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Post-Transcriptional Regulation of the DUSP6/MKP-3 Phosphatase by MEK/ERK Signaling and Hypoxia

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DUSP6/MKP-3 is a cytoplasmic dual-specificity phosphatase specific for the MAP kinases ERK1/2. Previous data have shown that the MEK/ERK axis exerts a retro-control on its own signaling through transcriptional and post-translational regulation of DUSP6. We first confirm the key role of MEK/ERK in maintaining the levels of *dusp6* mRNA, while PI3K/mTOR, p38 MAPK, and JNK signaling pathways had no significant effects. We further show that regulation of *dusp6* mRNA stability plays a critical role in ERK-dependent regulation of *dusp6* expression. Luciferase reporter constructs indicated that MEK/ERK signaling increased the half-life of *dusp6* mRNA in a 3′untranslated region (3′UTR)-dependent manner. In addition, hypoxia, a hallmark of tumor growth, was found to increase both endogenous levels of *dusp6* mRNA and the stability of the luciferase reporter constructs containing its 3′UTR, in a HIF-1-dependent manner. Nevertheless, a basal ERK activity was required for the response to hypoxia. Finally, Tristetraprolin (TTP), a member of the TIS11 CCCH zinc finger protein family, and PUM2, an homolog of drosophila pumilio, two proteins regulating mRNA stability reduced the levels of endogenous *dusp6* mRNA and the activity of the *dusp6/3′UTR* luciferase reporter constructs. This study shows that post-transcriptional regulation is a key process in the control of DUSP6 expression.

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The MAP kinases ERK1/2 play an important role in the transduction of extracellular signals leading to changes in cell metabolism and in the expression of genes involved in various cellular responses including proliferation, differentiation, survival, or motility. Spatio-temporal regulation of the ERK1/2 pathway is crucial for determining cell fate in physiological and pathological contexts (reviewed in Chambard et al., 2007). Several types of retro-controls on ERK signaling exist, including recycling of cell surface receptors, dissociation of scaffolding complexes and action of phosphatases on the multiple kinases of the pathway. Although ERK1/2 are substrates for broad-specificity phosphatases, they also have specific inhibitors, the MAP kinase phosphatases (MKPs), which belong to the family of dual-specificity phosphatases DUSPs, so called as they dephosphorylate both tyrosine and serine/threonine residues. While some DUSPs are able to inactivate different MAPKs to the same extent, others are more specific to one type of MAPKs. This is the case of DUSP6/MKP-3, an ERK1/2 cytoplasmic phosphatase (Groom et al., 1996; Muda et al., 1998; Arkell et al., 2008). This specificity is insured by the fact that the interaction of DUSP6 with ERKs is an absolute requirement for the catalytic activation of the phosphatase through conformational changes (Camps et al., 1998; Fjeld et al., 2000).

Conserved among animals, DUSP6 is required for different processes during development: for eye patterning and differentiation of wing veins in *Drosophila*, axial patterning in the zebrafish, limb bud formation in the chick, and midbrain/hindbrain isthmus, maxilar-mandibular and ear formation in mice, among others (Dickinson et al., 2002; Klock and Herrmann, 2002; Rintelen et al., 2003; Tsang et al., 2004). Interestingly, the phenotype of *dusp6* mutants was generally reminiscent of FGF or FGFR knockouts, suggesting a critical role of this phosphatase in the regulation of signaling by this morphogen family. Besides its specific role during animal organogenesis, DUSP6 could also participate in tumorogenesis processes. In myeloma cell lines harboring an active mutant form of N-ras and in melanoma cell lines with either a B-raf V600E or a Q61R N-ras mutation, *dusp6* mRNA is up-regulated (Croonquist et al., 2003; Bloethner et al., 2005). On the contrary, *dusp6* expression is low in invasive phases of pancreatic ductal adenocarcinoma, compared to earlier phases of pancreatic intraepithelial neoplasia (Furukawa et al., 2003, 2005). Nevertheless, the mechanisms that lead to up-regulation or down-regulation of *dusp6* expression in cancer cells during tumor progression appear to be very dependent on the cancer type and remain unclear.

The expression levels of DUSP6 can depend on the transcriptional activity of the *dusp6* gene promoter, the stability and the rate of translation of its mRNA and the stability of the DUSP6 protein. At the protein level, previous studies from our group and others showed that stimulation with serum or PDGF-BB alone can induce a MEK-dependent phosphorylation of DUSP6 on Ser159, Ser174, and Ser197, which is followed by the degradation of the phosphatase by the proteasome (Marchetti et al., 2005; Jurek et al., 2009). DUSP6 is also phosphorylated upon activation of the mTOR pathway, a

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phosphorylation that reduces the half-life of the phosphatase as well (Bermudez et al., 2008). Finally, DUSP6 was also shown to interact with and to be phosphorylated by protein kinase CK2a/casein kinase 2, another ubiquitous kinase involved in the regulation of cell proliferation and survival (Castelli et al., 2004). Concerning the transcriptional regulation of *dusp6*, the ERK pathway consistently appears as a major regulator of mRNA expression, notably in a negative feed-back loop of FGF signaling (Eblaghie et al., 2003; Tsang et al., 2004; Smith et al., 2006). Luciferase reporter assays showed that the transcriptional activation of *dusp6* by FGF family members involves an Ets2-dependent mechanism dependent on the intron 1 of the phosphatase gene (Ekerot et al., 2008; Furukawa et al., 2008). If the MEK/ERK pathway appears as a major regulator of *dusp6* transcription, nothing is known about its role in *dusp6* mRNA stability, an important aspect of post-transcriptional gene regulation in physiological and pathological conditions.

In the present work we explored the molecular mechanisms regulating *dusp6* mRNA in two different cancer cell lines harboring different mutations in the MAPK pathway: the LS174 colon carcinoma which present a K-ras mutation and the A375 melanoma cell line carrying a B-raf activating mutation. We show here that the MEK/ERK but not the PI3K neither the p38 or JNK pathways regulate *dusp6* mRNA levels. Using a luciferase reporter cloned upstream of the 3′untranslated region (3′UTR) of *dusp6* mRNA, we showed that the MEK/ERK pathway is involved in the regulation of *dusp6* mRNA stability. Interestingly, hypoxia, a hallmark of growth of many solid tumors, induces a HIF-1-dependent increase in *dusp6* mRNA levels. Finally, we identified two factors affecting *dusp6* mRNA stability: Tristetraprolin (TTP) protein, the canonic member of the TIS11 family, and PUM2 which belongs to the PUF mRNA-binding proteins.

