



# DNA Demethylation Switches Oncogenic $\Delta$ Np63 to Tumor Suppressive TAp63 in Squamous Cell Carcinoma

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The *TP63* gene encodes two major protein variants; TAp63 contains a p53-like transcription domain and consequently has tumor suppressor activities whereas  $\Delta$ Np63 lacks this domain and acts as an oncogene. The two variants show distinct expression patterns in normal tissues and tumors, with lymphocytes and lymphomas/leukemias expressing TAp63, and basal epithelial cells and some carcinomas expressing high levels of  $\Delta$ Np63, most notably squamous cell carcinomas (SCC). Whilst the transcriptional functions of TAp63 and  $\Delta$ Np63 isoforms are known, the mechanisms involved in their regulation are poorly understood. Using squamous epithelial cells that contain high levels of  $\Delta$ Np63 and low/undetectable TAp63, the DNA demethylating agent decitabine (5-aza-2'-deoxycytidine, 5-dAza) caused a dose-dependent increase in TAp63, with a simultaneous reduction in  $\Delta$ Np63, indicating DNA methylation-dependent regulation at the isoform-specific promoters. The basal cytokeratin *KRT5*, a direct  $\Delta$ Np63 transcriptional target, was also reduced, confirming functional alteration of p63 activity after DNA demethylation. We also showed high level methylation of three CpG sites in the *TAP63* promoter in these cells, which was reduced by decitabine. DNMT1 depletion using inducible shRNAs partially replicated these effects, including an increase in the ratio of *TAP63*: $\Delta$ *NP63* mRNAs, a reduction in  $\Delta$ Np63 protein and reduced *KRT5* mRNA levels. Finally, high DNA methylation levels were found at the *TAP63* promoter in clinical SCC samples and matched normal tissues. We conclude that DNA methylation at the *TAP63* promoter normally silences transcription in squamous epithelial cells, indicating DNA methylation as a therapeutic approach to induce this tumor suppressor in cancer. That decitabine simultaneously reduced the oncogenic activity of  $\Delta$ Np63 provides a “double whammy” for SCC and other p63-positive carcinomas. Whilst a variety of mechanisms

may be involved in producing the opposite effects of DNA demethylation on TAp63 and  $\Delta$ Np63, we propose an “either or” mechanism in which *TAP63* transcription physically interferes with the ability to initiate transcription from the downstream  *$\Delta$ NP63* promoter on the same DNA strand. This mechanism can explain the observed inverse expression of p63 isoforms in normal cells and cancer.

**Keywords:**  $\Delta$ Np63, TAp63, alternative promoter usage, squamous cell carcinoma, keratinocytes, DNA methylation, decitabine

## INTRODUCTION

The *TP63* gene codes for two major protein variants using transcripts produced from two separate promoters. Unlike TAp63, which is transcribed from the upstream promoter (P1) and contains an N-terminal p53-like transactivation sequence,  $\Delta$ Np63 is initiated at a downstream promoter (P2) and the protein lacks the N-terminal transactivation domain. Thus,  $\Delta$ Np63 was originally thought to act as a transcriptional repressor, but was subsequently shown to contain an alternative transactivation domain, inducing expression of target genes involved in proliferation, survival, adhesion and differentiation of stratified epithelial cells and tissues such as breast, prostate and urothelium (1, 2).

TAp63 has tumor suppressor roles that reflect its p53-like activities of inducing apoptosis (3) or senescence (4), and inhibiting metastasis (5, 6). In contrast,  $\Delta$ Np63 promotes tumorigenesis (7, 8) and resistance to cytotoxic therapies (9). The tumor suppressor properties of TAp63 stimulated attempts to induce TAp63 as a therapeutic strategy, producing reduced cell viability and enhanced response to therapy (10). In particular, because TAp63 has p53-like properties, this approach is a viable option for replacing p53 tumor-suppressive activities in tumors with p53 mutation (10, 11). Similarly, reducing the oncogenic activity of  $\Delta$ Np63 causes tumor-suppressive activities for tumors that overexpress this protein (12, 13).

In human cancers,  $\Delta$ Np63 is commonly overexpressed in squamous cell carcinoma (SCC), whereas some B-cell lymphomas/leukemias express TAp63 (14, 15). These patterns of p63 isoforms in malignancy reflect their normal tissue expression patterns - TAp63 but not  $\Delta$ Np63 is present in oocytes and lymphocytes, whereas  $\Delta$ Np63 is the only form present in most adult squamous epithelia (1, 2, 16). In tumors that overexpress  $\Delta$ Np63, the level of TAp63 associates with improved patient survival in SCC of the cervix and triple-negative breast cancers, in keeping with a tumor suppressor role of TAp63 in these and other cancers (14, 17–20). However, how the  $\Delta$ Np63 and TAp63 isoforms are regulated to produce their tissue-specific expression patterns and their dysregulation in cancer is not known. Understanding the pathways involved in TAp63 and  $\Delta$ Np63 regulation is therefore important to enable manipulation of their levels for cancer treatment. The aim of a p63-based therapeutic approach for tumors with high levels of  $\Delta$ Np63 is to either decrease  $\Delta$ Np63 or to increase TAp63, either of which can reduce tumor cell growth on their own (10–13).

Here, we investigated DNA methylation at the *TP63* gene locus as a potential regulator of p63 isoform transcription. This notion was based in part on evidence from studies of leukemic cells (TAp63-positive,  $\Delta$ Np63-negative), where an inverse correlation exists between *TP63* mRNA levels and methylation at the P1 *TAP63* promoter (21, 22). In addition, hypomethylation at the P2  *$\Delta$ NP63* promoter in SCC (23–25), provides evidence for P2 promoter methylation as a repressive mechanism for  $\Delta$ Np63. Thus, we investigated whether the DNA methyltransferase inhibitor (DNMTi) decitabine (5-Aza-2'-deoxycytidine; 5dAza), can activate TAp63 in SCC by reducing P1 methylation without influencing  $\Delta$ Np63 levels from the already demethylated P2 promoter. Unexpectedly, we found that decitabine not only increased TAp63, but also caused a concomitant reduction in  $\Delta$ Np63 protein and mRNA. Genetic depletion of DNMT1 partially recapitulated these results. The data show for the first time that *TP63* transcription can be switched from oncogenic  $\Delta$ Np63 to tumor suppressor TAp63 and that this can be achieved using a clinically approved DNA demethylating agent.

## MATERIAL AND METHODS

All reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Control treatments were performed using the same volume of the corresponding solute (DMSO or water for decitabine or doxycycline, respectively).

### Cell Culture and Treatments

FaDu (human pharynx squamous cell carcinoma) and SCC-25 (human squamous cell carcinoma of the tongue) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Non-transformed squamous HaCaT cells (spontaneously immortalized human keratinocytes) were obtained from DKFZ (Heidelberg, Germany). FaDu and HaCaT cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% sodium pyruvate, and penicillin/streptomycin (all from Gibco, Thermo Fisher Scientific, MA, USA) at 37°C in 5% CO<sub>2</sub>. SCC-25 cells were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12, Gibco) with 10% FBS, 0.4 µg/ml hydrocortisone (Lonza, Basel, Switzerland), 1% sodium pyruvate, and penicillin/streptomycin. Cells were grown to 40–70% confluency before treatment according to the type and length of the experiment. Cell viability was determined using Resazurin (see the

supplementary material for details). Samples used for Western blotting and RT-qPCR were analyzed in at least three biological triplicates.

### Inducible DNMT1 Knockdown Cell Lines

Five individual TET-inducible TRIPZ plasmids containing shRNAs targeting *DNMT1* were obtained from Horizon Discovery (RHS4740-EG1786, Cambridge, UK). Plasmid DNAs were isolated using a Plasmid Maxi Kit (12162, Qiagen, Hilden, Germany) and used to produce viral particles in HEK293FT cells (ATCC). Lentiviruses were collected 48 and 96 h after transfection and transduced into HaCaT, FaDu and SCC-25 cells as described previously (12). Medium was replaced after 24 h, and selection in puromycin (1 µg/ml for HaCaT and FaDu and 0.05 µg/ml for SCC-25) started after a further 24 h. Medium containing puromycin was replaced every three days. Puromycin-resistant cells were expanded, shRNAs were induced with 2 µg/ml doxycycline and DNMT1 was assessed by Western blotting. Cell populations showing DNMT1 downregulation were single-cell sorted (BD FACS Aria III, Wokingham, Berks., UK) into 96-well plates and two individual clones were prepared for each cell line after further puromycin selection. Individual clones were re-tested for doxycycline-inducible DNMT1 knockdown by Western blotting. SCC-25 cell clones died during puromycin selection and no stable clones were obtained. Stable cell lines were obtained for HaCaT and FaDu and were routinely cultured in DMEM with 10% FBS containing 1 µg/ml puromycin. Doxycycline was added at 2 µg/ml final concentration to induce shRNA-mediated depletion of DNMT1 and the medium was replaced with freshly prepared medium every 24 h. Cells were analyzed after four or six days of continuous doxycycline and compared to the same cells grown in the absence of doxycycline.

