

Keratin 5/14-mediated cell differentiation and transformation are regulated by TAp63 and Notch-1 in oral squamous cell carcinoma-derived cells

SAUMYA S. SRIVASTAVA¹, HUNAIN ALAM², SONAM J. PATIL¹, RASHMI SHRINIVASAN¹,
SWETA RAIKUNDALIA¹, PRATIK RAJEEV CHAUDHARI^{1,3} and MILIND M. VAIDYA^{1,3}

¹Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre (TMC), Kharghar, Navi Mumbai, Raigad, Maharashtra 410210, India; ²Department of Molecular and Cellular Oncology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA;

³Homi Bhabha National Institute, Training School Complex, Anushaktinagar, Mumbai 400085, India

Received September 19, 2017; Accepted January 12, 2018

DOI: 10.3892/or.2018.6298

Abstract. Keratins 5/14 (K5/14) are intermediate filament proteins expressed in the basal layer of stratified epithelial cells and are known targets of p63. Previous research in our laboratory showed that upon K5/14 downregulation in oral squamous cell carcinoma (OSCC)-derived cells, there was an increase in intracellular Notch-1 levels and differentiation markers such as involucrin, keratin 1 and a decrease in tumorigenic potential *in vivo*. However, the molecules involved in the K14 regulated cell differentiation and transformation are not known to date. In order to understand the possible role of TAp63, we downregulated TAp63 in a K14-knockdown background. We observed that there was a decrease in the expression of Notch-1. Expression levels of differentiation markers such as involucrin, K1, loricrin and filaggrin were also decreased. Furthermore, TAp63 downregulation led to an increase in invasion, migration and *in vivo* tumorigenic potential of these cells. We observed a decrease in β -catenin signaling in K14-downregulated cells. Notably, when TAp63 was downregulated in K14-knockdown cells, there was increase in non-phospho β -catenin levels. Hence, this study indicates that TAp63 plays an important role in K14-downregulated cells possibly by regulating the Notch-1 expression. K14 regulates the expression of TAp63 which in turn regulates expression of Notch-1. The present study is a step forward in our quest

to understand the functional significance of molecules that regulate the process of differentiation and tumorigenesis in stratified epithelial cells.

Introduction

Keratins are cytoplasmic intermediate filaments expressed by epithelial tissues and are expressed in tissue- and differentiation-specific manners (1). They play an important role in maintaining cell viability and structure by providing mechanical support. In addition to their cytoprotective function, keratins are known to modulate important signaling pathways associated with protein synthesis, cell growth and cell differentiation (2). Studies have found that K5/14 null mice died 2 days after birth (3) and the loss of K14 cannot be compensated for by the ectopic expression of any other keratin, suggesting that K14 performs an important regulatory function (4,5). Previous research in our laboratory demonstrated that depletion of K14 leads to an increase in Notch-1 and differentiation markers such as K1 and K10 and a decrease in tumorigenic potential.

p63 is a member of the p53 tumor-suppressor family of proteins (6). There are two major isoforms of p63 dependent upon the differential promoter usage, which are TAp63 which has a N terminal transactivation domain and Δ Np63 which lacks the N terminal domain. Each is further subdivided into α , β , γ depending upon the C terminal splicing. Δ Np63 and its isoforms are known to regulate the proliferative potential of basal cells and initiate the process of differentiation (7), while TAp63 plays an important role in the maintenance of the basal cell population and prevents premature ageing of the epidermis (8). TAp63 acts as a tumor suppressor and is known to be downregulated in various types of cancers such as colon cancer (9) and was also found to promote epithelial-mesenchymal transition in MDCK cells (10).

In the present study, we aimed to elucidate the molecules involved in K14-regulated cell differentiation and decrease in tumorigenic potential. p63 is an important molecule to study with respect to K14 as Δ Np63 is known to be a direct regulator

Correspondence to: Dr Milind M. Vaidya, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre (TMC), Sector 22, Kharghar, Navi Mumbai, Raigad, Maharashtra 410210, India
E-mail: mvaidya@actrec.gov.in

Abbreviations: K14, keratin14; OSCC, oral squamous cell carcinoma; Ex/Em, excitation/emission; KD, knockdown

Key words: keratin 14, TAp63, Notch-1, differentiation, β -catenin

of K5/14 expression in basal keratinocytes (11). Furthermore, p63 is a known modulator of epidermal homeostasis (12,13).

In the present study, we showed that TAp63 is the isoform of p63 which undergoes upregulation after K14 depletion. Furthermore, to understand the role of TAp63 in K14-depleted cells, the TAp63 gene was knocked down using shRNA technology in K14-depleted cells. We report here that TAp63 regulates cell differentiation, cell motility and tumorigenesis in OSCC-derived cells possibly through Notch-1.

Materials and methods

Statement of ethics. All protocols for animal studies were approved by the Institutional Animal Ethics Committee (IAEC) (approval ID 28/2016) constituted under the guidelines of the 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)' of the Government of India.

Housing and monitoring of experimental animals and in vivo tumorigenicity assay. The animals were housed in a controlled environment at $23\pm 2^{\circ}\text{C}$, with 40-70% relative humidity and a dark/light cycle of 12 h each. The animals received autoclaved in-house-made food including natural ingredients such as roasted Bengal gram, casein, milk powder, vitamins, ground nut oil and sterile water *ad libitum*. The tumorigenic potential of cells was determined by subcutaneous injection in NOD-SCID mice. The cells were suspended in plain medium without serum, and 6×10^6 cells were injected subcutaneously into the dorsal flank of 6 to 8 week-old mice. Four mice were injected per clone and were observed for tumor formation over a period of approximately 2 months. Tumor volume was determined using a digital Vernier caliper (Advance, Alandi, India) and the volume was calculated by the modified ellipsoidal formula: [Tumor volume = $1/2$ (length \times width²)]. After 6-8 weeks, the animals were sacrificed so that the mean tumor diameter did not exceed 1.2 cm, as described in the AAALAC guidelines for Animal Welfare in Cancer Research (14). The mice were euthanized using CO₂ method and the tumor was removed (18).

