

# DLEC1, a 3p tumor suppressor, represses NF- $\kappa$ B signaling and is methylated in prostate cancer

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## Abstract

Deleted in lung and esophageal cancer 1 (*DLEC1*), located at 3p22-p21.3, is involved in the carcinogenesis of multiple cancers, but its role in prostate cancer (PrCa) remains unclear. Here, we studied the epigenetic alteration of *DLEC1* and its functions in prostate cancer. We found that *DLEC1* was highly expressed in normal prostate tissues, normal prostatic epithelium cell line (RWPE-1), and benign prostatic hyperplasia cell line (BPH-1), but frequently downregulated by promoter methylation in PrCa cell lines. Pharmacologic demethylation could restore *DLEC1* expression. *DLEC1* was downregulated in prostate tumor tissues compared with their adjacent non-malignant tissues. *DLEC1* was methylated in 76/110 primary tumors, but rarely in benign prostatic hyperplasia tissues. *DLEC1* methylation was associated with higher PSA levels

( $p=0.016$ ), higher Gleason scores ( $p=0.015$ ), and more advanced tumor stages ( $p=0.003$ ). Furthermore, *DLEC1* methylation was detected in 11/30 urine sediment samples from PrCa patients, but seldom in ones from BPH patients. Ectopic expression of *DLEC1* inhibited the colony formation of PrCa cells, through inducing cell apoptosis. *DLEC1* also suppressed PrCa cell migration. Moreover, *DLEC1* inhibited NF- $\kappa$ B transcription activity in PrCa and HEK293 cells. Taken together, our data demonstrate that *DLEC1* functions as a tumor suppressor but is frequently methylated in prostate cancer. *DLEC1* methylation is associated with prostate cancer progression, which could be a non-invasive epigenetic biomarker for PrCa diagnosis.

## Key messages

- Promoter methylation of *DLEC1* is a potential prognostic biomarker for PrCa.
- *DLEC1*, a functional tumor suppressor, is frequently methylated in PrCa.
- *DLEC1* suppresses prostate cancer growth and metastatic behavior.
- *DLEC1* mediates tumor-suppressive activities through NF- $\kappa$ B signaling.

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**Keywords** *DLEC1* · Prostate cancer · Tumor suppressor gene · Methylation · NF- $\kappa$ B

## Abbreviations

Aza	5-Aza-2-deoxycytidine
ATCC	The American Type Culture Collection
BGS	Bisulfite genomic sequence
BPH	Benign prostatic hyperplasia
DLEC1	Deleted in lung and esophageal cancer
DRE	Digital rectal examination
FBS	Fetal bovine serum
IHC	Immunohistochemistry

MSP	Methylation specific polymerase chain reaction
PrCa	Prostate cancer
RT-PCR	Reverse-transcription polymerase chain reaction
RP	Radical prostatectomy
TSA	Trichostatin A
TSG	Tumor suppressor gene
TUR	Transurethral resection

## Introduction

Prostate cancer (PrCa), as the most common male malignancy, accounted for the second leading cause of cancer death in men in the USA [1]. With an aging population, changing in lifestyle factors, and better screening methods, the incidence of PrCa detected in China has increased in recent years, which severely affects the life quality and life expectancy of men >50 years old. Epigenetic inactivation of tumor suppressor genes (TSG) by promoter methylation is well recognized to play a crucial role in prostate carcinogenesis [2–4], while the underlying molecular mechanisms involved in PrCa pathogenesis remain elusive.

Multiple TSGs with aberrant promoter methylation have been found in PrCa, such as *GSTP1*, *RASSF1A*, *RAR $\beta$* , *APC*, *AR*, *ESR1*, and *ESR2* [5–11]. These genes are associated with key cellular pathways, including DNA repair, cell cycle control, hormone response, signal transduction, and tumor invasion [6]. The aberrant promoter methylation of *GSTP1*, *PTGS2* has been shown related to PrCa initiation and progression, suggesting its potential as good biomarkers for early detection and prognosis prediction [10, 12]. Identification of TSGs with aberrant promoter methylation involved in PrCa carcinogenesis will benefit for uncovering molecular heterogeneity of PrCa and providing insights into its biomarker discovery. Due to the poor specificity of current methods (e.g., serum PSA), there is an urgent clinical need to identify more novel biomarkers to improve PrCa diagnosis and treatment.

Deleted in lung and esophageal cancer 1 (*DLEC1*) is located at 3p22-p21.3 and encodes a 1755-amino acid polypeptide. That chromosomal region has been shown to be a “hot spot” associated with multiple types of cancer development, including prostate cancer [13]. *DLEC1* was initially identified as a tumor suppressor gene for esophageal and lung cancers, being frequently inactivated by hemi-zygote deletion and promoter methylation [14]. More recent studies also demonstrated that *DLEC1* is silenced by promoter methylation in other common types of tumors, including ovarian, hepatocellular, gastric, renal, and breast carcinomas [15–19]. Furthermore, *DLEC1* methylation was identified as an independent marker of poor survival in patients with non-small cell lung carcinoma [20]. All these studies suggested that *DLEC1* serves as a tumor

suppressor gene and plays an important role in cancer development and progression, while there are no reports about *DLEC1* methylation and its biological functions in prostate cancer.

In this study, we investigated whether *DLEC1* was a newly identified TSG with aberrant promoter methylation for PrCa. To get this conclusion, we examined *DLEC1* expression and methylation in prostate tumor cell lines, primary tumors, and urine sediments and analyzed the relationship between its methylation and clinicopathological features in PrCa patients. We also investigated its tumor-suppressive functions and related mechanisms in PrCa cells.

## Materials and methods

### Tissue specimen collection

All prostate specimens, including 110 cases of primary PrCa samples and 26 cases of benign prostatic hyperplasia (BPH) samples, were obtained from the urology department, Peking University First Hospital, Beijing, People’s Republic of China, from January 2011 to December 2013. Clinicopathological features of patients were listed in Table 1. All prostate cancer specimens were collected from radical prostatectomy (RP) without adjuvant therapy, and BPH specimens were collected from transurethral resection (TUR), after obtaining patients’ written content, and defined by two urological pathology physicians. Tumor tissues were selected in areas rich in neoplastic cells. Normal kidney and prostate tissue RNA samples were purchased commercially (Stratagene, La Jolla, CA).