### DNA Methylation at the TAP63 Promoter

To investigate whether methylation at the P1 promoter could be a mechanism for regulating *TAP63* transcription, we searched CpG methylation profiles in the ENCODE project (<https://www.encodeproject.org/>) (26) in lymphocyte/leukemia cell lines that may express TAp63 and in epithelial cells that may express ΔNp63. To measure CpG methylation at the identified sites, cells were harvested with trypsin and genomic DNA isolated (QIAamp, Qiagen, Manchester, UK). Bisulfite conversion of 500 ng DNA was performed using EZ DNA methylation (Zymo, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite converted PCR primers (Generi Biotech, Hradec Kralove, Czech Republic) were designed according to MethPrimer 2.0 ([urogene.org/cgi-bin/methprimer2/MethPrimer.cgi](http://urogene.org/cgi-bin/methprimer2/MethPrimer.cgi)) (27) (**Supplementary Table S1**) to amplify a 134 bp region beginning 111 bp upstream of the *TAP63* transcription start site and containing three CpG sites. PCR was performed using Taq polymerase (Invitrogen, ThermoFisher Scientific, Waltham MA, USA): 95°C for 30 s and 40 cycles of 95°C for 15 s, 50°C for 20 s and 68°C for 90 s, followed by a final extension at 72°C for 3 min. PCR products were analyzed on 1.5% agarose gels and purified (QIAquick gel extraction kit, Qiagen) for Sanger sequencing (Eurofins Genomics, Benesov, Czech

Republic) using the forward or reverse PCR primer. CpG methylation was calculated as described (28, 29) using the peak heights of the bisulfite modified nucleotide at the CpG sites and measuring the average peak heights on either side.

To investigate methylation levels in clinical SCC samples, we retrieved publicly available data from the Gene Expression Omnibus repository (<https://www.ncbi.nlm.nih.gov/geo/>) for three independent patient cohorts; TCGA analysis of DNA methylation for lung SCC (GSE 68825, updated 2019); a separate cohort of lung SCC samples [GSE66045 (30)] and patients with oropharyngeal SCC [GSE124464 (31)]. All cohorts included matched normal tissue samples. Further details are provided in the supplementary materials.

### Western Blotting

See the supplementary material for details. Total proteins were separated using SDS-PAGE, blotted onto nitrocellulose and the membranes cut into horizontal strips using the molecular weight markers. Individual strips of the same blot were used to detect different proteins. Target proteins were identified using the mouse monoclonal antibodies ΔNp63 1.1 and TAp63 4.1 for p63 isoforms, as described previously (17, 32) and rabbit antibodies to DNMT1 (clone D63A #5032) and γ-H2AX (#9718, both from Cell Signaling Technology, Danvers, MA, USA). β-actin (clone C4, sc-47778, Santa Cruz Biotechnology, Dallas, TX, USA) was used as loading control. Proteins were detected with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Bucks, UK). Blots were quantified by densitometry using ImageJ (<https://imagej.nih.gov/ij/>) and normalized to β-actin.

### RNA Isolation and Reverse Transcription-Quantitative PCR

Total RNA was isolated using TRIzol and 500 ng were reverse transcribed using High Capacity cDNA Reverse Transcription (Applied Biosystems, ThermoFisher Scientific). PCR primers were obtained from Generi Biotech (**Supplementary Table S1**) and PCR was performed on a Fast Real-Time PCR System with Sybreen (Applied Biosystems): 95°C for 3 min, 50 cycles of 95°C for 5 s and 60°C for 25 s. At least three biological replicates were performed, and each cDNA sample was analyzed in technical triplicates. Mean cycle threshold (Ct) values were normalized to β-actin (*ACTB*) and transformed into relative mRNA levels (33).

### Statistical Analysis

Data were tested for normal distribution using Shapiro-Wilk tests with significance level ( $\alpha$ ) = 0.05 and examining skewness and excess kurtosis (<https://www.statskingdom.com/shapiro-wilk-test-calculator.html>). No experimental dataset showed a significant departure from normality. Therefore, these data are presented as mean ± SEM and statistical significance was determined using unpaired 2-tailed t-tests against control values. In **Figure 9**, where data showed a significant deviation from normality, Mann-Whitney test was used.  $p < 0.05$  was considered significant.

## RESULTS

### Decitabine Increased TAp63 mRNA and Protein Levels in SCC

Decitabine acts to prevent re-methylation of newly synthesized DNA and therefore progressively reduces DNA methylation over increased numbers of replicative cycles. By Western blotting, TAp63 was present at low or undetectable levels in untreated HaCaT, FaDu and SCC-25 cells, in keeping with previous observations in transformed and non-transformed squamous cells (16, 34). Treatment with decitabine at concentrations from 0.001  $\mu\text{M}$  to 10  $\mu\text{M}$  for four days increased TAp63 protein in all three cell lines in a dose-dependent manner at concentrations of 0.5  $\mu\text{M}$  and above. Accurate quantitation of TAp63 in Western blots is difficult due to the very low signals in untreated cells but was calculated as representing 45-fold to 86-fold induction at 10  $\mu\text{M}$  decitabine ( $p < 0.01$  for each cell line) (Figure 1A). These protein data correspond to the low levels of *TAP63* mRNA under normal growth conditions and the dose-dependent increases after treatment with decitabine at 0.5  $\mu\text{M}$  and higher concentrations in all three cell lines, rising to between 10-fold and more than 100-fold higher levels in the different cell lines after 10  $\mu\text{M}$  decitabine ( $p < 0.01$  for HaCaT and  $p < 0.001$  for FaDu and SCC-25) (Figure 1B).

### Decitabine Decreased $\Delta\text{Np63}$ mRNA and Protein Levels in SCC

Conversely to TAp63, Western blotting showed that decitabine caused a dose-dependent reduction of  $\Delta\text{Np63}$  protein, with a 2-fold to 20-fold reduction after 10  $\mu\text{M}$  decitabine for four days (Figure 2A). RT-qPCR also showed a 2-fold to 3-fold decrease in  $\Delta\text{NP63}$  mRNA levels at higher decitabine concentrations, although low concentrations showed a slight increase in all cell lines, which was significant in HaCaT cells ( $p = 0.045$  at 10 nM decitabine) (Figure 2B).

### Decitabine Decreased *KRT5* mRNA Levels

To evaluate whether decitabine repression of  $\Delta\text{Np63}$  and induction of TAp63 influenced p63 transcriptional activity, we monitored the basal cytokeratins *KRT5* and *KRT14*, which are markers of undifferentiated basal squamous cells and are direct  $\Delta\text{Np63}$  target genes (35), and of *KRT1* and *KRT10* that are markers of squamous cell differentiation. Decitabine caused a reproducible and dose-dependent decrease in *KRT5* mRNA levels (5-fold to 10-fold reduction at the highest concentration of decitabine,  $p < 0.05$  for each cell line) (Figure 3). Changes in other cytokeratins were inconsistent, with FaDu cells showing induction of *KRT1* and *KRT10* but no change in *KRT14* mRNA, whilst *KRT14* was decreased in SCC-25 without induction of *KRT1* or *KRT10* mRNAs (Supplementary Figure S1).

