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Supplementary Materials for

STIM1 Controls Endothelial Barrier Function Independently of Orai1 and Ca^{2+} Entry

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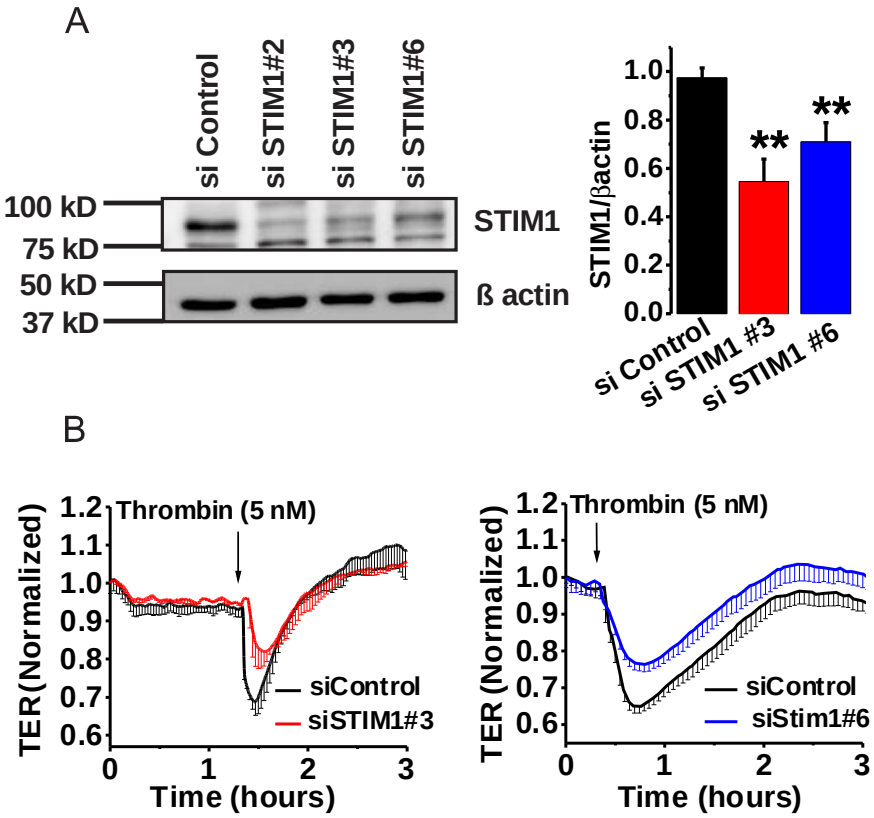


Fig. S1. Effect of different STIM1 siRNAs on the thrombin-mediated decrease in TER in HUVECs. (**A**) The efficiency of knockdown of the three STIM1 siRNAs as determined by Western Blotting of HUVECs. siSTIM1#2 is the one used for most data shown in the study. Two additional siRNA (siSTIM1#3 and siSTIM1#6) decreased STIM1 with different efficiencies. (**B**) The effectiveness of siSTIM1#3 and siSTIM1#6 on thrombin-induced changes in TER in HUVECs correlates with their effectiveness at knocking down STIM1. Data are representative of 3 independent transfections.

