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DUSP26 negatively affects the proliferation of epithelial cells, an effect not mediated by dephosphorylation of MAPKs

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ABSTRACT

Dual specificity phosphatases are characterised by their ability to dephosphorylate both phosphotyrosine and phosphoserine/threonine residues within the one substrate. The aim of this study was to characterise the phosphatase activity of the atypical dual specificity phosphatase, DUSP26 on MAP kinases, and to determine its expression, regulation and function in cancer cells. Overexpression and knockdown of DUSP26 in epithelial cells and in vitro phosphatase assays were used to demonstrate that, contrary to several published reports, DUSP26 does not act as a dual specificity phosphatase on ERK, JNK or p38 MAPKs. However, overexpression of DUSP26 in MCF10A epithelial cells suppressed colony formation and acinar growth in 3D culture, effects dependent on its phosphatase activity, while knockdown of DUSP26 in HOSE17.1 cells enhanced colony formation and cellular proliferation. *DUSP26* mRNA expression was reduced in neuroblastoma, brain and ovarian cancer cell lines. Consistent with epigenetic silencing of DUSP26, expression was enhanced by treatment of cells with 5-aza-2-deoxycitidine and trichostatin A, and a CpG island upstream of the *DUSP26* transcriptional start site was variably methylated in cancer cell lines. Together, these results help to clarify confusion in the literature relating to DUSP26 substrate specificity and support recent reports that substrates other than MAPKs are the primary substrates of this phosphatase. In addition, they indicate that DUSP26 may function as a tumour suppressor in particular cancers.

1. Introduction

Dual specificity phosphatases (DUSPs) comprise a large and heterogeneous subgroup of the type-1, cysteine-based protein tyrosine phosphatase (PTP) superfamily [1,2]. The unique feature that characterises DUSPs is their ability to dephosphorylate both tyrosine (Tyr) and serine/threonine (Ser/Thr) residues within the one substrate. The 61 DUSPs can be further divided into subgroups based on sequence similarity. One of the most extensively characterised subgroups comprises the mitogen-activated protein kinase phosphatases (MKPs). Individual MKPs exhibit substrate selectivity towards MAPKs ERK, JNK and/or p38 and by dephosphorylating the TxY activation motif of these kinases act as antagonists of associated signalling cascades. These phosphatases have been the subject of several reviews [3–10]. Interestingly, some MKPs are encoded at chromosomal regions that exhibit loss of heterozygosity, and these MKPs exhibit reduced expression in particular cancers. These include DUSP6, DUSP7, DUSP10, DUSP4 and DUSP16, and functional studies support a role for some of these MKPs as tumour suppressors [11–17].

The largest DUSP subgroup comprises the atypical DUSPs, which share some characteristics of the MKPs but are most similar to the low molecular weight phosphatase VH1, present in vaccinia virus [18]. Atypical DUSPs contain the consensus DUSP catalytic domain but lack the N-terminal CH2/Cdc25 homology domain found in MKPs. Originally, atypical DUSPs were presumed to play a similar role to MKPs, as critical regulators of MAPK cascades. Although true for some, it is now clear that many atypical DUSPs have alternative substrates and distinct physiological roles (reviewed in refs. [19,20]).

DUSP26 is a 24-kDa atypical DUSP first described in 2005 [21]. Since then, although several studies have been published regarding its MAPK substrate specificity and biological role [22–28], most are contradictory and a consensus has yet to emerge. Briefly, DUSP26 has been reported to function as a p38-specific phosphatase [21,23], as an ERK-specific phosphatase [22] and also as a positive regulator of JNK and p38 [24]. Another independent study reported that DUSP26 is not a MAPK phosphatase but instead modulates the PI3K/Akt pathway by an unknown mechanism [25]. Subsequent studies by the same group demonstrated that DUSP26 mediated reduced phosphorylation of ERK5, ERK1/2, Src and Akt in PC12 cells in response to EGF stimulation, possibly via downregulation of PI3K activity [26].

Abbreviations: TSA, Trichostatin A; 5'AZA, 5-aza-2-deoxycitidine; DUSP, dual specificity phosphatase; PTP, protein tyrosine phosphatase; MAPK, mitogen-activated protein kinase; pNPP, *p*-nitrophenyl phosphate; GFP, green fluorescent protein

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Recently, kinesin family member 3a (Kif3a) was identified as a novel DUSP26-interacting partner, and DUSP26 was shown to dephosphorylate kinesin superfamily-associated protein-3 (Kap3), a subunit of the Kif3a motor complex [27].

The biological roles of DUSP26 also require clarification. In anaplastic thyroid carcinoma, DUSP26 over expression enhances colony formation, while knockdown inhibits cell growth and increases apoptosis, results consistent with an oncogenic role [23]. Furthermore, in an independent study, overexpression of DUSP26 in PC12 cells inhibited NGF-induced neurite outgrowth, which suggests that DUSP26 may help to maintain an undifferentiated phenotype, an effect also characteristic of an oncogene. However, in contrast, overexpression of DUSP26 sensitised PC12 cells to cisplatin-induced apoptosis, consistent with a role as a tumour suppressor [25]. This hypothesis is supported by two recent studies, one demonstrating that DUSP26 acts via downregulation of the EGFR [26] and the other by enhancement of cell–cell adhesion [27].

DUSP26 is highly expressed in neuro-endocrine tissue and is absent in several human cancer cell lines including those derived from brain tumours, neuroblastomas [21,24,25], squamous tumours, lymphoma, colon cancer, non-small cell lung cancer, melanoma, prostatic carcinoma, breast carcinoma and cervical carcinoma [21,25]. Loss of expression also occurs in primary human glioblastoma tumours [27]. Interestingly, *DUSP26* is located at chromosomal region 8p12, which exhibits loss of heterozygosity in several malignancies, including bladder [17], breast [29], prostate [14], and ovarian cancers [15,30,31]. These data are consistent with a role for DUSP26 as a tumour suppressor. However, DUSP26 may have contrasting roles in specific cancers, as *DUSP26* is amplified and overexpressed in anaplastic thyroid carcinoma [23].

It is evident that further work is required to clarify the biochemical and biological activities of DUSP26. In this paper we present data regarding the biological function and epigenetic regulation of DUSP26 that support a tumour suppressive role. However, the complete absence of demonstrable activity against ERK, JNK or p38 MAPKs, even in cells where suppression of proliferation by DUSP26 is observed, indicates that 'atypical' functions must underpin this biological role.

