DUSP6/MKP3 is overexpressed in papillary and poorly differentiated thyroid carcinoma and contributes to neoplastic properties of thyroid cancer cells

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Abstract

Thyroid carcinomas derived from follicular cells comprise papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, poorly differentiated thyroid carcinoma (PDTC) and undifferentiated anaplastic thyroid carcinoma (ATC). PTC, the most frequent thyroid carcinoma histotype, is associated with gene rearrangements that generate RET/PTC and TRK oncogenes and with BRAF-V600E and RAS gene mutations. These last two genetic lesions are also present in a fraction of PDTCs. The ERK1/2 pathway, downstream of the known oncogenes activated in PTC, has a central role in thyroid carcinogenesis. In this study, we demonstrate that the BRAF-V600E, RET/PTC, and TRK oncogenes upregulate the ERK1/2 pathway’s attenuator cytoplasmic dual-phase phosphatase DUSP6/MKP3 in thyroid cells. We also show DUSP6 overexpression at the mRNA and protein levels in all the analysed PTC cell lines. Furthermore, DUSP6 mRNA was significantly higher in PTC and PDTC in comparison with normal thyroid tissues both in expression profile datasets and in patients’ surgical samples analysed by real-time RT-PCR. Immunohistochemical and western blot analyses showed that DUSP6 was also overexpressed at the protein level in most PTC and PDTC surgical samples tested, but not in ATC, and revealed a positive correlation trend with ERK1/2 pathway activation. Finally, DUSP6 silencing reduced the neoplastic properties of four PTC cell lines, thus suggesting that DUSP6 may have a pro-tumorigenic role in thyroid carcinogenesis.
Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, with an increasing incidence over the last few decades (Davies & Welch 2006). Most TC, including well-differentiated (WDTC), poorly differentiated (PDTC), and undifferentiated anaplastic (ATC) carcinomas, originates from thyrocytes. Papillary thyroid carcinoma (PTC), a WDTC histotype, is the most prevalent thyroid malignancy. It is usually associated with a good prognosis and therapeutic response; nevertheless, ~10% of patients present with recurrences and distant metastases. Four different alternative genetic lesions have been identified as driving oncogenic alterations in ~70% of PTCs, including RET/PTK or TRK rearrangements and BRAF or RAS mutations. All these PTC-associated genetic lesions constitutively activate the ERK1/2 pathway (Greco et al. 2009). PDTC, recently recognised as an independent histotype, presents morphological and behavioural characteristics intermediate between those of WDTC and ATC. Both PDTC and ATC can arise de novo or can evolve from pre-existing WDTC, particularly from PTC. Accordingly, WDTC-associated gene mutations are also found in small fractions of PDTC (BRAF and RAS mutations) and ATC (BRAF mutation) (Santoro et al. 2002, Santarpia et al. 2008).

The MAP-kinases/ERK1/2 pathway plays well-recognised roles in cell proliferation, differentiation, survival and motility. The activity of ERK1/2 is tightly regulated by many broad- and narrow-specificity phosphatases in physiological and pathological contexts (Chambard et al. 2007). MAP kinase phosphatase enzymes (MKPs), which belong to the family of dual-specificity phosphatases (DUSPs), inactivate different MAPK proteins, including ERK1/2. Among these, DUSP6/MKP3 cytoplasmic phosphatase displays a high specificity for ERK1/2 (Groom et al. 1996, Camps et al. 1998, Muda et al. 1998, Fjeld et al. 2000, Arkell et al. 2008). Recently, p38 and FOXO1 have been suggested as additional DUSP6 targets (Wu et al. 2010, Zhang et al. 2011).

DUSP6 is an evolutionarily conserved, strictly regulated gene required during development, whose product is subject to regulation at multiple levels, including mRNA transcription and stability, rate of translation, protein stability and enzymatic activity (Bermudez et al. 2010). The MEK-ERK1/2 pathway appears to be a major regulator of DUSP6, as activated ERKs induce DUSP6 mRNA transcription, and MEK-dependent phosphorylation of the DUSP6 protein is followed by proteasomal degradation (Bermudez et al. 2011).

The role of DUSP6 in neoplastic transformation is poorly defined, and either up- or downregulation of this phosphatase has been reported in different tumours. DUSP6 expression is low in invasive pancreatic adenocarcinoma, lung, oesophageal and nasopharyngeal carcinomas (Furukawa et al. 2003, 2005, Okudela et al. 2009). By contrast, DUSP6 is upregulated in myeloma cell lines with an active NRAS mutation, melanoma cell lines with BRAF or NRAS mutations, colon carcinoma and ERBB2-positive breast cancers (Croonquist et al. 2003, Bloethner et al. 2005, Lucci et al. 2010, Quyun et al. 2010). In addition, DUSP6 pro-survival functions have been hypothesised in HeLa (MacKeigan et al. 2005) and breast cancer cells (Lonne et al. 2009), and a tumour-promoting role has recently been suggested in glioblastoma cells (Messina et al. 2011).

As shown in the flowchart in Supplementary Figure S1, see section on supplementary data given at the end of this article, we investigated DUSP6 expression in PTC and PDTC, starting from the evidence that the RET/PTC1 oncogene, known to enhance ERK pathways in primary human thyrocytes (Borrello et al. 2005), concomitantly upregulates certain regulators of these pathways, including several DUSPs and SPRY2. We have shown that high levels of DUSP6 mRNA and protein are present in all the analysed PTC cell lines and in the majority of PTC and PDTC surgical samples. Unexpectedly, high levels of the protein were associated with high ERK1/2 activation in the analysed TCs. Functional experiments of DUSP6 silencing in four PTC cell lines that overexpress the phosphatase unveiled a protumorigenic role for DUSP6.

