

**Supplementary Table 1. Solutions for whole-cell recording**

Name	NaCl	CaCl <sub>2</sub>	MgCl <sub>2</sub>	Glucose	HEPES
External ( <b>Ca2</b> )	160	2	-	10	10
High Ca <sup>2+</sup> external ( <b>Ca20</b> )	124	20	-	10	10
Nominally Ca <sup>2+</sup> -free external ( <b>Mg2</b> )	160	-	2	10	10

Name	CsAsp	CsCl	Cs <sub>4</sub> BAPTA	HEPES
Ca <sup>2+</sup> - free internal	133	2	12	15

Solution names used in text are indicated in bold. Concentrations are in mM. High Ca<sup>2+</sup> external solution contained 19 mM sucrose. Ca<sup>2+</sup>-free internal solution contained 8 mM magnesium gluconate. pH of external and internal solutions was 6.6 and 7.2 respectively, adjusted by appropriate hydroxide. Osmolality of all solutions was 324 ± 1 mOsm. Gd<sup>3+</sup> was added as GdCl<sub>3</sub> (Aldrich).

**Supplementary Figure 1 Orai forms oligomers in resting S2 cells.** Reciprocal co-immunoprecipitation was performed on *Drosophila* S2 cells cotransfected with HA and Flag-tagged Orai subunits. Cells transfected with only HA-Orai or Flag-Orai are used as a control of the co-immunoprecipitation specificity. (IP = immunoprecipitation, Co-IP= Co-immunoprecipitation, IB= Immunoblot)

**Supplementary Figure 2 Homomeric nature of cross-linked Orai in intact S2 cells.**

Numbers represent assigned state of oligomerization: 1, monomer; 2, dimer; 3, trimer; 4, tetramer. **a**, Determination of Orai oligomeric structure using chemical cross-linking in living cells. **b**, Relative mobility (Rf) through SDS-PAGE of Orai cross-linked products in presence of DFDNB (measured in Figure 1a) as a function of the logarithm of the putative number of cross-linked subunits plotted on a semi-logarithmic scale. Initial assignment of the number of cross-linked subunits was determined from the apparent molecular mass of each band and tested by plotting the logarithm of the estimated number of cross-linked subunits against the relative mobility of each band on a 4-12% SDS-polyacrylamide gel. The linear relationship in the plot indicates that the relative mobility of each band corresponds to its assigned subunit stoichiometry. **c**, In vivo cross-linking pattern of GFP-Flag-Orai. To rule out the possibility of heteromultimeric species between Orai and a protein of the same apparent molecular weight, DFDNB cross-linking was performed on a GFP-Flag-Orai fusion protein (GFP-Orai) that we showed is a functional CRAC channel in patch-clamp experiments; and the size of the cross-linked products was analysed by SDS PAGE. S2 cells were transfected with GFP or GFP-Flag-Orai, and incubated with various amount of DFDNB for 10 min at 37°C. After lysis, cross-linked products were analyzed by SDS-PAGE on 4-8% tris-Acetate gradient gels. (\*, high order aggregates; n.s, non-specific signal). **d**, Relative mobility (Rf) through SDS-PAGE of GFP-Flag-Orai cross-linked products in presence of DFDNB (measured in **c**) as a function of the putative number of cross-linked subunits plotted on a semi-logarithmic scale.

**Supplementary Figure 3 Kinetics of the chemical cross-linking reaction.** **a**, Cross-linking reaction kinetics in cell lysates. DFDNB or BS3 was incubated with HA-Orai transfected cell lysates for 0 to 60 minutes at 37°C; the reaction was stopped by addition of 20 mM TrisHCl pH 7.5, and the cross-linked products analyzed by SDS-PAGE on 4-12% gradient gels. **b**, Cross-linking reaction kinetics in intact cells. Live HA-Orai transfected cells were treated with DFDNB for 0 to 60 minutes at room temperature, the reaction was stopped by addition of 20 mM TrisHCl pH 7.5, and after cell lysis the cross-linked products analyzed by SDS-PAGE on 4-12% gradient gels. The \* sign indicating high order oligomers or aggregates and subunit stoichiometry assignment refer to each panel.

**Supplementary Figure 4 Validation of the PFO-PAGE technique.** Numbers represent assigned state of oligomerization: 1, monomer; 2, dimer; 3, trimer; 4, tetramer; 6, hexamer. **a**, HA-Orai transfected S2 cells (left) and HEK293 cells (right) were solubilized in the presence of 1% NP-40 and then incubated with sample buffer containing different PFO concentration for 30 minutes at room temperature before electrophoresis as described previously. Neither the lysis condition nor the cellular context affected the predominance of Orai dimers. The \* indicates high-order aggregates of nonsolubilized proteins. **b**, Validation of the PFO-PAGE techniques on the rP2X2 channel expressed in HEK293 cells.  $\Delta$ 3N-rP2X2-Flag-transfected HEK293 cells solubilized in presence of 1% NP-40 (left) or total cell lysates (right) were analysed by PFO-PAGE. **c**, Orai dimers remain the main species regardless of the PFO solubilization conditions. HA-Orai-transfected S2 cells total cell lysates were incubated with sample buffer containing different PFO concentration for 30 min at different temperatures (left), or at room temperature for 5 to 120 min (right) before electrophoresis. Orai dimers remain the main species regardless of the solubilization conditions.

