

# *DeltaNp63alpha*-Mediated Induction of Epidermal Growth Factor Receptor Promotes Pancreatic Cancer Cell Growth and Chemoresistance

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## Abstract

Pancreatic ductal adenocarcinoma (PDAC) is highly resistant to current chemotherapy regimens, in part due to alterations in the p53 tumor suppressor pathway. p53 homolog p63 is a transcription factor essential for the development and differentiation of epithelial surfaces. However its function in cancer is controversial and its role in PDAC is not known. We discovered that  $\Delta Np63\alpha$  was the predominantly expressed p63 variant in pancreatic cancer cell lines.  $\Delta Np63\alpha$  protein and mRNA levels were high in T3M4, BxPC3 and COLO-357 pancreatic cancer cells and low in ASPC-1 and PANC-1 cells. Overexpression of  $\Delta Np63\alpha$  in PANC-1 cells and shRNA-mediated knockdown in T3M4 cells indicated that  $\Delta Np63\alpha$  promoted anchorage-dependent and -independent growth, motility and invasion, and enhanced resistance to cisplatin-induced apoptosis. Epidermal growth factor receptor (EGFR) signaling pathways contribute to the biological aggressiveness of PDAC, and we found that the motogenic effects of  $\Delta Np63\alpha$  were augmented in presence of EGF. Ectopic expression of  $\Delta Np63\alpha$  resulted in upregulation of EGFR and  $\beta 1$ -integrin in PANC-1 cells. Conversely,  $\Delta Np63\alpha$  knockdown had an opposite effect in T3M4 cells.  $\Delta Np63\alpha$  potentiated EGF-mediated activation of ERK, Akt and JNK signaling. Chromatin immunoprecipitation and functional reporter assays demonstrated that  $\Delta Np63\alpha$  activated EGFR transcription. 14-3-3 $\sigma$  transcription was also positively regulated by  $\Delta Np63\alpha$  and we have previously shown that 14-3-3 $\sigma$  contributes to chemoresistance in pancreatic cancer cell lines. Conversely, shRNA-mediated knockdown of 14-3-3 $\sigma$  led to abrogation of the  $\Delta Np63\alpha$  effects on cell proliferation and invasion. Thus, p53 homolog  $\Delta Np63\alpha$  enhances the oncogenic potential of pancreatic cancer cells through trans-activation of EGFR and 14-3-3 $\sigma$ .

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## Introduction

Studies in human disease demonstrate that most established tumors carry more than one genetic defect. Pancreatic ductal adenocarcinoma (PDAC) results from the successive accumulation of gene mutations [1]. Activating mutations in K-Ras oncogene and inactivation of tumor suppressors CDKN2A, p53 and SMAD4 are implicated in PDAC development and progression [2]. Genetically engineered mouse models have supported the “double-hit” hypothesis where introduction of either mutant p53 allele or biallelic deletion of *ink4a/Arf* in mice results in progression of pancreatic intraepithelial neoplastic lesions with local invasion and metastases [3,4]. p53 mutations are found in 60 to 70% of PDAC. By contrast, p63, an ancestral member of p53 family, is rarely mutated in cancer.

Six variants of p63 are generated through transcription from two distinct promoters and alternative splicing. Isoforms transcribed from P1 contain a full-length trans-activation domain (TAp63 $\alpha$ ,  $\beta$  and  $\gamma$ ). Transcription from P2 generates amino-terminally

truncated variants ( $\Delta Np63\alpha$ ,  $\beta$  and  $\gamma$ ), whose exact role in cancer is not clear. The  $\Delta Np63$  variant is overexpressed in a variety of human cancers, including tumors of squamous cell origin (head and neck, lung), breast and bladder [5]. In head and neck squamous cell carcinoma and “triple-negative” breast cancer cells,  $\Delta Np63$  suppresses p73-dependent apoptosis and thus promotes tumor survival [6,7]. By contrast, downregulation of  $\Delta Np63\alpha$  in urothelial carcinoma cell lines promotes cancer invasiveness [8], suggesting that the  $\Delta N$  variant may function in a cell type-specific manner.

The role of p63 in PDAC is poorly understood. Here we demonstrate that the  $\Delta Np63\alpha$  variant is expressed at variable levels in PDAC cell lines, and provide evidence that  $\Delta Np63\alpha$  promotes pancreatic cancer cell growth, migration, invasion and chemoresistance. Via direct transcriptional activation,  $\Delta Np63\alpha$  leads to the up-regulation of epidermal growth factor receptor (EGFR) and 14-3-3 $\sigma$ , sensitizing cancer cells to EGF and enhancing their oncogenic potential.

## Methods

### Cell lines

Human pancreatic cancer cell lines ASPC-1, BxPC3 and PANC-1; HEK293 human embryonic kidney cells and H1299 human lung carcinoma cells were purchased from American Type Culture Collection (Manassas, VA). COLO-357 and T3M4 human pancreatic cancer cell lines were a gift from Dr. R. Metzgar (Duke University, Durham, NC). Cell lines were grown in RPMI or DMEM (HEK293) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium).

### Plasmid constructs

The human and mouse  $\Delta$ Np63 $\alpha$  expression plasmids and (p53RE)<sub>5</sub>-tk-luciferase plasmid were reported previously [9]. TAp63 $\alpha$  expression plasmid was purchased from Open Biosystems (Huntsville, AL). pBV-14-3-3 $\sigma$  promoter BDS 2 3 $\times$  (p53 binding site)-luc plasmid was obtained from Addgene (Cambridge, MA). pGL3-EGFR promoter (p53 binding sites)-luc plasmid was a gift from Dr. L. Piri [10]. Sequence modifications within a coding of a DNA-binding domain of the human  $\Delta$ Np63 $\alpha$  expression plasmid and within EGFR promoter p53 binding site 1 were introduced using the Quick-Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

### Immunoblotting

Cells were lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Sodium phosphate, 1 mM NaVO<sub>3</sub>, 1 nM EDTA, 1 nM EGTA, supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and 1 mM PMSF). Proteins were analyzed by immunoblotting as previously described [11]. The following antibodies were used: p63 (4A4; Millipore, Billerica, MA; 1:500); TAp63 $\gamma$  (Li et al., 2006; 1:1,000), 14-3-3 $\sigma$  (Abcam, Cambridge, MA; 1:500), ERK-2 (C-14; Santa Cruz Biotechnology, Santa Cruz, CA; 1:10,000), p53 (DO-1; Santa Cruz Biotechnology; 0.4 µg/mL), EGFR (Calbiochem; 1:2,000), cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, ERK-1/2, phospho-ERK1/2 [T202/Y204], Akt, phospho-Akt [S473] (Cell Signaling Technology, Danvers, MA; 1:1,000), active JNK (Promega, Madison, WI), horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (BioRad; 1:5,000).

### Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 1 µg RNA by random hexamer priming using the Superscript III RT Kit (Invitrogen, Carlsbad, CA). Semi-quantitative RT-PCR was carried out using forward and reverse primers specific for p63 isoforms as previously published [12]. The following PCR cycling conditions were used: 94°C for 3 min, 35 cycles of 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1.5 min; and 72°C for 4 min.

For the analysis of  $\Delta$ Np63 and TAp63 transcript expression quantitative real time PCR (Q-PCR) was performed in a 7300 Sequence Detector using Universal PCR Master Mix according to manufacturer's instructions (Applied Biosystems, Foster City, CA), template cDNA and gene specific probes (Hs00978339\_m1 [ $\Delta$ Np63]; Hs00978349\_m1 [TAp63]; Applied Biosystems). All samples were analyzed in duplicates.

### Transient transfections, adenoviral infection and luciferase assays

PANC-1 and T3M4 cells were transiently transfected with ExGen 500 *in vitro* Transfection Reagent (Fermentas Life Sciences,

Glen Burnie, MD), using equal amounts of experimental or control plasmid. Cells were incubated for 48 hours and protein expression was verified by immunoblotting. The control and  $\Delta$ Np63 $\alpha$ -expressing adenovirus were used as described [13]. ASPC-1 and PANC-1 cells were infected with adenovirus at MOI of ~5 in complete medium for 24 hours.

For luciferase assays, PANC-1 cells were co-transfected with experimental plasmid or control and the luciferase reporter construct (pBV-14-3-3 $\sigma$  promoter BDS 2 3 $\times$  (p53 binding site)-luc; or pGL3-EGFR promoter-luc) along with pCMV $\beta$  vector (Clontech Laboratories, Mountain View, CA). The amount of DNA per transfection was kept constant by using empty pcDNA3.1 vector. Cells were harvested 48 hours post-transfection and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Relative light units were determined using a luminometer (LMaxII<sup>384</sup>, Molecular Devices, Sunnyvale, CA) for firefly luciferase.  $\beta$ -galactosidase activity was determined using a colorimetric method to normalize transfection efficiency [14].

### Lentiviral shRNA mediated gene silencing

Lentivirus containing shRNA targeting  $\alpha$ -specific, TAp63 specific, all p63 isoforms and sh control were described previously [15]. Lentivirus-based 14-3-3 $\sigma$ -targeting and control shRNA were also described by us previously [11]. Lentiviral particles were produced by four plasmid transfection system [11]. Knockdown of p63 isoforms and 14-3-3 $\sigma$  in T3M4 and PANC-1 cells was confirmed by Western blotting and Q-PCR.

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were plated in 96-well plates (3000 cell per well, 6 wells per sample) and cultured in the absence or presence of 10 µg/mL cisplatin (Sigma-Aldrich, St. Louis, MO) for 48 hours. MTT (Sigma-Aldrich) was added at a final concentration 0.55 µg/mL. After additional 4 hours incubation, absorbance at 570 nm was determined using an Emax precision microplate reader (Molecular Devices). We have previously observed that in pancreatic cancer cell lines the MTT assay correlates with cell growth as determined in a doubling and [<sup>3</sup>H]thymidine incorporation assays [16].

### Soft agar assays

Soft agar assays were set up in twelve-well plates, each well containing a bottom layer of 0.5% Difco agar noble (BD Biosciences), a middle layer of 0.3% agar including 1500 cells, and a top layer of 0.3% agar. The plates were incubated for 14 days. Next, 150 µL of 5 mg/mL MTT solution were added to each well. After incubation at 37°C for 4 hours plates were photographed and colonies were counted.

### Cell migration and invasion assays

Cell motility was assessed in *in vitro* wound healing and Transwell migration assays. For wound healing assays, cells were grown to confluency in 6-well tissue culture plates, and serum-starved for 12 hours. The resulting cell monolayer was scratched with a 10 µL pipette tip generating two parallel wounds and incubated for 18 hours in serum-free medium (SF; 0.1% bovine serum albumin) in the absence or presence 1 nM EGF (Millipore). Photographs were taken of each well at four marked locations under 40 $\times$  magnification at zero and 18 hours after wounding. The wound area of matched pictures was measured using the ImageJ software (National Institutes of Health). For Transwell migration assays, cell were suspended in 100 µL SF medium and

placed onto the upper compartment of Transwell chambers (8  $\mu$ m pore size, Corning Incorporated, Corning, NY). For invasion assays, cells were suspended in 500  $\mu$ L SF medium and placed onto upper compartment of Matrigel-coated Transwell chambers (8  $\mu$ m pore size, BioCoat Matrigel Invasion Chambers; BD Biosciences; Franklin Lakes, NJ). In both Transwell assays, the lower compartment was filled with 750  $\mu$ L SF medium in the absence or presence of 1 nM EGF. After 18–20 h, membranes were fixed in methanol, stained with toluidine blue (Fisher Scientific, Pittsburgh, PA) and counted using a light microscope.

