p53 and \( \Delta Np63\alpha \) Coregulate the Transcriptional and Cellular Response to TGF\( \beta \) and BMP Signals

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**Abstract**

The TGF\( \beta \) superfamily regulates a broad range of cellular processes, including proliferation, cell-fate specification, differentiation, and migration. Molecular mechanisms underlying this high degree of pleiotropy and cell-type specificity are not well understood. The TGF\( \beta \) family is composed of two branches: (i) TGF\( \beta \), activins, and nodals, which signal through SMAD2/3, and (ii) bone morphogenetic proteins (BMP), which signal through SMAD1/5/8. SMADs have weak DNA-binding affinity and, therefore, do not directly transcribe target genes. SMADs physically interact with transcriptional partners for SMAD proteins and thereby influence cellular responses to TGF\( \beta \) and BMPs. Suppression of p53 or overexpression of \( \Delta Np63\alpha \) synergistically enhance BMP-induced transcription. Mechanistically, p53 and \( \Delta Np63\alpha \) physically interact with SMAD1/5/8 proteins and co-occupy the promoter region of target genes in the mammary epithelium. Collectively, these results establish an integrated gene network of SMADs, p53, and \( \Delta Np63\alpha \) that contribute to EMT and metastasis.

**Implications:** This study identifies aberrant BMP activation as a result of p53 mutation or \( \Delta Np63\alpha \) expression. *Mol Cancer Res; 13(4); 732–42. ©2015 AACR.*

**Introduction**

The TGF\( \beta \) superfamily is composed of TGF\( \beta \)s, bone morphogenetic proteins (BMP), growth differentiation factors, the Mullerian hormone, activin, inhibin, and nodal (1). These ligands signal through heterotetrameric complexes of type I and type II serine/threonine kinase transmembrane receptors. Type II receptors are constitutively active and phosphorylate type I receptors resulting in their activation. Once activated, the type I receptors phosphorylate receptor-regulated SMADs (R-SMAD). BMPs bind to BMP-specific type I receptors; ALK2, ALK3, and ALK6, and signal through the R-SMADs; SMAD1, SMAD5, and SMAD8. Traditionally, it has been thought that TGF\( \beta \)s activate the TGF\( \beta \)-specific type I receptor, ALK5, which in turn phosphorylates the R-SMADs: SMAD2 and SMAD3. However, this view has been challenged by recent studies that demonstrate that TGF\( \beta \) can induce phosphorylation of SMAD1/5/8 in specific contexts (2, 3). Once R-SMADs are phosphorylated, they interact with SMAD4, a common partner SMAD for all TGF\( \beta \) superfamily ligands. This R-SMAD/SMAD4 complex translocates into the nucleus, and together with coactivators and corepressors, exerts effects on gene transcription (1).

The TGF\( \beta \) family plays diverse roles in regulating epithelial cells, including controlling cell fate decisions, proliferation, migration, and differentiation. TGF\( \beta \) exerts paradoxical effects during carcinogenesis, acting as a tumor suppressor in the early stages of tumor progression and switching to a prometastatic signal during later stages of tumorigenesis (4). Notably, TGF\( \beta \) has a well-characterized role in activating and maintaining epithelial-to-mesenchymal transition (EMT) in both physiologic and pathologic contexts (5). EMT is a process that involves the reversible trans-differentiation of epithelial cells to mesenchymal cells via activation of a network of transcription factors, miRNAs, and cytoskeletal components (6). The rapid and reversible switch to a mesenchymal phenotype renders cells more migratory and invasive. Recent reports have implicated EMT in tumor progression, chemotherapeutic resistance, the expansion of tumor stem cells, and the initiation of a metastatic cascade (6). Together, these studies suggest that EMT may be targeted for therapeutic benefit.

One of the most frequent genetic alterations in human cancer is mutation of the tumor suppressor gene TP53. Patients with Li-Fraumeni syndrome, a disorder associated with germline mutations in p53, exhibit a high incidence of breast tumors and sarcomas (7). Abundant evidence indicates that loss-of-function (LOF) p53 mutations can fundamentally alter the biologic outcomes of diverse cellular signaling pathways. For example, a spectrum of cancer-associated p53 LOF mutations converts TGF\( \beta \)
signaling outputs from tumor suppressive to tumor promoting. In many cellular contexts, p53 gain-of-function (GOF) mutations are required for TGF-β-induced migration and invasion (8). In addition, the TGFβ effector proteins, SMAD2/3, physically interact with GOF-mutant p53 and ΔNp63 (the predominant isoform of the p53 family member TP63) to exert a prometastatic phenotype. TP63, which is rarely mutated in human cancer, plays a critical role in epithelial specification and the preservation of stem cell regenerative capacity in stratified epithelial tissues (9). In addition, ΔNp63Δα has been shown to possess oncogenic and prosurvival activity in triple-negative breast cancers and squamous cell carcinomas of the head and neck (10, 11). The high degree of homology between the DNA-binding domain of ΔNp63α and that of p53 enables ΔNp63α isoforms to bind to p53-response elements (12). Studies have demonstrated that ΔNp63α isoforms are able to act as dominant negative suppressors of p53-dependent transcription (12).

