

MOL #89714

**β -arrestin1 and distinct CXCR4 structures are required for SDF-1 to down-regulate
CXCR4 cell-surface levels in neuroblastoma**

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Neurobiology of Disease: I.C.C., Molecular Pharmacology and Experimental Therapeutics:
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MOL #89714

SDF-1 uses β -arrestin1 & CXCR4 structures in neuroblastoma

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Abstract: 256 wds

Introduction: 983

Discussion: 1461 wds

44 pages

8 figures

59 references

LIST OF ABBREVIATIONS USED: (GPCR) G-Protein Coupled Receptor, (PMA) phorbol 12-myristate 13-acetate, (PLC) Phospholipase C, (PKC) Protein Kinase C, (SDF-1) Stromal Derived Factor-1, also known as CXCL12, (GRK) G-Protein Coupled Receptor Kinase.

MOL #89714

ABSTRACT

CXCR4 is a G protein-coupled receptor (GPCR) located on the cell-surface that signals upon binding the chemokine SDF-1 (also called CXCL12). CXCR4 promotes neuroblastoma proliferation and chemotaxis. CXCR4 expression negatively correlates with prognosis and drives neuroblastoma growth and metastasis in mouse models. All functions of CXCR4 require its expression on the cell-surface, yet the molecular mechanisms that regulate CXCR4 cell-surface levels in neuroblastoma are poorly understood. We characterized CXCR4 cell-surface regulation in the related SH-SY5Y and SK-N-SH human neuroblastoma cell lines. SDF-1 treatment caused rapid down-modulation of CXCR4 in SH-SY5Y cells. Pharmacologic activation of Protein Kinase C (PKC) similarly reduced CXCR4, but via a distinct mechanism. Analysis of CXCR4 mutants delineated two CXCR4 regions required for SDF-1 treatment to decrease cell-surface CXCR4 in neuroblastoma cells: the IL motif at residues 328 and 329, and residues 343-352. In contrast, and unlike CXCR4 regulation in other cell types, serines 324, 325, 338 and 339 were not required. Arrestin proteins can bind and regulate GPCR cell-surface expression, often functioning together with kinases such as GRK2. Using SK-N-SH cells which are naturally deficient in β -arrestin1, we showed that β -arrestin1 is required for the CXCR4 343-352 region to modulate CXCR4 cell-surface expression following treatment with SDF-1. Moreover, GRK2 overexpression enhanced CXCR4 internalization, via a mechanism requiring both β -arrestin1 expression and the 343-352 region. Together, these results characterize CXCR4 structural domains and β -arrestin1 as critical regulators of CXCR4 cell-surface expression in neuroblastoma. β -arrestin1 levels may therefore influence the CXCR4-driven metastasis of neuroblastoma as well as prognosis.

MOL #89714

INTRODUCTION

Neuroblastoma is the most common malignancy in infants, causing one case in every 100,000 children in the United States (Davidoff, 2012; Verissimo et al., 2011). High risk neuroblastoma patients undergo intensive chemotherapy, stem cell harvesting, and primary tumor resection (Davidoff, 2012), interventions that improve prognosis but cause significant morbidity, developmental delays, leukemia risk, and infertility. There is an urgent need for neuroblastoma treatments that spare normal tissue including normal developing hematopoietic and other stem cells. In addition to MYCN gene amplification, DNA ploidy changes, and expression of neurotrophin receptors (Huang et al., 2011; Raffaghello et al., 2009; Vasudevan et al., 2005)(Davidoff, 2012; Vasudevan et al., 2005), elevated expression of the CXCR4 chemokine receptor is a marker and proposed drug target for high risk neuroblastoma (Shim et al., 2009). CXCR4 expression is associated with neuroblastoma metastatic growth (Lieberman et al., 2012), clinical presentation in bone marrow and liver (Geminder et al., 2001; Meier et al., 2007; Raffaghello et al., 2009; Russell et al., 2004; Zhao et al., 2012), and poor prognosis (Russell et al., 2004). Unfortunately, pharmacological targeting of CXCR4 is complicated by the widespread expression of CXCR4 on normal cells. Here, we focus on characterizing the molecular mechanisms that regulate the expression of functional CXCR4 receptors specifically on the neuroblastoma cell surface, information which could potentially lead to more selective approaches for inhibiting the oncogenic activities of CXCR4 in neuroblastoma.

CXCR4 is a member of the superfamily of seven transmembrane-spanning G-protein coupled receptors (GPCRs) (Hanyaloglu and von Zastrow, 2008; Zlotnik and Yoshie, 2012) Wu et al., 2010). CXCR4 is also a chemotactic cytokine, or “chemokine” receptor, that functions to

MOL #89714

mediate cellular migration towards its chemokine ligand, SDF-1 (also called CXCL12) (Zlotnik and Yoshie, 2012). Like other GPCRs, CXCR4 requires expression at the cell-surface in order to bind its ligand. CXCR4 undergoes a conformational change upon binding extracellular ligand that activates the signal transduction function of one or more heterotrimeric G proteins associated with the cytoplasmic face of the receptor, including pertussis toxin-sensitive Gi/o-type G proteins (Zlotnik and Yoshie, 2012). CXCR4 is widely expressed, and has been implicated in regulating the normal migration patterns of immune (Kremer et al., 2011) and other cell types (Dziembowska et al., 2005) into regions that express SDF-1 including the lymph nodes, bone marrow, liver, lung, and brain. Similar to its chemotactic roles for many normal cells, CXCR4 chemotactic signaling drives cancer metastasis, including breast cancer (Liu et al., 2010), lymphoma (Wu et al., 2009), and neuroectodermal cancers (Domanska et al., 2013). The diverse physiologic roles of CXCR4 are highlighted by the effects of genetic knockout of either CXCR4 or SDF-1 in mice, which results in neonatal lethality with defective formation of the brain, gastrointestinal tract, vasculature, and immune system (Baird et al., 1999; Bajetto et al., 2001; Holman et al., 2011; Tachibana et al., 1998). The immune system, including lymphocyte migration and hematopoietic stem cell homing, is also acutely disrupted in humans and mice treated with CXCR4 antagonists (De Clercq, 2009; Modak et al., 2012).

Mechanisms for regulating CXCR4 cell-surface expression have until now primarily been characterized using epithelial (Barker and Benovic, 2011; Cheng et al., 2000) and immune cells (Kremer et al., 2011; Kumar et al., 2011) rather than neuroblastoma cells. For CXCR4 and other GPCRs, this process typically involves the clustering of ligated receptors into clathrin-coated pits on the plasma membrane followed by membrane invagination and then the pinching off of an endosome and the trafficking of the receptors into early endosomes. Following endocytosis,

MOL #89714

additional CXCR4 trafficking results in either receptor degradation or recycling back to the cell-surface (Kumar et al., 2011). Others have extensively characterized the CXCR4 structural domains and binding proteins important for CXCR4 endocytosis in non-neuronal cell types. These studies have often implicated as a critical control region the carboxyl-terminal “tail” region of CXCR4 that spans approximately the amino acid residues 319-352. For example, in HEK293 epithelial cells, the carboxyl-terminal region of CXCR4 was shown to contain serine phosphorylation sites for G-protein Coupled Receptor Kinases (GRKs), which mediate the direct binding of β -arrestin scaffold proteins responsible for linking CXCR4 to the endocytosis machinery (Busillo et al., 2010). Moreover, within this larger region of CXCR4, the IL residues at residues 324 and 325 and a specific serine at residues 338 were found to be important for CXCR4 endocytosis in HEK293 cells (Orsini et al., 2000). In contrast, the 319-352 region of CXCR4 was not required for ligand-dependent CXCR4 internalization in a leukemic cell line (Haribabu et al., 1997). Thus, the mechanisms responsible for mediating CXCR4 endocytosis may differ among cell types.

Here, we characterize the distinctive molecular mechanisms that control how SDF-1 treatment regulates and modulates the levels of cell-surface CXCR4 expression in neuroblastoma. For these studies, we employed the well-characterized human MYCN-amplified neuroblastoma and neuronal model cell line SH-SY5Y together with its parent line SK-N-SH, derived from a metastatic neuroblastoma tumor (Biedler and Spengler, 1976; Huang et al., 2011). We show evidence that, while Protein Kinase C (PKC) can activate CXCR4 internalization via phorbol 12-myristate 13-acetate (PMA), PKC is not required for SDF-1 to mediate CXCR4 internalization in neuroblastoma. The mechanistically-related Phospholipase Cs (PLCs) are similarly not required for SDF-1 – mediated CXCR4 endocytosis in neuroblastoma, nor are

MOL #89714

pertussis toxin (PTX) sensitive G-proteins. Moreover, we identify two portions of the carboxyl-terminal tail region of CXCR4 that are involved in SDF-1 mediated CXCR4 endocytosis, we show that one of these CXCR4 regions is important because it permits CXCR4 endocytosis regulation by β -arrestin1, and we show that CXCR4 endocytosis in neuroblastoma occurs independently of several specific CXCR4 phosphorylation sites that critically mediate CXCR4 endocytosis in other cell types. Furthermore, we show that GRK2 can enhance the ligand-dependent internalization of CXCR4 in these cells, via a mechanism requiring both the carboxyl-terminal tail region of CXCR4 and β -arrestin1 overexpression. Together, these results significantly increase understanding of the molecular mechanisms regulating cell-surface CXCR4 expression in neuroblastoma. Targeting these molecular mechanisms may permit the development of more selective CXCR4-focused treatments for high risk neuroblastoma patients.

MOL #89714

MATERIALS AND METHODS

Cell Lines

The related neuroblastoma cell lines SH-SY5Y and SK-N-SH were obtained from ATCC and grown in DMEM (1X) supplemented with 10 % FBS, 4.5 g/L D-glucose, 2 mM L-glutamine, and 110 mg/L sodium pyruvate, penicillin/streptomycin and 5 mM HEPES pH 7.4 (Life Technologies, Green Island, NY). Cells were grown until approximately 80 % confluence, then adherent cells were removed from the flasks using 1.5 mM EDTA in 1 x PBS and replated at one-fifth density. SH-SY5Y and SK-N-SH cells were derived from a human metastatic neuroblastoma tumor by Biedler et al. (Biedler and Spengler, 1976; Huang et al., 2011; Yusuf et al., 2013; Zhao et al., 2012). The NTera-2 cell line was obtained from ATCC and grown in DMEM and 10% FBS (Life Technologies, Green Island, NY), as previously described (Carlin and Andrews, 1985). The Jurkat human T lymphocyte cell line was maintained as previously described (Kremer et al., 2011; Kumar et al., 2006; Kumar et al., 2011). The KG1a leukemia cell line was grown and utilized for control experiments as previously described (Kremer et al., 2013).

