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ERK MAP kinase-dependent *SOCS-3* Gene Induction requires c-Jun, STAT3 and SP3 Transcription Factors

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Running Title: Regulation of *SOCS-3* Gene expression by ERK

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ABBREVIATIONS

Cyclic AMP, 3', 5' cyclic adenosine monophosphate; C/EBP, CCAAT/enhancer binding protein; ChIP, Chromatin immunoprecipitation; HUVEC, human umbilical vein endothelial cell; PKA, cyclic AMP-dependent protein kinase; SOCS-3, suppressor of cytokine signalling 3; siRNA, small interfering RNA; SEM, standard error of mean; STAT, Signal transducers and activators of transcription; EPAC, exchange protein activated by cyclic AMP; PGE_2, prostaglandin E_2; MSH, melanocyte stimulating hormone; AP-1, activator protein 1; SP1/SP3- Specificity proteins 1 and 3; ERK, extracellular signal regulated kinase; MAP kinase, microtubule associated kinase.

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ABSTRACT

SOCS-3 gene induction by cyclic AMP-elevating agents or the PKC-activator, PMA, in primary HUVECs was found to require PKCη– and PKCε-dependent ERK activation. The minimal, ERK-responsive element of the *SOCS-3* promoter was localised to a region spanning nucleotides -107 to the transcription start site and contains conserved binding sites for AP-1 and SP1/SP3 transcription factors, as well as proximal and distal STAT (pSTAT and dSTAT) binding elements. All three classes of transcription factor were activated in response to ERK activation. Moreover, representative protein components of each of these transcription factor binding sites, namely c-Jun, STAT3 and SP3, were found to undergo ERK-dependent phosphorylation within their respective transactivation domains. Mutational analysis demonstrated an absolute requirement for the SP1/SP3 binding element in controlling basal transcriptional activity of the minimal *SOCS-3* promoter. In addition AP-1, pSTAT and SP1/SP3 binding sites were required for ERK-dependent, PMA-stimulated *SOCS-3* gene activation. The dSTAT site appears to be important for supporting activity of the AP-1 site, since combined deletion of both sites completely blocks transcriptional activation of *SOCS-3* by PMA. Together these results describe novel, ERK-dependent regulation of transcriptional activity that requires co-dependent activation of multiple transcription factors within the same region of the *SOCS-3* gene promoter.

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INTRODUCTION

The suppressors of cytokine signaling (SOCS) constitute a family of eight related Src homology 2-(SH2) containing proteins, namely CIS and SOCS-1 to SOCS-7 (Krebs and Hilton, 2001). Only SOCS-1 and SOCS-3 proteins have been intensely studied and have been shown to function as end points in a classical negative feedback loop whereby activation of STAT transcription factors triggers the induction of SOCS proteins, which then bind and terminate signaling from activated cytokine receptors (Kubo et al., 2003). The SOCS-3 protein is known to inhibit signal transduction from various receptors, including IL-6Rα, IFN-γR, IL-12 receptor β2, G-CSF, erythropoietin and leptin receptors (Dalpke et al., 2008; Dimitriou et al., 2008). SOCS-3 exerts its negative feedback through at least two mechanisms: 1) by binding to JAK-phosphorylated receptors via an SH2 domain SOCS-3 inhibits JAK activity and, consequently, activation of STATs 1 and 3 (Sasaki et al., 1999), 2) SOCS-3 also targets SH2-bound proteins for ubiquitination and proteosomal degradation (Kamura et al., 1998; Zhang et al., 1999).

The importance of SOCS-3 negative feedback is highlighted by the fact that dysregulation of SOCS-3 and increased levels of STAT3 activation contributes to the development of cancer in multiple neoplasias, including cholangiocarcinoma, hepatocellular carcinomas and breast and lung cancer (Silver and Hunter, 2010). Indeed, SOCS-3 has been reported to be a tumour suppressor in breast cancer cells (Barclay et al., 2009) and methylation of CpG islands within the SOCS-3 promoter regions occurs frequently in a variety of cancers, including melanoma (Tokita et al., 2007), glioblastoma (Martini et al., 2008), head and neck squamous cell carcinoma (Weber et al., 2005) and cancers of the lung (He et al., 2003b) and gut (Tischoff et

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al., 2007), thereby preventing SOCS-3 induction and limiting its damping actions on cell growth. Despite this, inhibition of SOCS-3 induction in macrophages may actually be therapeutic for the suppression of tumor metastasis, since hyperactivation of STAT3 in these cells simultaneously exerts an anti-inflammatory as well as anti-tumor effects, through the concomitant suppression of IL-6 and TNFα production and increased production of monocyte chemotactic protein 2 (Hiwatashi et al., 2011).

SOCS-3 expression is increased, however, at sites of acute and chronic inflammation (White et al., 2011) and IL-6 has been reported to promote acute and chronic inflammatory disease in the absence of SOCS-3 (Croker et al., 2012). Moreover, conditional deletion of the *SOCS-3* gene in haematopoietic and endothelial cells of transgenic mice results in death caused by severe inflammatory lesions in the peritoneal and pleural cavities (Croker et al., 2008). Consequently, cell permeable forms of recombinant SOCS-3 have been used as therapy to effectively suppress pathogen-induced, acute inflammation, by reducing the production of inflammatory cytokines, attenuating liver apoptosis and limiting haemorrhagic necrosis (Jo et al., 2005). It is clear, therefore that by understanding and manipulating the molecular control of *SOCS-3* gene induction we may find novel therapies for diverse diseases ranging from chronic inflammation to cancer.

In this respect, we have found efficient induction of the *SOCS-3* gene by cyclic AMP in human umbilical vascular endothelial cells (HUVECs) and COS1 cells requires coincident activation of the ERK MAP kinase cascade and ERK-dependent phosphorylation of C/EBPβ on Thr-235 appears to be a prerequisite for efficient *SOCS-3* induction (Borland et al., 2009; Sands et al., 2006; Woolson et al., 2009c). In

this case the pathway leading from cyclic AMP to ERK in these cells is not known, however it does appear to be independent of activation of both PKA and the Rap1 guanine nucleotide exchange factor (GEF), EPAC1 (Woolson et al., 2009b). In the current study we use HUVECs and COS1 cells, both of which have been shown to exhibit SOCS-3 induction in response to elevations in intracellular cyclic AMP, to investigate how the ERK MAP kinase cascade serves to integrate these diverse cyclic AMP-regulated pathways during the regulation of *SOCS-3* promoter activity in an effort to determine how we could manipulate SOCS-3 protein production for therapeutic benefit. In this respect, we have investigated the individual roles of STAT, AP-1 and SP1/SP3 transcription factors in mediating *SOCS-3* induction in response to ERK activation. Given the central role that both the ERK MAP kinase cascade and SOCS-3 play in regulating inflammatory and cell proliferative responses, our findings cast new light on a potentially important new gene-regulatory signaling pathway.

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MATERIALS AND METHODS

Materials - Primary antibodies to PKCα, PKCδ, PKCη, PKCε, ERK, phospho-ERK (Thr202/ Tyr204), c-Jun, phospho-c-Jun (Ser 63), STAT3 and phospho-STAT3 (Ser 727) were obtained from New England Biolabs UK Ltd (Hertfordshire, UK). The anti-SP3 and anti-SOCS-3 antibodies were purchased from Santa Cruz Biotechnology (California, USA) and the anti-GAPDH antibody was from Applied Biosystems/Ambion (Texas, USA). The primary antibody that recognises ERK-phosphorylated SP3 (Ser 73) was a generous gift from Dr Giles Pagès (University of Nice Sophia Antipolis, France). Protein A sepharose beads**,** Enhanced chemiluminescence (ECL) reagents, secondary antibodies anti-rabbit-IgG-hourse-raddish-peroxidase conjugate and anti-mouse-IgG horse-raddish-peroxidase conjugate were bought from GE Healthcare (Buckinghamshire, UK). HUVECs and Endothelial Cell Growth Medium 2 were obtained from PromoCell (Heidelberg, Germany). HiPerFect transfection reagent was purchased from Qiagen (West Sussex, UK), and PGE_2, αMSH, Dulbecco's phosphate saline buffer from Sigma-Aldrich. Forskolin, rolipram, phorbol 12-myristate 13-acetate (PMA), MG132, and U0126, were obtained from Merck (Hertfordshire, UK).

Plasmids - Mouse SOCS-3 promoter constructs were a generous gift from Professor JG Bode (Heinrich-Heine University, Dusseldorf, Germany) with permission from Professor Shlomo Melmed (Ceders-Sinai Medical Center, California, USA). These included pGL3-SOCS3-2757Luc, which contains the promoter region -2757 to +929 of the murine SOCS3 gene fused to the coding region of firefly luciferase as described (Auernhammer et al., 1999), as well as promoter truncates pGL3-SOCS3-511Luc, -107Luc, -79Luc, -68Luc and -49Luc and pGL3-SOCS3-107Luc constructs mutated to

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disrupt the putative SP1/SP3, distal and proximal STAT binding regions (dSTAT and pSTAT, respectively) as described (Ehlting et al., 2005). The Quickchange site-directed mutatgenesis kit (Agilent) was used to introduce mutations into vectors pGL3-SOCS3-107Luc, pGL3-SOCS3-107-pSTAT, pGL3-SOCS3-107-SP1/SP3 and pGL3-SOCS3-107-pSTAT-SP1/SP3, using primers, 5'-GCCTTTCAGTGCAGAGTAAAGCTTAAACATTACAAGAAGACCGGCCGGGC-3' (forward) and 5'-GCCCGGCCGGTCTTCTTGTAATGTTTAAGCTTTACTCTGCACTGAAAGGC-3' (reverse), to disrupt the putative AP1 site ($^{-105}$GTGACTAA^{-99} to $^{-105}$AAGCTTAA^{-99}). Mutations were also introduced into vectors pGL3-SOCS3-107Luc, pGL3-SOCS3-107-pSTAT, pGL3-SOCS3-107-SP1/SP3, pGL3-SOCS3-107-pSTAT-SP1/SP3, using primers 5'-GCCTTTCAGTGCAGAGTAAAGCTTAAACATCCCAGGAAGACCGGCCGGGC-3' (forward) and 5'-GCCCGGCCGGTCTTCCTGGGATGTTTAAGCTTTACTCTGCACTGAAAGGC-3' (reverse), to disrupt both the putative AP1 binding site ($^{-105}$GTGACTAA^{-99} to $^{-105}$AAGCTTAA^{-99}) together with the putative dSTAT site ($^{-95}$TTACAAGAA^{-88} to $^{-95}$TCCCAGGAA^{-88}). The AP1-Luc (fn34), STAT-Luc, SP1/SP3-Luc (pAldGCB^{4}luc; (Ehlting et al., 2005)) and CRE-Luc reporter constructs were generous gifts from Professor Walter Kolch (University College Dublin, Republic of Ireland), Dr Timothy Palmer (University of Glasgow, Scotland), Professor Gerald Thiel (University of Saarland, Hamburg, Germany) and Professor Ferenc Antoni (University of Edinburgh, Scotland).

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Cell culture - HUVECS were grown in Endothelial Cell Growth Medium 2 (Promocell) at 37°C and 5% (v/v) CO_2 Cells were passaged weekly to a maximun of 6. COS1 cells were cultured at 37 °C in 5% (v/v) CO_2 in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 2 mM-glutamine and 2% (v/v) penicillin/streptomycin (Sigma-Aldrich).

Transfection of Cells with siRNA - The day before transfection HUVECs were seeded into 6 well plates at a density of approximately 2 x 10^5 cells/cm^2 and grown to approximately 90% confluence. Cells were then transfected with 200nM HP validated siRNAs (Qiagen) to either PKCα (catalogue number (1) SI00605927 or (2) SI00605934), PKCδ (catalogue number (1) SI02660539or (2) SI00301329), PKCη (catalogue number SI02224075), PKCε (catalogue number SI00287784) or non-targeting control oligonucleotides (AllStars Negative Control siRNA, catalogue number 1027280) using HiPerFect (Qiagen, Valencia, CA) transfection reagent, according to the manufacturers instructions. The following day cells were treated with pharmacological agents, harvested in SDS-PAGE sample buffer and analysed by Western blotting.

Western Blotting - For Western blotting cells were harvested by scraping directly into 200µl of SDS-PAGE sample buffer (20 mM Tris-HCl, pH 8.0, 2% (w/v) SDS, 2 mM EDTA, 20% (v/v) glycerol, 2.5% (v/v) β-mercaptoethanol, 0.01% (w/v) bromphenol blue), separated on 10% (w/v) resolving gels and electroblotted onto nitrocellulose membranes. Membranes were blocked in 5% (w/v) milk powder (or 5% (w/v) BSA for phospho-specific antibodies) in Tris-buffered saline containing 0.1% (v/v) Tween 20. Blots were incubated in primary antibodies overnight at 4°C followed

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by appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Blots were then developed using enhanced chemiluminescence reagent (GE Healthcare) according to the manufacturer's instructions.