Previously we reported that ΔNp63α induces BMP signaling via activation of BMP7 expression (13). Furthermore, inhibition of BMP signaling was sufficient to prevent tumorigenesis in an allograft mouse model of breast cancer (13). These results, coupled to reports that BMP7 is overexpressed in >70% of breast cancers, and raised questions about the molecular basis of BMP signaling activation in breast tumorigenesis. Here, we present data indicating that changes in either p53 or ΔNp63α status alter cellular responsiveness to BMP signals. We further investigate the involvement of BMPs and ΔNp63α in the crosstalk between p53 and TGFβ signaling, and elucidate the functional importance of this network in cancer cells. Our data indicate that ΔNp63α is a coactivator of BMP signaling and that wild-type p53 is a corepressor. We report that both ΔNp63α and p53 directly bind to canonical p53-binding elements located within the promoter of the prosurvival BMP target gene, inhibitor of differentiation 2 (ID2). In addition, we demonstrate that ΔNp63α and p53 physically interact with BMP-specific SMAD effector proteins.

Unexpectedly, we observed increased expression of canonical BMP target genes following TGFβ treatment. Full activation of these target genes by TGFβ required ΔNp63α activity and was synergistically enhanced with p53 loss. We also report that the master EMT initiator, Snail, is coregulated by TGFβ, Δp63α, and p53 in mammary epithelial cells. Using computational tools, we investigated the activity of 29 distinct transcriptional programs required for TGFβ-induced EMT and found that the p63-driven transcriptional program was the most active. In addition, the transcriptional targets of p53 and SMAD proteins were significantly enriched in genes upregulated during EMT, suggesting that these transcriptional programs are also active during EMT. Finally, we found that ΔNp63α shares a significant number of target genes with TGFβ-specific SMADs and that BMP signaling components are significantly enriched among ΔNp63α target genes. Collectively, our results support a model in which the p53 family complexes with TGFβ and BMP effector SMADs to influence transcriptional and cellular outputs. We hypothesize that the differential expression of these coregulatory molecules contributes to the pleiotropic nature of these signaling pathways.

Materials and Methods

Cell culture and reagents

Immortalized mammary epithelial cells (IMEC) and IMEC-sh-p53 cells were derived as previously described (14, 15). IMECs were cultured in MEGM complete media (Lonza CC-3051) with 50 µg/ml puromycin, bovine pituitary extract (BPE), and 100 U/ml penicillin/streptomycin. Treatments with TGFβ1 and A83-01 were performed without BPE and with the addition of 0.1%BSA. H1299 cells were cultured according to ATCC guidelines. Cells were serum starved for 3 hours before all TGFβ treatments.

Recombinant TGFβ1 (Milenyi) was used at 500 pmol/L. A83-01 (Tocris) was used at 2 µmol/l for 1 hour before TGFβ1 treatment, or as indicated. LDN193189 (Stemgent Technologies) was used at 1 µmol/L, unless otherwise indicated. Recombinant BMP7 (R&D systems) was used at 50 ng/mL.

qRT-PCR

Total cellular RNA was prepared using i-Script RT-qPCR sample preparation reagent (Bio-Rad) according to the manufacturer’s protocol. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed with iQ SYBR Green Super mix (Bio-Rad). Relative changes in gene expression were obtained using the 2−ΔΔCt method normalizing to the GAPDH housekeeping gene.

Western blot analysis

Cells were lysed in NETN lysis buffer [100 mmol/L Tris-Cl (pH 7.8), 1 mmol/L EDTA, 100 mmol/L NaCl, and 0.1% Triton X-100] with protease and phosphatase inhibitors (Roche). Protein concentration was determined by Lowry protein assay. Protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with antibodies directed against phospho-SMAD1/5/8 (Cell Signaling Technology), total SMAD1/5/8 (Cell Signaling Technology), p63 (4A4 clone; Lab Vision), p53 (Clone DO-7; Thermo Scientific), and β-actin (Cell Signaling Technology) primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were used. Blots were visualized by enhanced chemiluminescence (Millipore).

