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## Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai

Andriy V. Yeromin<sup>1,\*</sup>, Shenyuan L. Zhang<sup>1,\*</sup>, Weihua Jiang<sup>1</sup>, Ying Yu<sup>1</sup>, Olga Safrina<sup>1</sup>, and Michael D. Cahalan<sup>1</sup>

<sup>1</sup>Department of Physiology & Biophysics and Center for Immunology, University of California, Irvine, California 92697, USA

### Abstract

Recent RNA interference screens have identified several proteins that are essential for store-operated Ca<sup>2+</sup> influx and Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> (CRAC) channel activity in *Drosophila* and in mammals, including the transmembrane proteins Stim (stromal interaction molecule)<sup>1,2</sup> and Orai<sup>3–5</sup>. Stim probably functions as a sensor of luminal Ca<sup>2+</sup> content and triggers activation of CRAC channels in the surface membrane after Ca<sup>2+</sup> store depletion<sup>1,6</sup>. Among three human homologues of *Orai* (also known as *olf186-F*), *ORAI1* on chromosome 12 was found to be mutated in patients with severe combined immunodeficiency disease, and expression of wild-type Orai1 restored Ca<sup>2+</sup> influx and CRAC channel activity in patient T cells<sup>3</sup>. The overexpression of Stim and Orai together markedly increases CRAC current<sup>5,7–9</sup>. However, it is not yet clear whether Stim or Orai actually forms the CRAC channel, or whether their expression simply limits CRAC channel activity mediated by a different channel-forming subunit. Here we show that interaction between wild-type Stim and Orai, assessed by co-immunoprecipitation, is greatly enhanced after treatment with thapsigargin to induce Ca<sup>2+</sup> store depletion. By site-directed mutagenesis, we show that a point mutation from glutamate to aspartate at position 180 in the conserved S1–S2 loop of Orai transforms the ion selectivity properties of CRAC current from being Ca<sup>2+</sup>-selective with inward rectification to being selective for monovalent cations and outwardly rectifying. A charge-neutralizing mutation at the same position (glutamate to alanine) acts as a dominant-negative non-conducting subunit. Other charge-neutralizing mutants in the same loop express large inwardly rectifying CRAC current, and two of these exhibit reduced sensitivity to the channel blocker Gd<sup>3+</sup>. These results indicate that Orai itself forms the Ca<sup>2+</sup>-selectivity filter of the CRAC channel.

Orai and Orai1 possess four hydrophobic stretches that are predicted to span the membrane. On the basis of strategies used previously for several different ion channels, we made point mutations to investigate the most conserved loop between putative transmembrane segments (Supplementary Fig. 1a) and examined properties of ion selectivity, current–voltage rectification and block that are intimately associated with a pore-forming subunit. Wild-type or mutant Orai proteins were overexpressed together with wild-type Stim in S2 cells; messenger RNA and protein expression were verified by RT–PCR and western blotting (Fig. 1a and

Correspondence and requests for materials should be addressed to M.D.C. (mcahalan@uci.edu).

\*These authors contributed equally to this work.

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**Author Contributions** A.V.Y. was responsible for all patch-clamp experiments and analysis. S.L.Z. was responsible for all molecular biology and biochemistry experiments, with the assistance of W.J. Y.Y. and O.S. performed RT–PCR. M.D.C. provided advice and overall direction, and supervised project planning and execution.

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Supplementary Fig. 1b). In addition, by co-immunoprecipitation of the epitope-tagged wild-type proteins, we evaluated whether Stim and Orai are associated with each other before and after  $\text{Ca}^{2+}$  store depletion. When cells were cultured without stimulation, only limited interaction was seen. However,  $\text{Ca}^{2+}$  store depletion triggered by thapsigargin, which inhibits the SERCA (sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase) re-uptake pump, markedly increased Stim–Orai protein interaction when either protein was used to pull down the other by co-immunoprecipitation (Fig. 1a). This result shows that both transfected proteins are expressed and, moreover, that they interact, either directly or as part of a complex, after  $\text{Ca}^{2+}$  store depletion to initiate CRAC channel activation. During whole-cell recording on the co-transfected cells, dialysis of a  $\text{Ca}^{2+}$  chelator (BAPTA) into the cytoplasm activated a very large  $\text{Ca}^{2+}$ -selective inward current, as shown previously<sup>5</sup>. Using a pipette solution that maintains normal  $\text{Ca}^{2+}$  store content to prevent spontaneous channel opening, we confirmed that addition of inositol-1,4,5-trisphosphate ( $\text{IP}_3$ ) inside or thapsigargin (TG) outside resulted in greatly augmented CRAC current (Fig. 1b).

Expression of an Orai mutant with a glutamate-to-aspartate mutation at position 180 (Orai (E180D)), again together with wild-type Stim, generated a current that developed with the normal kinetics of CRAC current during passive  $\text{Ca}^{2+}$  store depletion, but also exhibited marked alterations in ion selectivity and rectification. In contrast to the normal inward CRAC current, Orai(E180D) expression produced a very large outward current and a smaller inward current that developed in parallel (Fig. 2a). Current–voltage ( $I$ – $V$ ) curves of the Orai(E180D)-induced current revealed an outwardly rectifying shape with a reversal potential of  $6.0 \pm 0.4$  mV ( $n = 14$  cells), in contrast to the characteristic inwardly rectifying  $I$ – $V$  curves with a reversal potential  $>50$  mV of native or wild-type Orai-induced CRAC current (Fig. 2b).

