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ORIGINAL ARTICLE

Phosphatidylserine enhances *IKBKAP* transcription by activating the MAPK/ERK signaling pathway

Maya Donyo, Dror Hollander, Ziv Abramovitch, Shiran Naftelberg and Gil Ast*

Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel

*To whom correspondence should be addressed. Tel: +972 36406893; Fax: +972 36409900; Email: gilast@post.tau.ac.il

Abstract

Familial dysautonomia (FD) is a genetic disorder manifested due to abnormal development and progressive degeneration of the sensory and autonomic nervous system. FD is caused by a point mutation in the *IKBKAP* gene encoding the IKAP protein, resulting in decreased protein levels. A promising potential treatment for FD is phosphatidylserine (PS); however, the manner by which PS elevates IKAP levels has yet to be identified. Analysis of ChIP-seq results of the *IKBKAP* promoter region revealed binding of the transcription factors CREB and ELK1, which are regulated by the mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) signaling pathway. We show that PS treatment enhanced ERK phosphorylation in cells derived from FD patients. ERK activation resulted in elevated *IKBKAP* transcription and IKAP protein levels, whereas pretreatment with the MAPK inhibitor U0126 blocked elevation of the IKAP protein level. Overexpression of either ELK1 or CREB activated the *IKBKAP* promoter, whereas downregulation of these transcription factors resulted in a decrease of the IKAP protein. Additionally, we show that PS improves cell migration, known to be enhanced by MAPK/ERK activation and abrogated in FD cells. In conclusion, our results demonstrate that PS activates the MAPK/ERK signaling pathway, resulting in activation of transcription factors that bind the promoter region of *IKBKAP* and thus enhancing its transcription. Therefore, compounds that activate the MAPK/ERK signaling pathway could constitute potential treatments for FD.

Introduction

Familial dysautonomia (FD) is a hereditary autosomal recessive neuropathy restricted to the Ashkenazi Jewish population, with a relatively high carrier frequency between 1 in 32 and 1 in 18 in Ashkenazi Jews of Polish descent (1–3). The clinical features of FD include progressive depletion of unmyelinated sensory and autonomic neurons, gastrointestinal and cardiovascular dysfunction, vomiting crises, pain and temperature insensitivity and recurrent pneumonia (4–6). FD is caused by a point mutation, a transition from T to C at position 6 of the 5′ splice site of intron 20 in the *IKBKAP* gene, which encodes the IκB kinase complex-associated protein (IKAP) (7,8). Although exon 20 is constitutively spliced in healthy individuals, the mutation results in alternative splicing of the exon in FD patients, causing a frameshift and a premature stop codon in the skipped isoform, which in turn leads to considerably reduced IKAP protein levels. Levels of exon 20 skipping vary depending on the tissues with the lowest inclusion level observed in the nervous system, probably accounting for the nervous system-specific manifestations of the disease (9,10). Cells derived from heterozygous individuals have lower *IKBKAP* mRNA and protein levels than do cells isolated from wild-type (WT) individuals (11), yet heterozygous individuals are asymptomatic, suggesting that a tissue-specific threshold of IKAP expression is required for normal development and maintenance. Therefore, it is likely that a therapy that increases the amount of the WT *IKBKAP* mRNA levels, in turn elevating the normal, functional IKAP protein, would be beneficial. Thus, unraveling the regulatory pathways influencing the *IKBKAP* gene expression could reveal new therapeutic targets.

IKAP function in the cell has been vastly investigated; it is most commonly considered as a subunit of the Elongator complex, assisting RNA polymerase II in transcription elongation in

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the nucleus (12–15), and was also implicated in cytosolic functions such as tRNA modification (16,17), exocytosis (18), cell adhesion, migration of cells and reorganization of actin in the cytoskeleton (19–21), regulation of the c-Jun N-terminal kinase (JNK) signaling pathway (22,23) and p53 activation (24). IKAP may also play a role in oligodendrocyte differentiation and/or myelin formation (25), nerve growth factor signaling and peripheral neuron target innervations (23,26) and was shown to be crucial for vascular and neural development during embryogenesis (26–28). Nevertheless, little is known concerning *IKBKAP* transcription regulation.

Several potential therapeutics have been tested for treatment of FD, such as kinetin (29,30), RECTAS (17) and tocotrienols (31,32); however, their therapeutic effects on FD patients remain to be seen. Phosphatidylserine (PS), an FDA-approved food supplement, was also tested for FD with promising results, both in cell lines derived from FD patients (11) and in an FD mouse model (33) and in a small-scale clinical trial in FD patients (34). PS is the major acidic phospholipid in human membranes, and it serves as a structural component of the plasma membrane and of various intracellular membranes (35,36). In the healthy human brain, PS is mostly in the form of 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoserine (37,38), and the docosahexaenoic acid (DHA) content of neuronal PS is of functional importance (39). However, the manner by which PS influences production of IKAP has yet to be identified.

In order to better design treatments for FD, we sought to unravel how *IKBKAP* transcription is regulated. Analysis of ChIP-seq results in the *IKBKAP* promoter region revealed binding of a group of transcription factors regulated by the mitogen-activated protein kinase (MAPK) signaling pathways, with ELK1 and CREB exhibiting the strongest correlations with the *IKBKAP* expression level. One of the MAPK pathways, the Ras/Raf/MEK/extracellular-regulated kinase (ERK) signaling cascade, couples signals from cell surface receptors to transcription factors, such as ELK1 and CREB, which regulate gene expression (40–42). ERK1 and 2 are serine/threonine kinases, activated by phosphorylation. Activated ERK1 and 2 modulate gene expression by phosphorylating transcription factors directly and by activating other protein kinases, which then phosphorylate proteins involved in gene expression (41). We show that PS treatment enhances ERK phosphorylation in cells derived from FD patients. MAPK/ERK activating substances also elevated WT *IKBKAP* transcript and IKAP protein levels, whereas treatment with the MAPK inhibitor U0126 prior to addition of PS blocked elevation of IKAP protein levels. Overexpression of either ELK1 or CREB activated transcription from the *IKBKAP* promoter, whereas knockdown of these transcription factors resulted in a decrease of the IKAP protein level. Taken together, our results demonstrate that PS activates the MAPK/ERK signaling pathway, which activates transcription factors that enhance *IKBKAP* transcription. In addition, IKAP-depleted cells display impaired cell migration (19–21), which could underlie the neuropathology of FD patients. Therefore, PS was also tested for effect on FD cell migration and was found to improve wound closure rate, possibly due to MAPK/ERK activation.