Reverse Transcriptase PCR – HUVECs were grown to 150, 000 cells/well in 6-well plates and transfected with siRNA oligonucleotides as described (Borland et al., 2009). Cells were then stimulated for 5 hours with diluent, a combination of 10μM forskolin plus 10μM rolipram or 10μM PMA for 5 hours. Cells were then washed twice with ice-cold PBS and then total RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Extracted RNA (2-10ng) was then converted to cDNA and amplified using the OneStep RT-PCR Kit (Qiagen), with a total reaction volume of 25μl, containing 0.4μM dNTPs and 0.6μM of primers (SOCS3-Forward 5'-CACATGGCACAAGCACAAGA-3', SOCS3-Reverse 5'-AAGTGTCCCCTGTTTGGAGG-3', Actin-Forward 5'-CTGGCACCCAGCACAATG-3' and Actin-reverse 5 '-GCCGATCCACACGGAGTACT-3'). The RT-PCR reaction was initiated by 1 cycle at 50°C for 30 mins, followed by 15 minutes at 95°C to activate the hot start DNA polymerase. The amplification reaction involved a denaturation step (94°C, 30sec), annealing step (50°C, 30sec) and 30 cycles of amplification (72°C, 1min) followed by a single amplification step (72°C, 10min). DNA fragements were visualised using 1.5-2% (w/v) agarose gels.

Dual Luciferase Reporter Assays - COS1 cells were grown on 12-well plates until around 80–90% confluence and then transfected with 0.125μg of *Renilla* luciferase (pGL4.74) together with 1.125 μg of with either AP1-Luc, STAT-Luc, CRE-Luc or

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SP1/SP3 reporter constructs or murine SOCS3-Luc promoter deletion/mutation constructs using the DOTAP (Roche) transfection agent. Cells were incubated with luciferase reporter constructs for 24 h, and then the medium was changed for Dulbecco's modified Eagle's medium after which the cell treatments were applied and incubated for a further 24 h. Cells were then harvested according to the protocols in the Promega Dual Luciferase Reporter Assay kit and analyzed using a BMG Labtech luminometer.

Densitometry and Statistical Analysis - Non-saturating immunoblots from multiple experiments were quantified densitometrically using ImageJ software. Statistical significance was determined by one-way ANOVA using GraphPad InStat Software.

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RESULTS

PKC Isoforms Regulate SOCS-3 Induction through the ERK MAP kinase Pathway in HUVECs

In an ongoing effort to delineate the signalling pathways that regulate the induction of the *SOCS-3* gene, we previously demonstrated that in COS1 cells PKC isoforms α and δ act in a cyclic AMP-activated gene regulatory pathway upstream of ERK MAP kinase (Borland et al., 2009). ERK then induces transcriptional activation of the *SOCS-3* gene through the phosphorylation of the transcription factor C/EBPβ on Thr-235, both in COS1 cells and in HUVECs (Borland et al., 2009; Woolson et al., 2009a; Woolson et al., 2009c). What is not known if whether PKC isoforms are required for ERK activation in HUVECs and hence contribute to anti-inflammatory signalling in this model of endothelial dysfunction (Sands and Palmer, 2005). HUVECs normally express the conventional PKC (cPKC; DAG and Ca^{2+} dependent), PKCα, the novel PKCs (nPKCs; DAG but not Ca^{2+}dependent), PKCδ, PKCη and PKCε and the atypical PKC (DAG and Ca^{2+} independent) PKCζ (Mellor and Parker, 1998). Of these, the nPKCs, PKCη and PKCε, are not expressed in COS1 cells (Borland et al., 2009), which prompted us to investigate the importance of PKCη and PKCε for *SOCS-3* gene regulation in HUVECs. Pre-incubation of cells with individual siRNAs towards PKCη or PKCε had little effect on the ability of cyclic AMP elevation with a combination of the adenylyl cyclase activator, forskolin, and the cyclic AMP-specific phosphodiesterase inhibitor, rolipram, (F/R) to increase SOCS-3 protein levels (results not shown). However, co-incubation with PKCη and PKCε siRNAs significantly impaired the ability of F/R to induce SOCS-3 mRNA, as determined by RT-PCR (Fig 1a), and SOCS-3 protein expression, as determined by Western blotting (Fig 1b), but not actin mRNA (Fig 1a), GAPDH protein (Fig1b) or PKCα protein

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(Fig 1b). Moreover, PKCη and PKCε siRNA blocked the ability of PGE_2, a physiological stimulus capable of elevating cyclic AMP in HUVECs (Sands et al., 2006), to induce SOCS-3 protein, indicating that these PKC isoforms can modulate to response of the *SOCS-3* gene to a physiological agonist (Fig 1c).

We next tested the involvement of PKCη and PKCε, or a combination of PKCα and PKCδ, in controlling ERK activation in HUVECs (Fig 2a). We monitored ERK using phospho-specific antibodies and found that, as with SOCS-3 induction, a combination of PKCη and ε siRNAs significantly inhibited ERK activation in response to F/R or the cell-permeable diacylglycerol-analogue, phorbol 12-myristate 13-acetate (PMA, 10μM; Fig 2a). Moreover, F/R- or PMA-stimulated ERK activation appeared to be also sensitive to inhibition by a combination of PKCα and PKCδ siRNAs (Fig 2a). Individually these two siRNAs had no significant effect on ERK activity or SOCS-3 induction (results not shown), however a combination of the two siRNAs effectively inhibited SOCS-3 induction in response to F/R and PMA (Fig 2b), as well as PGE_2 (Fig 2b). Together these observations suggest that, as in COS1 cells (Borland et al., 2009), both PKCα and PKCδ are required for SOCS-3 protein induction by cyclic AMP in HUVECs.

To support the idea that PKC isoforms are required for SOCS-3 induction in HUVECs, two chemical inhibitors of cPKC and nPKC, Gö 6983 (10μM) and Ro-31-7549 (10μM), were found to robustly inhibit ERK activation and SOCS-3 protein induction in response to either F/R or PMA (Fig 3a). Importantly, co-incubation with the inhibitor of ERK activation, U0126 (10μM), dramatically inhibited SOCS-3 induction in response to PGE_2, αMSH, F/R and PMA (Fig 3a and 3b), demonstrating

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that ERK is required for SOCS-3 induction by both cyclic AMP and PMA in HUVECs. Moreover, the fact that treatment with U0126 completely ablated SOCS-3 induction by PMA (Fig 1a) indicates that the ability of cPKCs and nPKCs to induce *SOCS-3* gene activity is through regulation of the ERK MAP kinase pathway. This indicates that in HUVECs, as in COS1 cells (Borland et al., 2009), the response of SOCS-3 to cyclic AMP stimulation is also dependent on PKC-regulated ERK activation. Moreover, the ERK MAP kinase cascade appears to be vital for coordinating signals from both cyclic AMP- and PKC-activated pathways to induce SOCS-3 expression and the nPKCs, PKCη, PKCε, and PKCδ, and the cPKC, PKCα, play a vital role in controlling this gene regulatory cross-talk.

Identification of the Minimal, PKC- and ERK-regulated SOCS-3 Promoter

Having determined a central role for ERK in regulating SOCS-3 protein expression in HUVECs, and previously in COS1 cells (Borland et al., 2009), we next sought to determine which region of the *SOCS-3* gene promoter is specifically targeted by PKC-regulated ERK. A deletion series of the murine *SOCS-3* promoter (Auernhammer et al., 1999), cloned into a promoterless firefly luciferase expression vector (pGL3-Basic), was transfected into COS1 cells and then stimulated in the presence or absence of 10μM PMA for 16 hours. COS1 cells were used in place of HUVECs for these experiments since they are comparably easier to transfect and SOCS-3 is regulated in an ERK- and PKC-dependent manner in these cells (Borland et al., 2009). PMA treatment was used because it induces a robust activation of ERK in both HUVECs (Fig 2a) and COS1 cells (Borland et al., 2009).

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Luciferase activities were measured in PMA-stimulated cells extracts and results demonstrated that the full-length promoter (-2757/+929), and two truncated promoters (-159/+929 and -107/+929), showed significant increases in promoter activity following PMA stimulation (Fig 4a). The -107/+929 region showed slightly higher activity than -159/+929 and -2757/+929 (Fig 3a), which probably represents the deletion of a repressor element between nucleotides -107 and -159. Further deletion beyond position -107 to position -79 and beyond resulted in a loss of PMA responsiveness, indicating that the PMA-responsive element lies between nucleotides -107 and -79, a region that contains a putative AP-1 and dSTAT transcripton factor binding site (Fig 4a and 4c).

Having determined that the mSOCS3/-107 luciferase construct contains the minimal PMA-responsive promoter we then tested whether this region was in fact regulated through activation of the ERK MAP kinase pathway. Cells were transfected with the mSOCS3/-107 minimal promoter construct and then stimulated with PMA in the presence or absence of the MEK inhibitor 10μM U0126 (Fig 4b). Results demonstrated that inhibition of ERK significantly reduced the ability of PMA to induce the activity of the minimal SOCS-3 promoter and also significantly reduced basal activity (Fig 4b). These results demonstrate that the ERK MAP kinase pathway is vital for the regulation of SOCS-3 transcriptional activity through interactions with a minimal segment of the *SOCS-3* promoter contained within -107 nucleotides relative to the transcription start site (+1). Of note, however, is the fact that U0126 did not completely abolish transcriptional activation of the minimal SOCS-3 promoter (Fig 4b) but is still highly effective at inhibiting SOCS-3 protein induction in response to PMA treatment and increased cyclic AMP in HUVECs (Fig 3a). This

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suggests that PMA may act through pathways in addition to the ERK cascade to induce SOCS-3 expression in COS-1 cells. A potential candidate pathway for this is the JNK MAP kinase pathway, which may also be important for SOCS-3 induction in these cells (Dunlop and Yarwood, unpublished observations).

ERK Regulates the Activity of AP-1 complex, STAT and SP1/SP3 Transcription Factors Analysis of the minimal mSOCS3 -107+929 promoter fragment reveals the presence of putative consensus sites for AP-1 complex, STAT (dSTAT and pSTAT) and SP1/SP3 transcription factors, which are conserved within the promoter sequences from both human and mouse (Fig 4c). Previously it has been demonstrated that the AP-1 site is required for promoter responsiveness to cyclic AMP analogues (Bousquet et al., 2001), the pSTAT site for responsiveness to IL-6 and LIF (Auernhammer et al., 1999; Ehlting et al., 2005) and the SP1/SP3 site is also required for the action of IL-6 (Ehlting et al., 2005), as well as PGE_2 (Barclay et al., 2007).

To confirm that these transcription factor binding sites can be activated in our cell system we transfected COS1 cells with specific luciferase reporter constructs and found that transcriptional activity could be elicited from each of the AP-1, STAT and SP1/SP3 reporters following either F/R or PMA treatment (Fig 5a). In contrast, using a CRE reporter to monitor the activation of the cyclic AMP-responsive transcription factor, CREB, showed that this was activated by F/R alone and not PMA (Fig 5b). This demonstrates that it is unlikely that CREB activation is responsible for the activation of the mSOCS3 -107/+929 promoter construct in response to PMA-stimulated ERK activation and that transcription factor interaction with the AP-1, STAT and SP1/SP3 sites may be involved. This idea is supported by the observation

that the activation of each of the AP-1, STAT and SP1/SP3 reporter constructs by PMA was significantly reduced by co-incubation with the inhibitor of ERK activation, 10μM U0126 (Fig 5c).

To further test the idea that ERK activates transcription factors, which then interact with the minimal SOCS-3 promoter, we used Western blotting of HUVEC cell extracts with phospho-specific antibodies to examine whether representative AP-1 complex, STAT or SP1/SP3 interacting proteins, namely c-Jun, STAT3 and SP3, are phosphorylated in their transactivation domains in an ERK-dependent manner (Fig 5d). It has previously been shown that ERK-dependent phosphorylation of c-Jun on Ser-63 (Pulverer et al., 1991), STAT3 on Ser-727 (Kuroki and O'Flaherty, 1999; Wen et al., 1995) and SP3 on Ser-73 (Pages, 2007) is required for full activity of each these transcription factors. We found that the inhibitor of ERK activation, U0126, effectively blocked phosphorylation of each of these transactivating sites as induced by PMA treatment (Fig 5d).