In a series of ion-substitution experiments, we evaluated the ion selectivity properties of current induced by Orai(E180D). Substitution of  $\text{Na}^+$  by choline while maintaining constant  $\text{Ca}^{2+}$  in the external solution—an experimental procedure that has little effect on native CRAC current<sup>10</sup> or CRAC current with overexpressed wild-type Orai (Fig. 2c)—reduced the inward current and shifted the reversal potential to hyperpolarized potentials (Fig. 2d), indicating that the Orai(E180D)-induced inward current is carried predominantly by  $\text{Na}^+$  rather than by  $\text{Ca}^{2+}$ . Next, different divalent cation species in the external solution were tested, resulting in a  $I$ – $V$  pattern of wild-type Orai-induced CRAC current similar to native CRAC current described previously<sup>10</sup>.  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  (20 mM in each case, while maintaining high external  $\text{Na}^+$ ) resulted in increased inward current (Fig. 2e), indicating that each of these divalent cations is permeant, whereas  $\text{Mg}^{2+}$  is relatively impermeant. In contrast, representative  $I$ – $V$  curves of Orai(E180D)-induced current (shown in Fig. 2f with the same colour code for comparison with Fig. 2e) reveal an entirely different pattern. A tenfold increase of  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]$ ) in the external solution significantly decreased the outward current and did little to the small inward current. Substitution of 2 mM external  $\text{Ca}^{2+}$  by 20 mM  $\text{Sr}^{2+}$  had little effect;  $\text{Mg}^{2+}$  increased the current; and  $\text{Ba}^{2+}$  significantly increased the inward current while decreasing the outward current (Supplementary Fig. 2a). These results indicate that divalent cations block the outward current (carried by  $\text{Cs}^+$ ) in the potency sequence  $\text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+} > \text{Mg}^{2+}$ , and the inward current (carried by  $\text{Na}^+$ ) in the potency sequence  $\text{Ca}^{2+} \approx \text{Sr}^{2+} > \text{Mg}^{2+} > \text{Ba}^{2+}$ . Together, these ion-substitution experiments indicate that the Orai (E180D) mutation transforms the CRAC channel from being selective for divalent cations to being a monovalent-selective channel that selects against, and indeed is blocked by, divalent cations.

CRAC channels share with voltage-gated  $\text{Ca}^{2+}$  channels the property of monovalent ion permeation when external divalent ions are removed<sup>11–15</sup>. Upon withdrawal of external divalent cations,  $\text{Na}^+$  carries significant current through CRAC channels, whereas  $\text{Cs}^+$  is far less permeant<sup>10,14,16,17</sup>. The monovalent CRAC current normally declines within 10 s, a

process termed depotentiation<sup>15</sup> that is due to removal of external  $\text{Ca}^{2+}$ . To check whether the Orai(E180D) mutation alters monovalent CRAC current, both wild-type- and Orai(E180D)-induced CRAC currents were recorded in divalent-free  $\text{Na}^+$  and  $\text{Cs}^+$  test solutions. Three clear differences can be discerned (Fig. 3). First, the inward  $\text{Na}^+$  current was much larger in the Orai (E180D) mutant and did not depotentiate to the same extent as either wild-type Orai (Fig. 3a, b) or native CRAC current in S2 cells<sup>10</sup>. Second, in contrast to the relatively small  $\text{Cs}^+$  current density in wild-type Orai ( $-1.6 \pm 0.6 \text{ pA pF}^{-1}$ ,  $n = 4$ ), Orai(E180D)-induced  $\text{Cs}^+$  current density was much larger ( $-191 \pm 46 \text{ pA pF}^{-1}$ ,  $n = 4$ ), similar in amplitude to the  $\text{Na}^+$  current ( $-238 \pm 43 \text{ pA pF}^{-1}$ ,  $n = 9$ ), and the large  $\text{Cs}^+$  current did not depotentiate (Fig. 3b). Third, the measured reversal potential in  $\text{Na}^+$  external solution was  $7.9 \pm 0.3 \text{ mV}$  ( $n = 9$ ) for Orai(E180D), whereas it was  $52.4 \pm 0.9 \text{ mV}$  ( $n = 5$ ) for wild-type Orai (Fig. 3c, d). Calculated from reversal potentials, the average permeability ratio  $P_{\text{Na}}/P_{\text{Cs}}$  changed from 8.0 for wild-type Orai to 1.4 as a result of the E180D mutation, indicating an increase in pore diameter or altered electrostatic interactions with cations.  $I-V$  curves for Orai(E180D)-induced current changed shape with varying external  $[\text{Ca}^{2+}]$  (Fig. 3e), revealing voltage-dependent block (Fig. 3f) that causes outward rectification in physiological saline. This examination of ion selectivity properties shows that a conservative mutation of glutamate to aspartate at position 180 of Orai transforms the CRAC channel from being  $\text{Ca}^{2+}$ -selective and inwardly rectifying to one that conducts  $\text{Na}^+$  or  $\text{Cs}^+$  and is outwardly rectifying due to voltage-dependent  $\text{Ca}^{2+}$  block.

Expression of the charge-neutralizing Orai(E180A) mutant completely abolished native CRAC current (Fig. 4a), indicating that alanine-containing subunits prevent ion conduction in hetero-multimeric CRAC channels by a dominant-negative action. In contrast, expression of Orai (D184A), Orai(D186A) or Orai(N188A) mutants with Stim resulted in increased CRAC current with unaltered  $I-V$  shape (Fig. 4b) and normal monovalent  $\text{Na}^+$  current that exhibited depotentiation and low  $\text{Cs}^+$  permeability (data not shown). Figure 4c summarizes the effect of each Orai mutant tested on CRAC current density. Expressed by itself or together with Stim, only Orai(E180A) potentially inhibited native CRAC current. The inward current density and  $I-V$  shape in cells expressing Orai(D184A), Orai(D186A) and Orai(N188A) mutants did not differ significantly from current observed in cells expressing wild-type Orai. Orai(E180D) was unique in producing very large outward currents as a result of altered ion selectivity.