Materials and methods

Antibodies and reagents

The following mouse MABs were used in blotting experiments: anti-DUSP6 from Abcam (Cambridge, UK), anti-MEK1/2 from Cell Signaling Technology (Beverly, MA, USA), anti-MAP kinase activated (pERK1/2) and anti-vinculin from Sigma-Aldrich. The following rabbit MAB was used in blotting experiments: anti-phospho-Akt (Ser473) from Cell Signaling Technology. The following rabbit polyclonal Abs were used in blotting experiments: anti-RET, anti-TRK, anti-RAF-B, anti-RSK-1, p-RSK-1/2 (Thr359/Ser363) and anti-p-Shc (Tyr 239/240) from Santa Cruz Biotechnology; anti-MAP kinase (ERK1/2) from...
Sigma–Aldrich; anti-phospho-MEK1/2 (Ser217/221), anti-Akt, anti-PARP and anti-cleaved PARP (Asp214) from Cell Signaling Technology; anti-NBS1 from Novus Biologicals (Littleton, CO, USA) and anti-Shc from Upstate (Lake Placid, NY, USA).

EGF was from Sigma–Aldrich. The MEK inhibitor UO126 was from Promega. Human BRAF-V600E cDNA cloned in the pMCEF vector, kindly donated by Dr R Marais (Wellbrock et al. 2004), was subcloned into the pRC-CMV vector. Human RET/PTC1 and TRK-T3 cDNAs were cloned in the pRC-CMV vector (Roccato et al. 2002).

Tumour samples

Thyroid samples were collected at the Department of Pathology at Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milano, Italy. All patients signed an informed consent for the experimental use of their tissue samples in this study, which was approved by the Independent Ethical Committee of INT.

The TCs were classified according to WHO Classification (Delellis et al. 2004), and the extent of disease was determined according to the pathological tumour node metastasis (pTNM) staging system (Sobin et al. 2009). Genetic lesions were characterised as described previously (Frattini et al. 2004). The non-neoplastic thyroid tissues were from patients with pathologies other than TC. Twenty frozen thyroid samples (including five non-neoplastic and 15 TCs) were selected for real-time RT-PCR analyses. An additional 15 formalin-fixed, paraffin-embedded (FFPE) thyroid samples (including two non-neoplastic, three PTCs, eight PDTCs and two ATCs) were investigated by immunohistochemical (IHC) analyses. Among these, five pairs of matched frozen tissues (including two non-neoplastic and three PDTCs) were analysed by western blot (WB).

RNA extraction and real-time RT-PCR analysis

Total RNA from thyrocytes was extracted using Nucleospin RNA II (Macherey-Nagel, Düren, Germany), following the manufacturer’s protocols. Total RNA from tissue specimens was extracted as described previously (Frattini et al. 2004). Total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). For each sample, 20 ng template was amplified in PCRs performed in triplicate on an ABI PRISM 7900 using the TaqMan Gene Expression Assay (Applied Biosystems). DUSP4, DUSP6, SPRY2, PLA2 and CSF2 were tested. PGK1 was used as a housekeeping gene. Data analyses were performed with the Sequence Detection System (SDS) 2.4 and the RQ Manager 1.2.1 programs, using the \(2^{-\Delta\Delta Ct}\) method with a relative quantification RQmin/RQmax confidence level set at 95%. The error bars display the calculated maximum (RQmax) and minimum (RQmin) expression levels that represent s.e.m. of the mean expression level (RQ value). The upper and lower limits define the region of expression within which the true expression level is likely to occur.

Microarray data analysis

The expression of ERK pathway attenuators DUSP5 and DUSP6 and that of DUSP4, DUSP10, SPRED2 and SPRY2, when available, was examined in two microarray datasets from thyroid tissues that comprised 69 PTCs and 13 non-neoplastic thyroids. One dataset, generated in our laboratory using cDNA microarray chips, contains the expression profile data of nine non-neoplastic thyroids and 34 PTCs collected at the Department of Pathology of our institute. The PTC collection includes 24 classical types and ten tall cell variants; 11 samples carry the BRAF-V600E mutation, seven samples carry RET/PTC rearrangements, two samples carry TRK rearrangements and for the remaining five samples, none of the above genetic lesions were detected. The details of gene expression analysis have been previously reported (Frattini et al. 2004). The other dataset examined was extracted from the NCBI Gene Expression Omnibus (GEO) database under series number GSE27155 (Giordano et al. 2005). It contained the expression profile performed using oligonucleotide DNA microarrays (U133A GeneChip, Affymetrix, Santa Clara, CA, USA) with thyroid samples, including four normal thyroid tissues and 35 PTCs corresponding to classical (25) and tall cell (10) types. Among PTC samples, 26 carried the BRAF-V600E mutation and eight had RET/PTC rearrangements. The log intensity value of probe sets corresponding to DUSP4 (204014_at, 204015_s_at), DUSP5 (209457_at), DUSP6 (208891_at, 208892_s_at 208893_s_at), DUSP10 (215501_s_at, 221563_at), SPRY2 (204011_at) and SPRED2 (12458_at, 212466_at) were considered. Because the multiple DUSP4, DUSP6, DUSP10 and SPRED2 probe sets displayed an identical trend in transcript level changes, the average log intensity levels of the different probe sets for the same gene are reported.

Cell culture, transfections and RNA interference

Primary thyrocyte cultures were established from non-neoplastic thyroid samples from patients undergoing
surgery at INT and were maintained in a nutrient mixture consisting of Ham’s F12 medium (custom-made by Invitrogen) containing 5% calf serum and bovine hypothalamus and pituitary extracts, as described previously (Curcio et al. 1994). Primary thyrocytes expressing the RET/PTC1 oncogene, obtained by infection with RET/PTC1 retroviral vector, have been described (Borrello et al. 2005). Immortalised cell lines were maintained in media supplemented with 10% calf serum. The human thyroid cell lines NIM-1, TPC1, B-CPAP, WRO, 8505C, KAT-4, KAT18, BHT101 and HTC/C3 were grown in DMEM; N-Thy-ori3-1 were grown in RPMI 1640; K1 in DMEM: Ham’s F12: MCDB; FTC133 in DMEM: Ham’s F12; and HOTHC in Ham’s F12.