Colony formation assay

Cell lines were plated at 500 to 1,000 cells per well in six-well plates. Cells were treated with 1 µmol/L LDN193189, 50 ng/mL HBBMP7, or vehicle control every 48 hours for 10 to 14 days. Colonies were fixed in 80% methanol and stained with 0.1% crystal violet solution.

Adenovirus generation

ΔNp63α and GFP were subcloned from pcDNA3.1 into pShuttleCMV plasmids and recombined using the AdEasy Adenoviral production system. Viral titers were generated by amplification in HEK-293Ad cells. Adenovirus stocks were diluted 1:1000 to infect cell lines.

Retrovirus generation

pLPC-ΔNp63α and pLPC-empty vector retroviral expression plasmids were a gift from Dr. Lief Ellisen (Massachusetts General Hospital). The pBMN-ID2-Flag retroviral expression plasmid was a gift from Dr. Mark Israel (Geisel School of Medicine at Dartmouth). Viral titers were generated by amplification in Platinum-A retroviral packaging cells (Cell BioLabs). To infect cells, normal growth media supplemented with 4 µg/mL polybrene were added to cells in a 1:1 ratio with viral supernatant for 24 hours.
Chromatin immunoprecipitation (ChIP)/re-ChIP

Cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C. Cells were lysed in 1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.1, and protease inhibitors. Lysates were sonicated using a Bioruptor for three 5-minute cycles (30 seconds on/30 seconds off). Before IP, lysates (~5 million cells) were diluted 1:10 in dilution buffer (1% Triton, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.1). Protein A/G Dynabeads (Life Technologies) were incubated with 2.5 μg of indicated antibodies (p63: A44 clone; Lab Vision, and p53: Clone DO-7; Thermo Scientific) and rotated overnight at 4°C before adding dilute chromatin. IP reactions were carried out overnight rotating at 4°C. To control for nonspecific binding, lysates were also immunoprecipitated with mouse IgG. Cross-linking was reversed by incubating lysates in 0.1 mol/L NaHCO3 + 1% SDS overnight at 65°C. DNA was isolated using a QIAgen PCR purification kit. Sequences of PCR primers appear in the Supplementary Data.

For the Re-ChIP, beads from the first cycle of p53 or p63 ChIP were incubated with 1 μmol/L dithiothreitol (DTT) at 37°C for 30 minutes to elute immune complexes. The elution was diluted 1:20 in dilution buffer (1% Triton, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.1) and reimmunoprecipitated with SMAD1/5/8 antibodies (Cell Signaling Technology) overnight at 4°C. Cross-linking was reversed by incubating lysates in 0.1M NaHCO3 + 1% SDS overnight at 65°C. DNA was isolated using a QIAgen PCR purification kit.

Immunofluorescence

Cells were fixed in Cytorich Red at RT for 30 minutes and subsequently permeabilized in 0.1% Triton-X-100/PBS for 20 minutes at room temperature. Cells were then blocked in 5% goat serum/PBS for 20 minutes at 37°C and incubated with anti-vimentin primary antibody (1:100, Lab Vision) in 1% goat serum/PBS at 37°C for 45 minutes. Cells were washed in PBS/0.1% Tween-20 and incubated with Alexa-Fluor–conjugated secondary antibody in 5% goat serum/PBS (anti-mouse-Alexa Fluor 555, 1:500, Invitrogen) for 15 minutes at 37°C. Cells were washed in PBS, and Phalloidin-Alexa Fluor 488 (Life Technologies) was added to cells at a 1:1,000 dilution for 20 minutes at room temperature. Cells were washed in PBS/0.1% Tween-20, mounted in Vectashield mounting media with DAPI (Vector Laboratories), and imaged by fluorescent microscopy.

Commununoprecipitation

H1299 cells were transfected with plasmids expressing ΔNp63α, wt p53, or GOF-mutant p53 (R175H and R273H). Commununoprecipitation was carried out using the Pierce Coimmunoprecipitation Kit according to the manufacturer’s protocol (Thermo Scientific). Briefly, cells were lysed in the provided lysis buffer. Lysates were precleared by incubating with control agarose resin for 30 minutes rotating at 4°C. Cleared lysates were incubated with 2 μg of antibody (per 5 million cells) or an isotype-matched IgG control rotating overnight at 4°C. Antibody/lysate complexes were incubated with Coupling Resin for 4 hours rotating at 4°C. The co-IP was eluted according to the manufacturer’s protocol.

Dual luciferase assay

 Luciferase experiments were carried out using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s protocol. H1299 cells were transfected with 50 ng of the CAGA-luciferase reporter containing 12 repeats of the core SMAD0-binding motif (CAGA) upstream of a minimal promoter that drives luciferase expression. They were cotransfected with 5 ng of Renilla reniformis luciferase under a thymidine kinase promoter (Rl-Ikr) and 500 ng of the indicated expression plasmids. Transfections were carried out using LipofectAMINE according to the manufacturer’s protocol. Forty-eight hours posttransfection, cells were treated with 500 pM TGFβ for 1 hour and then harvested.

