Biochemical and Functional Characterization of Orai Proteins*5

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Stimulation of immune cells triggers Ca2+ entry through store-operated Ca2+ release-activated Ca2+ channels, promoting nuclear translocation of the transcription factor NFAT. Through genome-wide RNA interference screens in Drosophila, we and others identified olf186-F (Drosophila Orai, dOrai) and dStim as critical components of store-operated Ca2+ entry and showed that dOrai and its human homologue Orai1 are pore subunits of the Ca2+ release-activated Ca2+ channel. Here we report that Orai1 is predominantly responsible for store-operated Ca2+ influx in human embryonic kidney 293 cells and human T cells and fibroblasts, although its paralogue Orai3 can partly compensate in the absence of functional Orai1. All three mammalian Orai are widely expressed at the mRNA level, and all three are incorporated into the plasma membrane. In human embryonic kidney 293 cells, Orai1 is glycosylated at an asparagine residue in the predicted second extracellular loop, but mutation of the residue does not compromise function. STIM1 and Orai1 colocalize after store depletion, but Orai1 does not associate detectably with STIM1 in glycerol gradient centrifugation or coimmunoprecipitation experiments. Glutamine substitutions in two conserved glutamate residues, located within predicted transmembrane helices of Drosophila Orai and human Orai1, greatly diminish store-operated Ca2+ influx, and primary T cells ectopically expressing mutant E106Q and E190Q Orai1 proteins show reduced proliferation and cytokine secretion. Together, these data establish Orai1 as a predominant mediator of store-operated calcium entry, proliferation, and cytokine production in T cells.

Ca2+ is a key second messenger in intracellular signaling pathways. In lymphocytes, specialized store-operated Ca2+ channels known as CRAC5 channels are required for sustained Ca2+ influx across the plasma membrane (1). The resulting prolonged elevation of intracellular free Ca2+ entry is essential for sustained nuclear translocation of the transcription factor NFAT, a small family of proteins whose activation is critical for a productive immune response (2). NFAT proteins reside in the cytoplasm of resting lymphocytes in a highly phosphorylated form and translocate to the nucleus upon dephosphorylation by the Ca2+/calmodulin-dependent serine/threonine phosphatase calcineurin (2, 3). In the nucleus, NFAT proteins bind to promoters and regulatory regions of a large number of cytokine genes and other activation-associated genes, thereby mediating the activation, proliferation, and differentiation of T cells, B cells, and other immune system cells.

Although the notion of Ca2+ influx through “store-operated” Ca2+ channels was first proposed in 1986 (4, 5), the molecular identity of the proteins involved in this process remained unknown until the advent of large-scale RNAi-based screens. The first components of the pathway to be identified were Drosophila Stim (dStim) and its human homologues STIM1 and STIM2 through large-scale (albeit not genome-wide) RNAi-based screens in Drosophila and HeLa cells, respectively (6, 7). STIM proteins are single-pass transmembrane proteins localized predominantly in the membrane of the endoplasmic reticulum (ER); they contain an N-terminal EF-hand located in the ER lumen and appear to function as sensors of ER Ca2+ entry through “store-operated” channels (8–10). Store-influx through “store-operated” channels is also predominant in the plasma membrane (10) but are more likely points of apposition of the ER and plasma membranes (7, 11, 12). It is thought that within these puncta, STIM1 communicates with and opens CRAC channels located in the plasma membrane (13).

More recently, genome-wide RNAi screens performed in Drosophila cells by our laboratory (14) and others (15, 16) have identified a CRAC channel component, olf186-F. We have renamed this protein Drosophila Orai (dOrai). Its three human homologues, Orai1, Orai2, and Orai3 (also known as CRACM1, CRACM2 and CRACM3 (15, 17), are encoded by the genes TMEM142A, TMEM142B, and TMEM142C (HUGO Gene Nomenclature Committee). Orai1 bears the causal mutation in
a severe combined immunodeficiency (SCID) syndrome characterized by a defect in CRAC channel function and T cell cytokine expression (14, 18, 19). Combined overexpression of dOrai and dSTIM in Drosophila cells or Orai1 and STIM1 in Jurkat T cells, RBL cells, or HEK293 cells results in a dramatic increase in IC\textsubscript{CRAC} (16, 20, 21). Amino acid substitutions in either of two conserved glutamate residues, located in predicted transmembrane segments of dOrai and Orai1, changed the properties of IC\textsubscript{CRAC}, suggesting strongly that these proteins are pore subunits of the CRAC channel (16, 17, 22, 23).

Here we compare the properties of the three mammalian Orai proteins. We show that all three are widely expressed at the mRNA level and all can be incorporated into the plasma membrane when ectopically expressed. Orai1 forms homodimers and homomultimers in cells and in detergent solutions, can heteromultimerize with Orai2 and Orai3 as judged by co-immunoprecipitation, and has a predominant role in store-operated Ca\textsuperscript{2+} entry in HEK293 cells and human T cells and fibroblasts when stores are depleted with thapsigargin. Immunocytochemical analysis shows that ectopically expressed Orai1 and STIM1 colocalize partially in thapsigargin-stimulated T cells. We have generated dominant-interfering forms of dOrai and human Orai1 by substituting glutamine residues in place of either of two highly conserved glutamates located in the first and third predicted transmembrane segments. We show that ectopic expression of the E106Q and E190Q mutants of Orai1 in primary murine T cells severely impairs store-operated Ca\textsuperscript{2+} influx, proliferation, and cytokine production, consistent with our previous conclusion that Orai1 is a major contributor to T lymphocyte function and the adaptive immune response (14).

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Human T cell lines from one control individual and from SCID patient 2 were immortalized by transformation with herpesvirus saimiri as described (24). Foreskin fibroblasts from the newborn SCID patient 2 and a healthy newborn (Hs27 cell line; ATCC, Manassas, VA) were immortalized by retroviral transduction with a telomerase expression plasmid (hTERT, a generous gift from S. Lessnick, Dana-Farber Cancer Institute, Boston, MA). The macrophage-hemocyte-like Drosophila cell line S2R\textsuperscript{+} was grown in Schneider’s medium with 10% fetal calf serum (Invitrogen) according to standard protocols.