**Table 1** Association of clinicopathological features with *DLEC1* methylation status in prostate cancer

Clinicopathological features	No. of methylated (%)	No. of unmethylated (%)	<i>p</i> value
Overall	75 (68.2)	35 (31.8)	
Age (years)	66.27±6.28	65.31±6.46	0.585
PSA level, ng/ml	15.97±1.31	11.74±1.65	0.016
Gleason score			
6	2 (33.3)	4 (66.7)	0.015
7	51 (66.2)	26 (33.8)	
8	6 (60.0)	4 (40.0)	
9	16 (94.1)	1 (5.9)	
Stage			
T2	21 (51.2)	20 (48.8)	0.003
T3	54 (78.3)	15 (21.7)	

## Cell lines and drug treatment

Prostate cancer cell lines studied include C4-2, PC3, DU145, and LNCap. The immortalized human prostatic epithelial cell line RWPE-1 and benign prostatic hyperplasia cell line BPH-1 were used as controls. All of these cell lines were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The PrCa cell lines and benign prostatic hyperplasia cell line were routinely maintained in RPMI1640 supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1 % penicillin G, and 1 % streptomycin. RWPE-1 was cultured in keratinocyte serum-free medium (K-SFM) supplied with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA). All these cell lines were cultured at 37 °C in humidified CO<sub>2</sub> (5 %) incubator. For demethylation treatment, PrCa cells were diluted to 1×10<sup>5</sup>/ml and incubated overnight. Fresh medium containing 10 μM 5-aza-2-deoxycytidine (Aza) (Sigma, St. Louis, MO) was changed every 24 h. After Aza treatment for 3 days or an additional 24-h treatment with 100-nM Trichostatin A (TSA), the cells were harvested for further analysis.

## DNA and RNA extraction

Total RNA was isolated from tissues and cell lines using TRI Reagent (Molecular Research Center, Cincinnati, OH), as previously described [21]. Genomic DNA was extracted according to the protocol supplied by QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

## Semi-quantitative RT-PCR and real-time PCR analysis

Semi-quantitative real-time PCR (RT-PCR) was used to detect gene expression with GoTaq® Green Master Mix (Promega, Madison, WI, USA). The primers used were *DLEC1A*: 5'-TCCGGATCCCTTGAAACAC-3' and *DLEC1B*: 5'-CCTCAGGAACAATTCGGTAA-3', which were performed for 36 cycles (94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s). Real-time PCR was performed to detect *DLEC1* expression in primary prostate tumors, according to the manufacturer's protocol (HT7500 system, Applied Biosystems). The primers used were *DLEC1LCF*: 5'-CACGGAAGAGGCATCG-3' and *DLEC1LCR*: 5'-ATAAGCCGGAAGTAGTGT-3'. Real-time PCR was done for 45 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s). *GAPDH* was used as an internal control.

## Bisulfite treatment and promoter methylation analysis

Sodium bisulfite conversion of genomic DNA was performed as described previously [22, 23]. Methylation-specific PCR (MSP) was used to detect promoter methylation of *DLEC1*.

The MSP primers used for *DLEC1* were *DLEC1m1*: 5'-GTTTCGTAGTTCGGTTTCGTC-3', *DLEC1m2*: 5'-CGAAATATCTTAAATACGCAACG-3', *DLEC1u1*: 5'-TAGTTTTGTAGTTGGTTTTGTT-3', and *DLEC1u2*: 50-ACAAAATATCTTAAATACACAACA-3'. The detailed methylation information was analyzed by bisulfite genomic sequencing (BGS). The BGS primers were *DLEC1BGS56*: 5'-GGGTTTAGTAGTTTGTAGTTAG-3' and *DLEC1BGS4*: 5'-CAACTACAACCCCAAATCCTAA-3'. The bisulfite-treated DNA was used as a template for MSP and BGS. Forty cycles of PCR were performed using GoTaq®Hot Start Green Master Mix (Promega, Madison, WI). The BGS PCR products were cloned into the pEASY-T5 zero vectors (TransGen Biotech Co., Ltd, Beijing), and 5–8 colonies were randomly chosen and sequenced.

## Urine sediments preparation and MSP analysis

The first voided urine samples after digital rectal examination (DRE) were collected from PrCa patients undergoing RP and BPH patients undergoing TUR. Urine sediments were immediately prepared after collection to obtain good sample quality according to Hessels's report [24]. In brief, the urine was centrifuged at 4 °C at 1000×g for 10 min. The obtained urine sediments was suspended in 1 ml of PBS buffer and transferred to a 1.5-ml microcentrifuge tube. After centrifuging at 4 °C at 10,000×g for 10 min, sediment was obtained and then stored at –70 °C. For bisulfite conversion, 1–13 μl of sediments were used and directly converted to bisulfited DNA according to the handbook of EZ DNA Methylation-Direct Kit (Zymo research, Inc., Irvine, CA). One microliter of bisulfite-treated DNA was used for MSP, conducted at 95 °C for 10 min, then 41 cycles (94 °C, 30 s; 58 °C, 30 s; 72 °C, 30 s), followed by 72 °C for 5 min.

## Immunohistochemistry

Nine paraffin-embedding primary prostate tumor and their paired adjacent non-malignant tissues were used to examine *DLEC1* expression by immunohistochemistry (IHC). The slides were deparaffinized in xylene, rehydrated in a concentration decreasing ethanol series, subsequently boiled for 2 min in citrate buffer (pH 6.0) for antigen retrieval, and stained by anti-*DLEC1* antibody produced in rabbit, a Prestige Antibody (Sigma, St. Louis, MO) in a 1:100 dilution. Antibody binding was visualized using ChemMate Envision kit (Dako, Hamburg, Germany). Immunostaining was assessed by two urological pathologists (He Qun and Shen Qi) in a blinded manner and scored from 0 to 3 depending on the intensity of staining (none=0; weak=1; moderate=2; strong=3).

### Colony formation assay

To assess tumor cell clonogenicity, colony formation assay was used. Briefly,  $1.5 \times 10^5$  cells were plated in a 12-well plate and incubated overnight. Then the cells were transfected with *DLEC1* expression construct or the empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 h, the transfectants were replated in triplicate in a 6-well plate and selected with G418 for 10–15 days. The best selective concentration of G418 for PC3, DU145, and LNCap cells were 0.1, 0.2, and 0.3 mg/ml, respectively. Visible colonies ( $\geq 50$  cells) were stained with gentian violet after methanol fixation and counted.

