

Original Paper

EFHB is a Novel Cytosolic Ca²⁺ Sensor That Modulates STIM1-SARAF Interaction

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Key Words

EFHB • SARAF • STIM1 • Orai1 • Calcium entry

Abstract

Background/Aims: STIM1 and Orai1 are the key components of store-operated Ca²⁺ entry (SOCE). Among the proteins involved in the regulation of SOCE, SARAF prevents spontaneous activation of SOCE and modulates STIM1 function. **Methods:** Cytosolic Ca²⁺ mobilization was estimated in fura-2-loaded cells using an epifluorescence inverted microscope. STIM1 interaction with Orai1, EFHB (EF-hand domain family member B, also known as CFAP21) and SARAF was detected by immunoprecipitation followed by Western blotting using specific antibodies. The involvement of EFHB in the translocation of NFAT to the nucleus was detected by confocal microscopy. **Results:** Here, we report the identification of EFHB as a new SOCE regulator. EFHB interacts with STIM1 upon store depletion and dissociates through a Ca²⁺-dependent mechanism. RNAi-mediated silencing as well as overexpression studies revealed that EFHB plays a relevant role in the interaction of STIM1 and Orai1 upon store depletion, the activation of SOCE and NFAT translocation from the cytosol to the nucleus. Silencing EFHB expression abolished the dissociation of SARAF from STIM1, which indicates that EFHB might play an important role in the dynamic interaction between both proteins, which is relevant for the activation of Orai1 channels upon Ca²⁺ store depletion and their subsequent modulation via slow Ca²⁺-dependent inactivation. **Conclusion:** Our results indicate that EFHB is a new SOCE regulator that modulates STIM1-SARAF interaction.

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Introduction

Store-operated Ca^{2+} entry (SOCE) is a mechanism initiated by the depletion of the intracellular Ca^{2+} stores, mainly the endoplasmic reticulum (ER), which participates in the regulation of a large number of cellular functions [1-3]. The key elements of SOCE are the ER Ca^{2+} sensor STIM1 and the Ca^{2+} -permeable channel Orai1, which interact forming the Ca^{2+} -selective CRAC (Ca^{2+} release-activated Ca^{2+}) channels, or the SOC (store-operated Ca^{2+}) channels in combination with TRPC1 [4-9]. STIM1 is a protein mostly located in the ER membrane with a single transmembrane domain. The N-terminus exhibits a canonical EF-hand motif, which, upon dissociation from intraluminal Ca^{2+} , leads to oligomerization and clustering of STIM1 into puncta at ER-plasma membrane (PM) junctions [10] as well as to the conformational change of its cytosolic region from a closed to an extended state [11]. The extended configuration of STIM1 exposes the STIM1-Orai1 activating region (SOAR) domain (amino acids 344-442) [12], which results in full activation of Orai1 [11]. Downstream the SOAR, STIM1 exhibits the C-terminal inhibitory domain (CTID; amino acids 448-530) that mediates the interaction of STIM1 with its regulator SARAF (SOCE-associated regulatory factor) [13]. SARAF is a 339-amino acid long protein, mainly located in the ER membrane, that interacts with the CTID region of STIM1 to mediate slow Ca^{2+} -dependent inactivation (SCDI) of Orai1 channels and prevents spontaneous activation of STIM1 [14]. CTID modulates the access of SARAF to the SOAR. At rest, when the intracellular stores are filled with Ca^{2+} , CTID facilitates the interaction of SARAF to SOAR to keep STIM1 in an inactive state. Depletion of the intracellular Ca^{2+} stores leads to an initial STIM1-SARAF dissociation, to allow the interaction of STIM1 with Orai1 channels and their activation, followed by STIM1-SARAF re-association that has been linked to the SCDI of Orai1 [13, 15].

The mechanism involved in the dissociation and subsequent re-association of SARAF with STIM1 remains unclear. The involvement of the SARAF-STIM1 re-interaction in SCDI suggests certain Ca^{2+} dependence; however, SARAF does not exhibit Ca^{2+} -binding domains. We have identified an EF-hand containing protein, EFHB (EF-hand domain family member B) as a regulator of the dynamic interaction between STIM1 and SARAF. Human EFHB is an 833-amino acid cytosolic protein with two EF-hand domains between amino acids 561-596 and 597-632. Our results indicate that EFHB is required for the STIM1-Orai1 interaction and full SOCE activation. Interestingly, we found that EFHB interacts with STIM1 upon Ca^{2+} store depletion, which is essential of the dissociation of SARAF from STIM1. EFHB dissociates from STIM1 in a Ca^{2+} -dependent manner in a time scale that parallels the re-association of SARAF with STIM1. These findings suggest a role for EFHB as a cytosolic Ca^{2+} -binding protein involved in the regulatory mechanism of STIM1 by SARAF.

Materials and Methods

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Thapsigargin (TG), rabbit polyclonal anti-Orai1 antibody (catalog number O8264, epitope: amino acids 288-301 of human Orai1), rabbit polyclonal anti- β -actin antibody (catalog number A2066, epitope: amino acids 365-375 of human β -actin), dimethyl BAPTA, and bovine serum albumin (BSA) were from Sigma (Madrid, Spain). Rabbit polyclonal anti-TMEM66 (SARAF) antibody (catalog number PA5-24237, epitope: amino acids 33-62 of the N-terminal region of human TMEM66) and Turbofect transfection reagent were from Thermo Fisher (Madrid, Spain). Mouse monoclonal anti-STIM1 antibody (Clone 44/GOK, epitope: amino acids 25-139 of human STIM1, catalog number 610954) was from BD Transduction Laboratories (Franklin Lakes, NJ, U.S.A.). Rabbit polyclonal anti-EFHB antibody (catalog number AP10904a, epitope: amino acids 1-30 of human EFHB (N-terminal)) was from Abgent (San Diego, CA, U.S.A.). Rabbit polyclonal anti-EFHB antibody (catalog number TA340363, epitope: amino acids 743-792 of human EFHB (C-terminal)) was from OriGene (Herford, Germany). Horseradish peroxidase-conjugated anti-mouse IgG antibody and anti-rabbit IgG antibody for IP (not recognizing the heavy and light chains of the immunoprecipitating antibody) were from

Abcam (Cambridge, U.K.). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Complete EDTA-free protease inhibitor tablets were from Roche (Madrid, Spain). Rabbit polyclonal anti-NFATc1 (H-110) antibody and Alexa Fluor-labeled goat anti-rabbit IgG were from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). Hoechst 33342 was from Invitrogen Life Technology (Paisley, Scotland). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, U. K.). Overexpression plasmid for V5-tagged EFHBΔ1-164 was from Dharmacon (Madrid, Spain). All other reagents were of analytical grade.

Plasmids construction

Plasmid construction was based on the previously published EFHB sequences (RefSeq Gene ID: 151651). To knockdown expression of EFHB, a pLKO.1-puro plasmid-based shRNA targeting the sequence: GCCAGAAGATATTGTCTTAAA (clone ID: TRCN0000055578; Sigma-Aldrich) was used (EFHB-shRNA). In addition, a non-targeting shRNA plasmid (NT-shRNA) that targets no known human sequence was used as a control. A primer containing the target sequence along with a stem loop followed by the reverse target sequence was annealed to a complementary primer and inserted into the EcoRI and AgeI sites of the pLKO.1-puro plasmid (Addgene; number 10878). The resulting hairpin consisted of the following sequence: 5'-CCGGCCAGAAGATATTGTCTTAACTCGAGTTTAAAGACAATATCTTCTGGCTTTT -3'. The correct insertion of the hairpin into pLKO.1 plasmid was finally checked by sequencing.

Cell culture and transfection

HEK-293 and HeLa cell lines were obtained from ATCC (Manassas, VA, USA), and cultured at 37 °C with a 5% CO₂ in DMEM supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin and streptomycin.