N-thy-ori3-1 cells were transiently transfected using the Cell Line Nucleofector Kit V (Lonza, Basel, Switzerland), program X-005, according to the manufacturer’s protocols. Knockdown of DUSP6 protein in TPC1, NIM-1, B-CPAP and K1 cells was performed by transfection with the ON-TARGET plus SMART pool for human DUSP6 or NON-TARGET small interfering RNA control (Thermo Scientific, Dharmacon, Inc., Chicago, IL, USA) using siIMPORTER transfection reagent (Millipore, Billerica, MA, USA), following the manufacturer’s instructions.

**WB analysis**

Total protein cell extracts were prepared as described previously (Degl’Innocenti et al. 2010).

For separation of nuclear and cytoplasmic proteins, cells were incubated in a hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM MgCl₂, 0.5% NP-40 and 0.5 mM dithiothreitol (DTT)) supplemented with protease and phosphatase inhibitors. Subsequently, nuclei were sedimented by centrifugation and lysed through sonication in a high-salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM EDTA, 1.5 mM MgCl₂, 25% glycerol and 0.5 mM DTT) supplemented with protease and phosphatase inhibitors.

**IHC analysis**

The IHC experiments were performed with the antibodies and under the conditions shown in Table 1 using adequate positive and negative controls. For the 15 cases analysed through IHC, representative sections were selected and immunophenotyped.

**DNA isolation and sequencing**

Genomic DNA from FFPE specimens was isolated using the Qiagen Tissue Kit (Qiagen) as described previously (Namba et al. 2003). We analysed exons 11 and 15 of BRAF through DNA amplification using specific primers (Davies et al. 2002, Namba et al. 2003). The primers for BRAF analysis included the exonic sequence and at least 50 nucleotides of the flanking intronic sequences. Amplified products were purified with the QIAamp Purification Kit (Qiagen) and then directly sequenced on an ABI PRISM 3100 automated capillary Genetic Analyzer (Applied Biosystems).

**Early branching morphogenesis assay**

Morphogenic properties of thyroid cells were evaluated by testing cells’ ability to aggregate and form branches in a few hours when layered on an artificial extracellular matrix (Matrigel; BD Biosciences, San Jose, CA, USA), as described previously (Cassinelli et al. 2009). Seventy-two hours after transfection, cells were suspended in serum-free medium and overlaid on the gelled Matrigel. After incubating at 37°C for 4 h, branches were

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**Table 1** Antibodies working dilution and staging procedure for immunohistochemical analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Staining procedure</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUSP6 (Abcam)</td>
<td>1:150</td>
<td>Incubation on 4°C. Development with streptavidin/HRP</td>
<td>TPC1 and B-CPAP cell lines</td>
</tr>
<tr>
<td>Anti-p42/44 MAPK (Cell Signaling Technology)</td>
<td>1:25</td>
<td>Incubation on 4°C. Development with streptavidin/HRP</td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td>Anti-phospho-p42/44 MAPK (Cell Signaling Technology)</td>
<td>1:25</td>
<td>Incubation on 4°C. Development with streptavidin/HRP</td>
<td>Colon carcinoma</td>
</tr>
<tr>
<td>TTF1 Dako (Glostrup, Denmark)</td>
<td>1:100</td>
<td>Incubation 1 h at 25°C. Development with ultra vision LP large volume detection system HRP polymer</td>
<td>Thyroid</td>
</tr>
</tbody>
</table>

All samples were pre-treated for antigen retrieval: 15 min at 95°C with citrate buffer pH 6.
photographed with a digital camera. Quantification of branches was performed by measuring the total length of structures per field in adjacent fields \((n=10)\). The data are reported as percentages of control ± S.D.

**Cell proliferation assays**

Twenty-four hours after transfection, siRNA-transfected and -untransfected control cells were seeded at 20,000 cells/cm² in 96-well plates in the presence of DMEM with 10% FBS. Six hours after seeding, the cells were serum starved and exposed to solvent or drug when indicated. Cell growth was evaluated by sulforhodamine B (SRB) colorimetric assay at the indicated times, as described previously (Degl’Innocenti et al. 2010). The experiments were performed in eight replicates.

**Cell migration and invasion assays**

Forty-eight hours after transfection, PTC cells were harvested and transferred into 24-well transwell chambers (Costar, Corning, Inc., Corning, NY, USA) in complete medium. For the migration assay, cells were seeded in the upper chamber. For the invasion assay, the transwell membranes were coated with Growth Factor Reduced Matrigel (12.5 μg in 60 μl/well) (BD Biosciences) and dried for 1 h. Cells were transferred onto the artificial basement membrane. After 24 h of incubation at 37°C, cells that invaded the Matrigel layer and/or migrated to the lower chamber were fixed in 95% ethanol, stained with a solution of 0.4% SRB in 1% acetic acid and counted under an inverted microscope. Assays were performed in triplicate, cells were counted in adjacent fields \((n=10)\) and data were reported as average cell number per field ± S.D.

**Apoptosis analysis**

Cells were fixed and stained with Hoechst 33341 (Sigma) as described previously (Cassinelli et al. 2009). Apoptosis was evaluated by counting Hoechst 33341-stained apoptotic bodies in adjacent fields \((n=10)\) and expressed as the average percentage ± S.D.