### Decitabine Reduced DNMT1 Levels and Caused Variable Levels of DNA Damage

Decitabine acts by covalent trapping of DNMTs on methylated DNA, leading to DNMT degradation and potentially causing DNA damage (36, 37). We confirmed that decitabine caused a dose-dependent reduction of soluble DNMT1 (Figure 4). In

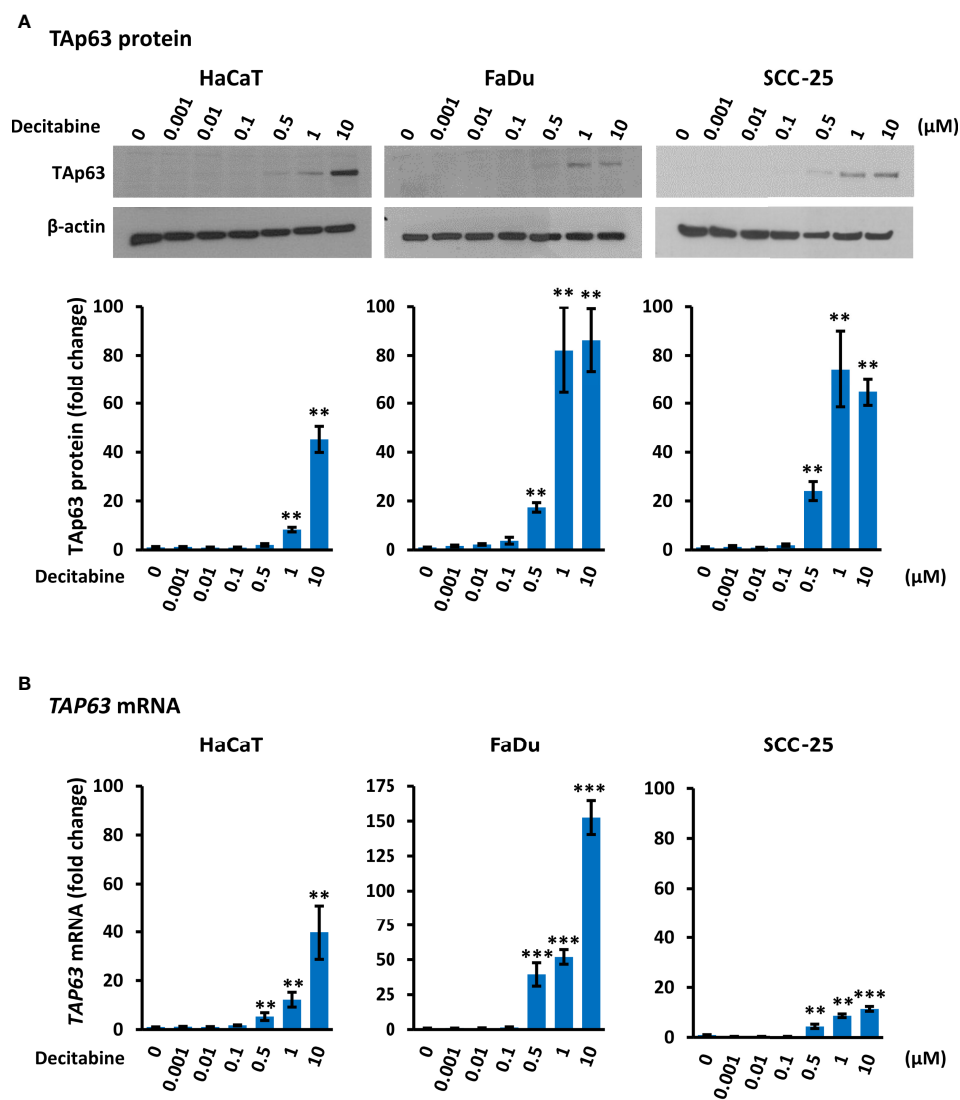
addition, high concentrations of decitabine increased the level of  $\gamma\text{-H2AX}$  as a marker of DNA double strand breaks (38) in FaDu cells which contain high levels of  $\gamma\text{-H2AX}$  under normal conditions, whereas HaCaT showed minimal  $\gamma\text{-H2AX}$  induction after decitabine and there was no apparent increase in SCC-25 cells (Supplementary Figure S2).

### DNMT1 Depletion Influences TAp63 and $\Delta\text{Np63}$

To determine whether DNMT1 levels directly regulate the balance of TAp63 and  $\Delta\text{Np63}$  isoforms, we created cell lines with inducible *DNMT1*-shRNAs. Of five vectors tested, two (201896523 and 201895794) showed DNMT1 depletion after shRNA induction by doxycycline for four days (Figure 5A). Stable clones of HaCaT and FaDu cells were subsequently prepared (SCC-25 cells died during subsequent puromycin selection/maintenance and stable clones could not be produced). HaCaT and FaDu *DNMT1*-shRNA cells were induced with 2  $\mu\text{g/ml}$  doxycycline for six days and analyzed for *TP63* isoform mRNAs, compared with the corresponding cells not exposed to doxycycline. These data showed an increase in *TAP63* and a decrease in  $\Delta\text{NP63}$  mRNAs, depicted as the change in their ratio after DNMT1 depletion (Figure 5B) albeit with reduced effects compared to decitabine (compare with Figure 1 and Figure 2). We also found a reduction in  $\Delta\text{Np63}$  levels by Western blotting, most noticeable in HaCaT cells but without a discernable increase in TAp63 protein, which remained undetectable by Western blotting (Supplementary Figure S3). However, *KRT5* mRNA was also reduced by *DNMT1*-shRNA, compatible with reduced  $\Delta\text{NP63}$  activity (Figure 5C). The lack of measurable increase in TAp63 protein presumably reflects the relatively small effect of shRNA, such that TAp63 levels are not sufficiently increased to overcome the Western blot sensitivity threshold, whereas a decrease in  $\Delta\text{Np63}$  is visible due to its high endogenous levels.

### Altered TAp63 Levels Correlate With CpG Methylation at the TAp63 Promoter

The above data suggest that TAp63 and  $\Delta\text{Np63}$  are regulated in squamous cells by DNA methylation. In particular, that DNMT inhibition increases TAp63 protein and mRNA implies that the P1 promoter is silenced by hypermethylation under normal conditions. To test this notion, we searched for evidence of differentially methylated CpG sites between cells that express TAp63 but not  $\Delta\text{Np63}$  and those that express  $\Delta\text{Np63}$  but not TAp63. We identified a series of CpG sites immediately upstream of P1 that show differential methylation profiles (Figure 6). These data show low/intermediate methylation of CpGs upstream of P1 in lymphocyte-derived cells that sometimes express TAp63 but not  $\Delta\text{Np63}$  (K562 lymphoblasts and EBV-transformed GM series lymphocytes), whilst epithelial cells that may express  $\Delta\text{Np63}$  but not TAp63 (HEEpiC, HMEC, MCF10A and PrEC) all show high methylation of the same CpGs. This association is not seen in CpGs downstream of the transcription start site (Figure 6), suggesting that CpG methylation immediately upstream of P1 may be involved in TAp63 regulation.