Results

MAPK-regulated transcription factors bind *IKBKAP* promoter

PS enhances transcription of the *IKBKAP* gene (11); however, the mechanism by which it influences transcription is unknown. To investigate the mechanism that controls *IKBKAP* expression at the transcriptional level, we examined a 2000 bp long region of the *IKBKAP* promoter using ChIP-seq data produced by the ENCODE Project Consortium 2012 (43). Among the transcription factors that bind in this region are a number of transcription factors that are regulated by the MAPK signaling pathways: ELK1, CREB, ATF1, c-Jun and JunD (Fig. 1A). To uncover additional evidence concerning the possible regulators of *IKBKAP* expression, we next analyzed transcriptomic data spanning different human nervous system tissues (45) to compute the correlations between *IKBKAP* expression levels and those of all other genes. We found high correlations between *IKBKAP* levels and those of *ELK1* ($R = 0.8$; ranked 531 of 18 093 genes) and *CREB* ($R = 0.49$; ranked 3469 of 18 093 genes) (Fig. 1B), suggesting that the transcription factors encoded by these genes positively regulate *IKBKAP* expression. *ATF1*, *c-Jun* and *JunD* exhibited low or negative correlations with *IKBKAP* levels ($R = 0.15$, -0.47, -0.53, respectively). ELK1, the best-characterized member of the ternary complex factor subfamily of ETS-domain transcription factors, is a direct target of all three classes of MAPK signaling cascades (46,47). CREB is implicated in brain functions such as neuronal survival and proliferation and is activated downstream of various kinase cascades stimulated by cAMP, Ras and/or Ca^{2+} signaling (48,49).

PS treatment elevates activated ERK and RSK in FD cells

In order to understand the involvement of the MAPK signaling cascade in FD, the activation of ERK1/2 and RSK, a kinase phosphorylated by ERK1/2 (41), was examined in an FD patient-derived immortalized fibroblast cell line. Cells were treated with several substances known to activate the MAPK signaling pathway: the widely used PKC-MAPK activator phorbol-12-myristate-13-acetate (PMA) (50); etorphine, a δ-opioid agonist (51) and the amino acid L-glutamic acid (glutamate), the most abundant excitatory neurotransmitter in the nervous system (52). Activation of ERK1/2 peaked after 5–10 min of treatment with 100 nM PMA, 0.1 μM etorphine or 50 μM glutamate, and activation of RSK peaked after 20 min of PMA treatment and after 5–10 min of etorphine or glutamate treatment (Fig. 2A–C). FD cells were also examined for MAPK activation after PS treatment. For our experiments, we used a lipid formulation enriched with PS-DHA (hereafter referred to as PS). Activation of ERK1/2 and RSK peaked after 5–10 min of treatment with 5 μg/ml PS (Fig. 2D). These results suggest that PS activates the MAPK/ERK signaling pathway.

MAPK activation elevates *IKBKAP* transcription and protein levels

Two cell lines generated from two different FD patients (designated *FD-A* and *FD-B*) were treated with a series of concentrations of PS, and RNA was extracted for analysis of *IKBKAP* mRNA levels. PS treatment elevated *IKBKAP* WT mRNA levels in the FD cell lines by 1.5–3-fold (Fig. 3A) in agreement with previously published results (11). Treatment of FD cells with MAPK/ERK-activating substances etorphine, glutamate and PMA also elevated *IKBKAP* WT mRNA levels significantly in the *FD-A* cell line by 1.8–2.6-fold. Etorphine, glutamate and PMA elevated *IKBKAP* WT mRNA levels in the *FD-B* cell line by 1.2–4.5-fold (Fig. 3B–D). We then evaluated IKAP protein levels in *FD-A* cells after treatment with etorphine, glutamate or PS, with or without pretreatment with the MAPK/ERK inhibitor U0126 (54). Treatment with each of the three substances elevated IKAP protein levels compared with the corresponding control-treated cells. Interestingly, pretreatment with the MAPK/ERK inhibitor U0126 decreased

