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# Store-operated calcium entry is dispensable for the activation of ERK1/2 pathway in prostate cancer cells☆

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ABSTRACT

STIM1, the endoplasmic reticulum $Ca^{2+}$ sensor that modulates the activity of plasma membrane $Ca^{2+}$ channels, becomes phosphorylated at ERK1/2 target sites during $Ca^{2+}$ store depletion triggered by thapsigargin or epidermal growth factor (EGF). This ERK1/2-dependent phosphorylation regulates STIM1 localization and dissociation from microtubules, and it is known that enhances the binding to ORAI1, a store-operated $Ca^{2+}$ entry (SOCE) channel, leading to the activation of this $Ca^{2+}$ influx pathway. However, there remained some evidence of a role for SOCE in the activation of ERK1/2, and here we assessed the contribution of SOCE to ERK1/2 activation by generating a STIM1-deficient cell line by CRISPR/Cas9 genome editing of the *STIM1* locus in prostate cancer PC3 cells. The genomic modification consisted of a 16 base-pair insertion in exon 5 of both alleles, therefore abrogating STIM1 synthesis. STIM1-KO cells did show a striking decrease in $Ca^{2+}$ influx in response to thapsigargin or EGF, a result that demonstrates that SOCE mediates $Ca^{2+}$ entry in PC3 cells during stimulation with EGF. Moreover, identical levels of total ERK1/2 were found in STIM1-KO cells and the parental cell line, and ERK1/2 activation was fully activated in KO cells, both in the presence and in the absence of extracellular $Ca^{2+}$, a result that supports that STIM1 and SOCE are not required for ERK1/2 activation. This activation was sensitive to Src kinase inhibition, but not to CAMKII nor PKC inhibition, a result that sets STIM1 and SOCE as downstream targets of the axis Src-Raf-MEK-ERK, rather than upstream regulators.

## 1. Introduction

STIM1 is a transmembrane protein resident at the endoplasmic reticulum (ER) that activates $Ca^{2+}$ entry through plasma membrane $Ca^{2+}$ channels under stimuli that trigger a transient decrease of the intraluminal [$Ca^{2+}$] [1,2]. STIM1-activated $Ca^{2+}$ entry pathway is therefore termed store-operated $Ca^{2+}$ entry (SOCE). The $Ca^{2+}$-sensitive EF-hand domain that STIM1 shows towards the intraluminal ER space facilitates the activation of this $Ca^{2+}$ entry pathway. The decrease of [$Ca^{2+}$] within this intracellular $Ca^{2+}$ store, triggered by a wide variety of stimuli that activate the phosphoinositide pathway, elicits a conformational change in STIM1 that enables the binding of this protein to different plasma membrane $Ca^{2+}$ channels, being ORAI1 the most widely studied and the $Ca^{2+}$ channel responsible for the $Ca^{2+}$-release-activated $Ca^{2+}$ current ($I_{CRAC}$) [3]. It is known that the $Ca^{2+}$ entry pathway regulated by STIM1-ORAI1 modulates critical cellular events, such as gene expression, cell cycle, proliferation, cell motility, and migration (reviewed in [4]).

Regarding cell motility, our group has reported a key role for STIM1-ORAI1 in the control of $Ca^{2+}$ entry at the leading edge of migrating cells, where this $Ca^{2+}$ mobilization enhances the reorganization of the cortical cytoskeleton required for the formation of filopodia and lamellipodia [5]. It is noteworthy that there is an enrichment of phosphorylated STIM1 at the leading edge of migrating cells, whereas the non-phosphorylated protein remains evenly diffused throughout the ER [5]. This polarized phospho-STIM1 was phosphorylated at the three ERK1/2 target sites, Ser575, Ser608, and Ser621, and it is known that this phosphorylation regulates STIM1 activation and localization. The phosphorylation of STIM1 by ERK1/2 enhances the dissociation of STIM1 from the end-binding protein 1 (EB1) [6], a protein that binds to

*Abbreviations:* EGF, epidermal growth factor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SOCE, store-operated calcium entry; Tg, thapsigargin

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the growing tip of microtubules [7]. At resting state STIM1 is transported through the surface of the ER network by this microtubule-dependent mechanism, but the dissociation from EB1 and microtubules is required for the clustering and relocalization of STIM1 in ER-plasma membrane junctions, where STIM1 activates ORAI1 [6,8,9]. Thus, ERK1/2-dependent phosphorylation of STIM1 is critical for SOCE, as it is required for the full activation of this $Ca^{2+}$ entry pathway [10], which is therefore sensitive to ERK1/2 inhibition [10,11]. STIM1 phosphorylation by ERK1/2, and the dissociation from EB1 have been reported for cells upon stimulation with epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1), two stimuli that enhance extracellular $Ca^{2+}$ entry [12,13].

However, there remained some evidence of a role for SOCE on ERK1/2 activation. Recent reports suggest that $Ca^{2+}$ entry through store-operated $Ca^{2+}$ channels is required for activating ERK1/2 in parotid acinar cells [14] and melanoma cell lines [15], and therefore a model where SOCE is an upstream regulator of ERK1/2 pathway has been proposed. To address these conflicting data between the two models of cross-regulation between SOCE and ERK1/2, we here evaluate the impact of STIM1 knock-out on ERK1/2 signaling pathway. The cellular model used for this study was the prostate cancer cell line PC3, because PC3 cells express high levels of EGF receptor [16,17], but they show low levels of ERK1/2 at resting state [18,19], conditions that enable the study of a wide range of levels of ERK1/2 activation in cells in culture. STIM1-KO PC3 cells were generated by CRISPR/Cas9 genome editing, and the results demonstrate that EGF triggers the activation of ERK1/2 in the absence of STIM1 as well as without extracellular $Ca^{2+}$. Because STIM1-KO cells are SOCE-deficient, our data prove that SOCE is not an upstream regulator of ERK1/2 activation in PC3 cells, and that upon stimulation with EGF in $Ca^{2+}$-free medium, Src kinases are major activators of the canonical Raf-MEK-ERK pathway. These findings are of particular importance since MAPKs regulate gene expression and have a role in cell growth, cell cycle and apoptosis, and this is why this pathway has been considered a pharmacological target in the treatment of a variety of cancers.

## 2. Materials and methods

### 2.1. Chemicals

PC3 cells were from the European Collection of Cell Cultures (ECACC) and distributed by Sigma-Aldrich (St. Louis, MO, USA). Flp-In T-Rex HEK293 cells from Life Technologies (Carlsbad, CA, USA); PD0325901 was purchased from Axon Medchem BV (Groningen, The Netherlands); EGF (ref. E4127), KN-62 and collagen type I were from Sigma-Aldrich (St. Louis, MO, USA); Gö 6976, Gö 6850 and SrcI1 were from Tocris (Bristol, UK); Fura-2-acetoxymethyl ester (fura-2-AM) was from Merck Millipore (Darmstadt, Germany); Thapsigargin (Tg) was from AbCam Biochemicals (Cambridge, UK); The luminol substrate was Supersignal West Femto from Thermo Fisher Scientific (Waltham, MA, USA); Polyethylenimine was purchased from Polysciences, Inc. (Eppelheim, Germany).