2. Materials and methods

2.1. Cell culture

COS7 (ATCC# CRL-1651), HEK293 cells (ATCC# CRL-1573) and Phoenix cells (ATCC# SD3444) were cultured in DMEM (Invitrogen, Mount Waverley, VIC, Australia) supplemented with 10% foetal calf serum (FCS) (Thermotrace, Noble Park, VIC, Australia), CHO cells (ATCC# CCL-61) were grown in HamsF12 (Invitrogen) and supplemented with 10% FCS. MCF10A-EcoR cells [32] were cultured in DMEM/F12 (Invitrogen), 5% (v/v) horse serum (Invitrogen), 20 ng/mL human recombinant EGF (236-EG R&D Systems, Minneapolis, MN, USA), 0.5 μg/mL hydrocortisone (H-0888 Sigma, Castle Hill, NSW, Australia), 100 ng/mL cholera toxin (C-8052 Sigma), 10 μg/mL bovine insulin (I-1882 Sigma), 50 units/mL penicillin G (Invitrogen) and 50 μg/mL streptomycin sulphate (Invitrogen). HOSE17.1 cells [33] were cultured in 50% M199 media (Invitrogen), 50% MCDB105 media with L-glutamine and 25 mM HEPES (M6395 Sigma), supplemented with 10% FCS (Thermotrace). Matched RNA and DNA from other cell lines used for RT-qPCR analysis were obtained from a cell line bank maintained by G. Lehrbach, Garvan Institute, Australia and RNA and DNA from brain cancer and neuroblastoma cell lines were kindly provided by Dr. M. Henderson, Children's Cancer Institute Australia, Randwick, Australia.

2.2. Antibodies and recombinant proteins

Anti-p-ERK, p-JNK and p-p38 antibodies were purchased from Cell Signalling Technology, Danvers, MA, USA (#9101, #9251 and #9211, respectively). Anti-ERK, JNK and p38 antibodies were also purchased from Cell Signalling Technology (#9102, #9252 and #9212, respectively). Anti-GAPDH (6c5) was purchased from Ambion, Austin, TX, USA (#4300) and anti-V5 antibody was purchased from Invitrogen (#R960-25). Recombinant active ERK, JNK and p38 were purchased from Cell Signalling Technology (#7416, #7418 or #7474, respectively). Recombinant GST-tagged DUSP1 was purchased from Upstate, Saratoga Springs, NY, USA.

2.3. Expression constructs

Human DUSP26 cDNA in vector pOTB7 was purchased from RZPD (German Science Centre for Genome Research, Berlin, Germany) and cloned by PCR into the Gateway entry vector pDONR221 (Invitrogen) using forward primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC-GAAGGAGATAGAACCATGTGCCCTGGTAACTGGCTT-3' and reverse primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATGCTTCCA-GACCCTGCCG-3'. DUSP26 cDNA was then cloned by LR recombination (Invitrogen) into the Gateway mammalian expression vector pcDNA3.1 (CMV/n-terminal V5), the Gateway prokaryotic expression vector pDEST15 (T7/N-terminal GST) and the Gateway-converted mammalian retroviral vector pMIG-GFP (pMSCV-IRES-GFP/N-terminal V5), which enables bi-cistronic expression of the gene of interest with GFP via an internal ribosomal entry site (IRES) [34]. pMIG-GFP/V5 was kindly provided by Dr. E. Caldon, Garvan Institute, Australia. Catalytically inactive DUSP26 (c/s_DUSP26) was generated by mutation of the critical cysteine at amino acid position 151 to serine (c/s) using two complimentary oligos with sequence: 5'-GAGGGAAGATCCTGGTGCA-TAGTGCTGTGGGCGTGAGC-3' and the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Expression vectors were sequence verified prior to use. pMIG plasmids encoding DUSP2 have been described previously [35]. The luciferase reporter plasmid pG5E4D38-Lux containing Gal4 elements, and the expression vector pSG/Gal-Elk encoding a fusion protein of the Gal4 DNA binding domain and the transcativation domain of ELK-1 were a kind gift of Prof. Peter Shaw (Nottingham) and have been described previously [36].

2.4. Overexpression and knockdown of DUSP26 in eukaryotic cells

COS7 cells were transfected using Polyfect (Invitrogen) according to the manufacturer's instructions. To generate DUSP26-containing retrovirus, the ecotropic packaging cell line Phoenix-EcoR were transfected 24 h after plating and at 70% confluency, with empty pMIG-GFP, pMIG_wt_DUSP26 or pMIG_c/s_DUSP26, using Fugene transfection reagent (Roche, Castle Hill, NSW, Australia) according to the manufacturer's instructions. The medium was changed after 24 h, and then the cells were incubated for a further 24 h. Retroviral supernatants were collected, passed through 0.45 μM Millipore filters (Millipore, North Ryde, NSW, Australia), and snap frozen at −80 °C until use. MCF10A cells were infected with the supernatant in the presence of Polybrene (Sigma) at 8 μg/mL then expanded for a further 4–7 days prior to sterile sorting by flow cytometry, using the FACS Vantage SE™ Cell Sorter (BD Biosciences) using FACSDiVa software version 4.1.2 (BD Biosciences) to obtain a 99.9% GFP-positive population. SMARTpool™ siRNA reagents specific for DUSP26 were purchased from Dharmacon (M-008027-01) (La Fayette, CO, USA). The non-targeting, RISC free fluorescent tagged Dharmacon siGLO™ was used as a control (D-001600-01-20). 20 nM siRNA was transfected into HOSE17.1 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.5. Generation of purified recombinant DUSP26 protein

BL21 Plys *E. coli* (Invitrogen) were transformed with Gateway pDEST15 vectors encoding GST only, wt_DUSP26 and c/s_DUSP26.

Recombinant protein expression was induced in mid log-phase cultures with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) and 2% w/v glucose for 1.5 h at 37 °C. Bacteria were pelleted and resuspended in 50 mM Tris–HCl (pH 8.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and the following protease inhibitors: 10 μg/mL aprotinin 10 μg/mL leupeptin and 1 mM PMSF. Bacterial lysates were sonicated at 4 °C, clarified by centrifugation at 17,500 × *g* and then incubated with glutathione sepharose 4B beads (GE Healthcare) overnight at 4 °C. Beads were washed five times in lysis buffer and then once in PBS before bound proteins were eluted using 0.5 M reduced glutathione in 1 M Tris–HCl pH 8.5. Purified protein was visualised by Coomassie blue gel staining following SDS-PAGE and quantified using a bovine serum albumin (BSA) concentration curve loaded on the same gel. Purified protein was stored in small aliquots at −80 °C until use.

2.6. Immunoprecipitation of V5-tagged DUSP26 protein

MCF10A cells stably expressing V5-tagged wt or c/s_DUSP26 and COS7 cells transiently transfected with vector, wt, or c/s_DUSP26 were lysed in normal lysis buffer containing 50 mM HEPES (pH7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM pyrophosphate and 100 mM NaF and clarified by centrifugation at 17,500 × *g*. 2.5 mg total protein from each sample was incubated overnight with approximately 40 μg anti-V5 antibody covalently coupled to CN-Br beads (A-7345 Sigma). The CN-Br beads were washed five times with lysis buffer and twice with ice cold PBS. Immunoprecipitated protein was visualised using the anti-V5 antibody and Western blotting as described.