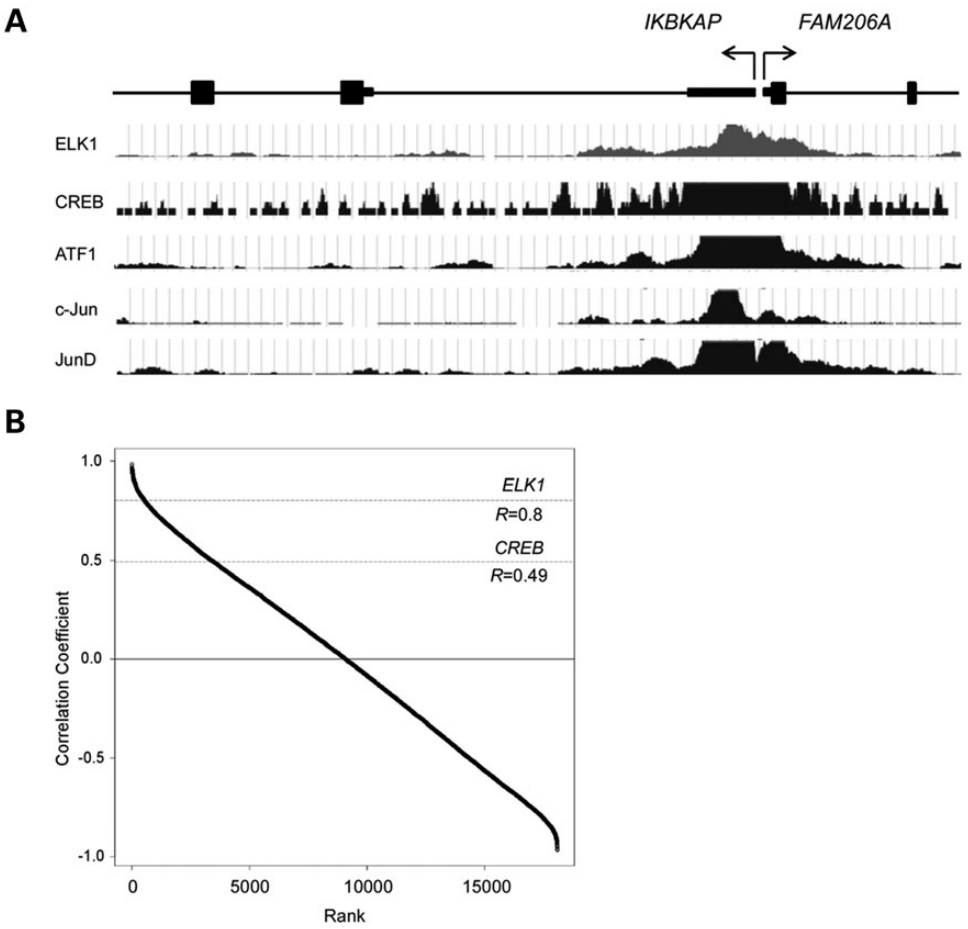

Figure 1. ELK1 and CREB bind the *IKBKAP* promoter, and their gene expression is correlated with *IKBKAP* levels. (**A**) ChIP-seq signals of transcription factors at the *IKBKAP* promoter region [data derived from (43)] adapted from the UCSC Genome Browser (44). Arrows indicate transcriptional direction at TSS. Black boxes indicate exons; narrow boxes indicate untranslated regions and wide boxes indicate coding exons. Black ChIP-seq signals represent data from K562 cells, and grey ChIP-seq signal represents data from HeLa cells. (**B**) Ranking (*x*-axis) of the coefficients of Pearson's correlations between *IKBKAP* expression levels and levels of all other genes in human nervous system tissues.

IKAP levels below those observed in the corresponding treated cells. These experiments reveal that PS, like the known MAPK/ERK activators etorphine and glutamate, induces expression from the *IKBKAP* gene in an FD cell line. These results imply that *IKBKAP* transcription is influenced by the MAPK/ERK signaling pathway and that the effect of PS on *IKBKAP* transcription is mediated through this pathway.

CREB and ELK1 influence transcription from the *IKBKAP* promoter

In order to explore whether CREB and ELK1 regulate *IKBKAP* transcription, we performed a luciferase assay using a reporter gene. We cloned about 2400 bp of the *IKBKAP* promoter into a pGL3 luciferase vector. We also cloned CREB and ELK1 coding sequences into p3xFLAG-CMV-10 expression vector, and one or both were co-transfected with the *IKBKAP* promoter vector into SHSY5Y or HeLa cells. Both transcription factors activated transcription from the *IKBKAP* promoter as luciferase activity was elevated by both CREB and ELK1 separately and combined in both cell lines (Fig. 4A). Interestingly, the combination of the two factors did not have an additive effect on luciferase activity, but rather resulted in an intermediate level between CREB and ELK1 activation.

Analysis of *CREB* and *ELK1* expression levels across 48 human tissues and cell lines revealed that the two genes have different expression profiles in most tissues and cell lines (Fig. 4B). A competition between the two factors could account for the intermediate luciferase activation exhibited when both factors are overexpressed. In support of this, putative binding sites for CREB and ELK1 are located only 260 bp apart in the *IKBKAP* promoter. Reciprocally, treating cells with siRNA directed against *ELK1* and *CREB* resulted in decreased IKAP protein levels (Fig. 4C).

Gardiner *et al.* (22) proposed a mechanism of interaction between the Elongator complex, the JIP/JNK module and microtubules, according to which JNK is activated by IKAP, later validated by Abashidze *et al.* (23). Also in support of this model, we observed that phospho-JNK levels were decreased in cells deficient in ELK1 and CREB. The lowest phospho-JNK level was observed in samples with the least IKAP protein levels (Fig. 4C).

PS treatment improves FD cell migration whereas MAPK inhibition decreases it

Cells with decreased IKAP levels have aberrant expression of genes involved in cell migration (55) and display defects in cell migration (19–21). In addition, ERK plays an important regulatory function in cell migration and wound healing (56,57). Therefore,