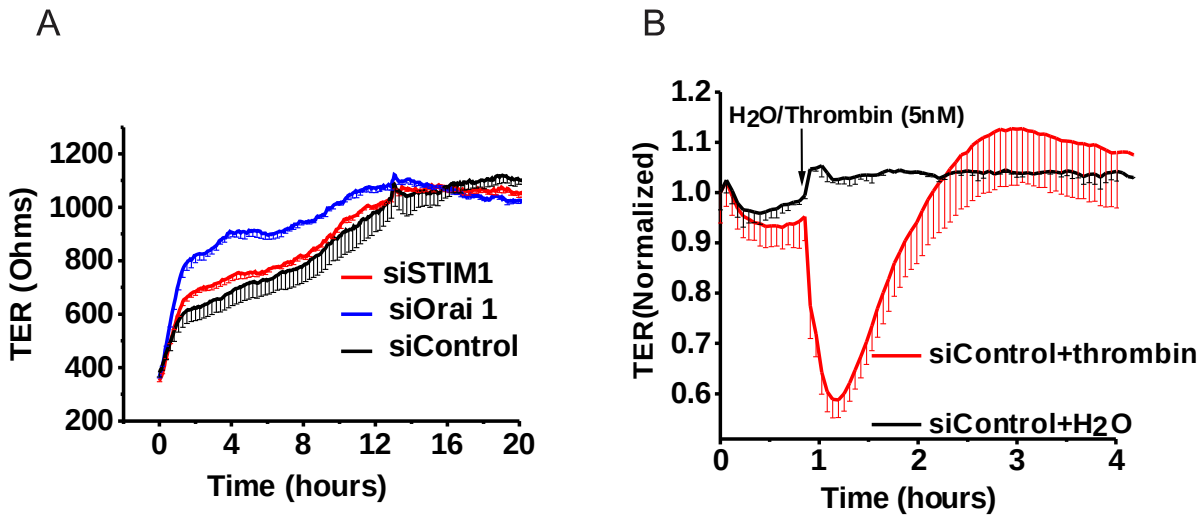


Fig. S2. STIM1 knockdown does not affect basal endothelial monolayer resistance. (**A**) HUVECs were transfected with either siRNA control (siControl), siRNA STIM1 (siSTIM1), or siRNA Orai1 (siOrai1), and after 3 days in culture, they were seeded on ECIS wells and TER was monitored for 20 hours. (**B**) Experiments with HUVECs transfected with control siRNA show that only the addition of thrombin caused a decrease in TER; vehicle addition (water) had no effect.