**Plasmids**—Full-length cDNAs for Orai1 (BC015369), Orai2 (BC069270), and Orai3 (BC022786) were purchased from OpenBiosystems (Huntsville, AL) and subcloned into pENTR11 (“Gateway” system; Invitrogen) for use in retroviral transductions. For Myc-tagged human Orai proteins, the c-Myc epitope tag (peptide sequence, EQKLISEEDL; nucleotide sequence, CGG CAC GGC AGC TAC CCA TAC GAT GTT CCA GAT TAC GCT) was placed in-frame immediately following the cDNA sequences of Orai1, 2, and 3, respectively (i.e. C-terminal Myc tag). The endogenous stop codon was replaced with GAG encoding the first E of the Myc tag; at the end of the Myc sequence a stop codon (TAG) was added to terminate translation. For retroviral expression plasmids encoding human Orai proteins, Orai1, 2, and 3 cDNAs with FLAG epitope tags introduced at the C terminus were cloned between the Xhol and EcoRI sites of pMSCV-CITE-eGFP-PGK-Puro. The endogenous termination codon was substituted with the coding sequence of the FLAG tag (AADYKDDDDK) followed by a TAG termination codon. For mutagenesis of Orai1, the QuikChange site-directed mutagenesis kit (Invitrogen) was used according to the manufacturer’s instructions. HA epitope tags were introduced within the second extracellular loop of Orai1 (22), Orai2, and Orai3 by first introducing SaII and MluI restriction enzyme sites between codons 206 and 207 of Orai1, codons 188 and 189 of Orai2, and codons 194 and 195 of Orai3, respectively, followed by ligation of a double-stranded oligonucleotide encoding the HA peptide flanked by a glycine-serine-glycine-serine linker on either side: 5'-TC GAC GGC AGC GGC AGC TAC CCA TAC GAT GTT CCA GAT TAC GCT GGC AGC GGC AGC A-3'.

For FLAG-tagged dOrai, the FLAG sequence was placed in-frame immediately after the coding sequence of dOrai. The endogenous stop codon was replaced with the first codon of the FLAG epitope tag; at the end of the flag sequence a TAG stop codon was added to terminate translation. S2R\textsuperscript{+} cells were stably transfected with wild-type or mutant dOrai subcloned into the expression plasmid pAc5.1 (Invitrogen), which adds a V5 tag to the C terminus of the coding sequences of dOrai. S2R\textsuperscript{+} cells were co-transfected with pAc5.1 dOrai and a hygromycin resistance gene under the control of a constitutively active promoter (pCoHygro; Invitrogen) at a ratio of 19:1. Cells were selected for 3–4 weeks with 300 \( \mu \)g/ml hygromycin, and stable clones were selected by visual inspection.

**Antibodies**—Anti-FLAG monoclonal antibody was purchased from Sigma (M2 clone), anti-HA monoclonal antibody (12CA5) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), and anti-STIM1 monoclonal antibody was purchased from BD Biosciences. Anti-Myc (9E10) monoclonal antibody was purified from hybridoma supernatants in our laboratory.

**Genome-wide Drosophila RNAi Screen**—The genome-wide RNAi screen in Drosophila was performed at the Drosophila RNAi Screening Center at Harvard Medical School as previously described (25–28). 10\(^4\) S2R\textsuperscript{+} cells stably expressing NFAT1-(1–460) GFP were added into each well of a 384-well plate containing 0.25 \( \mu \)g of double-stranded RNAs in 10 \( \mu \)l of serum-free medium and incubated for 1 h at 24 °C, followed by addition of 30 \( \mu \)l of complete medium. After incubation for 4 days at 24 °C, the cells were treated with 1 M thapsigargin for 10 min, fixed, and stained with 4',6-diamidino-2-phenylindole, and the coincident GFP and 4',6-diamidino-2-phenylindole images were acquired by an automated camera from three different locations in each well. A total of 63 384-well plates were analyzed, containing a total of \( \approx 23,770 \) wells into which individual double-stranded RNAs had been arrayed. The whole genome-wide screen was duplicated to improve the accuracy and check the reproducibility of the screen.

**Secondary RNAi Screen Measuring Thapsigargin-induced Ca\textsuperscript{2+} Entry**—The primary screen identified 16 candidates whose RNAi-mediated depletion interfered with NFAT nuclear import, including calmodulin and the catalytic (CanA-14P) and regulatory (CanB) subunits of calcineurin as expected (25). With the exception of calcineurin and calmodulin, the
Orai1 Mediates Store-operated Ca\(^{2+}\) Entry in T Cells

### TABLE 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ primer sequence</th>
<th>3’ primer sequence</th>
<th>Fluorogenic probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Orai1</td>
<td>CAGCTGCTGAATGGTGGAGG</td>
<td>AGCCACTGAAGGCTAGGCA</td>
<td>CTGGCACTCGACACGGTAGCTACCCA</td>
</tr>
<tr>
<td>Human Orai2</td>
<td>AAGTCGAAAGTGGGCAATG</td>
<td>TGGGACTCATCTGCTGGTACCTG</td>
<td>TCCATACCTGACCACCAGAAAGACCTT</td>
</tr>
<tr>
<td>Human Orai3</td>
<td>AGGTTGGGAGATCATGG</td>
<td>TGGTGGTCAGAAGGCAATG</td>
<td>TCCATACCTGACCACCAGAAAGACCTT</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>AGGCGGAGGATATGCTGAGA</td>
<td>GCCATGGTGGCCATGGT</td>
<td>TCCATACCTGACCACCAGAAAGACCTT</td>
</tr>
</tbody>
</table>