$\text{Gd}^{3+}$  very effectively suppresses native and wild-type Orai-induced CRAC current<sup>5</sup>. In addition to ion selectivity properties, block by  $\text{Gd}^{3+}$  was evaluated in all mutants (Fig. 4d). Two charge-neutralizing mutations, aspartate to alanine at positions 184 and 186, and, to a lesser degree, mutation of asparagine to alanine at position 188, significantly reduced the potency of  $\text{Gd}^{3+}$  block (Fig. 4d and Supplementary Fig. 2b). As shown previously<sup>5</sup>, wild-type Orai CRAC current was potentiated by 2-aminoethyl-diphenyl borate (2-PB) at a low concentration ( $5 \mu\text{M}$ ) and inhibited at a high ( $20 \mu\text{M}$ ) concentration (Supplementary Fig. 3a), similar to effects of 2-APB on native CRAC current in Jurkat and in S2 cells<sup>10,18</sup>. The effects of 2-APB on Orai(E180D)-induced CRAC current were complex but still showed potentiation and inhibition (Supplementary Fig. 3b). The time course of Orai(E180D) outward CRAC current showed an immediate decrease preceding potentiation by  $5 \mu\text{M}$  2-APB, and an immediate increase preceding inhibition during  $20 \mu\text{M}$  application ( $n = 3$ ). The pharmacological analysis provides support for the conclusion that the S1-S2 loop is involved in ion selectivity and block by  $\text{Gd}^{3+}$ , and may contribute to the complex effects of 2-APB on CRAC current.

Our results demonstrate that thapsigargin-triggered store depletion dynamically strengthens an interaction between Stim and Orai, supporting a model for CRAC channel activation in which Stim serves as the  $\text{Ca}^{2+}$  sensor to detect store depletion and as the messenger to activate CRAC channels in the plasma membrane. More importantly, we conclude that Orai is a bona fide ion channel, based on the following: (1) RNA-interference-mediated knockdown of Orai

expression suppresses thapsigargin-dependent  $\text{Ca}^{2+}$  influx and CRAC channel activity; (2) overexpression of Orai with or without Stim augments CRAC currents that exhibit biophysical properties identical to native CRAC current; and (3) mutations of negatively charged residues within the putative pore region of Orai significantly alter ion selectivity, current rectification and affinity to a charged channel blocker without altering channel activation kinetics. The marked alteration of these properties by a targeted point mutation provides definitive evidence that Orai embodies the pore-forming subunit of the CRAC channel.

The consensus sequence within the S1–S2 loop, 179**VEVQLD**xD186, contains the critical glutamate (bold) shown here to control ion selectivity properties of the CRAC channel, and two aspartates (underlined) that may help to attract  $\text{Gd}^{3+}$  (and  $\text{Ca}^{2+}$ ) towards the pore. It is not similar to pore sequences found in other channels. Unlike the pore regions of voltage-gated  $\text{Ca}^{2+}$  (CaV) channels, which contain a relatively long loop and a ring of critical glutamates from different domains that form a high-affinity  $\text{Ca}^{2+}$ -binding site<sup>19</sup>, the putative pore sequence of Orai is very short, and the key residue for ion selectivity (E180) is adjacent to the putative S1 segment. Nevertheless, because withdrawal of external divalent ions reveals permeability to monovalent cations in both CaV<sup>11,12</sup> and CRAC channels<sup>10,14,16,17</sup>, it is possible that the CRAC channel ion-selectivity filter is also formed by a ring of glutamates and that the mechanism of  $\text{Ca}^{2+}$  permeation is similar, although the single-channel conductance and maximum permeant ion size of the CRAC channel selectivity filter are smaller than that of the CaV channel<sup>10,17,20,21</sup>. Negatively charged side chains also contribute to  $\text{Ca}^{2+}$  selectivity of TRPV6; in this instance, aspartate (at position 541) is proposed to coordinate with  $\text{Ca}^{2+}$  ions and line the selectivity filter in a ring structure formed by four subunits<sup>22</sup>. The CRAC channel may be a multimer that includes several identical Orai subunits, as a non-conducting pore mutant (E180A) exerts a strong dominant-negative action on native CRAC current. Biochemical approaches and cysteine-scanning mutagenesis should be useful to elucidate better the unique pore architecture of the CRAC channel.

*Note added in proof:* While in proof, our attention was drawn to another paper reporting complementary findings regarding the control of ion selectivity in the CRAC channel<sup>23</sup>. In our study, the critical glutamate residue is 180 in the Orai protein encoded by the Invitrogen S2 cell complementary DNA (GenBank accession number DQ503470, as described<sup>5</sup>). The corresponding residue is 178 in the *Drosophila* genome database (accession number AY071273), and 106 in the human Orai1 homologue (accession number BC015369).

## Methods

### Molecular cloning and mutagenesis

Generation of pAc5.1/EGFP, pAc5.1/D-STIM, pAc5.1/D-STIM-V5-His and pAc5.1/olf186-F (GenBank accession number DQ503470) were as described<sup>5</sup>. *Orai* pore mutants were made by exchanging the corresponding codons (E180A, GAG to GCG; E180D, GAG to GAC; D184A, GAT to GCT; D186A, GAT to GCT; N188A, AAT to GCT; Supplementary Fig. 1) using the QuikChange site-directed mutagenesis kit (Stratagene). The pAc5.1/HA-olf186-F clone was made by adding the haemagglutinin tag via PCR and re-cloned into the *Xho*I and *Not*I sites of pAc5.1/V5-His B expression vector. Resulting clones were confirmed by sequencing. Description of the primers and conditions for cloning are available upon request. Both HA-olf186-F and Stim-V5-His were verified for normal function by whole-cell recording.