Materials and Methods

Reagents

Tetracycline, 4-hydroxytamoxifen (4-OHT) and rapamycin were obtained from Sigma (St. Quentin-Fallavier, France). PD184352 was a kind gift of Dr. Michiaki Kohno (Nagasaki University, Japan). SB203580 and SP600125 were purchased from Euromedex (Mundolsheim, France). 5,6-Dichlorobenzimidazole riboside (DRB) was from Sigma.

Plasmid constructs

The TK-luciferase-3′UTR*vegf* and the TK/luciferase constructs have been described previously (Ciais et al., 2004; Essafi-Benkhadir et al., 2007). The TK-luc-3′UTRdusp6 vector was constructed as follows: The region of *dusp6* 3′UTR (1,350 pb) was obtained by PCR using the oligos 3′UTR *dusp6*-sense-GAAAGACCCCACA-CCCTCC and 3′UTR *dusp6*-antisense-CCCTACTATGCCTA-CAAGTC, first cloned in TOPO-TA vector (Invitrogen, Cergy Pontoise, France) and subsequently cloned downstream of the TK-luc in the pBluescript II SK+ plasmid in *Bam*HI and *Not*I restriction sites. The pCDNA4/TO-TTP/myc-HisA vector, used for transient transfections and generation of tetracycline-inducible A375 clones for TTP was previously generated in our laboratory (Essafi-Benkhadir et al., 2007). The plasmid coding for PUM2 was a kind gift of Dr. Judith Kimble (University of Wisconsin, Madison) and has been previously described (Lee et al., 2007).

Cell culture and transfection

LS174 and A375 cells were grown in Dulbecco's modified Eagle's medium DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS) and were transfected with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations.

HEK293 cells expressing the chimera ΔRaf:ER cells (Cagnol et al., 2006) were cultured in DMEM medium without phenol red, supplemented with 7.5% of heat-inactivated FBS and transfected using the calcium phosphate method.

Inducible clones for TTP were obtained using A375 cells that expressed the Tetracycline repressor. Transfection was carried out with Lipofectamine™ 2000 (Invitrogen). Inducible clones were selected as already described (Essafi-Benkhadir et al., 2007). LS174 cells inducible for a shRNA against HIF-1α have been described previously described (Dayan et al., 2006).

Hypoxic conditions were obtained by growing cells in a sealed anaerobic workstation (Ruskinn Technology Biotrace International Plc, Bridgend, UK) at 1% O_2, 5% CO_2, 94% N_2 at 37°C and 95% humidity.

Immunoblotting

Cells were lysed in Laemmli buffer, proteins were quantified using bicinchoninic acid assay from Pierce Thermo Scientific (Rockford, IL) and separated by SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Immobilon-P from Millipore, Molsheim, France). Primary antibody included monoclonal anti-phospho-ERK1/2 (M8159 from Sigma), anti-DUSP6 was a kind gift of Dr. Stephen Keyse (University of Dundee, Scotland, UK), anti-ARD1 was a gift of Jacques Pouysségur, anti-phospho70 S6 kinase Thr389 (Cell Signaling Technology, Danvers, MA), rabbit serum against total ERK1/2, anti-myc (9E10, Sigma), anti-flag M2 (Sigma F1804) or rabbit serum against HIF-1α (kindly provided by Dr. Jacques Pouysségur and previously described in Richard et al., 1999). Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Promega (Charbonnières Les Bains, France). Immunoreactive bands were detected with the enhanced chemiluminescence detection system (ECL; Thermo Fisher Scientific, Brebières, France) on autoradiography films (Fujifilm, Dusseldorf, Germany). Staining was quantified using the Gene Tools software from Syngene (Cambridge, UK).

RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA were extracted with TRIzol Reagent (Invitrogen). Two micrograms of RNA were used for reverse transcription, using the Omniscript RT Kit (Qiagen, Hilden, Germany) with oligo(dT) to prime first-strand. Quantitative RT-PCR was performed in an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA), using Mastermix RT-SN2X-03+ for SYBR green dye (Eurogentec, Angers, France). The amplification of each gene was performed on cDNA templates using specific oligonucleotides: the PCR primers for *dusp6* and *rplp0* (ribosomal protein used as a control) were chosen on the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/): *dusp6* 12707568a3; *rplp0* 16933546a2.

The primers for *dusp5* and *dusp7* were designed using the Primer Express software:

dusp5-sense 5′-GGCCTTCGATTACATCAAGCA-3′,
dusp5-antisense 5′-TGGCCCATGAAGCCAAAG-3′,
dusp7-sense 5′-CGGCTGCCAGGCCTACTAC-3′,
dusp7-antisense 5′-TGGTCTCGCAGTGCTCAGAGT-3′.

For the amplification of *bnip3* the following primers were used, sense 5′-CACTTCAGCAATAATGGGAACGG-3′ and antisense 5′-TATCTTGTGGTGTCTGCGAGCG-3′. For *pai-p2*, the following primers were used; sense 5′-TCTCCCA-CAAACTATGGACCA-3′ and antisense 5′-TGCATTTGGATTCAGATTGCTCT-3′. The relative quantity for each gene was expressed in relation to the internal control gene *rplp0*. The relative gene expression was calculated using the $2^{-\Delta C_T}$ method where $\Delta C_t = 2^{-C_{T\,\text{geneofinterest}}} - 2^{-C_{T\,\text{Rplp0}}}$.

Luciferase assays

For luciferase assays, HEK293 or LS174 cells were serum starved for 16 h, treated or not with PD184352 or 4-OHT, when indicated. Cells were then lysed in a buffer containing 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100. Ten microliters of supernatant was used to measure luciferase activity in 96-well plates in a buffer with 20 mM Tricine, 1.07 mM $(MgCO_3)Mg(OH)_2$, $5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferine, and 530 μM ATP. Plates were read in a Robion Solaris luminometer (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) in order to normalize the luciferase activity with protein quantity present in the cell lysate of each condition.

Results

The MEK/ERK but not the PI3K or the p38/JNK pathways regulates *dusp6* mRNA levels

Ekerot et al. (2008) have shown that the induction of *dusp6* mRNA by the FGF family in NIH3T3 cells was dependent on the MEK/ERK pathway. We first investigated whether we could reproduce this finding in two cancerous cell lines, the LS174 colon carcinoma harboring a K-ras mutation, and the A375 melanoma carrying an activating mutation of B-raf, a downstream kinase of ras in the ERK signaling cascade. Although both types of mutations are assumed to result in over-activation of the MEK/ERK pathway, exponentially growing LS174 cells exhibit lower levels of ERK phosphorylation than A375 cells. Nevertheless, incubation with the MEK inhibitor PD184352 (PD) caused a rapid decrease in ERK activity and of endogenous *dusp6* mRNA levels in both cell lines, as measured by Q-PCR (Fig. 1A,B), reaching 98% and 90% decrease in A375 and in LS174 cells, respectively.