Plasmids, pharmaceuticals, and antibodies

The expression plasmid encoding the human CXCR4 fused to YFP (CXCR4-YFP) was previously described (Kumar et al., 2006). The CXCR4 mutations were constructed in this plasmid using site-directed mutagenesis and confirmed by DNA sequencing. Untagged CXCR4wt and CXCR4 Δ 322-352 containing GFP were also constructed in this manner, used in Supplemental Figure 1B. The expression plasmid encoding FLAG-tagged β -arrestin1 was a

MOL #89714

generous gift from Robert Lefkowitz (Luttrell and Lefkowitz, 2002). The expression plasmid encoding bovine GRK2 was a generous gift from Jon Willets (Morris et al., 2011) and the expression plasmid encoding bovine GRK2-K220R was from Adgene (plasmid number 14691; Cambridge, MA). Either 10^{-7} M SDF-1 α (R& D systems, Minneapolis, MN) or 10 ng/ml PMA (EMD Millipore, Billerica, MA) were used for cell stimulation. All cell stimulations were done at 37 °C for 60 min unless otherwise indicated. For inhibition of specific signaling pathways, cells were pretreated for 30 min with either 1 μ M UCN-01 (Sigma-Aldrich, St. Louis, MO) or 5 μ M U73122 (EMD Millipore, Billerica, MA) or 15 min with 100 ng/ml PTX Sigma-Aldrich, St. Louis, MO) or 1 hr with 0.6 M sucrose (Sigma-Aldrich, St. Louis, MO), and control cells were pretreated in parallel with equivalent amounts of vehicle (DMSO, Ethanol or inactive b-oligomer toxin, respectively). Activity of dissolved PTX was confirmed using an ERK activation assay as previously described (Kremer et al., 2013). A CXCR4 mAb conjugated to APC (R&D Systems (Minneapolis, MN) was used for flow cytometric detection of cell-surface CXCR4. β -arrestin1, GRK2, and ERK2 antisera used for immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA), β -actin antisera was from from Novus Biologicals (Littleton, CO).

Transient transfection of neuroblastoma cell lines

All reagents were first slowly brought to room temperature. For each transfection, 2 μ g plasmid DNA, 9 μ l of FuGene HD (Promega, Madison, WI), and 200 μ l of 1 % BSA in DMEM were combined and allowed to sit at room temperature for 20 min. The particular plasmid(s) included in each transfection varied as indicated. This mixture was then added to 35 x 10 mm cell culture dishes containing adherent neuroblastoma cells at approximately 50 % confluency together with 1 ml of conditioned cell culture media without penicillin/streptomycin. The dishes were placed at

MOL #89714

37 °C and analyzed 24 hrs later. Transfection efficiencies in different experiments, estimated from the percentage of YFP+ fluorescent cells as detected by flow cytometry, ranged from 1 to 40 %. For selected experiments, CXCR4-YFP expression and subcellular localization was also visualized in living, transfected cells using confocal microscopy as in (Kumar et al., 2011).

Assay of cell-surface CXCR4 regulation on neuroblastoma cells

Cells were pretreated with inhibitors in some cases, then CXCR4 endocytosis was stimulated by removing the culture media and adding 1 ml of conditioned media containing either SDF-1 or PMA. Following 2-60 min of incubation at 37 °C, cell-surface CXCR4 levels were assayed by flow cytometry as follows. Stimulated cells were washed in cold FACS buffer consisting of 1 X HBSS, 10 mg/ml BSA, 10 mM HEPES, and 0.2 % sodium azide. 20 µl of CXCR4-APC antibody (R&D Systems, Minneapolis, MN) was added to the cells and incubated for 20 min at 4 °C, then cells were washed twice with cold FACS buffer and fixed in 2 % paraformaldehyde before being analyzed on either a FACS Canto or FACS Caliber dual color flow cytometer (BD Biosystems, Bedford, MA). WinMDI software (Scripps Research Institute, La Jolla, CA) was used to analyze flow cytometric data; electronic gating was used to selectively analyze transfected (YFP+) cells. Similar results were obtained when non-YFP-tagged CXCR4 was used, although the detection of transfected cells was less precise (not shown).

MOL #89714

RESULTS

Endogenous CXCR4 is expressed on the surface of SH-SY5Y cells, and is rapidly down-regulated in response to treatment with SDF-1. SDF-1 binding has been shown to induce the endocytosis and intracellular trafficking of its receptor, CXCR4, in many cell types (Bardi et al., 2006; Ben-Baruch, 2009; Cheng et al., 2000; Kremer et al., 2011), however, the molecular mechanisms regulating this process have not previously been characterized in neuroblastoma. We began by assaying the expression of CXCR4 on the surface of SH-SY5Y neuroblastoma cells and by characterizing the effects of SDF-1 treatment. To quantitatively compare cell-surface CXCR4 levels, we used flow cytometry to assay the fluorescence associated with individual cells following the staining of the intact cells with fluorescently-labeled mAb specific for CXCR4. SH-SY5Y cells expressed endogenous CXCR4 on the cell-surface, with a mean fluorescence of CXCR4 staining that was greater than that of unstained control cells analyzed the same day (Fig 1A). In contrast, SH-SY5Y cells treated with SDF-1 for 60 min at 37 °C prior to staining for cell-surface CXCR4 showed distinctly lower mean fluorescence (Fig. 1A), indicating that CXCR4 was down-regulated from the cell-surface in response to SDF-1 treatment. Fig. 1B plots the mean CXCR4 cell-surface levels of SH-SY5Y cells analyzed at variable times after SDF-1 addition. The ability of SDF-1 to reduce the cell-surface expression of CXCR4 was seen as early as 10 min after SDF-1 treatment and was maximal at 60 min (Fig. 1B). Not all cell-surface CXCR4 was removed following SDF-1 treatment, most likely because of the existence of refractory receptors or because some endocytosed CXCR4 recycles back to the cell-surface (Kumar et al., 2011). Thus, SDF-1 treatment significantly reduced cell-surface CXCR4 on these neuroblastoma cells by 50-60 %.

MOL #89714

CXCR4 can signal through pertussis toxin (PTX) sensitive Gi/o type G proteins, therefore, we also determined the effects of PTX on this process. We found that the SDF-1 mediated internalization of CXCR4 expressed in SH-SY5Y cells was not significantly impaired by pretreatment with PTX as compared to the control inactive b-oligomer (Fig. 1C). As a control, we showed that the same aliquot of PTX significantly inhibited the SDF-1-dependent activation of ERK MAP kinase activity in a leukemic cell line (Fig. 1D), as expected based on a previous publication (Kremer et al., 2013). Thus, SDF-1 treatment induces the endocytosis of CXCR4 in neuroblastoma cells, and does so via a mechanism that is independent of the activity of PTX-sensitive Go/i - type G proteins.

Treatment with the PKC-stimulating drug PMA down-regulates CXCR4 on SH-SY5Y cells, while treatment with SDF-1 down-regulates CXCR4 on these cells via a mechanism independent of both PKC and PLC. Many GPCRs including CXCR4 signal by activating PLC (Bach et al., 2007; Hwang et al., 2005; Kremer et al., 2011; Li et al., 2000). PLC activity, in turn, can lead to PKC activation (Geisler, 2004), and PKC can phosphorylate and thereby induce the endocytosis of multiple GPCRs. We therefore tested the effects of the pan-PKC activator drug, PMA, on CXCR4 cell-surface regulation in neuroblastoma cells. We found that PMA treatment of SH-SY5Y cells elicited CXCR4 cell-surface down-regulation to a similar extent as did SDF-1 treatment (Fig. 2A). The PKC inhibitor drug UCN-01 blocked the ability of PMA to down-regulate CXCR4 cell-surface expression, indicating that the effects of PMA depend on PKC, and that increased PKC activity significantly decreases CXCR4 cell-surface expression in neuroblastoma cells (Fig 2B). Nevertheless, SDF-1 treatment did not similarly require PKC activity in order to down-regulate cell-surface CXCR4 expression in SH-SY5Y cells. UCN-01 had no effect on the ability of SDF-1 treatment to cause down-regulation of cell-

MOL #89714

surface CXCR4, even though UCN-01 abrogated PMA-mediated down-regulation of CXCR4 in the same experiments (Fig. 2C & 2D). Additionally, pretreatment with the pan-PLC inhibitor drug U73122 had no effect on SDF-1 – mediated down-regulation of cell-surface CXCR4 in SH-SY5Y cells (Fig 2E). Yet the same aliquot of U73122 efficiently blocked SDF-1 – stimulated ERK activation in the Jurkat cell line (Fig. 2F), as expected (Kremer et al., 2011). Thus, while PMA is capable of eliciting down-regulation of CXCR4 from the surface of neuroblastoma cells, neither PKC nor PLC activity are required for the mechanism by which SDF-1 treatment results in the down-regulation of cell-surface CXCR4 expression in neuroblastoma cells.

