

Supplementary Materials for  
**Direct Binding Between Orai1 and AC8 Mediates Dynamic Interplay  
Between Ca<sup>2+</sup> and cAMP Signaling**

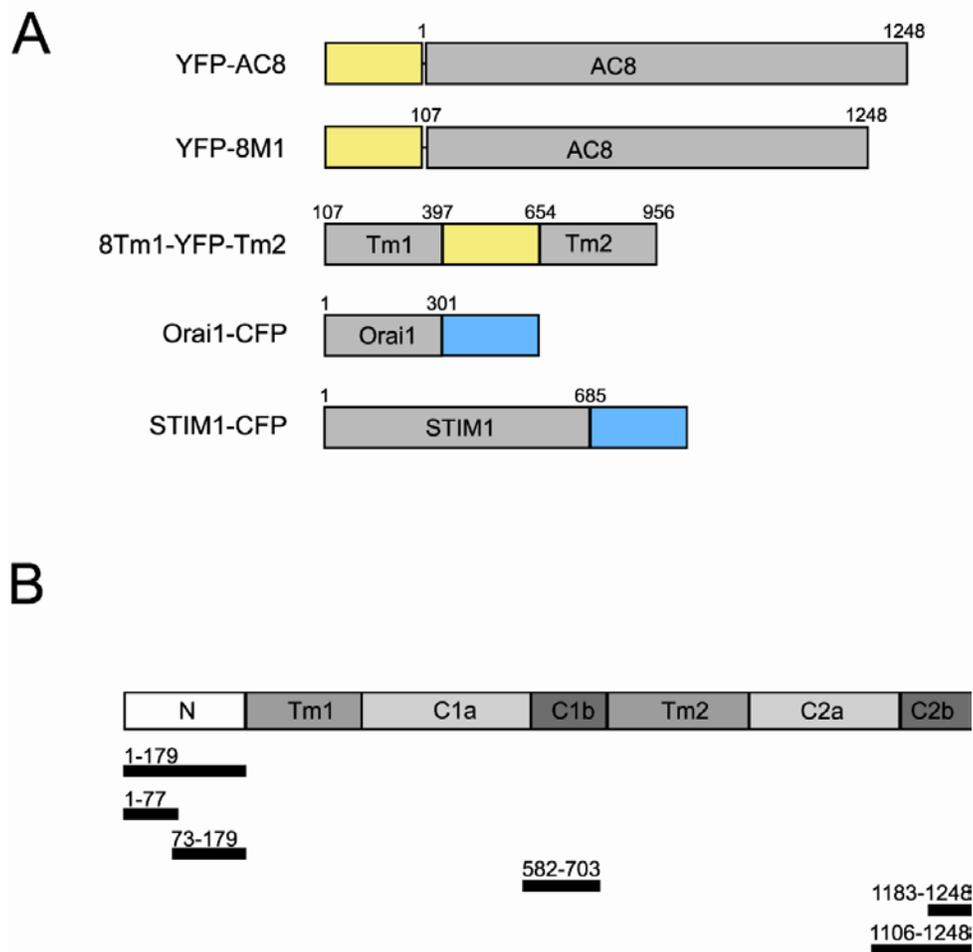
Debbie Willoughby, Katy L. Everett, Michelle L. Halls, Jonathan Pacheco, Philipp Skroblin, Luis Vaca, Enno Klussmann, Dermot M. F. Cooper\*