### Formaldehyde crosslinking, chromatin immunoprecipitation (ChIP) and PCR amplification

For DNA-histone crosslinking,  $2 \times 10^6$  cells were incubated with 1% formaldehyde in complete medium for 10 minutes. Cells were washed twice in ice-cold PBS and lysed in SDS Lysis Buffer (Millipore) complete with protease inhibitor cocktail (Roche). Protein lysates were sonicated to yield chromatin fragments of  $\sim 500$  bp as assessed by agarose gel electrophoresis. After pre-clearing, protein lysates were incubated at 4°C overnight with 2  $\mu$ g p63 $\alpha$  antibodies (H-129, Santa Cruz) or with rabbit IgG as isotype-specific control antibodies (Santa Cruz). Immunocomplexes were washed and DNA was purified using the ChIP Assay Kit (Millipore) and QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. PCR conditions and primers for 14-3-3 $\sigma$  consensus binding sites 1 and 2 were previously reported [17]. The following PCR primers and conditions were used for the EGFR promoter p53 binding site: forward primer 5' GGCCGCTGGCCTTGGGTC 3'; reverse primer 5' GCCGTGCGCGGTGGTTG 3'; 1 cycle of 94°C for 5 min, 43 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 50 sec, followed by 1 cycle of 72°C for 10 min. PCR was performed using HotStarTaq polymerase Kit (Qiagen), with use of Q-Solution in the EGFR PCR assay.

### Cisplatin-induced apoptosis

Pancreatic cancer cells were incubated for 24 h in complete medium in the absence or presence of 5 or 10  $\mu$ g/mL cisplatin for the initial 2 h, 6 h or the entire 24 h. Both floating and adherent cells were collected and subjected to immunoblotting.

## Results

### $\Delta$ Np63 $\alpha$ expression in pancreatic cancer cell lines

We found that p63 was differentially expressed in pancreatic cancer cell lines. p63 protein levels were highest in T3M4 and BxPC3 cells, intermediate in COLO-357 and below the detection level in ASPC-1 and PANC-1 cells (Fig. 1A). Since the p63 antibody (clone 4A4) recognized all six known isoforms of p63, we compared p63 mRNA transcript levels in the above cell lines. All the cell lines expressed low levels of TAp63 mRNA (Fig. 1B). By contrast,  $\Delta$ Np63 mRNA transcripts were relatively elevated in BxPC3, COLO-357, and T3M4 cells (Fig. 1B).

To further elucidate whether a particular splicing variant of p63 is expressed in pancreatic cancer cell lines, we employed semi-quantitative RT-PCR with p63 splicing variant-specific primers (Table S1). We confirmed that TAp63 $\alpha$  mRNA transcripts were expressed at relatively low and similar levels in all five cell lines. By contrast,  $\Delta$ Np63 $\alpha$  mRNA transcripts were only expressed in BxPC3, COLO-357, and T3M4 cells, whereas  $\Delta$ Np63 $\gamma$  mRNA was barely detectable in BxPC3 and T3M4 cells (Fig. 1C). Although p63 $\alpha$  can be detected in a specific PCR assay, isolated detection of p63 $\beta$  mRNA transcripts is unreliable since its C-terminus is not unique (Fig. S1). In our experiments, expression of

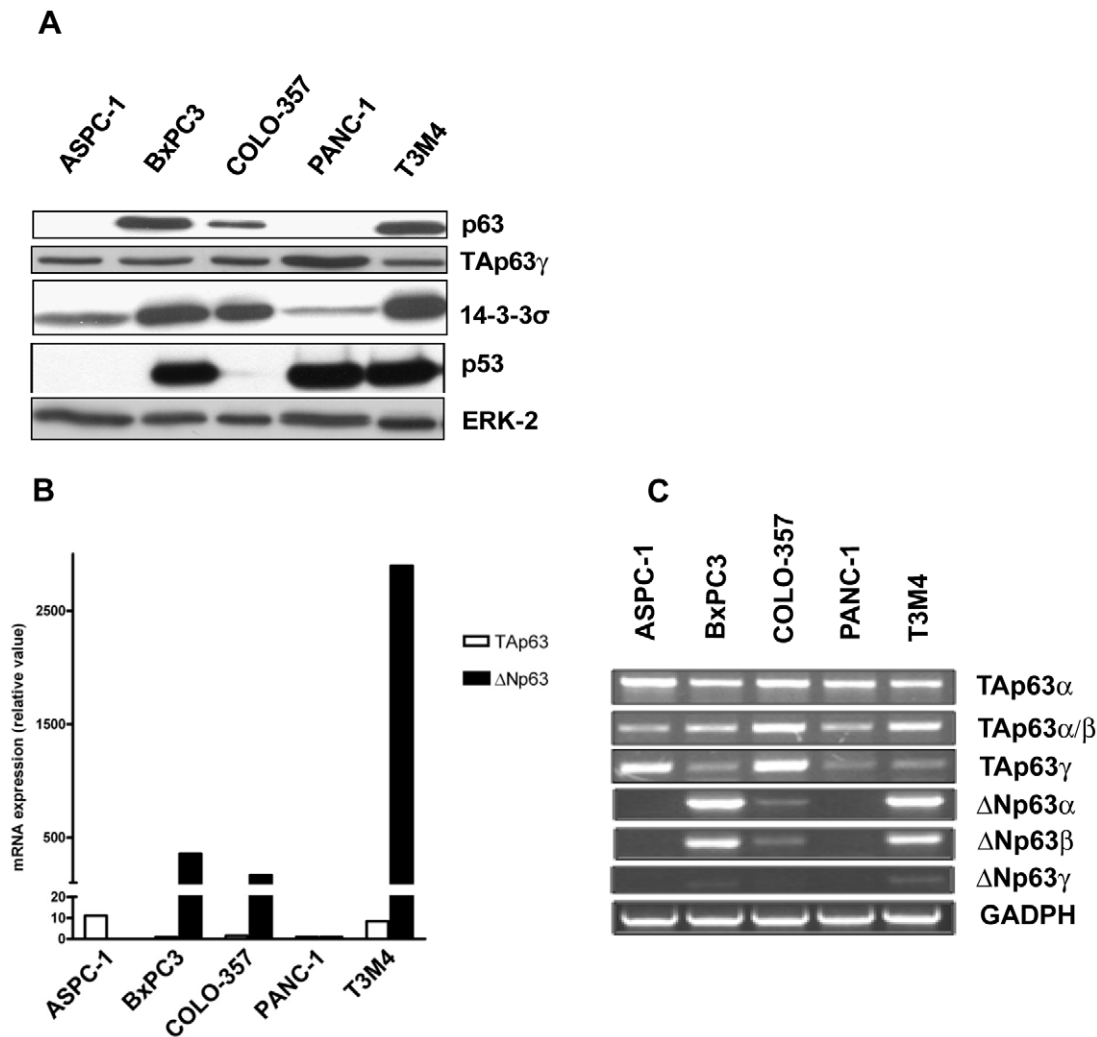
$\Delta$ Np63 $\beta$  mRNA transcript correlated with  $\Delta$ Np63 $\alpha$  expression, probably due to non-specific amplification of  $\Delta$ Np63 $\alpha$ . Since we detected p63 protein migration at  $\sim 68$  kDa, and  $\Delta$ Np63 $\beta$  protein migrates at  $\sim 52$  kDa [18–20], we concluded that  $\Delta$ Np63 $\alpha$  was the predominantly expressed p63 variant in pancreatic cancer cells.

### Oncogenic effects of $\Delta$ Np63 $\alpha$ in pancreatic cancer cells

To study the biologic effects of  $\Delta$ Np63 $\alpha$  in PDAC, we manipulated its expression in PANC-1 and T3M4 cells, which had low and high endogenous levels of  $\Delta$ Np63 $\alpha$ , respectively (Fig. 1). PANC-1 cells were transiently transfected with a  $\Delta$ Np63 $\alpha$ -expressing vector (PANC-1 $\Delta$ N), a TAp63 $\alpha$ -expressing vector (PANC-1TA), or control plasmid. Lentiviral-mediated shRNA was used to down-regulate p63 gene isoforms in T3M4 cells. Two different shRNA sequences, one complementary to a sequence within a p63 DNA-binding domain (sh DBD) and thus targeting all isoforms of p63, and another complementary to a sequence within sterile alpha motif domain (SAM) and thus targeting TAp63 $\alpha$  and  $\Delta$ Np63 $\alpha$  (sh p63 $\alpha$ ) were determined to reduce the  $\Delta$ Np63 $\alpha$  protein and transcript levels in T3M4 cells (Fig. 2A). While shRNA targeting trans-activation domain (sh TAp63) resulted in reduced TAp63 transcript levels, this did not translate into an appreciable change in the total p63 protein levels (Fig. 2A), confirming our observation that  $\Delta$ Np63 $\alpha$  variant predominates in pancreatic cancer cell lines.

Since anchorage-independent growth is a hallmark of malignant cells, we studied whether  $\Delta$ Np63 $\alpha$  has an effect on anchorage-independent growth and cell proliferation in PDAC. PANC-1 $\Delta$ N, but not PANC-1TA cells exhibited increased colony formation in soft agar assays and increased cell proliferation in MTT assays (Fig. 2B). Since T3M4 cells are incapable of forming colonies in soft agar, proliferation was studied in a clonogenic assay. As compared with control cells, proliferation of T3M4 cells was reduced in response to shRNAs that target the DBD or the  $\alpha$ -specific C-terminus and unchanged in response to sh TAp63 (Fig. 2C). Transient overexpression of mouse  $\Delta$ Np63 $\alpha$  cDNA, which carries a silent mutation in the region targeted by the  $\alpha$ -specific shRNA, resulted in a partial rescue of the proliferative ability of sh p63 $\alpha$  T3M4 cells, as compared with cells transfected with control vector (Fig. 2C).