Fluorescently-tagged CXCR4 can be overexpressed on the surface of SH-SY5Y cells, and is down-regulated from the cell-surface in response to treatment with SDF-1. Surface expression of CXCR4 has been shown to be low in several neuroblastoma cell lines (Carlisle et al., 2009). Nevertheless, CXCR4 overexpression is an indicator of worse outcomes for patients with neuroblastoma (Russell et al., 2004) and is also associated with site-specific metastasis particularly to the bone marrow (Ben-Baruch, 2009; Geminder et al., 2001; Meier et al., 2007). To mimic neuroblastoma with elevated CXCR4 and to facilitate our structure/function studies of CXCR4 down-regulation, we overexpressed CXCR4 in SH-SY5Y cells using transient transfection of an expression plasmid encoding wild-type CXCR4 tagged with fluorescent YFP (CXCR4wt). The YFP tag was used to facilitate detection of the transfected receptor. SH-SY5Y cells transiently-transfected with CXCR4wt expressed CXCR4 that was easily detected by confocal fluorescence microscopy, with CXCR4wt (green) visualized both on the cell-surface and within endosomal compartments of living cells (Fig. 3A). A fraction of endosomally-located GPCR is expected, representing receptor biosynthetic processing and constitutive receptor recycling (Hanyaloglu and von Zastrow, 2008). Flow cytometric analysis of cell-surface CXCR4

MOL #89714

confirmed that, compared to vector-transfected control cells that expressed only endogenous CXCR4, CXCR4^{wt} - transfected SH-SY5Y cells expressed approximately 30-fold higher levels of cell-surface CXCR4 (Fig. 3B). SDF-1 treatment efficiently down-regulated this transfected CXCR4^{wt}-YFP from the cell-surface (Fig 3C). Comparing the overexpressed CXCR4^{wt} with endogenous CXCR4 (in the vector-transfected cells), no statistically-relevant difference was noted in the extent of CXCR4 down-regulation in response to SDF-1 ($p > 0.5$). Thus, CXCR4 can be overexpressed in SH-SY5Y cells, and this overexpressed CXCR4 down-regulates from the cell-surface in a manner similar to endogenous CXCR4 when stimulated by SDF-1.

The cytoplasmic “tail” domain of CXCR4 consisting of the carboxyl-terminal amino acid residues 323-352 is not required for cell-surface expression in SH-SY5Y cells, but is required for SDF-1 treatment to down-regulate CXCR4 from the cell-surface. To investigate the CXCR4 structures required for the SDF-1 - mediated down-regulation of cell-surface CXCR4 in neuroblastoma, we mutated CXCR4 prior to overexpressing it in SH-SY5Y cells. Our results indicate that deleting most of this cytoplasmic carboxyl-terminal “tail” domain of CXCR4 (by removing amino acid residues 323-352 to create CXCR4 Δ 322-352) had little effect on the ability of CXCR4 to be expressed on the cell-surface of unstimulated cells (Fig. 5A, compare solid histograms). Yet unlike wild type CXCR4, CXCR4 Δ 322-352 failed to detectably down-regulate from the cell-surface upon SDF-1 treatment (Fig. 4A). In multiple experiments, CXCR4 Δ 322-352 did not decrease on the cell-surface following 60 min of SDF-1 treatment, in contrast to the wild-type CXCR4 (Fig 4B). Similar results were seen with 60 min PMA stimulation (Supplemental Figure 1B). Thus, the CXCR4 tail domain is required for SDF-1 treatment to reduce cell-surface levels of CXCR4 in SH-SY5Y neuroblastoma cells.

MOL #89714

Two distinct regions of the CXCR4 “tail” are both required for SDF-1 treatment to down-regulate CXCR4 cell-surface expression in SH-SY-5Y cells: the 342-352 region of CXCR4, and the CXCR4 IL motif. The crystal structure of CXCR4 showed that this receptor shares a similar structure with other GPCRs in that it consists of a tight bundle of seven transmembrane α helices, however, the carboxyl tail region of CXCR4 had no notable tertiary structural features (Wu et al., 2010). CXCR4 carboxyl terminal tail region does contain several defined phosphorylation and protein binding sites (Orsini et al., 2000). We therefore examined the effects of smaller mutations within the CXCR4 tail domain (Fig. 5A&B). Two progressively smaller truncation mutants were studied in addition to CXCR4 Δ 322-352: CXCR4 Δ 334-352 and CXCR4 Δ 342-352. All three deletion mutants displayed dramatic inhibition of SDF-1 - mediated receptor down-regulation (Fig 5C), indicating that the terminal 10 amino acid residues of CXCR4 (343-352) are minimally required for this down-regulation. Additionally, several point mutants within the CXCR4 tail domain were studied. Specifically, two serines at a time, including the confirmed 324 and 338 phosphorylation sites (Busillo et al., 2010; Orsini et al., 2000), were mutated to alanine in the mutants CXCR4-324/325 and CXCR4-338/339. Mutation of these serines did not significantly alter the ability of CXCR4 to internalize in response to SDF-1 treatment ($p > 0.5$ as compared to CXCR4wt) (Fig. 5C). In contrast to the lack of effect of these serine mutations, dual mutation of CXCR4 I328 and L329 to alanines (creating CXCR4-IL) significantly reduced the ability of SDF-1 to down-regulate the cell-surface levels of CXCR4 (Fig. 5C). This IL motif in CXCR4 was previously identified as homologous to the “dileucine motif” that regulates GPCR endocytosis in other cell types (Fischer et al., 2011; Guo and Jose, 2011; Orsini et al., 2000). Flow cytometric analysis indicated that these mutations did not inhibit the cell-surface expression of CXCR4, since of the CXCR4 mutants studied were expressed on

MOL #89714

the cell-surface at levels comparable to the wild-type CXCR4 construct (Supplemental Fig. 3A & B). We also analyzed the ability of CXCR4 to undergo ligand-dependent down-regulation from the cell-surface using another human neuronal tumor cell line, NTERA-2. The results show that, as in SH-SY5Y cells, wild-type CXCR4 endocytosed readily in response to SDF-1, while the CXCR4-IL and CXCR4-D322-352 mutants did not (Supplemental Figure 2A) ($n = 3 \pm$ S.E.M., $p < 0.05$). Taken together, the results in Fig. 5 delineate two distinct regions of the CXCR4 tail domain that are both required for SDF-1 treatment to reduce cell-surface expression of CXCR4 in neuronal tumor cells: the 343-352 region of CXCR4, and the CXCR4 IL motif.

β -arrestin1 expression is required for SDF-1 treatment to efficiently down-regulate cell-surface CXCR4 in the related SK-N-SH neuroblastoma cell line. The endocytosis of GPCRs can involve their clustering into clathrin-coated pits and clathrin-associated proteins including β -arrestins (Hanyaloglu and von Zastrow, 2008; Luttrell and Lefkowitz, 2002). To address the molecular and cellular mechanisms that mediate CXCR4 endocytosis in neuroblastoma, we first explored the role of clathrin using sucrose, which blocks clathrin-mediated receptor internalization (Hansen et al., 1993; Heuser and Anderson, 1989). We found that SDF-1 - mediated down-regulation of CXCR4 in SH-SY5Y cells was inhibited in the presence of sucrose (Fig. 6A), as was PMA mediated CXCR4 down-regulation (Supplemental Figure 1A). These results suggest a role for clathrin in both processes. Next, we asked if this process also involves β -arrestin. We discovered that a related variant of SH-SY5Y, the SK-N-SH cell line, expresses relatively reduced levels of β -arrestin1 protein (Fig. 6B). We therefore determined if this low β -arrestin1 expression affected CXCR4 internalization. Because SK-N-SH cells express only very low levels of endogenous CXCR4 as compared to SH-SY5Y cells, we assayed CXCR4 down-regulation in SK-N-SH vs. SH-SY5Y cells after transfection of both cell

MOL #89714

lines with wild type CXCR4. Significantly, the cell-surface CXCR4 was only poorly down-regulated upon SDF-1 treatment of SK-N-SH cells (Fig. 6C), as compared to SH-SY5Y cells (Figs. 2-4). In multiple experiments, the SDF-1 – stimulated CXCR4 down-regulation was significantly impaired in SK-N-SH cells as compared to SH-SY5Y cells, with 70 ± 7.3 % of CXCR4 still remaining on the surface of SDF-1 - stimulated SK-N-SH cells as compared to 44 ± 4.1 % for SH-SY5Y cells ($n = 3-7 \pm$ S.E.M., $p < 0.01$) (Fig. 6D). To determine if the relative deficiency of β -arrestin1 protein in SK-N-SH cells was responsible for the CXCR4 endocytosis defect in SK-N-SH cells, we transfected SK-N-SH cells with a β -arrestin1 expression plasmid. Adding β -arrestin1 significantly increased the efficiency of SDF-1 – dependent down-regulation of CXCR4 in SK-N-SH cells (Fig. 6E, compare with Fig. 6C). This result was significant; in multiple experiments, transfection of β -arrestin1 into SK-N-SH cells increased the down-regulation of CXCR4 in response to SDF-1 from 70 ± 7.3 % CXCR4 remaining on the cell-surface after SDF-1 treatment, to 41 ± 6.3 % ($n = 4 \pm$ S.E.M., $p < 0.05$) (Fig. 6D). Indeed, the down-regulation of CXCR4 in β -arrestin1 – expressing SK-N-SH cells was not statistically different from SH-SY5Y cells ($p > 0.05$) (Fig. 6D). As a control, we demonstrated that the same β -arrestin1 expression plasmid efficiently increased expression of β -arrestin1 protein detectable by western blotting after transfection into the Jurkat T cell line (Fig. 6F). A similar western blot could not be done on the transfected SK-N-SH cells due to the low transfection efficiency of these cells (data not shown). Nevertheless, the low transfection efficiency of SK-N-SH cells did not interfere with our flow cytometric assays of CXCR4 cell-surface levels on SK-N-SH cells because electronic gating permitted the selective analysis only of transfected cells. Together, these results indicate that β -arrestin1 expression is required for the efficient down-regulation of CXCR4 cell-surface expression in these neuroblastoma cells in response to SDF-1 treatment.

MOL #89714

The 343-352 region of CXCR4, but not the CXCR4 IL motif, is required for β -arrestin1 to regulate cell-surface levels of CXCR4 in SK-N-SH cells. Since β -arrestins can bind directly to the CXCR4 tail domain (Busillo et al., 2010; Orsini et al., 2000; Roland et al., 2003), we asked if specific CXCR4 carboxyl region structure(s) were required for the mechanism which β -arrestin1 expression enhances the down-modulation of CXCR4 from the cell-surface of the neuroblastoma cell lines. First, we showed that, as for SH-SY5Y cells that are replete with endogenous β -arrestin1, the β -arrestin1 - overexpressing SK-N-SH cells required the CXCR4 tail domain in order to down-modulate CXCR4 in response to SDF-1. This result was seen in multiple experiments and was statistically significant (Fig. 7A, compare CXCR4wt and CXCR4 Δ 322-352). Second, we showed that the more limited truncation mutant CXCR4 Δ 342-352 failed to be down-regulated from the cell-surface in response to SDF-1 when it was expressed in β -arrestin1 - overexpressing SK-N-SH cells (Fig. 7A&B). Thus, the ability of β -arrestin1 to enhance CXCR4 down-modulation requires the last 10 amino acid residues of CXCR4. Third, we showed that the CXCR4 serines 338 and 339 were not required for this response (Fig. 7A). The CXCR4 338 and 339 mutant was similarly deficient in causing internalization in SK-N-SH cells when compared to CXCR4 wildtype (Fig. 6D) in the absence of β -arrestin1 (data not shown) ($n = 3 \pm$ S.E.M., $p > 0.5$). Fourth, and in contrast to its behavior in SH-SY5Y cells, we found that the CXCR4-IL mutant expressed in β -arrestin1-transfected SK-N-SH cells was able to permit the down-regulation of cell-surface CXCR4 in response to SDF-1, and that this occurred to an extent that was not significantly different from the behavior of CXCR4wt-YFP ($n = 3 \pm$ S.E.M.; $p > 0.5$) (Fig. 7A). Together, these results indicate that the 343-352 region of CXCR4 is necessary for SDF-1 treatment to mediate the down-modulation of cell-

MOL #89714

surface CXCR4 in neuroblastoma cells via a mechanism requiring β -arrestin1, while the CXCR4 IL motif is required for CXCR4 down-regulation in SH-SY5Y cells for a distinct reason.