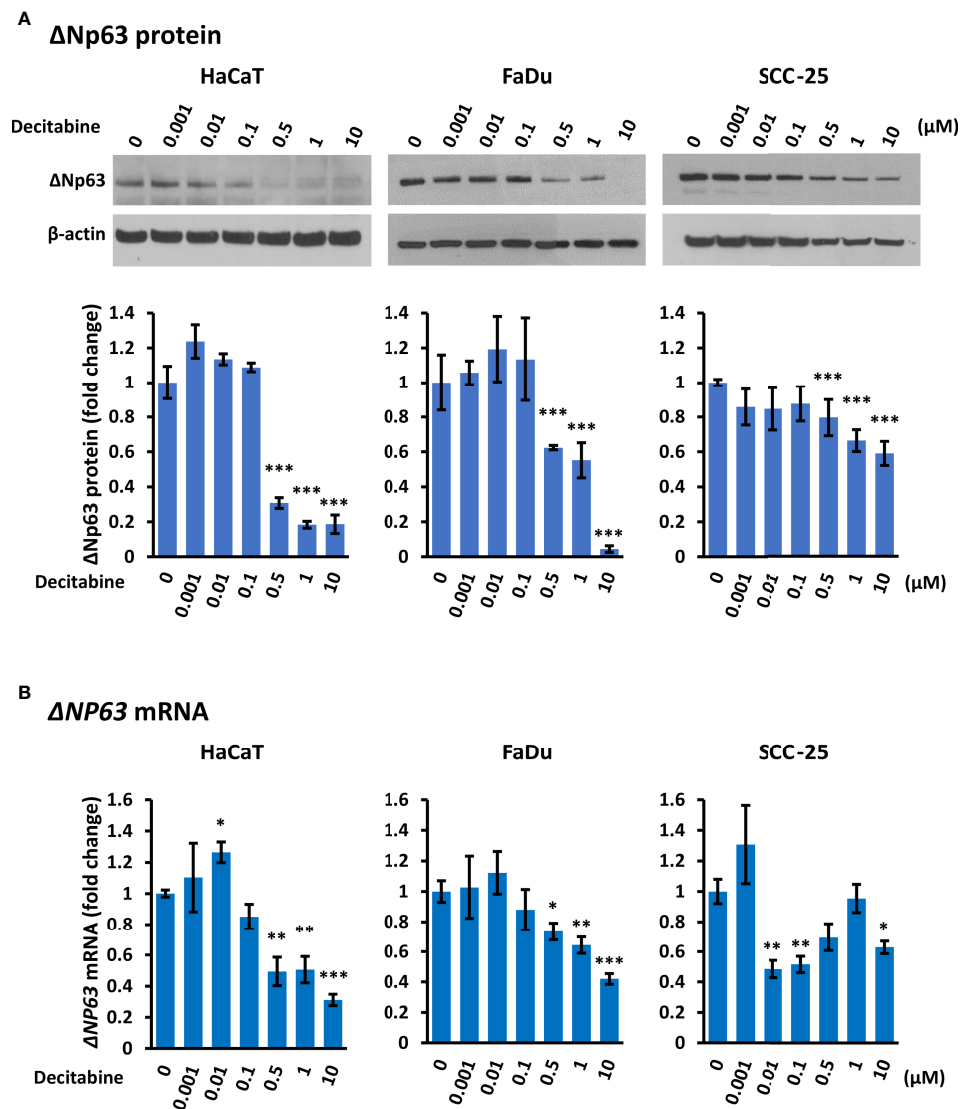


**FIGURE 1** | Decitabine increases TAp63 protein and mRNA levels in squamous cells. **(A)** Representative Western blots of TAp63 in HaCaT, FaDu and SCC-25 cells cultured in the presence of the indicated concentration of decitabine for four days.  $\beta$ -actin is shown as loading control. Densitometry data normalized to  $\beta$ -actin are shown below, indicating fold change  $\pm$  SEM compared to control cells (0, DMSO only;  $n = 3$  to 5 biological replicates, each with technical triplicates). **(B)** TAP63 mRNA levels normalized to *ACTB* mRNA ( $n = 3$  to 5 biological replicates, each with technical triplicates). Data are shown as fold change  $\pm$  SEM compared to control cells (0, DMSO only). Note the different y axis range for FaDu cells. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to control.

Based on this information, we used PCR to amplify a 134 bp region immediately upstream of P1 using bisulfite modified DNA from control cells and cells treated for four days with decitabine at concentrations that do not change (10 nM) or that do change p63 isoform levels (10  $\mu$ M). PCR products were sequenced with each primer to assess methylation changes at the three CpGs within the amplicon, designated as A, B, and C, from the most distal to the most proximal to the *TAP63* transcription start site. Under normal growth conditions, HaCaT, FaDu and SCC-25 cells showed high level cytosine methylation at all three CpG sites, with approximately 80% methylation at the distal CpG and > 90% methylation at the two sites closest to the transcription start site. High but not low

dose decitabine for four days decreased methylation at all three CpG sites (Figure 7).

We also analyzed specific DNMT1 depletion by doxycycline treatment of HaCaT and FaDu *DNMT1*-shRNA cells and sequencing of the distal CpG site. Two independent *DNMT1*-shRNA clones were used for each cell line and the data are summarized in Figure 8 by the average change of non-methylated CpG after doxycycline-mediated DNMT1 depletion for six days compared to the matched controls (*DNMT1*-shRNA HaCaT and FaDu cells without doxycycline). The magnitude of the effect of DNMT1 depletion is compared to the same average change in non-methylated CpG in parental HaCaT and FaDu cells after 10  $\mu$ M decitabine for four days compared to DMSO only



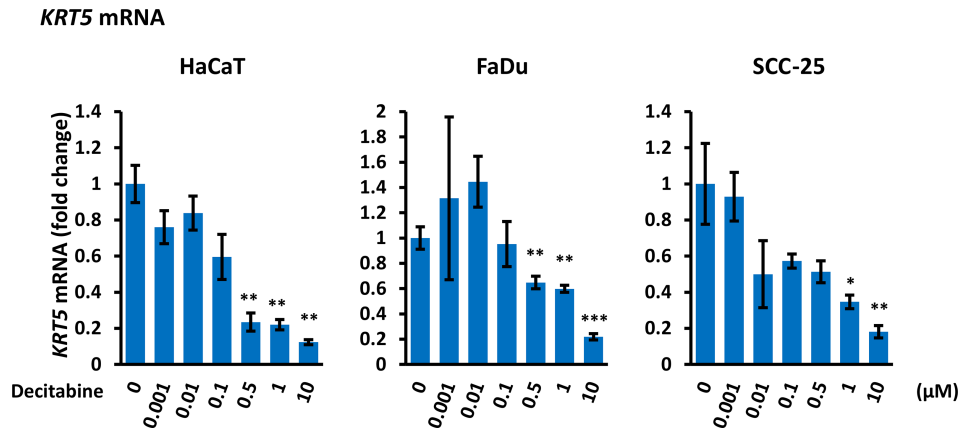
**FIGURE 2** | Decitabine reduces  $\Delta$ Np63 and mRNA levels in squamous cells. **(A)** Representative Western blots and densitometric quantitation of  $\Delta$ Np63 in HaCaT, FaDu and SCC-25 cells treated with the indicated concentrations of decitabine for four days.  $\beta$ -actin is shown as loading control. Densitometry data represent fold change  $\pm$  SEM compared to untreated cells (0, DMSO only;  $n = 3$  for each dose in each cell line) and normalized to  $\beta$ -actin. **(B)**  $\Delta$ NP63 mRNA levels normalized to *ACTB* mRNA ( $n = 3$  to 5 biological replicates, each with technical triplicates). Data are shown as fold change  $\pm$  SEM compared to untreated cells (0, DMSO only). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to control.

(derived from data in **Figures 7A, B**). *DNMT1*-shRNA caused an average 6.2% increase in demethylation in contrast to the average 23.5% increase in demethylation after decitabine (**Figure 8**).

## The TAP63 Promoter Is Highly Methylated in Primary SCC Tumor Samples

To investigate whether methylation at the *TAP63* promoter is seen only in the cell lines studied here or is a common finding in clinical SCC samples, we also analyzed publicly available methylation data in three independent patient cohorts that included normal tissue samples and tumor samples (two datasets of lung SCC and one of oropharyngeal SCC (OPSCC). Analysis of the two CpG sites

present on the array that lie immediately upstream of the *TAP63* transcription start site (cg04483101 and cg04489243) shows high level methylation in normal oropharyngeal mucosa and normal lung in all cohorts, and these values are not significantly altered in OPSCC or the lung SCC samples (**Figures 9A–C**). GSE60645 also contained 81 samples of adenocarcinoma (AC), showing maintained high level P1 methylation at cg04483101 but a decreased methylation for cg04489243 in AC compared to normal lung samples (**Figure 9A**). The levels of methylation at the two sites closest to the  $\Delta$ NP63 promoter (P2) previously reported to inversely correlate with  $\Delta$ Np63 levels (25) are also provided (cg13518031