remaining 13 candidates were re-evaluated in a secondary screen using double-stranded RNAs listed on the *Drosophila* RNAi Screening Center Web site (26–28). PCR fragments (size up to 600 bp) were used as templates for in vitro transcription reactions, followed by DNase I treatment to remove the template DNA. After purification, double-stranded RNA (5 μg) was transfected into S2R+ cells in 12-well plates. After 4 days of incubation, Ca\(^{2+}\) influx in S2R+ cells was measured by flow cytometry. Cells were detached from the dish with trypsin (CellGro, Herndon, VA) and then loaded with the Ca\(^{2+}\) indicator dyes Fluo-4-AM and Fura-Red (2 μM each; Invitrogen) for 45 min at room temperature in Schneider’s medium containing 10% fetal calf serum. Immediately prior to flow cytometry, cells were resuspended in Ringer solution containing no added Ca\(^{2+}\) and analyzed for basal levels of intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) on a FACSCalibur flow cytometer (BD Biosciences). After 30 s, thapsigargin (3 μM) was added to deplete ER Ca\(^{2+}\) stores. An equal volume of Ringer solution containing 4 mM Ca\(^{2+}\) was added to the samples (final [Ca\(^{2+}\)]\(_i\) 2 mM), and [Ca\(^{2+}\)]\(_i\) levels were monitored for 300 s. The ratio of Fluo-4 and Fura-Red emission was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**Northern Blots**—Northern blotting was performed on the First Choice Human Northern Blot I (Ambion, Foster City, CA) according to the manufacturer’s protocol. The probes were PCR-labeled using Strip-EZ PCR probe synthesis and removal kit (Ambion) according to the manufacturer’s instructions. The primers used to generate probes for the 3’-untranslated regions of the human Orai genes were as follows: Orai1, CCCCTTCCAGT-GCTTTTGCCCTTA and GTGTCAACACACACATGTACA-CCT; Orai2, AGGGACTGTGTTGCTAAAGGCTTT and CGTACCTTTGCAACGGCTCAAAAT; and Orai3, TTGTTGG-AGACCTTCTAGTGTACATT and ACAAAGTTTTGGCGCATGTCGTGG. For Northern blot analysis of murine tissues, total RNA was extracted using TRIzol reagent (Invitrogen), and 20 μg of RNA was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto Nytran SuperCharge membrane (Schleicher and Schuell Bioscience). Gene products and their human orthologues are grouped into functional categories. Only dOrai and dSTIM were unambiguously identified as regulators of store-operated Ca\(^{2+}\) influx in a secondary flow cytometry-based screen (14). ***, change in cell morphology prevented an accurate assessment of store-operated Ca\(^{2+}\) influx.

**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene name</th>
<th>FlyBase Gene no.</th>
<th>Human (Homologene)</th>
<th>Description</th>
<th>NFAT localization(\ast)</th>
<th>TG-stimulated Ca(^{2+}) influx(\ast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM</td>
<td>0000253</td>
<td>C2M2</td>
<td>Calmodulin</td>
<td>C(\ast)</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>CanB</td>
<td>0010014</td>
<td>PPP3R2</td>
<td>Calcinerin, regulatory subunit</td>
<td>C(\ast)</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>CanA-14F</td>
<td>0030758</td>
<td>PPP3CA</td>
<td>Calcinerin, catalytic subunit</td>
<td>C(\ast)</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>Nuclear transport</td>
<td>0022213</td>
<td>CSE1L</td>
<td>Recycler of importin-α</td>
<td>C</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Fx2/Ket</td>
<td>000986</td>
<td>KPNB1</td>
<td>Importin-β</td>
<td>C</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Kap-α3</td>
<td>0027338</td>
<td>KPN4 A</td>
<td>Importin-α</td>
<td>C &amp; N</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Cytokines</td>
<td>0000044</td>
<td>ACTR</td>
<td>Actin</td>
<td>C &amp; N</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Scra**</td>
<td>0004243</td>
<td>ANLN</td>
<td>Anillin, actin-binding protein</td>
<td>C &amp; N</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Peo**</td>
<td>0030241</td>
<td>PRCI</td>
<td>Protein regulator of cytokinesis 1</td>
<td>C &amp; N</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Pav**</td>
<td>0011692</td>
<td>KIF23, MLKP1</td>
<td>Kinesin family member 23</td>
<td>C &amp; N</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Calcium Influx</td>
<td>0045073</td>
<td>STIM1, 2</td>
<td>Calcium</td>
<td>C</td>
<td>Blocked</td>
<td></td>
</tr>
<tr>
<td>Off186-F (dOrai)</td>
<td>0041585</td>
<td>FLI1446 (Orai1, 2, 3)</td>
<td>Pore subunit of CRAC channel</td>
<td>C</td>
<td>Blocked</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0033260</td>
<td>CUL4B</td>
<td>E3 ubiquitin ligase subunit</td>
<td>C</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Noi</td>
<td>0014366</td>
<td>SF3A3</td>
<td>Splicing factor 3a, subunit 3</td>
<td>C &amp; N</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>CG7214</td>
<td>0031940</td>
<td>None*</td>
<td>Unknown function</td>
<td>C &amp; N</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>0030730</td>
<td>None*</td>
<td>Found only in insects</td>
<td>C &amp; N</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

\(\ast\) NFAT subcellular localization and Ca\(^{2+}\) influx in cells treated with dsRNA for 4 days and then with thapsigargin for 10 min. C, cytoplasmic; N, nuclear; n.t., not tested.

As also previously reported in Ref. 25.

No human homologue in Homologene.

HEK293 cells/well were seeded in 12-well plates and transfected the next day with control or experimental siRNAs (Dharmacon, Inc., Lafayette, CO) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol. Cells were resuspended and the transfection procedure was repeated after 24 h to increase the efficiency of knock down. Cells were harvested for real-time PCR or [Ca\(^{2+}\)]\(_i\) measurement 3 days after transfection. The siRNA sequences correspond to Orai1, UCAAGUUGUUAGCCAUAGA; Orai2, CUCAACCACUCGGUAA; Orai3, UUGAAGCUGUGAG-
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FIGURE 1. Orai family members are widely expressed at the mRNA level, but only Orai1 siRNA decreases store-operated Ca\textsuperscript{2+} entry in HEK293 cells. A and B, Northern analysis for expression of mRNAs encoding Orai1, 2, and 3 in human (A) and mouse (B) tissues and cells. GAPDH was used as the loading control. C and D, RNAi-mediated depletion shows that Orai1 is a major regulator of store-operated Ca\textsuperscript{2+} entry in HEK293 cells. C, HEK293 cells were transfected with siRNAs against Orai1, 2, or 3 or irrelevant sequence (Ctrl), and knockdown efficiency was determined 3 days later by quantitative reverse transcription PCR. Each siRNA depletes the target mRNA, but knock down of Orai3 results in an unexpected 3-fold increase in Orai1 mRNA. Error bars indicate mean and S.D. of at least two independent knockdown experiments, each performed in triplicate. D, Ca\textsuperscript{2+} influx was examined by single-cell video imaging. To stimulate influx, intracellular Ca\textsuperscript{2+} stores were depleted with 1 μm thapsigargin (TG) in the absence of extracellular Ca\textsuperscript{2+} (0 Ca\textsuperscript{2+}) followed by re-addition of 2 mM Ca\textsuperscript{2+} (2 Ca\textsuperscript{2+}). Knock down of Orai1 impairs store-operated Ca\textsuperscript{2+} entry, whereas knock down of Orai2 or Orai3 has little or no effect. The same control trace was used for all three panels.