We were surprised to note that although PMA promoted phosphorylation of Ser-727 of STAT3, we did not detect much increase above basal of Tyr 705 (results not shown), which is usually required for full activation of STAT3 (Darnell et al., 1994). This suggests that PMA stimulation leads either to the serine phosphorylation of a pool of STAT3 transcription factors that are already basally phosphorylated on tyrosine or that phosphorylation of Ser-727 is sufficient for the activation of STAT3 in HUVECs. Regardless of the mechanism involved, it is clear that stimulation of HUVECs, with either F/R or PMA, leads to recruitment of STAT3 to the *SOCS-3* promoter, as detected by chromatin immunoprecipitation (results not shown).

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Activation of STAT reporter constructs by F/R and PMA is clearly measurable in COS1 cells (Figs 5a and 5c). Together these results demonstrate that ERK-regulated transcription factors capable of interacting with AP-1, STAT or SP1/SP3 consensus sites are strong candidates for transcriptional regulation of the minimal *SOCS-3* promoter. We were also intrigued to note, however, that phosphorylation of c-Jun in response to PMA was also blocked by a chemical inhibitor of Jun N-terminal kinase (JNK) MAP kinase, SP600125 (10μM), whereas STAT3 and SP3 were insensitive. This suggests for the first time that JNK, in addition to ERK, may play an important role in regulating *SOCS-3* gene activity in HUVECs. Indeed, we found that SP600125 (10μM) is an effective inhibitor of cyclic AMP-induced SOCS-3 protein induction in these cells (Fig 5e).

Interactions between AP-1, STAT and SP1/SP3 Transcription Factor Binding Sites are required for ERK-dependent Regulation of the SOCS-3 Promoter

To further test the requirement for AP-1, STAT and SP1/SP3 transcription factor binding sites for the regulation of the minimal, ERK-responsive SOCS3 promoter (-107/+929), we introduced disruptive point mutations, individually or in combination, into each of these consensus binding motifs to test their requirement for PMA-induced transcriptional activation in COS-1 cells.

In the first set of experiments we examined the impact of disrupting the putative SP1/SP3 binding site, either alone or in combination with the other consensus motifs (Fig 6a). In agreement with others (Barclay et al., 2007; Ehlting et al., 2005), we found that ablation of the SP1/SP3 site alone was sufficient to significantly reduce basal transcriptional activity and severely blunt induction of the minimal *SOCS-3*

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promoter (Fig 6a). Mutation of either of the AP-1, dSTAT or pSTAT sites in combination with the SP1/SP3 site caused a further, significant reduction in basal transcriptional activity of the promoter region and effectively ablated the ability of PMA to induce transcriptional activation (Fig 6a). These results demonstrate that the SP1/SP3 transcription factor binding site is vital for basal and ERK-stimulated regulation of the *SOCS-3* promoter, possibly through supporting the activity and promoter recruitment of STAT and AP-1 complex transcription factors.

In the next set of experiments we sought to determine the individual roles of AP-1 and STAT consensus sites in regulating promoter activity (Fig 6b). Disruption of the pSTAT site alone caused a significant reduction in PMA-stimulated transcription activity, with little noticeable effect on basal activity of the promoter (Fig 7b). Ablation of the dSTAT site, alone or in combination with the pSTAT site, appeared to have little effect on PMA-induced promoter activation (Fig 6b). There was, however, a definite requirement for AP-1 complex transcription factors, since mutation of this site caused a marked decrease in transcriptional activation by PMA (Fig 6b). Surprisingly, co-deletion of the AP-1 site with the dSTAT, but not the pSTAT, site abolished the ability of PMA to induce activation of the *SOCS-3* promoter (Fig 6b). These results demonstrate the importance of the AP-1 and pSTAT sites for the ERK-dependent regulation of the *SOCS-3* promoter and that the activity of the AP-1 site appears to depend on transcription factor interaction with the dSTAT site (Fig 6b).

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DISCUSSION

Our previous work using COS1 cells demonstrated that down-stream signalling from cyclic AMP-activated EPAC1 to the *SOCS-3* gene appears to involve a pathway including Rap1 (Borland et al., 2009; Sands et al., 2006; Yarwood et al., 2008), phospholipase C (PLC) ε and protein kinase C isoforms α and δ (Borland et al., 2009). Our work here expands on th ese findings and further demonstrates that in HUVECs cPKCs and nPKCs, namely, PKCs α, δ, η and ε, play a vital role in governing the induction of *SOCS-3* gene expression through coordinated regulation of the ERK MAP kinase pathway following elevations in intracellular cyclic AMP. We demonstrated the involvement of PKC isoforms and ERK in *SOCS-3* induction by cyclic AMP using relatively high concentrations (10μM) of two indoylmalemide-based PKC inhibitors, Gö 6983 and Ro-31-7549, in addition to the MEK inhibitor, U0126 (Fig 3a). Whereas the specificity of the PKC inhibitors at this concentration may be questionable, since they have been shown to also be able to inhibit other kinases, including S6K1 and GSK3β (Davies et al., 2000), the use of specific siRNAs (Figs 1 and 2) provides more compelling evidence for the involvement of specific PKC isoforms. U0126, on the other hand, has been shown to display a much more impressive selectivity profile (Davies et al., 2000) and we can be confident that it achieves on-target effects here, particularly because we found that it inhibits known ERK-phosphorylation sites in STAT3, SP3 and c-Jun (Figure 5d). Similarly, the JNK inhibitor, 10 μM SP600125, was shown to block the known Ser-63 JNK-phosphorylation site in c-Jun, as well as SOCS-3 induction by cyclic AMP (Fig 5e), despite having well known off-target effects (Bain et al., 2003). Future work will be necessary, however, to unequivocally determine the involvement of the JNK cascade in the regulation of *SOCS-3* induction.

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Deletion analysis of the murine *SOCS-3* gene has previously been used to isolate the minimal functional promoter, which contains an AP-1 site, between nucleotides -105 and -99 from the start of transcription (Barclay et al., 2007; Bousquet et al., 2001), that is reported to bind c-*fos* and JunB (Bousquet et al., 2001), a GC-rich region (-58 to -52), which has the potential to bind either SP1 (Barclay et al., 2007) or SP3 (Ehlting et al., 2005) transcription factors and two binding sites for STAT transcription factors (Auernhammer et al., 1999; Ehlting et al., 2005), one proximal to the transcription start site (pSTAT; -72 to -64) and one distal (dSTAT; -95 to -87). Activation of the *SOCS-3* promoter following cytokine stimulation appears to involve the pSTAT site, which appears to be able to bind STATs 1, 3 or 5, depending on cell context (Emanuelli et al., 2000; He et al., 2003a; Yang et al., 2010). The function of the dSTAT site remains to be determined. *SOCS-3* responsiveness to IL-6 has also been shown to require SP3 transcription factor binding to the GC-rich region, in addition to STAT3 interaction with the pSTAT site (Ehlting et al., 2005; Yang et al., 2010). In contrast, activation of the *SOCS-3* promoter by cyclic AMP appears to be independent of STAT binding, but rather relies on the AP-1 site (Bousquet et al., 2001), or SP-1 interaction with the GC-rich region (Barclay et al., 2007), again depending on the cell type studied.

We found that once activated in HUVECs, ERK induces phosphorylation and activation of AP-1, STAT and SP1/SP3 family transcription factors. Of these, the AP-1 and pSTAT sites appear to be vital for full transcriptional activation by ERK, whereas the SP1/SP3 region appears to be responsible for maintaining basal promoter activity and for supporting the activity of the STAT and AP-1 sites. The molecular basis for this ERK-dependent cooperation between transcription factor binding sites

remains to be determined, but may involve the recruitment of enzymes, such as acetyltransferases, that are required for full transcription factor activity at the other sites. In this respect, c-Jun, STAT3 and SP3 have all been shown to require acetylation at specific lysine residues to achieve their full activity (Ammanamanchi et al., 2003; Vries et al., 2001; Yuan et al., 2005). It has also been demonstrated that the co-activator acetyltransferase, CREB binding protein (CBP) p300, is recruited to the *SOCS-3* promoter following ERK activation (Baker et al., 2008; Qin et al., 2007). Clearly further work needs to be done in this area to determine whether CBP p300, or another acetyltransferase, is required for the regulation of transcription factors targeting the *SOCS-3* promoter. Moreover, the role of transcription factor methylation should also be investigated in light of a recent study demonstrating that STAT3 is reversibly dimethylated following recruitment to the *SOCS-3* promoter, leading to termination of it's activity (Yang et al., 2010). In this case it is thought that phosphorylation of Ser-727 on STAT3, which we show here to be ERK-dependent in HUVECs, is thought to provide a binding site for the H3K4 methyltransferase SET9 (Yang et al., 2010), however this hypothesis requires to be formally tested.

An additional consideration is what is the role of C/EBPβ and C/EBPδ in this process? We have shown that both transcription factors are required for effective *SOCS-3* induction in HUVECs and that C/EBPβ is also phosphorylated and activated by ERK (Borland et al., 2009; Woolson et al., 2009a; Woolson et al., 2009c; Yarwood et al., 2008). ChIP analysis demonstrated that C/EBPβ interacts with the human *SOCS-3* promoter at approximately -2000 (~3000bp from ATG) from the putative transcription start site (Yarwood et al., 2008), which is quite distant from the minimal promoter region identified in this study. We predict, therefore, that there is an

additional C/EBP binding site located within the -107/+929 promoter region, perhaps located within the ERK-responsive AP-1 site we identified. This is a credible proposition since it has been demonstrated that C/EBPβ and c-Jun physically interact to regulate induction of the TNFα gene in myelomonocytic cells (Zagariya et al., 1998); whether the same is true for the *SOCS-3* gene remains to be determined. Overall this work point towards a central role for ERK-regulated transcription factors in regulating the induction of the *SOCS-3* gene in response to elevations in intracellular cyclic AMP. These findings may have consequences for a broad range of signaling scenarios where understanding of the molecular basis controlling *SOCS-3* gene induction may have therapeutic benefit; these range from stimulating ERK-dependent *SOCS-3* induction in vascular endothelial cells to combat chronic inflammation (Parnell et al., 2011), to suppression of *SOCS-3* in macrophages to combat the progression of cancer (Hiwatashi et al., 2011).

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Yarwood, S.J., Wiejak, J. and Borland,G.

Conducted experiments: Wiejak, J., Boland, G., Gao, S. and Dunlop, J.

Performed data analysis: Yarwood, S.J.

Wrote manuscript: Yarwood, S.J.

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FOOTNOTES

JW and JD contributed equally to this work

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LEGENDS FOR FIGURES

Figure 1 Involvement of the PKC isoforms η and ε in cyclic AMP-dependent SOCS-3 Induction in HUVECs

a) HUVECs were treated with a combination of PKCη- and PKCε-specific siRNAs (Sequence 1) and then stimulated with either F/R or PMA for 5 hours. Total RNA was then extracted from cells and subjected to one-step RT-PCR, with specific primers towards SOCS-3 or actin, as described in Materials and Methods. Amplified DNA fragments were visualised by agarose gel electrophoresis.

b) HUVECs were treated with control (cntrl) siRNA or a combination of PKCη- and PKCε-specific siRNAs. Cells were then stimulated with F/R or PMA for 5 hours in the presence of MG132 (10μM). Cell lysates were then prepared and immunoblotted with antibodies to SOCS-3, PKCη, PKCε, PKCα and GAPDH, as indicated.

c) Densitometric units were obtained from SOCS-3 immunoblots from three separate experiments and presented as a histogram in the *lower panel*. Significant differences in SOCS-3 expression in PKC siRNA-treated cells compared with cntrl siRNA cells are indicated #, $p<0.05$, as are significant increases in SOCS-3 expression relative to DMSO-treated cells, *, $p<0.05$.

Figure 2 Involvement of PKC isoforms in cyclic AMP-dependent ERK activation in HUVECs

a) HUVECs were treated with combinations of PKCα- and PKCδ-specific siRNAs (Sequences 1 and 2), or a combination of PKCη- and PKCε-specific siRNAs, and then stimulated with F/R or PMA as described above. Cell extracts were immunoblotted with anti-phospho ERK (Thr202/Tyr204) or total ERK antibodies and

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immunoblots from three separate experiments were quantified and densitometric units (n=3) presented in the histogram in the *lower panel*. Significant differences in ERK phosphorylation in siRNA-treated cells compared with control siRNA cells with equivalent treatment are indicated *, $p<0.05$, **, $p<0.01$ or ***, $p<0.001$.

b) HUVECs were treated with control (cntrl) siRNA, PKCα-specific siRNA (Sequence 1 and 2), PKCδ-specific siRNA (Sequence 1 and 2) or a combination of PKCα- and PKCδ-specific siRNAs. Cells were then stimulated with F/R or PMA for 5 hours in the presence of MG132 (10μM). Cell lysates were then prepared and immunoblotted with antibodies to SOCS-3, PKCα, PKCδ, PKCε and GAPDH, as indicated. In the *lower panel* SOCS-3 immunoblots from three separate experiments were quantified and densitometric units (n=3) are presented as a histogram in the *lower panel*. Significant differences in SOCS-3 expression in siRNA-treated cells compared with control siRNA cells with equivalent treatment are indicated #, $p<0.05$. Significant increases in SOCS-3 expression relative to DMSO-treated cells are also indicated *, $p<0.05$.