**Supplementary Figure 5 C-Stim constitutively activates  $\text{Ca}^{2+}$  influx through CRAC channels in S2 cells.** **a**, Schematic representation of full-length Stim and C-Stim (EF = EF hand; SAM = sterile- $\alpha$ -motif; TM = transmembrane domain, CC = coiled-coil). **b-d**, Intracellular fura-2 calcium measurement of GFP (control) and C-Stim transfected S2 cells. After 2 min in 0 mM calcium solution, adding back 2 mM calcium triggered  $\text{Ca}^{2+}$

influx in C-Stim-transfected cells (c) but not in control cells (b). The constitutive activation of the endogenous CRAC channels by C-Stim expression leads to an increase in the resting calcium level which is inhibited by the application of gadolinium ( $Gd^{3+}$ , 5 nM) (d). e, Resting calcium levels in S2 cells: control, transfected with C-Stim, and transfected with C-Stim in presence of  $Gd^{3+}$ . f, The subcellular localization of C-Stim was analyzed in S2 cells expressing low or high amount of C-Stim. C-Stim displays a plasma membrane-like staining at low expression level (left panel) suggesting that the overexpressed C-Stim may interact with the endogenous Orai subunits; some intracellular staining appears when the expression level is higher (right panel). This change in localization pattern is probably dependent on the relative expression between the overexpressed C-Stim and the endogenous Orai subunit.

**Supplementary Figure 6  $Ca^{2+}$  dependence and run-down of CRAC current constitutively induced by C-Stim.** a, CRAC current densities upon break-in are shown for cells transfected with Stim + GFP-Orai (left) and C-Stim + GFP-Orai (right);  $p=0.008$ . The maximal current density in cells co-transfected with Stim+GFP-Orai was  $27.7\pm 4.4$  pA/pF ( $n=11$  cells). The pre-activated current density in cells co-transfected with C-Stim+GFP-Orai was  $13.4\pm 2.5$  pA/pF ( $n=17$  cells). b, The sustained pre-activated CRAC current recorded with a high resistance pipette is augmented by increasing extracellular  $[Ca^{2+}]$ . c, I-V curves obtained when indicated in (b) with 2 mM (red) and 20 mM (blue) extracellular  $[Ca^{2+}]$ . d, Diffusion of C-Stim into the patch-pipette underlies the rundown of C-Stim-induced constitutive CRAC current. Typical time courses of CRAC currents in cells transfected with C-Stim and GFP-Flag-Orai, during whole-cell recording using either low-resistance (blue,  $R_p = 2.2$  M $\Omega$ ) or high-resistance (red,  $R_p = 11$  M $\Omega$ ) patch pipettes. e, Changes in CRAC current amplitude during the first 60 s after break-in for two sets of pipettes with average resistance of  $10.0 \pm 0.7$  M $\Omega$  (small pipettes,  $n=13$ , red circle) and  $2.3 \pm 0.1$  M $\Omega$  (large pipettes,  $n=9$ , blue oval). The bars indicate the mean values of current change for each set of cells. The arrows indicate cells presented in Fig. 2a, b.

**Supplementary Figure 7 Subcellular localization of GFP-Flag-Orai, Stim, and C-Stim.** a, Subcellular localization of Orai (top panel), full-length Stim (middle panel) or C-

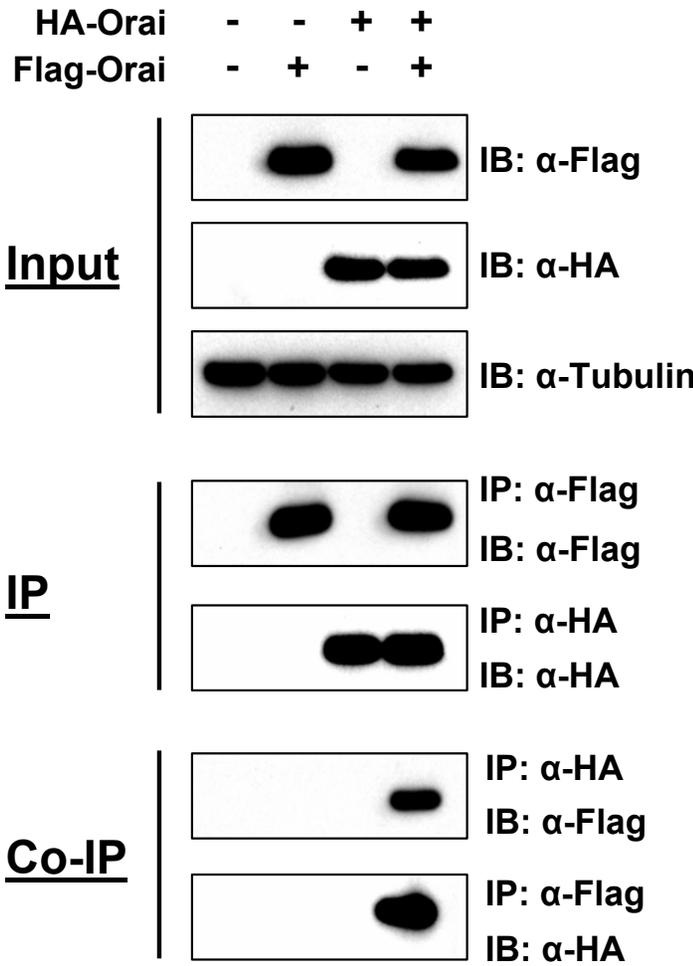
Stim (bottom panel) transfected alone in resting and store-depleted (2  $\mu$ M TG for 15 min) S2 cells. After store depletion, Stim alone but not Orai alone forms puncta. **b**, Subcellular localization of Orai co-transfected with full-length Stim (top panel) or C-Stim (bottom panel) in resting and store-depleted (2  $\mu$ M TG for 15 min) S2 cells. Both Stim and Orai colocalized in puncta after TG-induced store-depletion. The cytosolic C-terminal fragment of Stim is associated with the plasma membrane where it colocalizes with Orai but does not form puncta.