**Statistical analyses**

Statistical analyses and graphs were generated using GraphPad Prism version 5.0 (La Jolla, CA, USA). Comparison between two groups was performed with the two-tailed Student’s \(t\)-test or the Mann-Whitney \(U\) test, as stated in the figure legends. Three or more groups were analysed with the Kruskal–Wallis test with Dunn’s multiple comparison post-test. \(P<0.05\) was considered significant. Asterisks indicate *\(P<0.05\), **\(P<0.01\) and ***\(P<0.001\).

**Results**

**The RET/PTC1 oncogene upregulates attenuators of ERK pathways in primary human thyrocytes**

Using human primary thyrocytes exogenously expressing the RET/PTC1 oncogene as an in vitro model of PTC, we have previously shown through microarray analysis
(U133 GeneChips, Affymetrix) that RET/PTC1 induces the expression of a large set of genes, including genes involved in inflammation and tumour invasion. Their induction is strictly dependent on the presence of the RET/PTC1 major docking site, Tyr451 (Borrello et al. 2005).

In this work, we have further analysed the previously obtained gene expression profiles of uninfected human primary thyrocytes and of RET/PTC1- or RET/PTC1-Y451F-infected cells (Borrello et al. 2005). This novel analysis of expression profiles revealed the oncogene-induced upregulation of several MAPK pathway attenuators, including SPRY2, SPRED2, DUSP4, DUSP5, DUSP6 and DUSP10 (Fig. 1A).

By real-time RT-PCR analysis, we have now validated the expression of selected genes (Fig. 1B). The mRNAs of DUSP4, DUSP6 and SPRY2 were found to be up to 200-fold more abundant in RET/PTC1- with respect to RET/PTC1-Y451F-infected or uninfected thyrocytes. The established RET/PTC1’s transcripational target PLAU and CSF2 genes have been used as controls (Borrello et al. 2005, Guarino et al. 2009). These findings suggest that RET/PTC1 concomitantly activates the ERK1/2 pathway and several potential regulators of this pathway, both largely dependent on the RET multi-docking site.

Attenuators of ERK pathways in PTC gene datasets and in TC cell lines

To assess whether the ERK pathway attenuators upmodulated in vitro by RET/PTC1 could be overexpressed in PTC clinical samples, we examined two microarray datasets of thyroid tissues for a total of 13 non-neoplastic thyroids and 69 PTCs, including different subtypes. For both datasets, information about the genetic alteration of the neoplastic samples was available.

In the first dataset, a cDNA array generated in our laboratory (Frattini et al. 2004), only DUSP5 and DUSP6 could be investigated. All six of the MAPK feedback genes could be investigated in the second dataset downloaded from GEO (series number GSE27155) (Giordano et al. 2005). These analyses (Fig. 2) indicate that DUSP4, DUSP5, DUSP6 and SPRED2 expression is significantly higher in PTC compared with non-neoplastic thyroid, while DUSP10 and SPRY2 are expressed at similar levels. With regard to PTC histotypes, no difference could be observed between PTC NOS (not otherwise specified) and the more aggressive tall cell variant for the analysed genes (data not shown). Upregulation of DUSP4, DUSP5 and DUSP6 in PTCs confirms published data from additional independent microarray studies (Huang et al. 2001, Chevillard et al. 2004, Jarzab et al. 2005, Griffith et al. 2006, Delys et al. 2007, Eszlinger et al. 2007, Salvatore et al. 2007, Arora et al. 2009, Fontaine et al. 2009). The expression profiles of ERK pathway attenuators indicate that DUSP4-5-6 overexpression is significant in PTC irrespective of their genetic lesion (Supplementary Figure S2, see section on supplementary data given at the end of this article).

To confirm this finding, the mRNA levels of DUSP4, DUSP6 and SPRY2 were analysed by real-time RT-PCR in cell lines representative of PTC, follicular thyroid

### Figure 2
Gene expression levels of ERK pathway attenuators in PTC. Two different datasets were examined. Data are reported as scatter plots of log values and medians. P values were determined by the Mann–Whitney U test. *P < 0.05, **P < 0.01, ***P < 0.001: statistically significant results in comparison with non-neoplastic thyroid samples. (A) INT dataset. DUSP5 and DUSP6 relative gene expression values in PTC (n = 34) and non-neoplastic thyroid (n = 9) were measured as log2 ratios. The details of this gene expression analysis have been previously reported (Frattini et al. 2004). (B) GSE27155 dataset (Giordano et al. 2005). The relative gene expression values of the MAPK attenuators DUSP5, 6, 4, 10, SPRY2 and SPRED2 in PTC (n = 35) and non-neoplastic thyroid (n = 4) were measured as log10 values.
carcinoma (FTC) and ATC harbouring different genetic alterations (Supplementary Table S1, see section on supplementary data given at the end of this article), in comparison with immortalised normal thyrocytes (N-thy-ori3-1). As shown in Supplementary Figure S3, see section on supplementary data given at the end of this article, these three genes were expressed poorly or not at all in immortalised N-thy-ori3-1 cells. The overexpression of DUSP6 (200- to 600-fold) was observed in all PTC but not in FTC cell lines (panel A). DUSP4 was overexpressed in almost all the ATC and in two of four PTC cell lines (panel B). SPRY2 was moderately overexpressed in all the PTC lines (panel C). Taken together, these results indicate that PTC cells overexpress several members of the DUSP family compared with N-thy-ori3-1 cells. DUSP6 was distinctly overexpressed in the PTC histotype, and ERK1/2 pathway-specific mechanisms have been further investigated.

**DUSP6 upregulation by PTC-associated oncogenes depends on ERK1/2 pathway activation**

The effect of different PTC-related oncogenes on DUSP6 expression was investigated in N-thy-ori3-1 cells exogenously expressing BRAF-V600E, RET/PTC1 or TRK-T3. All PTC-associated oncogenes induced DUSP6 upregulation compared with mock-transfectants (Fig. 3A). Accordingly, the inhibition of RET/PTC1 by the RET-targeting agent RPI-1 (Cassinelli et al. 2009) in TPC1 cell line was associated with abrogation of DUSP6 expression (data not shown). The strongest DUSP6 modulation was induced by BRAF-V600E, in keeping with observations of PTC cell lines that endogenously express this oncogene.