Transcription factor target gene enrichment analysis

EMT time course data were downloaded from the Gene Expression Omnibus (GEO) database (GSE17708). In this previously published dataset, Sartor and colleagues treated human A549 lung adenocarcinoma cells with 5 ng/mL TGFβ for 0, 0.5, 1, 2, 4, 8, 16, 24, and 72 hours to induce EMT (16). Each time point was performed in triplicate. Gene expression was profiled using Affymetrix HG_U133_plus_2 arrays with 54675 probe-sets, applying standard techniques (16). Gene expression data were normalized and compared to identify differentially expressed genes between each time point after EMT induction and time 0. Upregulated genes were defined as genes that increased in expression by >1.5-fold, and conversely, downregulated genes were defined as genes that decreased in expression by >1.5-fold. Target genes for 29 human transcription factors were downloaded from the CHEA database (17), which were identified based on ChIP-CHIP, ChIP-Seq, or ChIP-PET data.

To calculate enrichment in upregulated genes, we calculated the number of target and non-target genes in upregulated genes (denoted as P1 and P0), and the number of target and nontarget genes in all other genes (genes that are not upregulated; denoted as C1 and C0). Then, we calculated the enrichment ratio as [P1/(P1+P0)]/[C1/(C1+C0)]. A ratio > 1 indicates enrichment of target genes of a transcription factor in upregulated genes; a ratio <1 indicates depletion of target genes of a transcription factor in upregulated genes. Similarly, we examined the enrichment of transcription factor target genes in downregulated genes at each time point. The significance of enrichment is calculated by using the Fisher exact test.

Overlapping analysis between gene sets

The overlapping between TP63 and SMAD3 target genes was examined and the significance of overlap was calculated using the Fisher exact Test. Similarly, the enrichment of TP63 targets in BMP pathway genes was analyzed. TP63 targets were defined based on Vigano and colleagues (18) and Perez and colleagues (19), which were downloaded from the CHEA database (17) and MsigDB database (20), respectively. SMAD3 targets were defined based on Koinuma and colleagues (21) data downloaded from the CHEA database. The BMP pathway gene set was defined by MsigDB.

Statistical analysis

Quantitative data are displayed as mean values of triplicate points. Error bars represent the SEM. P-values <0.05 are considered significant.

Results

P53 family members regulate canonical BMP signaling

Previously we reported a regulatory relationship between TP63 and BMP signaling in which ΔNp63α, the predominant TP63...
gene product, promotes expression of BMP7 in the mammary epithelium (13). Other studies indicate that suppression of BMP7 inhibits proliferation of p53-deficient, but not p53 wild-type, breast cancer cell lines (22). Together, these reports suggest that expression and activity of p53 family members influence BMP signaling. We observed that shRNA-mediated suppression of p53 in an hTERT-IMEC line sharply increased phosphorylation of SMAD1/5/8, indicating enhanced BMP signaling (Fig. 1A). Consistent with the opposing activities of p53 and ΔNp63α, siRNA-mediated suppression of ΔNp63α reduced P-SMAD1/5/8 levels (Fig. 1B), and ΔNp63α overexpression elevated P-SMAD1/5/8 (Fig. 1C). These results predict that p53 and ΔNp63α will differentially regulate canonical BMP target genes.

Inhibitor of differentiation genes, ID1, ID2, ID3, and ID4, are established transcriptional targets of BMP signaling that coordinate cellular responses to BMP activation primarily by maintaining proliferative capacity and governing cell fate decisions (23). In cancer, ID proteins inhibit differentiation and promote survival and metastatic colonization (24). ID2 has been shown to be highly expressed in several tumor types (25) and behaves as an oncogene by inhibiting anti-proliferative activity of the retinoblastoma protein, RB (26). Previous studies indicated that p53 represses ID2 expression in neural progenitor cells as a mechanism to suppress proliferation of this cell population (27). To determine the effects of p53 loss on BMP-induced transcription, we measured the induction of ID2 mRNA levels in response to BMP
stimulation in IMECs and IMECs with stable p53 knockdown. Results indicated that suppression of p53 significantly enhanced induction of ID2 by BMP7 (Fig. 1D). Stimulation of IMECs with recombinant BMP7 in the presence of overexpressed \( \Delta Np63\alpha \) also synergistically enhanced ID2 transcription (Fig. 1E). Furthermore, analysis of breast cancer cell lines in the Cancer Cell Line Encyclopedia revealed that BMP7 mRNA overexpression or amplification is a significantly more common event in p53-mutant (62%) versus p53 wild-type (28%) breast cancer cells (Fig. 1F). Together, these results indicate that loss of p53 or gain of \( \Delta Np63\alpha \) leads to increased sensitivity to BMP signaling, suggesting that tumors with these genetic lesions may be more responsive to BMP signaling inhibitors.