Figure 3 Involvement of ERK in SOCS-3 Induction in HUVECs

a) HUVECs were stimulated for 5 hours with MG132 (10μM) plus either a combination of 10μM forskolin plus 10μM rolipram (F/R) or 10μM PMA or in the presence or absence of the PKC inhibitors 10μM Ro-31-7549 (RO) or 10μM Gö 6983 or the MEK inhibitor, 10μM U0126. Cell extracts were then prepared and immunoblotted with the indicated antibodies.

b) HUVECs were stimulated for 5 hours with MG132 and either 10μM PGE_2, 10μM αMSH or 10μM PMA, in the presence or absence of the ERK inhibitor, U0126

(10μM), and then immunoblotted with anti-SOCS-3 and GAPDH antibodies as indicated.

Figure 4 Identification of the Minimal ERK-responsive *SOCS-3* Promoter Region

a) COS1 cells were transfected with firefly luciferase reporter constructs containing truncates of the murine SOCS3 promoter. In addition, cells were co-transfected with *Renilla* luciferase vector to normalise luciferase activity and to correct for transfection efficiency. Cells were then stimulated for 16 hours with 10μM PMA, after which cells were harvested and luciferase activities determined. The relative positions of putative transcription factor binding sites identified in Figure 3 are shown in the schematic on the *left hand side* of the histogram. Results are expressed means±SEM of absolute relative light units (RLUs) from three separate experiments and significant differences relative to cells stimulated with diluent alone are indicated ***, $p<0.001$. The significant difference in luciferase activity between reporter construct mSOCS-3 -107 and mSOCS-3 -79 is also indicated ###, $p<0.0001$.

b) COS1 cells were transfected with the luciferase reporter vector, mSOCS-3 -107, which represents the minimal PMA-responsive region of the murine *SOCS-3* promoter. Cells were then stimulated with either diluent (DMSO) or 10μM PMA, in the presence or absence of the ERK inhibitor U0126 (10μM). Significant differences in luciferase activity between diluent- and PMA-treated cells and between diluent- and U0126-treated cells are shown, ***, $p<0.001$ and ###, $p<0.001$ (n=3), respectively.

c) Sequence alignment of the comparative coding regions of the murine (mSOCS3; Entrez Gene ID 12702; Genbank Accession AF117732) and human

(hSOCS; Entrez Gene ID 9021) SOCS-3 genes was carried out using CLC Workbench Software and an abbreviated comparison of the first 1.2 kbp of the 5'-regulatory regions of each sequence is shown here. The transcription initiation site within the murine *SOCS-3* promoter is indicated as +1 (Auernhammer et al., 1999). The corresponding initiation site within the human *SOCS-3* promoter has yet to be formally identified therefore numbering of this sequence was begun at the translation initiation codon. The proximal (pSTAT) and distal (dSTAT) STAT-responsive elements, previously identified (Auernhammer et al., 1999) within the murine promoter, are indicated as is the GC-rich, specificity protein 1/3 (SP1/SP3) binding site (Barclay et al., 2007; Ehlting et al., 2005). The putative AP-1 site identified by Barclay et al (Barclay et al., 2007) and the TATA box are also shown.

Figure 5 Cyclic AMP or PMA Treatment Leads to ERK-dependent Activation of AP-1, STAT and SP1/SP3 Transcription Factors

a) COS1 cells were co-transfected with *Renilla* luciferase vector and the indicated firefly luciferase reporter constructs. Cells were then stimulated for 16 hours with either a combination of 50μM forskolin and 10μM rolipram (F/R) or 10μM PMA. Cells were then harvested and luciferase activities determined. Significant differences relative to diluent-treated cells are indicated,**, $p<0.01$ or ***, $p<0.001$.

b) COS1 cells that had been transfected with a CRE, firefly luciferase reporter construct were stimulated for 16 hours with either F/R or PMA. Luciferease activities were then determined and plotted as a histogram. Significant differences in luciferase activities relative to diluent-stimulated cells are indicated, ***, $p<0.001$. Non-significant changes in F/R treated cells are also indicated (n.s.).

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c) COS1 cells were co-transfected with *Renilla* luciferase vector and either an AP-1, STAT or SP1/SP3 reporter construct. Cells were then stimulated for 16 hours with 10μM PMA, in the presence or absence of 10μM U0126. Luciferase activities (Relative Light Units) were then determined and plotted in a histogram of means±SEM for three separate experiments. Significant increases in activity in PMA-treated cells relative to diluent-stimulated are indicated, ***, $p<0.001$. Significant decreases in luciferase activity in U0126-treated cells are also indicated, ###, $p<0.001$.

d) HUVECs were stimulated for 30 minutes with F/R or PMA in the presence or absence of the JNK inhibitor, SP600125 (10μM), or the ERK inhibitor, U0126 (10μM). Cells extracts were then prepared and immunoblotted with the indicated phospho-specific or "total" protein antibodies to c-Jun, STAT3 and SP3 transcription factors. Results are representative of an individual experiment carried out on three separate occasions.

e) HUVECs were stimulated for the indicated times with MG132 (10μM) plus F/R in the presence or absence of the JNK inhibitor, SP600125 (10μM). Cells extracts were then prepared and immunoblotted for SOCS-3 and β-tubulin as indicated. Results are representative of an individual experiment carried out on three separate occasions.

Figure 6 Role of AP-1, STAT and SP1/SP3 Transcription Factor Binding Sites in the Response of the *SOCS-3* Promoter to PMA-promoted ERK Activation

a) COS1 cells were co-transfected with *Renilla* luciferase vector, to normalise luciferase activity, together with firefly luciferase reporter constructs containing the minimal ERK-responsive element of the murine SOCS3 promoter, mSOCS-3 -107, in

which various combinations of the putative AP-1, STAT and SP1/SP3-binding sites had been mutated, as described in the *Experimental Procedures* section. The relative positions of mutated transcription factor binding sites are shown in the schematic on the *left hand side* of the histogram. Cells were then stimulated with 10μM PMA for 16 hours and luciferase activities determined. Results are expressed as relative light units (RLUs) and respresent means±SEM for three separate experiments. Significant differences relative to cells stimulated with diluent alone are indicated, ***, $p<0.001$, as are differences between mutated promoters and wild-type promoter, mSOCS-3-107, ###, $p<0.001$.

b) COS1 cells were co-transfected with *Renilla* luciferase vector together with wild-type *SOCS-3* promoter construct, mSOCS-3 -107 or mSOCS-3 -107 that had been mutated to ablate various combinations of transcription factor binding sites as indicated on the *left hand* side of the histogram. Following 16 hours treatment with 10μM PMA cells were harvested and luciferase activities determined. Significant differences relative to diluent-treated cells (***, $p<0.001$) or wild-type mSOCS-3-107 (###, $p<0.001$) are indicated.

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Figure 1

a)

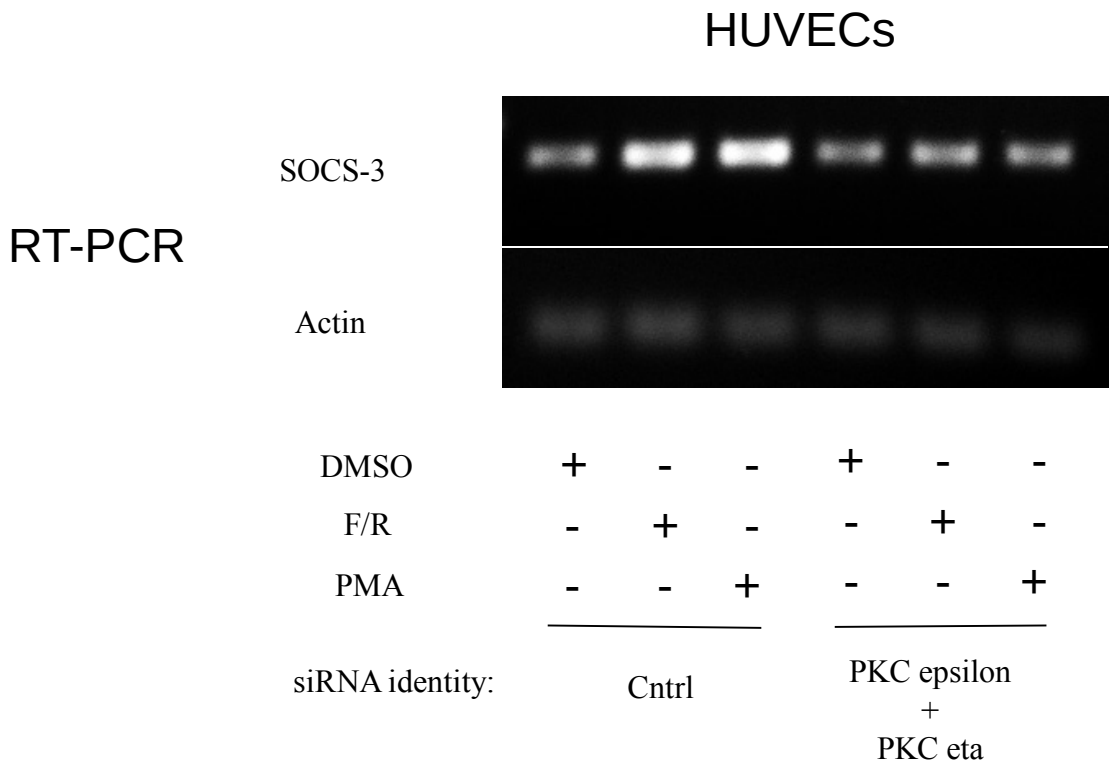

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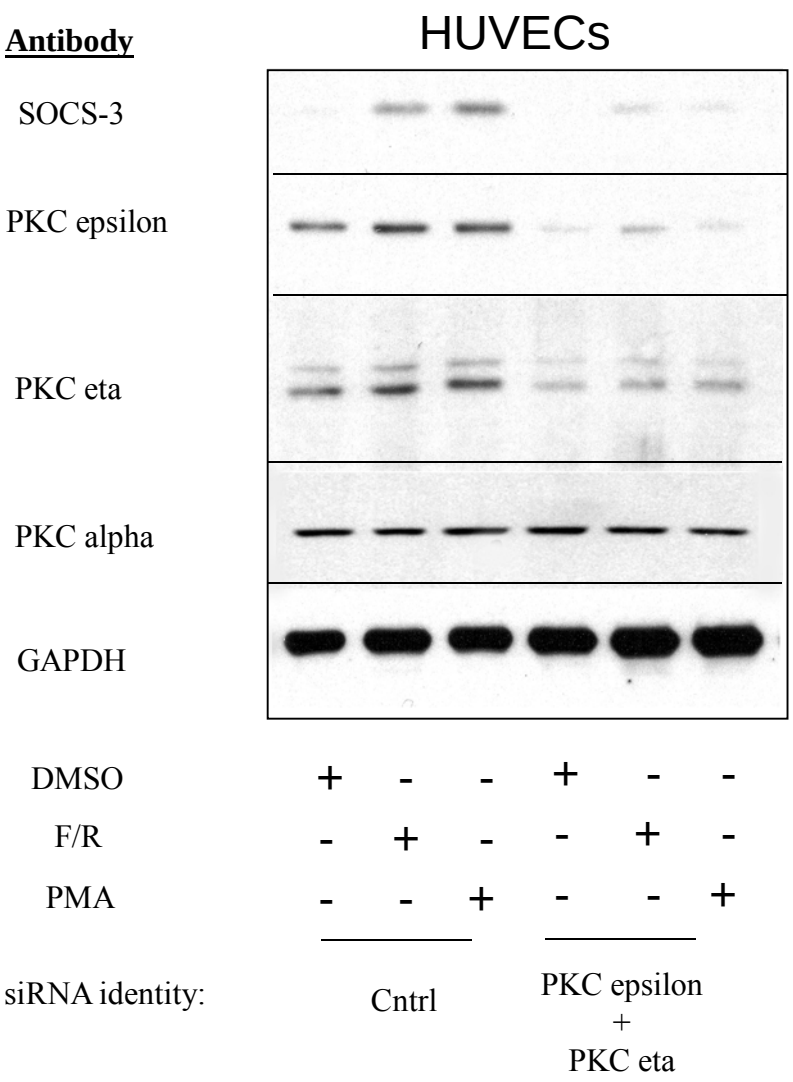
Figure 1

b)



c)