We then wonder whether other signaling pathways, particularly downstream of the PI3 kinases (PI3K), targets of active Ras, could be involved in the regulation of *dusp6*. No change in *dusp6* mRNA was observed after blocking PI3K with a specific pharmacological inhibitor, LY294002, in A375 and LS174 cells (Fig. 1A,B). Finally, because some cross-talk mechanisms exist between the different MAP kinases, we analyzed whether p38 MAPK and JNK could be involved in the process. Cell treatment with SP600125, an inhibitor of JNK, blocked the phosphorylation of the JNK substrate ATF2 but had no effect on *dusp6* mRNA levels (Fig. 1). Blockade of p38 MAP kinase by SB203580 did not have any effect either (not shown). Finally, we also confirmed the finding by Ekerot et al. that stimulating a ΔRaf:ER chimera, which allows direct activation MEK, was sufficient to induce *dusp6* expression (Fig. 1C). While ERK activation diminished after 8 h, *dusp6* mRNA still accumulates, suggesting that the increase of *dusp6* mRNA by MEK/ERK may require a certain delay. In the same conditions, we observed an early increase of DUSP6 protein levels, around 1 h, followed by a decrease of these levels at later time points, in spite of the strong increase in DUSP6 mRNA. This differential regulation is likely to involve phosphorylation and proteosomal degradation of DUSP6, as described in our previous report (Marchetti et al., 2005).

The MEK/ERK pathway stabilizes *dusp6* mRNA

These changes in the amounts of *dusp6* mRNA could reflect a modification of *dusp6* transcription, as documented in Ekerot et al. (2008) but also changes in *dusp6* mRNA stability, as described previously for *dusp1* (Lin et al., 2007; Emmons et al., 2008; Kuwano et al., 2008). Time-course experiments of either LS174, A375, or the non-transformed HEK293 cells with the transcription inhibitor DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) have allowed determining that *dusp6* mRNA has a short half-life ranging from 20 to 40 min according to the cell type (Fig. 2A). Thus, *dusp6* mRNA appears as a labile mRNA. Its short half-life may constitute a rapid and efficient manner to control ERK phosphorylation in the cytosol. Blocking MEK activity with PD184352 further reduced *dusp6* mRNA stability: its half-life diminished from 25 to 8 min in A375 cells and from 37 to 8 min in LS174 cells (Fig. 2A). If ΔRaf:ER HEK293 cells hardly express any detectable levels of *dusp6* mRNA in the absence of 4-OHT, the half-life of this mRNA was also strongly diminished by PD184352 in 4OHT-stimulated conditions (Fig. 2B). These data indicate that the MEK/ERK pathway regulates not only the transcription as shown by Ekerot et al. (2008) but also the stability of *dusp6* mRNA.

mRNA 3′UTR are classically involved in the regulation of their stability. Therefore, we cloned the 3′UTR of *dusp6* mRNA downstream of a minimal thymidine kinase (TK) promoter and a luciferase reporter gene (luc). ΔRaf:ER HEK293 cells were transiently transfected with either TK-luc or TK-luc-3′UTR*vegf*, used as negative and positive controls, respectively (Essafi-Benkhadir et al., 2007) and TK-luc-3′UTR*dusp6*. For each reporter gene, the 100% value was chosen as the value corresponding to the untreated control situation. Whereas the TK-luc construct presented a basal luciferase activity that did not change following the different treatments, stimulation of the MEK/ERK pathway by 4-OHT caused a strong increase in the luciferase activity generated by TK-luc-3′UTR*vegf* (Fig. 2C) as previously reported (Essafi-Benkhadir et al., 2007). The same treatment also produced a strong increase in luciferase activity generated by the TK-luc-3′UTR*dusp6* construct. In both cases, 4OHT-induced increment was blocked by pre-treating cells with the MEK inhibitor PD184352 (Fig. 2C). Together these results suggest that the MEK/ERK signaling cascade is able to stabilize *dusp6* mRNA via its 3′UTR region.

Hypoxia, a hallmark of tumor growth, increases *dusp6* mRNA stability

We next investigated whether hypoxia, another characteristic of tumor development could affect *dusp6* mRNA regulation. Previous reports have shown that hypoxia induces the expression of DUSP1, a phosphatase that inactivates ERK, p38, and JNK in the nucleus (Laderoute et al., 1999; Seta et al., 2001). Studies in different cell models including arterial endothelial cells and neurons showed that *dusp6* mRNA levels are up-regulated in hypoxic conditions (Mishra and Delivoria-Papadopoulos, 2004; Manalo et al., 2005). We confirmed these findings in A375 and LS174 cells cultured at 1% of oxygen during 4–48 h. Maximal induction was observed after 24 h and is shown on Figure 3A. The levels of HIF-1α, the subunit of the major transcription factor HIF1 expressed and stabilized following hypoxia, were enhanced in the same conditions (see Western blot in Fig. 3A). The moderate but reproducible induction of *dusp6* mRNA following hypoxia was comparable to that of *vegf* mRNA, an important gene of the angiogenic response (Bando et al., 2003; Manalo et al., 2005). Importantly, MEK activity remains necessary for maintaining *dusp6* mRNA levels, even in hypoxic conditions, as evidenced by results obtained in the presence of the inhibitor PD184352 in both cell lines.

In silico analysis did not reveal the presence of overt hypoxia responsive elements (HREs) in the 5′ region or the introns of the *dusp6* gene. The effects of hypoxia could be indirect and mediated either by the induction of a factor enhancing *dusp6* transcription or regulating the stability of its mRNA. To test the second hypothesis, luciferase experiments using the TK-luc-3′UTR*dusp6* plasmid were performed in low oxygen conditions. Hypoxia strongly increased the luciferase activity generated by the TK-luc-3′UTR*vegf*, while it had no effect on

