Supporting Information

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SI Text

Molecular Cloning. For experiments in mammalian cells, mutations were cloned into Flag-CXCR4, CXCR4-*Renilla* luciferase (CXCR4-Rluc), and CXCR4-YFP using the QuikChange Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's directions. HA-CXCR4 and T7-CXCR4 mutants were obtained by swapping the Flag tag (M-DYKDDDDK) for an HA tag (M-YPYDVPDYA) or T7 tag (M-ASMTGGQQMG) using the Flag-CXCR4 constructs, also by QuikChange. For disulfide cross-linking experiments in *Spodoptera frugiperda* (Sf9) insect cells, a Flag-tagged CXCR4 construct [CXCR4-1 in Wu et al. (1)] in pFastBac1 was generated as previously described (1). CXCL12 with its native signal sequence and a C-terminal HA tag (YPYDVPDYA) was also subcloned into pFastBac1 for coexpression in Sf9 cells. Cysteine mutations were introduced into Flag-CXCR4 and CXCL12-HA using QuikChange [referred to below as Flag-CXCR4(Cys), CXCL12-HA(Cys), and CXCL12-HA(P2G-Cys] in the case of a construct that also has a P2G mutation that converts CXCL12 into an antagonist (2).

Cell Culture and Transfection. CHO-Gα_{15} cells were maintained in DMEM/F12 nutrient mixture (Gibco) supplemented with 10% (vol/vol) FBS and 700 μg/mL G418. CXCR4 Tet-On cells were obtained by transfecting HEK293 cells with pACMV-Tet-On-Flag-CXCR4 and selecting for stably transfected cells with the addition of 700 μg/mL G418. These cells were maintained in DMEM with Glutamax (Invitrogen), 10% (vol/vol) tetracycline-free FBS (Gibco), and 700 μg/mL G418. HEK293T cells were maintained in DMEM with Glutamax and 10% (vol/vol) FBS. All mammalian cells were cultured at 37 °C with 5% CO_2.

Transient transfections of CHO-Gα_{15} stable cells were carried out using the TransIT CHO transfection kit according to the manufacturer's instructions, with the following modifications: 4-μL transfection reagent and 0.5 μL of the CHO Mojo booster reagent were used per every 1 μg of DNA to be transfected. These cells were plated in DMEM/F12 containing 10% (vol/vol) FBS and 0.25% DMSO 24 h before transfection, and the media was switched to DMEM/F12 with 10% (vol/vol) FBS just before transfection. Transient transfections of both HEK293 CXCR4 stable cells and HEK293T cells were performed using the TransIT-LT1 reagent (Mirus Bio), according to manufacturer's instruction.

Sf9 cells were cultured in ESF 921 media (Expression Systems) in vented Erlenmeyer flasks (Corning) at 27 °C with shaking. High-titer recombinant baculovirus (>10^9 viral particles per mL) was obtained using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Briefly, recombinant baculoviruses were generated by transfecting 5 μL of recombinant bacmid containing the target gene sequence into 2.5 mL of Sf9 cells at a density of 1.2×10^6 cells/mL using 3 μL of Xtreme Gene Transfection Reagent (Roche) and 100 μL of Transfection Medium (Expression Systems). Cell suspensions were incubated for 96 h with shaking at 27 °C. P0 viral stocks were then isolated and used to generate P1 viral stocks. Viral titers were quantified by flow cytometry following cell staining with PE-conjugated anti-gp64 antibody (Expression Systems). Sf9 cells at a density of 2–2.6×10^6 cells/mL were coinfected with P1 virus of both Flag-CXCR4(Cys) and CXCL12-HA(Cys) or CXCL12-HA(P2G-Cys) at a multiplicity of infection of 5. Biomass was harvested between 44 and 48 h postinfection.

Quantification of Protein Surface Expression by Flow Cytometry. For testing WT and mutant CXCR4 surface expression, mammalian cells were washed in PBS containing 0.5% BSA (FACS buffer). For Flag-tag detection, staining was carried out in a 50× dilution (2 μg/mL) of anti-DDDDK (Clone M2) conjugated to SureLight APC or mouse IgG1 isotype control conjugated to SureLight APC (Columbia Biosciences) for 45 min on ice. For T7-tag detection, staining was carried out in a 10× dilution of anti-T7 conjugated to SureLight APC (Columbia Biosciences). For HA-tag detection, staining was carried out in a 10× dilution of anti–HA-APC (Miltenyi Biotec) (Clone GG8-1F3.3.1). For simultaneous tag detection, cells were costained with two antibodies against tags of interest (either Flag and HA, or T7 and HA) using the same concentrations as above, also for 45 min on ice. For endogenous CXCR4 detection, cells were stained in a 50× dilution of anti–CXCR4-PE (clone 1D9) or $IgG_{2a,\kappa}$ isotype control-PE (BD Biosciences), also for 45 min on ice. Cells were then washed 3× in FACS buffer before analysis, which was carried out using a Guava bench top miniflow cytometer (Millipore).