**Enhanced BMP signaling mediates increased clonogenicity resulting from p53 inactivation and \( \Delta Np63\alpha \) overexpression**

The previous results indicate that BMP signaling is negatively regulated by p53 and positively regulated by \( \Delta Np63\alpha \). TP53 is a negative regulator of self-renewal in hematopoietic, neural, and mammary stem/progenitor cells (28–30), whereas \( \Delta Np63\alpha \) is required for prolonged proliferative capacity and self-renewal of epithelial stem cell populations. This suggested the possibility that enhanced BMP signaling mediates the
impaired p53 will display increased sensitivity to anti-BMP of cells that lose p53. These data suggest that tumors with p53 loss is suppressed by LDN193189 (Fig. 1K), indicating and J). In addition, the increased proliferation associated with indicate that BMP signaling mediates the enhanced clonogenic activities of p53 and ΔNp63α, but not p53, binds a second site in the ID2 promoter – 1203 bp upstream of the transcription start site (Fig. 2D and G). Studies using the promoter of CDKN1A (p21<sup>CIP1/WAF1</sup>), Fig. 2B and E) serve as a positive control as both p53 and ΔNp63α have been reported to directly bind to the p21 promoter (32). The ID2 site at –821 was previously shown to be bound by ΔNp63α in human keratinocytes (33). Together, these data demonstrate that p53 and ΔNp63α occupy regulatory regions within the ID2 gene, suggesting a mechanism by which p53 family members regulate transcription of ID2 and possibly other BMP targets.

P53 and ΔNp63α directly bind to the promoter region of the BMP target gene, ID2

The previous results indicated that p53 is able to repress BMP-induced transcription and that ΔNp63α is able to enhance BMP transcriptional activity. We therefore sought to determine whether p53 and ΔNp63α regulate BMP signaling via direct transcriptional regulation of BMP targets. Analysis of a region of the ID2 gene, spanning from 3 kb upstream of the transcription start site to 1 kb downstream, identified two putative p53/p63-binding elements (Fig. 2A). Chromatin immunoprecipitation (ChIP) studies indicated that one of these elements, ID2 –821, is directly bound by both p53 and ΔNp63α (Fig. 2C and F). ΔNp63α, but not p53, binds a second site in the ID2 promoter – 1203 bp upstream of the transcription start site (Fig. 2D and G). Studies using the promoter of CDKN1A (p21<sup>CIP1/WAF1</sup>), Fig. 2B and E) serve as a positive control as both p53 and ΔNp63α have been reported to directly bind to the p21 promoter (32). The ID2 site at –821 was previously shown to be bound by ΔNp63α in human keratinocytes (33). Together, these data demonstrate that p53 and ΔNp63α occupy regulatory regions within the ID2 gene, suggesting a mechanism by which p53 family members regulate transcription of ID2 and possibly other BMP targets.

Figure 3.
P53 and ΔNp63α physically interact with BMP-specific SMADs. A-D, coimmunoprecipitation of H1299 cells transfected with plasmids expressing ΔNp63α wt p53 or GOF mutant p53 (R175H and R273H). Cell lysates were immunoprecipitated with anti-p63 or p53 antibodies and immunoblotted with the indicated antibodies. Input lanes represent 5% of the total cell lysate.

E, ChIP and reChIP analyses in IMEC cells across the ID2 promoter. P53 and P63 antibodies were used for the first immunoprecipitation, and SMAD1/5/8 directed antibodies were used for the re-ChIP. F, proposed model of interaction between SMADs, p53, and p63.

activities of p53 and ΔNp63α on stem cell populations. ShRNA-mediated suppression of p53 or ectopic expression of ΔNp63α lead to a marked increase in clonogenic capacity (Fig. 1G and H, respectively). Under both conditions, the increased clonogenicity was sensitive to LDN193189, a potent and selective inhibitor of BMP type I receptor kinases. These results indicate that BMP signaling mediates the enhanced clonogenic capacity that is the result of p53 loss or ΔNp63α gain (Fig. 1I and J). In addition, the increased proliferation associated with p53 loss is suppressed by LDN193189 (Fig. 1K), indicating that BMP signaling contributes to the increased proliferation of cells that lose p53. These data suggest that tumors with impaired p53 will display increased sensitivity to anti-BMP therapeutics. This is consistent with our previous report that basal breast cancers, which have the highest rates of p53 loss of function mutations, also have the highest levels of BMP signaling activity (31).
indicating that this region was significantly enriched by p63 or p53-ChIP. In addition, this region could be further enriched by SMAD1/5/8 re-ChIP (Fig. 3E). This result suggests that this site is co-occupied by p63, p53, and SMAD1/5/8 in IMEC cells.

