Filamin A Modulates Store-Operated Ca^{2+} Entry by Regulating STIM1 (Stromal Interaction Molecule 1)–Orai1 Association in Human Platelets

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Objective—Here, we provide evidence for the role of FLNA (filamin A) in the modulation of store-operated calcium entry (SOCE).

Approach and Results—SOCE is a major mechanism for calcium influx controlled by the intracellular Ca^{2+} stores. On store depletion, the endoplasmic reticulum calcium sensor STIM1 (stromal interaction molecule 1) redistributes into puncta at endoplasmic reticulum/plasma membrane junctions, a process supported by the cytoskeleton, where it interacts with the calcium channels; however, the mechanism for fine-tuning SOCE is not completely understood. Our results demonstrate that STIM1 interacts with FLNA on calcium store depletion in human platelets. The interaction is dependent on the phosphorylation of FLNA at Ser^{2152} by the cAMP-dependent protein kinase. Impairment of FLNA phosphorylation and knockdown of FLNA expression using siRNA increased SOCE in platelets. Similarly, SOCE was significantly greater in FLNA-deficient melanoma M2 cells than in the FLNA-expressing M2 subclone A7. Expression of FLNA in M2 cells attenuated SOCE, an effect prevented when the cells were transfected with the nonphosphorylatable FLNA S2152A mutant. Transfection of M2 cells with the STIM1(K684,685E) mutant reduced the STIM1–FLNA interaction. In platelets, attenuation of FLNA expression using siRNA resulted in enhanced association of STIM1 with the cytoskeleton, greater STIM1–Orai1 interaction, and SOCE. Introduction of an anti-FLNA (2597–2647) antibody attenuated the STIM1–FLNA interaction and enhanced thrombin-induced platelet aggregation.

Conclusions—Our results indicate that FLNA modulates SOCE and then the correct platelet function, by fine-tuning the distribution of STIM1 in the cytoskeleton and the interaction with Orai1 channels.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38: 386-397. DOI: 10.1161/ATVBAHA.117.310139.)**

Key Words: calcium signaling ■ filamins ■ ion channel ■ phosphorylation ■ stromal interaction molecule 1

Store-operated calcium entry (SOCE) is a major mechanism for calcium influx triggered by depletion of the intracellular Ca^{2+} stores, a phenomenon that is sensed by the proteins of the STIM family, with a predominant role of the STIM1 (stromal interaction molecule 1).[1] STIM1 is a protein with a single transmembrane domain located in the endoplasmic reticulum (ER),[2] the lysosomal like acidic Ca^{2+} stores,[3] and the plasma membrane.[4,5] On Ca^{2+} store depletion, that is, by inhibition of the Ca^{2+}-ATPase SERCA using thapsigargin, ER-resident STIM1 forms oligomers and distributes into puncta at the ER/plasma membrane (PM) junctions.[6–9] Activation of Ca^{2+} release–activated channels (CRAC) requires that STIM1 undergoes a conformational change from a closed to an extended state, which facilitates the communication with the channels.[10] Redistribution of STIM1 into puncta involves the association with the cytoskeleton and movement along the microtubules.[11] Furthermore, maneuvers aimed at disorganizing or stabilizing the cytoskeleton have resulted in impairment of the STIM1–Orai1 interaction and SOCE.[12,13]

FLNA (Filamin A) is a 2647-amino acid–long actin-cross-linking protein, with 2 N-terminal actin-binding domains followed by 24 immunoglobulin-like repeats.[14] FLNA is mostly located in the membrane cytoskeleton, underneath the PM, where it has been found to regulate a variety of cytoskeleton-related processes, including receptor clustering and cross talk among different receptors and the actin cytoskeleton.[14] FLNA has been found to be phosphorylated by PKA (protein kinase A),[15] and FLNA phosphorylation at Ser^{2152}, located in the Ig20 repeat, by PKA is essential for many cellular functions, such as cytoskeleton remodeling, cell migration, and platelet aggregation.[16–18] Mouse models with deficient expression of FLNA have been found to show platelets that fail to spread, have

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Nonstandard Abbreviations and Acronyms	
FLNA	filamin A
PKA	protein kinase A
SOCE	store-operated calcium entry
STIM1	stromal interaction molecule 1

decreased α-granule secretion and protein tyrosine phosphorylation, and exhibit hyperaggregability and thrombocytopenia because of platelet loss,[19,20] which indicates that FLNA plays a functional role in platelets.

Here, we have investigated the possible function of FLNA on the STIM1–Orai1 interaction and SOCE. We have found that on Ca^{2+} store depletion, STIM1 interacts with FLNA in human platelets in a Ca^{2+}-dependent manner, which requires phosphorylation at FLNA Ser^{2152}. FLNA expression knockdown results in clustering of STIM1 in the cytoskeletal fraction and enhancement of STIM1–Orai1 interaction, as well as SOCE and platelet function. These findings indicate that FLNA plays an essential role in the cross talk between STIM1 and Orai1 and modulates SOCE, avoiding Ca^{2+} overload, and then ensuring a correct platelet function.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Identification of FLNA as a New STIM1-Associated Protein

To ascertain whether FLNA is associated with STIM1 on Ca^{2+} store depletion, human platelets were stimulated with 1 µmol/L thapsigargin in the presence of 1 mmol/L extracellular Ca^{2+} or left untreated and lysed after 30 seconds. Whole-cell lysates were precleared with protein A-agarose and immunoprecipitated with anti-STIM1 antibody. We identified a band >150 kDa whose association with STIM1 was modified after treatment with thapsigargin with apparent molecular mass of ≈280 kDa (Figure 1A). The 280-kDa band was analyzed by MALDI TOF/TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry at the Biotechnology National Center (Madrid, Spain) yielding the results presented in the Table. The protein with the highest score was identified as FLNA, and the score provided for the remaining proteins was relatively low; hence, we analyzed the FLNA–STIM1 interaction.

FLNA Coimmunoprecipitates With STIM1 in a Ca^{2+}-Dependent Manner

Immunoprecipitation and subsequent Western blotting were conducted in platelets suspended in a medium containing 1 mmol/L Ca^{2+} and in platelets heavily loaded with dimethyl-BAPTA (1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), to prevent Ca^{2+}, but not store depletion, -dependent signals, that were suspended in a Ca^{2+}-free medium (125 µmol/L EGTA added). Platelets were stimulated with thapsigargin or left untreated. The analysis revealed the presence of FLNA in resting BAPTA-loaded and control cells (Figure 1B, top; n=6).

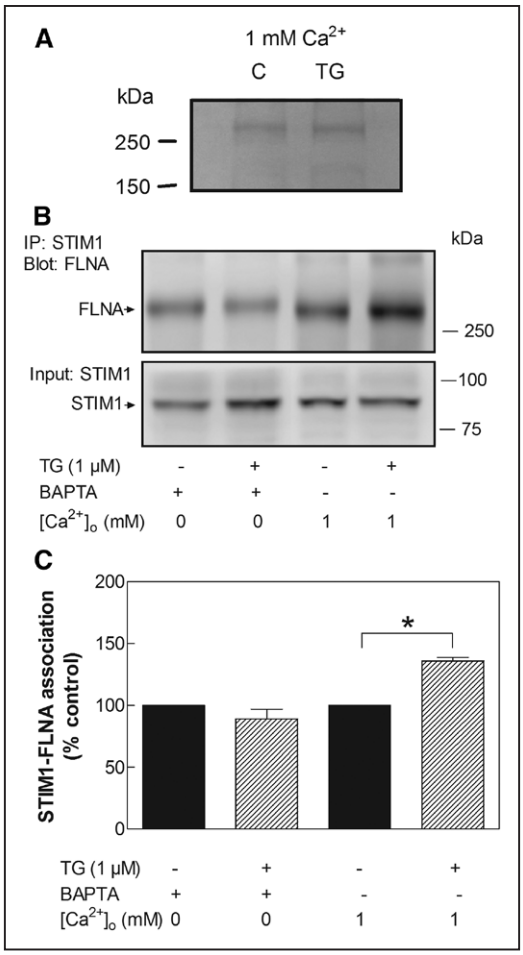

Figure 1. FLNA (filamin A) coimmunoprecipitates with STIM1 (stromal interaction molecule 1) in a Ca^{2+}-dependent manner. **A**, Human platelets were treated with 1 µmol/L thapsigargin (TG) for 30 s and lysed. Platelet lysates were precleared by incubation for 1 h with protein A-agarose and then were immunoprecipitated with anti-STIM1 antibody. Immunoprecipitated proteins were separated by SDS/PAGE followed by silver staining of the gel as described in the Materials and Methods section in the online-only Data Supplement. Positions of molecular-mass markers are shown on the **left**. **B** and **C**, Platelets were loaded with dimethyl BAPTA or left untreated and suspended in a Ca^{2+}-free medium ($[Ca^{2+}]_o$=0 mmol/L) or a medium containing 1 mmol/L Ca^{2+}, as indicated. Cells were then stimulated with 1 µmol/L TG for 30 s or the vehicle and then lysed. Platelet lysates were immunoprecipitated with anti-STIM1 antibody, and proteins were separated by SDS/PAGE followed by Western blotting with anti-FLNA antibody (**B**, **top**). Membranes were reprobed with anti-STIM1 antibody (**B**, **bottom**) as described in the Materials and Methods section in the online-only Data Supplement. Positions of molecular-mass markers are shown on the **right**. **C**, Bar graphs represent the quantification of STIM1–FLNA coimmunoprecipitation. Values are mean±SEM of 6 independent experiments. * P<0.05, Student t test) compared with the STIM1–FLNA association in resting cells.

