SUPPLEMENTAL MATERIAL

EXPERIMENTAL PROCEDURES

Channel expression in Xenopus laevis oocytes and preparation of membranes. For expression in *Xenopus laevis* oocytes, the coding sequence of the Kir3.1 chimera was PCR-amplified and inserted between the BamH1 and HindIII restriction sites of the dual-function expression vector pXooM (58). Kir3.1, Kir3.1*, Kir3.4 and M2R cDNAs were in the pGEMHE plasmid vector. Synthesis of capped cRNAs was performed by *in vitro* transcription using the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). Injected oocytes (50μl/oocyte) were kept at 19 °C for 2 days to allow for expression of the heterologous proteins and subsequently used for electrophysiological recordings or preparation of membranes. For isolation of the membrane fraction, oocytes were homogenized in 5mM Tris-HCl pH: 8.0, 1mM EDTA, 1mM EGTA containing 10mM $NaVO_4$, 10mM NaF and complete protease inhibitor cocktail (Roche, Indianapolis, IN), homogenates were centrifuged for 5min at 5000 RPM (4^o C) and supernatants were subsequently ultracentrifuged at 100,000g for 40min at 4°C in a Beckman Maxima Ultracentrifuge (Beckman, Brea, CA). The pellets were re-suspended by sonicating them with a 1:1 mixture of bovine brain PE (10 mg/ml) and PS (10 mg/ml) and reconstituted in lipid bilayers as described for the Kir3.1 chimera.

Two-electrode voltage clamp: Recombinant Kir3 channels and M2 receptors were expressed in *Xenopus laevis* oocytes as previously described (22, 55). cRNA was produced with T7 RNA polymerase using the Ambion kit. In all experimental groups, cRNA of the M2 receptors was injected at 1 ng/oocyte and the ratio of M2R to the combination of all Kir3 constructs injected was maintained at 1 : 2. Recordings in *Xenopus laevis* oocytes were performed 2–4 days following cRNA injection. Whole-oocyte currents were measured by conventional two-microelectrode voltage clamp with a GeneClamp 500 amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Electrodes were filled with 3 M KCl dissolved in 1% agarose to prevent the leakage of KCl into the oocytes. The electrodes had a resistance of less than 1 MΩ. Oocytes were constantly perfused with a high-potassium solution (HK) containing in mM: 96 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.4). M2 receptors expressed in the oocytes were activated by perfusion with high-potassium solution, containing 5 μM ACh (ACh). At the end of each experiment, high-potassium solution containing 5 mM $BaCl_2$ (Ba) was used to inhibit the Kir3 currents. Current amplitudes were measured at –100 mV. Data acquisition and analysis were achieved using pClamp8 (Axon Instruments/Molecular Devices, Sunnyvale, CA) and Origin 6 (Microcal/Origin, Clemente, CA) software.

Single-channel recording in oocytes: The cRNA of Kir3.1* was injected at 5-10 ng/50 nl for single channel recordings. Single channel currents were recorded from devitellinized oocytes under a standard excised inside-out patch-clamp configuration (56) with an Axopatch 200 amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Single-channel currents were sampled at 5 kHz and filtered at 1 kHz. pClamp was used to drive stimulus protocols and digitize currents. A standard pipette solution, ND96K and FVPP + 20 mM NaCl, was used in both solutions to prevent channel rundown. Recordings were performed 3-5 days following injection. Recording pipettes with a resistance of 1~1.5 MΩ in the both solution were frequently employed. Gβγ and short chain diC8 PIP_2 were applied to the intracellular side of excised patches. All experiments were performed at room temperature (~22-25^o C).
Data analysis. Single-channel current amplitude was determined by generating amplitude histograms for selected segments and fitted to Gaussian functions. Single-channel conductance values were determined by the slope of current-voltage (I-V) curve between –200 to + 200mV, where I-V data could be well fitted to a linear line. Channel open probability (P_o) was determined with continuous recordings of at least 2,000ms (total 1-3min) using pClamp software. The channel number used was the maximal number of simultaneous channel openings observed.

Confocal fluorescence microscopy: The C terminus of the Kir3.1-chimera was tagged with enhanced green fluorescent protein (Clontech, Mountain View, CA) by subcloning into pEGFP-N3 plasmid vector.

