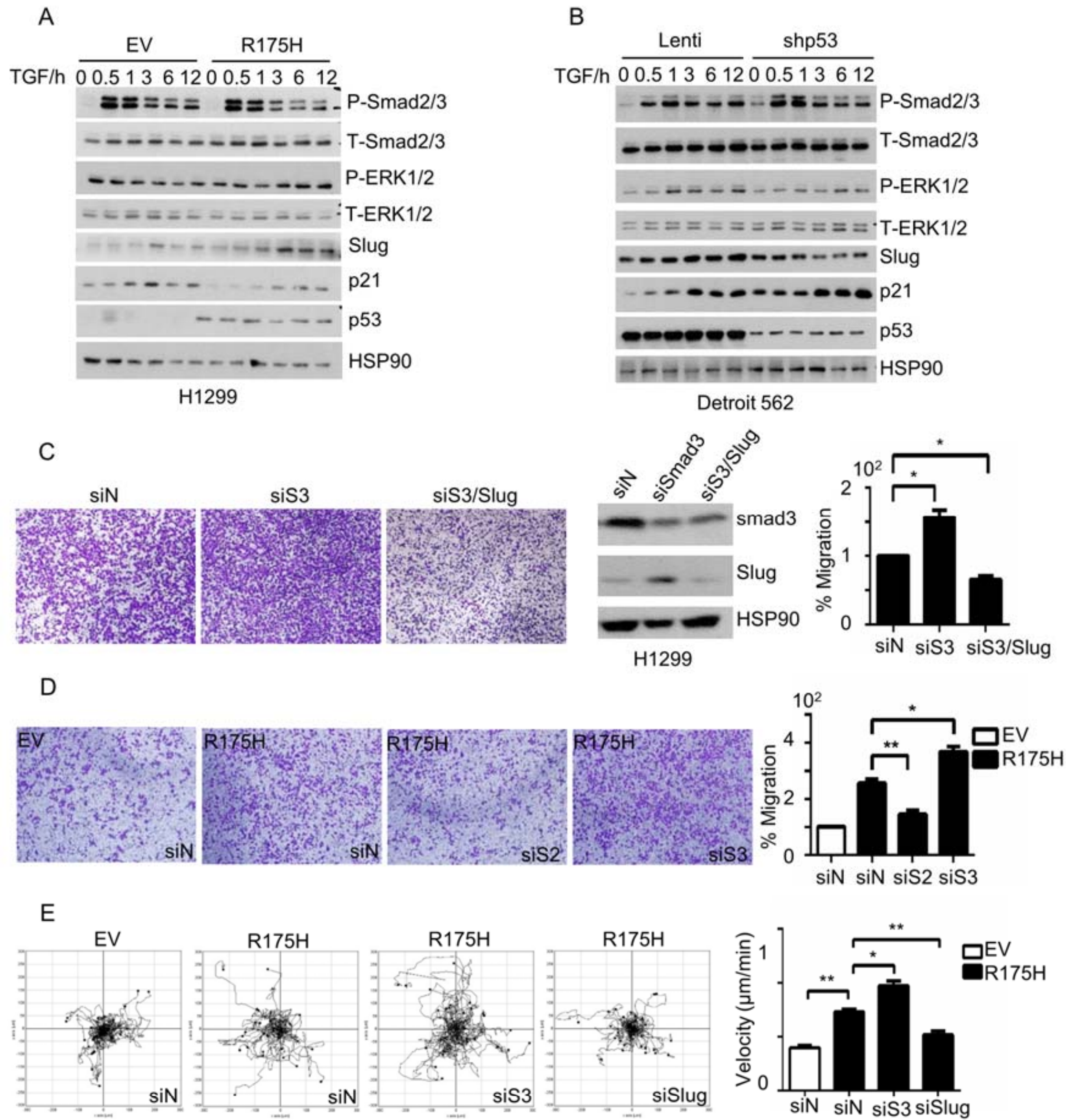

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Signal Transduction:

Mutant p53 Promotes Tumor Cell Malignancy by both Positive and Negative Regulation of TGF-β Pathway

Lei Ji, Jinjin Xu, Jian Liu, Ali Amjad, Kun Zhang, Qingwu Liu, Lei Zhou, Jianru Xiao and Xiaotao Li

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