2.7. In vitro phosphatase assays

Purified wt_DUSP26_GST, c/s_DUSP26_GST or GST only (1 μg) was assayed in 50 μl of phosphatase buffer; 50 mM imidazole pH7.5, 20 mM *p*-nitrophenyl phosphate (pNPP) (N4744 Sigma) and 5 mM DTT at 37 °C for the indicated times (Fig. 2). DUSP1_GST (Upstate) was used as a positive control. V5-tagged wt_DUSP26 and c/s_DUSP26 immunoprecipitates from COS7 and MCF10A cells were similarly assayed. 1/3 of the immunoprecipitated antibody/bead mixture was incubated with 50 μl phosphatase buffer for the indicated times (Supplementary Fig. 1). Immunoprecipitates from cells transfected with the empty vector were used as an additional negative control. The amount of pNPP hydrolysed was measured by absorbance at 405 nm with a microplate reader. For in vitro phosphatase assays using purified recombinant MAPKs, 200 ng ERK, JNK or p38 (#7416, #7418 and #7474, Cell Signalling) was incubated with 1 μg of purified recombinant wt_DUSP26_GST, c/s_DUSP26_GST, GST only (negative control) or DUSP1_GST (positive control) for 90 min at 37 °C in 10 mM Tris–HCl pH 7, in a total volume of 30 μl.

2.8. Luciferase assays

COS7 cells were co-transfected with 1.5 μg pcDNA3.1 vector only, pcDNA3.1 containing wt_DUSP26 or c/s_DUSP26 along with 0.5 μg of the aforementioned Gal-luc/Gal-ELK reporter system and 0.2 μg of the β-galactosidase expression vector pCMVβ (Clonetech) using Polyfect (Quiagen, Doncaster, VIC, Australia) according to the manufacturer's instructions. One day after transfection the cells were serum starved for 12–16 h, then stimulated with 100 ng/mL EGF (R&D Systems) for 9 h. Cells were harvested in Tropix lysis buffer provided by the Galacto-*Star™* (Applied Biosystems) chemiluminescence kit. The lysates were clarified by centrifugation at 17,500 × *g* and 5 μl aliquots of the supernatants were added to two 96-well plates in duplicate. The lysate on one plate was incubated for 30 min at room temperature with 100 μl of GalactonStar™ (Applied Biosystems, Scoresby, VIC, Australia) then production of β-galactosidase was measured using a TopCount™ scintillation reader. Luciferase activity was similarly measured using a TopCount™ scintillation reader immediately following the addition of 50 μl Luciferine (Promega, Annandale, NSW, Australia). Mean luciferase activity was normalised using the corresponding β-galactosidase activity and then expressed relative to the untreated control.

2.9. Western blotting

For MAPK activation experiments, HOSE17.1 and COS7 cells were deprived of serum for 16 h and MCF10A cells were cultured for 16 h in media deprived of EGF and insulin and containing 0.4% horse serum. Following starvation cells were treated with either 100 ng/mL EGF for 5 min, 400 mM sorbitol for 3 h or 50 μg/mL anisomycin for 30 min. Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) containing 50 mM Tris–HCl pH 7.4, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 137. 5 mM NaCl, 1% (v/v) glycerol, 0.5 mM EDTA, 10 μg/mL aprotinin (A-1153 Sigma), 10 μg/mL leupeptin hemisulphate (#2167 Auspep, Melbourne, VIC, Australia), 1 mM phenylmethylsulphonylfluoride (PMSF) (Sigma) and 1 mM of sodium orthovanadate (Sigma). Equivalent amounts of cell lysates (20–30 μg) were then resolved by SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked in 5% (w/v) skim milk powder in 0.1% (v/v) Tween 20/Tris-buffered saline and immunoblotted with antibodies as described above. Specific proteins were visualised by ECL™ chemiluminescence (Perkin-Elmer, Rowville, VIC, Australia). Densitometry was performed using Image J software (http://rsbweb.nih.gov/ij/).

2.10. Growth of MCF10A cells in 3D, colony formation and proliferation assays

MCF10A cells were grown on reduced growth factor Matrigel (354230, BD Biosciences, North Ryde, NSW, Australia) to generate 3D acini, as previously described [37]. Briefly, a uniform layer of cold Matrigel was first coated onto chamber slides (BD Falcon, Castle Hill, NSW, Australia) then solidified at 37 °C. MCF10A cells suspended in growth medium + 2% (v/v) Matrigel were then laid over the Matrigel at a density of 1000 cells/mL. 200 μl growth media was added to each chamber and the medium was replaced every 4 days. Quantification of individual acini diameter was performed using Image J software. Colony forming assays were performed by plating MCF10A and HOSE17.1 cells at low density in triplicate dishes. After 10–14 days the colonies were fixed and stained using Diff Quick staining protocol according to the manufacturer's instructions (Lab Aids Pty, Ltd, Narrabeen, NSW, Australia). Quantification of colony formation was performed Image J software (http://rsbweb.nih.gov/ij/). For proliferation assays, cells were plated in triplicate dishes at low density 24 h after siRNA transfection and maintained in culture for 8 days. Cell counts were performed daily in duplicate.

2.11. Reverse-transcriptase quantitative PCR

cDNA was generated using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Quantitative PCR was performed using TaqMan® Gene Expression Assays (Applied Biosystems) for DUSP26 (Hs00225167_m1) and then normalised to GAPDH expression (Hs00266705_g1) (or Mm99999915_g1 for murine cells). Each reaction was repeated in triplicate using 4.5 μl diluted cDNA mixed with 5 μl TaqMan® Gene Expression PCR Universal Master Mix and 0.5 μl of the appropriate TaqMan® Primer/Probe Gene Expression Assay. Relative expression was determined using Applied Biosystems ABI Prism 7900HT software.

2.12. Epigenetic assays

Cell lines were treated for 24 h with 5-aza-2-deoxycitidine (5'AZA) and/or trichostatin A (TSA), each at 5 μM. Expression of DUSP26

mRNA was determined 4–5 days after treatment using RT-qPCR with TaqMan™ primers and probes (Applied Biosystems) as described. For methylation analysis, 500 ng DNA was bisulphite treated for 6–8 h as previously described [38]. DNA was then ethanol precipitated and resuspended in 30 µl water and stored at −20 °C until further use. Methylation primers were designed to amplify both methylated and unmethylated bisulphite-converted DNA from the *DUSP26* CpG island which resides 2 kb upstream of the translational start site. Two sets of primers (A and B) were designed to perform nested PCR (each set with two outer and one inner primer) to obtain a specific and abundant amplicon for direct sequencing. Primers used were F1A_TTTGTAATTGGTGTAGTTTTGAGAATAT, R2A_CAAAYGCTAYGCTAAACCTCAA R3A_ CTATTTCTACCCAAAACAAATTAACAA and F1B_ TTTGTTAATTTGTTTTGGGTAGAAAT, R2B_ TTCTATCCAAAAATACAACCCTAAAC and R3B_ ATYGCCTTTATAACAAATCCATAACTA.

