Supplemental Data

Selectivity In The Use of $G_{i/o}$ Proteins Is Determined by the DRF Motif In CXCR6 And Is Cell-type Specific

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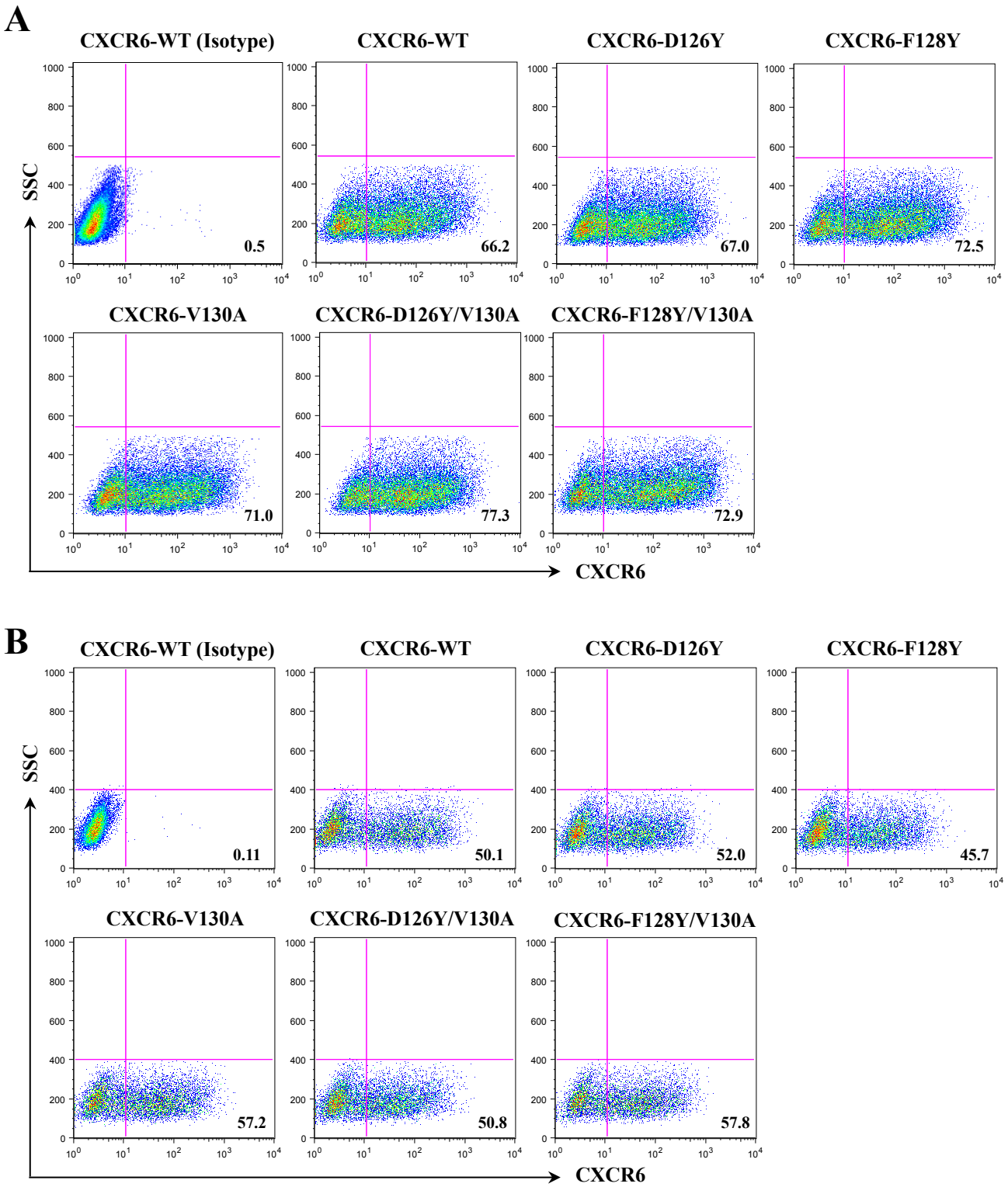
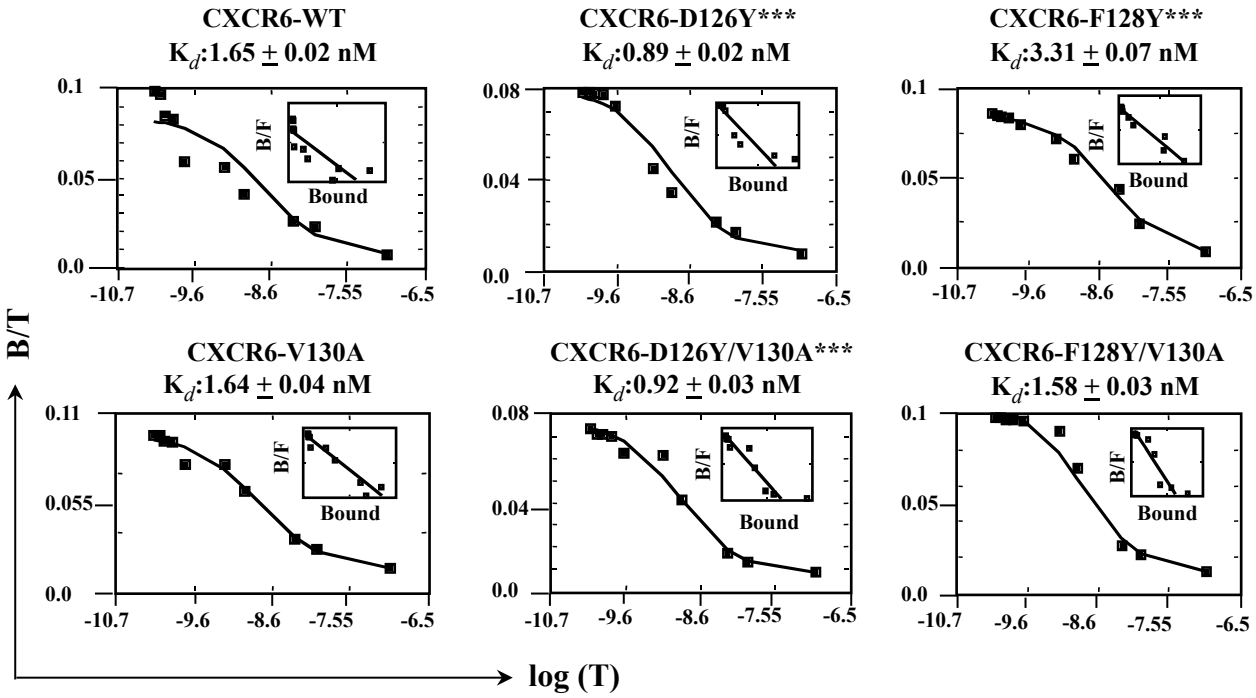
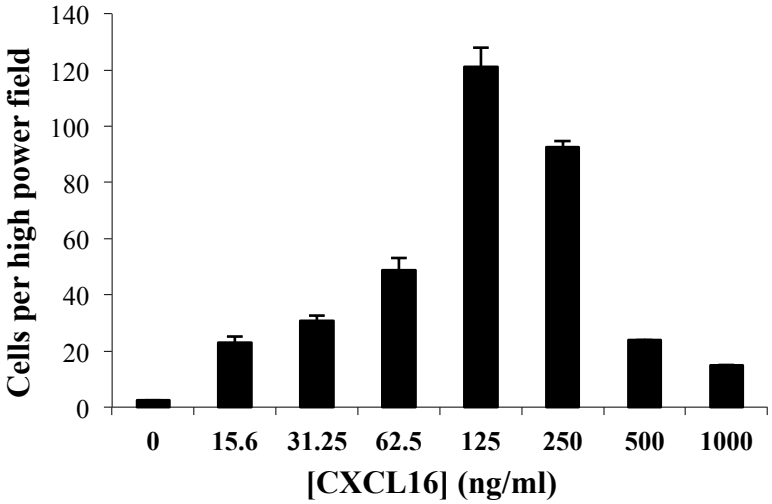
Molecular Pharmacology