**TGFβ, ΔNp63α, and p53 coregulate canonical BMP-induced transcription**

Recent studies have challenged the traditional view that TGFβ signals exclusively through SMAD2/3, whereas BMPs signal through SMAD1/5/8. TGFβ has been reported to phosphorylate and activate SMAD1/5/8 in endothelial and epithelial cell populations via ALK1 and ALK5 receptor complexes, respectively (2, 39). Consistent with these reports, we found that TGFβ induces the rapid phosphorylation of SMAD1/5/8 as early as one hour after treatment (Fig. 4A). This induction is sensitive to A83-01, an inhibitor, but not LDN193189, indicating that the phosphorylation of SMAD1/5/8 by TGFβ is carried out by ALK5. Analysis at 48 hours indicated that TGFβ-induced phosphorylation of SMAD1/5/8 was stable and persistent and had become partially sensitive to LDN193189 (Fig. 4B).

In addition, siRNA-mediated suppression of ALK5 dramatically reduced phosphorylation of SMAD1/5/8 in response to TGFβ, further demonstrating that TGFβ-induced phosphorylation of SMAD1/5/8 is mediated by an ALK5-containing receptor complex (Fig. 4C). TGFβ also induced expression of canonical BMP target genes, ID1 and ID2 (Fig. 4E and F) as well as the canonical BMP target gene, Pai-1 (Fig. 4D). These inductions were sensitive to A83-01, and resistant to LDN193189 (Fig. 4E–D) demonstrating that TGFβ directly induces phosphorylation of SMAD1/5/8, and subsequently induces canonical BMP transcriptional responses, through ALK5 kinase activity.

On the basis of our model in which ΔNp63α and p53 act as transcriptional partners for SMADs, we investigated whether ΔNp63α and p53 influenced TGFβ-induced transcription of canonical BMP targets. siRNA-mediated suppression of ΔNp63α significantly reduced TGFβ-dependent induction of ID1 and ID2, demonstrating that ΔNp63α is required for full activation of these genes by TGFβ (Fig. 4G–I). We also tested the influence of ΔNp63α on the transcriptional activation of two well-characterized TGFβ target genes, Pai-1 and Snail. Similarly, we found that ΔNp63α was required for full activation of the pro-EMT regulator, Snail, by TGFβ (Fig. 4K). As Snail induction alone is sufficient to induce EMT, these data suggest that ΔNp63α may play a critical role in TGFβ-induced EMT (Fig. 4K, ref. 40). In contrast, ΔNp63α suppression did not alter TGFβ induction of Pai-1, suggesting that ΔNp63α coregulates only a subset of Smad target genes (Fig. 4I).

In these assays, the suppression of ΔNp63α is transient, and under these conditions, growth and survival of the cells are not impacted. Conversely, stable shRNA knockdown of wt p53 synergistically enhanced TGFβ-mediated induction of Pai-1, ID1, and Snail, and a similar trend was observed for ID2 (Fig. 4L–P). These data suggest that p53 is a corepressor and ΔNp63α is a coactivator of TGFβ-mediated transcription of a subset of target genes. In contrast, mutants of p53 (R175H and R273H) did not alter TGFβ-mediated induction of Pai-1, ID2, or Snail (Fig. 4Q–S), suggesting that the ability of p53 to coregulate a subset of TGFβ target genes may contribute to its tumor suppressor activity.

To further investigate the role of p53 and ΔNp63α as coregulators of Smad signaling, we performed Dual Luciferase assays using the CAGA-luciferase reporter containing 12 repeats of the core SMAD-binding motif (CAGA) upstream of a minimal promoter that drives luciferase expression. As expected, we found that TGFβ induces the expression of this reporter, and that ΔNp63α enhances the activity of this reporter both endogenously and under TGFβ stimulation (Fig. 2H). This supports our data that ΔNp63α is a coactivator to TGFβ-mediated Smad signaling. We found that both wt p53 and GOF-mutant p53 do not enhance the activity of this reporter both in the presence or absence TGFβ treatment (Fig. 2H).