Plasmids and cell lines. Human tongue SCC-derived cell line AW13516 was established in our institute (15). This cell line was cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; HyClone Thermo Scientific, Lafayette, CO, USA) and antibiotics (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO₂ incubator at 37°C. The establishment and characterization of HaCaT cells were as described previously (16). TAp63 shRNA was designed and cloned into the Plko1.Neo plasmid (plasmid #13425; Addgene, Cambridge, MA, USA). Efficient knockdown was generated by transducing a lentiviral-based Plko1.neo vector encoding shRNA and an empty vector in K14-depleted HaCaT (D2 clone) and AW13516 (K7 clone) cell lines. The shRNA sequences for TAp63 are as follows: TAp63A, 5'-GGAACAGCCTATATGTTTCAGTTC-3' and TAp63B, 5'-GCCCAGAGCACACAGACAAACTC-3'.

Lentiviral-mediated transduction. 293FT cells were cultured until achieving 50% confluency in complete Dulbecco's

modified Eagle's medium (DMEM; Life Technologies). Calcium phosphate precipitation method was used for co-transfection of the lentiviral transfer and packaging vectors (17). For transduction, the viral supernatant along with polybrene (8 $\mu\text{g/ml}$) was added to the 50% confluent OSCC cells. After 24 h, the supernatant was replaced with complete media. Furthermore, the stable clones were selected in puromycin (0.5 $\mu\text{g/ml}$) and G418 (500 $\mu\text{g/ml}$), purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting. The whole cell extracts were prepared in SDS lysis buffer with protease inhibitor cocktail (Sigma-Aldrich). For Notch-1 inhibition, K14-depleted cells were treated with γ -secretase inhibitor (DAPT; N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; Merck-Millipore, Darmstadt, Germany) at concentrations of 150 and 250 nM for 72 h and then cell lysates were prepared. After blocking, the blot was incubated with the diluted primary antibody for 1 h at room temperature (RT) on a rocker. The blot was then washed thrice with TBST [0.1% Tween-20 (v/v), 150 mM NaCl, 10 mM Tris-HCl pH 8.0] followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse; A4416 or anti-rabbit; A-0545; Sigma-Aldrich) for 1 h at RT on a rocker followed by TBST washes. The blots were developed using ECL chemiluminescence reagent (WesternBright™ ECL; Advansta, East Sussex, UK) according to the manufacturer's protocol (18). The primary antibodies for K14 (1:8,000; mouse monoclonal, clone LL002; cat. no. MCA890) and involucrin (1:1,000 dilution ratio; mouse monoclonal, clone SY5; cat. no. 5390-9950) were obtained from AbD Serotec (a Bio-Rad Company, Raleigh, NC, USA). The non-phospho β -catenin (1:1,000; rabbit monoclonal antibody D28UY; cat. no. 19807) and Notch-1 antibodies (1:1,000; rabbit monoclonal, clone D1E11; cat. no. 3608) were purchased from Cell Signaling Technology (Danvers, MA, USA). Vimentin (1:1,000; mouse monoclonal, clone V9; cat. no. V-6630), β -actin (1:8,000; mouse monoclonal; cat. no. AC-74) and were obtained from Sigma-Aldrich. Total β -catenin (1:1,000; mouse monoclonal; cat. no. Sc7963) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). TAp63 (1:1,500; mouse polyclonal; cat. no. Ab53039) was obtained from Abcam (Cambridge, MA, USA). E-cadherin antibody (1:500; mouse monoclonal; cat. no. 610182) was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

RT-PCR and RT-qPCR. For RT-PCR and RT-qPCR, RNA was isolated with the TRI reagent and cDNA was prepared using Revert Aid First Strand cDNA synthesis kit according to the manufacturer's protocol (MBI Fermentas, Amherst, NY, USA). All SYBR-Green-based RT-qPCRs were performed on the ABI 7900HT fast real-time based systems (Applied Biosystems, Foster City, CA, USA) as previously described (17). The SYBR-Green Master Mix was purchased from Applied Biosystems. The relative levels were calculated using the $\Delta\Delta\text{Ct}$ method and the relative expression fold ($2^{-\Delta\Delta\text{Ct}}$) was calculated (17). The respective gene expression was normalized to GAPDH. The primers used for PCR amplification are as follows: TAp63 forward, 5'-TGGTGCACAAACAAGATTG-3' and reverse, 5'-ATAGGGACTGGTGACGAGG-3'; ΔNp63 forward, 5'-TGTACCTGGAAACA

ATGCCCCA-3' and reverse, 5'-GACGAGGAGCCGTTCTGAATCT-3'; GAPDH forward, 5'-CTTCTTTTTCGTCGCCAGCC-3' and reverse, 5'-GAGTTAAAAGCAGCCCTGGTGA-3'; involucrin forward, 5'-GAAACAGCCAACTCCACTGC-3' and reverse, 5'-ATTCTTGCTCAGGCAGTCCC-3'; vimentin forward, 5'-GTCAGCAATATGAAAGTGTGGC-3' and reverse, 5'-GGTAGTTAGCAGCTTCAACGG-3'; K1 forward, 5'-TGACAAGGTGAGGTTCCCTGG-3' and reverse, 5'-GTTGGTCCACTCTCCTTCGG-3'; loricrin forward, 5'-GATCTGCCACCAGACCCAG-3' and reverse 5'-CCCCTGGAAAACACCTCCAA-3'; E-cadherin forward, 5'-CTTTGACGCCGAGAGCTACA-3' and reverse, 5'-TTTGAATCGGGTGTGCGAGG-3'; Snail forward, 5'-CCAGTGCCTCGACCACTATG-3' and reverse, 5'-CTGCTGGAAGGTAACTCTGGA-3'; Twist forward, 5'-TCTACCAGGTCTCCAGAG-3' and reverse, 5'-CTCCATCCTCCAGACCGAGA-3'.

Phenotypic assays for cell transformation. For the invasion assay, 2×10^5 cells were seeded on a Matrigel (1 mg/ml; BD Biosciences) coated insert and incubated for 16 h (17). Transwell migration assay was performed similar to the invasion assay but without coating the insert with Matrigel. At the 15th h, 4 μ g/ml calcein AM (Life Technologies) was added to the lower chamber and incubated for 1 h at 37°C. The cells on the upper chamber were carefully removed with a cotton swab at 16th h. Fluorescence was measured at a wavelength of 488/535 (Ex/Em) on a bottom reading fluorescent plate reader (Berthold Technologies, Bad Wildbad, Germany).

