ROLE OF SDF-1/CXCR4 SIGNALING IN REGULATION OF PKA ACTIVITY DURING CELL MIGRATION

by

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Abstract

Cell migration plays an important role in development and the immune system, and the signaling pathways governing this process are regulated in a spatial and temporal manner. One important signaling molecule is cAMP-dependent Protein Kinase (PKA), which has been shown to be a key regulator of migration. In migrating cells, PKA activity exists in a gradient that is highest at the leading edge, where it phosphorylates proteins that promote migration. Although the functions of PKA in migration have been identified, upstream regulators of PKA-mediated cell migration have yet to be defined.

A candidate for regulation of PKA during migration is the chemokine receptor CXCR4, which has been shown to induce cell migration and signal through PKA-phosphorylatable proteins upon ligation with stromal derived factor-1 (SDF-1). I have created a novel CXCR4 deficient Jurkat cell line, JC4, and show that Jurkat cell migration towards SDF-1 is CXCR4-dependent. As well, we show through biochemical studies that the SDF-1/CXCR4 signaling pathway is an upstream regulator of PKA activation in CHO-CXCR4 cells, J774 macrophages, and Jurkat T-cells. Furthermore, the SDF-1/CXCR4 signaling pathway is an upstream regulator of PKA activation in migrating cells. This is supported by the observation that there is a CXCR4-dependent increase in PKA-phosphorylated substrates at the periphery of J774 macrophages and protrusions of Jurkat T-cells upon SDF-1 stimulation. Furthermore, SDF-1/CXCR4 signaling establishes a PKA activity gradient in migrating Jurkat cells that is highest at the leading edge. These studies define a regulatory role for the SDF-1/CXCR4 signaling pathway in promoting PKA-mediated cell migration.

Preface

This dissertation is composed of original and unpublished work from the author, Daniel He, with the exception of the plasmid constructs in Figure 12A which were designed by Dr. Chinten James Lim. All other figures are the product of my original work.

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List of Abbreviations

A-kinase activity reporter	AKAR
A-kinase anchor protein	AKAP
Actin depolymerizing factor	ADF
Adenomatous polyposis coli	APC
Adenylyl cyclase	AC
All-trans retinoic acid	ATRA
Analysis of variance	ANOVA
Atrophin interacting protein 4	AIP4
Basic fibroblast growth factor	bFGF
Bcl-2-associated death promoter	BAD
Bicinchoninic Acid	BCA
Bovine serum albumin	BSA
Calmodulin	CaM
Chinese Hamster Ovary	CHO
Clustered regularly interspaced short palindromic repeats	CRISPR
CRISPR-associated	Cas
Cyan fluorescent protein	CFP
Cyclic adenosine monophosphate	cAMP
Differential interference contrast	DIC
Dimethyl sulfoxide	DMSO
Dulbecco's modified eagle medium	DMEM
Epidermal growth factor	EGF
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Fluorescein isothiocyanate	FITC
Fluorescence activated cell sorting	FACS
Fluorescence resonance energy transfer	FRET
Focal adhesion kinase	FAK
Forskolin	Fsk

G protein-coupled receptor	GPCR
G protein receptor kinase	GRK
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
GTPase activating protein	GAP
Guanine nucleotide exchange factor	GEF
Human immunodeficiency virus	HIV
Human umbilical vein endothelial cell	HUVEC
Hypoxia inducible factor 1	HIF-1
Integrin-linked kinase	ILK
Integrin cytoplasmic domain-associated protein	ICAP1
Interferon	IFN
Interleukin	IL
Intracellular adhesion molecule	ICAM
Liver-enriched inhibitory protein	LIP
Look up table	LUT
Lymphocyte function-associated antigen	LFA
Macrophage colony-stimulating factor	M-CSF
Major histocompatibility complex	MHC
Microtubule organizing center	MTOC
Mitogen activated protein kinase	MAPK
Myosin light chain	MLC
Myosin light chain kinase	MLCK
Nuclear respiratory factor-1	NRF-1
Numerical aperture	NA
p21-activated kinase	PAK
PAK-interacting exchange-factor-β	β-PIX
Parathyroid hormone	PTH
Phosphoinositide 3-kinase	PI3K
Pleckstrin homology	PH
Phosphate buffered saline	PBS
Phosphatidylinositol (3,4)-bisphosphate	PtdIns(3,4)P_2

Phosphatidylinositol (3,4,5)-trisphosphate	PIP_3
Platelet-derived growth factor	PDGF
Phospholipase C	PLC
Polyacrylamide Gel Electrophoresis	PAGE
Protein Kinase A	PKA
Protein Kinase B	PKB
Protein Kinase C	PKC
Protein kinase inhibitor peptide	PKI
Rap1-GTP-interacting adapter molecule	RIAM
Region of interest	ROI
Regulator of G protein signaling	RGS
Rho-associated, coiled-coil-containing protein kinase	ROCK
Roswell Park Memorial Institute medium	RPMI
SH2 domain-containing inositol phosphatase 1	SHIP1
Sodium dodecyl sulfate	SDS
Stromal derived factor-1	SDF-1
Tetramethylrhodamine	TRITC
Toll-like receptor 2	TLR2
Transforming growth factor	TGF
Tris-buffered saline	TBS
Tumor necrosis factor	TNF
Vascular cell adhesion molecule	VCAM
Vascular endothelial growth factor	VEGF
Vasodilator-stimulated phosphoprotein	VASP
Very late antigen	VLA
von Hippel-Lindau	VHL
WASP-family verprolin-homologous protein	WAVE
Wiskott-Aldrich syndrome protein	WASP
Yellow fluorescent protein	YFP
Yin-Yang 1	YY1

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To my parents, for their love and support

1. Introduction

1.1 Cell Migration

Cell migration is an important process in development, immunity, and disease, involving many signaling events that are tightly regulated in a spatial and temporal manner. It can vary from single cell migration to a coordinated multicellular form known as collective cell migration, where one or more cells lead trailing cells that are tightly linked to each other. These forms of migration serve different purposes in the body: collective migration is involved in processes such as tissue repair, while single cell migration is important in leukocyte trafficking in immunity as well as neural crest cell migration in morphogenesis[1].

Within single cell migration, there can be amoeboid and mesenchymal migration (Figure 1A). Rounded cells that extend and retract pseudopodia move in a rapid, gliding fashion known as amoeboid migration, and this is most commonly seen in leukocytes[1,2]. There is also an alternative form of amoeboid migration involving a propulsive mechanism driven by actin polymerization[2] as seen in zebrafish germ cells[3], *Dictyostelium*[4,5], and neutrophils[6,7]. Mesenchymal migration, seen in fibroblasts[8] and cancer cells[9], differs from amoeboid in that it is highly dependent on focal interactions with the extracellular matrix (ECM) at the leading edge and generally proceeds in a much slower rate[1]. Despite these differences in mechanism, cell migration is a cyclic repetition of polarization, membrane protrusion, and retraction of the cell rear[1] (Figure 1B).

Cell migration is a complex interplay of cytoskeletal components, membrane receptors, adaptor proteins, and signaling kinases. The spatial and temporal localization of these molecules are crucial to the efficient migration of a cell. Of particular importance are the Rho family of small GTPases which are active when GTP-bound, a process mediated by guanine nucleotide exchange factors (GEFs) and inhibited by GTPase activating proteins (GAPs). The Rho GTPases Cdc42, Rac, and RhoA have been extensively studied in cell migration[10]. The following sections will investigate each phase of cell migration with respect to its signaling pathways, with a specific focus on the signaling molecule protein kinase A (PKA).

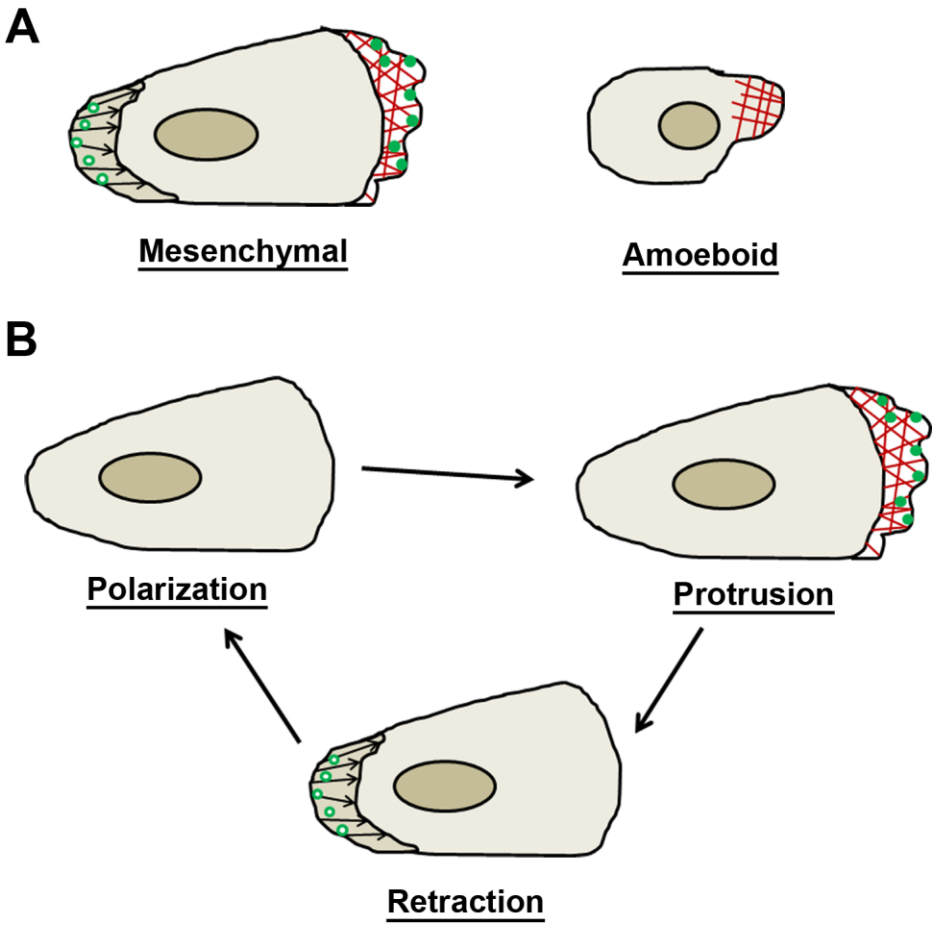

Figure 1. Overview of cell migration. (A) Two main forms of single cell migration. Mesenchymal migration is seen in spread, slow moving cells, while amoeboid migration is characterized by rounded cells that migrate rapidly. Amoeboid migration is less dependent on adhesion formation in comparison to mesenchymal migration. (B) Three main steps in cell migration. Establishment of a leading "front" edge and a trailing "rear" edge is referred to as polarization, and it is mediated by Cdc42 signaling. Another step is protrusion, where the actin cytoskeleton undergoes reorganization to push the cell forward. The protrusive pushing motion is obtained through polymerization of new filaments (red lines), as mediated by Rac and Cdc42. As well, new adhesions (green dots) are being formed within the protrusions. Lastly, the cell must retract in order to move forward, and this is mostly composed of signaling events such as Rho activation that lead to disassembly of adhesions (empty green dots).

1.1.1 Protrusion

The process by which cells extend protrusions in the direction of movement is dependent on the actin cytoskeleton, as its polymerization and organization provide the forward pushing of the cell membrane. Monomeric G-actin is polymerized to form filamentous F-actin, and these filaments display a polarity with a growing "barbed" end that incorporates new G-actin and a "pointed" end that serves as the origin of the filament[11].

Actin polymerization is dependent on the pool of available monomeric G-actin, which is buffered by G-actin-binding proteins such as profilin[12] and thymosin β4[11,13]. Available G-actin can come from de novo synthesis, as β-actin RNA has been found in protrusions[14], but it can also come from recycled G-actin from the depolymerization of the pointed end of actin filaments. Depolymerization can be spontaneous due to filament aging or by actin depolymerizing factor (ADF)/cofilin, which catalyzes filament severing[15,16]. The action of cofilin is regulated by LIM kinase through phosphorylation[17] as well as coronin[18], which also protects actin from cofilin severing[19].

Actin filaments can be organized in bundles or a more branched, dendritic form depending on the actin-binding proteins it associates with. Bundles are laterally bound actin filaments that can be parallel (both barbed ends in the same direction) or antiparallel (barbed ends are at opposite ends)[20]. This is mediated by the actin cross-linking proteins α-actinin, fascin, fimbrin, and myosin II[21–23].

At the growing barbed end of actin filaments, polymerization is regulated by promoters and capping proteins. Promoter proteins bind to the barbed end in order to recruit G-actin monomers through profilin for filament assembly as well as protect the barbed end from binding of regulatory capping proteins. Examples of promoter proteins include formin, which can also serve as a nucleation site for formation of new filaments[24], and vasodilator-stimulated phosphoprotein (VASP)[25]. This mechanism is likely to predominate in the formation of filopodia, which contain longer bundled actin filaments[26]. Capping proteins serve as antagonists to this mechanism by binding to the barbed end and blocking polymerization[27]. These include gelsolin[28], brevin[29], capZ[30], capG[31], adducin[32], severin[33], villin[34], adseverin[35], advillin[36], and supervillin[37].

Actin nucleators bind to the pointed end of actin filaments in order to provide a scaffold for polymerization at the barbed end. One major nucleator is the seven-subunit Arp2/3 complex, which is activated by the WASP/WAVE (Wiskott-Aldrich syndrome protein; WASP-family verprolin-homologous protein) family of proteins. The Arp2/3 complex binds to the side of an actin filament and promotes the polymerization of a new daughter filament. This results in the branched actin filament organization commonly seen in lamellipodia[38].

There are two main types of protrusions that contribute to cell migration, and they can be differentiated by their actin polymerization. Filopodia are thin protrusions characterized by long parallel actin bundles and serve as sensors to probe the environment. In contrast, lamellipodia contain branched short actin filaments, and this actin geometry allows for the extension of a broad protrusion more suited as the basis for directional migration. Following the lamellipodia is the lamellum, which differs in that its actin is bundled rather than branched[20,39–41].

The master regulators of protrusion are the Rho family of small GTPases. RhoA plays an important role in migration through its downstream effector ROCK (Rho-associated, coiled-coil-containing protein kinase). Cofilin activity is inhibited by LIM kinase phosphorylation[42], and LIM kinase is activated by p21-activated kinase 1 (PAK1, which is activated by Rac and Cdc42)[43] and ROCK[44]. Formins are activated by binding the Rho GTPases RhoA and Cdc42[45]. Lastly, Rho GTPases regulate the WASP/WAVE proteins: Cdc42 (in conjunction with PIP2) bind and activates WASP proteins[46], while Rac does the same to WAVE proteins[47].

1.1.2 Polarization

Polarization in a migrating cell refers to the differences in morphology and signaling between the leading “front” edge and the trailing “rear” edge, which can be spontaneous or induced by extracellular cues such as chemoattractants. Typically these cues are presented in a concentration gradient, which induces the redistribution of the binding receptors and downstream signaling events involved in establishing polarity and migration. In spontaneous polarization, cells form the “rear” first by localizing inhibitory protrusive signals and reorganizing actin into bundles in order to create zones with no protrusive activity. This mechanism has been shown to be dependent on myosin II[20].

The Rho GTPase Cdc42 is the major signaling molecule in regulating cellular polarity during migration. Cdc42 activity is highest at the tip of the leading edge in migration[48]. The Ras-related GTPase Rap1 is required for its activation[49], and positive feedback loops involving the β-PIX/Scribble complex[50] and PAK1[51] can sustain localized Cdc42 activation. Cdc42 signals through Par3, Par6, PKCζ, GSK-3, and adenomatous polyposis coli (APC) in order to localize the microtubule organizing center (MTOC) and Golgi apparatus towards the leading edge[52], which aids in facilitating delivery of protrusion-promoting proteins to the cellular front[53]. This is particularly important in larger cells undergoing mesenchymal migration as diffusion or actin-based transport is not enough to ensure adequate protein transport to the front. Certainly, in smaller cells such as leukocytes undergoing amoeboid migration, the MTOC and Golgi localization is not seen. Hence, Cdc42 is crucial in maintaining protrusions at the leading edge through localization of the MTOC and Golgi apparatus[53].

Chemoattractant stimulation results in the localized activation of phosphoinositide 3-kinase (PI3K) at the leading edge, which generates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and phosphatidylinositol (3,4)-bisphosphate ($PtdIns(3,4)P_2$)[54,55]. This results in activation of Rho GTPases at the leading edge, as PIP_3 and $PtdIns(3,4)P_2$ recruit certain Rho GEFs as well as Akt/PKB through binding to the Pleckstrin homology (PH) domain[56–59]. These Rho GEFs can activate Cdc42 and Rac, both of which stimulate actin polymerization in membrane protrusion as described in the previous section. In addition to activation of PI3K at the leading edge, there is activation of PTEN at the trailing edge in order to maintain the PIP_3 gradient within the cell as PTEN degrades PIP_3 through hydrolyzation[60]. In neutrophils and lymphocytes, particularly in leukemic cell lines lacking PTEN such as Jurkat[61,62], this is mediated by SHIP1 (SH2 domain-containing inositol phosphatase 1)[63–65]. Therefore, the intracellular PIP_3 gradient serves to localize and activate important molecules regulating polarization.

1.1.3 Adhesion in Cell Migration

Adhesion to the ECM plays an important role in cell migration as it provides tractional forces and activates signaling pathways required for the cell to pull itself forward. This section will focus on the types of adhesions, important signaling pathways, and the major adhesion receptor called integrins.

Classification of Adhesions

Cellular adhesions during migration can be classified into subtypes. The lamellipodium first forms nascent adhesions, which are small, linked to dendritic actin, and transient – they may continue to mature into stable adhesions or disassemble[66–68]. Another type of adhesion are focal complexes, which are larger, more mature than nascent adhesions and located at the leading edge of protrusions between the lamellipodium and lamellum[69,70]. Like nascent adhesions, they may also mature into more stable adhesions or disassemble, and their formation is dependent on Rac and myosin[70]. Both nascent adhesions and focal complexes are found in motile, fast moving cells such as leukocytes, which correlates with their high turnover rate as these cells continually form protrusions during their migration. Focal adhesions are the most mature type of adhesion, and are generally seen in stationary if not slow migrating cells[71,72]. They are developed from focal complexes, which mature through elongation along an actin "template", and can be distributed throughout the cell[20,39,41]. The purpose of focal adhesions is to provide a link between the actin cytoskeleton and the ECM, and this will be covered in the following section.

Finally, highly motile and invasive cells contain actin-rich circular adhesions at their periphery, and these are termed podosomes and invadopodia[73,74]. Although they are similar in structure, podosomes are found in macrophages and dendritic cells, while the term invadopodia is reserved for invasive cancer cells[20,39]. These structures have actin-rich cores that also contain actin polymerizing and regulatory proteins (cortactin, gelsolin, WASP, Rho GTPases, Arp2/3) to promote protrusion. In podosomes, the core is surrounded by a ring of integrins and adhesion proteins such as paxillin, focal adhesion kinase (FAK), and Pyk2, which differs from invadopodia as these components are located within the core[75,76].

Adhesion Assembly and Disassembly

Adhesion formation is driven by actin polymerization. Maturation of the adhesion requires elongation along actin filaments bundled by α-actinin, and this step requires paxillin, vinculin, and myosin II[67,77]. Regulation of assembly is largely by Rho GTPases. As previously mentioned, Cdc42 and Rac promote actin polymerization through WASP/WAVE proteins[46,47], while Rho activates myosin II, leading to increased contractility of adhesions[78]. Cdc42 and Rho also regulate formins, which are responsible for actin elongation[45–47]. Rho GTPases are further

regulated through activation and inhibition by GEFs and GAPs, and these are recruited by molecules such as FAK[79] and paxillin[80].

Adhesions incur rapid turnover rates within the protrusion. Nascent adhesions that are rich in paxillin have a high turnover rate, indicating a possible role in disassembly at the front[81]. For focal adhesions, disassembly is regulated by FAK and Src, as cells deficient in either have an increased number of adhesions[82]. FAK/Src signaling activates p190Rho-GAP, which decreases Rho activity, and also recruits Rac and PAK to mediate adhesion turnover[83]. PAK reduces the activity of myosin light chain kinase (MLCK), which phosphorylates the myosin light chain (MLC) to activate myosin II[84]. Reduced myosin II results in loss of large adhesions[85]. Therefore, during adhesion turnover, FAK/Src mediates a change from Rho-mediated adhesion maturation to Rac-mediated adhesion turnover.

Integrins: Major Adhesion Receptors

Integrins are αβ heterodimeric transmembrane receptors that have an ECM-binding extracellular head domain and a cytoplasmic tail domain that links to the actin cytoskeleton. There are 18 α- and 8 β-integrin subunits in mammals, and each combination has its own binding specificity for ECM ligands. For example, fibronectin is bound by α4β1, α5β1, and αvβ3; collagen is bound by α1β1 and α2β1; and laminin is bound by α2β1, α3β1, and α6β1. Furthermore, on the cell surface, the immunoglobulin superfamily cell adhesion molecule ICAM-1 (intracellular adhesion molecule-1) is bound by αLβ2 (LFA-1, lymphocyte function-associated antigen-1) and αMβ2, and VCAM-1 (vascular cell adhesion molecule-1) is bound by α4β1 (VLA-4, very late antigen-4)[39]. Both LFA-1 and VLA-4 are highly expressed on lymphocytes.