### 2.2. Antibodies and DNA constructs

Antibodies against phosphorylated ERK1/2 (Thr202/Tyr204), anti-total-ERK1/2, anti-phospho-p38 (Thr180/Tyr182), anti-total p38, anti-phospho-c-Jun (Ser63), anti-CAMKII alpha, anti-phospho CAMKII (Thr286), and anti-phospho-Src (Tyr416) were from Cell Signaling Technology Inc. (Danvers, MA, USA). The rabbit polyclonal anti-STIM1 (N-terminus) antibody was from ProSci Inc. (Poway, CA, USA). Mouse monoclonal anti-STIM1 (C-terminus) antibody was from BD Biosciences (San Jose, CA, USA). Antibodies against phospho-Ser575-STIM1, phospho-Ser608-STIM1, and phospho-Ser621-STIM1 were produced in collaboration with the Division of Signal Transduction Therapy (DSTT), University of Dundee (Dundee, UK), as described elsewhere [6,12,13,20]. Anti-total-c-Jun antibody was from the DSTT, University of Dundee (Dundee, UK). Anti-total Src kinase (clone GD11) was from Sigma-Aldrich. Mouse monoclonal anti-GAPDH antibody was from AbCam Biochemicals (Cambridge, UK). All secondary horseradish peroxidase (HRP)-labelled antibodies were from Thermo Fisher Scientific (Waltham, MA USA).

For transient transfections, Stim1 cDNA (mouse Stim1, NM_009287) was cloned into the pCMV5-Flag1 vector (University of Dundee) as a *Bam*HI-*Not*I vector by adding those restriction sites to the Stim1 cDNA by PCR. DNA used for transfection was purified from *E. coli* DH5α using ZymoPURE Plasmid kits according to the manufacturer's protocol. Transfection of cells with DNA constructs was performed with 1–3 μg plasmid DNA per 10-cm dish and polyethylenimine in serum-containing medium, 18–24 h prior to the beginning of the experiments.

### 2.3. Culture and treatment of cells

PC3 and HEK293 cells were cultured on 3.5- or 10-cm-diameter dishes in DMEM medium with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, in a humidified atmosphere of air/5% $CO_2$ at 37 °C. Cell culture dishes and glass coverslips were treated with collagen type I solution (0.01%) for a minimum of 30 min at 37 °C before plating cells. In all experiments, FBS was removed 12 h prior to the beginning of the experiments. Unless otherwise stated, cells were treated in $Ca^{2+}$-containing or $Ca^{2+}$-free HBSS (Life Technologies). The composition of $Ca^{2+}$-free HBSS was as follows: 138 mM NaCl; 5.33 mM KCl; 0.34 mM $Na_2HPO_4$; 0.44 mM $KH_2PO_4$; 4.17 mM $NaHCO_3$; 5.55 mM glucose; 4 mM $MgCl_2$ and 0.1 mM EGTA (pH 7.4). The composition of $Ca^{2+}$-containing HBSS was as follows: 138 mM NaCl; 5.33 mM KCl; 0.34 mM $Na_2HPO_4$; 0.44 mM $KH_2PO_4$; 4.17 mM $NaHCO_3$; 5.55 mM glucose; 0.49 mM $MgCl_2$; 0.41 mM $MgSO_4$; 1.26 mM $CaCl_2$ (pH 7.4). EGF (50 μg/ml, as stock solution) was dissolved in PBS + 0.1% BSA, and single-use aliquots were stored at − 80 °C. Tg was dissolved in DMSO and stored as 1 mM stock solution at − 80 °C.

For the treatment of cells with kinase inhibitors, prior to the stimulation with EGF, cells were incubated with PD0325901 for 10 min, KN-62 for 30 min, Gö 6976, Gö 6850, and SrcI1 for 20 min. Then, cells were stimulated with EGF in HBSS in the presence of the inhibitors. All inhibitors were dissolved in DMSO and stock solutions stored at − 80°C.

### 2.4. Immunoblot

Immunoblot for phosphorylated STIM1 was performed as indicated previously [6,12,13,20]. Cells were lysed in the following buffer: 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Nonidet P40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride. Then, samples were sonicated with five 10-second pulses using a Branson Digital Sonifier with a setting of 45% amplitude. Samples were clarified with 0.75–1 ml of ice-cold lysis buffer/dish and centrifugation at 20,000*g* for 15 min at 4 °C. Protein concentration was determined using the Coomassie Protein Assay Reagent (Thermo Fisher Scientific).

Lysates (20–40 μg) were subjected to electrophoresis on polyacrylamide gels (4–12% acrylamide) and subsequent electroblotting to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in blocking buffer: TBS-T (Tris-buffered saline buffer, pH 7.5, with 0.2% Tween-20) containing 10% (w/v) non-fat milk. The phospho-specific anti-STIM1 antibodies were pre-incubated for 30 min at room temperature with the non-phosphorylated peptides (LVEKLPDSPALAKK for the anti-phospho-Ser575; PSVPPGGSPLLDSS for the anti-phospho-Ser608; SHSLSPSSPDPDTPS for the anti-phospho-Ser621), at the following ratio: 1 μg/ml antibody + 10 μg/ml non-phosphopeptide. Then, the membranes were incubated overnight with the phospho-specific STIM1 antibody diluted in blocking solution

(1 μg/ml) at 4 °C, washed, and then incubated with anti-sheep IgG HRP-conjugated secondary antibody (1:10,000 dilution) for 1 h at room temperature. Total STIM1 immunoblot (phosphorylated and non-phosphorylated) was performed after overnight incubation at 4 °C with 1 μg/ml anti-STIM1 antibody (ProSci, Inc. or BD Biosciences) diluted in blocking solution. HRP-conjugated secondary antibodies were used at 1:20,000 dilution for 1 h at room temperature in blocking solution.

Immunoblot for phospho-ERK1/2, phospho-p38, phospho-c-Jun, phospho-CAMKII, and phospho-Src were performed with overnight incubation with the antibodies diluted in TBS-T (at 1 μg/ml dilution) containing 5% BSA, followed by 1 h incubation with HRP-conjugated secondary antibody. After developing with luminol substrate, membranes were stripped, blocked with 10% non-fat milk, and assessed for total ERK1/2, total p38, or total CAMKII with the antibodies that recognize both the phosphorylated and the non-phosphorylated proteins (1 μg/ml dilution in TBS-T containing 5% BSA), followed by a 1 h-incubation with HRP-conjugated secondary antibodies. To detect total levels of c-Jun, total Src kinase, and GAPDH, blocked membranes were incubated overnight at 4 °C with the anti-c-Jun or the anti-Src antibodies diluted in TBS-T (1 μg/ml) containing 10% non-fat milk, followed by a 1 h-incubation with HRP-conjugated secondary antibodies.

Luminol substrate was added and membranes were exposed for 1–3 min to chemiluminescence films. Developed films were scanned, and the signal was quantified by volumetric integration using Image J.