### Annexin V Apoptosis assay

The PrCa cells transfected with *DLEC1*-expressing vectors or empty vectors were harvested after transfection for 48 h. Then the cells were fixed in 75 % ethanol, stained with Annexin V-FITC and 7-AAD (BD Biosciences, Bedford, MA), and sorted by Becton Dickinson LSRII (BD Biosciences, Bedford, MA) according to the manufacturer's protocol. The data were analyzed using Flow Jo software (BD Biosciences), and the Annexin V-FITC-positive cells were counted as apoptotic cells.

### Wound-healing assay

The cell motility was assessed by scratch wound healing assay. DU145 cells ( $2\text{--}3 \times 10^6$  per well) were plated in a 6-well plate for 1 day and then transfected with *DLEC1* expression vectors or the control vectors for 24 h. The cell layers were washed with PBS after carefully scratching by sterile tips. After incubation for 0, 24, and 48 h, photos were taken. The assays were performed in triplicate.

### Transwell migration assay

The PrCa cells (DU145 and LNCap) were placed on the upper layer of a cell permeable membrane after transfection with *DLEC1* expression vector or control vector for 24 h. Following another 24–48 h incubation, the cells migrated through the membrane were stained with 1 % Crystal Violet and counted.

### Western blot

PrCa cells were transiently transfected with *DLEC1* expression vector or empty vector for 48 h then lysed in ice-cold lysis buffer containing 137 nmol/L of NaCl, 2 mmol/L of EDTA, 1 % Triton X, 10 % glycerol, 50 mmol/L of NaF, 1 mmol/L of DTT, and a protease

inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with the following antibodies after blocking with 5 % milk: cleaved PARP (Cell Signaling Technology Inc., Beverly, MA), Flag (Sigma, St. Louis, MO), and NF- $\kappa$ B/p65 (Cell Signaling Technology Inc., Beverly, MA). The blots were visualized using the enhanced chemiluminescence (ECL) Western blotting detection system (Thermo Scientific).

### Dual-luciferase reporter assay

Luciferase reporters of several key signaling pathways, including *p53bs-luc*, *p21p-luc*, *NF- $\kappa$ Bbs-luc*, and *MYCp-luc*, were used to screen for a signaling pathway modulated by *DLEC1*. Cells were transiently co-transfected with *DLEC1* expression vector, phRL-TK, the luciferase reporters, or empty vector. After 48 h, the cells were lysed and the luciferase activities were measured using Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega, Madison, WI, USA). To normalize transfection efficiency, phRL-TK luciferase activities were measured as an internal control. At least three independent experiments were carried out, and each experiment was repeated in triplicates.

### Statistical analysis

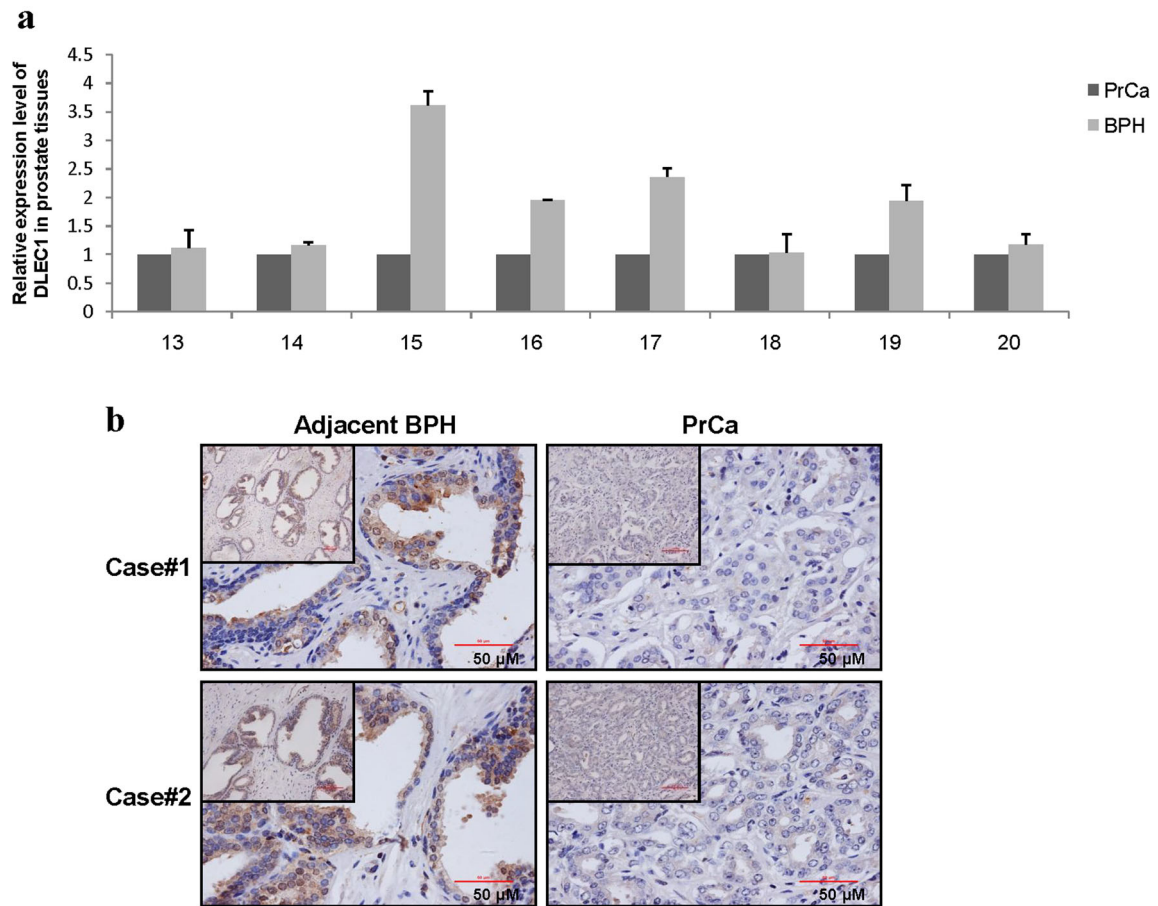
Statistical analyses were done using the two-tailed *t* test, Fisher exact test, or chi-square test to determine *p* values, in which  $<0.05$  is considered significant.