Cell adhesion can be modified through integrin clustering (avidity) or integrin activation (affinity)[86]. Integrins exist in a closed, bent conformation, but can be activated via inside-out signaling to an open conformation with high binding affinity for their respective matrix ligands[87]. This is mediated by talin and kindlin, which both bind to the cytoplasmic tail of β-integrins via their FERM domain[88,89]. Though the mechanism of kindlin is unknown, electrostatic interactions between talin and the plasma membrane result in rotation of the talin/β-integrin complex, which leads to separation of the α and β subunits into an open conformation and thus integrin

activation[90,91]. Talin-mediated integrin activation is regulated by Rap1, which recruits talin to the cytoplasmic tail of β subunits through its effector RIAM (Rap1-GTP-interacting adapter molecule)[92]. As well, PtdInsP kinase Iγ[93,94] and FAK[95] have both been implicated in talin recruitment to the membrane. Inhibition of talin-mediated integrin activation is mostly through competitive binding with other proteins such as filamin[96] and ICAP1 (integrin cytoplasmic domain-associated protein 1)[97], though phosphorylation of the talin-binding motif NPXY in β subunits by Src family kinases also inhibits talin binding[98,99]. The importance of integrin activation is highlighted by the localization of αvβ3 and α4β1 integrins to the leading edge of endothelial cells[100] and SDF-1-stimulated T cells[101], respectively, during migration, where they promote assembly of new adhesions.

As previously mentioned, integrins are the link between the ECM and the actin cytoskeleton. The cytoplasmic tail of integrins does not directly bind to actin, but rather through various actin-binding proteins such as talin[102]. Talin also binds vinculin[103,104], which can bind actin and the actin nucleating Arp2/3 complex[105]. Another actin-binding molecule, α-actinin, binds to β1 subunits[106] as well as vinculin[107]. Finally, filamin can bind to β1 and β7 subunits[108]. These complex interactions form stable adhesions required for cellular migration, and are tightly regulated through phosphorylation and binding of regulatory proteins.

Signaling within integrin-containing adhesions involves a large number of proteins that interact with each other; this is referred to as the adhesome[109]. One important molecule in the adhesome is the tyrosine kinase FAK, which is indirectly bound to β integrins through talin[95,110,111]. FAK controls activation of RhoA through binding RhoA-specific GEFs and GAPs important in maintaining stability of focal adhesions[79,112]. Furthermore, FAK activates paxillin, another protein that binds to integrins, through tyrosine phosphorylation to promote adhesion assembly[113,114]. This opens binding sites on paxillin for other adhesion-promoting proteins, such as vinculin[115], and signaling molecules, particularly activators of Rac[80]. For example, p130Cas can bind to both FAK and paxillin, and recruits CrkII/DOCK1, which is a Rac activator[116,117]. Integrin-mediated signaling is not limited to FAK, and it is comprised of many events that can affect cell migration, proliferation, and survival[109].

1.1.4 Retraction

The final step of migration is retraction of the rear, which requires adhesion disassembly and contraction of the cytoskeleton. Adhesion disassembly at the rear is mediated by similar proteins involved in disassembly at the front such as FAK and Src, which mediate adhesion turnover through MLCK and extracellular signal-regulated kinase (ERK1/2)[84]. Integrins can undergo endocytosis and be recycled in a clathrin-dependent matter[118–120]. As well, the calcium-dependent protease calpain mediates the proteolysis of FAK and talin[121–123]. Finally, contraction of the actomyosin skeleton (actin and myosin II) is mediated by the RhoA/ROCK pathway, which activates myosin II through phosphorylation of its MLC[124–128].

1.1.5 Protein Kinase A in Cell Migration

While the bulk of research into cell migration has been focused on Rho GTPases, another important signaling molecule is cyclic AMP-dependent protein kinase (PKA). Like the Rho GTPases, PKA is tightly regulated spatially and temporally in cell migration. The following sections will review the structure, function, and regulation of PKA during cell migration.

Structure

PKA is a heterotetrameric serine/threonine kinase containing two regulatory (R) and two catalytic (C) subunits. There are two types of PKA (type I and type II) that are determined by the type of R subunits, of which there are four (RIα, RIβ, RIIα, RIIβ) in addition to three C subunits (Cα, Cβ, Cγ). The N-terminus dimerization domain on each R subunit mediates the heterotetrameric assembly of PKA. The PKA holoenzyme is kept inactive by the R subunits, which bind to the catalytic cleft of the C subunit through a pseudosubstrate (RI subunits) or a true substrate (RII subunits) motif. Upon binding of two cyclic adenosine monophosphate (cAMP) molecules to the C-terminus of each R subunit, the C subunit is released from the R subunit, allowing for its serine/threonine kinase activity to occur[129].

Regulation

A-kinase anchoring proteins (AKAPs) bind to PKA through an N-terminal dimerization domain on the R subunit dimer. AKAPs are restricted to specific locations within the cell through a targeting domain, and serve to tether inactive PKA holoenzymes to promote localized substrate

phosphorylation in response to elevations in cAMP. In cell migration, AKAPs such as AKAP-Lbc, ezrin and WAVE1 can bind to the actin cytoskeleton and promote formation of protrusions. Hence, AKAPs regulate the spatial localization of PKA activity[130].

PKA in Cell Migration

The activation of PKA has been shown to be required for cell migration through filopodia and lamellipodia formation[131,132], actin filament assembly[133], and stress fiber dissolution[134]. The effects of PKA on migration are tightly regulated in a spatial manner as PKA activity is shown to be highest at the leading edge, a process that is dependent on α4β1 and α5β1 integrins[135]. Furthermore, AKAPs (and thus type I and II PKA) are also localized to the leading edge of migrating cells, and cells establish a front-to-back cAMP gradient during migration[135–137].

Specific downstream targets of PKA activity have been identified at the leading edge of migrating cells. The VASP family proteins are promoters of actin filament assembly, and localize to focal adhesions, filopodial tips, and lamellipodia. With respect to VASP family proteins, the activity of PKA diminishes their actin-nucleating mechanisms[138]. Alpha4 integrins are highly expressed on immune cells, neural crest cells, and smooth and skeletal muscle cells, and they play a major role in immunity and development. As previously mentioned, α4 integrins are key mediators of cell migration and are localized to the front of migrating cells. The α4 integrin is PKA-phosphorylated on its cytoplasmic tail at Ser988[139]. This serves to disrupt the interaction between paxillin and α4, and thereby promoting migration through negative regulation of lamellipodia formation[132,140]. Furthermore, α4 integrin serves as an AKAP for type I PKA to promote migration[137].

PKA also has numerous effects on Rho GTPases, which are important signaling molecules in cell migration as they regulate polarization, protrusions, and adhesions. Active GTP-bound Rac1 serves as an AKAP for inactive type II PKA[131,141]. This association allows for PKA to phosphorylate GTP-bound Rac1 and Rac1-GTP activated-PAK, an effector of Rac1 signaling, in response to G protein-coupled receptor (GPCR)-triggered cAMP elevation (e.g. through β-adrenergic receptor). The PKA-mediated PAK activation further contributes to signaling by enhancing ERK1/2 activation[141]. Cdc42 is not directly phosphorylated by PKA, but its activation

is PKA-dependent[142]. Lastly, at the leading edge of migrating cells, PKA activity regulates protrusion/retraction cycles through RhoA phosphorylation[143].

1.2 The SDF-1 Chemokine and its Receptor CXCR4

Chemokines are small chemotactic cytokines (8-10 kDa) that guide directed migration of leukocytes. They function to regulate homeostatic leukocyte trafficking as well as recruit leukocytes to sites of inflammation. At least 47 chemokines have been identified and are categorized based on the position of the first two cysteine residues near the N-terminus: CXC, which have a single amino acid residue between the cysteine residues; CC, which have two adjacent cysteine residues; C, which only have one cysteine residue; and CX3C, which have three amino acid residues between the cysteine residues. The chemoattractant effect of chemokines is achieved through their binding to chemokine receptors, of which 18 have been discovered and are named based on the types of chemokine they bind; for example, CC receptors bind CC chemokines. Chemokine receptors are G-protein coupled receptors, which possess seven transmembrane domains and signal through G proteins. Binding of the chemokine to its receptor dissociates the heterotrimeric G protein (consisting of Gα and Gβγ), causing the activation of signaling pathways involving the Rho family of GTPases[144].

Stromal derived factor-1 (also known as CXCL12), or SDF-1, is a CXC chemokine that was originally discovered as a stimulatory factor in B cell lymphopoiesis and is constitutively expressed in many organs such as liver, heart, and brain[145–148]by endothelial, dendritic, and stromal cells[149,150]. The receptor for SDF-1, CXCR4, is a seven-transmembrane domain GPCR and is highly expressed in hematopoietic cells, though it is also expressed in various types of stem cells as well as endothelial cells[151]. SDF-1 has six splice variant isoforms: α, β, γ, δ, ε, and ϕ/θ[147,148]. In adult humans, SDF-1α is the most commonly found isoform and is constitutively expressed in various organs (along with β), while γ is found mainly in the heart, and δ, ε, and ϕ/θ are mostly expressed in the pancreas[147]. The widespread expression of both SDF-1 and CXCR4 contributes to the pairing's homeostatic function in the body, as the extravasation of T and B cells into secondary lymphoid organs is dependent on SDF-1/CXCR4[144]. As well, the potent chemoattractive effect of SDF-1 on progenitor cells is important in organ homeostasis and wound healing[151]. SDF-1/CXCR4 signaling is also crucial to hematopoiesis and organogenesis in

development, as evidenced by the embryonic lethality of SDF-1 or CXCR4 knockouts. This section aims to provide a review on the current knowledge of the SDF-1/CXCR4 signaling axis.

1.2.1 SDF-1/CXCR4 Signaling

As previously mentioned, CXCR4 is a G-protein-coupled receptor. SDF-1-bound CXCR4 functions as a GEF, which mediates the exchange of GTP for GDP on the Gα subunit and causes the dissociation of the heterotrimeric G protein of Gα and Gβγ, allowing for G protein signaling[152] (Figure 2). The Gα subunit family can be classified into four groups ($G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q}$, and $G\alpha_{12/13}$), and SDF-1/CXCR4 can signal through all of them[153]. A brief overview of CXCR4 can be found in Figure 2.

A key feature of chemokine receptor signaling is an increase in intracellular Ca^{2+} ions. SDF-1/CXCR4 signals through phospholipase Cβ (PLCβ) to induce calcium flux[154,155]. Although $G\alpha_{q}$ and Gβγ are the only G proteins capable of activating PLCβ, CXCR4-mediated calcium flux is inhibited by the $G\alpha_{i}$ inhibitor pertussis toxin, suggesting that $G\alpha_{q}$, Gβγ, and $G\alpha_{i}$ are all involved in this process[153,154,156,157]. Calcium has been shown to function in CXCR4-mediated chemotaxis and may contribute to growth and proliferation as well[158,159].

The main function of SDF-1/CXCR4 signaling is chemotactic cell migration. Both $G\alpha_{i}$ and Gβγ signal through PI3K and Akt to phosphorylate Pyk-2, FAK, Nck, paxillin, Crk, Crk-L, and p130Cas, which are all components of focal adhesions and are involved in chemotaxis[153]. PI3K also mediates an increase in activation of Src family kinases (Src, Fyn, Lck, and Lyn), which have all been shown to be involved in SDF-1/CXCR4-mediated chemotaxis through interactions with proteins such as SHP2, cbl, ShcA, ZAP-70, and vav[160–165]. Mitogen activated protein kinase (MAPK) pathways regulate chemotaxis through both ERK1/2, which is activated by both $G\alpha_{i}$ and Gβγ through Ras/Raf, and p38 MAPK, which is activated by protein kinase C (PKC)[153,166,167].

The Rho family of GTPases, of which Rac, Rho, and Cdc42 are the most studied, are highly involved in SDF-1/CXCR4-mediated chemotaxis. Through $G\alpha_{i}$ and Gβγ, SDF-1/CXCR4 activates Rac which acts downstream to activate LIM kinase 1, which further phosphorylates

cofilin[168]. At the leading edge of migrating cells, Rac drives the formation of lamllipodia, which are actin-rich membrane ruffles[41]. Rho, important in stress fiber formation and focal adhesion stability at the rear of migrating cells, is activated by CXCR4 in a $G\alpha_{13}$-dependent manner[169]. As well, Cdc42, a protein regulating formation of lamellipodia and filopodia, signals through WASP and Arp2/3 in CXCR4 signaling, and this is mediated by $G\alpha_i$[170]. Lastly, the $G\alpha_{12/13}$ subunits mediate Rho-guanine nucleotide exchange factors, which are crucial for CXCR4-mediated chemotaxis through their activation of Rho proteins[152].

SDF-1/CXCR4 signaling has also been shown to promote cell survival and proliferation. CXCR4 mediates phosphorylation of Akt through PI3K, resulting in the upregulation of vascular endothelial growth factor (VEGF), a promoter of angiogenesis[171]. Akt and ERK1/2, both activated by SDF-1/CXCR4 signaling, have been shown to phosphorylate (and thus inactivate) the proapoptotic protein BAD (Bcl-2-associated death promoter)[172,173]. Similarly, Akt and ERK1/2 can upregulate the expression of the survival proteins Bcl-2, Bcl-xL, Notch-1, and survivin through activation of β-catenin and NF-κB[172,173].

Finally, CXCR4 can also signal in a manner independent of G proteins. CXCR4 forms homodimers and heterodimers with other chemokine receptors, which may serve to activate the Jak/STAT signaling pathway[174,175]. SDF-1-bound CXCR4 is phosphorylated by G-protein receptor kinases (GRKs), allowing for the binding of β-arrestin. This activates MAPK pathways important in chemotaxis, as the CXCR4-β-arrestin complex binds Raf, activating ERK1/2, and β-arrestin itself activates p38 MAPK[176–179].

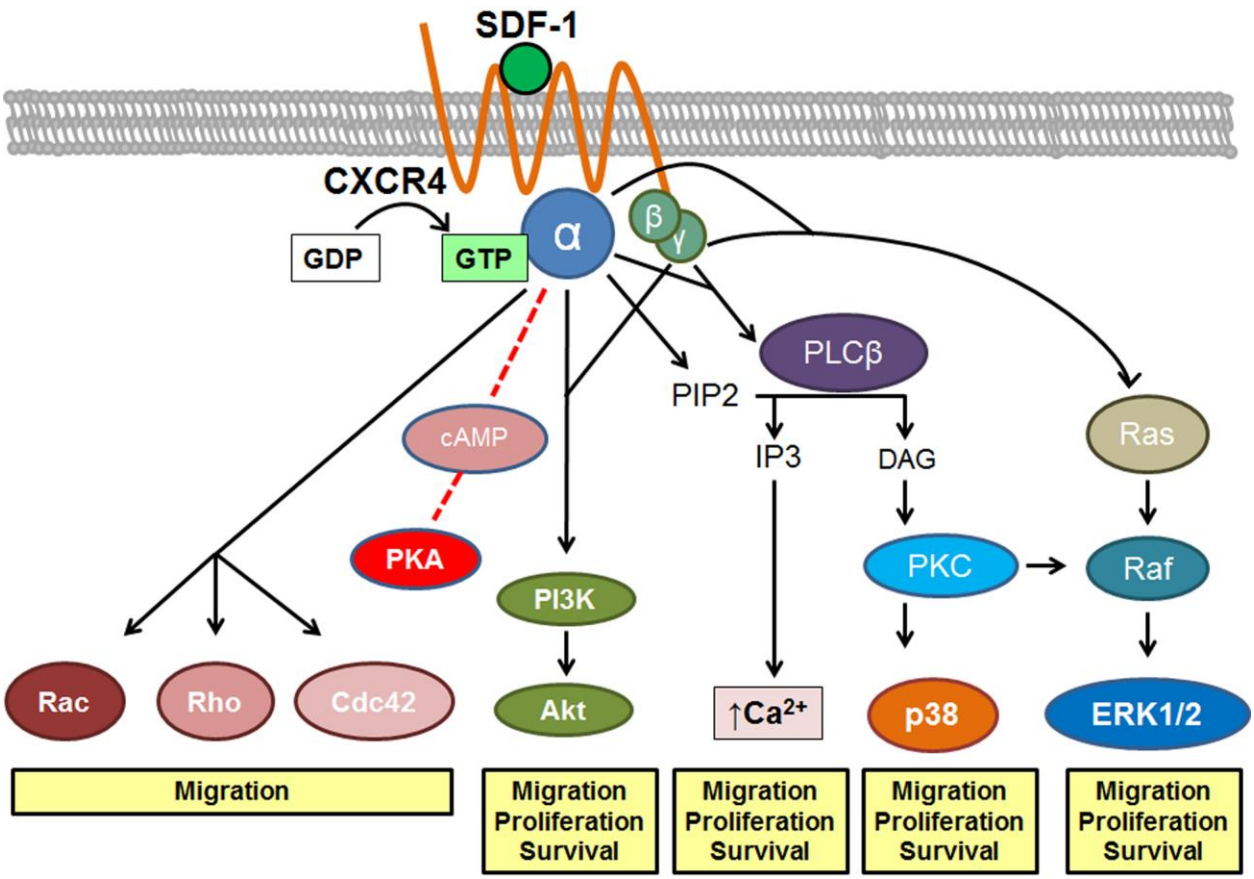

Figure 2. Overview of SDF-1/CXCR4 signaling. SDF-1-bound CXCR4 acts a guanine nucleotide exchange factor to facilitate the exchange of bound GDP for GTP on the Gα subunit, allowing for the heterotrimeric G protein to dissociate and perform its downstream signaling. The Rho GTPases Rac, Rho, and Cdc42 are main regulators of CXCR4-mediated cell migration, although Akt, calcium flux, p38 MAPK, and ERK1/2 also contribute to migration in addition to proliferation and survival.

SDF-1/CXCR4 Signaling and cAMP-Dependent Protein Kinase (PKA)

Adenylyl cyclases (ACs) are enzymes that catalyze the production of cAMP from ATP, and they are regulated by G proteins. There are four groups categorizing mammalian transmembrane AC isoforms: Group I consists of AC1, 3, 8; Group II consists of AC2, 4, 7; Group III consists of AC5, 6; and Group IV consists of AC9[180]. All AC isoforms are stimulated by the $G\alpha_s$ subunit, but the four groups differ by their regulation. Group I is inhibited by the $G\alpha_i$ (for AC1) and $G\beta\gamma$ families, but is also stimulated by Ca^{2+} through the action of calmodulin (CaM). Group II and III

are both stimulated by Gβγ, but Group III is inhibited by $G\alpha_i$ as well as Ca^{2+}. Finally, Group IV is inhibited by Ca^{2+} through calcineurin[180]. Given the diverse role of G protein subunits in regulation of cAMP activity, it is no surprise that the literature surrounding the effects of SDF-1/CXCR4 signaling on cAMP levels is conflicting.

SDF-1/CXCR4 signaling was initially thought to be limited to $G\alpha_i$[153,181], but it was later shown that CXCR4 could signal through the other Gα subunit families as well[182]. As mentioned above, $G\alpha_i$ is an inhibitor of cAMP levels, and much of the literature describes CXCR4-mediated suppression of cAMP. Downregulation of cAMP levels by SDF-1/CXCR4 has been shown to contribute to tumor growth in glioma, glioblastoma, and medulloblastoma[183,184].

However, SDF-1/CXCR4 signaling can also upregulate cAMP levels. SDF-1 acts as an antirepellent in dorsal root ganglion axonal growth by elevating cAMP levels and activating PKA[185]. This antirepellent effect is mediated by AC8, which is activated by CaM, and is dependent on $G\alpha_i$, $G\alpha_{q/11}$, and Gβγ[186,187]. Finally, SDF-1 ligation to CXCR4 has been found to stimulate activity of the PKA catalytic subunit in naïve CD4+ T lymphocytes[188].

Diseases can also use CXCR4 to upregulate PKA. The viral human immunodeficiency virus (HIV) protein gp120 binds to CXCR4 and enhances outwards potassium currents in a PKA-dependent manner[189]. Frimbriae of *Porphyromonas gingivalis* bind to CXCR4, causing activation of PKA signaling which inhibits TLR2-related inflammatory responses[190]. In breast cancer cells, CXCR4 was found to downregulate expression of MHC class II proteins through a PKA-dependent mechanism[191].