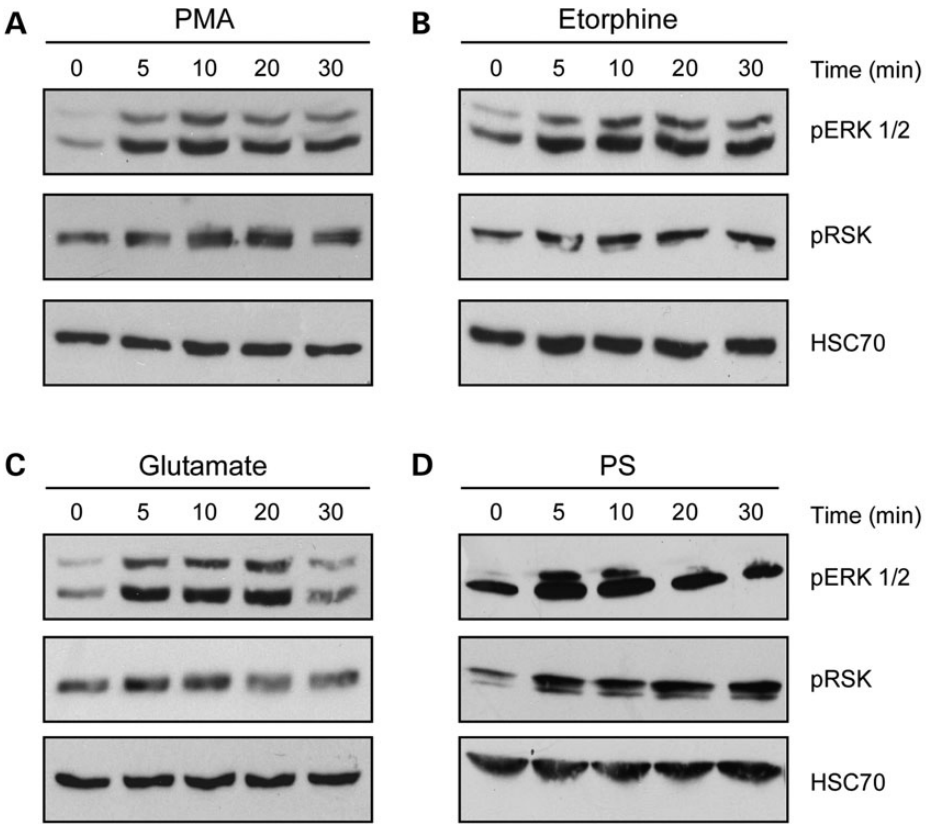

Figure 2. PS activates ERK and RSK. FD cells were treated with (**A**) 100 nM PMA, (**B**) 0.1 μM etorphine, (**C**) 50 μM glutamate or (**D**) 5 μg/ml PS. Cells were lysed and proteins were extracted at different times after treatment. Proteins were separated on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and probed with antibodies. Phosphorylated ERK1/2 (upper and lower bands, respectively) and RSK were detected using anti-phospho-ERK1/2 and anti-phospho-RSK antibodies. The HSC70 protein level was evaluated as loading control.

we examined the influence of PS and of inhibition of MAPK/ERK pathway on FD cell migration in a wound healing assay. FD cells were cultured until confluent and inflicted by a straight scratch simulating a wound. Wound closure in cells with different treatments was monitored using an IncuCyte Zoom microscope; scratch size was measured as a function of time as previously described (58,59). Cells treated with PS exhibited a significantly higher wound closure rate when compared with control cells (one-sided Student's *t*-test; $P \leq 0.04$ between 2 and 28 h after treatment), and a delay in wound closure was observed in cells treated with U0126 ($P \leq 0.04$ from 21 h after treatment) and in cells treated with both PS and U0126 (Fig. 5). These results indicate that PS improves the cell migration phenotype aberrant in FD cells, possibly due to MAPK/ERK activation.

Discussion

PS enhances *IKBKAP* WT mRNA transcription and consequently increases IKAP protein levels in FD cell lines and in a mouse model (11,33). The goal of this study was to elucidate the molecular mechanism underlying this effect. We found that substances that activate the MAPK/ERK signaling pathway significantly increase WT *IKBKAP* mRNA and protein levels in FD cells; inhibition of this signaling pathway resulted in a significant decrease in IKAP protein. Correspondingly, we demonstrated that PS enhanced ERK and RSK activation, and inhibition of the MAPK pathway blocked the effect of PS on IKAP protein levels. These results indicate that *IKBKAP* transcription is regulated through the MAPK/ERK signaling pathway and that PS treatment increases levels of IKAP due to MAPK activation.

Most MAPKs are cytoplasmic proteins that following activation phosphorylate cytoplasmic and membrane proteins (23) or translocate to the nucleus where they phosphorylate and regulate nuclear proteins such as transcription factors (60,61). The MAPK/ERK signaling cascade is often aberrantly activated in malignantly transformed cells and certain cancer types (62–67). Notwithstanding, activation of ERK1/2 was shown to protect neural cells against amyloid peptide-induced neurotoxicity (68) and neurons of the suprachiasmatic nucleus against glutamate exitotoxicity (69). ERK1 and 2 are also necessary for neural cell adhesion molecule-stimulated neurite outgrowth of importance for brain development and neuroplasticity (70). Furthermore, ERK1/2 deletion in the embryonic peripheral nervous systems of mice results in dorsal root ganglia (DRG) degeneration and the absence of all peripheral projections; ERK1/2 signaling is essential at multiple stages of Schwann cell development and required for peripheral nervous system myelination (71). Recent work has revealed that IKAP plays a specific role in neurite outgrowth in DRGs of the chick embryo (23), indicating that IKAP may share molecular functions with ERK.

In our analysis of publically available ChIP-seq data, we found that the MAPK-regulated transcription factors CREB and ELK1 bind the *IKBKAP* promoter, and our analysis revealed *CREB* and *ELK1* genes to be positively correlated with the transcription level of *IKBKAP*. We also showed that overexpression of these transcription factors activated the *IKBKAP* promoter, whereas