## Results

### *DLEC1* is downregulated in primary prostate tumor samples

We firstly evaluated *DLEC1* expression in prostate tumor tissues. Quantitative RT-PCR was used to evaluate messenger RNA (mRNA) expression level of *DLEC1* in eight cases of primary prostate cancer and their paired adjacent non-malignant tissues. Compared with the adjacent non-malignant tissues, *DLEC1* was significantly downregulated in prostate tumor tissues ( $p < 0.05$ ; Fig. 1a). The protein expression of *DLEC1* was further detected by IHC in another nine cases of paired prostate tumor samples. Results showed that *DLEC1* was highly expressed in adjacent non-malignant tissues, but reduced in prostate tumor tissues ( $p < 0.05$ ; Fig. 1b). These results indicate that *DLEC1* may be a potential TSG for prostate cancer.





**Fig. 1** *DLEC1* mRNA and protein expression in prostate tissues. **a** Real-time PCR was performed to detect mRNA expression levels of *DLEC1* in eight paired human prostate cancer tissues and their adjacent non-

malignant prostate tissues. \* $p < 0.05$ . **b** Expression of *DLEC1* in representative adjacent non-malignant tissues and prostate cancer tissues by immunohistochemistry. \*\* $p < 0.01$

*DLEC1* is frequently silenced by promoter methylation in PrCa cell lines

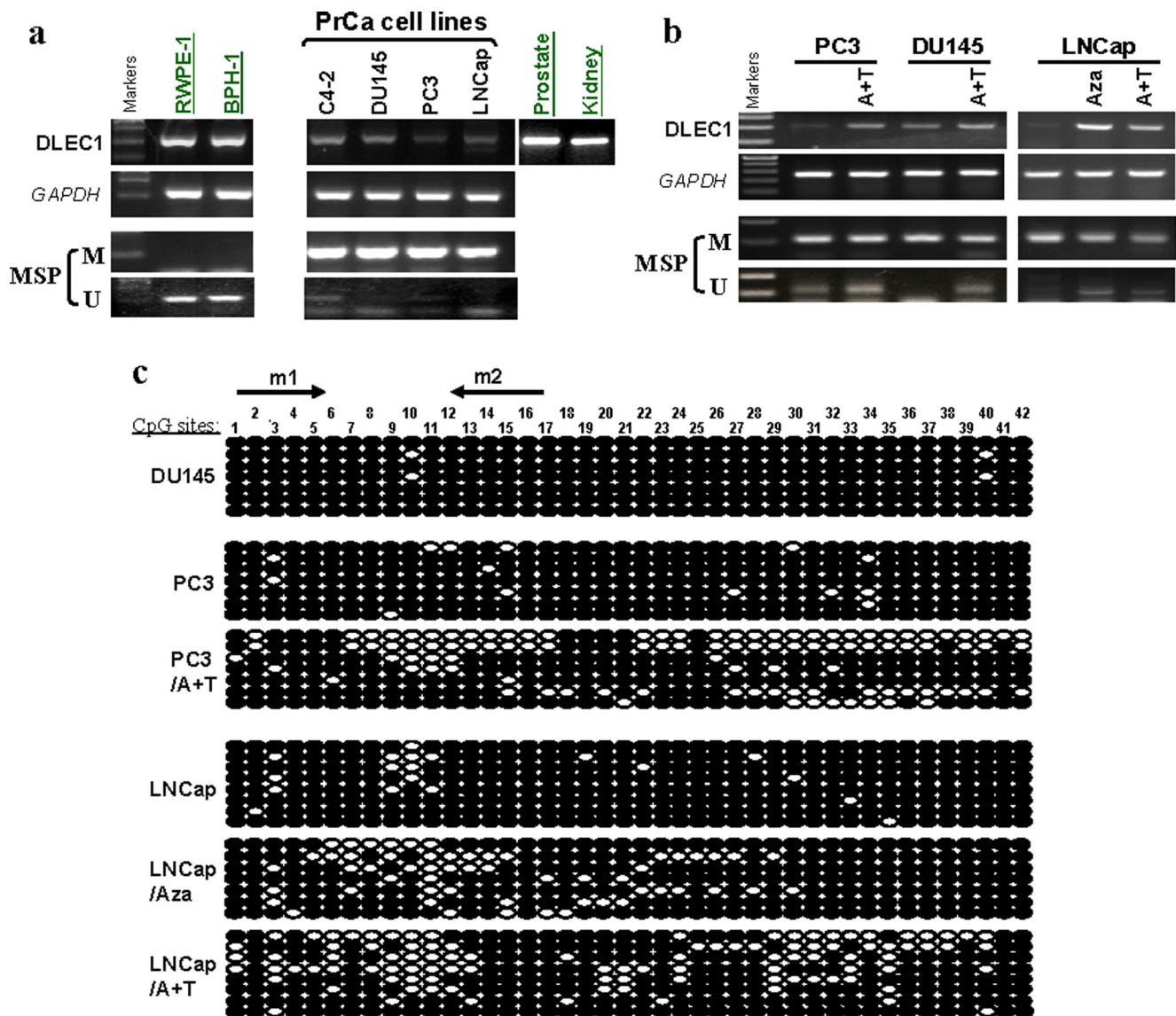
We then examined mRNA expression level of *DLEC1* in PrCa cell lines (C4-2, DU145, PC3, and LNCaP) and found that *DLEC1* was silenced or reduced in these cell lines. In contrast, *DLEC1* was highly expressed in normal human prostate tissue, immortalized prostate epithelial cell line RWPE-1, and benign prostatic hyperplasia cell line BPH-1 (Fig. 2a). As described in our previous report, there is a typical CpG island at promoter and exon 1 region of *DLEC1* [18]. We thus hypothesize that the promoter methylation may be responsible for *DLEC1* downregulation or silencing in PrCa cells. We next examined *DLEC1* methylation in PrCa cell lines. MSP showed that *DLEC1* was completely methylated in DU145, C4-2, LNCap, and PC3 cells, whereas unmethylated in RWPE-1 and BPH-1 cells (Fig. 2a), consistent with its silencing. BGS analysis further confirmed the MSP results (Fig. 2c). These data suggest that aberrant promoter methylation contributes to *DLEC1* silencing in PrCa cells.