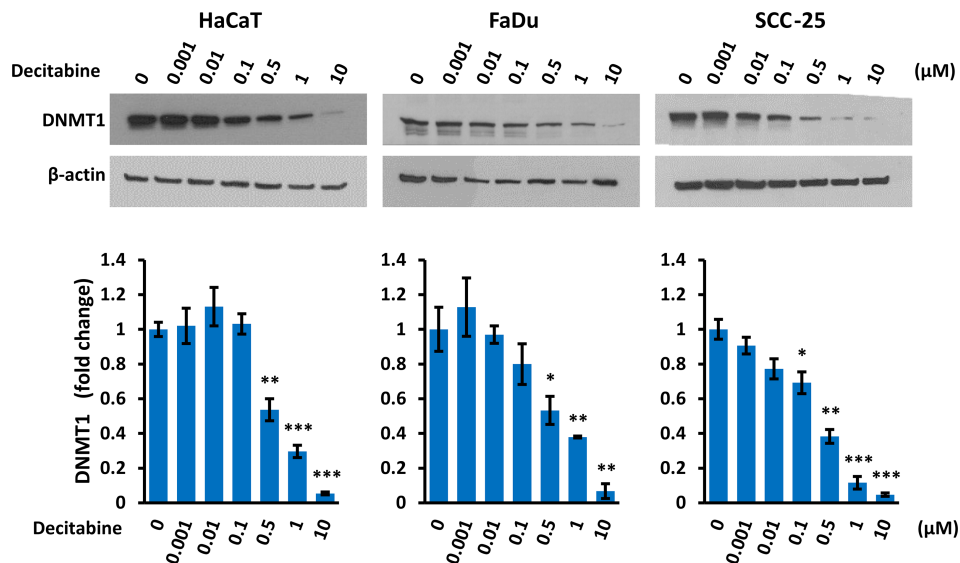
**FIGURE 3 | Decitabine decreases KRT5 mRNA.** Cells were treated with the indicated concentrations of decitabine for four days and analyzed for KRT5 mRNA by RT-qPCR. Data are shown as fold change ± SEM compared to control cells (0; DMSO only) and are normalized to ACTB mRNA (n = 3 biological replicates with technical triplicates for each concentration in each cell line). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 compared to untreated cells.

and cg06520450). Unlike P1 methylation, P2 methylation at both sites is decreased in lung SCC but is maintained at a high level in AC compared to normal lung (Figures 9A, B). These data are in keeping with observations that some lung AC show p63 positivity using pan-p63 antibodies, but are not positive with antibodies to ΔNp63 (1, 39), suggesting that lung AC may express TAp63 but not ΔNp63 due to altered DNA methylation at P1 but not P2. In the oropharynx, P2 promoter methylation levels are relatively low in normal tissue samples [reflecting the high level of ΔNp63 protein and mRNA levels in normal oral mucosa (1, 2, 16, 34)] and methylation at cg06520450 is further decreased in OPSCC (Figure 9C). These data strengthen and extend our findings of

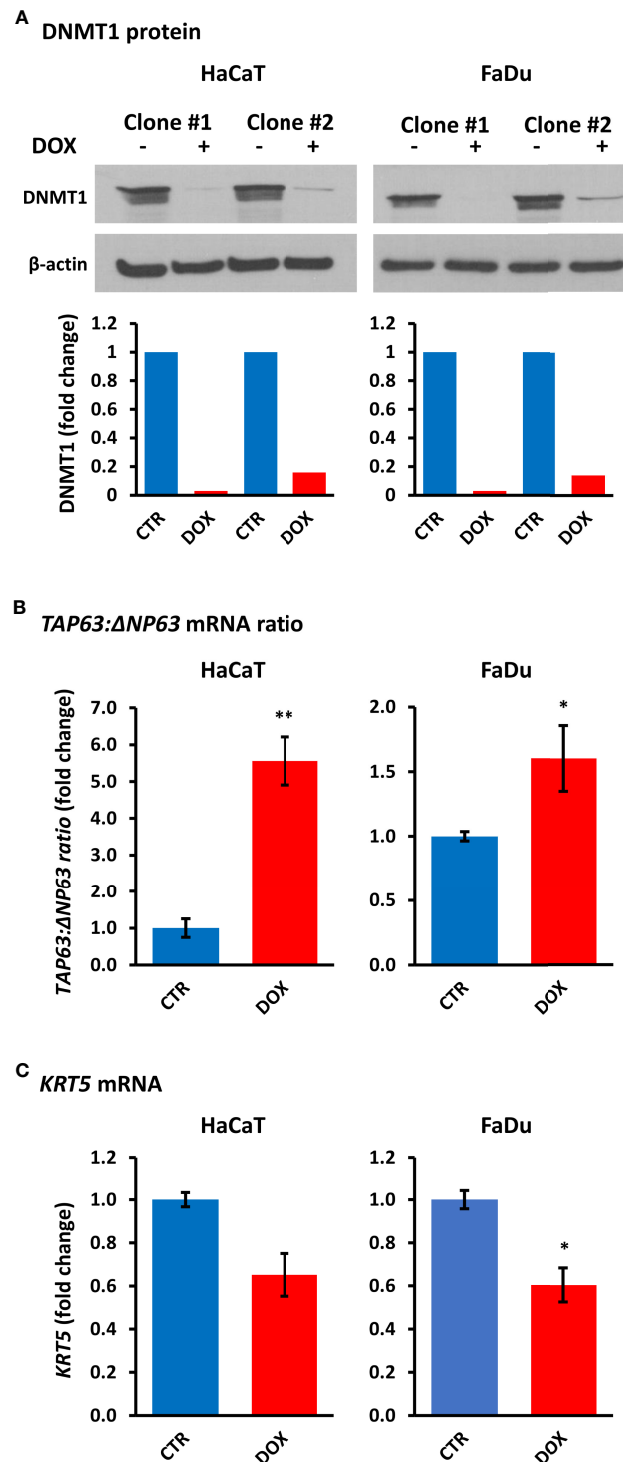
high TAP63 P1 methylation *in vitro* in squamous cell lines used in our experimental studies, implying that clinical demethylation of these sites would similarly increase TAp63 activity in SCC patients.

## DISCUSSION

Methylation of cytosine in CpG motifs is a major epigenetic modification that acts to repress gene transcription and is associated with chromatin compaction and inaccessibility. DNA methylation is carefully controlled during development

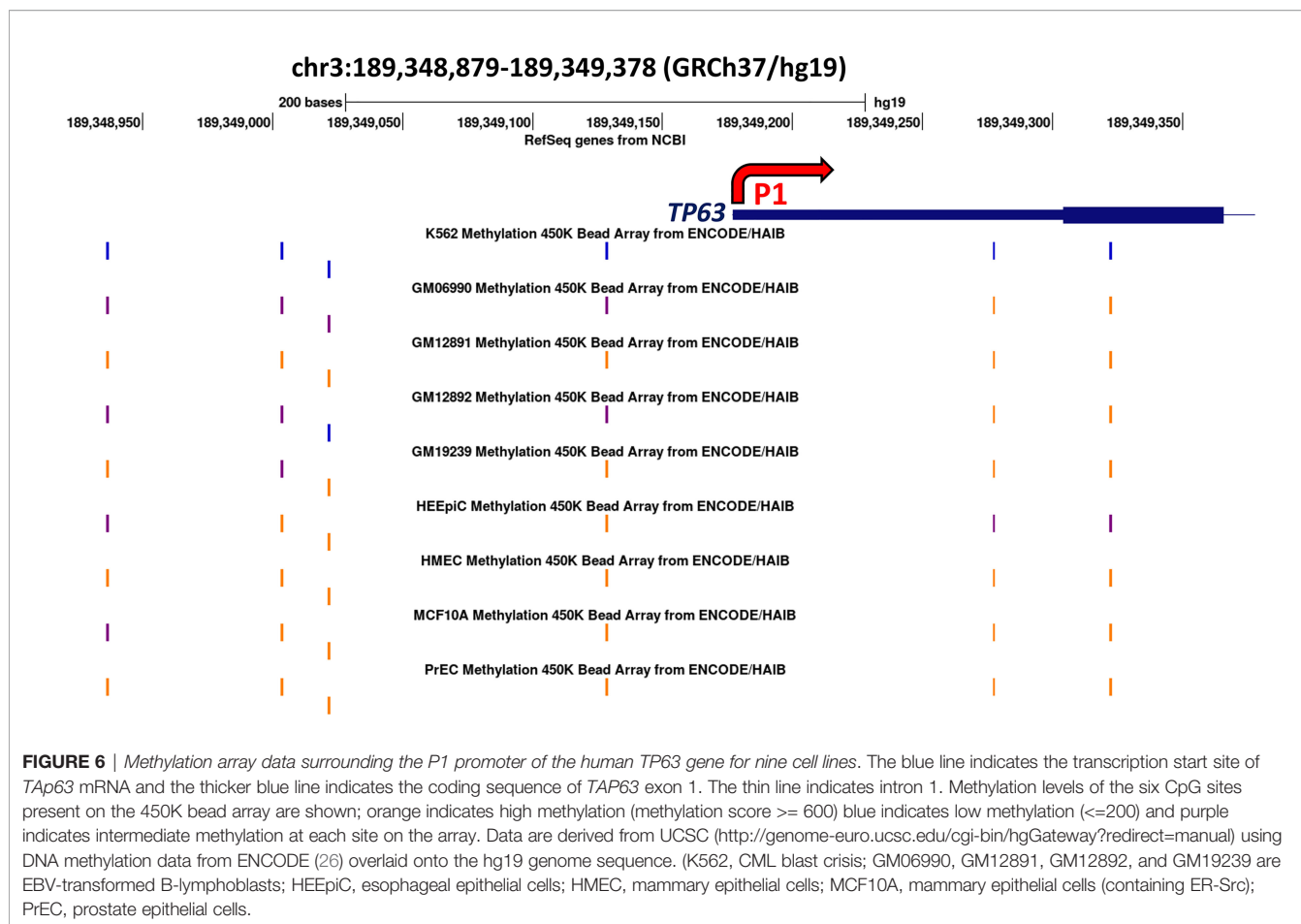


**FIGURE 4 | Decitabine decreases DNMT1.** Representative Western blots and densitometry of DNMT1 in cells treated with the indicated concentration of decitabine for four days. Data are shown as fold change ± SEM compared to untreated cells (0; DMSO only) and are normalized to β-actin (n = 3 biological replicates for each concentration in each cell line). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001 compared to control cells.