\*To whom correspondence should be addressed. E-mail: dmfc2@cam.ac.uk

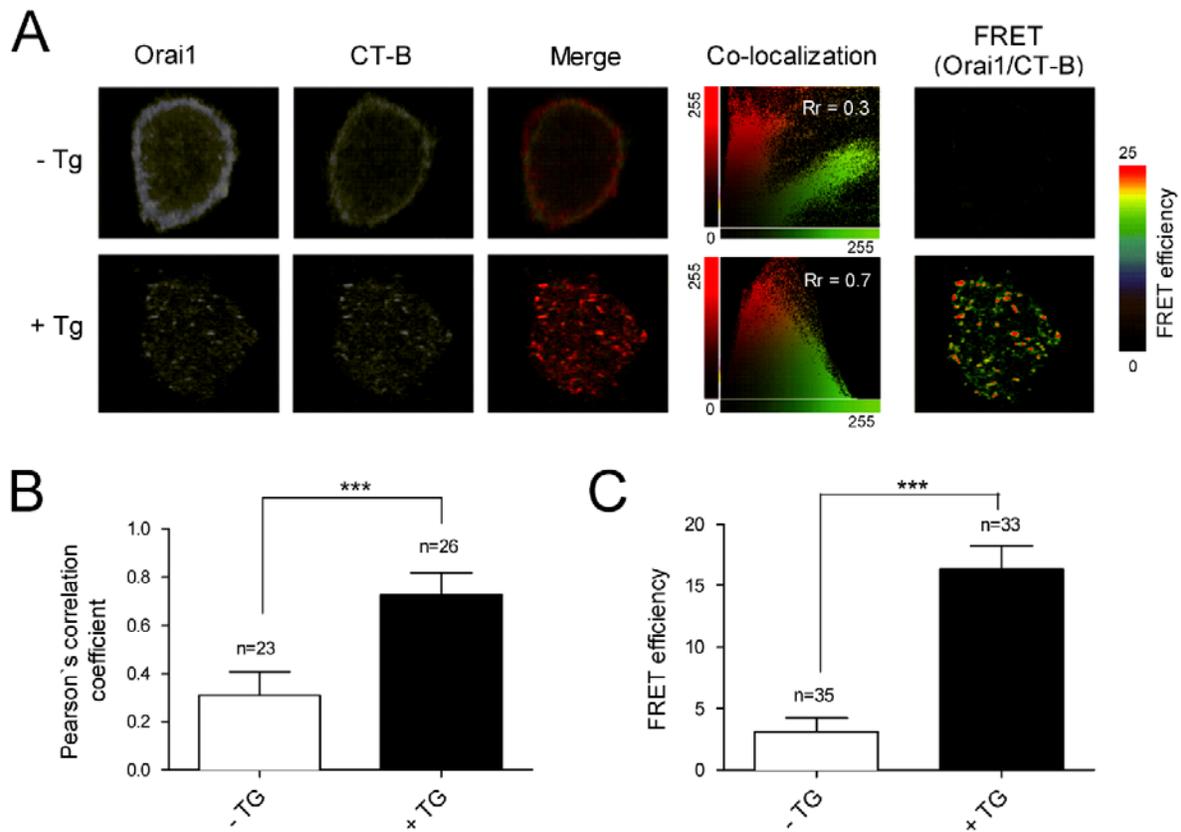
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**The PDF file includes:**