Generation of Stable CHO-Gα_{15} Cell Line for CXCR4 Mutant Characterization. Plasmid vector for human G$\alpha_{15/16}$ in pcDNA3.1$^+$ was purchased from the Missouri S&T cDNA Resource Center and transfected into Chinese hamster ovary cells (CHO-K1, ATCC) using the TransIT-CHO transfection kit (Mirus Bio). After 24 h, cells were transferred into selection media containing 600 μg/mL G418 (Gibco). After 1 wk of selection, the surviving cells were reseeded into a 96-well plate at a density of ∼1 cell per well, and single-cell colonies were allowed to grow for 2 wk, with fresh G418-containing cell culture media supplemented every 2 d. Colonies were analyzed by Western blot using rabbit polyclonal antibody against G$\alpha_{15/16}$ (Abcam) and HRP-conjugated goat anti-rabbit antibody (Thermo) (Fig. S1*M*), and by Ca^{2+} mobilization upon transient transfection with CXCR4 (Fig. S1 *N*–*P*), for Gα_{15} expression. The clone with the highest expression and best signal-to-noise ratio (L12) was chosen for all experiments (referred to as CHO-Gα_{15}).

Protein Purification for Disulfide Cross-Linking Experiments. For screening of cross-linked species between Flag-CXCR4 and CXCL12-HA cysteine mutants, biomass was thawed and lysed in hypotonic buffer [10 mM Hepes pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, and EDTA-free protease inhibitor mixture (Roche)] followed by 40 strokes of Dounce homogenization and centrifugation at 50,000 × *g* at 4 °C for 30 min. Purified membranes were subjected to two additional rounds of Dounce homogenization and centrifugation in a high salt buffer (10 mM Hepes pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 1 M NaCl, and EDTA-free protease inhibitor mixture). Following the last centrifugation, membrane pellets were resuspended and homogenized in hypotonic buffer supplemented with 30% glycerol (vol/vol) and flash-frozen at −80 °C until further use. Purified membranes were thawed on ice and mixed with an equal volume of 2× solubilization buffer [100 mM Hepes pH 7.5, 800 mM NaCl, 1.5% (wt/vol) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 0.3% cholesteryl hemisuccinate (CHS, Sigma)], incubated for 3 h at 4 °C, and then centrifuged at 25,000 × *g* for 30 min. The supernatant was incubated overnight at 4 °C with TALON IMAC resin (Clontech) and 20 mM imidazole. After binding, the resin was washed with twenty column volumes of wash buffer [25 mM Hepes pH 7.5, 400 mM NaCl, 0.025% DDM, 0.005% CHS, 10% (vol/vol) glycerol]. Complexes were eluted in six column volumes of wash buffer supplemented with 250mM Imidazole pH 8.0.

1. Wu B, et al. (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330(6007):1066–1071.

2. Crump MP, et al. (1997) Solution structure and basis for functional activity of stromal cell-derived factor-1; Dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J* 16(23):6996–7007.