2.13. Statistical analysis

For statistical analyses, a two-sided Student's *t*-test was used (Microsoft Excel, WA, USA). Differences were considered to be statistically significant at $P<0.05$.

3. Results

3.1. DUSP26 does not act as a dual specificity phosphatase on MAPKs in COS7 cells

We sought to determine if DUSP26 could dually dephosphorylate ERK, JNK or p38 and to clarify multiple conflicting reports in the literature on the MAPK substrate specificity of DUSP26. First, MAPK phosphorylation was determined in COS7 cells overexpressing DUSP26. Following transfection with constructs encoding V5-tagged wild-type (wt) DUSP26 or the catalytically inactive (c/s) DUSP26 mutant, cells were serum starved and then stimulated with EGF or sorbitol to activate ERK or JNK and p38 respectively. An equal level of wt and c/s DUSP26 protein expression was confirmed by Western blotting with antibodies specific to the V5 tag (Fig. 1a–c). Transfection of His–tagged wt DUSP2 or catalytically inactive c/s_DUSP2 was performed in parallel as a positive control for ERK dephosphorylation [9,39,40]. Cell lysates were then Western blotted with phospho-specific antibodies that recognise ERK, JNK or p38 when these kinases are dually or mono-phosphorylated on the activation loop TxY motif. This revealed that despite a clear reduction in the phosphorylation of ERK in COS7 cells expressing wt DUSP2 (Fig. 1d), DUSP26 does not exhibit DUSP activity towards any of these MAPKs (Fig. 1a–c). The phosphatase activity of transfected V5-tagged wt DUSP26 was confirmed using an in vitro assay with *p*-nitrophenyl phosphate (pNPP) as a substrate. V5-tagged wt DUSP26 and c/s DUSP26 were immunoprecipitated from COS7 cells and incubated with pNPP as described in Materials and methods. The wt_DUSP26 exhibited readily demonstrable phosphatase activity, but there was no hydrolysis of pNPP by the vector only or c/s_DUSP26 negative control immunoprecipitates (Supplementary Fig. 1a, c). In addition, constructs encoding V5-tagged wt DUSP26 or c/s DUSP26 were transfected into HEK293 cells and phospho-specific MAPK antibodies were used to demonstrate that DUSP26 does not affect ERK, JNK or p38 phosphorylation in these cells (Supplementary Fig. 2a–c).

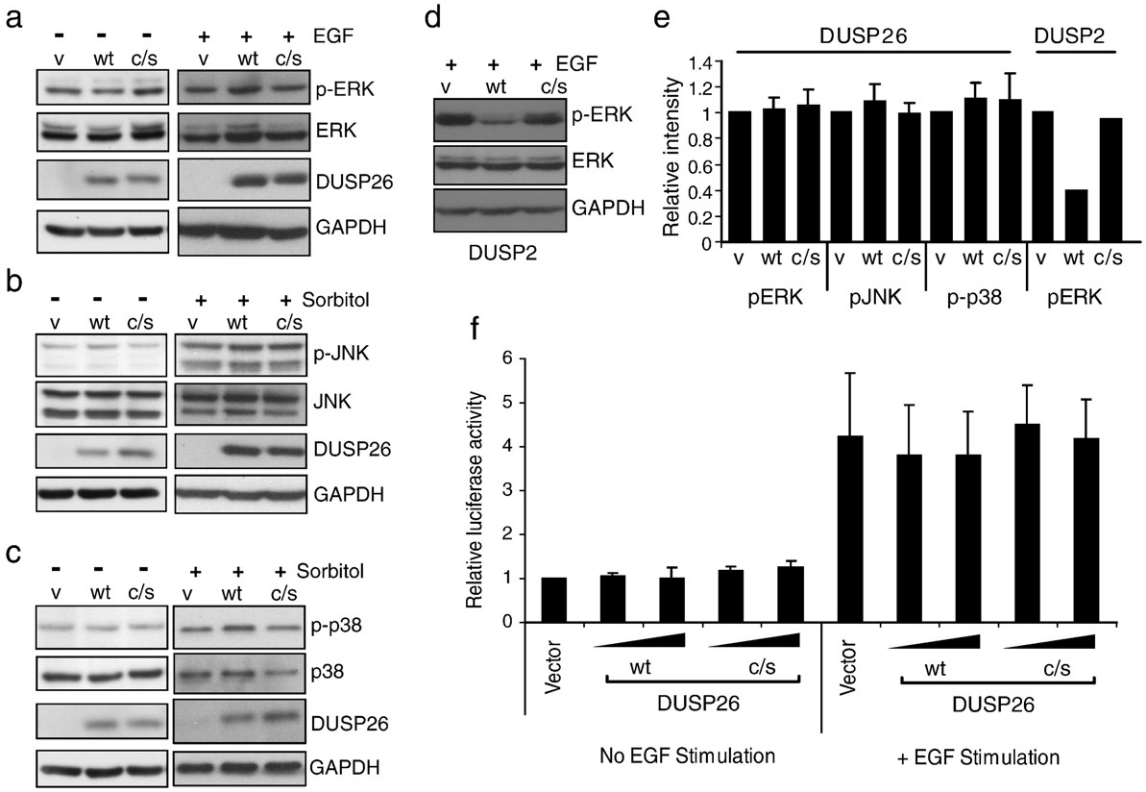

Fig. 1. DUSP26 does not dually dephosphorylate MAPKs in COS7 cells. (a–c) Effect of DUSP26 on MAPK phosphorylation. COS7 cells were transfected with empty vector or constructs encoding wt DUSP26 or c/s DUSP26 and then stimulated as indicated. Cell lysates were then blotted with phospho-specific antibodies against p-ERK (a), p-JNK (b) and p-p38 (c). Western blot images are representative examples of at least three independent experiments. (d) A DUSP2/PAC1 construct was transfected to provide a positive control for ERK dephosphorylation. (e) Densitometry was performed on the Western blots from stimulated COS7 cells and MAPK phosphorylation was normalised for protein expression and expressed relative to the value for vector-transfected cells, which was arbitrarily set at 1.0. Data represent the mean of at least three independent experiments; error bars indicate the standard error. (f) DUSP26 does not affect nuclear MAPK activity. An ELK reporter was co-transfected with vector or plasmids encoding wt DUSP26 or c/s DUSP26 into COS7 cells. In addition, a β-galactosidase construct was included as a transfection efficiency control. Cells were serum starved and then stimulated with EGF. Normalised luciferase activity is expressed relative to untreated, empty vector-transfected cells. Data represent the mean of four independent experiments, each performed in duplicate. Error bars indicate the standard error.