Because we have demonstrated that the RET/PTC1 multi-docking site, responsible for MEK-ERK1/2 pathway activation, is necessary for DUSP6 upregulation, and the highest DUSP6 expression was present in BRAF-V600E cells, we next evaluated DUSP6 expression levels in TPC1 and K1 cells treated with the MEK inhibitor UO126. DUSP6 mRNA was strongly downregulated in drug-treated cells compared with control cells (Fig. 3B), suggesting that DUSP6 overexpression might be a compensatory mechanism in response to inhibition of the ERK1/2 pathway. DUSP6 protein was also downregulated in response to treatment with the MEK inhibitor in both TPC1 and K1 cells (panel B, right). DUSP6-specific antibody detected two protein bands corresponding to translation products initiating at different ATG codons, and the larger protein was more greatly affected by treatment (Fig. 3B), as previously shown (Zhang et al. 2010).

**Figure 3**
DUSP6 upregulation via the ERK1/2 pathway by PTC-related oncogenes, DUSP6 overexpression and ERK1/2 pathway activation in PTC cell lines. (A) Left panel: real-time RT-PCR analysis of the DUSP6 gene in N-thy-ori3-1 cells transiently transfected with the indicated oncogenes. Right panel: lysates from corresponding N-thy-ori3-1 transfected cells analysed by WB with the indicated antibodies. (B) Left panel: real-time RT-PCR analysis of the DUSP6 gene in K1 or TPC1 PTC cell lines treated with vehicle or UO126. Right panel: total protein extracts obtained from corresponding K1- or TPC1-treated cells analysed by WB. (C) Analysis of the MAPK signalling pathway in N-thy-ori3-1 and in selected PTC cell lines. The membranes were then stripped and re-probed with the respective anti-protein antibodies. (D) Total, cytoplasmic and nuclear extracts were concomitantly analysed with the indicated phospho-specific antibodies. Anti-NBS1 and anti-vinculin blots are shown as nuclear and cytoplasmic controls respectively. In all the WB analyses, an anti-vinculin blot is shown as a protein loading control.
DUSP6 protein was variably overexpressed in all the analysed PTC cells (Fig. 3C). Accordingly, selected components of the MAP kinase pathway were found to be more highly phosphorylated in all the analysed PTC cell lines, compared with N-thy-ori3-1. Within the ERK signalling cascade, MEK1/2, the upstream activators of ERK1/2, was found to be phosphorylated in \( \text{BRAF}^{\text{V600E}} \)- and, to a lesser extent, in \( \text{RET/PTC1} \)-expressing cells. The ERK effectors RSK1/2 were mostly activated in \( \text{BRAF}^{\text{V600E}} \) expressing cells. Overall, the expected inverse correlation between DUSP6 expression and ERK1/2 activation was not observed. Moreover, nuclear/cytoplasmic fractionation of proteins was performed, and DUSP6 displayed an exclusive cytoplasmic localisation, as expected, and total and phosphorylated ERK1/2 proteins were mostly cytoplasmic (Fig. 3D).

**DUSP6 mRNA and protein expression in human thyroid carcinoma surgical samples**

We next analysed DUSP6 mRNA expression by real-time RT-PCR in five non-neoplastic thyroids and 15 PTC biopsies, including nine primary tumours from pT1 to pT4 stage and six nodal metastases. The genetic and histological characterisation of the PTCs cases is reported in Supplementary Table S2. As shown in Fig. 4A, DUSP6 transcripts were significantly higher in PTCs than in non-neoplastic thyroids. According to our results in TC patient datasets and cell lines (Fig. 2 and Supplementary Figure S2), the overexpression of DUSP6 in PTCs cases was independent of the harboured genetic lesion (Fig. 4B). The same PTC samples were grouped into primary tumours, divided into stage 1–2 and stage 3–4, and nodal metastases (panels C and D). DUSP6 transcript levels remained significantly upregulated compared with non-neoplastic thyroids for the pT3-T4 and nodal subclasses and trended upwards with tumour stage and in nodal metastases vs primary tumours.

To further investigate the expression of DUSP6 in TC, we performed IHC analysis for the detection of DUSP6 protein and ERK phosphorylation in a series of TC samples characterised by increasing aggressiveness. The cases analysed included two non-neoplastic samples, three PTCs (one NOS, one tall cell and one follicular variant), tyrosine kinase rearrangement. Unknown: genetic lesions other than those mentioned above. (C) The same specimens as panel (A) showing PTC classified as primary tumour (primary) or nodal metastasis (nodal). (D) The same specimens as panel (A) classified according to tumour staging. pT1: tumours $<1$ cm and limited to the thyroid; pT2: tumours larger than 1 cm but not more than 4 cm in greatest dimension and limited to the thyroid gland; pT3: tumours more than 4 cm and limited to the thyroid; pT4: tumours displaying local extra-thyroid spread; nodal: nodal metastasis.
eight PDTCs, and two ATCs. Of note, three PDTCs and one ATC retained a papillary component, suggesting that they were derived from pre-existing PTCs. The results are reported in Table 2. Very low expression of DUSP6 was found in normal tissues and in ATCs, whereas DUSP6 was upregulated in the three PTCs and in 7/8 PDTCs, despite its heterogeneous levels.