CXCR4 and Integrins

SDF-1/CXCR4 signaling is important in the recruitment of T and B cells to secondary lymphoid organs as well as progenitor cell homing to the bone marrow[192]. This follows the leukocyte extravasation model, which involves 1) leukocyte rolling 2) activation of integrins 3) firm adhesion and 4) diapedesis. SDF-1 has been shown to activate the integrins VLA-4 (α4β1) and LFA-1 (αLβ2) in human CD34+ cells[193], which causes firm adhesion of lymphocytes to the respective ligands VCAM-1 and ICAM-1[194,195]. Furthermore, activated VLA-4 and CXCR4 are

also localized at the leading edge of migrating T cells[101]. The following section reviews previous literature suggesting cooperativity of SDF-1/CXCR4 and integrin signaling.

Activation of VLA-4 by SDF-1 is $G\alpha_i$-dependent and requires localization of CXCR4 into lipid rafts[196], though adhesion is unaffected by phosphorylation of ERK1/2 or Akt[197,198]. The signaling molecules Lck[199], RhoA[200], Rac1[200], Vav1[201–203], Syk[204], FAK[205], Rap1[101,205], and PLC[206] are all necessary for SDF-1-mediated lymphocyte adhesion to VCAM-1. Vav1, a GEF for the Rho family of GTPases, is a particularly important molecule in SDF-1-mediated adhesion to VCAM-1. Vav1 is constitutively associated with talin and SDF-1 signals to dissociate this complex through binding of ZAP-70 to Vav1, which is then tyrosine phosphorylated. Upon dissociation from Vav1, talin binding to β1 integrin is promoted (partly through upregulation of PIP2 by PIPKIγ90), and this association is a necessary step for β1 integrin activation[88]. Furthermore, vinculin associates with talin upon SDF-1 stimulation, and phosphorylated Vav1 activates Rac1 (which is regulated by RGS10[207]); these stabilize and strengthen adhesion, respectively, to VCAM-1 via VLA-4[201–203].

SDF-1/CXCR4 signal through $G\alpha_i$ and $G\alpha_{q/11}$ to activate LFA-1[208], but this is not dependent on lipid raft dependent clustering of CXCR4[196]. SDF-1-induced LFA-1 adhesion to ICAM-1 is dependent on H-Ras[209], PI3K[209,210], Cdc42[208], RhoA[208,211], myosin[208], and Rap1[212,213], while downregulation of LFA-1 binding appears to be mediated by Raf-1[209], ERK1/2[209], and Lyn[214–216]. This activation has been found to be both through affinity and avidity: SDF-1 causes LFA-1 to change to its open, active conformation[217], and it also increases LFA-1 clustering which is dependent on the conserved integrin motif GFFKR[218].

Upon adhesion to the endothelium, leukocytes undergo transmigration through endothelial cells and continue to migrate following a chemokine gradient. SDF-1-stimulated transmigration of lymphocytes through activated bone marrow endothelial cells and HUVECs is VLA-4 and LFA-1 dependent[193,219,220]. Within the extracellular matrix, SDF-1-mediated migration is also integrin dependent: VLA-2 is required for migration on collagen type I and laminin, while VLA-4 and VLA-5 are required for migration on fibronectin[221]. Furthermore, SDF-1-mediated migration within a 3D-ECM-like gel (collagen, laminin, fibronectin) has been shown to require VLA-4 and

VLA-5[193]. Integrin-linked kinase (ILK), a kinase that associates with β1 and β3 integrins, is necessary for SDF-1-mediated migration and functions upstream of Akt phosphorylation and Rac1 activation[222]. SDF-1 activates Lyn kinase, which is a positive regulator of cell movement and negatively regulates adhesion through downregulation of LFA-1 binding to ICAM-1[208,214,216]. Hence, post-transmigration, SDF-1-mediated chemotaxis is largely VLA-4 and VLA-5 dependent.

SDF-1-mediated integrin activation is not limited to hematopoietic cells. Small cell lung cancer and melanoma cells have both shown increased VCAM-1 binding via VLA-4 in response to SDF-1[223–225]. Surface expression of αVβ3 integrin is upregulated by SDF-1 in prostate cancer[226] and chondrosarcoma[227], while adhesion to various extracellular matrix components in prostate[226,228], lung[229], and renal[230] cancer can be mediated by the α3, α5, β1, and β3 integrin subunits. Finally, upregulation of αVβ6 integrin in ovarian carcinoma and colorectal cancer mediates tumor cell invasion[231,232].

Regulation of SDF-1/CXCR4 Signaling

The expression of CXCR4 is regulated by many types of molecules that can promote its transcription, likely through nuclear respiratory factor-1 (NRF-1) and Sp-1[233,234], or suppress it, likely through Yin-Yang 1 (YY1)[233,235,236]. Growth factors (bFGF[237,238], VEGF[238], EGF[239]), second messengers (calcium[233], cAMP), and cytokines (IL-2[233], IL-4[240], IL-7[240], IL-10[241], IL-15[240], TGF-1β[241,242]) can all increase CXCR4 expression. Neuregulin 1 can increase CXCR4 expression through upregulation of liver-enriched inhibitory protein (LIP), which has been shown to bind to the CXCR4 promoter[243]. CXCR4 expression can also be suppressed by the transcription factor Yin-Yang 1 (YY1)[236], and this is induced by cytokines such as IL-1β[244], IL-4[241], IL-13[241], TNF-α[244], and IFN-γ[244]. Furthermore, post-translational modifications to CXCR4 include glycosylation (Asn11)[245,246] and tyrosine sulfation (Tyr21)[247,248], and these regulate SDF-1/CXCR4 ligation.

Regulation of SDF-1 expression is stimulated by all-trans retinoic acid (ATRA) through the transcription factor C/EBPβ[249,250]. The pro-inflammatory cytokine IL-17A can directly stimulate SDF-1 expression, as well as indirectly via IL-6 and M-CSF[251]. IL-1β, PDGF-BB, VEGF, TNF-

α, and PTH have all been shown to upregulate expression of SDF-1, while TGF-β_1 decreases it[252].

SDF-1/CXCR4 signaling undergoes GPCR regulation through desensitization, internalization, and degradation. CXCR4 is phosphorylated by GRK6 and GRK3, which causes recruitment of β-arrestin and β-arrestin-2 to uncouple CXCR4 from G proteins and therefore desensitize signaling[176,179,253]. PKC mediates CXCR4 internalization[254], and the phosphorylated residues are necessary for binding of CXCR4 by the E3 ubiquitin ligase atrophin interacting protein 4 (AIP4), causing the degradation of CXCR4 through ubiquitination[255–257].

G proteins exist in their activated state due to the exchange of GDP for GTP on the Gα subunit, causing the dissociation of the Gα subunit from the Gβγ subunit. However, the Gα subunit has intrinsic GTPase activity, and regulators of G protein signaling (RGS) proteins promote this GTP hydrolysis to shut off G protein signaling[258]. RGS1[259,260], RGS3[261], RGS4[262], RGS13[263,264], RGS16[264–266], and RGS18[261] have been implicated in the downregulation of CXCR4-mediated signaling in developing T cells, progenitor B cells, and megakaryocytes.

1.2.2 SDF-1/CXCR4 in Disease

CXCR4 is well known for its role in HIV infection. The HIV glycoprotein gp120 binds to CD4 and a coreceptor, which can be either CCR5 or CXCR4, for viral entry into CD4+ T cells[235,267,268]. However, CXCR4 is implicated in other diseases such as cancer, and aberrant CXCR4 signaling usually results in increased migration of cells.

WHIM syndrome is an immunodeficiency disorder characterized by warts, hypogammaglobulinemia, infection, and myelokathexis[269]. This is a result of autosomal dominant mutations in the CXCR4 gene, leading to a C-terminal truncation of 10-19 amino acid residues[270–272]. The resulting phenotype is increased leukocyte migration towards SDF-1[271–273], calcium influx[270,273], and actin polymerization[272], and decreased desensitization[270,271] and internalization[272]. However, WHIM syndrome is not limited to CXCR4[272] and may result from mutations in regulatory proteins associated with CXCR4 such as GRK3[274], GRK6[275,276], and β-arrestin-2[275].

The SDF-1/CXCR4 signaling axis plays an important role in cancer as CXCR4 is overexpressed in more than 20 different types[277–279]. CXCR4 expression is normally low or absent in non-hematopoietic cells, but is often higher in the corresponding tumor cells[181,278,280–283]. This may be due to the activation of hypoxia inducible factor 1 (HIF-1) as a result of the hypoxic tumor microenvironment, which has been shown to increase CXCR4 expression in cancers such as breast[284–286], ovarian[284], bone[287,288], leukemia[289–292], lymphoma[259], myeloma[293], stomach[294], colorectal[295,296], and glioblastoma[297]. Similarly, mutations in the tumour-suppressor von Hippel-Lindau (VHL) gene are found to mediate upregulation of CXCR4 in renal cell carcinoma[298,299]. These mutations result in a loss of VHL activity, which normally degrades HIF-1. The angiogenic protein VEGF has also been found to promote CXCR4 expression in breast cancer[300] and glioblastoma[297]. Two fusion gene oncoproteins, PAX3-FKHR and RET/PTC, have been identified as promoters of CXCR4 expression in rhabdomyosarcoma[301–303] and breast cancer[304] cells, respectively.

As the primary function of CXCR4 is to regulate lymphocyte trafficking, its expression on cancer cells has many implications for metastasis. Muller *et al.* originally discovered that CXCR4 expression was high in breast cancer cells and could mediate metastasis and invasion[305]. Since then, CXCR4 has been found to be associated with tumor metastasis in other cancers such as prostate[306], lung[307], ovarian[308], gastric[309], and pancreatic[310]. *In vitro*, blockade or knockdown of CXCR4 is found to inhibit metastasis in breast[311,312], prostate[306], lung[313], ovarian[308], thyroid[314], brain[315], and liver cancer[316]. Coupled with the fact that SDF-1 is highly expressed in liver, lung, brain, and bone marrow, all of which are common sites of metastasis[145–147,317], it is likely that the SDF-1/CXCR4 signaling axis plays a large role in the dissemination of tumor cells.

1.2.3 CXCR7: An Alternative Receptor for SDF-1

Although SDF-1 and CXCR4 were originally thought to be an exclusive ligand/receptor pairing, the receptor CXCR7 (also known as RDC-1) has been identified as another binding receptor for SDF-1[318]. CXCR7 is found on both T and B lymphocytes, and can also bind the chemokine CXCL11[318–321]. Like CXCR4, expression of CXCR7 has been associated with proliferation and metastasis in prostate[322], breast[319,323,324], lung[323,325], and pancreatic[326,327] cancer. However,

despite sharing sequence similarities and ligands, CXCR7 does not function like a typical chemokine receptor. It fails to induce Ca^{2+} mobilization, and signal through ERK1/2 and PI3K in a G-protein independent fashion[318,328–332]. Moreover, studies surrounding its ability to induce chemotaxis have yielded mixed results. Instead, CXCR7 has been shown to promote adhesion and survival[319], and may function as a decoy receptor to reduce the amount of available SDF-1 in an effort to enhance the effect of transcriptional changes in SDF-1 in the environment[333]. Furthermore, CXCR7 recruits and signals through β-arrestin, suggesting that it is a regulator of CXCR4-mediated signaling[333].

1.3 Hypothesis and Research Aims

1.3.1 Hypothesis

PKA-mediated phosphorylation of proteins at the leading edge is a crucial regulator of cell migration, but upstream regulators of PKA activity have yet to be elucidated. A possible candidate for upstream regulation of PKA is the chemokine receptor CXCR4, which is a key mediator of leukocyte migration and has been shown to activate many known molecules of cell migration such as the Rho GTPases Rac and Cdc42. As well, CXCR4 binding of its ligand SDF-1 leads to downstream activation of integrins, which have been shown to mediate PKA activation at the leading edge. Although SDF-1/CXCR4 signaling has been associated with regulation of cAMP/PKA, there is conflicting evidence regarding the exact relationship as studies have found both up- and down-regulation. However, these studies have focused on global activation of cAMP and PKA activity instead of the spatiotemporal activation shown to be important in cell migration. We therefore hypothesize that **SDF-1/CXCR4 signaling is an upstream regulator of cAMP/PKA activity important for directional cell migration.**

1.3.2 Research Aims

1) To characterize the requirement of SDF-1/CXCR4 signaling in directed cell migration.

2) To determine the spatiotemporal regulation of PKA activation by SDF-1/CXCR4 signaling.

2. Materials and Methods

2.1 Cell Culture

2.1.1 Cell Lines

Experiments were conducted using the cell lines Chinese Hamster Ovary (CHO-K1, ATCC, Manassas, VA), J774 murine macrophages (ATCC, gift from Dr. Zakaria Hmama, UBC), and Jurkat T-lymphoblasts (ATCC). Adherent (CHO, J774) and suspension (Jurkat) cells were passaged in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) and Roswell Park Memorial Institute medium (RPMI) (Sigma-Aldrich), respectively, both of which were supplemented with 10% FBS (Life Technologies, Carlsbad, CA), penicillin and streptomycin (Life Technologies), and non-essential amino acids (Life Technologies). All cells were incubated at 37°C with 5% CO_2 and kept between passage 2 and 20.

2.1.2 Fluorescence Activated Cell Sorting (FACS)

Surface expression of CXCR4 in CHO, J774, and Jurkat cells was determined through flow cytometry. Cells were incubated with anti-CXCR4 antibody (Biolegend, San Diego, CA) at a 1:50 dilution for 1 hour, washed, and then incubated for another hour with Alexa Fluor® 488 goat anti-mouse (Life Technologies) at a 1:200 dilution. Flow cytometry was performed with the BD FACSCanto™ (BD Biosciences, Franklin Lakes, NJ) and data was analyzed using FlowJo (Tree Star, Ashland, OR), where viable cells were gated and analyzed for surface CXCR4 expression. Incubation without anti-CXCR4 antibody served as the negative control for J774 cells.

2.1.3 Cell Transfection

In a 6-well tissue culture plate, $2x10^5$ CHO cells per well were seeded and grown to 70-90% confluence, then transfected with 2μg of plasmid DNA using the FuGENE transfection reagent (Promega, Madison, WI). For Jurkat cells, approximately $1.5x10^6$ cells were transfected via electroporation using the X-001 program for the Amaxa Nucleofector 2b Device (Lonza, Basel, Switzerland). Cells transfected transiently were left to grow overnight, while stable cell line

transfections were placed under hygromycin (250μg/mL) or G418 (500μg/mL) selection 48 hours post-transfection.

2.1.4 Plasmids and Plasmid Construction

The pcDNA3.1-Hygro(+)-CXCR4 plasmid was generated through ligation of a cut CXCR4 sequence (from restriction digest of a pcDNA3.1-Amp(+)-CXCR4 plasmid, received from Dr. Jurgen Kast, UBC) into pcDNA3.1-Hygro(+) (Invitrogen). The FRET biosensor constructs pmAKAR3.3 and pmAKAR3.3(TA) were a generous gift from Dr. Jin Zhang (Johns Hopkins University).

Construction of CRISPR-Cas Plasmid for Disruption of CXCR4 Expression

The CRISPR-Cas system is a genome editing tool designed to target specific sequences of DNA. CRISPR-Cas is a form of immunity found in bacteria, whereby DNA from phage and plasmids is acquired by the bacteria and reduced to clustered regularly interspaced short palindromic repeats (CRISPR). The nuclease Cas9 uses guide RNA to bind to target DNA and cause double stranded breaks, which are repaired with the error-prone non-homologous end joining mechanism[334].

To use the CRISPR-Cas system, we developed a plasmid containing a genomic target sequence for human CXCR4, which was determined based on the proximity to the closest exon splice site and the sequence preceding the amino acids NGG. Two oligonucleotides against the target site were developed (forward: 5'-CACCGTACACCGAGGAAATGGGCTC-3' and reverse: 5'-AAACGAGCCCATTTCCTCGGTGTAC) according to the indicated specifications for the plasmid backbone (pX330, Addgene, Cambridge, MA). After BbsI (New England Biolabs, Ipswich, MA) digest of pX330, the oligonucleotides were ligated into the pX330 backbone, creating the pX330-hCXCR4 construct.

Clonal Isolation of CXCR4 Null Clones

The pX330-hCXCR4 construct was transfected into Jurkat cells, and sorted for negative CXCR4 expression by FACS one week post-transfection. The polyclonal population of CXCR4-negative Jurkat cells was then diluted into a 96-well plate at a density of 0.5 cells per well (200μL in each well). Three clones were selected and confirmed for negative CXCR4 expression by flow

cytometry. Two clones were discarded, as a surface expression analysis of integrins α4, α5, α6, and β1 revealed lower β1 expression compared to the parental Jurkat cell line. The remaining clone, referred to as the JC4 cell line, was used for experiments as indicated.

Sequencing Analysis of the CXCR4 Null JC4 Cell Line

Genomic DNA was isolated using the EZ-10 Spin Column Animal DNA Mini-Preps Kit (BS628, Bio Basic, Markham, ON). PCR amplification (Platinum Pfx DNA polymerase, Life Technologies) of the CXCR4 locus was performed using the forward 5'-CTCCCTTCTCCCCTCTTCCC-3' and reverse 5'-TGCCTCACTGACGTTGGC-3' primers, which were selected to be 500bp upstream and downstream of the CRISPR guide RNA target site. The optimized PCR conditions for amplification were determined to be at 33 cycles with denaturing, annealing, and extension temperatures of 94, 58, and 68 degrees Celsius for 15, 30 and 120 seconds, respectively. The PCR product was run on a 1% agarose gel and the 1000 bp amplified PCR fragment was cut out and purified using a gel extraction kit (BS654, Bio Basic). Sequencing of the amplified PCR fragment was conducted by the CORE Facility at CMMT (Vancouver, BC) using the Applied Biosystems 3130xL Genetic Analyzer (Life Technologies) with the forward 5'-CCCTCCCTGGGCGAAAAAC-3' and reverse 5'-GCTTCCTTGGCCTCTGACTG-3' sequencing primers.

2.1.5 Reagents

Recombinant SDF-1α (Prospec, East Brunswick, NJ), isoproterenol (Sigma-Aldrich), and AMD3100 (Sigma-Aldrich) were solubilized in H_2O at stock concentrations of 20μg/mL, 400nM, and 10mg/ml, respectively. Forskolin (Fsk, LC Labs, Woburn, MA) and H-89 (LC Labs) were both solubilized in DMSO at a concentration of 20mM. Cells were stimulated with recombinant SDF-1α at 400ng/mL (50nM), isoproterenol at 1nM, AMD3100 at 25μM, Fsk at 50μM, and H-89 at 25μM.

2.2 Protein Analysis by SDS-PAGE and Immunoblotting

2.2.1 Cell Lysates

Cells were routinely lysed using a buffer consisting of 10mM PIPES (Sigma-Aldrich), 50mM NaCl (Sigma-Aldrich), 150mM sucrose (Sigma-Aldrich), 50mM NaF (Sigma-Aldrich), 40mM

$Na_4P_2O_7 \bullet 10H_2O$ (Thermo Fisher Scientific, Waltham, MA), 1.0% triton X-100 (Sigma-Aldrich), and protease inhibitors (Roche, Basel, Switzerland). Lysate protein concentration was determined by Bicinchoninic Acid (BCA) assay (Pierce, Rockford, IL) and then normalized to 2mg/mL per sample.

2.2.2 Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE loading buffer (15µL of 6X concentrate) was added to 75µL of cell lysates and heated for 5 minutes at 95°C. Samples were centrifuged to remove condensation and then loaded (15µL) into individual wells of a discontinuous 10% SDS-PAGE gel, along with a protein ladder of mixed molecular weights (BioRad, Hercules, CA). The loaded gel was electrophoresed at 80V for 30 min, 100V for 30 min, and then 120V for 40 min.

2.2.3 Blot Transfer to Nitrocellulose

Transfer of SDS-PAGE separated proteins to nitrocellulose membrane was performed using the Trans-Blot turbo transfer system (BioRad). Cold transfer buffer-soaked components were stacked in the following order: (1) filter paper stack, (2) nitrocellulose membrane, (3) SDS-PAGE gel, (4) filter paper stack. Transfer was performed using the Turbo Mixed MW protocol at 25V for 7 minutes.

2.2.4 Immunoblot Detection of Target Proteins

Upon completion of transfer, the nitrocellulose membrane was blocked with 5% milk diluted in tris-buffered saline (TBS) for 1 hour to prevent nonspecific antibody binding. For antibodies directed against phosphorylated substrates, NaF was added to TBS in order to inhibit phosphatase activity. After blocking, the membrane was washed with TBS for 5 minutes and then blotted with primary antibodies diluted in TBS with Tween (TBS-T). GAPDH (Biolegend), PKA-catalytic subunit α (Cell Signaling, Danvers, MA), phospho-PKA substrate (RRXS*/T*) (Cell Signaling), and phospho-p44/42 MAPK (Erk1/2) (Cell Signaling) antibodies were diluted (1:1000) into a 5% BSA/TBS-T solution. All antibodies were blotted overnight at 4°C on a shaker except for GAPDH, which was blotted for 1 hour at room temperature. Anti-vinculin antibody (Sigma-Aldrich) was diluted (1:1000) into 2.5% milk/TBS-T for 1 hour at room temperature.