Previous reports have shown that DUSP26 predominantly exhibits nuclear expression with some expression in the cytoplasm [21,22,24]. In order to investigate the effect of DUSP26 on the phosphorylation and activation of nuclear MAPKs, an ELK1 luciferase reporter assay was used. ELK1 is a transcription factor predominantly downstream of ERK [41,42], but it is also moderately activated subsequent to JNK and p38 signalling [43,44]. Plasmids encoding wt DUSP26 or c/s DUSP26 were co-transfected with the ELK1 luciferase reporter construct and a plasmid encoding β-galactosidase for normalisation of transfection efficiency into COS7 cells. Cells were serum starved and stimulated with EGF. Upon growth factor stimulation, there was a significant increase in luciferase expression; however, there was no change in reporter activity in cells transfected with DUSP26, even at high concentrations of transfected plasmid (Fig. 1f). Similarly, there was no change in reporter activity in HEK293 or CHO cells transfected with DUSP26 (Supplementary Fig. 2d and e, respectively). Since this assay indirectly measures MAPK activity, rather than phosphorylation, these data indicate that DUSP26 is unlikely to mono-dephosphorylate MAPKs leading to diminution of their signalling via ELK1.

3.2. DUSP26 does not dually dephosphorylate MAPKs at the TxY motif in vitro

To determine if DUSP26 could directly dephosphorylate ERK, JNK or p38 in vitro, GST-tagged wt DUSP26 and c/s DUSP26 were expressed and purified from *E. coli* as described in Materials and methods. Phosphatase activity of purified recombinant GST-tagged wt DUSP26 was confirmed using *p*-nitrophenyl phosphate (pNPP) as a substrate. The degree of pNPP hydrolysis by wt DUSP26 was comparable to that by DUSP1 and there was no hydrolysis of pNPP by the GST only or c/s DUSP26 controls (Fig. 2a).

Active recombinant MAPKs ERK, JNK and p38 were then incubated with recombinant wt DUSP26, c/s DUSP26, GST only or DUSP1. Using phospho-specific antibodies and Western blotting, we determined that although DUSP1 could effectively dephosphorylate ERK, JNK and p38, wt DUSP26 does not exhibit DUSP activity towards these MAPKs in vitro (Fig. 2b).

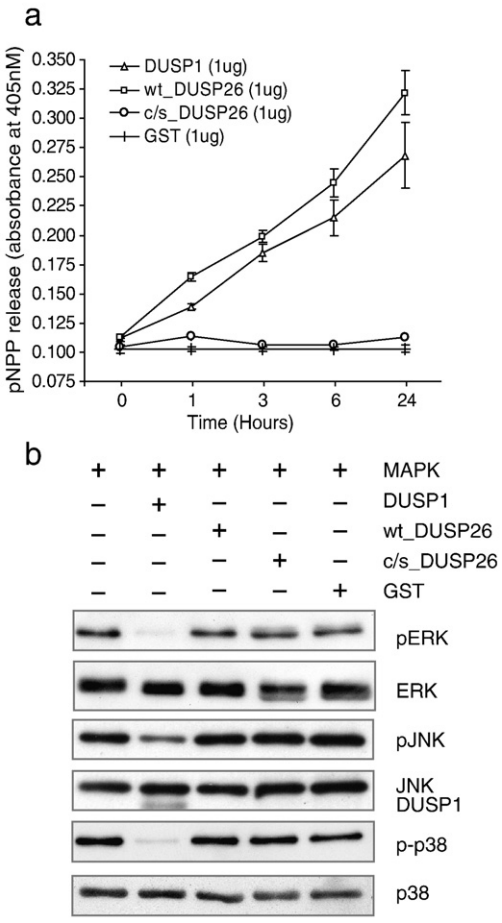

Fig. 2. DUSP26 does not dually dephosphorylate MAPKs in vitro. (a) DUSP26 has intrinsic phosphatase activity against pNPP. Purified recombinant GST-tagged wt DUSP26, c/s DUSP26, GST only or GST-tagged DUSP1 were incubated with pNPP. Error bars represent standard error of three independent reactions. (b) Activity of DUSP26 against MAPKs in vitro. Recombinant ERK, JNK or p38 were incubated with purified GST only, DUSP1-GST, wt DUSP26-GST or c/s DUSP26-GST. Western blotting with phospho-specific antibodies was then used to determine the relative amount of ERK, JNK and p38 phosphorylation. Western blots shown are representative examples of three independent experiments.

3.3. DUSP26 does not dephosphorylate MAPKs in MCF10A or HOSE17.1 cells, despite anti-proliferative effects

DUSP26 expression is absent in several epithelial tumour types including breast, prostate and colon [21,25]. To investigate the consequences of DUSP26 overexpression and knockdown in epithelial cells, colony formation assays were performed using the normal immortalised breast epithelial cell line MCF10A, stably overexpressing wt DUSP26 or c/s DUSP26, as well as normal immortalised human ovarian surface epithelial cells HOSE17.1, transfected with DUSP26-specific siRNA. Overexpression of DUSP26 in MCF10A cells was confirmed by Western blotting (Fig. 3a), and RT-qPCR analysis demonstrated that DUSP26-specific siRNA reduced DUSP26 mRNA expression by approximately 75% compared with control siRNA and mock-transfected control cells (Fig. 3b). Phosphatase activity of stably expressed wt DUSP26 was confirmed using pNPP as a substrate, as previously described for COS7 cells (Supplementary Fig. 1b, c).

MCF10A cells overexpressing wt DUSP26 generated fewer colonies compared with vector only control cells or cells expressing c/s DUSP26 (Fig. 3c). When grown on an enriched basement membrane, MCF10A cells form acini with a hollow lumen, modelling development of glandular architecture in vivo (described previously in ref. [37]). To determine the effect of DUSP26 expression on growth of MCF10A 3D acini, cells stably overexpressing wt DUSP26 or c/s DUSP26 were cultured on Matrigel™ as described [37]. Acini overexpressing wt DUSP26 were markedly smaller than those expressing c/s DUSP26 or the vector only control (Fig. 3d). Consistent with this result, HOSE17.1 cells generated more colonies when transfected with DUSP26 siRNA compared with the control siRNA-transfected cells (Fig. 3e). In addition, the doubling time of DUSP26-deficient HOSE17.1 cells was significantly less than that of the control siRNA-transfected cells, indicating an increased proliferation rate in the DUSP26-deficient HOSE17.1 cells (Fig. 3f).