Figure 5 shows representative cases including one non-neoplastic thyroid, three PDCTs (panel A) and one PTC/PDTC case showing two histologically distinct components (panel B). IHC analysis showed that DUSP6 was highly expressed in cases 10 and 13 compared with non-neoplastic thyroid and was found in the cytoplasm, as expected. ERK1/2 total proteins were expressed at similar levels in non-neoplastic and tumour tissues and displayed both nuclear and cytoplasmic localisation. Phosphorylated-ERK1/2 proteins were absent in non-neoplastic thyroids, in 1/3 PDTCs (case 9, displaying low DUSP6) and in 2/2 ATC. By contrast, pERK1/2 was easily detected in the other ten PDTC and PTCs samples (cases 10 and 13 are shown). The marker of thyroid differentiation thyroid transcription factor 1 (TTF1) was analysed as a control (Bejarano et al. 2000; Table 2 and Fig. 5A). Interestingly, case 7 presented two histologically distinct tumour components: PDTC and PTC tall cell. BRAF gene sequence analysis from the dissected FFPE sample revealed the presence of \( \text{BRAF-}^\text{V600E} \) in the PTC area (data not shown). In the latter, DUSP6 was strongly upregulated, and ERK1/2 was markedly activated compared with the \( \text{BRAF-}^\text{wt} \) area (Fig. 5B).

Furthermore, to confirm the IHC results and to extend the analysis to other components of the ERK pathway, five thyroid samples for which matched frozen tissue was available were subjected to WB with antisera to DUSP6 and to total and phosphorylated ERK1/2 and MEK proteins (Supplementary Figure S4, see section on supplementary data given at the end of this article). DUSP6 expression levels and ERK phosphorylation in thyroid samples by WB analysis correlated with those obtained by IHC, as shown in Fig. 5A. MEK1/2 were found activated in the PDTC sample (case 10) displaying the highest ERK1/2 phosphorylation level. Altogether, our analyses of surgical specimens unveiled DUSP6 protein overexpression in

Table 2  DUSP6 protein expression in thyroid carcinoma specimens.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age</th>
<th>Gender</th>
<th>BRAF genotype</th>
<th>Specimen</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>DUSP6</th>
<th>TTF1</th>
<th>Clinical outcome (length of follow-up)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Non-neoplastic thyroid</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Very low</td>
<td>+</td>
<td>–</td>
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<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Non-neoplastic thyroid</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Very low</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>F</td>
<td>( \text{BRAF-}^\text{V600E} )</td>
<td>PTC (Tall Cell v.)</td>
<td>pT2</td>
<td>Nx</td>
<td>Mx</td>
<td>High</td>
<td>+</td>
<td>NED (5 years)</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>F</td>
<td>( \text{wt} )</td>
<td>PTC (NOS)</td>
<td>pT3</td>
<td>N1a</td>
<td>Mx</td>
<td>High</td>
<td>+</td>
<td>Lost to follow up</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>F</td>
<td>( \text{wt} )</td>
<td>PTC (follicular v)</td>
<td>pT3</td>
<td>Mx</td>
<td>N1a</td>
<td>Moderate</td>
<td>+</td>
<td>NED (5 years)</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>F</td>
<td>( \text{wt} )</td>
<td>PDTC( ^a )</td>
<td>pT4b</td>
<td>N1b</td>
<td>Mx</td>
<td>Moderate</td>
<td>+</td>
<td>Progression (1 year)</td>
</tr>
<tr>
<td>7</td>
<td>74</td>
<td>F</td>
<td>( \text{BRAF-}^\text{V600E} ) and ( \text{wt} )</td>
<td>PDTC( ^a )</td>
<td>pT4a</td>
<td>N1a</td>
<td>Skeleton</td>
<td>High (( \text{BRAF-}^\text{V600E} )), Moderate (( \text{BRAF-}^\text{wt} ))</td>
<td>+</td>
<td>NED (1 year)</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>F</td>
<td>( \text{wt} )</td>
<td>PDTC( ^a )</td>
<td>pT3</td>
<td>N1a</td>
<td>Mx</td>
<td>High</td>
<td>+</td>
<td>NED (5 years)</td>
</tr>
<tr>
<td>9</td>
<td>81</td>
<td>M</td>
<td>( \text{wt} )</td>
<td>PDTC</td>
<td>pT4b</td>
<td>N1b</td>
<td>Mx</td>
<td>Very low</td>
<td>+</td>
<td>Progression (1 year)</td>
</tr>
<tr>
<td>10</td>
<td>74</td>
<td>M</td>
<td>( \text{wt} )</td>
<td>PDTC</td>
<td>pT4b</td>
<td>N1b</td>
<td>Mx</td>
<td>Moderate-high</td>
<td>+</td>
<td>NED (4 years)</td>
</tr>
<tr>
<td>11</td>
<td>73</td>
<td>M</td>
<td>( \text{wt} )</td>
<td>PDTC</td>
<td>pT3</td>
<td>N1b</td>
<td>Mx</td>
<td>Skeleton</td>
<td>High</td>
<td>NED (1 year)</td>
</tr>
<tr>
<td>12</td>
<td>73</td>
<td>F</td>
<td>( \text{wt} )</td>
<td>PDTC</td>
<td>pT3</td>
<td>N1b</td>
<td>Mx</td>
<td>Lung</td>
<td>High</td>
<td>NED (4 years)</td>
</tr>
<tr>
<td>13</td>
<td>74</td>
<td>M</td>
<td>( \text{wt} )</td>
<td>PDTC</td>
<td>pT3</td>
<td>N1b</td>
<td>Mx</td>
<td>Skeleton</td>
<td>High</td>
<td>Progression (1 year)</td>
</tr>
<tr>
<td>14</td>
<td>54</td>
<td>F</td>
<td>ND</td>
<td>ATC( ^a )</td>
<td>pT4b</td>
<td>N1a</td>
<td>Mx</td>
<td>Very low</td>
<td>+</td>
<td>Died of disease</td>
</tr>
<tr>
<td>15</td>
<td>68</td>
<td>F</td>
<td>ND</td>
<td>ATC( ^a )</td>
<td>pT4a</td>
<td>N1a</td>
<td>Mx</td>
<td>Very low</td>
<td>–</td>
<td>Died of disease</td>
</tr>
</tbody>
</table>

ND, not determined; pTNM, pathological tumor-node-metastasis staging; Nx, regional lymph nodes cannot be assessed; Mx, distant metastasis cannot be assessed; TTF1, thyroid transcription factor 1; NED, not evidence of disease.