$\Delta$ Np63 $\alpha$  is known to regulate the adhesion program in mammary epithelial cells and keratinocytes [15]. Given the importance of cell adhesion in tumor invasion and progression, we studied the effects of  $\Delta$ Np63 $\alpha$  on the motility and invasive capabilities of the pancreatic cancer cells. In wound-healing assays, PANC-1 $\Delta$ N cells demonstrated enhanced motility in SF conditions, while overexpression of TAp63 $\alpha$  had no effect (Fig. 2D). Since activation of the EGFR pathway is associated with increased tumor aggressiveness and decreased survival in PDAC [21], we sought to determine the effect of EGF on motility. EGF stimulation enhanced migration in PANC-1 $\Delta$ N and control cells (Fig. 2D). Similarly, T3M4 cells expressing sh p63 $\alpha$ , but not sh TAp63, exhibited reduced motility both at baseline and in presence of EGF (Fig. 2E). Transient overexpression of mouse  $\Delta$ Np63 $\alpha$  in T3M4 cells expressing sh p63 $\alpha$  resulted in a partial rescue of the motility phenotype (Fig. 2F). Manipulation of  $\Delta$ Np63 $\alpha$  expression levels had no effect on the invasion of pancreatic cancer cells in Matrigel chambers in SF conditions (Fig. 2G, H). However, stimulation with EGF led to a dramatic increase in the invasive capability of PANC-1 $\Delta$ N cells (Fig. 2G). Consistent with the above findings, shRNA-mediated suppression of  $\Delta$ Np63 $\alpha$ , but not TAp63, significantly reduced the ability of T3M4 cells to invade through Matrigel in response to EGF stimulation (Fig. 2H).



**Figure 1. Expression of 14-3-3 $\sigma$ , p63 isoforms and p53 in pancreatic cancer cell lines.** A, Protein levels of p63, 14-3-3 $\sigma$ , TAp63 $\gamma$  and p53 in pancreatic cancer cell lines. B, mRNA expression of TAp63 and  $\Delta$ Np63 isoforms of p63 in pancreatic cancer cell lines. Total RNA isolated from PDAC cell lines was reverse-transcribed and subjected to real-time PCR with probes specific for  $\Delta$ N and TA isoforms of p63. Results were normalized to 18S levels, and expressed as mean of two independent experiments done in duplicates in which similar results were obtained. C, mRNA expression of p63 splicing variants in pancreatic cancer cell lines. Levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. doi:10.1371/journal.pone.0026815.g001

Collectively, our results indicate that  $\Delta$ Np63 $\alpha$  enhances the colony-forming, proliferative and invasive capacity of the pancreatic cancer cells.

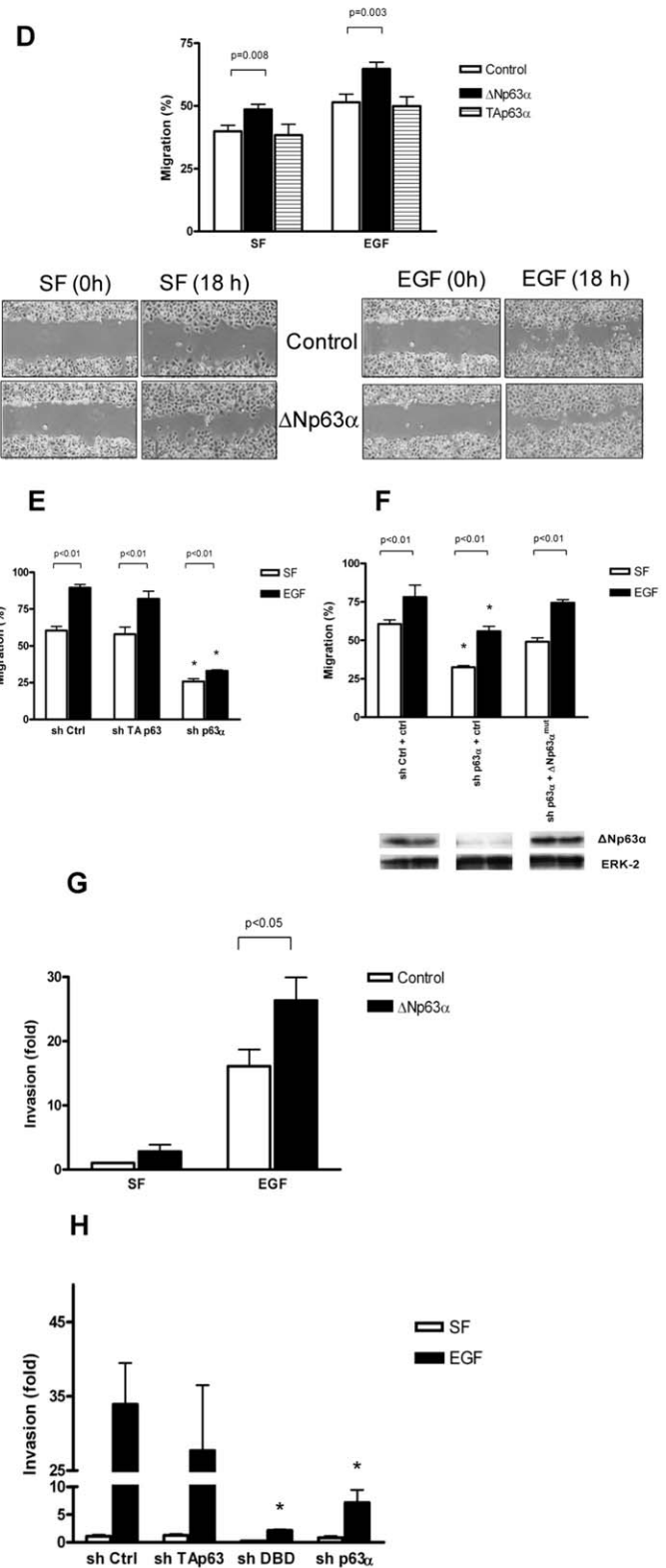
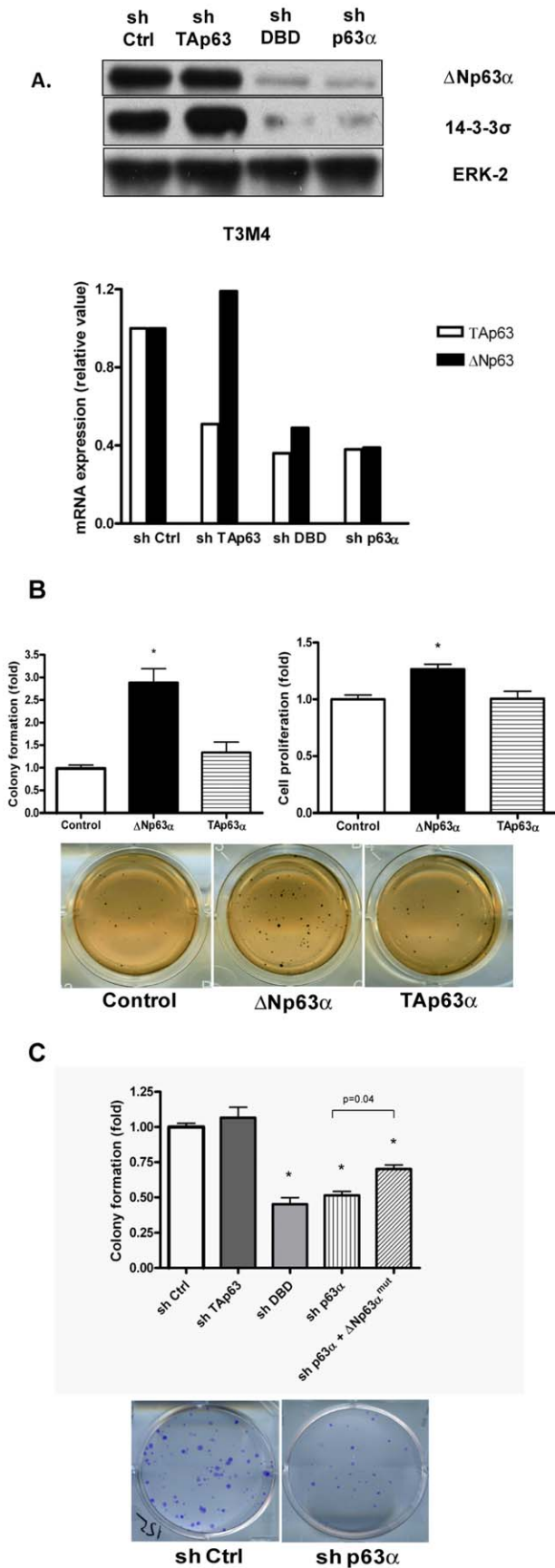
#### $\Delta$ Np63 $\alpha$ upregulates EGFR and sensitizes pancreatic cancer cells to the effects of EGF

Compared with the normal pancreas, EGFR protein and mRNA are expressed at high levels in pancreatic cancer [22]. EGFR mRNA expression predicts decreased survival and poor response to chemotherapy in patients with PDAC [23]. In our work,  $\Delta$ Np63 $\alpha$  dramatically potentiated pancreatic cancer cell motility and invasive abilities in the presence of EGF. To explain this phenomenon, we studied the effect of  $\Delta$ Np63 $\alpha$  on EGFR levels. We determined that engineered expression of  $\Delta$ Np63 $\alpha$  in PANC-1 cells enhanced EGFR expression, while ectopic TAp63 $\alpha$  had no effect (Fig. 3A). Consistent with this observation, we found that EGFR levels were decreased in T3M4 cells in response to shRNA-mediated suppression of  $\Delta$ Np63 $\alpha$  (Fig. 3B), thus documenting a direct correlation between  $\Delta$ Np63 $\alpha$  and EGFR

expression in pancreatic cancer cell lines. To study the effects of  $\Delta$ Np63 $\alpha$  overexpression on EGFR signaling, we assessed activation of downstream kinases upon EGF stimulation. Treatment of PANC-1- $\Delta$ N cells with EGF resulted in enhanced phosphorylation of ERK, Akt and JNK (Fig. 3C). In those cells, all three kinases exhibited increased level of activation at as early as 10 minutes after cell stimulation, and ERK activity persisted for 60 minutes.

Next, we employed a tyrosine kinase inhibitor to assert that the effects of  $\Delta$ Np63 $\alpha$  were mediated through the EGFR pathway. Erlotinib is a reversible inhibitor of the EGF signaling, which associates with the ATP-binding site of the receptor. Incubation of PANC-1 cells with 1  $\mu$ M erlotinib attenuated the  $\Delta$ Np63 $\alpha$ -mediated enhancement of colony formation, motility and invasion (Fig. S2), thus excluding a possibility of a non-specific effect. By contrast, p63 protein levels were not affected when cells were incubated with either EGF or erlotinib (data not shown).