We next sought to determine if the biological effects demonstrated in MCF10A and HOSE17.1 cells were mediated by dephosphorylation of MAPKs by DUSP26. MCF10A cells overexpressing DUSP26 or siRNA-transfected HOSE17.1 cells were serum starved overnight prior to being stimulated with EGF, sorbitol or anisomycin. Western blotting indicated that there was no change in the phosphorylation of ERK, JNK or p38 upon manipulation of DUSP26 expression in either system (Supplementary Fig. 3).

3.4. Loss of DUSP26 mRNA expression in cancer cell lines

Since DUSP26 suppressed cell proliferation, we characterised the expression pattern of this phosphatase in cancer cell lines versus normal cell line or tissue controls, hypothesising that DUSP26 may exhibit loss of expression consistent with tumour suppressor activity. DUSP26 mRNA expression was reduced in ovarian cancer cell lines compared with the immortalised human ovarian surface epithelial cell line HOSE17.1. Similarly, DUSP26 mRNA expression was much lower in neuroblastoma cell lines, as compared with normal adrenal

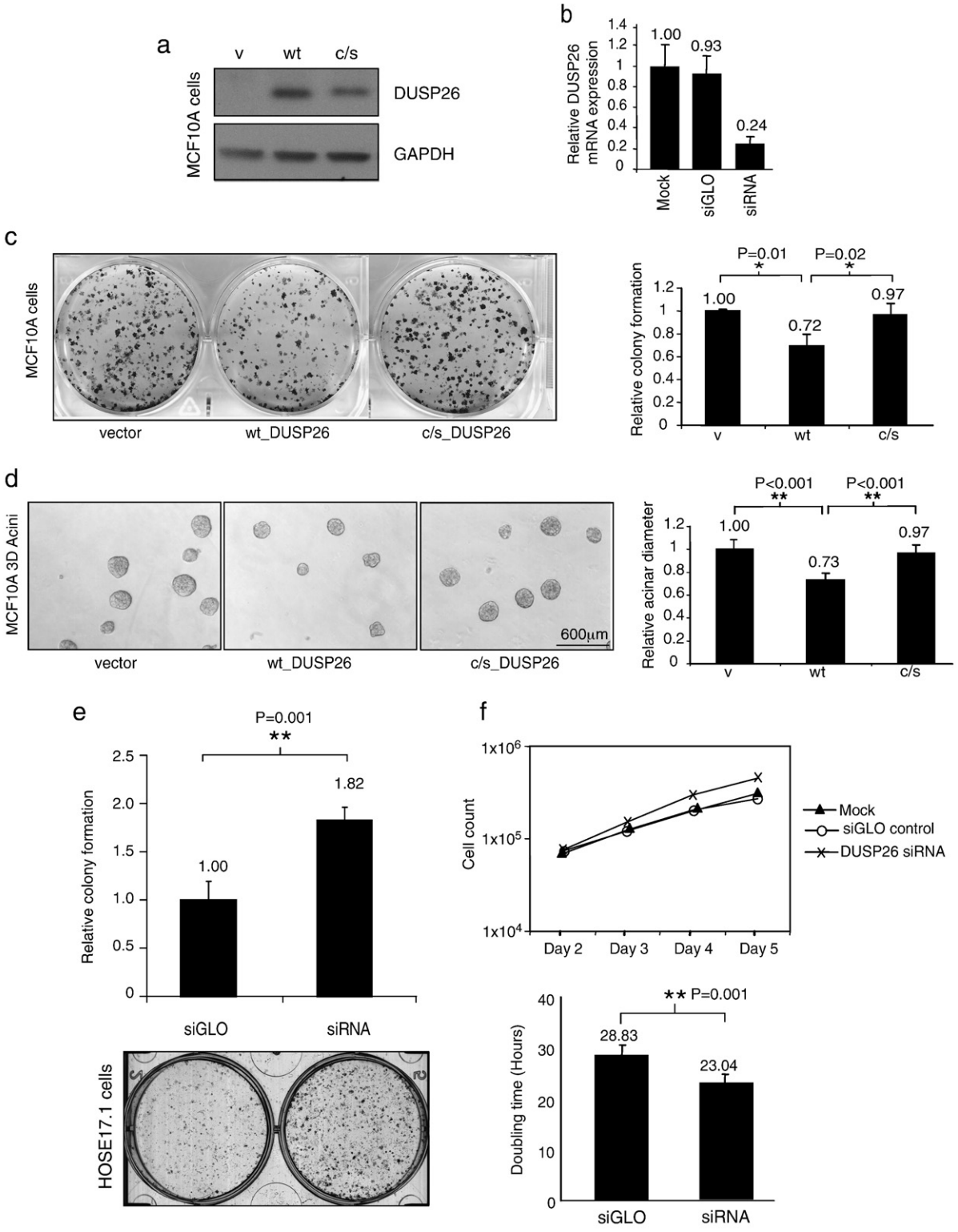

Fig. 3. Effect of DUSP26 overexpression and knockdown in epithelial cells. (a) Western blotting with V5-tag antibody demonstrating overexpression of DUSP26 in MCF10A cells. (b) RT-qPCR analysis demonstrating knockdown of DUSP26 mRNA in HOSE17.1 cells. Overexpression of DUSP26 in MCF10A cells results in reduced colony forming ability (c) and smaller acini when cells are grown in 3D (d). Data represent the mean of at least three independent experiments and are expressed relative to the value for control cells, which is arbitrarily set at 1.0. (e) Transfection of HOSE17.1 cells with DUSP26-specific siRNA results in an enhanced colony forming ability (e) and increased proliferation rate (f). Error bars indicate the standard error. Images shown are representative examples of at least three independent experiments.

gland. Expression was also reduced in three medulloblastoma cell lines and one glioblastoma cell line compared with normal brain. While DUSP26 was not expressed in normal mouse astrocytes (Fig. 4), it remains possible that it is expressed in the neural progenitor cells thought to be the 'cell of origin' for glioblastoma [45].