GRK2 participates in regulating CXCR4 cell-surface levels and internalization.

Since several reports suggest that β -arrestins associate with GRKs and that these proteins function together to regulate GPCRs (Busillo et al., 2010; Morris et al., 2011; Willets et al., 2001), we asked if GRK2 also participates in regulating β -arrestin1-dependent CXCR4 internalization in neuroblastoma cells. GRK2 was endogenously expressed in both SH-SY5Y and SK-N-SH cells (Fig. 8A). Interestingly, overexpressing GRK2 in SH-SY5Y cells via transient transfection of an expression plasmid (Fig. 8C) significantly reduced the cell-surface levels of CXCR4 even in unstimulated neuroblastoma cells. Moreover, this effect of GRK2 was not detectably different when comparing CXCR4_{wt} vs. CXCR4-D342-352 (Fig. 8B) (compare $59 \% \pm 0.8 \%$ vs. $61 \% \pm 7.7 \%$, $p > 0.5$, $n = 3$). In contrast to this effect of overexpressing GRK2, overexpressing β -arrestin1 did not alter the levels of unstimulated CXCR4 on these cells (Fig. 6D). Despite the differences in CXCR4 cell-surface levels on unstimulated cells, it is clear that the SDF-1-mediated internalization of wild-type CXCR4 is significantly more complete when GRK2 was overexpressed (Fig 8D & E) ($n = 3 \pm$ S.E.M.; $p < 0.05$). Fig. 8D shows an examples of a typical experiment, and Fig. 8E shows the mean results of multiple such experiments. Moreover, GRK2 overexpression did not detectably increase the internalization of the CXCR4 Δ 342-352 mutant (Fig. 8E) ($n = 3 \pm$ S.E.M., $p > 0.1$), suggesting that this effect of GRK2 also requires β -arrestin1 expression. Consistent with this idea, we found that GRK2 overexpression in the β -arrestin1-deficient SK-N-SH cell line failed to significantly increase the internalization of wild-type CXCR4 unless β -arrestin1 was also expressed (Fig. 8F). In addition, overexpressing a point-mutated, kinase-inactive GRK2 (Willets et al., 2001) in SH-SY5Y cells

MOL #89714

had no effect on either CXCR4 cell-surface levels of unstimulated cells or SDF-1 – induced CXCR4 internalization, indicating that these effects of GRK2 require its kinase activity (Supplemental Fig. 4). Thus, the carboxyl-terminal 343-352 residues of CXCR4 and β -arrestin1 are required for GRK2 to enhance the ligand-dependent internalization of CXCR4 in neuroblastoma cells.

DISCUSSION

Here, we characterize two distinct structural regions of CXCR4 that are both necessary for SDF-1 to modulate the cell-surface levels of CXCR4 in neuroblastoma cells. In addition, we show that one of these CXCR4 structural regions (encompassing the CXCR4 amino acid residues 343-352) is further regulated by expression of the β -arrestin1 scaffold protein and GRK2 kinase in these cells, while the other CXCR4 structural region (the IL motif) regulates CXCR4 cell-surface levels in neuroblastoma cells via an independent mechanism. Moreover, we showed that ligand-dependent internalization of CXCR4 in these cells does not require activity of PTX sensitive G proteins. In addition, we showed that activation of an endogenous kinase, PKC, potently down-regulates CXCR4 cell-surface levels in neuroblastoma cells in the absence of SDF-1. Finally, and in contrast to previous reports in other cell types (Kremer et al., 2011), we found that CXCR4 cell-surface regulation in neuroblastoma was unaffected by either a PLC inhibitor drug or mutation of several specific CXCR4 serine residues.

β -arrestin proteins bind to various regions of GPCR cytoplasmic tail domains and regulate endocytosis, frequently in response to GRK-mediated receptor phosphorylation (Busillo et al., 2010; Kelly et al., 2008; Luttrell and Lefkowitz, 2002). Interestingly, we found that β -

MOL #89714

arrestin1 expression varied significantly between the SH-SY5Y and SK-N-SH neuroblastoma cell lines. Moreover, we found that the lack of β -arrestin1 expression in SK-N-SH cells contributed significantly to the failure of SDF-1 to down-modulate CXCR4 cell-surface levels in these cells. We further showed that CXCR4 cell-surface regulation in SK-N-SH cells was rescued by introduction of β -arrestin1, that β -arrestin1 regulation of CXCR4 levels in both SH-SY5Y and SK-N-SH cells requires the 343-352 region of CXCR4, and that GRK2 overexpression further enhanced ligand-dependent CXCR4 internalization via a mechanism similarly requiring the 343-352 region. This 343-352 CXCR4 region may bind directly to β -arrestin1 in neuronal cells, since it is contained within a larger CXCR4 tail region previously characterized as a β -arrestin binding site in epithelial cells (Cheng et al., 2000; Roland et al., 2003). In further support of our results, both β -arrestin1 and 2 were previously shown to conditionally associate with the CXCR4 tail region upon SDF-1 stimulation of HEK293 cells, and did so via mechanism requiring a similar carboxyl-terminal region CXCR4 (Busillo et al., 2010). In contrast, our results suggest that another β -arrestin binding site reportedly located within the 3rd intracellular loop of CXCR4 (Roland et al., 2003), which was intact in all our CXCR4 mutants, either does not bind β -arrestin1 in neuroblastoma cells or else plays no role in regulating CXCR4 endocytosis in these cells.

Our results significantly improve understanding of CXCR4 regulation in neuroblastoma and may lead to better therapies for this disease. All known effects of CXCR4 require its expression on the cell-surface, which is required for CXCR4 to bind SDF-1 and initiate signal transduction. Yet until now, the molecular mechanisms involved in regulating the cell-surface levels of CXCR4 and other GPCRs, which typically vary among different cell types, have mainly been studied in cell types other than neuroblastoma (Bhandari et al., 2007; Holman et al., 2011;

MOL #89714

Kelly et al., 2008). We focused here on characterizing the molecular mechanisms responsible for the SDF-1 – dependent modulation of CXCR4 cell-surface expression in neuroblastoma cells, since this type of CXCR4 down-modulation has been shown to functionally lower cellular responses to SDF-1 (Kumar et al., 2011) and since aggressiveness and poor prognosis in neuroblastoma correlates with SDF-1/CXCR4 signaling in these cells (Domanska et al., 2013; Geminder et al., 2001).

Our results indicate that CXCR4 cell-surface regulation in neuroblastoma may involve some mechanisms that are relatively tissue-specific, which may be helpful for designing future therapies for this disease. For example, we found no role for the CXCR4 phosphorylation sites 324 and 338 in regulating CXCR4 cell-surface levels in neuroblastoma cells, in contrast to previous reports in epithelial cells (Orsini et al., 2000). In another example, we found evidence that neuroblastoma cells and lymphocytes differ significantly in phosphoinositide pathway regulation of cell-surface CXCR4. We showed that a PLC inhibitor drug which impairs SDF-1-dependent down-modulation of CXCR4 in T lymphocytes (Kremer et al., 2011) had no effect on CXCR4 cell-surface regulation in neuroblastoma. This latter result is particularly interesting, because it suggests that there may be a way to therapeutically modulate CXCR4 in neuroblastoma without disrupting normal immune functions. We identified a critical role for β -arrestin1 as a required modulator of CXCR4 cell-surface levels in neuroblastoma cells that may also be relatively specific for this cell type. β -arrestin1 was previously reported to be dispensable for ligand-dependent CXCR4 internalization in epithelial cells, with this process requiring β -arrestin2 instead (Bhandari et al., 2007; Orsini et al., 2000). Thus, future therapies designed to specifically enhance expression of β -arrestin1 may be able to selectively inhibit CXCR4 cell-surface expression and function on neuroblastoma. Our results additionally suggest that β -

MOL #89714

arrestin1 expression, together with GRK2, may serve as useful markers in combination with CXCR4 for predicting metastatic risk in neuroblastoma, helping to regulate the migration, proliferation, invasion, and metastasis of neuroblastoma that is dependent on CXCR4 (Lieberman et al., 2012; Raffaghello et al., 2009). Other β -arrestin1 binding proteins might also modulate CXCR4 expression and function in neuroblastoma, for example ESCRT-0, STAM-1, and/or AIP-4 (Bhandari et al., 2007; Malik and Marchese, 2010).

Neuroblastoma derives from the neural crest (Verissimo et al., 2011) and neuroblastoma cell lines including SH-SY5Y have been used as models for studying the molecular pathways involved in neural development and degeneration, such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis (Agholme et al., 2010; Cheung et al., 2009; Emanuelsson and Norlin, 2012). Neural crest cells, which migrate via CXCR4, differentiate into Schwann cells, melanocytes, enteric neurons, and chromaffin cells (Verissimo et al., 2011), and can be dysregulated to form other neuroectodermal tumors such as melanoma and small cell lung cancer (Raffaghello et al., 2009). SDF-1 and CXCR4 have been implicated in the development, proliferation, and migration of neuronal and glial precursors (Bajetto et al., 2001; Stumm and Holtt, 2007) and the etiology and recovery of the nervous system during disease (Carbajal et al., 2011). Yet the molecular regulation of CXCR4 is still poorly understood in these diseases. Our results describing the roles of β -arrestin1, GRK2, and specific CXCR4 structural elements may also be relevant for these related neuroectodermal cells, tumors, and diseases.

