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Tumor necrosis factor- α -mediated suppression of dual-specificity phosphatase 4: crosstalk between NF κ B and MAPK regulates endothelial cell survival

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Abstract

We investigated the effects of tumor necrosis factor- α (TNF- α) exposure on mitogen-activated protein kinase signalling in human microvascular endothelial cells. TNF- α caused a significant suppression of a dual specificity phosphatase, DUSP4, that regulates ERK1/2 activation. Thus, we hypothesized that suppression of DUSP4 enhances cell survival by increasing ERK1/2 signalling in response to growth factor stimulation. In support of this concept, TNF- α preexposure increased growth factor-mediated ERK1/2 activation, whereas overexpression of DUSP4 with an adenovirus decreased ERK1/2 compared to an empty adenovirus control. Overexpression of DUSP4 also significantly decreased cell viability, lessened recovery in an *in vitro* wound healing assay, and decreased DNA synthesis. Pharmacological inhibition of NF κ B activation or a dominant-negative construct of the inhibitor of κ B significantly lessened TNF- α -mediated suppression of DUSP4 expression by 70–84 % and attenuated ERK activation, implicating NF κ B-activation in the TNF- α -mediated suppression of DUSP4 that contributes to ERK1/2 signalling. Taken together, our findings show that DUSP4 attenuates ERK signalling and reduces cell viability, suggesting that the novel crosstalk between NF κ B and MAPK pathways contributes to cell survival.

Keywords

Dual specificity phosphatase; MAP kinase signalling; NF κ B; endothelial cells; cellular proliferation; DNA synthesis

Introduction

Cell survival following exposure to inflammatory cytokines is dependent on the balance between pathways that mediate proliferation and survival and those that cause cell senescence, apoptosis, and death. These responses can be influenced by the duration and strength of cytokine activation, and the heterogeneity of signalling mediators expressed in different cell types. Notably, a complement of intracellular kinases and phosphatases modulate cellular responses following cytokine- or growth factor-mediated receptor stimulation [1,2]. MAPK proteins are essential components of many signal transduction

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pathways [3], including those of growth factors and cytokines, such as TNF- α . MAPK signalling is divided into three general pathways, ERK, JNK, and p38 [4,5]. ERK1/2 activation promotes cell survival and proliferative responses [4], whereas the other two MAPK pathways are commonly grouped together as stress-activated protein kinase (SAPK) that are upregulated in response to cytokines and other environmental stresses [3,4,6]. Although activation of these SAPK kinases may also play a role in cell survival, their prolonged activation has been shown to inhibit cell proliferation and promote apoptosis [7–9].

Dual-specificity phosphatases (DUSPs or MAP Kinase Phosphatases, MKPs) are known to modulate MAPK effects by dephosphorylation and/or sequestration of the ERK, JNK and p38 kinases [2]. Members of this family of phosphatases have overlapping specificities for various MAPKs. In our previous studies of human microvascular endothelial cells (HMVEC), we found that TNF- α exposure decreased the expression of DUSP4 [10], a MAPK phosphatase that preferentially regulates ERK1/2 [11,12]. Consistent with its role in regulating MAPK activation, we found that siRNA-mediated knockdown of DUSP4 enhanced ERK1/2 phosphorylation in response to TNF- α [10]. Other studies suggest that DUSP4 may also target phospho-JNK, as well, although this finding has not been consistently observed [13,11,12]. Similarly, the role of DUSP4 in modulating cell survival and growth is also unresolved and may vary in a cell-specific manner. Thus, repression of DUSP4 has been linked to cell growth in some cancer cells [14,15], whereas other studies have shown that excess MAPK-stimulation following knockdown of DUSP4 unexpectedly decreases rates of proliferation of embryonic fibroblasts [16]. Here, we analyze the effects of DUSP4 on modulating cell growth and survival in human endothelial cells. We hypothesized that suppression of DUSP4 by TNF- α enhances cell survival. To test this hypothesis, we determined whether pretreatment of cells with TNF- α augmented growth factor-mediated ERK1/2 and JNK phosphorylation, and determined whether excess DUSP4 attenuated these signalling effects to decrease cell survival.