### Whole-cell recording

Cells transfected (Amaxa) with *Orai* and *Stim* constructs were identified by fluorescence of co-transfected GFP. Patch-clamp experiments were performed at room temperature in the standard whole-cell recording, using conditions similar to those reported previously<sup>10</sup>. The

membrane capacitance of S2 cells selected for recording was  $11.6 \pm 0.4$  pF ( $n = 131$  cells). Membrane potentials were corrected for a liquid junction potential of 10 mV between the pipette and bath solution. The series resistance ( $2\text{--}7$  M $\Omega$ ) was not compensated. The membrane potential was held at  $-10$  mV, and 220-ms voltage ramps from  $-130$  to 90 mV alternating with 220-ms pulses to  $-130$  mV were delivered every 2 s. Only cells with high input resistance ( $>2$  G $\Omega$ ) were selected for recording. External and pipette solution recipes are listed in Supplementary Table 1. Analysed data are presented as mean  $\pm$  s.e.m.

### Co-immunoprecipitation

Two days after transfection,  $5 \times 10^6$  cells were treated with either Ringer's solution or zero- $\text{Ca}^{2+}$  Ringer's solution plus 1  $\mu\text{M}$  thapsigargin using solutions described<sup>6</sup> for 15 min at room temperature. Cells transfected with *Stim-V5-His* only or *HA-olf186-F* only were used as controls. Cell extracts were then prepared using RIPA lysis buffer (Upstate) according to the manufacturer's instructions. The extracts were pre-cleared with protein A/G beads (Pierce) and protein concentration was determined, using the Pierce BCA Protein Reagent Kit. The cell lysate was then diluted to approximately 1 mg ml<sup>-1</sup> total protein with PBS and mixed with either anti-HA monoclonal antibodies (1  $\mu\text{g}$  per 100  $\mu\text{g}$  total protein, Santa Cruz) or anti-V5 monoclonal antibodies (1  $\mu\text{g}$  per 100  $\mu\text{g}$  total protein, Invitrogen) for 4 h at 4 °C. Equal amounts of samples were mixed with either normal mouse IgG or vehicle as negative controls. Protein A/G beads were subsequently added (1  $\mu\text{l}$  per 1  $\mu\text{g}$  IgG) to rock overnight at 4 °C. Proteins were eluted by boiling in 4 $\times$  sample buffer (Invitrogen). Samples were resolved by SDS-PAGE and analysed by standard western blotting techniques. Anti-HA monoclonal antibodies (Santa Cruz) were used at a dilution of 1:200. Anti-V5 monoclonal antibodies (Invitrogen) were used at a dilution of 1:2,500. Proteins were detected by developing with the SuperSignal (Pierce) detection system. Samples treated with normal mouse IgG or vehicle showed no detectable signal for both *Stim-V5-His* and *HA-olf186-F* (data not shown), indicating that the immunoprecipitation and co-immunoprecipitation are specific.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

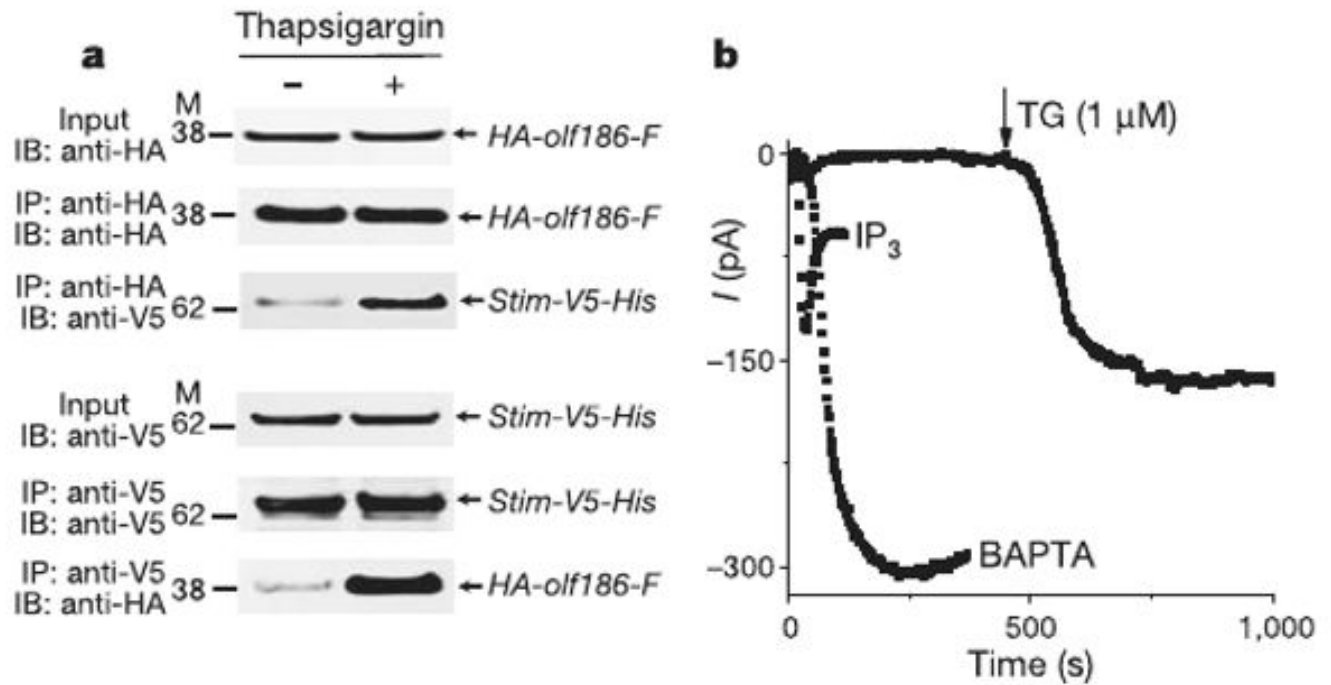
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### References