Cells were transfected with expression plasmids for Orai1, YFP-Stim1, Stim1 (kindly provided by Dr. Romanin), Orai1E106Q (kindly provided by Dr. Capiod) or the commercial V5-tagged EFHBΔ1-164 mutant, as well as with the shEFHB, siSARAF, EGFP-SARAF or scramble plasmids as described previously [16-18] using Turbofect transfection reagent and were used 48 h after transfection. Plasmids were used for silencing experiments at 1 µg/mL.

Measurement of cytosolic free-calcium concentration ([Ca²⁺]_i)

Cells were loaded with fura-2 by incubation with 1 µM fura 2/AM for 30 min at 37 °C. Coverslips with cultured cells were mounted on a perfusion chamber and placed on the stage of an epifluorescence inverted microscope (Nikon Eclipse Ti-2, Amsterdam, The Netherlands) with image acquisition and analysis system for videomicroscopy (Nikon NIS-Elements AR, Amsterdam, The Netherlands). Cells were continuously superfused with HEPES-buffered saline (HBS) containing (in mM): 125 NaCl, 5 KCl, 1 MgCl₂, 5 glucose, 25 HEPES, and pH 7.3, supplemented with 0.1% (w/v) BSA. Cells were alternatively excited with light from a xenon lamp passed through a high-speed monochromator (Optoscan ELE 450, Cairn Research, Faversham, U.K.) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (sCMOS camera (Zyla 4.2, Andor, Belfast, U.K.) and recorded using NIS-Elements AR software (Nikon, Amsterdam, The Netherlands). Fluorescence ratio (F_{340}/F_{380}) was calculated pixel by pixel and data are presented as $\Delta F_{340}/F_{380}$, as previously described [19]. TG-evoked Ca²⁺ release and influx was measured as the integral of the rise in fura-2 fluorescence ratio above basal for 2½ min after the addition of TG in the absence or presence of extracellular Ca²⁺, respectively.

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as described previously [20]. Briefly, 500 µL aliquots of cell suspension (4 × 10⁶ cell/mL) were lysed with an equal volume of ice-cold 2 × NP-40 buffer, pH 8, containing 274 mM NaCl, 40 mM Tris, 4 mM EDTA, 20% glycerol, 2% nonidet P-40, 2 mM Na₃VO₄ and complete EDTA-free protease inhibitor tablets. Aliquots of cell lysates (1 mL) were immunoprecipitated by incubation with 1 µg of anti-STIM1 or 2 µg of anti-Orai1 antibody and 25 µL of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 10% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of Orai1, STIM1, EFHB and SARAF was achieved by incubation for 2 h with anti-STIM1 antibody diluted 1:500 in TBST, for 1 h with anti-Orai1

antibody diluted 1:500 in TBST, or overnight with the anti-SARAF or anti-EFHB antibodies diluted 1:1000 in TBST. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG antibody or horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted 1:10000 in TBST and then exposed to enhanced chemiluminescence reagents for 4 min. The density of bands was measured using C-DiGit Chemiluminescent Western Blot Scanner and ImageJ software (NIH, U.S.A.). Data were normalized to the amount of protein recovered by the antibody used for the immunoprecipitation.

Confocal immunofluorescence

HeLa cells transfected with shEFHB or scramble plasmid were seeded on coverslips and stimulated in the absence or presence of 1 μ M TG for 2 and 24 h. Treatments were performed in DMEM (supplemented as described in the cell culture section) at 37°C and 5% CO₂. For measurements of NFATc1 nuclear accumulation, experiments were performed as previously described [21]. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature (RT), permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, and blocked with 2% bovine serum albumin in PBS for 2 h. Primary antibodies, rabbit polyclonal anti-NFATc1 antibody (1:250) was incubated overnight at 4 °C and Alexa Fluor-labeled secondary antibody, goat anti-rabbit IgG (1:600) was incubated by 2 h at RT. The nuclei were stained with the nucleic acid dye Hoechst 33342 (4 μ M) and incubated together with the secondary antibody. Cells were examined at 60 \times magnification using a Bio-Rad MRC 1024ES laser scanning confocal microscope (Bio-Rad Lab, Life Sciences Division, Hercules, CA, U.S.A.). The specificity of immune staining was confirmed by the absence of staining when primary or secondary antibodies were omitted. Hoechst 33342 and Alexa Fluor were excited at 405 nm and 488 nm and the emitted light was collected through 450/50-nm and 525/50-nm bandpass filters, respectively. Multiple fields for each coverslip were acquired and images analyzed using ImageJ software (NIH, Bethesda, MD, U.S.A.). Blue fluorescent Hoechst images were used to generate a mask to define the nuclear area. The ratio between the mean fluorescence intensity of NFATc1 (green) in the nuclear area and the cytosol of each cell was determined.

Statistical Analysis

Analysis of statistical significance was performed using one-way analysis of variance. For comparison between two groups Student's *t* test was used. *P* < 0.05 was considered to be significant for a difference.

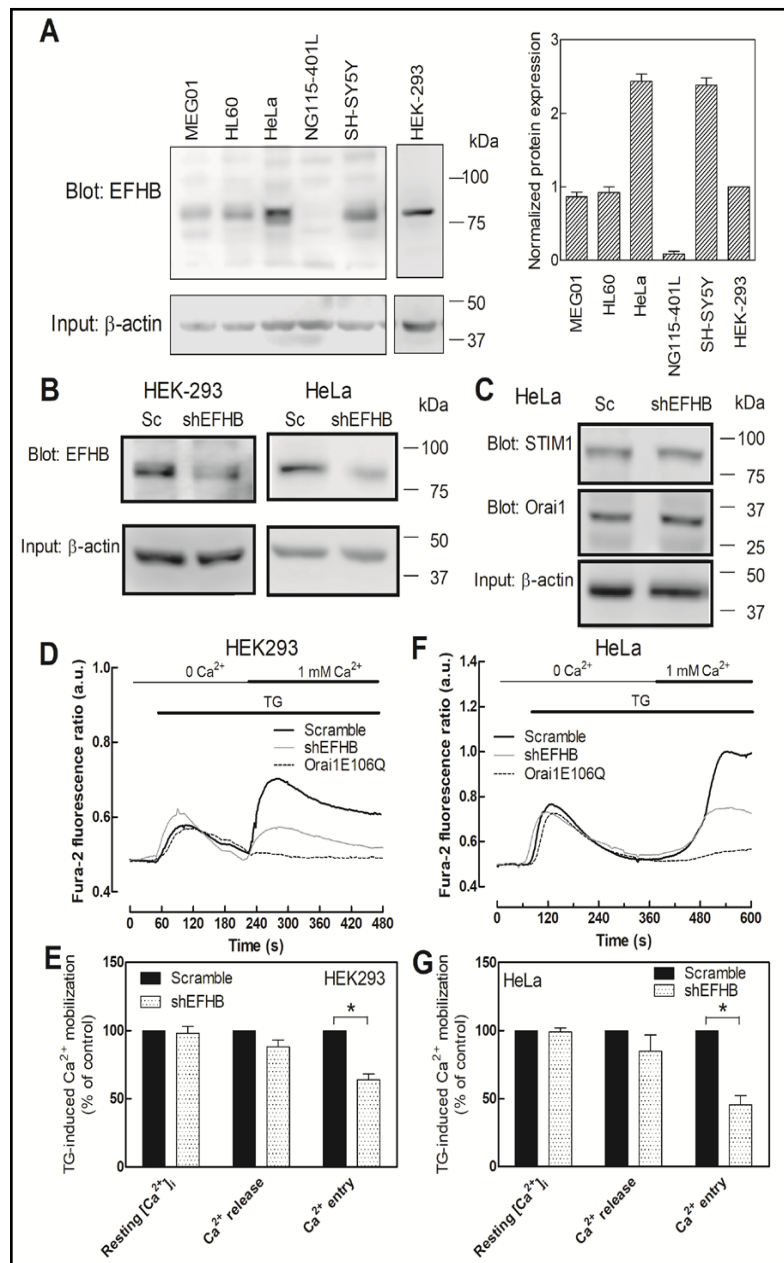
Results

EFHB is a widely expressed protein that modulates store-operated Ca²⁺ entry

Western blot analysis of whole cell lysates from a number of human cell types, including MEG-01, HL60, HeLa, SH-SY5Y and HEK-293 cells, with a specific anti-human EFHB (N-terminal) antibody revealed a broad expression of EFHB at the protein level in a wide variety of unrelated cell lines (Fig. 1A; *n* = 3). Analysis of EFHB expression in the different cell lines, normalized to the β -actin content and expressed as fold-increase over the expression level in HEK-293 cells, shows a greater expression of EFHB in HeLa and SH-SY5Y cells. However, we were almost unable to detect EFHB expression in the NG115-401L cell line, a mouse neuroblastoma and rat glioma hybrid (Fig. 1A). The latter might be attributed to a low expression of EFHB at the protein level in this cell line, which would be in line with the lack of expression of STIM1 [22-23], or to the failure of the antibody used to detect non-human EFHB.

