Figure Supplementary 1

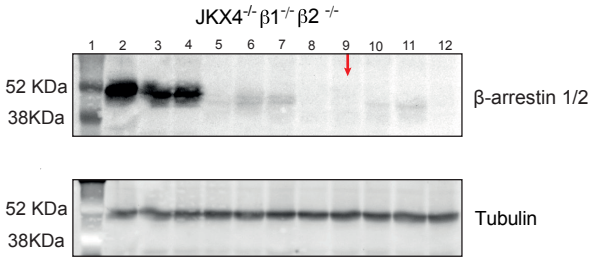

Figure Supplementary 1. **Generation of JKX4$^{-/-}$ cells lacking β-arrestin1 and β-arrestin2**. Representative western blot analysis of distinct JKX4$^{-/-}$β1$^{-/-}$β2$^{-/-}$ clones generated by CRISPR-Cas9 editing and probed with an anti-β-arrestin1/2 monoclonal antibody. Lane 1: molecular weight marker; lane 2: JKX4$^{-/-}$ cell lysate; lane 3: JKX4$^{-/-}$β 1$^{-/-}$ cell lysate; Lines 4–12: lysates from independent JKX4$^{-/-}$β1$^{-/-}$β2$^{-/-}$ clones. As a loading control, the membrane was reblotted with an anti-tubulin monoclonal antibody. The red arrow indicates the JKX4$^{-/-}$β1$^{-/-}$β2$^{-/-}$ clone selected for subsequent experiments.