Pharmacologic demethylation restores *DLEC1* expression in PrCa cell lines

To determine whether *DLEC1* expression was directly repressed by promoter methylation, PrCa cell lines with methylated and reduced *DLEC1* (DU145, PC3, and LNCap) were treated with DNA methylation inhibitor Aza alone or combined with the HDAC inhibitor TSA. As shown in Fig. 2, *DLEC1* expression was restored after demethylation treatments, accompanied by an increase of unmethylated alleles and a decrease of methylated alleles. Detailed BGS analysis further confirmed *DLEC1* demethylation (Fig. 2c). These results suggest that promoter methylation directly mediates *DLEC1* downregulation in PrCa cells.

*DLEC1* methylation in primary prostate tumors

*DLEC1* methylation was further analyzed in 110 primary PrCa samples and 26 BPH samples. MSP analysis showed that *DLEC1* methylation was detected in 76



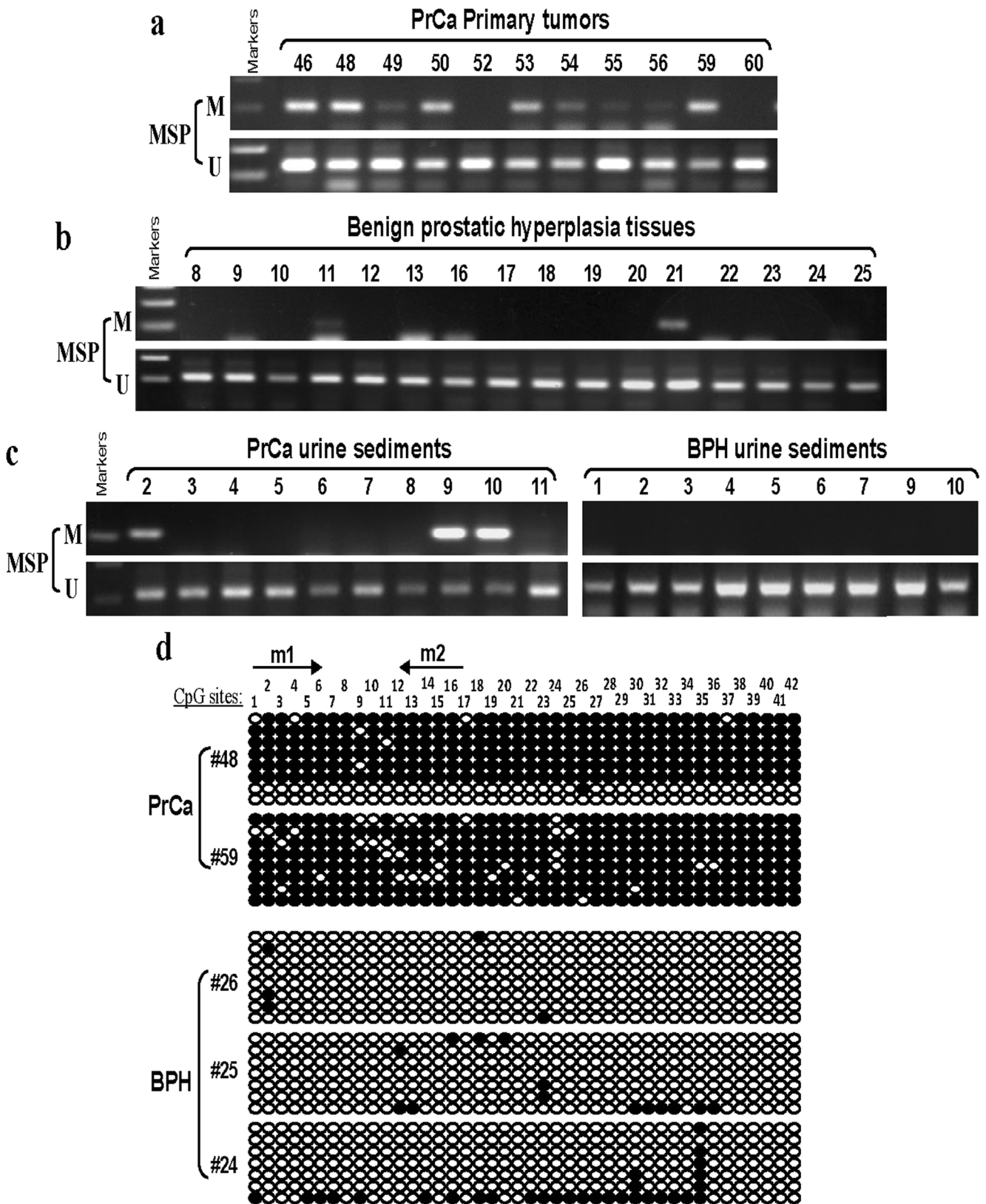
**Fig. 2** **a** *DLEC1* is frequently silenced and methylated in PrCa cell lines (C4-2, PC3, DU145, and LNCap), but highly expressed and unmethylated in immortalized normal prostate epithelial cell line RWPE-1 and benign prostatic hyperplasia cell line BPH-1. *M* methylated, *U* unmethylated. **b** Pharmacological demethylation with

Aza (*A*) alone or combined with TSA (*A+T*) restored *DLEC1* expression in PrCa cell lines (PC3, DU145, and LNCap). **c** Detailed BGS analyses of the *DLEC1* promoter in PrCa cell lines with or without demethylation treatment. *Filled circle* represents the methylated while *open circle* the unmethylated

out of 110 PrCa tissues, but seldom in BPH tissues (2 out of 26) (Fig. 3a, b). In addition, the high-resolution BGS results showed densely methylated CpG sites in two representative primary tumors, but only scattered methylated alleles in three representative BPH tissues (Fig. 3d). Thus, *DLEC1* methylation is a frequent event in PrCa tumorigenesis. We further detected *DLEC1* methylation in urine samples from 30 PrCa patients and 30 BPH patients. Results showed that *DLEC1* was methylated in 11 out of 30 urine sediments of PrCa samples but rarely in BPH urine sediments samples (Fig. 3c), indicating that *DLEC1* methylation is a potential non-invasive marker for PrCa.