For soft agar assay, 1 ml of the basal layer was made by adding equal volumes of 2X complete IMDM and 2% low melting agarose. Cells (1,000) in complete medium containing 0.4% low melting agarose were seeded over the basal layer. Plates were fed with complete medium on every alternate day and incubated at 37°C in a 5% CO₂ atmosphere for 15 days. Opaque and dense colonies were observed and counted microscopically (Zeiss Aiovert 200M; Zeiss, Oberkochen, Germany) on day 15.

Immunohistochemistry (IHC). Mouse tumor tissues were fixed in 10% formalin buffer and processed for histology. Immunohistochemistry was performed as previously reported (19). As per the protocol, the tissues were subjected to microwave treatment for antigen retrieval. The sections were then blocked with pre-immune horse serum for 30 min at RT followed by incubation in primary antibody K14 (1:100) and the respective secondary antibody which was coupled with biotin (Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA). Signals were detected by an avidin-biotin based immunoperoxidase technique. The expression of protein in IHC staining was quantified by visual assessment of the microscopic field by counting a total of 100 cells per field and for each section, in an upright microscope (Leica Biosystems, Wetzlar, Germany).

Densitometric quantification and statistical analysis. The densitometric quantification of the western blots was performed using ImageJ software (NIH, Bethesda, MD, USA). Band intensities were normalized with respect to their loading controls. All the statistical analysis was performed using the Graph Pad Prism Software (version 5.0). Two groups of data

were statistically analyzed by t-test. Two-way ANOVA using Bonferroni test was performed for tumor volume analysis. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Increased expression of TAp63 in K14-knockdown cells. p63 is known to regulate transcription of K14. In order to assess the expression of p63 in K14-depleted cells, we performed western blotting and reverse transcription. We observed that there was no alteration in Δ Np63 at the mRNA and protein level (Fig. 1A and B). However, there was an increase in TAp63 at the transcript level (Fig. 1A). Furthermore, an 2.25-fold increase in TAp63 protein level in the K14-knockdown (K14 KD) cells was also observed (Fig. 1B).

Generation of TAp63 knockdown in K14-depleted cells. TAp63 was stably downregulated in the K14-depleted cells. Two clones (T2 and T3) obtained from HaCaT cells demonstrated significant levels of reduction in TAp63 at the transcript level as seen by RT-PCR (Fig. 1C). TAp63 was downregulated 80 and 86% in T2 and T3, respectively, at the protein level as compared to the vector control (HA-NeoVc) (Fig. 1D). Ha-NeoVc stands for plko1.neo empty vector in HaCaT K14 KD cells. TAp63 KD clones (KT3.1, 3.2 and 4.4) obtained from AW13516 cells also showed reduced TAp63 expression at the mRNA level in RT-PCR (Fig. 1C). There was an 88, 70 and 82% reduction in TAp63 expression at the protein level in KT3.1, 3.2 and 4.4, respectively (Fig. 1D) as compared to AW-NeoVc (vector control in AW-13516 K14 KD cells). There were no significant changes observed in the levels of K5, K14 and Δ Np63 respectively after depletion of TAp63 in these cells, as seen by western blotting (Fig. 5D).

TAp63 knockdown results in downregulation of Notch-1 and cell differentiation markers. We assessed the levels of differentiation markers by western blotting and RT-qPCR. Involucrin, K1, loricrin and filaggrin were downregulated 4.0-, 2.6-, 1.8- and 1.5-fold, respectively, at the mRNA level (Fig. 2A) (P<0.005). There was an 25 and 22% decrease in the levels of involucrin at the protein level in the T2 and T3 clones, respectively (Fig. 2B). Similarly, there was a 38 and 42% decrease in involucrin in KT3.1 and KT4.4, respectively, as compared to AW-NeoVc (Fig. 2B).

p63 is known to regulate the process of differentiation in keratinocytes through Notch-1 (20). We observed an 2.7- and 3.3-fold decrease in Notch-1 expression in T2 and T3, respectively (Fig. 2C) (P<0.002). Consistently, a 41% decrease in activation of Notch-1 intracellular domain (NICD) at the protein level was observed in the TAp63KD HaCaT clones as compared to HA-NeoVc. There was a 24 and 40% decrease in NICD in KT3.1 and KT4.4, respectively (Fig. 2D). These observations are consistent with a previous study, which demonstrated that TAp63 is known to activate transcription of members of the Notch-1 pathway which in turn promotes transcription of differentiation-related genes (21).

Downregulation of TAp63 leads to changes in the transformation potential of AW13516 cells and in vivo