### 2.5. Generation of STIM1-KO PC3 cells using CRISPR/Cas9 genome editing

The human *STIM1* locus (ENSG00000167323) shows three verified transcriptional variants: NM_001277961.1, NM_001277962.1, and NM_003156.3. As we described in a recent report [5], the first coding exon shared between the published transcripts was exon 3, but there remained some evidence of a shorter 512 residue variant initiating further downstream, and exon 5 was therefore chosen as the CRISPR target site. The guide pair (sense 5′-AGATGACAGACCGGAGTCAT and antisense 5′-AGTCCCTGTCATGGTGGTGT) was subsequently identified using the Sanger Institute CRISPR webtool (http://www.sanger.ac.uk/htgt/wge/find_crisprs). Complementary oligos with *Bbs*I compatible overhangs were designed according to the Zhang method [21] and these dsDNA guide inserts ligated into *Bbs*I-digested target vectors; the antisense guide was cloned into the spCas9 D10A expressing vector pX335 (Addgene plasmid #42335) and the sense guide into the puromycin selectable plasmid pBABED P U6 (University of Dundee) [22,23]. The specificity of these constructs for the generation of STIM1-KO cells has been proved and reported elsewhere [5]. PC3 cells were co-transfected with 1 μg of each plasmid using polyethylenimine in a 10-cm dish. Following 48 h of puromycin selection (2 μg/ml), the cell culture was extended 24 h without puromycin, and the cell pool was subsequently single-cell sorted by FACS. Clones were analysed for STIM1 depletion by immunoblotting and genomic DNA sequencing.

Genomic DNA was isolated, and the region surrounding the target site of the guide RNAs amplified by PCR (forward primer: 5′-CAAGAGCTAGAAGTGTTCCTGGG; reverse primer: 5′-CTTTGGTTTCC ATGGCACAGC). Resulting PCR products were subcloned using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies) and 12 colonies picked for each clonal line and sequenced to verify indels. Sequencing of exon 5-PCR fragments from the selected STIM1-KO clone revealed a unique modification, a 16 base-pair insertion that was common to the two alleles, confirming the successful KO of the *STIM1* locus.

### 2.6. Cytosolic free calcium concentration measurement

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was measured basically as described elsewhere [5,10,12,13,24,25] in fura 2-AM-loaded cells, using an inverted microscope Nikon Ti-E equipped with micro-incubation platform DH-40i (Warner Instruments). Excitation fluorescence wavelengths were selected with 340/26 and 387/11 nm filters, and emission fluorescence with a 510/10 nm filter (Semrock). All measurements were performed at 35–36 °C. Excitation/emission conditions were controlled by the NIS-Elements AR software. Depletion of $Ca^{2+}$ stores was triggered by incubating cells with 1 μM Tg in $Ca^{2+}$-free HBSS. SOCE was measured by monitoring the increase of the $[Ca^{2+}]_i$ after the addition of 2 mM $CaCl_2$ to the Tg-containing medium.

### 2.7. Statistical analysis of data

Statistical analyses were done using the unpaired *t*-test. Differences between groups of data were taken statistically significant for $p < 0.05$. The *p*-values are represented as follows: (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

## 3. Results

### 3.1. MAPK signaling pathways in the absence of extracellular $Ca^{2+}$

To assess the role of extracellular $Ca^{2+}$ influx on the activation of MAPK signaling pathways we designed a preliminary experiment to downregulate these pathways by a simple FBS deprivation step. In Fig. 1A we show the level of phospho-ERK1/2, phospho-p38 MAPK, and phospho-c-Jun, as well as the level of total ERK1/2, p38 MAPK, and c-Jun, in PC3 cells after 6-12 h of FBS deprivation in DMEM. This test showed that residues Thr180/Tyr182 of p38 MAPK were efficiently dephosphorylated (i.e., p38 was inactivated) after 6 h of deprivation, and that 12 h of FBS deprivation is required to inactivate JNK1/2 kinases (monitored by the phosphorylation of its substrate c-Jun). ERK1/2 remained inactivate in all cases, consistent with low ERK1/2 activity in basal conditions due to the absence of PTEN phosphatase in the PC3 cell line [26] and the consequent activation of PKB [27], which negatively regulates ERK1/2 pathway. In this case, a positive control with HEK293 cells treated with thapsigargin was run together with PC3 samples to confirm the phospho-specificity of the immunoblot. As a result, we selected 12 h of FBS deprivation as the experimental condition to evaluate the activation of MAPKs by EGF.

EGF stimulation of PC3 cells was carried out with a 10-min incubation in the presence of 5–100 ng/ml EGF in the absence of extracellular $Ca^{2+}$ (see buffer composition above), and the results showed that maximal activation was attained with 50 ng/ml EGF (Fig. 1B), a concentration within the range of concentrations used by other groups. In addition, PD0325901, a well-known inhibitor of MEK1/2 and therefore a specific inhibitor of the activation of ERK1/2 [28], fully inhibited the immuno-reactive signal (Fig. 1C), validating the results of the immunoblot and the observed activation of ERK1/2 in response to EGF without extracellular $Ca^{2+}$ shown in Fig. 1. The other two MAPKs, p38 MAPK and JNK, were also evaluated in these specific conditions (EGF + no extracellular $Ca^{2+}$), and the results indicate that both kinases became activated upon EGF stimulation (Fig. 1C).

A downstream target for ERK1/2 is STIM1 [6,10,29], the master regulator of the store-operated $Ca^{2+}$ entry pathway [1,2,30]. To evaluate the ERK1/2 activation in the absence of extracellular $Ca^{2+}$ we also tested the phosphorylation level of its substrate, STIM1, with phospho-specific antibodies generated against the three known ERK1/2-target residues in STIM1 sequence: Ser575, Ser608, and Ser621 [6,10,12,13,20]. For this purpose, PC3 cells were transfected for the transient expression of Flag-STIM1, FBS-starved for 12 h, and treated with 50 ng/ml EGF in $Ca^{2+}$-free HBSS for 10 min (Fig. 2). The results confirmed that ERK1/2 was activated by EGF, and that STIM1 became phosphorylated in the absence of extracellular $Ca^{2+}$ at the three ERK1/2 target sites, being this phosphorylation fully inhibited by PD0325901. As a control of this assay, HEK293 cells overexpressing Flag-STIM1 were treated with 1 μM thapsigargin in $Ca^{2+}$-free HBSS, conditions known to trigger ERK1/2 activation and STIM1 phosphorylation [6,10].