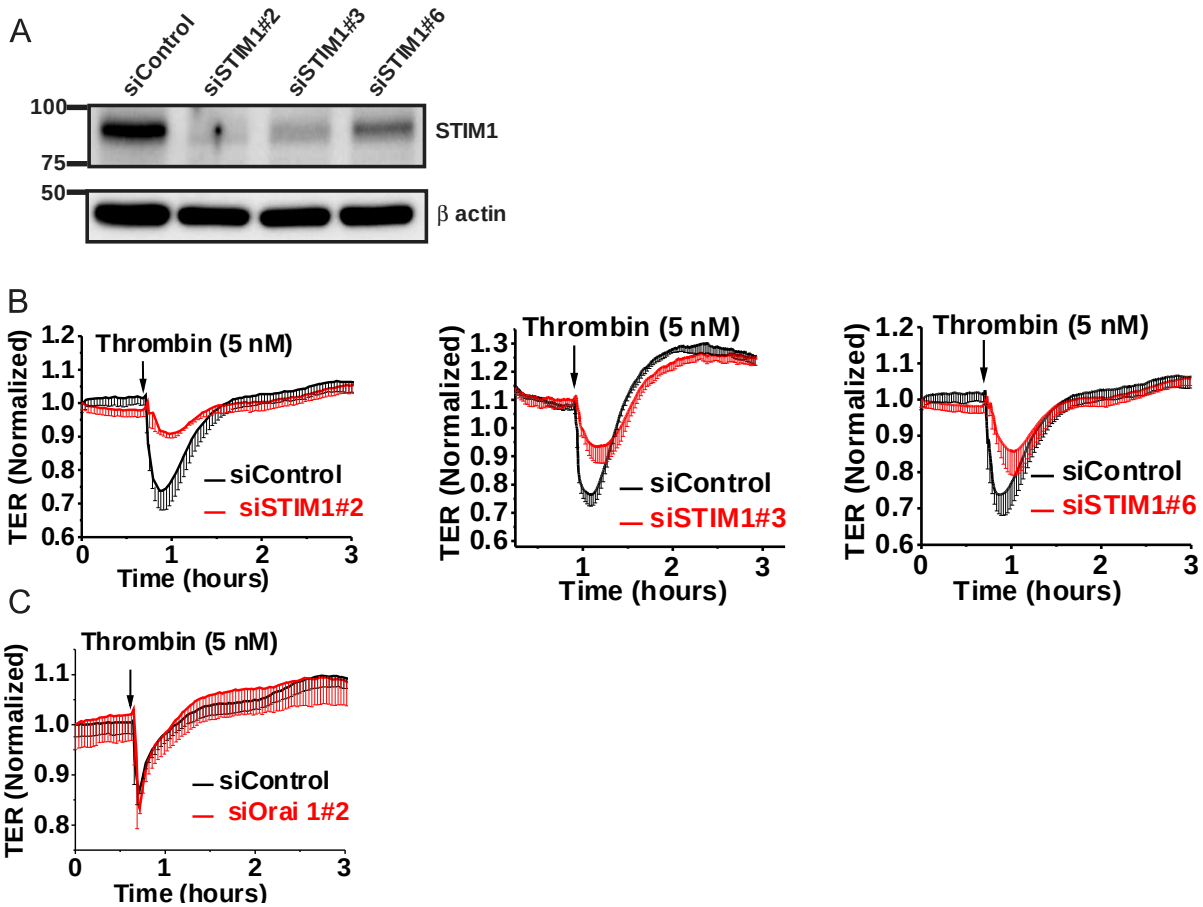


Fig. S3. STIM1 knockdown, but not Orai1 knockdown, inhibited the decrease in TER of HDMECs in response to thrombin. (**A**) Western bloting shows the efficiency of STIM1 knockdown in HDMECs. (**B**) The effect of STIM1 knockdown with the indicated siRNA on the thrombin-induced decrease in TER of HDMECs. (**C**) The effect of Orai1 knockdown on the thrombin-induced decrease in TER in HDMECs. All experiments are representative of 3 independent transfections.

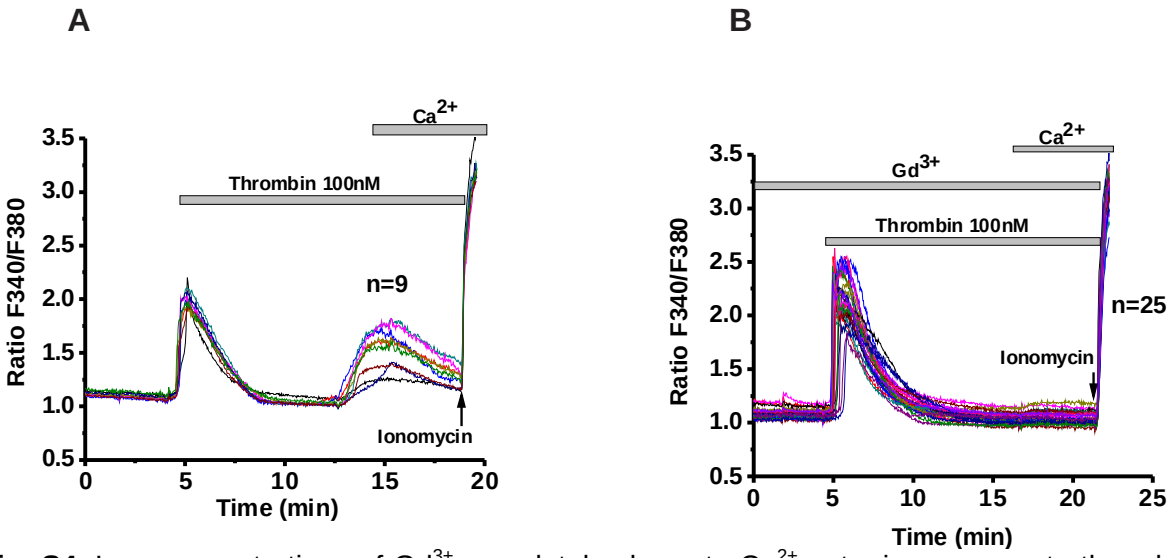
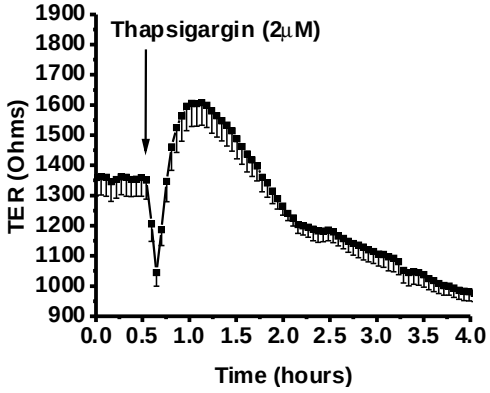