**FIGURE 5 |** *DNMT1* depletion alters *TP63* isoform mRNA levels and decreases *KRT5* mRNA levels. **(A)** Representative Western blots of DNMT1 in two independent clones of HaCaT and FaDu *DNMT1*-shRNA cells cultured for four days in the absence (-) or presence (+) of 2  $\mu$ g/ml doxycycline (DOX).  $\beta$ -actin is shown as loading control. Densitometric quantitation of DNMT1 normalized to  $\beta$ -actin is shown below for each clone. **(B)** Relative changes in the ratio of *TAP63* to  $\Delta$ *NP63* mRNA levels without induction (CTR) or after six days of 2  $\mu$ g/ml doxycycline (DOX). Data were normalized to *ACTB* mRNA for each clone in each cell line and are shown as the average change for the two independent clones for each cell line. **(C)** *KRT5* mRNA levels in response to *DNMT1*-shRNA induction. Plots show fold change  $\pm$  SEM compared to non-induced cells.  $n = 3$  to 5 biological replicates; \* $p < 0.05$ ; \*\* $p < 0.01$ .



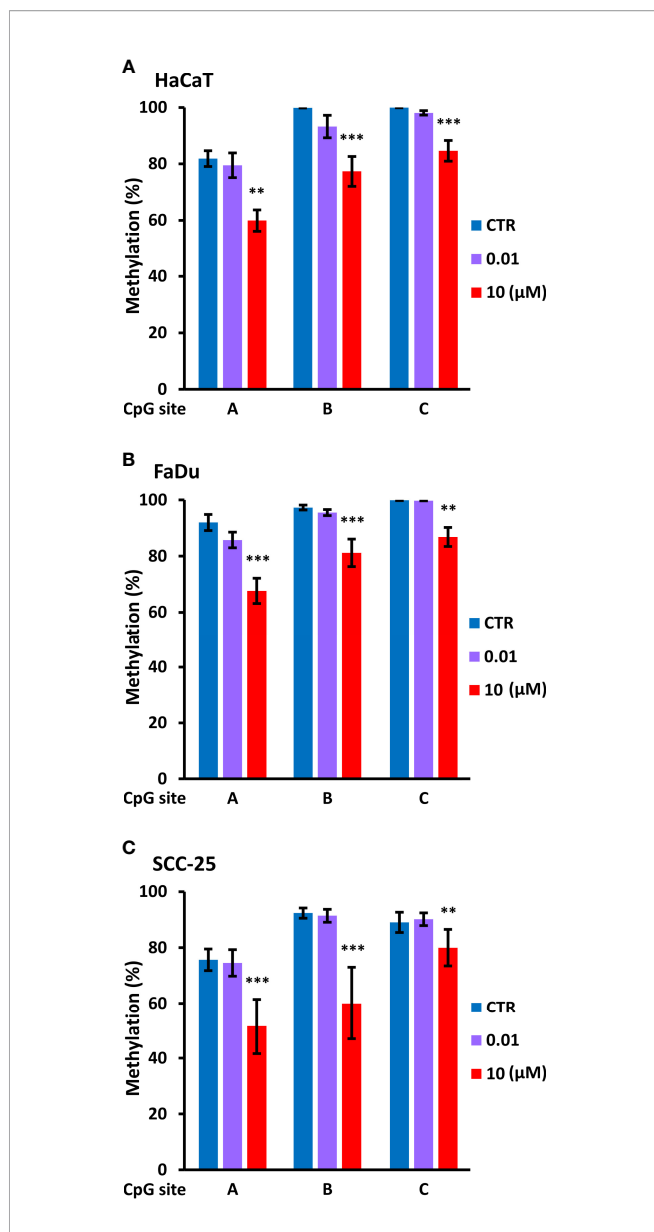


and cell differentiation and is dysregulated in many cancers, where hypermethylation of tumor suppressor genes is a common finding to inhibit their expression (40–42). In SCC, genes that are commonly hypermethylated to repress their expression and tumor-suppressive activities include CDKN2A and RASSF1 (growth arrest), MGMT (DNA repair), and DAPK (apoptosis) (43, 44). In general, CpG methylation is initiated by DNMT3 enzymes, whereas DNMT1 maintains DNA methylation as cells divide. However, this is an over-simplification and it is known that DNMT3 may also maintain and/or remodel methylation patterns (45, 46). In addition, although promoter DNA methylation is associated with transcriptional silencing, DNA methylation in the gene body is associated with gene expression (47).

Here, we examined whether p63 variants, TAp63 and  $\Delta$ Np63, are subject to regulation by DNA methylation.  $\Delta$ Np63 is present at high levels in normal squamous cells and is often increased further in SCC, sometimes but not always due to chromosome 3 amplification, with an average more than 200-fold higher level of  $\Delta$ Np63 than TAp63 mRNA in SCC (1, 25). This increase in  $\Delta$ Np63 mRNA is associated with hypomethylation at the P2 promoter and intronic sites closest to the  $\Delta$ Np63 transcription start site (23–25). In contrast, lymphomas and leukemias [that

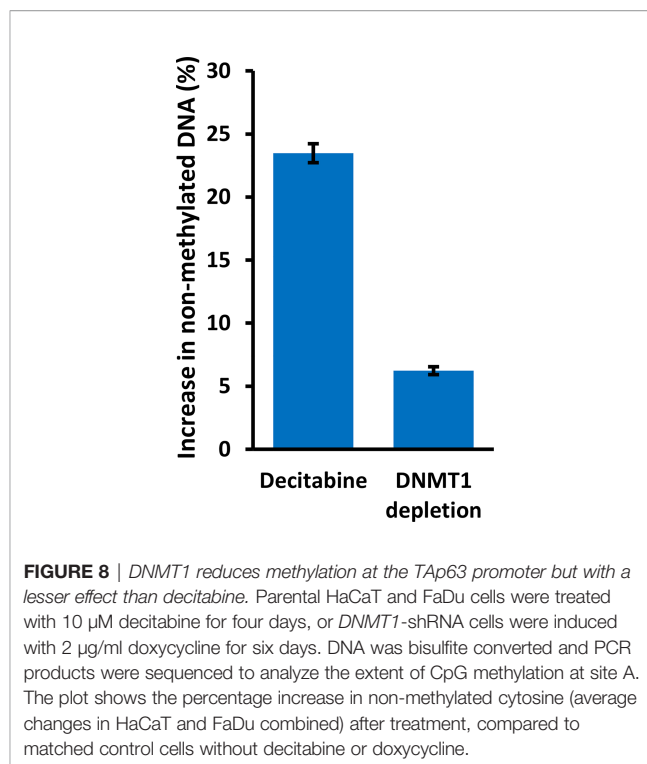
express TAp63 but not  $\Delta$ Np63 (21, 48)] exhibit hypomethylation at P1 in association with increased TP63 levels (21, 22). Therefore, we hypothesized that TAP63 may be repressed and  $\Delta$ NP63 activated in squamous cells by differential promoter methylation. Reducing DNA methylation is then expected to de-repress TAP63 transcription, allowing transcriptional activation of this tumor suppressor protein, but not to influence  $\Delta$ Np63 mRNA levels from the endogenously hypomethylated P2 regions.