We evaluated whether EFHB plays a relevant role in the activation of SOCE by transfecting HEK-293 and HeLa cells with EFHB-shRNA or scramble plasmid. As depicted in Fig. 1B, 48 h after transfection of the shEFHB plasmid the expression of EFHB at the protein level was significantly reduced in both cell types as compared to cells treated with scramble plasmid (*p* < 0.05; *n* = 3). EFHB expression silencing was without effect on STIM1 and Orai1 expression at the protein level in HeLa cells (Fig. 1C; *n* = 3). Treatment of HEK-293 and HeLa cells with 1 μ M thapsigargin (TG) in a Ca²⁺-free medium resulted in a transient rise in fura-2 340/380 fluorescence ratio due to passive Ca²⁺ release from the intracellular

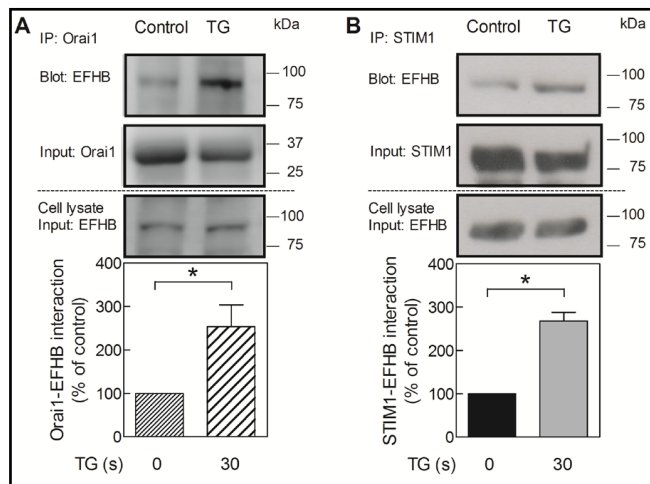
Fig. 1. EFHB is required for TG-induced Ca^{2+} mobilization. A, MEG01, HL60, HeLa, NG115-401L, SH-SY5Y and HEK-293 cells were lysed and subjected to Western blotting with anti-EFHB(N-terminal)antibody, followed by reprobing with anti- β -actin antibody for protein loading control. Bar graphs represent EFHB expression normalized to the β -actin content and expressed as fold-increase over the expression level in HEK-293 cells. B-G, HEK-293 and HeLa cells were transfected with shEFHB or scramble plasmid (Sc), as indicated. After 48 h cells were lysed and subjected to Western blotting with anti-EFHB (N-terminal) antibody followed by reprobing with anti- β -actin antibody for protein loading control (B). C, HeLa cells were transfected with shEFHB or scramble plasmid (Sc). After 48 h cells were lysed and subjected to Western blotting with anti-STIM1 or anti-Orai1 antibody followed by reprobing with anti- β -actin antibody for protein loading control. D and F, HEK-293 and HeLa cells were transfected with shEFHB, scramble plasmid



(Sc) or co-transfected with YFP-Stim1 and Orai1E106Q expression plasmid, as indicated. Forty eight hours after transfection, fura-2-loaded HEK-293 (D) and HeLa cells (F) were perfused with a Ca^{2+} -free medium (100 μM EGTA added) and then stimulated with TG (1 μM) followed by readdition of external Ca^{2+} (final concentration 1 mM) to initiate Ca^{2+} entry. Data are original traces representative of 40-80 cells/day/5 days. Values are expressed as described in methods. E and G, Bar graphs represent TG-induced Ca^{2+} mobilization in HEK-293 (E) and HeLa cells (G) transfected with shEFHB or scramble plasmid. Data are expressed as mean \pm S.E.M. and presented as percentage of control (cells treated with scramble plasmid). * represents $p < 0.05$ as compared to TG-induced Ca^{2+} mobilization in scramble-treated cells.

stores. Readdition of Ca^{2+} to the extracellular medium resulted in a further increase in fura-2 fluorescence ratio indicative of SOCE (Fig. 1D and F). Silencing EFHB expression by cell transfection with shEFHB significantly inhibited SOCE in HEK-293 and HeLa cells by 40 and 55%, respectively, without having any effect on the resting $[\text{Ca}^{2+}]_i$ or the release of Ca^{2+} from

Fig. 2. EFHB interacts with STIM1 and Orai1 upon Ca^{2+} store depletion. HEK-293 cells were co-transfected with expression plasmids for V5-EFHB Δ 1-164 and STIM1 or Orai1. Cells were left untreated (control) or stimulated with TG (1 μ M) for 30 s and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-Orai1 (A) or anti-STIM1 antibody (B) and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-EFHB (C-terminal) antibody. Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. Cell lysates were also subjected



to Western blotting with anti-EFHB (C-terminal) antibody, as indicated. The panels show results from one experiment representative of 3 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Bar graphs represent the quantification of Orai1-EFHB or STIM1-EFHB interaction in resting and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as percentage of control (cells not stimulated with TG). * represents $p < 0.05$ as compared to controls.

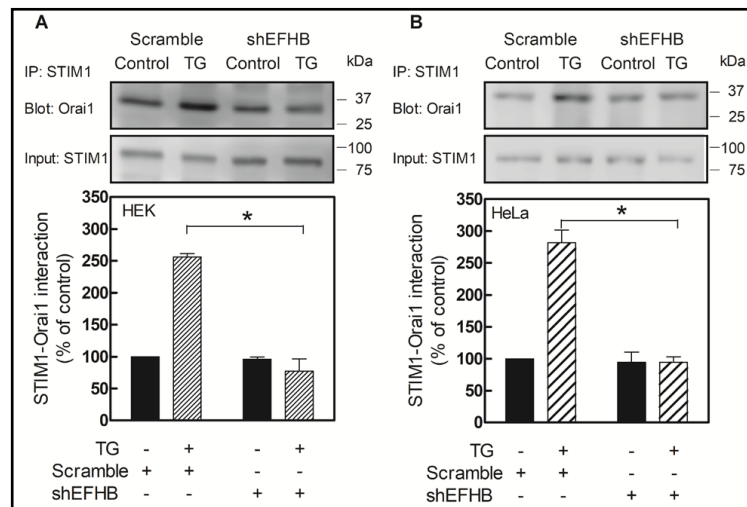
the intracellular stores (Fig. 1D-G; $p < 0.05$; 40-80 cells/day/5 days). Co-transfection of YFP-Stim1 and the pore-dead Orai1E106Q mutant confirmed that TG-induced Ca^{2+} entry was dependent on Orai1 (Fig. 1D and F; 40 cells/day/3 days). Although some EFHB expression was still detectable after shEFHB transfection, these findings indicate for the first time that EFHB plays an important role in the activation of SOCE.