After primary antibody blotting, the nitrocellulose membrane was washed three times with TBS-T for 5 minutes each, then incubated at room temperature for 1 hour with a secondary antibody (DyLight goat anti-rabbit or goat anti-mouse, Pierce) solution of 2.5% milk diluted in TBS-T. The membrane was then washed another three times with TBS-T for 5 minutes each, and then left in TBS solution. Bound antibody detection was performed using the Licor Odyssey machine to scan the membrane in the 700 and/or 800 channel (depending on the secondary antibody used). The Licor Odyssey densitometry function was used to determine densitometry values of the visible bands.

2.3 Microscopy and Cell Imaging

The Olympus IX-81 inverted microscope (Olympus, Richmond Hill, ON) was used for all microscopy methods. Live imaging was performed under humidified 37°C and 5% CO_2 conditions (Weather Station, Precision Control LLC). Using the Metamorph version 7.5 software (Molecular Devices, Sunnyvale, CA), light microscopy images were acquired using the 20X 0.45 NA and 40X 0.75 NA dry objectives, while immunofluorescence and tagged fluorescent protein images were acquired using the 60X 1.35 NA oil objective.

2.3.1 Fibronectin Coating

In a 12-well plate, 18mm glass coverslips (Electron Microscopy Sciences, Hatfield, PA) were coated with 750µL of 10µg/mL human fibronectin (Fn) (purified from plasma) and left at 4°C overnight. Coverslips were blocked with 1% BSA/PBS (Bovine serum albumin from Thermo Fisher Scientific) and washed with PBS (phosphate buffered saline, Sigma-Aldrich) prior to cell seeding. Adherent cells were plated at a density of $2x10^5$ cells/mL, while suspension cells were plated at a density of $4x10^5$ cells/mL.

2.3.2 Light Microscopy for Cell Migration

Live imaging of cells was performed using differential interference contrast (DIC) microscopy. Imaging of global SDF-1 stimulation was performed on cells adhered on Fn-coated coverslips mounted in a silicone gasket (Chamlide CMB, Quorum Technologies, Guelph, ON). Images were taken every 15 seconds at 100 ms exposure for 1 hour.

The Bioflux 200 system (Fluxion Biosciences, San Francisco, CA) was used for directional SDF-1 chemoattractant-mediated migration assays. The 24-well plates (P/N 910-0009, Fluxion Biosciences) consist of 8 channels, each of which is connected to three wells. The solutions in two of the wells run parallel to each other under pressure-induced flow, and converge into the third "out" well. In our experiments, we pipetted blank media in one well and blank media with SDF-1 in the other well. The channels were coated with 100μg/mL fibronectin overnight and washed with PBS the next day. Cells were momentarily flowed (2.3 dyn/cm^2) through the system at a concentration of 3-4x10^6 cells/mL and left to adhere for 2 hours. SDF-1 stimulation of cells was initiated by introduction of flow at 1 dyn/cm^2 using the BioFlux 200. Images pre- and post-SDF-1 stimulation were acquired every 1 minute at 100 ms exposure for 1 hour.

The position of cells was tracked and analyzed using the MTrackJ plugin for ImageJ (NIH, Bethesda, MD). We measured the width of the channel (1760μm) and divided it by half to determine the locations over which the blank media and SDF-1 in blank media would flow. Since we were monitoring chemotactic migration, only the cells in the channel under flow with blank media were tracked as they displayed the greatest migration towards the SDF-1 gradient.

2.3.3 Immunofluorescence Staining and Imaging

J774 cells were plated on 18mm coverslips coated with fibronectin, fixed with 3.7% formaldehyde, and permeabilized with 0.1% TX-100. Nonspecific antibody binding sites were blocked with 2% BSA in TBS. Cells were probed using phospho-PKA substrate antibody (Cell Signaling) with goat anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR) and rhodamine-conjugated phalloidin (Sigma-Aldrich,). Images were acquired in the FITC and TRITC channels for phospho-PKA substrates and rhodamine-phalloidin, respectively, and optically sectioned 21 times spaced 0.3μm apart. For acquisition of FITC images, the excitation filter 485/20 and emission filter 525/30 were used, while the excitation filter 560/25 and emission filter 607/36 were used for TRITC (Semrock, Rochester, NY).

2.3.4 Fluorescence Resonance Energy Transfer (FRET) Imaging

Jurkat cells transfected with pmAKAR were plated on Fn-coated coverslips or within the Bioflux flow chamber one day post-transfection. Images of YFP and CFP were acquired using the DV2

Multichannel Imaging System (Photometrics, Tucson, AZ) with the 505dcxr beam splitter (Dual-View, Optical Insights LLC, Tucson, AZ) containing a 438/24 CFP excitation filter, 457/22 CFP emission filter, and a 530/20 YFP emission filter (Chroma Technology, Bellows Falls, VT). The lamp (X-Cite Exacte, Lumen Dynamics, Mississauga, ON) intensity was set at 10%, and images were acquired every 20 seconds for global SDF-1/Fsk stimulation or every 30 seconds for directional SDF-1 stimulation with an exposure time of 300 ms at 2x2 binned resolution.

Analysis of acquired FRET images was performed using ImageJ v1.46j. The YFP and CFP images were background corrected by subtracting the value of the empty space surrounding the target cell. Then, the YFP image was divided by the CFP image to compute the FRET ratiometric values using the ImageJ plugin "Ratio Plus". The ratiometric images were pseudocoloured by applying the Ratio LUT (Look Up Table) plugin to indicate regions of high and low FRET ratios. Cell response to Fsk was analyzed through mean measurements of the FRET ratio in select ROIs (regions of interest). Comparison of protrusion against cell body was determined with bisecting line (10 pixels wide) measurements of the mean FRET ratio.

2.4 Statistical Analysis

Data are expressed as mean ± standard deviation. For analysis of immunofluorescence data, one-way ANOVA with post-hoc Newman-Keuls multiple comparison test was used to determine statistical significance. For all other experiments, data was analyzed using the student's t-test to determine statistical significance. All analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Characterization of SDF-1/CXCR4 Signaling in CHO Cells

3.1.1 Expression of CXCR4 in CHO cells

While planning our investigations into SDF-1/CXCR4 signaling, we identified CHO cells as a cell line that was well studied as a model of migration yet lacked CXCR4 expression.
CHO cells have been used to investigate the actin-ECM relationship as well as signaling pathways in migration. Previous studies incorporating CXCR4 into CHO cells have confirmed known signaling pathways upon SDF-1 stimulation, including calcium mobilization, and ERK and Akt/PKB phosphorylation[335–338]. As described in 'Materials and Methods', I created a clonal CHO cell line expressing CXCR4 and confirmed surface expression of CXCR4 through flow cytometry (Figure 3).

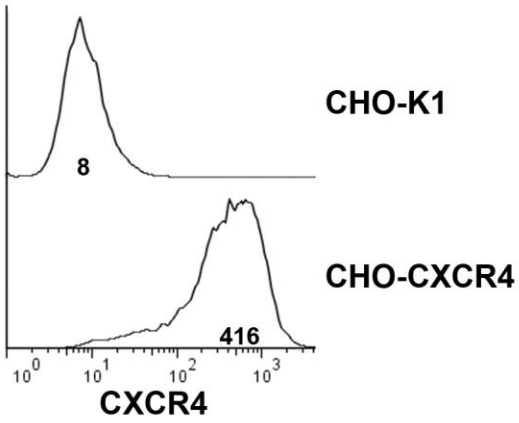

Figure 3. Comparison of surface CXCR4 expression between CHO and CXCR4-transfected CHO cells by flow cytometry. CHO cells were transfected with CXCR4 and hygromycin-selected, then sorted for surface CXCR4 expression by FACS and cloned. Median fluorescence intensity is indicated below the histogram peaks.

3.1.2 SDF-1/CXCR4 Signaling in CHO-CXCR4 Cells

To assess the downstream signaling pathways activated by SDF-1 ligation to CXCR4, a time course stimulation of CHO-CXCR4 cells with SDF-1 was performed and lysates were immunoblotted with an antibody directed against PKA-phosphorylated substrates as a measure

of PKA activity. Efficiency of the antibody was confirmed through stimulation of CHO-CXCR4 cells with the cAMP (and thus PKA) stimulator Fsk. SDF-1-stimulated CHO-CXCR4 cells displayed increased levels of PKA activity 5 and 10 minutes post-stimulation (Figure 4). An antibody detecting phosphorylated p44/42 MAPK (ERK1/2) was also used to confirm SDF-1 stimulation of CXCR4-related signaling pathways, as this relationship has been well documented in previous literature[339,340].

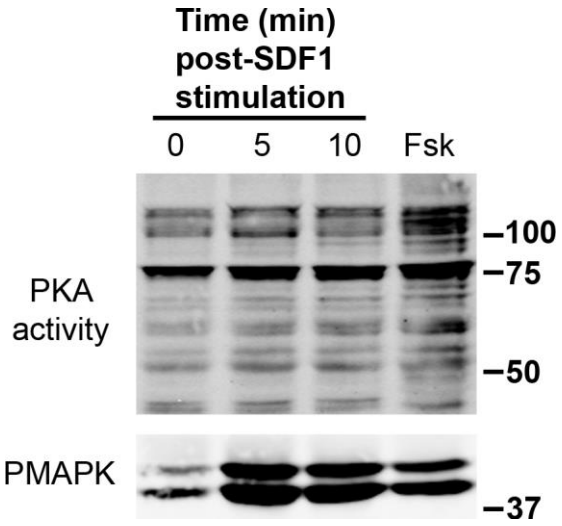

Figure 4. Time course of SDF-1 stimulation for CHO-CXCR4 cells. CHO-CXCR4 cells were plated on cell culture dishes and stimulated with SDF-1 (50nM) for 5 and 10 minutes, and Fsk (50μM, 10 min). Lysates were immunoblotted for PKA-phosphorylated substrates (PKA activity) and phosphorylated p44/42 MAPK (PMAPK). Molecular weights are indicated by the numbers to the right.

Since PKA-phosphorylated substrates were increased upon SDF-1 stimulation, I next wanted to confirm specific effects of SDF-1/CXCR4 on PKA activity. I compared stimulation of CHO and CHO-CXCR4 cells with SDF-1, a combination of SDF-1 and the PKA inhibitor H89, the CXCR4 antagonist AMD3100, as well as the cAMP stimulator Fsk. PKA-phosphorylated substrates were increased upon SDF-1 stimulation in CHO-CXCR4 cells but not CHO cells, and that this stimulation could be reversed in the presence of H89 and AMD3100 (Figure 5). These findings suggest that PKA activity is upregulated in CHO-CXCR4 cells through SDF-1/CXCR4-dependent signaling mechanisms.

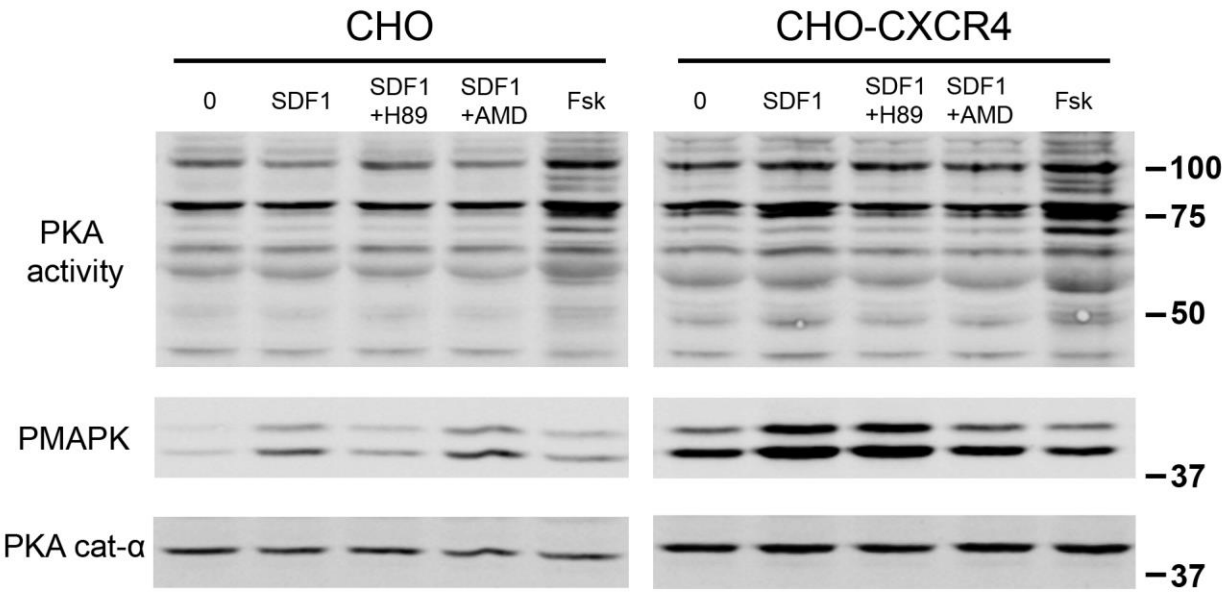

Figure 5. Comparison of CHO and CHO-CXCR4 response to SDF-1. CHO and CHO-CXCR4 cells were plated on cell culture dishes and stimulated with SDF-1 (50nM, 10 min), SDF-1 and H89 (25μM, 10 min), AMD3100 (25μg/ml) and Fsk (50μM, 10 min). Lysates were immunoblotted for PKA-phosphorylated substrates (PKA activity), phosphorylated p44/42 MAPK (PMAPK), and PKA catalytic subunit α (PKA cat-α). Molecular weights are indicated on the right.

3.1.3 Migration of CHO-CXCR4 Cells in Response to SDF-1

Although an increase in CXCR4 signaling was detected through measurement of PKA activity and phosphorylated MAPK, no changes were observed in the phenotypes (membrane ruffling, protrusions, or migration) of SDF-1 stimulated CHO-CXCR4 cells under live DIC imaging (data not shown). Therefore, we decided to investigate CXCR4 signaling in leukocyte cell lines since they naturally express CXCR4.

3.2 Characterization of SDF-1/CXCR4 Signaling in J774 Macrophages

3.2.1 Expression of CXCR4 in J774 Cells

J774 is a highly motile murine macrophage cell line that has been used in many investigations of macrophage chemotaxis. We selected them for their spread morphology while adherent, which

would provide for ease of analysis for microscopy experiments. Surface CXCR4 expression of J774 cells was confirmed by comparing median fluorescence intensity against a secondary antibody control. J774 cells expressed high levels of CXCR4 compared to the reagent control (Figure 6).

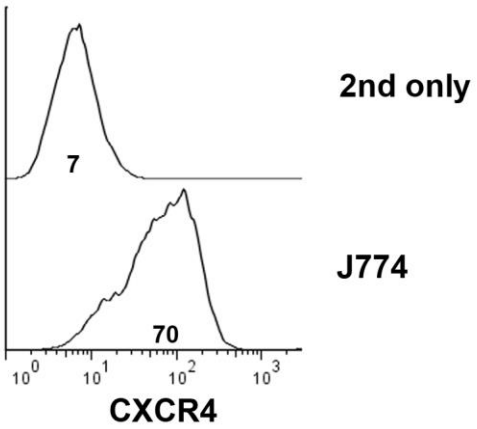

Figure 6. Expression of surface CXCR4 in J774 cells compared to a secondary antibody-only control by flow cytometry. Median fluorescence intensity is indicated below the histogram peaks.

3.2.2 SDF-1 Stimulates J774 Membrane Ruffling and Cell Migration

Since there was no migratory phenotype in SDF-1-stimulated CHO-CXCR4 cells, we decided to investigate the effect of SDF-1 stimulation on J774 murine macrophage cells. Live imaging of SDF-1-stimulated J774 cells revealed a phenotype characterized by peripheral membrane ruffling (Figure 7A). To better visualize the ruffling activity, a kymograph was created indicating the changes in intensity over time upon SDF-1 stimulation (Figure 7B).

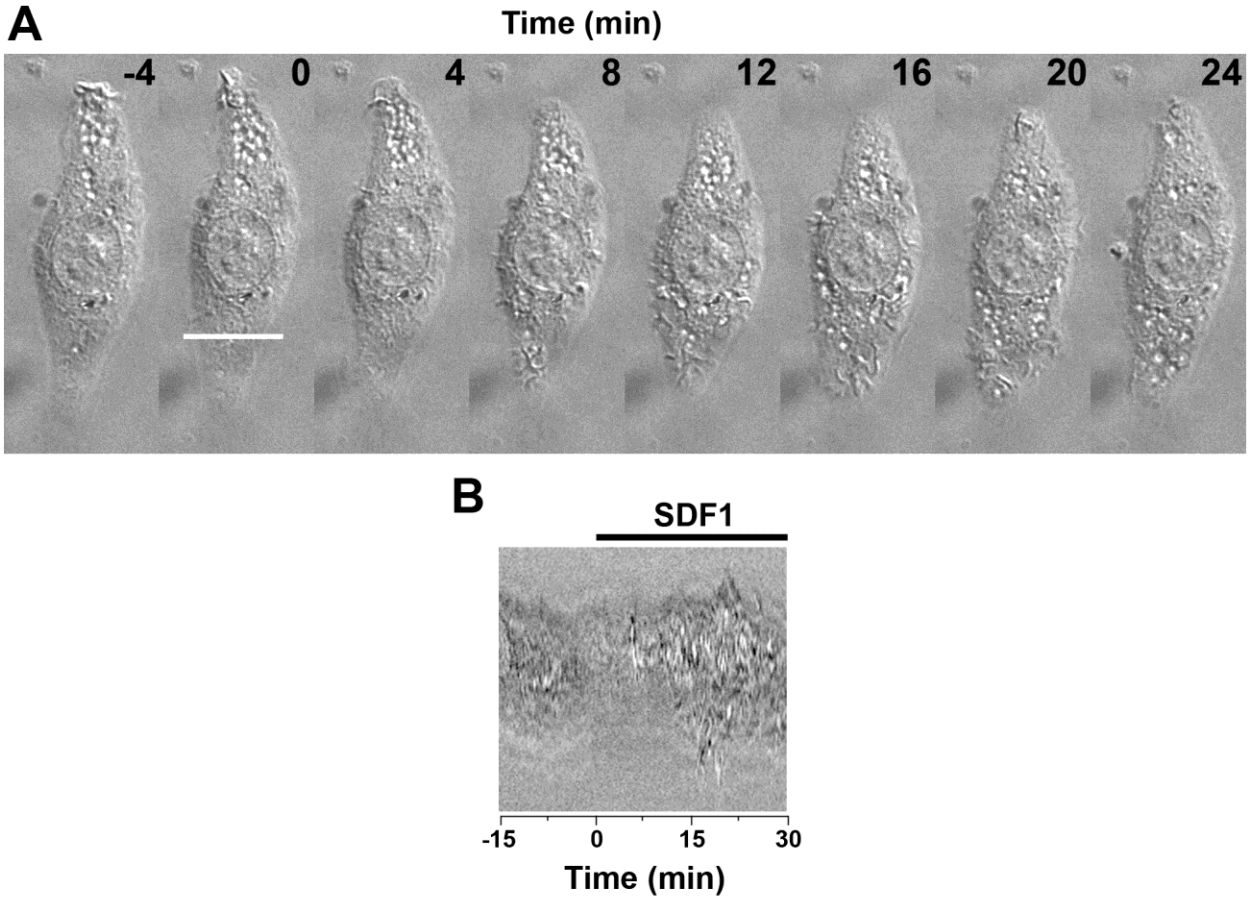

Figure 7. Live microscopy of J774 cells stimulated with SDF-1. (A) Time course of a J774 cell globally stimulated with SDF-1 (50nM). J774 cells were plated on fibronectin and imaged for 15 minutes pre-SDF-1 stimulation and 30 minutes post-SDF-1 stimulation. Numbers indicate time in minutes post-stimulation. (B) Kymograph of SDF-1-stimulated membrane ruffling in J774 cells. This was generated from the line drawn across an area of membrane ruffling indicated by the white line in Figure 7A.

As J774 cells exhibited a ruffling phenotype upon global SDF-1 stimulation, we next sought out to see if J774 cells would respond to SDF-1 presented in a directional manner. To define an assay where SDF-1 would act as a chemoattractant, I used the Fluxion Bioflux system to create a gradient of SDF-1. As seen in Figure 8A, J774 cells plated on fibronectin within the Bioflux plate channel are subject to flow (1 dyn/cm^2) of two streams of liquid: one with SDF-1 diluted in normal media, and the other with normal media only (DMEM). For unstimulated controls, both

streams contained normal media. In order to quantify the extent of cell migration, the (X,Y) position of the macrophage cell body was tracked. Within the system, the introduction of flow did not affect the horizontal positioning of J774 cells as there was no difference between unstimulated and SDF-1 stimulated cells (Figure 8B). J774 cells exhibited migratory activity towards SDF-1 as there was a significant difference in the vertical (Y) displacement towards SDF-1 when compared to the unstimulated control (Figure 8C, D). As seen in the inset of Figure 8A, the J774 cells extended protrusions in the direction of SDF-1, and these protrusions were high in membrane ruffling.