Previous reports suggested that  $\Delta$ Np63 $\alpha$  is able to regulate cell adhesion proteins in mammary epithelial cells [15]. Since integrin-mediated tumor cell interactions with the extracellular matrix play



**Figure 2. Effect of  $\Delta$ Np63 $\alpha$  on anchorage-independent growth, motility, and invasion in pancreatic cancer cells.** A, T3M4 cells were infected with GFP-expressing virus, or p63-specific shRNA complementary to DBD, TA and  $\alpha$ -specific domains of p63. Whole-cell protein lysates were subjected to immunoblotting (top panel). Total RNA was isolated, reverse-transcribed and subjected to real-time PCR with probes specific for  $\Delta$ N and TA isoforms of p63. Results were normalized to 18S levels (bottom panel). Knockdown of p63 isoforms was routinely monitored during the subsequent experiments. B,  $\Delta$ Np63 $\alpha$  stimulates anchorage-independent growth of PANC-1 cells in soft agar assay (left and bottom panels) and cell proliferation in MTT assay (right panel). PANC-1 cells were transiently transfected with  $\Delta$ Np63 $\alpha$ -expressing vector, TAp63 $\alpha$  or control vector. Cells were plated in soft agar at a density of 1500/well of a 12-well plate, four wells per sample. Colonies were counted after 14 days of incubation. For MTT assay cells were plated in 96-well plates, six wells per sample. MTT was added after incubation for 48 h. Data are the mean  $\pm$  SE of four independent experiments. \*,  $p < 0.001$  compared with control. C, Downregulation of  $\Delta$ Np63 $\alpha$  slows proliferation of T3M4 cells in a clonogenic assay. Reconstitution of  $\Delta$ Np63 $\alpha$  partially restores the proliferative ability. Cells were plated on 6 well plates at a density of 500 cells/well. 14 days later, plates were fixed in 3:1 methanol:glacial acetic acid and stained with 2% crystal violet. Data are the mean  $\pm$  SE of three independent experiments done in triplicates. \*,  $p < 0.01$  compared with control (sh ctrl). D–F, Effect of p63 on cell motility measured in wound-healing assays in PANC-1 (D) and T3M4 cells (E, F). Cells were incubated in serum-free (SF) conditions in the absence or presence of 1 nM EGF for 18 h after making a scratch. Quantitative analysis of the images was performed. Ectopic expression of mouse  $\Delta$ Np63 $\alpha$  in sh p63 $\alpha$  T3M4 cells resulted in a partial restoration of cell motility (F); corresponding  $\Delta$ Np63 $\alpha$  protein levels shown below. Data are the mean  $\pm$  SE of at least three independent experiments. Representative pictures shown (magnification  $\times 40$ ). \*,  $p < 0.001$  compared with control (sh ctrl). G and H, Effect of  $\Delta$ Np63 $\alpha$  on the invasion in Matrigel chambers. PANC-1 (G) or T3M4 (H) cells were plated in Matrigel chambers ( $5 \times 10^4$ /ml) and incubated in absence (SF) or presence of 1 nM EGF for 18 hours. Effect was normalized to invasion of control in SF conditions. \*,  $p < 0.001$  compared with control (sh ctrl). doi:10.1371/journal.pone.0026815.g002

a critical role in determining the invasive phenotype in PDAC, we investigated whether  $\Delta$ Np63 $\alpha$  had an effect on integrin expression in pancreatic cancer cells. Ectopic expression of  $\Delta$ Np63 $\alpha$  in PANC-1 cells increased expression of  $\beta 1$ -integrin, whereas the expression of  $\alpha 3$ - and  $\alpha 5$ -integrins was unchanged (Fig. 3A). Conversely, shRNA-mediated suppression of  $\Delta$ Np63 $\alpha$  led to a decrease in  $\beta 1$ -integrin in T3M4 cells (Fig. 3B).

Thus,  $\Delta$ Np63 $\alpha$  contributes to the oncogenic potential of pancreatic cancer cells, at least in part, through upregulation of EGFR and  $\beta 1$ -integrin.

### $\Delta$ Np63 $\alpha$ contributes to chemotherapy resistance in pancreatic cancer cells

Since PDAC is notoriously resistant to chemotherapy agents, we studied the role of  $\Delta$ Np63 $\alpha$  in chemotherapy resistance. It has been previously shown that cisplatin induces degradation of  $\Delta$ Np63 $\alpha$  via stimulation of I $\kappa$ B kinase [24]. Consistent with those data, incubation of pancreatic cancer cell lines with 10  $\mu$ g/ml of cisplatin resulted in degradation of endogenous  $\Delta$ Np63 $\alpha$ . This effect of cisplatin was evident after 6 hours of incubation and was accompanied by increased apoptosis in all tested cell lines (Fig. 4A). We then investigated whether ectopic expression of  $\Delta$ Np63 $\alpha$  would contribute to resistance to cisplatin-induced apoptosis in PDAC. PANC-1 cells were incubated for 24 hours in complete medium supplemented with 5  $\mu$ g/mL cisplatin for 2, 6, or 24 hours. PANC-1 $\Delta$ N cells exhibited a mild attenuation of apoptosis compared with control cells, as manifested by decreased PARP and caspase cleavage (Fig. 4B). This decrease in cisplatin-induced apoptosis correlated with an increase in viability of PANC-1 $\Delta$ N cells as determined in an MTT assay, while PANC-1TA cells exhibited a slightly reduced survival (Fig. 4C). By contrast, T3M4 cells expressing sh p63 $\alpha$  were sensitized to cisplatin-induced apoptosis, as demonstrated by an increased cleavage of PARP and caspase (Fig. 4D). Thus,  $\Delta$ Np63 $\alpha$  attenuates the sensitivity of pancreatic cancer cells to cisplatin.

### $\Delta$ Np63 $\alpha$ upregulates 14-3-3 $\sigma$ in pancreatic cancer cell lines

Next we studied the mechanisms of  $\Delta$ Np63 $\alpha$ -mediated chemoresistance in PDAC. We have previously shown that 14-3-3 $\sigma$  dramatically enhances resistance to cisplatin-induced apoptosis in pancreatic cancer cell lines [11]. Westfall et al. demonstrated that  $\Delta$ Np63 $\alpha$  acts as a transcriptional repressor of the 14-3-3 $\sigma$  promoter in human epidermal keratinocytes [25]. By contrast, we observed that expression of  $\Delta$ Np63 $\alpha$  correlated with 14-3-3 $\sigma$  protein expression in pancreatic cancer cells (Fig. 1A). Additionally,

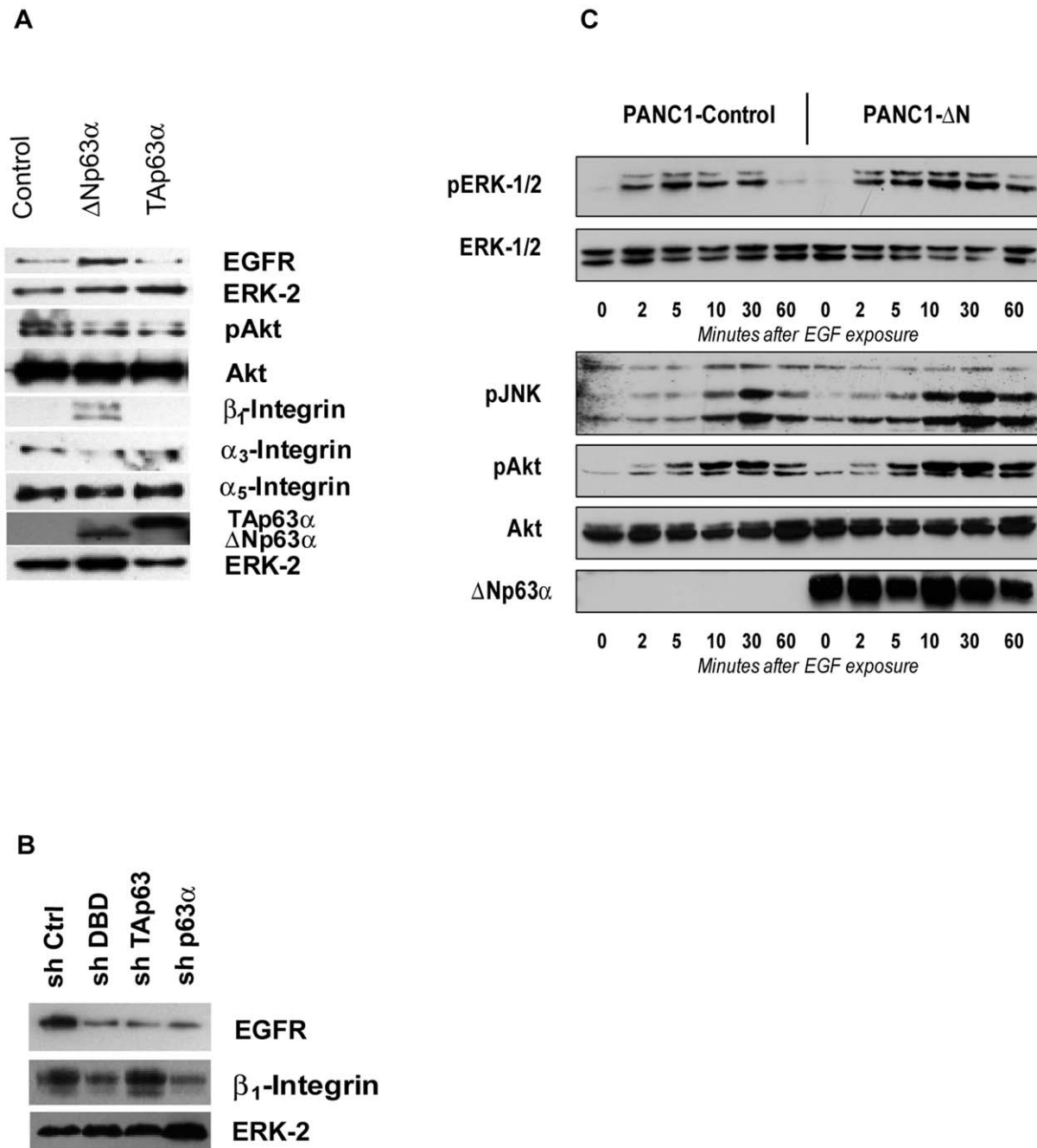
silencing  $\Delta$ Np63 $\alpha$  was accompanied by reduction in 14-3-3 $\sigma$  protein level in T3M4 cells (Fig. 2A). 14-3-3 $\sigma$  is a p53 transcriptional target, yet BxPC3, PANC-1 and T3M4 cells are known to carry mutant p53 whose transcriptional activity is defective [26], while COLO-357 cells express low p53 protein levels (Fig. 1A). Hence, 14-3-3 $\sigma$  expression is unlikely to be dependent on p53 in PDAC.

To determine if  $\Delta$ Np63 $\alpha$  modulates 14-3-3 $\sigma$  expression in pancreatic cancer cells, we introduced  $\Delta$ Np63 $\alpha$  cDNA via adenoviral infection in ASPC-1 and PANC-1 cells, which express low levels of 14-3-3 $\sigma$  and  $\Delta$ Np63 $\alpha$ . We observed an increase in 14-3-3 $\sigma$  protein levels in the  $\Delta$ Np63 $\alpha$  overexpressing cells compared to the control-infected cells (Fig. 5A). This  $\Delta$ Np63 $\alpha$ -mediated increase in 14-3-3 $\sigma$  was not associated with alterations of protein levels of the other 14-3-3 isoforms (Fig. 5A). By contrast, manipulation of 14-3-3 $\sigma$  levels in PANC-1 and T3M4 cells did not have an effect on  $\Delta$ Np63 $\alpha$  (Fig. 5B and 5C).