EFHB interacts with STIM1 and Orai1 upon Ca^{2+} store depletion and is required for STIM1-Orai1 association

To ascertain the mechanism by which EFHB modulates SOCE, we raised the question whether EFHB supports the interaction between STIM1 and Orai1 upon Ca^{2+} store depletion. To address this issue, we first explored the possible interaction of EFHB with STIM1 and Orai1 by looking for co-immunoprecipitation in whole cell lysates from HEK-293 cell co-expressing either STIM1 or Orai1 and the commercial EFHB construct lacking the N-terminal 164 amino acids. As depicted in Fig. 2A, a detectable interaction between STIM1 and EFHB Δ 1-164 was found in resting cells, which was significantly increased to 270% in cells stimulated with 1 μ M TG for 30 s ($p < 0.05$; $n = 4$). Similarly, immunoprecipitation of whole cell lysates with anti-Orai1 antibody followed by Western blotting with anti-EFHB (C-terminal) antibody revealed a detectable interaction between both proteins in resting cells and a significant increase to 253% in the Orai1-EFHB interaction upon treatment with TG (Fig. 2B; $p < 0.05$; $n = 4$). These observations clearly indicate that, while there is a detectable interaction between EFHB and both STIM1 and Orai1 in resting cells, this association is significantly enhanced in response to depletion of the intracellular Ca^{2+} stores. In addition, the studies reported above demonstrate that the EFHB N-terminal 164 amino acids are not essential for the interaction with STIM1 and Orai1.

Next, we explored the involvement of EFHB in the interaction between STIM1 and Orai1 by co-immunoprecipitation in HEK-293 cells co-expressing STIM1 and Orai1 as well as in HeLa cells. As shown in Fig. 3, Ca^{2+} store depletion by TG enhances the interaction between STIM1 and Orai1 in both cell types, a response that was abolished by cell transfection with shEFHB ($p < 0.05$; $n = 6$). These findings provide insights into the mechanism by which EFHB modulates SOCE.

Fig. 3. EFHB expression knockdown attenuates store depletion-induced STIM1-Orai1 interaction. HEK-293 cells co-expressing STIM1 and Orai1 (A) and HeLa cells (B) were transfected with shEFHB or scramble plasmid, as indicated. Cells were left untreated or stimulated with TG (1 μ M) for 1 min and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-



Orai1 antibody. Membranes were reprobed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Bar graphs represent the quantification of STIM1-Orai1 association in resting and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as percentage of control (resting cells transfected with scramble plasmid). * represents $p < 0.05$ as compared to TG-treated cells transfected with scramble plasmid.

We have further tested the role of EFHB in STIM1-Orai1 interaction and SOCE in HEK-293 cells co-expressing STIM1, Orai1 and the commercial EFHB construct EFHB Δ 1-164. First of all, we evaluated the role of EFHB Δ 1-164 overexpression (Fig. 4A; $n = 3$) in TG-induced STIM1-Orai1 interaction by looking for co-immunoprecipitation in whole cell lysates. As shown in Fig. 4B, TG-evoked STIM1-Orai1 interaction was found to be significantly greater in cells overexpressing EFHB Δ 1-164 ($p < 0.05$; $n = 6$), which confirms the role of EFHB in the STIM1-Orai1 association observed after transfection with shEFHB (Fig. 3). Remarkably, expression of EFHB Δ 1-164 significantly enhanced TG-induced SOCE without having any effect on the resting fura-2 fluorescence ratio or on TG-induced Ca^{2+} release (Fig. 4C; $p < 0.05$; 40-50 cells/day/5 days). The latter findings indicate that EFHB is not involved either in the maintenance of the resting cytosolic Ca^{2+} level, the accumulation of Ca^{2+} into the intracellular stores or the permeability of the ER membrane to Ca^{2+} . The observations reported above further indicate that EFHB plays an important role in the activation of SOCE probably by supporting the STIM1-Orai1 interaction.

As an independent third approach to evaluate the possible action of EFHB on SOCE, we tested the ability of EFHB to interfere with the Ca^{2+} -dependent translocation of the nuclear factor of activated T cells (NFAT) to the nucleus, a mechanism that has been found to be activated by SOCE [7]. We evaluated the translocation of NFATc1 to the nucleus by confocal immunofluorescence using specific antibodies in HeLa cells. As depicted in Fig. 5, in resting cells NFATc1 shows a predominant cytosolic distribution. Treatment with 1 μ M TG in the presence of 1 mM extracellular Ca^{2+} induced the translocation of NFATc1 into the nucleus at least during the first 24 h after addition of TG. Cells transfected with shEFHB exhibited attenuated NFATc1 translocation in response to Ca^{2+} store depletion both 2 and 24 h after treatment with TG (Fig. 5; $p < 0.05$; $n = 50-100$ cells/3-4 different experiments). These findings suggest that EFHB is required for the translocation of NFATc1 to the nucleus upon Ca^{2+} store depletion.