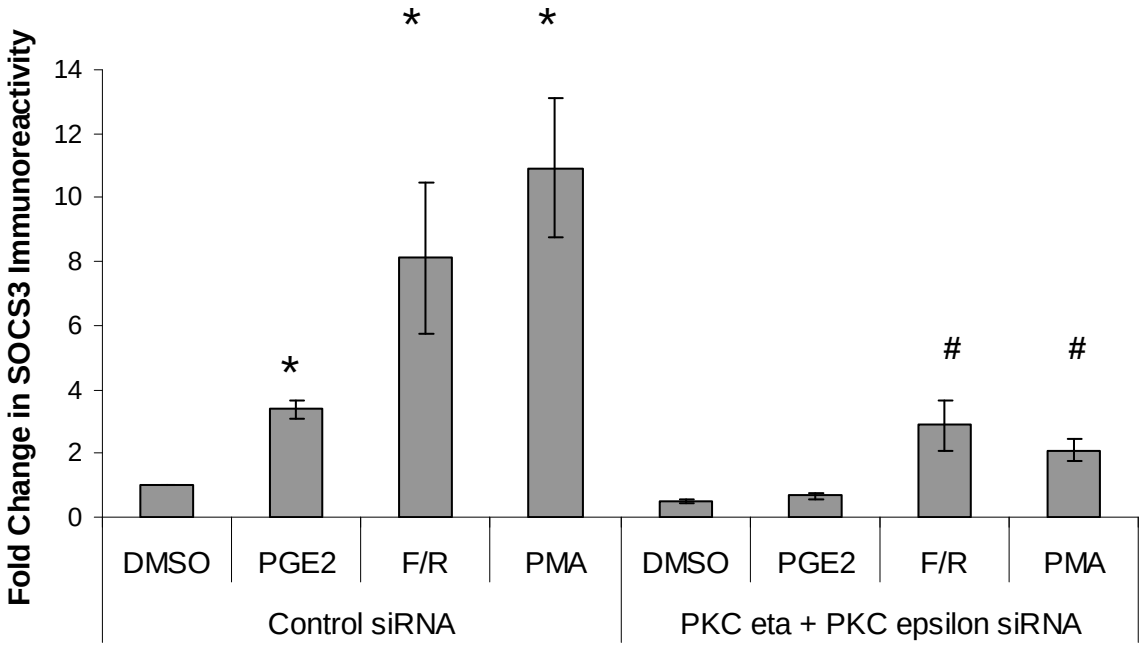


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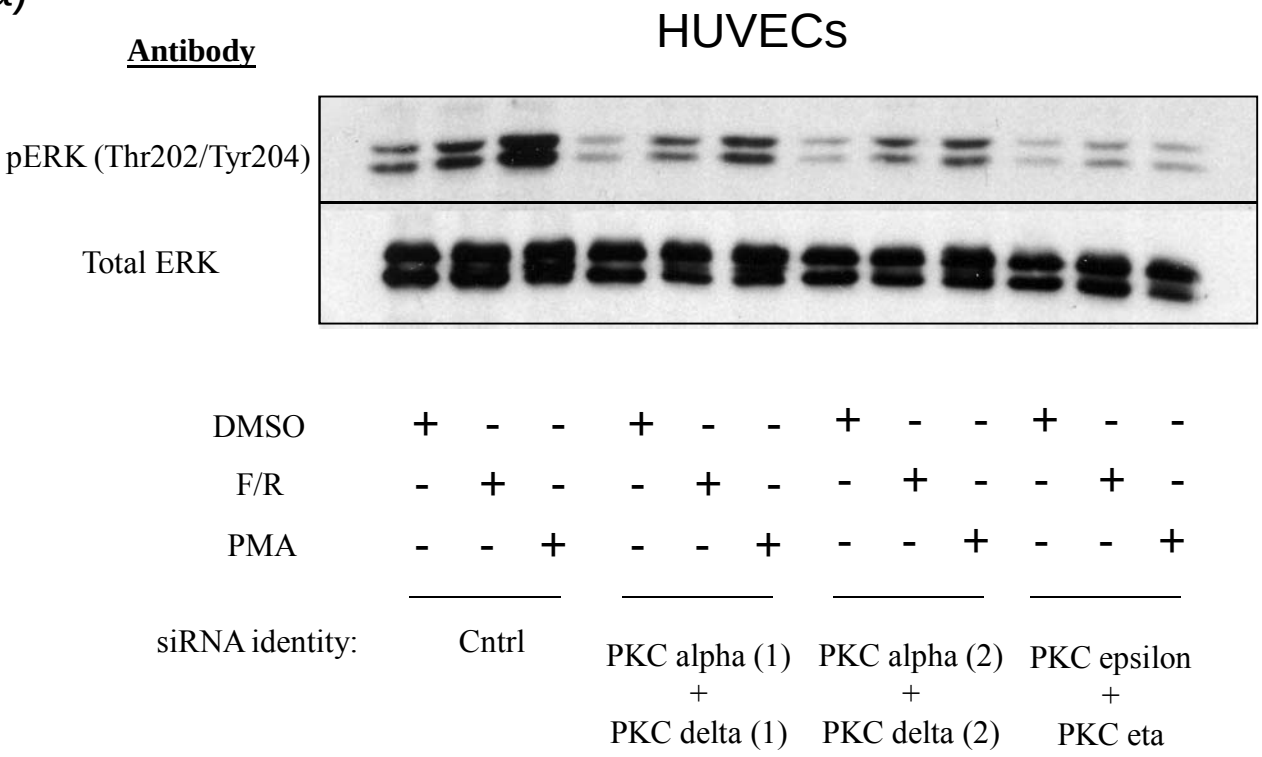
Figure 2

a)



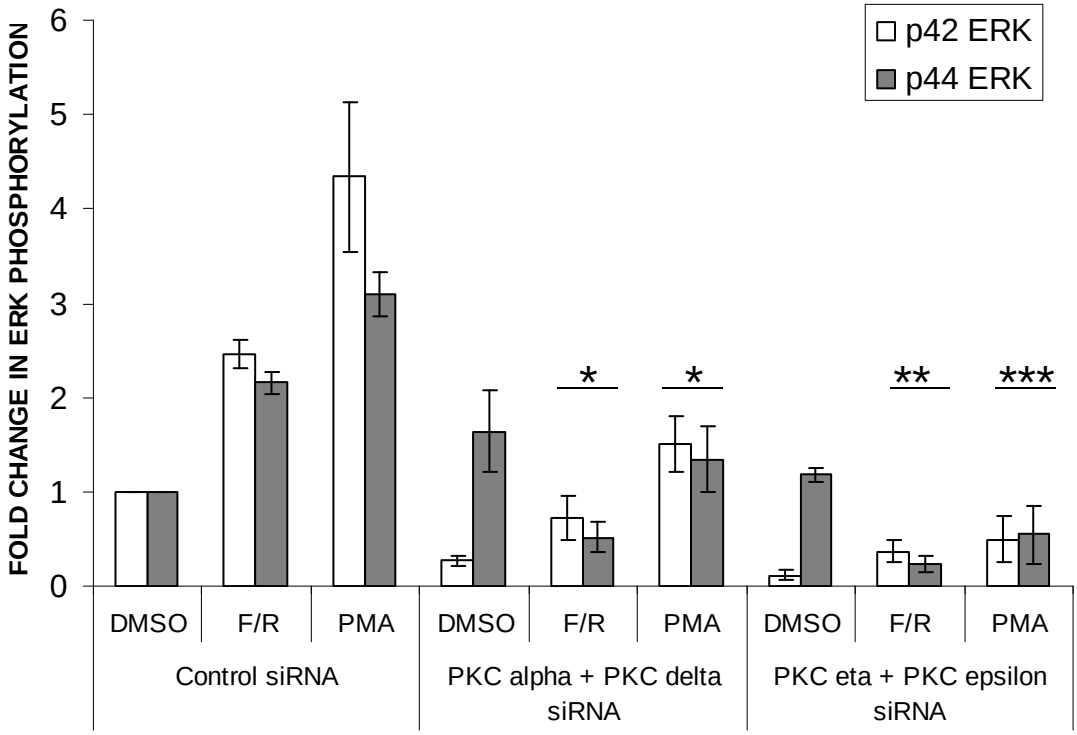


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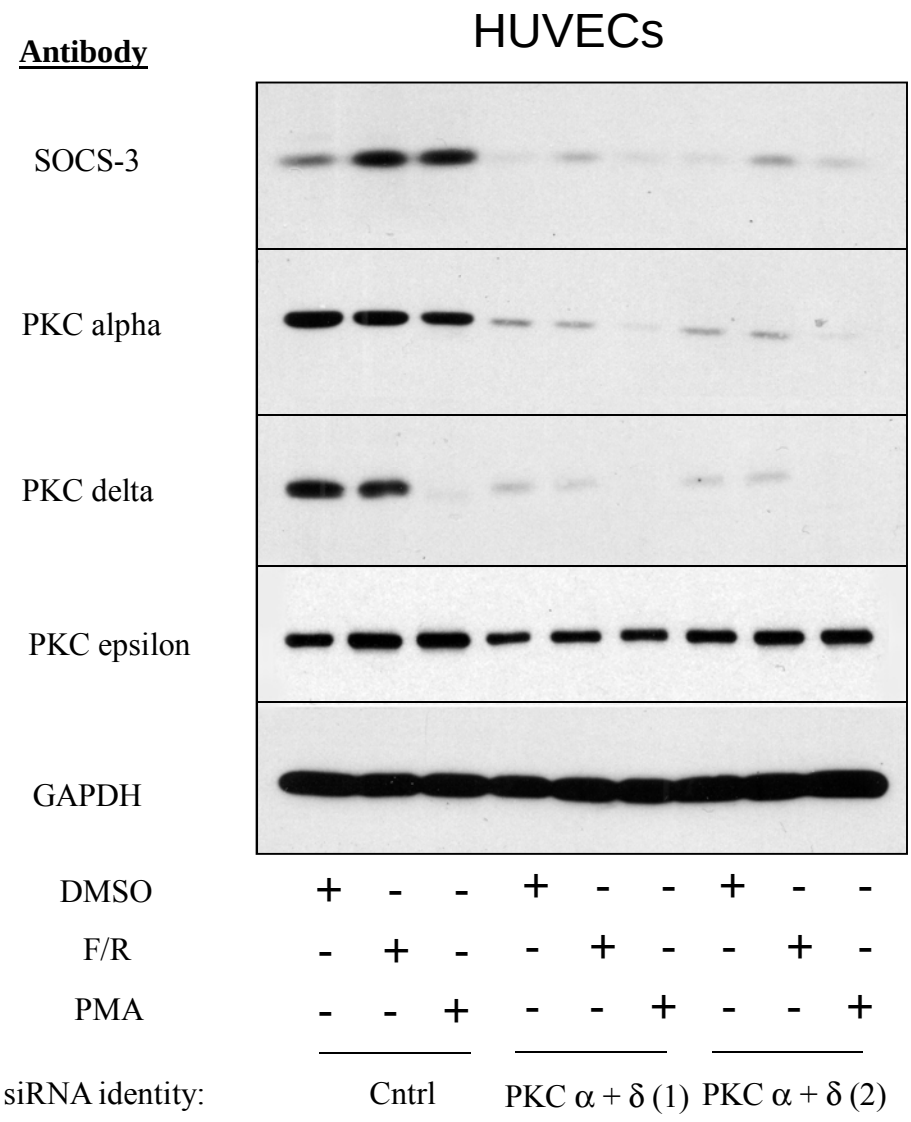
Figure 2

b)