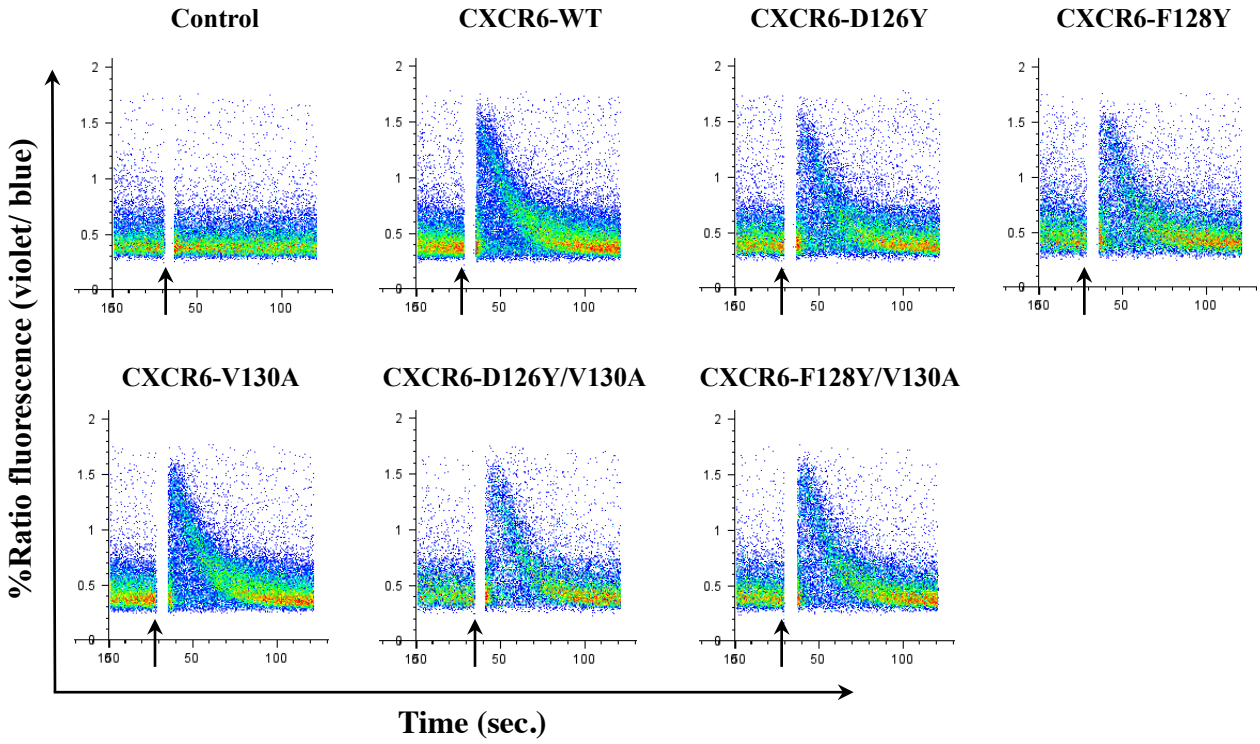
Supplemental Figure 1. **Transfecting with variable amounts of DNAs yields equal surface expression of CXCR6-WT and mutant receptors.** HEK-293T cells (A) or Jurkat E6-1 cells (B) were transfected with variable amounts of DNAs encoding wild-type and mutant CXCR6 receptors in order to produce equal surface expression. Cells were stained either with PE-conjugated isotype control or with anti-human CXCR6-PE. Cells transfected with CXCR6-WT and stained with isotype control were used to draw the gate. Numbers show the percentages of positive cells. Data shown for each cell type are from one of more than twenty experiments.