Because our results pointed out that extracellular $Ca^{2+}$, and

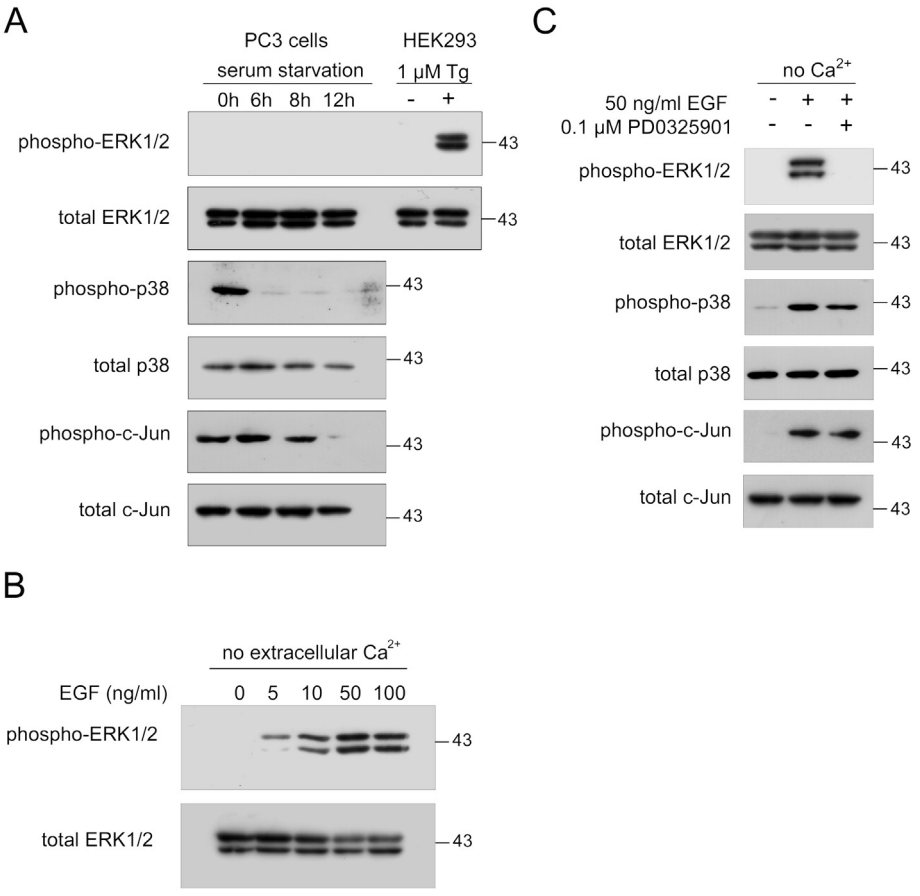


**Fig. 1.** ERK1/2 activation in the absence of extracellular $Ca^{2+}$. Panel A: PC3 cells were FBS starved for 6–12 h and clarified lysates (20 μg protein/lane) were assessed for MAPK activation by immunoblot. ERK1/2 was evaluated by monitoring phosphorylation at Thr202/Tyr204; p38 MAPK by monitoring phosphorylation at Thr180/Tyr182; JNK activation was evaluated by monitoring the phosphorylation level of its substrate c-Jun at Ser63. Total protein (loading control) was evaluated with a non-phosphospecific antibody. As an additional control of the immunoblot, HEK293 cells were treated with 1 μM thapsigargin (Tg) in $Ca^{2+}$-free medium to trigger ERK1/2 activation. Panel B: PC3 cells were FBS starved for 12 h and then treated with 5–100 ng/ml EGF in $Ca^{2+}$-free HBSS to evaluate ERK1/2 activation by immunoblot as in panel A (20 μg protein/lane). Panel C: PC3 cells were FBS starved for 12 h and then treated with 50 ng/ml EGF in $Ca^{2+}$-free HBSS with or without 0.1 μM PD0325901. Activation of ERK1/2, p38 MAPK and JNK was evaluated as in panel A (20 μg protein/lane). Immunoblots are representative of 3 independent experiments.

therefore $Ca^{2+}$ entry, were dispensable for ERK1/2 activation, we monitored the effect of the presence of extracellular $Ca^{2+}$ on this activation. For that, PC3 cells were FBS-starved for 12 h, and stimulated with EGF in $Ca^{2+}$-free or $Ca^{2+}$-containing HBSS. The results (Fig. 3) demonstrated that there is no significant increase of this activation in the presence of extracellular $Ca^{2+}$, further supporting that $Ca^{2+}$ entry pathways are dispensable in this activation in response to EGF. As stated above, it has been previously suggested that SOCE could be an upstream activator for ERK1/2 [14,15], although in those reports cells were stimulated with FBS, thapsigargin or carbachol. However, our results were supporting a different model in which SOCE is not required for ERK1/2 activation. To study the real contribution of SOCE to this signaling pathway we designed a set of experiments to avoid SOCE activation by generating a STIM1-deficient PC3 cell line, as SOCE fully depends on the activity of this protein.

### 3.2. Generation of a STIM1-KO PC3 cell line by CRISPR/Cas9 genome editing

Using CRISPR/Cas9-mediated gene edition we designed a strategy to knock-out *STIM1* locus in PC3 cells (shown in Fig. 4A). The selected clone was found to have a 16 bp insert at exon 5, which led to a premature stop codon, confirmed by genomic DNA sequencing. As a consequence, modified PC3 cells did not show detectable expression of STIM1 protein, which was tested with two different antibodies, raised against N-terminal and C-terminal epitopes (Fig. 4B). To evaluate further whether STIM1 loss caused a significant effect on SOCE, we challenged STIM1-KO PC3 cells with thapsigargin in $Ca^{2+}$-free HBSS to deplete intracellular $Ca^{2+}$ stores and measured $Ca^{2+}$ entry after addition of $CaCl_2$ to the assay medium (Fig. 4C, left panel). This experiment revealed a striking decrease in SOCE, indicating the successful knock-out of STIM1 activity. We also evaluated $Ca^{2+}$ entry level in response to EGF in both PC3 and STIM1-KO PC3 cells, and the results demonstrated that STIM1 mediate extracellular $Ca^{2+}$ entry in parental PC3 cells in response to EGF (Fig. 4C, right panel), and that $Ca^{2+}$ entry in STIM1-KO cells was negligible. Therefore the STIM1-KO PC3 cell line is an adequate experimental tool to evaluate the role of STIM1 and SOCE on cell signaling and particularly on SOCE-dependent ERK1/2 activation.

### 3.3. ERK1/2 activation does not require STIM1-dependent $Ca^{2+}$ influx

PC3 cells and STIM1-KO PC3 cells (SOCE-deficient) were assessed for MAPK activation in response to EGF in both $Ca^{2+}$-free and $Ca^{2+}$-containing HBSS. The results in Fig. 5A show that ERK1/2 activation in response to EGF was even larger in STIM1-KO compared to wild-type cells (for n = 4 independent experiments) in the absence of extracellular $Ca^{2+}$, and that therefore SOCE is not required for ERK1/2 activation. Indeed, the intensity of ERK1/2 activation was the same in the absence or presence of $Ca^{2+}$ in the extracellular medium assay, i.e., there was no $Ca^{2+}$-dependent enhancement of ERK1/2 activation in the presence of $Ca^{2+}$ in the medium assay, conditions where a notable $Ca^{2+}$ influx was detected (see Fig. 4C), further confirming that SOCE is dispensable for ERK1/2 activation in PC3 cells.

Because thapsigargin triggers the depletion of intracellular $Ca^{2+}$ stores, we also tested the contribution of $Ca^{2+}$ release from the ER on the activation of ERK1/2, in cells treated with thapsigargin. The activation of ERK1/2 in response to thapsigargin was the same in STIM1-KO and wild-type cells (Suppl. File 1), and significantly weaker than that observed in cells treated with EGF, suggesting that the contribution of $Ca^{2+}$-release from the ER on the activation of ERK1/2 is minimal, and further supporting that STIM1 and SOCE are dispensable for the activation of ERK1/2, even in cells treated with thapsigargin.