In addition to MAPK stimulation, TNF- α also activates NF- κ B in endothelial cells to upregulate adhesion molecules, such as ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), and E-selectin. In many cell types, NF- κ B activation also promotes cell survival via its transcriptional upregulation of anti-apoptotic genes that antagonize caspase pathways of programmed cell death. NF- κ B pathways can also attenuate cellular oxidant accumulation to mitigate JNK activation, thereby lessening cell death. [17]. Thus, to understand better the mechanism of TNF- α -mediated DUSP4 suppression, we determined whether NF- κ B pathways were involved in this response. In this report, we show a new connection between NF- κ B and MAPK pathways in which NF- κ B-activation suppresses DUSP4 to enhance endothelial cell survival.

Materials and methods

Chemicals and reagents

Pyrrolidine dithiocarbamate (PDTC) was obtained from Sigma-Aldrich (St. Louis, MO, USA); and the MEK1/2 inhibitor, U0126 was purchased from Cell Signalling (Danvers, MA, USA). TNF- α was obtained from R&D Systems (Minneapolis, MN, USA). Recombinant adenoviral vectors expressing DUSP4 (AdDUSP4), the empty vector (AdCtrl), or a dominant negative I κ B were obtained from Vector Biolabs (Philadelphia, PA, USA).

Cell culture

Primary human microvascular endothelial cells (HMVEC) were maintained in endothelial basal medium 2 supplemented with EGM-2 MV (Lonza, Walkersville, MD, USA) unless otherwise noted.

Western blots

Cells were washed in PBS and then lysed in cell lysis buffer with protease inhibitors, or nuclear and cytoplasmic samples were prepared with a nuclear extraction kit (Active Motif, Carlsbad, CA, USA). Protein samples were separated on 4–15% SDS-polyacrylamide gels (BioRad, Hercules, CA, USA), and transferred to PVDF membranes, using a semi-dry transfer system. Following washes in TBST (50 mM Tris-HCl, pH7.4; 150 mM NaCl; 0.05% Tween-20), blots were blocked with TBST supplemented with 5% nonfat dry milk (Cell Signalling, Danvers, MA) for 1 h. After three 15 min washes with TBST, membranes were then incubated overnight at 4°C with antibodies to ICAM-1, VCAM-1, NF- κ B p65, I κ B α , DUSP4 (Santa Cruz, Santa Cruz, CA, USA), phospho-ERK1/2, ERK1/2, JNK, phospho-JNK (Cell Signalling, Danvers, MA), or rabbit anti- α -actin antibody (Sigma-Aldrich) in TBST supplemented with 5% nonfat dry milk. The following day, blots were rinsed five times with TBST, followed by a 1 h incubation with HRP-conjugated secondary antibodies (Cell Signaling, Danvers, MA) in TBST with 5% nonfat dry milk. After 5 rinses in TBST, the ECL detection system (GE Healthcare Life Sciences, Piscataway, NJ, USA) was used for visualization on X-Ray films, and the VersaDoc system (BioRad) and QuantityOne program were used for imaging and analysis, respectively.

RNA isolation and qRT-PCR

Total RNA was extracted with the RNeasy kit (Qiagen, Germantown, MD, USA) using an optional DNase I step to remove residual DNA. Oligo(dT) primers were used to synthesize cDNA from 0.4–1 μ g of each total RNA sample using the Advantage RT-for-PCR Kit (Clontech, Mountain View, CA, USA), according to the manufacturer's protocol. An Applied Biosystems PRISM 7900 HT Sequence Detector (Life Technologies) was used for quantitative real-time PCR and data analysis by the C_t method that compares the amount of target gene to an endogenous control (actin), followed by normalization to a control sample to obtain relative levels of gene expression using conditions previously described [10]. Commercially available TaqMAN assay kits (Life Technologies) were used for the detection of DUSP4 (Hs00154826_m1) or actin (4352935E).

Cell viability, death, and proliferation assays

For cell counting, cells grown in 6-well plates were trypsinized, washed, and then mixed 1:1 with trypan blue and counted on a hemocytometer 48 h after infection with AdDUSP4 or control. Alternatively, cells were grown in 96-well plates, exposed to adenovirus constructs for 48 h, then treated with a tetrazolium salt, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], and an electron coupling reagent, phenazine methosulfate. The conversion of the tetrazolium salt to a soluble formazan product involves dehydrogenase enzymes that are active in living cells. The formazan product was measured at A₄₉₀. Fluorescent peptides were used in the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, WI, USA) to quantify cell viability and death simultaneously by measuring the relative activity of an intracellular protease in viable cells and one released by dead cells. For the assay of migration/wound healing, cells grown in 6 well plates were exposed to adenoviruses for 24 h, then a scratch was made in the monolayer using a pipet tip. Images (40X) were taken at fixed points on the plate to document the area of clearing at time 0 and 18 h, and ImageJ was used to outline and quantitate the cleared area. To measure cell proliferation capacity, DNA synthesis was