Fig. S4. Low concentrations of Gd^{3+} completely abrogate Ca^{2+} entry in response to thrombin in HDMECs. (**A**) Ca^{2+} imaging of HDMECs stimulated with thrombin in Ca^{2+}-free medium to visualize first Ca^{2+} release from internal stores, followed by the addition of Ca^{2+} (2 mM) to visualize Ca^{2+} entry. The Ca^{2+} pore-forming chemical ionomycin is added at the end of the experiment. (**B**) Ca^{2+} imaging of HDMECs preincubated with Gd^{3+} (10 µM) show a complete absence of Ca^{2+} entry following the addition of thrombin and Ca^{2+} to the medium. Each colored trace represents a single cell. Results are representative of 3-4 independent experiments.

A



B

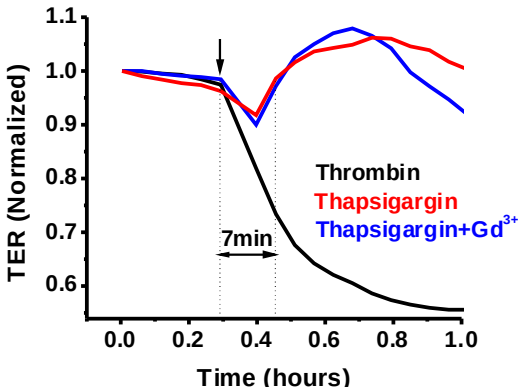


Fig. S5. The effects of thapsigargin on TER are distinct from those of thrombin and are SOCE-independent. (**A**) Thapsigargin was added to HUVECs monolayer and change in TER was monitored. (**B**) Normalized TER in HUVECs in response to thapsigargin, thrombin, or thapsigargin-treated cells preincubated with Gd^{3+} (10 μM). The down arrow shows the addition of thapsigargin or thrombin. The dashed lines and double-headed arrow indicate the duration of the initial TER drop in response to thapsigargin (~7 min). Data are representative of 5 independent experiments.