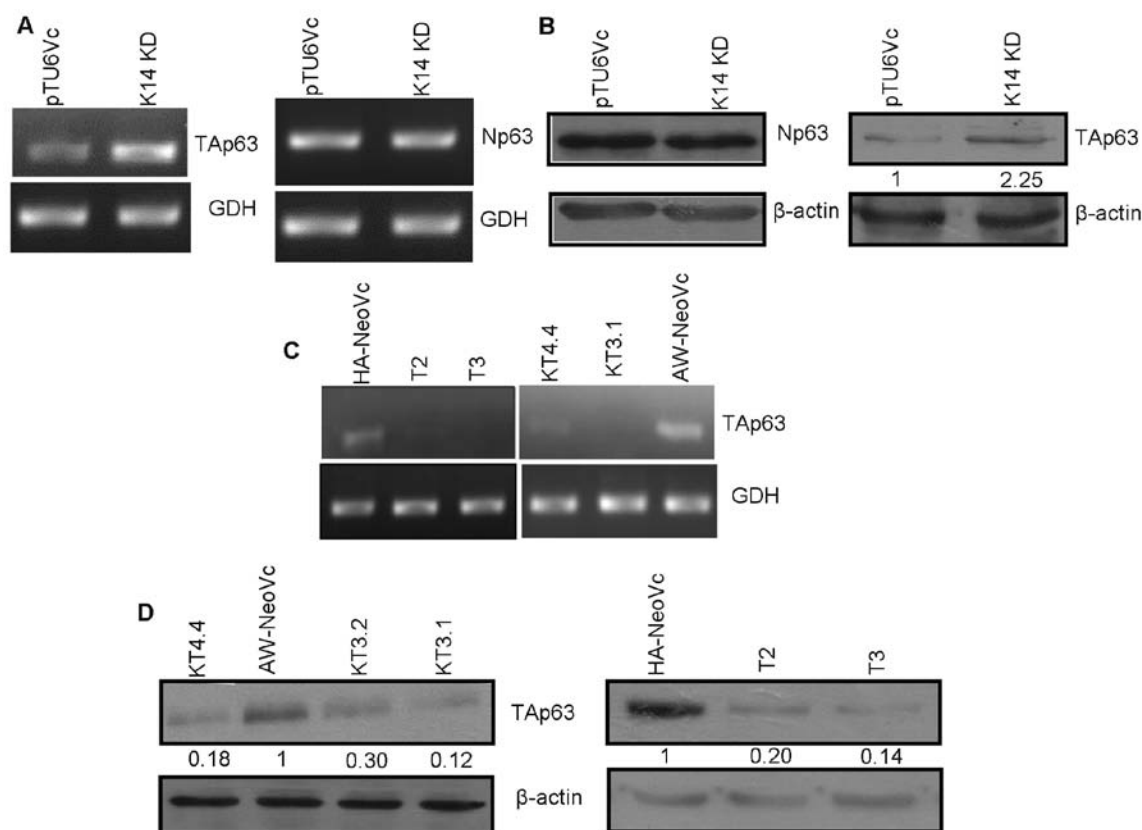


Figure 1. Generation of TAp63-knockdown clones in K14-depleted HaCaT and AW13516 cells. (A) RT-PCR of TAp63 and Δ Np63 in K14 KD cells. (B) Western blot analysis of TAp63 and Δ Np63 respectively in K14 KD and pTU6Vc. (C) mRNA levels of TAp63 in TAp63 KD clones and their respective vector control in HaCaT and AW13516 cells. (D) Western blot analysis of TAp63 in the above mentioned clones. The number below the blots indicate the relative intensity of the bands using densitometric analysis. GDH stands for the GAPDH gene and Np63 symbolizes Δ Np63.

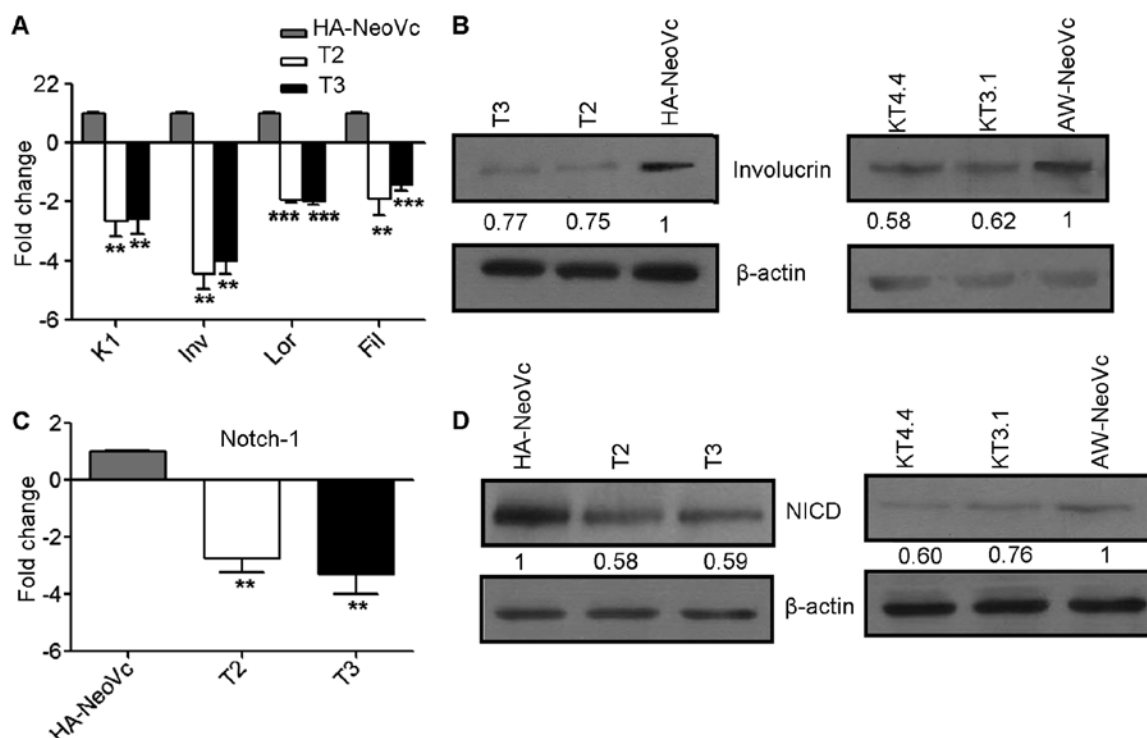


Figure 2. TAp63 KD mediates a decrease in cell differentiation markers and Notch-1. (A) Graphical representation of RT-qPCR analysis of involucrin, loricrin and filaggrin as determined in the above mentioned clones. (B) Western blot analysis of stable TAp63-knockdown clones (T2 and T3); KT3.1, KT4.4 and their respective vector control (HA-NeoVc and AW-NeoVc) with involucrin antibody. (C) RT-qPCR analysis of Notch-1 in T2 and T3 clones. (D) Western blot analysis of NICD in TAp63 KD clones and their respective vector controls. ** $P < 0.005$, *** $P < 0.0001$. The graph data represents the mean \pm SEM of three independent experiments. The number below the blots indicates relative intensity of the bands using densitometric analysis.