MOL #89714

ACKNOWLEDGEMENTS

We thank Dr. Allan Bieber (Mayo Clinic, Rochester, MN) for his help in the critique and editing of this manuscript.

MOL #89714

AUTHORS' CONTRIBUTIONS

Participated in research design: Clift, Bamidele, Kremer, Hedin

Conducted experiments: Clift, Rodriguez-Ramirez, Bamidele

Contributed new reagents or analytic tools: Clift, Bamidele, Kremer, Hedin

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Wrote or contributed to the writing of the manuscript: Clift, Hedin

MOL #89714

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MOL #89714

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MOL #89714

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MOL #89714

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MOL #89714

FOOTNOTES

This work was supported by a National Multiple Sclerosis Society Collaborative Research Center Award (to M. Rodriguez, Mayo Clinic, and K.E.H.); The Alma B. Stevenson Endowment Fund for Medical Research (to K.E.H.); Mayo Clinic Robert D. and Patricia E. Kern Predoctoral Fellowship for Neuroscience Research (to I.C.C.).

MOL #89714

FIGURE LEGENDS

FIGURE 1: Endogenous CXCR4 is expressed on the surface of SH-SY5Y cells, and is rapidly down-regulated in response to treatment with SDF-1 independent of G α i. SH-SY5Y neuroblastoma cells were treated with either nothing (“Unstim.”) or SDF-1 at 37 °C for the indicated times. Cell-surface CXCR4 levels were assayed on individual cells via flow cytometry after staining of intact cells with APC-conjugated CXCR4 mAb. A) Representative flow cytometric data from one experiment, showing the decreased cell-surface CXCR4 levels on neuroblastoma cells following 60 min treatment with SDF-1. B) Summary of multiple experiments performed as in A for various times of SDF-1 treatment. Each point denotes the mean CXCR4 cell-surface level of SDF-1 - treated as compared to unstimulated cells for three independent experiments \pm S.E.M.; ***, significantly different from results using unstimulated cells, $p < 0.001$. C) The G α i/o inhibitor, PTX, fails to inhibit the ability of SDF-1 to decrease cell-surface CXCR4 on neuroblastoma cells. SH-SY5Y cells were pretreated with either inactive control toxin (b-oligomer) or PTX. Cells were then stimulated with SDF-1 for 60 min and cell-surface CXCR4 was assayed as in A & B. A summary of multiple experiments is shown; bars denote the mean CXCR4 cell-surface level of SDF-1- treated as compared to unstimulated cell for 3 independent experiments \pm S.E.M.; Results of cells treated with PTX were not significantly different than control cells, $p > 0.5$. D) Positive control for C, showing that PTX pretreatment inhibits ERK activation in the KG1a cell line in response to SDF-1. Bars denote the mean ERK activation of 3 independent experiments \pm S.E.M.; **, significantly different from vehicle treated cells, $p < 0.01$.

MOL #89714

FIGURE 2: Treatment with the PKC-stimulating drug PMA down-regulates CXCR4 on SH-SY5Y cells, while treatment with SDF-1 down-regulates CXCR4 on these cells via a mechanism independent of both PKC and PLC.

A) PMA stimulates the down-regulation of cell-surface CXCR4 on neuroblastoma cells. SH-SY5Y cells were treated with either nothing, SDF-1, or 10 ng/ml PMA for 60 min, then assayed for cell-surface CXCR4 levels as in Fig. 1. A summary of multiple experiments is shown; bars denote the mean CXCR4 cell-surface level of SDF-1- treated or PMA- treated cells as compared to unstimulated cells for 3 independent experiments \pm S.E.M.; ***, significantly different from unstimulated cells, $p < 0.001$. B) The PKC inhibitor UCN-01 blocks the effects of PMA on neuroblastoma cells. SH-SY5Y cells were pretreated with either vehicle (DMSO) or UCN-01. Cells were then stimulated with PMA for the indicated times, and cell-surface CXCR4 was assayed as in A. Each point denotes the mean CXCR4 cell-surface level of SDF-1 - treated as compared to unstimulated cells for 3 independent experiments \pm S.E.M.; *, significantly different from unstimulated cells, $p < 0.05$. C&D) UCN-01 does not block the ability of SDF-1 to decrease cell-surface CXCR4 on neuroblastoma cells. C, Representative experiment in which SH-SY5Y cells were pretreated with UCN-01 as in B. Cells were then stimulated with either SDF-1 or PMA for 60 min, and cell-surface CXCR4 levels were measured as in Fig. 1. D, Summary of multiple experiments as in C, in which cells were pretreated with either vehicle (DMSO) or UCN-01 then stimulated with SDF-1 for the indicated times. Each point denotes the mean CXCR4 cell-surface level of SDF-1 - treated as compared to DMSO - treated cells for 3 independent experiments, \pm S.E.M.; **, significantly different from unstimulated cells, $p < 0.01$. E) The PLC inhibitor, U73122, fails to inhibit the ability of SDF-1 to decrease cell-surface CXCR4 on neuroblastoma cells. SH-SY5Y cells were pretreated with either vehicle (ethanol, EtOH) or U73122. Cells were then stimulated with SDF-1 for 60 min and

MOL #89714

cell-surface CXCR4 was assayed as in A. A summary of multiple experiments is shown; bars denote the mean CXCR4 cell-surface level of SDF-1- treated as compared to unstimulated cell for 3 independent experiments \pm S.E.M.; Results of cells treated with U73122 were not significantly different than control cells, $p > 0.5$. F) Positive control for E, showing that U73122 pretreatment inhibits ERK activation in the Jurkat T cell line in response to SDF-1. Bars denote the mean ERK activation of 3 independent experiments \pm S.E.M.; ***, significantly different from vehicle treated cells, $p < 0.001$.

FIGURE 3: Fluorescently-tagged CXCR4 can be overexpressed on the surface of SH-SY5Y cells, and is down-regulated from the cell-surface in response to treatment with SDF-1. To address the role of CXCR4 structural domains on CXCR4 endocytosis, we first tested the effects of overexpressing wild type CXCR4 tagged with fluorescent YFP (CXCR4wt) in SH-SY5Y cells. A) Typical confocal fluorescent microscopic image of a transfected SH-SY5Y cell, in which CXCR4wt (green) is seen localized to both the cell-surface and within endosomal compartments. B) SH-SY5Y cells were transiently-transfected with plasmids encoding either YFP alone (vector control) or CXCR4wt-YFP. Cell-surface CXCR4 levels of YFP-expressing cells were assayed 24 hrs later following CXCR4 staining as in Fig. 1; electronic gating of YFP+ cells was used to compare CXCR4 levels only on transfected cells. C) SH-SY5Y cells transfected as in B were stimulated with SDF-1 for 60 min, then cell-surface CXCR4 levels on transfected cells were assayed by flow cytometry as in B. A summary of multiple experiments is shown; bars denote the mean CXCR4 cell-surface levels of SDF-1- treated as compared to unstimulated cells for 3 independent experiments \pm S.E.M.

MOL #89714

FIGURE 4: The cytoplasmic “tail” domain of CXCR4 consisting of the carboxyl-terminal amino acid residues 322-352 is not required for cell-surface expression in SH-SY5Y cells, but is required for SDF-1 treatment to down-regulate CXCR4 from the cell-surface. SH-SY5Y cells were transiently-transfected with plasmids encoding either YFP-tagged wild-type (wt) CXCR4 or a YFP-tagged mutant version of CXCR4 that lacks the “tail” domain (Δ 322-352). 24 hrs later, the cells were assayed as in Fig. 3B&C to determine if SDF-1 treatment can down-regulate YFP-tagged CXCR4. A) Results of a representative experiment, indicating that 60 min of SDF-1 treatment down-regulates cell-surface CXCR4wt but not CXCR4 Δ 322-352. B) Summary of multiple experiments performed as in A; bars denote the mean CXCR4 cell-surface levels of SDF-1 - treated as compared to unstimulated cells for 3 independent experiments \pm S.E.M.; **, significantly down-regulated as compared to unstimulated cells, $p < 0.01$.

FIGURE 5: Two distinct regions of the CXCR4 “tail” are both required for SDF-1 treatment to down-regulate CXCR4 cell-surface expression in SH-SY-5Y cells: the 342-352 region of CXCR4, and the CXCR4 IL motif. A) Cartoon of CXCR4 showing the amino acid residues of the 322-352 “tail” domain. Triangles denote the positions of CXCR4 deletion mutants; the IL motif at residues 328 and 329 is underlined. B) The carboxyl-terminal amino acid residues of each CXCR4 mutant are shown. C) SH-SY5Y cells were transiently-transfected with plasmids encoding either YFP-tagged wild-type (wt) CXCR4 or the indicated YFP-tagged mutant CXCR4 construct. 24 hrs later, SDF-1 - stimulated down-regulation of YFP-tagged CXCR4 was assayed as in Fig. 3B&C. A summary of the results of multiple experiments is shown; each bar denotes the mean CXCR4 cell-surface levels of SDF-1- treated as compared to

MOL #89714

unstimulated cells expressing each CXCR4 construct for 3-5 independent experiments \pm S.E.M.; *, significantly different from wild-type (wt) CXCR4, $p < 0.05$.

FIGURE 6: β -arrestin expression is required for SDF-1 treatment to efficiently down-regulate cell-surface CXCR4 in the related SK-N-SH neuroblastoma cell line.