In cells suspended in a medium containing 1 mmol/L Ca^{2+}, coimmunoprecipitation of STIM1 and FLNA was significantly enhanced by 40% on treatment with thapsigargin (Figures 1B and 1C; P<0.05; n=6). By contrast, in BAPTA-loaded cells thapsigargin was unable to increase the STIM1–FLNA interaction (Figure 1B and 1C; P<0.05; n=6), which indicates that the STIM1–FLNA association requires rises in $[Ca^{2+}]_c$. Reprobing of the membranes with anti-STIM1 antibody confirmed a similar content of this protein in all lanes (Figure 1B, bottom).

Table. Protein Identification by MALDI-TOF Mass Spectrometry of the 280-kDa Band

	Accession	Mass	Score	Description
1	P21333	283 301	763	Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4
2	Q14315	293 407	56	Filamin-C OS=Homo sapiens GN=FLNC PE=1 SV=3
3	P50990	60 153	44	T-complex protein 1 subunit theta OS=Homo sapiens GN=CCT8 PE=1 SV=4
4	P49642	50 327	41	DNA primase small subunit. OS=Homo sapiens GN=PRIM1 PE=1 SV=1
5	Q7Z406	228 701	39	Myosin-14 OS=Homo sapiens GN=MYH14 PE=1 SV=2
6	Q9Y490	271766	39	Talin-1 OS=Homo sapiens GN=TLN1 PE=1 SV=3
7	Q96PX9	141 576	39	Pleckstrin homology domain-containing family G member 4B OS=Homo sapiens GN=PLEKHG4B PE=2 SV=4
8	Q9H9G7	98 495	37	Protein argonaute-3 OS=Homo sapiens GN=AGO3 PE=1 SV=2
9	Q9HCK5	98 175	36	Protein argonaute-4 OS=Homo sapiens GN=AGO4 PE=1 SV=2
10	Q96EK9	38 820	36	Protein KTI12 homolog OS=Homo sapiens GN=KTI12 PE=1 SV=1
11	P26196	54 781	35	Probable ATP-dependent RNA helicase DDX6 OS=Homo sapiens GN=DDX6 PE=1 SV=2
12	Q9HCX3	77 450	34	Zinc finger protein 304 OS=Homo sapiens GN=ZNF304 PE=2 SV=2
13	Q9NQ75	87 888	34	Cas scaffolding protein family member 4 OS=Homo sapiens GN=CASS4 PE=1 SV=2
14	P05423	44 539	34	DNA-directed RNA polymerase III subunit RPC4 OS=Homo sapiens GN=POLR3D PE=1 SV=2
15	Q7RTP6	225 297	32	Protein-methionine sulfoxide oxidase MICAL3 OS=Homo sapiens GN=MICAL3 PE=1 SV=2

GN indicates gene name; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OS, organism name; PE, protein existence; and SV, sequence version.

FLNA–STIM1 Interaction Requires Phosphorylation of FLNA at Serine 2152

FLNA phosphorylation at residue 2152 has been reported to play a crucial role in its biological functions.[21–23] It is well recognized that FLNA can be phosphorylated at Ser^{2152} by PKA,[15,24] a relevant event for its function.[18,25] To investigate whether phosphorylation at Ser^{2152} is required for the STIM1–FLNA interaction, we first explored whether Ca^{2+} store depletion is able to induce FLNA phosphorylation and whether this event is Ca^{2+} dependent, as for the STIM1–FLNA interaction (see above). As shown in Figure 2A (top) and 2C, thapsigargin modifies FLNA phosphorylation at Ser^{2152} in a time-dependent manner. An increase in FLNA phosphorylation was detected after 10 seconds of treatment, which reached a maximum within 30 seconds with a 1.44±0.04 (SEM)-fold increase and then decreased, reaching the resting level after 120 seconds of stimulation. In BAPTA-loaded cells, thapsigargin was unable to induce FLNA phosphorylation at Ser^{2152}, which strongly supports that this event requires changes in $[Ca^{2+}]_c$ (Figures 2B and 2C; $P<0.05$; n=6). Membranes were reprobed with anti-β-actin antibody for protein loading control (Figures 2A and 2B, bottom).

We have further explored whether FLNA phosphorylation at Ser^{2152} evoked by store depletion is dependent on PKA, as previously reported under other conditions,[24] by using KT-5720, which impairs PKA activation in human platelets.[26] As depicted in Figures 2D and 2F, preincubation of platelets for 30 minutes at 37°C with 1 µmol/L KT-5720 abolished thapsigargin-induced FLNA phosphorylation at least during 120 seconds ($P<0.05$; n=6). By contrast, inhibition of the brefeldin A–sensitive guanine nucleotide-exchange 2 has been found to lead to FLNA phosphorylation; hence, we have explored the effect of brefeldin A on FLNA Ser^{2152} phosphorylation in human platelets. As depicted in Figures 2E and 2F, treatment with brefeldin A (100 µmol/L) for 60 minutes significantly enhanced FLNA phosphorylation at Ser^{2152} both in nonstimulated cells and in cells stimulated with thapsigargin for 10, 30, and 60 seconds ($P<0.05$).

Next, we explored whether phosphorylation at Ser^{2152} is relevant for the association between FLNA and STIM1. As shown in Figures 3A and 3B, treatment of human platelets with KT-5720 abolished the STIM1–FLNA interaction evoked by thapsigargin without modifying the association at rest (Figures 3A and 3B; $P<0.05$; n=6). These findings strongly support that phosphorylation at Ser^{2152} is a prerequisite for the interaction of FLNA with STIM1.

As expected, preincubation of human platelets with KT-5720 abolished the coimmunoprecipitation of FLNA phosphorylated with STIM1 (Figures 3C and 3D; $P<0.05$; n=6).

Phosphorylation of FLNA at Serine 2152 Modulates Store-Operated Ca^{2+} Entry

Because FLNA phosphorylated at Ser^{2152} associates with STIM1 on store depletion, we have explored its role in SOCE. As shown in Figure 3E, treatment of platelets with thapsigargin in a Ca^{2+}-free medium resulted in a prolonged elevation of $[Ca^{2+}]_c$, because of Ca^{2+} release from intracellular stores. The subsequent addition of Ca^{2+} (1 mmol/L) to the external medium induced a sustained increase in $[Ca^{2+}]_c$, indicative of SOCE. Preincubation with KT-5720 was unable to alter thapsigargin-evoked Ca^{2+} release from the stores but significantly enhanced SOCE by 45% (the integrals of the rise in $[Ca^{2+}]_c$ after the addition of $CaCl_2$ were 74 072±6442 and 107 404±1750 nM·s in the absence and presence of KT-5720; Figure 3F; $P<0.05$). These findings suggest that impairment of the association between STIM1 and FLNA, by dephosphorylation of