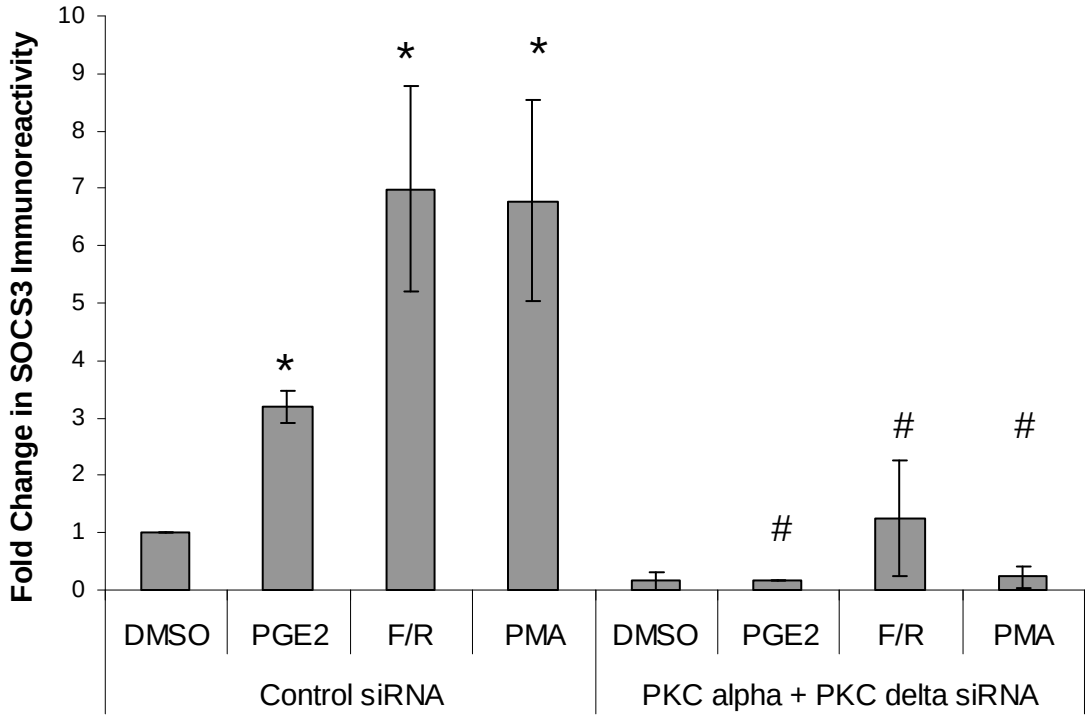


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Figure 3

a)

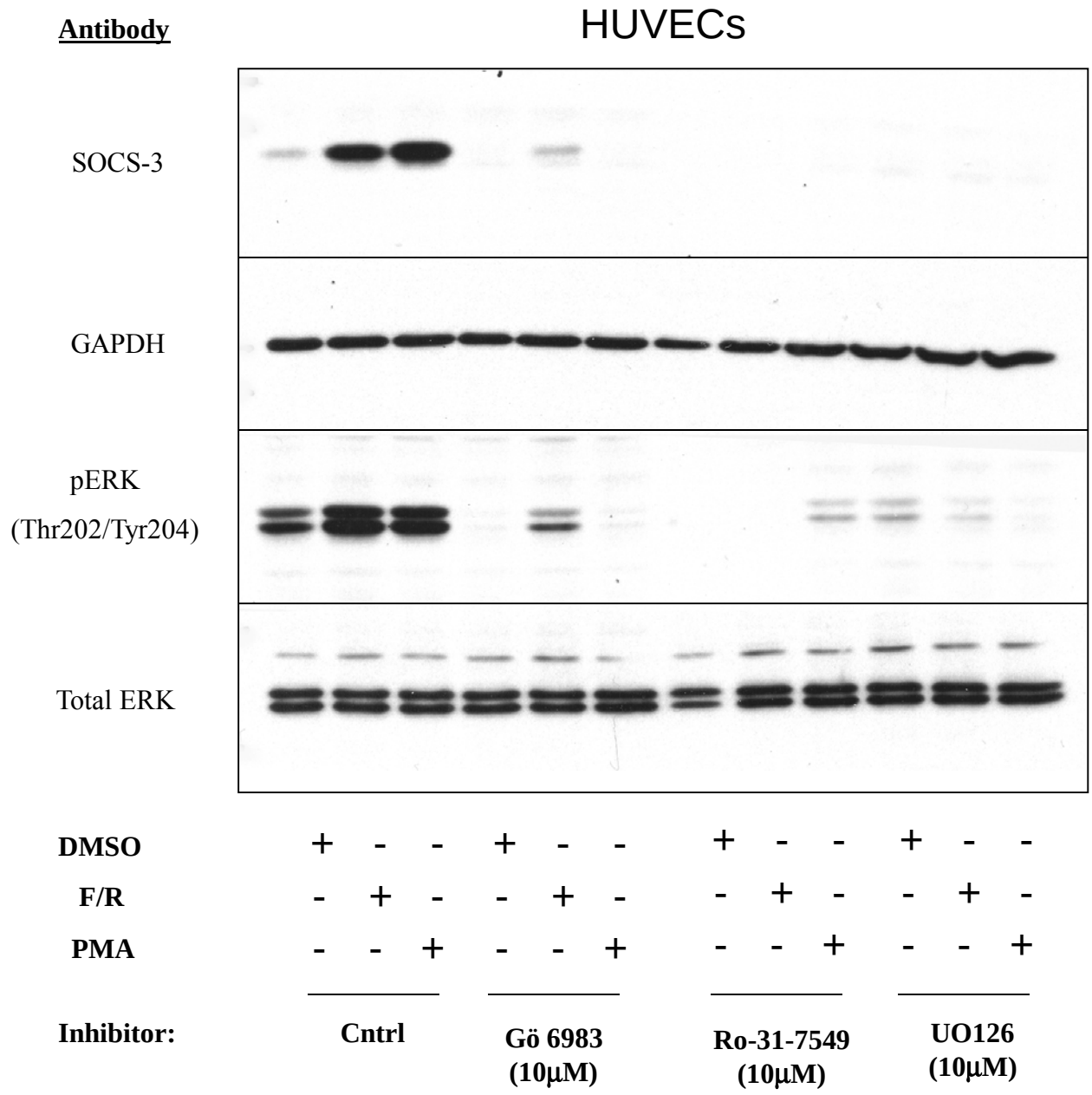

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Figure 3

b)

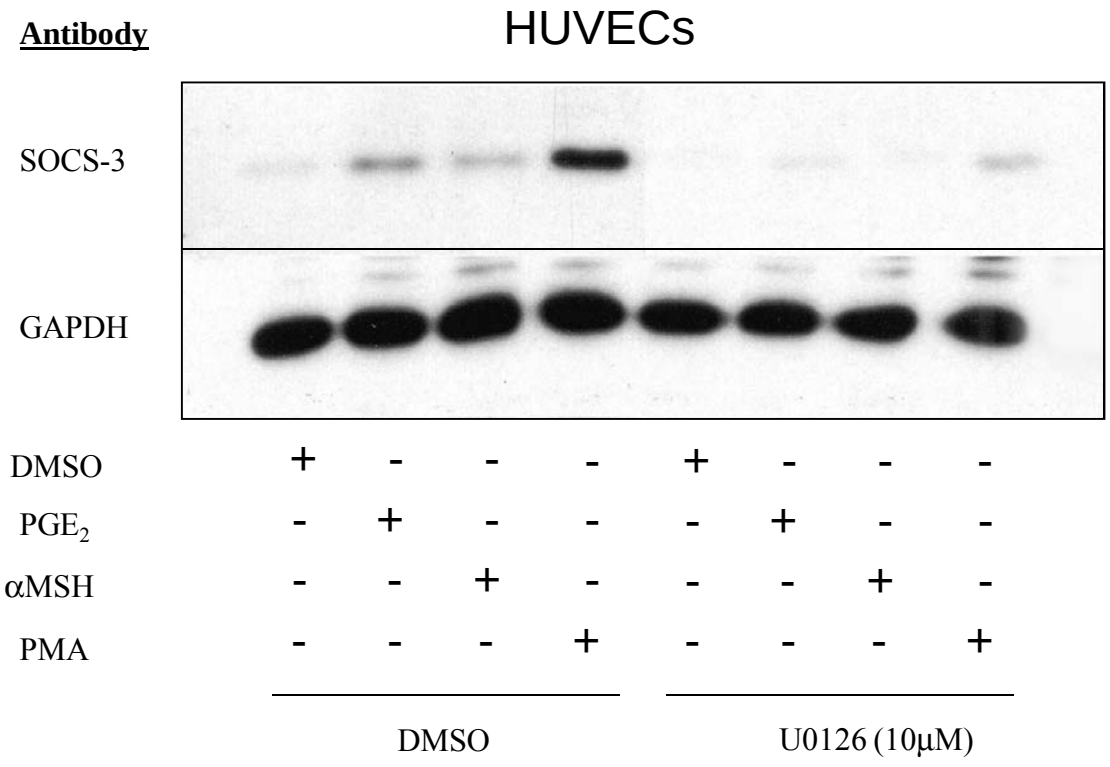

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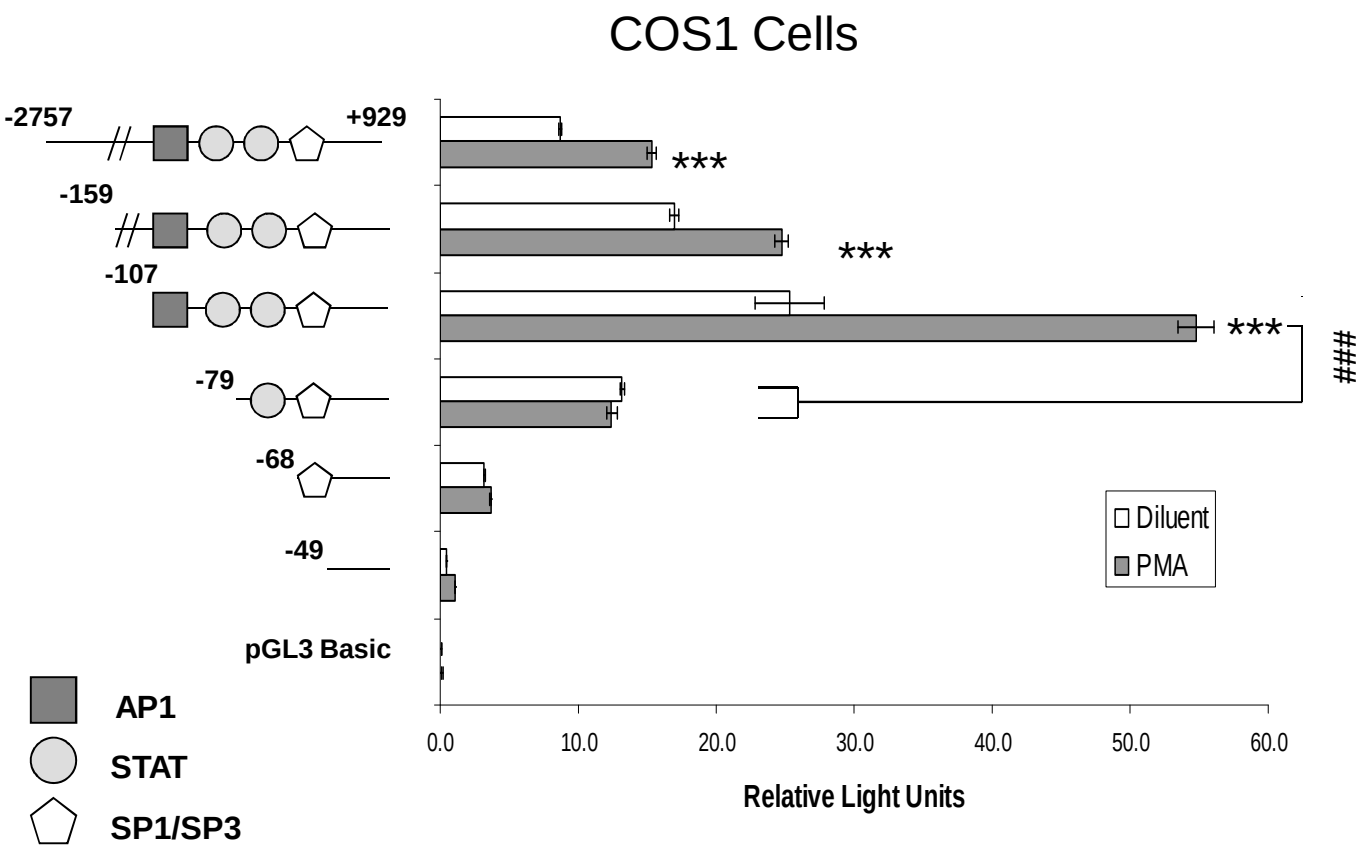
Figure 4

a)



b)