*DLEC1* methylation is associated with poor prognosis of PrCa patients

We further evaluated the relationship between *DLEC1* methylation and clinicopathological features of PrCa patients. As shown in Table 1, there was no significant difference between *DLEC1* methylation and age, while *DLEC1* methylation was detected in patients with higher PSA levels ( $p=0.016$ ), higher Gleason scores ( $p=0.015$ ), and more advanced tumor stages ( $p=0.003$ ), which showed a statistically significant difference. These results indicate that *DLEC1* methylation is correlated with poor prognosis of PrCa.



**Fig. 3** MSP analysis of *DLEC1* methylation in representative **a** prostate tumor (*PrCa*) tissues, **b** benign prostatic hyperplasia (*BPH*) tissues, and **c** urine sediments. **d** High-resolution methylation mapping of CpG sites in

the *DLEC1* promoter by BGS in representative *PrCa* tissues and *BPH* tissues



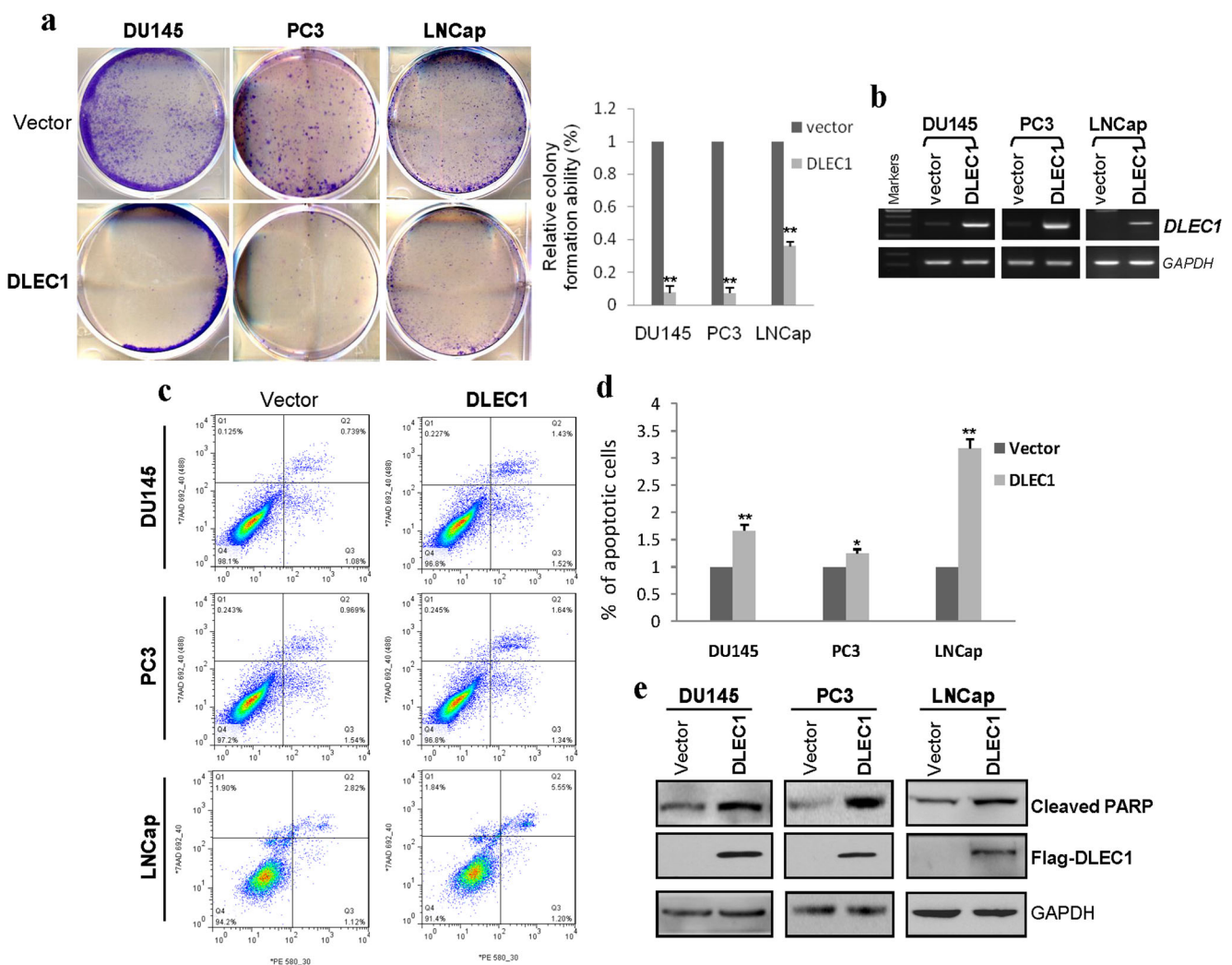
### *DLEC1* is a functional tumor suppressor in PrCa cells

To investigate the tumor-suppressive functions of *DLEC1* in PrCa, we examined the colonogenicity of PrCa cells by colony formation assay. *DLEC1* expression vector and empty vector were transfected to PrCa cell lines (PC3, DU145, and LNCap) with reduced *DLEC1*. The abilities of colony formation were significantly inhibited in *DLEC1*-expressing prostate tumor cells compared with control ones (Fig. 4a). Re-expression of *DLEC1* mRNA in these cells was confirmed by RT-PCR (Fig. 4b).

To examine whether *DLEC1*-mediated growth inhibition was the result of apoptosis, cell apoptosis was determined in PrCa cell lines (DU145, PC3, and LNCap) by Annexin V-PE and 7-AAD double staining. Results

showed that the number of total apoptotic cells significantly increased in the cells transiently transfected with *DLEC1* expression vector compared with their controls (Fig. 4c, d). We further checked the classic apoptotic marker cleaved PARP by Western blot and found that cleaved PARP was obviously increased in *DLEC1*-expressing DU145, PC3, and LNCap cells (Fig. 4e).