**Supplementary Figure 8 Reciprocal co-immunoprecipitation of Stim/C-Stim and Orai with or without store depletion by TG.** Molecular weights (Mw) are indicated on the left and are the same for all panels. IP = immunoprecipitation, Co-IP = co-immunoprecipitation, IB = immunoblot, # = IgG heavy chains.

**Supplemental video 1 Example of two-step bleaching in an oocyte expressing GFP-FLAG-Orai alone.** The image sequence shows a 3x3  $\mu\text{m}$  region of oocyte membrane observed by TIRF microscopy; bar is 0.5  $\mu\text{m}$ . The trace to the right shows corresponding measurements of average fluorescence within the region of interest marked by the circle

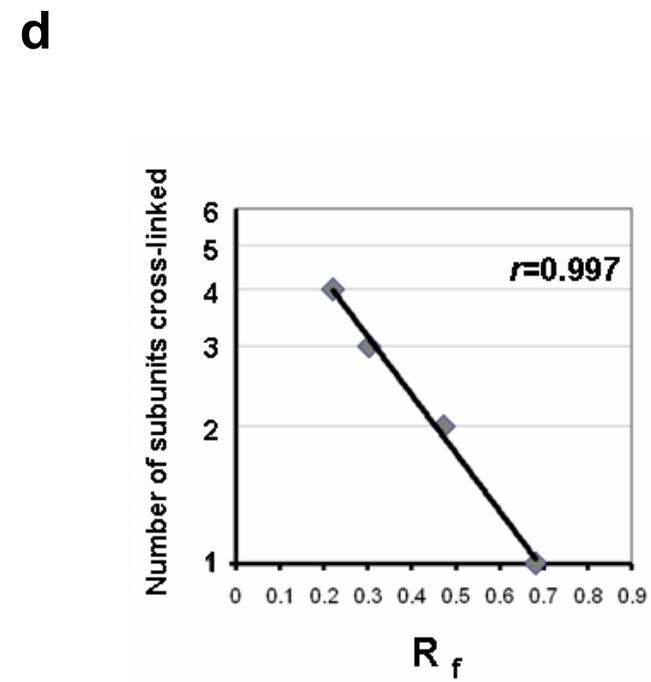
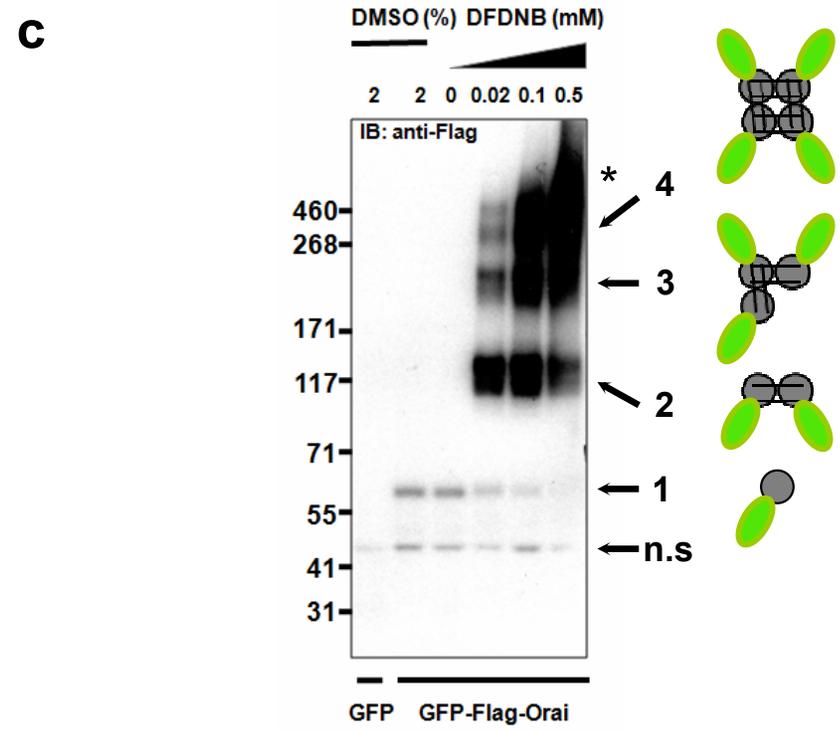
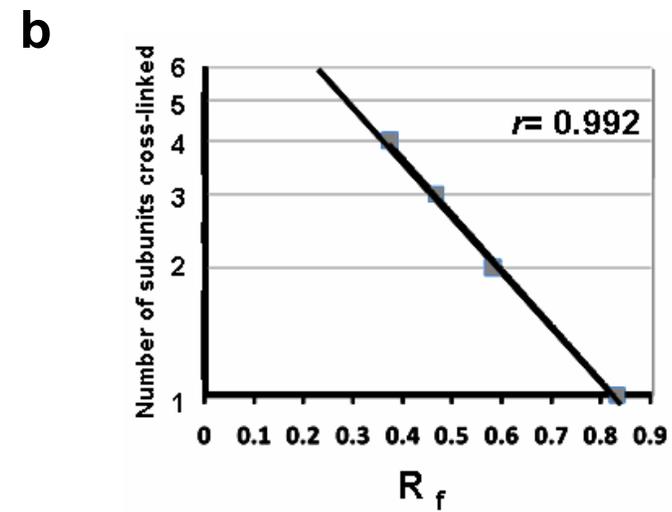
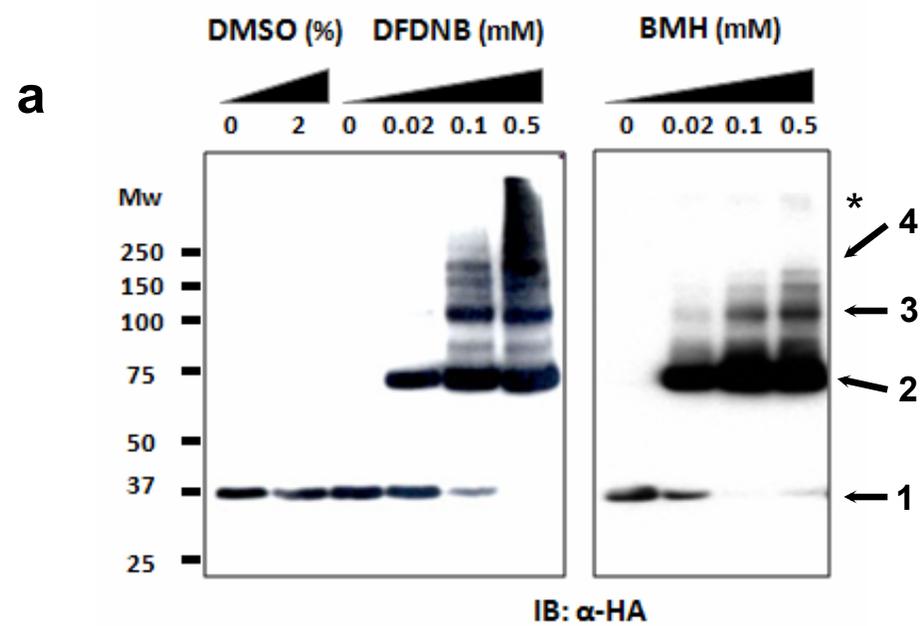
**Supplemental video 2 Example of four-step bleaching in an oocyte expressing GFP-FLAG-Orai together with C-Stim.** The image sequence shows a 3x3  $\mu\text{m}$  region of oocyte membrane observed by TIRF microscopy; bar is 0.5  $\mu\text{m}$ . The trace to the right shows corresponding measurements of average fluorescence within the region of interest marked by the circle.