In keeping with this concept, the universal demethylating agent decitabine (5-dAza) increased TAp63 protein and mRNA levels, with between 45- and 80-fold higher protein and up to 150-fold higher TAP63 mRNA after decitabine. These changes were associated with decreased cytosine methylation at each of three CpGs lying immediately upstream of the TAP63 transcription start site. At the same time,  $\Delta$ Np63 protein and mRNA levels were reduced by decitabine, shifting the isoform ratio even further towards tumor suppression. The effects on  $\Delta$ NP63 mRNA are surprising and difficult to explain by demethylation at the  $\Delta$ NP63 gene promoter. One relatively simple explanation is that activating the upstream promoter hinders transcription from the downstream promoter, and transcription from the downstream promoter is more efficient



**FIGURE 7 | Decitabine reduces DNA methylation at the *TAp63* promoter.** The indicated cell lines were untreated (CTR, DMSO only) or treated with 0.01  $\mu$ M or 10  $\mu$ M decitabine for four days. DNA was extracted and bisulfite converted, and PCR products were sequenced to analyze CpG methylation at each of the three individual CpG sites (A–C). Plots show percentage methylation ( $n = 2$  to 3 biological replicates). Statistical comparisons compare each CpG site in control cells with the same site in decitabine treated cells. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

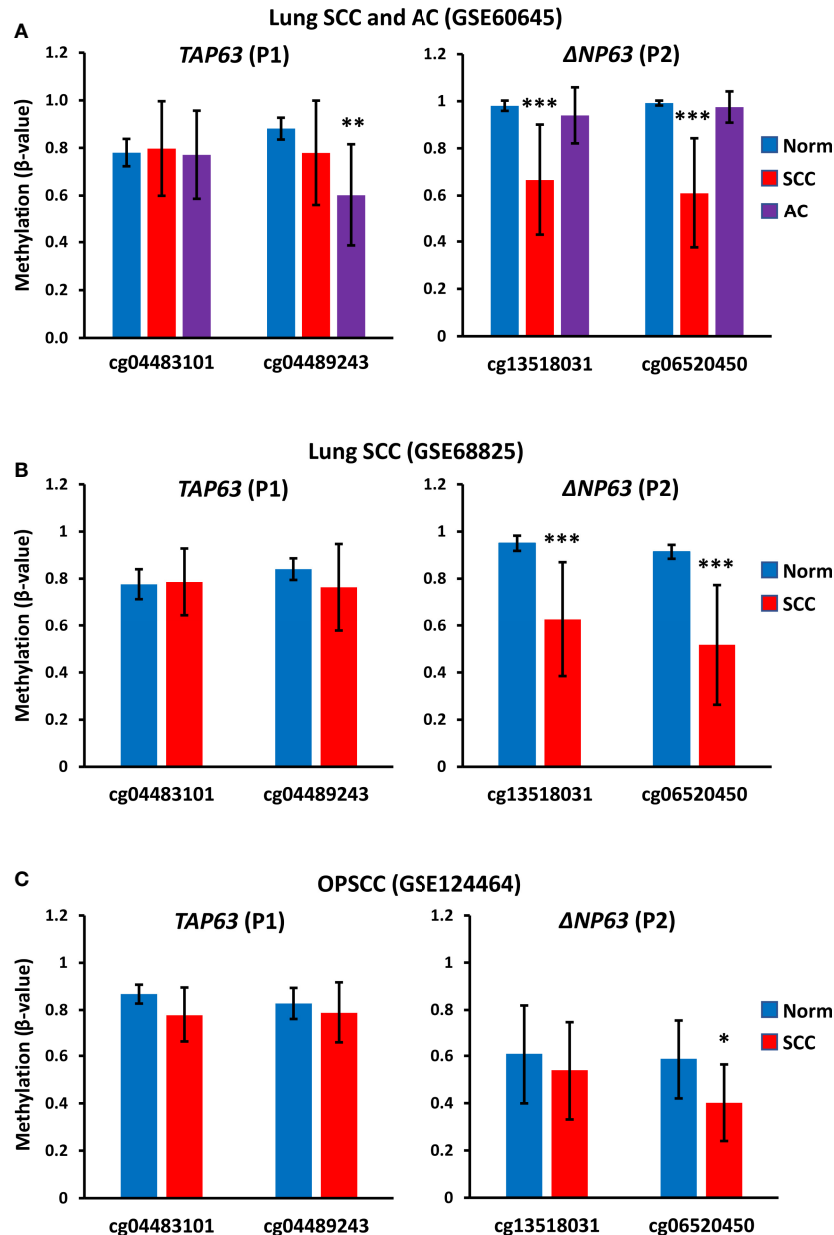
in the absence of upstream promoter usage and transcriptional read-through at the downstream promoter. This mechanism takes into account the physical difficulty in transcribing through an active downstream promoter on the same gene if the downstream promoter is occupied by transcription factors, RNA polymerase and associated proteins and can explain how *TAP63* and  $\Delta$ *NP63* mRNAs show inverse responses to methylation changes in SCC cells and the regulation of



**FIGURE 8 | DNMT1 reduces methylation at the *TAp63* promoter but with a lesser effect than decitabine.** Parental HaCaT and FaDu cells were treated with 10  $\mu$ M decitabine for four days, or *DNMT1*-shRNA cells were induced with 2  $\mu$ g/ml doxycycline for six days. DNA was bisulfite converted and PCR products were sequenced to analyze the extent of CpG methylation at site A. The plot shows the percentage increase in non-methylated cytosine (average changes in HaCaT and FaDu combined) after treatment, compared to matched control cells without decitabine or doxycycline.

isoform-specific transcription in epithelial cells *versus* lymphocytes (Figure 10A).

An alternative explanation is that  $\Delta$ Np63 acts as a transcriptional repressor of *TAp63* (49), and that DNA damage caused by high concentrations of decitabine (36, 37) reduces  $\Delta$ Np63 by ubiquitin-mediated degradation (50–52) to allow *TAP63* transcription. In addition to DNA damage as a cause of reduced  $\Delta$ Np63 protein after decitabine, this agent is known to cause growth arrest and apoptosis along with caspase activation in malignant cells including SCC cells (53–55) (and see Supplementary Figure S4 of cell growth data in our study), suggesting that these proteolytic pathways may contribute to the reduced  $\Delta$ Np63 protein levels observed after decitabine. However, reduced protein levels of  $\Delta$ Np63 by post-translational degradation through caspases, the proteasomal pathway or any other mechanism does not directly account for transcriptional lowering of  $\Delta$ *NP63* mRNA. In contrast, decitabine reciprocally changed  $\Delta$ *NP63* and *TAP63* mRNA levels, indicating transcriptional regulation rather than protein degradation as at least part of the mechanism for reduced  $\Delta$ Np63 protein levels. Moreover, although an increase in *TAP63* was reported in one study of  $\Delta$ Np63-specific knockout mice (49), this was not observed in another study using the same transgenic mice (56) or in a similar  $\Delta$ Np63-specific knockout mouse (57). Thus, whilst downregulation of  $\Delta$ Np63 protein by DNA damage or apoptosis may contribute to *TAp63* induction, the evidence is weak. We also showed variable induction of DNA damage after decitabine, measured by  $\gamma$ -H2AX, and the level of damage did not correlate with the effects of decitabine on *TAp63* or  $\Delta$ Np63. In addition, the *DNMT1*-shRNA experiments indicate a direct