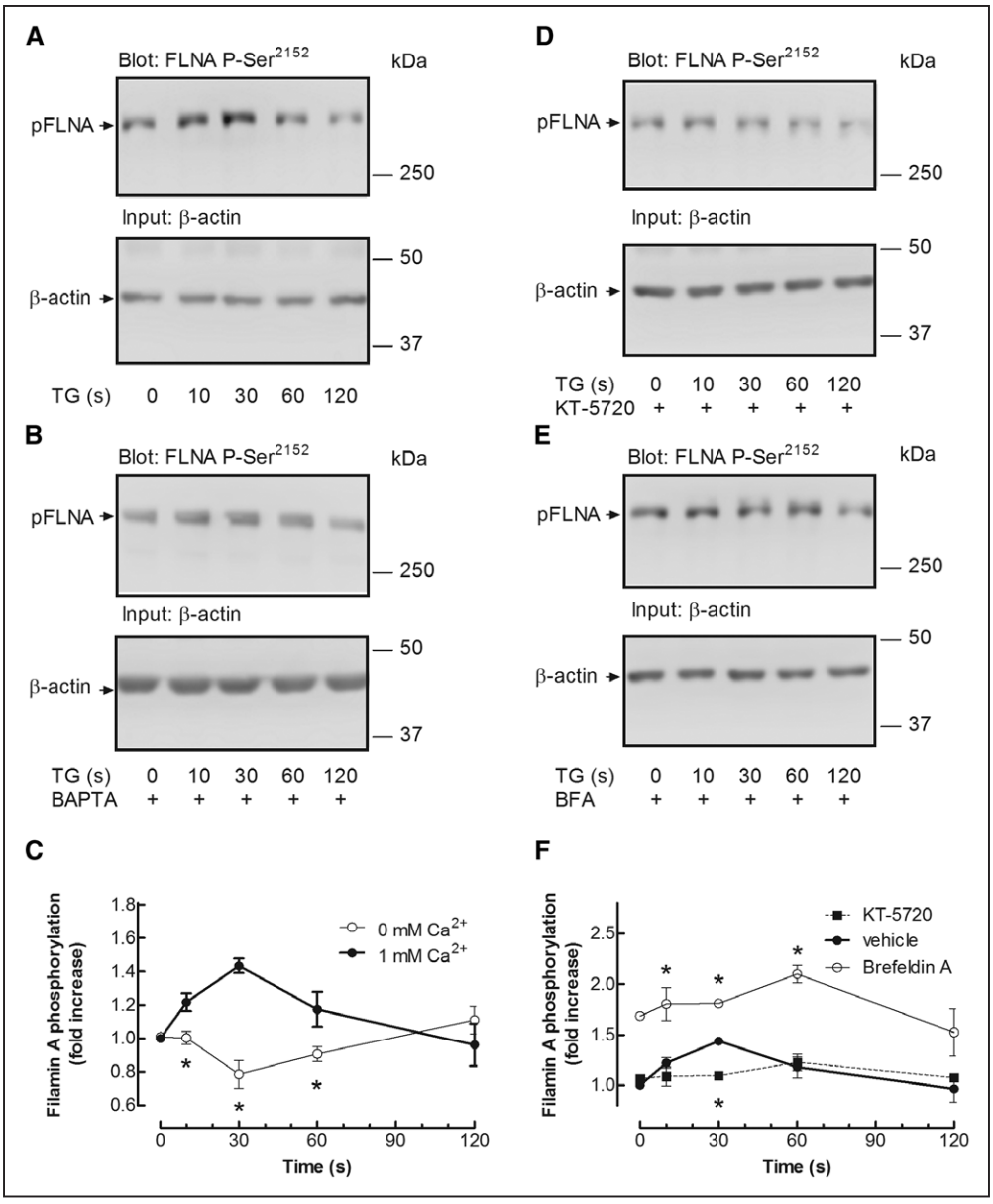

Figure 2. Phosphorylation of FLNA (filamin A) at serine 2152 is Ca^{2+}- and PKA-dependent process. **A–C**, Platelets were loaded with dimethyl BAPTA (**B**) or left untreated (**A**) and suspended in a Ca^{2+}-free medium (**B**) or a medium containing 1 mmol/L Ca^{2+} (**A**). Cells were then stimulated with 1 μmol/L thapsigargin (TG) for various periods of time (10–120 s) and lysed. Proteins were separated by SDS/PAGE followed by Western blotting with anti-phospho-FLNA (FLNA P-Ser^{2152}) antibody (**top**) or anti-β-actin antibody (**bottom**) as described in the Materials and Methods section in the online-only Data Supplement. Positions of molecular-mass markers are shown on the **right**. **C**, Graph represents the quantification of FLNA phosphorylation. Values are mean±SEM of 6 separate experiments. *P<0.05 (ANOVA combined with the Dunnett test) compared with FLNA phosphorylation in cells not loaded with BAPTA. **D–F**, Platelets were pretreated at 37°C for 30 min with 1 μmol/L KT-5720 (**D**) or for 60 min with 100 μmol/L brefeldin A and then stimulated with 1 μmol/L TG for various periods of time (10–120 s) in the presence of 1 mmol/L extracellular Ca^{2+} and lysed. Proteins were separated by SDS/PAGE followed by Western blotting with anti-phospho-FLNA (pSer2152) antibody (**top**) or anti-β-actin antibody (**bottom**) as described in the Materials and Methods section in the online-only Data Supplement. Positions of molecular-mass markers are shown on the **right**. **F**, Graph represents the quantification of FLNA phosphorylation. Values are mean±SEM of 6 separate experiments. *P<0.05 (ANOVA combined with the Dunnett test) compared with FLNA phosphorylation in cells treated with vehicle.

the latter, results in a significant increase in SOCE. To further explore the role of FLNA phosphorylation at Ser^{2152} on SOCE, we tested the effect of brefeldin A. As shown in Figure 3E, platelet treatment with brefeldin A significantly attenuated SOCE without having any effect on thapsigargin-induced Ca^{2+} release, which further supports the role of FLNA phosphorylation at Ser^{2152} on the activation of SOCE (the integral of the rise in $[Ca^{2+}]_c$ after the addition of $CaCl_2$ in the presence of brefeldin A was 62 208±3066 nM·s; Figure 3F; P<0.05; n=6).

FLNA Plays a Relevant Role in the Modulation of Store-Operated Ca^{2+} Entry

The use of KT-5720 and brefeldin A provides indirect evidence for the involvement of FLNA on SOCE. Hence, we

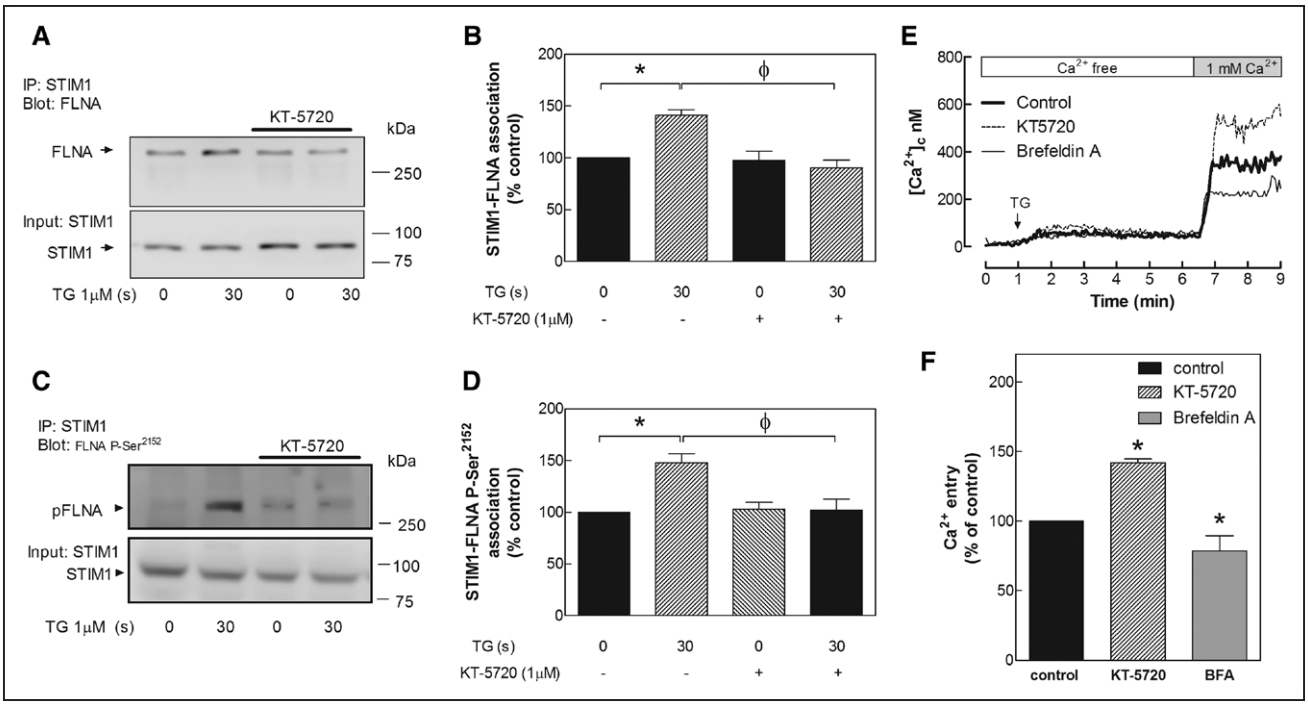

Figure 3. FLNA (filamin A) phosphorylation at serine 2152 is essential for its interaction with STIM1 (stromal interaction molecule 1) and the modulation of store-operated Ca^{2+} entry. **A** and **B**, platelets were pretreated for 30 min with 1 μmol/L KT-5720 at 37°C or the vehicle in a medium containing 1 mmol/L Ca^{2+}. Cells were then stimulated with 1 μmol/L thapsigargin (TG) for 30 s or the vehicle and then lysed. Platelet lysates were immunoprecipitated with anti-STIM1 antibody, and proteins were separated by SDS/PAGE followed by Western blotting with anti-FLNA antibody (**A**, **top**). Membranes were reprobed with anti-STIM1 antibody (**A**, **bottom**) as described in the Materials and Methods section in the online-only Data Supplement. Positions of molecular mass markers are shown on the **right**. **B**, Bar graphs represent the quantification of STIM1–FLNA coimmunoprecipitation. Values are mean±SEM of 6 independent experiments. *P<0.05 compared with the STIM1–FLNA association in resting cells and $^{\Phi}P$<0.05 compared with the STIM1–FLNA association in cells not treated with KT-5720 (ANOVA combined with the Dunnett test). **C** and **D**, Platelets were pretreated for 30 min with 1 μmol/L KT-5720 or the vehicle in a medium containing 1 mmol/L Ca^{2+}. Cells were then stimulated with 1 μmol/L TG for 30 s or the vehicle and then lysed. Platelet lysates were immunoprecipitated with anti-STIM1 antibody followed by Western blotting with antiphospho-FLNA (pSer2152) antibody (**C**; **top**). Membranes were reprobed with anti-STIM1 antibody (**C**; **bottom**) as described in the Materials and Methods section in the online-only Data Supplement. Positions of molecular-mass markers are shown on the **right**. **D**, Bar graphs represent the quantification of STIM1-phospho-FLNA coimmunoprecipitation. Values are mean±SEM of 6 independent experiments. *P<0.05 compared with the STIM1-phospho-FLNA association in resting cells and $^{\Phi}P$<0.05 compared with the STIM1-phospho-FLNA association in cells not treated with KT-5720 (ANOVA combined with the Dunnett test). **E**, Fura-2-loaded human platelets were preincubated for 30 min with 1 μmol/L KT-5720, for 60 min with 100 μmol/L brefeldin A or the vehicle at 37°C. Cells were then stimulated with TG (1 μmol/L) in a Ca^{2+}-free medium (125 μmol/L EGTA added), and 5½ min later, $CaCl_2$ (final concentration 1 mmol/L) was added to the medium to initiate Ca^{2+} entry. $[Ca^{2+}]_c$ was monitored as described in the Materials and Methods section in the online-only Data Supplement. Traces are representative of 6 independent experiments. **F**, Bar graphs indicating the percentage of Ca^{2+} entry under the different conditions relative to their control (vehicle was added). Ca^{2+} entry was determined as described in the Materials and Methods section in the online-only Data Supplement. Values are means±SEM; significant values indicate differences compared with TG-treated cells in the absence of inhibitors. *P<0.05 (ANOVA).

have more directly evaluated the role of FLNA in SOCE by using interference RNA-based technology. siRNA has previously been proven to be an efficient technique for silencing the expression of platelet proteins.[27] In human platelets, transfection with FLNA siRNA significantly attenuated the expression of FLNA at the protein level after 24 hours (Figure 4A; P<0.05; n=3). To explore the role of FLNA on SOCE, platelets were transfected with FLNA siRNA, scramble target sequence (siRNA A), or left untreated. As shown in Figure 4B, the resting, as well as thapsigargin-evoked Ca^{2+} release from the stores, was not affected by any of the experimental maneuvers (Figures 4B and 4C); however, attenuation of FLNA expression significantly enhanced SOCE (the integrals of the rise in $[Ca^{2+}]_c$ after the addition of $CaCl_2$ were 146 748±20 634, 67 548±12 357 and 61 796±10 092 nM·s in cells transfected with FLNA siRNA, siRNA A, or left untreated, respectively; Figures 4B and 4D; P<0.05; n=6). These observations provide evidence for a relevant role of FLNA in the modulation of SOCE.