# Supplementary Figure 1



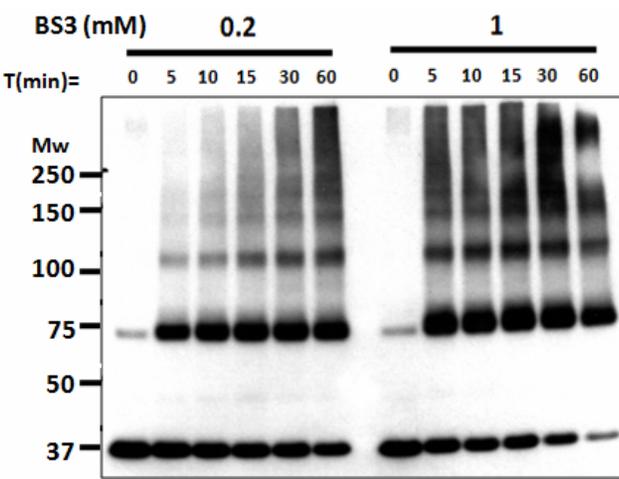
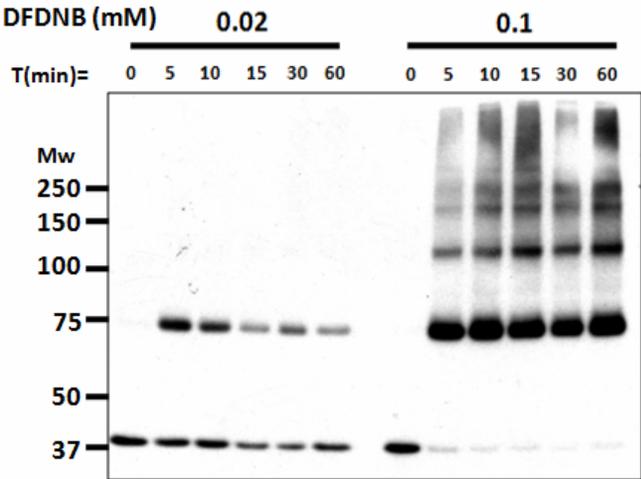
Supplementary Figure 2