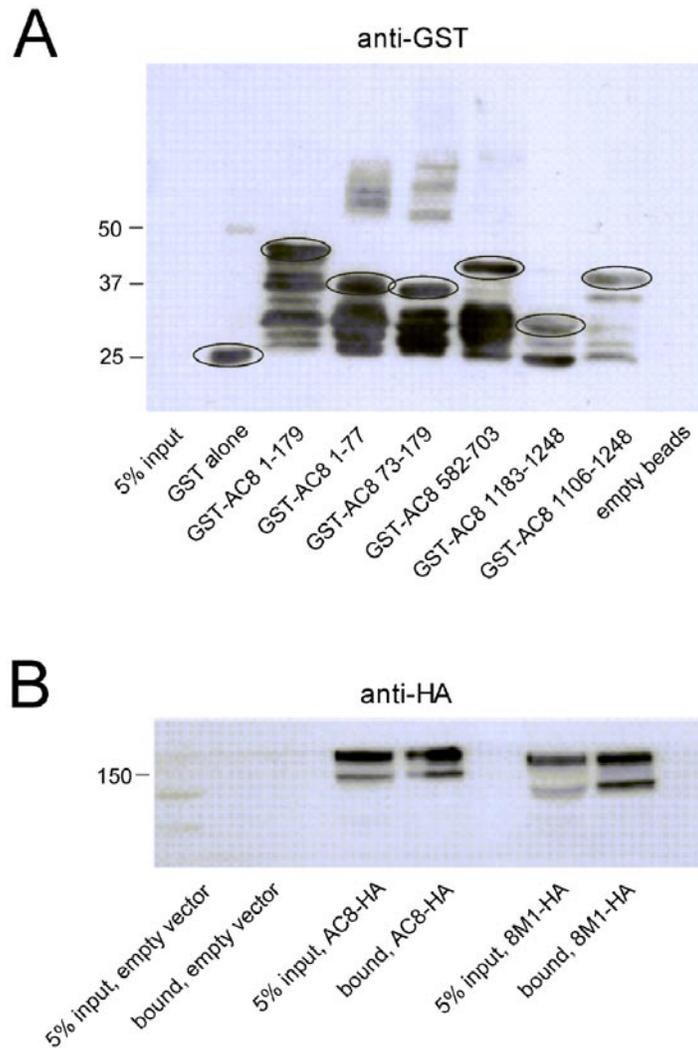
- Fig. S1. Constructs used to study AC8 and Orai1 interaction.
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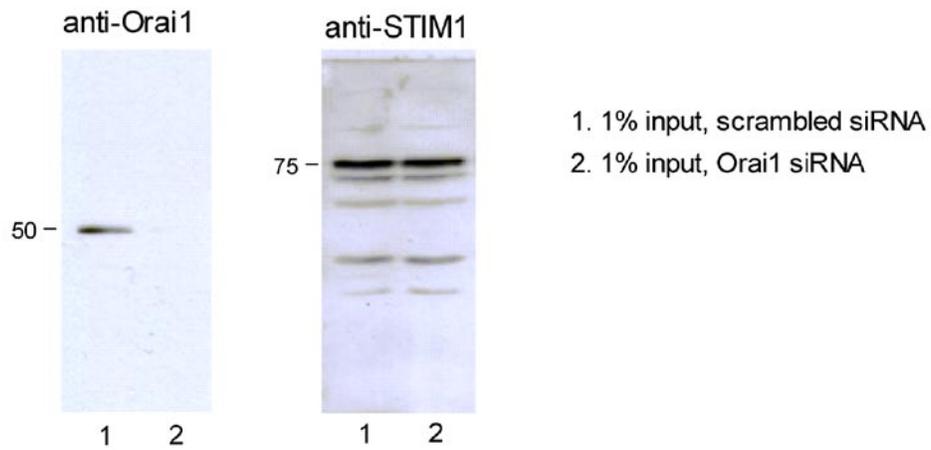
**Fig. S1. Constructs used to study AC8 and Orai1 interaction.** **A**, YFP-tagged wild-type AC8, and two AC8 variants used for FRET studies with C-terminally CFP-tagged full-length Orai1 and STIM1. YFP-8M1 lacks the first 106 N-terminal residues of AC8. 8Tm1-YFP-Tm2 contains the full-length N-terminus and transmembrane (Tm) domains of AC8 but lacks the catalytic C1 and C2 domains, and has YFP sandwiched between residues 397 and 654 of AC8. **B**, Cytosolic fragments of AC8 from the N-terminus (residues 1-179, 1-77, and 73-179), C1 domain (residues 582-703), and C2 domain (residues 1183-1248, 1106-1248) used for GST-pull down studies.



**Fig. S2. Colocalization between Orai1 and Cholera toxin B subunit is enhanced following thapsigargin treatment.** *A*, LG-TIRFM images from wild type HEK293 cells expressing Orai1-CFP and the lipid raft marker, cholera toxin B subunit (CT-B) conjugated to Alex Fluor 594. Correlation plots reveal enhanced pixel colocalization for Orai1-CFP and CT-B after addition of 1 $\mu$ M thapsigargin (Tg). Efficiency of FRET between Orai1-CFP and CT-B is shown in pseudocolour. *B*, Bar chart of mean  $\pm$  s.e.m. values for Pearson's correlation coefficient as a measure of colocalization, \*\*\* $p < 0.001$ . *C*, Bar chart of mean  $\pm$  s.e.m. calculated FRET efficiencies for Orai1 and CT-B  $\pm$  Tg, \*\*\* $p < 0.001$ , n values are given above bars.



**Fig. S3. Relative levels of GST-tagged AC8 fragments.** *A*, Exemplar GST blot for AC8 pull-down peptides used against Orai1 and STIM1 as presented in Fig. 3A and 3B. Bands identified as GST-tagged AC8 fragments have been *circled*. *B*, Exemplar control HA blot for Orai1 and STIM1 HA affinity co-immunoprecipitations presented in Fig. 3C and 3D. Data show comparable amounts of AC8-HA and 8M1-HA in cell lysate. All blots are representative of at least 4 repeats.