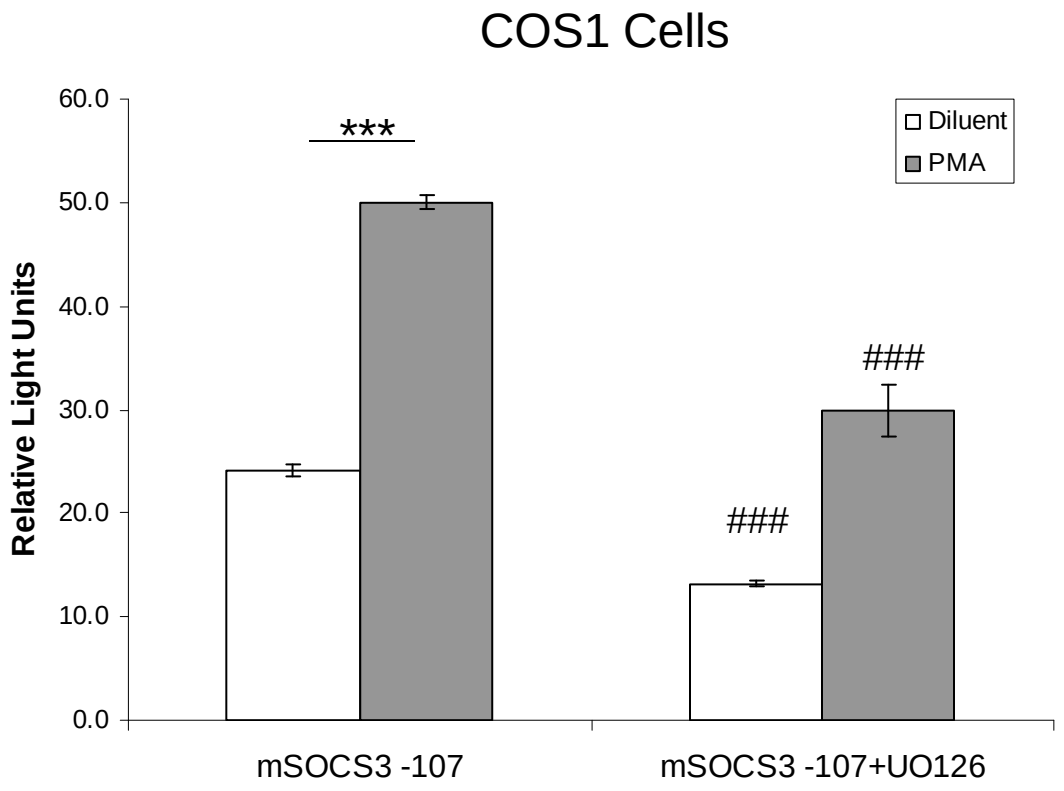


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Figure 4

c)

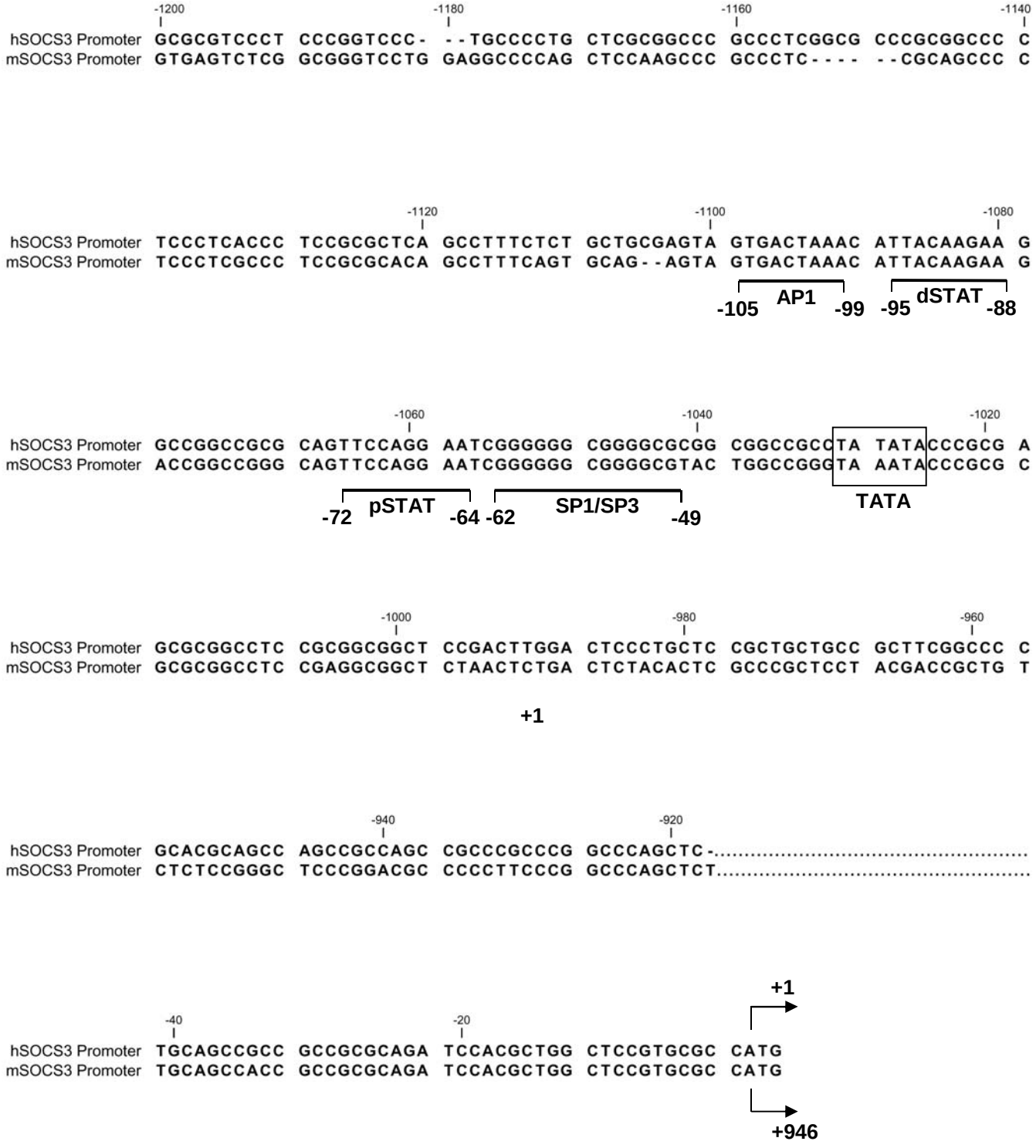

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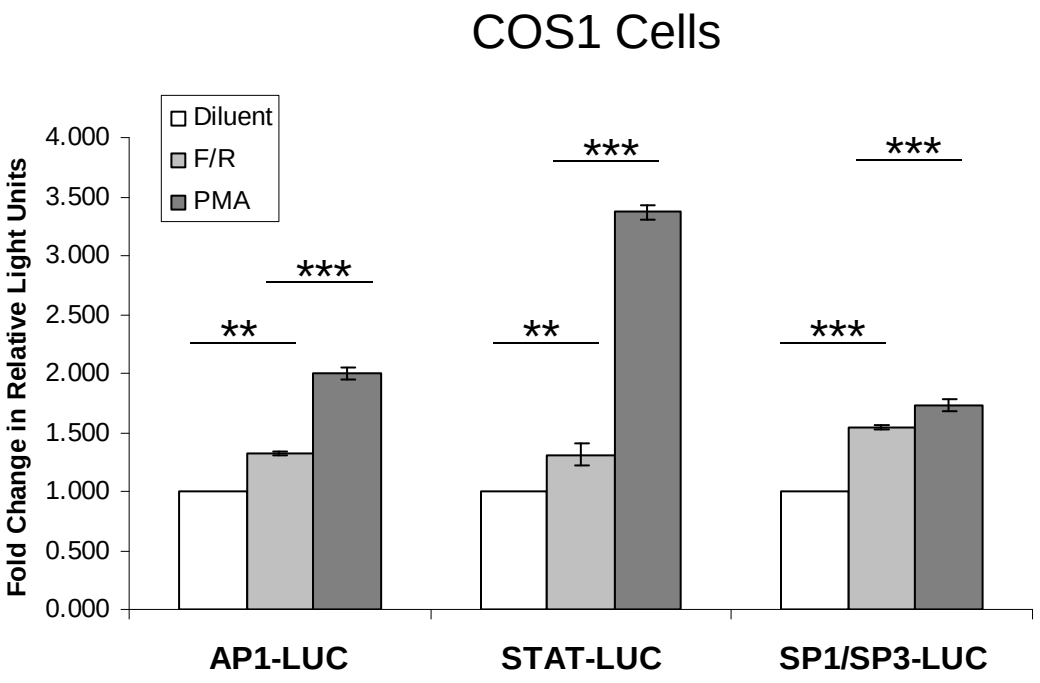
Figure 5

a)



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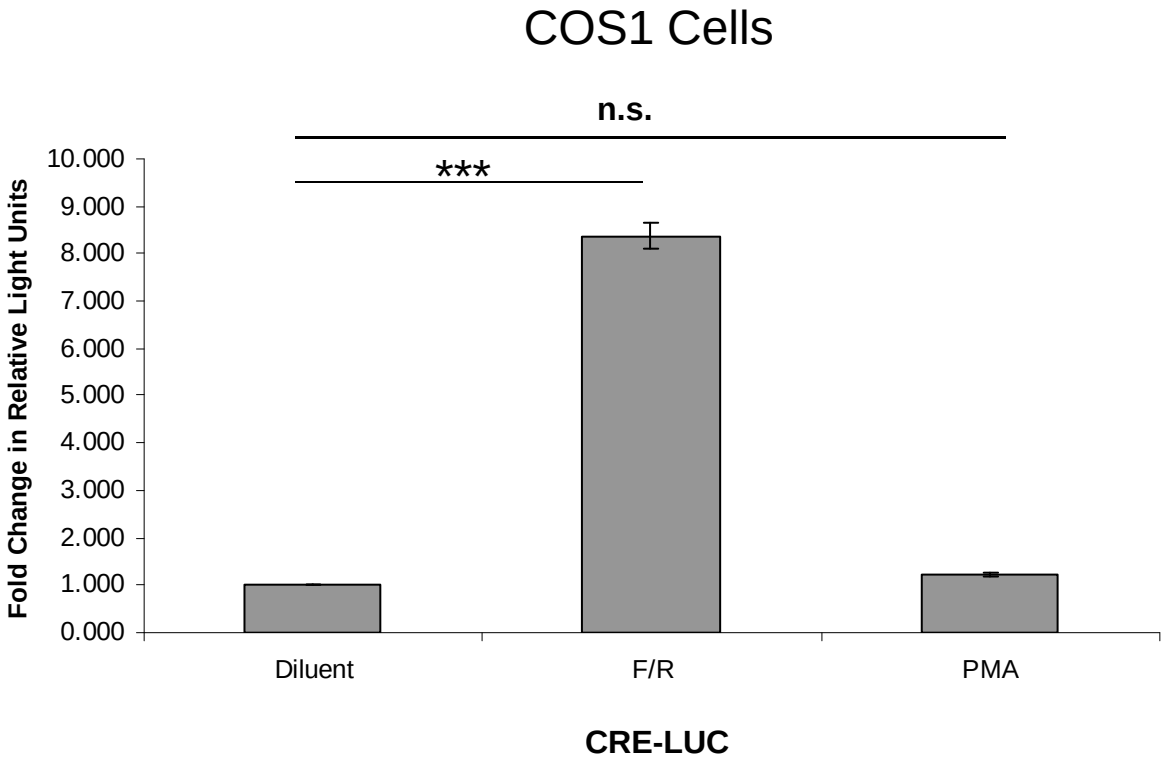