Intact cells

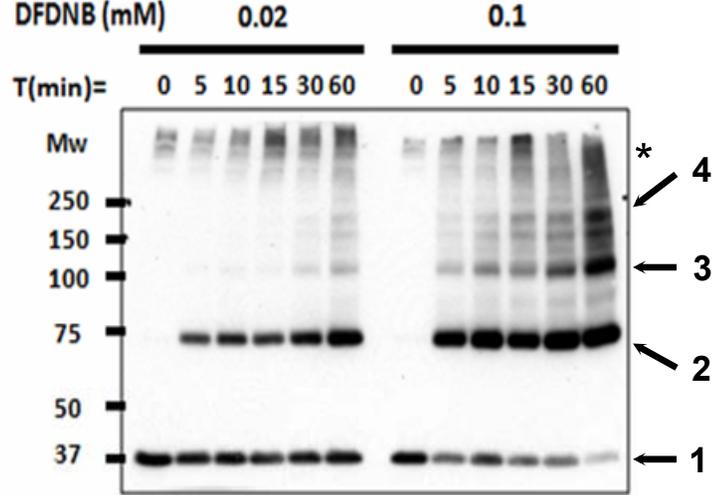


Supplementary Figure 3

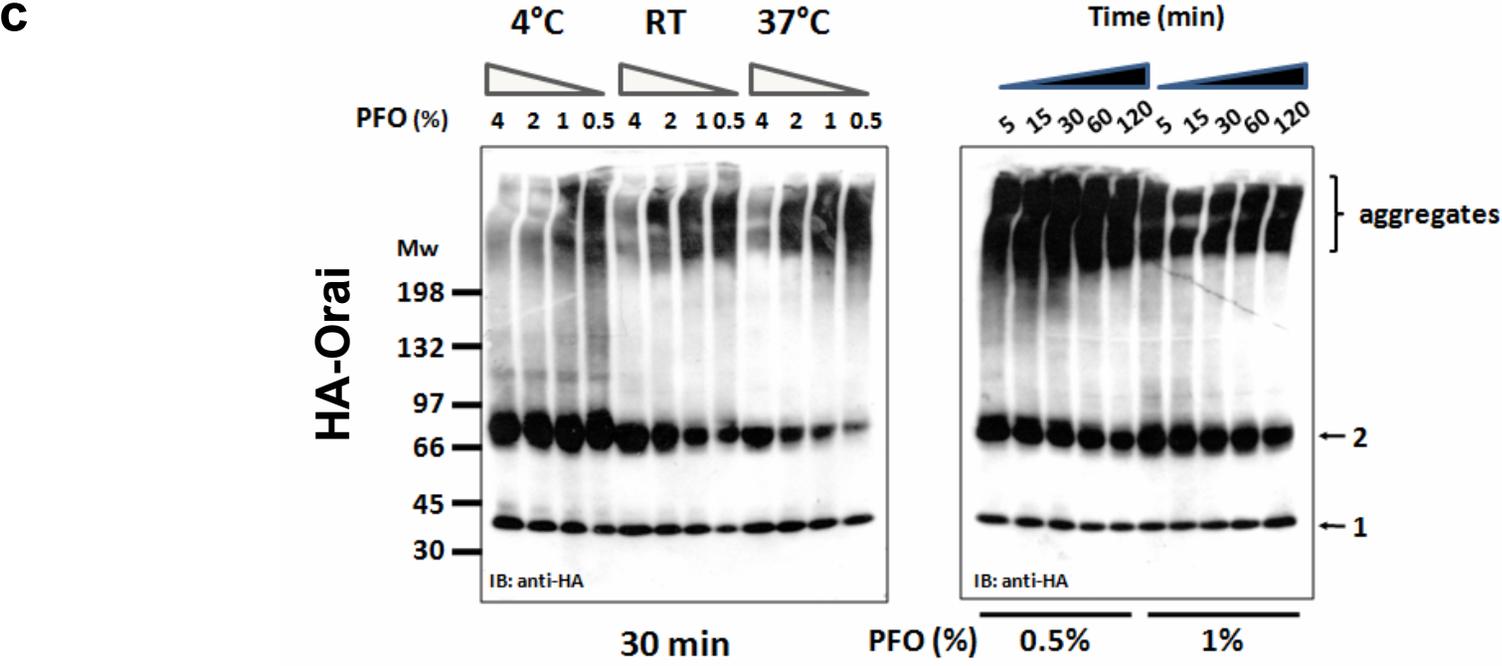
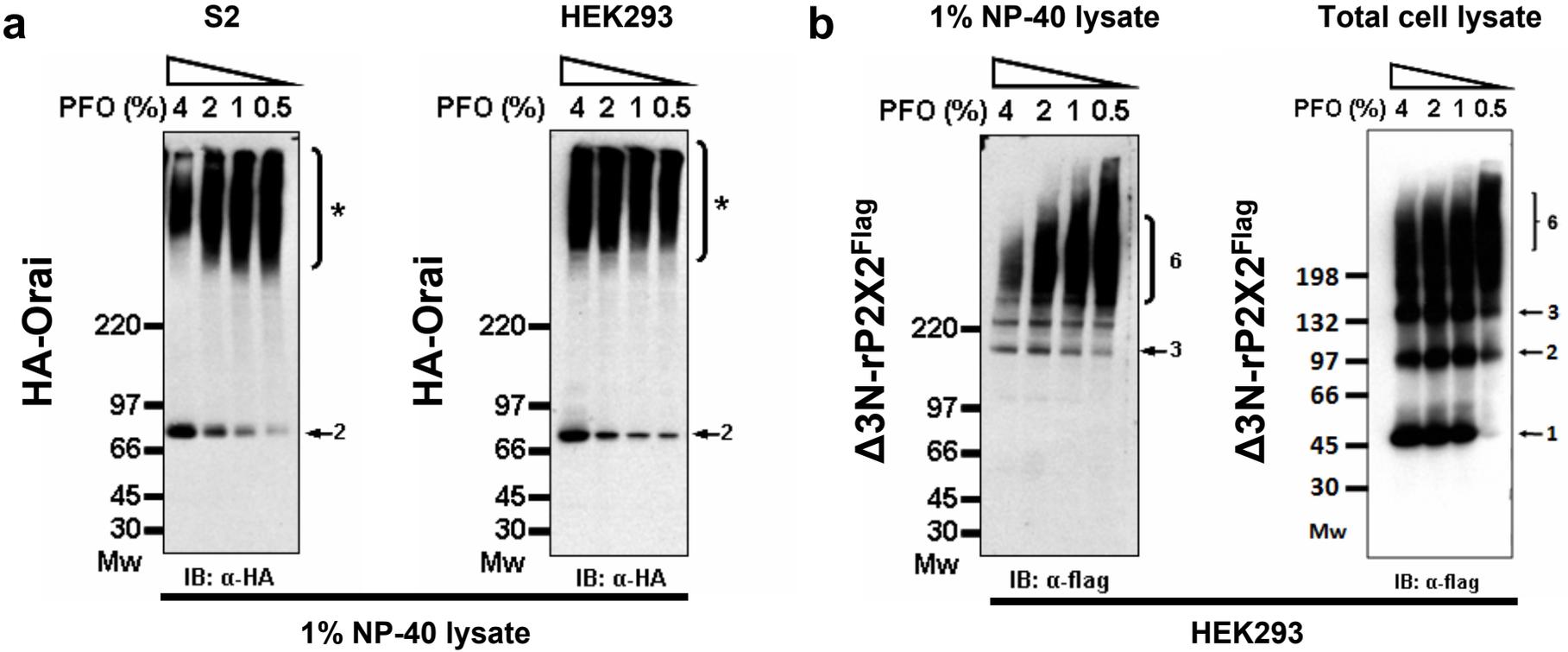
**a Cell lysates**