FLNA Modulates the Location of STIM1 in the Cytoskeletal Fraction and the Interaction With Orai1

Because FLNA is a cytoskeletal protein, we have explored whether it modulates the association of STIM1 with the platelet cytoskeleton. As shown in Figure 5A, attenuation of FLNA expression significantly enhanced the location of STIM1 in the cytoskeletal fraction (P<0.05; n=6). Consistent with this, the expression of STIM1 in the cytosolic and membrane fraction was reduced in cells transfected with FLNA siRNA (Figure 5B; P<0.05; n=6). These findings indicate that FLNA modulates the association of STIM1 with the cytoskeleton. As we have previously reported that the cytoskeleton plays a relevant role in the interaction between STIM1 and Orai1,[12]

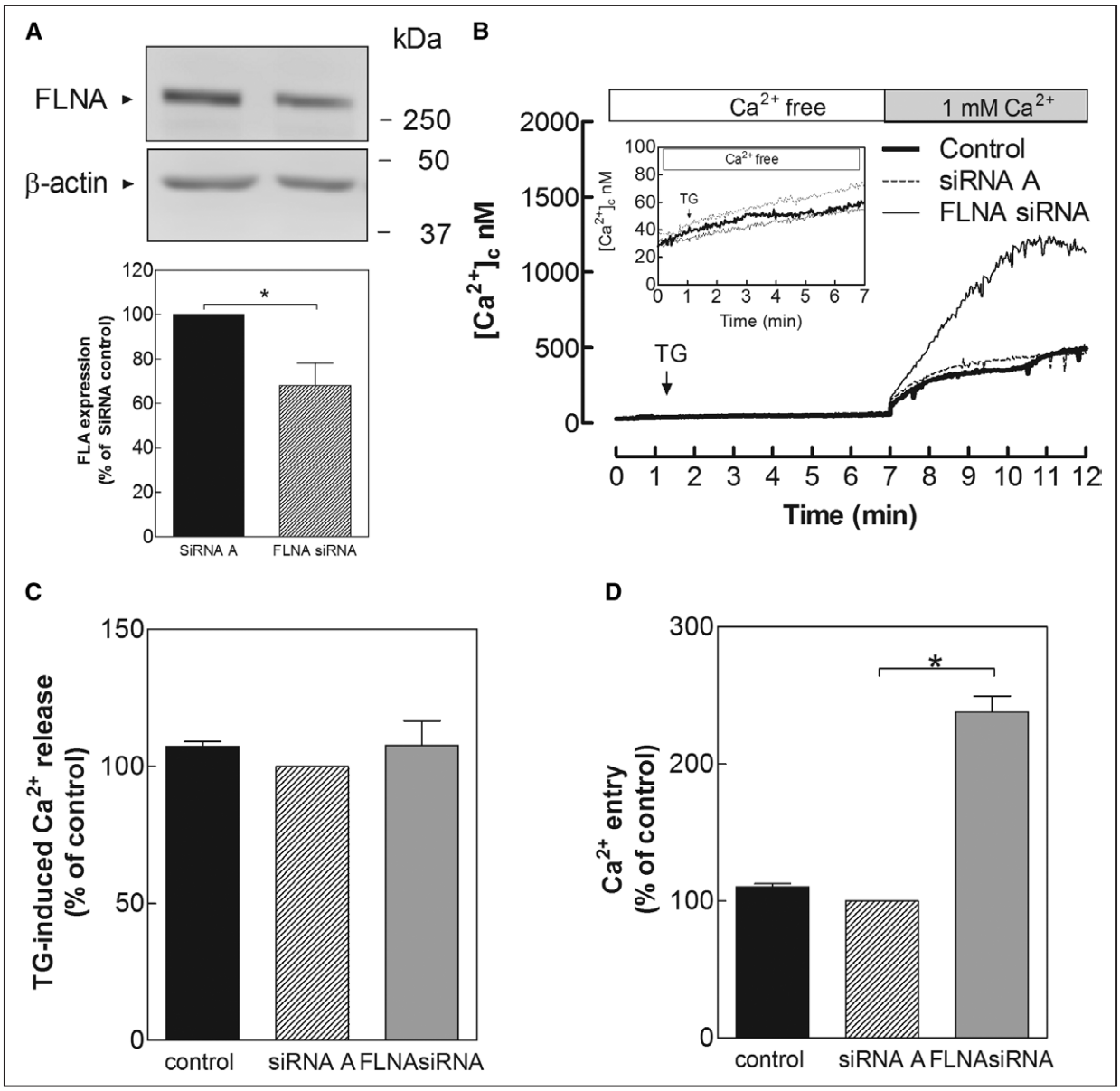

Figure 4. FLNA (filamin A) knockdown enhances store-operated Ca^{2+} entry. Human platelets were transfected with FLNA siRNA or scrambled siRNA (siRNA A). **A**, Twenty-four h after transfection, cells were lysed and subjected to Western blotting with anti-FLNA antibody, followed by reprobing with anti-β-actin antibody for protein loading control. *P<0.05, Student t test. **B**, Platelets were transfected with FLNA siRNA or scrambled siRNA (siRNA A). Twenty-four h later, cells were loaded with fura-2. Cells were then stimulated with thapsigargin (TG; 1 μmol/L) in a Ca^{2+}-free medium (125 μmol/L EGTA added), and 5½ min later, $CaCl_2$ (final concentration 1 mmol/L) was added to the medium to initiate Ca^{2+} entry. $[Ca^{2+}]_c$ was monitored as described in the Materials and Methods section in the online-only Data Supplement. Traces are representative of 6 independent experiments. **C** and **D**, Bar graphs indicating the percentage of Ca^{2+} release and entry under the different conditions relative to their control (cells transfected with siRNA A). Ca^{2+} release and entry were determined as described in the Materials and Methods section in the online-only Data Supplement. Values are means±SEM; significance values indicate differences compared with control (cells transfected with siRNA A). *P<0.05, ANOVA combined with the Dunnett test.

we have investigated the role of FLNA in the association of STIM1 with Orai1 in platelets transfected with FLNA siRNA or siRNA A and then stimulated with thapsigargin or left untreated. After immunoprecipitation with anti-STIM1 antibody, Western blotting revealed the presence of Orai1 in samples from resting platelets transfected with scramble siRNA, which was significantly enhanced by 32±9% on treatment with thapsigargin (Figure 5C; P<0.05; n=6). Interestingly, both resting and thapsigargin-evoked STIM1–Orai1 association was significantly greater in platelets transfected with FLNA siRNA (Figure 5C; P<0.05; n=6), indicating that FLNA regulates SOCE by modulating the association of STIM1 with the cytoskeleton and, subsequently, STIM1–Orai1 interaction.

We have further explored the role of FLNA on ex vivo platelet aggregation in response to the physiological agonist thrombin. Human platelets transfected with siRNA A or FLNA siRNA for 24 hours were stimulated with 1 U/mL thrombin in a medium containing 1 mmol/L Ca^{2+}. The percentage of aggregation on stimulation with thrombin was 52±7% in cells transfected with siRNA A and 73±5% in cells transfected with