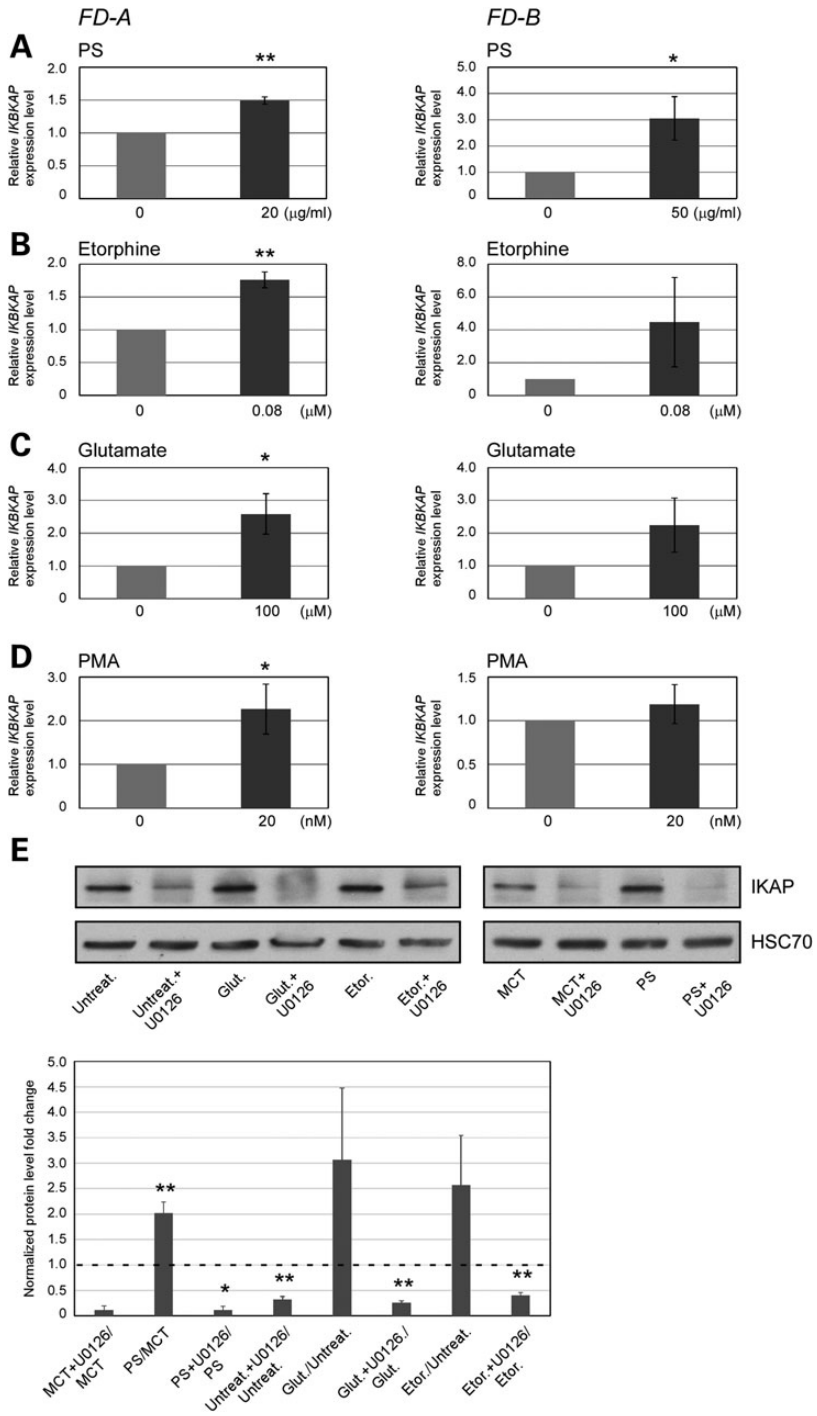

Figure 3. MAPK/ERK activation correlates with *IKBKAP* mRNA and protein levels in FD cell lines. (**A–D**) FD cell lines (FD-A and FD-B) treated with (A) PS, (B) etorphine, (C) glutamate or (D) PMA. Cells were harvested after 24 h. qRT–PCR was used to evaluate WT *IKBKAP* transcript levels. The most consistent concentration in three biological replicates is exhibited; error bars represent standard error of the mean. Asterisks denote statistically significant differences (*$P \leq 0.05$ and **$P \leq 0.01$) relative to control; Student's *t*-test. (**E**) FD-A cells were treated for 14 days with 0.08 μM etorphine, 100 μM glutamate or 5 μg/ml PS with or without pretreatment for 1 h with 2 μM U0126. Cells treated with MCT were used as control for PS treatment. Untreated cells were used as controls for etorphine and glutamate treatments. Proteins were harvested and separated on 10% SDS–PAGE. Membranes were probed with anti-IKAP antibody; HSC70 was evaluated as loading control. Fold change in IKAP levels following treatments relative to control-treated cells, normalized to loading control, was determined using ImageJ (53) and represents three biological replicates; error bars represent standard error of the mean. Asterisks denote statistically significant differences (*$P \leq 0.05$ and **$P \leq 0.01$) relative to control; Student's *t*-test.

reduction in CREB and ELK1 levels decreased IKAP protein levels. These results establish that the MAPK/ERK signaling cascade regulates *IKBKAP* transcription. Interestingly, the downregulation of both transcription factors was required in order to reduce IKAP protein levels. We speculate that this could be due to a compensatory effect of other transcription factors when CREB or ELK1 levels are reduced. This hypothesis is supported by the findings of Odrowaz and Sharrocks (72), who identified a large set of