\( ^a \)The original specimen from these patients contained areas showing PTC component.
most PTC/PDTC cases and a positive correlation with ERK1/2 pathway activation.

**DUSP6 biological effects**

The functional role of **DUSP6** in PTC was investigated through its silencing in the PTC cell lines TPC1, NIM-1, K1 and B-CPAP. As described in Supplementary Table S1, TPC1 carry the RET/PTC1 and the other cell lines the **BRAF**-V600E oncogene. We have previously shown that TPC1 cells display morphogenic properties that are abrogated by treatment with the **RET** inhibitor RPI-1 (Cassinelli et al. 2009). TPC1 cells transiently transfected with **DUSP6** small interfering RNAs (siDUSP6), which drastically reduced the DUSP6 expression level (Fig. 6A), showed a significant reduction in morphogenic capability compared with non-targeting siRNA-transfected cells. Biochemical analyses showed, subsequent to **DUSP6** silencing, a marked reduction of ERK1/2 phosphorylation (50–70% in repeated experiments). PARP analysis (panel A) and apoptotic bodies count (data not shown) suggest that siDUSP6 does not cause apoptosis. The proliferation rate of siDUSP6-transfected TPC1 cells was lowered only 5 days after transfection (Fig. 6B and data not shown). In a separate series of experiments (Fig. 6C), **DUSP6** silencing significantly reduced the ability of TPC1 cells to migrate into the lower chamber of a transwell and to invade the Matrigel layer. Parallel biochemical analysis showed that **DUSP6** silencing, in addition to lowering pERK levels, consistently lowers pMEK and, to a lesser extent, p52 and p66 SHC protein phosphorylation. Interestingly, siDUSP6 slightly decreased RET/PTC1 phosphorylation without affecting the activation of the RTK most active in TPC1 cells, including HGFR, EGFR and AXL (Supplementary Figure S5, see section on supplementary data given at the end of this article).

NIM-1 cells, previously demonstrated to depend on RAF/MEK/ERK activation for proliferation (Degl’Innocenti et al. 2010), were similarly transfected with siDUSP6 or non-targeting siRNA (Fig. 7). As a further control, untransfected cells were treated with UO126. As expected, the MEK inhibitor significantly reduced NIM-1 cell growth.

**Figure 5**

IHC analysis of DUSP6, pERK and ERK of representative cases reported in Table 2. Serial sections were immunolabelled with the indicated antisera. Representative tumour areas are shown. Original magnification 100×. (A) IHC analysis of non-neoplastic thyroid and PDTC surgical samples.

The indicated numbers (#2, #9, #10 and #13) refer to patients reported in Table 2. (B) IHC analysis of distinct areas of the PTC/PDTC case #7 with different histology and with or without the genetic lesion **BRAF-V600E**.
DUSP6 overexpression in thyroid carcinoma

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Biological effects of DUSP6 silencing on TPC1 cells. (A) Early tubulogenesis assay performed with untransfected (NT), non-targeting siRNA-transfected or siDUSP6-transfected TPC1 cells. Representative images (original magnification 10×) and total length quantifications of branched structures are shown. The data are reported as percentages of the control ± i.o. Protein extracts from corresponding TPC1 cells were analysed by WB with the indicated antibodies. Anti-vinculin blots are shown as protein loading controls. (B) TPC1 cell growth evaluated by the SRB proliferation assay performed with untransfected (NT), non-targeting siRNA-transfected or siDUSP6-transfected cells at day 5 from transfection. (C) Migration assay and invasion assay performed with non-targeting siRNA-transfected or siDUSP6-transfected TPC1 cells. Assays were performed in triplicate, and cells were counted in adjacent fields (n = 10). The data are reported as the average cell number per field ± i.o. Representative images of SRB-stained cells are shown below (original magnification 40×). Protein extracts from TPC1 transfected cells were analysed by WB with the indicated antibodies (right panel). *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test are indicated in the figure.

Because untreated NIM-1 cells have invasive but not morphogenic capabilities (data not shown), the effects of siDUSP6 on NIM1 cells were assessed through migration and invasion assays (panel C). NIM-1 cells transfected with siDUSP6 showed significantly reduced ability to migrate and to invade the Matrigel layer compared with non-targeting siRNA-transfected cells. Similarly, DUSP6 silencing significantly reduced the invasive behaviour of the PTC cell lines K1 and B-CPAP (Supplementary Figure S6, see section on supplementary data given at the end of
**Figure 7**

Biological effects of DUSP6 silencing on NIM-1 cells. (A) NIM-1 cells untransfected (NT), non-targeting siRNA-transfected (non-targeting), siDUSP6-transfected (siDUSP6), untransfected cells exposed to solvent (DMSO) or to UO126 (UO126) were grown for up to 72 h in medium without FBS. Cell proliferation was evaluated with the SRB assay. Representative growth curves from one experiment are shown (left panel). Apoptosis was further evaluated by counting apoptotic nuclei in adjacent fields (n = 10), as condensed and fragmented nuclei upon Hoechst 33341 staining under fluorescent microscope (indicated by arrows). Relative quantification is shown in the right panel; data were expressed as the average percentages ± S.D. (C) Migration assay and invasion assay performed with non-targeting siRNA-transfected or siDUSP6-transfected NIM-1 cells. Assays were performed in triplicate, and cells were counted in adjacent fields (n = 10). The data were reported as average cell numbers per field ± S.D. Representative images of SRB-stained cells are shown below (original magnification 40×).