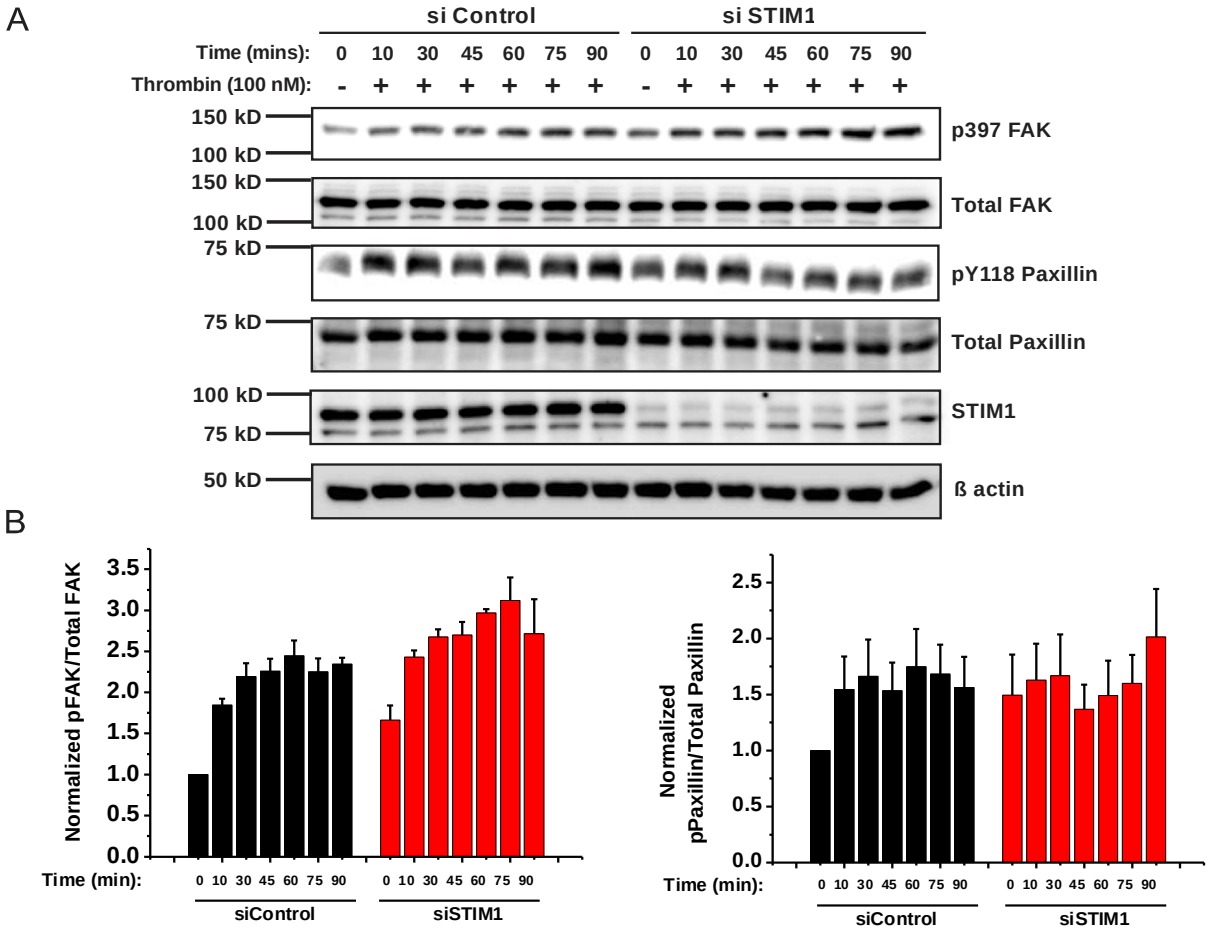


Fig. S6. STIM1 knockdown in HUVECs increased basal phosphorylation of FAK and paxillin. (**A**) Western blot representative of 4 independent transfections with similar results. The indicated proteins were detected by Western blotting with specific antibodies. p397 FAK is FAK phosphorylated on Tyr^{397}; pY118 paxillin is paxillin phosphorylated on Tyr^{118}. (**B**) Quantification of FAK and Paxillin phosphorylation from 4 independent experiments. Basal FAK and paxillin phosphorylation is higher in siSTIM1 compared to siControl ($p<0.05$).

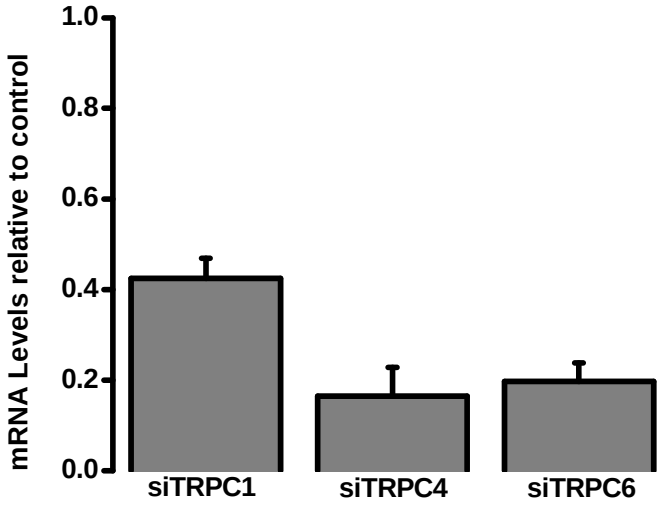


Fig. S7. Effectiveness of TRPC1, TRPC4, or TRPC6 knockdown in HUVECs. Transcripts were measured with quantitative reverse-transcriptase polymerase chain reaction 3 days after transfection of HUVECs with siRNA against TRPC1, TRPC4, or TRPC6.