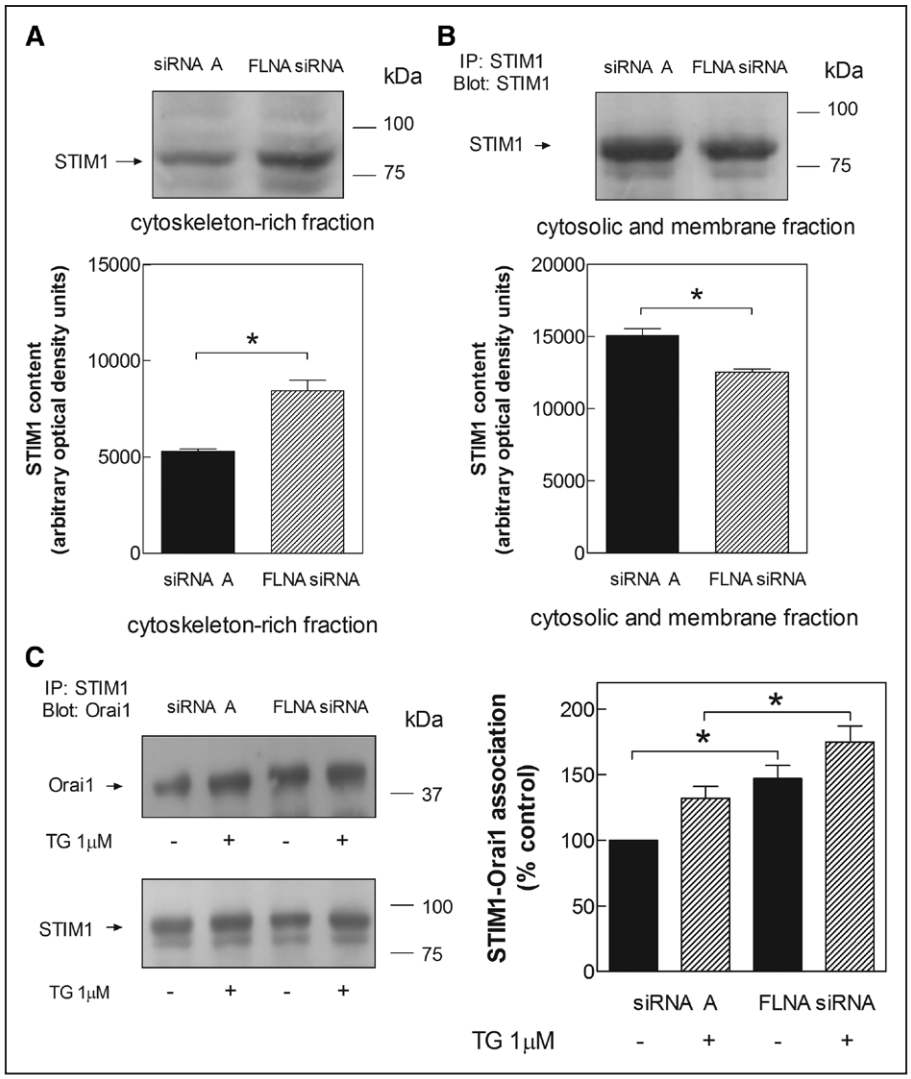

Figure 5. FLNA (filamin A) knockdown modulates the association of STIM1 (stromal interaction molecule 1) with the cytoskeleton and its interaction with Orai1. Human platelets were transfected with FLNA siRNA or scrambled siRNA (siRNA A). Twenty-four h after transfection, cells were lysed with triton buffer to separate cytoskeletal fraction (**A**) and cytosolic and membrane fraction (**B**), as indicated. The pellet (cytoskeletal fraction) was subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-STIM1 antibody. The supernatant (cytosolic and membrane fraction) was immunoprecipitated with the anti-STIM1 antibody followed by Western blotting with the same antibody. The image shows results from 1 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 in the cytoskeletal or cytosolic and membrane fractions. Results are presented as arbitrary optical density units and expressed as mean±SEM; significant values indicate differences compared with control (cells transfected with siRNA A). *P<0.05, Student t test. **C**, Platelets were transfected with FLNA siRNA or scrambled siRNA (siRNA A). Twenty-four h after transfection, cells were stimulated with 1 μmol/L thapsigargin (TG) for 30 s and lysed. Platelet lysates were immunoprecipitated with anti-STIM1 antibody, and proteins were separated by SDS/PAGE followed by Western blotting with anti-Orai1 antibody (**top**). Membranes were reprobed with anti-STIM1 antibody (**bottom**) for protein loading control. Positions of molecular-mass markers are shown on the **right**. Bar graphs represent the quantification of STIM1-Orai1 coimmunoprecipitation. Values are mean±SEM of 6 independent experiments. *P<0.05, ANOVA combined with the Dunnett test.

FLNA siRNA (P<0.05; n=6), indicating that FLNA modulates platelet aggregation probably by regulating Ca^{2+} signaling.

FLNA Modulates SOCE in Melanoma Cells Through Phosphorylation at Residue 2152

To confirm the regulatory role of FLNA in SOCE observed in human platelets, we have used the FLNA-deficient melanoma cell line M2 and the FLNA-expressing M2 subclone A7.[28] As shown in Figure 6A, top, FLNA was strongly detected in A7 cells but was undetectable in the M2 cell line. In A7 cells, our results indicate that treatment with the agonist endothelin-1 (10 nM) for 30 and 60 seconds significantly enhances FLNA phosphorylation at Ser^{2152} (Figure 6B; P<0.05; n=6), in agreement with previous studies reporting that G protein–coupled receptors induce recruitment and phosphorylation of FLNA, an event that has long been known to be essential for the regulation of the structure and dynamics of the actin cytoskeleton.[29]

Treatment of A7 and M2 cells with thapsigargin in a Ca^{2+}-free medium resulted in a transient increase in $[Ca^{2+}]_c$ because of Ca^{2+} release from the intracellular stores. Subsequent addition of Ca^{2+} to the extracellular medium resulted in a further elevation in $[Ca^{2+}]_c$ indicative of SOCE. As shown in

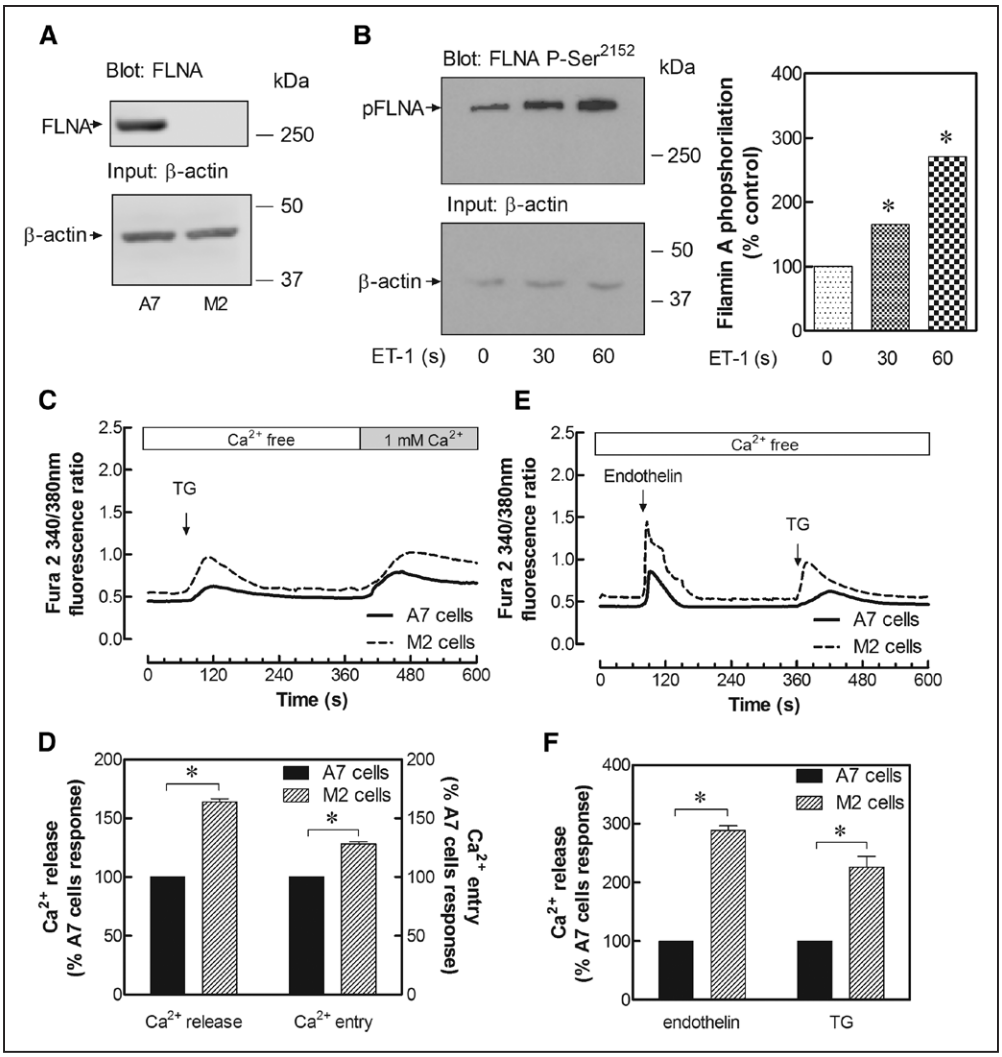

Figure 6. FLNA (filamin A) plays a relevant role in Ca^{2+} release and entry in melanoma cells. **A**, A7 and FLNA-deficient M2 melanoma cells were lysed and subjected to Western blotting with anti-FLNA antibody, followed by reprobing with anti-β-actin antibody for protein loading control. **B**, A7 cells were stimulated with 10 nM endothelin-1 and lysed. Cell lysates were subjected to Western blotting with antiphospho-FLNA (pSer2152) antibody or anti-β-actin antibody, as indicated. Bar graphs represent the percentage of FLNA phosphorylation as compared with nonstimulated (control) cells. **C**–**F**, Fura-2-loaded A7 and M2 melanoma cells were stimulated with thapsigargin (TG; 1 μmol/L) in a Ca^{2+}-free medium (125 μmol/L EGTA added) and 5½ min later $CaCl_2$ (final concentration 1 mmol/L) was added to the medium to initiate Ca^{2+} entry (**C** and **D**) or were stimulated with 10 nM endothelin-1 in a Ca^{2+}-free medium (125 μmol/L EGTA added) followed by addition of 1 μmol/L TG (**E** and **F**). $[Ca^{2+}]_c$ was monitored as described in the Materials and Methods section in the online-only Data Supplement. Traces are representative of 6 independent experiments. Bar graphs indicate the percentage of Ca^{2+} release and entry under the different conditions relative to their control (response in A7 cells). Values are means±SEM; significance values indicate differences compared with the response in FLNA-expressing A7 cells. *P<0.05, Student t test.