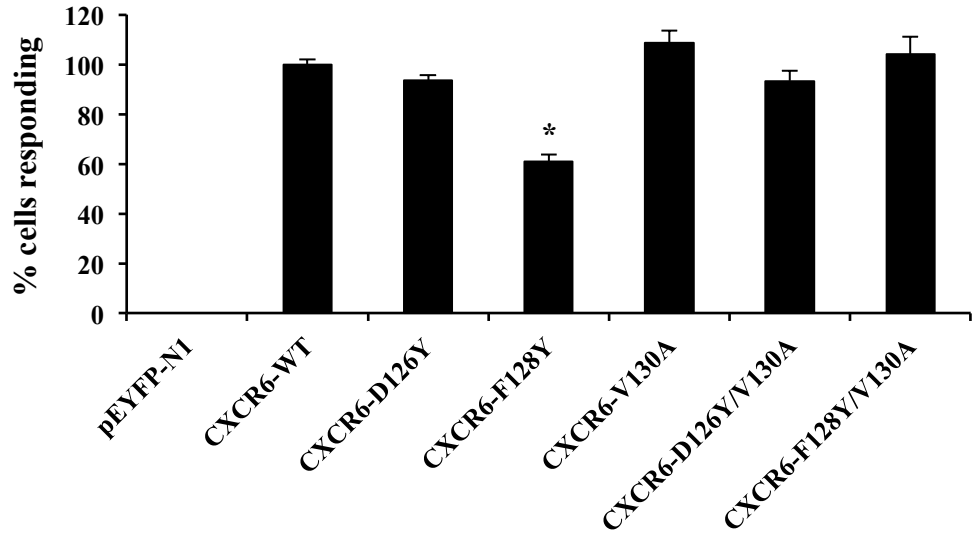


Supplemental Figure 2. **For CXCR6 expressed on HEK-293T cells, mutations in the D$^{3.49}$R$^{3.50}$F$^{3.51}$I$^{3.52}$V$^{3.53}$ sequence alter the affinity for CXCL16**. HEK-293T cells were transfected with variable amounts of DNAs encoding wild-type and mutant CXCR6-YFP receptors, in order to produce equal surface expression (see Supplemental Figure 1). Binding of ^{125}I-labelled CXCL16 was measured at room temperature in the presence of increasing concentrations of unlabeled CXCL16 as described in Materials and Methods. Measurements at each concentration of unlabeled CXCL16 were done in triplicate and averaged. B, T and F represent bound, total and free ligand, respectively. Insets show Scatchard analyses. The K_d values shown are mean ± SEM from three independent experiments one of which is shown here for each receptor. The asterisks indicate a significant difference from HEK-293T cells expressing the wild-type receptor. ***, P <0.001. Cpm bound in the absence of added competitor ranged from 5255-6796, ≥ 95% of which was specific and represented approximately 10% of the total cpm.

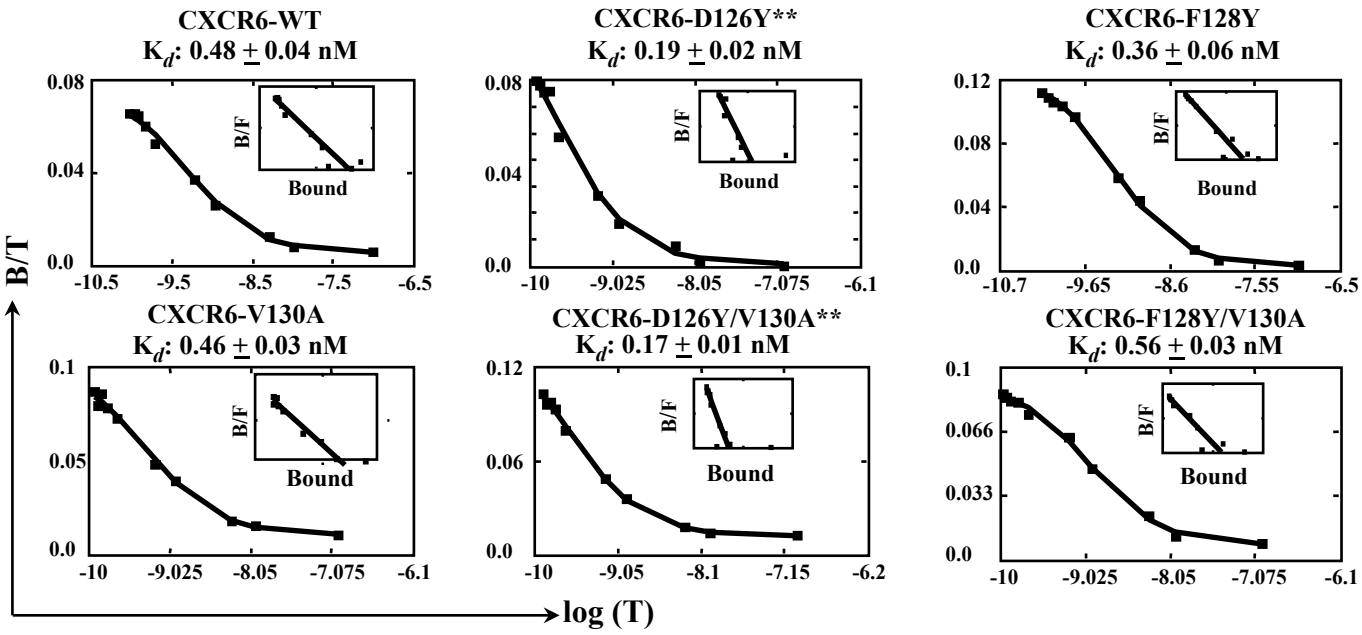


Supplemental Figure 3. Bell-shaped response for migration of HEK-293T cells expressing wild-type CXCR6 to increasing concentrations of CXCL16. Migration of HEK-293T cells expressing wild-type receptor was measured using a microchemotaxis chamber containing CXCL16 in the lower wells as described in Materials and Methods. Data shown are means ± SEM from three independent experiments.