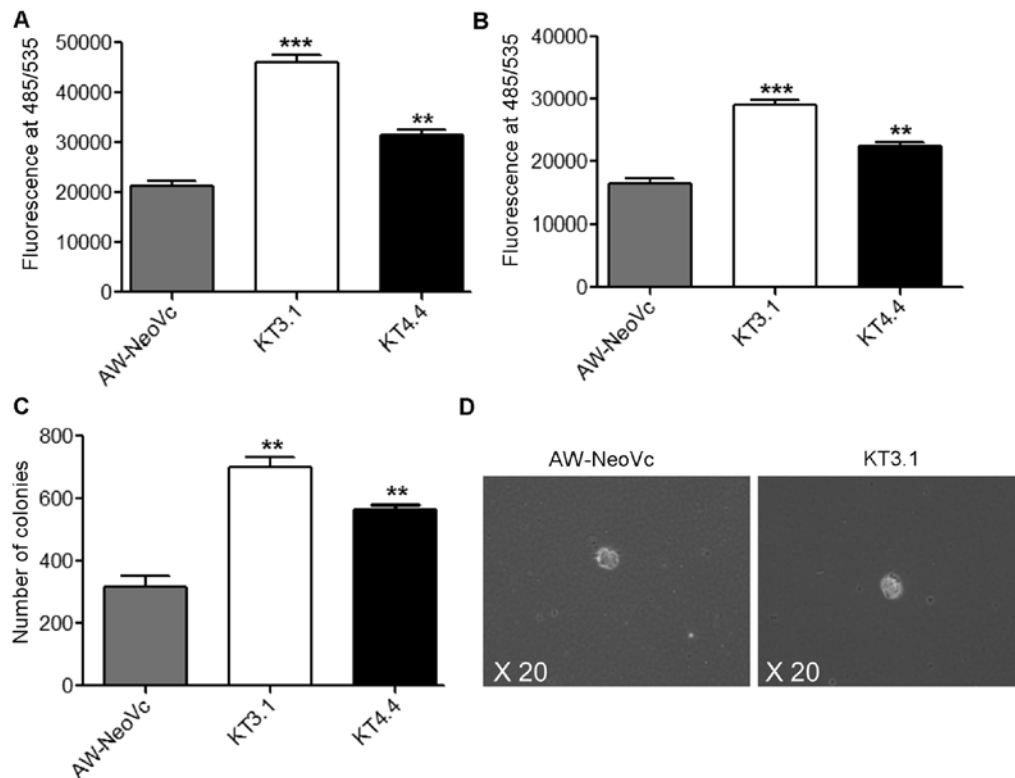


Figure 3. Loss of TAp63 leads to an increase in migration, invasion and transformation *in vitro*. (A and B) The graphs show the fluorescence of migrated and invaded cells, respectively, which was read at wavelengths of 485/535 nm (Ex/Em) in KT3.1, KT4.4 and AW-NeoVc. (C) The graphical representation of the number of colonies formed on soft agar by TAp63KD clones. (D) The representative image of the number of colonies formed on soft agar in AW-NeoVc and KT3.1, respectively. ***P<0.0001, **P<0.002. The graphical data represents the mean \pm SEM of three independent experiments.

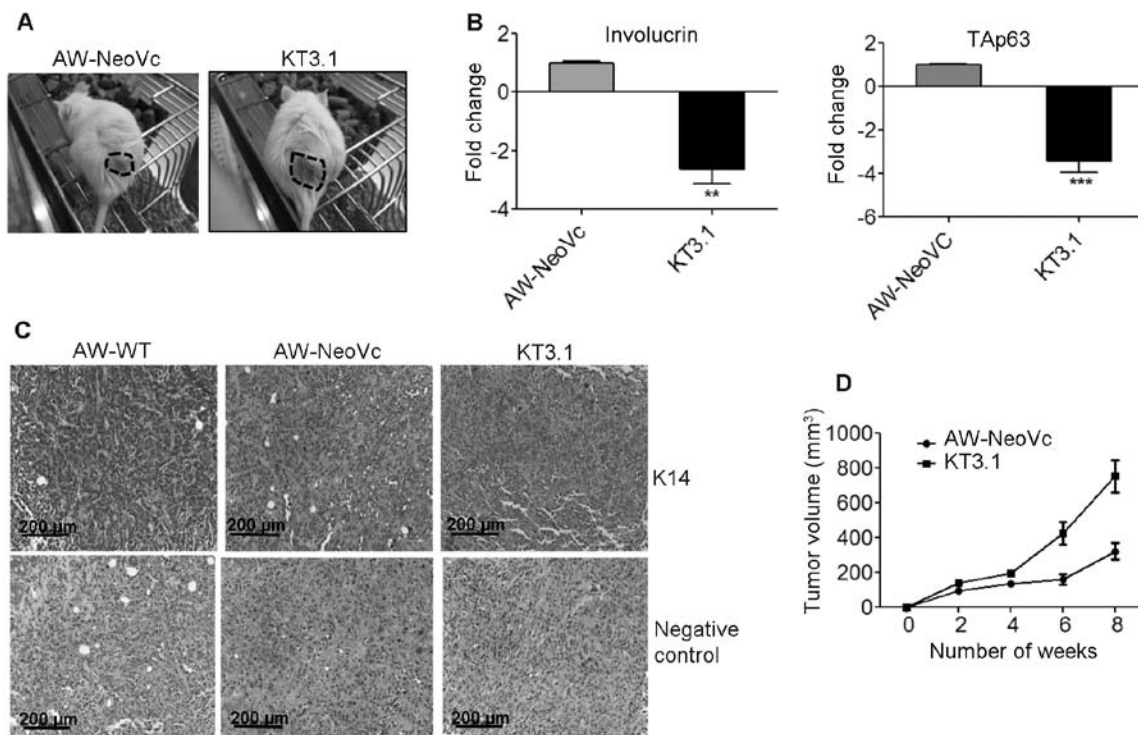


Figure 4. Downregulation of TAp63 leads to an increase in tumorigenicity. (A) The representative image of the NOD-SCID mice bearing tumor formed from KT3.1 and AW-NeoVc cells after 8 weeks of injection. The tumors are indicated by dotted circles. (B) RT-qPCR analysis of TAp63 and involucrin in RNA isolated from tumors of KT3.1 and AW-NeoVc NOD-SCID mice. (C) The upper panel shows IHC staining of K14 in tumors isolated from wild-type (AW-WT), AW-NeoVc and KT3.1 NOD-SCID respectively and the lower panel shows tissue sections incubated with serum from non-immunized mice. (D) The graph represents the tumor volume plotted against time in weeks for AW-NeoVc and KT3.1. It represents the mean \pm SEM for four animals injected. ***P<0.0001, **P<0.002.