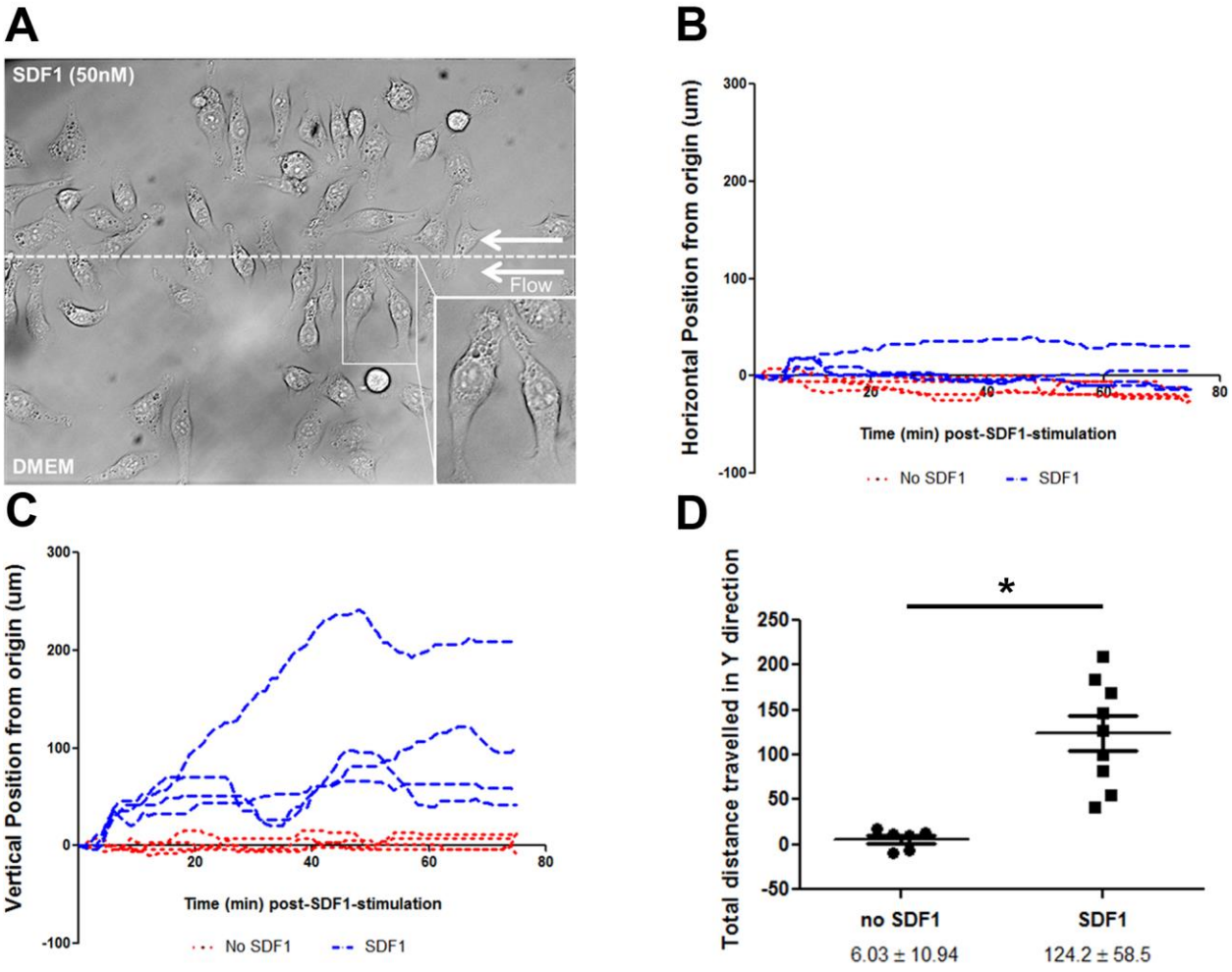

Figure 8. Migration of J774 cells towards SDF-1. (A) J774 cells were plated on 100ug/ml fibronectin inside the channel of the BioFlux plate for 2 hours, then introduced to SDF-1 (50nM) under flow (1 dyn/cm2) as indicated by the arrows. Images were acquired every 1

minute by time lapse microscopy. Inset, Image of J774 cells extending protrusions towards SDF-1. (B) The horizontal position of J774 cells was tracked over time after introduction to flow. The dotted blue line represents horizontal position of SDF-1 stimulated cells, while the dotted red line indicates horizontal position of unstimulated cells. (C) Tracking of the vertical position of J774 cells in response to SDF-1. (D) Comparison of total vertical distance travelled between unstimulated and SDF-1 stimulated J774 cells. Mean values are indicated below the treatment titles (n = 9 cells). Asterisk indicates statistical significance ($p < 0.05$) determined by unpaired t-test.

3.2.3 J774 Cells Exhibit Increases in PKA Activity in Response to SDF-1

Since J774 cells migrated towards SDF-1, I next examined the signaling pathways activated in SDF-1-stimulated J774 cells. To determine effects of SDF-1 stimulation on PKA activity in J774 cells, a time course stimulation of SDF-1 on J774 cells was performed and lysates were immunoblotted for PKA-phosphorylated substrates, phosphorylated p44/42 MAPK (ERK1/2), and vinculin (as a loading control) (Figure 9A). Isoproterenol (iso), a stimulator of cAMP synthesis and thus PKA activity, was used as a positive control as J774 cells do not respond to Fsk[341]. Maximal induction of PKA activity occurred around 6 minutes, as indicated by densitometry values (Figure 9B).

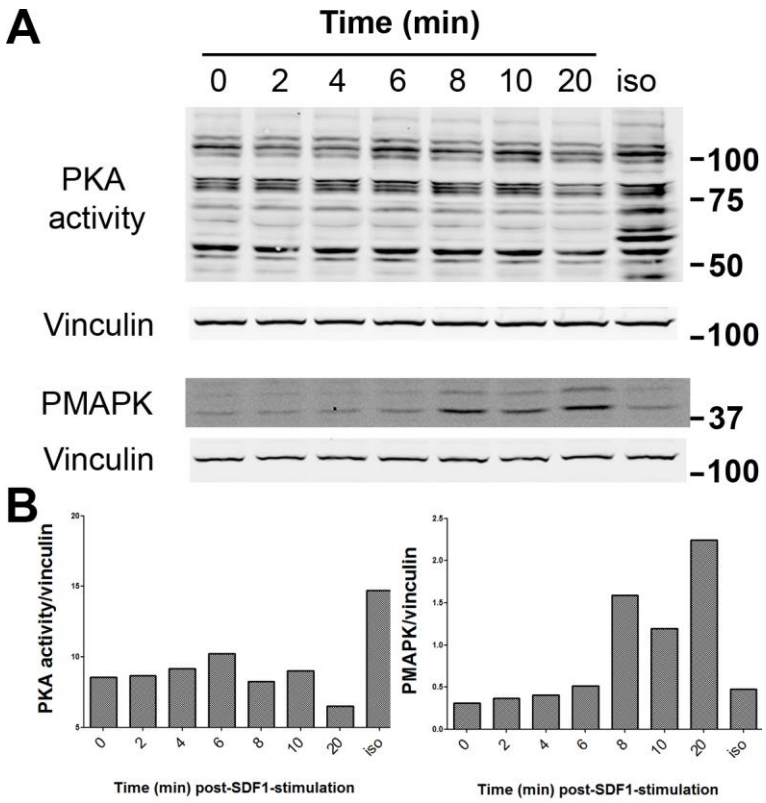

Figure 9. Time course protein immunoblot of SDF-1-stimulated J774 cells. (A) J774 cells were plated on 60mm cell culture dishes and stimulated with SDF-1 (50nM) and isoproterenol (1μM, 5 min). SDF-1 stimulation was performed in a time course of the indicated time points. Lysates were immunoblotted for PKA-phosphorylated substrates (PKA activity), phosphorylated p44/42 MAPK (PMAPK), and vinculin. Molecular weights are indicated on the right. (B) Quantification of protein immunoblot with densitometry. The densitometry values of PKA-phosphorylated substrates and phosphorylated MAPK were normalized to the vinculin loading control.

In order to confirm that the increases in PKA-phosphorylated substrates were CXCR4-dependent, we considered two possibilities. The first was the use of CRISPR-Cas system to knock out CXCR4; however, this system involves transfection of a DNA plasmid into J774 cells, which do not respond well to transfection (to be discussed later). The second possibility was to use the CXCR4 antagonist AMD3100, which we were able to use successfully in CHO-CXCR4 cells (Figure 5). In testing AMD3100 on J774 and Jurkat cells, I was unable to prevent

stimulation of PKA and phosphorylated MAPK when using SDF-1 in conjunction with AMD3100 (data not shown). Upon literature review, we found that another group found similar results *in vitro* while using AMD3100 as it was a partial agonist for CXCR4[342]. Although CXCR4-dependent stimulation of PKA activity in J774 cells could not be confirmed, I was able to reverse the increase in PKA-phosphorylated substrates through co-stimulation of SDF-1 with the PKA inhibitor H89 (Figure 10), indicating that the increase in PKA-phosphorylated substrates measured by the antibody was PKA-specific.

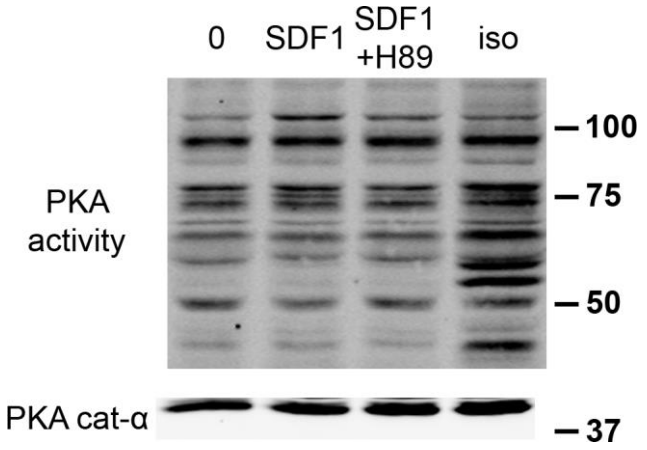

Figure 10. PKA activity of SDF-1-stimulated J774 cells. J774 cells were plated on 60mm cell culture dishes and stimulated with SDF-1 (50nM, 6 min), SDF-1 and H89 (25μM, 6 min) and isoproterenol (1μM, 5 min). Lysates were immunoblotted for PKA-phosphorylated substrates (PKA activity) and PKA catalytic subunit α (PKA cat-α), which was a loading control. Molecular weights of proteins are indicated on the right.

3.2.4 PKA activity is increased at the periphery of SDF-1 stimulated J774 cells

We initially planned to use the FRET biosensor for PKA activity, pmAKAR, to visualize the spatiotemporal activation of PKA in SDF-1-stimulated J774 cells. However, as there was very low transfection efficiency of pmAKAR in J774 cells (using either FuGene or electroporation), we opted to monitor PKA activity through immunofluorescence staining.

To visualize the localization of PKA activity in J774 cells, unstimulated, SDF-1 stimulated, and SDF-1 and H89 stimulated J774 cells were fixed and stained for PKA activity using

immunofluorescence (using an antibody for PKA-phosphorylated substrates) as well as F-actin (using Rhodamine-phalloidin). Our results indicate that there was localized PKA activity and F-actin polymerization at the periphery of SDF-1-stimulated J774 cells (Figure 11A). To compare the differences in peripheral staining between treatments, mean fluorescence intensity at the periphery was measured and normalized the values against the intensity of the cell body (Figure 11B). SDF-1-stimulated cells showed significantly more F-actin staining and PKA-phosphorylated substrates at the periphery compared to the control, or SDF-1 and H89 treated cells.

As indicated earlier, I was unable to perform controls for CXCR4 specificity. However, the lack of actin and PKA-phosphorylated substrate staining in the periphery of SDF-1 and H89 co-stimulated J774 cells suggests that the increase in PKA-phosphorylated substrates at the periphery of SDF-1-stimulated J774 cells was PKA-specific.

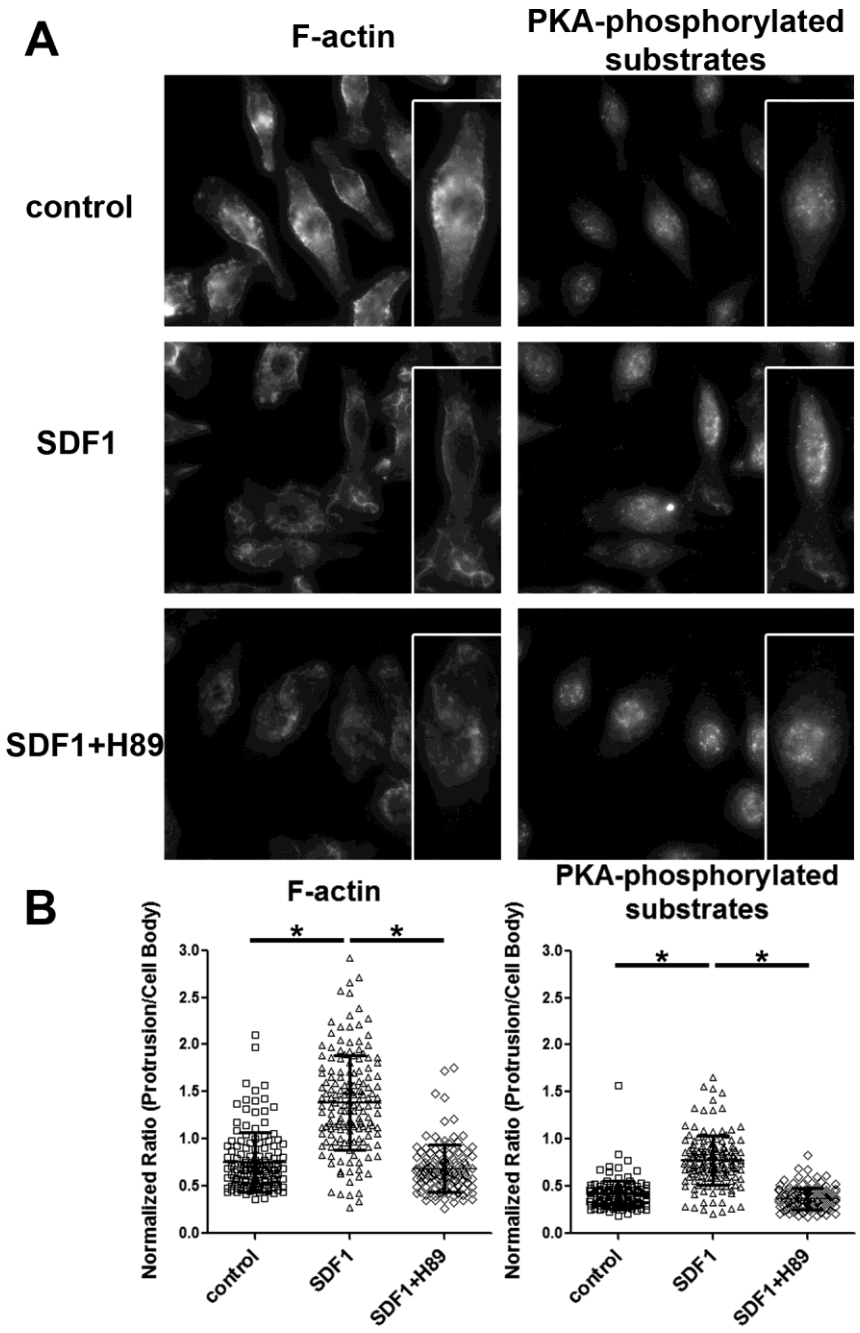

Figure 11. Visualization of PKA activity in SDF-1-stimulated J774 cells by immunofluorescence. (A) J774 cells were plated on fibronectin-coated glass coverslips and stimulated with SDF-1 (50nM, 6 min), and SDF-1 and H89 (25μM, 6 min). Fluorescence staining was performed with an antibody against PKA-phosphorylated substrates (1:300 dilution) and Rhodamine-phalloidin (1:300 dilution). Insets, image of enlarged representative single cell (B) Comparison of normalized fluorescence intensity of

phosphorylated PKA substrates and F-actin between treatments. The fluorescence intensity of the protrusion and cell body was measured for each cell (n = 144 for control, n = 164 for SDF-1, n = 133 for SDF-1+H89), and the normalized fluorescence intensity is obtained from dividing the protrusion by the cell body. Statistical analysis between treatments was performed using one-way ANOVA, and significance ($p < 0.05$) is indicated by the asterisks. Error bars indicate the SD of the mean of one set of observations (n = 1).

3.3 Characterization of SDF-1/CXCR4 Signaling in Jurkat Cells

3.3.1 Generation of CXCR4-negative Jurkat Cells Using CRISPR-Cas

Jurkat T-cells are a widely used cell line in many studies related to CXCR4 signaling. Since Jurkat cells naturally express CXCR4, we used the CRISPR-Cas system to generate CXCR4-negative Jurkat cells. We designed a CRISPR-Cas guide RNA construct that targeted exon 2 of the CXCR4 gene, which would cause double stranded breaks in the genomic DNA and be repaired by the error prone non-homologous end joining mechanism. After transfection of Jurkat cells with the CRISPR-Cas construct against CXCR4, I generated a clonal cell line that lacked surface CXCR4 expression (henceforth termed JC4) as confirmed by flow cytometry analysis (Figure 12A). In order to verify the mutation in CXCR4, genomic DNA of JC4 was sequenced and I found that there was a 2 base pair deletion (g.406_7del) in exon 2 (Figure 12B). This caused a shift creating a premature stop codon that resulted in a 20 amino acid product (Figure 12C).

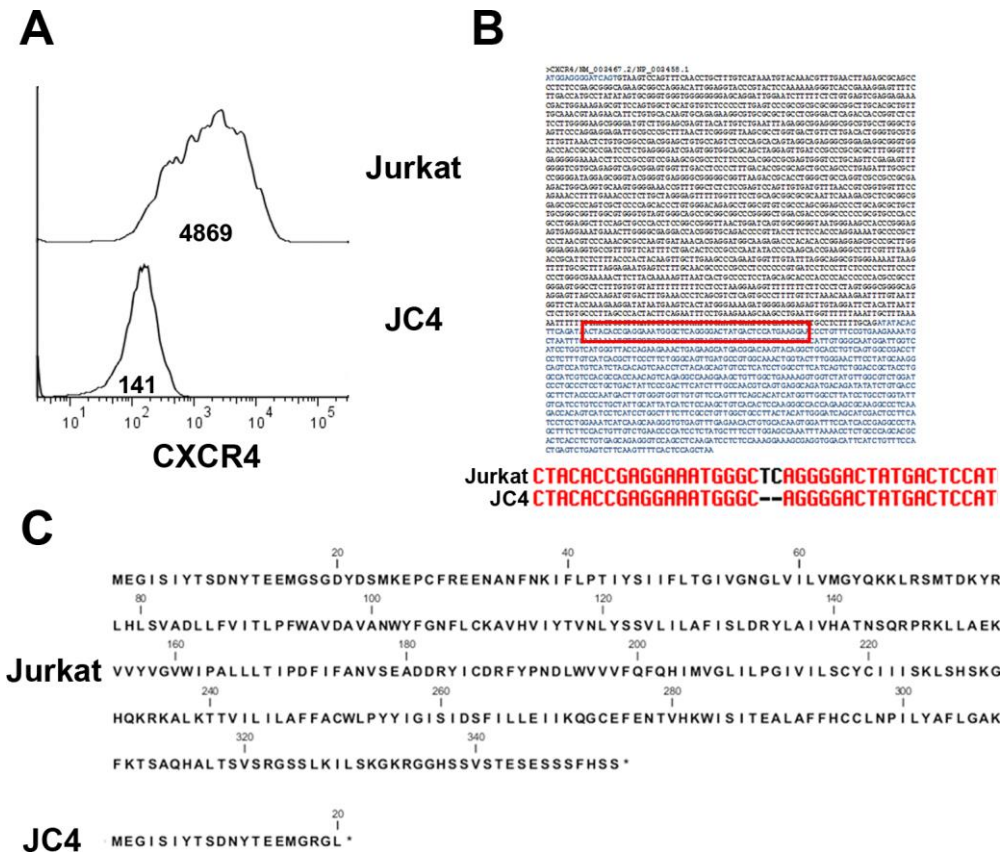

Figure 12. Creation of JC4, a CXCR4-negative Jurkat cell line. (A) Surface CXCR4 expression of Jurkat and JC4 cells. JC4 cells were generated from Jurkat cells transfected with a CRISPR-Cas construct targeting exon 2 of CXCR4. Median fluorescence intensity is indicated below the histogram peaks. (B) Mutation of CXCR4 in JC4 cells. Top, genomic DNA of CXCR4. Bottom, 2 base pair deletion found in genomic CXCR4 DNA of JC4 cells. (C) Comparison of the CXCR4 amino acid sequence between Jurkat and JC4 cells.