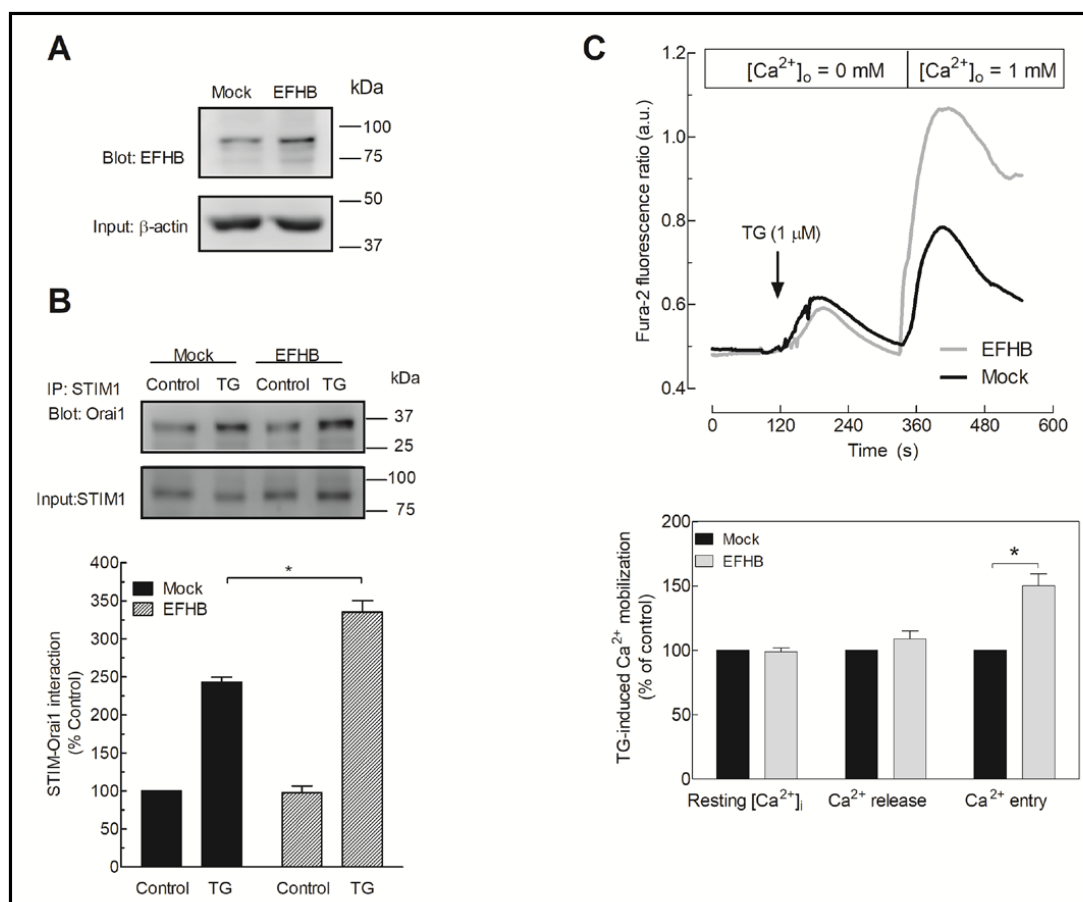


Fig. 4. EFHB overexpression enhances TG-evoked STIM1-Orai1 interaction and Ca²⁺ entry. HEK-293 were co-transfected with STIM1, Orai1 and either the expression plasmid for EFHB Δ 1-164 (EFHB) or empty vector (Mock), as indicated. A, Immunoblot analysis of EFHB expressed in HEK-293 cells before or after treatment with expression plasmid for EFHB Δ 1-164 using anti-EFHB (C-terminal) antibody. Membranes were re probed with anti- β -actin antibody for protein loading control. B, After 48h cells were left untreated or stimulated with TG (1 μ M) for 1 min, lysed and whole cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody. Immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-Orai1 antibody. Membranes were re probed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Bar graphs represent the quantification of STIM1-Orai1 interaction in control (untreated) and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as percentage of control (resting mock-treated cells). * represents $p < 0.05$ as compared to TG-treated mock-treated cells. C, Forty eight hours after transfection fura-2-loaded cells were perfused with a Ca²⁺-free medium (100 μ M EGTA added) and then stimulated with TG (1 μ M) followed by reintroduction of external Ca²⁺ (final concentration 1 mM) to initiate Ca²⁺ entry. Data are original traces representative of 40-50 cells/day/5 days. Values are expressed as described in methods. Bar graphs represent TG-induced Ca²⁺ mobilization in cells transfected with EFHB Δ 1-164 expression plasmid or empty vector. Data are expressed as mean \pm SEM and presented as percentage of control (mock-treated cells). * represents $p < 0.05$ as compared to TG-induced Ca²⁺ entry in cells transfected with empty vector.

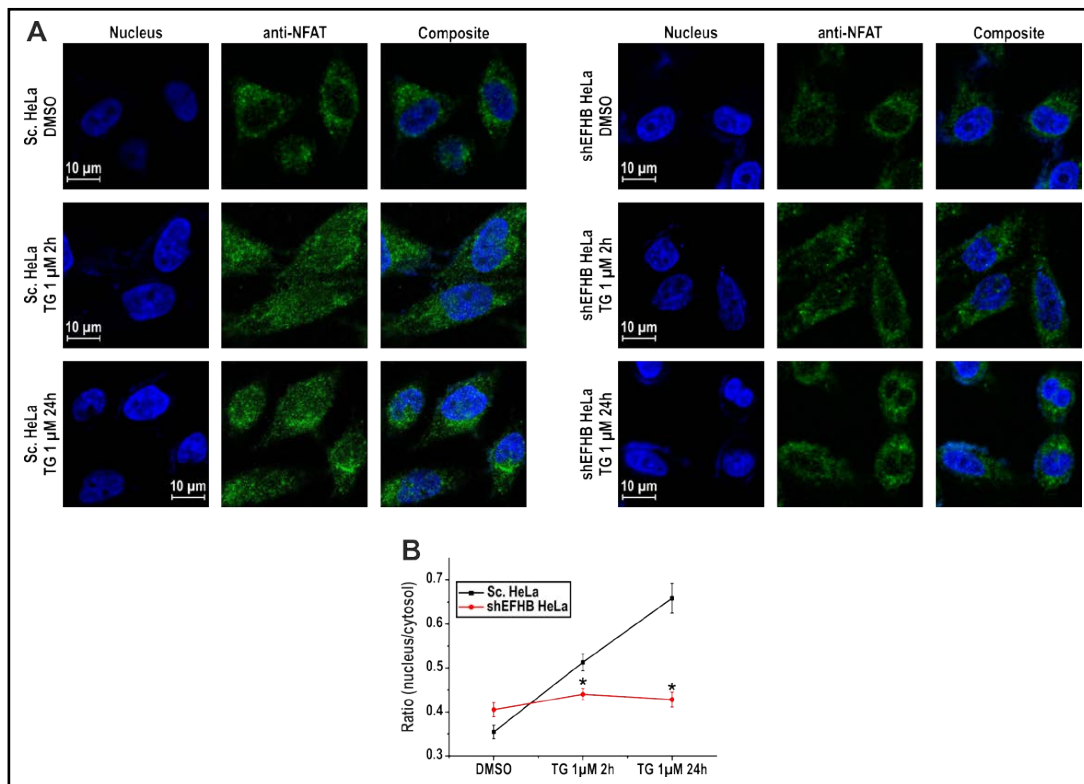
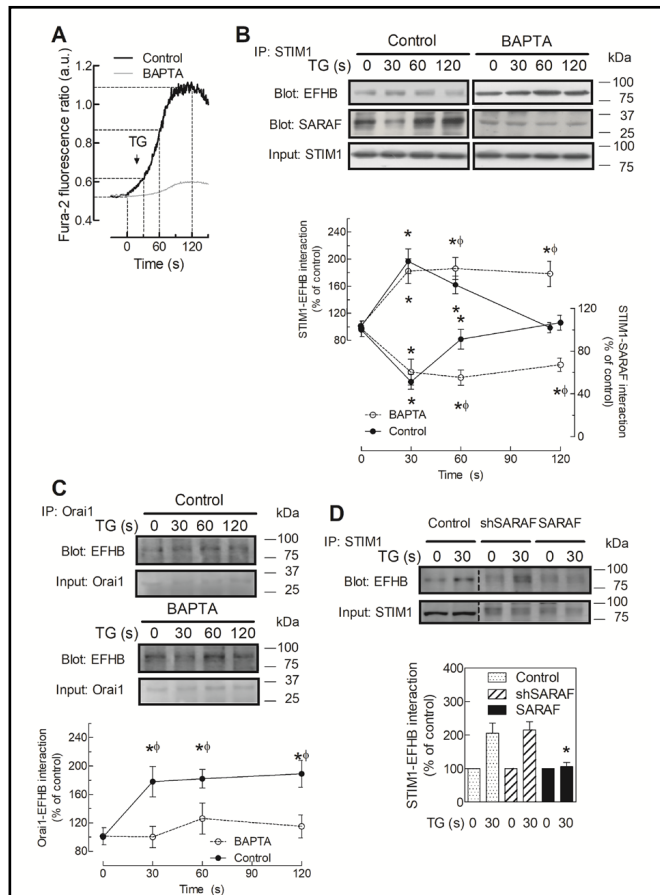


Fig. 5. EFHB is required for NFAT translocation. A, Confocal immunofluorescence images of NFATc1 localization in HeLa cells transfected with shEFHB or scramble plasmid, as indicated, before, 2 h and 24 h after exposure to 1 μ M TG in a 1 mM Ca^{2+} -containing bath solution. B, Time course of the nuclear/cytosolic NFATc1 fluorescence ratio in cells transfected with shEFHB or scramble plasmid. * represents $p < 0.05$ as compared to nuclear/cytosolic NFATc1 fluorescence ratio in cells transfected with scramble plasmid. Data are presented as the mean \pm SEM of 50-100 cells from 3-4 different experiments of each condition.

Calcium dependence of the STIM1 interaction with EFHB and SARAF upon store depletion