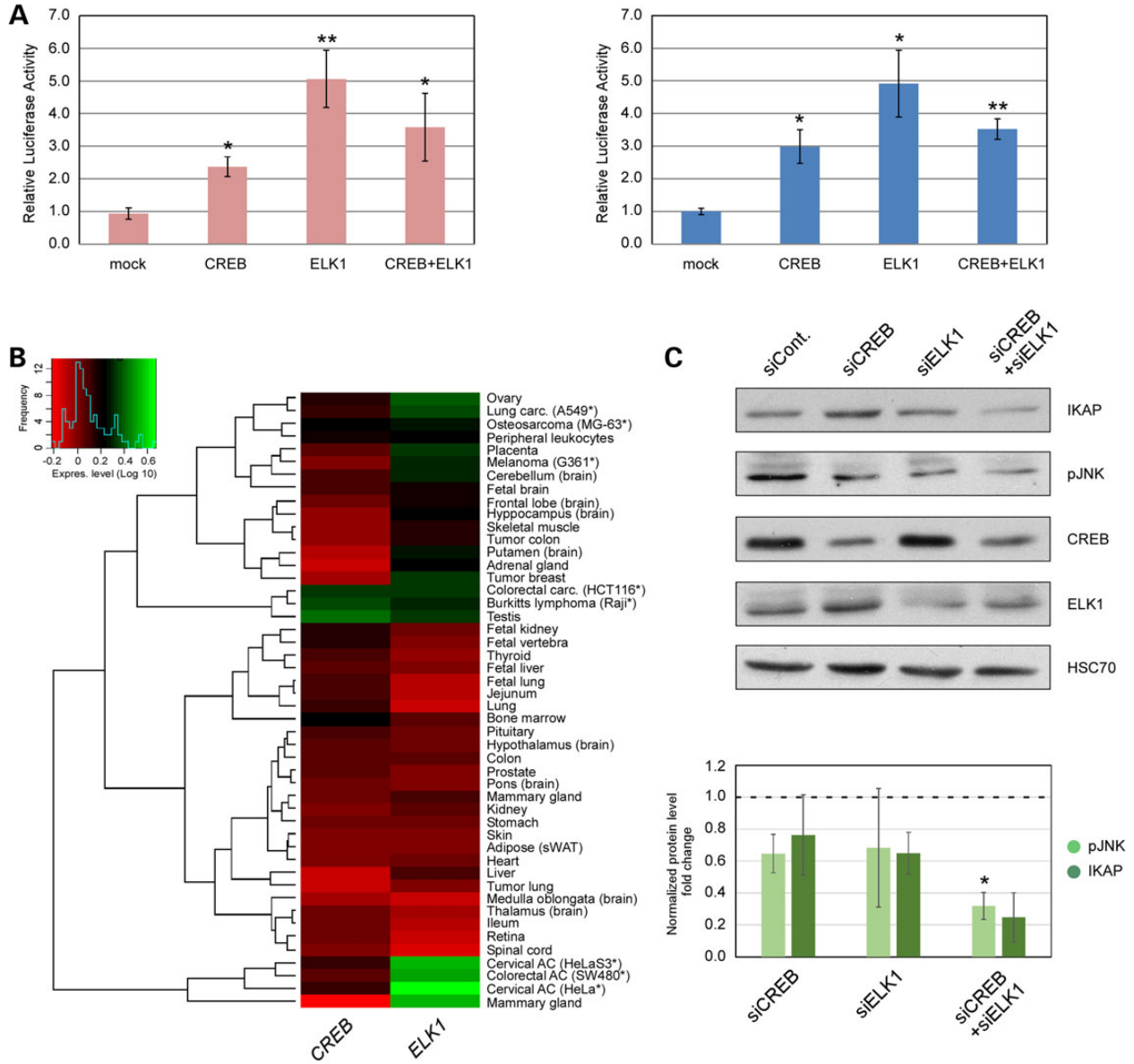

Figure 4. CREB and ELK1 influence transcription from the *IKBKAP* promoter. (**A**) Luciferase activity measurement in SHSY5Y (left) and HeLa (right) cells co-transfected with *IKBKAP* promoter-luc construct and with vectors for expression of either CREB or ELK1 or both. Luciferase activity was measured 48 h after transfection. The results were normalized to *Renilla* luciferase activity and are shown as fold enrichment levels over mock-treated cells. Error bars represent the standard error of the mean calculated from five biologically independent experiments with SHSY5Y cells and four with HeLa cells, each representing the average of two replicates. Asterisks denote statistically significant differences (*$P \leq 0.003$ and **$P \leq 0.0002$) relative to control (*IKBKAP* reporter in the presence of empty p3xFLAG-CMV-10); Student's *t*-test. (**B**) *CREB* and *ELK1* expression levels in 48 human tissues and cell lines. Cell lines are marked by asterisks. The color key includes a histogram of the gene expression level distribution. (**C**) SHSY5Y cells were transfected with control-siRNA or siRNA directed against the mRNA-encoding CREB or ELK1. Cells were harvested 72 h after transfection, and proteins were separated in 10% SDS–PAGE. Membranes were probed with the indicated antibodies; HSC70 was evaluated as loading control. Fold change in IKAP and pJNK levels following siRNA directed against *CREB* and/or *ELK1*, normalized to loading control, was determined using ImageJ and represents three biological replicates; error bars represent standard error of the mean. Asterisks denote statistically significant differences (*$P \leq 0.05$), relative to control; Student's *t*-test.

'redundant' ELK1-regualted genes. These genes are controlled by ELK1 and other transcription factors and do not exhibit gene expression changes upon siRNA-mediated ELK1 knockdown. The same phenomenon could also account for CREB.

It should be mentioned that 60 bp upstream of the *IKBKAP* transcription start site (TSS) is the TSS of *FAM206A*, a gene that is transcribed from the opposite DNA strand from the *IKBKAP* gene (Fig. 1A). CREB and ELK1 binding to that genomic region may also affect transcription of *FAM206A*. Little is known about the *FAM206A* gene function.