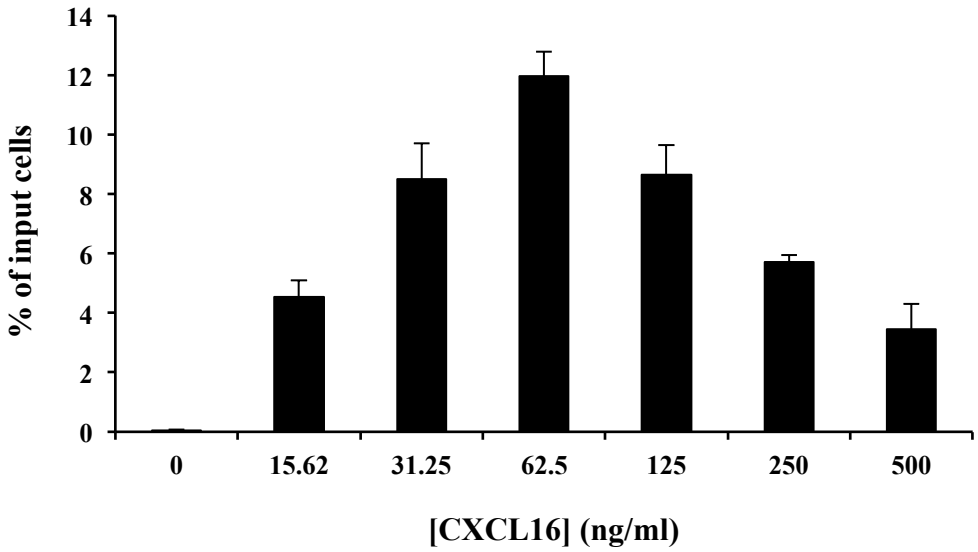




Supplemental Figure 4. **Changing a non-canonical to canonical amino acid diminishes CXCR6 activity in HEK-293T cells.** Cells were transfected with 2-5 μg of DNAs encoding wild-type and mutant CXCR6-YFP proteins in order to produce equal surface expression (see Supplemental Figure 1). Cells were loaded with indo-1/AM and the %'s of the YFP^+ cells mobilizing intracellular calcium at the time of peak response to 1 μg/ml of CXCL16 added at the times indicated by the arrows were determined on a flow cytometer. Results were normalized to the value for cells expressing wild-type CXCR6 from one experiment, which was set to 100. Bars show means ± SEM from three experiments including the one shown in the upper panel. . *, P <0.05 versus CXCR6-WT.



Supplemental Figure 5. **For CXCR6 expressed on Jurkat E6-1 cells, mutants containing D126Y have increased affinity for CXCL16.** Experiments using Jurkat E6-1 cells were done and analyzed as for HEK-293T cells in Supplemental Figure 2. Cells were transfected with variable amounts of DNAs encoding wild-type and mutant CXCR6-YFP receptors, in order to produce equal surface expression (see Supplemental Figure 1). The K_d values shown are means $\pm$ SEM from three independent experiments, one of which is shown for each receptor. The asterisks indicate a significant difference from Jurkat E6-1 cells expressing the wild-type receptor. **, P <0.01. Cpm bound in the absence of added competitor ranged from 5432-6467, $\geq$ 95% of which was specific and represented approximately 10% of the total cpm.



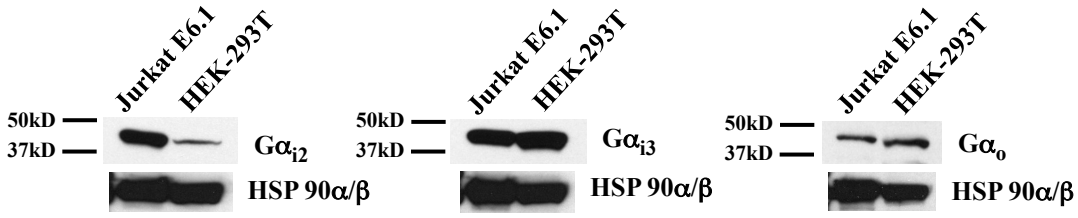
Supplemental Figure 6. Bell-shaped response for migration of Jurkat E6-1 cells expressing wild-type CXCR6 to increasing concentrations of CXCL16 . Jurkat E6-1 cells expressing wild-type receptor, were analyze for chemotaxis using various concentration of CXCL16 in lower wells of a Transwell plate as described in Materials and Methods. The means were obtained for the % of input cells migrating in duplicate wells of a Transwell plate. Data shown are means ± SEM from two independent experiments.