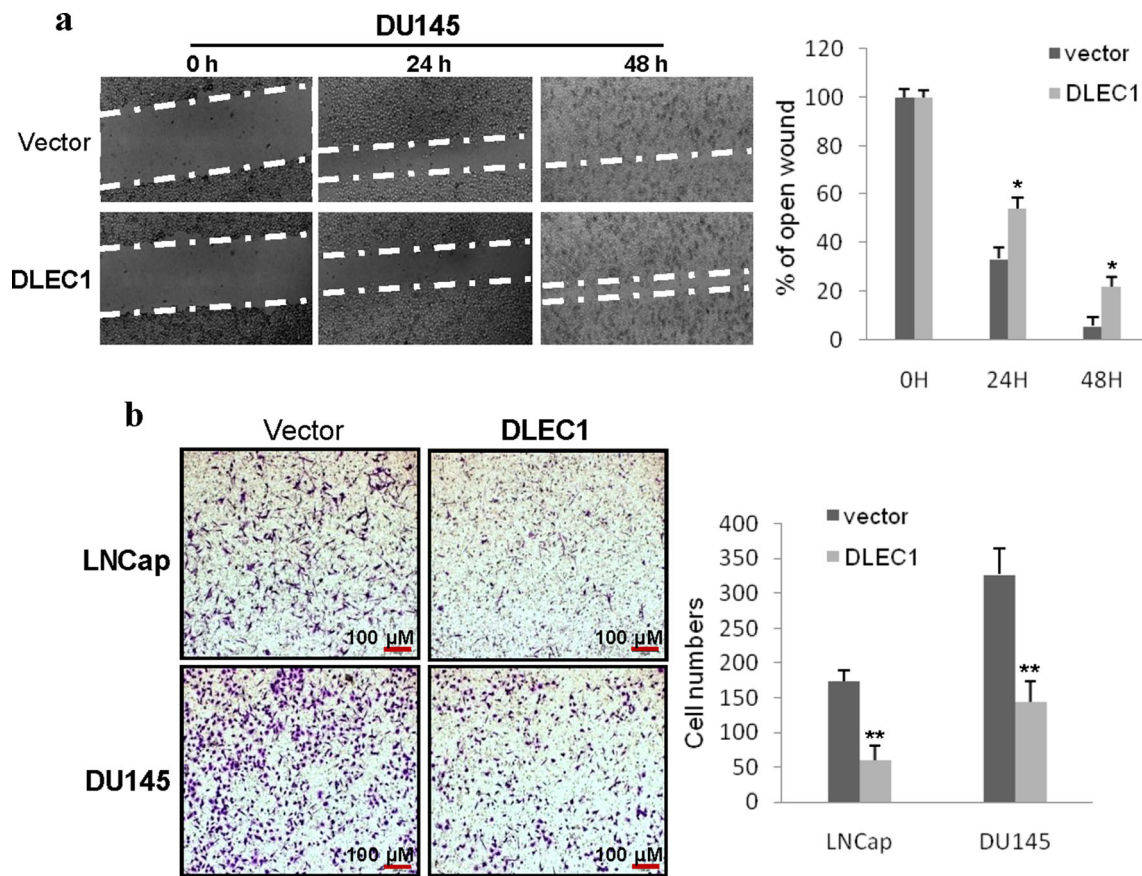
We further evaluated the migration ability of *DLEC1* in PrCa cells by wound healing and transwell migration assays. The scratch wound healing assay showed that *DLEC1*-expressing DU145 cells closed the wound much slower than controls (Fig. 5a). The transwell migration assay showed that ectopic expression of *DLEC1* significantly inhibited the migration abilities of DU145 and LNCap cells. These data suggest that *DLEC1* acts as a



**Fig. 4** Tumor suppressive functions of *DLEC1* in PrCa cells. **a** *DLEC1* inhibits tumor cell growth by colony formation assay. Quantitative analyses of colony numbers in three replicates were shown as values of mean  $\pm$  s.d., \*\* $p < 0.01$ . **b** *DLEC1* expression was detected by RT-PCR in *DLEC1*- or vector- transfected cells, with *GAPDH* as a control. **c** *DLEC1* induced apoptosis of prostate tumor cells (PC3, DU145, and LNCap) by

Annexin V-PE and 7-AAD double staining. **d** Representative flow cytometry plots. \* $p < 0.05$ , \*\* $p < 0.01$ . **e** Western blot showed that increased cleaved PARP in *DLEC1*-expressing DU145, PC3, and LNCap cells. *DLEC1* expression was probed by Flag-tag antibody, *GAPDH* was used as a control





**Fig. 5** a Wound healing assay showed that *DLEC1* suppressed migration in *DLEC1*-transfected DU145 cells. Pictures were captured at 0, 24, or 48 h after the scratch. \* $p < 0.05$ . b *DLEC1* inhibited migration ability of

DU145 and LNCap cells as measured by Transwell migration assay. \*\* $p < 0.01$

functional TSG in PrCa cells through inhibiting tumor cell colonogenicity and migration.