3.3.2 Jurkat Cells Display Chemotactic Migration Towards SDF-1

To determine the ability of Jurkat cells to migrate towards SDF-1, I used the Bioflux machine to introduce a SDF-1 gradient and compared fibronectin-adhered Jurkat cells with the CXCR4 deficient JC4 cells. To track cell movement, the position of cells with (X,Y) coordinates was coordinated (Figure 13A). The angle of migration was determined (Figure 13B) and shown to be significant between SDF-1-stimulated Jurkat and JC4 cells (Figure 13C). As T-cells are less adhesive than macrophages and have the ability to roll in response to shear flow, both Jurkat and JC4 cells had horizontal (X) displacement in the direction of flow (Figure 13D). Jurkat cells

showed significantly higher vertical (Y) displacement in the direction of SDF-1 compared to JC4 (Figure 13E), indicating that CXCR4 is the required receptor for SDF-1-mediated cell migration.

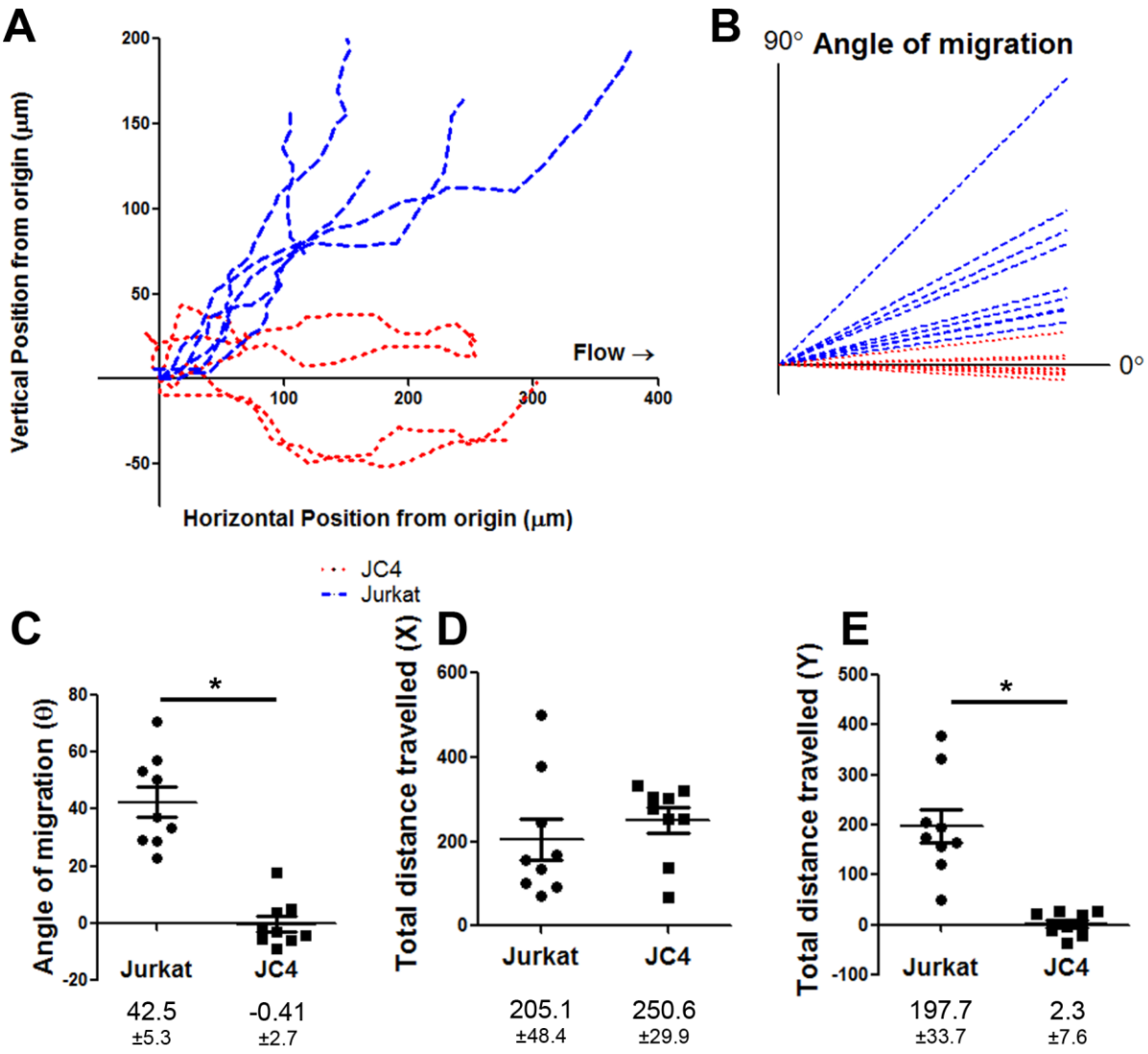

Figure 13. Tracking of SDF-1-stimulated Jurkat cells. (A) The (X,Y) coordinates of SDF-1-stimulated cells was tracked every 1 minute by time lapse microscopy. The blue dotted line indicates Jurkat cells, while the red dotted line indicates JC4 cells. Migration towards SDF-1 is indicated by high Y-axis values. (B) Average angle of migration. Values were determined through interpolation of time points into a line from which the angle was calculated from the slope. (C, D, E) Comparisons between Jurkat and JC4 for X-axis, Y-

axis, and angle of migration. Error bars indicate the SD. Statistical significance ($p < 0.05$), indicated by the asterisk, was determined through the unpaired t-test ($n = 9$ cells).

3.3.3 SDF-1 Stimulates PKA Activity in Jurkat Cells

Because I was able to confirm migratory response of Jurkat cells towards SDF-1, I next investigated signaling pathways. To determine the temporal activation of PKA in Jurkat cells, SDF-1 stimulation of Jurkat cells was performed in a time course. Lysates were immunoblotted for PKA-phosphorylated substrates, phosphorylated p44/42 MAPK (ERK1/2), and GAPDH, which served as the loading control (Figure 14A), and densitometry was performed in order to quantify the changes in PKA-phosphorylated substrates and phosphorylated MAPK (Figure 14B). The amount of PKA-phosphorylated substrates seems to peak around 6-8 minutes, which appears to coincide with the peak of phosphorylated MAPK.

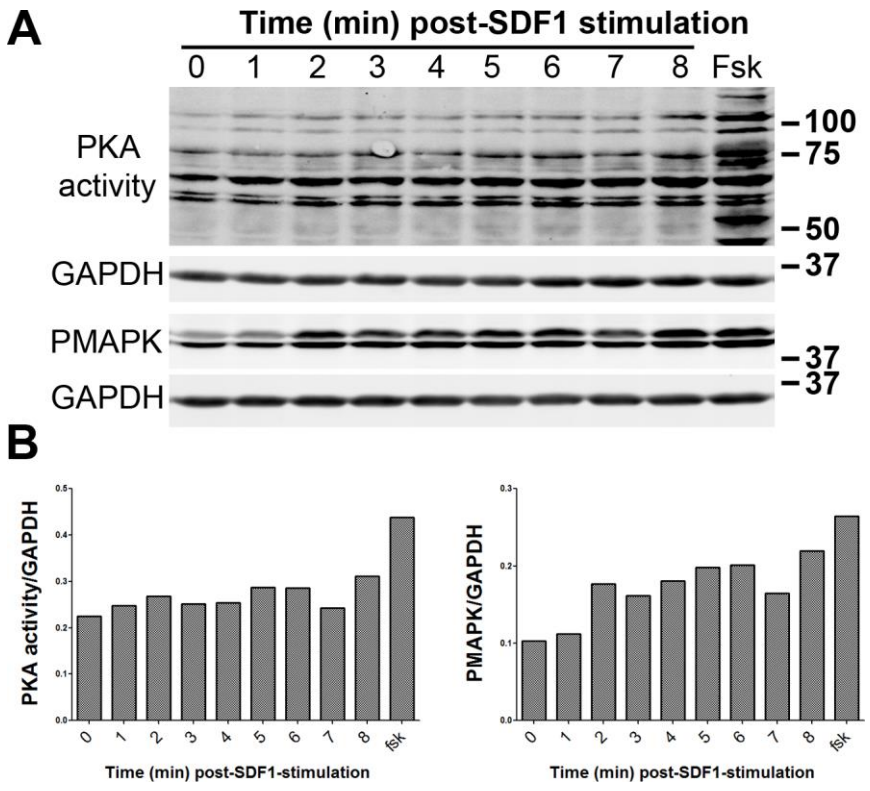

Figure 14. Time course protein immunoblot of SDF-1-stimulated Jurkat cells. (A) Jurkat cells were stimulated with SDF-1 (50nM, indicated time points) and Fsk (50μM, 5 min). Lysates were immunoblotted for PKA-phosphorylated substrates (PKA activity),

phosphorylated p44/42 MAPK (PMAPK), and GAPDH. Molecular weights are displayed to the right. (B) Quantification of protein immunoblot with densitometry. The densitometry values of PKA-phosphorylated substrates and phosphorylated MAPK were normalized to the GAPDH loading control.

Next, Jurkat and JC4 cells were stimulated with SDF-1 for 8 minutes and lysates were subject to protein immunoblot analysis for PKA-phosphorylated substrates, phosphorylated p44/42 MAPK (ERK1/2), and GAPDH as a loading control (Figure 15A). MAPK was phosphorylated in response to SDF-1 for Jurkat cells but not JC4, indicating that CXCR4 signaling pathways were successfully knocked out. Jurkat cells displayed an increase in PKA activity compared to no increase in JC4 cells, confirming that the SDF-1-stimulated increase in PKA-phosphorylated substrates (Figure 15B).

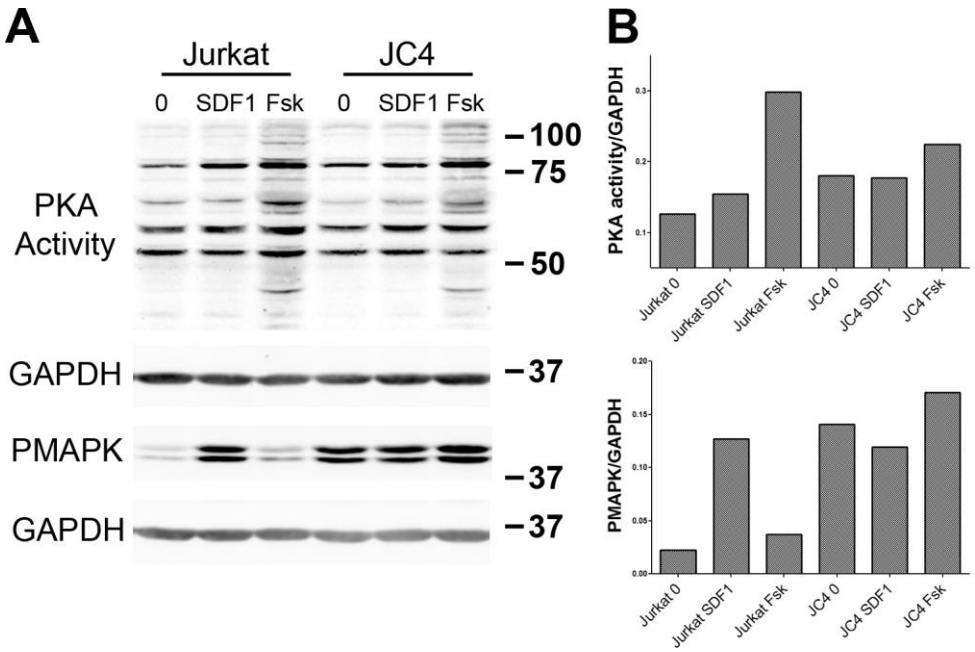

Figure 15. Comparison of response to SDF-1 stimulation by Jurkat and JC4 cells. (A) Jurkat and JC4 cells were stimulated with SDF-1 (50nM, 8 minutes) and Fsk (50μM, 5 min). Lysates were immunoblotted for PKA-phosphorylated substrates (PKA activity), phosphorylated p44/42 MAPK (PMAPK), and GAPDH. Molecular weights are indicated on the right. (B) Quantification of Jurkat and JC4 response to SDF-1. Densitometry values

of PKA-phosphorylated substrates and phosphorylated MAPK were normalized to the GAPDH loading control.

3.4 Fluorescence Resonance Energy Transfer (FRET) for Visualization of PKA Activity

3.4.1 Establishing PKA Imaging Using A-Kinase Activity Reporter

Because we wanted to monitor the spatiotemporal regulation of PKA activity in cells, I employed the use of the A-Kinase Activity Reporter (AKAR), a FRET biosensor for PKA activity[343]. It consists of YFP and CFP molecules tethered to each other through a linker sequence consisting of a PKA substrate and a phosphopeptide-binding FHA domain. At rest, excitation of CFP at 436 nm produces a CFP emission at 480 nm. Phosphorylation of the substrate by PKA results in phospho-substrate binding by the FHA domain, causing a conformational change that brings the YFP molecule into proximity with the CFP molecule. Once CFP is excited, there is an energy transfer that occurs from CFP to YFP, resulting in the emission of YFP at 535 nm. The resulting CFP and YFP images are recorded and processed using a ratiometric method of YFP over CFP to indicate FRET efficiency, and thus PKA activity (Figure 16A).

AKARs may be targeted to specific cellular compartments. For our investigations, we chose to use the plasma membrane AKAR (pmAKAR), which contains the CaaX box sequence from K-Ras for plasma membrane localization. Because we are studying the properties of cell migration, we expected much of the PKA activity to be localized at or near the membrane. There is also a non-phosphorylatable control construct for pmAKAR, termed pmAKAR(TA) as the phosphorylatable threonine is replaced by an alanine residue[344].

To establish PKA imaging, CHO-CXCR4 cells transfected with pmAKAR and pmAKAR(TA) were stimulated with Fsk. Because Fsk upregulates cAMP levels within the cell, the activity of PKA is also increased, and the pmAKAR construct should be able to detect the change. Indeed, CHO-CXCR4 cells transfected with pmAKAR show a marked increase in FRET efficiency as measured by YFP/CFP. The efficiency of the control pmAKAR(TA) construct was also confirmed, as it did not respond to increases in PKA activity (Figure 16B).

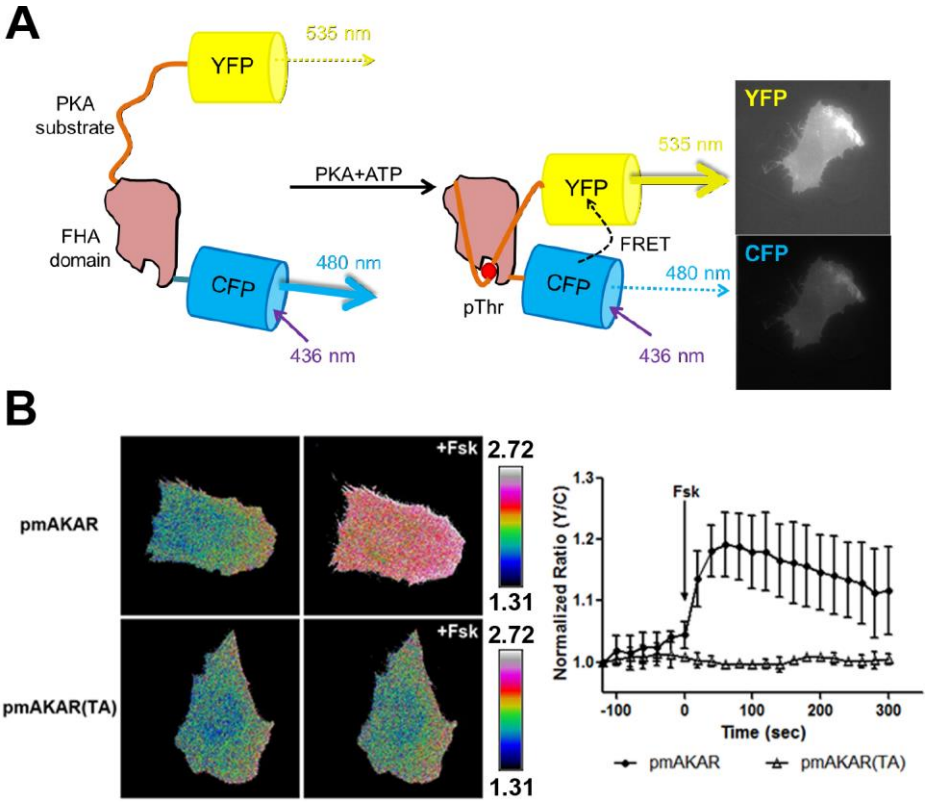

Figure 16. FRET imaging using a biosensor for PKA activity. (A) Mechanism of the FRET biosensor for PKA activity. Phosphorylation of the PKA substrate of AKAR causes a conformational change. Excitation of CFP results in an energy transfer from CFP to YFP. The resulting YFP and CFP images are recorded and further analyzed using a ratiometric method. (B) CHO cells were transfected with the plasma membrane localized AKAR (pmAKAR) and non-phosphorylatable control construct pmAKAR(TA), adhered onto fibronectin, and then stimulated with Fsk (50µM). Images were acquired every 20 seconds. Left, Pseudocoloured FRET images representing the YFP/CFP emission ratio of CHO cells expressing the indicated constructs, 1 minute pre- and post-stimulation with Fsk. The range of FRET ratios is indicated in the colour bar to the right. Right, Average FRET values of the whole cell were plotted as mean and SD against the indicated time points (n = 3 cells).

3.4.2 SDF-1 Induces Protrusions in Jurkat Cells with High PKA Activity

To visualize PKA activity upon SDF-1 stimulation, Jurkat cells were transfected with pmAKAR and pmAKAR(TA). Jurkat cells expressing either construct were globally stimulated with SDF-

1, which stimulated the cells to produce protrusions. In pmAKAR-transfected cells, these protrusions were high in PKA, while the pm-AKAR(TA)-transfected cell, which is unresponsive to changes in PKA, displayed no difference (Figure 17A). To quantify the differences in FRET ratio between the protrusion and cell body, the FRET ratio was measured across the cell body and protrusion as indicated by the dotted line. The average FRET ratio value of the protrusion was normalized to the cell body and compared between pmAKAR- and pmAKAR(TA)-transfected cells. SDF-1-stimulated Jurkat-pmAKAR cells displayed a significantly higher ratio of protrusion to cell body compared to pmAKAR(TA) protrusions, which confirms that the SDF-1-mediated increase in PKA is not due to an artifact of imaging (Figure 17B).

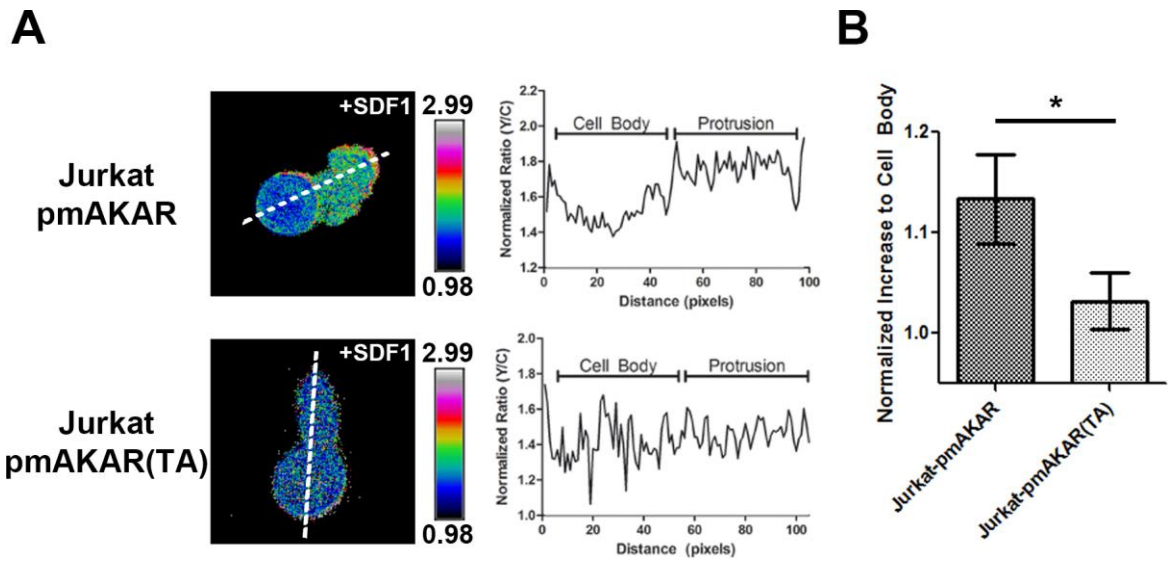

Figure 17. SDF-1 stimulation of Jurkat cells induces protrusions that are high in PKA activity. (A) Comparison of SDF-1-stimulated Jurkat cells transfected with pmAKAR or pmAKAR(TA). Jurkat cells were adhered onto fibronectin and stimulated with SDF-1 (50nM). Left, Pseudocoloured FRET images with the corresponding colour bar indicating high and low PKA activity. Images were taken 10 and 23 minutes post-stimulation for Jurkat-pmAKAR and Jurkat-pmAKAR(TA), respectively. Right, Line profile analysis of mean FRET ratio across the cell. Measurements were taken along the dotted line indicated in the pseudocoloured FRET images. (B) Normalization of line profile analysis. Mean FRET ratio measurements of the protrusion were normalized to measurements of the cell

body. Error bars indicate the SD of the mean (n = 3 cells). Statistical significance ($p < 0.05$), indicated by the asterisk, was determined through the unpaired t-test.