A

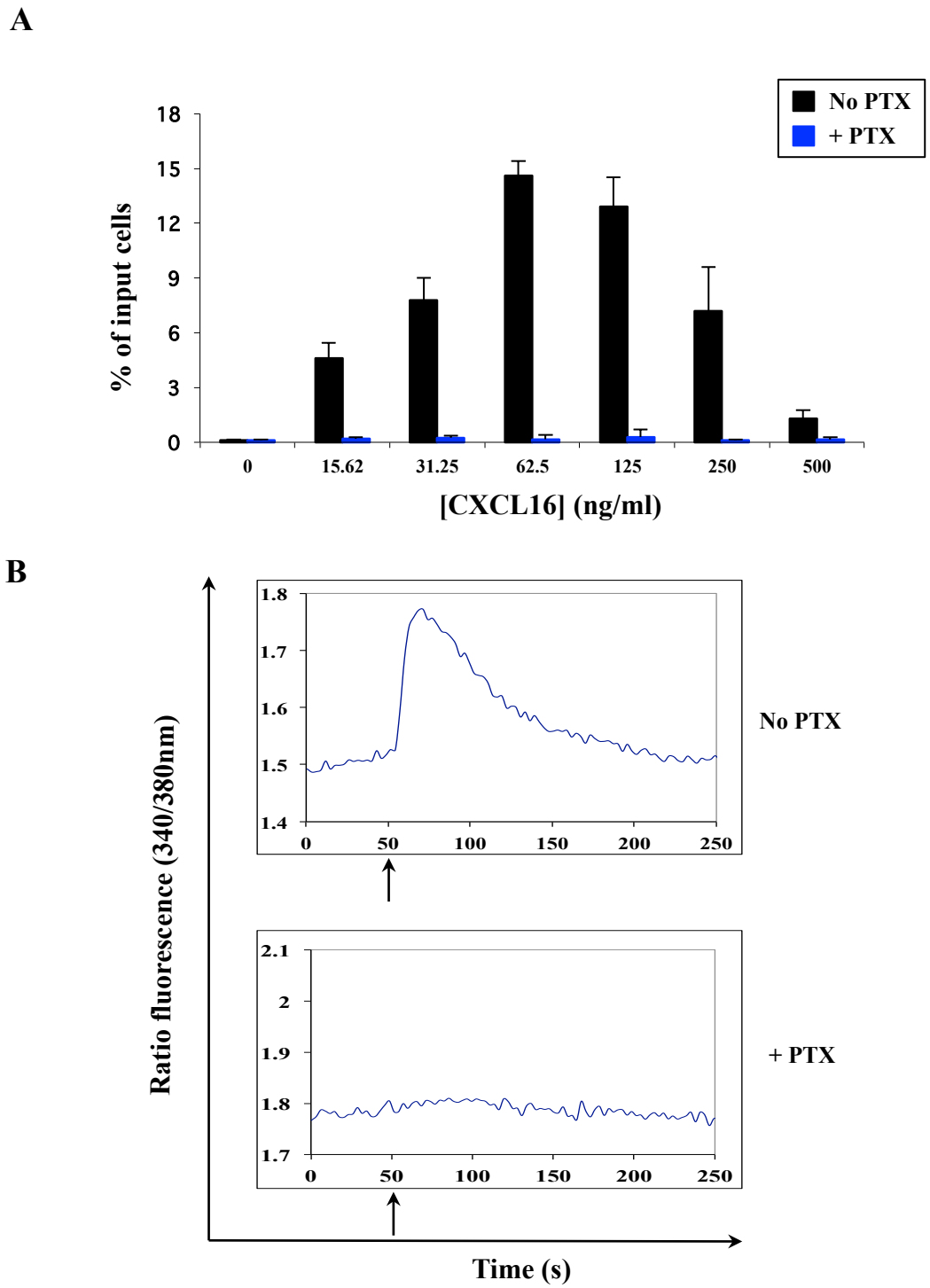
HEK-293T: $G\alpha_{i2} > G\alpha_{i3} = G\alpha_{13} > G\alpha_q = G\alpha_{11} > G\alpha_{12} > G\alpha_{i1} > G\alpha_o >> G\alpha_{14} > G\alpha_{16}$
3.7 1.1 1.9 1.0 1.3 2.4 3.8 14.9 8.9

Jurkat E6-1: $G\alpha_{i2} > G\alpha_{13} = G\alpha_q > G\alpha_{i3} > G\alpha_{16} > G\alpha_{12} > G\alpha_o > G\alpha_{14} > G\alpha_{11} >>> G\alpha_{i1}$
7.9 1.0 1.7 1.6 2.5 2.0 2.0 2.1 727