For mammalian cell expression, HEK-293 cells were grown on coverslips and transfected using lipofection with Kir3.1 chimera-EGFP alone or Kir3.1 chimera-EGFP and Kir3.4. One day after the transfection, cells were fixed in 2.5% paraformaldhyde. Coverslips were washed and mounted onto coverslides using ProLong Gold antifade reagent (invitrogen, Carlsbad, CA). Cells were visualized using a Leica TCS2 AOBS laser scanning confocal microscope.

FIGURE LEGENDS

Fig. S1. Purification of the Kir3.1 chimera. (*A*) In the final step of the purification, the eluate obtained from cobalt affinity chromatography was run over a Sephacryl S-200 gel filtration column to yield a chromatogram containing three peaks. Peak A eluted at the exclusion volume (36 ml) of the column. (*B*) A 15% SDS-PAGE analysis of selected fractions stained with coomassie blue shows that peak B contains the majority of the chimera. Two main bands marked by arrows can be observed. As assessed by in gel-digestion and mass spectrometry, both bands correspond to the Kir3.1 chimera. The faster band migrates with an apparent molecular weight of 34 kDa, which matches well with the calculated mass (36 kDa) of the chimera monomer. The upper band migrates with an apparent molecular weight above the 212 kDa marker and likely corresponds to the chimera tetramer. In-gel digestion and mass spectrometry confirmed the identity of both bands.

Fig. S2. Electron microscopy. (*A*) Plot of the Euler angle distribution of the particles used in the 3D reconstruction. Every particle is represented by a triangle in a tilted transparent semi-sphere. Particles are grouped by the default angular step (10 deg) used by the reconstruction algorithm. Two regions lacked complete angular coverage: The first one (surrounded by a dotted line) is the result of the 4-fold symmetry (not applied during the reconstruction process) of the volume. The second one (top of the sphere) coincides with the z-axis and it is due to enrichment of the experimental particle images in a lateral orientation. The lack of angular coverage in this region could cause a decrease in the resolution of the volume along this z-axis. (*B*) Gallery of experimental class averages (same as in Fig. 1D) generated by MLF2D compared to selected backprojections (with white Gaussian noise added and boxed in dotted lines) from the final 3D reconstruction. (*C*) The Fourier shell correlation (FSC) suggests a resolution of 24 Å according to the 0.5 criterion.

Fig. S3. Expression in *Xenopus* oocytes. (*A*) *Xenopus* oocytes were injected with M2R-cRNA alone (Control), or (*B*) coinjected with cRNA for M2R (1 ng) in combination with cRNA for Kir3.1 chimera (2 ng,); (*C*) Kir3.4 (2 ng); (*D*) Kir3.4 (1 ng) and Kir3.1 chimera (1 ng); and (*E*) Kir3.4 (1 ng) and Kir3.1 (1ng). All currents were recorded at -100 mV. Basal currents were recorded in the presence of HK solution. M2Rs were activated with 5 μM ACh.

Fig. S4. Expression in HEK-293 cells. Kir3.1 chimera was C-terminally tagged with EGFP. Localization was monitored by confocal microscopy for (*A*) Kir3.1 chimera-GFP alone and (*B*) co-expression with Kir3.4.

Fig. S5. Single-channel conductance of Kir3.1* measure in inside-out patches or bilayers using membranes from *Xenopus* oocytes. (*A*) Single-channel current traces from an inside-out patch at –180, 0 and +180 mV for GIRK1* when expressed in oocytes. (*B*) Current-voltage relationships for GIRK1* when expressed in oocytes. Single-channel conductance was 15.95 ± 0.72 ps (n = 7). (*C*) Single-channel current traces for Kir3.1* expressed in oocyte membranes and reconstituted in a lipid bilayer. Membrane potentials were held as indicated at -80, 0, and +80 mV. (*D*) Current-voltage relationships for Kir3.1* obtained in bilayer. Single-channel conductance was 19.30 ± 2.10 ps (n = 3).

Key words: G protein K^+ channel, Kir3.1 chimera, Gβγ subunits, PIP_2, electron microscopy

Fig. S1 Leal-Pinto et al.