CAACAU. Knock down efficiency was quantified by quantitative PCR analysis using the Taqman method. Briefly, RNA was purified with TRIzol LS reagent (Invitrogen) and oligo(dT)-primed for first-strand cDNA synthesis (Superscript II kit; Invitrogen) according to the manufacturer’s instructions. Quantitative 5′-nuclease fluorogenic real-time PCR (Taqman) was performed with an iCycler IQ (Bio-Rad). Primers for PCR spanned an intron to exclude contamination with genomic DNA. The amplified cDNA was normalized to GAPDH and expressed as percentage relative to samples treated with control siRNAs (“scrambled” siRNA from Dharmacon). Sequences of primers and probes are tabulated in Table 1.

Western Blots and Immunoprecipitation—HEK293 cells were stably or transiently transfected with mammalian expression vectors. 48 h after transfection, 10⁷ cells were harvested in PBS and lysed in 1 ml of Triton lysis buffer (10% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and then centrifuged at 100,000 × g for 1 h at 4 °C before preclearing for 2 h at 4 °C with 25 μl of packed protein G-Sepharose. Lysates were immunoprecipitated overnight at 4 °C with anti-FLAG resin (Sigma). In some experiments, cells were pre-treated with 2 μg/ml tunicamycin (Sigma) for 18 h before harvesting and lysis. Immunoprecipitates were washed four times in lysis buffer and resolved by 10% SDS-PAGE, and proteins were detected by immunoblotting. S2R+ cells were transiently transfected with Drosophila expression vectors. After 48 h, cells were lysed in Triton lysis buffer, precipitated with anti-FLAG resin, and analyzed by immuno blotting with antibodies to FLAG, Myc, or V5 epitope tags.

Glycerol Gradient Analysis—2 × 10⁷ Jurkat T cells, stably transfected with FLAG-tagged Orai1, were left untreated or treated with thapsigargin for 10 min in PBS. Cells were lysed in Triton lysis buffer, cleared by centrifugation, and diluted to 0.5% Triton X-100. The lysates were applied to 10–40% glycerol cushion and centrifuged at 55,000 rpm for 12 h at 4 °C. A total of 42 fractions were collected from the top, and the fractions between 10 (top) and 40 (bottom) were analyzed by immunoblotting with anti-FLAG antibody. Molecular weights were estimated from the results of parallel experiments with size marker proteins.

Dithiobis(succinimidyl propionate) Cross Linking—10⁷ HEK293 cells, stably expressing FLAG-tagged Orai1, were left untreated or treated with thapsigargin for 10 min and then harvested in PBS and cross-linked with 1 mM dithiobis(succinimidyl propionate) for 1 h on ice, followed by quenching with 20 mM Tris-Cl, pH 7.5. Cells were lysed in Triton lysis buffer (see above), precleared, and immunoprecipitated overnight at 4 °C with anti-FLAG resin (Sigma). Immunoprecipitates were washed four times in lysis buffer and resolved by 10% SDS-PAGE, and Orai1 was detected by immunoblotting with anti-FLAG antibodies.

T Cell Differentiation—Murine CD4+ cells were purified by magnetic bead separation (Dynal, Invitrogen) from spleen and lymph nodes of young (3–5-week-old) C57BL/6 mice following the manufacturer’s protocols. The purity of CD4+ T cells in different preparations was >95%. Cells were stimulated with anti-CD3 and anti-CD28 under Th1 conditions and expanded in IL-2-containing medium as previously described (29).

Intracellular Cytokine Staining and Proliferation Assays—For intracellular cytokine staining, T cells were stimulated with...
10 nm phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for 4 h. Brefeldin A (10 μg/ml; Sigma) was included during the final 2 h of stimulation. Cells were fixed with 4% paraformaldehyde in PBS for 8 min at 25 °C, washed twice with PBS, and permeabilized in saponin buffer (PBS, 0.5% saponin, 1% bovine serum albumin, and 0.1% sodium azide). Cells were washed three times in saponin buffer and twice in PBS, and were analyzed with a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software. Phycoerythrin anti-IL-2 and allophycocyanin anti-IFN-γ antibodies were purchased from eBioscience (San Diego, CA). BrdUrd incorporation assays were performed according to the manufacturer’s instructions (BD Biosciences). Briefly, T cells were stimulated with 10 nM PMA and 1 μM ionomycin for 30 min and cultured in medium without IL-2 to assess autocrine proliferation. The next day, cells were pulsed with BrdUrd for 60 min, stained with phycoerythrin-conjugated anti-BrdUrd monoclonal antibody (Pharmingen), and analyzed with a FACSCalibur flow cytometer (BD Biosciences) to measure BrdUrd incorporation.

**Immunocytochemistry and Confocal Imaging**—Jurkat T cells were transfected with C-terminal FLAG-tagged Orai1, Orai2, or Orai3 (Fig. 3A) or co-transfected with FLAG-tagged Orai1 and Myc-tagged STIM1, and allowed to grow for 48 h. The cells were allowed to attach on a poly-L-lysine-coated chamber slide and either left untreated or treated with 1 μM thapsigargin for 10 min. The cells were fixed with 3% paraformaldehyde, permeabilized with wash buffer containing 0.5% Nonidet P-40, and incubated with anti-Myc (9E10) or anti-FLAG (M2; Sigma) antibodies and then with Cy5 or Cy3-labeled secondary antibodies. For surface staining (Fig. 3B), HEK cells transiently expressing Orai1, Orai2, or Orai3 with an HA tag introduced into the TM3-TM4 loop were plated overnight in chamber slides, fixed with 3% paraformaldehyde for 20 min at room temperature, and then blocked and treated with primary and secondary antibodies in PBS with 10% serum. Immunofluorescence was analyzed by confocal imaging using a Radiance 2000 laser-scanning confocal system (Bio-Rad) on a BX50BWI Olympus microscope using a 60 oil immersion objective lens.