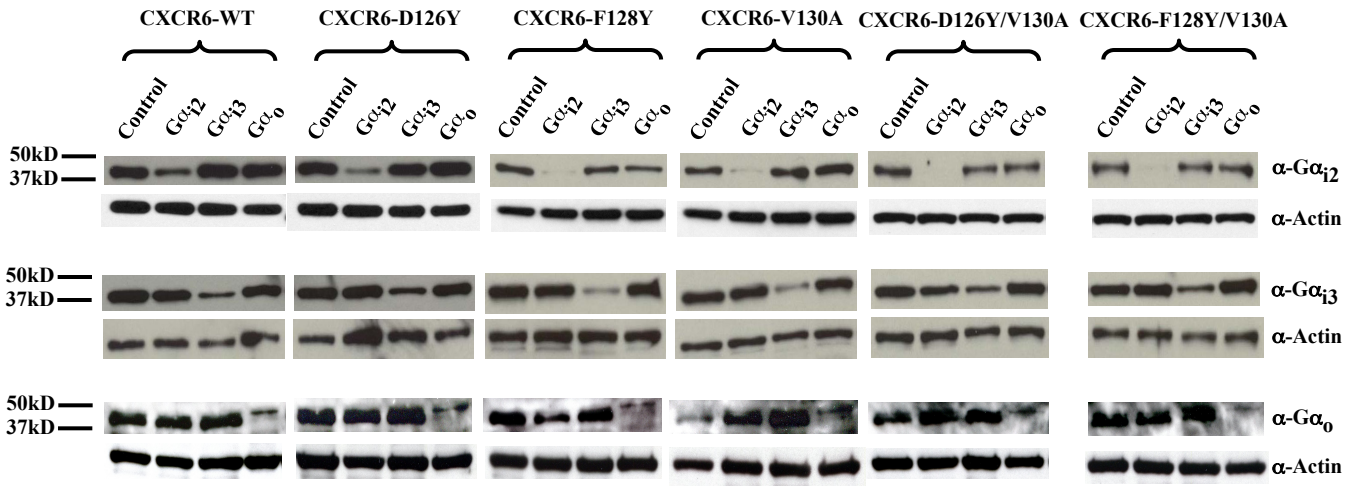
B



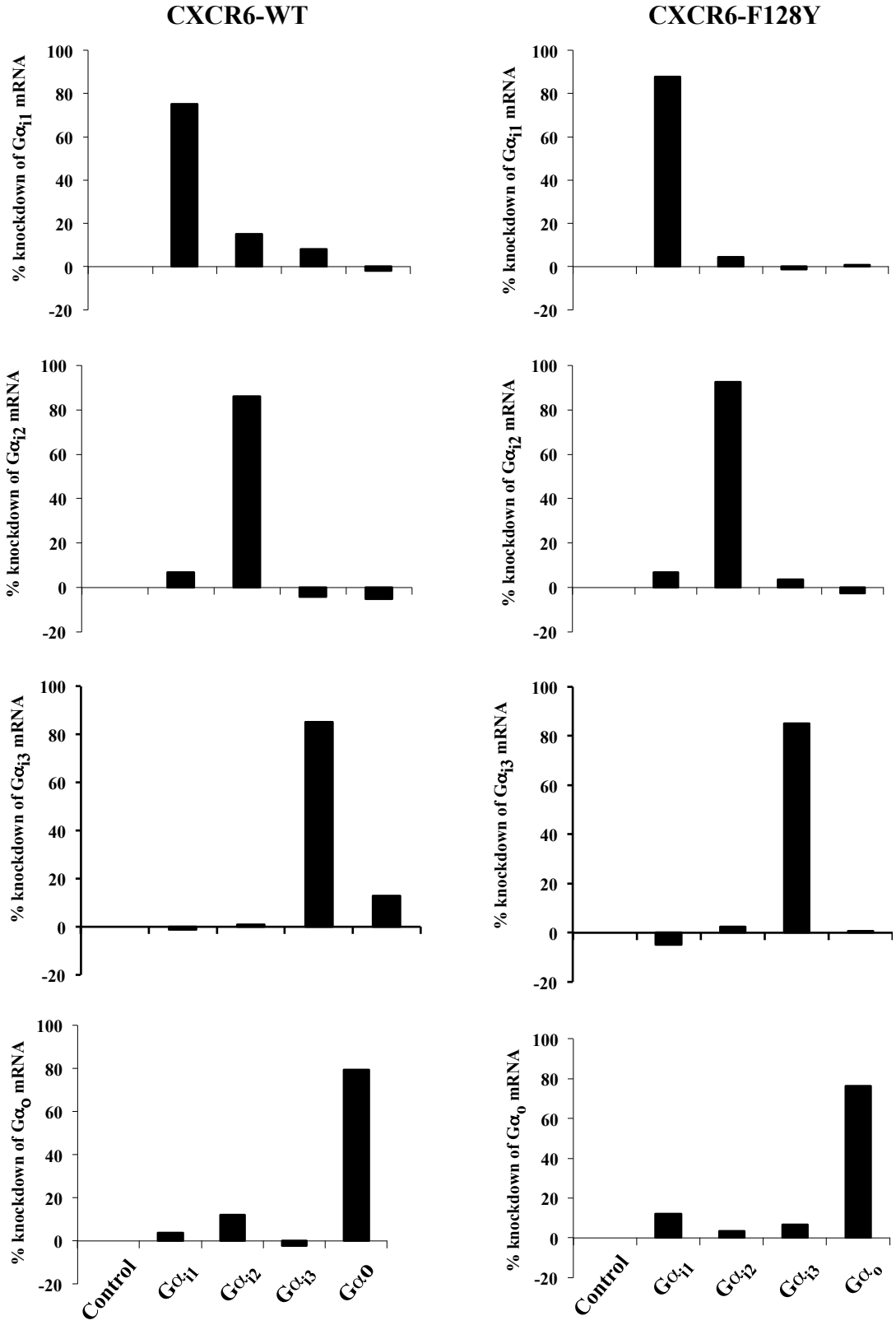
Supplemental Figure 7. **Relative amounts of Gα mRNAs and proteins in HEK-293T and Jurkat E6-1 cells.** (A) HEK-293T and Jurkat E6-1 cells were analyzed by semi-quantitative real-time RT-PCR for the expression of mRNAs for the indicated G protein α-subunits. Based on calculated ΔC_T values for each G protein α-subunit vs. GAPDH, their rank orders of expression were determined. Numbers under the > symbols represent the fold difference in expression between the G protein α-subunit on the left and its nearest neighbor on the right. Data shown are from a single experiment and are representative of three experiments. (B), equal amount (15 μg) of cell lysates from Jurkat E6-1 and HEK-293T cells were resolved by SDS PAGE and analyzed by Western blotting for the presence of the indicated G protein α-subunits and HSP 90α/β, which is displayed as a loading control. Positions of molecular mass markers (kD) are shown beside the blots. Data shown are from a single experiment and are representative of two experiments.



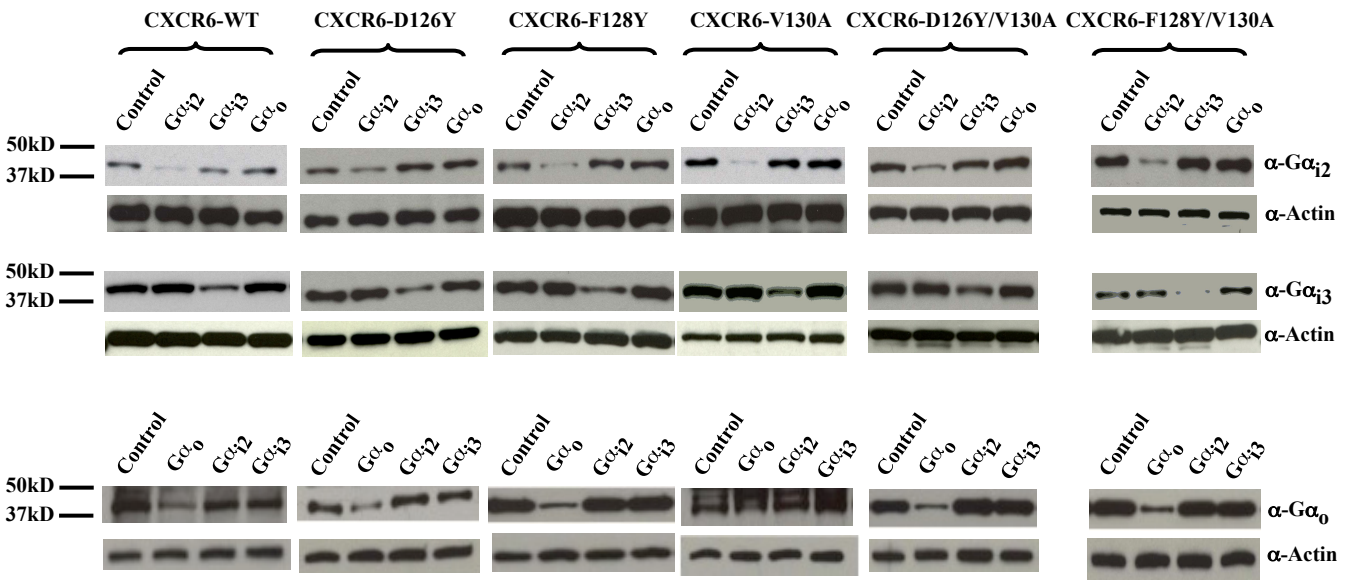
Supplemental Figure 8. Pertussis toxin inhibits CXCR6-mediated chemotaxis and calcium flux in Jurkat E6-1 cells. (A) Jurkat E6-1 cells expressing wild-type CXCR6 were pretreated with pertussis toxin (500 ng/ml) or left without treatment for 3 hours at 37^0C and analyzed for chemotaxis using various concentrations of CXCL16 in lower wells of a Transwell plate as described in Materials and Methods. The means were obtained for the % of input cells migrating in duplicate wells of a Transwell plate. Data shown are means ± SEM from two independent experiments. (B) Cells treated as in (A), were loaded with Fura-2/AM and assayed for intracellular calcium mobilization on a spectrofluorometer in response to 1 μg/ml CXCL16 added at the times indicated by the arrows as described in Materials and Methods. Data shown are from a single experiment and are representative of three experiments.