**Fig. S4. Specificity of Orai1 and STIM1 antibodies and Orai1 knockdown.** *Left-hand blot*, Western blot analysis was used to confirm knockdown of band identified as endogenous Orai1 (~ 50kDa) following treatment with Orai1-selective siRNA compared to a scrambled siRNA control. *Right-hand blot*, expression of band identified as endogenous STIM1 (~ 75 kDa) was unaffected by Orai1 knockdown. Representative of n=4 blots.

# A

Orai1 amino acids 1-87 (spots A1-B4):

A1 MHPEPAPPPSRSSPELPPSGGSTTS  
**A2** APPPSRSSPELPPSGGSTTS **GSRRS**  
**A3** RSSPELPPSGGSTTS **GSRRSRRRRSG**  
**A4** LPPSGGSTTS **GSRRSRRRRSGDGEP**  
**A5** GSTTS **GSRRSRRRRSGDGEP**  
**A6** **GSRRSRRRRSGDGEP**GAPPPPSAV  
A7 RRRSGDGEPGAPPPPSAVTYPDW  
A8 DGEPGAPPPPSAVTYPDWIGQSY  
A9 GAPPPPSAVTYPDWIGQSYSEVMS  
A10 PPSAVTYPDWIGQSYSEVMSLNEHS  
B1 TYPDWIGQSYSEVMSLNEHSMQALS  
B2 IGQSYSEVMSLNEHSMQALSWRKLY  
B3 SEVMSLNEHSMQALSWRKLYLSRAK  
B4 VMSLNEHSMQALSWRKLYLSRAK

Orai1 amino acids 141-173 (spots B5-B7):

B5 STSILPNIEAVSNVHNLNSVKESPH  
B6 PNIEAVSNVHNLNSVKESPHERMHR  
B7 EAVSNVHNLNSVKESPHERMHRHIE

Orai1 amino acids 256-301 (spots B8-C3):

B8 HFYRSLVSHKTDRQFQELNELAEFA  
B9 LVSHKTDRQFQELNELAEFARLQDQ  
B10 TDRQFQELNELAEFARLQDQLDHRG  
C1 QELNELAEFARLQDQLDHRGDHPLT  
C2 LAEFARLQDQLDHRGDHPLTPGSHY  
C3 AEFARLQDQLDHRGDHPLTPGSHYA