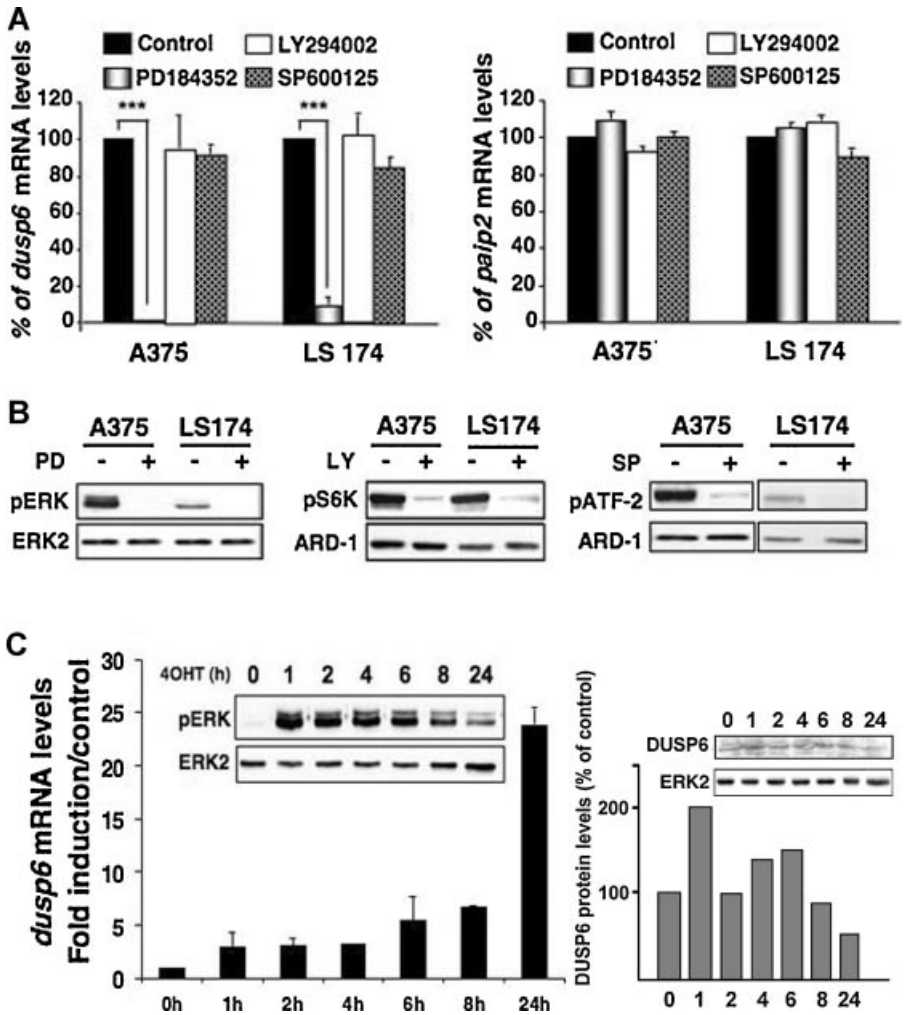

Fig. 1. The MEK/ERK pathway has a critical role in the regulation of *dusp6* mRNA. A: Exponentially growing A375 and LS174 cells were treated in the absence or presence of 5 μM of PD184352 (PD), 15 μM LY294002 (LY), or 10 μM SP600125 (SP) for 30 min; RNA were extracted and amounts of *dusp6* and *paip2* mRNA were measured by RT-quantitative PCR. mRNA levels are expressed in percentage of non-treated control cells, set at 100%. **B:** The efficiency of pharmacological treatments was controlled by analysis of phosphoERK, phospho-S6 kinase, and phosphoATF2 following treatment with PD184352, LY294002, or SP600125, respectively. **C:** ΔRaf:ER HEK293 cells were serum starved for 16 h and then stimulated with 1 μM of 4-OHT for the indicated times. RNA was prepared at each time point and *dusp6* mRNA levels analyzed by RT-QPCR. *dusp6* mRNA levels before 4-OHT treatment were arbitrary set at 1 and values for subsequent time points are indicated as fold induction compared to time 0 h. Activation of the ΔRaf1:ER chimera with 4-OHT also resulted in a strong and sustained increase in pERK levels, as seen by Western blot. Levels of DUSP6 proteins were analyzed in the same conditions, using total ERK as loading control (right part) and setting levels of DUSP6 in unstimulated cells at 1. Statistics were calculated with the Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

the TK-luc control cells (Fig. 3B). Although the increase was less important, hypoxia also enhanced the luciferase activity of the TK-luc-3′UTR*dusp6* reporter, suggesting that *dusp6* mRNA is also stabilized following hypoxia (Fig. 3B). Hence, although we cannot rule out a potential transcriptional effect of hypoxia on the *dusp6* gene, our results indicate that hypoxia-induced regulation of *dusp6* also occurs through mRNA stabilization.

Hypoxia-induced *dusp6* mRNA stability is HIF-1 and MEK/ERK dependent

Since HIF-1 is a major actor of gene expression following hypoxia, we checked whether it was involved in *dusp6* mRNA regulation. HIF-1α was silenced in LS174 cells expressing an inducible shRNA (Dayan et al., 2006). As shown in Figure 3C tetracycline-induced shRNA caused an almost total down-regulation of HIF-1α in both normoxic and hypoxic conditions. In the absence of tetracycline, HIF-1α shRNA-LS174 inducible cells show a response to hypoxia comparable to that of the parental LS174 cells. Hypoxia increased the levels of *bnip3* mRNA, a classical target of HIF-1, up to sevenfolds, and the levels of endogenous *dusp6* mRNA by twofolds. When HIF-1α shRNA was induced by tetracycline, the up-regulation of both genes was completely blocked.

The potential role of HIF-1 was also investigated on the 3′UTR constructs. shHIF-1α LS174 cells were transfected with luciferase reporter plasmids. Luciferase activity was measured following shRNA induction (or not), and following normoxic versus hypoxic conditions (Fig. 3D). HIF-1α down-regulation prevented the increase in luciferase activity of the TK-luc-3′UTR*dusp6* construct following hypoxia while it had no effect on the control TK-luc construct.

Finally, similar experiments were performed in the absence or presence of the MEK inhibitor PD184352. The response of