Next, we determined whether upregulation of 14-3-3 $\sigma$  by  $\Delta$ Np63 $\alpha$  is of biological significance in PDAC. Using a lentiviral based approach we silenced endogenous 14-3-3 $\sigma$  in PANC-1 cells. In such cells, enforced expression of  $\Delta$ Np63 $\alpha$  failed to upregulate 14-3-3 $\sigma$  (data not shown). Consistent with our previous observations, loss of 14-3-3 $\sigma$  did not affect migration abilities of PANC-1 cells in the presence of EGF [[11], Fig. 5E]. Similarly,  $\Delta$ Np63 $\alpha$ -mediated enhancement in cell motility was not influenced by the lack of 14-3-3 $\sigma$  (Fig. 5E). By contrast, 14-3-3 $\sigma$  was required for  $\Delta$ Np63 $\alpha$ -mediated anchorage-independent growth and invasion in presence of EGF (Fig. 5D and 5F). Thus,  $\Delta$ Np63 $\alpha$  upregulates 14-3-3 $\sigma$  in pancreatic cancer cells which then contributes to  $\Delta$ Np63 $\alpha$ -mediated anchorage-independent growth and invasion.

### $\Delta$ Np63 $\alpha$ is a transcriptional activator of EGFR and 14-3-3 $\sigma$ in pancreatic cancer

$\Delta$ Np63 $\alpha$  is a regulator of the transcriptional activities of the p53 family member proteins. As such,  $\Delta$ Np63 $\alpha$  may inhibit transcription via its dominant negative effect on p53 and TAp63 $\gamma$  [27]. In addition, the  $\Delta$ N variant is able to trans-activate p53 target genes via binding to p53 responsive elements within the corresponding gene promoters. While TAp63 $\gamma$  is a potent gene trans-activator due to the absence of an auto-inhibitory domain [27], recent work demonstrated that it has a very low ability to bind the p53 responsive element within the EGFR promoter, and in fact represses promoter activity [28]. Since pancreatic cancer cell lines expressed very low levels of the TAp63 isoform, and since we found no correlation between  $\Delta$ Np63 $\alpha$  and TAp63 $\gamma$  expression in those cell lines (Fig. 1A), the dominant negative effect of  $\Delta$ Np63 $\alpha$



**Figure 3.  $\Delta$ Np63 $\alpha$  upregulates EGFR expression and signaling in pancreatic cancer cells.** A, Ectopic expression of  $\Delta$ Np63 $\alpha$  results in increased expression of EGFR and  $\beta$ 1-integrin in PANC-1 cells. After cells were incubated for 48 hours, total protein lysates were subjected to immunoblotting. A representative image of three independent experiments is shown. B, Downregulation of  $\Delta$ Np63 $\alpha$  in T3M4 cells results in decreased expression of EGFR and  $\beta$ 1-integrin. A representative image of three independent experiments is shown. C, PANC-1 $\Delta$ N and PANC-1 control cells were stimulated with EGF during indicated time periods. Protein lysates were subjected to immunoblotting. A representative image of three independent experiments is shown.  
doi:10.1371/journal.pone.0026815.g003

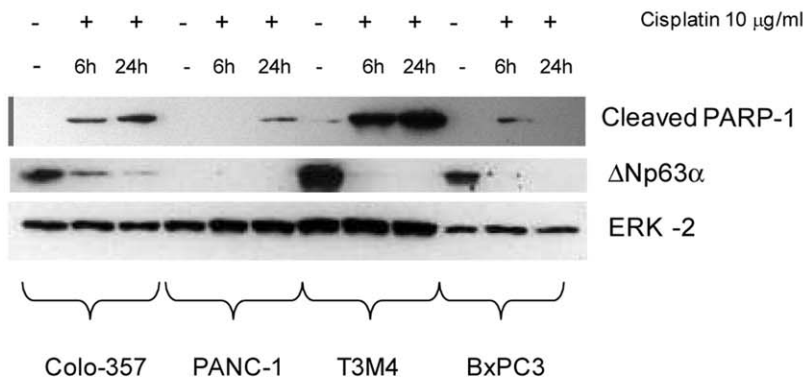
was an unlikely explanation of the changes in EGFR and 14-3-3 $\sigma$  expression. Both EGFR and 14-3-3 $\sigma$  promoters contain well-conserved p53 binding sites, suggesting they are transcriptional targets of  $\Delta$ Np63 $\alpha$  in PDAC. The 14-3-3 $\sigma$  promoter is known to be unmethylated in pancreatic cancer cell lines [29].

$\Delta$ Np63 $\alpha$  requires an intact DNA-binding domain for its transcriptional activity [18]. We generated a mutant vector where we introduced a missense mutation into human  $\Delta$ Np63 $\alpha$

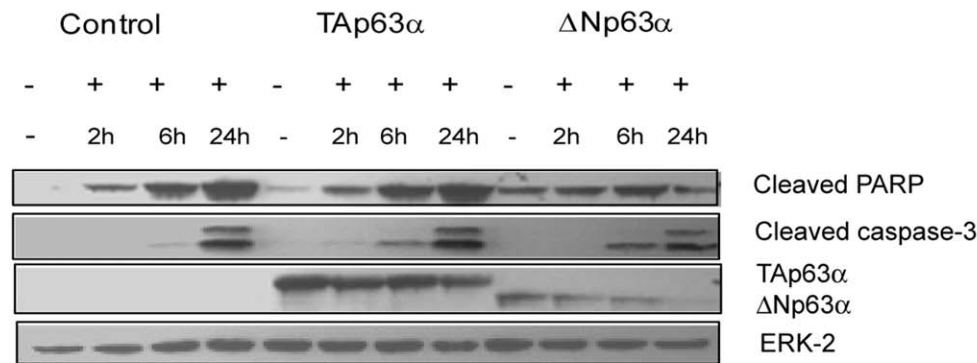
cDNA sequence resulting in a single amino acid substitution at position 202 ( $\Delta$ Np63 $\alpha$ <sup>DBDmut</sup>; Fig.S3). Enforced expression of  $\Delta$ Np63 $\alpha$ <sup>DBDmut</sup> in PANC-1 cells resulted in a detectable  $\Delta$ Np63 $\alpha$  protein, but failed to upregulate 14-3-3 $\sigma$  (Fig. 6A) indicating that the ability of  $\Delta$ Np63 $\alpha$  to upregulate 14-3-3 $\sigma$  is dependent upon its ability to bind to DNA.

We tested if  $\Delta$ Np63 $\alpha$  was able to activate EGFR promoter in an *in vitro* luciferase assay. The details of the p53 binding sites within