measured by monitoring 5-bromo-2-deoxyuridine (BrDU) incorporation. Briefly, cells in 96-well plates were treated for 48 h with virus, and then exposed to BrDU for 3 h. After fixing the cells, DNA was denatured, and the anti-BrDU antibody was used to detect BrDU incorporated into the DNA. Quantitative measurements involved the use of an HRP-linked secondary antibody and a TMB (3,3',5,5'-tetramethylbenzidine) substrate that could be quantitated by absorbance. Absorbance values were adjusted for relative cell number by using a cell viability assay on cells treated in parallel.

Results

DUSP4 suppression by TNF- α

TNF- α causes a sustained, significant decrease in DUSP4 gene expression in HMVEC that is evident at 2 h and sustained throughout the 48 h time course (Fig. 1A). To determine the mechanism by which TNF- α suppresses DUSP4, we first examined whether U0126, a known inhibitor of MEK1/2, the MAP kinase kinase that phosphorylates ERK, affects DUSP4 expression. Preincubation of HMVEC with U0126 significantly decreased DUSP4 expression in the absence of TNF- α , suggesting that basal levels of DUSP4 may be maintained, in part, via steady-state activation of the ERK pathway (Fig. 1B). U0126 pretreatment did not alter the ability of TNF- α to suppress DUSP4.

As TNF- α -mediated receptor signalling also involves activation of NF- κ B, we next used PDTC, an NF- κ B inhibitor, to determine whether NF- κ B activation contributes to TNF- α -mediated suppression of DUSP4. PDTC effectively attenuated TNF- α -mediated upregulation of ICAM-1 and VCAM-1 adhesion molecule expression (Fig. 2A). Under these conditions, PDTC significantly lessened the suppressive effect of TNF- α on DUSP4 expression by 70–100% (Fig. 2B). Since PDTC may have additional effects as an antioxidant, to confirm a role for NF- κ B in DUSP4 suppression, we tested whether a dominant negative I κ B (I κ B DN) was capable of blocking TNF- α -mediated DUSP4 suppression. As shown in Figure 2C (lower panel), unlike the endogenous I κ B, I κ B DN is not degraded following TNF- α exposure, and its presence attenuates NF- κ B-mediated effects, as illustrated by the reduction of TNF- α -mediated adhesion molecule upregulation in I κ B DN treated cells and the reduction of TNF- α -mediated migration of p65 to the nucleus (Fig. 2C upper panel and Fig. 2D, respectively). Concomitantly, I κ B DN significantly attenuates the TNF- α -mediated repression of DUSP4 by approximately 85% (Fig. 2E,F), confirming that NF- κ B-activation mediates DUSP4 suppression.

TNF- α enhances growth factor signalling via NF κ B

Previously, we reported that suppression of DUSP4 by siRNA-mediated knockdown augments acute TNF- α -mediated ERK1/2 signalling and contributes to TNF- α -mediated VCAM-1 upregulation [10]. To study further the role of DUSP4 suppression in cell signalling, we pretreated HMVEC with TNF- α for 24 h, and then examined the effects of exposure to this cytokine on growth factor-mediated ERK1/2 activation. To do so, TNF- α -treated and untreated cells were incubated in media lacking serum and growth factors for 1–3 h prior to re-exposing them to complete media, replete with serum and growth factors. Under these conditions, growth factor stimulation caused a robust activation of ERK1/2 that was augmented by pretreatment with TNF- α (Fig. 3A). JNK1/2 signalling was also upregulated by TNF- α exposure. To determine the role for NF- κ B pathways in the enhanced MAPK-activation, cells were treated with I κ B DN under conditions shown to attenuate DUSP4 suppression in Fig. 2. Pretreatment with I κ B DN decreased growth factor-mediated ERK1/2 phosphorylation and lessened the enhanced growth factor-mediated ERK1/2 phosphorylation caused by TNF- α (Fig 3B), consistent with a role for NF- κ B-mediated DUSP4 suppression in modulating the MAPK-signalling. Similarly, forced overexpression

of DUSP4 with an adenovirus construct lessened growth factor-mediated MAPK activation, confirming that DUSP4 effectively suppresses MAPK signalling in endothelial cells (Fig. 3C). Interestingly, pre-exposure to TNF- α lessened the expression of the recombinant DUSP4 protein (Fig. 3D), similar to the effects of TNF- α on the endogenous DUSP4. I κ B DN attenuated the TNF- α -mediated suppression of recombinant DUSP4, suggesting that an NF- κ B-dependent mechanism regulates this suppression (Fig. 3D).