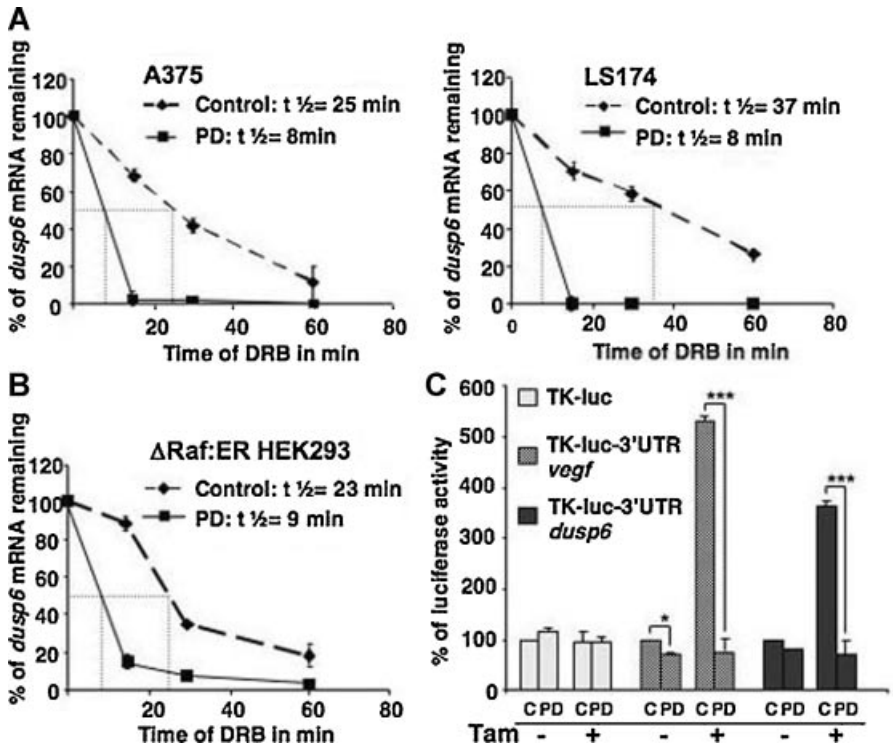

Fig. 2. ***dusp6*** **mRNA stability is regulated by the MEK/ERK pathway. A: LS174 and A375 cells were treated or not with 5 μM of PD184352 for 1 h and then with 25 μg/ml of DRB, an inhibitor of transcription. Amounts of remaining *dusp6* mRNA were measured at different times after DRB treatment by RT-QPCR. *dusp6* mRNA levels before DRB treatment were taken as 100%. Dotted lines indicate the half-life times of *dusp6* mRNA in cells with and without PD184352 treatment. B: *dusp6* mRNA half-life time is strongly decreased after blocking MEK activity in HEK293 ΔRaf:ER. Cells were stimulated with 4-OHT for 24 h in order to obtain an increased pool of *dusp6* mRNA. Cells were then incubated or not with 5 μM of PD184352 and after with 25 μg/ml of DRB. RNA was prepared at different times after DRB application. Half-life time is indicated for control and PD-treated cells. C: Luciferase activity reporter of the 3′UTR of *dusp6* is modified by MEK/ERK activity. HEK293 ΔRaf:ER cells were transfected with 500 ng/ml of TK-luc or TK-luc-3′UTR-*vegf* or TK-luc-3′UTR *dusp6* and then serum starved for 16 h. Cells were treated with 5 μM of PD184352 for 1 h and then the ΔRaf:ER was stimulated with 1 μM 4-OHT for 4 h. Statistics were calculated with the Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).**

the TK-luc-3′UTR*vegf* and the TK-luc-3′UTR*dusp6* constructs to hypoxia requires MEK activity (Fig. 4). This was the case in both LS174 cells, in which hypoxia stimulates ERK activity, and in A375 cells in which hypoxia has no effect on ERK signaling (Fig. S1).

Two mRNA regulating proteins, Tristetraprolin and PUM2, decrease the half-life of *dusp6* mRNA via its 3′UTR

Our group and others have shown that *vegf* mRNA regulation involved proteins of the TIS11 CCCH zinc finger family (Ciais et al., 2004; Essafi-Benkhadir et al., 2007). Hence, TTP binding on the *vegf* mRNA 3′UTR-induced reduction of its half-life. Since the 3′UTR region of *dusp6* mRNA contains a potential AU rich sequence (ARE) for binding of TIS11 family members (Fig. 5A), we tested whether TTP may promote *dusp6* mRNA degradation, using A375 clones in which TTP expression can be induced by tetracycline. Since TTP expression causes the death of some cell lines (Jackson et al., 2006), a range of small concentrations of tetracycline was tested. We did not observe any cell death up to a few hours of treatment with 20 ng/ml tetracycline, while it readily induced the expression of TTP-myc (Fig. 5B). In these conditions, TTP expression caused a significant decrease in *vegf* and *dusp6* mRNA levels (Fig. 5C). Importantly, the effect of TTP expression was not due to a toxic effect as the mRNA levels of another gene, neuropilin-1, a co-receptor of VEGF expressed in many cells including melanomas (Graeven et al., 2000) was enhanced. This is not surprising as reducing VEGF expression is often correlated with an increase in the expression of its receptors. TTP effect was also not related to an inhibition of the MEK/ERK pathway, as shown on Figure 5B.

Next, we verified that the effect of TTP occurred through the 3′UTR of *dusp6* mRNA using luciferase reporter assays. ΔRaf:ER HEK293 cells were transfected either with TK-luc as a negative control, TK-luc-3′UTR*vegf* as a positive control or TK-luc-3′UTR*dusp6*, simultaneously with increasing amounts of a plasmid allowing transient expression of TTP. TTP expression strongly reduced, in a dose-dependent manner, the luciferase activity of the TK-luc-3′UTR*vegf* and had no effect on the activity of the TK-luc control construct as already described (Essafi-Benkhadir et al., 2007). As for TK-luc-3′UTR*vegf* construct, TTP induced a dose-dependent inhibition of the activity linked to TK-luc-3′UTR *dusp6* construct (Fig. 5D). The inhibitory effect of TTP was obtained whether or not the cells were stimulated with 4OHT (not shown).

PUF proteins are other important players in the regulation of mRNAs stability through direct binding to 3′UTR of target mRNAs (Spassov and Jurecic, 2003). The founder members of this family, Drosophila Pumilio (Pum) and *C. elegans* FBF (fem-3-binding factor) have been associated with maintenance of pluripotency in stem cells, through degradation of MAPK mRNAs (Lee et al., 2007). Screening experiments reported an