**P63 and Smad transcriptional networks are robustly active during epithelial to mesenchymal transition**

EMT is a critical mechanism for mediating tumor metastasis. It was first described as a developmental process responsible for remodeling during gastrulation, cardiac morphogenesis, and neural crest formation (41). EMT is defined as a reversible change in cell adhesion proteins, resulting in changes in cell polarity and cytoskeletal structures. It is typically marked by decreased levels of epithelial markers, including E-cadherin, MUC1, and laminin-1, and a corresponding increase in mesenchymal markers, including vimentin, N-cadherin, and fibronectin (41). Blocking E-cadherin in transformed cells has been shown to increase their invasive and metastatic potential by decreasing cell–cell adhesion in vivo (42). The transcription factors Twist1, Zeb1, Snail, and Slug have been identified as master initiators of EMT. Under certain conditions, overexpression of any one of these transcription factors is sufficient to induce EMT in a variety of cell types (40). Having discovered that Snail is coregulated by ΔNp63α, p53, and TGFβ, we investigated the role of cross-talk between p53 and TGFβ family members in regulating the EMT state. For these studies, we used MCF10A cells, an immortalized but nontransformed mammary epithelial cell line, due to the ability of these cells to undergo EMT when stimulated with TGFβ. We treated MCF10A cells with TGFβ and observed changes in morphology characteristic of EMT after 72 hours of treatment; a loss of epithelial/cuboidal-like shape and a switch to a more mesenchymal/fibroblast-like morphology (Fig. 5A). Conversely, cells took on a cuboidal epithelial morphology when treated with the TGFβR1 (ALK5) kinase inhibitor, A83-01 (Fig. 5A). Staining of actin filaments with FITC-phalloidin highlights the mesenchymal morphology seen after TGFβ treatment (Fig. 5B) and enhanced expression of the mesenchymal marker vimentin is also observed.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** TGFβ, ΔNp63α, and p53 coregulate canonical BMP-induced transcription. A, Western blot analysis of MCF10As pretreated with vehicle, 2 μmol/L A83-01 or 1 μmol/L LDN-193189 for 10 minutes, followed by treatment with vehicle or 500 μmol/L TGFβ for 1 hour and (B) 48 hours. Blots were probed with P-SMAD5/5 antibody. Total SMAD5/5 and β-actin are used as loading controls. C, MCF10A cells transfected with ALK5 siRNA or a scrambled control siRNA and treated with vehicle or TGFβ. Blots were probed with P-SMAD5/5, total SMAD5/5, P-SMAD2, and total SMAD2 antibodies. β-actin is used as a loading control. D–F, qPCR analysis of Pai-1, ID1, and ID2 expression in MCF10A cells pretreated with A83-01 or LDN193189 for 1 hour before treatment with vehicle or 500 μmol/L TGFβ for 48 hours. qPCR analysis of ΔNp63α, p53, ID1, ID2, Pai-1, and Snail levels in MCF10A cells (G–I) transiently transfected with ΔNp63α siRNA or scrambled control siRNA for 72 hours followed by treatment with vehicle or TGFβ for 1 hour, (L–P) stably transfected with p53 or empty vector siRNA and treated with vehicle or TGFβ for 1 hour. *P < 0.05. Q–S, qPCR analysis of ID2, Pai-1, and Snail levels in MCF10A cells transiently transfected with GOF-mutant p53 plasmids (R175H and R273H) followed by treatment with vehicle or TGFβ for 1 hour.
qPCR-based analysis demonstrates induction of pro-EMT effectors as well as EMT markers in response to TGFβ (Supplementary Fig. S1). To investigate the function of p63, p53, and SMADs in regulating EMT, we performed transcriptional network analysis of gene expression data present within the GEO database (GSE17708) of A549 lung cancer cells treated with TGFβ to induce EMT over a 72-hour time course (16). Expression levels of genes were compared between each time point after EMT induction and time point $t = 0$. Genes with $>1.5$-fold increase in expression were defined as upregulated genes, and genes with $>1.5$-fold decrease in expression were defined as downregulated genes. We mined publicly available human ChIP-Seq, ChIP-ChIP, and ChIP-PET data in the CHEA database (17) to obtain lists of target genes for 29 transcription factors. We then integrated these data by performing statistical analyses to determine whether targets of a transcription factor were overrepresented in the differentially expressed genes during EMT. A diagram of the experimental design is displayed in Fig. 5C. At each time point, the overlap between up and downregulated genes and transcription factor target genes was examined (see methods for details). SMAD3 and p63 were identified as being highly active during TGFβ-induced EMT, as evidenced by their transcriptional targets being enriched in upregulated genes. Strikingly, TP63 was the most active transcription factor of the 29 transcription factors for which data were available in the CHEA database (Supplementary Fig. S2). These results suggest that p63-directed transcriptional networks contribute to EMT.