**RESULTS**

**Candidates Identified in the Drosophila RNAi Screen**—We recently performed two genome-wide RNAi screens in Drosophila to identify regulators of the signal transduction pathway leading from [Ca2+]i influx to calcineurin activation and thence to nuclear import of the transcription factor NFAT (14, 25). The first screen was intended to find proteins whose depletion by RNAi resulted in nuclear accumulation of an NFAT-GFP fusion protein in resting cells; hundreds of candidates
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emerged, including NFAT kinases and candidates with known or presumed roles in Ca\textsuperscript{2+} homeostasis (25). The second screen was designed to find proteins whose RNAi-mediated depletion prevented nuclear accumulation of NFAT-GFP in thapsigargin-stimulated cells (14). Of the more than 21,000 gene candidates evaluated, this screen identified only 16 candidates whose depletion interfered robustly with NFAT nuclear import. Only two of these candidates, dOrai and dSTIM, were unambiguously identified as regulators of store-operated Ca\textsuperscript{2+} influx in a secondary flow cytometry-based screen (14). The remaining candidates included calcineurin subunits/ regulators (25) and proteins involved in nuclear transport as expected, four candidates (actin, Pav, ScrA, and Feo) with roles in cytokinesis (30–32), two candidates (Cul4 and Nol) whose role in NFAT regulation is not immediately obvious, and two candidates (Thr and the CG7214 gene product) that do not have conserved human homologues (Table 2). We focus here on Drosophila and human Orai.

Orai1 Is the Predominant Contributor to Store-operated Ca\textsuperscript{2+} Entry in Human T Cells and Fibroblasts—Drosophila Orai has three human homologues, TMEM142A (chromosome 12) encoding Orai1, which has been clearly validated as a contributor to I\textsubscript{CRAC} (14, 15, 17, 22); TMEM142B (chromosome 7) encoding Orai2; and TMEM142C (chromosome 16) encoding Orai3. Northern analysis of human and murine tissues showed that all three Orai family members are expressed in multiple tissues (Fig. 1, A and B). Orai1 and Orai3 transcripts are widely expressed, Orai2 transcripts are prominent in kidney, lung, and spleen, and Orai3 appears to be the only family member that is strongly expressed at the mRNA level in brain (Fig. 1, A and B). Orai1 and Orai3 transcripts of 1.5 and 2.2 kb matched the size of NCBI model mRNAs NM_032790 (Orai1) and NM_152288 (Orai3), but Orai2 had multiple transcripts that were longer than 2.5 kb, the size of NCBI model mRNA NM_032831.

We used RNAi-mediated depletion to ask whether Orai2 and Orai3 resembled Orai1 in regulating store-operated Ca\textsuperscript{2+} entry. HEK293 cells and Jurkat T cells were transfected with siRNAs directed against Orai2 and Orai3 and then cultured for 3 days before analysis of Ca\textsuperscript{2+} influx. Orai1 was used as a positive control. Depletion of Orai1 decreased thapsigargin-stimulated Ca\textsuperscript{2+} influx as expected (11, 14, 15, 22, 33), whereas depletion of Orai2 and Orai3 had little or no effect (Fig. 1, C and D; data not shown for Jurkat T cells). The siRNAs were effective in diminishing transcript levels of the corresponding mRNAs in HEK293 cells, as shown by reverse transcription PCR (Fig. 1C). Unexpectedly, however, depletion of Orai3 caused a large increase in mRNA levels of Orai1, possibly due to a compensatory feedback mechanism (Fig. 1C). We therefore independently evaluated Orai function by measuring the ability of Orai proteins to restore store-operated Ca\textsuperscript{2+} entry when expressed in cells from I\textsubscript{CRAC}−deficient SCID patients (19) bearing an R91W mutation in Orai1 (14). As shown previously (14), Orai1 efficiently complemented store-operated Ca\textsuperscript{2+} influx in SCID T cells and fibroblasts; in contrast, Orai2 and Orai3 reconstituted poorly or not at all (Fig. 2 and supplemental Fig. S1A). However, SCID fibroblasts transduced with Orai3 showed a small amount of store-operated Ca\textsuperscript{2+} entry, which was blocked by La\textsuperscript{3+} (supplemental Fig. S1A) and eliminated by mutation of the conserved glutamate residue in the first predicted transmembrane domain of Orai3 (E81Q, corresponding to the E106Q mutation in Orai1 described below; Fig. 2B). Moreover, combined overexpression of Orai3 and STIM1 resulted in substantial reconstitution of Ca\textsuperscript{2+} entry in SCID fibroblasts, whereas combined overexpression of Orai2 and STIM1 had little effect (supplemental Fig. S1B). Combined overexpression of Orai1 and STIM1 did not increase store-operated Ca\textsuperscript{2+} entry significantly above the level observed with Orai1 alone (supplemental Fig. S1B and data not shown).

One explanation for the weaker reconstitution by Orai2 and Orai3 could have been that these proteins were poorly expressed relative to Orai1 or were not inserted into the plasma membrane. However, immunocytochemistry of