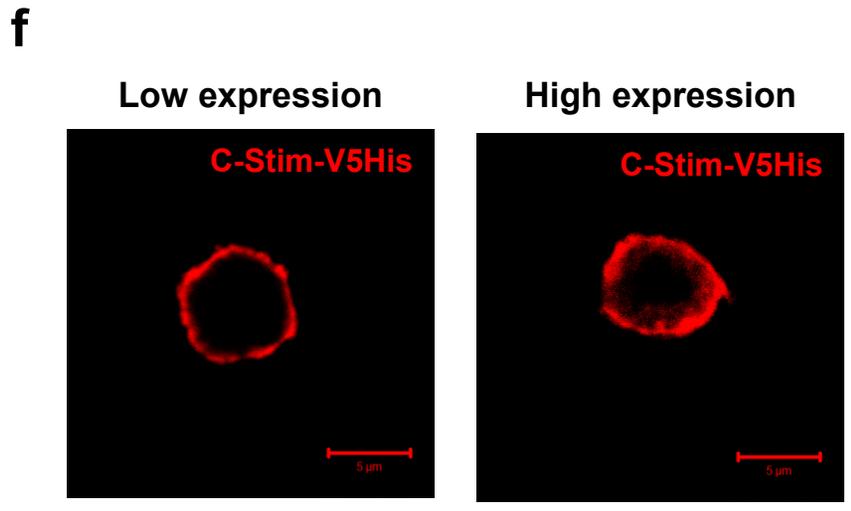
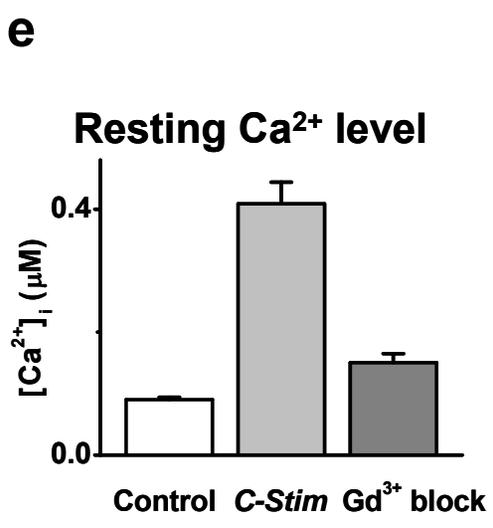
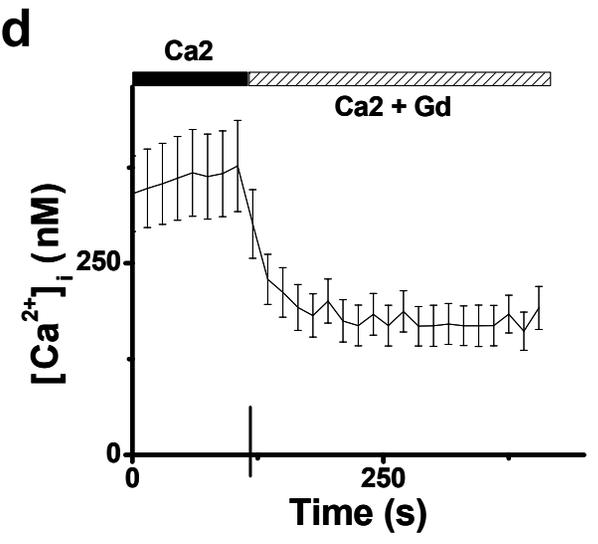
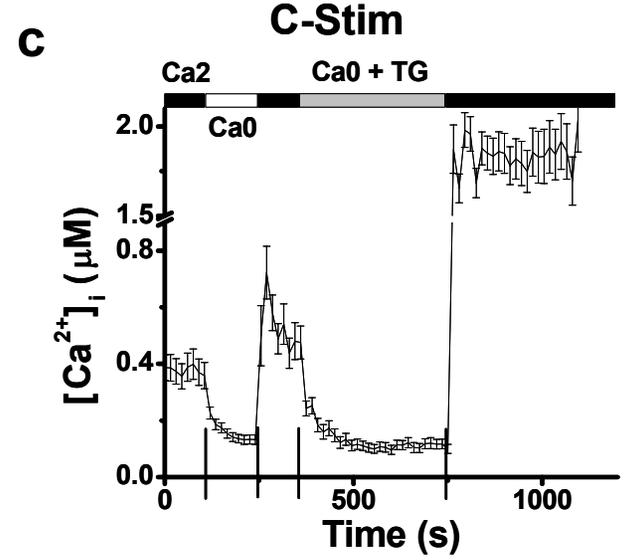
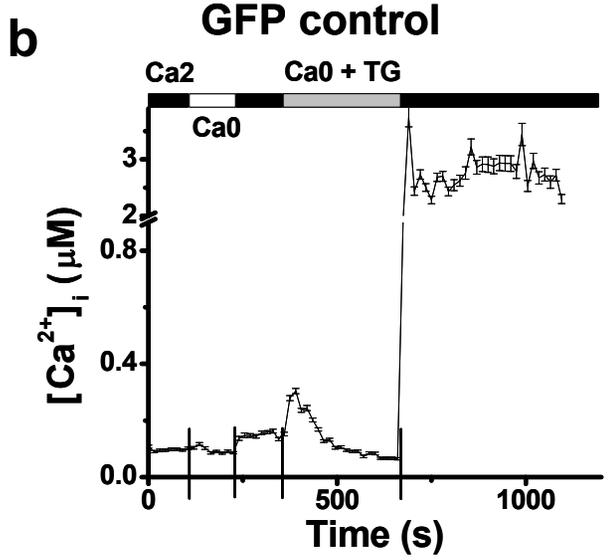
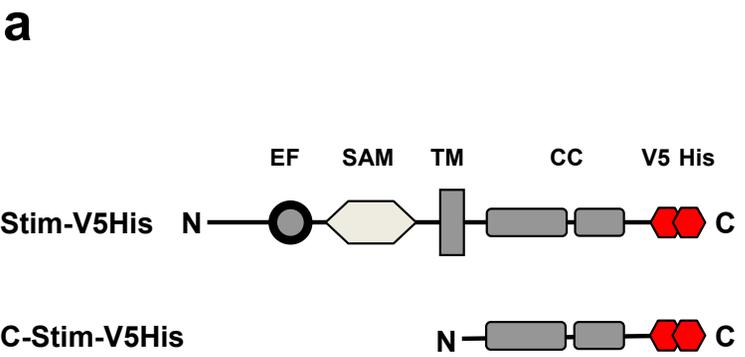
**b Intact cells**