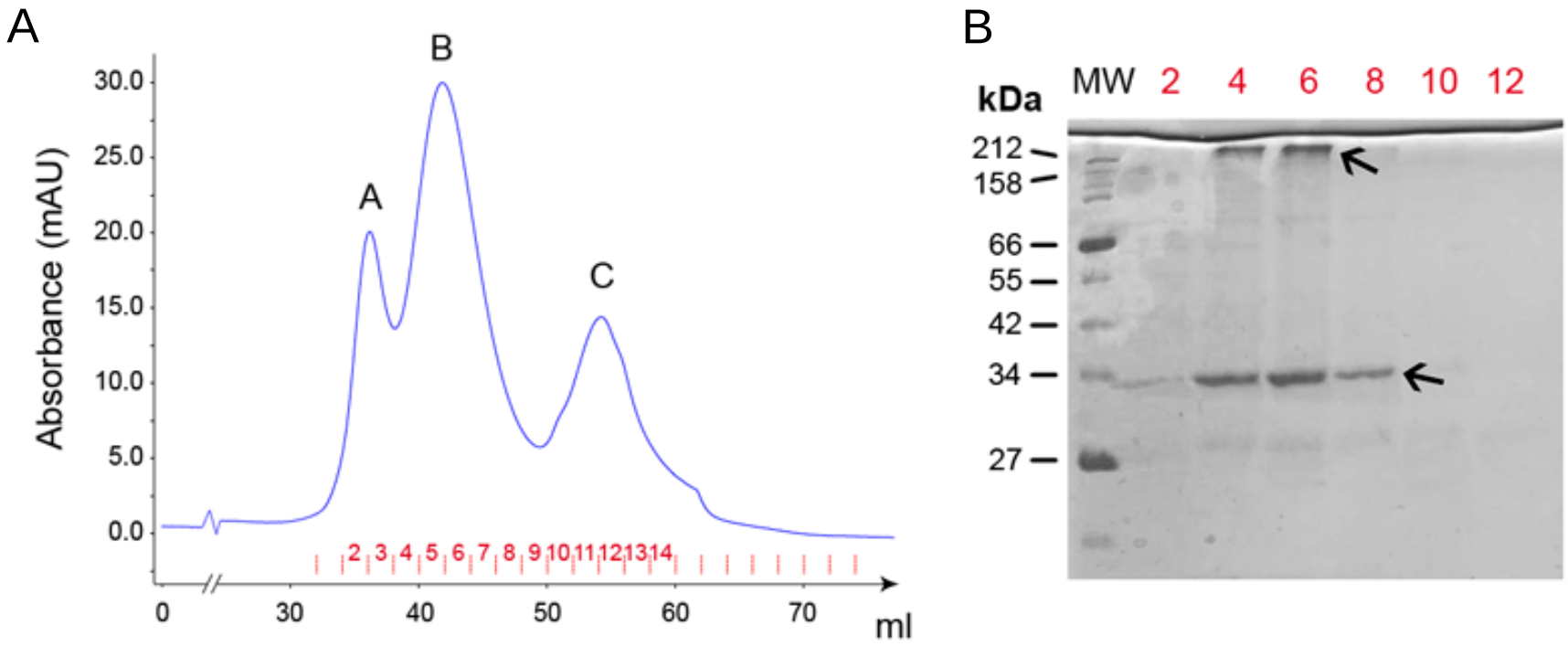


Fig. S2 Leal-Pinto et al.