![FIGURE 3. All three Orai proteins can localize to the plasma membrane. A, Jurkat T cells were transiently transfected with FLAG-tagged Orai proteins, and subcellular localization was assessed by immunocytochemistry in permeabilized cells, either untreated or treated for 10 min with thapsigargin. An antibody to the ER resident protein ERP72 was used to visualize the endoplasmic reticulum. B, Orai1 and Orai3 bearing HA epitope tags in the predicted TM3-TM4 loop were expressed in HEK293 cells together with GFP and shown to be localized at the plasma membrane by immunocytochemistry of non-permeabilized cells with an anti-HA antibody. This result was previously reported for Orai1 (22).](image-url)
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FIGURE 4. Glycosylation site mutants of Orai1 are localized at the plasma membrane and can reconstitute store-operated Ca\(^{2+}\) entry in SCID fibroblasts. A, Orai1 is glycosylated, whereas Orai2 and Orai3 are not. HEK293 cells stably transfected with FLAG-tagged Orai family members were left untreated or treated with 2 \(\mu\)g/ml tunicamycin (Tunic.) for 18 h, following which cell lysates were immunoprecipitated with anti-FLAG beads and the migration positions of the FLAG-Orai proteins in SDS gels were determined by Western blotting with anti-FLAG antibody. The asterisk (Orai1*) indicates the glycosylated form. A nonspecific band is indicated (ns). B, a non-glycosylated mutant of Orai1 (NVS >IIV) can reconstitute store-operated Ca\(^{2+}\) entry in SCID fibroblasts. Top panel, HEK293 cells were transfected with Myc-tagged wild-type Orai1 or Orai1 in which residues 223–225 (NVS) were replaced with IVI. The migration positions of the Myc-Orai proteins were determined by Western blotting with anti-Myc antibody. Gly, glycosylated (wild-type); NG, non-glycosylated (NVS >IIV). Middle panel, The Myc-tagged NVS >IIV mutant of Orai1 was expressed in HEK293 cells using a bicistronic IRES-GFP retroviral vector. Immunocytochemistry with an anti-Myc antibody shows that the mutant protein is expressed at or near the plasma membrane. Bottom panel, Ca\(^{2+}\) influx was examined by single-cell video imaging in GFP\(^{-}\) SCID fibroblasts after retroviral transduction with the NVS >IIV mutant of Orai1 in a bicistronic IRES-GFP retroviral vector. The NVS >IIV mutant reconstitutes store-operated Ca\(^{2+}\) entry to SCID fibroblasts as effectively as wild-type Orai1. C, a second non-glycosylated mutant of Orai1 (N223A) can reconstitute store-operated Ca\(^{2+}\) entry in SCID fibroblasts. Top panel, FLAG-tagged Orai1, in which asparagine residue 223 was replaced with alanine, was expressed in Jurkat T cells using a bicistronic IRES-GFP retroviral vector. Immunocytochemistry of GFP\(^{-}\) cells with an anti-FLAG antibody shows that the mutant protein is expressed at or near the plasma membrane. Bottom panel, Ca\(^{2+}\) influx was examined by single-cell video imaging in GFP\(^{-}\) SCID fibroblasts after retroviral transduction with empty vector, wild-type Orai1, or the N223A mutant of Orai1 in a bicistronic IRES-GFP retroviral vector. The N223A mutant restores Ca\(^{2+}\) influx as effectively as wild-type Orai1.

tagged proteins expressed in Jurkat T cells and HEK293 cells confirmed that all three Orai proteins were expressed and localized at or near the plasma membrane, showing little or no overlap with the ER marker ERP72 (Fig. 3A and data not shown); localization was not grossly altered after store depletion with thapsigargin (Fig. 3A). To confirm plasma membrane localization, we generated versions of all three Orai proteins bearing HA epitope tags in the predicted TM3-TM4 loop as previously described for Orai1 (22) and expressed them in HEK293 cells. Immunocytochemical analysis demonstrated that the HA tag is extracellular, that is, accessible to antibody staining in intact, unpermeabilized cells (Fig. 3B). Thus, all three Orai proteins can be inserted into the plasma membrane. Taken together, these data show unambiguously that in HEK293 cells and human SCID T cells and fibroblasts in which store depletion has been induced with thapsigargin, Orai1 is the major regulator of store-operated Ca\(^{2+}\) influx, whereas Orai3 can complement partially and Orai2 has a lesser role.

Orai1 Is Glycosylated in HEK293 Cells, but Glycosylation Is Not Necessary for Its Function—Orai1 has a putative N-glycosylation motif (NVS) in its extracellular loop between predicted transmembrane segments 3 and 4. This motif is absent in Orai2 and 3 (supplemental Fig. S2). We showed that this motif is indeed glycosylated in Orai1 by examining anti-FLAG immunoprecipitates from lysates of HEK293 cells stably expressing FLAG-tagged Orai1, 2, and 3. Orai1 migrated on SDS gels as a fuzzy band with an apparent molecular mass of ~45 kDa, significantly larger than the molecular mass (~33 kDa) deduced from its amino acid sequence; however, treatment of the Orai1-expressing cells with tunicamycin, an inhibitor of the first step of glycosylation in the ER, caused the Orai1 band to shift to a position close to the predicted size (Fig. 4A, panels 1 and 2). In

tor of store-operated Ca\(^{2+}\) entry in SCID fibroblasts.
contrast, Orai2 and Orai3 migrated at positions close to their predicted molecular masses of 28 and 32.5 kDa, respectively, and their migration properties were not changed by tunicamycin treatment (Fig. 4A, panels 3 and 4). Glycosylation is not required for Orai1 function, however: both the NVS and N223A mutations abolished the glycosylation of Orai1 without affecting its localization within the cell (22) (Fig. 4B, top and middle panels), but the mutant proteins were at least as effective as wild-type Orai1 at reconstituting store-operated Ca\(^{2+}\) influx in SCID patient cells (Fig. 4B, bottom, and Fig. 4C and data not shown).