Both CREB and ELK1 facilitate expression of genes known to have important roles in neuroprotection against various neurotoxins and injuries (73–77). In addition, in a large-scale screen using FD-induced pluripotent stem cells, Lee *et al.* (78) showed that compounds that elevated *IKBKAP* expression also elevated phospho-CREB levels; a number of these compounds had been reported by others to activate MAPK/ERK signaling. MAPK signaling pathways are also implicated in Parkinson's disease, which is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (79). Activation of ERK1/2

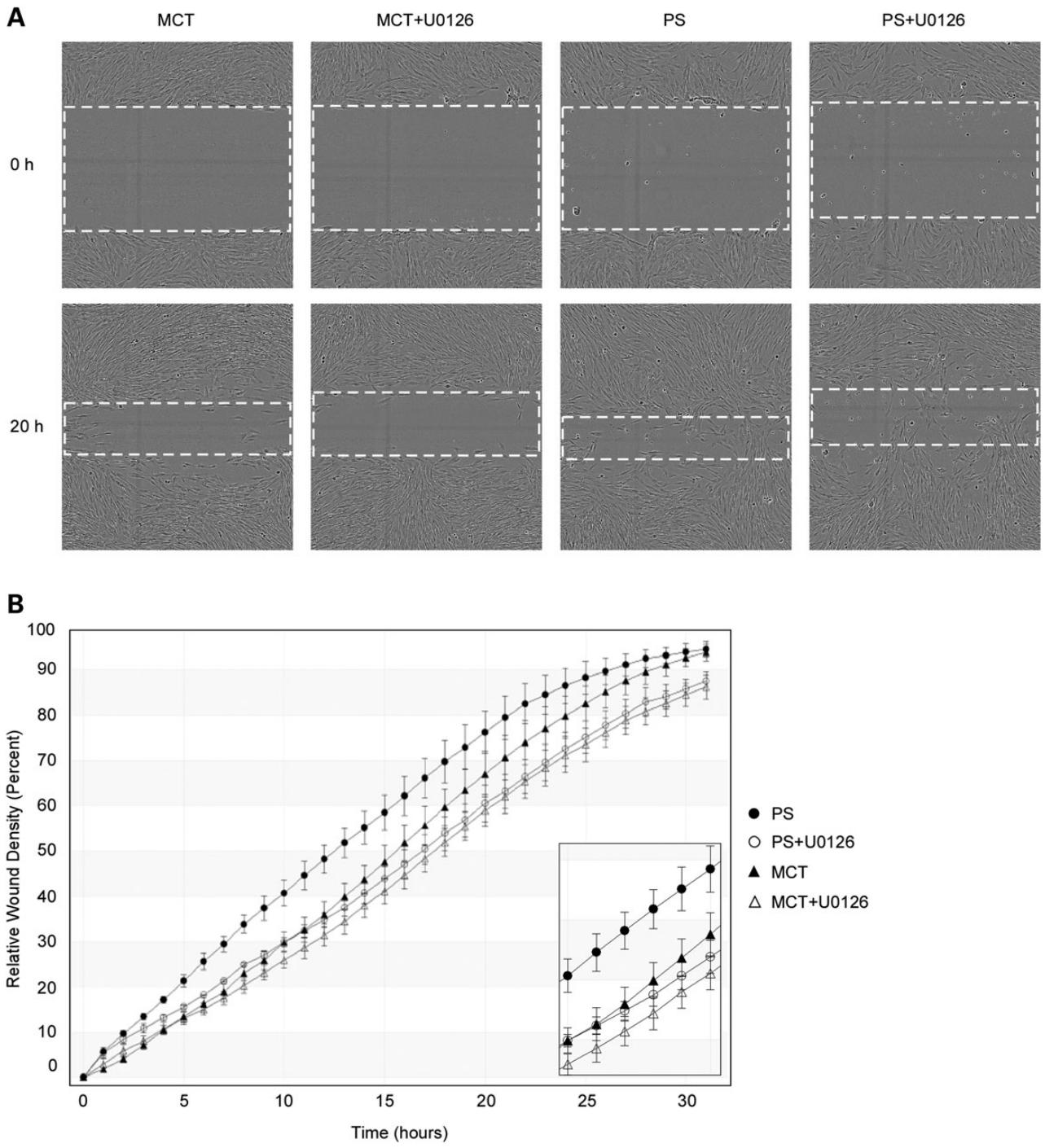

Figure 5. PS treatment accelerates wound healing in FD cells. FD cells were seeded in a 96-well plate and cultured until confluent. All wells were inflicted by a wound across the cell layer, and selected wells were incubated with U0126 for 30 min. PS or solvent was then added, and cell migration was monitored using an IncuCyte Zoom microscope (Essen Bioscience). (**A**) Representative images of FD cells with different treatments at the indicated time points after wounding. Wound size is marked by a dashed rectangle. (**B**) Relative wound density during time period until wound closes without U0126 treatment, calculated using the IncuCyte Zoom image analysis software. On the bottom right- an enlarged image of time period between 10 and 15 h.

protects dopaminergic neurons against toxin-induced apoptosis (80,81), although sustained ERK activation was also shown to have cytotoxic effect in neuronal cell lines (82–84). Thus, activation of ERK1/2 can be neuroprotective as well as cytotoxic depending on kinetics of ERK activation and cellular localization (85,86). Intriguingly, upstream activators of MAPK/ERK, such as PKC, have remarkable neuroprotective effects that could prove beneficial in the treatment of neurodegenerative diseases (87). In fact, binding of endogenous PS along with calcium ions causes PKC to translocate from the cytosol to the membrane, a step that is required for its activation (36,87,88). This may be how the exogenous PS used in this work activates MAPK/ERK signaling. It is noteworthy that PS treatment of FD mice resulted in the downregulation of several genes known to be associated with

Parkinson's disease (33), implying that PS might be beneficial for patients with Parkinson's disease.

Finally, we demonstrated how PS treatment enhanced migration of FD cells and that MAPK/ERK inhibition blocked this effect. FD cell lines are characterized by improper regulation of the cell cycle, as previously published results indicate that exit from G1 into the S phase is impaired in these cells. PS treatment releases FD cells from cell cycle arrest and results in cell cycle distribution similar to that of the control cells (11). In different cell types, activation of the MAPK/ERK signaling pathway improves both cell migration (56,57) and proliferation (89). Taken together, these results suggest that PS treatment improved both defected phenotypes through activation of the MAPK/ERK signaling pathway.