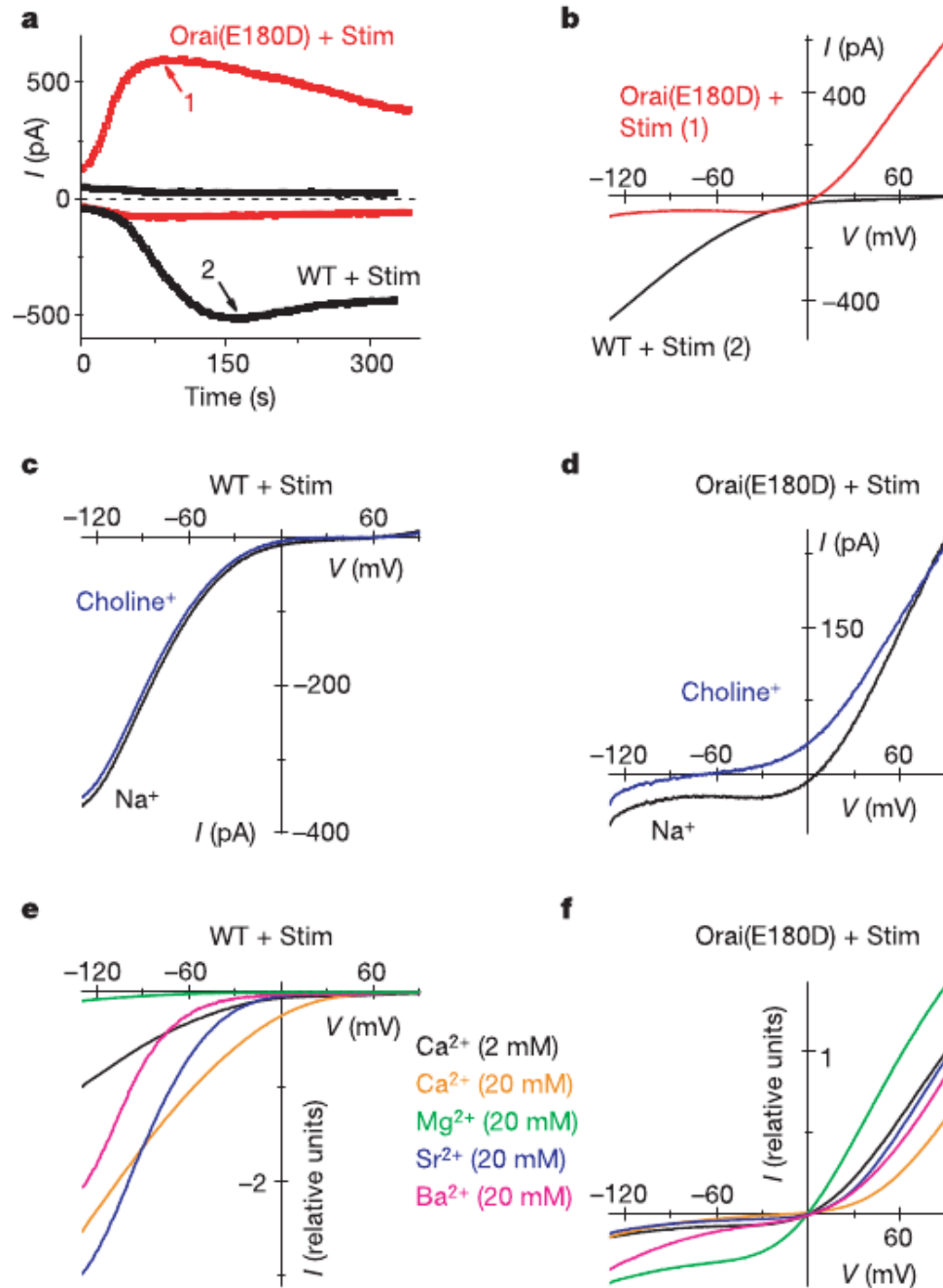
1. Liou J, et al. STIM is a  $\text{Ca}^{2+}$  sensor essential for  $\text{Ca}^{2+}$ -store-depletion-triggered  $\text{Ca}^{2+}$  influx. *Curr Biol* 2005;15:1235–1241. [PubMed: 16005298]
2. Roos J, et al. STIM1, an essential and conserved component of store-operated  $\text{Ca}^{2+}$  channel function. *J Cell Biol* 2005;169:435–445. [PubMed: 15866891]
3. Feske S, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 2006;441:179–185. [PubMed: 16582901]
4. Vig M, et al. CRACM1 is a plasma membrane protein essential for store-operated  $\text{Ca}^{2+}$  entry. *Science* 2006;312:1220–1223. [PubMed: 16645049]
5. Zhang SL, et al. Genome-wide RNAi screen of  $\text{Ca}^{2+}$  influx identifies genes that regulate  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channel activity. *Proc Natl Acad Sci USA* 2006;103:9357–9362. [PubMed: 16751269]
6. Zhang SL, et al. STIM1 is a  $\text{Ca}^{2+}$  sensor that activates CRAC channels and migrates from the  $\text{Ca}^{2+}$  store to the plasma membrane. *Nature* 2005;437:902–905. [PubMed: 16208375]