Figure 6C and 6D, SOCE was significantly greater in FLNA-deficient M2 cells (the integrals of the rise in fura-2 fluorescence ratio after the addition of $CaCl_2$ were 58.4±2.1 and 44.6±1.4 au·s in M2 and A7 cells, respectively; P<0.05; n=6). These observations are consistent with the results obtained in human platelets and further support that FLNA regulates the activation of SOCE. Thapsigargin-evoked Ca^{2+} efflux from the internal stores was also found to be greater in M2 cells (the integrals of the rise in fura-2 fluorescence ratio after the addition of thapsigargin were 67.5±1.1 and 36.1±0.9 au·s in M2 and A7 cells, respectively; P<0.05; n=6). This observation might be a consequence of the greater SOCE in these cells, although we cannot exclude a possibility of a modulatory role of FLNA on Ca^{2+} store refilling by SERCA. To assess this possibility, cells were stimulated with endothelin-1 in a Ca^{2+}-free medium followed by addition of thapsigargin to estimate the ability of the cells to store Ca^{2+}. As depicted in Figure 6E and 6F, treatment of A7 and M2 cells with endothelin-1 (10 nM) induced a transient increase in $[Ca^{2+}]_c$, subsequent addition of thapsigargin induced a further rise in $[Ca^{2+}]_c$ indicative of the amount of Ca^{2+} accumulated in the stores. Both, endothelin-1–evoked and thapsigargin-evoked responses were greater in M2 cells (the integrals of the rise in fura-2 fluorescence ratio after the addition of endothelin-1 were 28.4±1.6 and 11.4±1.9 ua·s in M2 and A7 cells, respectively, and that of the thapsigargin-induced responses were 13.6±1.2 and 6.2±0.5 ua·s in M2 and A7 cells, respectively; P<0.05; n=6), which indicates that the ability to refill

the stores is greater in FLNA-deficient M2 and suggests that FLNA might regulate SERCA activity in these cells.

We have further explored whether phosphorylation of FLNA at Ser^{2152} is involved in the modulation of SOCE in melanoma cells as reported above in human platelets. To investigate this possibility, M2 cells were transfected with expression plasmids for wild-type FLNA or the nonphosphorylatable FLNA S2152A mutant (Figure 7A). As shown in Figure 7B and 7C, expression of FLNA in M2 cells significantly attenuated SOCE (the integrals of the rise in fura-2 fluorescence ratio after the addition of $CaCl_2$ were 42.2±2.2 and 28.5±2.2 ua·s in M2 and FLNA-expressing M2 cells, respectively; P<0.05; n=6), which further confirms the modulatory role of FLNA on SOCE. By contrast, expression of the nonphosphorylatable FLNA mutant was without effect on thapsigargin-induced Ca^{2+} release and entry (the integral of the rise in fura-2 fluorescence ratio after the addition of $CaCl_2$ was 40.7±3.2 ua·s in FLNA S2152A-expressing M2 cells; n=6), thus suggesting that the role of FLNA on these processes entirely depends on phosphorylation at Ser^{2152}. The attenuation of thapsigargin-evoked Ca^{2+} release from the stores induced by the expression of FLNA was also impaired by the S2152A mutation (the integrals of the rise in fura-2 fluorescence ratio after the addition of thapsigargin were 41.4±2.2, 32.1±1.4, and 40.3±3.2 ua·s in M2, FLNA-expressing M2, and FLNA S2152A-expressing M2 cells, respectively; P<0.05; n=6).

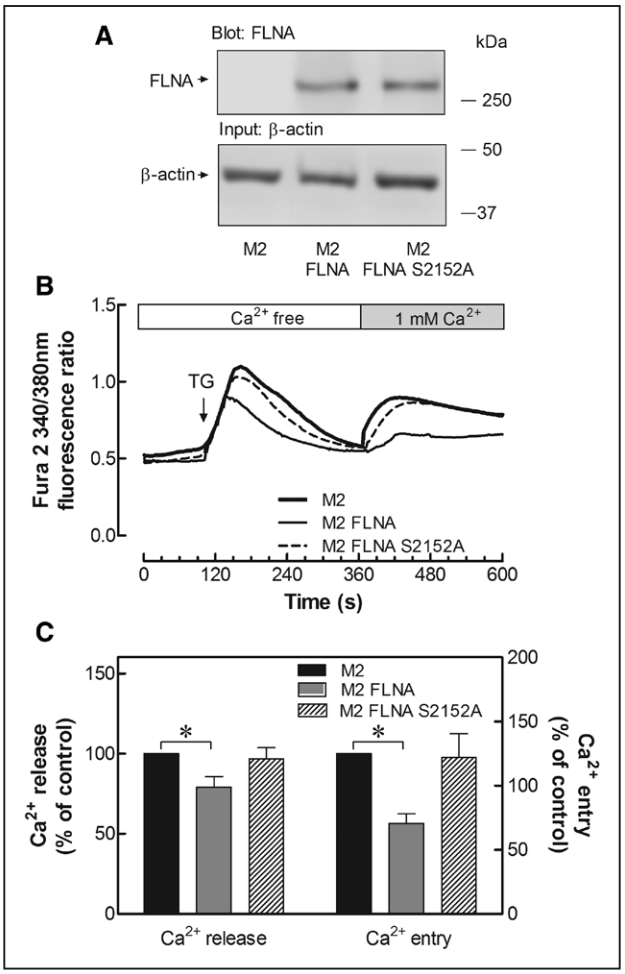

Figure 7. Modulation of store-operated calcium entry (SOCE) by filamin A (FLNA) requires phosphorylation at serine 2152 in melanoma cells. **A**, M2 melanoma cells were transfected with overexpression plasmids for or FLNA S2152A (FLNA S2152A) or empty vector, as control, and then were lysed and subjected to Western blotting with anti-FLNA antibody, followed by reprobing with anti-β-actin antibody for protein loading control. **B**, Wild-type M2 melanoma cells and M2 cells expressing FLNA or FLNA S2152A were stimulated with thapsigargin (TG; 1 µmol/L) in a Ca^{2+}-free medium (125 µmol/L EGTA added) followed by the addition of $CaCl_2$ (final concentration 1 mmol/L) to the medium to initiate Ca^{2+} entry. $[Ca^{2+}]_c$ was monitored as described in the Materials and Methods section in the online-only Data Supplement. Traces are representative of 6 independent experiments. **C**, Bar graphs indicate the percentage of Ca^{2+} release and entry under the different conditions relative to their control (cells transfected with empty vector). Values are means±SEM; significant values indicate differences compared with control. *P<0.05, ANOVA combined with the Dunnett test.

C-Terminal Domain of STIM1 and Filamin A Dimerization Are Essential for Their Interaction and Platelet Function

We have finally analyzed the location of the STIM1- and FLNA-binding sites. STIM1 shows a polybasic lysine-rich domain in the C-terminal region that is involved in the interaction with TRPC1 channels[30] and phosphoinositides at the plasma membrane.[31] Hence, we have investigated whether this region is responsible for the interaction with FLNA in M2 cells overexpressing pEYFP-STIM1 (full-length) or the pEYFP-STIM1(K684,685E) mutant together with FLNA. As shown in Figure 8A, our results indicate that coexpression of pEYFP-STIM1 and FLNA show detectable coimmunoprecipitation between both proteins at rest, which was enhanced by treatment with thapsigargin. Interestingly, STIM1–FLNA interaction was abolished in cells coexpressing FLNA and the STIM1(K684,685E) mutant, which strongly indicates that the lysine-rich region of STIM1 is required for FLNA interaction. Similarly, we have investigated the location of the relevant STIM1-interacting FLNA site. The C-terminal region of FLNA is especially important for the interaction with signaling molecules, and many of these interactions occur at the 24th dimerizing repeat of FLNA[32]; therefore, we have investigated whether this region is important for the interaction with STIM1. To assess this possibility, the anti-FLNA antibody, which recognizes the sequence between residues 2597 and 2647, was introduced into platelets by electropermeabilization, as described previously.[5] The internalization of the antibody was investigated in samples from electropermeabilized cells incubated with 4 µg/mL anti-FLNA (2597–2647) antibody, by immunoprecipitation without adding any additional anti-FLNA antibody and subsequent Western blotting with the anti-FLNA (2597–2647) antibody (Figure 8B). As shown in Figure 8C, interaction between STIM1 and FLNA was significantly attenuated in cells transjected with 4 µg/mL anti-FLNA (2597–2647) antibody (top, lanes 1 and 2) compared with cells transjected with a rabbit IgG (top, lanes 3 and 4) used as control because this is the nature of the anti-FLNA (2597–2647) antibody. The FLNA–STIM1 interaction was reduced by 82% and 74% in resting and thapsigargin-treated cells, respectively, in cells transjected with the anti-FLNA (2597–2647) antibody compared with control (cells transjected with rabbit IgG; P<0.05; n=6). Reprobing of the same membranes with anti-STIM1 antibody confirmed a similar protein loading in all lanes (Figure 8C, bottom). These findings indicate that the FLNA 2597 to 2647