DUSP4 mediates cell viability, and proliferation in HMVEC

We hypothesized that the ability of DUSP4 to modulate ERK1/2 signalling may regulate cell proliferation. To test this hypothesis, we monitored the effect of DUSP4 overexpression on cell viability in HMVEC. AdDUSP4-mediated overexpression significantly reduced viable cell number, as determined by trypan blue exclusion, by approximately 40–50% (Fig. 4A). To confirm these findings, cell viability was measured by an assay dependent on the metabolic conversion of MTS to formazan in living cells (Fig. 4B). Forced overexpression of DUSP4 significantly decreased cell viability in a dose-dependent manner as measured by each of these assays. To determine whether these changes in cell viability would alter cell migration and proliferation, we used an *in vitro* scratch/wound healing assay. Compared to control virus-treated cells, cells overexpressing DUSP4 filled 62% less of the cleared area than the control cells ($p < 0.0005$) (Fig. 4C). As closure of the clearing on the plate can be affected by cell migration as well as cell proliferation and death, we next measured cell death. We found that compared to control cells, there was more cell death in cells with excess DUSP4 (Fig. 4D). To determine whether DUSP4 overexpression also alters cellular proliferation, we monitored DNA synthesis by adding the thymidine analog BrDU to cells and monitoring its incorporation into DNA after 3 h. There was a significant decrease of BrDU incorporation into the DNA in cells overexpressing DUSP4 compared to control adenovirus-treated cells or non-virus-treated cells that was apparent even after correcting for the lower number of viable cells following overexpression of DUSP4 (Fig. 4E). Thus, cells overexpressing DUSP4 had only 20% of the BrDU incorporation compared to cells exposed to the control adenovirus, suggesting a decreased rate of DNA proliferation caused by DUSP4 overexpression. DUSP4 expression shows a dose-dependent effect on BrDU proliferation that parallels its ability to affect basal ERK1/2 phosphorylation under normal growth conditions (Fig. 4F,G). Co-incubation with TNF- α increased the levels of ERK stimulation, and suppressed DUSP4 expression (Fig. 4G). At the lower MOIs, DUSP4 augmented BRDU proliferation (Fig. 4F).

Discussion

DUSP4 is one of a group of MAPK phosphatases that has been reported to primarily dephosphorylate ERK, although it may also target JNK signalling [2]. Previous reports have suggested that transcription of the DUSP4 gene can be regulated by oxidative stress and TGF- β , which both upregulate its expression in some cell types [18,19]. The DUSP4 gene is also subject to transcriptional suppression following promoter hypermethylation [14], and the DUSP4 protein may be regulated by phosphorylation and proteasomal degradation [20]. In our previous study, we found that DUSP4 was downregulated following 2 h exposure to TNF- α . This suppression was augmented by deficiency of glutathione peroxidase-1 (GPx-1), an antioxidant enzyme, the lack of which also augmented NF- κ B responses [10]. In follow-up studies, we found that DUSP4 downregulation is sustained following longer exposure to TNF- α , even in cells containing normal amounts of GPx-1. Also, of the DUSPs, DUSP4 was the only phosphatase that was suppressed in both GPx-1-deficient and control cells following TNF- α exposure ([10] and unpublished). Interestingly, we found that TNF- α exposure suppresses DUSP4 for at least 48 h. Thus, here, we focus on the effects of DUSP4 in modulating endothelial cell survival and proliferation, and the mechanisms of DUSP4