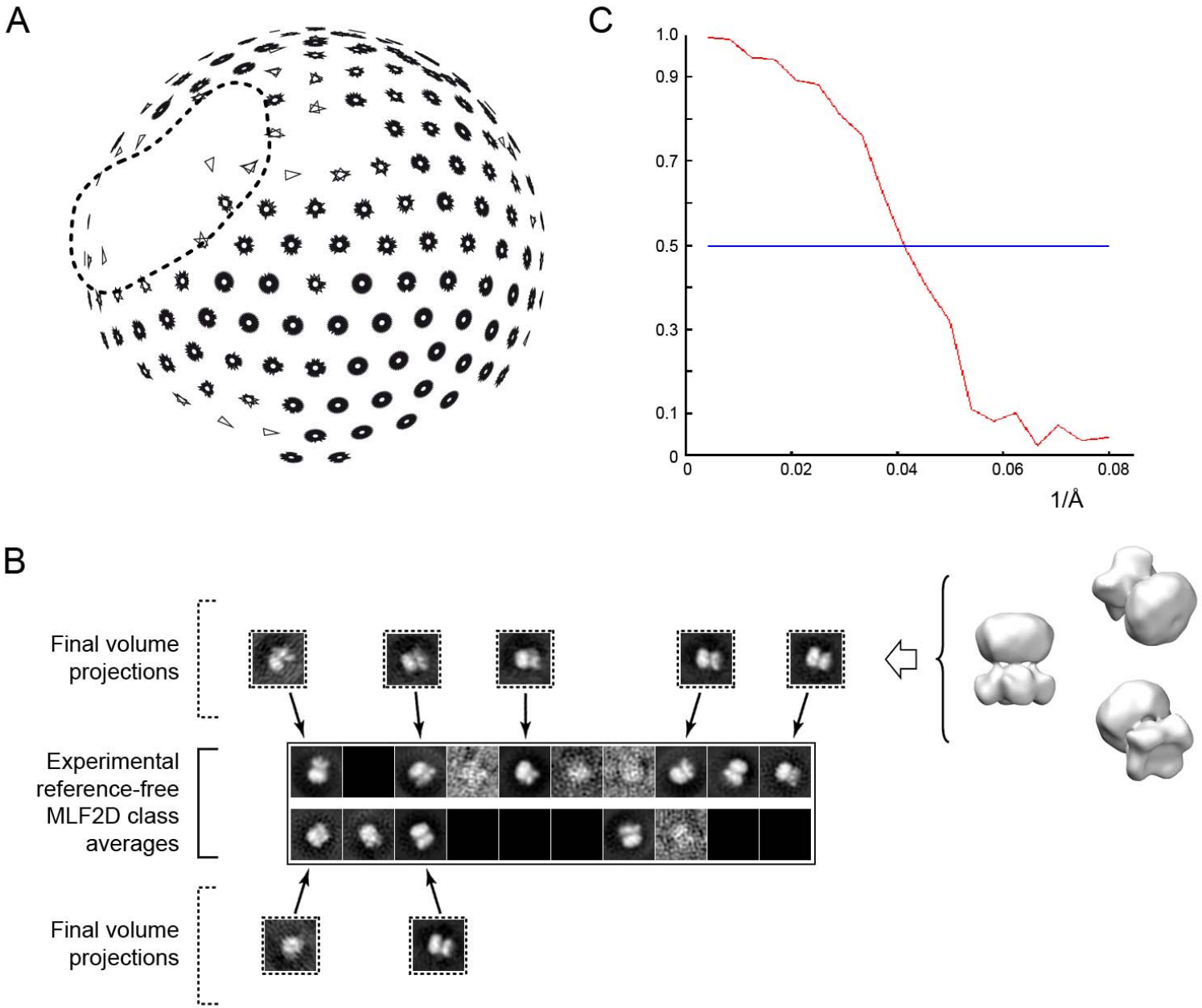


Fig. S3 Leal-Pinto et al.