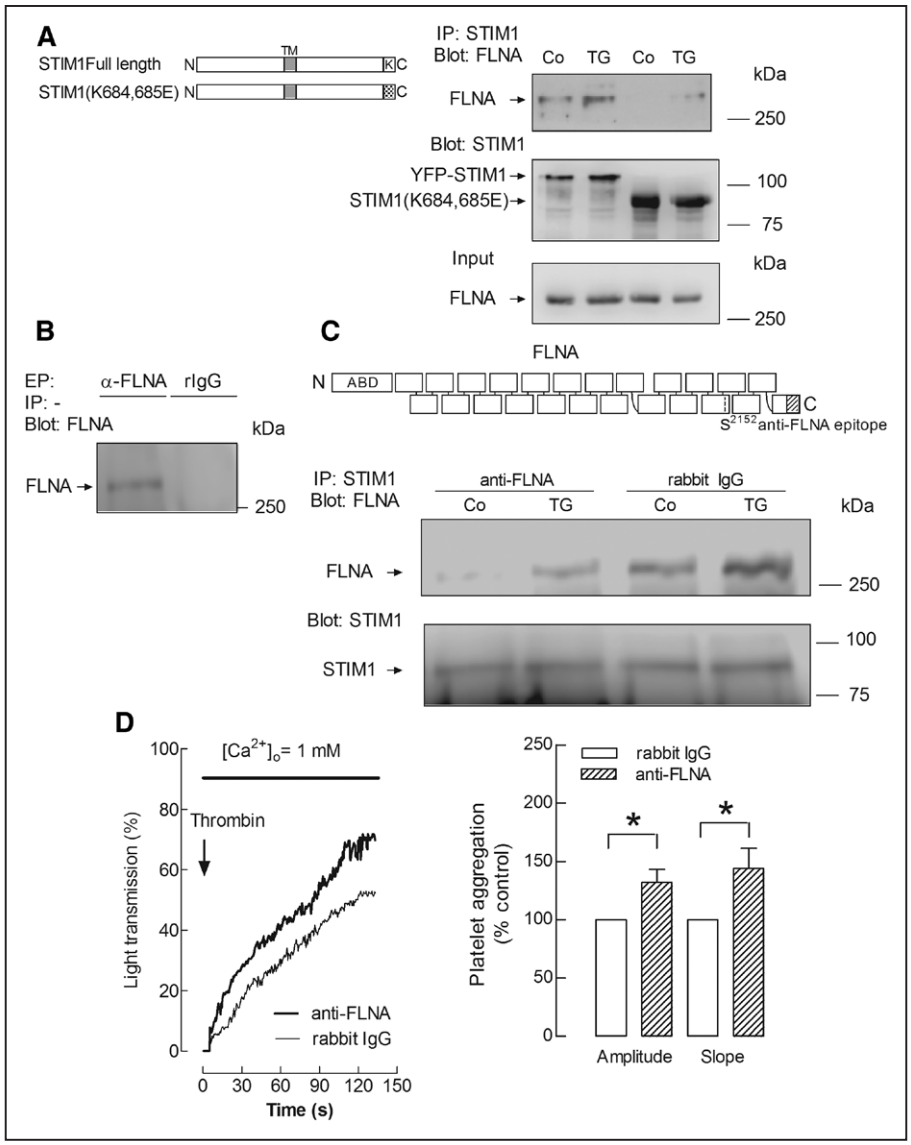

Figure 8. The C-terminal domains of STIM1 (stromal interaction molecule 1) and FLNA (filamin A) are essential for their interaction and platelet function. **A**, FLNA-deficient M2 melanoma cells were transfected with overexpression plasmids for STIM1 (lanes 1 and 2) or the STIM1(K684,685E) mutant (lanes 3 and 4) and FLNA or left untreated. Cells were then stimulated for 30 s with TG (1 μmol/L) or the vehicle (control; Co), as indicated, in a medium containing 1 mmol/L $CaCl_2$ and lysed. Cell lysates were immunoprecipitated with the anti-STIM1 antibody followed by Western blotting with the anti-FLNA antibody (**top**) and the anti-STIM1 antibody (**middle**; for protein loading control). The input with anti-FLNA antibody is shown in the **bottom**. The image shows results from 1 experiment representative of 5 others. Molecular masses indicated on the **right** were determined using molecular mass markers run in the same gel. **B**, Human platelets electropermeabilized (EP) in a Gene Pulser as described in the Materials and Methods section in the online-only Data Supplement were incubated in the presence of 4 μg/mL anti-FLNA antibody (α-FLNA) or 4 μg/mL rabbit IgG (rIgG), as indicated, for 60 min and then lysed. Whole cell lysates were immunoprecipitated in the absence of antibodies but adding protein A-agarose, and immunoprecipitated proteins were analyzed by Western blotting using anti-FLNA antibody. These results are representative of 3 independent experiments. **C**, Platelets (10^9 cells/mL) were electropermeabilized and incubated with 4 μg/mL rabbit IgG or with 4 μg/mL anti-FLNA (2597–2647) antibody for an additional 60 min at 37°C, as indicated. Cells were then incubated for 30 s in the absence or presence of 1 μmol/L TG in a medium containing 1 mmol/L $CaCl_2$ and lysed. Whole-cell lysates were immunoprecipitated (IP) with anti-STIM1 antibody. Immunoprecipitates were analyzed by Western blotting (WB) using anti-FLNA antibody (**top**) and reprobed with anti-STIM1 antibody (**bottom**). Positions of molecular mass markers are shown on the **right**. These results are representative of 6 independent experiments. ABD, actin-binding domain. The structural domains of FLNA showing the location of the epitope recognized by the anti-FLNA (2597–2647) antibody is depicted on **top**. **D**, Human platelets were electropermeabilized and incubated with 4 μg/mL rabbit IgG or with 4 μg/mL anti-FLNA (2597–2647) antibody for an additional 60 min at 37°C, as indicated in the legend to Figure 6B. Platelets were then suspended in a medium containing 1 mmol/L $CaCl_2$ and then stimulated with 2 U/mL thrombin. Traces shown are representative of 6 separate experiments. Bar graphs indicate the amplitude and slope of platelet aggregation in response to thrombin in cells treated with anti-FLNA or rabbit IgG, as control.

sequence, located in the dimerizing repeat, is relevant for the STIM1–FLNA interaction and suggest that dimerization of FLNA is necessary for the interaction.

Because introduction of the anti-FLNA (2597–2647) antibody impairs the STIM1–FLNA interaction, we have investigated whether this interaction is important for platelet

aggregation. As shown in Figure 8D, introduction of the anti-FLNA (2597–2647) antibody into platelets significantly enhanced the amplitude and slope of thrombin-induced platelet aggregation compared with transjection of a rabbit IgG. These findings indicate that the STIM1–FLNA interaction plays a relevant functional role in human platelets.

Discussion

SOCE is a complex mechanism for Ca^{2+} influx controlled by the Ca^{2+} stores. Although the central event is based on the interaction between STIM1 and the store-operated channels at ER–PM junctions, many proteins fine-tune the amplitude of Ca^{2+} entry, an essential process for different cellular functions, including platelet physiology,[33,34] B-cell response,[35] or neuronal differentiation.[36] Key modulators of STIM1–Orai1 interaction include CRACR2A, STIMATE, SARAF, septins, or ORMDL3, among others.[37] More precisely, STIM1–Orai1-supporting proteins include CRACR2A, which stabilizes the SOCE signalplex, STIMATE, required for the activation and translocation of STIM1, and septins, involved in the organization of membrane microdomains important for STIM1–Orai1 interaction. Among the negative modulators of SOCE are SARAF, ORMDL3 or golli, which prevent excessive Ca^{2+} influx. [37] The SOCE signalplex is supported by the cytoskeleton,[11–13] which is rapidly remodeled on store depletion.[38] By looking for new modulators of STIM1 function, we have identified FLNA as a protein that coimmunoprecipitates with STIM1 on Ca^{2+} store depletion in human platelets. Our results indicate that the interaction between FLNA and STIM1 is Ca^{2+} dependent and requires FLNA phosphorylation at Ser^{2152}. Phosphorylation of FLNA at Ser^{2152} acts as a chemomechanical switch that modulates downstream cellular processes.[18] We have found that impairment of FLNA phosphorylation enhances SOCE, meanwhile inhibition of FLNA dephosphorylation leads to the opposite effect, thus suggesting that FLNA might be a negative modulator of SOCE in platelets. We have confirmed the role of FLNA in SOCE in platelets using siRNA. Platelets carry out mRNA translation, a process that is relevant for platelet-mediated thrombosis and hemostasis,[39] and siRNA has been demonstrated to be efficiently introduced into platelets to modulate protein expression in these cells.[27] Our results indicate that 24 hours after transfection of the FLNA siRNA, FLNA expression was significantly reduced. Further transfection periods led to a significant reduction in cell viability (data not shown). Interestingly, attenuation of FLNA expression revealed that FLNA is a modulator of SOCE in human platelets. This function was confirmed in the FLNA-deficient melanoma cell line M2 where SOCE was greater than that in the FLNA-expressing M2 subclone A7. To our knowledge, this is the description of a role for FLNA in the modulation of SOCE. We also found that FLNA modulates Ca^{2+} uptake into the intracellular stores by SERCA, whereas this effect seems to be specific for melanoma cells, because this function was not observed in human platelets, we cannot exclude a possible role of FLNA on SERCA in platelets that can be difficult to detect because of the low rate of Ca^{2+} efflux from the stores in these cells on treatment with thapsigargin. Using melanoma cells, we have also confirmed that phosphorylation of FLNA at Ser^{2152} is essential for the regulation of SOCE, in agreement with the observations in human platelets.

Our results indicate that the C-terminal regions of STIM1 and FLNA play a relevant role in the interaction with each other, as mutation of the STIM1 lysine-rich domain and interference with the FLNA 2597 to 2647 region impair the STIM1–FLNA association.

Finally, we have investigated the regulatory mechanism of FLNA. Because FLNA is an actin-binding protein, we have initially investigated its role in the location of STIM1 in the cytoskeletal fraction. We have found that FLNA expression knockdown enhances the association of STIM1 with the cytoskeleton and subsequently increases the interaction of STIM1 with Orai1, leading to a dramatic increase in SOCE. Furthermore, impairment of FLNA–STIM1 interaction results in enhanced platelet aggregation in response to agonists. These findings strongly suggest that FLNA might be essential for an appropriate association of STIM1 with the cytoskeleton, which, in turn, is necessary to fine-tune the interaction with the store-operated channels and SOCE and also to modulate platelet function. Previous studies in a mouse model with deficient expression of FLNA have revealed that platelets exhibit hyperaggregability and thrombocytopenia because of platelet loss.[19,20] Furthermore, a recent study has reported that a patient carrying a mutation that results in a 100-amino acid–long FLNA C-terminal extension (p.Ter2648SerextTer101) showed normal platelet count but upregulated platelet function.[40] Our results provide evidence that a deficient expression or function of FLNA in platelets might alter Ca^{2+} homeostasis, which, in turn, might underlie platelet function disorders.

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Disclosures

None.