Figure Supplementary 2

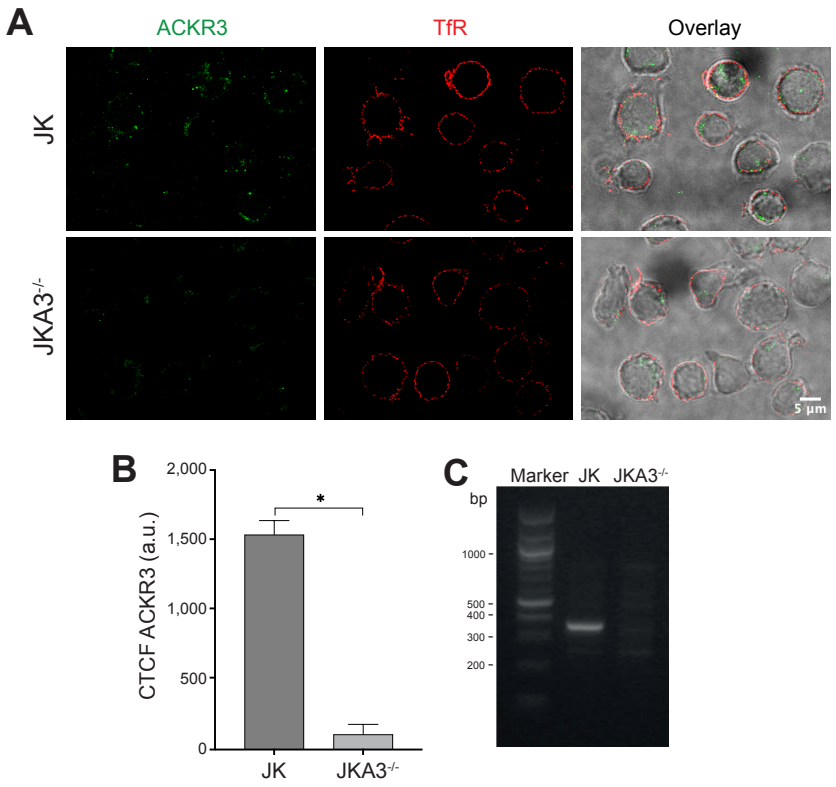

Figure Supplementary 2. Generation of ACKR3-deficient (JKA3$^{-/-}$) cells. (A) Representative confocal microscopy images of JK and JKA3$^{-/-}$ cells stained with an anti-ACKR3 monoclonal antibody (mAb) followed by anti-mouse IgG-Alexa Fluor 488 (green), and with anti-transferrin receptor (TfR) mAb-Alexa Fluor 647 (red). The figure shows the brightfield image and the merged fluorescence channels (from left to right: 488 nm channel, 638 nm channel, and merged image overlaid on brightfield). Scale bar: 5 µm. **(B)** Quantification of ACKR3 labeling normalized to the isotype control mAb. Data are presented as the mean ± SD ($n = 3$; $*P \leq 0.05$). **(C)** 2% agarose gel showing size-based separation of PCR products obtained after amplification of the genomic DNA region identified by sequencing as mutated in the selected clones. Lane 1: base pair marker; lane 2: JK control; lane 3: JKA3$^{-/-}$ cells.