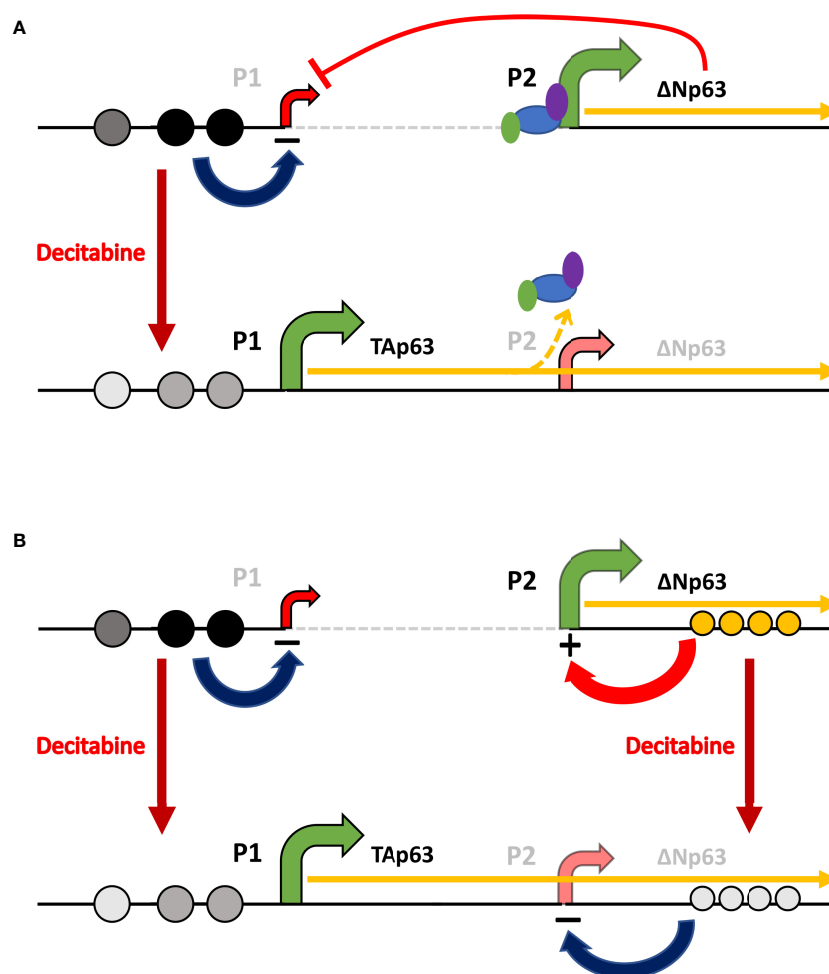


**FIGURE 9** | *TAP63* promoter methylation levels in primary SCC tumor samples. Graphs show data for two probes (cg04483101 and cg04489243) that lie immediately upstream of the *TAP63* promoter (P1) (see Figure 6) and for two probes closest to the *ΔNP63* promoter (P2) identified in (25). **(A)** Data from GSE60645 containing normal lung (Norm; n=12), lung SCC (n=22) and lung adenocarcinoma (AC, n= 81). **(B)** Data from the cancer genome atlas analysis of lung SCC (GSE68825; normal lung (n=43) and lung SCC (n=96). **(C)** Data from GSE124464 of oropharyngeal SCC (OPSCC) (n=64) and normal oral mucosa (n=5). Graphs represent mean  $\beta$ -values  $\pm$  SD.  $\beta$ -values range from 0 (no methylation) to 1 (complete methylation). \*p < 0.05, \*\*\*p < 0.001, Mann-Whitney test.

effect of reduced methylation in causing reduced  $\Delta$ Np63 protein levels.

Another potential explanation for decreased  $\Delta$ NP63 mRNA after decitabine is altered maintenance of DNA hydroxymethylation in enhancer regions (Figure 10B). DNMT3A promotes hydroxymethylation in the center of active enhancers, while DNMT3B promotes methylation across the gene body, and both are essential for  $\Delta$ NP63 transcription (58). Against

this theory, hypomethylation at the P2 promoter is seen in SCC in association with  $\Delta$ Np63 (23, 24), and hypomethylation of two intron sites nearest the  $\Delta$ NP63 transcription start site is also associated with the levels of  $\Delta$ NP63 transcription in SCC (25). Thus, the available evidence indicates that demethylation at these sites increases  $\Delta$ Np63, whereas we find that global demethylation, which would include demethylation at these sites, decreases  $\Delta$ Np63. However, it is also important to note that DNA methylation is



**FIGURE 10** | Schematic of the potential mechanisms involved in reciprocal regulation of *TAp63* and  $\Delta Np63$ . **(A)** In squamous cells, where  $\Delta Np63$  is highly expressed from the P2 promoter, methylation of CpG sites (dark gray or black circles) at P1 inhibits *TAP63* transcription and reduced methylation at these sites after decitabine (lighter gray circles) allows *TAP63* production. As the *TAP63* mRNA is elongated, it unavoidably transcribes through the P2 region thereby reducing transcription initiation at this site, perhaps by displacing transcription factors and RNA Polymerase II (colored ovals). **(B)** In addition, methylation/hydroxy-methylation within the gene body (orange circles) may activate  $\Delta Np63$  transcription, which is reduced after decitabine.

interrelated with histone methylation/acetylation, providing a further layer of complexity to epigenetic regulation (58–60). Additional investigations of DNA methylation/hydroxymethylation and histone methylation/acetylation profiles at the  $\Delta NP63$  promoter, enhancers and gene body will be required to distinguish between these potential mechanisms of action.

Our experiments using *DNMT1* depletion with two *DNMT1*-shRNAs that effectively reduced *DNMT1* levels showed that both constructs increased *TAP63* and decreased  $\Delta NP63$  and *KRT5* mRNA levels, albeit with lesser changes than decitabine. These data provide independent validation for the role of DNA hypomethylation in activating *TAp63* and repressing  $\Delta Np63$  in SCC. That the effect of *DNMT1* depletion is lower than that of decitabine indicates that molecules other than *DNMT1* are involved. This lower level of demethylation is an expected result, reflecting the continued presence of *DNMT1* protein

after mRNA inhibition together with the maintained activity of other *DNMT* enzymes that would not be operative in the presence of decitabine but are not inhibited by *DNMT1*-specific shRNA and are therefore able to maintain and/or renew methylation at previously modified CpGs to at least some extent (45).

The alternative hypotheses for reciprocal isoform regulation after demethylation with decitabine are not mutually exclusive and it may be that a combination of mechanisms is involved. Whilst the precise mechanism(s) involved in the inverse relationships of *TAp63* and  $\Delta Np63$  in SCC is uncertain, it will be important to determine whether de-methylation induces *TAp63* in all cancers, including tumor types lacking p63 expression but containing mutant p53, where *TAp63* may replace p53 activity for therapeutic gain (10, 11, 13), or if *TAp63* activation occurs only in cells with an already active

*TP63* gene. These latter tumor types include not only SCC but also other common epithelial tumors such as breast, bladder and prostate that contain  $\Delta Np63$  either in the majority of cells or in the specific subset of cancer stem cells (1, 2, 8, 32, 61–63). In this respect, it has been reported that decitabine alters *TAP63* and  $\Delta Np63$  mRNA levels in an inconsistent and non-reciprocal fashion in bladder cancer cell lines (64), different from our results in SCC. However, it must be noted that the findings of high level *TAP63* and absent  $\Delta Np63$  in normal bladder cells are the opposite of a subsequent study showing abundant  $\Delta Np63$  mRNA and protein and absent or minimal *TAP63* mRNA and undetectable TAp63 protein in normal urothelial cells (65). Similarly, analyses of primary bladder cancer samples and a larger panel of bladder cancer cell lines also showed absent or extremely low levels of TAp63 protein and mRNA compared to  $\Delta Np63$  protein and mRNA, including some of the cell lines previously reported to contain abundant *TAP63* mRNA (65, 66), casting doubt on the validity of the findings after decitabine. Clearly, further experiments using the improved isoform-specific reagents now available will be required to determine the expression patterns of TP63 isoforms in this cancer type.

In conclusion, we have shown that inhibiting DNA methylation causes a switch in the relative levels of p63 isoforms, potentially converting  $\Delta Np63$ -mediated tumor promotion and therapy resistance (7–9, 12, 13) towards TAp63-mediated tumor suppression (3, 4, 6, 10, 11). By identifying DNA hypermethylation at the *TAP63* promoter in SCC, these data add this gene to the list of tumor suppressors that are epigenetically silenced in malignancy (40–42). Moreover, our data indicate that demethylation at this locus simultaneously reduces transcription of the related  $\Delta Np63$  isoform, indicating the potential for additive effects in SCC. In addition to the direct effects of p63 isoform switching for cell growth/survival, reciprocal TAp63 and  $\Delta Np63$  regulation would be expected to further increase the response to cancer therapeutics compared to the effects of  $\Delta Np63$  reduction or TAp63 induction alone (9–11, 13, 67).

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## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

ZP and PC designed the research approaches. ZP, VH and VT performed experiments. ZP, BV and PC analyzed and interpreted data. ZP and PC wrote the manuscript. All authors contributed to manuscript revision and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2022.924354/full#supplementary-material>

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**Conflict of Interest:** BV is a consultant for Moravian Biotechnology.

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