Thus, additional mechanisms should explain the activation of the Raf-MEK-ERK pathway by EGF in a $Ca^{2+}$ influx-independent fashion.

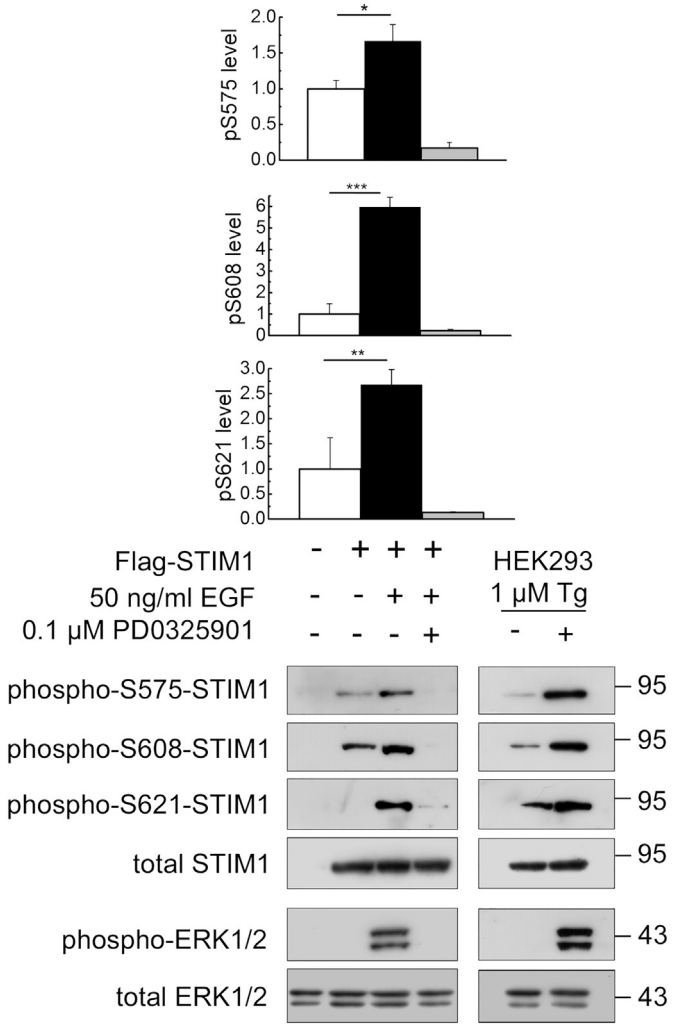


**Fig. 2.** $Ca^{2+}$ influx-independent phosphorylation of the ERK1/2 substrate STIM1. PC3 cells were transfected for the transient expression of Flag-STIM1, and 24 h after transfection cells were FBS starved for 12 h and subsequently treated with EGF as in Fig. 1, panel C. Lysates (40 μg protein/lane) were evaluated for the level of phospho-STIM1 (pS575, pS608, and pS621), using total STIM1 level as loading control. As an additional control of the experiment, the level of ERK1/2 activation (phospho-ERK1/2) was evaluated as in Fig. 1. In parallel, HEK293 cells stably expressing Flag-STIM1 were treated with 1 μM Tg in $Ca^{2+}$-free HBSS. The data are presented as mean ± standard deviation (s.d.) from 3 independent experiments. Statistical analysis was done using the unpaired t-test. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

Because it has been suggested that for some stimuli, such as thapsigargin [31], the release of $Ca^{2+}$ from the intracellular stores and the subsequent activation of extracellular $Ca^{2+}$ entry activate the $Ca^{2+}$/calmodulin-dependent kinase II (CAMKII), we also monitored the activation of this kinase. Using the experimental conditions described above, we treated PC3 cells with EGF (50 ng/ml) in $Ca^{2+}$-free HBSS supplemented with 0.1 mM EGTA (Fig. 5B). The experiment was performed in STIM1-KO and wild-type cells. Activation of CAMKII was monitored following its phosphorylation at Thr286, a specific residue described as the initial site of $Ca^{2+}$-dependent autophosphorylation in response to transient changes in intracellular $Ca^{2+}$ concentrations in a variety of cells (reviewed in [32]). In parallel, we evaluated the phosphorylation level of CAMKII in response to thapsigargin in the presence of extracellular $Ca^{2+}$, conditions known to activate CAMKII, as a positive control of the experiment. As a result, we did not observed activation of CAMKII during the treatment of cells with EGF in the absence of $Ca^{2+}$, ruling out the participation of this pathway in the activation of ERK1/2 in these conditions. This conclusion was supported by the fact that KN-62, which fully inhibited CAMKII activation triggered by thapsigargin in our control experiment (see Fig. 5B, top right panel), did not have any effect of the activation of ERK1/2 triggered EGF (Fig. 5B).

### 3.4. Src kinase activates EGF in a $Ca^{2+}$-independent manner

In the evaluation of the contribution of upstream kinases to the activation of ERK1/2 by EGF we find two additional candidates: protein kinase C, PKC [33,34] and sarcoma viral oncogene homolog (Src) family members [35,36]. Following a similar strategy, we used specific inhibitors for PKC: Gö 6976, which inhibits the $Ca^{2+}$-dependent PKCα and PKCβ1 isoforms, and Gö 6850 (also known as GF 109203X), an inhibitor of the PKCα, β1, and the $Ca^{2+}$-independent PKCδ, ε, and ζ isoforms. As a control of the experiment we monitored phospho-c-Jun levels, a downstream target of PKC, to evaluate PKC inhibition. The results demonstrated that Gö 6976 and Gö 6850 did not inhibited EGF-triggered activation of ERK1/2 (Fig. 5C), ruling out the involvement of PKCα/β in the $Ca^{2+}$-independent activation of ERK1/2.

We also monitored the effect of SrcI1, an inhibitor of the Src family members Src, Lck, Csk, and Yes [28]. As a control we assessed the phosphorylation of Src at Tyr416, an autophosphorylation site for the positive regulation of its activation [37]. SrcI1 strongly inhibited the activation of ERK1/2 triggered by EGF (Fig. 6A–C), a result that confirmed that Src activated the canonical Raf-MEK-ERK pathway. Although the suggested concentration of SrcI1 to achieve full inhibition in a cell-based assay is within 1–2 μM range, the effect of SrcI1 in the inhibition of ERK1/2 activation was evident at 0.5 μM (Fig. 6C), which fits well with the reported $IC_{50}$ in an in vitro kinase assay for this specific compound [28].