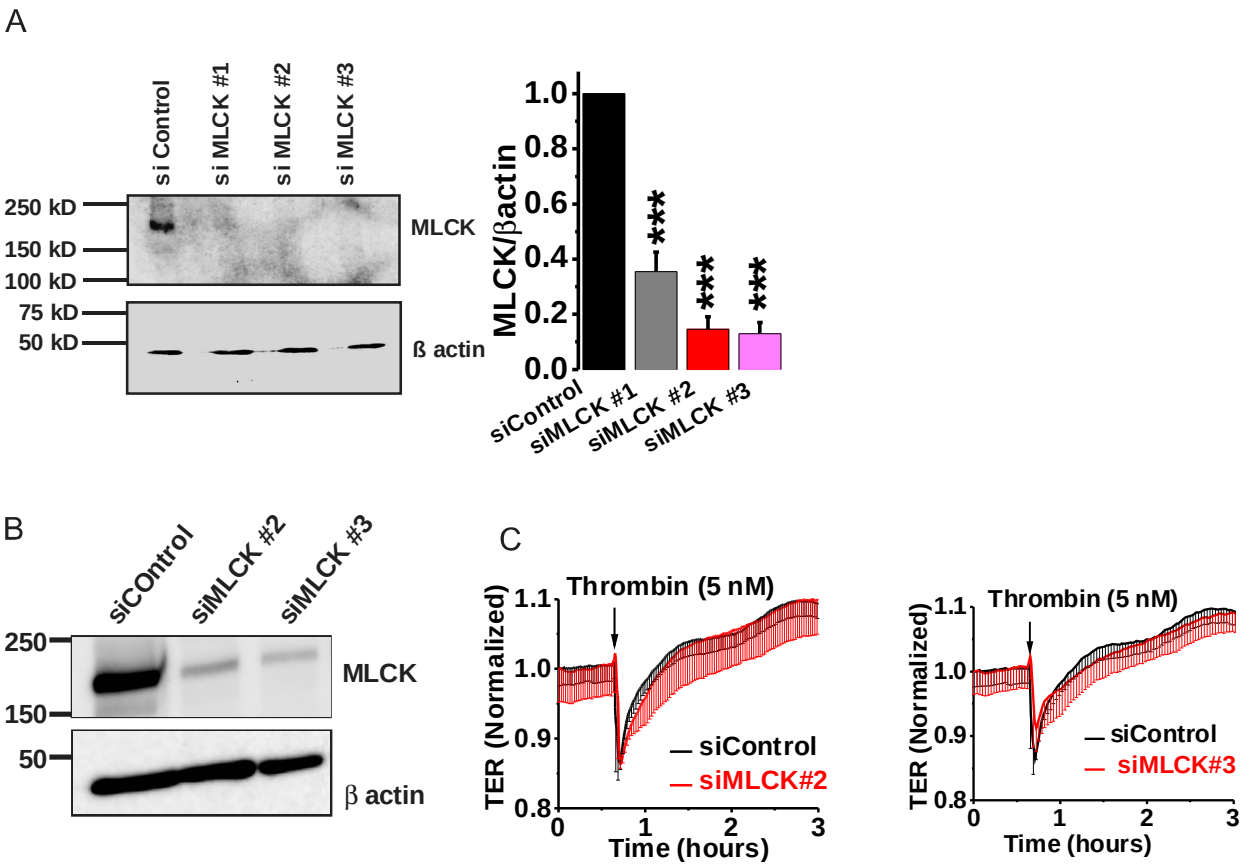


Fig. S8. MLCK knockdown in HUVECs and HDMECs has no effect on the thrombin-induced decrease in TER. (**A**) Western blots show the effectiveness of MLCK knockdown with three different siRNAs targeting MLCK in HUVECs and results of 3 independent experiments are quantified. siMLCK#1 was not used for TER experiments because it was toxic to the cells . (**B**) Western blots show the effectiveness of MLCK knockdown with two different siRNAs targeting MLCK in HDMECs. (**C**) The thrombin-induced decrease in TER in HDMECs transfected with siMLCK#2 or siMLCK#3. Data are representative of n=3 independent transfections.