7. Mercer JC, et al. Large store-operated calcium-selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor. *Stim1 J Biol Chem*. June 28;2006 10.1074/jbc.M604589200published online
8. Peinelt C, et al. Amplification of CRAC current by STIM1 and CRACM1 (Orai1). *Nature Cell Biol* 2006;8:771–773. [PubMed: 16733527]
9. Soboloff J, et al. Orai1 and STIM reconstitute store-operated calcium channel function. *J Biol Chem* 2006;281:20661–20665. [PubMed: 16766533]
10. Yeromin AV, Roos J, Stauderman KA, Cahalan MD. A store-operated calcium channel in *Drosophila* S2 cells. *J Gen Physiol* 2004;123:167–182. [PubMed: 14744989]
11. Almers W, McCleskey EW. Non-selective conductance in calcium channels of frog muscle: calcium selectivity in a single-file pore. *J Physiol (Lond)* 1984;353:585–608. [PubMed: 6090646]
12. Hess P, Tsien RW. Mechanism of ion permeation through calcium channels. *Nature* 1984;309:453–456. [PubMed: 6328315]
13. Hoth M, Penner R. Calcium release-activated calcium current in rat mast cells. *J Physiol (Lond)* 1993;465:359–386. [PubMed: 8229840]
14. Lepple-Wienhues A, Cahalan MD. Conductance and permeation of monovalent cations through depletion-activated  $\text{Ca}^{2+}$  channels (ICRAC) in Jurkat T cells. *Biophys J* 1996;71:787–794. [PubMed: 8842217]
15. Zweifach A, Lewis RS. Calcium-dependent potentiation of store-operated calcium channels in T lymphocytes. *J Gen Physiol* 1996;107:597–610. [PubMed: 8740373]
16. Kozak JA, Kerschbaum HH, Cahalan MD. Distinct properties of CRAC and MIC channels in RBL cells. *J Gen Physiol* 2002;120:221–235. [PubMed: 12149283]
17. Bakowski D, Parekh AB. Monovalent cation permeability and  $\text{Ca}^{2+}$  block of the store-operated  $\text{Ca}^{2+}$  current  $I_{\text{CRAC}}$  in rat basophilic leukemia cells. *Pflugers Arch* 2002;443:892–902. [PubMed: 11889590]
18. Prakriya M, Lewis RS. Potentiation and inhibition of  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels by 2-aminoethyl-diphenyl borate (2-APB) occurs independently of  $\text{IP}_3$  receptors. *J Physiol (Lond)* 2001;536:3–19. [PubMed: 11579153]
19. Ellinor PT, Yang J, Sather WA, Zhang JF, Tsien RW.  $\text{Ca}^{2+}$  channel selectivity at a single locus for high-affinity  $\text{Ca}^{2+}$  interactions. *Neuron* 1995;15:1121–1132. [PubMed: 7576655]
20. Prakriya M, Lewis RS. Separation and characterization of currents through store-operated CRAC channels and  $\text{Mg}^{2+}$ -inhibited cation (MIC) channels. *J Gen Physiol* 2002;119:487–507. [PubMed: 11981025]
21. Zweifach A, Lewis RS. Mitogen-regulated  $\text{Ca}^{2+}$  current of T lymphocytes is activated by depletion of intracellular  $\text{Ca}^{2+}$  stores. *Proc Natl Acad Sci USA* 1993;90:6295–6299. [PubMed: 8392195]
22. Voets T, Janssens A, Droogmans G, Nilius B. Outer pore architecture of a  $\text{Ca}^{2+}$ -selective TRP channel. *J Biol Chem* 2004;279:15223–15230. [PubMed: 14736889]
23. Prakriya M, et al. Orai1 is an essential pore subunit of the CRAC channel. *Nature*. August 20;2006 10.1038/nature05122advance online publication



**Figure 1. Orai interacts with Stim and generates increased store-operated currents in S2 cells**

**a**, Representative co-immunoprecipitation of Stim and Orai ( $n = 3$ ). Input: total cell lysate, showing an equal amount of samples prepared for immunoprecipitation. Cells were transfected with *HA-olf186-F* (Orai) and *Stim-V5-His* (Stim) constructs, as indicated. M refers to molecular weight markers (kDa). **b**, Activation of CRAC current (at  $-130$  mV) by three methods: IP<sub>3</sub> ( $10$  μM IP<sub>3</sub> added to high-Ca<sup>2+</sup> pipette solution; current density  $-11.4 \pm 3.1$  pA pF<sup>-1</sup>,  $n = 5$ ); thapsigargin (high-Ca<sup>2+</sup> pipette solution and thapsigargin added to bath;  $-7.6 \pm 2.5$  pA pF<sup>-1</sup>,  $n = 2$ ); or passive store-depletion (Ca<sup>2+</sup>-free pipette solution;  $-28.7 \pm 1.8$  pA pF<sup>-1</sup>,  $n = 27$ ). All three methods produced substantially greater CRAC current density than passive store depletion in control cells ( $-2.5 \pm 0.4$  pA pF<sup>-1</sup>,  $n = 18$ ).