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Figure 5

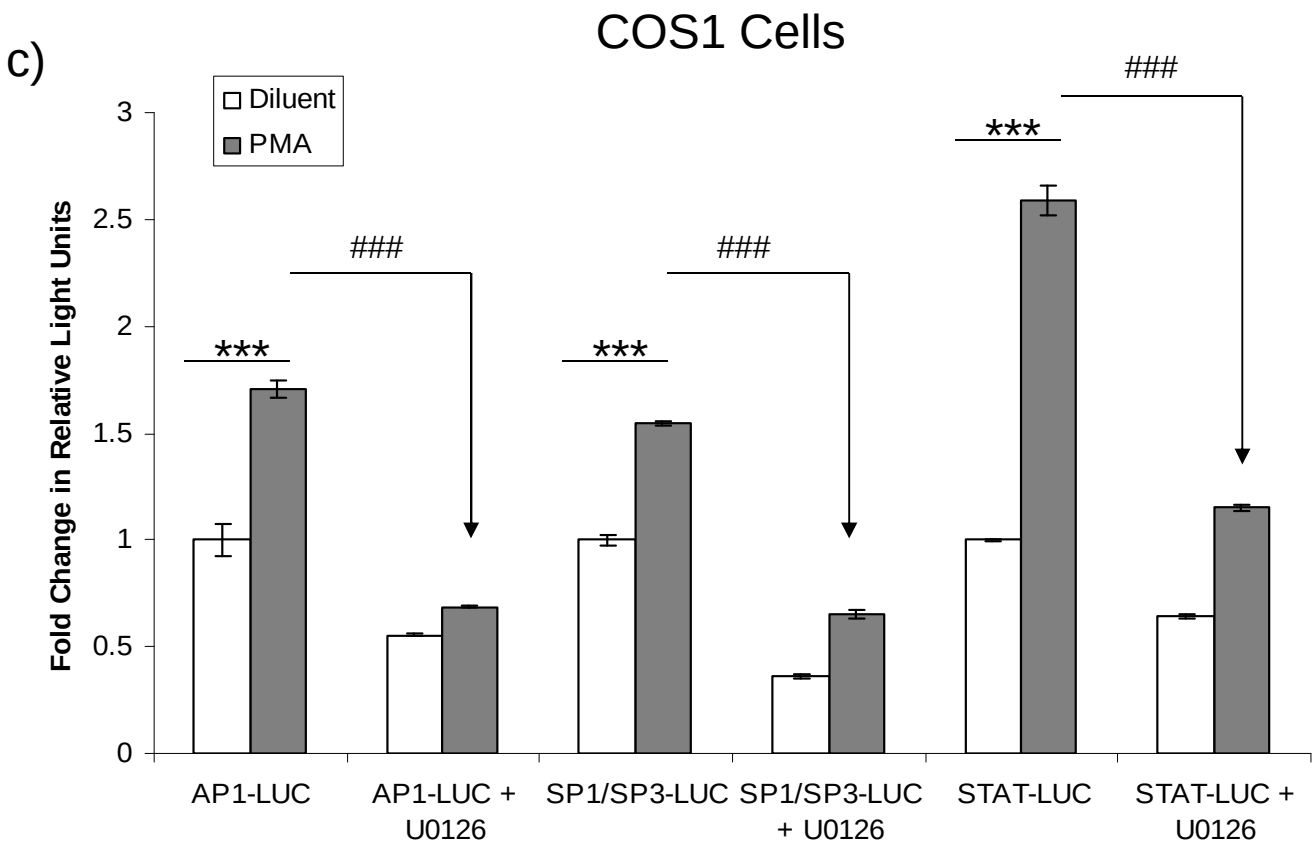

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Figure 5

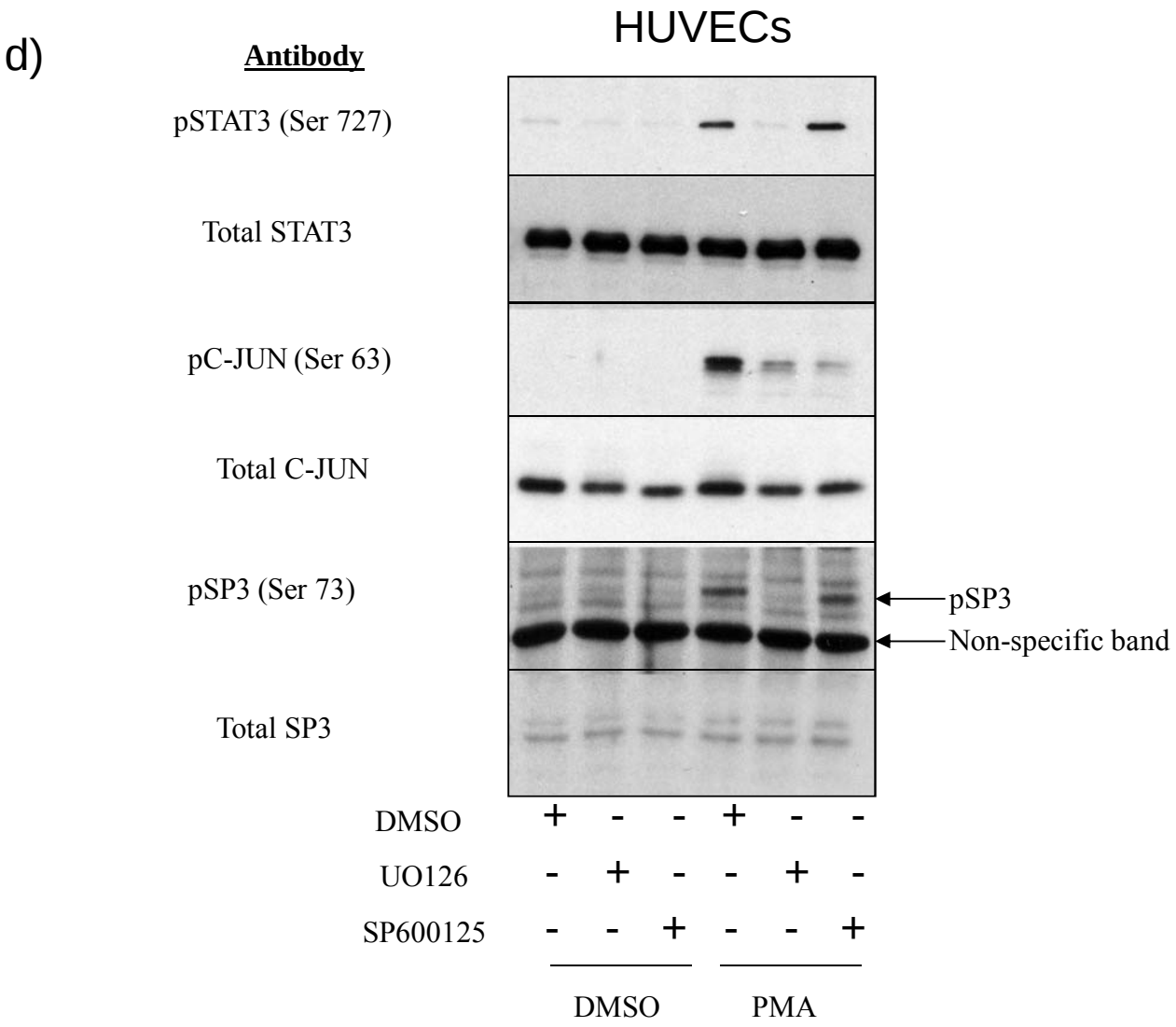

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Figure 5

e)

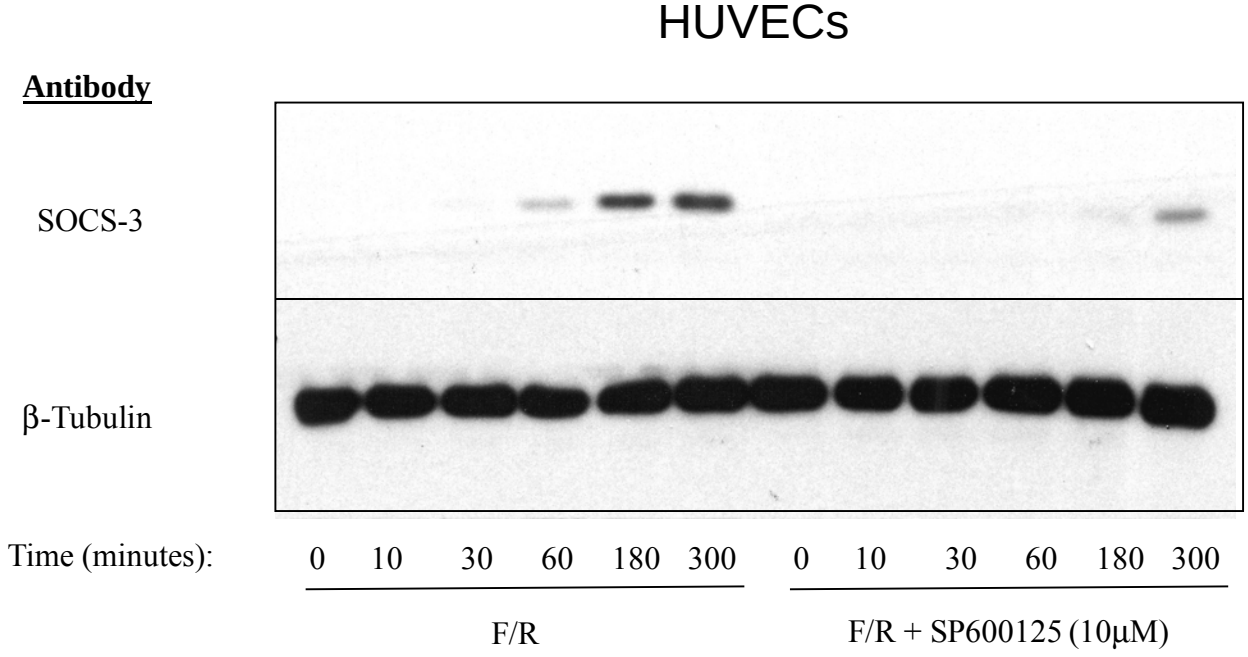

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Figure 6

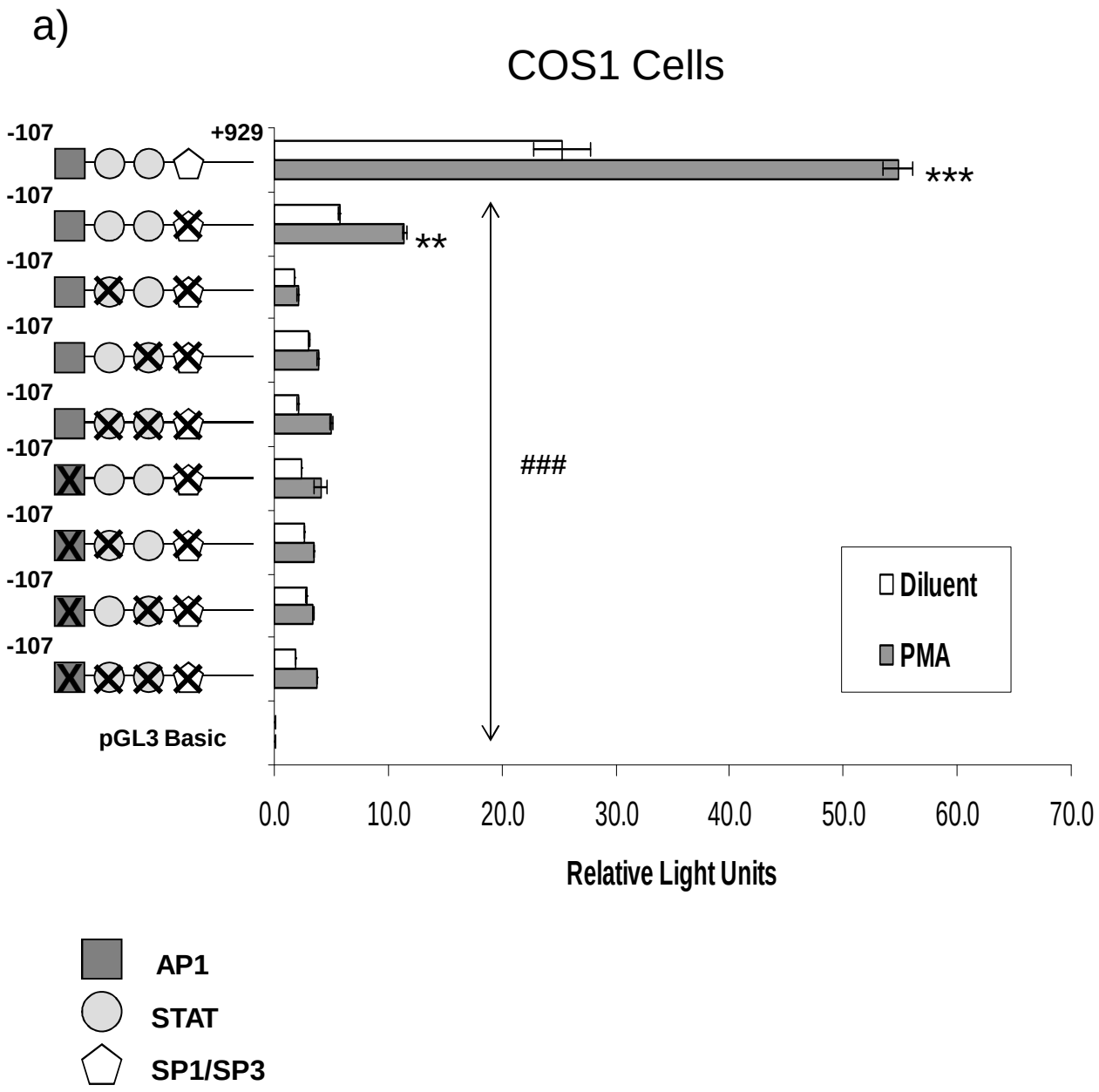

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Figure 6