Table S1: Antibodies used in the study with corresponding dilutions.

Antibody Name	Species (Host)	Source	Dilution
Primary Antibodies			
Western Blotting			
Anti-STIM1	Mouse	BD Transduction Laboratories	1:250
Anti-Orai 1	Rabbit	Alomone Labs	1:2000
Anti-MLCK (clone K36)	Mouse	Sigma	1:4000
Anti Beta Actin	Mouse	Sigma	1:35000
Anti p397 FAK	Rabbit	Invitrogen	1:1000
Anti total FAK	Rabbit	Santa Cruz Biotechnology	1:1000
Anti pY118 Paxillin	Rabbit	Cell Signaling	1:500
Anti total Paxillin	Mouse	BD Transduction Laboratories	1:500
Immunofluorescence			
Anti- Alpha Tubulin	Mouse	Sigma	1:200
Alexa Fluor 594 phalloidin	Mushroom toxin	Molecular Probes	1:200
Anti VE- Cadherin	Goat	Santa Cruz Biotechnology	1:100
Anti-diphos MLC	Rabbit	Clements *et al*. (AJP. Lung cellular and molecular physiology **288**, L294 (2005)	1:100
Secondary Antibodies			
Anti-rabbit IgG -HRP	Rabbit	Calbiochem	1:10000
Anti-mouse IgG -HRP	Mouse	Calbiochem	1:10000
Alexa Fluor 594 donkey anti-mouse IgG	Mouse	Invitogen	1:800
Alexa Fluor 488 donkey anti-mouse IgG	Mouse	Invitrogen	1:800

Table S2: Sequences of the primers and siRNA sequences used throughout the study.

PCR Primers

Target	Forward (5'-3')	Reverse (3'-5')
TRPC1	GATGCATTCCATCCTACACT	TACACAGTCCTTCTGCTCCT
TRPC4	GGACTTCAGGACTACATCCA	ACGCAGAGAACTGAAGATGT
TRPC6	GAACTTAGCAATGAACTGGCAGT	CATATCATGCCTATTACCCAGGA
STIM1	AAC TGC TTA GCA CCC CTG GC	ATG ACC TTG CCC ACA GCC TT
Orai1	AGGTGATGAGCCTCAACGAG	CTGATCATGAGCGCAAACAG
MLCK	CAA CAA ACA ACA GAG AAG ACG G	AGT CTT CTG AAG GAC CGG G
Actin	GCG GGA GGG TAC TGA G	TCC ATG TCA TCC ACG TCG TCA
hGAPDH	AACTGCTTAGCACCCCTGGC	ATGACCTTGCCCACAGCCTT

siRNA sequences

siRNA	Sequence
TRPC1#1	GAGAAATGCTGTTACCATA
TRPC1#2	GCGACAAGGGTGACTATTA
TRPC4#1	GGTCAGACTTGAACAGGCA
TRPC4#2	GGCTCAGTTCTATTACAAA
TRPC6#1	GTGTGGATTACATGGGCCA
TRPC6#2	GGACCAGCATACATGTTTA
STIM1 # 2	AAGGGAAGACCUCAAUUACCAUU
STIM1 # 3	GGUGGUGUCUAUCGUUAUU
STIM1 # 6	UCUCUUGACUCGCCAUAAU
Orai1 # 1	CGUGCACAAUCUCAACUCGUU
Orai1 # 2	CUGUCCUCUAAGAGAAUAAUU
MLCK # 1	GCUGCUAGAUUUGACUGCAAGAUUG
MLCK # 2	AGUCCCGCCACUUCCAGAUAGACUA
MLCK # 3	GAUGAGGACGGGAACUGCUCUUUAA
siControl	UGGUUUACAUGUCGACUAAUU