3.4.3 PKA Activity is Localized to the Leading Edge in Migrating Jurkat Cells

Since PKA activity was high in globally stimulated SDF-1-induced protrusions in Jurkat cells, we sought to investigate the effects of SDF-1 on PKA activity during directional migration. Using the Bioflux system, pmAKAR-transfected Jurkat cells were stimulated with SDF-1 in a directional manner. Jurkat cells migrating towards SDF-1 exhibited high levels of PKA activity within their protrusions (Figure 18A). A line profile analysis (as seen in Figure 17A) shows increased mean FRET ratio in the protrusions of a migrating cell compared to the cell body (Figure 18B). Furthermore, comparison of the normalized FRET ratios of the protrusion compared to the cell body (as seen in Figure 17B) showed that the protrusions consistently had increased PKA activity compared to the cell body (Figure 18C).

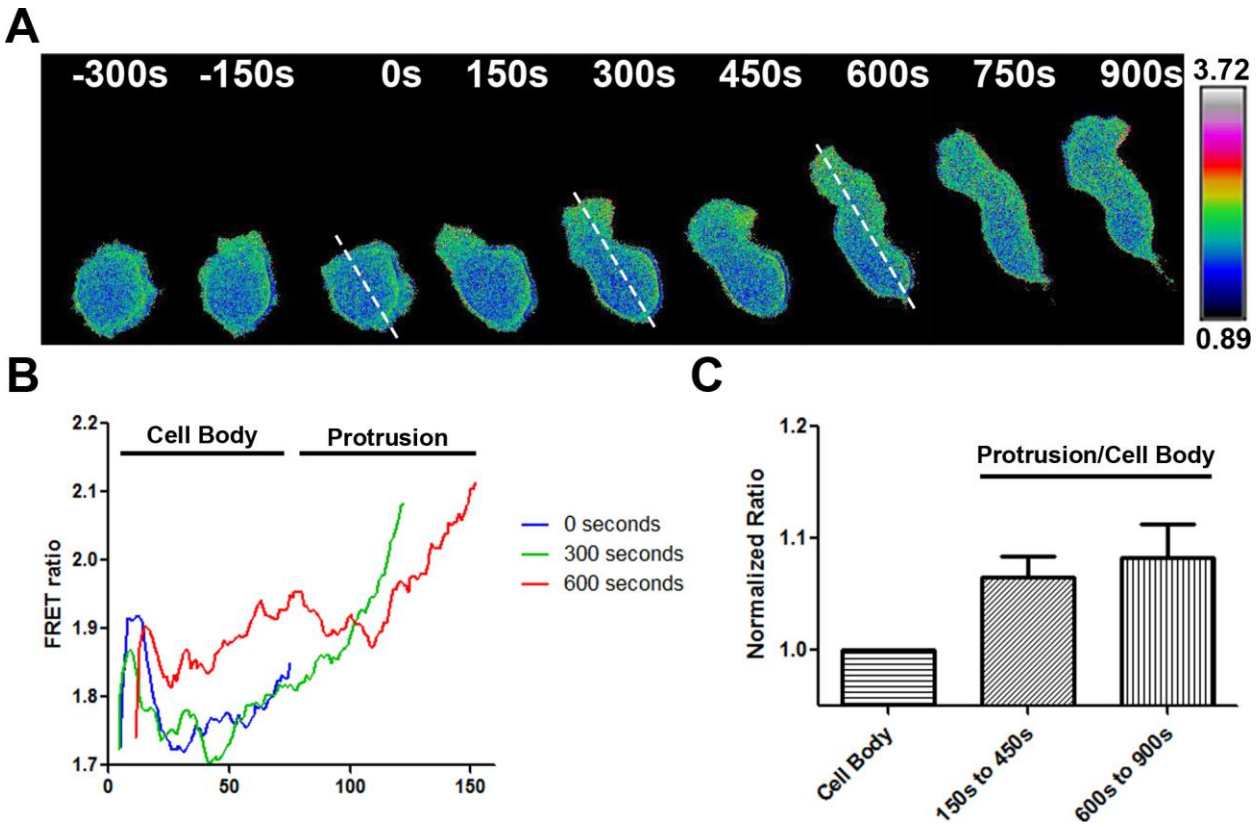

Figure 18. Directional migration of pmAKAR-transfected Jurkat cells towards SDF-1. (A) Time lapse microscopy of Jurkat-pmAKAR cells migrating towards SDF-1 (50nM), which

was added at t=0. Images were acquired every 30 seconds pre- and post-SDF-1-stimulation. (B) Line profile analysis of Jurkat-pmAKAR migration towards SDF-1. Mean FRET ratio was measured over the length of a bisecting line, as indicated by the dotted white line in Figure 16A, through the migrating cell. (C) Comparison of protrusion to cell body ratios pre- and post-SDF-1 stimulation. FRET ratios of the protrusion and cell body were obtained by line profile analysis and averaged in 5 minute intervals. If no protrusion was present, normalization would be set to 1. Error bars indicate SD of the mean (n = 3 cells).

4. Discussion

4.1 Overview

In cell migration, the cyclical process of protrusion, translocation, and retraction is dependent on highly localized signaling pathways. One of these signaling molecules is PKA, whose activity exists in a front-to-back gradient within a migrating cell. At the leading edge, PKA phosphorylates migration-promoting proteins such as α4 integrins and VASP, and also indirectly mediates activation of migration related kinases such as the Rho GTPases Rac and Cdc42. Although downstream effectors of PKA have been investigated, upstream regulators of PKA activity have yet to be elucidated. We postulated that, given its role in leukocyte migration and activation of migration-related signaling pathways, SDF-1/CXCR4 signaling is an upstream regulator of PKA activity. Despite conflicting literature regarding the role of SDF-1/CXCR4 signaling and regulation of PKA activity, the present study reveals activation of PKA in SDF-1-stimulated CHO, J774, and Jurkat cell lines, all of which expressed CXCR4. Although we were unable to observe a migratory phenotype in CHO cells, J774 cells showed increased peripheral ruffling that was high in PKA activity. Furthermore, SDF-1 stimulation of Jurkat cells in a directional assay revealed establishment of a gradient of PKA activity that was highest at the leading edge. Collectively, these observations indicate that SDF-1/CXCR4 signaling regulates PKA activity, and suggest a role for SDF-1/CXCR4 signaling in promoting PKA-mediated cell migration.

4.2 Activation of PKA in SDF-1/CXCR4 Signaling

4.2.1 SDF-1/CXCR4 Signaling in CHO Cells

We initially investigated SDF-1/CXCR4 signaling in CHO cells as they were a well-studied model of migration lacking CXCR4 expression (for ease of CXCR4-negative control experiments). Activation of CXCR4-mediated signaling pathways by SDF-1 such as calcium mobilization, Akt/PKB activation, and p44/42 MAPK (ERK1/2) phosphorylation have all been previously described in CXCR4-expressing CHO cells[335,337,338,345]. A clonal CHO cell line expressing a high level of surface CXCR4 expression was developed, CHO-CXCR4, and its signaling functionality was confirmed through increases in phosphorylated p44/42 MAPK

(ERK1/2) upon SDF-1 stimulation. Furthermore, I found that SDF-1 stimulation of CHO-CXCR4 cells caused an increase in PKA-phosphorylated proteins and thus PKA activity.

Despite MAPK phosphorylation and upregulation of PKA activity by SDF-1/CXCR4 signaling, two processes that have been linked to promotion of cell migration, no morphological changes were observed in SDF-1-stimulated CHO-CXCR4 cells. Although numerous investigations have studied the intracellular signaling pathways in CHO-CXCR4 cells, only one study has reported SDF-1 directed migration within a transwell assay[346]; however, this assay was performed without a negative control for CXCR4. While some studies have successfully obtained a migratory phenotype in chemokine receptor (formyl peptide receptor 1[347], CCR5[348], and CXCR3[349]) transfected CHO cells, characterization of other chemokine receptors transfected in CHO cells (CCR2[350], CCR3[350,351], CXCR2[352], CXCR4[338]) have notably performed migration assays with blood cells rather than the transfected CHO cells. This presents the likely possibility that specific chemokine receptor signaling pathways present in leukocytes are absent in CHO cells.

4.2.2 SDF-1/CXCR4 Signaling in J774 Macrophages

As I was unable to elicit a migratory phenotype in CHO cells, we decided to investigate the murine macrophage cell line J774. The advantages to this are threefold: J774 cells natively express CXCR4[353]; they have been reported to migrate towards SDF-1[354]; and their spread morphology upon substrate adhesion allows for observation of a leading edge during migration[355]. J774 surface expression of CXCR4 was confirmed and directional migration of J774 cells towards SDF-1. Furthermore, an increase in phosphorylated p44/42 MAPK (ERK1/2) and PKA-phosphorylated substrates was also observed upon SDF-1 stimulation, which is the first time activation of these signaling pathways have been demonstrated in SDF-1-stimulated J774 cells.

A limitation in our J774 experiments is the lack of a proper negative control for CXCR4. I attempted to use AMD3100, a reported antagonist of CXCR4, in conjunction with SDF-1. However, AMD3100 appeared to cause stimulation of PKA activity and MAPK phosphorylation (data not shown), which was unexpected as AMD3100 appeared to inhibit SDF-1 in CHO cells. Subsequent literature review revealed that AMD3100 is a partial agonist of CXCR4 *in vitro*,

stimulating MAPK and Akt/PKB phosphorylation[356,357]. Indeed, the ability of AMD3100 to inhibit CXCR4 signaling in CHO cells but not J774 cells further suggests that expression of CXCR4 in CHO cells is not sufficient to fully re-create signaling events. Another possible control would have been to create a CXCR4-negative J774 cell line using the CRISPR-Cas method, but I did not attempt this given our difficulties in transfecting J774 cells.

4.2.3 SDF-1/CXCR4 Signaling in Jurkat T-cells

One of the main functions of SDF-1/CXCR4 signaling is the leukocyte extravasation model of lymphocyte trafficking. Many of the studies contributing to this knowledge have used the Jurkat T-cell line in researching related mechanisms such as integrin activation and signaling events promoting migration. In order to confirm that our observations from Jurkat SDF-1 stimulation experiments were CXCR4-specific, we used the CRISPR-Cas system to create JC4, a stable Jurkat cell line lacking CXCR4 expression as verified by flow cytometry. Although there are Jurkat derivative cell lines engineered to lack expression of certain proteins such as α4 integrin and CD47, JC4 is a novel cell line as there have been no other Jurkat derivatives lacking CXCR4 expression previously reported. While comparing Jurkat cells with JC4 cells, I determined that directional migration towards SDF-1 is CXCR4-dependent. Furthermore, I found that SDF-1 stimulation of Jurkat cells caused CXCR4-dependent phosphorylation of MAPK and upregulation of PKA-phosphorylated substrates (and thus PKA activity) in immunoblot analysis. To further this investigation into CXCR4-dependent upregulation of PKA activity, we are currently reconstituting CXCR4 expression in JC4 cells in order to create a clonal JC4-CXCR4 cell line.

Previous studies surrounding SDF-1/CXCR4 signaling and cAMP/PKA activity have found both up and downregulation. CXCR4 is a GPCR and can associate with the $G\alpha_i$ subunit, which inhibits cAMP production. CXCR4-mediated suppression of cAMP levels have been shown to promote tumor growth in brain cancers, but elevation of cAMP by SDF-1 is also shown to contribute to axonal growth. Furthermore, CD4+ T lymphocytes show increased PKA catalytic subunit activity in response to SDF-1. Here I have shown an increase in PKA activity upon SDF-1 stimulation in three different cell lines: CHO-CXCR4, J774, and Jurkat. Our method of detecting PKA activity involves probing for proteins that have been phosphorylated by PKA.

While this is not a direct measurement of cAMP concentration or PKA activity, it allows us to confirm downstream action of PKA on other proteins. As the antibody detected all PKA-phosphorylated substrates, we were able to observe increases in intensity at specific molecular weights: the largest increase in Jurkat cells was found at around 75 kDa; J774 cells displayed a large increase at around 100 kDa; and CHO-CXCR4 cells displayed increases at both 75 kDa and 100 kDa. These molecular weights may serve as a future guideline to identify PKA-specific targets in SDF-1/CXCR4 signaling.

4.3 Localization of PKA Activity in SDF-1/CXCR4 Signaling

Having found an increase in PKA activity in SDF-1-stimulated J774 and Jurkat cells, we decided to investigate the spatiotemporal localization of PKA activity in SDF-1/CXCR4 signaling. Since I was unable to obtain expression of the FRET biosensor for PKA activity, pmAKAR, in J774 cells, I used immunofluorescence and identified significant peripheral staining of PKA activity and F-actin in SDF-1-stimulated J774 cells. Our results are not dissimilar to previous studies, as chemokine-induced peripheral F-actin staining has been previously described in fMLP stimulation of J774 cells[355]. As our experiments involved global SDF-1 stimulation, we cannot discern whether the PKA activity was localized at the leading edge, as seen in previous studies of PKA-mediated migration. Fixation of directionally SDF-1-stimulated J774 cells was also not possible due to logistics involving the Bioflux system. However, live imaging of global SDF-1 stimulation of J774 cells revealed membrane ruffling at the periphery, which was similar to the ruffling seen at the leading edge of directionally SDF-1-stimulated J774 cells. Moreover, the immunofluorescent staining of PKA activity was also localized with concentrated F-actin staining at the periphery, and membrane ruffles are actin-rich structures[358] whose formation is driven by PKA activation[359]. This suggests that PKA activity may play a role in mediating J774 leading edge dynamics such as membrane ruffling during SDF-1-mediated cell migration.

To determine the spatiotemporal localization of PKA in SDF-1/CXCR4 signaling, we employed the use of pmAKAR, the PKA FRET biosensor localized to the plasma membrane. This biosensor is sensitive to increases in PKA activity and has been used for many investigations into PKA activity localization. Using this approach, I transiently expressed pmAKAR in Jurkat cells and stimulated them with SDF-1. I observed that SDF-1-stimulated Jurkat-pmAKAR cells

exhibited an increased number of protrusions that were high in PKA activity. These increases in PKA activity were confirmed to not be artifacts of imaging as they were not present in SDF-1-stimulated Jurkat-pmAKAR(TA) cells (the nonphosphorylatable control FRET biosensor for PKA). Moreover, directional SDF-1 stimulation showed PKA activation at the leading edge of Jurkat-pmAKAR cells.

PKA activation has been shown to contribute to many signaling processes at the leading edge in cell migration such as microfilament assembly, protrusion formation, activation of Rac and Cdc42, and inhibition of Rho. Furthermore, PKA-phosphorylated substrates and PKA itself are localized to cell protrusions. As such, there is a gradient of PKA activity in a migrating cell that is highest at the leading edge, and this activation has been shown to be adhesion-dependent. Here, we show CXCR4-dependent activation of PKA within cell protrusions and at the leading edge of migrating Jurkat cells. These findings are agreeable with previous investigations. PKA activation in migrating cells is dependent on integrin adhesion, and SDF-1/CXCR4 signaling promotes integrin activation to a higher affinity binding state. Additionally, CXCR4 and PKA share similar downstream targets in their promotion of migration such as activation of Rac and Cdc42. However, it remains to be seen whether PKA activation is a necessary step in SDF-1/CXCR4 mediated migration. Nevertheless, activation of PKA may be a possible mechanism by which SDF-1/CXCR4 signaling promotes cell migration.

4.4 Future Directions

The goal of our research was to determine the role of SDF-1/CXCR4 signaling in regulation of PKA activation. Through the use of a FRET biosensor for PKA activity, we have shown leading edge PKA activation by SDF-1/CXCR4 signaling. Future investigations would aim to determine whether PKA activation is a necessary mechanism of SDF-1/CXCR4-mediated cell migration. Previous studies have demonstrated the importance of PKA in cell migration and protrusion formation through inhibition of PKA activity or localization. These include the competitive PKA inhibitors PKI[136], Rp-cAMP[140], and H-89[131] as well as the PKA-AKAP interaction inhibitors StHt31 (Type II)[136] and AKB-RI (Type I)[137]. Pre-incubation of these compounds with Jurkat-pmAKAR cells prior to SDF-1 stimulation would aid in elucidating the necessity of PKA activation in SDF-1/CXCR4signaling.

If PKA activation is a necessary mechanism of SDF-1/CXCR4-mediated cell migration, what are its targets? Both LIM kinase and VASP, two proteins involved in actin filament assembly at the leading edge, can be PKA-phosphorylated[360,361]. Another possibility may be the α4 integrin, whose phosphorylation by PKA is required for directional cell migration[137]. Finally, PKA phosphorylation of Rap1 is necessary for its migration promoting functions at the leading edge[362]. These proteins have been linked with CXCR4 in previous studies. LIM kinase is activated by CXCR4 through Pak1, CXCR4 mediates PKG-phosphorylation of VASP, and CXCR4 activates α4 integrins and Rap1. Given that CXCR4 has been shown to associate with these targets, it may be possible that they are also targets of CXCR4-mediated PKA activation.

Although we show increased activation of PKA by way of phosphorylated substrates and FRET imaging, it remains to be seen how CXCR4 upregulates PKA activity at the leading edge and in protrusions. Cyclic AMP levels within a migrating cell also exist in a front-to-back gradient and contribute to localized increases in PKA activity at the leading edge[135]. Since PKA activation is dependent on cyclic AMP, it would be worthwhile to examine cAMP levels upon SDF-1 stimulation. This would be investigated by measuring global cAMP levels in SDF-1-stimulated cells as well as use of a previously developed FRET biosensor for cAMP levels to visualize localization of cAMP levels within a migrating cell[363]. This investigation would confirm whether or not CXCR4-mediated PKA activation was a result of increased cAMP synthesis.

Finally, our finding that CXCR4 upregulates PKA activity presents a possibility that PKA activation is a characteristic of chemokine signaling. Since the main function of chemokines is to induce chemotaxis, establishment of the front-to-back gradient of PKA activity may be a necessary requirement for cell migration. CXCR4-dependent activation of PKA is not the first time a chemokine receptor has been linked with PKA upregulation, as CXCR3 and its ligand IP-10 have been shown to increase PKA activity, though this study was performed on endothelial cells[364]. Therefore, we could stimulate leukocytes with various chemokines and detect PKA activity through protein immunoblot detection of phosphorylated substrates as well as FRET biosensors for PKA activity.

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Appendix A

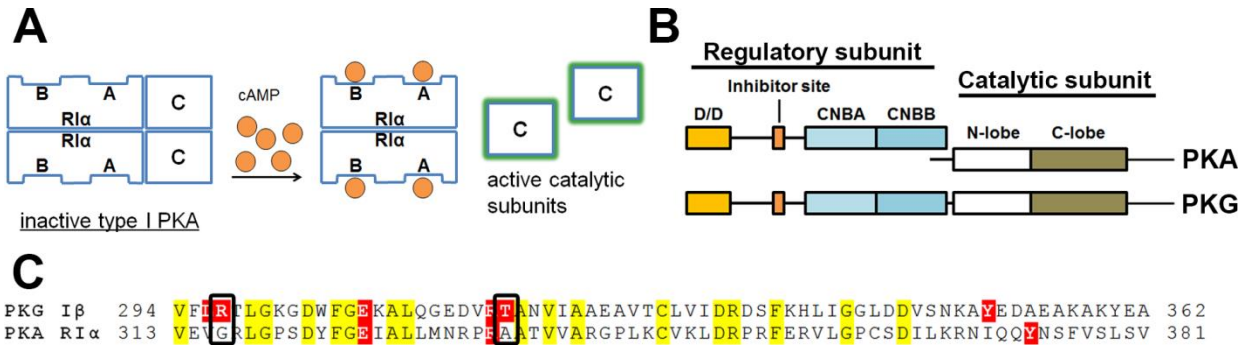

Figure A1. Comparison of PKA and PKG. (A) Activation of PKA. The PKA holoenzyme exists as a tetramer composed of two regulatory subunits that bind two catalytic subunits. Upon binding of cAMP to the regulatory subunits, the catalytic subunits are released from the regulatory subunits and become active. (B) Comparison of protein domain structures of PKA and PKG. For regulatory functions, both kinases contain dimerization/docking (D/D) domains for localization by anchoring proteins such as AKAPs and GKAPs, inhibitor sites that bind to the active site cleft of the catalytic subunit, and two cyclic nucleotide binding domains designated as A (CNBA) and B (CNBB). The catalytic subunits are also well conserved, consisting of a small N-terminal lobe for ATP binding and a larger C-terminal lobe for catalytic activity. In contrast to the tetrameric structure of PKA, PKG exists as a singular structure containing a regulatory and catalytic subunit. (C) Amino acid sequence comparison of PKG and PKA regulatory subunit cyclic nucleotide binding B domains. The conserved residues are highlighted in yellow, while the residues interacting with cyclic nucleotides are highlighted in red. For the B domain of PKG, Arg297 and Thr317 regulate selectivity of binding for cGMP. Hence, mutation of the PKA RIα subunit B domain (G316R, A336T) should confer cGMP selectivity in addition to retaining cAMP selectivity (unpublished data from Dr. Choel Kim's group). The equivalent mutation in the A domain (T190R, A210T) should also confer cGMP selectivity for the PKA RIα subunit, though its cAMP selectivity is unknown.