## 4. Discussion

The Raf-MEK-ERK pathway is involved in the regulation of cell cycle, cell growth, and apoptosis [38]. Multiple stimuli can trigger the activation of Raf proteins, which is mediated mainly by Ras, PKC, or members of the Src kinase family [36,38–41]. Consequently this pathway has attracted the attention of the current research in order to design strategies to prevent malignant cell growth. The Raf-MEK-ERK is also closely connected to the phosphoinositide 3-kinase (PI3K)-PTEN-Akt pathway, because this latter pathway negatively regulates Raf protein activity. Thus, the absence of PTEN in PC3 cells leads to relatively low basal levels of ERK1/2 activity, although the pathway is fully

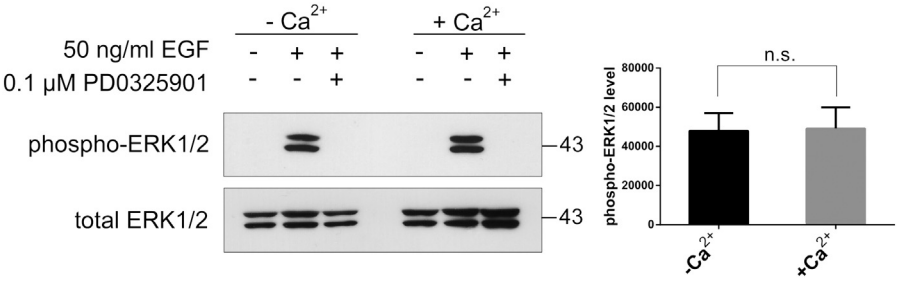


**Fig. 3.** $Ca^{2+}$ influx is dispensable for ERK1/2 activation. Left: PC3 cells were FBS starved for 12 h and treated with 50 ng/ml EGF in $Ca^{2+}$-free HBSS (− $Ca^{2+}$) or $Ca^{2+}$-containing HBSS (+ $Ca^{2+}$). Lysates (20 μg protein/lane) were evaluated for the level of ERK1/2 activation, using total ERK1/2 levels as loading control. Blots are representative of 4 independent experiments. Right: Quantification of ERK1/2 phosphorylation from experiments shown in left panel. Data are presented as mean ± s.d. Statistical analysis of data was done using the unpaired t-test. (n.s.) $p > 0.05$.

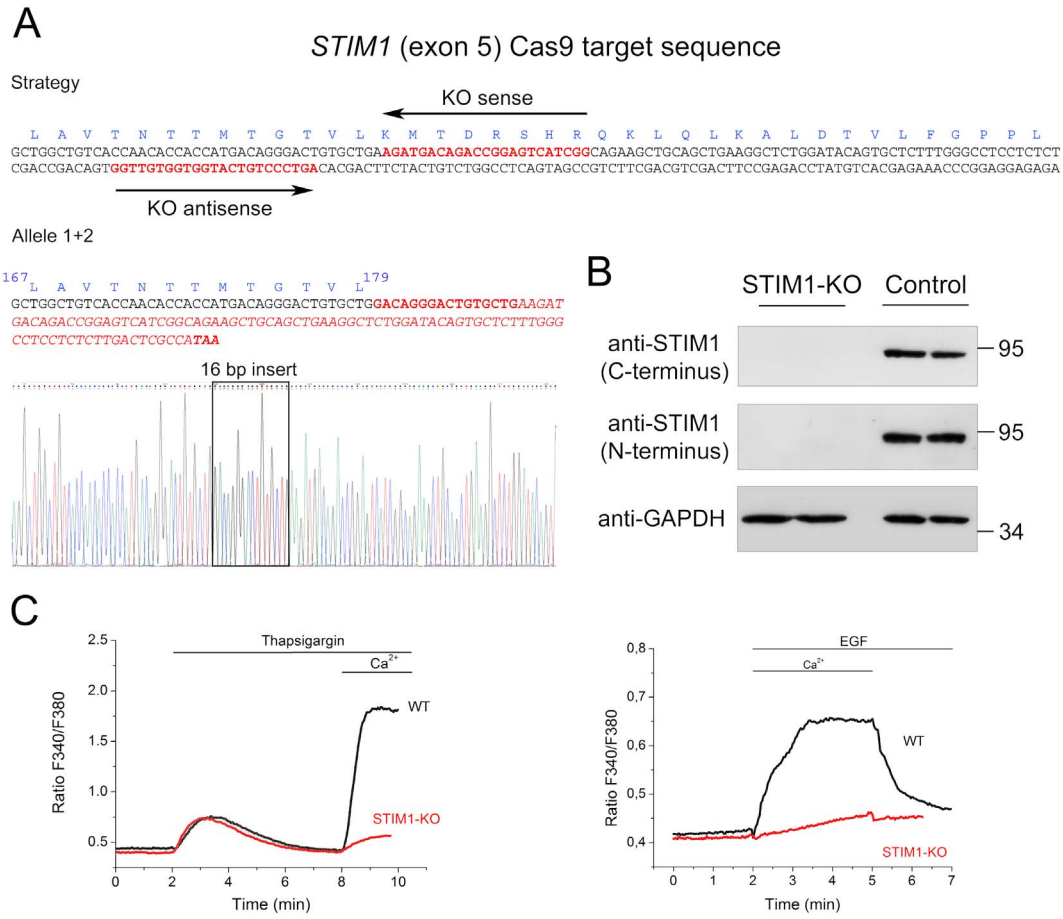


**Fig. 4.** Knock-out of STIM1 expression by CRISPR/Cas9 D10A gene editing. Panel A: Strategy for gene editing using CRISPR/Cas9 D10A in PC3 cells. A pair of guide RNAs (KO sense and antisense) was designed to trigger a double nick at exon 5 of *STIM1* locus. Sequencing a PCR product from the genomic DNA at the target site revealed a 16 base-pair insertion (in bold red font). Frameshifting sequence is denoted in red font, with premature stop codon at the end of the sequence. Bottom: Genomic DNA sequencing chromatogram from STIM1-KO cells, showing the location of the 16 bp insert. Panel B: The selected clone of cells was assessed for STIM1 expression by immunoblot, using two different anti-STIM1 antibodies generated against C-terminal and N-terminal epitopes. Anti-GAPDH antibody was used as loading control. Panel C: (Left) Fura-2-loaded cells were incubated in a $Ca^{2+}$-free HBSS (assay medium), and 1 μM thapsigargin (Tg) was added for 6 min. $Ca^{2+}$ (2 mM $CaCl_2$) was added to evaluate the extent of $Ca^{2+}$ entry in KO cells (red line) and control cells (black line). Data are presented as the mean ± s.d. of 3 independent experiments (n = 25 cells for KO; n = 25 cells for wild-type). (Right) (c) Fura-2-loaded cells were incubated in $Ca^{2+}$-free HBSS. EGF (50 ng/ml) + 2 mM $CaCl_2$ was added to the cells to evaluate the extent of $Ca^{2+}$ entry in STIM1-KO (red line) and control cells (black line). After 6 min, the assay medium was replaced by $Ca^{2+}$-free HBSS + 50 ng/ml EGF to monitor the drop in $Ca^{2+}$ entry in the absence of extracellular $Ca^{2+}$. Data are presented as the mean ± s.d. of 3 independent experiments (n = 25 cells for KO; n = 20 cells for wild-type). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activatable by EGF.