Common mechanisms of gene silencing in cancer include loss of heterozygosity and epigenetic silencing. In particular, epigenetic silencing due to hypermethylation of CpG islands located in the promoter region of tumour suppressor genes is a common and early event in carcinogenesis [46]. The *DUSP26* gene contains a CpG island

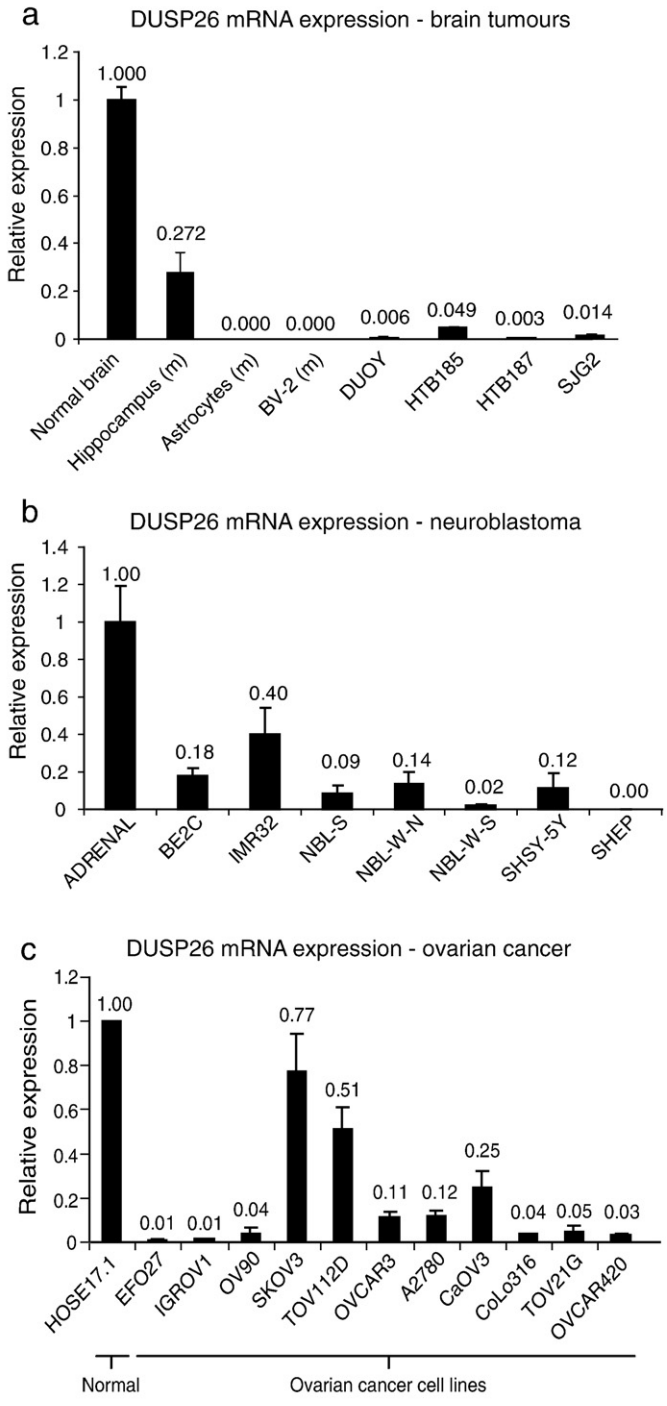

Fig. 4. DUSP26 expression in brain tumour, neuroblastoma and ovarian cancer cell lines. RT-qPCR determined that DUSP26 mRNA expression is reduced in brain cancer (a), (m = mouse tissue), neuroblastoma (b), and ovarian cancer cell lines (c) compared with normal controls, which are arbitrarily set at 1.0. Data represent the mean of three independent reactions; error bars indicate standard error.

600 bp in length and located 100 bp upstream of the transcriptional start site (Fig. 5a).

We sought to determine if DUSP26 could be epigenetically regulated. Treatment of ovarian cancer cell lines with the methyltransferase inhibitor (5'AZA) and/or the histone de-acetylase inhibitor trichostatin A (TSA) followed by RT-qPCR analysis demonstrated that DUSP26 expression significantly increased in all cell lines after treatment with 5'AZA and in the majority of cell lines, re-expression increased further upon treatment with both 5'AZA and TSA, suggesting that DUSP26 expression can be epigenetically regulated by both methylation and histone modification (Fig. 5b).

To determine if the DUSP26 CpG island is methylated in cancer cell lines, the *DUSP26* promoter associated CpG island was amplified from sodium bisulphite-treated cell line DNA using PCR primers that amplify DNA containing both converted (to uracil, indicating non-methylated) and non-converted (indicating methylated) cysteine residues. Direct DNA sequencing of the resulting amplicon determined that the *DUSP26* CpG island was methylated at specific CpG sites to a variable extent in the majority of the cancer cell lines (Fig. 5c). In most of the cell lines, hypermethylation could be correlated with loss of gene expression (Fig. 4). For example, of the neuroblastoma cell lines, the highest level of methylation was present in the cell line SHEP, which displays the least DUSP26 expression and heavy methylation of the brain cancer cell line SJG2 is consistent with low to absent expression of DUSP26 (Fig. 4). There was no methylation in the normal ovarian cell line HOSE17.1 which expresses DUSP26; however, methylation in the ovarian cancer cell lines was variable and did not always correspond with relative expression levels suggesting that methylation is not the only mechanism by which loss of DUSP26 expression occurs. Also, consistent with re-expression following treatment with TSA, it is likely that chromatin modification also plays a role in the epigenetic regulation of DUSP26.

4. Discussion

Despite the growing interest and research focused on DUSP26 since it was first characterised in 2005 [21], its functional characteristics including its biological role and substrate specificity remain unclear. Atypical DUSPs have the most varied substrate specificity of all the protein tyrosine phosphatases and have been shown to target alternative protein substrates such as glucokinase (DUSP12) [47], signal transducer and activator of transcription (STAT) proteins (DUSP3) [48] and upstream MAPK activators (DUSP22) [49,50]. Atypical DUSPs have also been shown to target non-protein substrates such as mRNA (DUSP11) [51]. It is becoming clear that, unlike typical MKPs, atypical DUSPs have a variety of non-MAPK substrates; however, few generalisations can be made.

The results presented here using cell-based and in vitro assays indicate that DUSP26 does not act as a dual specificity phosphatase on the MAPK proteins ERK, JNK or p38, despite reports to the contrary that suggest DUSP26 can dephosphorylate p38 [21,52], p38 and ERK [23], ERK and JNK [22]. While in some contexts DUSP26 appears to modulate the activity of particular proteins in the MAPK and PI3K signalling cascades [25,26], the mechanism(s) by which this occurs is not fully understood. One possibility is that in order to target MAPKs, DUSP26 requires additional binding partners that act as scaffolds, tethering DUSP26 to its target(s), and these binding partners are present or active only in specific cell types. However, this seems unlikely given that relatively large amounts of recombinant DUSP26 did not dephosphorylate MAPKs in vitro. Alternatively, DUSP26 phosphatase activity may be regulated by 'substrate-modulators' that are also present in a cell-specific context. It has been shown that the catalytic activity of some DUSPs can be enhanced upon binding to their MAPK substrates [53,54]. Consequently, the phosphatase activity of DUSP26 in vivo may be dependent on the presence of other proteins that upon binding to the enzyme alter the conformation of the catalytic domain and direct its substrate specificity towards particular MAPKs. Another possibility is that physiological DUSP26 substrates lie upstream of PI3K or MAPKs and affect their activity indirectly. In this context, the recent study linking DUSP26 with Kif3a and Kap3 is of interest, since intact microtubule-based transport is required for NGF-induced MAPK signalling in PC12 cells [55]. Finally, the effect of DUSP26 may differ depending on the specific hierarchy of signalling cascades that are activated or on the kinetics of these