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Highlights

- FLNA (filamin A) plays an essential role in the cross talk between STIM1 (stromal interaction molecule 1) and Orai1.
- FLNA regulates store-operated calcium entry and the correct platelet function.
- FLNA phosphorylation at serine 2152 is required for the regulation of store-operated calcium entry.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Filamin A Modulates Store-Operated Ca^{2+} Entry by Regulating STIM1 (Stromal Interaction Molecule 1)–Orai1 Association in Human Platelets

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Materials and methods

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes® (Leiden, The Netherlands). Thapsigargin (TG), KT-5720, brefeldin A, thrombin, endothelin-1, apyrase, prostaglandin I_2, rabbit polyclonal anti-Orai1 antibody (catalog number O8264), rabbit polyclonal anti-β-actin antibody (catalog number A2066), and rabbit polyclonal anti-phospho-FLNA (pSer2152) antibody (catalog number SAB4503819) were from Sigma-Aldrich® (Madrid, Spain). PrimeScript™ RT-PCR Kit and SYBR® Premix Ex Taq™ were from Takara Bio Inc. (Otsu, Shiga, Japan). Turbofect transfection and Lipofectamine RNAiMAX were from Invitrogen® (Carlsbad, CA, USA). Mouse monoclonal anti-STIM1 antibody (Clone 44/GOK) was from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Rabbit polyclonal anti-FLNA (2597-2647) antibody was from Bionova (Madrid, Spain). Protein A-agarose was from Millipore (Billerica, MA, USA). Trizol, enhanced chemiluminescence detection reagents and FLNA monoclonal antibody (clone FLMN01 PM6/317) were from Thermo-Fisher (Waltham, MA, USA). FLNA siRNA, control siRNA-A and RT-PCR primers were from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase-conjugated anti-mouse IgG antibody and anti-rabbit IgG antibody were from Jackson laboratories (West Grove, PA, USA). All other reagents were of analytical grade.

Platelet preparation

Platelets were prepared as previously described,[1] as approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Platelet-rich plasma was supplemented with acetylsalicylic acid (100 μM) and apyrase (40 μg/mL) and platelets were then collected by centrifugation at 350×*g* for 20 min and resuspended in HEPES-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 HEPES, 10 d-glucose, 5 KCl, 1 $MgSO_4$ and supplemented with 0.1% (w/v) BSA and 40 μg/mL apyrase. For dimethyl BAPTA loading, platelets were incubated for 30 min with 10 μM dimethyl BAPTA/AM.

Platelet transfection

FLNA siRNA and negative control (siRNA-A) were transfected into platelets according to the method described by Hong et al.[2] Platelets (2×10^8 in 1 mL) were suspended in modified Tyrode's buffer (containing (in mM): 138 NaCl, 5.5 dextrose, 12 $NaHCO_3$, 0.8 $CaCl_2$, 0.4 $MgCl_2$, 2.9 KCl_2, 0.36 Na_2HPO_4, and 20 HEPES, pH 7.4) supplemented with 1 μM prostaglandin I_2 and were transfected with 400 nM siRNA by using the cationic lipid transfection reagent Lipofectamine RNAiMAX.

Platelet aggregation

The amplitude and slope of aggregation in washed platelets were monitored using a Chronolog (Havertown, PA, USA) aggregometer at 37 °C under stirring at 1200 rpm [3].

Reversible electroporation procedure

The platelet suspension was transferred to an electroporation chamber containing antibodies at a final concentration of 4 μg/mL, and the antibodies were transjected according to published methods. [1,4] Reversible electropermeabilization was performed at 4 kV/cm at a setting of 25-microfarad capacitance and was achieved by seven pulses using a Bio-Rad Gene Pulser Xcell electroporation system (Bio-Rad). Following electroporation, cells were incubated with antibodies for an additional 60 min at 37 °C and were centrifuged at 350 × *g* for 20 min and resuspended in HBS prior to the experiments.

Cell culture and transfection

M2 (FLNA-deficient) and the isogenic A7 (FLNA-expressing) melanoma cell lines were provided by Dr Mañé (Department of Immunology and Oncology, Centro Nacional de Biotecnologia/Consejo Superior de Investigaciones Cientificas, Madrid, Spain). Cells

were cultured at 37ºC with a 5% CO_2 in minimum essential medium (MEM) supplemented with 5% fetal calf serum and 100 U/mL penicillin and streptomycin, as described.[5]
Cells were transfected with expression plasmid for pcDNA3-myc-FLNA WT and pcDNA3-myc-FLNA S2152A, provided by Dr. Blenis (Harvard University, Boston, MA, USA), as well as with full-length STIM1 and STIM1(K684, 685E) or scramble plasmid, using Turbofect transfection reagent®, as described previously.[6-7]

Immunoprecipitation and Western blotting
Immunoprecipitation and Western blotting were performed as described previously.[6] Briefly, cell lysates were immunoprecipitated by incubation with 2 µg of anti-STIM1 antibody and 25 µL of protein A-agarose overnight at 4 °C on a rocking platform. Immunoprecipitates were resolved by 10% SDS-PAGE and separated proteins electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Immunodetection of FLNA, FLNA phosphorylated at Ser^{2152}, β-actin, Orai1 and STIM1 was achieved by overnight incubation at 4ºC with anti-FLNA, anti-FLNA-phospho-Ser^{2152} or anti-STIM1 antibodies diluted 1:250 in TBST, or by incubation for 1h with anti-Orai1 or anti-β-actin antibody, diluted 1:200 or 1:2000 in TBST, respectively.

Quantitative RT-PCR

Total RNA isolation was perfomed using Trizol reagent and single-strand cDNA synthesis was performed using PrimeScript™ RT-PCR Kit.. The primers used for PCR analisis were: β-actin sense: 5'-AGC GAG CAT CCC CCA AAG TT-3'; β-actin antisense: 5'- GGG CAC GAA GGC TCA TCA TT-3' and a commercial primer for FLNA from Santa Cruz Biotechnology (Dallas, TX, USA). SYBR green qRT-PCR was performed using SYBR® Premix Ex Taq™ in an Applied Biosystems STEPONE Real-Time thermal cycler (Life Technologies Corporation, Carlsbad, CA, USA). PCR products were obtained using the following cycling conditions: 96 °C for 2 min, followed by 35 cycles of 96 °C for 15 s, 55–60 °C for 25 s and finished with 72 °C for 10min. mRNA abundance was calculated by the comparative CT (ΔΔCT) method using the formula: $RQ=2^{-\Delta\Delta CT}$. The amount of mRNA transcripts were normalized to β-actin expression and represented as mean expression relative to cells transfected with siRNA A ± S.E.M.

Silver staining
500 µL aliquots of platelet suspension (2×10^9 cells/mL) were immunoprecipitated with 2 µg of mouse anti-STIM1 or 2 µg of unconjugated rabbit anti-mouse IgG 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 stained with silver by using a procedure compatible with MALDITOF/TOF mass spectrometry. Following electrophoresis, gels were fixed in 50% ethanol and 10% acetic acid solution for 30 min and sensitized with 30% ethanol, 12.7 mM sodium thiosulfate and 0.82 M sodium acetate. Silver staining was performed by incubating gels with 0.25% silver nitrate solution for 10 min. After staining, gels were washed and developed in 2.5% sodium carbonate and 0,015% formaldehyde solution for 2 min.

Subcellular fractionation
Human platelet fractionation was carried out as described previously.[1] TG-treated and non-treated control cells (2×10^8 cells/mL) were immediately lysed with an equal volume of 2× Triton buffer (2% Triton X-100, 2 mM EGTA, 100 mM Tris/HCl (pH 7.2), 2 mM Na_3VO_4 and complete EDTA-free protease inhibitor tablets) at 4 °C for 30 min. Platelet lysate was centrifuged at 16,000 × *g* for 5 min. The supernatant (cytosolic and membrane fraction) was immunoprecipitated with anti-STIM1 antibody, and the pellet (cytoskeleton-rich fraction) was solubilized into Laemmli's buffer, boiled for 5 min. Both fractions were subjected to Western blotting with anti-STIM1 antibody.

Measurement of cytosolic free-calcium concentration ($[Ca^{2+}]_c$) in single cells

M2 and A7 cells were loaded with fura-2 and changes in $[Ca^{2+}]_c$ was performed as described previously using an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY, USA) with image acquisition and analysis system for videomicroscopy (Hamamatsu Photonics, Hamamatsu, Japan). Cells were alternatively excited at 340/380 nm and fluorescence emission at 505 nm was detected using a cooled digital CCD camera and recorded using Aquacosmos 2.5 software (Hamamatsu Photonics, Hamamatsu, Japan).[8] TG-induced Ca^{2+} entry and SOCE were estimated as the integral of the rise in fura-2 fluorescence 340/380 nm ratio above basal for 3 min after the addition of TG or $CaCl_2$, respectively.

Measurement of cytosolic free-calcium concentration ($[Ca^{2+}]_c$) in cell suspensions

Cells were loaded with fura-2 and fluorescence was recorded using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm, as described previously.[1] Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to Grynkiewicz et al.[9] TG-induced Ca^{2+} release and entry were estimated using the integral of the rise in $[Ca^{2+}]_c$ for 3 min after the addition of TG or $CaCl_2$, respectively.

Platelet aggregation

The percentage of aggregation in washed platelets were monitored using a Chronolog aggregometer (Havertown, PA, USA) at 37 °C under stirring at 1200 rpm as described previously.[4]

Statistical Analysis

Normality of the data was tested by using the Shapiro-Wilk normality test which reported a P value over 0.05 for the data tested. Analysis of statistical significance was performed using Student's unpaired *t*-test. For multiple comparisons, one-way analysis of variance combined with the Dunnett´s tests was used.

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