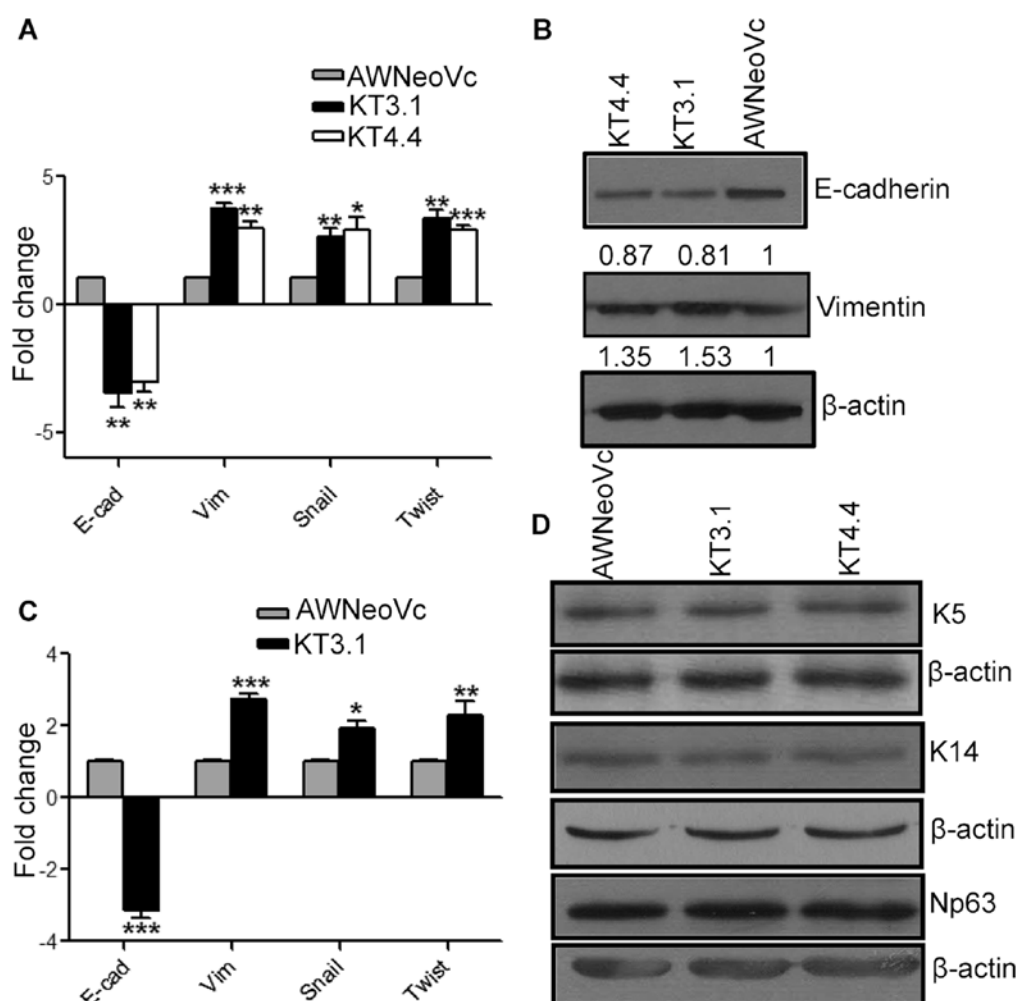


Figure 5. TAp63 downregulation leads to an increase in EMT. (A) The graph represents RT-qPCR analysis of E-cadherin, vimentin, snail and twist in KT3.1, KT4.4 and AW-NeoVc. (B) Western blot analysis of EMT markers E-cadherin and vimentin in KT3.1, KT4.4 and AW-NeoVc. (C) The graph represents the RT-qPCR analysis of E-cadherin, vimentin, snail and Twist in tumors obtained from KT3.1 and AW-NeoVc NOD-SCID mice, respectively. (D) Western blot analysis of K5, K14 and Δ Np63 in TAp63 KD clones and AWNeoVc. Np63 stands for Δ Np63. The graph data represent the mean \pm SEM of three independent experiments. The number below the blots indicates the relative intensity of each bands using densitometric analysis. *** $P < 0.0001$, ** $P < 0.005$, * $P < 0.05$.

tumorigenicity. Knockdown of TAp63 in K14-depleted cells caused an increase in cell migration and invasion. In the Transwell migration assay, TAp63 KD clones KT3.1 and KT4.4 showed an 50 and 37.5% increase in migration, respectively, as compared to the vector control AW-NeoVc (Fig. 3A) ($P < 0.001$). There was an 40 and 30% increase in invasion in the TAp63 KD clones KT3.1 and KT4.4, respectively, as compared to the vector control AW-NeoVc (Fig. 3B) ($P < 0.002$). However, there were no changes in the migration and invasion in HaCaT TAp63 KD clones as compared to HA-NeoVc (data not shown), since HaCaT is an immortalized and not a transformed cell line. Furthermore, there was an increase in the number of soft agar colonies by 40% in KT3.1 and 22% in KT4.4 compared to AW-NeoVc (Fig. 3C and D) ($P < 0.002$). The tumorigenic potential of TAp63 KD cells was determined by subcutaneous injection into NOD-SCID mice ($n=4$). The volume of the tumors formed in the TAp63 KD mice (KT3.1) was significantly higher as compared to the vector control (AW-NeoVc) (Fig. 4A and D). IHC studies illustrated a decrease in the expression of K14 in the tumor samples obtained from the mice injected with AW-NeoVc

and KT3.1 as compared to the wild-type (AW-WT) tumor samples (Fig. 4C). The TAp63 antibody was not effective in IHC. Therefore, RT-qPCR was performed to analyze the levels of TAp63 in RNA samples derived from the respective tumors. At the mRNA level, the TAp63 level in KT3.1 was 3.4-fold less when compared to AW-NeoVc in the tumor tissues (Fig. 4B) ($P < 0.001$). The involucrin mRNA level was also 2.5-fold lower in the tumor samples derived from KT3.1 when compared to AW-NeoVc (Fig. 4B) ($P < 0.002$).