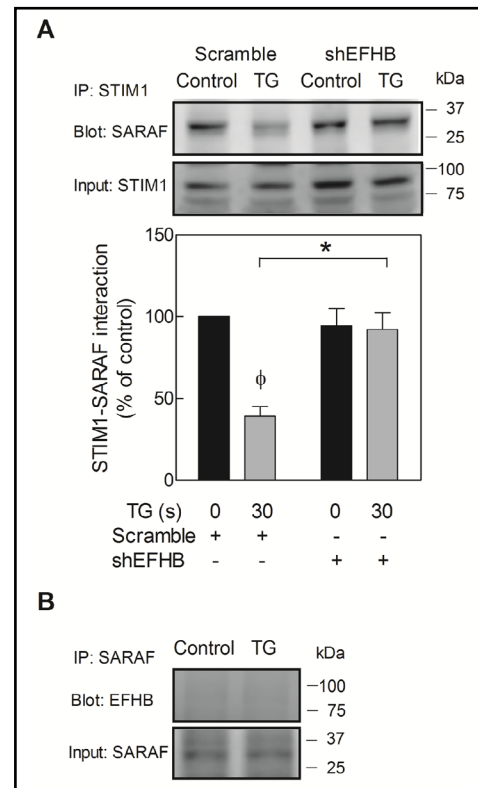
Since EFHB exhibits two putative EF-hand motifs, we further explored the Ca^{2+} dependence of the STIM1-EFHB interaction. HeLa cells, endogenously expressing STIM1 and EFHB, heavily loaded with the Ca^{2+} chelator dimethyl BAPTA were used for this study so as to prevent Ca^{2+} -, but not store depletion-, dependent responses. As shown in Fig. 6A, dimethyl BAPTA loading impaired TG-induced Ca^{2+} mobilization in the presence of 1 mM extracellular Ca^{2+} (35 cells/day/3 days). Immunoprecipitation of whole cell lysates with anti-STIM1 antibody followed by Western blotting with anti-EFHB antibody revealed a detectable interaction between both proteins in resting cells. Treatment of HeLa cells with TG resulted in a significant increase in the STIM1-EFHB interaction that reached a maximum after 30 s and then decreased, reaching the resting level 120 s after addition of the SERCA inhibitor, TG (Fig. 6B; $p < 0.05$; $n = 5$). Cell loading with dimethyl BAPTA did not significantly modify the STIM1-EFHB interaction under resting conditions (Fig. 6B). In cells loaded with BAPTA, TG induced an increase in the STIM1-EFHB interaction, which reached a maximum after 30 s of stimulation and then was sustained for at least 90 s. As a result, the interaction between STIM1 and EFHB induced by treatment with TG for 60 and 120 s was significantly greater in BAPTA-loaded cells (Fig. 6B; $p < 0.05$; $n = 5$). These observations strongly suggest that while the interaction of STIM1 with EFHB is independent on the rises in cytosolic free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), the dissociation between both proteins is Ca^{2+} dependent. These findings are supported by the mobilization of Ca^{2+} induced by TG in these cells. The fura-2 fluorescence ratio (340/380 nm) of resting HeLa cells was estimated as 0.52

Fig. 6. Role of cytosolic Ca^{2+} on the interaction of STIM1 with EFHB and SARAF. A, Fura-2-loaded dimethyl BAPTA-loaded HeLa cells, suspended in a Ca^{2+} -free medium (100 μM EGTA added), and control HeLa cells, suspended in a medium containing 1 mM Ca^{2+} , were stimulated with TG (1 μM). Traces are representative of 35 cells/day/3 days. Values are expressed as described in methods. B, Dimethyl BAPTA-loaded HeLa cells, suspended in a Ca^{2+} -free medium (100 μM EGTA added), and control HeLa cells, suspended in a medium containing 1 mM Ca^{2+} , were treated in the absence or presence of TG (1 μM) for 30, 60 and 120 s, as indicated, and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-EFHB or anti-SARAF antibody. Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 4 others. Molecular masses indicated on the right



were determined using molecular-mass markers run in the same gel. Data represent the quantification of STIM1-EFHB or STIM1-SARAF interaction in resting and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as percentage of control (cells not loaded with BAPTA and not stimulated with TG). * represents $p < 0.05$ as compared to their relative controls. ϕ represents $p < 0.05$ as compared to cells not loaded with dimethyl BAPTA. C, Dimethyl BAPTA-loaded HeLa cells, suspended in a Ca^{2+} -free medium (100 μM EGTA added), and control HeLa cells, suspended in a medium containing 1 mM Ca^{2+} , were treated in the absence or presence of TG (1 μM) for 30, 60 and 120 s, as indicated, and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-Orai1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-EFHB antibody. Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 3 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Data represent the quantification of Orai1-EFHB interaction in resting and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as percentage of control (cells not loaded with BAPTA and not stimulated with TG). * represents $p < 0.05$ as compared to their relative controls. ϕ represents $p < 0.05$ as compared to BAPTA-loaded cells. D, HeLa cells were transfected with siSARAF, SARAF overexpression plasmid or scramble plasmid (Control) and then treated in the absence or presence of TG (1 μM) for 30 s, as indicated, and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-EFHB antibody. Membranes were reprobbed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 2 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Data represent the quantification of STIM1-EFHB interaction in resting and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as the percentage of cells not stimulated with TG. * represents $p < 0.05$ as compared to control cells.

Fig. 7. EFHB expression knockdown impairs store depletion-induced STIM1-SARAF dissociation. A, HeLa cells were transfected with shEFHB or scramble plasmid, as indicated. Cells were left untreated (control) or stimulated with TG (1 μ M) for 30 s and lysed. Whole cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-SARAF antibody. Membranes were reprobed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 5 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. Bar graphs represent the quantification of STIM1-SARAF interaction in control (untreated) and TG-treated cells. Results are recorded as arbitrary optical density units, expressed as mean \pm S.E.M. and presented as percentage of control (resting cells transfected with scramble plasmid). * represents $p < 0.05$ as compared to cells transfected with scramble plasmid stimulated with TG. ϕ represents $p < 0.05$ as compared to resting cells transfected with scramble plasmid. B, HeLa cells were treated with 1 μ M TG for 30 s or left untreated and lysed. Whole cell lysates were immunoprecipitated with anti-SARAF antibody and immunoprecipitates were subjected to 10% SDS-PAGE and subsequent Western blotting with specific anti-EFHB antibody. Membranes were reprobed with the antibody used for immunoprecipitation for protein loading control. The panels show results from one experiment representative of 2 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel.



± 0.01 u.a. Cell treatment with 1 μ M TG in the presence of 1 mM extracellular Ca^{2+} increases the fura-2 fluorescence ratio to 0.62 ± 0.03 , 0.86 ± 0.04 and 1.09 ± 0.07 u.a. at 30, 60 and 120 s, respectively (Fig. 6A). These data indicate that about 83% of the Ca^{2+} response to TG takes place between 30 and 120 s after its addition, and the rise in $[Ca^{2+}]_c$ might support the dissociation of EFHB from STIM1.

SARAF has been presented as a regulator of STIM1 function that prevents STIM1 spontaneous activation, induces STIM1 deoligomerization upon Ca^{2+} store refilling and modulates SOCE [14]. SARAF interacts with STIM1 at rest [13-15] but upon store depletion it firstly dissociates from and then re-associates with STIM1 [13, 15]. The latter has been suggested to be involved in the activation of slow Ca^{2+} -dependent inactivation of SOCE [13, 24]. We have previously reported that, in MEG-01 cells, maximal STIM1-SARAF dissociation occurs approximately 30 s after the initiation of Ca^{2+} store depletion with TG and the STIM1-SARAF association returns to the resting level between 60 and 120 s after the addition of TG [15]. Here, we confirm the dynamic interaction of SARAF with STIM1 in HeLa cells. As shown in Fig. 6B, treatment of HeLa cells with TG evoked a significant initial decrease in SARAF-STIM1 interaction that reached a minimum of 51% of the resting level 30 s after stimulation ($P < 0.001$; $n = 5$). The interaction between SARAF and STIM1 then increased, reaching the basal level 120 s after stimulation. Interestingly, cell loading with BAPTA impaired the recovery of the SARAF-STIM1 interaction after 30 s of treatment with TG, without having any significant effect on the initial dissociation induced by store depletion (Fig. 6B; $n = 5$). These findings indicate that SARAF-STIM1 dissociation is not Ca^{2+} dependent while the secondary re-interaction between both proteins seems to be clearly dependent on the rises in $[Ca^{2+}]_c$.

We have also explored the time course of the EFHB-Orai1 interaction by looking for coimmunoprecipitation of HeLa lysates with anti-Orai1 antibody followed by Western blotting with anti-EFHB antibody. As shown in Fig. 6C, treatment of HeLa cells with 1 μM TG in a medium containing 1 mM Ca^{2+} significantly increased the EFHB-Orai1 interaction, as reported in HEK-293 cells (Fig. 2), which was maintained for at least 120 s ($P < 0.05$; $n = 4$). Interestingly, cell loading with dimethyl BAPTA impaired TG-evoked increase in the EFHB-Orai1 association (Fig. 6C; $P < 0.05$; $n = 4$), thus suggesting that this event depends on the rises in $[\text{Ca}^{2+}]_c$.