suppression (in cells replete with GPx-1). Our previous studies also indicated that siRNA-mediated knockdown of DUSP4 augments TNF- α -mediated ERK1/2 signalling and contributes to VCAM-1 upregulation [10]. Consistent with these findings, we found that growth factor-mediated ERK1/2 and JNK signalling is augmented by pre-exposure to TNF- α , conditions that suppress DUSP4 expression via an NF- κ B mechanism (Fig. 5). Thus, PDTC, an NF- κ B inhibitor, or I κ B DN, which blocks NF- κ B activation, prevents TNF- α -mediated DUSP4 suppression (Fig. 2B, E, F). Blocking NF- κ B pathways subsequently attenuates ERK1/2 activation (Fig. 3B). Activation of ERK1/2 contributes to cell survival, as our findings show that overexpression of DUSP4 decreases cell viability and proliferation (Fig. 4), while decreasing ERK1/2 phosphorylation (Figs. 3B,C & 4G) and increasing cell death (Fig. 4D). These findings tie the TNF- α -mediated suppression of DUSP4 to functional changes in MAPK signaling, and highlight a new linkage between NF- κ B pathways and MAPK signalling that can modulate endothelial cell proliferation and survival. Interestingly, NF- κ B-inhibitors have been developed as therapeutic agents to lessen cardiovascular disease and other diseases that have an inflammatory component [21–23]; however, NF- κ B also stimulates pathways that are anti-apoptotic and promote cellular proliferation, such as the MAPK pathway studied here. Thus, potentially, an NF- κ B inhibitor may have unexpected consequences on the growth and survival of endothelial cells, due, in part, to an attenuation of DUSP4 suppression.

Little is known about the role of DUSP4 in endothelial cells. One recent study suggested that overexpression of DUSP4 protects cells against apoptosis caused by TNF- α exposure in human umbilical vein endothelial cells (HUVECs) putatively by lessening JNK phosphorylation [24]. Surprisingly, in this previous study of HUVECs, overexpression of DUSP4 had no effect on ERK1/2 activation in response to TNF- α . In our previous study, we found that DUSP4 was decreased in response to short term TNF- α stimulation, especially in endothelial cells that lacked GPx-1 [10]. Furthermore, our studies found that alterations in DUSP4 expression modulated VCAM-1 expression but did not change the expression of ICAM-1 following TNF- α exposure. Thus, in this current study, we focused on the ability of DUSP4 to modulate growth factor-mediated responses and cellular proliferation in human microvascular endothelial cells. We found that DUSP4 modulated both ERK and JNK stimulation following acute activation by growth factors (after their withdrawal) (Fig. 3), and also modulated basal MAPK phosphorylation states under normal growth conditions (Fig. 4G). Our studies suggest that DUSP4 is anti-angiogenic, as the presence of excess DUSP4 suppresses both ERK and JNK, inhibits cell growth by reducing DNA synthesis, and increases cell death in endothelial cells. Further research is necessary to determine whether this anti-angiogenic property can be exploited to modulate therapeutically neovascularization in disease states such as cancer.

In other cell types, DUSP4 upregulation in response to TGF β stimulation has been shown to decrease ERK1/2 activation to reduce overall cell survival by promoting apoptosis [19]. In addition, forced overexpression of DUSP4 has been found to increase senescence in fibroblasts, in part, by decreasing MAPK-signalling [25]. Consistent with its role in regulating cell survival, several studies have implicated DUSP4 as a tumor suppressor gene, as its deletion has been found in breast, prostate, and lung cancers [26,27,15]. In gliomas, hypermethylation of the DUSP4 gene has been linked with its transcriptional suppression, and the resulting decreased expression of DUSP4 coincides with increased ERK1/2 signalling and cellular proliferation [14]. In resistant basal-like breast cancer cells, hypermethylation of the DUSP4 gene and lowered levels of its expression similarly correlated with enhanced ERK1/2 signalling and increased cell survival [28]. In contrast to these studies that suggest DUSP4 expression serves to limit cell growth and survival, deficiency of DUSP4 was found to reduce proliferation and enhance apoptosis in mouse embryonic fibroblasts from DUSP4 knockout mice [16]. These opposing effects on cell