To investigate global coregulation of ANP63α and SMAD3 target genes, we analyzed their target gene sets identified from ChIP-CHIP data by Vigano and colleagues and Koinuma and colleagues.
respectively (18, 21). We found that ΔNp63α shares a significant number of target genes (17 out of 40) with SMAD3 (Fig. 5I). In addition, a significant number of these shared genes were enriched during TGFβ-induced EMT indicating that a subset of ΔNp63α and SMAD3 coregulated genes are important for maintaining EMT (Supplementary Fig. S3). We validated the predicted SMAD3 and ΔNp63α shared target genes by real-time PCR analysis. We were able to confirm that cytokeratin14, CDKN1A, and ITGα3 are shared TGFβ and ΔNp63α direct target genes, thereby validating our computational analyses (Fig. 5J–L). Because of lack of human SMAD1/5/8 data available in the CHEA database, we utilized BMP (PID_BMPPATHWAY) and ΔNp63α (PEREZ_TP63_TARGETS) gene sets from the Molecular Signatures Database (MSigDB). Gene sets in MSigDB are compiled based on pathway databases and previously published reports. We discovered a significant enrichment of p63 targets in the BMP pathway genes (Supplementary Fig. S4). These data indicate the presence of a complex network of SMAD proteins, p53, and ΔNp63α that regulates EMT, and further supports the hypothesis that ΔNp63α acts as a coactivator to a subset of BMP and TGFβ target genes.

Discussion

BMP signaling is activated in multiple tumor types and has been implicated in a diverse range of both tumor-suppressive and oncogenic activities (43, 44). BMPs, like TGFβ, exert context-dependent effects on cancer cell populations (44). Here, we report that p53 LOF mutations enhance cellular responsiveness to BMP signaling. Our results suggest that p53 family member status may predict sensitivity to anti-BMP therapeutics.

SMAD proteins have weak DNA-binding affinity and require the association of other transcription factors to increase DNA-binding and transcriptional activity. CBP/p300 (45) and Runx (46) have been shown to behave as coactivators for R-SMADs in certain cellular contexts; however, coactivators of BMP-mediated transcription involved in many cellular responses are unknown. Our results reveal that ΔNp63α and p53 act as transcriptional partners for SMAD1/5/8. We present for the first time that ID2 is a direct positively regulated ΔNp63α target gene, and a direct negatively regulated p53 target gene in mammary epithelial cells. We propose that p53 and ΔNp63α physically interact with BMP or TGFβ-specific SMADs and co-occupy the regulatory regions of target genes to exert synergistic or antagonistic effects on transcription. Future studies are needed to elucidate the role of other p53 family members in regulating SMAD-directed transcription and cellular outputs. The transcriptional responses will likely depend on the presence and abundance of each protein in the complex. It is unclear whether these proteins compete with each other for binding sites or whether they cooperate together. The balance between these proteins and their affinities for DNA-binding remains to be determined. For example, it will be important to discern if TGFβ-regulated SMADs are displaced from p53/p63 complexes in favor of BMP-specific SMADs in the context of high BMP ligand stimulation, and vice versa.

During development, EMT is essential for the maintenance of epithelial plasticity allowing for the massive alterations in cell polarity and migration that occur during gastrulation and organogenesis (6, 47). During cancer progression, this developmental program is aberrantly reactivated leading to an aggressive phenotype marked by decreased proliferation, increased motility, acquisition of stem-like characteristics, and acquired resistance to conventional and targeted therapies (48). Here, we determine the role of p63, p53, and SMAD proteins in regulating the transcriptional changes observed during EMT. BMPs have been shown to both promote and reverse EMT depending on the particular cell type and context (49, 50). We report that during EMT, TGFβ can directly induce phosphorylation of SMAD1/5/8 through the ALK5 receptor and turn on expression of canonical BMP target genes in a ΔNp63α- and p53-dependent manner. Finally, we show that the transcriptional targets of p53, SMADs, and ΔNp63α are significantly enriched in differentially expressed genes during EMT. This is the first report showing ΔNp63α transcriptional outputs contribute significantly to the gene expression changes observed during EMT. The subset of ΔNp63α transcriptional targets responsible for regulating EMT remains to be elucidated. Future work will shed light on the presence and activity of this cross-talk in other cellular outputs influencing cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. DiRenzo, Amanda L. Balboni
Development of methodology: P. Cherukuri, C. Cheng, J. DiRenzo, Amanda L. Balboni
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Cherukuri, C. Cheng, J. DiRenzo, Amanda L. Balboni
Writing, review, and/or revision of the manuscript: M. Ung, A.J. DeCastro, J. DiRenzo, Amanda L. Balboni
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Cherukuri, A.J. DeCastro, J. DiRenzo, Amanda L. Balboni
Study supervision: J. DiRenzo

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Balboni et al.
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