#### *DLEC1* inhibits *NF-κB* signaling in PrCa cells

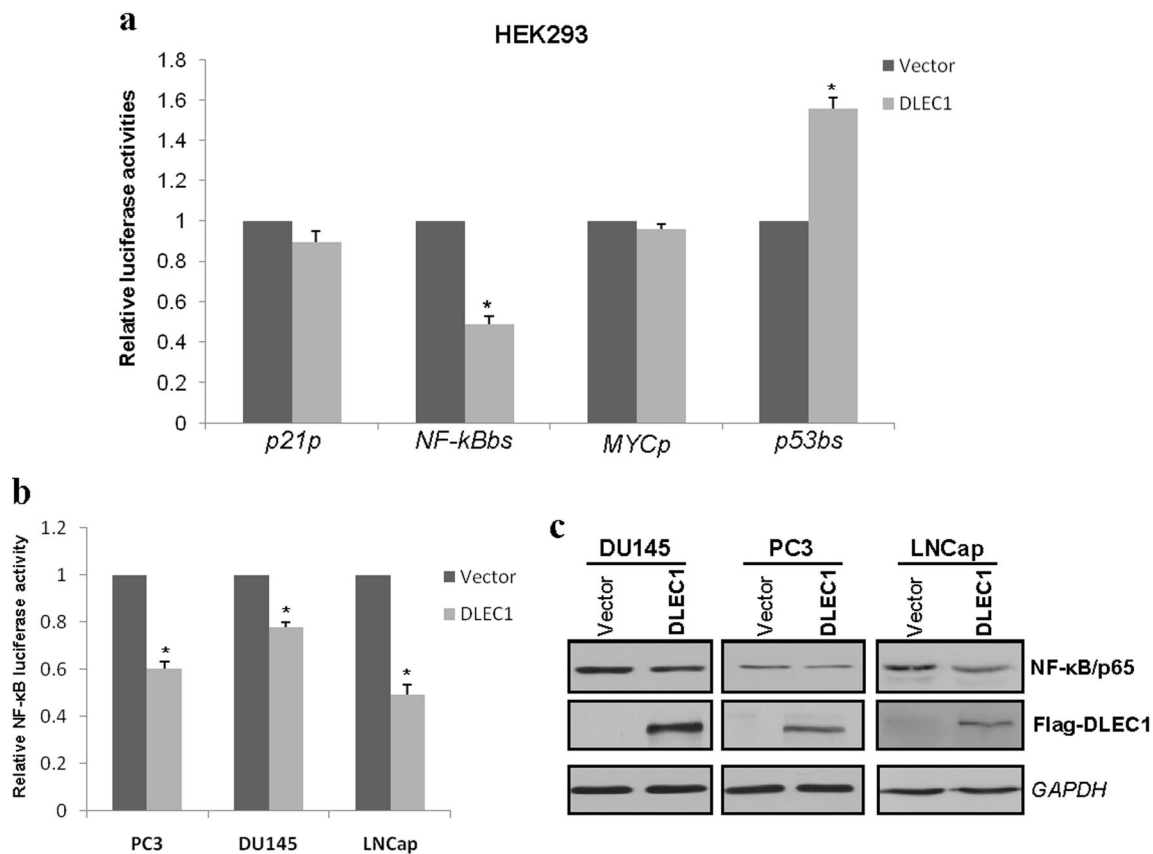
To elucidate the molecular mechanisms of *DLEC1* in tumor suppression in PrCa, we examined several signaling pathways involved in apoptosis (*p53bs*), cell cycle (*p21*), and cell proliferation (*NF-κBs*, *MYCp*) in HEK293 by luciferase reporter assay. *DLEC1* could activate *p53* signaling and downregulate *NF-κB* signaling pathway in HEK293 cells (Fig. 6a). We then detected *NF-κB* transcriptional activity in PrCa cell lines and found that *NF-κB* signaling was also significantly inhibited in *DLEC1*-expressing DU145, PC3, and LNCap cells (Fig. 6b), together with a decreased *NF-κB* (*p65*) protein level (Fig. 6c).

#### Discussion

Despite improved screening methods for PrCa and advances in the treatment of progressive disease, PrCa

remains the most common cause of cancer death for men. Thereby, early detection and monitoring of this disease may improve its prognosis and treatment. DNA methylation has been recognized as a hallmark of cancer, which helps to uncover the molecular mechanisms underlying tumor development and provide molecular screening strategies to identify cancer-specific diagnostic and prognostic tools [25, 26]. Recently, DNA methylation detection in tissues and body fluids could serve as feasible tumor markers [27–30].

In this study, we identified *DLEC1* as a functional TSG silenced by promoter methylation in PrCa, which implies a potential biomarker for PrCa. Silencing of *DLEC1* by promoter CpG methylation has been identified to be involved in multiple carcinogenesis including nasopharyngeal, esophagus, lung, gastric, hepatocellular, colon, and renal [14–19], suggesting that it could be an epigenetic biomarker for the molecular diagnosis and prognosis prediction of these tumors. We found that *DLEC1* was frequently silenced by promoter methylation in PrCa cell lines but not in BPH cells and normal prostate epithelial cells. After being treated with demethylation drugs in *DLEC1*-methylated PrCa cells, we found that



**Fig. 6** *DLEC1* inhibited *NF-κB* signaling in PrCa cells. **a** The effect of *DLEC1* on several signaling pathways in HEK293 cells as measured by luciferase reporter assay. **b** *NF-κB* transcriptional activity was inhibited in *DLEC1*-expressing PrCa cells by dual-luciferase reporter assay in three

replicates,  $*p < 0.05$ . **c** *NF-κB/p65* downregulation was detected by Western blot in *DLEC1*-expressing DU145, PC3, and LNCap cells. *DLEC1* expression was detected by Flag antibody. *GAPDH* was used as a control

pharmacologic demethylation could reactivate its expression. *DLEC1* methylation was also frequently detected in PrCa patients, but rarely in BPH tissues. Moreover, *DLEC1* methylation in PrCa was associated with serum PSA level, Gleason score, and advanced stage, suggesting its potential as a prognostic predictor for PrCa. Remarkably, *DLEC1* methylation was examined in PrCa urine sediments samples, indicating that it could be a non-invasive tumor marker for PrCa. Furthermore, a large-scale clinical validation in body fluids from PrCa patients is currently underway in our department to investigate the role of *DLEC1* in prostate cancer metastasis and confirm it as a methylation marker for PrCa diagnosis.

Recent studies showed that *DLEC1* as a TSG participates in the pathogenesis of multiple cancers and regulates cell proliferation [14, 17]. In this study, the tumor-suppressive functions of *DLEC1* were found in PrCa cells, including the inhibition of cell proliferation, migration, and induction of apoptosis. As the programmed cell death can also be measured by loss of colony-forming ability, thus, *DLEC1* likely inhibits colony formation of prostate tumor cells partially through inducing apoptosis. Moreover, the molecular mechanism of

*DLEC1* was further explored. *DLEC1* inhibited *NF-κB* signaling in PrCa cells, suggesting that *DLEC1* may exert its tumor-suppressive function through regulating *NF-κB* signaling pathway. Aberrant *NF-κB* activation has been reported to be associated with the initiation and progression of many cancers, including prostate cancer. *NF-κB* signaling activation was implicated in the progression of prostate cancer, which might be employed as a novel therapeutic target [31–34]. As LNCaP/C4-2 is an ideal model of prostate cancer progression, a further in-depth investigation of the mechanisms of *DLEC1* in prostate cancer metastasis will be carried out using LNCaP/C4-2 model.

In summary, we found that *DLEC1* is a functional TSG in PrCa, and its frequent methylation is associated with tumor poor prognosis of PrCa, which could serve as a potential tumor marker for PrCa.

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