There are many downstream targets of ERKs, including transcription factors, additional kinases, such as the ribosomal S6 kinase (RSK), or cytoskeletal proteins [42,43]. In addition, early observations reported that SOCE was negatively regulated by inhibitors of ERK1/2, such as U0126 or PD184352, and that this inhibition was PI3K-independent but sensitive to inhibitors of Ras [11]. After the finding that STIM1 is the intraluminal $Ca^{2+}$ sensor in the ER that activates SOCE, it was later demonstrated that STIM1 is a direct target of ERK1/2 in vitro and in vivo, and that this phosphorylation regulates STIM1 activity and binding to microtubules [6,10], leading to the conclusion that SOCE is an additional downstream target of ERK1/2. However, differences between cell types might be found since they rely on different levels of Raf-MEK-ERK, PI3K-PTEN-Akt, and $Ca^{2+}$ signals (monophasic increases, repetitive waves, etc.). Thus, it is possible to find some discrepancy in the recent literature regarding the interdependence of ERKs and SOCE. For instance, EGF triggers a $Ca^{2+}$ influx-independent activation of ERK1/2 in adenocarcinoma endometrial cells [12]. A similar activation was reported in HEK293 cells under stimulation with IGF-1 [13]. In agreement with these results, STIM1 knock-down did not modify phosphorylation levels of MEK1/2-ERK1/2 in gastric cancer cells [44], suggesting that STIM1 is not an upstream regulator of ERKs in cancer progression. Similarly, retinal epithelial cells (ARPE-19) proliferation and migration is sensitive to STIM1 and ORAI1 knock-down, but the reduced expression of STIM1 and ORAI1 did not have any negative effect on the activation of ERK1/2 in response to EGF [45].

On the other hand, it has been reported that $Ca^{2+}$ entry regulates ERK1/2 activation in melanoma cells. Using an experimental design based on STIM1 or ORAI1 silencing by siRNA, SOCE-CAMKII-Raf-ERK signaling pathway was proposed to be controlling cell proliferation [15]. Similarly, the activation of ERK1/2 by SOCE has been described in parotid acinar cells, a finding that was based on the inhibition of SOCE by the blocker 2-APB [14]. The genetic manipulation of cultured cells using genome editing tools, such as CRISPR/Cas9 techniques, offers the possibility to knock-out gene expression without loss of genomic stability due to the small indels created at the target sites. In this report we demonstrate that genetically engineered PC3 cells lacking STIM1 expression, and consequently without $Ca^{2+}$ entry through SOC channels in response to thapsigargin or EGF, are able to activate ERK1/2 in response to EGF similarly to what is observed for STIM1-expressing cells. In addition to the activation of ERK1/2 in the absence of extracellular $Ca^{2+}$ this result demonstrates that, in PC3 cells, ERK1/2 activation is $Ca^{2+}$ influx-independent. It is important also to emphasize that there is

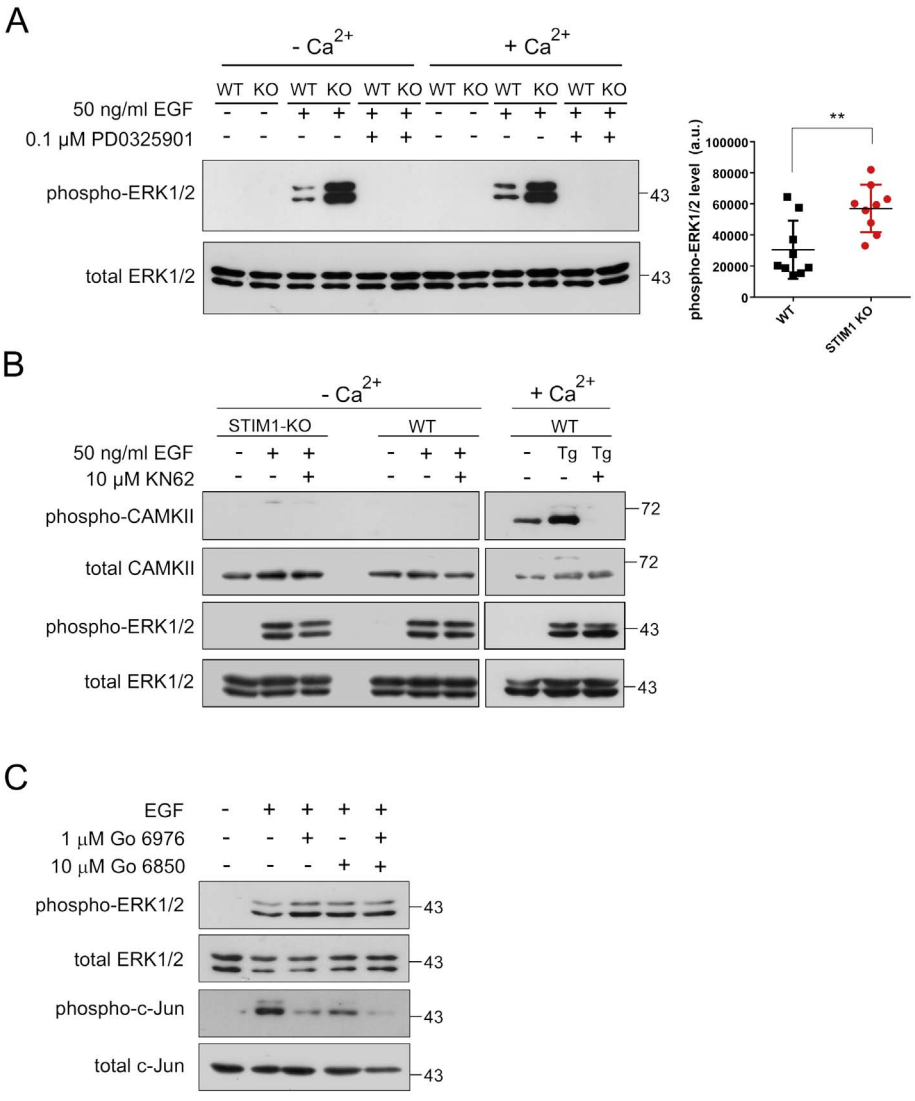


**Fig. 5.** ERK1/2 activation in STIM1 deficient cells. Panel A: (Left) PC3 cells, either wild type (WT), or STIM1-KO (KO) were FBS starved for 12 h and subsequently treated with EGF in $Ca^{2+}$-free HBSS (− $Ca^{2+}$) or $Ca^{2+}$-containing HBSS (+ $Ca^{2+}$). Lysates (20 μg protein/lane) were evaluated for the level of phospho-ERK1/2, using total ERK1/2 as loading control. PD0325901 (0.1 μM) was added to block ERK1/2 activation. (Right): Quantification of ERK1/2 activation in the absence of extracellular $Ca^{2+}$ from 6 independent experiments (n = 9) using immunoblots as in left panel. Data are presented as a dot plot with mean ± standard deviation (s.d.). (**) $p < 0.01$. Panel B: Cells were treated as in panel A, in the presence of 10 μM KN-62. The level of phospho-CAMKII (Thr286) was assessed to evaluate CAMKII activation, using total CAMKII as loading control (20 μg protein/lane). In parallel, ERK1/2 activation was evaluated in the same samples (bottom). The validation of the immunoblot phospho-specificity against phospho-CAMKII was performed with PC3 cells treated with 1 μM Tg with or without KN-62 (right panel). Immunoblots are representative of 2 independent experiments. Panel C: Cells were FBS starved and pre-incubated for 20 min with Gö 6976 or Gö 6850 before adding EGF to cell cultures. EGF treatment was performed in $Ca^{2+}$-free HBSS. Lysates (30 μg protein/lane) were assessed for the level of ERK1/2 activation (phospho-ERK1/2) using total ERK1/2 as loading control. Inhibition of c-Jun phosphorylation was evaluated as a control of PKC inhibition, using total c-Jun as loading control. Immunoblots are representative of 2 independent experiments.

no alteration of total ERK1/2 expression in STIM1-KO cells, making the determination of phospho-ERK1/2 a suitable method for the determination of the activation of this pathway.