A) SH-SY5Y cells were pretreated either with or without 0.6 M sucrose, then assayed for SDF-1 - dependent down-regulation of endogenous cell-surface CXCR4 as in Fig. 1. The results of 3 independent experiments are shown \pm S.E.M.; *, significantly different from untreated, $p < 0.05$. B) Compared to SH-SY5Y cells, SK-N-SH cells are deficient in expression of β -arrestin1 protein. Top gel: western blot of whole cell lysates of SH-SY5Y cells and their variant neuroblastoma cell line SK-N-SH. As a control, the same blot was stripped and re-blotted for Actin (bottom gel). C) Results of a representative experiment, showing that SDF-1 treatment is defective in stimulating down-regulation of cell-surface CXCR4 in SK-N-SH cells. D) The indicated cell lines were transiently-transfected, with either CXCR4wt alone or CXCR4wt plus a β -arrestin1 expression plasmid, as indicated. 24 hrs later, the SDF-1 - stimulated down-regulation of YFP-tagged CXCR4 was assayed as in Fig. 3B&C. Bars denote the mean CXCR4 cell-surface levels of SDF-1- treated as compared to unstimulated cells for 3-7 independent experiments \pm S.E.M. A comparison of SDF-1 stimulated down-regulation of cell-surface CXCR4 in SH-SY5Y and SK-N-SH cells is statistically different (**, $p < 0.01$). Co-transfection with β -arrestin1 significantly enhanced the ability of SDF-1 treatment to down-regulate cell-surface CXCR4 on SK-N-SH cells (*, $p < 0.05$) and is not statistically different from SH-SY5Y cells transfected with YFP-tagged wt CXCR4 alone ($p > 0.05$). E) Results of a representative experiment as in D, for SK-N-SH cells transiently-transfected with CXCRwt and β -arrestin1. F) β -arrestin1 protein is

MOL #89714

overexpressed 24 hrs after transient transfection of the β -arrestin1 expression plasmid, shown using Jurkat T cell line and immunoblotting whole cell lysates as in B (top gel). Control cells were transiently transfected with plasmid vector alone. The same blot was stripped and reblotted for Actin as a control (bottom gel).

FIGURE 7: The 342-352 region of CXCR4, but not the CXCR4 IL motif, is required for β -arrestin to regulate cell-surface levels of CXCR4 in SK-N-SH cells. SK-N-SH cells were transiently-transfected with β -arrestin1 plus either YFP-tagged wild-type (wt) or mutant CXCR4. 24 hrs later, the SDF-1 - stimulated down-regulation of YFP-tagged CXCR4 was assayed as in Fig. 3C. A) Summary of multiple experiments as in B. Bars denote the mean CXCR4 cell-surface levels of SDF-1 - treated as compared to unstimulated cells for 3 independent experiments \pm S.E.M. Deletion of either the entire tail domain (Δ 322-352) or the final 10 amino acid residues (Δ 342-352) significantly inhibited the down-regulation of CXCR4 associated with β -arrestin1; *, significantly different from CXCR4wt ($p < 0.05$). B) Results of a representative experiment performed as in A.

FIGURE 8: GRK2 participates in regulating CXCR4 cell-surface levels and internalization. A) Western blot of whole cell lysates of SH-SY5Y cells and their variant neuroblastoma cell line SK-N-SH showing endogenous GRK2 expression levels as compared to ERK2 control. B) The indicated cell lines were transiently-transfected with either CXCR4wt alone or CXCR4wt plus a GRK2 expression plasmid or CXCR4 Δ 342-352 alone or CXCR4 Δ 342-352 plus a GRK2 expression plasmid, as indicated. Including the GRK2 expression plasmid significantly reduced CXCR4 surface levels regardless of CXCR4 construct.

MOL #89714

Bars denote the mean CXCR4 cell-surface levels as compared to unstimulated without GRK2 addition for 3 independent experiments \pm S.E.M.; ***, $p < 0.001$. C) Positive control western blot of whole cell lysates of Jurkat T cells transfected with the GRK2 expression plasmid. D) Representative flow cytometric data from one experiment, showing that the decrease in cell-surface CXCR4 levels on SH-SY5Y cells following 60 min treatment with SDF-1 is enhanced by GRK2 overexpression. E) Pooled data as in D; bars denote the mean CXCR4 cell-surface levels of SDF-1 - treated cells as compared to unstimulated cells for 3 independent experiments \pm S.E.M. ***, $p < 0.001$. F) SK-N-SH cells were transiently-transfected with YFP-tagged CXCR4wt and either GRK2 or GRK2 and β -arrestin1 expression plasmids. 24 hr later, the SDF-1 - stimulated down-regulation of YFP-tagged CXCR4 was assayed as in Fig. 3C. Down-regulation of YFP-tagged CXCR4 was not significantly different from GRK2 transfected cells, $p > 0.1$; ***, down-regulation of YFP-tagged CXCR4 was significantly different from GRK2 and β -arrestin1 transfected cells, $p < 0.001$.

Figure 1

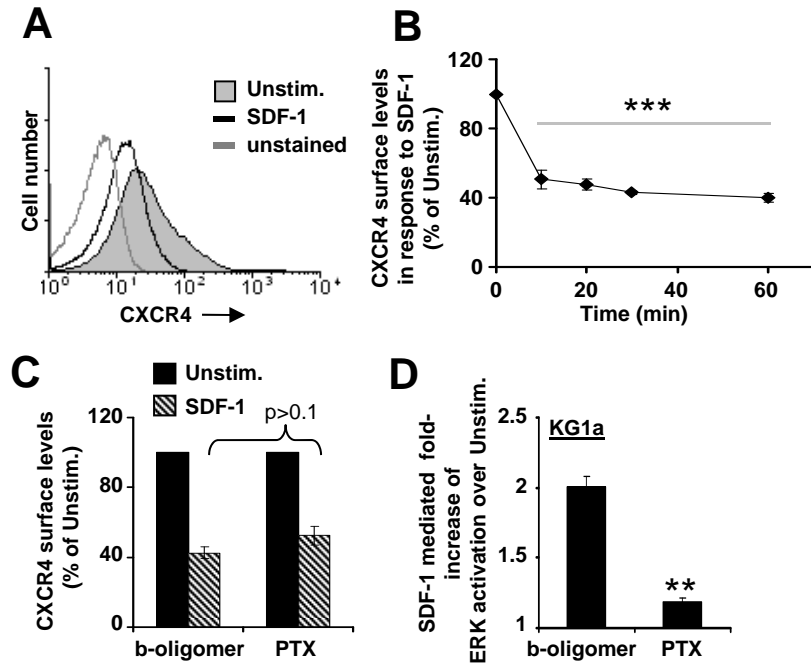


Figure 2

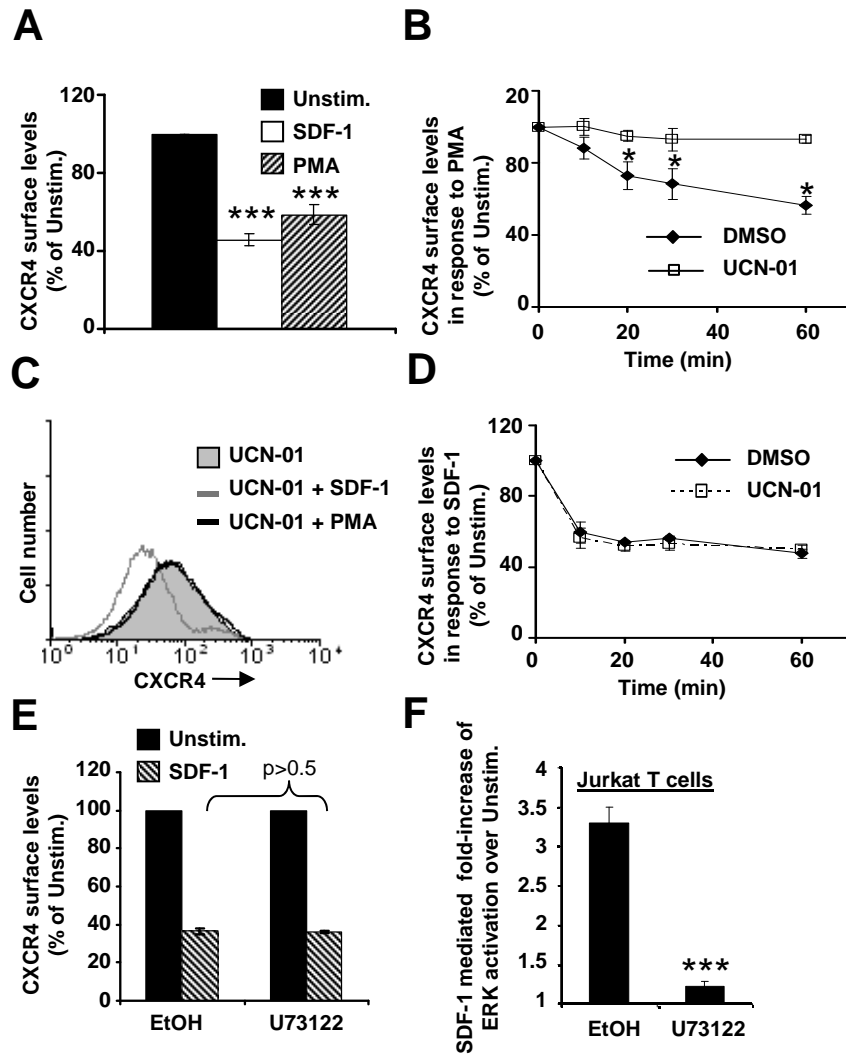


Figure 3

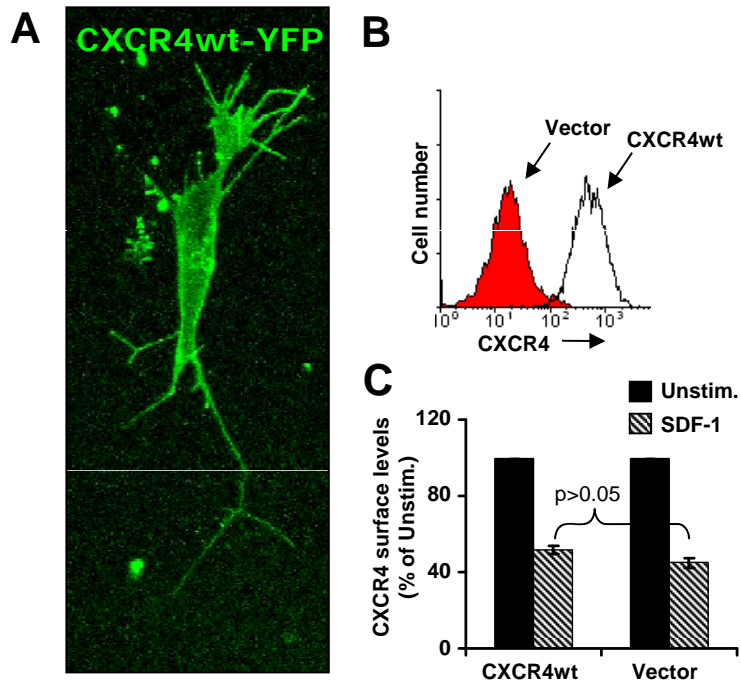
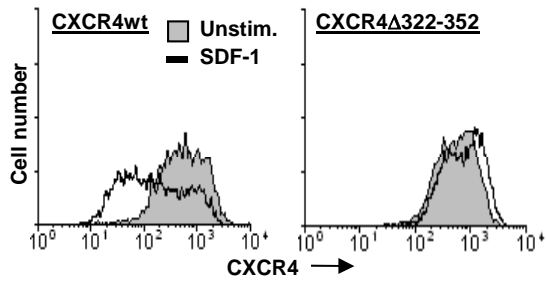