**Protein-Protein Interactions and Multimerization Status of Orai1**—We examined the possibility that Orai1 formed multimers. HEK293 cells stably expressing FLAG-tagged Orai1 were transiently transfected with Myc-tagged Orai1. The stably expressed FLAG-Orai1 protein migrated principally as the fully glycosylated 45-kDa form (Fig. 5A, panel 1). In this experiment as in occasional other experiments, two Orai1 bands were observed with sizes corresponding to monomeric (45 kDa) and dimeric (90 kDa) forms. A nonspecific band is indicated (ns). D, Orai1 exists as a higher-order complex in intact cells prior to thapsigargin treatment. HEK293 cells stably expressing FLAG-tagged Orai1 were left untreated or were treated with thapsigargin for 10 min and cross-linked in the presence of the cell-permeant reversible cross linker dithiobis(succinimidyl propionate) (1 mM, 1 h on ice). Cell lysates were analyzed by SDS gel electrophoresis followed by immunoblotting with anti-FLAG antibody. Immunoblotting with STIM1 antibody did not reveal STIM1-Orai1 association in either resting or thapsigargin-treated cells (data not shown).
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**FIGURE 6. Dominant-interfering versions of Drosophila Orai and human Orai.** A, Drosophila Orai can form multimers. S2+ cells stably expressing V5-tagged Orai were transiently transfected with FLAG-tagged Orai, and cell lysates were analyzed directly (−) or immunoprecipitated with anti-FLAG resin or anti-HA resin as a control. Interactions between the differently tagged dOrai proteins were determined by immunoblotting with anti-V5 and anti-FLAG antibodies. B, schematic representation of Drosophila Orai (GenBank\(^\text{TM}\) accession number AY071273). The positions of conserved glutamate residues in or near the transmembrane domains are indicated. The numbers in parentheses are the corresponding residues in human Orai1 (also see supplemental Fig. S2). C, store-operated Ca\(^{2+}\) influx in S2+ cells stably expressing wild-type dOrai and the E→Q mutants. Ca\(^{2+}\) influx was analyzed by flow cytometry. The E178Q and E262Q mutants exert a dominant-interfering effect in cells expressing endogenous dOrai. Plotted is the ratio of the mean fluorescence intensity (MFI) of two Ca\(^{2+}\) indicator dyes, Fluo-4 and Fura-Red. D, suppression of store-operated Ca\(^{2+}\) influx in wild-type human fibroblasts, retrovirally transduced with E106Q and E190Q mutant Orai1 in bicistronic IRES-GFP vectors. Intracellular Ca\(^{2+}\) stores were depleted with 1 \(\mu\text{M}\) thapsigargin (TG) in the absence of extracellular Ca\(^{2+}\), followed by readdition of 20 \(\mu\text{M}\) Ca\(^{2+}\). Ca\(^{2+}\) influx was analyzed by single-cell video imaging of Fura2-labeled, GFP\(^+\) cells. More than 30 GFP\(^+\) cells were analyzed in each experiment.

immunocytochemistry showed that STIM1 and Orai1 colocalized at least partially in thapsigargin-treated Jurkat cells (supplemental Fig. S3), consistent with recent findings that STIM1 and Orai1 colocalize after store depletion (13, 34) at sites of Ca\(^{2+}\) entry (13).

**Generation of Dominant-interfering Versions of Drosophila Orai—** The fact that Orai1 existed as at least a dimer in cell lysates suggested that inactive mutant versions of the protein might exert a dominant-interfering effect, by sequestering essential components or by forming mixed dimers or higher-order multimers with native wild-type subunits. We evaluated this hypothesis initially in Drosophila cells, first confirming that Drosophila Orai1 was a multimer in coimmunoprecipitation experiments (Fig. 6A) and then testing the effect of glutamine substitutions in four highly conserved glutamate residues located within or near transmembrane regions (Glu-178, Glu-221, Glu-245, and Glu-262; numbering based on GenBank\(^\text{TM}\) accession number AY071273; Fig. 6B and supplemental Fig. S2). We generated S2+ cells stably expressing V5-tagged dOrai proteins with glutamine substitutions at each of these conserved residues and assessed store-operated Ca\(^{2+}\) entry by flow cytometry (Fig. 6C). Expression of the E178Q and E262Q mutants led to a strong decrease in store-operated Ca\(^{2+}\) entry, expression of the wild-type and E245Q proteins led to at most a 2-fold decrease, and expression of the E221Q mutant had almost no effect compared with a vector control (Fig. 6C and data not shown).

**Dominant-interfering Versions of Human Orai1 Block Lymphocyte Function—** We used the information from the Drosophila experiments to construct dominant-interfering versions of human Orai1. We generated mutant proteins in which glutamates Glu-106 and Glu-190 of human Orai1, corresponding to Glu-178 and Glu-221 of dOrai and the E→Q mutants, were substituted with glutamine. When expressed in wild-type human fibroblasts and primary mouse CD4\(^+\) T cells, the mutants were expressed at levels comparable with wild-type Orai1 and were substituted with glutamine. When expressed in wild-type human fibroblasts and primary mouse CD4\(^+\) T cells, the mutants were expressed at levels comparable with wild-type Orai1 and were substituted with glutamine. When expressed in wild-type human fibroblasts and primary mouse CD4\(^+\) T cells, the mutants were expressed at levels comparable with wild-type Orai1 and were substituted with glutamine. When expressed in wild-type human fibroblasts and primary mouse CD4\(^+\) T cells, the mutants were expressed at levels comparable with wild-type Orai1 and were substituted with glutamine.
FLAG-tagged wild-type Orai1 or the E106Q or E190Q mutants of Orai1, together with GFP, in a bicistronic vector in which an internal ribosomal entry site (IRES) was followed by GFP cDNA. Ca\(^{2+}\) influx was evaluated by single-cell video imaging of murine T cells, retrovirally transduced with wild-type and mutant Orai1 in bicistronic IRES-GFP vectors. Ca\(^{2+}\) stores were depleted with 1 μM ionomycin (Iono) in the absence of Ca\(^{2+}\), followed by readdition of 2 mM Ca\(^{2+}\). For each experiment, ~100 GFP\(^{+}\) T cells were analyzed. B, proliferation of transduced T cells assayed by BrdUrd incorporation. T cells were stimulated for 6 h with 10 nM PMA and 1 μM ionomycin, transferred to IL-2-free medium, and pulsed with BrdUrd. BrdUrd incorporation was determined by flow cytometry. C, T cells were stimulated with 10 nM PMA and 1 μM ionomycin for 4 h, and cytokine (IL-2 and IFN-γ) expression by GFP\(^{+}\) T cells was assessed by intracellular staining and flow cytometry.

**FIGURE 7. Dominant-interfering versions of human Orai1 block lymphocyte function.** A, dominant-interfering versions of human Orai1 diminish store-operated Ca\(^{2+}\) influx in murine CD4\(^{+}\) T cells. Ca\(^{2+}\) influx was evaluated by single-cell video imaging of murine T cells, retrovirally transduced with wild-type and mutant Orai1 in bicistronic IRES-GFP vectors. Ca\(^{2+}\) stores were depleted with 1 μM ionomycin (Iono) in the absence of Ca\(^{2+}\), followed by readdition of 2 mM Ca\(^{2+}\). For each experiment, ~100 GFP\(^{+}\) T cells were analyzed. B, proliferation of transduced T cells assayed by BrdUrd incorporation. T cells were stimulated for 6 h with 10 nM PMA and 1 μM ionomycin, transferred to IL-2-free medium, and pulsed with BrdUrd. BrdUrd incorporation was determined by flow cytometry. C, T cells were stimulated with 10 nM PMA and 1 μM ionomycin for 4 h, and cytokine (IL-2 and IFN-γ) expression by GFP\(^{+}\) T cells was assessed by intracellular staining and flow cytometry.