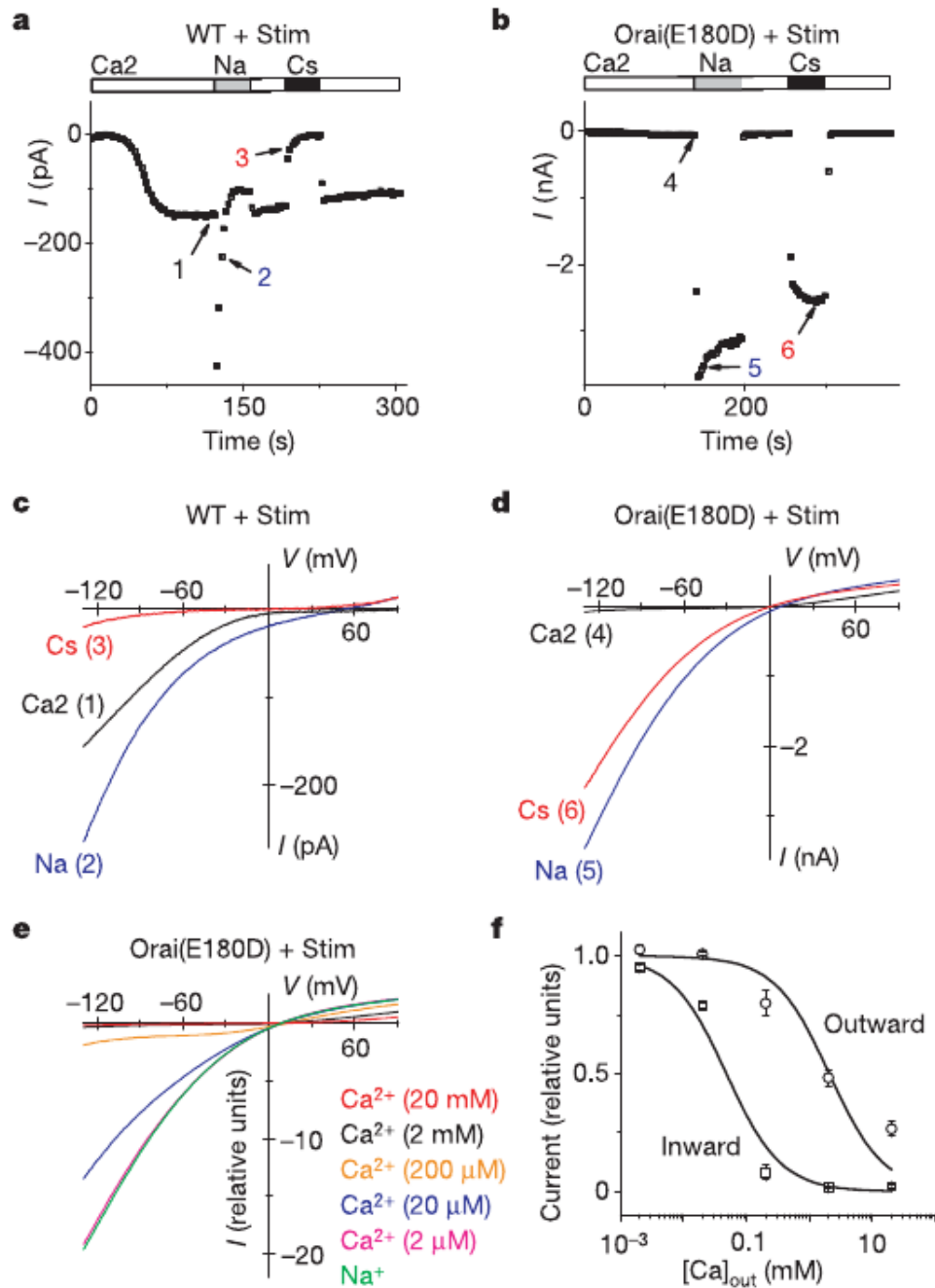


**Figure 2. Mutation E180D of Orai alters ion selectivity of the CRAC current**

**a**, Time courses of inward current at  $-130$  mV and outward current at  $90$  mV in representative cells overexpressing wild-type Orai (WT, black) or Orai(E180D) mutant (red).  $\text{Ca}^{2+}$ -free internal solution. **b**,  $I$ - $V$  curves at times indicated in **a**. **c**,  $I$ - $V$  curves of wild-type Orai-induced current in  $\text{Ca}^{2+}$  solution containing  $160$  mM  $\text{Na}^{+}$  and  $2$  mM  $\text{Ca}^{2+}$  and in choline external solution with  $1.1$  mM  $\text{Na}^{+}$  and  $2$  mM  $\text{Ca}^{2+}$ . (Complete solution recipes are indicated in Supplementary Table 1.) **d**, Representative  $I$ - $V$  curves for Orai(E180D) with the same solutions as **c** ( $n = 3$ ). **e**, Divalent cation selectivity of wild-type Orai CRAC current.  $I$ - $V$  curves are normalized to current values at  $-130$  mV in  $\text{Ca}^{2+}$  solution. Test solutions contained  $20$  mM test divalent and  $124$  mM  $\text{Na}^{+}$ . External solutions for **e** and **f** are labelled according to colour. **f**,  $I$ - $V$  curves (not