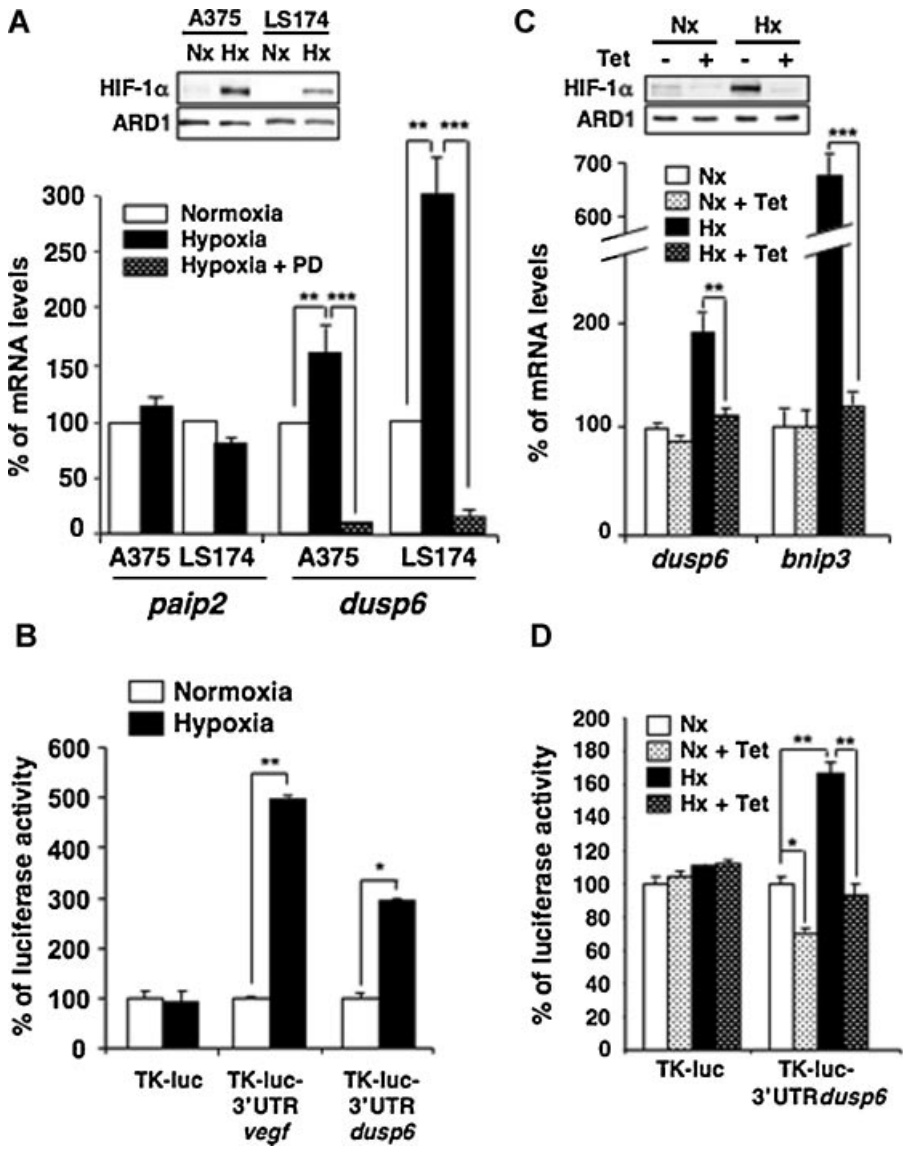

Fig. 3. Hypoxia increases *dusp6* mRNA levels and luciferase activity of TK-luc-3′UTR*dusp6* in a HIF-1α-dependent manner. A: Cells were grown in 1% oxygen for 24 h. Levels of HIF-1α were detected by Western blot in normoxia (Nx) and hypoxia (Hx) in A375 and LS174 cells. The protein ARD1 was taken as a loading control. When indicated, cells were also treated with 5 μM PD184352. Levels of *dusp6* and *paip2* mRNA were measured by RT-QPCR and expressed in relation to levels in normoxia, taken as 100%. B: Cells were transfected with 500 ng of either TK-luc, TK-luc-3′UTR*vegf*, or TK-luc-3′UTR*dusp6*, grown for 24 h in hypoxia and processed for luciferase activity measurement. The value in normoxia was taken as 100%. * Significant differences based on the Student's *t*-test. C: HIF-1α was silenced in LS174 cells using a tetracycline(tet)-inducible shRNA system, as checked by Western blot. The levels of *dusp6* and *bnip3* mRNA were measured by RT-QPCR in LS174 cells. The 100% value was set as the normoxic condition in the absence of tetracycline. D: HIF-1α shRNA LS174 cells were transfected with either TK-luc or TK-luc-3′UTRdusp6, and grown in normoxia or hypoxia, and in the presence or the absence of tetracycline. Luciferase activity was measured, taking the value in normoxia without tetracycline as 100%. Statistics were calculated with the Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

interaction of PUM2 with *dusp6* mRNA (Galgano et al., 2008) and two PUM consensus binding motives are found in the 3′UTR of *dusp6* sequence (see Fig. 5A). Transfection of HEK293 cells with a vector encoding a M2-tagged PUM2 (Fig. 5E) caused a 30% decrease of endogenous *dusp6* mRNA, while it had no significant effect on the mRNA of PAIP2 which does not contain PUM-binding sequence in its 3′UTR (Fig. 5F). Moreover, PUM2 over-expression decreased the luciferase reporter activity generated by the TK-Luc-3′UTR*dusp6*, in a dose-dependent manner, reaching 86% of inhibition, while it had no effect on the activity of the control TK-luc construct (Fig. 5G). This effect was observed whether the ΔRaf:ER chimera was stimulated or not by 4-OHT (not shown).

Discussion

Previous reports had shown that the MEK/ERK pathway was involved in the regulation of the *dusp6* gene at the transcriptional level, through an Ets2-binding site located in intron 1 of the gene (Ekerot et al., 2008; Furukawa et al., 2008). However, those studies did not address the regulation of *dusp6* at the post-transcriptional level. Here we confirm the importance of the ERK pathway in *dusp6* expression and further show that the MEK/ERK pathway stabilizes the *dusp6* mRNA through its 3′UTR, although other regions may be involved as well. Hypoxia, a hallmark of tumor growth and a condition involved in some aspects of development was also found to

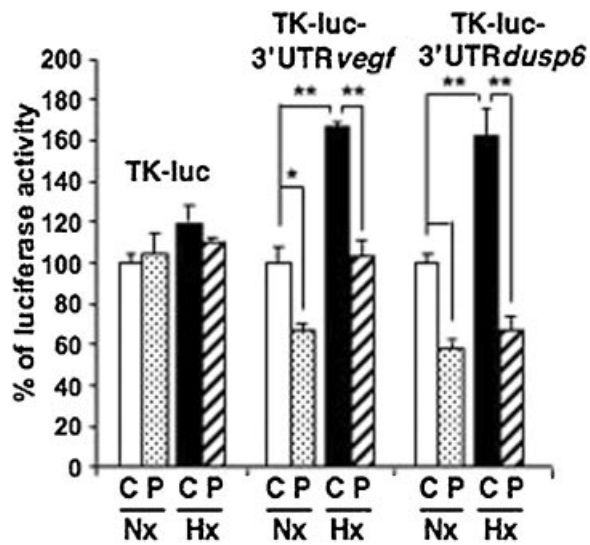

Fig. 4. Hypoxia-induced increase in TK-luc-3′UTR stability is MEK dependent. LS174 cells were transfected with either 500 ng of TK-luc as negative control or TK-luc-3′UTR*dusp6* or TK-luc-3′UTR*vegf* taken as a positive control. Cells were incubated in the absence (C) or in the presence of 5 μM of PD184352 (P), and cultured in hypoxia for 24 h. Luciferase activity was measured, taking the normoxic condition without PD184352 as 100%. Statistics were calculated with the Student's *t*-test (P* < 0.05; ***P* < 0.01; ****P* < 0.001).**