growth and survival caused by the lack of this same phosphatase may reflect underlying differences in compensatory mechanisms that exist in various cell types, such as cell-specific expression of other DUSP family members and/or the relative abundance of other proteins in the MAPK signalling networks. In our study, we found that an increase in DUSP4 dose-dependently suppresses phospho-ERK. In preliminary studies, we find that DUSP4's suppression of ERK1/2 phosphorylation is inversely linked with the upregulation of p38 phosphorylation, suggesting a possible pathway that contributes to the deleterious effects of DUSP4 overexpression on endothelial cell survival. It is well known that high intensity, prolonged activation of p38 contributes to inhibition of cell growth and apoptosis [29]. Furthermore, stress caused by lack of ERK1/2 and the resulting upregulation of p38 pathways may perpetuate the attenuation of ERK1/2 signalling by promoting the dephosphorylation of MEK1/2, the MAPK kinases that activate ERK [30,31]. Thus, we suggest that TNF- α -mediated suppression of DUSP4 by NF- κ B contributes to the survival of HMVEC by augmenting ERK1/2 pathways of activation and preventing the upregulation of inhibitory pathways, such as those mediated by p38. The ultimate effect of TNF- α on endothelial cell survival or death, however, may depend on the balance among all survival and apoptotic/death factors, of which DUSP4 is one of many. Nonetheless, we found that DUSP4 expression dose-dependently suppresses DNA proliferation and increases cell death, suggesting conditions that enhance its expression decrease cell viability.

TNF- α has many effects on gene expression and signal transduction that together regulate cell growth and viability. NF- κ B activation, downstream of TNF- α , mediates some of these pro-survival responses by increasing the expression of anti-apoptotic and anti-oxidant factors [32,17]. Our findings further link NF- κ B with the regulation of MAPK pathways via a new pathway involving suppression of DUSP4 (Figure 5). Conditions that inhibit TNF- α -mediated suppression of DUSP4 also decrease ERK1/2 activation, and the expression of DUSP4 inversely correlates with cell proliferation. The precise molecular basis for the TNF- α -induced, NF- κ B-dependent suppression of DUSP4 is a matter of speculation and ongoing research. NF- κ B is known to suppress gene expression by many mechanisms, including those that directly inhibit transcription, through the formation of p50-p50 dimers [33]. In addition, recent findings have suggested a role for NF- κ B in promoting epigenetic silencing in a promoter-specific manner [34,35]. The DUSP4 promoter, however, has no NF- κ B consensus binding site. Other studies have suggested indirect mechanisms by which NF- κ B represses gene expression, such as through the NF- κ B-mediated upregulation of microRNAs [36–38] to decrease target gene mRNA and protein. Our finding that the recombinant DUSP4 is suppressed by NF- κ B, similar to the endogenous DUSP4, lends support for a post-transcriptional mechanism, most likely involving transcript stability or miRNA, that modulates DUSP4 downstream of TNF- α exposure. Taken together, our findings show that excess DUSP4 reduces cell viability, suggesting that the novel crosstalk between NF- κ B and MAPK pathways to enhance ERK1/2 signalling may contribute to cell survival.

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Fig. 1. TNF- α suppresses DUSP4 expression in HMVEC. (A) HMVEC were exposed to 10 ng/ml TNF- α for 2–48 h. Transcript levels were determined by quantitative RT-PCR using actin as an endogenous control gene. ANOVA, $p < 0.0001$; * $p < 0.05$ by pairwise, post-hoc analysis vs. no TNF- α . (B) Transcript levels were determined in HMVEC pre-exposed to 10 μ M UO126, the MEK1/2 inhibitor, or DMSO (veh) and then exposed to 10 ng/ml TNF- α for 4h. ANOVA $p < 0.0001$; * $p < 0.05$ by pairwise, post-hoc analysis vs. no TNF- α . UO126 treated groups were not significantly different from each other or the TNF- α -treated group.

Fig. 2.

NF B suppresses DUSP4 expression. (A,B) HMVEC were treated with 0–200 μM PDTC, an NF B inhibitor, followed by 10 ng/ml TNF- for 2 h before RNA isolation or 4 h for protein harvesting. (A) Western blots for the adhesion molecules ICAM-1 and VCAM-1, and actin. (B) Transcript levels were determined by qRT-PCR using actin as an endogenous control. Suppression of DUSP4 mRNA was determined by comparing the levels of DUSP4 mRNA in the presence and absence of 10 ng/ml TNF- . ANOVA, $p < 0.0005$, $*p < 0.05$ by pairwise post-hoc comparison vs. 0 μM PDTC. (C–F) HMVEC were pretreated for 48 h with control adenovirus (AdCtrl) or adenovirus with a dominant negative IkB (AdIkB (DN) or AdDN), followed by exposure to 10 ng/ml TNF- for 15 min to 4 h. (C) Western blots for the adhesion molecules ICAM-1 and VCAM-1, as well as actin (upper panel, 4 h time point); or for IkB and actin (lower panel, 15 m time point). (D) Nuclear extracts were harvested from cells following 15 m exposure to TNF- . Western blots for NF B p65 subunit and actin are shown. (E,F) Transcript levels were determined by TaqMAN qRT-PCR using actin as an endogenous control. (E) Relative levels of DUSP4 transcript. ANOVA, $p < 0.00001$; $*p < 0.05$ by post-hoc, pairwise comparison, with vs. without TNF- . (F) Suppression of DUSP4 mRNA was calculated by comparing the levels of DUSP4