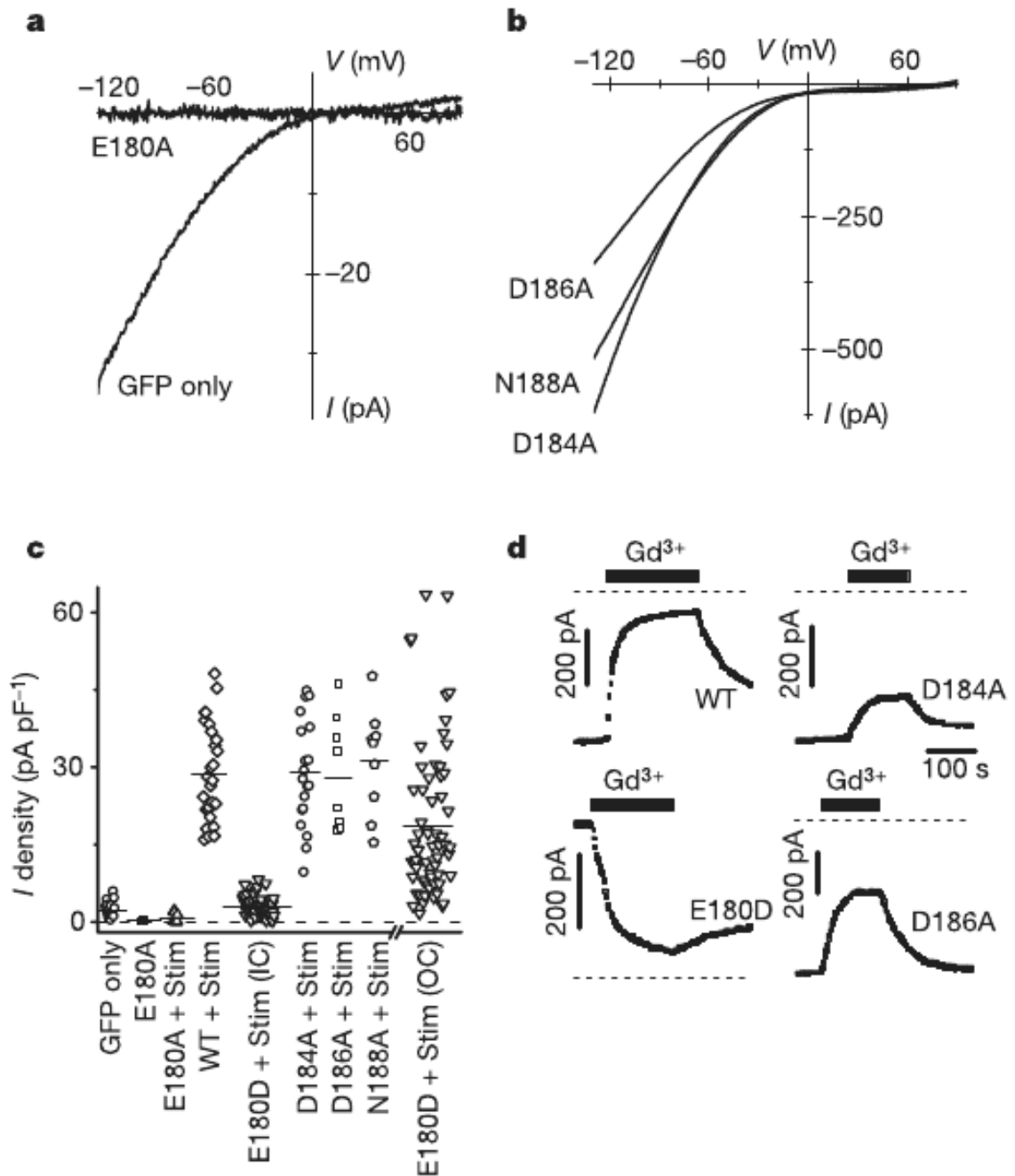
leak-subtracted) for Orai(E180D) with the same divalent cations, normalized to currents at 90 mV in  $\text{Ca}^{2+}$  solution.



**Figure 3. Monovalent current in the absence of divalent ions exhibits altered ion selectivity in the Orai(E180D) mutant**

**a**, Time course of inward current in a cell expressing wild-type Orai. Bars indicate external solution exchange. **b**, Same as **a** but for Orai(E180D). **c**,  $I$ - $V$  curves for wild-type Orai at times indicated in **a**. **d**,  $I$ - $V$  curves for Orai(E180D) at times indicated in **b**. **e**,  $I$ - $V$  curves of Orai (E180D)-induced current in the presence of varying external  $[Ca^{2+}]$ . **f**,  $[Ca^{2+}]$  dependence of Orai(E180D) CRAC current at  $-130$  (squares) and  $90$  mV (circles), scaled to the current in divalent-free solution.  $Ca^{2+}$ -dependent block (mean  $\pm$  s.e.m.) was fitted by the function  $y = 1 / (1 + [Ca^{2+}] / IC_{50})$ , where  $IC_{50}$  is the calculated half-blocking  $Ca^{2+}$  concentration for inward current at  $-130$  mV ( $48 \pm 13 \mu M$ ;  $n = 13, 8, 4, 6$  and  $2$  cells for  $20, 2, 0.2, 0.02$  and  $0.002$  mM

free external  $[Ca^{2+}]$ , respectively) and outward current at 90 mV ( $2.05 \pm 0.78$  mM;  $n = 13, 8, 4, 5$  and  $3$  cells for  $20, 2, 0.2, 0.02$  and  $0.002$  mM free external  $[Ca^{2+}]$ , respectively).



**Figure 4. Charge-neutralizing mutations in the S1-S2 loop**

**a**, *I-V* curves in a cell expressing the Orai(E180A) mutant (without Stim) compared to control.

**b**, *I-V* curves for Orai(D184A), Orai(D186A) and Orai(N188A) mutants. **c**, CRAC current

density in transfected S2 cells. Each point represents the maximal CRAC current density ( $\text{pA pF}^{-1}$ ) in a single cell, plotted as absolute values (from left to right): GFP-transfected control;

Orai(E180A) ( $P = 1.7 \times 10^{-4}$  relative to control); Orai(E180A) plus Stim ( $P = 8.2 \times 10^{-3}$ );

wild-type Orai plus Stim; Orai(E180D) plus Stim, inward current; Orai(D184A) plus Stim;

Orai(D186A) plus Stim; Orai(N188A) plus Stim; and Orai(E180D) plus Stim, outward current.

**d**, Suppression of CRAC current by 5 nM  $\text{Gd}^{3+}$  in wild-type Orai and the D184A, E180D and

D186A Orai mutants. Bars indicate time of  $Gd^{3+}$  application; dashed lines indicate the zero-current level.