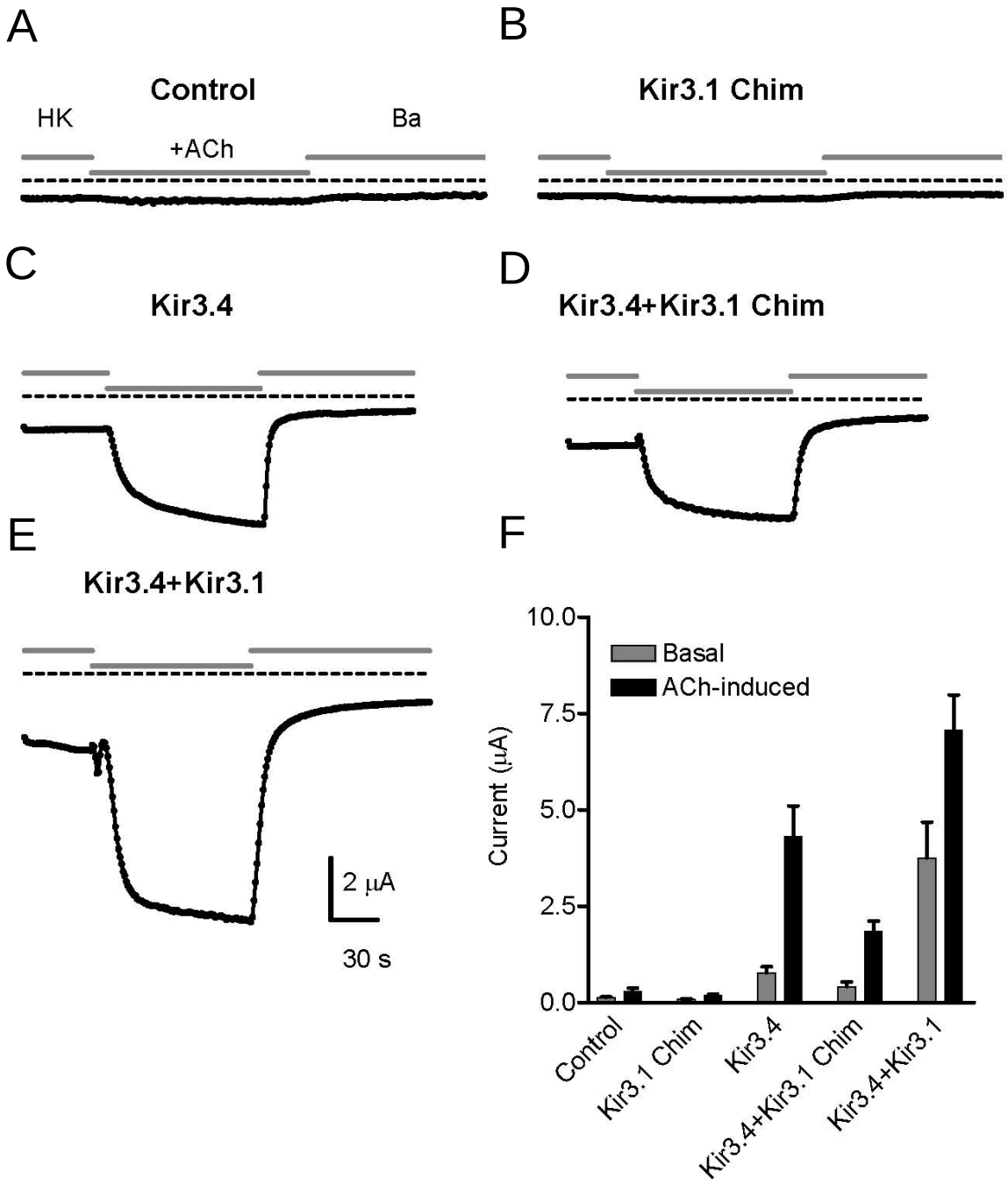
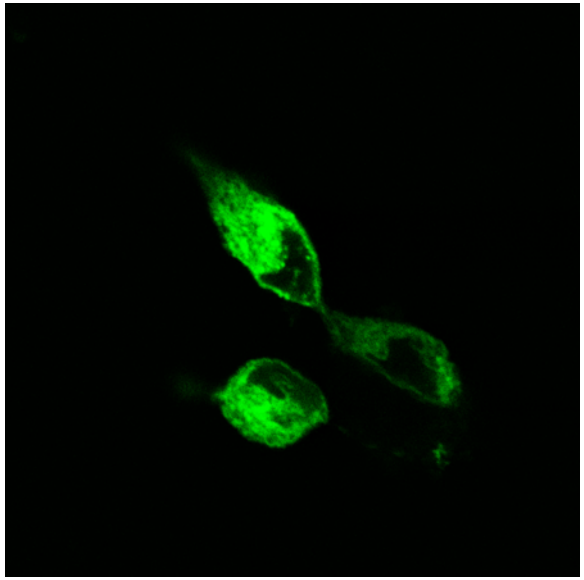


Fig. S4 Leal-Pinto et al.

A

Kir3.1 Chim-GFP



B

Kir3.4+Kir3.1 Chim-GFP

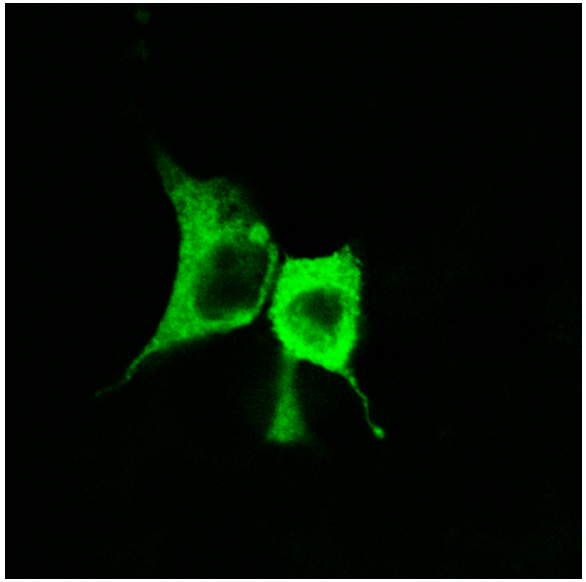


Fig. S5 Leal-Pinto et al.