Figure Supplementary 3

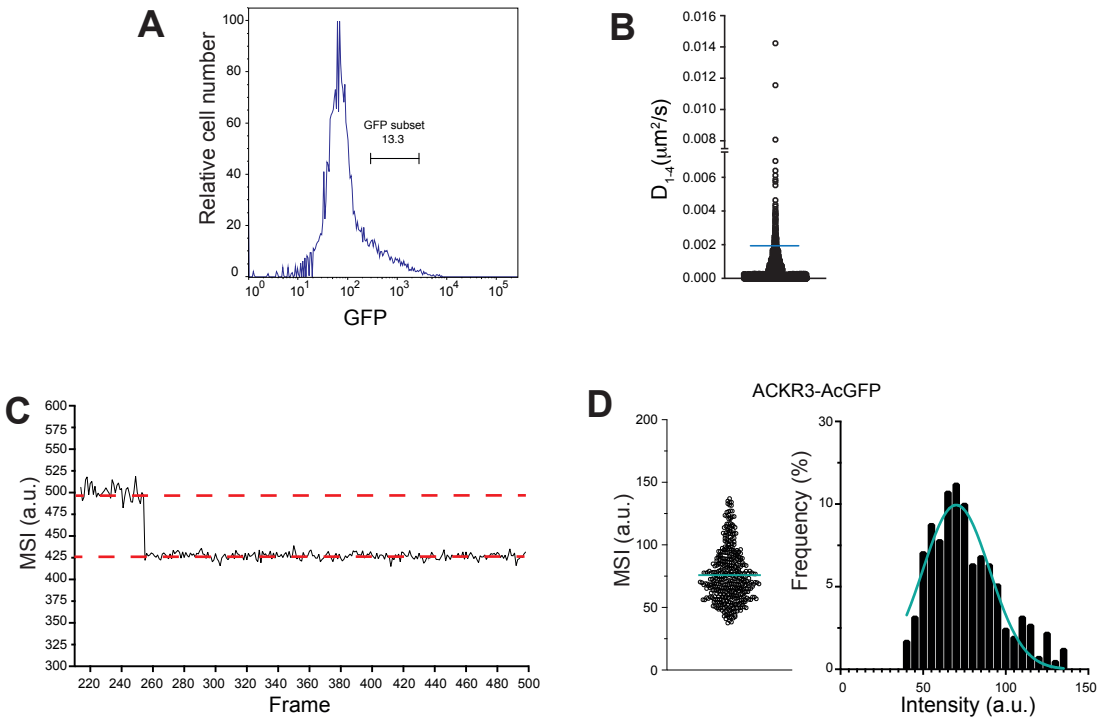

Figure Supplementary 3. Characterization of cells used for TIRF-M experiments. (A) FACS-sorted population (GFP^{subset}) of cells transiently transfected with ACKR3-AcGFP. GFP intensities correspond to low receptor expression levels suitable for single-particle tracking (SPT) experiments. **(B)** Diffusion coefficient values ($D_{1\text{-}4}$) of monomeric AcGFP protein (2,103 trajectories) on glass, used to define the diffusion threshold corresponding to immobile particles (0.002 $\mu m^2\ s^{-1}$). **(C)** Representative photobleaching step analysis showing a single, discrete loss of AcGFP fluorescence intensity, corresponding to one photobleaching step. **(D)** Mean spot intensity (MSI) values (a.u.) derived from trajectories of ACKR3-AcGFP containing a single photobleaching step (410 trajectories) The corresponding Gaussian fit was used to determine reference monomer intensity for ACKR3-AcGFP (70 a.u.).