mRNA in the presence and absence of 10 ng/ml TNF- reported in (E). ANOVA, $p < 0.0001$, $*p < 0.05$ by pairwise post-hoc comparison vs AdCtrl or no virus.

Fig. 3.

DUSP4 and ERK signalling. Western blots are shown. (A) HMVEC were pretreated for 24 h with and without 10 ng/ml TNF- α , then cells were exposed to media without serum or growth factors for 1.5 h. Cells were then re-exposed to complete media with serum and growth factors (GF), and harvested at 0–30 m following GF replacement. (B) HMVEC were exposed to control adenovirus (AdCtrl) or adenovirus with a dominant negative I κ B (AdI κ B (DN) or Ad(DN) in figures), then exposed to TNF- α , and GF for 15 m as described in (A). Note that the (tagged) I κ B (DN) has a higher MW than the endogenous I κ B. (C) HMVEC were treated with control adenovirus (AdCtrl) or adenovirus for DUSP4 (AdDUSP4) for 48 h, then exposed to GF as described above. (D) Cells expressing AdDUSP4 were co-incubated with AdCtrl or AdI κ B (DN), with or without 10 ng/ml TNF- α for 48 h.

Fig. 4.

DUSP4 decreases cell proliferation. HMVEC were exposed to various levels of control adenovirus (AdCtrl) or an adenovirus for DUSP4 (AdDUSP4) for 48 h. Each assay was repeated in 3–4 independent assays with 2–9 replicas per assay. (A) Cells grown in 6 well plates were trypsinized, washed, and counted on a hemocytometer following trypan blue exposure. ANOVA, $p < 0.0001$; * $p < 0.05$ by post-hoc, pairwise comparison with either no virus or AdCtrl conditions. (B) Cells growth assay in 96 well plates. Reduced formazan product (A_{490}) provides a measure of viable cells. ANOVA, $p < 0.0001$; * $p < 0.05$ by post-hoc, pairwise comparison vs all other conditions. # $p < 0.05$ vs all but matched AdCtrl. (C) Wound closure assay. The ability of cells to close a scratch/wound was quantitated by comparing the wound area/total area at time 0 and 18 h using ImageJ software. Graph

represents the relative amount of closure in AdCtrl vs DUSP4 treated cells at 18 h. $p < 0.0005$, by t-test). (D) Cell death assay. Cell death was measured relative to live cells in 96 well plates. ANOVA, $p < 0.00001$; * $p < 0.05$ by post-hoc, pairwise comparison vs. no virus or AdCtrl conditions. (E,F) 5-bromo-2-deoxyuridine (BrDU) was added for 3 h to measure DNA synthesis. (E) Cells were cultured for 48 h in the presence or absence of adenovirus prior to BrDU exposure. ANOVA, $p < 0.0005$; * $p < 0.05$ by post-hoc, pairwise comparison vs. no virus or AdCtrl conditions. (F) Cells were exposed to virus and or TNF- α , prior to BrDU exposure. ANOVA, $p < 0.00001$; * $p < 0.05$ by post-hoc, pairwise comparison, with vs. without TNF- α . (G) Cells were cultured for 48 h in the presence or absence of Adenovirus and TNF- α . Cell lysates were used in Western blotting.

Fig. 5.

NF κ B regulates DUSP4 to modulate cell growth and survival. DUSP4 modulates ERK and JNK signaling via its phosphatase activity. Following TNF- α exposure, NF κ B suppresses DUSP4 to enhance growth-factor mediated ERK and JNK activation. The crosstalk between NF κ B and MAPK may promote cell proliferation and survival by suppressing the phosphatase DUSP4 to enhance ERK and JNK signalling. Conditions that increase DUSP4 expression suppress MAPK signaling to decrease cell proliferation and survival.