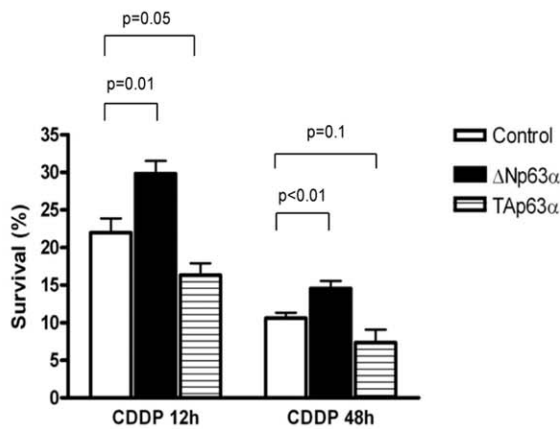
**A**



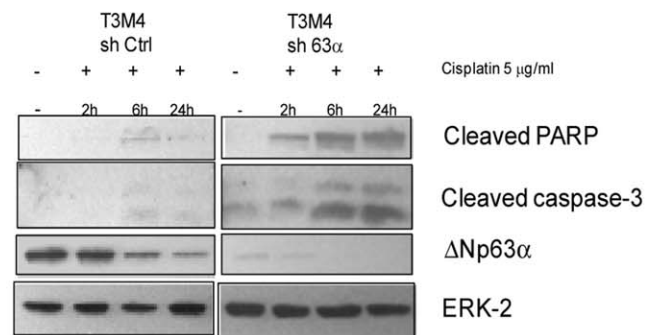
**B**



**C**

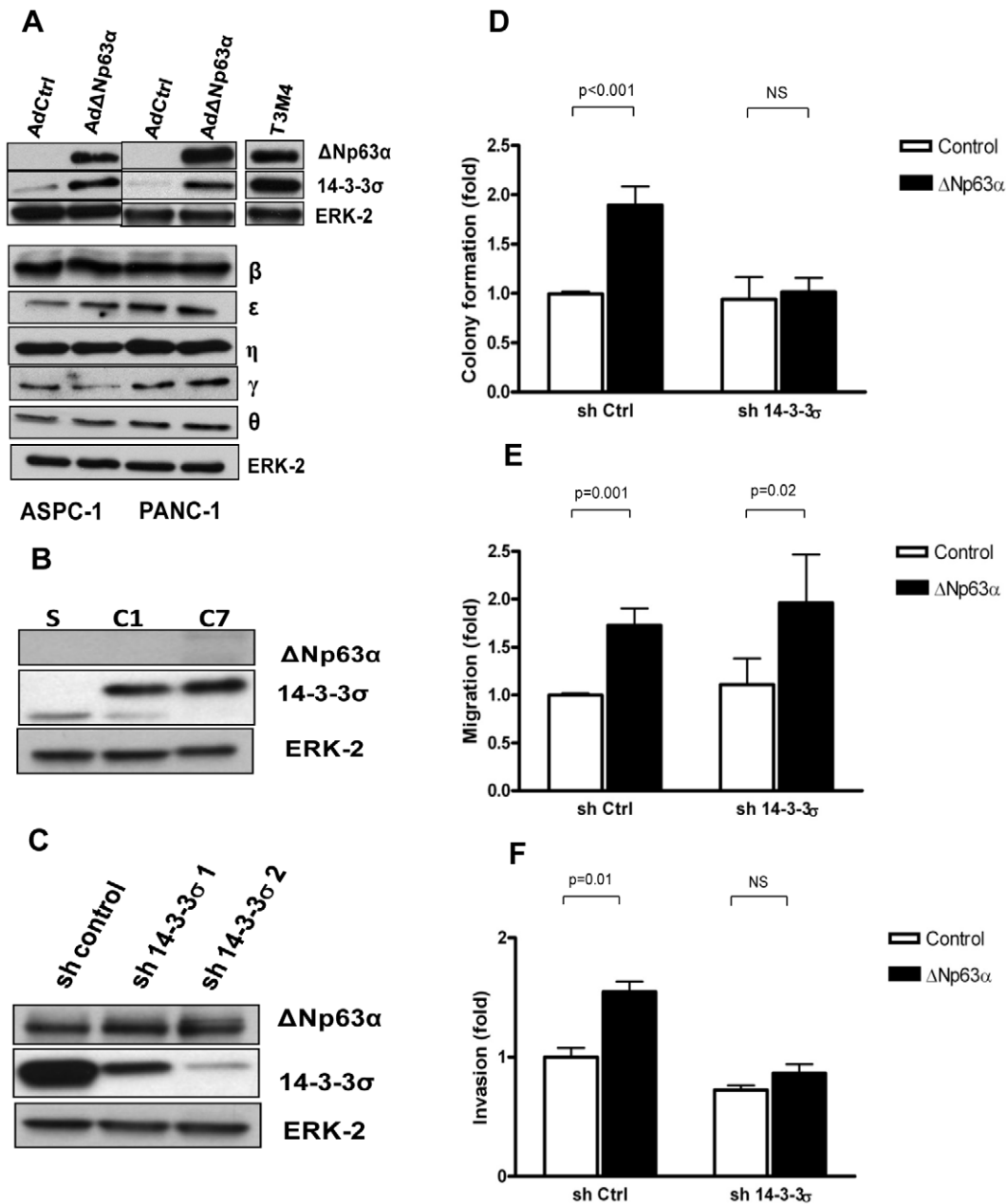


**D**



**Figure 4. ΔNp63α contributes to chemoresistance in pancreatic cancer cells.** A, Effect of cisplatin on ΔNp63α in PDAC. Cells were incubated with 10 µg/mL cisplatin for the initial 6 or full 24 hours. Apoptosis was assessed by monitoring PARP cleavage. B–D, Effect of ΔNp63α on cisplatin-induced apoptosis in PANC-1 (B, C) and T3M4 cells (D). Cells were incubated with 5 µg/mL of cisplatin during indicated time periods. Cells were pelleted after 24 h of incubation, proteins isolated and run on SDS-PAGE gel. Apoptosis was assessed by monitoring PARP and caspase-3 cleavage. A representative image of at least three independent experiments is shown. PANC-1 cells were incubated with 10 µg/mL of cisplatin for the first 12 hours or full 48 hours. MTT assay was done as described in materials and methods. Data are the mean ± SE of three independent experiments. doi:10.1371/journal.pone.0026815.g004

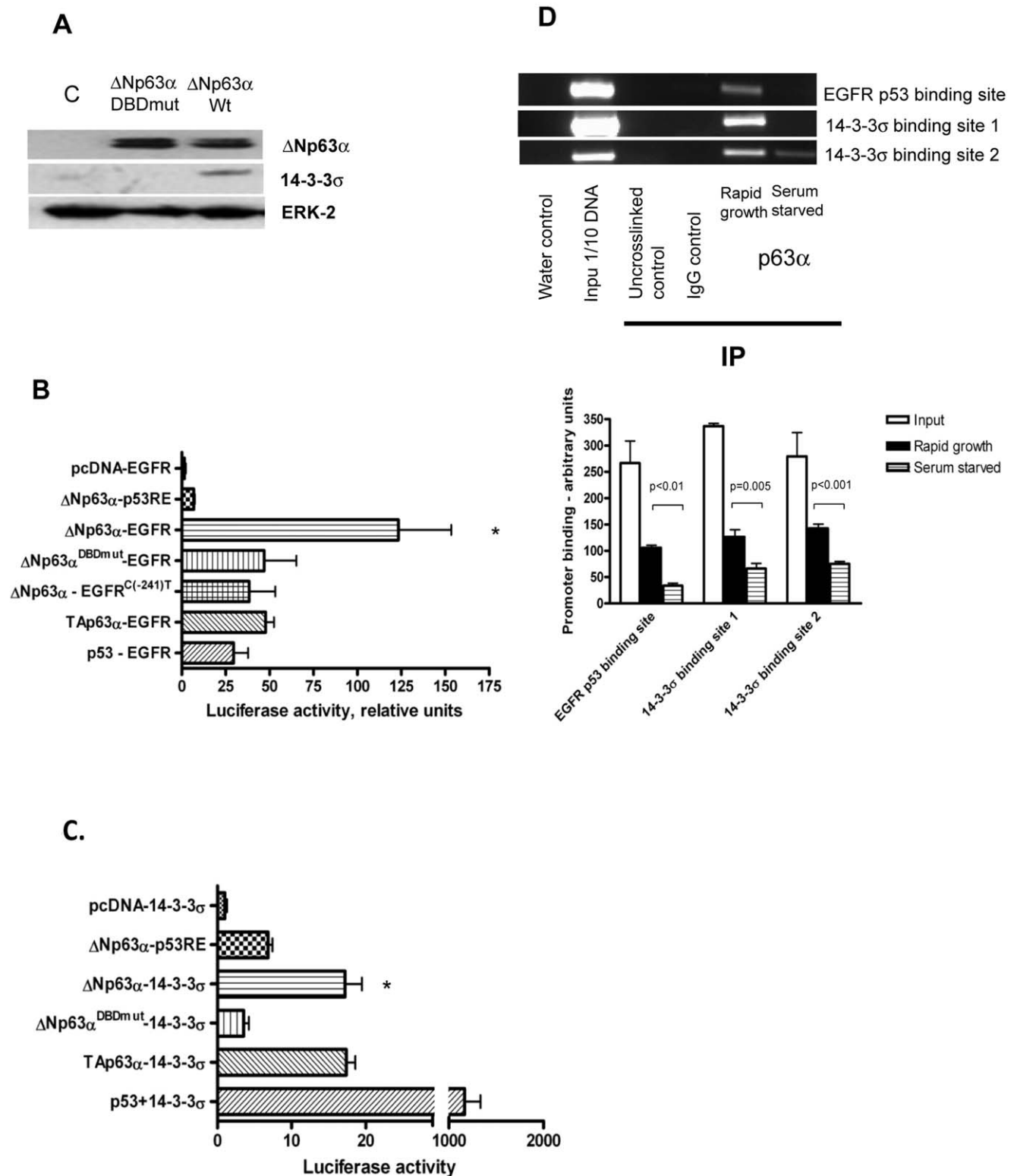




**Figure 5.  $\Delta$ Np63 $\alpha$  upregulates 14-3-3 $\sigma$  in pancreatic cancer cells.** A, ASPC-1 and PANC-1 cells were infected with adenovirus containing either empty vector or full length  $\Delta$ Np63 $\alpha$ , and whole cell protein lysate was prepared 48 hours after infection and probed with anti- $\Delta$ Np63 $\alpha$  and 14-3-3 $\sigma$  and other 14-3-3 isoforms. B, Overexpression of 14-3-3 $\sigma$  had no effect on  $\Delta$ Np63 $\alpha$  levels. PANC-1 cells were transfected with an empty vector (Sham) or with the full-length human 14-3-3 $\sigma$  cDNA that was tagged with HA. C, Silencing 14-3-3 $\sigma$  does not affect  $\Delta$ Np63 $\alpha$ . T3M4 cells were infected with lentivirus containing control or 14-3-3 $\sigma$  specific shRNAs (sh 14-3-3 $\sigma$  1 and 2), whole cell lysates were prepared 72 hrs post infection and probed for  $\Delta$ Np63 $\alpha$  and 14-3-3 $\sigma$ . D-F. 14-3-3 $\sigma$  is required for the oncogenic effects of  $\Delta$ Np63 $\alpha$  in PDAC. PANC-1 cells were infected with control sh RNA or sh 14-3-3 $\sigma$  2. Subsequent to that, cells were transiently transfected with  $\Delta$ Np63 $\alpha$ -expressing vector or vector control. D, Anchorage-independent growth. Cells were incubated in soft agar as described in the methods. Data are the mean  $\pm$  SE of three independent experiments. E, Cells ( $5 \times 10^4$ /well) were subjected to a migration assay in Transwell chambers in the presence of EGF (1 nmol/L) for 18 hours in duplicates. Data are the mean  $\pm$  SE of three independent experiments. F, Cells were subjected to invasion in Matrigel chambers in the presence of EGF (1 nmol/L) for 20 h in duplicates. Data are the mean  $\pm$  SE of three independent experiments. doi:10.1371/journal.pone.0026815.g005

the EGFR promoter and the corresponding luciferase vector were published previously [10]. Ectopic expression of  $\Delta$ Np63 $\alpha$  resulted in a dramatic increase of the activity of EGFR reporter plasmid in PANC-1 cells (Fig. 6B). The effect of  $\Delta$ Np63 $\alpha$  significantly exceeded that of wild-type p53 and TAp63 $\alpha$ . Next, we introduced a single nucleotide mutation within the p53 BS 1 of the EGFR

promoter. Specifically, cytosine was replaced with thymidine at position -241 (EGFR<sup>C(-241)T</sup>). As a further confirmation of our findings, co-transfection of a mutant EGFR reporter plasmid with wild-type  $\Delta$ Np63 $\alpha$ , or wild-type EGFR reporter plasmid and mutant  $\Delta$ Np63 $\alpha$ <sup>DBDmut</sup> resulted in attenuated luciferase activity (Fig. 6B). Consistent with this observation, ectopic expression of



**Figure 6.  $\Delta Np63\alpha$  is a transcriptional activator of EGFR and 14-3-3 $\sigma$  in pancreatic cancer cells.** A,  $\Delta Np63\alpha^{DBDmut}$  does not upregulate 14-3-3 $\sigma$ . B and C,  $\Delta Np63\alpha$  upregulates EGFR and 14-3-3 $\sigma$  promoter in a luciferase assay. PANC-1 cells were transfected with wildtype EGFR, mutant EGFR or 14-3-3 $\sigma$  promoter luciferase construct as indicated, along with pCMV $\beta$  plasmid, with or without wildtype p53,  $\Delta Np63\alpha$ ,  $\Delta Np63\alpha^{DBDmut}$ , or TAp63 $\alpha$ . At 48–72 h after transfection, the luciferase activity was determined. The transfection efficiency was standardized against  $\beta$ -galactosidase activity. Results are indicative of four independent experiments. \*,  $p < 0.0001$ . D, p63 binds EGFR and 14-3-3 $\sigma$  promoters in T3M4 cells. Binding is decreased upon serum-starvation. T3M4 cells were grown in medium containing 10% FBS (rapidly growing cells) or in serum-free medium (predominantly resting cells). Cells were pelleted and ChIP was performed as described in materials and methods. Uncrosslinked protein lysate, protein lysate immunoprecipitated with an irrelevant antibody, and a protein lysate immunoprecipitated with p63 antibody, but where irrelevant DNA sequences located ~3000 bp downstream of the 14-3-3 $\sigma$  or EGFR promoter regions were amplified served as negative controls (the latter not shown). doi:10.1371/journal.pone.0026815.g006

$\Delta$ Np63 $\alpha$  resulted in a significant increase of the 14-3-3 $\sigma$  reporter plasmid activity in PANC-1 cells, which was attenuated when a  $\Delta$ Np63 $\alpha$ <sup>DBDmut</sup> expression vector was co-transfected in place of wild type  $\Delta$ Np63 $\alpha$  (Fig. 6C).