Figure Supplementary 4

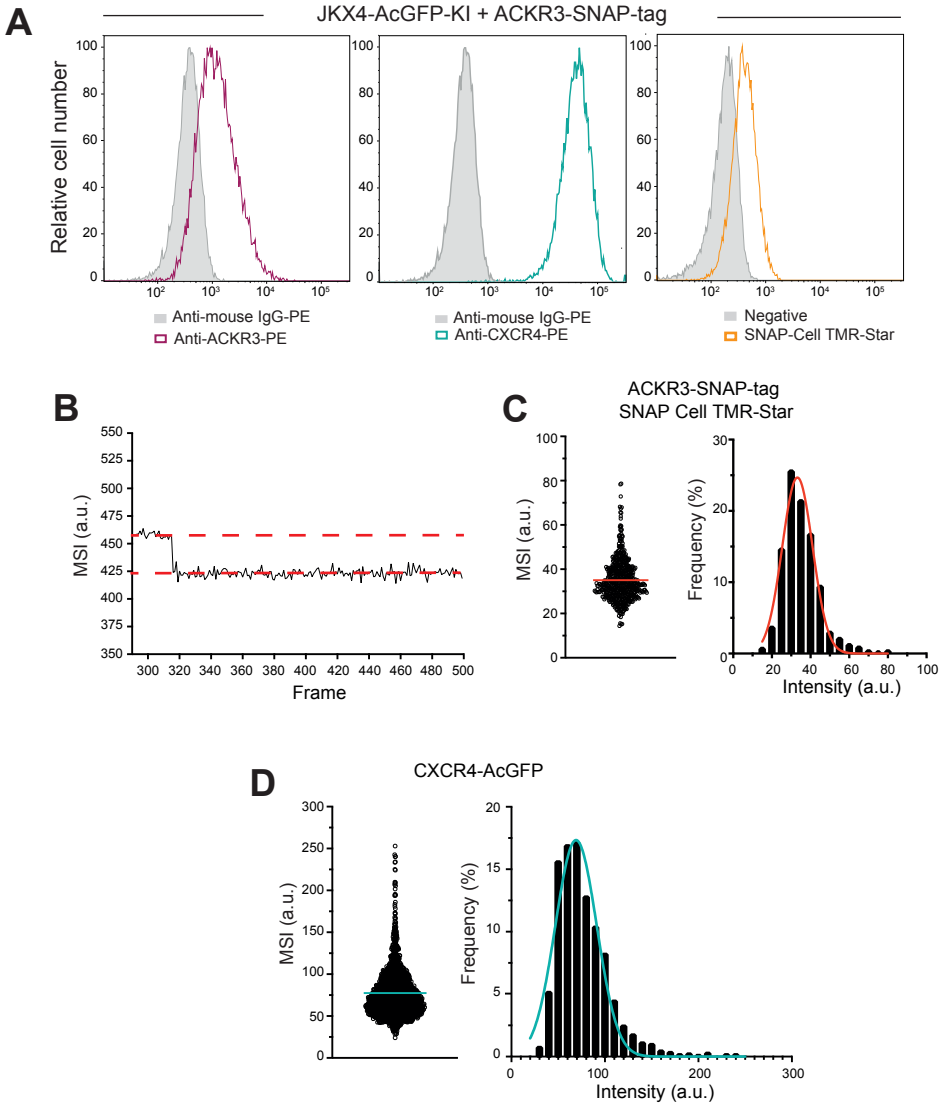

Figure Supplementary 4. Characterization of cells used for dual TIRF-M experiments. (A) Representative flow cytometry histograms of JKX4-AcGFP-KI cells transiently transfected with ACKR3-SNAP-tag and stained with an anti-ACKR3 monoclonal antibody (mAb) (left), an anti-CXCR4 mAb (middle) or after staining with SNAP-Cell TMR-Star (right) (n = 3). **(B)** Representative photobleaching step analysis showing a single, discrete loss of SNAP-Cell TMR-Star fluorescence intensity corresponding to one photobleaching step. **(C)** Mean spot intensity (MSI) values (a.u.) from trajectories of ACKR3-SNAP-Cell TMR-Star that contain a single photobleaching step (575 trajectories) along with the corresponding Gaussian fit used to determine the monomer reference intensity (33 a.u.). **(D)** MSI values (a.u.) from trajectories of CXCR4-AcGFP that contain a single photobleaching step (1,703 trajectories) along with the corresponding Gaussian fit used to determine the monomer reference intensity (70 a.u.).

Figure Supplementary 5

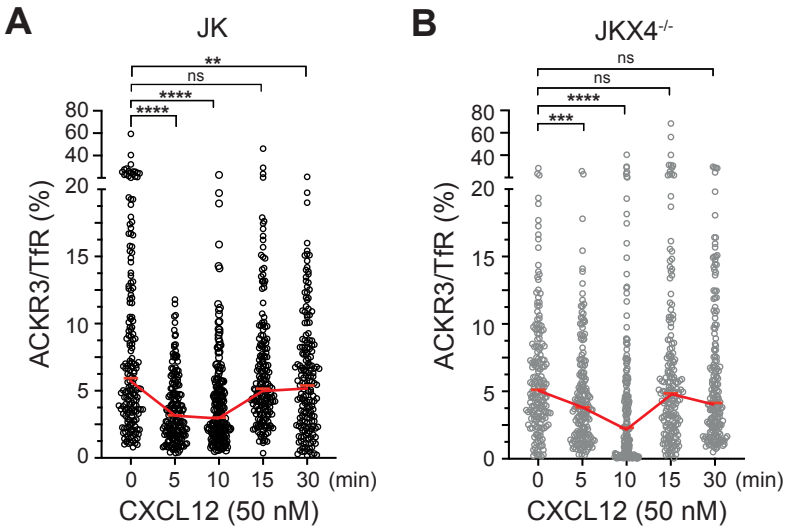

Figure Supplementary 5. ACKR3 internalization is not affected by CXCR4 expression. Cell surface expression of ACKR3 in **(A)** JK and **(B)** JKX4$^{-/-}$ cells following stimulation with CXCL12 (50 nM) at different time points and determined as in Figure 1D. Normalization of ACKR3 labeling to transferrin receptor labeling is shown. Red line indicates median values in each condition. Each circle represents one cell (180 cells per condition). (n = 3; ns = not significant; **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