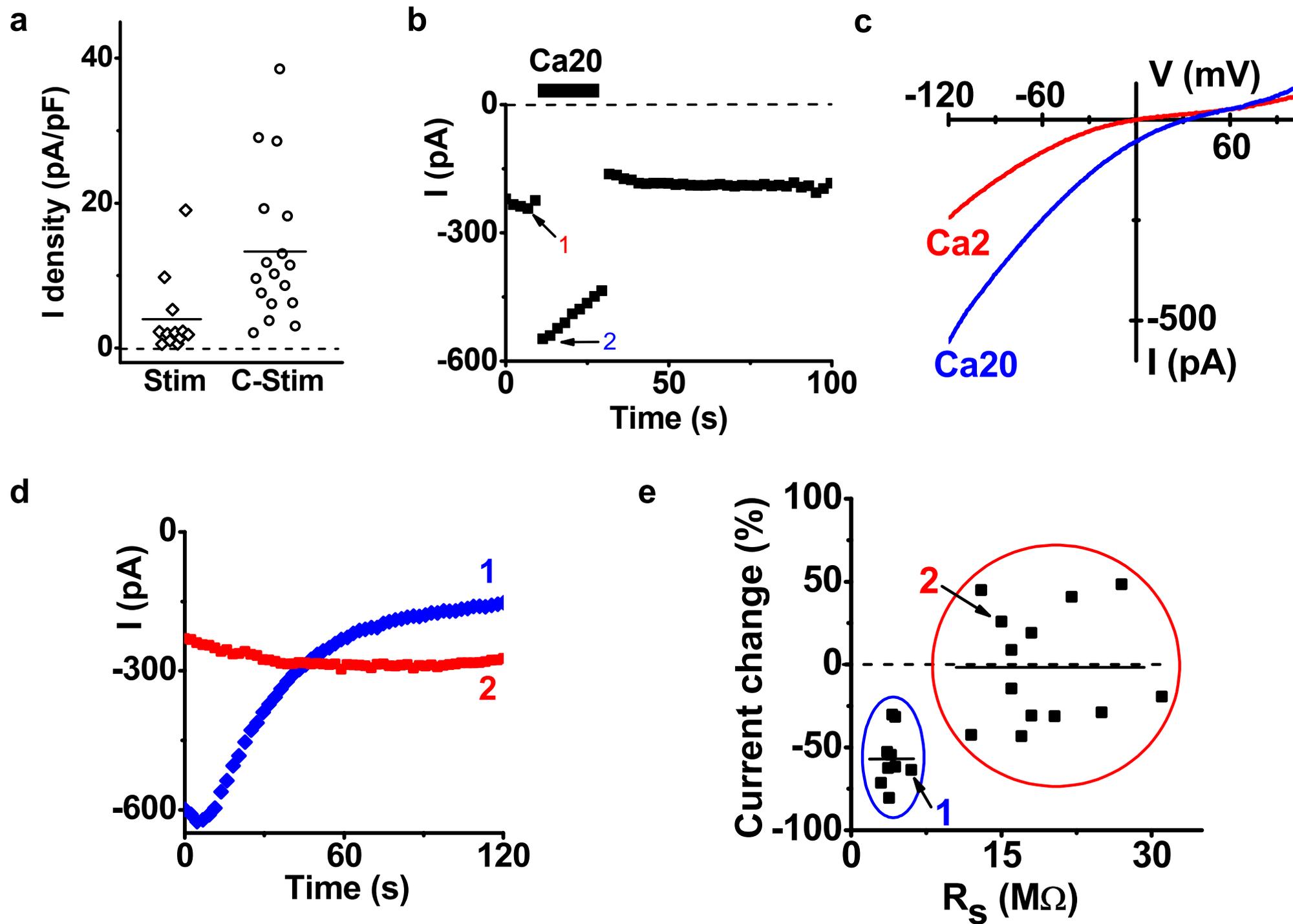
Supplementary Figure 4



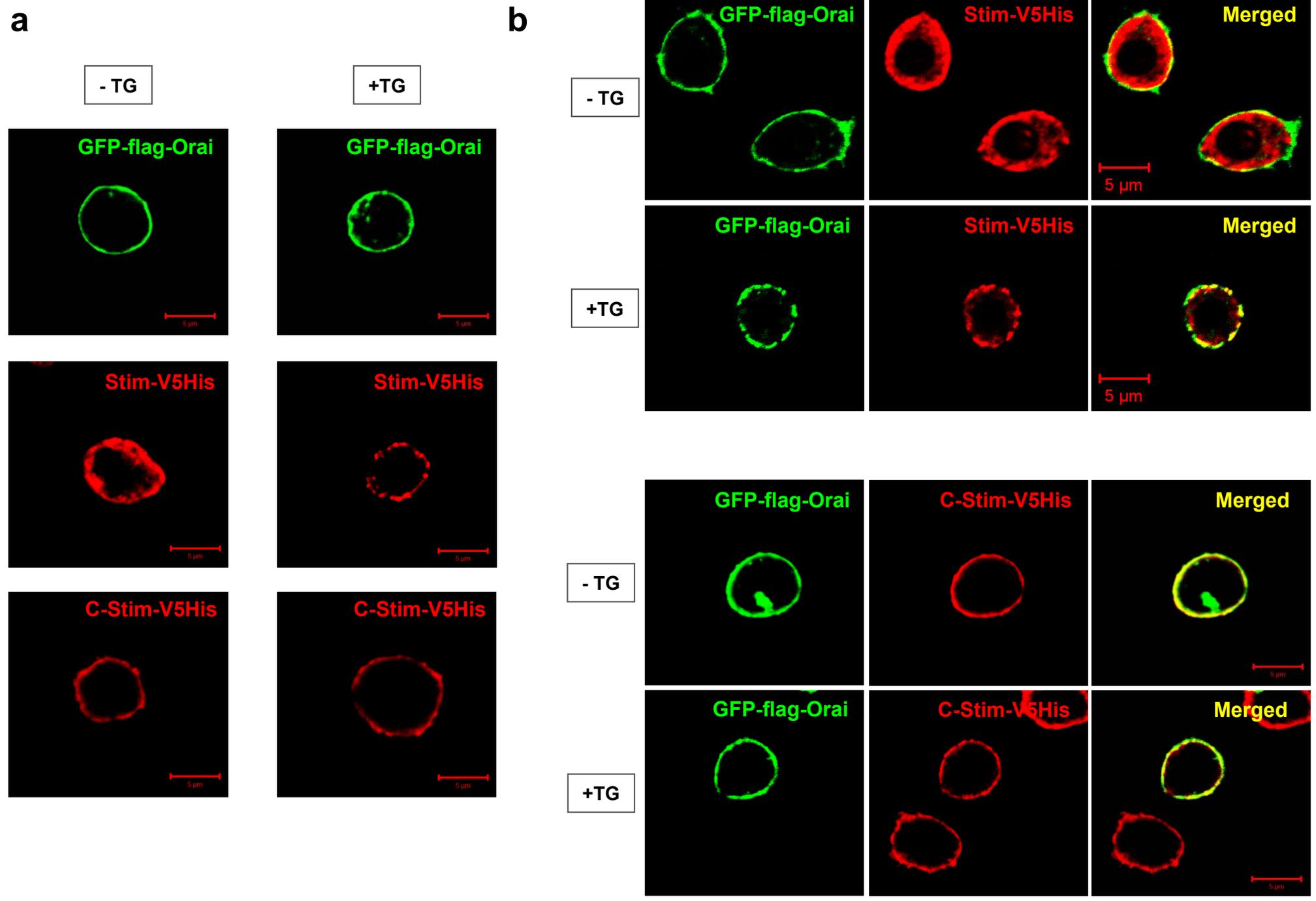
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8