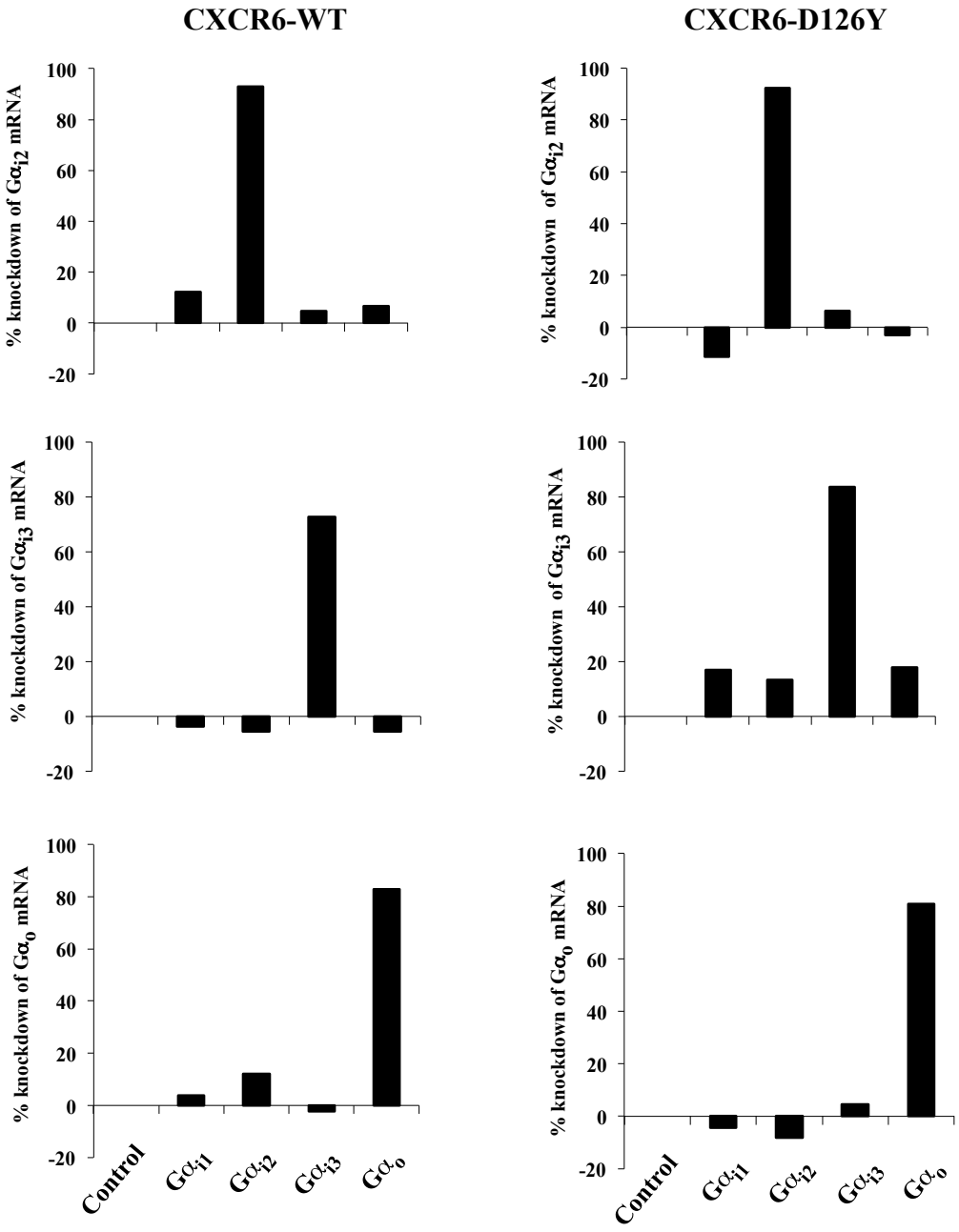
Supplemental Figure 9. **Knockdown of individual G protein α-subunits using siRNAs in HEK-293T cells**. HEK-293T cells co-transfected with CXCR6-WT or mutant plasmids and the siRNAs as indicated above the lanes were harvested 72 hours later and analyzed by Western blotting for the presence of the G protein α-subunits as indicated on the right and actin, which is displayed as a loading control. Positions of molecular mass markers (kD) are shown beside the blots. Data shown are from a single experiment and are representative of three experiments.