Next, we tested whether  $\Delta$ Np63 $\alpha$  is capable of binding EGFR and 14-3-3 $\sigma$  promoters at their corresponding p53 binding sites. Our ChIP experiments revealed that  $\Delta$ Np63 $\alpha$  exhibited significant binding to p53 binding sites in the endogenous EGFR and 14-3-3 $\sigma$  promoters in T3M4 cells (Fig. 6D). Chromatin binding depended on the growth phase of T3M4 cells as serum starvation resulted in a significant decrease in promoter occupancy by  $\Delta$ Np63 $\alpha$  (Fig. 6D), while  $\Delta$ Np63 $\alpha$  protein expression was unchanged (data not shown).

We next sought to determine whether the ability of  $\Delta$ Np63 $\alpha$  to trans-activate EGFR and 14-3-3 $\sigma$  promoters was unique to pancreatic cancer. We studied HEK293 cells, a transformed non-malignant cell line, and H1299 cells, a non-small cell lung cancer cell line [30]. Similar to our pancreatic cancer cells, H1299 cells carry a mutant p53. While  $\Delta$ Np63 $\alpha$  is known to be expressed in non-small cell lung cancer [31], H1299 cells predominantly express the TAp63 variant [30].

Both HEK293 and H1299 cells expressed lower levels of p63 and 14-3-3 $\sigma$  compared to T3M4 pancreatic cancer cells (Fig. 7A). Engineered expression of  $\Delta$ Np63 $\alpha$  in these cells did not lead to an increase in either EGFR or 14-3-3 $\sigma$  protein levels (Fig. 7A). Similarly,  $\Delta$ Np63 $\alpha$  did not activate the EGFR promoter in either cell line in an *in vitro* luciferase reporter assay (Fig. 7B, left panels). By contrast,  $\Delta$ Np63 $\alpha$  transactivated the 14-3-3 $\sigma$  promoter in H1299 cells, albeit to a lesser degree than in pancreatic cancer cells (Fig. 7B, top right panel). In HEK293 cells,  $\Delta$ Np63 $\alpha$  repressed the 14-3-3 $\sigma$  promoter, similar to what was previously reported in human keratinocytes [25], whereas in H1299 cells enforced expression of  $\Delta$ Np63 $\alpha$  led to an increase in 14-3-3 $\sigma$  mRNA transcript levels (Fig. 7C).

$\Delta$ Np63 has been shown to enhance proliferative capacity in normal and malignant cells of epithelial origin [32–34]. While  $\Delta$ Np63 $\alpha$  stimulated anchorage-independent growth, invasion and migration of pancreatic cancer cells and sensitized them to EGF, such effects were not observed in H1299 cells (Fig. 7D). Collectively, these data indicate that  $\Delta$ Np63 $\alpha$  binds and trans-activates the EGFR and 14-3-3 $\sigma$  promoters in pancreatic cancer cells and that these actions of  $\Delta$ Np63 $\alpha$  do not necessarily occur in other cell types.

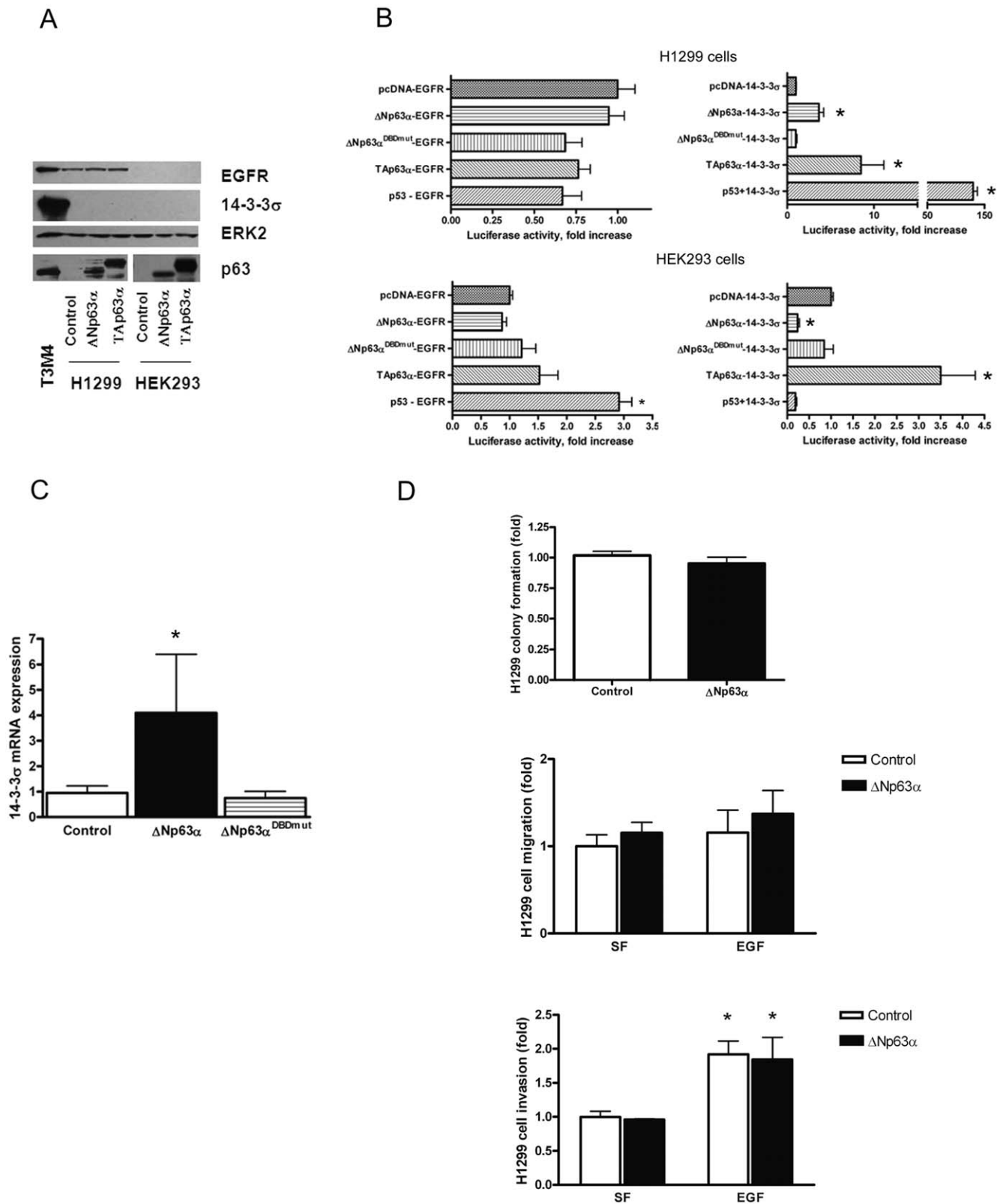
## Discussion

Genetic defects which result in inactivation of tumor suppression genes and accumulation of oncogenic alleles promote development and progression of PDAC. p53 is a prototypic tumor suppressor that is vital in cell growth control. However, normal p53 function is commonly lost in PDAC. p53 homolog p63 possesses an N-terminal trans-activation domain (TA), a DNA-binding domain (DBD) and a C-terminal oligomerization domain (OD). Studies in knockout mice demonstrated that despite structural similarity with p53, p63 has distinct functional properties [35]. Moreover, variability of expression of six known p63 isoforms among normal and malignant tissues suggests that the functional role of p63 is dependent on the cellular context. TAp63 isoforms may carry an anti-oncogenic potential as they mediate Ras-induced cellular senescence, antagonize tumorigenesis *in vivo* and suppress development of metastases through regulation of microRNA network [36,37]. The role of  $\Delta$ Np63 in cancer is less clear. Recent studies have indicated that  $\Delta$ Np63 $\alpha$  is necessary and sufficient to by-pass oncogene-induced senescence suggesting that

it plays a critical role in the very early steps of cancer initiation [38]. Overexpression of  $\Delta$ Np63 is found in cancers of epithelial origin (lung, head and neck), where it promotes tumor survival [7].  $\Delta$ Np63 has been shown to enhance the proliferative capacity of both epithelial stem cells and cancer cells, and loss of p63 reduced the proliferative rate of MCF-7 breast cancer cells [32,33]. However, the role of p63 in PDAC is not known. Here we report that  $\Delta$ Np63 $\alpha$  is the predominantly expressed p63 isoform in pancreatic cancer cell lines. We demonstrate that ectopic expression of  $\Delta$ Np63 $\alpha$  in PANC-1 cells, which have low p63 mRNA transcript and protein levels, resulted in enhancement of anchorage-independent growth, cell motility and invasion. Conversely, shRNA-mediated suppression of  $\Delta$ Np63 $\alpha$  in T3M4 cells resulted in the opposite effect, i.e. decreased proliferation, pro-migratory and pro-invasive responses. In earlier reports, down-regulation of endogenous p63 led to enhanced apoptosis in head and neck squamous cell cancer cell lines irrespective of the p53 gene status [7]. By contrast, exogenous  $\Delta$ Np63 $\alpha$  induced cell cycle arrest and apoptosis in p53-null non-small cell lung cancer cells [39]. In our studies, neither engineered expression of  $\Delta$ Np63 $\alpha$ , nor downregulation of endogenous  $\Delta$ Np63 $\alpha$  induced apoptosis in p53-mutant pancreatic cancer cells (data not shown). While migration and invasion are both paramount to embryonic development and wound healing in normal tissues, in cancer those processes are involved in local tumor invasion and metastasis. Hence, these data underscore the importance of  $\Delta$ Np63 $\alpha$  in tumor progression in PDAC.

Tyrosine kinase and serine/threonine kinase pathways regulate multiple cellular processes and are major effectors in PDAC development [40]. EGFR is a 170 kDa transmembrane glycoprotein of the ErbB family of tyrosine kinase growth factor receptors. EGFR activates Ras/Raf/MAPK-ERK and PI-3 kinase/Akt pathways leading to increased cell proliferation, reduced apoptosis, increased angiogenesis, enhanced motility, invasion and metastasis [41]. EGFR signaling blockade via a dominant negative mechanism leads to reduction in mitogenic activity in pancreatic cancer cells through decreased activation of MAPK pathway [42]. In our experiments,  $\Delta$ Np63 $\alpha$  enhanced EGF-mediated mitogenic effects and potentiated EGFR signaling as evidenced by enhanced phosphorylation of ERK and Akt and increased activation of JNK in PANC-1  $\Delta$ N cells. We and others previously reported that EGF is capable of promoting cancer cell growth through JNK activation [42,43]. Suppression of  $\Delta$ Np63 $\alpha$  in T3M4 cells resulted in a remarkable attenuation of EGF-mediated invasion. Collectively, these observations suggest that PDAC requires  $\Delta$ Np63 $\alpha$  to fulfill its pro-invasive potential in response to EGF stimulation.