**DISCUSSION**

We have performed a genome-wide RNAi screen in *Drosophila* cells to identify candidates whose knock down inhibits NFAT nuclear translocation. A remarkably small number of protein candidates emerged from this screen, including calcineurin and nuclear transport proteins as expected. As previously described, we identified the CRAC channel pore subunit dOrai (olf186-F) and the putative ER Ca\(^{2+}\) sensor dStim as key regulators of store-operated Ca\(^{2+}\) entry (14, 22); similar results were reported by others using Ca\(^{2+}\)-based screens (6, 7, 15–17, 20, 23). The small number of candidates emerging from our NFAT-based screen is in surprising contrast to the large numbers of candidates obtained in the two Ca\(^{2+}\)-based screens that also identified dOrai. The most likely explanation for the difference is that even low sustained [Ca\(^{2+}\)]\(_{i}\), increases lead to NFAT nuclear localization (34); thus, candidates whose depletion decreased Ca\(^{2+}\) influx only partially or only at early times would not be scored in our screen.

Notably, only dStim and dOrai were identified in three independent genome-wide RNAi screens in *Drosophila*, our NFAT-based screen as well the two Ca\(^{2+}\)-based screens (Refs. 14–16 and this report). The splicing factor Noi emerged as a candidate both in our screen and in the screen performed by Vig et al. (15) but was excluded as a regulator of store-operated Ca\(^{2+}\) entry in our secondary screen. The kinesin family member Pav was identified both in our screen and by Zhang et al. (16) and could potentially participate in STIM relocalization. Unfortunately, however, Pav depletion in *Drosophila* cells caused a severe mitotic arrest phenotype, presumably reflecting the known role of Pav in cytokinesis (30), and thus we were unable to establish a direct role for Pav in store-operated Ca\(^{2+}\) influx. Depletion of the SNARE protein Syntaxin 5, which is involved in vesicle fusion, was shown by Zhang et al. (16) to inhibit store-operated Ca\(^{2+}\) influx by 2- to 3-fold, but this protein was not identified in our screen or in that performed by Vig et al. (15). The robust identification of dOrai and dStim in all three screens is consistent with the finding that combined overexpression of dOrai and dStim in *Drosophila* cells greatly increases store-operated Ca\(^{2+}\) entry (16). The implication is that other components are not limiting, possibly because they are stable proteins that are difficult to deplete by RNAi.

It has been shown that STIM1 and Orai1 colocalize after store depletion (13, 34) and that the sites of colocalization coincide with sites of Ca\(^{2+}\) entry (13). In addition, there have been recent reports of physical interactions between ectopically expressed STIM and Orai: a resting interaction of human STIM1 with human Orai1 was observed in cells whose Ca\(^{2+}\) stores are replete (17), and store depletion was reported to induce a further association of *Drosophila* Sttim with *Drosophila*...
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In our hands, co-immunoprecipitation experiments have failed to demonstrate a tight association between human Orai1 and STIM1 in any of several detergent and salt conditions tested (Fig. 5 and data not shown). The interaction, if direct, may be of low affinity (rapidly dissociating) or may involve intermediary proteins.

We previously described SCID patients whose T lymphocytes exhibit a multiple cytokine deficiency, secondary to a severe defect in CRAC channel function (18, 19). We have shown that the causal molecular defect in these patients is a point mutation in the CRAC channel pore subunit Orai1 (14, 22). Reconstitution of SCID cells with wild-type Orai1 restores store-operated Ca\textsuperscript{2+} entry and CRAC channel function (Ref. 14 and this report); however, because the retroviral transduction efficiency of the SCID T cells is very low (1–2%), we were unable to monitor cytokine expression directly in the reconstituted cells. To define the relation of Orai1 activity to lymphocyte function, we instead overexpressed dominant-interfering versions of human Orai1 (E106Q and E190Q) in primary murine T lymphocytes and showed that these proteins interfered simultaneously with store-operated Ca\textsuperscript{2+} entry, T cell proliferation, and cytokine production (Fig. 7). Together these data emphasize that Orai1 is a major regulator of store-operated Ca\textsuperscript{2+} influx in human and murine T cells. Given that Orai1 is widely expressed, the most likely explanation for the immune-specific phenotype of the SCID patients is that T cells are much more dependent on store-operated Ca\textsuperscript{2+} entry through CRAC channels than on other cell types such as neurons, heart and muscle cells, which utilize voltage-gated, ligand-gated, and diverse Ca\textsuperscript{2+} channels to mediate Ca\textsuperscript{2+} influx.

The predominant role of Orai1 in store-operated Ca\textsuperscript{2+} entry in T cells and fibroblasts has been demonstrated through siRNA-mediated knock down in HEK293 cells (1); likewise, if direct, may be of low affinity (rapidly dissociating) or may involve intermediary proteins.

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What are the roles of Orai2 and Orai3 in store-operated Ca\textsuperscript{2+} entry? We have used several parallel approaches to ascertain the contributions of these two Orai proteins relative to Orai1. The predominant role of Orai1 in store-operated Ca\textsuperscript{2+} entry in T cells and fibroblasts has been demonstrated through siRNA-mediated knock down in HEK293 cells (Fig. 1), by expressing all three Orai proteins in SCID patient cells (Fig. 2 and supplemental Fig. S1), and by showing that overexpression of two dominant-interfering Orai1 mutants, E106Q and E190Q, in primary T cells blocks store-operated Ca\textsuperscript{2+} entry as well as proliferation and cytokine production (Figs. 6 and 7). However, our results suggest strongly that Orai3 is also functional in store-operated Ca\textsuperscript{2+} entry. First, the small increase in store-operated Ca\textsuperscript{2+} entry observed in SCID cells reconstituted with Orai3 is not observed when the E81Q mutant of Orai3 is used instead (Fig. 4). Second, combined expression of STIM1 and Orai3 in SCID Cells. Thus, the E81Q mutant of Orai3 may be of low affinity (rapidly dissociating) or may involve intermediary proteins.

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