increase the stability of *dusp6* mRNA in a HIF-1 and MEK/ERK-dependent manner. The MAPK phosphatase DUSP1 is also regulated by hypoxia as previously described (Takano et al., 1995; Seta et al., 2001; Bernaudin et al., 2002; Liu et al., 2003, 2005; Mishra and Delivoria-Papadopoulos, 2004). Data from the literature suggest that it is also the case for DUSP6, in human pulmonary arterial cells, in tissues of animals exposed to hypoxia and in melanoma cells cultured in 3D spheroids, which are assumed to be hypoxic at their core (Mishra and Delivoria-Papadopoulos, 2004; Ghosh et al., 2005; Manalo et al., 2005). Here we show that low levels of oxygen cause an increase in *dusp6* mRNA levels in the melanoma A375 and the colon adenocarcinoma LS174 cell lines, as well as in cervical carcinoma HeLa cells (Fig. S2). shRNA studies indicated that the transcription factor HIF-1, was necessary for *dusp6* mRNA increase following hypoxia.

HIF-1 activates the transcription of a myriad of genes involved in glucose homeostasis, angiogenesis, vascular permeability, and inflammation, by binding to specific sequences in the genes, called the HRE. HREs are enhancer elements containing the consensus core sequence 5′-(A/G)CGT(G/C)(G/C)-3′, localized at various positions and orientations in promoter and intronic sequences of hypoxia-regulated genes such as erythropoietin, VEGF, glucose transporter 1, or glycolytic enzymes. In silico analysis performed with the Genomatix program did not reveal any canonical HRE in the 5′ promoter region or in the intronic sequences of the *dusp6* gene. However, several potential HRE were detected in its 3′ region although their biological significance remains to be tested. Alternatively, the role of HIF-1 in *dusp6* transcription may be indirect, through the induction of a HIF-1 target gene coding for a protein promoting *dusp6* expression at the transcriptional or post-transcriptional level.

Since it cannot be ruled out that hypoxia, directly or indirectly, regulates *dusp6* through transcriptional activation of the *dusp6* promoter, it has a clear effect on the stability of *dusp6* mRNA, as demonstrated by experiments undertaken with the 3′UTR of the phosphatase gene. HIF-1α was required for this effect as well. Forced expression of HIF-1 increased the stability of several mRNAs, including that of *vegf* (Liu et al., 2002) or hepatocyte growth factor (Chu et al., 2009). One obvious explanation would be that HIF-1 stimulates the transcription of proteins involved in mRNA stability. In addition, a basal MEK/ERK activity was required for the hypoxic response of *dusp6* to occur. The fact that hypoxia either induces a decrease or an increase of phosphorylated ERK levels in A375 and in LS174 cells, respectively, suggests that this requirement for MEK activity is independent of the potential ERK signaling pathway triggered by hypoxia. This is likely to be related to previous findings that ERK signaling enhances HIF-1 transcriptional activity and its accumulation in the nucleus (Richard et al., 1999; Mylonis et al., 2006). Alternatively, ERK may be required for the action of a potential downstream target of HIF-1 that would regulate *dusp6* mRNA, or more simply, be necessary for the stabilization of *dusp6* mRNA per se, independently of hypoxia.

Interestingly, we have found that the expression of another cytoplasmic ERK phosphatase, *dusp7*, remained unchanged following hypoxia. In contrast, the same conditions strongly increased the expression of *dusp5*, a nuclear phosphatase for ERKs, p38, and JNK (Fig. S3). This may be especially relevant considering the importance of *dusp5* in vascular development (Pramanik et al., 2009). It also indicates that selectivity and specificity exist in the response of DUSPs to hypoxia.

We have identified two mRNA regulatory proteins decreasing the levels of *dusp6* mRNA, TTP, and PUM2. TTP is a tandem CCCH zinc finger protein that was identified as an immediate early gene because of its rapid induction by mitogens in fibroblasts. It belongs to the TIS11 family (tetradecanoyl phorbol-acetate-inducible-sequence11b), which binds to AU rich elements (AREs) in mRNAs and increases their rates of deadenylation and destruction. It has been involved in the regulation of inflammatory genes but recent studies have revealed that it targets other functional families and could regulate tumor angiogenesis (Essafi-Benkhadir et al., 2007; Brennan et al., 2009). When expressed in A375 cells, TTP decreases both *dusp6* mRNA endogenous levels and luciferase activity of the 3′UTR of *dusp6*. This probably occurs through binding of TTP to the AU rich element found in the *dusp6* 3′UTR which corresponds to a typical pentamer 5′-AUUUA-3′, known as class I ARE (beginning at nucleotide 1,136 of the cDNA). The mRNA of another MAPK phosphatase, DUSP1, was also found to be a target of TTP (Lin et al., 2008). In a previous report from our group, it was shown that the destabilizing activity of TTP towards the 3′UTR of *vegf* mRNA was diminished upon MEK/ERK activation (Essafi-Benkhadir et al., 2007). It is thus likely that one way for ERKs to stabilize *dusp6* mRNA occurs through inhibition of TTP.

PUF mRNA-binding proteins are well conserved among animals and regulate in a negative way mRNAs, either by provoking mRNA destabilization or by preventing their translation. PUF proteins participate in the regulation of major developmental processes, such as anterior/posterior patterning in drosophila and germline switch from spermatogenesis to oogenesis in *C. elegans*. In addition, they are necessary to maintain stem cells pluripotency in different species (Spassov and Jurecic, 2003). In humans, two paralogous PUF proteins exist: PUM1 (Pumilio Homolog 1) and PUM2 (Pumilio Homolog 2). Using modified ribonucleoprotein immunoprecipitation microarrays confirmed by pull-down assays, Galgano et al. (2008) found 1424 mRNA interacting with PUM1 and 575 with PUM2, two of them being ERK2 itself and DUSP6. We sought for putative PUM-binding sites in the 3′UTR of *dusp6* and identified two core consensus sequences related to drosophila nano-response elements (NREs) and composed of eight nucleotides 5′-UGUANAU-3′, where N is any nucleotide. A related but slightly degenerated sequence was