Figure 4

A



B

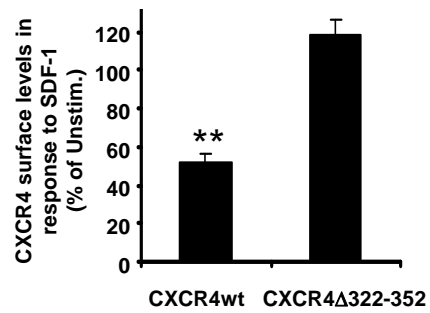
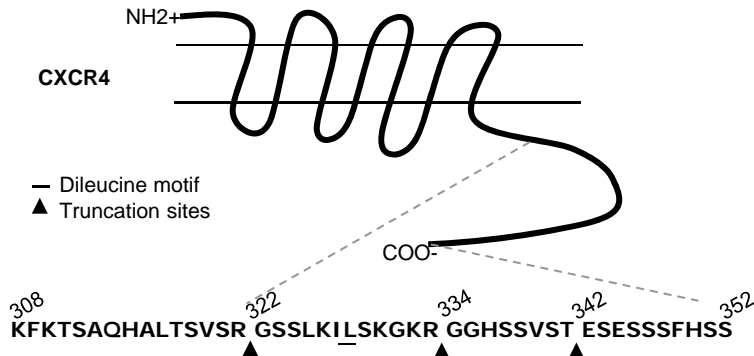


Figure 5

A



B

CXCR4wt:

KFKTSAQH~~ALT~~SVSR GSSLKILSKGKR GGHSSVST EESS~~SS~~FHSS (-YFP)

CXCR4 Δ 322-352:

KFKTSAQH~~ALT~~SVSR(-YFP)

CXCR4 Δ 334-352:

KFKTSAQH~~ALT~~SVSR GSSLKILSKGKR(-YFP)

CXCR4 Δ 342-352:

KFKTSAQH~~ALT~~SVSR GSSLKILSKGKR GGHSSVST(-YFP)

CXCR4-324/325:

KFKTSAQH~~ALT~~SVSR G~~AA~~LKILSKGKR GGHSSVST EESS~~SS~~FHSS (-YFP)

CXCR4-338/339:

KFKTSAQH~~ALT~~SVSR GSSLKILSKGKR GGH~~AA~~VST EESS~~SS~~FHSS (-YFP)

CXCR4-IL:

KFKTSAQH~~ALT~~SVSR GSSLK~~AA~~SKGKR GGHSSVST EESS~~SS~~FHSS (-YFP)

C

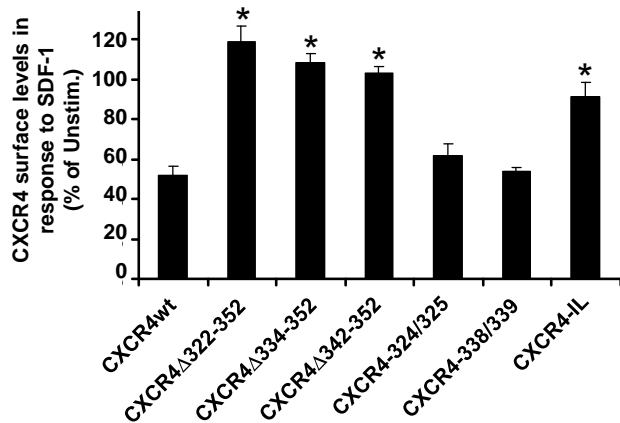


Figure 6

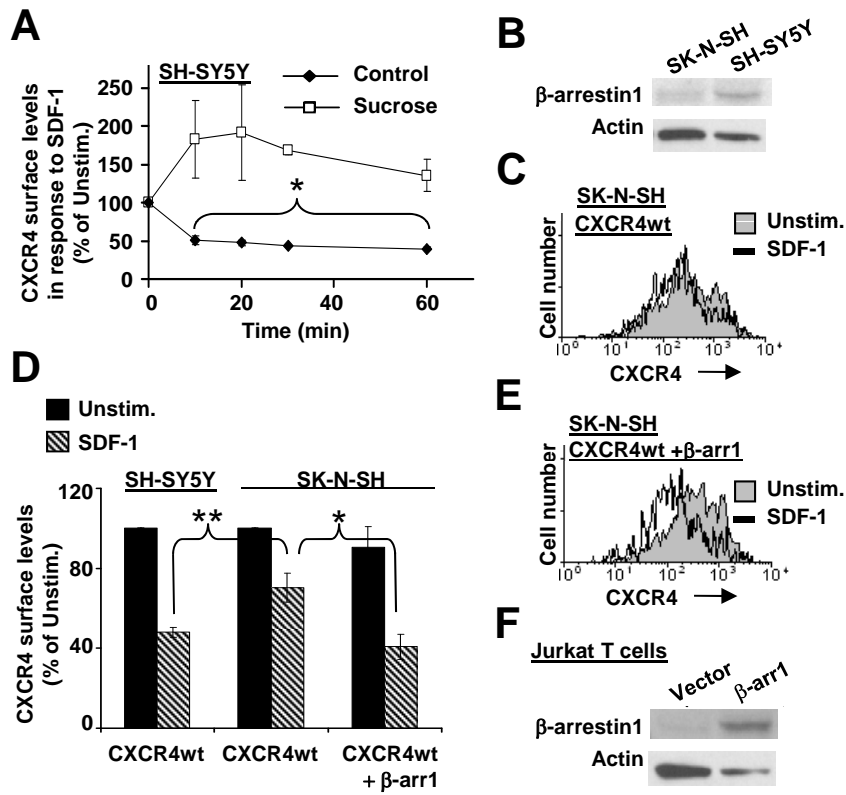


Figure 7

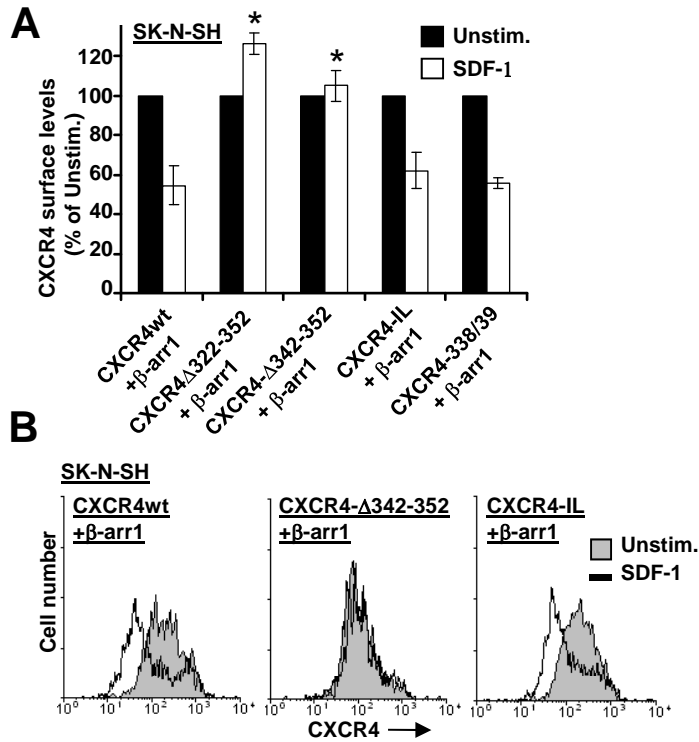
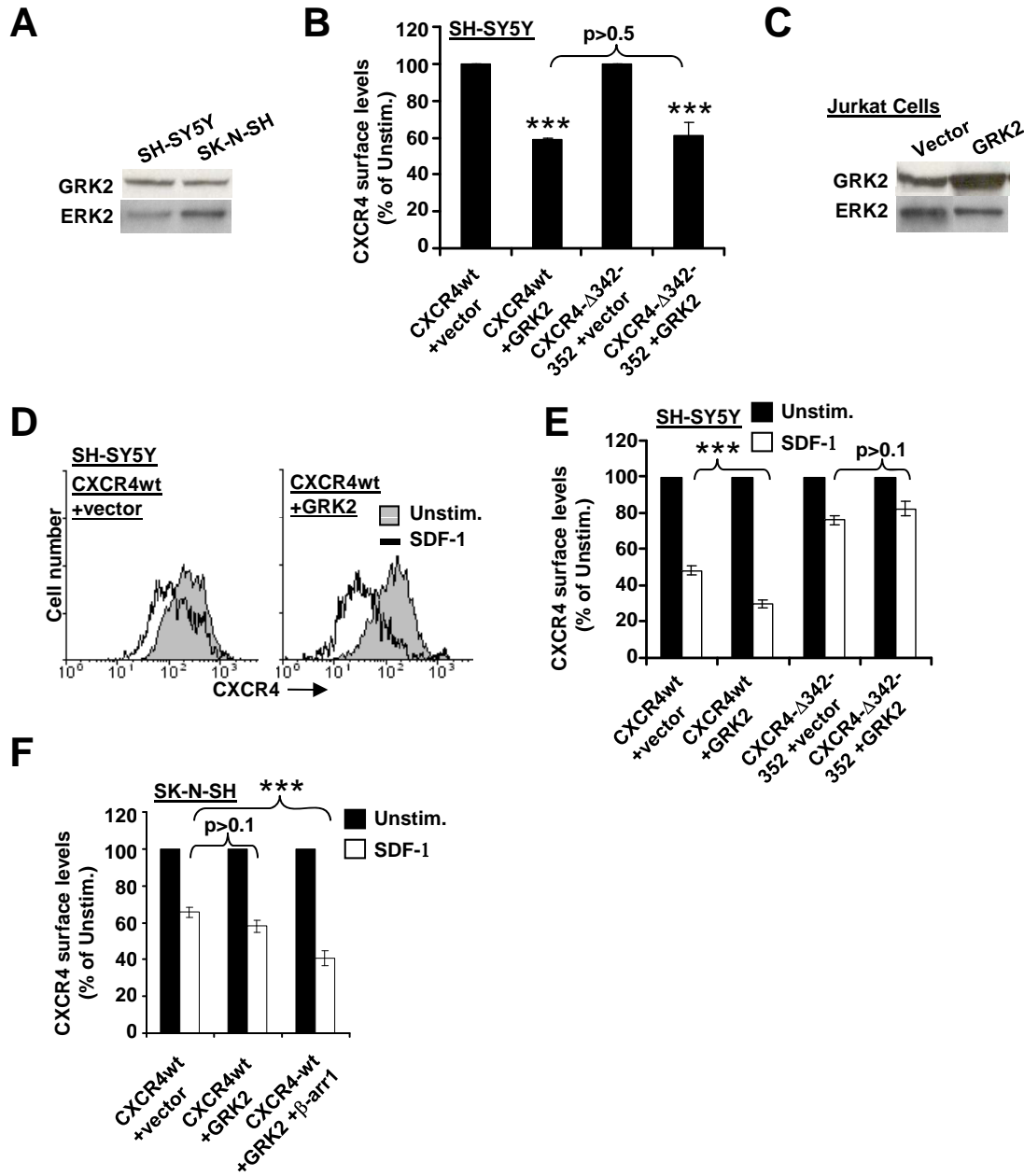
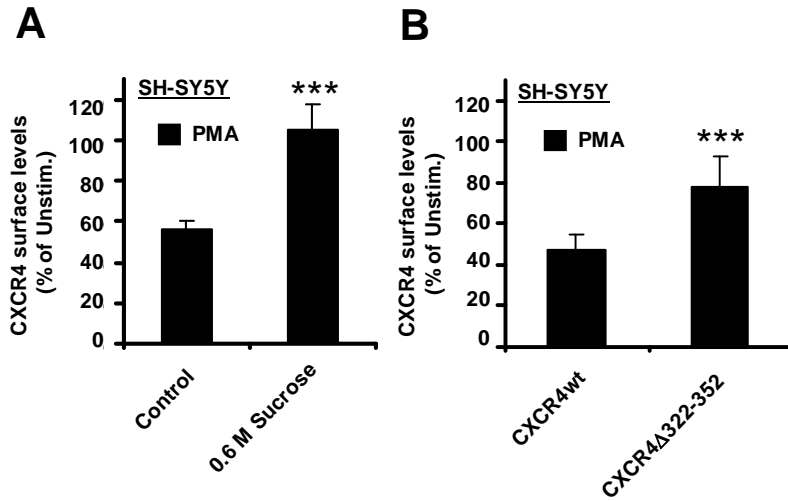