Furthermore, we have investigated whether SARAF expression interferes with the EFHB-STIM1 interaction. As shown in Fig. 6D, silencing SARAF expression did not significantly modify the interaction of EFHB with STIM1 in resting cells or cells stimulated with 1 μM TG for 30 s. By contrast, overexpression of SARAF significantly attenuated TG-induced EFHB-STIM1 interaction ($P < 0.05$; $n = 3$), which suggests that SARAF and EFHB might compete for the interaction with STIM1.

EFHB modulates the interaction of STIM1 with SARAF

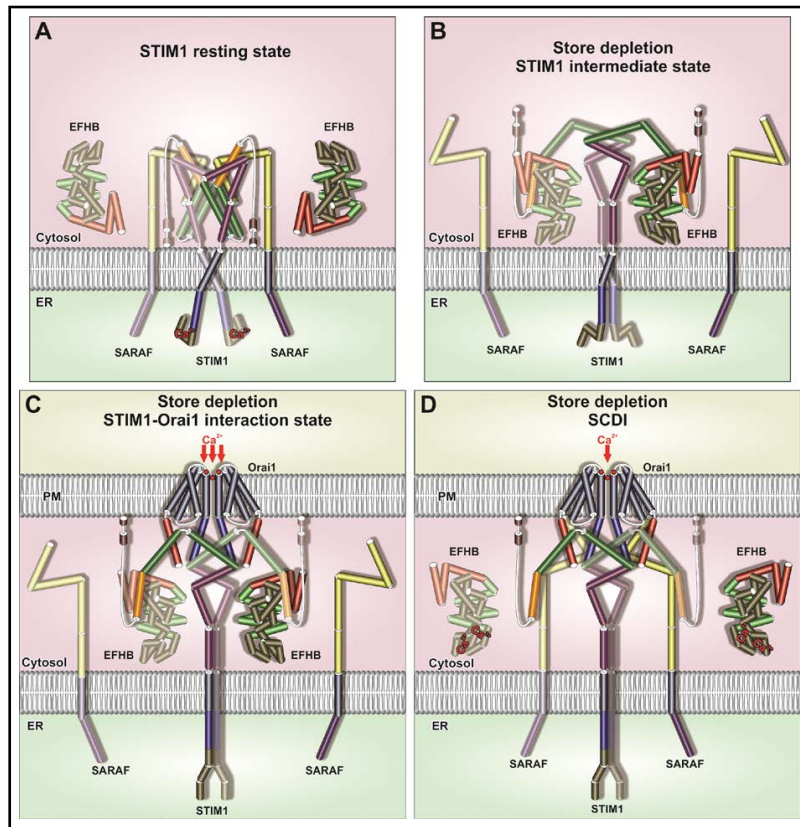
As depicted in Fig. 6, the dynamics of the interaction between STIM1 and both EFHB and SARAF is similarly regulated by Ca^{2+} store depletion and the rises in $[\text{Ca}^{2+}]_c$. Hence, we have assessed the role of EFHB in STIM1-SARAF interaction by testing the co-immunoprecipitation between both proteins in HeLa cells transfected with shEFHB or scramble plasmid. Immunoprecipitation of whole lysates from scramble-treated HeLa cells, at rest and upon stimulation with TG for 30 s, with anti-STIM1 antibody, followed by Western blotting with anti-SARAF antibody, revealed that the interaction between both proteins was significantly attenuated 30 s after addition of TG, confirming our previous studies [15] (Fig. 7A; $p < 0.05$; $n = 6$). Silencing EFHB expression by transfection of shEFHB abolishes TG-evoked SARAF-STIM1 dissociation (Fig. 7A; $p < 0.05$; $n = 6$), which indicates that EFHB is required for the dynamic STIM1-SARAF interplay upon store depletion. We have further explored the interaction between EFHB and SARAF in resting cells and cells stimulated with 1 μM TG; however, we have been unable to detect interaction between both proteins in resting or TG-treated cells (Fig. 7B; $n = 3$), which suggests that EFHB modulates the interaction of STIM1 with SARAF by direct association with STIM1.

Discussion

The present study identifies EFHB as a widely expressed protein that plays a relevant role in the activation of SOCE. A number of experimental data support this hypothesis. First of all, silencing EFHB expression at the protein level significantly inhibited SOCE in two unrelated cell types, HEK-293 and HeLa cells. Second, overexpression of an EFHB construct lacking the N-terminal 164 amino acids, therefore containing the EF-hand motifs, clearly enhances TG-induced SOCE in HeLa cells, without altering the resting cytosolic Ca^{2+} level or the ability of these cells to accumulate Ca^{2+} into the ER. Third, translocation of NFAT to the nucleus, a Ca^{2+} -dependent process activated by SOCE [7, 25, 26], has been found to be dependent on the expression of EFHB. Fourth, EFHB is able to interact with the key elements of SOCE, STIM1 and Orai1, and promotes STIM1-Orai1 interaction. Thus depletion of EFHB impairs the interaction between STIM1 and Orai1 while overexpression of this protein enhances the STIM1-Orai1 association. This latter finding suggests that EFHB plays an active role facilitating STIM1-Orai1 interaction.

Our results indicate that the interaction of STIM1 with EFHB is enhanced upon Ca^{2+} store depletion, reached a maximum 30 s after the addition of TG, and then returns to the resting level. Using cells heavily loaded with dimethyl BAPTA we have found that the interaction between STIM1 and EFHB induced by store depletion is independent on the rises in $[\text{Ca}^{2+}]_c$, while the subsequent dissociation is a Ca^{2+} -dependent mechanism. The time course of the interaction between STIM1 and EFHB resembles the association of STIM1 and its negative

Fig. 8. Tentative model for the role of EFHB in the STIM1-Orai1-SARAF interaction. A, Under resting conditions, ER-resident STIM1 is associated with SARAF, which prevents spontaneous activation of Orai1 channels in the PM. B, Upon Ca^{2+} store depletion, EFHB associates with STIM1 (intermediate state), thus promoting the dissociation of SARAF from STIM1. C, Dissociation of SARAF from STIM1 facilitates its interaction with and activation of Orai1 at ER-PM junctions. D, The rise in $[\text{Ca}^{2+}]_c$ due to Ca^{2+} entry through Orai1 channels induces the dissociation of EFHB from STIM1, which, in turn, facilitates the re-interaction of SARAF with STIM1 in order to mediate slow Ca^{2+} -dependent inactivation (SCDI) of Orai1 channels.



regulator, SARAF, previously reported [15], although occurring in opposite directions, which is compatible with the time course of SCDI. In agreement with previous studies in MEG-01 cells [15], we have found strong interaction between STIM1 and SARAF in HeLa cells that was reduced after store depletion, reaching a minimum 30 s after treatment with TG, and was followed by STIM1-SARAF re-interaction, which has been associated to the activation of SCDI [13]. Our results indicate that the initial dissociation between STIM1 and SARAF is independent on the rises in $[\text{Ca}^{2+}]_c$, while the re-interaction is Ca^{2+} dependent.

Our results also indicate that EFHB interacts with Orai1 in a Ca^{2+} -dependent manner. This interaction cannot be simply attributed to a non-specific detection secondary to the interaction of both proteins, Orai1 and EFHB, with STIM1 upon store depletion, as the initial step of the EFHB-STIM1 interaction is independent on the rises in $[\text{Ca}^{2+}]_c$. We have reported that Orai1 interacts with SARAF upon Ca^{2+} store depletion, which enhances Ca^{2+} entry through Orai1 [15]. The dynamics of the Orai1-SARAF interaction resembles the EFHB-Orai1 association [15] and both events might be related with an increase in Orai1 channel function.