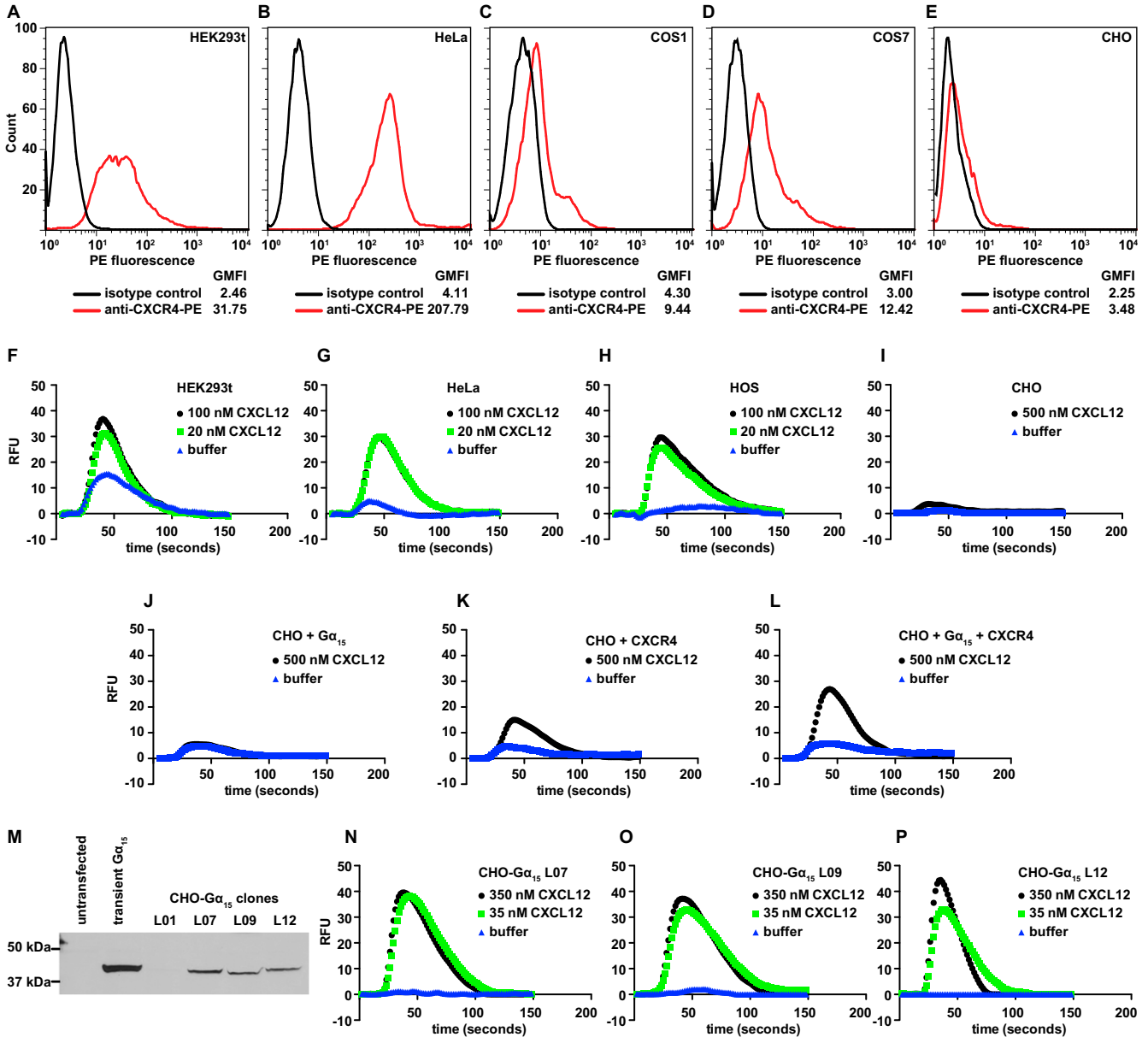

Fig. S1. Design of CXCR4-free cell line for CXCR4 mutant functionality testing. (*A–E*) Endogenous expression of CXCR4 was detected via flow cytometry following surface staining with anti–CXCR4-PE (clone 1D9; BD Biosciences). Endogenous CXCR4 was detected on the surface of (*A*) HEK293t cells, (*B*) HeLa cells, (*C*) COS1, and (*D*) COS7 cells, but not (*E*) CHO cells. (*F–I*) Ca^{2+} mobilization elicited by the indicated CXCL12 concentrations was also measured for several cell types. CXCL12 caused Ca^{2+} mobilization in untransfected (*F*) HEK293t, (*G*) HeLa, and (*H*) HOS cells, but not in (*I*) CHO cells. (*J–L*) CHO cells displayed robust CXCL12-induced Ca^{2+} mobilization after transfection with both $G\alpha_{15}$ and CXCR4. CHO cells that were transfected with (*J*) $G\alpha_{15}$ alone showed no appreciable Ca^{2+} mobilization, and those transfected with (*K*) CXCR4 showed an improved but still low response, whereas transfection with (*L*) both $G\alpha_{15}$ and CXCR4 produced robust CXCL12-induced Ca^{2+} mobilization. (*M–P*) Clone selection of stably $G\alpha_{15}$-transfected CHOs and resultant Ca^{2+} mobilization data. (*M*) Western blot detection of human $G\alpha_{15}$ in clonally selected populations of stably transfected CHO cells. Cells were harvested, normalized, pelleted by centrifugation, and lysed in G protein-specific lysis buffer [20 mM Hepes, pH 8.8, 100 mM NaCl, 5 mM $MgCl_2$, 1% sodium cholate, 1× Protease inhibitor (Sigma), 2 mM GTP, and 1 mM DTT]. Cell lysates were separated by SDS/PAGE, transferred to nitrocellulose membranes, blocked overnight, and probed with rabbit polyclonal antibody against $G\alpha_{15/16}$ (Abcam) followed by HRP-conjugated goat anti-rabbit antibody (Thermo) according to the manufacturer's instructions. (*N–P*) Three separate clones of CHO-$G\alpha_{15}$ stable cells all display robust CXCL12-induced Ca^{2+} mobilization upon transfection with CXCR4.

Table S1. Properties of the cell lines tested and generated to identify a background-free CXCR4 mutant testing system

Cell line	Endogenous CXCR4 expression*	Transfection efficiency†	Ca^{2+} mobilization‡: untransfected	Ca^{2+} mobilization: CXCR4 transfected
HEK293T	+++	+++	+++	+++
HeLa	+++	NT	++	NT
COS-1	++	NT	NT	NT
COS-7	++	NT	NT	NT
HOS	+	—	++	++
CHO-K1	—	++	—	+
CHO-$G\alpha_{15}$	—	++	—	++
Target properties	—	+++	—	+++

*Quantified by anti-CXCR4-PE staining and flow cytometry.

†Efficiency tested by screening a panel of transfection reagents: Roche X-tremeGene, Mirus TransIT-LT1, TransIT-2020, or TransIT-CHO, Fermentas ExGen-500, Invitrogen Lipofectamine 2000, LP9.

‡Tested by FLIPR4 Ca^{2+} mobilization assay. +++, excellent/highest among the cell lines tested; ++, good; +, moderate; —, poor/none; NT, not tested.