Downregulation of TAp63 leads to an increase in the expression of EMT markers. We observed changes in invasion *in vitro*, therefore, we assessed whether epithelial-mesenchymal transition (EMT) was involved in the TAp63-regulated invasion. We found that downregulation of TAp63 led to an 3.36-, 2.77- and 3.14-fold increase in the mRNA levels of vimentin ($P < 0.001$), snail ($P < 0.05$) and twist ($P < 0.001$) in the K14-depleted cells, respectively. A 2.91-fold downregulation of E-cadherin was noted at the mRNA level (Fig. 5A) ($P < 0.003$). In addition, there was a 19 and 13% decrease in E-cadherin protein expression in KT3.1 and KT4.4, respectively, as compared to AW-NeoVc.

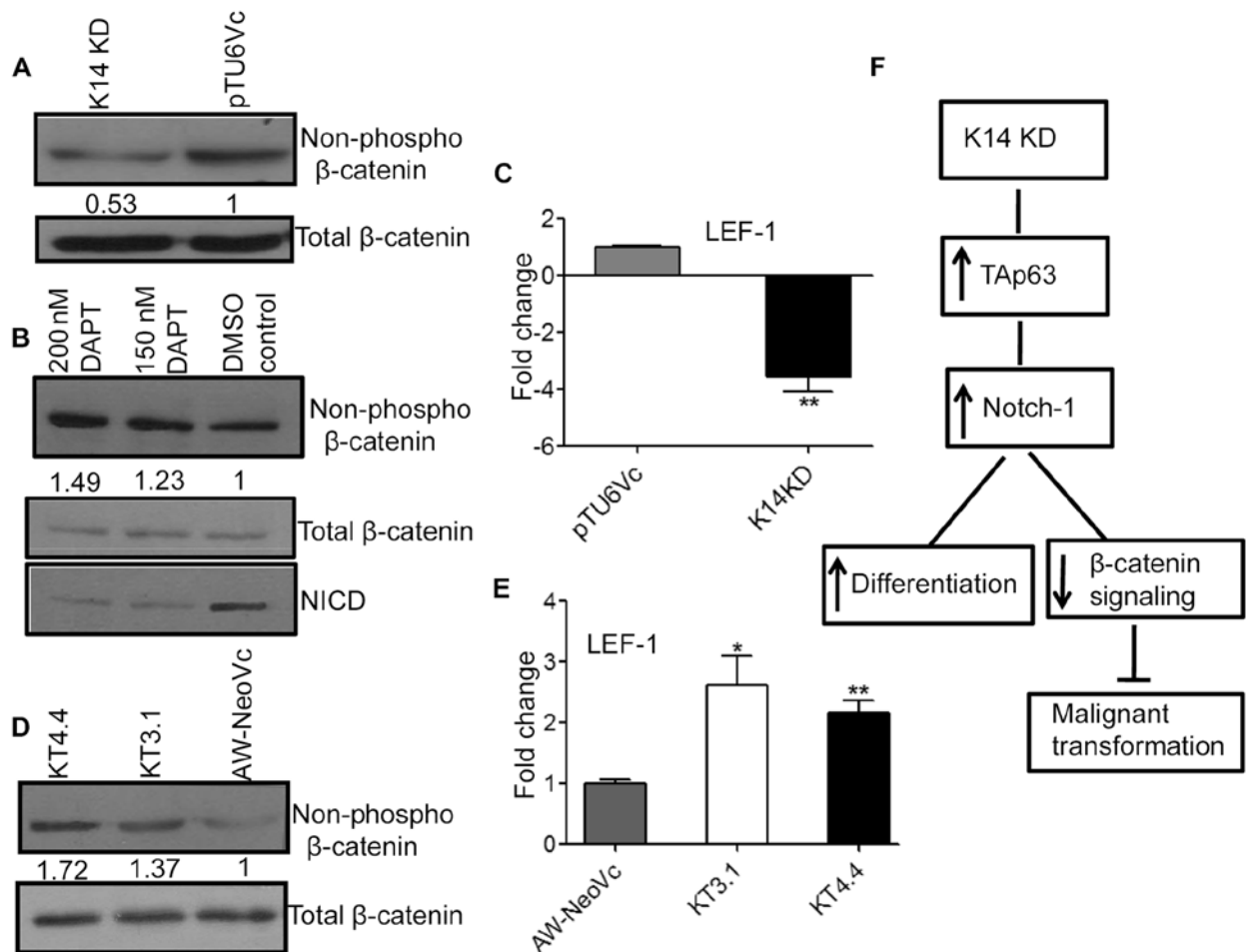


Figure 6. TAp63 and Notch-1 modulates β-catenin signaling in K14 downregulated cells and the schematic representation depicting the role of TAp63 and Notch-1 in K14 KD cells. (A) Western blot analysis of non-phospho β-catenin in the K14 KD and pTU6 cells. (B) Western blotting for non-phospho β-catenin after treatment of the K14 KD clone with 150 and 250 nM of Notch-1 inhibitor (DAPT) for 72 h. (C) The graph represents the RT-qPCR analysis of LEF-1 in K14 KD cells and pTU6. (D) The western blot analysis of non-phospho β-catenin in TAp63 KD clones KT3.1, KT4.4 and AW-NeoVc. (E) The graph represents RT-qPCR analysis of LEF-1 in TAp63KD and AW-NeoVc. (F) The model shows that K14 KD leads to an increase in TAp63 expression. TAp63 in turn regulates the process of malignant transformation and differentiation through Notch-1. The mechanism by which K14 regulates expression of TAp63 is yet to be explored (shown in dotted line). ↑ symbolizes increase in expression, ↓ symbolizes decrease in the expression or signaling, ⊥ stands for inhibition. The data represent the mean ± SEM of three independent experiments. Note: the number below the blots indicate the relative intensity of each bands using densitometric analysis. **P<0.005, *P<0.05.

Subsequently, there was an 1.53- and 1.35-fold increase in vimentin expression at the protein level in KT3.1 and KT4.4, respectively (Fig. 5B). Likewise, we observed an increase in the expression of vimentin, snail and twist in the KT3.1 tumor tissues at the mRNA level (P<0.05) and subsequently, there was downregulation of E-cadherin mRNA in the KT3.1 tumor tissues (Fig. 5C).