The interactions of STIM1 with EFHB and SARAF share common features, i.e. both mechanisms are triggered by Ca^{2+} store depletion and are similarly modulated by $[\text{Ca}^{2+}]_c$, which suggests that EFHB might be involved in the regulation of the interaction of SARAF with STIM1. To confirm this possibility, we explored the role of EFHB in the interaction between SARAF and STIM1 and found that EFHB expression silencing impaired the dissociation of both proteins, which provides for the first time evidence for a role for EFHB in the modulation of the STIM1-SARAF interaction. Although some EFHB expression was still detectable after treatment with shEFHB, our results indicate that full EFHB expression is required for STIM1-SARAF dissociation. According to these observations, we propose a model (Fig. 8) where, under resting conditions, Orai1 and STIM1 are located in the PM and the ER membrane, respectively, and STIM1 is associated with SARAF, which prevents spontaneous activation

of SOCE, while EFHB is located in the cytosol. Ca^{2+} store depletion induces the association of EFHB with STIM1, thus promoting the dissociation of SARAF, which facilitates the interaction of STIM1 with and activation of Orai1 at ER-PM junctions. The rise in $[\text{Ca}^{2+}]_c$ as a result of Ca^{2+} entry via Orai1 channels, might be sensed by EFHB through the EF-hand motifs, which might induce dissociation of this protein from STIM1 and, thus, re-interaction of SARAF with STIM1 in order to mediate SCDI of Orai1 channels. In summary, we have identified EFHB as a new regulator of SOCE that modulates the dynamic interaction of STIM1 with SARAF and Orai1.

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Author contributions: A.B.-E. and J.A.R. conceived the project. J.A.R. wrote the manuscript. L. A., J. J. L., I.J., J. S.-C., P. J. C. and A. B.-E. performed the experiments and analyzed the data. G. M. S. and T. S. helped write the manuscript and were involved in data discussion. All authors reviewed and approved the manuscript.

Disclosure Statement

The authors declare that there are no conflicts of interest.

References

- 1 Baba Y, Nishida K, Fujii Y, Hirano T, Hikida M, Kurosaki T: Essential function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. *Nat Immunol* 2008;9:81-88.
- 2 Stiber J, Hawkins A, Zhang ZS, Wang S, Burch J, Graham V, Ward CC, Seth M, Finch E, Malouf N, Williams RS, Eu JP, Rosenberg P: STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. *Nat Cell Biol* 2008;10:688-697.
- 3 Berna-Erro A, Jardin I, Salido GM, Rosado JA: Role of STIM2 in cell function and physiopathology. *J Physiol* 2017;595:3111-3128.
- 4 Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA: STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J Cell Biol* 2005;169:435-445.
- 5 Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD: STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. *Nature* 2005;437:902-905.
- 6 Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S, Worley PF: STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol* 2006;8:1003-1010.
- 7 Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A: A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 2006;441:179-185.
- 8 Mercer JC, Dehaven WI, Smyth JT, Wedel B, Boyles RR, Bird GS, Putney JW, Jr.: Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *J Biol Chem* 2006;281:24979-24990.

- 9 Ambudkar IS, de Souza LB, Ong HL: TRPC1, Orai1, and STIM1 in SOCE: Friends in tight spaces. *Cell Calcium* 2017;63:33-39.
- 10 Stathopoulos PB, Zheng L, Li GY, Plevin MJ, Ikura M: Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. *Cell* 2008;135:110-122.
- 11 Muik M, Fahrner M, Schindl R, Stathopoulos P, Frischauf I, Derler I, Plenk P, Lackner B, Groschner K, Ikura M, Romanin C: STIM1 couples to ORAI1 via an intramolecular transition into an extended conformation. *EMBO J* 2011;30:1678-1689.
- 12 Yuan JP, Zeng W, Dorwart MR, Choi YJ, Worley PF, Muallem S: SOAR and the polybasic STIM1 domains gate and regulate Orai channels. *Nat Cell Biol* 2009;11:337-343.
- 13 Jha A, Ahuja M, Maleth J, Moreno CM, Yuan JP, Kim MS, Muallem S: The STIM1 CTID domain determines access of SARAF to SOAR to regulate Orai1 channel function. *J Cell Biol* 2013;202:71-79.
- 14 Palty R, Raveh A, Kaminsky I, Meller R, Reuveny E: SARAF inactivates the store operated calcium entry machinery to prevent excess calcium refilling. *Cell* 2012;149:425-438.
- 15 Albarran L, Lopez JJ, Ben Amor N, Martín-Cano FE, Berna-Erro A, Smani T, Salido GM, Rosado JA: Dynamic interaction of SARAF with STIM1 and Orai1 to modulate store-operated calcium entry. *Sci Rep* 2016;6:24452.
- 16 Lopez E, Berna-Erro A, Salido GM, Rosado JA, Redondo PC: FKBP52 is involved in the regulation of SOCE channels in the human platelets and MEG 01 cells. *Biochim Biophys Acta* 2013;1833:652-662.
- 17 Dionisio N, Smani T, Woodard GE, Castellano A, Salido GM, Rosado JA: Homer proteins mediate the interaction between STIM1 and Ca_v1.2 channels. *Biochim Biophys Acta* 2015;1853:1145-1153.
- 18 Albarran L, Lopez JJ, Woodard GE, Salido GM, Rosado JA: Store-operated Ca²⁺ entry-associated regulatory factor (SARAF) plays an important role in the regulation of arachidonate-regulated Ca²⁺ (ARC) channels. *J Biol Chem* 2016;291:6982-6988.
- 19 Lopez JJ, Albarran L, Jardin I, Sanchez-Collado J, Redondo PC, Bermejo N, Bobe R, Smani T, Rosado JA: Filamin A Modulates Store-Operated Ca²⁺ Entry by Regulating STIM1 (Stromal Interaction Molecule 1)-Orai1 Association in Human Platelets. *Arterioscler Thromb Vasc Biol* 2018;38
- 20 Zbidi H, Jardin I, Woodard GE, Lopez JJ, Berna-Erro A, Salido GM, Rosado JA: STIM1 and STIM2 are located in the acidic Ca²⁺ stores and associates with Orai1 upon depletion of the acidic stores in human platelets. *J Biol Chem* 2011;286:12257-12270.
- 21 Awla D, Zetterqvist AV, Abdulla A, Camello C, Berglund LM, Spéjel P, Pozo MJ, Camello PJ, Regnér S, Gomez MF, Thorlacius H: NFATc3 regulates trypsinogen activation, neutrophil recruitment, and tissue damage in acute pancreatitis in mice. *Gastroenterology* 2012;143:1352-1360.
- 22 Csutora P, Peter K, Kilic H, Park KM, Zarayskiy V, Gwozdz T, Bolotina VM: Novel role for STIM1 as a trigger for calcium influx factor production. *J Biol Chem* 2008;283:14524-14531.
- 23 Albarran L, Berna-Erro A, Dionisio N, Redondo PC, Lopez E, Lopez JJ, Salido GM, Brull Sabate JM, Rosado JA: TRPC6 participates in the regulation of cytosolic basal calcium concentration in murine resting platelets. *Biochim Biophys Acta* 2014;1843:789-796.
- 24 Jardin I, Albarran L, Salido GM, Lopez JJ, Sage SO, Rosado JA: Fine-tuning of store-operated calcium entry by fast and slow Ca²⁺-dependent inactivation: Involvement of SARAF. *Biochim Biophys Acta* 2017;1865:463-469.
- 25 Frischauf I, Zayats V, Deix M, Hochreiter A, Jardin I, Muik M, Lackner B, Svobodova B, Pammer T, Litvinukova M, Sridhar AA, Derler I, Bogeski I, Romanin C, Etrich RH, Schindl R: A calcium-accumulating region, CAR, in the channel Orai1 enhances Ca²⁺ permeation and SOCE-induced gene transcription. *Sci Signal* 2015;8:ra131.
- 26 Cheng KT, Liu X, Ong HL, Swaim W, Ambudkar IS: Local Ca²⁺ entry via Orai1 regulates plasma membrane recruitment of TRPC1 and controls cytosolic Ca²⁺ signals required for specific cell functions. *PLoS Biol* 2011;9:e1001025.