**Discussion**

We have shown that PTC cell lines and the majority of PTC and PDTC specimens overexpress DUSP6/MKP3. Accordingly, DUSP6 displays tumour-promoting effects in TC cell lines. It is known that the ERK1/2 pathway is essential for thyroid carcinogenesis (Greco et al 2009) and that DUSP6 mediates one of the feedbacks to this pathway. We have demonstrated that BRAF, RET/PTC and TRK oncogenes activated in TC are able to upregulate DUSP6 expression, thus activating both the ERK1/2 pathway and its negative feedback mechanisms. Consistently, we found that other ERK signalling regulators are upregulated by RET/PTC1 and are overexpressed in PTC datasets. However, the ERK1/2-DUSP6 interplay is complex, as active ERK1/2 upregulates DUSP6 mRNA, but by favouring protein degradation, downregulates DUSP6 protein (Bermudez et al. 2011).

Analyses of public gene expression profiles and of our surgical samples concordantly suggest that most PTCs overexpress DUSP6 mRNA. Through IHC and biochemical analyses, we have demonstrated that DUSP6 protein was overexpressed in TC surgical samples compared with non-neoplastic thyroids and thyrocytes surrounding the tumour. This was a novel finding because DUSP6 has mainly been investigated at the RNA level in cancers.

Because it has been suggested that DUSP6 acts as a tumour suppressor gene in several carcinomas, it might also be hypothesised to have a similar function in TC progression. On the contrary, we have found DUSP6 overexpression even in more aggressive PTC variants and this article). Overall, our functional experiments in four PTC cell lines (summarised in Supplementary Figure S1) suggest that DUSP6 silencing counteracts malignant PTC cell phenotypes.
in PDTC, a histotype with features intermediate between WDTC and ATC. The only PDTC that did not overexpress DUSP6 showed basal levels of ERK1/2 pathway activation. The same was true for the two analysed ATCs: neither overexpress DUSP6, in accord with the literature on DUSP6 mRNA (Salvatore et al. 2007), and both show basal levels of pERK1/2 (data not shown). A positive correlation trend was found between DUSP6 expression and the activation of the ERK1/2 pathway components MEK, ERK1/2 and RSK. Thus, DUSP6 overexpression seems to be a read-out of ERK1/2 pathway activation instead of being its negative feedback. This was corroborated by a reported specific case displaying high pERK and DUSP6 levels in a BRAF-V600E-positive tumour area and low pERK and DUSP6 in a BRAF-wt tumour area.

Upregulation of DUSP6 has been reported in tumours of different histotypes (e.g. Quyun et al. 2010). In addition, a tumour-promoting role for DUSP6 has recently been suggested in glioblastoma cells (Messina et al. 2011).

We performed functional experiments in four PTC cell lines (TPC1, NIM-1, K1 and B-CPAP). DUSP6 silencing in TPC1 cells resulted in a reduction of branched morphogenesis, consistent with inhibition of the epithelial to mesenchymal transition. This finding is in agreement with the reported identification of DUSP6 as one of the GDNF-induced genes regulated by the RET proto-oncogene during ureteric bud branching morphogenesis (Lu et al. 2009). Of note, DUSP6 was necessary but not sufficient to induce branching morphogenesis because the other three PTC cell lines do not show this ability. Interestingly, DUSP6 silencing significantly reduced the invasive ability of all four PTC cell lines. In addition, NIM-1 cell proliferation was reduced and apoptosis was enhanced. Furthermore, in both NIM-1 and TPC1 cells, the steady state level of ERK1/2 was not enhanced by DUSP6 silencing, as might be expected by lowering a negative feedback regulator. This apparent discrepancy might be the result of the complex network involving forward and feedback regulators of RTK and ERK1/2 pathways (Wortzel & Seger 2011). How the shutdown of DUSP6 may lower pERK1/2 and the thyroid cell lines’ invasive and migratory abilities remains to be elucidated, especially considering the hundreds of protein substrates of these kinases. Of note, our results suggest a possible backward effect of DUSP6 on RET/PTC1 protein activation in TPC1 cells. Furthermore, in NIM-1 cells, we showed that DUSP6 silencing, by contrast with ERK1/2 chemical inhibition, enhances apoptosis and lowers pAKT, thus confirming the known interplay between ERK and AKT pathways in the thyroid (Miller et al. 2009). Although we cannot exclude the role of additional DUSP6 targets, the phosphorylation of p38, recently indicated as a novel DUSP6 target, is not enhanced through DUSP6 silencing (data not shown).

Although further studies are needed, our work clearly points to DUSP6 overexpression as a possible player in thyroid malignancy. High DUSP6 expression levels in PTC were confirmed by Lee et al. (2012) in work published during the review process of our manuscript.

Overall, our work suggests that dissecting the role of ERK1/2 pathway components may allow a better understanding of the complex network involved in thyroid carcinogenesis, possibly providing useful information to design appropriate targeted therapies.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0078.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
M G Borrello and D Degl’Innocenti designed the study with the collaboration of S Pilotti, A Greco, E Seregni and M A Pierotti. P Romeo, G Cassinelli, V Catalan and C Lanzi conceived, performed and analysed data of the in vitro and functional experiments. D Degl’Innocenti conceived, performed and analysed data of real-time experiments. M Sensi performed dataset analysis. E Tarantino and F Perrone performed histopathological analysis evaluated by S Pilotti. E Seregni collected clinical data. All authors were involved in writing the paper, especially M G Borrello, D Degl’Innocenti, S Pilotti, P Romeo, M Sensi, A Greco, C Lanzi and M A Pierotti.

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References
Research

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