Notch-1 regulates β-catenin signaling in the K14 KD cells. The available literature has shown that inactivation of Notch-1 activates β-catenin signaling and its downstream target lymphoid enhancing factor-1 (LEF-1) which leads to an increase in tumorigenesis (22). Notch-1 was upregulated in the K14 KD cell lines and in addition there was a decrease in tumorigenic potential of these cells. Thus, we hypothesized that β-catenin could be one of the targets that maybe involved in the K14-regulated decrease in tumorigenic potential. To assess this hypothesis, we examined the β-catenin levels in K14 KD cells. There was a decrease in the non-phospho levels

of β-catenin (active pool) in the K14 KD cells as compared to the vector control (Fig. 6A). The total β-catenin levels remain unchanged. There was a 3.5-fold decrease in LEF-1 expression at the mRNA level in the K14 KD cells as compared to pTU6Vc (Fig. 6C) (P<0.001). LEF-1 is a downstream target of β-catenin.

To further confirm that β-catenin levels in K14 KD cells are modulated by Notch-1, we treated the AW-K7 clone with Notch-1 inhibitor DAPT. There was a 1.5-fold increase in non-phospho β-catenin (active pool) levels with 200 nM of DAPT in the AW-K7 clone as compared to the DMSO-treated AW-K7 clone (control) (Fig. 6B). This verifies our hypothesis that β-catenin levels are regulated by Notch-1 in K14-depleted cells.

However, TAp63 knockdown clones KT3.1 and KT4.4 displayed a 1.37- and 1.72-fold increase in non-phospho β-catenin (active pool) levels as compared to the vector control (Fig. 6D). Subsequently, there was a 2.3-fold increase in LEF-1 expression at the mRNA level in the TAp63 KD clones (Fig. 6E).

Discussion

K14 is expressed in the basal cells of stratified epithelia and as the cells move upward to the suprabasal layer, the expression of K14 is replaced by expression of K1 and K10. This suggests that keratins play an important regulatory role in cell differentiation (23). A previous study by our laboratory showed that depletion of K14 initiates the process of cell differentiation. An increase in Notch-1 expression and a decrease in tumorigenicity was also observed in K14 depleted cells (24). Yet, the molecules involved in the K14-regulated differentiation and malignant transformation remain unknown.

p63 is a master regulator of epithelial cell proliferation and differentiation. We showed here that K14-downregulated cells exhibited an increase in TAP63 levels at both the mRNA and protein levels. Upon TAP63 KD, we observed a decrease in differentiation markers such as involucrin, K1, filaggrin and loricrin. Furthermore, in the present study, upon downregulation of TAP63 in K14 KD cells, there was a decrease in the expression in the Notch-1 level, suggesting that Notch-1 is regulated by TAP63. A previous report demonstrated that there is a complex interplay between Notch-1 and p63 in regulating the switch between cell differentiation and stemness. p63 plays a dual role by suppressing Notch-1 in the basal cell compartment and then synergizes with Notch-1 signaling in the early stages of differentiation (20). Our data is consistent with this report, as we showed that upon K14 downregulation, there was an increase in TAP63 expression and an increase in intracellular levels of Notch-1, which further regulates genes involved in differentiation.

TAP63-depleted cells further exhibited an increase in cell migration, invasion and tumorigenicity in the K14-downregulated cells. Our observations suggest that in K14-downregulated cells, TAP63 modulates the process of cell transformation and tumorigenicity. An increase in invasion could be related to EMT. Lin *et al* showed that p63 regulates metastasis in colon cancer cells by regulating EMT (9). We were curious to ascertain whether TAP63 KD cells which show a less differentiated and more invasive phenotype, exhibit EMT. Our results demonstrated an increase in the expression of vimentin and a decrease in the expression of E-cadherin at both the transcript and protein levels in the TAP63 KD AW13516 cells. Similar results were obtained in RNA samples isolated from tumor tissues of mice injected with the TAP63 KD cells.

Alam *et al* found an increase in the expression of Notch-1 and a decrease in tumorigenicity *in vivo* in K14 KD cells (24). We were curious to ascertain whether there were any changes in β -catenin and its downstream signaling molecules in K14 KD cells. We found that there was a decrease in the non-phospho pool of β -catenin (active) in K14 KD cells as seen by western blotting and subsequent decrease in LEF-1 at the mRNA level. Furthermore, when we treated the cells with DAPT (Notch-1 inhibitor), there was an increase in the active pool of β -catenin. However, when we observed β -catenin levels in TAP63 KD cells, there was an increase in expression of non-phospho β -catenin at the protein level and LEF-1 at the mRNA level. Thus, we can infer that Notch-1 regulates tumorigenesis in K14-depleted cells and Notch-1 expression in turn is modulated by TAP63. Furthermore, how β -catenin levels regulate

tumorigenesis warrants further investigation. Reports available in the literature demonstrate that inhibition of Notch-1 leads to development of squamous cell carcinoma in which β -catenin signaling is upregulated (25,26).

In summary, the present study demonstrated that TAP63 plays an important role in K14-regulated cell differentiation. Knockdown of TAP63 led to an increase in cell motility, invasion and tumorigenesis *in vivo*. TAP63 may modulate tumorigenic potential and differentiation of AW13516 cells through Notch-1 (Fig. 6F). Romano *et al* showed that gene expression of K5/14 is regulated by p63 (11). In the present study, we demonstrated that upon K14 downregulation, there was an increase in the expression of TAP63. The mechanism by which K14 regulates TAP63 is yet to be discovered. Further studies are required to investigate the interplay between p63 and K14 and to decipher the mechanism underlying the regulation of p63 by K14.

Acknowledgements

The present study was supported by the Department of Science and Technology (DST, India) (grant no. LS-630/2013) which also provided a fellowship to Dr Saumya S. Srivastava. We thank Dr Crismita D'Mello and Dr Richa Tiwari for their experimental suggestions and Mrs. Sharda Sawant for help with the IHC analysis. We thank Mr Sridhar Nadkar, Ms. Silvania Charles and Ms. Zinia D'Souza for assistance in the animal experiments.

Competing interests

The authors declare that they have no competing interests.

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