# B

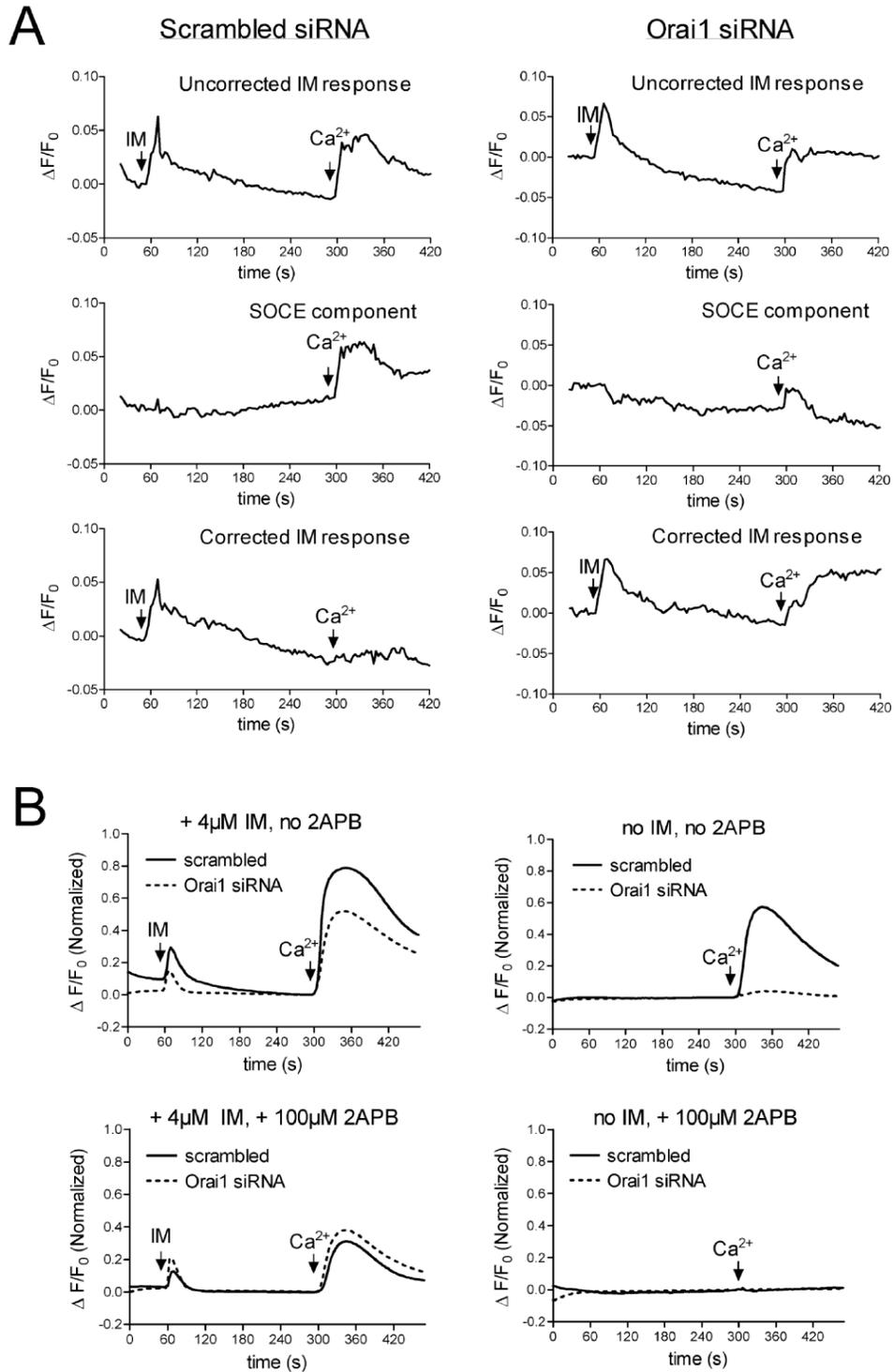
Single Ala substitutions:

A1 LPPSGGSTTSGSRRSRRRRSGDGEP  
A2 APPSGGSTTSGSRRSRRRRSGDGEP  
A3 LAPSGGSTTSGSRRSRRRRSGDGEP  
A4 LPASGGSTTSGSRRSRRRRSGDGEP  
A5 LPPAGGSTTSGSRRSRRRRSGDGEP  
A6 LPPSAGSTTSGSRRSRRRRSGDGEP  
A7 LPPSGASTTSGSRRSRRRRSGDGEP  
A8 LPPSGGATTSGSRRSRRRRSGDGEP  
A9 LPPSGGSATSGSRRSRRRRSGDGEP  
B1 LPPSGGSTASGSRRSRRRRSGDGEP  
B2 LPPSGGSTTAGSRRSRRRRSGDGEP  
B3 LPPSGGSTSASRRSRRRRSGDGEP  
B4 LPPSGGSTSGARRSRRRRSGDGEP  
B5 LPPSGGSTSGARSRRRRSGDGEP  
B6 LPPSGGSTSGRASRRRRSGDGEP  
B7 LPPSGGSTSGRRARRRRSGDGEP  
B8 LPPSGGSTSGRRSARRRRSGDGEP  
B9 LPPSGGSTSGRRSRARRSGDGEP  
C1 LPPSGGSTSGRRSRRASGDGEP  
C2 LPPSGGSTSGRRSRRRAGDGEP  
C3 LPPSGGSTSGRRSRRRSADGEP  
C4 LPPSGGSTSGRRSRRRSGAGEPP  
C5 LPPSGGSTSGRRSRRRSGDAEPP  
C6 LPPSGGSTSGRRSRRRSGDGAPP  
C7 LPPSGGSTSGRRSRRRSGDGEAP  
C8 LPPSGGSTSGRRSRRRSGDGEP

Double Ala substitutions:

C9 LPPSGGSTTSGSRRSRRRRSGDGEP  
D1 AAPSGGSTTSGSRRSRRRRSGDGEP  
D2 LAASGGSTTSGSRRSRRRRSGDGEP  
D3 LPAAGGSTTSGSRRSRRRRSGDGEP  
D4 LPPAAGSTTSGSRRSRRRRSGDGEP  
D5 LPPSAASTTSGSRRSRRRRSGDGEP  
D6 LPPSGAATTSGSRRSRRRRSGDGEP  
D7 LPPSGGAATTSGSRRSRRRRSGDGEP  
D8 LPPSGGSAASGSRRSRRRRSGDGEP  
D9 LPPSGGSTAAGSRRSRRRRSGDGEP  
E1 LPPSGGSTTAASRRSRRRRSGDGEP  
E2 LPPSGGSTSAARRSRRRRSGDGEP  
E3 LPPSGGSTSGAARSRRRRSGDGEP  
**E4** LPPSGGSTSGS**AA**SRRRRSGDGEP  
E5 LPPSGGSTSGSRAARRRRSGDGEP  
E6 LPPSGGSTSGSRRRAARRRRSGDGEP  
**E7** LPPSGGSTSGSRRS**AA**RRSGDGEP  
**E8** LPPSGGSTSGSRRS**RAA**SGDGEP  
**E9** LPPSGGSTSGSRRS**RAA**AGDGEP  
F1 LPPSGGSTSGSRRSRRRAADGEP  
F2 LPPSGGSTSGSRRSRRRSAAAGEPP  
F3 LPPSGGSTSGSRRSRRRSGAAEPP  
F4 LPPSGGSTSGSRRSRRRSGDAAPP  
F5 LPPSGGSTSGSRRSRRRSGDGAPP  
F6 LPPSGGSTSGSRRSRRRSGDGEAA  
F7 LPPSGGSTSGSRRSRRRSGDGEP

**Fig. S5. Spot sequences for peptide array data presented in Fig. 4. A,** Peptide spots generating positive hits for interaction with GST-tagged AC8 N-terminus are shown in bold. **B,** Double alanine substitutions incorporated into the spot sequence for A4 (panel A) that successfully blocked interaction with GST-tagged AC8 N-terminus are in bold. Single alanine substitutions did not prevent interaction.



**Fig. S6. Isolation of the ionomycin-induced, non-specific  $\text{Ca}^{2+}$  entry signal in GCaMP2-AC8- and global GCaMP2-expressing cells.** *A*, 4  $\mu\text{M}$  ionomycin addition (IM) and subsequent addition of 0.5 mM external  $\text{Ca}^{2+}$  has the potential to induce both non-specific  $\text{Ca}^{2+}$  entry and SOCE in cells pre-treated with 200nM thapsigargin (Tg) (*top traces*). The contribution of SOCE

to the GCaMP2-AC8 signal was measured in cells pre-treated with Tg, but not exposed to IM prior to  $\text{Ca}^{2+}$  addition (*middle traces*). The *lower traces* represent GCaMP2-AC8 response to IM-induced non-specific  $\text{Ca}^{2+}$  entry corrected for any contribution from SOCE (result of subtracting the *middle trace* from the *top trace*). Data are plotted for GCaMP2-AC8 expressing cells treated with either scrambled siRNA or Orai1-selective siRNA. **B**, IM-induced non-specific  $\text{Ca}^{2+}$  entry was measured with a cytosolic (non-targeted) GCaMP2 sensor in HEK293 cells. All cells were pre-treated with 200nM Tg. *Top left*, combined SOCE and non-specific  $\text{Ca}^{2+}$  entry in IM-treated cells under control conditions or following Orai1 knockdown. *Top right*, contribution of SOCE was attenuated by Orai1 knockdown. *Bottom left*, IM-induced non-specific  $\text{Ca}^{2+}$  entry was isolated by treatment with 100  $\mu\text{M}$  2-aminoethoxydiphenyl borate (2APB), a potent SOCE inhibitor (see *bottom right*). A similar degree of non-specific  $\text{Ca}^{2+}$  entry was seen in control cells and in Orai1 knockdown cells. N values ranged from 30 to 57 cells for each condition.