Clinical trials of EGFR-targeting agents reported modest effects on patient survival in PDAC [44,45]. The therapeutic efficacy of these agents could be improved if regulation of EGFR signaling was better understood. Here we provide evidence of a functional interplay between  $\Delta$ Np63 $\alpha$  and EGFR in pancreatic cancer. Ectopic expression of  $\Delta$ Np63 $\alpha$ , but not TAp63 $\alpha$ , resulted in increased EGFR protein levels in PANC-1 cells. Conversely, downregulation of  $\Delta$ Np63 $\alpha$  in T3M4 cells led to a decreased expression of EGFR. It was initially felt that whereas TAp63 is a strong gene trans-activator,  $\Delta$ Np63 functions as a dominant negative isoform [27]. However, recent findings point out that  $\Delta$ Np63 is able to trans-activate p53 target genes as well as distinct targets [46,47]. TAp63 $\gamma$  has been reported to repress EGFR promoter activity in H1299 lung cancer cells [28]. However, we determined that  $\Delta$ Np63 $\alpha$  is a transcriptional activator of EGFR in pancreatic cancer cells, but not in HEK293 or H1299 cells. Moreover, compared to p53,  $\Delta$ Np63 $\alpha$  was a strong trans-activator of EGFR in a functional reporter assay, and ChIP experiments



**Figure 7. A distinct role of  $\Delta Np63\alpha$  in HEK293 and H1299 cells.** *A*,  $\Delta Np63\alpha$  does not affect EGFR or 14-3-3 $\sigma$  protein levels in HEK293 or H1299 cells. Representative blot of three independent experiments is shown. *B*,  $\Delta Np63\alpha$  fails to upregulate EGFR promoter but modulates 14-3-3 $\sigma$  promoter in a luciferase reporter assay. Cells were transfected with wildtype EGFR or 14-3-3 $\sigma$  promoter luciferase construct as indicated, along with pCMV $\beta$  plasmid, with or without wildtype p53,  $\Delta Np63\alpha$ ,  $\Delta Np63\alpha^{DBDmut}$ , or TAp63 $\alpha$ . At 48–72 h after transfection, the luciferase activity was determined. The transfection efficiency was standardized against  $\beta$ -galactosidase activity. Results are indicative of three independent experiments performed in duplicates. *C*,  $\Delta Np63\alpha$  increases 14-3-3 $\sigma$  mRNA transcript levels in H1299 cells. H1299 cells were transfected with  $\Delta Np63\alpha$ ,  $\Delta Np63\alpha^{DBDmut}$  or vector

control. At 36 hours total RNA was isolated, reverse-transcribed and subjected to real-time PCR with probe specific for 14-3-3 $\sigma$ . Results were normalized to 18S levels. Data are the mean of two independent experiments done in duplicates in which similar results were obtained. *D*,  $\Delta$ Np63 $\alpha$  does not enhance anchorage-independent growth, migration or invasion in H1299 cells. H1299 cells were transfected with  $\Delta$ Np63 $\alpha$  or vector control. Soft agar, transwell migration and invasion assays were performed as described above. \*,  $p < 0.05$  compared with control. doi:10.1371/journal.pone.0026815.g007

confirmed that  $\Delta$ Np63 $\alpha$  binds the EGFR promoter at the p53-binding site. By contrast, TAp63 $\alpha$  was a weak transcriptional activator of EGFR in a functional reporter assay. However, downregulation of TAp63 isoforms also decreased EGFR expression in T3M4 cells, suggesting that in those cells TAp63 isoforms could still be contributing to EGFR expression.

PDAC is notoriously resistant to conventional chemotherapy agents. This is partly explained by frequent inactivation of p53 in pancreatic tumors and thus unresponsiveness to genotoxic stress. Here we demonstrate that  $\Delta$ Np63 $\alpha$  further contributes to chemotherapy resistance in PDAC. While overexpression of  $\Delta$ Np63 $\alpha$  in PANC-1 cells resulted in a marginal increase in apoptotic death upon exposure to cisplatin, its downregulation in T3M4 cells led to a markedly increased sensitivity of those cells to cisplatin-induced apoptosis. Similar to our observations in pancreatic cancer cells, cisplatin induces proteosomal degradation of  $\Delta$ Np63 $\alpha$  in other cell types [24]. Additional mechanisms that contribute to cisplatin resistance in pancreatic cancer include overexpression of cyclin D1 [48] and multidrug resistance-associated proteins [49]. Moreover,  $\Delta$ Np63 $\alpha$  itself may promote chemoresistance through several mechanisms. For example, it can have a dominant negative effect on the proapoptotic partners within the p53 family, as suggested by earlier experiments performed in head and neck and breast cancer [6]. Although  $\Delta$ Np63 $\alpha$  contributed to chemoresistance through regulation of Akt1 expression in ovarian and head and neck cancer [50], we did not observe such effect in pancreatic cancer cells (Fig. 3A). However, we highlight a new potential mechanism of  $\Delta$ Np63 $\alpha$ -mediated chemoresistance in PDAC. In our earlier experiments, 14-3-3 $\sigma$  dramatically increased chemoresistance and enhanced the pro-invasive potential of pancreatic cancer cells [11]. Here we demonstrate that expression of 14-3-3 $\sigma$  protein correlates with  $\Delta$ Np63 $\alpha$  levels in those cells. While others have identified 14-3-3 $\sigma$  as a  $\Delta$ Np63 repression target in human embryonic keratinocytes [25], we established that  $\Delta$ Np63 $\alpha$  activates the 14-3-3 $\sigma$  promoter and upregulates 14-3-3 $\sigma$  protein expression in pancreatic cancer cells (and in H1299 lung cancer cells), thus decreasing sensitivity of pancreatic cancer cells to cisplatin-induced apoptosis. This discrepancy provides additional evidence that  $\Delta$ Np63 $\alpha$  actions are tissue-specific, and its function in cancer is distinct from its role in embryonic cells. We also found that 14-3-3 $\sigma$  promoter binding by  $\Delta$ Np63 was more pronounced in rapidly cycling as compared with resting T3M4 cells. This suggests that at least some  $\Delta$ Np63 $\alpha$  target sites may be poorly accessible in quiescent cells and require chromatin remodeling to occur prior to binding, further implicating cellular context in modulation of p63 function.

In our immunoprecipitation experiments we found no interaction between p63 and 14-3-3 $\sigma$  proteins (data not shown), however 14-3-3 $\sigma$  contributed to the mitogenic and motogenic effects of  $\Delta$ Np63 $\alpha$  in PDAC. Lentiviral knockdown of 14-3-3 $\sigma$  resulted in a dramatic attenuation of the effects of  $\Delta$ Np63 $\alpha$  on anchorage-independent growth and EGF-stimulated invasion of PANC-1 cells. Silencing of 14-3-3 $\sigma$  had no consequences on the effects of  $\Delta$ Np63 $\alpha$  on cell motility, leading to the conclusion that  $\Delta$ Np63 $\alpha$  affects migration through potentiation of the other signaling pathways, i.e. EGFR and integrins.

p63 controls the adhesion program in MCF-10A mammary epithelial tissues through transcriptional regulation of integrins

[15]. A number of integrin subunits have been shown to be upregulated in PDAC [51]. We demonstrate that  $\Delta$ Np63 $\alpha$  modulates expression of  $\beta$ 1-integrins, which are involved in regulation of cell adhesion and migration of tumor on stroma proteins, thus further implicating  $\Delta$ Np63 $\alpha$  in pancreatic carcinogenesis. Importantly, integrin and EGFR pathways are closely intertwined and orchestrate cancer growth and invasion. Integrin-mediated adhesion of cells to extracellular matrix induces EGFR activation in a ligand-independent manner, while EGFR regulates integrin signaling and is necessary for adhesion-induced activation of ERK and other signaling molecules [52]. Cross-talk between integrins and EGFR family members affects multiple aspects of tumor progression, including proliferation, migration and invasion [53]. For example, blockade of integrin  $\alpha_v\beta_5$  reverses the EGF-stimulated invasion and metastasis in pancreatic cancer cells [54]. On the other hand, overexpression of  $\beta$ 1-integrin has been shown to correlate with acquired resistance to EGFR inhibitors in lung cancer [55]. The unique ability of  $\Delta$ Np63 $\alpha$  to regulate both EGFR and  $\beta$ 1-integrin in PDAC renders it a particularly promising therapeutic target.

In conclusion,  $\Delta$ Np63 $\alpha$  is the predominantly expressed p63 variant in PDAC cell lines with oncogenic properties. In PANC-1 and T3M4 cells  $\Delta$ Np63 $\alpha$  enhanced anchorage independent growth, cell proliferation, and basal and EGF-stimulated motility and invasion and conferred chemoresistance. The oncogenic effects of  $\Delta$ Np63 $\alpha$  were mediated via transcriptional activation of EGFR and 14-3-3 $\sigma$ . Our observations indicate that targeting  $\Delta$ Np63 $\alpha$  and 14-3-3 $\sigma$  may present a strategy to potentiate the efficacy of EGFR-targeting therapies in PDAC.

## Supporting Information

### Table S1 p63 primer sequence and expected size of PCR products.

(DOC)

### Figure S1 Genomic structure of human p63 and location of primers and sh RNA complementary sites.

Genomic structure of human p63 on chromosome 3 and schematic representation of the six variants of p63. TA – trans-activation domain, DBD – DNA-binding domain, OD – oligomerization domain, SAM – sterile alpha motif, TID – transactivational inhibitory domain.

(TIF)

### Figure S2 Erlotinib attenuates $\Delta$ Np63 $\alpha$ -mediated enhancement of migration, invasion and anchorage-independent growth in PANC-1 cells.

*A*, Erlotinib attenuates  $\Delta$ Np63 $\alpha$ -mediated anchorage-independent growth of PANC-1 cells in soft agar assay. Soft agar assay was performed as described previously in presence of 1  $\mu$ M erlotinib or vehicle control. *B*, PANC-1 cells ( $5 \times 10^4$ /well) were subjected to a migration assay in Transwell chambers in presence of EGF (1 nmol/L) as described above, with or without 1  $\mu$ M erlotinib. *C*, PANC-1 cells ( $1 \times 10^4$ /well) were subjected to invasion assay in Matrigel chambers in presence of EGF (1 nmol/L) as described above, with or without 1  $\mu$ M erlotinib. \*,  $p < 0.05$  compared with control.

(TIF)

### Figure S3 $\Delta$ Np63 $\alpha$ <sup>DBDmut</sup> cDNA-expressing vector. Partial sequence of the $\Delta$ Np63 $\alpha$ cDNA shown (nucleotides

**601 to 612, within DBD).** Substituted nucleotide is shown in capital. (TIF)

## Acknowledgments

We would like to thank Dr. Lucia Pirisi-Creek for the pGL3-EGFR promoter (p53 binding sites)-luc plasmid.

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## Author Contributions

Conceived and designed the experiments: AVD DN JD MK. Performed the experiments: AVD DN ASN EVF LAH. Analyzed the data: AVD MK. Contributed reagents/materials/analysis tools: AVD JD MK. Wrote the paper: AVD MK.

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