In conclusion, our results identified for the first time the MAPK/ERK signaling cascade as a regulator of the *IKBKAP* gene transcription. Therefore, substances that activate this signaling pathway have potential in the treatment of FD. PS is such a substance as it activates ERK1/2. Accumulating data indicating that ERK1/2, and its substrates CREB and ELK1, have neuroprotective traits further warrants testing the use of the safe-to-use PS in FD patients and in patients with other neurodegenerative diseases.

Materials and Methods

Cell culture and chemicals

Human FD fibroblast cells were obtained from the appendices of FD patients and immortalized using telomerase activation. FD and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20 or 10% fetal calf serum (FCS), respectively, 0.29 mg/ml L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. SHSY5Y cells were cultured in RPMI 1640 supplemented with 10% FCS, 0.29 mg/ml L-glutamine and 40 μg/ml gentamycin sulfate. All cell culture materials were purchased from Biological Industries. All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2. PMA and U0126 were purchased from Calbiochem and dissolved according to the manufacturer's instructions. InCog™, a lipid composition containing PS-omega 3, DHA enriched, referred to here as PS, was dissolved in organic solvent medium chain triglycerides (MCTs). Both PS and MCT were obtained from Enzymotec. L-glutamic acid (Sigma-Aldrich, MO, USA) was dissolved in phosphate-buffered saline (PBS). Etorphine, kindly provided by Yosef Sarne's laboratory, was dissolved in doubly distilled water and titrated with HCl to complete dissolution. When cells were treated for 14 days, culture medium was replaced every 2–3 days.

RNA purification, RT–PCR and quantitative RT–PCR

Total RNA was extracted with TRI Reagent (Sigma-Aldrich) and reverse-transcribed by RT-AMV (Roche, Basel, Switzerland) with oligo(dT) reverse primer. A quantitative real-time PCR (qPCR) analysis of mRNA expression was performed on Mx3005P (Stratagene) using Absolute Blue QPCR SYBR Green ROX mix (Thermo Scientific, MA, USA). Primers detecting the WT IKBKAP isoform (inclusion of exon 20) were exon19-F (TTCACGGATTGTCACTGTTGTGCC) and exon20-R (TTGTCCAACCACTTCCGAATCTG). Primers for analysis of the *LZIC* mRNA used in normalization are LZIC-F (TGATACA GATGAATATGAAGAAACC) and LZIC-R (TCTACCAAAGTCATAT TTCCAGAC). Analysis was performed using the MxPro 4.01 software. All reactions were performed in triplicate.

Plasmid construction, transfection and luciferase assay

Human CREB and ELK1 coding sequences were amplified using KAPA HiFi HotStart (Biosystems, Barcelona, Spain) and cloned into pX3FLAG-CMV-10 expression vector. About 2400 bp of the *IKBKAP* promoter was cloned into pGL3 luciferase reporter vector. Constructs were verified by DNA sequencing. Cells were plated in 24-well plates for 24 h prior to transfection with 400 ng of each vector using GeneJammer Transfection Reagent (Agilent, CA, USA). Cells were harvested after 48 h, and luciferase activity in lysates was measured with a Dual-Luciferase Reporter Activity Assay (Promega) and normalized to *Renilla* luciferase activity.

Transfection of siRNAs into SHSY5Y cells was carried out using Lipofectamine RNAiMAX (Invitrogen, CA, USA), according to the manufacturer's protocol with 100 nM siCREB, siElk1 or control siGENOME non-targeting human siRNA pool #2 (all purchased from Dharmacon, CO, USA). Culture medium was replaced 24 h after transfection, and cells were harvested after additional 48 h.

Western blotting

Proteins were extracted by a hypotonic lysis buffer [50 mM Tris–HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 1 mM EDTA] containing protease inhibitor (Roche) and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Proteins were separated in 10% SDS–polyacrylamide gel electrophoresis (PAGE) and then electroblotted onto a Protran nitrocellulose transfer membrane (Whatman, Maidstone, UK). The following antibodies were used: anti-IKAP (AS-54494, Anaspec, CA, USA), anti-ELK1 (ab-131465, Abcam, Cambridge, UK), anti-CREB (#9197, Cell Signaling, MA, USA), anti-phospho-JNK (sc-12882, Santa Cruz Biotechnology, TX, USA), anti-phospho-ERK1/2 (sc-7383, Santa Cruz Biotechnology), anti-phospho-RSK provided by Roni Seger from Weizman Institute of Science (sc-12898-R, Santa Cruz Biotechnology) and anti-HSC70 (sc-7298, Santa Cruz Biotechnology).

Wound healing assay

FD cells were seeded in an ImageLock 96-well plate at a density of 27 000 cells/well and cultured until confluent. All wells were inflicted by a wound across the cell layer using WoundMaker™, washed with PBS and supplemented with fresh medium. Selected wells were incubated with U0126 for 30 min prior to addition of PS (final concentration 200 μg/ml) or solvent. Migration was monitored using an IncuCyte Zoom microscope (Essen Bioscience, MI, USA), with image acquisition every 60 min for 48 h. The IncuCyte Zoom image analysis software was used to quantify wound closure.

Bioinformatic analyses

Transcriptome-wide microarray data in 48 human tissues and cell lines was retrieved from Castle *et al.* (45) and analyzed to determine the profiles of expression of 18 093 genes. Pearson's correlations between the gene expression of *IKBKAP* and all other genes across all nervous system tissues were computed. Data processing and visualization were carried out using custom R (R Development Core Team 2014) scripts (90).

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Conflict of Interest statement. None declared.

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