Fig. 8

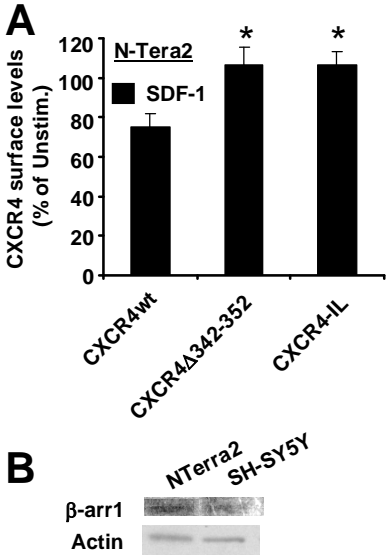


Supplemental 1



Supplemental Figure 1: **PMA mediated CXCR4 internalization is inhibited in the presence of sucrose and requires the CXCR4 intracellular tail.** SH-SY5Y neuroblastoma cells were treated with either nothing ("Unstim.") or PMA at 37 °C for 60 min. Cell-surface CXCR4 levels were assayed on individual cells via flow cytometry after staining of intact cells with APC-conjugated CXCR4 mAb. A) Surface levels of CXCR4 in PMA treated cells as a percentage of unstimulated ("Unstim.") in control and 0.6 M sucrose conditions. B) Surface levels of CXCR4 in PMA treated cells as a percentage of unstimulated ("Unstim.") in CXCR4wt GFP and CXCR4D322-352 GFP plasmid transfected overexpressions. Each point denotes the mean CXCR4 cell-surface level of PMA - treated as compared to unstimulated cells for three independent experiments \pm S.E.M.; ***, significantly different from results using unstimulated cells, $p < 0.001$.

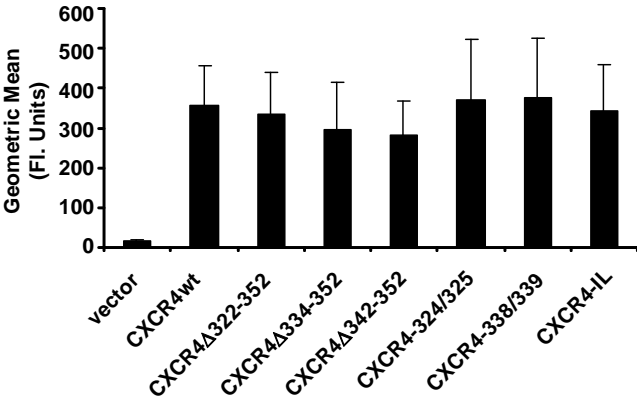
Supplemental 2



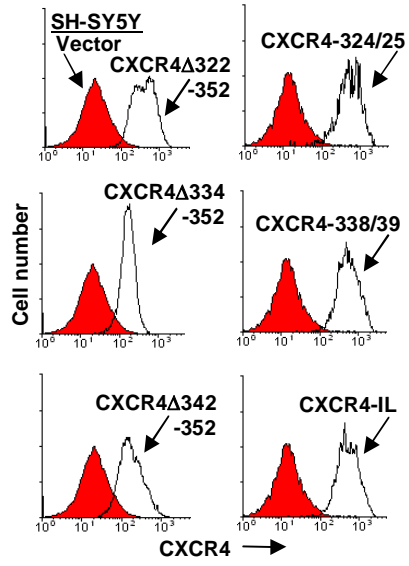
Supplemental Figure 2: **N-Tera2 cells contain β -arrestin1 and SDF-1 dependant CXCR4 internalization is inhibited by mutations to the CXCR4 tail region.** N-tera2 cells were treated with either nothing ("Unstim.") or SDF-1 at 37 °C for 60 min. Cell-surface CXCR4 levels were assayed on individual cells via flow cytometry after staining of intact cells with APC-conjugated CXCR4 mAb. A) Surface levels of CXCR4 in SDF-1 treated cells as a percentage of unstimulated ("Unstim.") in CXCR4wt-YFP, CXCR4 Δ 342-352, and CXCR4-IL plasmid transfected overexpressions. Each point denotes the mean CXCR4 cell-surface level of PMA - treated as compared to unstimulated cells for three independent experiments \pm S.E.M.; *, significantly different from results using unstimulated cells, $p < 0.05$. B) Western blot showing the expression of β -arrestin1 in N-Tera2 and SH-SY5Y cells compared to Actin control.

Supplemental 3

A

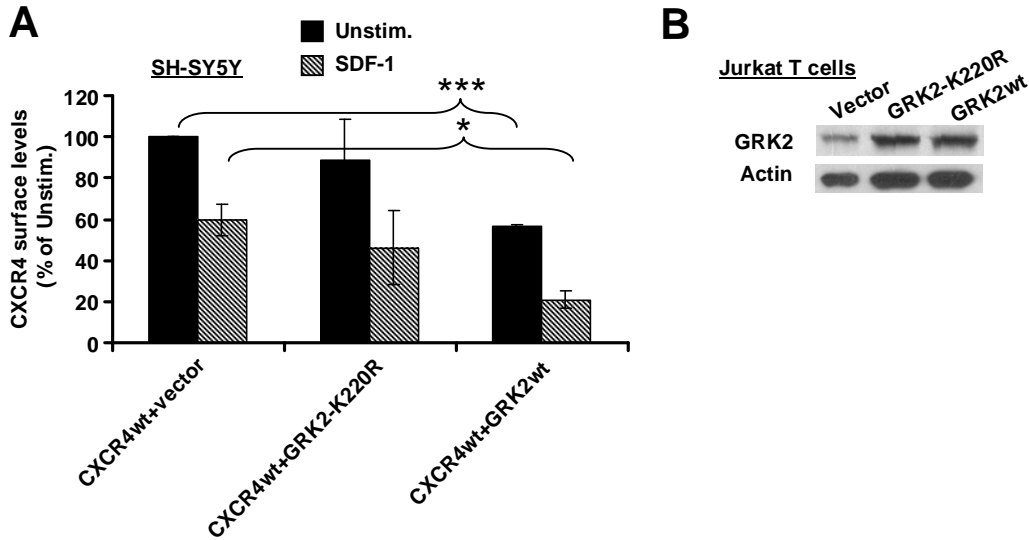


B



Supplemental Figure 3: **CXCR4 surface levels in CXCR4wt and mutant plasmid overexpression vectors.** Shown as the geometric mean of fluorescent units. A) Surface levels of CXCR4 in Vector transfected cells compared to CXCR4 plasmid overexpression mutants represented in mean geometric units; $n > 3$. B) Representative FACS plots comparing vector transfected cells to individual mutant overexpressions as in A.

Supplemental 4



Supplemental Figure 4: **GRK2 requires K220-mediated kinase activity to participate in regulating CXCR4 cell-surface levels and internalization.** A) SH-SY5Y cells were transiently-transfected with either CXCR4wt alone or CXCR4wt plus GRK2 or point-mutated, kinase-inactive GRK2 (GRK2-K220R) expression plasmids, as indicated. The GRK2 expression plasmid, but not GRK2-K220R, significantly reduced CXCR4 cell-surface levels, and also enhanced SDF-1 - dependent CXCR4 internalization. Bars denote the mean CXCR4 cell-surface levels compared to unstimulated without GRK2 addition for 3 independent experiments \pm S.E.M.; ***, $p < 0.001$; *, $p < 0.05$. B) Positive control western blot of whole cell lysates of Jurkat T cells showing that transfection with GRK2 or GRK2-K220R expression plasmids induce similar levels of GRK2 overexpression in comparison to actin control.