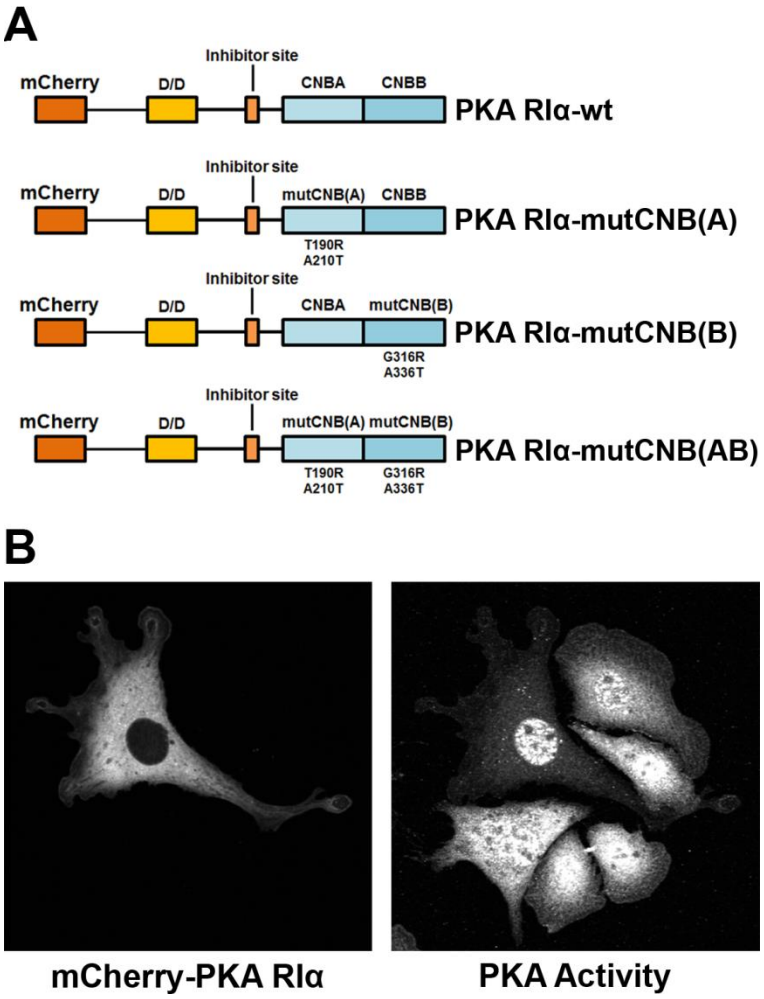

Figure A2. Mutation of the PKA RIα subunit and reconstitution in RIα$^{-/-}$ mouse embryonic fibroblasts (MEFs). (A) PKA RIα subunit constructs. In order to examine the cyclic nucleotide binding properties and activation of PKA, we created an A (T190R, A210T) domain mutant PKA RIα-mutCNB(A), a B (G316R, A336T) domain mutant PKA RIα-mutCNB(B), and a double A and B domain mutant PKA RIα-mutCNB(AB) using site-directed mutagenesis. We also tagged each construct with mCherry in order to be able to confirm reconstitution in MEFs lacking RIα (RIα$^{-/-}$). (B) Reconstitution of the PKA RIα subunit in RIα$^{-/-}$ MEFs. The PKA RIα subunit is the primary regulator of PKA activity in the cell. Therefore, the RIα$^{-/-}$ MEFs has higher basal PKA activity due to a higher amount of free and active catalytic subunits. To confirm reconstitution of PKA RIα activity, we fixed RIα$^{-/-}$ cells transfected to express the mCherry-tagged PKA RIα construct. We confirmed transfection of the PKA RIα subunit in the mCherry channel through confocal

microscopy. Using an antibody directed against PKA-phosphorylated substrates as a measure of PKA activity, we confirmed reconstitution of PKA RIα subunit function as the mCherry-expressing cell had a lower PKA activity compared to the non-reconstituted RIα$^{-/-}$ cells. This indicates that the expressed mCherry-PKA RIα subunit lowered the basal PKA activity levels by forming the functional holoenzyme through binding of the catalytic subunits.

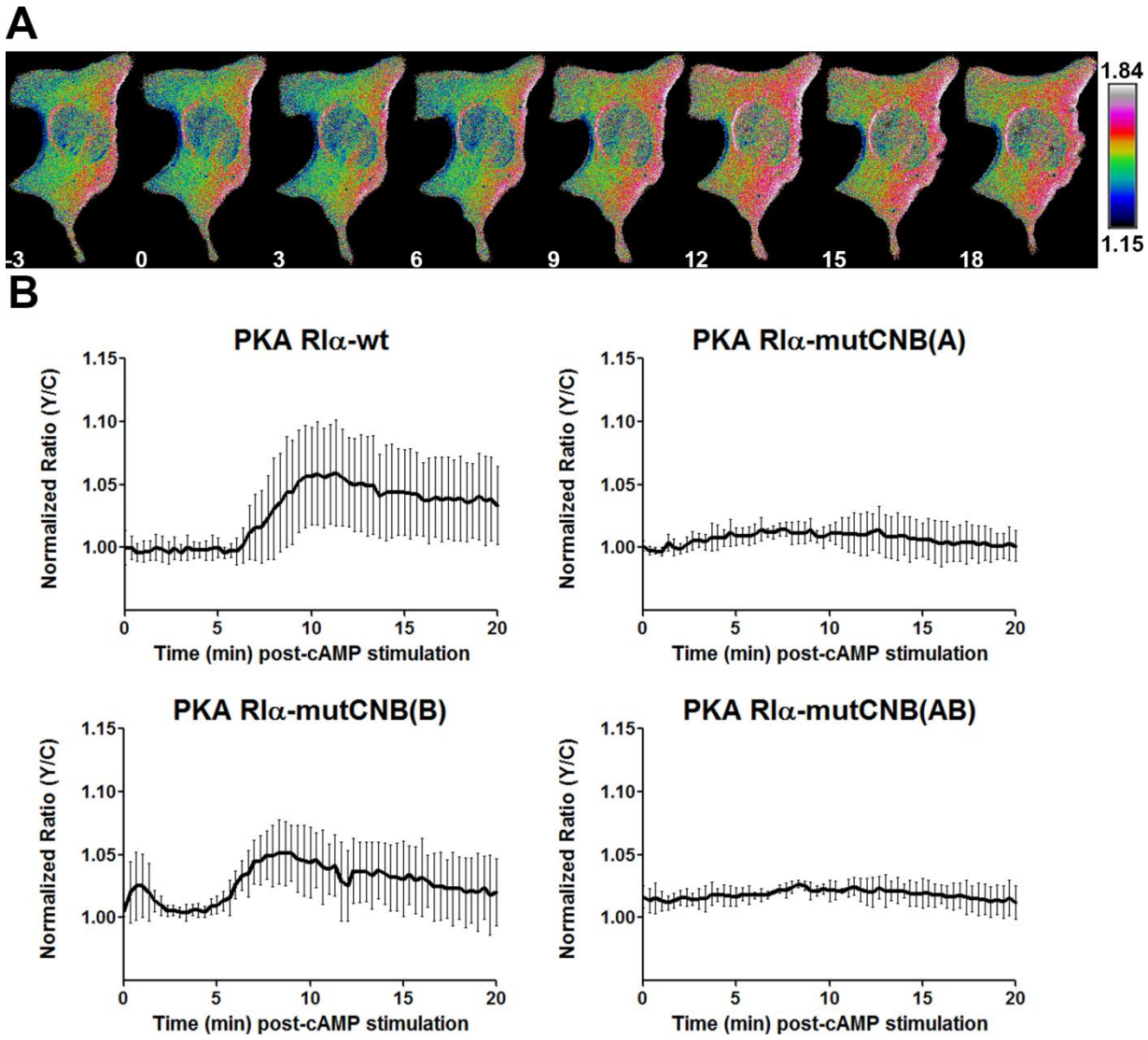

Figure A3. Cyclic AMP activation of PKA RIα constructs. (A) Visualization of cAMP activation of the wild type PKA RIα subunit (PKA RIα-wt) using FRET. RIα$^{-/-}$ MEFs were transfected with PKA RIα-wt and the FRET biosensor for cytoplasmic PKA activity NEC-AKAR, and then stimulated with 500µM 8-CPT-cAMP. YFP and CFP images were obtained every 20 seconds, and then processed ratiometrically (YFP/CFP) to produce FRET images. Here, the images are pseudocoloured using the "Ratio" LUT in ImageJ, whereby high and low PKA activity is indicated on the colour bar to the right. The time point in minutes post-cAMP stimulation of each image is indicated at the bottom left in white text. (B) Graphical representation of cAMP activation of the PKA RIα constructs. RIα$^{-/-}$ MEFs were transfected with the indicated PKA RIα subunit and NEC-AKAR. The

mean FRET ratio of a cytoplasmic area of each cell was measured at each time point and normalized to an average of the mean FRET ratio values obtained prior to cAMP stimulation. Both the PKA RIα-wt and PKA RIα-mutCNB(B) constructs showed increases in PKA activity upon cAMP stimulation. For all graphs, data is shown as the mean normalized FRET ratios of n = 4 cells. Error bars indicate SD of the mean.

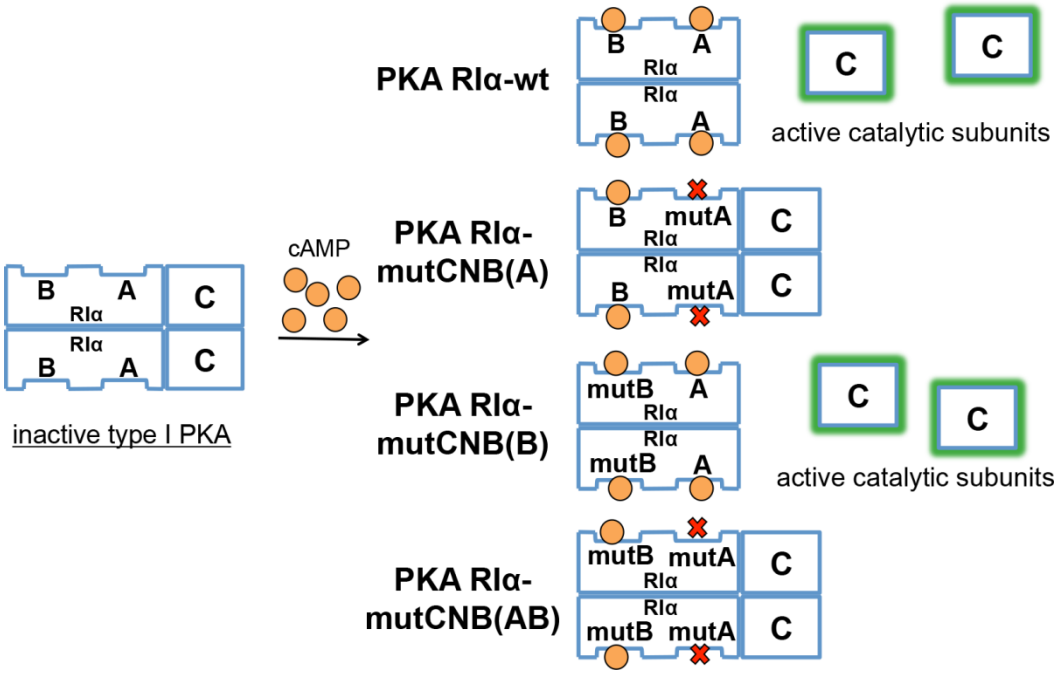

Figure A4. Summary of cAMP stimulation of PKA RIα constructs. Since both A and B domains of PKA RIα-wt bind cAMP, there is activation of PKA. We mutated the A domain (PKA RIα-mutCNB(A)) to be selective for cGMP, and found that cAMP stimulation did not cause PKA activation. However, mutating the B domain (PKA RIα-mutCNB(B)) for cGMP selectivity still resulted in activation of PKA, although unpublished data from Dr. Choel Kim's group suggests that cAMP binding is retained in PKA RIα-mutCNB(B). Finally, mutation of both the A and B domain (PKA RIα-mutCNB(AB)) results in no PKA activation upon cAMP stimulation. This data suggests that the A domain mutation is selective for cGMP and not cAMP, as stimulation with cAMP does not cause activation of PKA in PKA RIα-mutCNB(A) and PKA RIα-mutCNB(AB). However, as we were still able to observe PKA activation in PKA RIα-mutCNB(B), suggesting that the B domain mutation was able to bind cAMP.

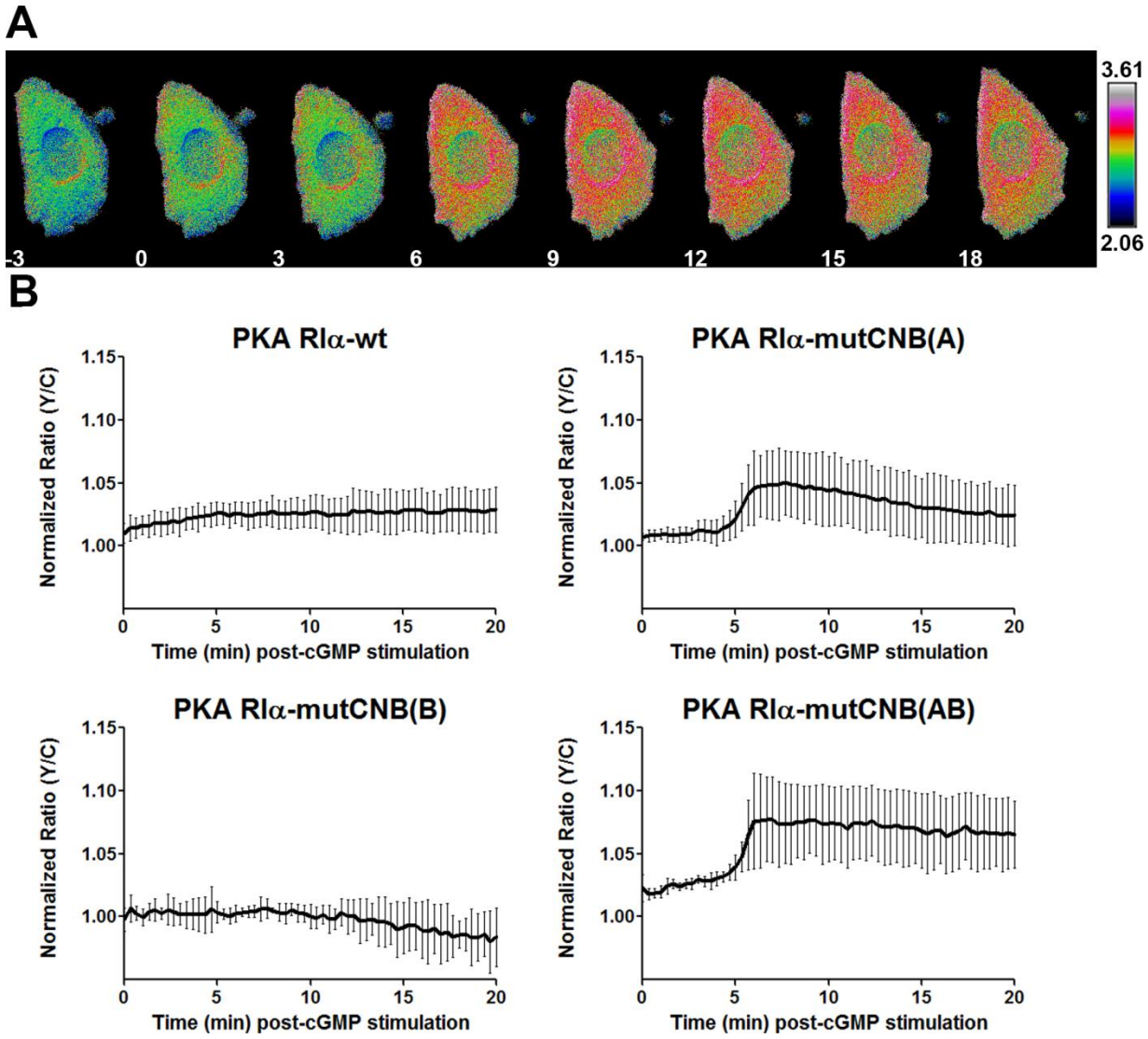

Figure A5. Cyclic GMP activation of PKA RIα constructs. (A) Visualization of cGMP activation of the double A and B domain mutated PKA RIα subunit (PKA RIα-mutCNB(AB)) using FRET. RIα$^{-/-}$ MEFs were transfected with PKA RIα-mutCNB(AB) and NEC-AKAR, and stimulated with 500μM 8-CPT-cGMP. YFP and CFP image acquisition was performed every 20 seconds and processed ratiometrically (YFP/CFP) to produce FRET images. A pseudocolour was applied to the above images using the "Ratio" LUT in ImageJ, and the colour bar to the right indicates high and low PKA activity. Time in minutes post-cGMP stimulation of each image is indicated in white text at the bottom left. (B) Graphical representation of cGMP activation of the PKA RIα constructs. RIα$^{-/-}$ MEFs were transfected with the indicated PKA RIα subunits and NEC-AKAR. Mean

FRET ratio of a cytoplasmic area of each cell was obtained at each time point and normalized to an average of the mean FRET ratio values obtained prior to cGMP stimulation. The PKA RIα-mutCNB(A) and PKA RIα-mutCNB(AB) constructs showed increases in PKA activity upon stimulation with cGMP. Data shown is the mean of normalized FRET ratios for n = 4 cells, and error bars indicate SD of the mean.

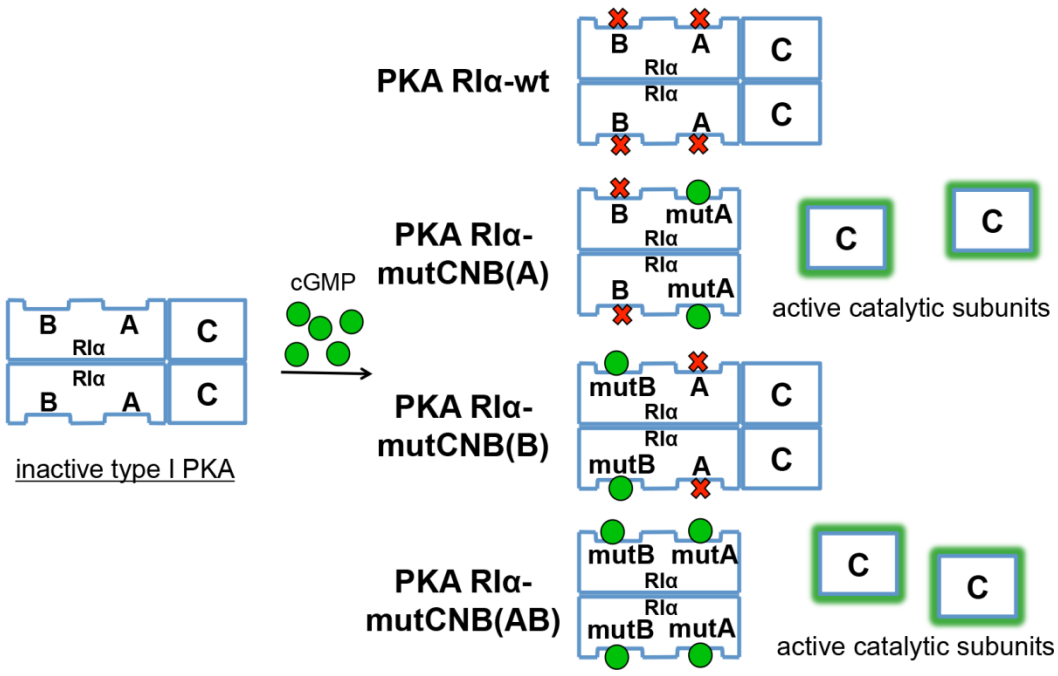

Figure A6. Summary of cGMP stimulation of PKA RIα constructs. As neither the A nor B domain of PKA RIα-wt are selective for cGMP binding, there was no PKA activation observed. When we mutated the A domain (PKA RIα-mutCNB(A)), we observed PKA activation upon cGMP stimulation despite the B domain lacking cGMP selectivity. Mutating only the B domain (PKA RIα-mutCNB(B)) to confer cGMP selectivity did not result in PKA activation when stimulated with cGMP. Lastly, mutation of both the A and B domains (PKA RIα-mutCNB(AB)) resulted in activation of PKA upon cGMP stimulation. Coupled with the cAMP activation data from Figures A3 and A4, this suggests that the A domain is the gatekeeper for PKA activation. Here, we only observed PKA activation with cGMP stimulation when the A domain was mutated to have cGMP selectivity, regardless of B domain selectivity. In Figure A3, we observed that PKA activation upon cAMP stimulation was only possible when the A domain had cAMP selectivity.