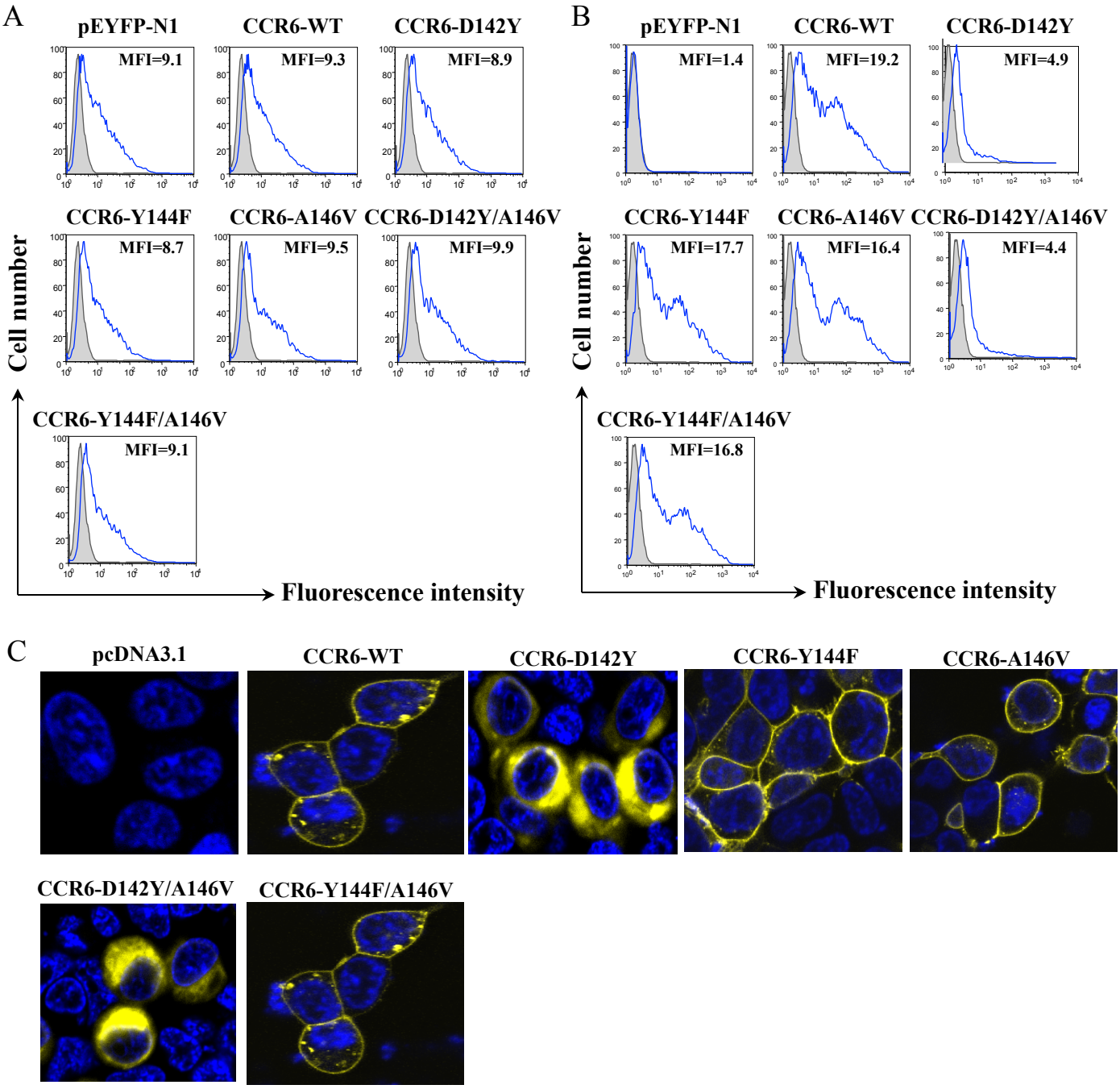
Supplemental Figure 10. **Knockdown of individual G protein α-subunit mRNAs in HEK 293T cells using siRNAs**. HEK 293T cells were co-transfected with CXCR6-WT or CXCR6-F128Y plasmids and the siRNAs as indicated below the bars, harvested 72 hours later and analyzed by real-time RT-PCR. Percent knockdown of the G protein α-subunit mRNAs indicated on the y-axes are shown as compared with cells transfected with control siRNAs. Means of duplicate assays are shown from a single experiment.



Supplemental Figure 11. **Knockdown of individual G protein α-subunits using siRNAs in Jurkat E6-1 cells**. Jurkat E6-1 cells co-transfected with CXCR6-WT or mutant plasmids and the siRNAs as indicated above the lanes were harvested 72 hours later and analyzed by Western blotting for the presence of the G protein α-subunits as indicated on the right and actin, which is displayed as a loading control. Positions of molecular mass markers (kD) are shown beside the blots. Data shown are from a single experiment and are representative of three experiments.



Supplemental Figure 12. **Knockdown of individual G protein α-subunit mRNAs in Jurkat E6-1 cells using siRNAs**. Jurkat E6-1 cells were co-transfected with CXCR6-WT or CXCR6-D126Y plasmids and the siRNAs as indicated below the bars, harvested 72 hours later, and analyzed by RT-PCR. Percent knockdown of the G protein α-subunit mRNAs indicated on the y-axes are shown as compared with cells transfected with control siRNAs. Means of duplicate assays are shown from a single experiment.



Supplemental Figure 13. **Replacing D142$^{3.49}$ with Y leads to redistribution of CCR6 in HEK-293T cells.** HEK-293T cells were transfected with equal amounts of pcDNA3.1, pEYFP-N1 and DNAs encoding CCR6-YFP wild-type and mutant receptors. Cells transfected with pcDNA3.1 were stained with a PE-conjugated isotype control antibody and other cells were stained with anti-human CCR6-PE. (A) YFP expression is shown for pcDNA3.1 transfected cells in the shaded histograms and cells transfected with pEYFP-N1 or CCR6 DNAs in non-shaded histograms outlined in blue. Mean fluorescent intensities (MFIs) of YFP expressing cells are shown. (B) CCR6 surface staining in duplicate panels for the same cells as in (A). Data are from one of more than three experiments. (C) Confocal microscopy of HEK-293T cells transfected with equal amounts of pcDNA3.1 or DNAs encoding CCR6-YFP wild-type and mutant receptors. The nuclei were stained with Hoechst 33342, shown in blue, and emission from the YFP fusion proteins is shown in yellow. Data shown are from a single experiment and are representative of three experiments.