Figure Supplementary 6

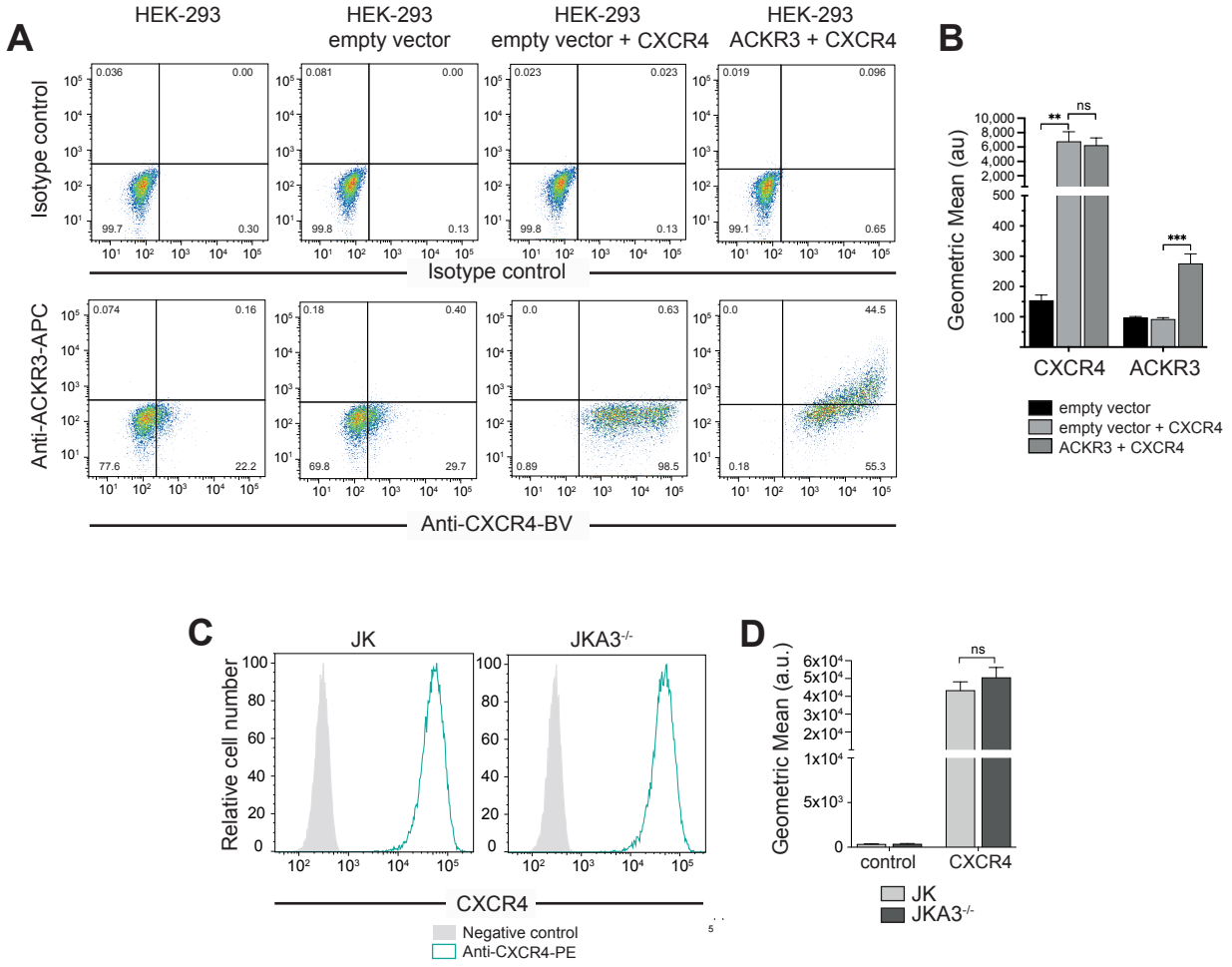

Figure Supplementary 6. Characterization of ACKR3 and CXCR4 expression in JK and HEK-293 cells. (A) Representative flow cytometry histograms of HEK-293 cells untransfected and transiently transfected with empty vector, empty vector+CXCR4, or ACKR3+CXCR4, and stained with isotype controls or with an anti-ACKR3-APC monoclonal antibody (mAb) (clone 11G8) and anti-CXCR4-BV mAb (clone 12G5). **(B)** Quantification of CXCR4 and ACKR3 membrane expression levels from cells in (A) (n = 3; ns = not significant; **P ≤ 0.01; ***P ≤ 0.001). **(C)** Representative flow cytometry histograms of JK (left) and JKA3$^{-/-}$ (right) cells stained with anti-CXCR4-PE mAb (clone 12G5). **(D)** Quantification of CXCR4 membrane expression levels in cells shown in (C) (n = 3; ns = not significant).