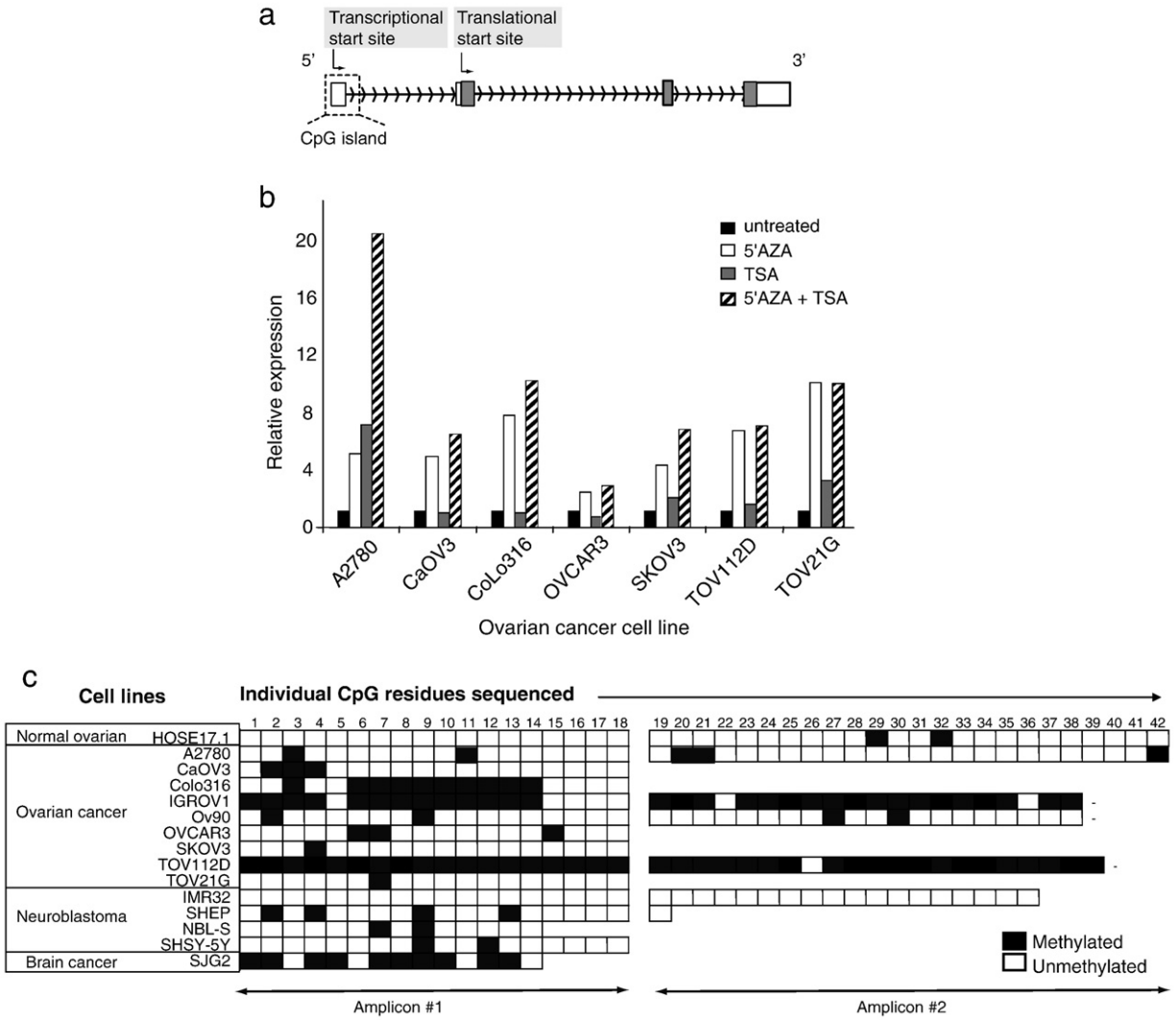

Fig. 5. Epigenetic modification of DUSP26. (a) Representative scale diagram of *DUSP26* genomic sequence. The CpG island is represented as an dotted box 600 bp in length and starting 100 bp upstream of the transcriptional start site. Exons are represented as white and grey boxes with the translated sequence in grey. (b) DUSP26 expression in ovarian cancer cell lines before and after treatment with 5'AZA and TSA. Values shown are calculated as an average of three individual reactions. (c) Direct sequencing of bisulphite-converted DNA across the DUSP26 CpG island identified specific CpG sites that were methylated (black boxes) or unmethylated (white boxes).

signalling cascades. Indeed, DUSP26 exerts contrasting effects on EGF versus NGF-induced ERK activation [25,26].

The role DUSPs play in the pathogenesis of various cancers is the subject of ongoing research. Many DUSPs are encoded at chromosomal regions that exhibit loss of heterozygosity and these DUSPs exhibit reduced expression in particular cancers. Functional studies support a role for some of these DUSPs as tumour suppressors [11–17]. Our results are consistent with a functional role for *DUSP26* as a tumour suppressor gene. Specifically, knockdown of DUSP26 in HOSE17.1 cells resulted in both an increased ability of these cells to form colonies and an increase in their proliferation rate. Conversely, overexpression of DUSP26 in MCF10A cells resulted in a decreased ability of these cells to form colonies, and the three dimensional acini that these cells form when grown on an enriched basement membrane were smaller. Importantly, these effects on cell proliferation were not mediated via modulation of MAPK pathways, indicating that DUSP26 must target other substrates involved in proliferative signalling and/or cell cycle control.

Interestingly, there have been reports suggesting that DUSP26 may have both tumour suppressive [26,27] and oncogenic [23] actions. This is not the first DUSP for which both oncogenic and tumour suppressor roles have been reported in different cell types. Overexpression of DUSP1 can result in positive effects on the growth of prostate cancer [56] but negative effects on the growth of breast cancer cells [57]. The molecular events that govern the growth, proliferation and survival of epithelial cells are complex and it is likely that the biological role(s) of these proteins is cell type and context specific.

Our results also demonstrate that DUSP26 expression is reduced/lost in certain cancer cell lines and that this loss of expression is at least partly due to epigenetic regulation. *DUSP26* is located at 8p12, a chromosomal region previously shown to exhibit loss of heterozygosity in several cancers including bladder [17], breast [29], prostate [14], and ovarian cancers [15,30,31] implying that tumour suppressor genes may exist in this region. Analysis of copy number at the *DUSP26* locus would determine if genomic deletion occurs in cancers that show loss of DUSP26 expression. In addition, it will be important to identify the physiological role of DUSP26 in brain and neuroblastoma tissue and to determine how its loss perturbs pathways that underpin tumour progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.03.014.

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