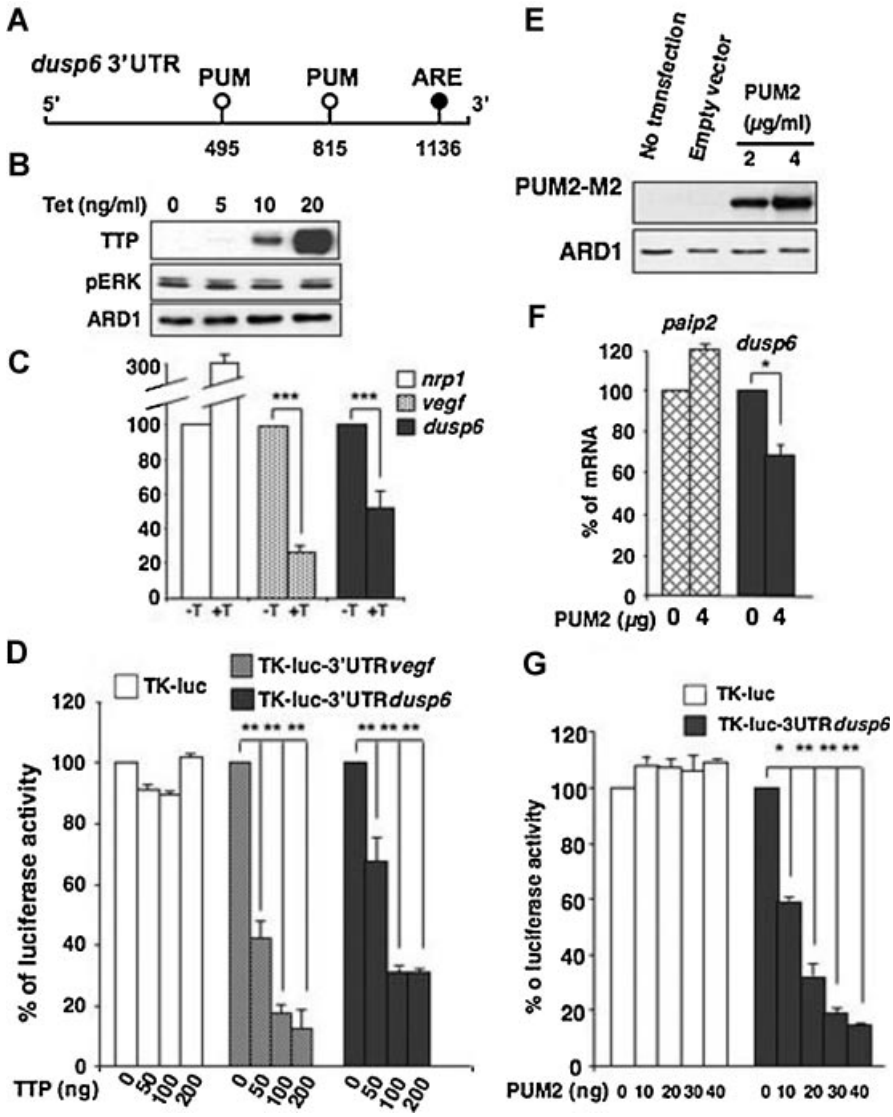

Fig. 5. TTP and PUM2 exerts an inhibitory effect on *dusp6* mRNA stability, via its 3′UTR. A: The 3′UTR of dusp6 mRNA contains consensus binding motives for PUM and TTP factors (ARE). B: A375 tetracyclin-inducible cells for TTP were treated with a dose range of tetracycline and samples were analyzed by Western blot. ARD1 levels were used as a loading control. C: *dusp6* mRNA levels were quantified by RT-quantitative PCR after the induction of TTP with 10 ng/ml of tetracyclin (T) for 24 h. Results shown here were obtained with one clone but were confirmed with another A375-independent clone. D: HEK293 ΔRaf:ER cells were transfected with 500 ng of either TK-luc, TK-luc-3′UTR*vegf*, or TK-luc-3′UTR*dusp6* and different amounts of the TTP expression vector as indicated (in ng). E: Cells were transfected with either 2 or 4 μg of a PUM2 expression vector (M2-tagged), using an empty vector as a control. Cell samples were then analyzed for expression of PUM2 by Western blot, using an anti-M2 flag. Levels of ARD1 were used as a loading control. F: HEK 293 cells were transfected or not with 4 μg of PUM2-expressing vector and endogenous *dusp6* and *paip2* mRNA levels were analyzed by RT-QPCR. The value obtained after transfection of the empty vector was set as 100%. G: HEK293 ΔRaf:ER cells were transfected with 500 ng of either TK-luc or TK-luc-3′UTR*dusp6* and with increasing amounts of the PUM2 vector as indicated. Luciferase activity was assessed 24 h after transfection. Statistics were calculated with the Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

also identified in the 5′ region of the 3′UTR, which may serve as a binding site for other members of the pumilio family. Our finding that expression of PUM2 decrease both the levels of endogenous *dusp6* mRNA and the activity of the luciferase reporter gene coupled to the 3′UTR of *dusp6*, confirms the biological significance of the co-precipitation of PUF factors with this mRNA (Galgano et al., 2008). This suggests that PUF factors may have a critical role in the regulation of ERK signaling, not only at the level of ERK mRNA as described previously, but also at the level of their physiological inhibitors DUSPs.

The present work and previous reports by our group and others indicate that the regulation of *dusp6* mRNA stability by its own target, that is, MAP kinases ERKs, takes place in a complex retro-control loop, which also includes transcriptional induction of the *dusp6* gene (Ekerot et al., 2008), as well as DUSP6 phosphorylation by ERKs which is followed by the degradation of the phosphatase by the proteasome (this work and Marchetti et al., 2005; Ekerot et al., 2008). Several members of the DUSPs family are also regulated by the MAPK pathways they eventually inhibit (Owens and Keyse, 2007) at transcriptional and post-translational levels. These processes are likely to be critical for a fine spatio-temporal regulation of MAPK signaling, which highly depends on the type of extracellular signal. In the context of cancer, mutations of either

tyrosine kinase receptors or downstream effectors such as Ras or Raf family members often lead to over-activation of MEK/ERK signaling. However, this over-activation must be limited to a certain threshold, since an excess of ERK activity may lead to cell-cycle arrest and even apoptosis (Pumiglia and Decker, 1997; Ravi et al., 1998; Cagnol et al., 2006). Up-regulation of *dusp6* expression may thus be a way for cancer cells to maintain a level of ERK activity compatible with survival and cycle progression. In this respect, it may explain why *dusp6* expression is up-regulated in many cancers harboring Ras or Raf mutations (Warmka et al., 2004; Bloethner et al., 2005).

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