Our results also show a higher activation of ERK1/2 in STIM1-KO cells. ERK1/2 phosphorylation status depends on the balance between upstream kinases and phosphatases, and a plausible explanation for this result is that the knock-out of STIM1 and the lack of SOCE can be partially inhibiting specific phosphatases with ERK1/2 as a target. An example of this kind of regulation is the serine/threonine-protein phosphatase 2B (PP2B or calcineurin), which becomes activated by SOCE [46], and therefore it is expected to be downregulated or inhibited by the lack of SOCE in STIM1-KO cells. More closely related with MAPKs, the dual specificity phosphatase MAPK phosphatase-1 (MPK-1) is known to be upregulated by $Ca^{2+}$ influx, and that MPK-1 expression is dependent on calcineurin [47], a mechanism that could explain the higher activation of ERK1/2 in STIM1-KO cells because of the lower dephosphorylation rate of ERK1/2.

On the other hand, Src kinase has been shown to phosphorylate Raf at Tyr340/341 in endothelial cells stimulated by vascular endothelial growth factor (VEGF), leading to MEK1-dependent protection from apoptosis [33], and we demonstrate here that Raf-MEK-ERK is activated in a Src kinase-dependent manner, and that specific inhibition of this upstream kinase is enough to prevent ERK1/2 activation. On the other hand, the phosphorylation of c-Src at Tyr416 was monitored to follow the activation of this kinase in cells treated with EGF in the absence of extracellular $Ca^{2+}$, a result that confirmed that Src activation is a $Ca^{2+}$-independent process, further confirming the unnecessary of $Ca^{2+}$ entry for the activation of the axis Src-Raf-MEK-ERK.

The consequences of these observations are important because they set SOCE downstream ERK1/2, but not upstream Raf proteins. The designing of inhibitors/modulators of signaling pathways to control or prevent malignant progression (i.e., epithelial-mesenchymal transition and cell migration) depends on the specific pathways activated in particular cancer cells. In prostate cancer cells, as in other aggressive cancer cells, it is observed a direct correlation between cellular aggressiveness and the fraction of cellular Src present in the active state [48], and Nam et al. reported that the inhibition of Src family kinases with dasatinib (BMS-354825) could be a therapeutic strategy on human prostate cancer cells since this inhibition suppresses cell adhesion, migration, and invasion [49]. However, inhibition of upstream regulators may lead to a number of side effects during chemotherapy, and designing inhibitors for specific downstream $Ca^{2+}$-dependent events, such as cell adhesion, migration, and invasion would be an alternative strategy. Thus, it is plausible that the inhibition of SOCE in cancer cells, with $Ca^{2+}$-independent activation of Raf-MEK-ERK and high levels of active Src, would target $Ca^{2+}$-dependent events only, including cell migration and metastasis, therefore reducing the number of adverse side effects.

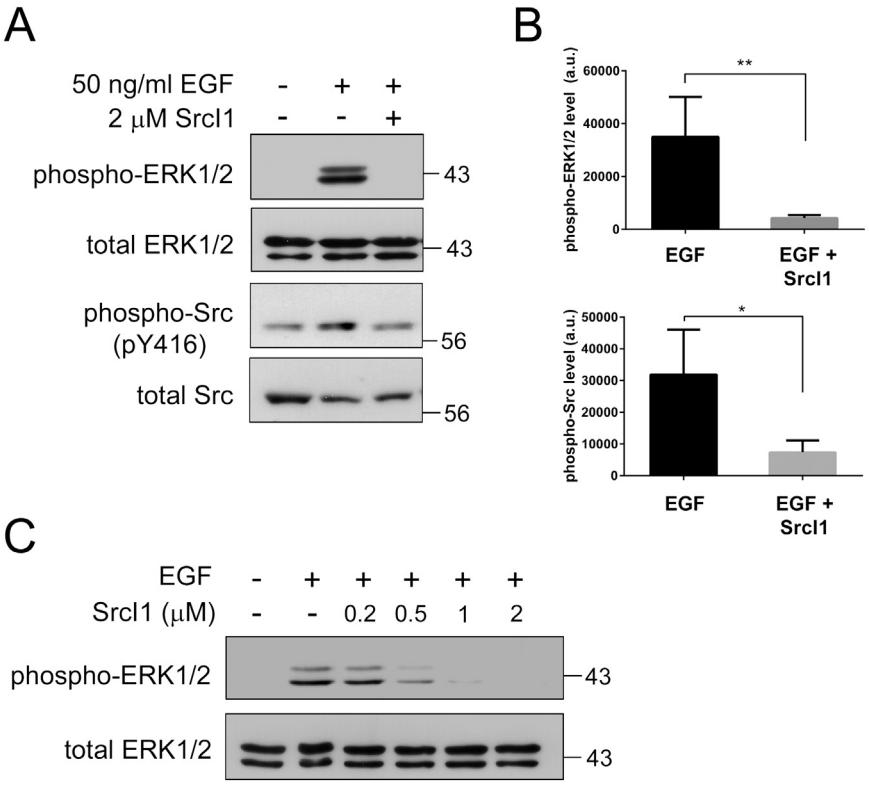


**Fig. 6.** Src kinase-dependent ERK1/2 activation. Panel A: PC3 cells were FBS starved and pre-incubated for 20 min with SrcI1 before adding EGF to cell cultures. EGF treatment was performed in $Ca^{2+}$-free HBSS. Lysates (30 μg protein/lane) were assessed for the level of ERK1/2 activation (phospho-ERK1/2) using total ERK1/2 as loading control. Src phosphorylation at Y416 was monitored as an assessment of Src activation status, using total Src as loading control. Panel B: Quantification of ERK1/2 and Src phosphorylation from 3 independent experiments performed as in panel A. Data are represented as mean ± s.d. Panel C: PC3 cells were pre-incubated with SrcI1 at the indicated concentrations for 20 min, and then treated with EGF in the presence of 0.2–2 μM SrcI1 in $Ca^{2+}$-free HBSS.

## 5. Conclusions

In conclusion, there is a considerable variation in data regarding the $Ca^{2+}$ dependence of ERK1/2 activation. Since the linear Raf-MEK-ERK pathway constitutes a simple pharmacological target, it is required to study upstream regulators and downstream substrates in the genetically diverse types of cancers. Our report, using STIM1-deficient cells, demonstrate that ERK1/2 activation is $Ca^{2+}$-influx independent and Src-dependent, pointing out the focus of a suitable pharmacological target on this kinase family when the control of ERK1/2 is required, but ruling out the participation of SOCE in the activation of ERK1/2.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2017.08.010.

## Author contributions

AML-G, FJM-R, and EP-G designed experiments; AM-L, CP-C and EP-G performed experiments; FJM-R and EP-G wrote the manuscript. All authors reviewed and approved the final manuscript.

## Conflict of interest

No competing interests declared.

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